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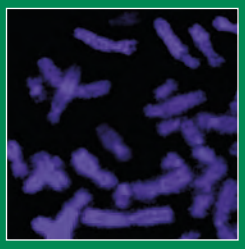


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Genetic and morphology analysis among the pentaploid F_1 hybrid fishes (*Schizothorax wangchiachii* ♀ × *Percocypris pingi* ♂) and their parents

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Triploid and pentaploid breeding is of great importance in agricultural production, but it is not always easy to obtain double ploidy parents. However, in fishes, chromosome ploidy is diversiform, which may provide natural parental resources for triploid and pentaploid breeding. Both tetraploid and hexaploid exist in Schizothorax *fishes, which were thought to belong to different subfamilies with tetraploid* Percocypris *fishes in morphology, but they are sister genera in molecule. Fortunately, the pentaploid hybrid fishes have been successfully obtained by hybridization of* Schizothorax wangchiachii *(♀, 2n = 6X = 148) ×* Percocypris pingi *(♂, 2n = 4X = 98). To understand the genetic and morphological difference among the hybrid fishes and their parents, four methods were used in this study: morphology, karyotype, red blood cell (* ***RBC****) DNA content determination and inter-simple sequence repeat (* ***ISSR****). In morphology, the hybrid fishes were steady, and between their parents with no obvious preference. The chromosome numbers of* P. pingi *have been reported as 2n = 4X = 98. In this study, the karyotype of* S. wangchiachii *was 2n = 6X = 148 = 36m + 34sm + 12st + 66t, while that the hybrid fishes was 2n = 5X = 123 = 39m + 28sm + 5st + 51t. Similarly, the RBC DNA content of the hybrid fishes was intermediate among their parents. In ISSR, the within-group genetic diversity of hybrid fishes was higher than that of their parents. Moreover, the genetic distance of hybrid fishes between* P. pingi *and* S. wangchiachii *was closely related to that of their parental ploidy, suggesting that parental genetic material stably coexisted in the hybrid fishes. This is the first report to show a stable pentaploid F_1 hybrids produced by hybridization of a hexaploid and a tetraploid in aquaculture.*

Keywords: distant hybridization, aquaculture, polyploidy, karyotype, inter-simple sequence repeat

Implications

Distant hybridization can integrate the advantages of both parents, resulting in heterosis and improved growth rates, survival rates and disease resistance. Similarly, odd-ploidy breeding is of great importance in agricultural production, but it is not always easy to obtain double ploidy parents. In this study, we found that the cross between *S. wangchiachii* and *P. pingi* could produce stable pentaploid hybrid fishes under the impact of odd-ploidy and distant hybridization. This is the first report to show a stable pentaploid generation produced by hybridization of natural polyploid fishes in aquaculture and provides a successful case of hybridization and a new breeding pathway in *Schizothorax* fishes even more polyploid fishes.

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Introduction

Triploid breeding has substantial economical and ecological benefits in aquaculture, it usually can reduce the energy distribution in reproductive development, leading to sterility and thereby improving the ability of nutritional growth, disease resistance, genetic containment and so on, but it is not always easy to obtain double ploidy parents (Valérie, 2008; Piferrer *et al.*, 2009; Liu, 2010; Zhang *et al.*, 2014). Compared with higher vertebrates, the genome duplication in fishes is more frequent (Vogel, 1998; Zhou *et al.*, 2001; Venkatesh, 2003; Meyer and Van de Peer, 2005), which has led to the diversiform chromosome number in fishes (Song *et al.*, 2012; Yang *et al.*, 2015), it may provide natural parental resources for odd-ploidy breeding.

Schizothorax fishes are important economic cold-water fishes and are widely distributed in and around the

Qinghai–Tibet Plateau, such as China, India and Pakistan and so on (Yue, 2000). The presence of both tetraploids and hexaploids within the *Schizothorax* genus has attracted the attention of evolutionary biologists (Yang *et al*., 2015; Wang *et al*., 2016) and our research team. Another protagonist is *Percocypris* fishes, which are also important economic cold-water fishes but are distributed only around the eastern Qinghai–Tibet Plateau (Yue, 2000). Morphologically, *Schizothorax* and *Percocypris* were once thought to belong to two different subfamilies (Yue, 2000). However, molecularly, they were shown to be sister genera in a recent study (Wang *et al*., 2013 and 2016).

Distant hybridization, defined as a cross between two different species or higher-ranking taxa, can integrate the advantages of both parents, resulting in heterosis and improved growth rates, survival rates and disease resistance (Liu, 2010). However, it may also result in a risk of low hybridization efficiency and a high progeny malformation rate due to hybrid incompatibility (Chen *et al*., 2016). *Schizothorax* fishes and *Percocypris* fishes are compatible parents for hybridization because of their great differences in morphological characters but only slight differences genetically. Therefore, our research team attempted a distant cross between *S. wangchiachii* and *P. pingi*, both with similar reproductive periods and that are distributed in the upper reaches of the Yangtze River and its tributaries (Ding, 1994; Yue, 2000), to obtain new fish varieties. Our research team observed the development of embryos, larvae and juveniles of the hybrid fishes and found that the embryos were well developed, with a fecundability of 95.33% and a hatching rate of 71.12%. Under the same conditions, the embryos could not develop normally by reciprocal cross. At the beginning of the blastocyst stage, the number of dead individuals increased, and 85.83% of the embryos died at the end of the gastrula stage. The causes were mainly the rupture of the yolk membrane and the overflow of the yolk material in the process of blastoderm downwards wrapped, resulting in the death of the embryo (Yang *et al*., 2018).

The karyotypes of *P. pingi* has been recorded as $2n = 4X = 98 = 42m + 30sm + 10st + 10t$ (Zan *et al*., 1984; Li *et al*., 2017). The karyotypes of many *Schizothorax* fishes have been reported (Supplementary Table S1), but the karyotypes of *S. wangchiachii* have not been reported. In terms of the number of chromosomes, *P. pingi* is a tetraploid species, but there are both tetraploid and hexaploid species of *Schizothorax* fishes. Studies have shown that homoploid hybridization and different ploidy-level hybridization have different hybridization results and breeding significance (Liu, 2010). Additionally, under the both impacts of distant hybridization and different ploidy level, the unreal hybridization and genetic instability may occur in hybrid offspring (Liu *et al*., 2007; Wang *et al*., 2017 and 2018). In summary, it is very important to carry out relevant genetic research for the future development of the hybrid fishes.

In this study, morphology, karyotypes, red blood cell (**RBC**) DNA content and inter-simple sequence repeat (**ISSR**) of the hybrid fishes and their parents were compared to understand the genetic composition and stability of the hybrid fishes. We provided basic genetic and morphology information on the hybrid fishes and their parents for aquaculture and further research on hybrid fishes and present a new pathway of breeding for *Schizothorax* fishes and potentially other polyploid fishes.

Materials and methods

Experimental fishes acquisition

In March 2016, the hybridization experiment was carried out in Xichang Jiahe Agriculture Co. Ltd in Xichang Sichuan's Xide County. Both the parental fishes of *S. wangchiachii* and *P. pingi* were 3 to 4 years old with a body mass of 1500 to 2000 g, bred in 25 m^2 fishponds with a water temperature of 14.5°C ± 1.0°C and a pH of 8.10 to 8.52. The sexually mature female *S. wangchiachii* and the male *P. pingi* were injected with human chorionic gonadotropin and luteinizing hormone-releasing hormone. After 48 to 72 h, the mature parental fishes were pressed gently on the abdomen, and the mature eggs and sperm were obtained naturally. Then, hybridization was conducted by dry fertilization. When the fertilized eggs fully absorbed water and expand, they were incubated evenly on the hatching net (2000 to 3000 eggs/m2) with a water temperature of 14.5°C ± 1.0°C and a pH of 8.10 to 8.81. Juvenile fishes were placed in the circulating water system at our laboratory breeding facility.

Morphology

P.pingi (122.03 ± 1.78 mm, 25.2 ± 1.05 g, n = 30), *S. wangchiachii* (106.78 ± 1.41 mm, 18.43 ± 0.74 g, n = 30) and the hybrid fishes (125.84 ± 2.71 mm, 29.22 ± 1.85 g, n = 30) were studied using traditional morphometrics, which includes meristic and measurable traits, and the examination standards were basically referred to Zou *et al*. (2008). The body shapes were photographed by a SLR camera (Canon EOS 100D, Japan), and the details of the head were photographed by a stereomicroscope (Nikon SMZ25) after fixation by Bouin's fixative. Ten meristic traits (lateral line scales, scales above the lateral line, scales below the lateral line, hard and soft rays of the dorsal fin, hard and soft rays of the pectoral fin, hard and soft rays of the pelvic fin and hard and soft rays of the anal fin) and 13 measurable traits (whole length, body length, body height, body width, head length, snout length, snout barbel length, maxillary barbel length, caudal peduncle length, caudal peduncle depth, eye diameter, interorbital width and the distance of eyeballs (± 0.01 mm)) of each fish were recorded. Where, whole length, body height, body width, head length and caudal peduncle length were divided by body length, caudal peduncle depth was divided by caudal peduncle length, interorbital width was divided by the distance of eyeballs and snout length, eye diameter, interorbital width, vsnout-barbel length and maxillary barbel length were divided by head length to eliminate the effect of different body sizes on

experimental results. Euclidean distance (**ED**) between individuals was calculated using the following formula:

$$\mathrm{ED}ij = \sqrt{\sum_{k=1}^{n} (Xik - Xjk)^2}$$

where ED_{ij} represent ED between two random individuals (*i* and *j*); within a group, *i* and *j* represent random two individuals in a group; between groups, *i* and *j* represent random two individuals from different groups; *k* represents *Z*-score of the ratio of different characters, which was calculated by SPSS 21.0.

Karyotype

Since karyotypes of *P. pingi* have been reported, only *S. wangchiachii* and the hybrid fishes were analyzed in this study. Mitotic chromosome preparations were obtained from the kidney tissues of *S. wangchiachii* (n = 10) and the hybrid fishes (n = 20), and an air-drying technique was adopted (Foresti *et al.*, 1993; Liu *et al.*, 2001). For each type of fish, 100 well-dispersed chromosomes were selected for counting and 10 clear, dispersed and good quality metaphase spreads were used for the analysis of karyotype that were classified by previous standards (Levan *et al.*, 1964; Liu *et al.*, 2001). The pairing of metaphase chromosomes through Adobe Photoshop CS6 and the length measurement of chromosomes by ImageJ. In the hybrid fishes, chromosomes were not arranged in pairs. Since their parents both were paleopolyploids, that already were diploidization in the long evolutionary process, their parents can only be considered as diploids with different numbers of chromosomes from the perspective of breeding.

Red blood cell DNA content

RBC DNA content was determined according to the technique described by Tao *et al.* (2012) with slight modifications. Peripheral blood was taken from the caudal veins of 5 *S. wangchiachii*, 5 *P. pingi*, and 10 hybrid fishes that were same with morphology. In addition, five *Schizothorax davidi* (2n = 4X = 98, 16.5 to38.9 g) were selected as a double control between *P. pingi* with same ploidy and congeneric *S. wangchiachii*. The blood samples were stained according to PI Stain (50 ug/ml, including RNase) (Leagene, Beijing, China). A BD Accuri C611000-type flow cytometer was used for detection. Under excitation light at 488 nm and Fluorescent Light 3 channel, fluorescence was collected, and then, a DNA histogram was obtained. The data analysis was conducted using FlowJo V10. The RBC DNA content of chicken (*Gallus sp.*, whose DNA content is 2.50 pg) was used as a standard reference, and the RBC DNA content of fishes was calculated by the following formula:

$$P_1 = (E_1 \times P_2)/E_2$$

where P_1 indicates the RBC DNA content of the fishes. P_2 indicates the RBC DNA content of cook. E_1 represents the absorbance of the RBC DNA content of cook and E_2 denotes the absorbance of the fishes.

Inter-simple sequence repeat

Fin tissues were, respectively, taken from eight hybrid fishes and their parents, which were from wild population. Nineteen ISSR-PCR primers were screened preliminarily for this investigation is shown in Supplementary Table S2 and were synthesized by the Beijing Genomics Institute. Genomic DNA was extracted from fin tissues using the Qiagen DNeasy Kit (Qiagen, Shanghai, China), according to the instructions of the manufacturer. PCR reactions were conducted in 25 ul volumes containing the following: 1 ul genomic DNA (100 ng/ul), 1 ul 10 uM of primer, ddH2O 10.5 ul and Tap Plus Master Mix 12.5 ul (Vazyme, Nanjing, China). The following conditions were used for PCR reactions: (1) pre-denaturation at 95°C for 4 min; (2) denaturation at 95°C for 45 s; (3) annealing at 51°C for 45 s; (4) elongation at 72°C for 1.5 min (repeated 2 to 4 stages 35 times) and a final elongation at 72°C for 7 min. The PCR products were size-separated by standard horizontal electrophoresis in 2% agarose gels (0.4 g agarose, 2.5 ul ethidium bromide, 40 ml 1 × TAE buffer (40 mmol/L Tris-HCL, 40 mmol/L acetic acid and 1 mmol/L EDTA)) and run with 1 × TAE buffer at 120 V for 90 min. The gels were viewed and photographed by GelDoc XR + Bio Imaging Systems (Bio-Rad, Shanghai, China). Only robust bands that were readily distinguishable were considered in this study.

According to the hypothesis of Apostol *et al.* (1996), each band was examined as a locus; the percentages of polymorphic loci = number of polymorphic loci of total loci × 100% were calculated. Each polymorphic ISSR band was given a score of 1 for presence or 0 for absence, and the data were transferred to a binary (1/0) data matrix. The data matrix was used to cluster and genetic distances (**GD**) analysis based on Nei' method (Nei and Li, 1979). GD between individuals were calculated using the following formula:

$$\mathrm{GD}_{ij} = 1 - 2N_{ij}/(N_i + N_j)$$

where GD_{ij} represent genetic distance between two individuals; N_{ij} represent the number of DNA amplification fragments shared between individuals *i* and *j*; N_i and N_j, respectively, are the number of DNA amplification fragments of individuals *i* and *j*.

Statistical analyses

Descriptive statistics are presented as the means ± SE by SPSS 21.0. In morphology, meristic and measurable traits between the hybrid fishes and their parents were analyzed by one-way ANOVA. The Tukey test was used to detect differences between the hybrid fishes and their parents. The ED within a group was calculated as the average ED between two individuals of the group. The ED between groups was calculated as the average of the EDs among all randomly paired individuals from different groups. The morphological clustering and ED were performed in each

Table 1 *Traditional metric and meristic characters of the hybrid fishes and their parents*

	P. ping	*S. wangchiachii*	Hybrid fish
Meristic characters			
Dorsal fin	III + 8	III + 8	III + 8
Pectoral fin	I + 15 to 17 (15.51 ± 0.11[a])	I + 15 to 17 (16.00 ± 0.13[b])	I + 16 to 18 (17.43 ± 0.13[c])
Pelvic fin	I + 9	I + 9	I + 9
Anal fin	III + 5	II + 5	II + 5
Scale lateral line	53.77 ± 0.4031[a] (49 to 58)	102.20 ± 0.6185[c] (96 to 108)	76.70 ± 0.5321[b] (69 to 82)
Scale above lateral line	9.50 ± 0.1045[a] (8 to 10)	23.00 ± 0.3108[c] (18 to 26)	14.97 ± 0.1947[b] (13 to 17)
Scale below lateral line	6.17 ± 0.1081[a] (5 to 7)	16.87 ± 0.1642[c] (16 to 19)	10.90 ± 0.1879[b] (9 to 13)
Metric characters			
WL/BL	1.20 ± 0.0023[a]	1.22 ± 0.0025[b]	1.26 ± 0.0029[c]
BW/BL	0.13 ± 0.0012[a]	0.12 ± 0.0003[b]	0.13 ± 0.0010[a]
BH/BL	0.21 ± 0.0019[a]	0.22 ± 0.0019[b]	0.21 ± 0.0015[a]
HL/BL	0.28 ± 0.0015[a]	0.22 ± 0.0015[c]	0.24 ± 0.0017[b]
CPL/BL	0.15 ± 0.0021[a]	0.16 ± 0.0026[b]	0.15 ± 0.0022[a]
CPD/CPL	0.62 ± 0.0096[a]	0.61 ± 0.0115[a]	0.63 ± 0.0113[a]
SL/HL	0.25 ± 0.0025[a]	0.27 ± 0.0031[b]	0.26 ± 0.0019[b]
ED/HL	0.23 ± 0.0031[a]	0.26 ± 0.0033[b]	0.26 ± 0.0030[b]
IW/DE	0.52 ± 0.0023[a]	0.68 ± 0.0017[c]	0.59 ± 0.0019[b]
IW/HL	0.24 ± 0.0017[a]	0.39 ± 0.0038[c]	0.32 ± 0.0038[b]
SBL/HL	0.25 ± 0.0027[a]	0.12 ± 0.0038[c]	0.22 ± 0.0033[b]
MBL/HL	0.25 ± 0.0035[a]	0.15 ± 0.0039[c]	0.27 ± 0.0043[b]

WL = Whole length; BL = Body length; BW = Body width; BH = Body height; HL = Head length; CPL = Caudal peduncle length; CPD = Caudal peduncle depth; SL = Snout length; ED = Eye diameter; IW = Interorbital width; DE = Distance of eyeballs; SBL = Snout-barbel length; MBL = Maxillary-barbel length. a,b Values within a row with different superscripts differ significantly at $P < 0.01$ based on Tukey test.

fish by SPSS 21.0. In ISSR, the genetic clustering and genetic distance were analyzed based on unweighted pair group method average by Popgene 1.32 (Yeh *et al.*, 1999). The calculation method used for genetic distance inside and outside the groups was the same as that used for the ED.

Results

Morphology

The morphology difference among the hybrid fishes and their parents was larger (Table 1 and Figure 1). In body color, the melanin of *P. pingi* was distributed evenly on each scale, whereas it was unevenly distributed in *S. wangchiachii*. Interestingly, melanin was only found on scales along the lateral lines and above in the hybrid fishes. In scale, *P. pingi* had larger scales than *S. wangchiachii*, and the hybrid fishes had intermediate scale size. The head of the *P. pingi* was relatively slender with a developed branchial membrane, a lower lip with higher radian, a lower jaw without keratinization, a terminal mouth, a deeper mouth crack, bulgier eyeballs and a longer snout barbel and maxillary barbel. Comparatively, the head of *S. wangchiachii* is relatively round with an undeveloped branchial membrane, lower lip with a lesser radian, lower jaw with keratinization, inferior mouth, shallower mouth crack, slightly bulging eyeballs and a shorter snout barbel and maxillary barbel. In general, the head traits of hybrid fishes were between their parents, but there were some special features, such as longer maxillary barbel than both parents, lower jaw without keratinization that was same as *P. pingi* and so on (Figure 1).

In 10 meristic traits, generally, the hybrids and their parents were similar, but the number of soft rays of the pectoral fin of hybrid fishes was much than those in both parents, and the hard rays of anal fin were the same in the hybrids as in *S. wangchiachii*. In 12 metric traits, most of the traits were significantly different ($P < 0.01$) among the hybrids and their parents, especially the traits of the head (Table 1).

In ED based on morphology, the morphological difference within a group of hybrid fishes was similar to that of their parents. Between groups, the morphological differences of hybrid fishes were generally intermediate between the two parents but with slightly greater similarity to *S. wangchiachii* (Table 2). However, the clustering showed that the hybrid fishes and *P. pingi* shared a common branch (Figure 2). Based on the above two results, this study suggests that the morphology of hybrid fishes was intermediate between parents without an obvious preference for one or the other.

Karyotype

According to the statistical results of chromosome number (Table 3), it can be preliminarily determined that the chromosome number of *S. wangchiachii* was 148, which is consistent with the reported chromosome number of hexaploid *Schizothorax* fishes (Supplementary Table S1). The chromosome number of the hybrid fishes was 123, which is half of the sum of the chromosomes of *P. pingi* and *S. wangchiachii*. No polyploidization or gynogenesis was found in the chromosome counting of 20 hybrids.

Table 2 *Euclidean distance based on morphology and genetic distance based on inter-simple sequence repeat (ISSR) among the hybrid fishes and their parents*

	Euclidean distance			Genetic distance		
Species	P	S	PS	P	S	PS
P	0.1873 ± 0.0891			0.0630 ± 0.0248		
S	0.6735 ± 0.0577	0.1931 ± 0.0984		0.9043 ± 0.0345	0.1076 ± 0.0349	
PS	0.4410 ± 0.0562	0.4154 ± 0.0756	0.1875 ± 0.0823	0.5457 ± 0.0227	0.3606 ± 0.0283	0.1364 ± 0.0416

P = *P. ping*; S = *S. wangchiachii*; PS = The hybrid fishes.

Figure 1 (colour online) External morphological comparison in the hybrid fishes and their parents. (a) The ventral head of *S. wangchiachii*; (b) the reverse head of *S. wangchiachii*; (c) the full view of *S. wangchiachii*; (d) The ventral head of hybrid fish; (e) the reverse head of hybrid fish; (f) the full view of hybrid fish; (g) the ventral head of *P. ping*; (h) the reverse head of *P. ping*; (i) the full view of *P. ping*. The white scale is 1 mm; the black scale is 10 mm.

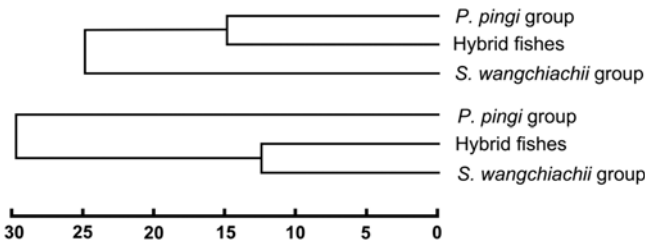

Figure 2 The morphology and genetic clustering in the hybrid fishes and their parents' groups. (a) The morphology clustering analysis based on Euclidean distance, and it was downsized based on Supplementary Figure S1 by showing only the first branch; (b) the genetic distance clustering based on inter-simple sequence repeat (ISSR). Both (a) and (b) were redrawn through Adobe Photoshop CS6 based on Supplementary Figure S1 and Supplementary Figure S2.

According to the statistical results of the karyotype (Figure 3, Supplementary Table S3 and Supplementary Table S4), the karyotype of *S. wangchiachii* was 2n = 6X = 148 = 36m + 34sm + 12st + 66t; the karyotype of *P. pingi* was 2n = 4X = 98 = 42m + 30sm + 10st + 16t; the karyotype of hybrid fishes was 2n = 5X = 123 = 39m + 28sm + 5st + 51t.

Red blood cell DNA content

The results of the DNA content determined by flow cytometry showed that (Table 4 and Figure 4), in the case of RBC DNA content of cook (2.50 pg) as a standard control, the average RBC DNA content of *S. davidi* was 4.20 ± 0.36 pg, the content of *P. pingi* was 4.28 ± 0.25 pg, the content of *S. wangchiachii* was 7.42 ± 0.38 pg and the content of the hybrid fishes was 5.53 ± 0.20 pg, which was minimally different from the expected value. No polyploidization or gynogenesis was found in the RBC DNA content of the 10 hybrid fishes.

Inter-simple sequence repeat

Nineteen ISSR primers were used to analyze the hybrid fishes and their parents, generating a total 178 bands, of which 149 were polymorphic. Partial electrophoretic results were presented in Supplementary Figure S3. The average polymorphism of ISSR makers was 83.7% (Supplementary Table S2). The genetic distance based on ISSR between 24 individuals of the hybrid fishes and their parents is shown in Supplementary Table S5.

In the genetic diversity study within a group based on ISSR (Table 2), the average value of *P. pingi* (0.0630 ± 0.0248) was 0.625 times higher than that of

Table 3 *Chromosome counts of S. wangchiachii and the hybrid fishes*

Species	Chromosome number	Individual number	Frequency of occurences (%)
S. wangchiachii	≤142	7	7
	144	22	22
	146	28	28
	148	40	40
	≥150	3	3
Hybrid fish	≤118	9	9
	120	14	14
	122	11	11
	123	59	59
	≥124	6	6

Table 4 *Red blood cell (RBC) DNA content of Chicken, S. davidi, the hybrid fishes and their parents*

Species	No. of chromosome	Fluorescence intensity (M)	DNA content /pg
Chicken	78	0.98 ± 0.07	2.50
S. davidi	98	1.66 ± 0.14	4.20 ± 0.36
P. ping	98	1.68 ± 0.10	4.28 ± 0.25
S. wangchiachii	148	2.91 ± 0.15	7.42 ± 0.38
Hybrid fish	123	2.18 ± 0.08	5.53 ± 0.20
Expected value	123	2.29	5.85

Expected value is half the average amount of RBC DNA content of both *P. ping* and *S. wangchiach*.

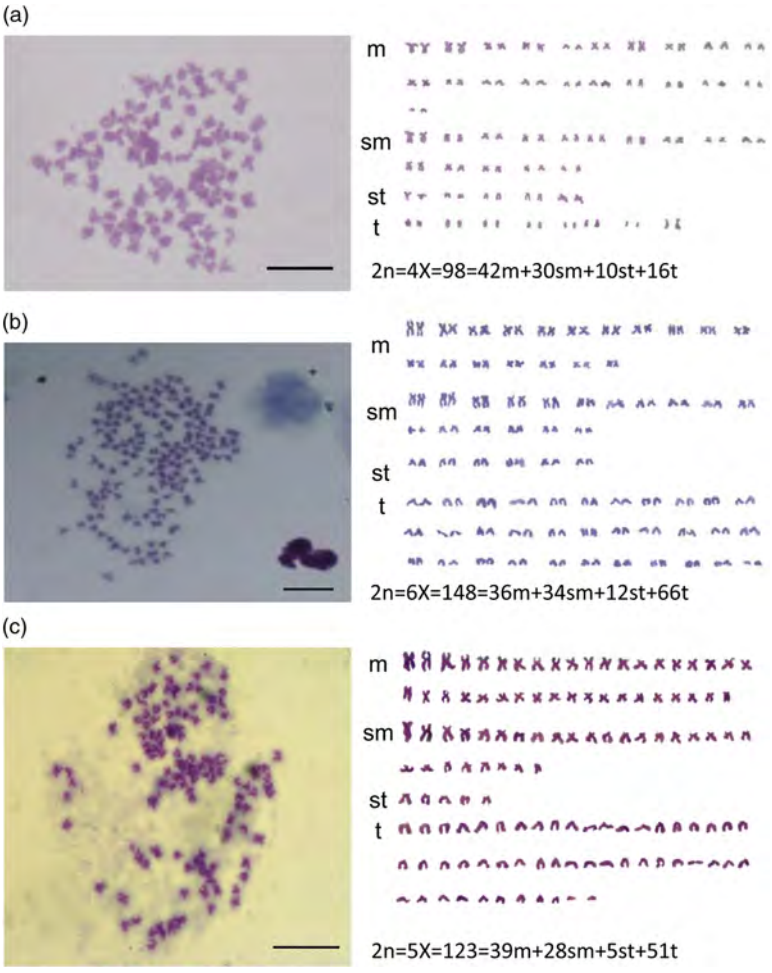

Figure 3 (colour online) The spread of metaphase chromosomes and the karyotype of the hybrid fishes and their parents. (a) The metaphase chromosomes and karyotype of *P. pingi* refer to Li *et al.* (2017); (b) The metaphase chromosomes and karyotype of *S. wangchiachii* in this study; (c) The metaphase chromosomes and karyotype of the hybrid fish in this study. The scale is 10 μm. m = metacentric chromosome; sm = submetacentric chromosome; st = subterminal chromosome; t = terminal chromosome.

S. wangchiachii (0.1076 ± 0.0349), which was related to their ploidy (0.6 times), and the genetic diversity of the hybrid fishes (0.1364 ± 0.0416) was higher than that of the parents.

In the genetic distance study between groups (Table 2), the average value between *P. pingi* and *S. wangchiachii* (0.9043 ± 0.0345) was much larger than that of their respective groups. Interestingly, the GD of hybrid fish between

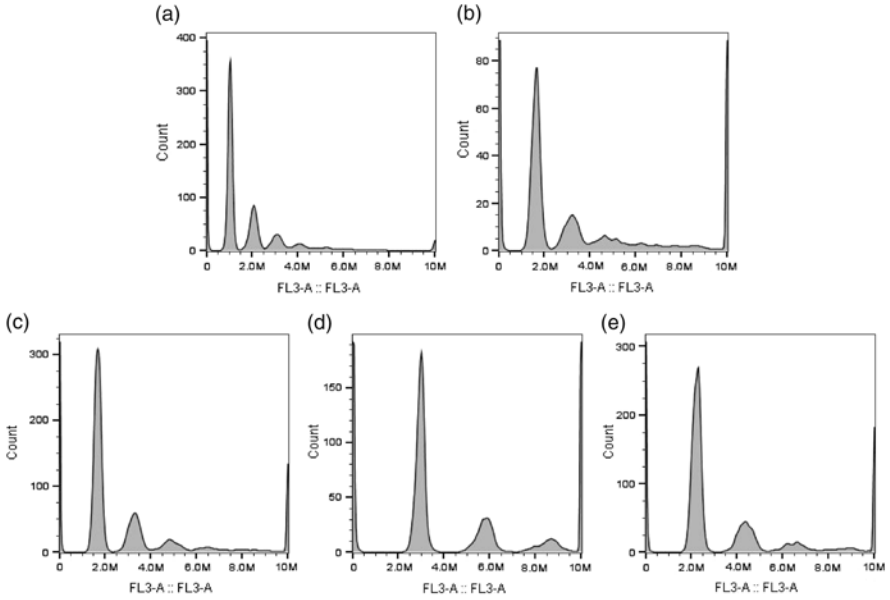

Figure 4 Flow cytometric RBC DNA content histograms of chicken, *S. davidi*, the hybrid fishes and their parents. (a) Chicken; (b) *S. davidi*; (c) *P. ping*; (d) *S. wangchiachii*; (e) the hybrid fishes. FL3-A = the area of fluorescent light 3 channel.

P. pingi and *S. wangchiachii* were 0.5457 ± 0.0227 and 0.3606 ± 0.0283, respectively, which are also an almost 0.6 times relationship. According to the cluster analysis (Figure 2), the hybrid fishes were similar to *S. wangchiachii* in genetics based on ISSR.

Discussion

Morphology

Within a group, there was morphologic diversity in three groups, which mainly came from the genetic diversity of parents and the recombination of parental genetic material in meiosis (Mugal *et al.*, 2013). The average ED in the hybrid fishes and their parents was similar, indicating that the parental genetic material in the hybrid fishes was normal combination without disorder. Female *S. wangchiachii* can provide more genetic material than male *P. pingi* in hybrid fish, whether from nuclear genes or mitochondrial genes. However, the morphology of hybrid fishes was intermediate between parents, and there was no obvious preference. Of course, this may be related to the selection of the quantitative indicators. On the other hand, this hybridization is a typical and successful hybridization in which the hybrid fishes consistently combine the genetic material of their parents. The traits such as lateral line scales, melanin distribution, structure of the lip, length of maxillary and snout barbels and so on can be used as an index to distinguish the hybrid fishes and their parents in aquaculture later.

Karyotype of S. wangchiachii

In this study, the karyotype formula of *S. wangchiachii* was $2n = 6X = 148 = 36m + 34sm + 12st + 66t$, and the chromosome number was consistent with other reported hexaploid *Schizothorax* fishes (Supplementary Table S1). Hexaploid *Schizothorax* fishes are derived from tetraploid (Wang *et al.*, 2016). Although the mechanism of origin of hexaploid in the *Schizothorax* fishes is still poorly understood, in artificial breeding, autotriploids and allotriploids can be produced by inhibiting the exclusion of a second polar body and distant hybridization (Valérie, 2008; Piferrer *et al.*, 2009), and even the latter can be produced naturally without artificial inhibition (Liu *et al.*, 2007; He *et al.*, 2012; Hu *et al.*, 2012; Xiao *et al.*, 2014). Similarly, this process could happen in nature. In particular, the reported hexaploid *Schizothorax* fishes were only distributed around the eastern Qinghai–Tibet Plateau in China (Yue, 2000), where may be the origin area of hexaploid *Schizothorax* fishes. Currently, FishBase contains 127 species (subspecies) of *Schizothorax* fishes, but only a few karyotypes have been reported. Therefore, it is not clear whether the hexaploid *Schizothorax* fishes are widely distributed or only distributed in the above water system. Therefore, more karyotype studies on *Schizothorax* fishes are needed in the future.

Karyotype of the hybrid fishes

The karyotype of the hybrid fishes was $2n = 5X = 123 = 39m + 28sm + 5st + 51t$, and the expected value of it was $2n = 5X = 123 = 39m + 32sm + 11st + 41t$, which was half the average of parental karyotypes. In above, the actual value and the expected value are not exactly equivalent, which may be caused by statistical errors or karyotype variation (Zhao *et al.*, 2006). However, this result still was a typical hybridization in general. In fish distant hybridization, different ploidies may be produced in hybrid offspring (Liu *et al.*, 2007; He *et al.*, 2012; Hu *et al.*, 2012; Xiao *et al.*, 2014). However, in this study, the hybridization is relatively stable, and no different ploidy progeny or gynogenetic progeny were found in the karyotype detection of 20 hybrid fishes and in the RBC DNA content detection of 10 hybrid fishes.

Triploid breeding is of great value in aquaculture. The most common induction methods are based on the suppression of the second meiotic division of fertilized eggs, but this approach is restricted to some extent by the uncertainty of 100% triploid progeny, parental reproductive behaviour, dysplasia of offspring, mosaicism and other factors. Another common method is based on the suppression of the second first cleavage to breed autotetraploids, which can interbreed with diploids to produce triploids. However, in practice, this has proved difficult to achieve in aquaculture, and there have been no reports of sexually fertile groups; these factors contribute to the high cost of autotetraploid breeding (Valérie, 2008; Piferrer *et al.*, 2009; Song *et al.*, 2012). In contrast to the above methods, natural distant hybridization without any induction can acquire stable, sexually fertile allotetraploid (Liu *et al.*, 2001) and autotetraploid groups (Qin *et al.*, 2014), but this approach is rare and requires long-term breeding. In this study, the hybrid fishes were pentaploid, which had the same breeding significance as triploid fishes, but were bred by natural parents, which greatly reduced breeding time. Tetraploid and hexaploid species exist in the *Schizothorax*, which is very rare in vertebrates and the large natural parents of pentaploid fish. The above tetraploid and hexaploid fish hybridization can be used to produce pentaploid fishes, and if the production characteristics are favorable, these hybrids can be directly put into production with a good development prospect.

In the homoploid distant hybridization of red crucian carp (2n = 100) (♀) × common carp (2n = 100) (♂), bisexual fertile F_3–F_{18} allotetraploid hybrids (4n = 200) were formed, and it can produce sterile triploid fish by backcrossing with their parents (Liu, 2010). In *Megalobrama amblycephala* (2n = 48) (♀) × *Xenocypris davidi* (2n = 48) (♂), allotriploid hybrids (3*n* = 72) can be obtained directly (Hu *et al.*, 2012). Could the homoploid hybridization in tetraploid *Schizothorax* and *Percocypris* fishes produce similar results? This is of great significance both in the future study of the origin of hexaploid *Schizothorax* fishes and in genetic breeding.

Red blood cell DNA content

The actual RBC DNA content in the hybrid fishes was not significantly different from the expected value, and the SE of hybrid fishes was lower than that of two parents, indicating that its genome was stable, and half that of *S. wangchiachii* and *P. pingi*. There was no significant difference in the average RBC DNA content between *S. davidi* and *P. pingi*, both of which had 98 chromosomes. According to the RBC DNA content of *S. davidi*, it is speculated that the RBC DNA content of *S. wangchiachii* should be 6.30 pg, but the actual content was 7.42 pg. In general, polyploidized species produce a large number of redundant genes, which tend to be lost, silenced or subfunctionalized in the process of evolution toward diploidization (Ozkan *et al.*, 2001; Ma and Gustafson, 2005). However, in this study, the RBC DNA content of *S. wangchiachii* was more than 1.5 times that of *S. davidi*, and the specific reason for this remains to be further studied.

Inter-simple sequence repeat

ISSR can efficiently detect polymorphisms, and are reproducible, stable and reliable. Unlike SSR, ISSR markers are generated using primers that amplify regions between SSR loci (Zeitkiewicz *et al.*, 1994).

Within a group, there was also some genetic diversity in ISSR in three groups, and the reason is similar to morphology. Interestingly, the genetic diversity of tetraploid *P. pingi* was 0.625 times than that of hexaploid *S. wangchiachii*, suggesting that the genetic diversity base ISSR gene may be related to ploidy, and in both species, the variational probability of sequence repeats on each chromosome may be similar. Different from morphology, in the hybrid fishes, the genetic diversity based on ISSR was higher than that of their parents, rather than between them, as assumed above. Suggesting that, on the one hand, parental genes were integrated in the hybrid fishes, on the another hand, recombination, insertion and loss of DNA fragments in connection with sequence repeats may occur in the hybrid fishes (Ye *et al.*, 2017). In addition, it was possible that the genetic material of the parents was diluted in the hybrid fishes, so that some bands of the parents were not obvious, leading to wrong judgment.

Between groups, the genetic distance of hybrid fishes between *P. pingi* and *S. wangchiachii* was almost 3 : 2, which was consistent with the parental ploidy. There are no sequence repeats satisfying ISSR primers in the complete mitochondrial sequence of *S. wangchiachii* (Chen *et al.*, 2013), and ISSRs segregate mostly as dominant markers following simple Mendelian inheritance. Taken together, above phenomenon showed that coexistence of parental genetic material in hybrid fishes without partiality.

Conclusion

In morphology, the average ED within a group in the hybrid fishes and their parents was similar; in karyotype, no different ploidy progeny or gynogenetic progeny were found; in RBC DNA content, the SE of hybrid fishes was normal compared to that of their parents, and no different ploidy progeny or gynogenetic progeny were found; in ISSR, the diversity of hybrid fishes was higher than their parents, this may be due to the normal interaction of parental genes. In summary, all results showed that the hybrid fishes were stable pentaploid. Therefore, in fish, it is feasible to cross natural tetraploid parents with hexaploid parents to produce stable pentaploid offspring. These findings provide a successful case of hybridization and a new breeding pathway in *Schizothorax* fishes even more polyploid fishes.

Supplementary material

To view supplementary material for this article, please visit https://dx.doi.org/10.1017/S1751731119001289

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Declaration of interest

The authors declare that they have no competing interests.

Ethics statement

The authors claim that none of the material in the paper has been published or is under consideration for publication elsewhere. The submission is original, and all authors are aware of the submission and agree to its publication in **Animal**. We declare that there is no conflict of interests regarding the publication of this paper.

Software and data repository resources

The authors declare that the data of this research are not deposited in any official repository.

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Genetic parameters and direct, maternal and heterosis effects on litter size in a diallel cross among three commercial varieties of Iberian pig

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The Iberian pig is one of the pig breeds that has the highest meat quality. Traditionally, producers have bred one of the available varieties, exclusively, and have not used crosses between them, which has contrasted sharply with other populations of commercial pigs for which crossbreeding has been a standard procedure. The objective of this study was to perform an experiment under full diallel design among three contemporary commercial varieties of Iberian pig and estimate the additive genetic variation and the crossbreeding effects (direct, maternal and heterosis) for prolificacy. The data set comprised 18 193 records for total number born and number born alive from 3800 sows of three varieties of the Iberian breed (Retinto, Torbiscal and Entrepelado) and their reciprocal crosses (Retinto × Torbiscal, Torbiscal × Retinto, Retinto × Entrepelado, Entrepelado × Retinto, Torbiscal × Entrepelado and Entrepelado × Torbiscal), and a pedigree of 4609 individuals. The analysis was based on a multiple population repeatability model, and we developed a model comparison test that indicated the presence of direct line, maternal and heterosis effects. The results indicated the superiorities of the direct line effect of the Retinto and the maternal effect of the Entrepelado populations. All of the potential crosses produced significant heterosis, and additive genetic variation was higher in the Entrepelado than it was in the other two populations. The recommended cross for the highest yield in prolificacy is a Retinto father and an Entrepelado mother to generate a hybrid commercial sow.

Keywords: prolificacy, diallel cross, heritability, crossbreeding, Iberian pig

Implications

Reproductive efficiency is a limiting factor in the production of Iberian pigs. This study showed that litter size in the Iberian pig can be increased through two non-exclusive strategies. The use of within-line selection is supported by the presence of sufficient additive genetic variation, and crossbreeding is supported by estimates of the crossbreeding effects derived from a full diallel experiment among three varieties (Entrepelado, Retinto and Torbiscal). In addition, the optimal hybrid sow can be produced by crossing a Retinto father and an Entrepelado mother.

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Introduction

The Iberian pig breed is recognized as one of the porcine populations that has the highest meat quality (Serra *et al.*, 1998; Gandemer, 2009), which is an important reason for its conservation because it has slower growth and lower feed efficiency (Barea *et al.*, 2011) and prolificacy (Silió *et al.*, 2001) than do other commercial pig populations. Furthermore, a profound transformation that has involved the replacement of many traditional producers by intensive management farms has affected Iberian pig production. Normative that regulates the Iberian pig products forces that the sow is Iberian, whereas the boar could be either Iberian, Duroc (**DU**) or hybrid between these two breeds (Boletín Oficial del Estado, 2014). In fact, a large proportion of Iberian pig farmers uses DU sires to obtain better growth rate and efficiency (Serrano *et al.*, 2008). Therefore, under intensive

management, improvement in the reproductive efficiency of Iberian sows is essential for its economic viability.

The number of weaned piglets per sow in a year (or numerical productivity) is one of the most important factors in the profitability of pig farms, and litter size is its most important component (Quinton *et al*., 2006; Dekkers *et al*., 2011). Two non-exclusive strategies can lead to the genetic improvement of litter size: (1) within-line selection and (2) appropriate crossbreeding between lines to exploit heterosis.

Traditionally, genetic improvement in litter size in Iberian pig populations has been limited (Fernández *et al*., 2008), and Iberian pig farmers have not used crossbred sows; rather, they have bred one of the varieties of the Iberian breed, exclusively (Martínez *et al*., 2000), which differed markedly from the practices used with other populations of commercial pigs in which selection efforts for prolificacy in maternal lines have been common and crossbreeding has been a standard procedure (Dekkers *et al*., 2011). Several studies have identified genetic variability for prolificacy within (Rodríguez *et al*., 1994, Fernández *et al*., 2008) and between (García-Casco *et al*., 2012) varieties of Iberian pig. Thus, within-line selection and the implementation of a maternal crossbreeding scheme are plausible strategies for genetic improvement; however, to identify the most appropriate strategy, reliable estimates of the heritability of prolificacy in each population and estimates of the crossbreeding parameters as defined by the Dickerson's model are required (Dickerson, 1969).

The objective of this study was to estimate the additive genetic variation (or heritability) and the crossbreeding effects (direct, maternal and heterosis) for prolificacy using data from an experiment based on a full diallel design (Hayman, 1954) among three contemporary commercial varieties of Iberian pig [Entrepelado (E), Retinto (R) and Torbiscal (T)] under intensive management conditions. The overarching objective of the study was to identify suitable genetic improvement strategies within a pyramidal breeding program among the Iberian pig populations.

Materials and methods

Animals and experimental design

The data set comprised 18 193 records for total number born (**TNB**) and number born alive (**NBA**) from 3800 sows that had been obtained from a full diallelic experiment among three varieties of the Iberian pig breed (Retinto x Retinto (**RR**), Torbiscal x Torbiscal (**TT**) and Entrepelado x Entrepelado (**EE**)) and their reciprocal crosses (Retinto × Torbiscal (**RT**), Torbiscal × Retinto (**TR**), Retinto × Entrepelado (**RE**), Entrepelado × Retinto (**ER**), Torbiscal × Entrepelado (**TE**) and Entrepelado × Torbiscal (**ET**)). The three varieties are recognized in Spain's official Iberian herd-book [Spanish Association of Iberian Purebred Pig Breeders (AECERIBER)]. A detailed description of their characteristics is provided by Ibáñez-Escriche *et al*. (2016). Purebred sows were located in two nucleus herds in intensive management systems (Herds 1 and 2), and the service boars for these sows were kept at an artificial insemination center. A third herd (HERD 3) was a commercial production herd in which all sows, purebred or crossbred, were mated with boars from a DU population following the standard production system of Iberian pigs under intensive management. The distribution of the data between herds and breed of service sire and the average and SD of NBA and TNB for all crosses are presented in Table 1. In addition, the pedigree was extended back up to 3 generations, and it consisted of 4609 individuals. The number of founders in each per population was 47 Entrepelado (13 sires and 34 dams), 80 Retinto (18 sires and 62 dams) and 107 Torbiscal (38 sires and 69 dams).

Statistical analyses

TNB and NBA were analyzed with a multiple population repeatability model following García-Cortés and Toro (2006), which can divide the additive genetic values into separate fractions depending on their genetic origin. The general model of the analysis was as follows:

$$\mathbf{y} = X\mathbf{b} + \sum_{i=E,R,T}^{3} Z_i\mathbf{a_i} + W\mathbf{p} + \mathbf{e}$$

where **y** is the vector of phenotypic records (TNB and NBA), **b** is the vector of systematic effects: order of parity (six levels: first, second, third, fourth, fifth and sixth and beyond), herd-year-season (85 levels), genetic line of service boar [four levels: E, R, T and DU] and the genetic line of the sow (L_E, L_R, L_T for E, T and R, respectively), genetic line of the mother of the sow (M_E, M_R and M_T for E, T and R, respectively) and heterosis (H_{ER}, H_{ET} and H_{RT} between E and R, E and T and R and T, respectively) effects following Dickerson's model (Dickerson, 1969). Furthermore, $\mathbf{a_i}$ is the vector of random additive genetic effects of pure and crossbred individuals of the *i*th line –E, R or T- (with 1934, 2748 and 1765 levels, respectively), **p** is the vector of the permanent effect of the sow (3800 levels) and **e** is the residuals vector; *X* and *W* are known incidence matrices that link fixed and permanent random effects with the vector **y**. Further, Z_E, Z_R and Z_T are the matrices that links data with the random additive effects of each line with dimensions 18 193 × 1934, 18 193 × 2748 and 18 193 × 1765 levels, respectively. The model allows for a different additive genetic variance component for each genetic origin, whereas the permanent environmental (**p**) and the residual effects (**e**) were assumed homogeneous between lines.

The model assumed flat prior distributions for systematic effects (**b**) and multivariate Gaussian distributions for the additive ($\mathbf{a_i}$), permanent environmental (**p**) and residual effects (**e**). The permanent environmental effects and the residuals were assumed identically and independently distributed; therefore, their prior distribution was as follows:

$$\mathbf{p} \sim N\left(0, I\sigma_p^2\right) \quad \mathbf{e} \sim N(0, I\sigma_e^2)$$

Table 1. *Number of recorded parities by Iberian pig breed of sow, service sire and herd, and the mean and SD of the NBA and the TNB*

	Sire									
	HERD 1			HERD 2			HERD 3		NBA	TNB
Dam (number of sows)	E	R	T	E	R	T	DU	Total	Mean (SD)	Mean (SD)
EE (707)	517	752	5	255	811	70	433	2843	7.73 (2.15)	7.95 (2.16)
ER (527)	–	–	–	–	–	–	2336	2336	8.31 (2.25)	8.53 (2.27)
ET (177)	–	–	–	–	–	–	942	942	7.81 (2.25)	8.02 (2.25)
RE (196)	–	–	–	–	–	–	806	806	8.55 (2.43)	8.84 (2.45)
RR (874)	1450	491	96	655	277	633	870	4472	8.05 (2.19)	8.39 (2.20)
RT (488)	–	–	–	–	–	–	2450	2450	8.31 (2.43)	8.60 (2.43)
TE (36)	–	–	–	–	–	–	193	193	8.34 (2.47)	8.69 (2.49)
TR (343)	–	–	–	–	–	–	1993	1993	8.29 (2.28)	8.53 (2.32)
TT (452)	197	808	58	109	507	247	232	2158	7.21 (2.12)	7.59 (2.13)
Total (3 800)	2 164	2 051	159	1 019	1 595	950	10 255	18 193	8.01 (2.27)	8.29 (2.28)

E = Entrepelado; R = Retinto; T = Torbiscal; DU = Duroc; EE = Entrepelado × Entrepelado; ER = Entrepelado × Retinto; ET = Entrepelado × Torbiscal; RE = Retinto × Entrepelado; RR = Retinto × Retinto; RT = Retinto × Torbiscal; TE = Torbiscal × Entrepelado; TR = Torbiscal × Retinto; TT = Torbiscal × Torbiscal; NBA = number born alive; TNB = total number born.

The assumed prior distribution for the additive effects was as follows:

$$\mathbf{a_E} \sim N\left(0, A_E\sigma^2_{a_E}\right) \quad \mathbf{a_R} \sim N\left(0, A_R\sigma^2_{a_R}\right) \quad \mathbf{a_T} \sim N\left(0, A_T\sigma^2_{a_T}\right)$$

where A_E, A_R and A_T are the partial relationship matrices generated by the founders of E, R and T, respectively. The calculation of those matrices was performed following the algorithm proposed by García-Cortés and Toro (2006), and they are illustrated with a tiny example in the Appendix. Prior distributions for the variance components $(\sigma^2_{(a_E)}, \sigma^2_{(a_R,)}\sigma^2_{(a_T)}, \sigma^2_p \text{ and } \sigma^2_e)$ were uniform.

The above model leads to the following mixed model equations:

$$\begin{bmatrix} X'X & X'Z_E & X'Z_R & X'Z_T & X'W \\ Z'_EX & Z'_EZ_E + A^-_E\frac{\sigma^2_e}{\sigma^2_{a_E}} & Z'_EZ_R & Z'_EZ_T & Z'_EW \\ Z'_RX & Z'_RZ_E & Z'_RZ_R + A^-_R\frac{\sigma^2_e}{\sigma^2_{a_R}} & Z'_RZ_T & Z'_RW \\ Z'_TX & Z'_TZ_E & Z'_TZ_R & Z'_TZ_T + A^-_T\frac{\sigma^2_e}{\sigma^2_{a_T}} & Z'_TW \\ W'X & W'Z_E & W'Z_R & W'Z_T & W'W + I\frac{\sigma^2_e}{\sigma^2_p} \end{bmatrix} \begin{bmatrix} \hat{\mathbf{b}} \\ \hat{\mathbf{a}}_\mathbf{E} \\ \hat{\mathbf{a}}_\mathbf{R} \\ \hat{\mathbf{a}}_\mathbf{T} \\ \hat{\mathbf{p}} \end{bmatrix} = \begin{bmatrix} X'y \\ Z'_Ey \\ Z'_Ry \\ Z'_Ty \\ W'y \end{bmatrix}$$

where $A^-_E, A^-_R \text{ and } A^-_T$ are the inverses of non-zero part of the population specific partial numerator relationship matrices (see the Appendix).

From that general full model (**LMH**), the following models were defined by fixing to zero the direct (**MH**), maternal (**LH**), heterosis (**LM**), direct and maternal (**H**), direct and heterosis (**M**) and maternal and heterosis effects (**L**). The analysis of each model was performed using a *Gibbs sampling* algorithm (Gelfand and Smith, 1990) with a single long chain of 1 000 000 iterations, following a 'burn in' period of 250 000 iterations.

Model comparisons

The models were compared using the deviance information criteria (**DIC**) (Spiegelhalter *et al.*, 2002) and the logarithm of the conditional predictive ordinate (**LogCPO**) (Gelfand, 1996).

Deviance information criteria: The DIC compares the global quality of two or more hierarchical models accounting for model complexity. For a particular model M, the DIC is defined as follows:

$$DIC_M = 2\bar{D}_M - D\left(\bar{\boldsymbol{\theta}}_\mathbf{M}\right),$$

where $\bar{D}_M$ is the posterior expectation of the deviance $D\left(\bar{\boldsymbol{\theta}}_\mathbf{M}\right)$ and $D\left(\bar{\boldsymbol{\theta}}_\mathbf{M}\right) = -2log\left(p\left(\mathbf{y}|\bar{\boldsymbol{\theta}}_\mathbf{M}\right)\right)$ is the deviance evaluated at the posterior mean estimate of the parameter vector $\left(\bar{\boldsymbol{\theta}}_\mathbf{M}\right)$. The computation of DIC comprises two terms, $\bar{D}_M$ is a measure of model fit and $\bar{D}_M - D\left(\bar{\boldsymbol{\theta}}_\mathbf{M}\right)$ is related to the effective number of parameters. Models that have the smallest DIC have the best global fit after accounting for model complexity.

Log marginal probability: If we consider the data vector $\mathbf{y} = (y_i, \mathbf{y}_{-i})$, where y_i is the ith datum and $\mathbf{y}_{-i}$ is the vector of data with the ith datum deleted, the conditional predictive distribution has a probability density equal to the following:

$$p(y_i|\mathbf{y}_{-i}) = \int p(y_i|\mathbf{y}_{-i}, \boldsymbol{\theta})p(\boldsymbol{\theta}|\mathbf{y}_{-i})d\boldsymbol{\theta},$$

where θ is the vector of unknown parameters and random effects in the model. Therefore, $p(y_i|\mathbf{y}_{-i})$ can be interpreted as the probability of each datum given the remaining data and is the conditional predictive ordinate (**CPO**) for the

Table 2. *Differences with the best model for DIC and the LogCPO for NBA and TNB among three varieties of Iberian pig*

	NBA		TNB	
Model	DIC	LogCPO	DIC	LogCPO
LMH	–	–	–	–
MH	6.3	−0.9	5.9	−0.4
LH	6.3	−5.0	5.7	−2.9
LM	23.8	−19.8	22.4	−20.2
L	26.3	−22.3	25.8	−23.0
M	26.6	−20.9	26.1	−23.3
H	7.5	−6.9	8.0	−4.8

LMH = full model with direct line; maternal and heterosis effects; MH = reduced model with maternal and heterosis effects; LH = reduced model with direct line and heterosis effects; LM = reduced model with direct line and maternal effects; L = reduced model with direct line effects; M = reduced model with maternal effects; H = reduced model with Heterosis effects; DIC=deviance information criteria; LogCPO = logarithm of the conditional predictive ordinate; NBA = number born alive; TNB = total number born.

i th datum. The pseudo log-marginal probability of the data is as follows:

$$\sum_i ln\ p(y_i|\mathbf{y}_{-i}).$$

A Monte Carlo approximation of the CPO (Gelfand, 1996) is $\sum_i ln\ \hat{p}(y_i|\mathbf{y}_{-i})$, where $\hat{p}(y_i|\mathbf{y}_{-i}) = N\left[\sum_{j=1}^{N}\frac{1}{p(y_i|\theta^j)}\right]^{-1}$, and N is the number of Markov chain Monte Carlo (**MCMC**) draws, and θ^j is the jth draw from the posterior distribution of the corresponding parameter. The higher the value of the LogCPO, the better the fit of the model to the data.

Results

For both NBA and TNB, the model with best fit with DIC and LogCPO was the complete LMH model, followed by a group of models (LH, MH and H) whose differences from the best model ranged from 5.9 to 8.0 units for DIC and from 0.4 to 6.9 for LogCPO (Table 2). The LM, L and M models had a worse adjustment and their differences from the LMH model ranged from 22.4 to 26.6 for DIC and from 19.8 to 23.3 for LogCPO.

The posterior mean estimates (± posterior SDs) of the additive genetic variance ranged from 0.371 ± 0.106 (T) to 0.665 ± 0.123 (E) and from 0.418 ± 0.115 (T) to 0.717 ± 0.126 (E) for NBA and TNB, respectively, and the posterior mean estimates (± posterior SDs) of the permanent environmental and residual variances were 0.361 ± 0.051 and 4.020 ± 0.048 for NBA and 0.371 ± 0.053 and 4.029 ± 0.048 for TNB (Table 3). The posterior mean (± posterior SDs) estimates for the population-specific heritabilities were 0.078 ± 0.021 (T), 0.084 ± 0.017 (R) and 0.131 ± 0.022 (E) for NBA and 0.086 ± 0.022 (T), 0.090 ± 0.017 (R) and 0.140 ± 0.022 (E) for TNB. The results from the MH, LH, LM, L, M and H models were similar (Supplementary Tables S1 and S2 for NBA and TNB, respectively).

The posterior mean (± posterior SD) estimates of the differences between L_E and L_R, L_E and L_T and L_R and L_T were −0.684 ± 0.317, 0.114 ± 0.324 and 0.798 ± 0.210 for NBA and −0.876 ± 0.327, −0.024 ± 0.334 and 0.852 ± 0.217 piglets for TNB (Table 4). In addition, the posterior mean (± posterior SD) estimates of the differences of M_E with M_R and M_T were 0.443 ± 0.123 and 0.450 ± 0.144 for NBA and 0.554 ± 0.117 and 0.533 ± 0.117 piglets for TNB. The differences between M_R and M_T were only 0.007 ± 0.104 and −0.021 ± 0.107. Those differences between the maternal effects were supported by the comparisons between reciprocals. The posterior mean (± posterior SD) estimates of the differences between ER and RE and between ET and TE were −0.443 ± 0.123 and −0.554 ± 0.125 and −0.450 ± 0.144 and −0.534 ± 0.147 for NBA and TNB, respectively. The differences between TR and RT were negligible (−0.007 ± 0.104 and 0.021 ± 0.107). Furthermore, the posterior mean (± posterior SD) estimates of the heterosis effects ranged from 0.600 ± 0.129 (H_{ET}) to 0.690 ± 0.092 (H_{RT}) for NBA and from 0.622 ± 0.131 (H_{ET}) to 0.666 ± 0.093 (H_{RT}) for TNB (Table 4). The results from the other models followed the same pattern (Supplementary Tables S3 and S4 for NBA and TNB, respectively).

The posterior mean and SDs of differences between each cross with respect to the RE ranged from –0.443 (with ER) to –1.566 piglets (with TT) piglets for NBA and from –0.464 (with TE) to –1.607 (with TT) for TNB, and the posterior probability that RE is the best cross given the LMH model was 0.985 and 0.991 for NBA and TNB, respectively (Table 5).

Discussion

The main advantage of the multiple population repeatability model proposed by García-Cortés and Toro (2006) is that it provides specific estimates of the additive variance component (and heritability) for each population of origin and, therefore, it can detect differences in their genetic variation. The additive variance (and heritability) of the E variety was markedly higher than that of the other two populations (R and T) in all models. The estimates of the E population were higher than those obtained in previous studies of other Iberian pig populations (Pérez-Enciso and Gianola, 1992; García-Casco *et al.*, 2012), and the estimates for R and T were closer to such published estimates; however, estimates of heritability over 0.10 are common in white pig (Bidanel, 2011; Putz *et al.*, 2015; Ogawa *et al.*, 2018) and in Iberian populations (Fernández *et al.*, 2008). The results of our study should be confirmed based on a larger database; however, given the results of our study, it is plausible that the response to selection in the E population under an appropriate breeding scheme to improve litter size might be greater than it would be in the other lines. Thus, the E variety is a good candidate for inclusion in a pyramidal scheme for the improvement of litter size.

Table 3. *Posterior mean (and posterior SD) estimates ['of'?] the additive (σ^2_{aE}, σ^2_{aR} and σ^2_{aT}), permanent environmental (σ_p^2), and residual variance (σ_e^2) components and population specific heritabilities (h_E^2, h_R^2, h_T^2) for NBA and TNB for each of three varieties of Iberian pig (Entrepelado (E), Retinto (R) and Torbiscal (T)) analyzed with full model with direct line, maternal and heterosis effect*

	NBA	TNB
σ_{aE}^2	0.665 (0.123)	0.717 (0.126)
σ_{aR}^2	0.401 (0.088)	0.439 (0.091)
σ_{aT}^2	0.371 (0.106)	0.418 (0.115)
σ_p^2	0.361 (0.051)	0.371 (0.053)
σ_e^2	4.020 (0.048)	4.029 (0.048)
h_E^2	0.131 (0.022)	0.140 (0.022)
h_R^2	0.084 (0.017)	0.090 (0.017)
h_T^2	0.078 (0.021)	0.086 (0.022)

NBA = number born alive; TNB = total number born.

Table 4. *Posterior mean (and posterior SD) estimates of the contrast between three varieties of Iberian pig for the direct line (L_E, L_R and L_T) and maternal (M_E, M_R and M_T) effects, and the heterosis (H_{ER}, H_{ET} and H_{RT}) effects on NBA and TNB under the full model with direct line, maternal and heterosis effects*

		NBA	TNB
Direct line	L_E *v.* L_R	−0.684 (0.317)	−0.876 (0.327)
	L_E *v.* L_T	0.114 (0.324)	−0.024 (0.334)
	L_R *v.* L_T	0.798 (0.210)	0.852 (0.217)
Maternal	M_E *v.* M_R	0.443 (0.123)	0.554 (0.147)
	M_E *v.* M_T	0.450 (0.144)	0.533 (0.117)
	M_R *v.* M_T	0.007 (0.104)	−0.021 (0.107)
Heterosis	H_{ER}	0.653 (0.098)	0.661 (0.099)
	H_{ET}	0.600 (0.129)	0.622 (0.131)
	H_{RT}	0.690 (0.092)	0.666 (0.093)

L_E, L_R and L_T are the direct line effects for Entrepelado, Retinto and Torbiscal, respectively. M_E, M_R and M_T are the maternal line effects for Entrepelado, Retinto and Torbiscal. H_{ER}, H_{ET} and H_{RT} are the heterosis effects between Entrepelado and Retinto, Entrepelado and Torbiscal and Retinto and Torbiscal, respectively. NBA=number born alive; TNB=total number born.

The results of the comparison of models indicated the importance of the direct line, maternal and heterosis effects based on both measures (LogCPO and DIC) because the LMH model had the best fit; however, the extent of this relevance was mixed. For example, the best model was followed closely by a group of models that share the effects of heterosis (LH, MH and H), which is reinforced by a posterior probability of a heterosis effect > 0 that was > 0.999 for all models and traits. Therefore, there was clear evidence of heterosis between each of the three potential crosses and small differences among them. A significant degree of heterosis was expected because it is common in crosses between pig populations (Haley *et al.* 1995; Cassady *et al.*, 2002; García-Casco *et al.*, 2012). The results were surprising, however, because previous studies (Fabuel *et al.*, 2004) have suggested that the genetic distance between E and R is lower

Table 5. *Posterior mean (and SD) of the differences of the crosses (Dif.) with the Iberian pig population with the best performance (RE) and the posterior probability (Prob.) of being the best cross under the LMH model for NBA and TNB*

	NBA		TNB	
	Dif.	Prob.	Dif.	Prob.
EE	−0.995 (0.196)	0.000	−1.09 (0.202)	0.000
ER	−0.443 (0.123)	0.000	−0.554 (0.125)	0.000
ET	−0.902 (0.175)	0.000	−1.000 (0.178)	0.000
RE	–	0.985	–	0.991
RR	−0.754 (0.189)	0.000	−0.777 (0.193)	0.000
RT	−0.471 (0.178)	0.003	−0.516 (0.183)	0.002
TE	−0.452 (0.164)	0.003	−0.464 (0.169)	0.003
TR	−0.463 (0.198)	0.009	−0.536 (0.203)	0.004
TT	−1.560 (0.226)	0.000	−1.607 (0.232)	0.000

EE = Entrepelado × Entrepelado; ER = Entrepelado × Retinto; ET = Entrepelado × Torbiscal; RE = Retinto × Entrepelado; RR = Retinto × Retinto; RT = Retinto × Torbiscal; TE = Torbiscal × Entrepelado; TR = Torbiscal × Retinto; TT = Torbiscal × Torbiscal; LMH = full model with direct line; maternal and heterosis effects. NBA = number born alive; TNB = total number born.

than the distance between either of them with T. Therefore, we expected a lower degree of heterosis between E and R than in crosses involving T.

The superiority of the LMH model over MH and the posterior distribution of the contrast between direct line effects in the LMH model confirmed the differences in the direct line effects on litter size. Numerous studies have reported differences in reproductive performance among swine populations (Bidanel, 2011); thus, it is not surprising that this variability occurs among the Iberian varieties because the genetic diversity in Iberian pig populations is as high as it is among white pig populations (Martínez *et al.*, 2000; Fabuel *et al.*, 2004). Over centuries, the Iberian pig has evolved adapting to harsh environmental conditions, and producers have subjected populations to 'empirical' selection in which adipogenic capacity and morphological criteria have played an important role. In addition, the small size of the herds and the degree of isolation between populations are other important factors. In general, the RR variety had the best line effects for prolificacy, and the E and T varieties had similar performances.

Furthermore, the best fit of LMH compared to the LH model and the posterior distribution of maternal effects provides evidence of its importance. The results indicated clearly the superiority (posterior probability > 0.99) of the maternal effect of the E variety over the R and T varieties, which was supported by the significant differences between reciprocals (RE *v.* ER and TE *v.* ET). In quantitative genetics, the importance of maternal effects is well recognized (Wilham, 1972; Wolf and Wade, 2009) and is typically found in growth traits in non-prolific species; however, few studies have reported on the maternal effects of sows on the litter size of their daughters. In addition, the estimates of the maternal effect on NBA and TNB were small (Southwood and Kennedy, 1990; Ferraz and Johnson, 1993; Irgang *et al.*, 1994), which differs

considerably from the results of our study, where the estimates of the differences between the maternal effects of the Dickerson's model of the E variety compared to those of the R and T varieties were significant. Given the information available, the possible cause of that phenomena cannot be disentangled; however, recent studies on the genetic determinism of litter size in prolific species suggest a very complex panorama, which might permit speculation about a possible reason. The influence of maternal care on the reproductive performance of adult females has been investigated in rats (Cameron, 2011), which suggests that maternal behavior is transmitted to the next generation through epigenetic modifications such as methylation and histone acetylation, which is reflected in the variability in the expression of the estrogen receptor alpha. In addition, that phenomena is consistent with recent evidence of the effects of DNA methylation (Hwang *et al.*, 2017) and the presence of imprinted genes (Coster *et al.*, 2012) on litter size in pigs. Furthermore, apparently epistasis is a very important source of genetic variation in litter size in mice (Peripato *et al.*, 2004) and swine (Noguera *et al.*, 2009). Another possibility is the effects of mitochondrial DNA, the variation of which has recently been associated with the maturation of oocytes in bovids (Srirattana *et al.*, 2017) and with the reproductive performance of daughters in pigs (Tsai *et al.* 2016).

Despite the uncertainty about the biological basis of the results of this study, it has identified the clear advantage of crossbred over purebred individuals in litter size traits in the Iberian breed. Therefore, the implementation of a pyramidal scheme to provide crossbred sows to the producers is strongly recommended. Among the varieties investigated, the R population had the greatest direct line effect, and the E population had the greatest maternal effect and can provide a distinct advantage if it is used as the maternal line. The recommended cross for the practical implementation of a pyramidal scheme is R as the paternal and E as the maternal lines, which is supported by the posterior probability of being the best cross for NBA and TNB.

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Declaration of interest

The authors declare that they have no competing interests.

Ethics statement

The research ethics committee of the Institute of Agrifood Research and Technology (IRTA) approved all of the management and experimental procedures involving live animals, which were performed in accordance with the Spanish Policy of Animal Protection RD1201/05, which complies with the European Union Directive 86/609 for the protection of animals used in experimentation.

Software and data repository resources

None of the data were deposited in an official repository. FORTRAN code is available from the corresponding author under reasonable request.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731119001125.

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Appendix : Partial numerator relationship matrices and their generalized inverses

In order to illustrate the procedure of definition of the partial numerator relationship matrices required for the implementation of the procedure of García-Cortes and Toro (2006), we have defined the following pedigree with purebred and crossbred individuals between three populations (A, B and C):

Given this genealogical information, the partial numerator relationship matrix for the A population is:

Individual	Sire	Dam	Population
1	0	0	A
2	0	0	A
3	0	0	B
4	0	0	B
5	0	0	C
6	0	0	C
7	1	2	A
8	1	4	A × B
9	3	4	B
10	1	6	A × C
11	3	6	B × C
12	5	6	C

$$A_A = \begin{bmatrix} 1 & 0 & 0 & 0 & 0 & 0 & 0.5 & 0.5 & 0 & 0.5 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 & 0 & 0.5 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0.5 & 0.5 & 0 & 0 & 0 & 0 & 1 & 0.25 & 0 & 0.25 & 0 & 0 \\ 0.5 & 0 & 0 & 0 & 0 & 0 & 0.25 & 0.5 & 0 & 0.25 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0.5 & 0 & 0 & 0 & 0 & 0 & 0.25 & 0.25 & 0 & 0.5 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \end{bmatrix}$$

The inverse of the non-zero part of this matrix (A_A^-) can be calculated following the rules described by García-Cortés and Toro (2006), given that $A_A^- = (I - P)' D_X^- (I - P)$. P is a matrix that relates progeny to parent and D_X is recursively obtained by the algorithm described by Quaas (1976). Therefore:

$$A_A^- = \begin{bmatrix} 3.5 & 0.5 & - & - & - & - & -1 & -2 & 0 & -2 & - & - \\ 0.5 & 1.5 & - & - & - & - & -1 & 0 & 0 & 0 & - & - \\ - & - & - & - & - & - & - & - & - & - & - & - \\ - & - & - & - & - & - & - & - & - & - & - & - \\ - & - & - & - & - & - & - & - & - & - & - & - \\ - & - & - & - & - & - & - & - & - & - & - & - \\ -1 & -1 & - & - & - & - & 2 & 0 & 0 & 0 & - & - \\ -2 & 0 & - & - & - & - & 0 & 4 & 0 & 0 & - & - \\ - & - & - & - & - & - & - & - & - & - & - & - \\ -2 & 0 & - & - & - & - & 0 & 0 & 0 & 4 & - & - \\ - & - & - & - & - & - & - & -- & - & - & - & - \\ - & - & - & - & - & - & - & - & - & - & - & - \end{bmatrix}$$

Note that the inverse of the full A_A matrix does not exist because of the existence of the non-zero part.

The partial numerator relationship matrix for the B population is

$$A_B = \begin{bmatrix} 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0.5 & 0.5 & 0 & 0.5 & 0 \\ 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0.5 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0.5 & 0 & 0 & 0 & 0 & 0.5 & 0.25 & 0 & 0.25 & 0 \\ 0 & 0 & 0.5 & 0.5 & 0 & 0 & 0 & 0.25 & 1 & 0 & 0.25 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0.5 & 0 & 0 & 0 & 0 & 0.25 & 0.25 & 0 & 0.5 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \end{bmatrix}$$

With the following inverse:

$$A_B^- = \begin{bmatrix} - & - & - & - & - & - & - & - & - & - & - & - \\ - & - & - & - & - & - & - & - & - & - & - & - \\ - & - & 3.5 & 0.5 & - & - & - & -2 & -1 & - & -2 & - \\ - & - & 0.5 & 1.5 & - & - & - & 0 & -1 & - & 0 & - \\ - & - & - & - & - & - & - & - & - & - & 0 & - \\ - & - & - & - & - & - & - & - & - & - & - & - \\ - & - & - & - & - & - & - & - & - & - & - & - \\ - & - & -2 & 0 & - & - & - & 4 & 0 & - & 0 & - \\ - & - & -1 & -1 & - & - & - & 0 & 2 & - & 0 & - \\ - & - & - & - & - & - & - & - & - & - & - & - \\ - & - & -2 & 0 & - & - & - & 0 & 0 & - & 4 & - \\ - & - & - & - & - & - & - & - & - & - & - & - \end{bmatrix}$$

Finally, the partial numerator relationship for the C population is

$$A_C = \begin{bmatrix} 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0.5 & 0.5 & 0.5 \\ 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0.5 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0.5 & 0 & 0 & 0 & 0 & 0.5 & 0.25 & 0.25 \\ 0 & 0 & 0 & 0 & 0.5 & 0 & 0 & 0 & 0 & 0.25 & 0.5 & 0.25 \\ 0 & 0 & 0 & 0 & 0.5 & 0.5 & 0 & 0 & 0 & 0.25 & 0.25 & 1 \end{bmatrix}$$

And its inverse:

$$A_C^- = \begin{bmatrix} - & - & - & - & - & - & - & - & - & - & - & - \\ - & - & - & - & - & - & - & - & - & - & - & - \\ - & - & - & - & - & - & - & - & - & - & - & - \\ - & - & - & - & - & - & - & - & - & - & - & - \\ - & - & - & - & 3.5 & 0.5 & - & - & - & -2 & -2 & -1 \\ - & - & - & - & 0.5 & 1.5 & - & - & - & 0 & 0 & -1 \\ - & - & - & - & - & - & - & - & - & - & - & - \\ - & - & - & - & - & - & - & - & - & - & - & - \\ - & - & - & - & - & - & - & - & - & - & - & - \\ -- & - & - & - & -2 & 0 & - & - & - & 4 & 0 & 0 \\ - & - & - & - & -2 & 0 & - & - & - & 0 & 4 & 0 \\ - & - & - & - & -1 & -1 & - & - & - & 0 & 0 & 2 \end{bmatrix}$$

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Structural changes in the small intestine of female turkeys receiving a probiotic preparation are dose and region dependent

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*Gut microbiota have been shown to play a critical role in the maintenance of host health. Probiotics, which regulate gut microbiota balance, could serve as an effective alternative to antibiotic growth promoters. Since changes in the gastrointestinal tract, caused by a variety of different strains, groups and amounts of microorganisms, may be reflected in its histological structure, the aim of the present study was to examine the effects of rising doses of a mixed probiotic preparation on the structure and development of the small intestine of female turkeys. Eighty, three-day-old, healthy, female turkeys (Big-6 breed) were used in the current (16-week) study. The turkeys were randomly allocated to four weight-matched (59.70 ± 0.83 g) groups (*n *= 20), according to probiotic treatment dose (0, 10^7 cfu•g^{-1}, 10^8 cfu•g^{-1} or 10^9 cfu•g^{-1}, in 500 g•1000 kg^{-1}) (cfu – a colony-forming unit). Three, non-genetically modified strains of probiotic cultures obtained from poultry, four bacterial and one yeast culture, were used. Histomorphometric analysis of the structure of the small intestinal wall of the duodenum and jejunum was performed. All probiotic doses used in the current study exerted a beneficial effect on the histological structure of the small intestine; however, the observed effect was dose and region dependent. Significant increases in villi height, crypt depth, villi and crypt width, mucosa thickness, epithelial height, enterocyte number, absorption surface and intestinal ganglia geometric indices were observed, specifically in the duodenum of birds receiving an intermediate dose of probiotic (10^8 cfu•g^{-1}). The probiotic doses used in the current study differed significantly in their effect on the small intestine (*P* < 0.01), with the intermediate dose (10^8 cfu•g^{-1}) significantly improving 58% of the parameters assessed, compared to the control. The duodenum was more susceptible to the favourable effects of the probiotic than the jejunum (56% v. 31% improvement in the parameters assessed) (*P* < 0.01). The weakest favourable effect was observed in the group that received the highest dose of probiotic.*

Keywords: gut microbiota, poultry, duodenum, jejunum, histomorphometry

Implications

This study extends the available knowledge on the use of mixed bacterial and yeast probiotic preparations in the livestock industry. The study is focused on identifying the optimal dose of the mixed bacterial and yeast probiotic preparation used, in order to improve the welfare of birds in a poultry production setting, which may in turn reduce animal losses and thus increase cost effectiveness. Knowledge on the exact composition of the probiotic preparation and the areas of the gut in which the effects of the probiotic preparation are localised may contribute to the reduction of costs in a poultry breeding/production setting.

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Introduction

From 1 January 2006, the European Union banned the use of antibiotics as growth promoters for farm animals (livestock) as a consequence of the growing concern about the development of antimicrobial resistance and the transference of antibiotic resistance genes from animal to human microbiota. However, the removal of these compounds from livestock diets has put tremendous pressure on livestock and poultry producers to find an effective alternative that would perform the same function, especially in the breeding of young animals (Agboola *et al.*, 2014; Hanczakowska *et al.*, 2017). Thus there is a need to investigate the use of alternative products and strategies which would promote the growth of

production animals, as well as carry out a prophylactic/therapeutic function, simultaneously. The abovementioned goals may be achieved through the use of specific feed additives, dietary raw materials or probiotics, which have been shown to favourably affect animal performance and welfare (Soccol *et al*., 2013; Agboola *et al*., 2014). The favourable effects of various feed additives, dietary raw materials and probiotics are thought to be attributed to their ability to regulate gut microbiota balance, which in turn plays a critical role in maintaining host health (Sekirov *et al*., 2010). Probiotics, which are viable microorganisms with no pathogenic properties consisting of either yeast or bacteria, are commonly added to food to improve the microbial balance in the gastrointestinal tract of the host. Probiotics also play a role in the intermediate control of host metabolism, as well as the provision of certain nutrients and regulatory substances, and hence, are associated with the beneficial effects for human and animal health (Marteau *et al*., 2001; Soccol *et al*., 2013). Probiotics favourably alter the balance of intestinal microflora by inhibiting the growth of harmful bacteria, producing metabolic substrates (e.g. vitamins and short-chain fatty acids) and stimulating the immune system in a non-inflammatory manner (Rahimi *et al*., 2009; Agboola *et al*., 2014; Hanczakowska *et al*., 2017). Dietary probiotics make use of several modes of action within the gastrointestinal tract of animals including competition with pathogenic bacteria for intestinal adhesion sites and nutrients, production of antimicrobial substances including bacteriocins and microbial enzymes, modification of environmental conditions in the intestine by lowering pH through the increased production of organic acids, enhancement of the intestinal immune function and the enhancement of epithelial barrier integrity (Rahimi *et al*., 2009; Rawski *et al*., 2016). All the abovementioned probiotic-induced changes within the gastrointestinal tract may be reflected in its histological structure. Probiotics affect the histology and ultrastructure of the gastrointestinal tract, as well as the synthesis and secretion of mucus (Deplancke and Gaskins, 2001; Awad *et al*., 2006 and 2009; Rahimi *et al*., 2009). Available data on changes in the intestinal morphology of turkeys also support the concept that poultry gut health and function, and ultimately bird performance, can be improved by probiotics (Rahimi *et al*., 2009; Agboola *et al*., 2014). Gastrointestinal tract microstructure development has been linked to faecal microbiota numbers. There is a large diversity of bacterial (*Lactobacillus* sp. *Pediococcus* sp., *Streptococcus* sp., *Bacillus* sp., *Bifidobacterium* and *Enterococcus faecium*) and yeast (*Saccharomyces* sp.) strains, which have been studied at different doses, and they have been found to have a stimulating or modulating effect on structural features and the maturation of the gastrointestinal tract (de los Santos *et al*., 2007; Awad *et al*., 2009; Kabir, 2009; Aliakbarpour *et al*., 2012; Soccol *et al*., 2013; Chamani, 2016; Rawski *et al*., 2016). Therefore, the current study was designed to test the hypothesis whether there is a relationship between the improvement of the intestinal structure and an increase in the dose of probiotic preparation. Thus, the aim of the present study was to examine the effects of elevated doses of a probiotic preparation on the structure of the small intestine of female turkeys and to establish an optimal dose, if possible.

Materials and methods

Animal breeding and experimental design

The experiment was performed on 80 three-day-old, healthy, female turkeys (Big-6 breed), randomly allocated to four weight-matched (59.70 ± 0.83 g) groups, according to probiotic treatment dose. Each group consisted of 20 birds divided into four replicates with five birds each. The animals were kept under standard rearing conditions (litter maintenance system) with optimal air temperature level depending on the age of the birds. The turkeys had free access to fresh water and appropriate feed was supplied *ad libitum* in accordance with the stage of production cycle. The birds were fed with a diet corresponding to the four periods of rearing (Supplementary Material S1, Table S1), as previously described (Tomaszewska *et al*., 2016), for a total of 16 weeks. Diets were mixed and pelleted under temperature conditions not exceeding 45°C (since high temperatures may have killed the probiotic cells). At the age of 16 weeks, all birds were slaughtered by cutting the carotid arteries (Tomaszewska *et al*., 2016). Mean weekly body weight gain, mean daily feed intake and average feed conversion ratio were calculated.

Probiotic culture and supplementation

The birds were administered with the probiotic culture (JHJ, Sp. z o.o. Gizałki, Poland), including four strains of bacterial isolates: *Lactococcus lactis* IBB500, *Carnobacterium divergens* S1, *Lactobacillus casei* LOCK 0915 and *Lactobacillus plantarum* LOCK 0862, and one yeast isolate: *Saccharomyces cerevisiae* LOCK 0141, all of poultry gastrointestinal origin (EFSA 2015). The microorganisms were non-genetically modified strains. The *L. lactis* IBB500 strain is deposited in the Polish Collection of Microorganisms (PCM, Wroclaw, Poland), the *C. divergens* S1 strain is deposited in the Collection of Industrial Microorganisms (IAFB, Warsaw, Poland) and the other three strains are deposited in the Centre of Industrial Microorganisms Collection (LOCK, Lodz, Poland).

Experimental groups differed according to the concentration of probiotic spores (if any) added to the diet at every stage of fattening: Control – 0, Group 1 – 10^7 cfu•g^{-1}, Group 2 – 10^8 cfu•g^{-1}, Group 3 – 10^9•cfu g^{-1}, (cfu – a colony-forming unit) in an amount of 500 g•1000 kg^{-1} throughout the experimental period.

Histology preparation and histomorphometric analysis

At the end of the experiment all birds were weighed and sacrificed, and samples of small intestine segments (duodenum and jejunum) were obtained and processed for histology as previously described (Dobrowolski *et al*., 2012). Briefly, 25-mm long segments of duodenum (1.5 cm after stomach) and jejunum (from the middle portion of small intestine) were

Table 1 *The effect of different doses a of dietary probiotic preparation on the body weight and rearing results of female turkeys*

Parameter	Control	Group 1	Group 2	Group 3	Pooled SE	*P*
Final body weight [g]	8220 ± 441[a]	8175 ± 491[a]	8160 ± 439[a]	8246 ± 470[a]	102.9	>0.05
Mean weekly body weight gain [g]	511 ± 57[a]	509 ± 40[a]	508 ± 101[a]	513 ± 59[a]	14.4	>0.05
Mean daily feed intake [g]	194 ± 4[a]	196 ± 5[a]	193 ± 6[a]	191 ± 5[a]	1.1	>0.05
Average feed conversion ratio [kg/kg BWbw]	2.6 ± 0.2[a]	2.7 ± 0.1[a]	2.7 ± 0.1[a]	2.6 ± 0.1[a]	0.03	>0.05

The results are presented as mean (SD). Control – animals not receiving probiotic preparation, Group 1 – 10^7 cfu•g^{-1}, Group 2 – 10^8 cfu•g^{-1} and Group 3 – 10^9•cfu g^{-1} (cfu – a colony-forming unit).
Values with different superscripts within a single row are significantly different from one another ($P < 0.05$).

collected into a 35°C saline solution and gently cut open longitudinally, along the mesentery line. Samples were then placed flat (not stretched) into a standard histological cassette in such a way that the mucosa had no contact with any of the cassette walls, and fixed in 4% buffered formaldehyde (pH 7.0) for 24 h. After dehydration in graded ethanol solutions, samples were trimmed into three pieces (10 × 5 mm), allowing us to cut intestinal cross sections on the longer border of the samples. Following clearance in xylene, samples were then embedded in paraffin using a tissue processor (STP 120, Thermo Scientific, Waltham, MA, USA). Twenty cross sections (with 10 mm interval between each five-slice section), 4 mm thick, were then cut with a microtome (Microm HM 360, Microm, Walldorf, Germany) from every sample of the small intestine (Dobrowolski *et al*., 2012). Goldner's trichrome staining was used to differentiate the small intestine wall layers (Suvarna *et al*., 2013; Dobrowolski *et al*., 2016). Microscopic (two-dimensional) images of bright field (magnification ×50, ×200 and ×400) were collected using a confocal microscope (AXIOVERT 200 M, Carl Zeiss, Jena, Germany) equipped with a colour digital camera (AxioCam HRc, Carl Zeiss, Jena, Germany) and a halogen lamp (Dobrowolski *et al*., 2016). Microscopic pictures of particular segments of small intestine were subjected to further histomorphometric analysis. The structure of the small intestinal wall was examined under microscopic observation and with the use of graphic analysis software (ImageJ 1.51, National Institutes of Health, Bethesda, MD, USA; available at: http://rsb.info.nih.gov/ij/index.html). The following parameters were analysed: the thickness of the inner and outer muscle layer, mucosa and submucosa thickness, crypt depth (defined as the depth of the invagination between adjacent villi, from the bottom of the crypt to the base of the villus) and width (measured in the middle of the crypt depth), villi height (from the tip of the villus to the villus–crypt junction) and width (measured in the middle of the villus height), total crypt number (opened and closed crypts), number of open (showing mitoses and an open internal space, with access to the intestinal lumen) and closed crypts (showing no mitoses and a closed internal space), number of villi per millimetre of mucosa, enterocyte height (measured as the distance from brush border membrane to the basolateral membrane) and number of enterocytes per 100 mm of villus epithelium (Dobrowolski *et al*., 2012). Only vertically oriented villi and crypts were measured. Small intestinal absorptive surface was also determined according to Kisielinski *et al*. (2002) (Supplementary Material S1). Villi height to crypt depth ratio was also calculated. Meissner as well as Auerbach plexus features including mean sectional area of nerve ganglia, ganglia perimeter, shape factor (where 0 refers to the rod-shaped objects and 1 refers to objects that are round or spherical in shape), minimal radius and diameter, mean diameter, sphericity and convexity were also analysed (Dobrowolski *et al*., 2012; Tomaszewska *et al*., 2012).

Statistical analysis

All results are expressed as mean (SD). Differences between means were tested with the use of one-way ANOVA and a *post hoc* Tukey's honest significant difference test as a correction for multiple comparisons. Normal distribution of data was examined using the W Shapiro–Wilk test and equality of variance was tested by the Brown–Forsythe test. When data were not normally distributed and/or unequal variance of data, the Kruskal–Wallis test was used to analyse the difference between means and *post hoc* tests for mean range for every pair of groups. The two-sided significance level (*P*-value) of less than 0.05 was considered statistically significant. All statistical analyses were carried out using STATISTICA (data analysis software system), version 12. StatSoft, Inc. (2014). STATISTICA Power Analysis tool was used to calculate the sample size and the statistical power as a function of the error and the type and size of the effect for the one-way ANOVA. The effect size was calculated and interpreted according to Cohen's *f*, as described by Kotrlik (Kotrlik *et al*., 2011) (Supplementary Material S1, Tables S2 and S3). The general effect of probiotic dose on the small intestine parameters was calculated as a count of improvements (compared to the control group), compared using a χ^2 test.

Results

Body weight, body weight gain, feed intake and feed conversion ratio

No significant differences in body weight, body weight gain, feed intake and feed conversion ratios were observed between treatment groups (Table 1).

Histomorphometric analyses

Duodenum. Several intestinal indices were significantly increased in the duodenum of birds receiving the lowest dose

Table 2 *The effect of different doses of a dietary probiotic preparation on the histomorphometry of the duodenum of female turkeys*

Parameter	Control	Group 1	Group 2	Group 3	Pooled SE	*P*
Thickness of the outer muscle layer [μm]	52.4 ± 17.2^{a}	65.9 ± 9.7^{b}	72.3 ± 15.3^{b}	47.9 ± 6.2^{a}	1.1	<0.01
Thickness of the inner muscle layer [μm]	202.8 ± 41.4^{a}	201.6 ± 12.1^{a}	240.5 ± 62.6^{b}	215.2 ± 13.8^{a}	2.8	<0.01
Thickness of the submucosa [μm]	33.5 ± 4.3^{b}	42.0 ± 7.4^{c}	25.7 ± 5.0^{a}	25.9 ± 3.2^{a}	0.5	<0.01
Thickness of the mucosa [μm]	1699.0 ± 121.2^{a}	2204.3 ± 119.2^{b}	2201.5 ± 146.3^{b}	2238.7 ± 541.3^{b}	26.7	<0.01
Crypt depth [μm]	237.4 ± 63.9^{a}	226.9 ± 36.9^{a}	427.7 ± 118.8^{b}	261.2 ± 69.6^{a}	7.3	<0.01
Villi height [μm]	1440.6 ± 323.0^{a}	1763.4 ± 161.2^{b}	1862.6 ± 161.5^{b}	2156.3 ± 311.9^{c}	27.1	<0.01
Crypt width [μm]	47.4 ± 8.7^{a}	65.4 ± 12.7^{c}	55.7 ± 11.4^{b}	76.7 ± 11.3^{d}	1.3	<0.01
Villi width [μm]	85.8 ± 14.5^{a}	81.7 ± 10.4^{a}	115.3 ± 30.7^{b}	112.2 ± 18.4^{b}	1.7	<0.01
Total number of crypts/mm	12.9 ± 1.3^{a}	13.1 ± 1.6^{a}	15.5 ± 1.3^{b}	12.9 ± 1.9^{a}	0.1	<0.01
Number of open crypts/mm	3.6 ± 0.8^{a}	5.4 ± 1.4^{b}	5.8 ± 1.1^{b}	7.1 ± 1.3^{c}	0.1	<0.01
Number of closed crypts/mm	9.1 ± 2.2^{c}	7.8 ± 1.5^{b}	9.9 ± 1.8^{c}	5.8 ± 1.12^{a}	0.2	<0.01
Number of villi/mm	8.3 ± 1.7^{a}	8.1±1.2^{a}	7.8 ± 1.4^{a}	8.2 ± 0.5^{a}	0.1	=0.27
Enterocyte height [μm]	28.3 ± 3.3^{b}	23.8 ± 2.4^{a}	38.9 ± 4.3^{c}	45.2 ± 3.9^{d}	0.6	<0.01
Number of enterocytes/100 [μm]	18.7 ± 2.4^{a}	36.3 ± 9.0^{c}	24.1 ± 3.7^{b}	16.6 ± 1.2^{a}	0.6	<0.01
Villus/crypt ratio	6.0 ± 1.6^{b}	7.7 ± 1.2^{c}	4.7 ± 1.3^{a}	8.9 ± 2.5^{d}	0.2	<0.01
Absorption surface [μm^2]	27.0 ± 5.3^{a}	27.5 ± 5.0^{ab}	30.0 ± 4.5^{b}	26.4 ± 5.2^{a}	0.4	<0.01

The results are presented as mean (SD). Control – animals not receiving the probiotic preparation, Group 1 – 10^7 cfu•g^{-1}, Group 2 – 10^8 cfu•g^{-1} and Group 3 – 10^9•cfu g^{-1} (cfu – a colony-forming unit).
Values with different superscripts within a single row are significantly different from one another ($P < 0.05$).

of the probiotic preparation (10^7 cfu•g^{-1}) (Group 1), compared to the control group, namely: outer muscle thickness (26%), submucosa (25%) and mucosa thickness (30%), villi height (22%), crypt width (38%), number of open crypts (50%), total number of enterocytes (94%) and villus to crypt ratio (28%) ($P < 0.01$ in all cases). The number of closed crypts and enterocyte height were significantly decreased in Group 1 compared to the control group by 14% and 16% ($P = 0.01$ and $P < 0.01$), respectively (Table 2). Birds in Group 2 (receiving a higher dose of the probiotic preparation – 10^8 cfu•g^{-1}) displayed significantly increased outer (38%, $P < 0.01$) and inner muscle thickness (19%, $P = 0.03$), mucosa thickness (30%, $P < 0.01$), crypt depth (80%, $P < 0.01$), villi height (29%, $P < 0.01$), crypt (18%, $P = 0.02$) and villi width (34%, $P < 0.01$), total crypt number (20%, $P < 0.01$), number of open crypts (61%, $P < 0.01$), enterocyte height (37%, $P < 0,01$), total enterocyte number (29%, $P < 0,01$), as well as small intestine absorptive surface (11%, $P = 0.04$), compared to the control group. On the contrary, submucosa thickness (23%, $P < 0.01$) and villus to crypt ratio (22%, $P < 0.01$) were significantly decreased compared to that observed in the control group, with no marked change in the number of closed crypts (Table 2).

Interestingly, less duodenal parameters were affected by the highest dose of the probiotic preparation (10^9 cfu•g^{-1}), since only mucosa thickness (32%), villi height (50%), crypt (62%) and villi width (31%), number of open crypts (97%), enterocyte height (60%) and villus to crypt ratio (48%) were significantly elevated in birds from Group 3 compared to the control group ($P < 0.01$ in all cases). Villi height, crypt width, number of open crypts, enterocyte height and the villus to crypt ratio in birds from Group 3 were the greatest of all the birds studied. Submucosa thickness and the number of closed crypts were significantly decreased in birds from Group 3 compared to the control group by 23% and 36% ($P < 0.01$), respectively (Table 2).

Birds from Group 2 had significantly thicker inner muscle layers and significantly deeper crypts compared to birds in Groups 1 (19% thicker and 88% deeper; $P < 0.01$) and 3 (12% thicker and 64% deeper; $P = 0.02$ – inner muscle layer; $P < 0.01$ – crypts).However, submucosa thickness was significantly decreased (by 63% ($P < 0.01$) in Groups 2 and 3 compared to Group 1.

Villi height was significantly increased in birds from Group 3 compared to those from Group 1 (by 22%, $P < 0.01$) and Group 2 (by 16%, $P = 0.03$). Birds from Group 1 had 17% ($P = 0.04$) wider crypts than those in Group 2, but 15% ($P = 0.04$) narrower crypts than those in Group 3. Villi width was significantly increased ($P < 0.01$) in birds from Groups 2 (by 41%) and 3 (by 37%) *v.* that observed in those from Group 1. The total number of crypts was highest (both $P < 0.01$) in birds from Group 2 compared to those from Groups 1 (by 18%) and 3 (by 20%). Female turkeys receiving the highest dose of the probiotic preparation (10^9 cfu•g^{-1}) had more open crypts than those from Groups 1 (by 31%, $P < 0.01$) and 2 (by 22%, $P = 0.02$). Birds from Group 3 also had significantly less closed crypts (by 26%, $P < 0.01$) compared to those from Group 1.

The greatest enterocyte height was observed in birds from Group 3, with enterocytes that were 16% ($P = 0.04$) higher than those observed in birds from Group 2 and 90% ($P < 0.01$) higher than those observed in birds from Group 1. However, enterocyte number was greatest in birds from Group 1, with over 50% more enterocytes than that observed in birds from Group 2 ($P < 0.01$), and twice as much as that observed in birds from Group 3 ($P < 0.01$).

Jejunum. Over half of the histological parameters that were analysed in the jejunum were significantly elevated in birds

Table 3 *The effect of different doses of a dietary probiotic preparation on the histomorphometry of the jejunum of female turkeys*

Parameter	Control	Group 1	Group 2	Group 3	Pooled SE	*P*
Thickness of the outer muscle layer [μm]	40.9 ± 15.5^{a}	59.5 ± 11.1^{b}	75.5 ± 12.1^{c}	69.9 ± 11.0^{c}	1.5	<0.01
Thickness of the inner muscle layer [μm]	198.1 ± 53.8^{a}	236.3 ± 44.1^{b}	293.4 ± 38.7^{c}	280.1 ± 26.6^{c}	4.4	<0.01
Thickness of the submucosa [μm]	33.1 ± 6.5^{a}	43.2 ± 7.4^{c}	37.3 ± 3.8^{b}	46.9 ± 5.8^{d}	0.6	<0.01
Thickness of the mucosa [μm]	1685.8 ± 139.2^{a}	1917.5 ± 131.3^{c}	1802.4 ± 242.5^{b}	1687.6 ± 195.0^{a}	15.1	<0.01
Crypt depth [μm]	264.2 ± 59.9^{a}	300.1 ± 66.0^{a}	456.6 ± 202.3^{b}	268.7 ± 67.2^{a}	10.2	<0.01
Villi height [μm]	1497.6 ± 139.7^{b}	1653.5 ± 117.7^{c}	1696.3 ± 179.1^{c}	1401.3 ± 155.0^{a}	14.0	<0.01
Crypt width [μm]	44.9 ± 10.9^{a}	52.3 ± 7.8^{b}	49.3 ± 11.9^{ab}	52.4 ± 11.5^{b}	0.8	<0.01
Villi width [μm]	121.6 ± 23.2^{c}	77.4 ± 18.9^{a}	78.9 ± 15.9^{a}	107.8 ± 16.4^{b}	2.1	<0.01
Total number of crypts /mm	15.1 ± 2.7^{c}	12.4 ± 1.5^{a}	14.4 ± 2.6^{bc}	13.1 ± 2.4^{ab}	0.2	<0.01
Number of open crypts /mm	4.4 ± 1.0^{a}	4.4 ± 1.4^{a}	5.9 ± 1.5^{b}	7.0 ± 1.2^{c}	0.1	<0.01
Number of closed crypts /mm	10.6 ± 2.8^{c}	7.9 ± 2.0^{b}	8.5 ± 2.8^{b}	6.1 ± 2.4^{a}	0.2	<0.01
Number of villi /mm	5.8 ± 0.9^{a}	6.6 ± 2.4^{a}	9.8 ± 1.4^{c}	7.8 ± 0.8^{b}	0.2	<0.01
Enterocyte height [μm]	19.3 ± 3.6^{a}	26.4 ± 4.7^{b}	29.9 ± 2.6^{c}	27.5 ± 4.0^{b}	0.4	<0.01
Number of enterocytes /100 [μm]	16.9 ± 3.1^{a}	17.9 ± 3.4^{a}	20.4 ± 3.6^{b}	17.9 ± 1.3^{a}	0.2	<0.01
Villus/crypt ratio	5.7 ± 1.1^{b}	5.4 ± 1.0^{b}	4.0 ± 1.4^{a}	5.1 ± 1.0^{b}	0.1	<0.01
Absorption surface [μm^2]	26.0 ± 3.9^{a}	30.0 ± 2.7^{b}	33.3 ± 6.2^{c}	24.1 ± 3.9^{a}	0.4	<0.01

The results are presented as mean (SD). Control – animals not receiving the probiotic preparation, Group 1 – 10^7 cfu•g^{-1}, Group 2 – 10^8 cfu•g^{-1} and Group 3 – 10^9•cfu g^{-1} (cfu – a colony-forming unit).
Values with different superscripts within a single row are significantly different from one another ($P < 0.05$).

receiving the lowest dose of the probiotic preparation (10^7 cfu•g^{-1}) compared to the control group, namely: outer (45%) and inner muscle thickness (19%), submucosa (31%) and mucosa (14%) thickness, as well as villi height (10%), crypt width (16%), enterocyte height (37%) and small intestine absorptive surface (15%) ($P < 0.01$ in all cases). Villi width, total crypt number and the number of closed crypts were significantly decreased in birds from Group 1 compared to the control group, by 36%, 18% and 25% ($P < 0.01$), respectively (Table 3). Birds from Group 2, which received a higher dose of the probiotic preparation (10^8 cfu•g^{-1}), displayed significantly greater outer (85%, $P < 0.01$) and inner muscle thickness (48%, $P < 0.01$), submucosa (13%, $P = 0.03$) and mucosa (7%, $P = 0.04$) thickness, crypt depth (73%, $P < 0.01$), villi height (13%, $P < 0.01$), number of open crypts (34%, $P < 0.01$), total villi number (69%, $P < 0.01$), total number of enterocytes (21%, $P < 0.01$), enterocyte height (55%, $P < 0.01$) and small intestine absorptive surface (28%, $P < 0.01$) compared to the control group. Villi width, the number of closed crypts, as well as the villus to crypt ratio were significantly decreased in birds from Group 2 (by 35%, 23% and 30%, respectively (all $P < 0.01$)) compared to the control group (Table 3).

Less jejunal parameters were affected by the highest dose of the probiotic preparation (10^9 cfu•g^{-1}). Only seven parameters, including outer (71%) and inner (41%) muscle layer thickness, submucosa thickness (42%), number of open crypts (59%), villi number (34%) and enterocyte height (42%) ($P < 0.01$ in all cases), as well as the width of the crypts (17%, $P = 0.02$) were all significantly elevated compared to that observed in the birds from the control group. Villi height and width, total number of crypts and the number of closed crypts were significantly decreased in birds from Group 3 compared to the control group, by 6% ($P = 0.04$), 11% ($P = 0.02$), 13% ($P = 0.02$) and 42% ($P < 0.01$), respectively (Table 3).

The thickness of the outer and inner muscle layers was significantly higher in birds from Groups 2 (by 27% and 24%, respectively, $P < 0.01$) and 3 (by 17% and 19%, respectively, $P < 0.01$ – outer muscle layer; $P = 0.04$ – inner muscle layer), compared to that observed in birds from Group 1. Birds from Group 3, which received the highest does of the probiotic preparation, displayed significantly thicker submucosa layers compared to those in Groups 1 (by 9%, $P = 0.04$) and 2 (by 26%, $P < 0.01$). The submucosa (16%, $P < 0.01$) and mucosa layers (6%, $P = 0.03$) were significantly thicker in birds from Group 1 compared to those from Group 2.

Birds from both Groups 1 and 2 had significantly longer villi than those in Group 3, by an average of 20% (both $P < 0.01$). Conversely, the villi width was significantly lower in both these groups compared to those from Group 3, by an average of 38% ($P < 0.01$). Crypt depth was significantly greater in birds from Group 2 compared to that observed in birds from Groups 1 (52%) and 3 (70%) ($P < 0.01$ in both cases). Total crypt number was significantly higher in birds from Group 2 (16%, $P < 0.01$) than those in Group 1. Furthermore, the number of open crypts was higher in birds from Group 2 (34%, $P < 0.01$) than those in Group 1; however, still lower (by 19%, $P = 0.03$) than that observed in Group 3. Additionally, birds from Group 3 had the lowest number of closed crypts compared to all other probiotic treatment groups, by an average of 35% ($P < 0.01$). Birds from Group 2 had significantly increased total villi and enterocyte numbers, as well as enterocyte height compared to birds from Groups 1 and 3, by an average of 37% ($P < 0.01$), 14% ($P = 0.03$) and 11% ($P = 0.04$), respectively. The absorptive surface of the jejunum was significantly higher in Groups

Table 4. *The effect of different doses of a dietary probiotic preparation on the morphology of duodenal Meissner and Auerbach plexuses in the small intestine of female turkeys*

Parameter	Control	Group 1	Group 2	Group 3	Pooled SE	*P*
Meissner plexus						
Mean sectional area of nerve ganglia [μm^2]	1485 ± 399^{a}	2188 ± 975^{bc}	2544 ± 703^{c}	1841 ± 342^{ab}	97.2	<0.01
Ganglia perimeter [μm]	271 ± 86^{a}	236 ± 61^{a}	255 ± 121^{a}	233 ± 48^{a}	10.7	=0.58
Shape factor	0.30 ± 0.08^{a}	0.49 ± 0.13^{b}	0.56 ± 0.17^{b}	0.45 ± 0.12^{b}	0.02	<0.01
Minimal radius [μm]	8.7 ± 1.8^{a}	13.3 ± 4.7^{b}	15.5 ± 2.9^{b}	12.3 ± 2.1^{b}	0.5	<0.01
Minimal diameter [μm]	17.4 ± 3.7^{a}	26.6 ± 9.4^{bc}	30.1 ± 5.7^{c}	24.6 ± 4.2^{b}	1.0	<0.01
Mean diameter [μm]	36.7 ± 6.8^{a}	46.0 ± 11.1^{b}	50.5 ± 12.7^{b}	43.0 ± 4.4^{ab}	1.3	<0.01
Sphericity	0.034 ± 0.023^{a}	0.080 ± 0.039^{b}	0.087 ± 0.039^{b}	0.072 ± 0.029^{b}	0.005	<0.01
Convexity	0.88 ± 0.05^{a}	0.94 ± 0.05^{ab}	0.95 ± 0.04^{b}	0.92 ± 0.06^{ab}	0.007	<0.01
Auerbach plexus						
Mean sectional area of nerve ganglia [μm^2]	2878 ± 891^{a}	4202 ± 1539^{ab}	4586 ± 1817^{b}	5597 ± 1440^{b}	223	<0.01
Ganglia perimeter [μm]	377 ± 158^{ab}	313 ± 105^{a}	462 ± 183^{b}	480 ± 46^{b}	18.9	<0.01
Shape factor	0.34 ± 0.14^{a}	0.56 ± 0.18^{b}	0.45 ± 0.13^{b}	0.39 ± 0.14^{a}	0.02	<0.01
Minimal radius [μm]	13.4 ± 3.8^{a}	23.8 ± 6.9^{c}	22.2 ± 7.1^{bc}	18,3 ± 2.7^{ab}	0.9	<0.01
Minimal diameter [μm]	26.9 ± 7.5^{a}	47.7 ± 13.8^{c}	44.4 ± 14.1^{bc}	36.6 ± 5.4^{ab}	1.7	<0.01
Mean diameter [μm]	52.5 ± 10.1^{a}	73.9 ± 26.9^{b}	82.2 ± 26.0^{b}	70.8 ± 9.4^{ab}	2.9	<0.01
Sphericity	0.037 ± 0.014^{ab}	0.037 ± 0.005^{ab}	0.039 ± 0.001^{a}	0.030 ± 0.001^{b}	0.001	<0.01
Convexity	0.90 ± 0.05^{ab}	0.95 ± 0.04^{b}	0.91 ± 0.07^{ab}	0.87 ± 0.08^{a}	0.008	<0.01

The results are presented as mean (SD). Control – animals not receiving probiotic preparation, Group 1 – 10^7 cfu•g^{-1}, Group 2 – 10^8 cfu•g^{-1} and Group 3 – 10^9•cfu g^{-1} (cfu – a colony-forming unit).
Values with different superscripts within a single row are significantly different from one another ($P < 0.05$).

1 and 2 compared to the birds from Group 3, by an average of 23% ($P < 0.01$). Villus to crypt ratio was lowest in birds from Group 2.

Nervous innervation

Duodenum. Duodenal Meissner and Auerbach plexus ganglia of female turkeys receiving the lowest dose of the probiotic preparation (10^7 cfu•g^{-1}) had a significantly increased shape factor (by 63% and 65%, respectively, $P < 0.01$), minimal radius (by 53% and 78%, respectively, $P < 0.01$), minimal diameter (by 53% and 77%, respectively, $P < 0.01$), as well as mean diameter (by 25%, $P = 0.02$) and 41% ($P < 0.01$), respectively), compared to the control group (Table 4). Mean sectional area of nerve ganglia and sphericity were also significantly raised, by 135% ($P < 0.01$) and 47% ($P = 0.02$), respectively, but only in the Meissner plexus. No significant changes in mean sectional area of nerve ganglia, its perimeter as well as the sphericity and convexity indices were observed in the Auerbach plexus (Table 4). The intermediate dose of the probiotic preparation (10^8 cfu•g^{-1}) exerted a similar effect on the nervous innervation of the duodenum as that observed in the control group (with no probiotic preparation administration). However, significantly higher mean sectional area of nerve ganglia was observed in birds from Group 2, in both the Meissner and Auerbach plexuses, compared to the control group (by 71% and 59%, respectively, both $P < 0.01$). Birds from Group 2 also displayed a significant increase (by 8%, $P < 0.01$) in convexity in the Meissner plexus compared to the control group. No signs of hypertrophy or hyperplasia of neurons were observed.

Fewer changes in nervous innervation of the small intestine were observed in female turkeys receiving the highest dose of the probiotic preparation (10^9 cfu•g^{-1}). Significantly increased shape factor (50%), minimal radius (41%) and minimal diameter (41%) were observed in the Meissner plexus ($P = 0.02$ in all cases) compared to the control group (Table 4). Mean sectional area of nerve ganglia in the Auerbach plexus was almost twice as large as that observed in birds from the control group. However, no swollen ganglia were observed in any of the groups assessed.

Jejunum. Significantly fewer changes with regard to nervous innervation were observed in jejunum compared to the duodenum (Table 5). The lowest does of the probiotic preparation (10^7 cfu•g^{-1}) caused a significant increase in shape factor (by 24%, $P = 0.04$) and sphericity (by 177%, $P < 0.01$) in the Meissner plexus of birds from Group 1 compared to the control group. The intermediate dose of the probiotic preparation (10^8 cfu•g^{-1}) caused a 49% ($P < 0.01$) increase in the mean sectional area of nerve ganglia in the Meissner plexus, compared to that observed in the control group. The highest dose of the probiotic preparation (10^9 cfu•g^{-1}), surprisingly, decreased the minimal radius (by 31%, $P = 0.01$) and convexity (by 7%, $P = 0.02$) in the Auerbach plexus compared to that observed in the control group and the remaining treatment groups (Table 5).

The general effect of probiotic dose

The greatest stimulating effect was observed in animals receiving the moderate dose (10^8 cfu•g^{-1}), where over 58% of parameters were significantly improved compared to the control, which corresponded to over 44% of all

Table 5 *The effect of different doses of a dietary probiotic preparation on the morphology of jejunal Meissner and Auerbach plexuses in the small intestine of female turkeys*

Parameter	Control	Group 1	Group 2	Group 3	Pooled SE	*P*
Meissner plexus						
Mean sectional area of nerve ganglia [μm^2]	800 ± 207^{ab}	597 ± 100^{a}	1191 ± 447^{c}	939 ± 290^{bc}	46.1	<0.01
Ganglia perimeter [μm]	142 ± 33^{ab}	106 ± 26^{b}	181 ± 63^{a}	168 ± 43^{a}	6.6	<0.01
Shape factor	0.55 ± 0.09^{a}	0.68 ± 0.14^{b}	0.50 ± 0.16^{a}	0.45 ± 0.14^{a}	0.02	<0.01
Minimal radius [μm]	9.0 ± 2.0^{ab}	9.7 ± 3.2^{ab}	10.4 ± 1.8^{b}	8.2 ± 1.8^{a}	0.3	=0.05
Minimal diameter [μm]	18.0 ± 4.0^{ab}	19.5 ± 6.4^{ab}	20.8 ± 3.6^{b}	16.3 ± 3.7^{a}	0.6	=0.05
Mean diameter [μm]	30.5 ± 5.6^{ab}	26.3 ± 6.3^{a}	34.8 ± 5.3^{b}	29.8 ± 4.6^{ab}	0.8	<0.01
Sphericity	0.13 ± 0.06^{a}	0.36 ± 0.25^{b}	0.12 ± 0.08^{a}	0.14 ± 0.24^{a}	0.03	<0.01
Convexity	0.95 ± 0.03^{a}	0.96 ± 0.02^{a}	0.94 ± 0.04^{a}	0.92 ± 0.07^{a}	0.006	=0.06
Auerbach plexus						
Mean sectional area of nerve ganglia [μm^2]	2652 ± 572^{a}	2985 ± 1677^{a}	3336 ± 1604^{a}	2668 ± 602^{a}	159	=0.39
Ganglia perimeter [μm]	372 ± 183^{a}	308 ± 156^{a}	299 ± 112^{a}	358 ± 91^{a}	18.1	=0.39
Shape factor	0.41 ± 0.11^{ab}	0.50 ± 0.18^{b}	0.51 ± 0.18^{b}	0.29 ± 0.08^{a}	0.02	<0.01
Minimal radius [μm]	15.9 ± 4.7^{b}	15.7 ± 3.9^{b}	17.5 ± 5.5^{b}	11.0 ± 1.8^{a}	0.6	<0.01
Minimal diameter [μm]	31.7 ± 9.3^{a}	31.4 ± 7.8^{a}	34.9 ± 11.0^{a}	22.1 ± 3.6^{a}	1.2	<0.01
Mean diameter [μm]	60.0 ± 20.1^{a}	53.4 ± 14.7^{a}	56.3 ± 11.4^{a}	46.8 ± 5.7^{a}	1.9	=0.07
Sphericity	0.06 ± 0.04^{a}	0.12 ± 0.09^{ab}	0.20 ± 0.24^{b}	0.03 ± 0.02^{a}	0.02	<0.01
Convexity	0.92 ± 0.05^{b}	0.94 ± 0.04^{b}	0.92 ± 0.06^{b}	0.86 ± 0.05^{a}	0.007	<0.01

The results are presented as mean (SD). Control – animals not receiving probiotic preparation, Group 1 – 10^7 cfu•g^{-1}, Group 2 – 10^8 cfu•g^{-1} and Group 3 – 10^9•cfu g^{-1} (cfu – a colony-forming unit).
Values with different superscripts within a single row are significantly different from one another ($P < 0.05$).

ameliorated parameters in the study. Moreover, duodenum (56% of ameliorated parameters) turned to be significantly ($P < 0.01$) more susceptible for probiotics than jejunum (31% of ameliorated parameters). Surprisingly, the weakest stimulating effect was observed in the highest used dose of probiotics (10^9 cfu•g^{-1}), where only 30% of all parameters were ameliorated, especially compared to the moderate one ($P < 0.01$).

Discussion

Animals develop in association with complex commensal microbiota communities, which are essential for precise host organ function. Gastrointestinal microbiota, in particular, play a key role in overall host development, immunity, nutrient conversion, maintenance of host health and they serve as a barrier against exogenous pathogens (Sekirov *et al.*, 2010; Montalban-Arques *et al.*, 2015). Alternative products and strategies that aid in the maintenance of animal gut health are constantly being investigated in order to prevent or reduce the prevalence of pathogens in livestock. This is specifically important nowadays, where the development of antibiotic resistance is on the increase (Yang *et al.*, 2009; Soccol *et al.*, 2013; Agboola *et al.*, 2014). The use of probiotics as an effective alternative approach could improve gut microbial balance, and therefore, the natural defence of livestock against pathogenic bacteria (Soccol *et al.*, 2013). The appropriate probiotic dose for use in livestock is also becoming of importance (Yang *et al.*, 2009).

Several aspects of host–microbiota interactions that promote functional and structural maturation of the gastrointestinal tract have been examined recently, including peristaltic motility, intestinal absorptive surface, blood supply, nutrient acquisition, microbial balance and gut integrity. The health-promoting effects of probiotics are dependent on the type and number of the different strains of bacteria or yeasts used in the probiotic preparation, especially in mixed preparations (Saarela *et al.*, 2002; Yang *et al.*, 2009; Soccol *et al.*, 2013; Agboola *et al.*, 2014; Rawski *et al.*, 2016). It must be stressed that all probiotic strains are different and their identification, characteristics and host relevance are important factors to consider when extrapolating results observed in different host species, which has to be done with great caution and makes the translation of data more complex (Saarela *et al.*, 2002; Chamani, 2016). Nevertheless, assessment of gastrointestinal tract morphology and histology is a broadly utilised technique in the investigation of the health-promoting effects of probiotic preparations, since intestinal morphology may be facilitated by the gut microflora and the digestive function of the small intestine is closely related to its mucosal architecture and villi structure (Liu *et al.*, 2008; Rahimi *et al.*, 2009; Sekirov *et al.*, 2010; Agboola *et al.*, 2014; Rawski *et al.*, 2016). However, studies on the structural changes and development of the gastrointestinal tract in response to different doses of mixed bacteria and yeast preparations are scarce, especially in a turkey model.

Data from the detailed histomorphometric analyses performed in the present study confirm the effects of probiotics observed in previous studies. The significantly increased villi height, crypt depth, mucosa thickness and crypt and villi width observed in the present study, following administration of the probiotic preparation, are in agreement with results from previous studies (Pelicano *et al.*, 2005; Awad *et al.*, 2009; Rahimi *et al.*, 2009; Agboola *et al.*, 2014;

Chamani, 2016; Rawski *et al*., 2016). Improvement in epithelium features, villus to crypt ratio and small intestine absorptive surface were also in agreement with previous studies (de los Santos *et al*., 2007). However, the current study is unique in that it is the first study which compares, in detail, the effects of different doses of a mixed bacteria and yeast probiotic preparation on the development and structure of the small intestine of female turkey poults. Moreover, the results presented here also show, for the first time, an improvement in the nervous innervation of the small intestine following administration of the mixed probiotic preparation. In contrast to previous studies, more duodenal parameters were significantly improved in birds receiving the intermediate dose of the probiotic preparation compared to that observed in the jejunum, which was less susceptible to the favourable effects of the probiotic preparation (de los Santos *et al*., 2007).

The probiotic preparation used in the present study did not affect the body weight gain, feed intake or feed utilisation, which was also observed in previous studies (Chamani, 2016; Hanczakowska *et al*., 2017). However, Markovic *et al*. (2009) observed an increase in body weight gain after administration of yeast additives to broilers (Markovic *et al*., 2009). Alternatively, in a study by Agboola *et al*. (2014), supplementation with probiotics increased the body weight of turkey poults; however, there was no influence on the performance of the turkeys, thus feed intake, feed conversion ratio and protein intake were not significantly changed (Agboola *et al*., 2014). Intestinal bacteria have been suggested to regulate body weight through their effects on the host's metabolic, neuroendocrine and immune functions, with some studies reporting a reduction in total body and abdominal fat (Florou-Paneri *et al*., 2013); however, these effects may largely depend on the variety of bacterial specific strains, their proportions and the doses used in these preparations. These issues still remain to be investigated. In the present study, the specific probiotic preparation was chosen based on the colonisation of a particular section of the intestine, since in the turkey duodenum, *Lactobacillus* sp. prevails and the rest of the bacterial strains that were used are largely present in further parts of the small intestine. The yeasts were used as a result of their nutritional content, their resistance to the acidic stomach environment and their immunomodulatory activities, which complement those of the naturally occurring microflora.

In conclusion, all doses of the probiotic preparation used in the present study exerted a beneficial effect on the histological structure of the small intestine; however, the observed effects were dose and region dependent. The structural development of particular parts of the gastrointestinal tract proved to be greatly accelerated by the 10^8 cfu•g^{-1} dose of the mixed probiotic preparation. Investigations into why the highest dose (10^9 cfu•g^{-1}) of the probiotic preparation had the worst effect, as well as the mechanism of action of the probiotic preparation, will be carried out in future studies. Presented detailed data on the effects of different doses of probiotics on the histomorphometry of the small intestine, especially in turkey poults, may enrich poultry breeding practices.

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Declaration of interest

None.

Ethics statement

The present study and the experimental procedures carried out were approved by the Local Ethics Committee on Animal Experimentation of the University of Life Sciences in Lublin, Poland.

Software and data repository resources

The data presented are not deposited in any official repository.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731119001149.

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animal

The potential application of plant wax markers from alfalfa for estimating the total feed intake of sheep

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Estimating the feed intake of grazing herbivores is critical for determining their nutrition, overall productivity and utilization of grassland resources. A 17-day indoor feeding experiment was conducted to evaluate the potential use of Medicago sativa *as a natural supplement for estimating the total feed intake of sheep. A total of 16 sheep were randomly assigned to four diets (four sheep per diet) containing a known amount of* M. sativa *together with up to seven forages common to typical steppes. The diets were: diet 1,* M. sativa + Leymus chinensis + Puccinellia distans*; diet 2, species in diet 1* + Phragmites australis*; diet 3, species in diet 2* + Chenopodium album + Elymus sibiricus*; and diet 4, species in diet 3* + Artemisia scoparia + Artemisia tanacetifolia. *After faecal marker concentrations were corrected by individual sheep recovery, treatment mean recovery or overall recovery, the proportions of* M. sativa *and other dietary forages were estimated from a combination of alkanes and long-chain alcohols using a least-square procedure. Total intake was the ratio of the known intake of* M. sativa *to its estimated dietary proportion. Each dietary component intake was obtained using total intake and the corresponding dietary proportions. The estimated values were compared with actual values to assess the estimation accuracy. The results showed that* M. sativa *exhibited a distinguishable marker pattern in comparison to the other dietary forage species. The accuracy of the dietary composition estimates was significantly (*$P < 0.001$*) affected by both diet diversity and the faecal recovery method. The proportion of* M. sativa *and total intake across all diets could be accurately estimated using the individual sheep or the treatment mean recovery methods. The largest differences between the estimated and observed total intake were 2.6 g and 19.2 g, respectively, representing only 0.4% and 2.6% of the total intake. However, they were significantly (*$P < 0.05$*) biased for most diets when using the overall recovery method. Due to the difficulty in obtaining individual sheep recovery under field conditions, treatment mean recovery is recommended. This study suggests that* M. sativa*, a natural roughage instead of a labelled concentrate, can be utilized as a dietary supplement to accurately estimate the total feed intake of sheep indoors and further indicates that it has potential to be used in steppe grassland of northern China, where the marker patterns of* M. sativa *differ markedly from commonly occurring plant species.*

Keywords: alkanes, long-chain alcohols, Mongolian sheep, intake, *Medicago sativa* supplement

Implications

Developing methodologies to assess the nutrition of grazing herbivores is crucial for sustainable grazing management. Studies have attempted to estimate the feed intake of herbivores by administering controlled amounts of supplements labelled with external alkanes. However, the practical application of this remains challenging due to labelling procedure complexities and associated varying faecal recoveries. Our study firstly reports the utilization of alfalfa, which is characterized by distinct marker patterns in comparison to common plant species in steppe grassland, to accurately estimate the feed intake of indoor sheep. This study provides a potential method for monitoring the nutrition of grazing herbivores.

Introduction

Estimating the feed intake of grazing herbivores is critical for determining their nutrition, overall productivity and utilization of grassland resources. Various techniques, such as

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the cage technique, behavioural observations and indigestible markers (e.g. TiO_2 and Cr_2O_3; Holechek *et al*., 1982; Gordon, 1995), have been developed for estimating feed intake. However, assessing feed intake under grazing conditions remains a challenge, and these earlier methods were not always considered reliable due to the inherent complexity of grassland conditions or other numerous sources of errors (Dove and Mayes, 2005; Smit *et al*., 2005).

By the late 1990s, a plant wax marker technique was developed to estimate feed intake and dietary composition by dosing animals with a known amount of even-chain synthetic alkanes and relating the marker pattern in the faeces to marker patterns found in herbivorous forage (Dove and Mayes, 1991; Undi *et al*., 2008). Based on this technique, Dove and Oliván (1998) proposed that total feed intake could be estimated if animals were fed with a controlled amount of a dietary supplement. This method followed the logic that if (a) the amount of the supplement fed to the animals was known and (b) the proportion of this supplement in the diet could be estimated using the plant wax marker technique, then total feed intake could possibly be estimated (Dove and Oliván, 1998). However, many previously used supplements, such as solvent-extracted oilseed meal and wheat, contained little or no alkanes, and therefore beeswax, as one plant wax marker, was added to these supplements for estimating their dietary proportions (Dove and Oliván, 1998; Elwert *et al*., 2004). Faecal recovery values from diets containing different dietary proportions of beeswax-labelled supplements varied due to the weaker bonds between the surface of the supplements and beeswax alkanes, while a stronger bond exists between roughages and their native cuticular alkanes (Elwert and Dove, 2005). This may lead to poor estimation of diet composition (Morais *et al*., 2011; Ferreira *et al*., 2018) and subsequently, feed intake. Therefore, in order to avoid adding beeswax to the supplements and to decrease the variation in faecal recovery values, it is necessary that other supplements containing high concentrations of natural plant wax markers are developed for estimating feed intake.

The estimation of dietary composition using the plant wax marker technique relies on the hypothesis that the profiles of the plant wax markers differ markedly among the other forage species in the diet (Dove and Mayes, 1991). Therefore, according to this hypothesis, using a supplement that exhibits distinguishable marker patterns among dietary forage species could increase the accuracy of diet composition estimation. *Leymus chinensis* grassland is the most important grazing land in northern China where livestock farming contributes predominantly to the livelihood of the local people (Ma *et al*., 2014). Accordingly, studying the intake and dietary composition of the grazing livestock is of essential economic importance for the sustainable development of local animal husbandry. In this grassland, Lin *et al.* (2012) found that the majority of consumed forage species by grazing sheep showed distinctly different long-chain alcohol (**LCOH**) patterns as compared to *Medicago sativa*. Specifically, C26-alcohol or C28-alcohol was the most abundant LCOH of these forage species, while C30-alcohol was the predominant LCOH of *M. sativa*. *Medicago sativa* is widely used as a supplemental protein feed for herbivores (Horney *et al*., 1996) and supports a high level of performance (Turner *et al*., 2005). Hence, it seems that *M. sativa* has potential use as a natural supplement for estimating the dietary components of herbivores and thus their total intake.

In this study, a 17-day indoor feeding experiment was carried out in northern China. We tested whether the dietary proportion of *M. sativa* and subsequently, total feed intake, could be accurately estimated in diets containing other plant species that commonly appear in typical steppes. We hypothesized that *M. sativa* could be used as a tool for estimating feed intake because it exhibits a distinct plant wax marker pattern in comparison to other forage species present in the diets. If the method works well, this study could possibly provide a basis for estimating total feed intake under field conditions.

Material and methods

This study was performed at the National Grassland Ecosystem Research Station (longitude 115°46′N, latitude 41°44′N) in Hebei province China. This area is characterized as a typical temperate zone where *L. chinensis* is the dominant grassland species. *Phragmites australis*, *Puccinellia distans and Artemisia scoparia* also appear frequently in the grasslands.

Animals and diets

A total of 16 Mongolian sheep (6 to 8 months old, BW 30 ± 0.7 kg (mean ± standard error)) were randomly selected and housed separately in metabolism cages in a 17-day experiment. The sheep were randomly allocated to four diets (four sheep per diet) containing between two and four types of forage, that is, Gramineae (*L. chinensis*, *P. distans*, *P. australis* and *Elymus sibiricus*), Leguminosae (*M. sativa*), Chenopodiaceae (*Chenopodium album*) and Asteraceae (*A. scoparia* and *Artemisia tanacetifolia*). The forage species selected for the experimental diets followed the advice of local herdsmen; thus they were regarded as the typical dietary components of sheep in this region. Considering that *M. sativa* exhibits a distinguished plant wax marker pattern and is also rare in the local experimental area, it was thus used as the supplement in this study.

Medicago sativa was harvested within a day at the initial bloom stage from the local monoculture grassland. It was then naturally air-dried, ground, mixed with water and then processed into pellet feed, which was made prior to the feeding experiment. During the entire process, we ensured that no impurities were added into the pellets. Fresh forages were separately collected daily from local natural grasslands and chopped into particles of less than 2 cm length to avoid that sheep would select particular forage species or leaves or stems (Lin *et al*., 2007). All the dietary components were weighed separately according to the required dietary

Table 1 *Proportion (%) and actual intake (g DM/day) of each component, nutritive value and dry matter digestibility in diets 1, 2, 3 and 4 that offered to sheep*

Dietary component	Diet 1		Diet 2		Diet 3		Diet 4	
	Proportion	Intake	Proportion	Intake	Proportion	Intake	Proportion	Intake
Medicago sativa	21.3	157	21.7	157	21.0	157	21.6	157
Leymus chinensis	41.9	307	42.5	307	35.6	266	22.7	165
Puccinellia distans	36.8	270	22.6	163	12.8	95	10.7	78
Phragmites australis	/	/	13.1	95	10.9	81	8.7	63
Chenopodium album	/	/	/	/	15.1	113	12.4	90
Elymus sibiricus	/	/	/	/	4.6	35	4.8	35
Artemisia scoparia	/	/	/	/	/	/	10.0	73
Artemisia tanacetifolia	/	/	/	/	/	/	9.1	66
Total	100	734	100	722	100	747	100	726
MEI (MJ/kg $BW^{0.75}$/day)	0.48		0.46		0.49		0.48	
CPI (g/kg $BW^{0.75}$/day)	5.97		5.99		6.36		6.24	
DMD (%)	58.9		59.2		61.2		61.6	

MEI=metabolizable energy intake; **CPI**=crude protein intake; **DMD**=dry matter digestibility.
Metabolizable energy (ME)=0.82×(gross energy – faecal energy), gross energy and faecal energy were measured by adiabatic bomb calorimetry.
/ indicates no data.

composition and their water contents, which were determined before the feeding experiment. The fresh forages were then mixed thoroughly and prepared for feeding to the sheep the following day. The proportion and actual intake of each forage and the nutritive value and DM digestibility of each diet are presented in Table 1. Gross energy of each diet and faecal energy of each sheep were measured by adiabatic bomb calorimetry (Parr 6400, USA) and metabolizable energy (ME) in each diet was calculated by the equation (National Research Council (NRC), 2007): ME = 0.82 × (gross energy – faecal energy). ME intake in each diet could meet the maintenance requirements of sheep for energy (0.45 MJ/kg $BW^{0.75}$/day; NRC, 2007).

During the 10-day adaptation period, we adjusted the amount of dried *M. sativa* pellets and fresh forages offered to the sheep according to the residues to ensure that the residual feed amount was less than 5% (Brosh *et al.*, 2003). During the 7-day sampling period, a given amount (156 g DM/day) of *M. sativa* pellets were supplied to the sheep in each diet twice a day (at 0900 h and 1600 h), and after the offered pellets were consumed entirely, sheep were fed the mixed fresh forages. They had free access to water and mineral blocks during the experimental period. Fixed plastic feed containers were placed in front of the metabolism cages to supply the feed, and the containers were large enough to minimize the spoilage and spilling of the rations. Moreover, supervising technicians were present all day to collect the spilled feed and return it to the corresponding container. This process ensured minimal forage residue (Lin *et al.*, 2012). Residues were therefore not evaluated for their botanical compositions or chemical compositions. Nylon mesh was placed under each metabolism cage to collect all the faeces of each sheep.

Sample collection and preparation

Forage samples of each species were separately collected daily when weighing the dietary components, following which they were immediately dried at 65°C for 48 h in a forced-air oven to measure their water content. Total faecal output was collected from the nylon mesh daily at 0800 h. Fresh faeces were weighed and a representative sample (20% of the total) was collected. Residues were also recorded daily at 1900 h when the sheep had finished eating. After collection, the faeces and residue samples were immediately dried at 65°C for 48 h in a forced-air oven to measure their water content and subsequently compute the actual intake and faecal output (in unit of DM). Furthermore, subsamples of plants and faeces were pooled per forage species and sheep, respectively, and then ground through a 1 mm mesh for alkane and LCOH analysis.

Extraction and analysis of alkanes and alcohols

Alkane and LCOH concentrations of the individual plant species and faecal samples were extracted and analysed according to the methods of Lin *et al.* (2007 and 2009). Briefly, the method involved saponification (KOH), followed by solvent separation, purification through silica-gel columns and analysis by gas chromatography. The details are provided in Supplementary Material S1.

Calibration

Standard solutions, containing a combination of synthetic alkanes (C22-, C23-, C25-, C27-, C29-, C31-, C33- and C34-alkanes) dissolved in heptane, or alcohols (C20-, C22-, C24-, C26-, C27-, C28- and C30-alcohols) (Dr Ehrenstorfer GmbH, Augsburg, Germany) dissolved in heptane and ethanol at the volume ratio of 1 : 1, were made respectively at five concentrations (0.01, 0.02, 0.04, 0.08 and 0.16 mg/ml) according to Lin *et al.* (2012). The response factors for the concentrations of individual alkanes and LCOHs were computed from the peak areas according to the previously built linear regression curve between peak areas and standard solutions with varying concentrations. Extraction rate was calculated from internal standards (C22- and

C34-alkanes or C27-alcohol) and subsequently, the alkane and LCOH concentrations in the samples were calculated by referring to the extraction rates of C22- and C34-alkanes and C27-alcohol, respectively, as described in detail by Lin *et al.* (2012).

Calculations

Considering that the average concentrations of C23-, C25- and C33-alkanes and C20-alcohols were less than 50 mg/kg DM across all diet components, these markers were thus discarded in the diet composition estimation. This processing procedure could increase the accuracy of the diet composition estimation (Brosh *et al.*, 2003; Lin *et al.*, 2007). Moreover, one marker type may be insufficient for estimating the diet composition of diets 3 and 4. Therefore, faecal concentrations of both alkanes (C27-, C29- and C31-alkanes) and LCOHs (C22-, C24-, C26-, C28- and C30-alcohols) were utilized to estimate the sheep dietary composition across the four diets.

The faecal recovery rate of each alkane or LCOH was the ratio of the amount of alkane or LCOH in the faeces to the actual amount consumed in the ration (Lin *et al.*, 2012). Alkane and LCOH concentrations in the faeces were corrected using each of the three methods: (1) using recovery data for the individual sheep (individual sheep recovery), (2) using the mean recovery of sheep within a diet (treatment mean recovery) and (3) using the mean recovery across all dietary treatments (overall recovery). After correcting for faecal recovery, the diet composition was estimated using a non-negative least-square procedure ('Eatwhat' software, Dove and Moore, 1995).

Total intake was estimated using the 'supplement' method (Elwert and Dove, 2005). Specifically, the estimated proportion of supplement (P_s) in the diet and the known intake of supplement (I_s) offered to sheep were used for estimating total intake. The equation was:

$$\text{Total intake} = \frac{I_s}{P_s}$$

Estimated individual intakes of forages were calculated using the estimated proportion of a given forage (P_f) in the diet and the estimated total intake. The equation was:

$$\text{Intake of forage f} = \left(\frac{I_s}{P_s}\right) \times P_f$$

The accuracy of the diet composition estimates across all diet components was evaluated using the Kulczyński similarity index (**KSI**, in %), and this index calculated the intersection between the observed and estimated diet composition according to different faecal recovery methods (Lin *et al.*, 2012):

$$\text{KSI} = 100 \times \frac{\sum 2c_i}{\sum (a_i + b_i)}$$

where a_i and b_i are the observed and estimated diet composition of component i, respectively; c_i is the lesser proportion of component i in the observed and estimated diet composition (i.e. if $a_i > b_i$, then $c_i=b_i$; if $a_i < b_i$, then $c_i=a_i$); and (a_i+b_i) is the sum of proportions of each forage component in the observed and estimated diet composition.

The accuracy of *M. sativa* proportion and total intake were evaluated using the mean prediction error (**MPE**; Elwert and Dove, 2005), which was calculated by summing the square of the differences between the estimated and observed values, dividing by the replicates in the treatment and taking the square root of the quotient:

$$\text{MPE} = \sqrt{(1/n \times \sum (\text{estimated} - \text{observed})^2)}$$

The accuracy of individual species intake was evaluated by the mean relative prediction error (**MRPE**, in %; Elwert and Dove, 2005):

$$\text{MRPE} = \sqrt{\left(1/n \times \sum \left(\frac{\text{estimated} - \text{observed}}{\text{observed}}\right)^2\right)}$$

Statistical analysis

Statistical analysis was conducted using SAS (V. 8.1) (SAS Institute Inc., Cary, NC, USA). To assess differences among forage species according to their alkane and LCOH concentration profiles, log-transformed [log (1+x)] concentrations of individual alkanes and LCOHs of each species were subjected to principal component analysis (**PCA**). Most of the between-species variance in alkane and LCOH profiles was explained by the first two principal components. The correlation matrix was used to calculate PCA scores. In the graph, principal component score 1 (**PC1**) was plotted against principal component score 2 (**PC2**), and different plant species are represented by the points on the graph. One-way ANOVA followed by Duncan's method was used to assess the effect of diet composition on the faecal recovery rates of alkanes and LCOH. Paired *t*-tests were used to compare the differences between the observed and estimated values of the proportions of *M. sativa* and total intake. Moreover, the influence of diet and method for faecal recovery, and their interactions, on KSI values were assessed by two-way ANOVA.

Results

Marker profiles of the plant species

Medicago sativa contained the highest concentration of C31-alkanes and C30-alcohols, while the concentrations of C26- and C28-alcohols were generally lower than in all the other forages (Table 2). PCA revealed that the first two principal components accounted for the largest differences between forages, explaining 71% (PC1 and PC2 explained 44.9% and 26.1%, respectively) of the total variance among forage

Table 2 *Concentration (mg/kg DM) of individual n-alkanes and long-chain alcohols within each forage species consumed by sheep*

	n-alkanes(mg/kg DM)							Long-chain alcohols(mg/kg DM)						
Species	C23	C25	C27	C29	C31	C33	Total[1]	C20	C22	C24	C26	C28	C30	Total[1]
Leguminosae														
Medicago sativa	5	14	34	132	306	22	472	19	45	48	75	260	2861	3244
Gramineae														
Leymus chinensis	1	9	14	27	77	19	118	14	20	59	340	363	231	993
Puccinellia distans	10	27	39	80	158	26	277	35	19	30	1478	485	380	2373
Phragmites australis	10	21	38	33	33	10	104	33	42	35	241	1860	2143	4279
Elymus sibiricus	8	16	27	132	193	44	352	23	20	91	1600	391	271	2353
Chenopodiaceae														
Chenopodium album	6	29	99	345	125	36	569	26	39	119	612	1753	734	3218
Asteraceae														
Artemisia scoparia	1	16	65	201	132	25	398	90	186	436	174	227	279	1116
Artemisia tanacetifolia	33	56	116	278	177	22	571	65	232	439	199	567	352	1557

[1] Total concentration of alkanes was calculated from C27-alkane, C29-alkane and C31-alkane and total concentration of long-chain alcohols was calculated from C22-alcohol, C24-alcohol, C26-alcohol, C28-alcohol and C30-alcohol. These above-mentioned markers were used in the estimation of diet composition.

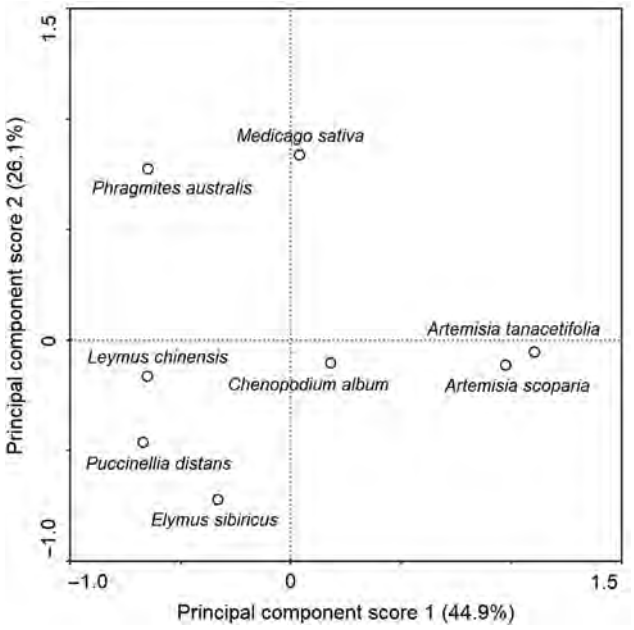

Figure 1 Biplots of the first and second principal component scores from the principal component analysis using the concentrations of C27, C29 and C31 alkane markers and C22, C24, C26, C28 and C30 long-chain alcohol markers in eight forage species in the sheep diets.

species. Furthermore, *M. sativa* was separated from most of the forages in the diet in both PC1 and PC2 (Figure 1).

Recovery of alkanes and long-chain alcohols in sheep faeces

Alkane recovery rates, ranging from 0.71 (C27-alkane) to 0.75 (C31-alkane, Figure 2a), were not significantly influenced by diet composition and increased with increasing alkane carbon-chain length. However, with the exception of C30-alcohol, the faecal recovery values of all other

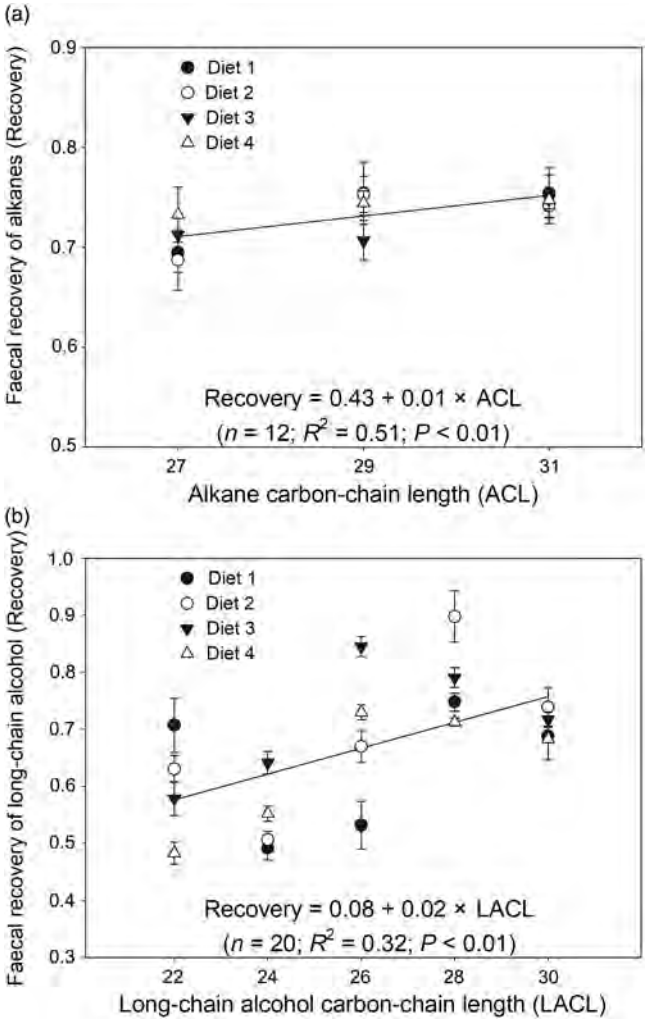

Figure 2 Relationships between (a) alkane carbon-chain length (ACL) or (b) long-chain alcohol carbon-chain length (LACL) and their faecal recovery (Recovery, mean ± standard error) of sheep fed diets 1, 2, 3 and 4.

Table 3 *Observed and estimated proportions of dietary component (mean ± standard error) in diets 1, 2, 3 and 4 using faecal recovery methods based on individual sheep recovery, treatment mean recovery and overall recovery as well as the mean prediction error (MPE) of* Medicago sativa

Diet	Recovery method	Diet composition (%)								MPE of *M.sativa*
		Leymus chinensis	*Puccinellia distans*	*Phragmites australis*	*Chenopodium album*	*Elymus sibiricus*	*Artemisia scoparia*	*Artemisia tanacetifolia*	*M. sativa*	
1	Observed	41.9	36.8						21.3	/
	Individual[1]	41.9 ± 0.03	36.8±0						21.3±0	0.03
	Treatment[1]	41.8±2.66	36.9±4.54						21.3±1.98	3.43
	Overall[1]	55.7±1.44	22.8±3.28						21.5±1.98	3.43
2	Observed	42.5	22.6	13.1					21.7	/
	Individual[1]	42.8±0.09	22.5±0.05	13.1±0.04					21.7±0	0.02
	Treatment[1]	42.6±2.99	22.6±2.17	13.2±2.21					21.7±0.52	0.90
	Overall[1]	43.9±2.76	19.5±2.07	17.2±2.28					19.5±0.36*	2.33
3	Observed	35.6	12.8	10.9	15.1	4.6			21.0	/
	Individual[1]	36.2±0.05	13.4±0.09	10.6±0	15.1±0.03	3.9±0.07			20.9±0	0.07
	Treatment[1]	34.4±3.23	9.3±3.50	11.9±2.38	14.9±0.85	8.5±4.93			21.0±0.41	0.71
	Overall[1]	29.4±2.80	2.4±1.49	16.5±1.87	9.3±0.83	23.9±3.01			18.7±0.38*	2.42
4	Observed	22.7	10.7	8.7	12.4	4.8	10.0	9.1	21.6	/
	Individual[1]	23.2±0.15	11.3±0.64	8.5±0.16	12.5±0.10	3.9±0.71	10.2±0.39	8.8±0.41	21.5±0.03	0.07
	Treatment[1]	22.1±3.29	5.3±3.90	10.1±1.50	11.4±1.13	10.6±4.04	6.8±2.78	12.3±3.28	21.6±0.33	0.57
	Overall[1]	17.8±3.13	10.5±3.84	5.7±1.22	15.2±0.53	8.1±3.43	19.2±1.19	0	23.6±0.34*	2.14

[1] Diet compositions were estimated using individual sheep recovery, treatment mean recovery and overall recovery methods.
*The estimated proportion of *M. sativa* differed significantly ($P<0.05$) from the observed value;
/ indicates no data

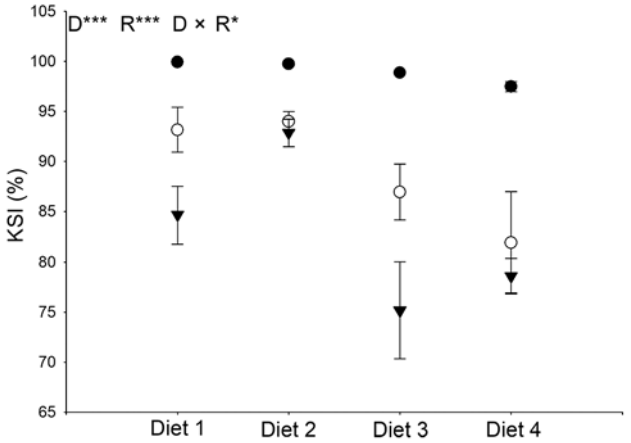

Figure 3 Kulczyński similarity index (KSI, %, mean ± standard error) between the observed and estimated diet composition of diets 1, 2, 3 and 4 using faecal recovery based on the recovery of individual sheep (closed circles), treatment mean (open circles) and overall diets (closed triangle). D: diet; R: faecal recovery correction; D × R: the interaction of diet and faecal recovery correction. Asterisks * and *** indicate significant differences at the levels of $P < 0.05$ and 0.001, respectively.

LCOHs were significantly ($P < 0.05$) influenced by diet composition. Faecal recovery values of the LCOHs, ranging from 0.55 to 0.79, exhibited an increasing tendency with increasing carbon-chain length (Figure 2b).

Estimation of diet composition, total feed intake and individual component intake

When the diet composition was estimated using individual sheep recovery, the differences between the observed and estimated proportions were negligible (0% to 0.8%, Table 3). Using the treatment mean recovery method, the differences between the observed and estimated diet composition were larger (≤5.8%, Table 3) than that using individual sheep recovery. When the diet composition estimates were based on overall recovery, the differences between the observed and estimated proportions increased further (≤19.2%, Table 3). The results of the two-way ANOVA showed that diet, faecal recovery method and their interactions significantly ($P < 0.05$) affected KSI values (Figure 3). The overall KSI values of diet 1 (92.6%) and diet 2 (95.5%) were significantly ($P < 0.001$) larger than that of diet 3 (87.0%) and diet 4 (86.0%). Kulczyński similarity index values decreased significantly ($P < 0.001$) from individual sheep recovery (99.0%), to treatment mean recovery (89.0%) and to overall recovery (82.8%).

When the diet composition was estimated using individual sheep or treatment mean recovery, the differences between the estimated and observed proportions of *M. sativa* and total intake were not significant ($P > 0.05$; Tables 3 and 4). Moreover, the greatest differences between estimated and observed total intake based on individual sheep recovery and treatment mean recovery across all diets were 2.6 g and 19.2 g, respectively, representing only 0.4% and 2.6% of the total intake, respectively (Table 4). However, when overall recovery was used, three of four estimated proportions of *M. sativa* and total intake differed significantly ($P < 0.05$) from the observed values. Except for diet 1, the MPE values of total intake estimated by overall recovery were greater than the corresponding values obtained using individual sheep or treatment mean recovery.

Table 4 *Observed and estimated total intake (g DM/day; mean±standard error) and mean prediction error (MPE) in diets 1, 2, 3 and 4 using faecal recovery methods based on individual sheep recovery, treatment mean recovery and overall recovery*

	Diet 1		Diet 2		Diet 3		Diet 4	
Recovery method	Total intake	MPE	Total intake	MPE	Total intake	MPE	Total intake	MPE
Observed	734	/	722	/	747	/	727	/
Individual sheep recovery	735±0	1.2	722±0	0.5	750±0	2.6	728±0.8	2.2
Treatment mean recovery	753±62.0	109.1	723±17.4	30.2	748±15.1	26.2	726±11.1	19.3
Overall recovery	746±60.9	106.1	806±15.0*	87.8	841±17.5*	99.0	664±9.6*	64.8

*The estimated total intake that differed significantly ($P<0.05$) from the observed value.
/ indicates no data.

For individual component intake within a diet, MRPE values increased generally from individual sheep recovery to treatment mean recovery, and to overall recovery (Table 5). According to the treatment mean or overall recovery estimates, the mean MRPE values of diet 3 or 4 were two- to four-fold higher than the corresponding values of diet 1 or 2. The MRPE values for the estimated intake of *E. sibiricus, P. distans, A. scoparia* and *A. tanacetifolia* were always larger than the corresponding values of the other dietary components.

Discussion

In the present study, *M. sativa* showed distinct patterns from the other forage species across all diets (Figure 1). Our study demonstrated that accurate estimations of *M. sativa* proportions as well as total feed intake based on individual sheep or treatment mean recovery could be obtained across all diets. However, considering that individual sheep recovery is challenging under field conditions, treatment mean recovery is thus more preferable (Charmley and Dove, 2007; Elwert *et al.*, 2008). Overall, our study showed that *M. sativa* may be used as a natural supplement for accurately estimating total feed intake under grazing conditions.

Alkanes and long-chain alcohols concentrations of Medicago sativa

Compared to the other dietary components, *M. sativa* contained the highest concentration of C31-alkanes and C30-alcohols, but generally lower concentrations of C26- and C28-alcohols (Table 2), which was also observed by Lin *et al.* (2012) and may explain the separation of *M. sativa* from most of the dietary components in the PCA (Figure 1). Furthermore, the estimation of diet composition using the alkane technique relies on the hypothesis that the profiles of the markers differ markedly among forage species (Dove and Mayes, 1991). Therefore, *M. sativa* has the potential to be successfully distinguished from other species in the diet.

Recovery of alkanes and long-chain alcohols in the faeces

Mean faecal recovery rates of alkanes (0.71 to 0.75) and LCOHs (0.55 to 0.79) were within the ranges (0.58 to 0.90 and 0.37 to 0.90 for alkanes and LCOHs, respectively) of those observed in the previous studies (Dove and Mayes, 2005; Charmley and Dove, 2007; Lin *et al.*, 2012). Our results also confirmed the previous findings that the faecal recovery rates of alkanes or LCOHs increase with increasing carbon-chain lengths in sheep (Lin *et al.*, 2007; Dove and Charmley, 2008; Ferreira *et al.*, 2018).

In our study, faecal alkane recovery rates were not affected by diet composition, as reported previously (Dove and Mayes, 1991; Elwert *et al.*, 2004; Dove and Charmley, 2008). However, except for C30-alcohols, the effect of diet composition on the faecal recovery rates of other LCOHs was significant ($P < 0.05$), which contradicted earlier studies (Dove and Charmley, 2008; Lin *et al.*, 2012). A possible explanation is that there were substantial differences between these studies in terms of diet composition, such as forage species, number of diet components and the proportion of each component, as earlier studies reported that faecal marker recovery was significantly influenced by forage species (Lin *et al.*, 2007; Elwert *et al.*, 2008) and diet digestibility (Ferreira *et al.*, 2015 and 2018). In our study, the digestibility among the four diets did not differ significantly ($P = 0.24$, Table 1), and thus, the forage species consumed by the animals might be the primary factor accounting for the variations in faecal marker recoveries for LCOHs (Lin *et al.*, 2012). Introducing new forages to the existing diet could obviously modify the proportion of markers contributed by each forage (Elwert *et al.*, 2004). Our results highlighted that diet composition exerted a significant impact on faecal recovery for LCOHs.

Estimation of diet composition, total feed intake and individual component intake

The variance in faecal marker recovery increased progressively from individual sheep to treatment mean, and to overall recovery (Charmley and Dove, 2007), and a more accurate estimation of dietary composition could be obtained using faecal recovery values with less variance (Ferreira *et al.*, 2018). Therefore, the accuracy of the estimated diet composition and dietary proportions of *M. sativa* decreased significantly from individual sheep recovery to treatment mean recovery, and to overall recovery. The accurate estimation of *M. sativa* proportion and feed intake relies on accurate diet

Table 5 *Observed and estimated individual component intake (g DM/day; mean±standard error), mean relative prediction error (MRPE, %) and the mean of MRPE (%, averaged across dietary components within a diet except for* Medicago sativa*) in diets 1, 2, 3 and 4 using faecal recovery methods based on individual sheep recovery, treatment mean recovery and overall recovery*

		Diet components														
		Leymus chinensis		*Puccinellia distans*		*Phragmites australis*		*Chenopodium album*		*Elymus sibiricus*		*Artemisia scoparia*		*Artemisia tanacetifolia*		
Diet	Recovery method	Intake	MRPE	Intake	MRPE	Intake	MRPE	Intake	MRPE	Intake	MRPE	Intake	MRPE	Intake	MRPE	Mean of MRPE
1	Observed	307	/	270	/											/
	Individual[1]	308±0.2	0.2	270±0	0.2											0.2
	Treatment[1]	310±14.0	7.9	286±51.4	33.5											20.7
	Overall[1]	413±27.8	38.0	176±34.3	41.3											39.6
2	Observed	307	/	163	/	95	/									/
	Individual[1]	309±0.6	0.7	162±0.4	0.6	95±0.3	0.7									0.7
	Treatment[1]	309±26.8	15.2	163±18.3	19.5	94±13.7	25.1									19.9
	Overall[1]	354±25.7	21.2	157±19.1	20.6	137±16.0	53.2									31.7
3	Observed	266	/	95	/	81	/	113	/	35	/					/
	Individual[1]	271±0.4	1.9	100±0.7	5.7	79±0	1.9	113±0.2	0.4	29±0.5	16.7					5.3
	Treatment[1]	258±27.5	18.2	70±26.7	55.3	89±17.3	38.2	111±6.9	10.7	63±36.4	197.2					63.9
	Overall[1]	248±26.9	18.8	20±13.1	82.2	138±15.1	77.5	78±7.3	32.7	200±24.1	486.2					139.5
4	Observed	165	/	78	/	63	/	90	/	35	/	73	/	66	/	/
	Individual[1]	169±1.2	2.7	83±4.8	12.1	62±1.1	3.5	91±0.8	1.7	29±5.1	31.3	74±2.9	7.2	64±2.9	8.0	9.5
	Treatment[1]	160±24.0	25.4	39±29.5	82.3	73±10.2	31.9	83±8.6	18.3	75±28.7	183.2	49±20.4	58.3	89±23.2	70.2	67.1
	Overall[1]	118±21.3	36.2	70±25.9	58.4	30±7.8	46.1	101±4.7	15.4	53±21.8	119.3	128±8.9	77.6	0	100.0	64.7

[1] Diet compositions were estimated using individual sheep recovery, treatment mean recovery and overall recovery methods.
/ indicates no data.

composition estimates, which did require the correction of faecal marker concentrations for their incomplete faecal recovery (Dove and Charmley, 2008). Our results showed that total feed intake could be accurately estimated by using individual sheep recovery or the treatment mean recovery method (Table 4). However, as dietary composition significantly affected the faecal recovery values for LCOHs, the overall recovery of LCOHs containing the variances in recovery attributable to diet deviated largely from the treatment mean recovery. Therefore, overall recovery only accurately estimated one of the four diets. Estimations based on individual sheep recovery were most accurate; however, this is difficult to determine under field conditions (Charmley and Dove, 2007; Elwert *et al.*, 2008). Although treatment mean recovery led to a small reduction in accuracy in comparison to individual sheep recovery, this had a negligible effect, as the largest difference between estimated and observed total intake across all diets was only 19.2 g, representing only 2.6% of total intake. Treatment mean recovery is thus recommended.

In this study, the accuracy of both diet composition and individual component intake estimates in diets 3 and 4 declined markedly in comparison to diets 1 and 2, which was possibly explained by the increasing similarity in wax marker patterns among forage species with more forage species in the diet (Dove and Mayes, 2005). In diet 3, a strong correlation ($r = 0.99$) was found between the marker patterns of *P. distans* and *E. sibiricus*, which might explain why the KSI values of diet 3 were lower in comparison to diet 2. This trend is similar to that reported by Dove and Charmley (2008) in a study that differentiated *Phalaris* from ryegrass with similar LCOH patterns and thus reduced KSI values. Additionally, *A. scoparia* and *A. tanacetifolia* shared similar marker profiles as they belong to the same genus (Lin *et al.*, 2007), which further decreased the KSI values of diet 4. In contrast to the above-mentioned species, *M. sativa* could be clearly distinguished from other species in the diet, and subsequently, its dietary proportion could be accurately estimated using the treatment mean recovery method despite seven other forages being present in the diet. This indicates that *M. sativa* can be recommended as a natural supplement for accurately estimating total feed intake, exhibiting potential use in naturally grazed grasslands. Additionally, the estimated intakes of *E. sibiricus* and *P. distans* deviated largely from the observed values, but the estimated total intakes of *E. sibiricus* and *P. distans* were similar to the corresponding observed values. A similar phenomenon was also observed for *A. scoparia* and *A. tanacetifolia* in diet 4 (Table 4). From this aspect, when species with similar marker profiles are present in one diet, the total intake of these species rather than individual species intake could be accurately estimated (Lin *et al.*, 2007).

This study describes a first step towards the establishment of a field-based method for estimating feed intake. *Medicago sativa* may be used for estimating grazing animal intake using the following guidelines: (a) *M. sativa* is fed individually and quantitatively, (b) the faecal marker concentrations of grazing animals should be corrected according to the faecal marker recoveries of indoor animals given similar diets and (c) rectal grab samples should be obtained from individual animals twice daily (Ma *et al.*, 2014) as the faecal marker concentration in the grab sample is representative of the total faecal output (Morais *et al.*, 2011). Moreover, our study suggested that not only *M. sativa* but also other forage species could be used for accurately estimating herbivore intake provided that the supplemented forage is characterized by distinct patterns of plant wax markers.

As similar dietary proportions of *M. sativa* were utilized in this study, it is unknown whether faecal recovery values would vary in diets containing different proportions of *M. sativa*. Meanwhile, the ultimate aim of this study is to apply this method to the field. Therefore, further studies, such as those utilizing various dietary proportions of natural supplements and comparing the method using even-chain synthetic alkanes under field conditions, are required to validate the reliability of this method.

In summary, our results revealed that *M. sativa*, a natural roughage instead of a labelled concentrate, demonstrates potential use as a dietary supplement for accurately estimating the total feed intake of indoor sheep using the plant wax marker technique. The treatment mean recovery method could be used as a reliable tool for estimating sheep feed intake, and this technique has potential to be applied in steppe grassland of northern China where the marker patterns of *M. sativa* and commonly occurring plant species are markedly different.

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Declaration of interest

None.

Ethics statement

This experiment obtained permission from the China Agricultural University Laboratory Animal Care Advisory Committee.

Software and data repository resources

Data were not deposited in an official repository.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731119001381.

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animal

Effect of supplementing sheep diets with macroalgae species on *in vivo* nutrient digestibility, rumen fermentation and blood amino acid profile

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In this study, a brown macroalgae species, Saccharina latissima, *processed to increase its protein concentration, and a red macroalgae species,* Porphyra spp.*, were used to evaluate their* in vivo *digestibility, rumen fermentation and blood amino acid concentrations. Four castrated rams were used, whose diets were supplemented with a protein-rich fraction of* S. latissima*, a commercial* Porphyra spp. *and soybean meal (SBM). Our results show that the protein digestibility of a diet with* S. latissima *extract was lower (0.55) than those with* Porphyra spp. *(0.64) and SBM (0.66). In spite of the higher nitrogen (**N**) intake of diets containing* Porphyra spp. *and SBM (20.9 and 19.8 g N/day, respectively) than that with* S. latissima *(18.6 g N/day), the ratio of N excreted in faeces to total N intake was significantly higher in the diet with* S. latissima *than those with* Porphyra spp. *and SBM. This reflects that the utilization of protein in* S. latissima *was impaired, possibly due to reduced microbial activity. The latter statement is corroborated by lower volatile fatty acid composition (25.6, 54.8 and 100 mmol/l for* S. latissima, Porphyra spp. *and SBM, respectively) and a non-significant tendency for lower ammonia concentration observed in diets with* S. latissima *and* Porphyra spp. *compared to SBM. It is important to note that the* S. latissima *used in this trial was rinsed during processing to remove salt. This process potentially also removes other water-soluble compounds, such as free amino acids, and may have increased the relative fraction of protein resistant to rumen degradation and intestinal absorption. Furthermore, the phlorotannins present in macroalgae may have formed complexes with protein and fibre, further limiting their degradability in rumen and absorption in small intestines. We recommend that further studies explore the extent to which processing of macroalgae affects its nutritive properties and rumen degradability, in addition to studies to measure the intestinal absorption of these macroalgae species.*

Keywords: *Saccharina latissima*, *Porphyra spp.*, digestibility, *in vivo*, ruminant

Implications

The protein concentration of seaweed varies greatly from species to species. Those with low-to-medium protein level require processing to remove the salt and to increase protein concentration. This process applied to *Saccharina latissima* species appeared to have reduced the nutrient availability, reflecting lower protein digestibility than that of *Porphyra spp.* and soybean meal. Both rumen fermentation and intestinal absorption were impaired in animals fed *S. latissima*. If the limitations in processing can be fathomed, *S. latissima* may present a potential biomass source that is abundant in nature; however, the challenges in its palatability and chemical composition should be addressed.

Introduction

Future projections estimate an increase in the import of soybean in Norway by 35% in 2050 compared to 1961 to 1990 levels, due to increasing demand for food and protein feed (Özkan Gülzari *et al.*, 2017). This threatens the competitiveness of ruminant production systems and urges the need for alternative protein sources with local origin. For this purpose, macroalgae (also known as seaweed) present a significant and yet potential biomass source. Currently, the brown macroalgae *Saccharina latissima* constitutes the major

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cultivated species in Europe. Despite its relatively low protein concentration (50–150 g/kg DM), large-scale cultivation may provide significant amounts of protein for use in animal feed. Red macroalgae contain a higher protein concentration than brown macroalgae, but cultivation technology does not yet exist for native, Northern European species. However, red macroalgae (e.g. *Porphyra spp.*) are the largest source of food among macroalgae, for example, nori in Japanese sushi (Makkar *et al.*, 2016), and contain up to 347 g/kg DM protein (Tayyab *et al.*, 2016).

A number of studies have investigated the digestibility of brown and red macroalgae. An *in vitro* trial measuring the organic matter (**OM**) digestibility of *S. latissima* reported digestibility as high as 0.97 in sheep (Makkar *et al.*, 2016). A previous study by Ramin *et al.* (2017) found that increasing the proportion of a protein-enriched fraction of *S. latissima* in an *in vitro* trial increased both the OM digestibility and utilizable protein concentration. *Porphyra spp.* were tested by Tayyab *et al.* (2016) *in situ*, and their crude protein concentration was found to be comparable to oilseed by-products such as sunflower meal and rapeseed meal. However, given that the aforementioned studies are either *in vitro* or *in situ*, there seems to be a lack of studies investigating the *in vivo* digestibility of macroalgae, and the extent to which they may provide comparable nutrient characteristics to soybean meal (**SBM**) for ruminants. To fill this gap, in this study we evaluated the *in vivo* digestibility, rumen fermentation parameters and amino acid composition in plasma of a diet supplemented with *S. latissima* protein extract or *Porphyra spp.*, and compared them with a diet containing SBM. Due to its high salt and iodine-concentration, the biomass of *S. latissima* was exposed to a simple processing by which salt concentration was removed and protein concentration was enriched. The *Porphyra spp*, a commercial product produced in large quantities in Asia, were included for comparison; and an extracted SBM was used as reference.

We hypothesise that the protein digestibility and utilization of diets containing extracted *S. latissima* and purchased *Porphyra spp.* are similar to that of SBM, and better than the diet without any additional protein source.

Materials and methods

Experimental design and animals

An *in vivo* digestibility trial was run with four wethers of Norwegian White Sheep, 30 months of age and 80 to 88 kg live body weight, using four diets in a 4×4 Latin square design. The four diets were a control, a diet with protein enriched fraction of *S. latissima* (**SW1**), a diet with *Porphyra spp.* (**SW2**) (CoDo International Limited, Qingdao, China) and a diet with extracted SBM (Champion Soyapellets, Felleskjøpet, Lillestrøm, Norway). The trial was run for four periods from October to November 2017. Each period consisted of 8 days adaptation in individual pens followed by 7 days in individual metabolism crates for daily collection of urine, faeces and feed refusal, if any.

Feeding

Animals were stalled and fed a control diet containing hay, oats and mineral/vitamin pellets in individual pens for 10 days before the experiment started. Body weight was measured every other day to adjust the maintenance requirements for hay. The DM intake was restricted during the trial and corresponded to maintenance requirements of adult rams in energy as calculated according to the body weight at pre-trial period. Diets with protein enrichment (SW1, SW2 and SBM) were planned to be isocaloric and isonitrogenous (Jarrige, 1988). Adjustments were made to the pre-trial rations where oats and mineral/vitamin pellets were removed to balance the diet. In addition, to increase the palatability of those who refused to eat the macroalgae, the protein feed was mixed with a fixed amount of sugar cane molasses (Felleskjøpet Agri, Sandnessjøen, Norway) at the time of feeding. The control group also received the same amount of molasses. The chemical composition of feed ingredients and the formulation and nutritive value of the individual feed ingredients are presented in Tables 1 and 2, respectively (also see Özkan Gülzari *et al.*, 2018a). The SW1 included 3.8 g/kg DM phlorotannins as phloroglucinol, which was not analysed for other feed ingredients. Note that even though the diets were designed to be isonitrogenous, chemical analyses showed that some variation in nitrogen (**N**) concentration existed.

Animals were fed their daily allowance in two equal portions at 0800 and 1600 h. Water was freely accessible through individual drinkers in each pen and metabolism crate. Blood and rumen fluid samples were taken on the last day of each collection period. Blood samples were taken before feeding in the morning, and again 3–4 h after morning feeding. Rumen fluid samples were taken 3–4 h after morning feeding. After sampling for blood at noon, animals were handled back to the stable and were offered their adaptation diet of next period in the afternoon feeding. Any feed refusal was collected every morning during the collection period and weighed, and 10% of the total waste was stored at −18°C until further analyses.

Preparation and chemical analyses of seaweed species

Porphyra spp. were purchased in the form of powder (dried and milled) and was used without further processing. The product was stored in a clean and dry place until use. Cultivated *S. latissima* was harvested at the coast of Sør-Trøndelag County, Norway, in May–June 2016. Seawater was drained, and small stones and other impurities were removed manually. The biomass was stored in plastic bags at −20°C until further processing. To produce the protein-enriched product, 2×375 kg biomass wet weight, 750 kg in total, was milled and washed in water (60°C–70°C) to reduce the salt concentration. Alginate lyase was added for a partial degradation of alginate, to facilitate the subsequent separation by centrifugation. The solid phase ('sludge') after centrifugation was dried in a pilot-scale Forberg® Dryer (Forberg International AS, Oslo, Norway) at 40°C (product temperature during drying).

Table 1 *Chemical composition of individual feed ingredients fed to wethers (g/kg DM unless specified otherwise)*

	Feed ingredients									
	Hay		Molasses		SW1		SW2		SBM	
Item	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Ash	43	(0.5)	132	(0.4)	221	(2.7)	87	(3.7)	77	(19.1)
Kjeldahl-Nitrogen	13.3	(0.33)	10.9	(0.09)	35.8	(0.66)	60.9	(0.50)	82.1	(3.18)
Amino acids	57	(6.1)	20	(1.9)	163	(4.0)	282	(2.4)	468	(25.5)
Crude fat	10.5	(0.79)	2.0	(0.34)	18.5	(3.32)	2.5	(1.06)	16.3	(4.3)
aNDFom[1]	615	(8.6)	0	–	408	(25.1)	431	(13.5)	182	(24.0)
ADF[2]	364	(5.0)	0	–	352	(8.5)	66	(7.0)	90	(5.7)
Crude fibre	308	(4.4)	0	–	152	(2.1)	36	(6.1)	64	(3.9)
Gross energy[3], MJ/kg DM[4]	19.0	(0.08)	15.7	(0.03)	15.7	(0.05)	18.8	(0.07)	19.5	(0.51)
Iodine, mg/kg DM	–		–		1230		1.5		–	

SW1=processed *S. latissima*; SW2=*Porphyra spp.*; SBM=soybean meal.
[1] Neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash.
[2] Acid detergent fibre expressed inclusive of residual ash.
[3] Gross energy calculated from gross energy determined with bomb calorimetry of diet ingredients. MJ: megajoule.
[4] Dry matter.

Table 2 *Formulation, chemical analysis and nutritive value of experimental diets fed to wethers averaged over four periods*

	Experimental diets			
Item	Control	SW1	SW2	SBM
Ingredients (g/kg DM[1])				
Hay	961	799	854	888
Protein feed[1]	0	162	108	72
Molasses	39	39	38	40
Sum	1000	1000	1000	1000
Chemical analysis (g/kg DM)				
Organic matter	953	925	949	951
Kjeldahl-Nitrogen	13.2	16.8	18.3	18.1
Amino acids	56	73	80	85
aNDFom[2]	591	557	571	559
ADF[3]	349	347	318	330
Crude fibre	296	271	267	278
Gross energy (MJ/kg DM)[4]	18.9	18.3	18.8	18.9
Iodine (mg/kg DM)	0.04	180	0.20	0.04

SW1=diet containing processed *S. latissima*; SW2=diet containing *Porphyra spp.*; SBM=diet containing soybean meal.
[1] Dry matter.
[2] Neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash.
[3] Acid detergent fibre expressed inclusive of residual ash.
[4] Gross energy calculated from gross energy determined with bomb calorimetry of diet ingredients. MJ: megajoule.

The salt concentration in the processed biomass (SW1) was reduced from 440 to 180 g/kg DM and the iodine concentration from 5.9 to 1.2 g/kg DM. The protein concentration as total amino acids was increased from 89 to 186 g/kg DM.

Collection and storage of samples

Feed samples were collected during the adaptation and collection periods for 15 days in each period by taking a handful of each feed ingredient every day and storing them in plastic boxes, except for hay which was stored in a cardboard box to prevent humidification. At the end of each collection period, 2×100 g samples of SW1, SW2, SBM and 2×200 g samples of hay were prepared. Molasses samples were taken as 2×100 g at the end of each collection period. All feed samples were stored at −18°C until further processing.

Faeces and urine were collected and weighed from each animal on a 24-h basis after feeding each morning and stored at 2°C–3°C until the end of the collection period (7th day). Urine was collected in individual buckets. To each collection bucket for urine, 100 ml sulphuric acid was added before collection started, in order to avoid loss of ammonia. A pH indicator strip non-bleeding pH 0–6.0 (Teststrips, pH, MColorpHast™, product number: 1.09531.0001, VWR International, Merck KGaA, Darmstadt, Germany) was used to measure the acidity of urine every day. After 7 days of collection period, pooled samples of urine and faeces were weighed again as control and 10% of the control weight was taken as a subsample and stored at −18°C until further processing. The rest of the urine and faeces samples were discarded.

At the end of each collection period (7th day), blood and rumen fluid samples were taken from each animal. Blood samples were collected from the jugular vein before (at approximately 0800–0830 h) and approximately 4 h after morning feeding (at approximately 1300–1330 h) to Vacuette® EDTA tubes (product number: 454021, Greiner Bio-One, GmbH, Kremsmünster, Austria). Plasma was separated by centrifugation at 2000 rpm for 20 min, after which aliquots of two samples were removed and pooled into a glass vial to give one sample per wether per period. Plasma was stored at −80°C until further chemical analyses.

Rumen fluid samples were taken within 3–4 h after morning feeding at approximately 1200–1300 h via the esophagus, using two flexible polyvinyl chloride tubes, with diameters of around 3 and 1 cm, respectively, of which

Table 3 *Amino acid concentration in experimental rations fed to wethers*

Item	Experimental diets			
	Control	SW1	SW2	SBM
Essential AA[1] (g/kg DM[2])				
Histidine	1.1	1.5	1.6	2.0
Isoleucine	2.8	3.6	3.7	4.3
Leucine	5.0	6.5	6.9	7.3
Lysine	3.3	4.3	4.8	5.4
Methionine	0.6	1.0	0.8	0.8
Phenylalanine	3.3	4.4	4.5	5.1
Threonine	2.2	2.9	3.2	2.7
Tryptophan	0.6	0.5	0.8	0.5
Valine	3.9	5.1	5.9	5.3
Non-essential AA (g/kg DM)				
Alanine	4.5	6.1	7.9	5.8
Arginine	2.8	3.9	4.6	4.9
Aspartic acid	5.4	6.6	6.9	8.7
Cystine	0.6	1.2	2.0	0.8
Glutamic acid	6.7	8.5	8.7	12.3
Glycine	3.3	4.6	4.5	4.4
Proline	0.6	1.2	2.0	0.8
Serine	3.9	5.1	5.6	6.0
Tyrosine	1.7	2.2	2.4	2.6

SW1=diet containing processed *S. latissima*; SW2=diet containing *Porphyra spp.*; SBM=diet containing soybean meal.
[1] Amino acid(s).
[2] Dry matter.

the latter was connected to a vacuum pump and sucked approximately 50 ml rumen fluid from each animal. Collected samples of rumen fluid were filtered through an absorbent gauze and pH was measured after filtering using a pH indicator strip non-bleeding pH 7.5–14 (Teststrips, pH, MColorpHast™, product number: 1.09532.0001, VWR International, Merck KGaA, Darmstadt, Germany). Three subsamples of each rumen fluid sample were made for analysing ammonia, lactic acid and volatile fatty acids (**VFAs**) by mixing 4 ml rumen fluid with 4 ml hydrochloric acid (**HCl**) 0.5M; 4 ml rumen fluid with 0.5 ml solution A; and 0.8 ml rumen fluid with 0.5 ml solution A, respectively. Solution A included 20 g/l metaphosphoric and 4 g/l crotonic acids in 0.5M HCl for deprotonization of samples whilst HCl was used to acidify the medium. Any leftover rumen fluid was transferred to glass vials and stored as reserve. Both subsamples and the reserve rumen fluid samples were stored at −80°C until further processing.

Processing and chemical analyses of samples

Feed samples, except for molasses, were ground to pass 1-mm screen using a Tecator Cyclotec Sample Mill® (Foss Analytical Co., Ltd., Suzhou, China), and were analysed for amino acids and iodine (as described below), and feed composition (Norwegian University of Life Sciences: LabTek, **NMBU**, Ås, Norway). Feed refusal was oven dried for 48 h, weighed and reweighed after storage at ambient temperature for 24 h. Frozen faeces samples were course-ground and freeze-dried for 48 h, using a Labconco FreeZone 4.5 Plus® (Kansas City, Missouri, USA) freeze-drier at a temperature and vacuum ranging between −80°C and −86°C, and 0.52 and 0.97 mbar, respectively. The freeze-dried samples were weighed immediately and again after 24 h storage in ambient temperature. The samples were then ground to pass 1-mm screen using a Tecator Cyclotec Sample Mill® (Foss Analytical Co., Ltd., Suzhou, China). The final weight of the samples was measured, and subsamples were taken and stored in plastic zipped bags at −18°C until further chemical analyses at LabTek, NMBU, for composition.

Feed, feed refusal and faeces samples were analysed for DM (103°C for at least 4 h to constant weight), ash (550°C for at least 4 h), Kjeldahl-N (KjeltecTM 8400; Foss Electric, Hillerød, Denmark), crude fat (accelerated solvent extraction, ASE™ 350 Accelerated Solvent Extractor, Dionex, USA) and gross energy (PARR 1281 Bomb Calorimeter, Moline, Illinois, USA). Neutral detergent fibre was determined with an ANKOM200 fibre analyser (ANKOM Technology, Fairport, New York, USA) according to Mertens (2002) using sodium sulphite, alpha amylase and ash correction (**aNDFom**), and acid detergent fibre (**ADF**) was determined according to Method 973.18 (AOAC, 2000) with the modification that the samples were not washed with acetone and were corrected for ash. Iodine in macroalgae was determined according to Roleda *et al.* (2018) who used the HPLC method to extract iodine by dry alkaline incineration (Nitschke and Stengel, 2015). The polyphenolic concentration in the *S. latissima* extract was determined according to Roleda *et al.* (2019) using phloroglucinol as standard reference.

Amino acid concentration in feed ingredients was analysed by an HPLC system (Agilent Infinity 1260, Agilent Technologies) coupled to an online post-column derivatization module (Pinnacle PCX, Pickering laboratories, Mountain View, California, USA), using ninhydrin (Trione) as a derivatizing reagent and Na+-ion exchange column (4.6×110 mm, 5 μm). Eighteen standard amino acids, ammonia and taurin were quantified from standard curves measured with amino acid standards (Pickering Laboratories, Mountain View, California, USA) (Table 3).

The frozen urine samples were thawed and subsamples were stored at 4°C until further chemical analyses at Vitas AS (Oslo, Norway) for iodine, and at LabTek, NMBU, for Kjeldahl N (Kjeltec 2460; Foss Electric, Hillerød, Denmark). Urine was diluted and homogenized before the samples were analysed for their iodine concentration by inductively coupled plasma mass spectrometry (Agilent 7900 ICP-MS, Japan). Unknowns were calibrated against known standards from Sigma-Aldrich.

Plasma samples were sent without further processing to University of Helsinki for amino acid analysis. Plasma amino acid concentrations were determined as described by Puhakka *et al.* (2016). Briefly, plasma samples were precipitated using 10% sulphosalisylic acid and analysed by ultra-performance liquid chromatography, equipped with an

Table 4 *Effect of diet on total digestibility in wethers (least square means)*

	Experimental diets					
	Control	SW1	SW2	SBM	SEM[5]	*P* value[6]
n	4	2	4	4		
Intake (g DM[1]/day)	1104	1106	1139	1094	0.36/0.55	
Faecal excretion (g DM/day)	352	360	354	337	10.6/16.2	
Digestibility (coefficient)						
Dry matter	0.68	0.67	0.69	0.69	0.0095/0.0146	
Organic matter	0.70	0.69	0.71	0.71	0.0096/0.0146	
Nitrogen	0.55[b]	0.55[b]	0.64[a]	0.66[a]	0.0087/0.0134	**
aNDFom[2]	0.69	0.70	0.70	0.69	0.0122/0.0186	
ADF[3]	0.64	0.62	0.61	0.62	0.0201/0.0306	
Crude fibre	0.68	0.66	0.66	0.68	0.0154/0.0236	
DE/GE[4]	0.66	0.65	0.67	0.67	0.0103/0.0157	

SW1=diet containing processed *S. latissima*; SW2=diet containing *Porphyra spp.*; SBM=diet containing soybean meal.
[1] Dry matter.
[2] Neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash.
[3] Acid detergent fibre expressed inclusive of residual ash.
[4] Ratio of digestible energy/gross energy, where digestible energy was calculated as the difference between gross energy and energy in excreted faeces.
[5] Standard error of the mean for Control, SW2 and SBM, and SW1, respectively.
[6] $^{**}P<0.01$.
[a,b] Values within a row with different superscripts differ significantly at $P<0.05$.

Ethylene Bridged Hybrid C_{18} column, and a photodiode array detector to detect amino acids.

The three subsamples of rumen fluid per animal and period were sent without further processing to University of Helsinki for ammonia and VFA analyses. Ammonia and VFA from rumen fluid samples were analysed according to McCullough (1967) and Lamminen *et al.* (2017), respectively.

Statistical analyses

Data were analysed using Proc MIXED in SAS (Statistical Analysis System Institute Inc., 2011) for a 4×4 Latin square design according to the following model:

$$Yijkl = \mu + Pi + Tj + sk + Eijk$$

where *Yijkl* is the dependent variable, μ the overall mean, *Pi* the effect of period *i*, *Tj* the effect of diet *j*, *sk* the effect of sheep *k* and *Eijk* the residual error. Period and diet were considered fixed effects, using the 'Repeated' statement to account for within-animal time-dependent correlations. The optimal covariance structure was assessed for each dependent variable with attention to the corrected Akaike's information
criterion. Degrees of freedom were estimated by using the formula of Satterthwaite. In two out of four periods, the animals refused to eat some of the SW1 supplement. The data from these sheep were excluded from the data analysis ($n = 14$). Differences between least squares means of response variables were estimated with Tukey's test. Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$. All reported values are least squares means.

Results

Total track digestibility

The digestibility of DM, OM, aNDFom, ADF, crude fibre (**CF**) and energy did not differ among the diets (Table 4). Nitrogen digestibility of diets containing SW2 and SBM was similar, which was higher than those of Control and SW1 ($P=0.002$). Nitrogen digestibility of the protein feeds, as calculated by difference, were 0.74, 0.89 and 0.97 for SW1 (SEM: 0.024), SW2 and SBM (SEM: 0.023), respectively. See also Özkan Gülzari *et al.* (2018a and 2018b) for preliminary results presented in conferences.

Nitrogen excretion

Total N intake (g/day) was similar in diets containing SW2 and SBM (20.9 and 19.8, respectively), which was higher than that of SW1. The latter was also found to be higher than that of Control (Table 5). Results show that relatively more N was excreted in faeces than in urine in SW1 than in SW2, SBM and Control diets ($P = 0.014$). Despite the significant difference in urine N excretion, the proportion of urine N excreted of the total N intake did not differ between SW1 and SW2.

Ammonia and volatile fatty acid composition in rumen fluid

The ammonia concentration in the rumen fluid tended ($P = 0.05$) to be highest in the diet containing SBM (7.36 mmol/l) and lowest in the diet containing SW1 (2.29 mmol/l). Urea concentration was similar in diets containing SW1, SW2 and SMB, and significantly higher in SW2 and SBM than in Control. There was a strong effect of diet on rumen fermentation. The SW1 reduced the rumen

Table 5 *Intake and excretion of nitrogen in urine and faeces of wethers*

	Experimental diets					
	Control	SW1	SW2	SBM	SEM[2]	*P* value[3]
n	4	2	4	4		
N[1] intake (g/day)	14.5	18.6	20.9	19.8		
Faecal N (g/day)	6.6[b]	8.3[a]	7.5[ab]	6.8[b]	0.17/0.26	*
Urine N (g/day)	5.8[c]	8.2[b]	10.6[a]	10.9[a]	0.26/0.39	***
Urine N excreted/total N intake (g/g)	0.40[b]	0.44[ab]	0.51[a]	0.55[a]	0.017/0.026	*
Urine N excreted/digested N (g/g)	0.74	0.79	0.79	0.84	0.033/0.051	
Faecal N excreted/total N intake (g/g)	0.46[a]	0.45[a]	0.36[b]	0.34[b]	0.009/0.013	**
Total N Excreted/total N intake (g/g)	0.85	0.89	0.87	0.90	0.019/0.029	

SW1=diet containing processed *S. latissima*; SW2=diet containing *Porphyra spp.*; SBM=diet containing soybean meal.
[1] Nitrogen.
[2] Standard error of the mean for Control, SW2 and SBM, and SW1, respectively.
[3] * $P<0.05$, ** $P<0.01$, *** $P<0.001$.
[a,b,c] Values within a row with different superscripts differ significantly at $P<0.05$.

Table 6 *Rumen fermentation products of wethers fed on four different diets*

	Experimental diets					
	Control	SW1	SW2	SBM	SEM[2]	*P* value[3]
n	4	2	4	4		
Ammonia, mmol/l	3.44	2.29	3.47	7.36	0.80/1.22	(*)
VFA[1] total, mmol/l	70.0[ab]	25.6[b]	54.8[b]	100[a]	15.1/20.0	*
VFA proportion, mmol/mol						
Acetic acid	701	707	707	705	6.8/10.3	
Propioic acid	173	151	151	158	7.7/11.3	
Butyric acid	85	93	96	93	6.1/8.6	
Isobutyric acid	3.0	4.4	3.9	5.1	0.76/1.17	
Isovaleric acid	2.9	4.0	3.6	4.8	0.74/1.14	
Valeric acid	5.6	5.6	5.9	6.3	0.56/0.85	
Caproic acid	29	33	32	27	3.4/5.1	
Acetic : Propinoic (ratio)	4.1	4.7	4.7	4.5	0.19/0.28	
pH	8.0	8.3	8.6	7.8	0.24/0.36	

SW1=diet containing processed *S. latissima*; SW2=diet containing *Porphyra spp.*; SBM=diet containing soybean meal.
[1] Volatile fatty acids.
[2] Standard error of the mean for Control, SW2 and SBM, and SW1, respectively.
[3] (*) $P<0.10$ and * $P<0.05$.
[a,b] Values within a row with different superscripts differ significantly at $P<0.05$.

fermentation greatly compared to SBM, where VFA profiles were 25.6 and 100 mmol/l, respectively. Nevertheless, the fermentation pattern was not affected, as the molar proportions of acetic acid, propionic acid and butyric acid compositions (mmol/mol) did not differ among diets (Table 6).

Amino acid composition in blood plasma

The diet composition had only negligible effect on plasma amino acid levels (Table 7). The plasma concentration of 1-Methyl-Histidine (**1MH**) was higher with the control diets than with the diets with extra protein. The concentration of methionine, glutamate and α-aminobutyric acid tended ($P < 0.10$) to be higher in SW1 than in SW2 and SBM diets.

Discussion

Total tract digestibility and N excretion

The low N digestibility in SW1 compared to SW2 and SBM can be explained by both the impaired rumen fermentation and the limited absorption of rumen-undegradable-containing compounds in the small intestines.

Macroalgae contain phlorotannins, phenolic compounds forming insoluble complexes with protein, whereby preventing protein degradation (Burtin, 2003). Phlorotannins are known to reduce digestion (Arnold and Targett, 1998). Wang *et al.* (2009) studied the effects of phlorotannins extracted from brown macroalgae on the rumen bacterial population and fermentation, and found that phlorotannins

Table 7 *Plasma amino acid composition of wethers fed on four different diets*

	Experimental diets					
	Control	SW1	SW2	SBM	SEM[2]	*P* value[3]
n	4	2	4	4		
Essential AA[1] (μmol/l)						
Histidine	54	52	46	52	3.6/5.5	
Isoleucine	88	80	76	82	7.2/11.1	
Leucine	100	104	92	89	7.5/9.6	
Lysine	126	114	106	107	16.8/23.6	
Methionine	19	19	16	17	1.4/1.6	(*)
Phenylalanine	40	42	44	38	2.1/2.8	
Threonine	117	152	93	111	13.1/20.0	
Tryptophan	31	34	32	31	1.5/2.3	
Valine	199	187	181	186	15.4/23.5	
Non-essential AA (μmol/l)						
Arginine	139	140	145	150	23.4/31.7	
Alanine	264	271	194	210	13.3/33.6	
Aspartic acid	7.5	9.1	6.4	7.6	0.96/1.15	
Glutamic acid	78	89	61	67	5.3/9.9	(*)
Glutamine	335	353	288	346	31.6/48.3	
Glycine	438	347	310	407	40.4/61.7	
Proline	81	82	65	73	7.6/10.8	
Serine	79	72	56	74	7.0/10.7	
Citrulline	129	118	116	128	18.1/24.8	
Ornithine	73	80	62	69	8.5/11.9	
Taurine	53	56	61	43	3.2/4.8	(*)
Tyrosine	56	58	48	53	4.3/6.6	
Hydroxyproline	20	20	17	19	2.1/3.0	
3-Methylhistidine	6.8	7.8	6.3	6.5	0.51/0.60	
1-Methylhistidine	78[a]	64[b]	65[b]	65[b]	21.3/21.5	*
Aspartic acid	7.5	9.1	6.4	7.6	0.96/1.15	
Sarcosine	7.0	7.4	6.3	6.5	0.56/0.78	
β-Alanine	2.8	2.8	2.6	2.6	0.11/0.16	
α-aminoadipic acid	5.2	3.9	4.0	4.1	0.66/0.75	(*)
α-amino-N-butric acid	12.2	15.0	9	11	1.4/1.9	(*)
Cystathionine	3.9	5.4	4.3	4.1	0.67/0.89	
Cystine	8.0	10.0	8.5	8.0	1.3/1.9	
Essential amino acids	894	887	815	848	79.2/118.2	
Essential amino acids/total amino acids (ratio)	0.332	0.341	0.362	0.338	0.0061/0.0092	(*)

SW1=diet containing processed *S. latissima*; SW2=diet containing *Porphyra spp.*; SBM=diet containing soybean meal.
[1] Amino acid(s).
[2] Standard error of the mean for Control, SW2 and SBM, and SW1, respectively.
[3] (*) $P<0.10$ and * $P<0.05$.
[a,b] Values within a row with different superscripts differ significantly at $P<0.05$.

inhibited the growth of *Fibrobacter succinogenes* and increased the non-cellulolytic bacteria *Selenomonas ruminantium*, *Ruminobacter amylophilus* and *Prevotella bryantii*. A study by Gaillard *et al.* (2018) reported that low ruminal degradability of amino acids of *Laminaria*, a brown seaweed genus related to *Saccharina*, was probably associated with phlorotannins. We did not analyse the phlorotannins in *Porphyra spp.*, but the 3.8 g/kg DM phlorotannins in SW1 may have bound and limited the digestibility of protein (Clark *et al.*, 1987). By binding the proteins and carbohydrates, tannins may leave less of the nutrients available for rumen microbiota, but also inhibit microbial enzyme activity, or directly affect rumen microorganisms, resulting in reduced rumen degradation (Frutos *et al.*, 2004).

Poor carbohydrate utilization of bacteria may have further compromised the digestibility of nutrients in animals fed SW1. Given that the soluble fraction is more rapidly degraded in the rumen than the insoluble fraction (Clark *et al.*, 1987), it can be interpreted that less of the N in the SW1 was in the soluble fraction than that of SW2 and SBM.

Tannin-protein complexes may have been formed in the rumen pH 3.8–8, but they would have been expected to dissociate in the abomasum where pH levels reduce below 3.5 or in duodenum where pH is above 8 (Frutos *et al.*, 2004).

If this is the reason for the rumen-undegraded N not to be absorbed in the small intestines in our study, this may be because the tannins interact with the membrane proteins of intestinal mucosa (Frutos *et al.*, 2004). This could be investigated by the mobile nylon-bag N disappearance technique (Frydrych, 1992). However, the physical properties of powder form SW1 and SW2 would result in loss of biomass not because they would be ingested but because they would pass through the pores of the nylon bag.

Nitrogen digestibility of SW2 was similar to that of SBM, due possibly to its high rumen degradability and high absorption of rumen-escapable proteins in the small intestine. An earlier study by Tayyab *et al.* (2016) suggests for *Porphyra spp.* that digestibility of escape protein is quite high, and 50% of the protein is degraded in the small intestine. It can be noted that similar amino acid composition in blood plasma for all diets may indicate that the quality of protein was similar among different diets, and that it mainly consisted of microbial protein. The latter statement is based on the fact that the control diet did not include any additional protein source.

Higher ash concentration in SW1 (221 g/kg DM) than in SW2 and SBM may also have affected the digestibility figures. Macroalgae with high concentrations of iodine and ash (e.g. SW1) need to be added to the diet at a low rate, but low inclusion may thwart their positive effects. Arieli *et al.* (1993) reported that supplementing sheep diets with *Ulva* seaweed species gave rise to reduced concentration of digested energy at 9.1 megajoule: **MJ**/kg DM, due mainly to the high ash concentration of seaweed (207 g/kg). It reduces its nutritive value, and therefore its potential as a ruminant protein supplement (Arieli *et al.*, 1993). However, the digestible energy concentration of different diets did not differ in this study and was on average 13.8 MJ/kg DM, due possibly to the low inclusion rate in the diet. The ash concentrations of the diets in this study were much lower than the 105.5 g/kg DM of a diet containing *Ulva* species in the study of Arieli *et al.* (1993), which seems to explain the higher digestibility of the diet in our study than in that of Arieli *et al.* (1993). To the best of authors' knowledge, there are no *in vivo* studies generating data where *S. latissima* was fed to sheep or ruminants, but the high iodine concentration may explain the resistance to consume the SW1 by two of the animals, even though the iodine concentration was significantly reduced (from 6 to 1.2 g/kg DM) by processing.

Reduced bacterial activity was probably the reason for the lower ammonia levels in SW1 than in SW2 and SBM in this study; however, the difference was not significant. Clark *et al.* (1987) stated that feeding cows with alimentary protein sources that were not easily degraded in the rumen may result in low ammonia, amino acids and peptides for ruminal microbiota. Low ammonia concentration as well as a ratio of 0.9 : 1.02 for the excretion of N in urine as a fraction of digested N in sheep fed *Ulva* seaweed species was reported to indicate low rumen degradability of the protein concentration in seaweed (Arieli *et al.*, 1993). A lower ratio (0.79) of the N excreted in urine compared to total N intake (g/g) in the current study than that reported by Arieli *et al.* (1993) may be due to the difference in protein level in the diet (low but sufficient protein concentration in our study).

Higher production of VFAs in SBM than in the diets supplemented with seaweed suggests rapid and extensive fermentation in the rumen. Even though the difference was not significant, lower total VFA profile in SW1 than SBM indicated that the predation of rumen protozoa on bacteria was altered, causing more of the bacteria to leave the rumen and therefore reducing microbial N per unit of apparently digested OM (Clark *et al.*, 1987).

Clark *et al.* (1987) calculated that 3.7 g of methionine and 18.4 g of lysine from microbial protein escape the rumen for every kg of OM fermented in the rumen. It is important to note that higher concentrations of amino acids present in the diet do not always translate into greater amounts of amino acids passing to the small intestines. Eventually, what determines the amount of amino acids passing to the small intestines is the amino acid concentration of protein that escapes ruminal fermentation (Clark *et al.*, 1987). In our study, even though greater amounts of lysine and methionine were detected in plasma for the SW1 than for SW2 and SBM, the difference was not significant. Despite the higher concentrations of lysine in diets containing SW2 and SBM than in SW1, greater lysine concentrations in blood plasma of SW1 indicate that the utilization of lysine in SW1 was hindered. This is further discussed by Gaillard *et al.* (2018) who reported that methionine and lysine in a brown seaweed species *Laminaria* were not degraded in the rumen, although they became available in the small intestines.

Seaweed harvesting time and processing on digestion parameters

Several factors have an impact on the protein concentration of seaweed. For the brown macroalgae, the protein concentration is highest in winter and early spring, before onset of the accumulation of storage carbohydrates during late spring and summer. Maximum protein coincides with maximum salt (ash) concentration (Schiener *et al.*, 2015). The *S. latissima* used in the current work was harvested in May–June with approximately 10% DM and contained almost no laminaran, but ~10% mannitol. In late summer or autumn, the dry weight is ~20%, and the soluble carbohydrates (laminaran and mannitol) constitute more than 30% of DM, with correspondingly lower protein and ash concentrations.

Cultivated seaweeds are harvested in spring, due to fouling and degradation during the summer. Processing to reduce their salt concentration will therefore be required for a high inclusion rate in animal feed. This processing also reduces the concentration of nutrients, such as free amino acids and other water-soluble N-containing compounds.

Adaptation period as a barrier

The adaptation period before the collection may have been a factor affecting the low digestibility. It is important to note that the carry-over effects between periods were modelled in the statistical analyses. As opposed to the 14-day adaptation period

in Carvalho *et al.* (2005) and Milis *et al.* (2005), in this study the rams were adapted to the diet for 8 days. A longer adaptation period may affect the adaptation of rumen microbiota to the new diet, resulting in increased levels of rumen fermentation. However, since the rumen microbiota responds to the diet changes differently, a longer adaptation period is not always justified. An example to this is the study by Fernando *et al.* (2010) who found significantly high number of *Megasphaera elsdenii*, *Streptococcus bovis*, *Selenomonas ruminantium* and *Prevotella bryantii*, but gradually reduced populations of *Butyrivibrio fibrisolvens* and *Fibrobacter succinogenes* when animals were adapted to high-concentrate diet for 7 days. Similarly, in a review on *in vivo* measurement of forage digestibility, Rymer (2000) indicates that animals need 4–12 days to adapt to diets, and that normally 6–8 days would be required (Omed, 1986). Further, Nicholson *et al.* (1956) reported that an adaptation period of 7 days would suffice when a constant hay to concentrate ratio was maintained in spite of a varying protein level. It can be also noted that the ability of protozoon in the rumen to adapt to the seaweed is also associated with the genetics of the animals (Orpin *et al.*, 1985). However, given that some sheep, for example, Orkney breed on North Ronaldsay in the United Kingdom, graze principally on seaweed, the ability to degrade seaweed by Orkney sheep renders the adaptation of the microbial population as more of a factor than genetics (Greenwood *et al.*, 1983). When decisions are made to increase the length of adaptation, a further consideration should be given to the trade-offs between the expected results and the compromise made for animal welfare, as well as the cost of an additional day in the adaptation.

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Declaration of interest

None.

Ethics statement

This study was conducted in accordance with the regulation for use of animals in experiments, adopted by the Norwegian Ministry of Agriculture and Food, and approved by the Ethics Commission on Animal Use by the Norwegian Food and Safety Authority, application number (Forsøksdyrforvaltningen tilsyns- og søknadssystem: FOTS (The experimental animal administration supervisory and application system) ID 8838, date 31 August 2017). It complies with the EU Directive 2010/63/EU on the use of experimental animals, which was incorporated to the European Economic Area Agreement in May 2015.

Software and data repository resources

None of the data were deposited in an official repository.

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animal

The effect of cultivated mixed-species green fodder on intake, milk production and milk composition of housed dairy goats

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*The majority of New Zealand dairy goat farmers utilise cultivated green-fed fodder dominated by perennial ryegrass (Lolium perenne L.) and white clover (Trifolium repens L.), but evidence from other ruminant species suggests that milk production may be improved when using a more diverse array of species within the green fodder. The aim of this experiment was to determine whether feeding lactating dairy goats a mixed-species green fodder (***MF***, consisting of perennial ryegrass, timothy (Phleum pratense L.), prairie grass (Bromus willdenowii Kunth), white clover, red clover (Trifolium pratense L.), lucerne (Medicago sativa L.), chicory (Cichorium intybus L.) and plantain (Plantago lanceolata L.) improves dietary intake, milk yield and composition compared with a standard ryegrass and white clover green fodder (***SF***). Thirty-six mid-lactation goats were housed indoors in pairs and split into two groups (A and B). The trial was split into three periods – firstly a uniformity period of 6 days, in which all goats were fed a combination of both green fodder types, followed by two treatment periods (P1 and P2) of 12 days, respectively. For P1, group A was fed MF and group B was fed SF, and then the group diets were switched for P2. Goats fed MF had 13% greater dry matter intake and 7% greater milk yield than goats fed SF. In addition, the milk protein and fat concentration of goats fed MF were 4% greater than for those fed SF, whereas there was no effect on milk lactose concentration. There was no treatment effect on the levels of protein, glucose, urea or non-esterified fatty acids in the blood of the goats. An effect of green fodder type on milk fat profile was demonstrated, with proportions of pentadecylic acid (C15:0), cis-vaccenic acid (C18:1 c11), linoleic acid (C18:2 n6) and α-linolenic acid (C18:3 n3) being increased in response to MF consumption. In contrast, iso-C15 and iso-C17 proportions were lesser. In summary, this study demonstrated that goats fed MF increased green fodder intake and milk production compared with goats fed SF. The green fodder type affected the fatty acid profile of goat's milk, with MF increasing the levels of beneficial polyunsaturated omega fatty acids (linoleic and α-linolenic acids).*

Keywords: chicory, fatty acids, legumes, plantain, temperate grasses

Implications

This study investigated the effect of feeding a mixed-species green fodder on intake and milk production in dairy goats. Goats were housed indoors and fed either fresh-cut mixed-species green fodder (comprising grasses, legumes and herbs) or standard green fodder (consisting of perennial ryegrass and white clover). When fed mixed-species green fodder, goats consumed 13% more dry matter than when they were fed standard green fodder, leading to a 7% increase in milk yield. Feeding mixed-species green fodders also affected the proportions of beneficial fatty acids in milk. The findings demonstrate that mixed-species green fodders improve dairy goat milk production.

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Introduction

In New Zealand, dairy goats are predominantly housed in free stall barns and fed a diet based on either fresh-cut green fodder or fodder silage (Prosser and Stafford, 2017). The diet is further supplemented with concentrate feeds, minerals and vitamins to optimise health and milk production (Prosser and Stafford, 2017). To date, there is little information available regarding the most appropriate fodder strategy for dairy goats within a cut-and-carry system. Most dairy goat farmers have adopted fodder based on cultivated pastures used in New Zealand dairy cow-grazing systems, which are dominated by perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.). Goats are considered opportunistic feeders and are adept at utilising a far more diverse range of forages compared with cows (Avondo *et al.*, 2008a). New Zealand farmers' accounts suggest that goats exhibit improved performance when offered a more

diverse diet, and New Zealand dairy goat farmers have supported fodder diversity, with the addition of other grass, legume and herb species as a strategy to increase milk production. Although several studies have demonstrated a positive effect of feeding mixed pastures on both cow and sheep milk production (Pembleton *et al.*, 2016; Kenyon *et al.*, 2017; Minneé *et al.*, 2017), there is no scientific evidence to validate these observations in lactating dairy goats. Another advantage of including a more diverse range of species, such as red clover (*Trifolium pratense* L.), lucerne (*Medicago sativa* L.), chicory (*Cichorium intybus* L.) and plantain (*Plantago lanceolata* L.), within a fodder is better nutritive values of these species compared with perennial ryegrass during dry summer periods typically seen in the main goat-farming regions of New Zealand (Brown *et al.*, 2003; Moorhead and Piggot, 2009).

A majority of research investigating goat dry matter intake (**DMI**) of forages and the effects on milk production have predominantly focused on conserved feeds, which is the favoured method of feeding in intensive dairy goat systems in most other countries (reviewed in Goetsch *et al.*, 2011). Several studies have examined the effects of green forages on the DMI of dairy goats but under grazing rather than housed systems (Collins and Nicol, 1986; Avondo *et al.*, 2008b; Lefrileux *et al.*, 2012). Huguet *et al.* (1979) investigated the feeding of different grass varieties in an experiment analogous to a cut-and-carry system and demonstrated that lactating goats performed better when fed Italian ryegrass (*Lolium multiflorum* Lam.) compared with the other grass species examined.

To our knowledge, there is no research showing the benefits of mixed-species green fodder (**MF**) on dairy goat intake and milk production compared with more conventional green fodder under experimental conditions analogous to New Zealand dairy goat farming systems. The aim of this experiment was to test the hypothesis that offering a diverse multi-species green fodder mix would increase dietary intake and milk production in mid-lactation dairy goats.

Materials and methods

Green fodder establishment and management

The fodder swards for the experiment were sown in autumn (April 2016) at Ruakura Research Centre, Hamilton, New Zealand (37°46′41.1″S 175°18′49.2″E; average annual rainfall 1072 mm) into a 7-ha block on light-medium sedimentary soils (pH 5.9 and an Olsen P 35). Three hectares were sown with standard ryegrass and white clover green fodder (**SF**) – perennial ryegrass (cultivar (**cv**.) Base AR 37 at 25 kg/ha) and white clover (cv. Tribute at 2.5 kg/ha and cv. Mainstay at 2.5 kg/ha); and 4 ha were sown with MF – perennial ryegrass (cv. Base AR 37 at 10 kg/ha), timothy (*Phleum pratense* L. cv. Charlton at 4 kg/ha), prairie grass (*Bromus willdenowii* Kunth cv. Atom at 6 kg/ha), white clover (cv. Tribute at 3 kg/ha), red clover (cv. Sensation at 4 kg/ha), lucerne (cv. Torlesse at 8 kg/ha), chicory (cv. Choice at 1 kg/ha) and plantain (cv. Tonic at 1 kg/ha). To reduce the risk of gastrointestinal worm infection and feed avoidance by the goats, no animals were grazed on these fields from sowing. The fodder was mechanically harvested from sowing until the end of the trial at target covers of 2400 ± 200 kg DM/ha with a consistent residual of 1600 kg DM/ha. To maintain soil fertility, 40 kg/ha NutriGro 13 k (N 13.5, P 11.2, K 12.5, S 4.9 plus Co, Se) was applied after each harvest. During the experiment the green fodder was cut once a day at approximately 1400 h using a SIP DISC S ALP 300 disc rotary mower (SIP Strojna Industrija d.d, Šempeter v Savinjski dolini, Slovenia). A portion was collected using a Bergmann Royal Loader Wagon (Ludwig Bergmann GmbH, Goldenstedt, Germany) and fed the same day at afternoon milking (1500 h). The remainder was left in the paddock overnight, collected using a Bergmann Royal Loader Wagon and fed the following morning (0700 h).

Animal management

The experiment was conducted in mid-lactation during spring to early summer (October to December) of 2016 with 36 multiparous Saanen goats (mean live weight (**LW**), 60.2 kg (range 44.0 to 75.5 kg); mean body condition score (**BCS**), 2.5 (range 2.0 to 3.0); mean milk yield, 3.5 kg/day (36 g/kg fat and 29 g/kg protein) and mean days of milking, 118 (range 109 to 123) at the start of experiment) that were sourced from a commercial dairy herd and housed at the Ruakura Goat Research Facility (Hamilton, New Zealand).

The goats were housed indoors in individual pens (3 m^2) for 30 days before the start of the experiment to allow for acclimatisation to their new surroundings. At the beginning of the experimental period, the goats were paired, and the size of pens was increased to 6 m^2 to accommodate the pairs. This pairing was an ethical requirement, to allow sufficient space to move per goat. Pairing was based on current LW, milk production and intake to minimise variation between pairs. For the entire experiment, goats were milked twice a day (approximately 0700 h and 1500 h). While the goats were out of the pens for milking, feed refusals were collected and a new ration of fresh fodder was offered at 120% of expected intake. A formulated mineral–vitamin mix was sprinkled over each pair's fodder allocation in each feed bin within the pen – 60 g NaCl, 30 g lime flour, 8 g Image goat mineral and vitamin premix (Image Holding Limited, Auckland, New Zealand), 2 g magnesium, 2 g vitamin B_{12}, 0.5 g biotin, 1 g yeast extract, 0.4 mg iodine and 0.4 mg selenium per pair per day. In addition, the goats were each fed 215 g DM of pelleted supplement at each milking (900 g/kg maize, 100 g/kg wheat, 14.8 MJ/kg metabolisable energy (**ME**); 88 g/kg crude protein (**CP**) by DM).

Experimental design

Prior to allocation to treatments, the experiment began with a uniformity period (day 1 to 6) in which goat pairs (experimental unit) were fed a combination of both green fodder

treatments (MF at morning and SF at afternoon) to allow the goats to adapt to the treatments and being housed in pairs. The pairs were then assigned into two groups (A and B), which were balanced for milk yield and composition, DMI, LW and BCS, as measured during the uniformity period. The study was designed as a simple cross-over experiment split over two treatment periods (P1, days 7 to 18; P2, days 19 to 30). The treatment periods were further split into an adaptation period (day 7 to 13 for P1 and days 19 to 25 for P2) and a measurement period (days 14 to 18 for P1 and days 26 to 30 for P2) in which data were collected. During P1 group A was assigned the MF treatment and group B was assigned the SF treatment. The treatments were switched for P2 (group A fed SF and group B fed MF).

Feed intake

The weight of fresh feed offered to and refused by each goat pair was recorded at each feeding. A sample (~150 g) was collected at each feeding of both fresh green fodder offered and refusals from the previous feeding. These samples were oven-dried (65°C for 48 h) to determine DM percentage. The goats were offered a total of 430 g DM per day of supplement pellets, with no refusals. Daily DMI was calculated for each pair of goats by subtracting the refused DM from the offered DM plus supplement pellet DM.

Fodder botanical composition and nutritive characteristics

During the measurement periods (days 14 to 18 for P1 and days 26 to 30 for P2), subsamples of fodder from each feeding and refusals were collected for determining botanical composition. Samples were mixed thoroughly and a portion containing approximately 400 pieces was selected, which was between 50 and 100 g depending on the gross botanical composition. This was separated into categories: perennial ryegrass, timothy, prairie grass, other grasses, white clover, red clover, lucerne, chicory, plantain, other non-grass species and dead matter. Each sample category was oven-dried (65°C for 48 h) and the percentage of each component was calculated on a DM basis.

A subsample of fodder from each feeding during the measurement periods was stored at −20°C and then freeze-dried and ground. These samples were pooled into representative samples for MF and SF spanning each measurement period and were analysed for fodder quality and fatty acid profile.

Green fodder quality analysis was conducted by an International Accreditation New Zealand–accredited laboratory (Hill Laboratories Ltd, Hamilton, New Zealand). Attributes measured were CP (Dumas combustion using conversion factor nitrogen × 6.25; AOAC 968.06; AOAC, 1990), acid detergent fibre (**ADF**; modified National Forage Testing Association method adapted for Ankom auto analyser; Ankom Technology, Macedon, New York, USA; National Forage Testing Association, 1997), neutral detergent fibre (**NDF**; National Forage Testing Association method adapted for Ankom auto analyser; Ankom Technology; National Forage Testing Association, 1997), ash (AOAC 942.05; AOAC, 1990), digestible organic matter in dry matter (**DOMD**; calculated from organic matter digestibility *in vitro*; Australian Fodder Industry Association (**AFIA**) pepsin-cellulase procedure using AFIA standard equation; Method 1.7R; AFIA, 2011), ME (DOMD × 0.016; AFRC, 1993), soluble sugars (80:20 ethanol:water extraction and colorimetric determination; Dubois *et al.*, 1956; Hall *et al.*, 1999), starch (removal of free sugars, enzymic hydrolysis of starch, colrimetric determination of glucose; Dubois *et al.*, 1956; Hall *et al.*, 1999), crude fat (**CF**; petroleum spirit extraction by Ankom auto analyser, AOCS Official Procedure AM-5-04; AOCS, 2017), non-structural carbohydrate (100 − (CP + ash + CF + NDF)).

Fatty acids from the fodder samples were isolated by a method modified from Toledo *et al.* (2002). Briefly, 2 ml of sample was mixed with 4 ml 2-propanol, and 3 ml hexane was added. The sample was mixed vigorously and the hexane layer was removed. The sample was evaporated to dryness and then resuspended in 4 ml hexane. Extracted fatty acids were methylated by a method modified from Christie (1989). Briefly, 100 µl 0.5 M sodium methoxide was added to the fatty acid sample, which was then incubated for 10 min with periodic mixing. Glacial acetic acid (5 µl) was added and the sample was mixed before the addition of anhydrous calcium chloride. The sample was mixed vigorously and then incubated at room temperature for 60 min. The top layer was collected and analysed by gas chromatography with flame ionising detection using Shimadzu GC 2010 (Shimadzu, Kyoto, Japan).

Milk yield and composition

Daily milk yield was recorded using in-line milk meters (TruTest, Auckland, New Zealand). Milk samples were collected at each milking in the final 2 days of the uniformity period (days 5 and 6) and for each day of the measurement period (days 14 to 18 for P1 and days 26 to 30 for P2). Samples were analysed by infrared spectrometry for fat, protein and lactose concentration and by flow cytometry for somatic cell count (**SCC**; Fossomatic equipment, LIC Herd Testing Station, Hamilton, New Zealand). Milk samples from 1 day of each measurement period (day 16 for P1 and day 28 for P2) were collected for analysis of fatty acid profiles following the method described above.

Live weight and body condition score

Live weights and BCS of goats were recorded after morning milking during the uniformity period (day 5) and at the end of treatment periods – P1 (days 17 and 18) and P2 (days 29 and 30). The BCS of goats was assessed on a scale of 1 to 5, where 1 signifies emaciated and 5 obese (National Animal Welfare Advisory Committee, 2012).

Blood glucose, protein, urea, and non-esterified fatty acids

On the final day of each measurement period (day 18 for P1 and day 30 for P2), goats were restrained and blood was collected from the jugular vein after morning milking and prior to being returned to their pens. Blood was collected from each goat into sodium fluoride (grey top) and plain (red

Table 1 *Botanical composition of mixed-species green fodder and standard green fodder fed to dairy goats during measurement periods (days 14 to 18 for measurement period 1 and days 26 to 30 for measurement period 2)*

	Mixed-species green fodder			Standard green fodder			
Fodder species	Offered (g/kg DM)	Refused (g/kg DM)	SED[1]	Offered (g/kg DM)	Refused (g/kg DM)	SED[1]	Between-treatment SED[1]
Grass species	531	532	20 (ns)	876	853	24 (ns)	33***
Perennial ryegrass	400	413	16 (ns)	843	844	26 (ns)	
Timothy	6	5	1 (ns)	–	–	–	
Prairie grass	117	101	17 (ns)	–	–	–	
Other grasses	9	13	2*	13	11	3 (ns)	
Non-grass species	460	443	21 (ns)	127	119	22 (ns)	
Legumes	119	122	15 (ns)	112	109	21 (ns)	26 (ns)
White clover	66	79	5*	112	109	21 (ns)	
Red clover	51	39	4*	–	–	–	
Lucerne	3	4	1 (ns)	–	–	–	
Herbs	329	316	25 (ns)	5	3	3 (ns)	17***
Chicory	176	155	17 (ns)	1	0	1 (ns)	
Plantain	153	160	18 (ns)	5	3	2 (ns)	
Other	11	6	3 (ns)	9	7	3 (ns)	
Dead	9	25	3***	17	26	3**	

[1] Significance is reported in parentheses after the SED, where ns=$P>0.05$,*$P<0.05$,**$P<0.01$,***$P<0.001$.

top) vacutainers. Grey top samples were kept chilled on ice for 1 h and centrifuged at 1500×**g** for 10 min at 4°C. The plasma was collected into aliquots and then stored at −20°C. Red top samples were incubated at room temperature for 2 h to allow clotting to occur and spun at 1500×**g** for 10 min at 20°C. Serum was collected into aliquots and then stored at −20°C. Plasma and serum samples were analysed at the New Zealand Vet Pathology Laboratory (Hamilton, New Zealand) for the following metabolites. Plasma glucose concentrations were measured with a colorimetric assay (Beckman Coulter Glucose Kit; Beckman Coulter Inc., Brea, CA, USA) using the Beckman Coulter AU680 Chemistry Analyzer (Beckman Coulter). Serum concentrations of non-esterified fatty acids (**NEFAs**) were measured with a colorimetric assay (Wako Non-Esterified Fatty Acid kit; FUJIFILM Wako Diagnostics U.S.A. Corporation, Mountain View, CA, USA) using the Beckman Coulter AU680 Chemistry Analyzer with reference to the Wako NEFA ACS-ACOD method for use on automated analysers. Total protein in serum was measured with a colorimetric assay (Beckman Coulter Total Protein Kit) using the Beckman Coulter AU680 Chemistry Analyzer. Total urea in serum was measured with a colorimetric assay (Beckman Coulter Urea Nitrogen Kit) using the Beckman Coulter AU680 Chemistry Analyzer.

Statistical analysis

Differences in botanical composition between offered and refused diets were determined by two-way ANOVA (Genstat 18; VSN International Limited, Hemel Hempstead, UK) with treatment and period as factors, and blocked for feeding (date and time). Differences in gross botanical composition (grass, legume, herb) between the treatment diets were analysed by two-way ANOVA (Genstat 18) with treatment and period as factors. The differences in nutritive quality and fatty acid content between diets were determined by two-way ANOVA (Genstat 18) with treatment and period as factors. Differences between DMI, milk yield, milk composition, LW, BCS, $\log_{10}$-transformed SCC, individual fatty acids in the milk and blood metabolites (glucose, protein, urea, and NEFA) were analysed by two-way ANOVA (Genstat 18) with pair as the experimental unit, treatment and period as factors and blocked for pair. Differences between means were considered significant at $P<0.05$.

Results

As expected, there was a substantial difference in botanical composition of MF and SF during the measurement periods (Table 1). The proportion of legume was similar between treatments, but approximately one-third of MF comprised herb species, and this treatment consisted of less grass compared with SF (Table 1). The botanical composition of MF refusals revealed a greater proportion of other grasses, white clover and dead material and a lesser proportion of red clover compared with MF (Table 1). Whereas, for SF only the proportion of dead material was higher in the refusals compared with the feed offered (Table 1). The levels of CP, NDF, ADF, ME, ash, soluble sugars, starch, non-structural carbohydrates, DOMD and CF measured in the MF and SF samples were similar (Table 2). However, the DM% of MF was lesser than that of SF (Table 2). There were also differences in the fatty acid profile between green fodder types, with the proportion of lauric acid (C12:0) and linoleic acid (C18:2 *n6*) being greater in MF compared with SF, and the proportion of α-linolenic acid (C18:3 *n3*) being lesser in MF compared with SF (Table 2).

Table 2 *Chemical composition (g/kg of DM unless otherwise stated) of green fodder and supplements (pellets) fed to dairy goats during measurement periods (days 14 to 18 for measurement period 1 and days 26 to 30 for measurement period 2)*

Chemical composition	Mixed-species green fodder	Standard green fodder	SED	P-value[1]	Pellets
DM (g/kg)	187	252	17	***	860
CP	142	136	4	ns	87
NDF	420	424	13	ns	80
ADF	317	299	13	ns	24
Metabolic energy (MJ/kg DM)	11.7	11.9	0.1	ns	14.8
Ash	107	106	3	ns	18
Soluble sugars	121	125	5	ns	39
Starch	26	22	9	ns	688
Non-structural carbohydrate	300	303	14	ns	783
DOMD[2] (g/kg)	730	747	6	*	929
Crude fat	31	33	1	ns	33
Triglycerides	15	16	0.4	ns	ND[3]
Fatty acid (proportion of total fatty acids detected; g/kg)					
C12:0	25	16	3	*	ND
C16:0	189	186	5	ns	ND
C18:0	18	18	2	ns	ND
C18:1 *c9*	18	16	3	ns	ND
C18:2 *n6*	149	117	2	***	ND
C18:3 *n3*	601	646	5	***	ND

[1] ns=$P>0.05$,*$P<0.05$,***$P<0.001$.
[2] Digestible organic matter in dry matter.
[3] Not determined.

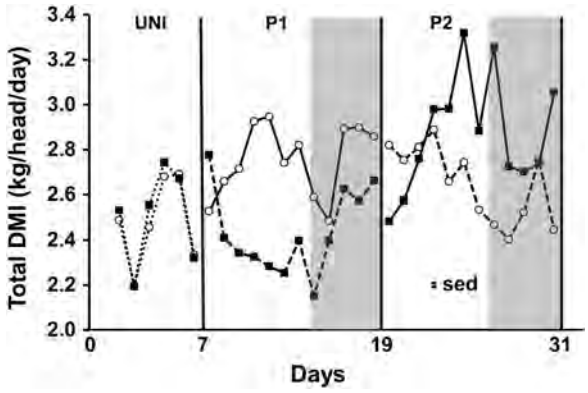

Figure 1 Mean daily dry matter intake (DMI) of green fodder and concentrate by lactating dairy goats housed in pairs and fed standard green fodder (SF; perennial ryegrass and white clover) or mixed-species green fodder (MF; perennial ryegrass, timothy, prairie grass, white clover, red clover, lucerne, chicory and plantain). During the uniformity period (UNI) goats were fed a combination of both SF and MF. During treatment period 1 (P1) group A (open circles, solid line) were fed MF and group B (closed squares, dashed line) were fed SF. During treatment period 2 (P2) group A (open circles, dashed line) were fed SF and group B (closed squares, solid line) were fed MF. Shaded areas indicate 5-day measurement periods. Bar represents SED between treatments (SF and MF).

When the goats were fed MF their total DMI (green fodder plus supplement) was 13% higher (Figure 1 and Table 3) and their milk yield was 7% higher than when the goats were fed SF (Figure 2 and Table 3). In addition, the BCS of goats was lesser when they were fed MF compared with SF (Table 3). No effect of treatment on LW was observed (Table 3).

The concentrations of milk protein and milk fat were 1.1 and 1.0 g/kg higher, respectively, when the goats were fed MF compared with SF (Table 3). No difference between treatments for milk lactose concentration was observed. Overall yield of milk solids was greater (protein increased 13 g/head per day, fat increased 12 g/head per day and lactose increased 13 g/head per day) for goats on MF compared with SF (Table 3). Somatic cell count was not affected by the different green fodder diets (Table 3).

Levels of blood protein, glucose, urea and NEFA were not affected by green fodder type (Table 3). Green fodder type did, however, affect the fatty acid profile of milk (Table 4). The proportions of pentadecylic acid (C15:0), palmitic acid (C16:0), *cis*-vaccenic acid (C18:1 *c11*), linoleic acid (C18:2 *n6*) and α-linolenic acid (C18:3 *n3*) were higher when goats were fed MF compared with SF. Whereas, caproic acid (C6:0), myristic acid (C14:0), stearic acid (C18:0), arachidic acid (C20:0), *iso*-C15, *iso*-C17, ginkgolic acid (C17:1) and oleic acid (C18:1 *c9*) proportions were lower in the milk of goats fed MF compared with SF.

Discussion

The DMI of goats was higher when they were offered a more diverse fodder species mix. Our results demonstrated that the increase in milk production observed was predominantly driven by increased DMI. The underlying mechanism for increased DMI in goats on MF diet was not investigated in this study but could be explained by previous works in other ruminant species. Herbs such as plantain and chicory and clover species are rapidly degradable in the rumen in comparison to grass species typically used in New Zealand farms (Burke *et al.*, 2000). Gregorini *et al.*

Table 3 *Mean daily feed intake, milk yield and composition, and blood metabolites of goats fed either mixed-species green fodder or standard green fodder and supplemented with concentrate during measurement periods (days 14 to 18 for measurement period 1 and days 26 to 30 for measurement period 2)*

Item	Mixed-species green fodder	Standard green fodder	SED	P-value[1]
DM intake (kg DM/head per day)	2.82	2.5	0.02	***
Green fodder (kg DM/head per day)	2.39	2.07	0.02	***
Supplement (kg DM/head per day)	0.43	0.43	–	–
Milk yield (kg/head per day)	4.12	3.85	0.03	***
Live weight (kg)	64.3	64.4	0.28	ns
Body condition score	2.4	2.5	0.04	*
Milk component concentration				
Fat (g/kg)	30.7	29.7	0.3	**
Protein (g/kg)	29.8	28.7	0.1	***
Lactose (g/kg)	46.3	46.4	0.1	ns
Milk component yield				
Fat (g/head per day)	126	114	1	***
Protein (g/head per day)	123	110	1	***
Lactose (g/head per day)	191	178	1	***
SCC[2] (log_{10} cells/ml)	5.56	5.56	0.03	ns
Blood metabolites				
Protein (g/L)	49.4	50.4	2.3	ns
NEFA[3] (mmol/L)	0.111	0.106	0.01	ns
Urea (mmol/L)	3.15	2.88	0.19	ns
Glucose (mmol/L)	2.76	2.74	0.05	ns

[1] ns=$P>0.05$, *$P<0.05$, **$P<0.01$, ***$P<0.001$.
[2] Somatic cell count.
[3] Non-esterified fatty acid.

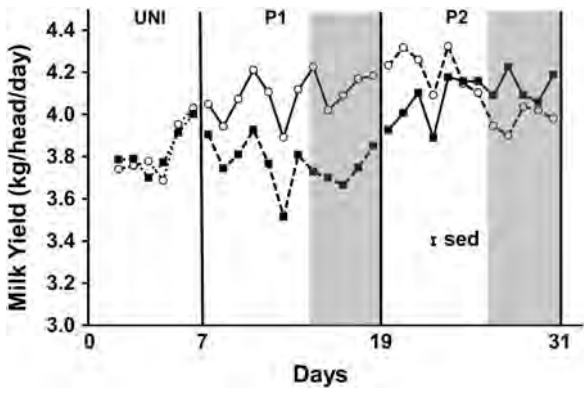

Figure 2 Mean daily milk yield from dairy goats housed in pairs and fed standard green fodder (SF; perennial ryegrass and white clover) or mixed-species green fodder (MF; perennial ryegrass, timothy, prairie grass, white clover, red clover, lucerne, chicory and plantain). During the uniformity period (UNI) goats were fed a combination of both SF and MF. During treatment period 1 (P1) group A (open circles, solid line) were fed MF and group B (closed squares, dashed line) were fed SF. During treatment period 2 (P2) group A (open circles, dashed line) were fed SF and group B (closed squares, solid line) were fed MF. Shaded areas indicate 5-day measurement periods. Bar represents SED between treatments (SF and MF).

(2013) demonstrated that cows grazing chicory and plantain, compared with perennial ryegrass, showed increased mastications per bite and reduced ruminative chewing. Their results suggest that the herbage particle size swallowed, when feeding on chicory and plantain compared with perennial ryegrass, was smaller and therefore may contribute to an increase in rumen turnover rate (Gregorini *et al.*, 2013). Hence, the high proportion of herbs and legumes in MF compared with SF could have facilitated a more rapid emptying of the rumen, stimulating greater intakes on MF.

The botanical analysis of refused feed demonstrated that the goats were selective in what fodder species they ate when given a more diverse choice – more of red clover and less of white clover and other grasses. In addition, the goats also avoided dead material in both treatment feeds. Goats have an innate ability to pick out different plant species and parts of plants containing high concentrations of protein or having higher digestibility (Provenza *et al.*, 2003). Therefore, a diverse diet, like the MF tested in this study, at an *ad libitum* allowance could potentially increase the ability to select dietary components conducive to higher intakes and milk production. A similar effect has been demonstrated in grazing lactating cows offered adjacent monocultures of perennial ryegrass, plantain and white clover (Pembleton *et al.*, 2016) and in grazing lactating sheep offered adjacent monocultures of perennial ryegrass and white clover (Champion *et al.*, 2004). Given an opportunity, ruminants naturally adjust intakes of different pasture/fodder species, which can positively influence overall milk production (Champion *et al.*, 2004; Pembleton *et al.*, 2016). However, whether the resulting increase in DMI when goats were offered MF compared with SF was due to the increased diversity of species alone or due to the presence of specific species within the mix is not clear and requires further research.

The two green fodder treatments investigated in this experiment had similar nutritive attributes, as demonstrated

Table 4 *Fatty acid profiles of milk from goats fed either mixed-species green fodder or standard green fodder*

Fatty acid	Mixed-species green fodder (g/kg)	Standard green fodder (g/kg)	SED	P-value[1]
Saturated				
C4:0	25.5	25.8	0.32	ns
C6:0	23.3	23.8	0.17	*
C8:0	25.2	25.9	0.38	ns
C10:0	96.5	98.1	1.64	ns
C12:0	40.1	40.8	1.05	ns
C14:0	111.2	113.2	0.84	*
C15:0	9.9	9.2	0.2	**
C16:0	284.7	275.8	3	**
C17:0	5.54	5.48	0.07	ns
C18:0	87.5	91.9	1.8	*
C20:0	1.3	1.4	0.02	*
Branched chain				
iso-C14	1.13	1.07	0.03	ns
iso-C15	2	2.3	0.05	***
iso-C16	2.2	2.3	0.07	ns
iso-C17	6.1	6.6	0.13	**
anteiso-C17	3.4	3.5	0.06	ns
Unsaturated				
C10:1	1.78	1.79	0.05	ns
C16:1	4.2	4.3	0.06	ns
C17:1	1.8	1.9	0.05	*
C18:1 *t9*	1.7	1.6	0.05	ns
C18:1 *t11*	17.2	18.2	0.67	ns
C18:1 *c9*	156.1	161	1.93	*
C18:1 *c11*	4.3	3.9	0.15	*
C18:2 *n6*	14.7	12.5	0.35	***
C18:3 *n3*	9.3	7.6	0.25	***
CLA[2]	8.5	8.7	0.36	ns

[1] ns=$P>0.05$,*$P<0.05$,**$P<0.01$,***$P<0.001$.
[2] Conjugated linoleic acids.

by chemical analyses of the feed and blood metabolites of the goats on each diet. Therefore, the observed increase in milk yield was most likely a direct consequence of increased DMI (Table 3). Along with a higher milk yield, the yields of all milk components also increased when the goats were fed MF. Increased milk production in response to feeding a more diverse pasture, including herbs and legumes compared with ryegrass-dominant pastures, has previously been demonstrated in cows (Waugh *et al.*, 1998; Chapman *et al.*, 2008; Roca-Fernández *et al.*, 2016) and sheep (Hutton *et al.*, 2011). Dairy cows grazing a mixed-species pasture, comprising grass, clover and herbs, had higher DMI and milk yields compared with cows grazing either perennial ryegrass alone or a perennial ryegrass and white clover mix (Roca-Fernández *et al.*, 2016). Dairy cows showed increased milk production when grazing chicory compared with ryegrass-based pastures (Waugh *et al.*, 1998), and Chapman *et al.* (2008) demonstrated that cows grazing a chicory and white clover pasture in late summer showed higher milk production than cows grazing predominantly grass pastures. Similarly, Hutton *et al.* (2011) established that ewes grazing a mixed pasture (consisting of chicory, plantain and red and white clover) in spring produced more milk and exhibited higher weight gains for both the ewe and her lamb compared with ewes grazing a ryegrass/white clover pasture. In addition, Harris *et al.* (1998) demonstrated that lactating dairy cows were more productive as the proportion of white clover in their diet was increased and that of perennial ryegrass was lowered.

The reason for higher protein and fat concentrations in milk from goats fed MF compared with SF was not determined in this study but may be related to a higher total energy and protein intake in goats due to a higher intake of DMI. A similar, although small, increase in milk protein and fat concentration in response to increased protein and energy intake has previously been reported in cows (Emery, 1978; Cragle *et al.*, 1986). However, it remains to be demonstrated whether goats can elicit the same response.

It has been well established that diet can affect the fatty acid profile of milk produced by ruminants (reviewed in Chilliard *et al.*, 2003), with some evidence of forage species effects (reviewed in Dewhurst *et al.*, 2006). The results of this study on goats demonstrate that a lower proportion of grass or a higher proportion of herbs within the green fodder diet increased the concentrations of linoleic and α-linolenic acids, which are both essential fatty acids. These findings are consistent with what has been demonstrated in cows feeding a diverse pasture (Soder *et al.*, 2006) or silage containing a higher proportion of legumes over grasses (Dewhurst *et al.*, 2003a and 2003b). Cows cannot synthesise these long-chain unsaturated fatty acids and therefore must obtain them from dietary sources. In the diets, the proportions of fatty acids within the two green fodder treatments were different, with MF being higher in lauric and linoleic acids and lower in α-linolenic acid compared with SF.

Oleic acid can either be derived directly from the diet or synthesised within the mammary gland from stearic acid. Another important source of oleic acid in milk is the adipose tissue, which will be mobilised by the mammary gland during periods of decreased energy intake (Jakobsen, 1999). This is plausible, as the proportion of this fatty acid was higher in goat's milk when they were fed SF, of which they ate a lesser amount.

Odd and branched chain fatty acids in milk fat are predominantly derived from rumen bacteria (Fievez *et al.*, 2012). It has been demonstrated that changes in the proportions of these fatty acids in cows are related to relative proportions of cellulolytic and amylolytic bacteria present in the rumen. It has also been determined to be a marker indicating changes in the rumen microbiome that occur when the diet is switched from predominantly forage-based to concentrate-based as well as for dietary imbalances such as subacute ruminal acidosis (**SARA**). The present study demonstrated an increase in pentadecylic acid and a decrease in ginkgolic acid, *iso*-C15:0 and *iso*-C17:0 in response to MF treatment relative to SF treatment. These changes in milk fat

composition suggest a shift towards amylolytic bacteria enrichment or the onset of SARA in response to the MF diet. The reason for this shift is unclear as both diets are similar in nutritive levels and may be a consequence of the difference in plant species within the diet being processed differently within the rumen. Again, further investigation is required to understand how the rumen and its microbiome process different plant species.

The diet also had a small effect on the proportions of several saturated fatty acids, with palmitic acid proportion being higher when the goats were fed MF, and caproic, myristic, stearic and arachidic acid proportions being higher when the goats were fed SF. The short- to mid-chain fatty acids (caproic and myristic) are predominantly produced by *de novo* synthesis within the mammary gland, whereas *de novo* synthesis of palmitic acid accounts for 50% of its presence in milk, the remainder being provided by the diet. Long-chain fatty acids in milk fat are predominantly derived from the diet, stearic acid being the product of biohydrogenation of unsaturated fatty acids, including linoleic and α-linolenic acid. Although these differences are present in the milk fat profiles between the treatment diets, overall the differences were relatively minor and probably did not significantly affect the attributes of the milk.

Conclusion

To our knowledge, this is the first study to investigate the effects of diverse multi-species green fodder on the DMI, milk production and milk fatty acid profiles of goats. Under these experimental conditions, feeding MF compared with SF stimulated a higher DMI in goats. The underlying mechanism for this increase was not determined. Feeding goats a diversity of green fodder mix altered milk fatty acid profiles, with increase in beneficial n-3 and n-3 fatty acids in response to the MF diet. Overall, the increased sward diversity of MF had a positive effect on goat milk production and represents an important additional management tool to increase milk production in green fodder-based dairy systems.

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Ethics statement

All animal manipulations were conducted in compliance with the rules and guidelines of the AgResearch Ruakura Animal Ethics Committee (Hamilton, New Zealand).

Declaration of interest

Authors declare no conflict of interest pertaining to the research described in this manuscript.

Software and data repository resources

None of the data were deposited in an official repository.

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Rubber seed oil and flaxseed oil supplementation alter digestion, ruminal fermentation and rumen fatty acid profile of dairy cows

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*Rubber seed oil (**RO**) that is rich in polyunsaturated fatty acids (**FA**) can improve milk production and milk FA profiles of dairy cows; however, the responses of digestion and ruminal fermentation to RO supplementation in vivo are still unknown. This experiment was conducted to investigate the effect of RO and flaxseed oil (**FO**) supplementation on nutrients digestibility, rumen fermentation parameters and rumen FA profile of dairy cows. Forty-eight mid-lactation Holstein dairy cows were randomly assigned to one of four treatments for 8 weeks, including basal diet (CON) or the basal dietary supplemented with 4% RO, 4% FO or 2% RO plus 2% FO on a DM basis. Compared with CON, dietary oil supplementation improved the total tract apparent digestibility of DM, neutral detergent fibre and ether extracts (*$P < 0.05$*). Oil treatment groups had no effects on ruminal digesta pH value, ammonia N and microbial crude protein (*$P > 0.05$*), whereas oil groups significantly changed the volatile fatty acid (**VFA**) profile by increasing the proportion of propionate whilst decreasing total VFA concentration, the proportion of acetate and the ratio of acetate to propionate (*$P < 0.05$*). However, there were no differences in VFA proportions between the three oil groups (*$P > 0.05$*). In addition, dietary oil supplementation increased the total unsaturated FA proportion in the rumen by enhancing the proportion of* trans*-11 C18:1 vaccenic acid (**VA**), cis-9,* trans*-11 conjugated linoleic acid (**CLA**) and α-linolenic acid (**ALA**) (*$P < 0.05$*). These results indicate that dietary supplementation with RO and FO could improve nutrients digestibility, ruminal fermentation and ruminal FA profile by enhancing the VA, cis-9, trans-11 CLA and ALA composition of lactating dairy cows. These findings provide a theoretical basis for the application of RO in livestock production.*

Keywords: vegetable oils, linolenic acid, digestion, ruminal metabolism, lactating cows

Implications

Rubber seed oil (**RO**) is a by-product of the rubber industry and rich in polyunsaturated fatty acids. This is the first *in vivo* study investigating the effects of diet RO on nutrient digestibility, rumen fermentation and rumen fatty acid profile of dairy cows. Our results indicate the potential of RO as a feedstuff in dairy cow diets to improve nutrients digestibility, ruminal fermentation and ruminal fatty acid profile by enhancing *trans*-11 C18:1, *cis*-9, *trans*-11 conjugated linoleic acid and α-linolenic acid. These findings provide a theoretical basis for the application of RO in livestock production.

Introduction

Vegetable oils rich in polyunsaturated fatty acids (**PUFA**) have been used in dairy cow diets to alter the milk fatty acids (**FA**) composition and especially to enhance PUFA content (Loor *et al.*, 2002; Bu *et al.*, 2007), which has been shown to have potential health benefits, including anticarcinogenic properties (Roche *et al.*, 2001). Rubber seed oil is a by-product of the rubber industry with the main origin of RO being in the Guangdong, Guangxi and Yunnan provinces of China. The levels of total unsaturated fatty acid (**UFA**) and PUFA in RO can amount to 83% and 59%, respectively (Pi *et al.*, 2016). Compared to the flaxseed oil (**FO**), which is a common vegetable oil rich in α-linolenic acid (**ALA**), RO is rich in ALA. In FO, ALA contributes

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approximately 55% of the oil's total FA; whereas, in RO, the ALA content is lower at 22%. It is notable that previous rat toxicological and brine shrimp tests showed that RO had no acute toxicity effects and was not found to contain any hazardous linamarin (Abdullah & Salimon, 2010). In addition, our previous study reported that RO and FO supplementation (as a source of ALA) can increase milk production and alter milk fat FA composition by increasing vaccenic acid (**VA**), conjugated linoleic acid (**CLA**) and ALA and decreasing the concentration of saturated fatty acid (**SFA**) (Pi *et al*., 2016), suggesting the potential of RO as a feedstuff in dairy cows dietary.

The process of biohydrogenation (**BH**) reduces the rumen outflow of PUFA and contributes to the accumulation of *cis* and *trans* isomers in ruminant products, including CLA and *trans* monoene. Hence, the extent and type of the rumen BH process will determine both the amounts and structures of FA leaving the rumen. In addition, PUFAs can be toxic to rumen microorganisms (Jenkins, 1993) that play an important role in the digestion and metabolism of nutrients. Thus, the FA structure present in the rumen may influence digestion physiology. Studies have shown that high dietary oil levels could decrease the counts of ruminal bacteria and protozoa (Dohme *et al*., 2001; Yang *et al*., 2009). On the other hand, supplementing ruminant diets with oil seed and vegetable oils had a negative effect on ruminal digestion of neutral detergent fibre (**NDF**) and organic matter (**OM**) (Jenkins, 1993; Martin *et al*., 2008; Lunsin *et al*., 2012b). Thus, such negative changes may limit the amount of RO that can be incorporated in the diet of dairy cows. However, information on the effect of dietary supplementation of RO on nutrient digestibility of dairy cows is scarce.

Feeding vegetable oils rich in PUFA can change rumen fermentation by enhancing the molar percentage of propionate (Jalc and Ceresnakova, 2002; Yang *et al*., 2009). *In vitro* studies have even demonstrated that the acetate to propionate ratio changed significantly by adding unsaturated C18 fatty acid (Li *et al*., 2012; Gao *et al*., 2016). In addition, a previous *in vitro* study using a rumen-simulation technique found that 4% RO supplements significantly changed the rumen fermentation pattern by decreasing both the acetate level and the ratio of acetate and propionate (Shi, 2014), suggesting that PUFA-enriched RO impacts on rumen fermentation. However, to date, there are no studies investigating the effect of RO on rumen fermentation *in vivo*. Therefore, the objective of this study was to investigate the responses of nutrient digestibility (total tract), rumen fermentation characteristics and rumen FA profile of lactating dairy cows to diet supplementation with RO and FO. We hypothesise that dietary supplementation with RO could also improve the nutrients' digestibility, change the rumen fermentation pattern and enhance *cis*-9, *trans*-11 CLA and ALA profiles in ruminal fluid in comparison with FO.

Material and methods

The animal experiment was performed at Tianjin Mengde Dairy Farm (Tianjin, China). All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Chinese Academy of Agricultural Sciences (Beijing, China).

Cows, experimental design and treatments

The details of the animal experimental design were reported in our previous study (Pi *et al*., 2016). Briefly, 48 mid-lactation healthy Chinese Holstein dairy cows (163 ± 25.3 days in milk, 29.6 ± 2.42 kg of milk/day and parity 1.8 ± 1.25) were randomly assigned to one of four treatments ($n = 12$) in a completely randomized design. The total duration of the experiment was 8 weeks, during which the cows were fed a basal diet (CON) or the basal diet supplemented with 4% RO, 4% FO or 2% rubber seed oil plus 2% flaxseed oil (**RFO**) on a DM basis. The FO was supplied by Huajian Axunge Co., Ltd (Shanxi, China), and the RO was supplied by Kunming Institute of Botany, Chinese Academy of Sciences (Kunming, China). The ingredients and chemical composition of the diets are presented in Table 1. The diet was formulated to meet or exceed the nutrient demand according to the Feeding Standards of Dairy Cattle, China NongYe HangYe Biaozhun/Tuijian-34. During the experimental period, cows were housed in a mechanically ventilated barn divided into four plots with 12 cows each and fed individually, with free access to fresh water. At the beginning of this experiment, cows were gradually adjusted to the experimental diets over a 1-week period. Diets were fed as a total mixed ration (**TMR**) three times daily (05:30, 13:30 and 18:00 h) to ensure <10% refusals. The oils were stored at 4°C and were added fresh as the final component after mixing the other dietary ingredients. The TMR containing oil supplements was made once daily and stored under shade until fed at later time. Cows were milked three times daily (at 05:00, 13:00 and 21:00 h) with individual milk yields recorded at each milking.

Sampling, measurements and analysis

The quantity of daily feed offered and refused was recorded for individual cows. Samples of TMR were collected daily and frozen at −20°C before subsequent analysis. Orts were sampled twice weekly from each cow, composited for each treatment, and frozen at −20°C for further analysis. Weekly representative samples of TMR from each treatment were analysed for DM content by oven drying at 60°C to a constant weight.

Faecal samples (approximately 100 g wet weight) were collected from the rectum of each cow every 6 h on day 53, 54 and 55 of the experiment, resulting in 12 representative faecal samples per cow according to Zhou *et al*. (2015). Samples from each time point were divided into two portions, and samples were pooled across sampling times for each cow. A 10% volume of 6 M hydrochloric acid was added to one pooled portion from each cow immediately after

Table 1. *Ingredient and chemical composition of the experimental diets for dairy cows*

Items	Treatment[1]			
	CON	RO	FO	RFO
Diet ingredient, % of DM				
Alfalfa hay	19.9	19.9	19.9	19.9
Chinese wildrye	3.8	3.8	3.8	3.8
Corn silage	24.6	24.6	24.6	24.6
Corn	17.8	16.2	16.2	16.2
Flaked corn	6.3	5.7	5.7	5.7
Soybean meal	11.5	10.5	10.5	10.5
Soy hulls	2.5	2.3	2.3	2.3
Distillers dried grains with solubles (**DDGS**)	3.8	3.5	3.5	3.5
Double-low rapeseed meal (**DLRM**)	3.9	3.6	3.6	3.6
Molasses (30%)	2.5	2.5	2.5	2.5
Rubber seed oil	–	4.0	–	2.0
Flaxseed oil	–	–	4.0	2.0
Sodium bicarbonate	0.5	0.5	0.5	0.5
Dicalcium phosphate	0.3	0.3	0.3	0.3
Salt	0.5	0.5	0.5	0.5
Calcium carbonate	0.9	0.9	0.9	0.9
Vitamin-mineral premix[2]	1.2	1.2	1.2	1.2
Chemical, % of DM (unless otherwise noted)				
DM	49.7	50.6	50.6	50.6
NE_L, Mcal/kg of DM[3]	1.61	1.73	1.73	1.73
CP	16.6	16.1	16.1	16.1
NDF	35.0	34.4	34.4	34.4
ADF	21.8	21.5	21.5	21.5
Ash	8.02	7.99	8.09	8.11
Ca	1.10	1.06	1.06	1.06
P	0.36	0.35	0.35	0.35
NFC[4]	37.59	35.14	35.09	35.13
EE	2.79	6.37	6.32	6.26
FA, % of total FA reported				
C14:0	0.39	0.23	0.20	0.20
C16:0	18.16	12.33	10.05	11.08
C16:1	0.55	0.36	0.24	0.31
C17:0	0.17	0.10	0.10	0.10
C18:0	2.58	5.23	2.91	3.98
C18:1 *cis*-9	21.97	21.70	19.38	20.50
C18:2 *cis*-9, *cis*-12	44.66	40.18	27.12	33.38
C18:3	9.06	18.33	38.59	29.18
C20:0	0.51	0.39	0.27	0.32
C20:1	0.23	0.21	0.20	0.20
C22:0	0.44	0.25	0.26	0.26
C22:2	0.46	0.22	0.22	0.21

RO = rubber seed oil; FO = flaxseed oil; DM = dry matter; NE_L = net energy for lactation; CP = crude protein; NDF = neutral detergent fibre; ADF = acid detergent fibre; NFC = non-fibre carbohydrate; EE = ether extracts; FA = fatty acids.
[1] Cows were fed a basal diet (control; CON) or basal diet supplemented with either 4.0% rubber seed oil (RO), 4.0% flaxseed oil (FO) or 2.0% rubber seed oil + 2.0% flaxseed oil (RFO). The CON diet was also used for feeding during the pre-trial period.
[2] Contained (per kg of DM): a minimum of 313 500 IU of vitamin A; 104 500 IU of vitamin D; 5000 IU of vitamin E; 780 mg of Cu; 780 mg of Fe; 780 mg of Mn; 3900 mg of Zn; 30 mg of Se; 50 mg of I; 65 mg of Co.
[3] Calculated value (based on China Standard NY/T 34, 2004).
[4] NFC = 100 − % NDF − % CP − % EE − % Ash.

sampling for subsequent nitrogen analysis. All faecal samples were dried at 60°C for 48 h, ground using a Wiley mill (Arthur H. Thomas Co.) and passed through a 1-mm mesh screen. During each 3-day collection period, TMR and ort samples were collected daily and stored at −20°C for subsequent analysis. Total-tract nutrient apparent digestibility (**TTAD**) was calculated using acid-insoluble ash (**AIA**) as an internal marker based on the concentration of AIA in the diet and faeces (Van Keulen and Young, 1977). The crude protein (CP; N × 6.25) content of feed samples was determined using the macro-Kjeldahl nitrogen test (AOAC International, 2000; method 976.05) with a Kjeltec digester 20 and a Kjeltec System 1026 distilling unit (Tecator AB). The contents of NDF and ADF were determined using procedure A by Van Soest *et al.* (1991) using heat-stable amylase (type XI-A of *Bacillus subtilis*; Sigma-Aldrich, St. Louis, MO), but without sodium sulphite for the NDF. The ash content was determined by incineration at 550°C overnight, and the OM content was calculated by AOAC International (2000; method 942.05). The ether extract (**EE**) content was determined using a Soxtec system HT6 apparatus (Tecator AB) according to AOAC International (2000; method 920.39).

Samples of ruminal fluid (approximately 100 ml) were collected 3 h after morning feeding on day 49 using an oral stomach tube (Shen *et al.*, 2012). The first 10 ml sample was discarded to eliminate saliva contamination that would falsely elevate the pH, volatile fatty acid (**VFA**) and ammonia nitrogen (N) levels. Ruminal liquor samples were strained through four layers of cheesecloth, and pH was determined immediately after collection (6250 membrane pH meter, Yibo Instruments Corp., Shanghai, China). Samples for ammonia-N analysis were acidified with 1 mol/l HCl, and those for VFA analysis had 1 ml of 25% meta-phosphoric acid added to 5 ml of rumen fluid to deproteinize the sample. All the samples were then stored at −20°C until analysis. Ammonia-N concentration was determined using the sodium salicylic acid and hypochlorous acid spectrophotometric method (Feng & Gao, 1993). Microbial crude protein (**MCP**) concentration was analysed using a spectrophotometer (UV-2450), according to the method of Makkar *et al.* (1982). The VFA concentration was determined by gas chromatography (model 6890, Series II; Hewlett Packard Co., Avondale, PA, USA) as described by Liu *et al.* (2009). Ruminal FA profile analysis was conducted using the method described in Pi *et al.* (2016). The methane production was calculated according to the method of Moss *et al.* (2000) as follows: methane production (mmol/l) = 0.45 × acetate (mmol/l) − 0.275 × propionate (mmol/l) + 0.4 × butyrate (mmol/l). The correlation coefficient of the prediction of methane production from VFA concentrations is 0.882.

Statistical analysis

All the data were analysed as a completely randomised design using the MIXED procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC). The statistical model included cow as a random effect and treatment as a fixed effect. The MIXED statistical model is as follows:

Table 2. *Effects of dietary rubber seed oil and flaxseed oil supplementation on nutrient intake and total tract apparent digestibility of dairy cows*

Items	Treatments[1]				SEM[2]	*P*- value[3]		
	CON	RO	FO	RFO		C *v.* oil	FO *v.* RO	RFO *v.* FO + RO
Intake, kg/day								
DM	20.21	20.53	19.84	19.92	0.263	0.34	0.56	0.45
NDF	7.27	7.01	6.94	7.00	0.114	0.68	0.75	0.87
ADF	4.53	4.39	4.34	4.38	0.074	0.77	0.46	0.67
CP	3.45	3.28	3.25	3.28	0.068	0.98	0.82	0.63
OM	19.12	18.75	18.55	18.72	0.281	0.22	0.58	0.44
EE	0.58	1.31	1.27	1.27	0.021	**	0.43	0.65
Digestibility, %								
DM	73.27	72.85	78.92	77.42	0.566	**	**	0.06
NDF	46.84	48.08	56.53	53.38	0.828	**	**	0.31
ADF	39.57	39.56	41.84	40.98	0.969	0.79	0.26	0.60
CP	71.81	72.80	74.33	73.53	0.988	0.53	0.31	0.88
OM	76.78	75.12	80.01	78.59	0.556	0.32	*	0.42
EE	86.07	90.79	93.93	91.93	0.571	**	*	0.61

RO = rubber seed oil; FO = flaxseed oil; DM = dry matter; NDF = neutral detergent fibre; ADF = acid detergent fibre; CP = crude protein; OM, organic matter; EE, ether extracts.
[1] Cows were fed a basal diet (control; CON) or basal diet supplemented with either 4.0% rubber seed oil (RO), 4.0% flaxseed oil (FO) or 2.0% rubber seed oil + 2.0% flaxseed oil (RFO). The control diet was also used for feeding during the pretrial period.
[2] SEM = Standard error of least squares means.
[3] CON *v.* oil = CON *v.* oil (RO, FO, RFO); RO *v.* FO = RO *v.* FO; RFO *v.* RO + FO = RFO *v.* RO plus FO.
*$P < 0.01$, **$P < 0.001$.

$$Y_{ij} = \mu + T_i + e_{ij},$$

where Y_{ij} = observation from animals, μ = overall mean, T_i = treatment effect ($i = 1$–4), and e_{ij} = random error.

Orthogonal contrasts included the following: CON *v.* oil supplemented diets to test the effect of oil supplementation; RO *v.* FO to test the effect of high rubber seed oil *v.* high flaxseed oil; and RFO *v.* RO + FO to test the additive effect of rubber seed oil and flaxseed oil. The significance level was declared at $P < 0.05$. Correlation analyses were assessed by Pearson's correlation test using GraphPad Prism version 5.00 (GRAPHPAD Software, San Diego, CA, USA). Significant correlation was considered at $P < 0.05$.

Results

Milk yield, nutrients intake and total tract apparent digestibility

The results of milk yield were reported in our previous study (Pi *et al.*, 2016). Briefly, oil supplementation significantly increased milk yield by 10.98% (14.02%, 10.98% and 7.95% for RO, FO and RFO, respectively) ($P < 0.05$). In addition, milk fat content was decreased in dairy cattle fed the RO, FO or RFO treatments (3.19% on average) compared with the CON (3.62%) ($P < 0.05$). However, oil treatment had no effect on milk fat yield ($P > 0.05$). Milk lactose yield was increased in dairy cattle fed the RO, FO or RFO treatments (1.40, 1.39 and 1.37 kg/d, respectively) compared with the CON (1.29 kg/d) ($P < 0.05$).

There were no differences in DM, NDF, ADF, CP and OM intake between the CON group and the oil groups; however, all the oil treatment groups showed increased intake of EE ($P < 0.05$) (Table 2). In addition, there were no differences in nutrient intake among the oil groups ($P > 0.05$). For TTAD, oil treatment groups significantly increased the TTAD of DM, NDF and EE compared with the CON group. However, there were no differences in the TTAD of ADF, CP and OM between the CON group and the oil groups ($P > 0.05$). In addition, the TTAD of DM, NDF, OM and EE were higher in the FO group than RO group ($P < 0.05$). Feeding the blend of RO and FO (RFO) did not result in any significant differences in TTAD of nutrients compared with feeding them separately ($P > 0.05$).

Ruminal fermentation parameters

Results of the ruminal fermentation parameters are shown in Table 3. Compared with the CON group, oil groups had no effect on pH, the concentrations of ammonia-N and MCP ($P > 0.05$). Oil groups significantly decreased the concentration of total VFA, the proportion of acetate and the ratio of acetate and propionate, but increased the proportion of propionate and valerate compared with CON ($P < 0.05$). However, there were no differences in the rumen fermentation parameters between the three oil groups ($P > 0.05$). In addition, feeding the blend of RO and FO (RFO) also showed no significant differences in rumen fermentation parameters compared with feeding them separately ($P > 0.05$). Oil groups significantly decreased the production of methane compared with the control group ($P < 0.05$).

Ruminal fluid and milk fatty acid profiles

The FA composition in ruminal fluid is shown in Table 4. Compared with CON, oil groups significantly increased the

Table 3. *Effects of dietary rubber seed oil and flaxseed oil supplementation on rumen fermentation characteristics of dairy cows*

Items	Treatments[1]				SEM[2]	*P*-Value[3]		
	CON	RO	FO	RFO		C *v.* oil	FO *v.* RO	RFO *v.* FO + RO
pH	6.33	6.36	6.38	6.35	0.037	0.09	0.70	0.26
Ammonia-N, mg/dl	14.28	8.99	9.19	12.93	1.908	0.09	0.94	0.11
MCP, mg/ml	1.15	1.12	1.14	1.08	0.077	0.62	0.80	0.58
Rumen VFA, mmol/l								
TVFA	120.89	99.71	105.71	106.05	5.399	*	0.17	0.33
Acetate	78.59	59.74	62.01	61.93	3.221	**	0.32	0.65
Propinate	28.22	27.98	30.80	29.98	1.961	0.58	0.23	0.36
Butyrate	10.21	8.49	8.61	9.47	0.599	0.45	0.22	0.51
Isobutyate	0.68	0.72	0.66	0.81	0.063	0.34	0.56	0.37
Valerate	2.25	2.57	2.55	2.75	0.197	0.55	0.24	0.51
Isovalerate	1.16	1.12	1.09	1.33	0.067	0.04	0.09	0.16
Molar proportion, mM/100 mM TVFA								
Acetate	66.70	59.88	58.69	58.34	0.934	**	0.35	0.40
Propionate	22.61	28.10	28.97	28.36	0.892	**	0.53	0.89
Butyrate	8.26	8.08	8.24	8.88	0.369	0.74	0.75	0.12
Isobutyate	0.67	0.73	0.63	0.81	0.042	0.58	0.40	0.29
Valerate	2.03	2.58	2.58	2.59	0.124	***	0.98	0.96
Isovalerate	1.10	1.15	1.05	1.26	0.047	0.63	0.45	0.18
Acetate: propionate ratio	2.95	2.16	2.05	2.07	0.128	**	0.54	0.83
Methane, mmol/L	31.69	22.58	22.88	23.41	1.150	**	0.68	0.77

RO = rubber seed oil; FO = flaxseed oil;MCP = microbial crude protein; VFA = volatile fatty acid; TVFA = total volatile fatty acid.
[1, 2, 3] For further description, check footnotes of Table 2.
*$P < 0.05$, **$P < 0.001$, ***$P < 0.01$.

levels of VA, *cis*-9, *trans*-11 CLA, ALA, total UFA and total monounsaturated fatty acids (**MUFA**) in rumen, while markedly decreasing the content of total SFA ($P < 0.05$). The concentrations of *cis*-9, *trans*-11 CLA, total UFA and total PUFA in the ruminal fluid of FO groups were higher than that in RO ($P < 0.05$). There were no significant differences seen from offering compared with feeding the oils separately ($P > 0.05$). The composition of FAs in milk was reported in our previous study (Pi *et al.*, 2016). Briefly, the proportions of short-chain fatty acids (C4:0, C6:0, C8:0, C10:0, C12:0 and C13:0) and medium-chain fatty acids (C14:0, C14:1, C15:0, C16:0 and C16:1) were lower ($P < 0.05$) in milk from cows fed RO, FO, or RFO compared to CON. The proportion of long-chain fatty acids (C18:0, *trans*-9 C18:1, VA, *cis*-9 C18:1, *cis*-9, *trans*-11 CLA and ALA) in milk fat were increased ($P < 0.05$) in cows fed RO, FO or RFO compared with CON.

Correlation analysis

The correlation relationships between rumen FA and milk FA profiles were evaluated in this study (Figure 1). The results showed that ruminal VA content was positively correlated with *cis*-9, *trans*-11 CLA content in milk ($P < 0.001$) (Figure 1a). Both the contents of *cis*-9, *trans*-11 CLA and ALA in rumen were positively correlated with these FA profiles in milk ($P < 0.001$) (Figure 1b and Figure 1c).

Discussion

Previous studies showed that high levels of dietary oil rich in UFA can influence the rumen BH (Loor *et al.*, 2004; Maia *et al.*, 2010) and cause negative effects on nutrient digestion and fermentation due to the hydrophobic and amphiphilic nature of oils (Onetti *et al.*, 2001; Martin *et al.*, 2008; Lunsin *et al.*, 2012b). However, the effects of dietary RO supplementation on rumen digestion and metabolism are unknown. Thus, this is the first *in vivo* study investigating the effects of diet RO and FO on nutrient digestibility, rumen fermentation and rumen FA profile of dairy cows. We observed that supplementation with oils improved the TTAD of nutrients (DM, EE and NDF) and shifted the rumen fermentation towards propionate reflected by the higher level of propionate and decreased ratio of acetate and propionate. In addition, the oils used also altered the rumen FA profiles by increasing the levels of VA, *cis*-9, *trans*-11 CLA and ALA and decreasing the total SFA level.

Rubber seed oil and flaxseed oil improved the total tract apparent digestibility of nutrients

Kholif et al. (2018) and Kholif et al. (2016) reported that flaxseed oil supplementation at approximately 2.4% of DM improved DM, EE and NDF digestibility. In addition, one *in vitro* study also observed that diet supplementation of plants oils at 3% of DM could enhance the digestibility of NDF and EE using a rumen-simulation technique (Vargas

Table 4. *Effects of dietary rubber seed oil and flaxseed oil supplementation on rumen fatty acid proportions of dairy cows (g/100 g total FA)*

	Treatments[1]					*P* -value[3]		
Items	CON	RO	FO	RFO	SEM[2]	C *v.* oil	FO *v.* RO	RFO *v.* FO + RO
C8:0	0.42	0.19	0.83	1.56	0.144	*	*	**
C12:0	0.27	0.15	0.19	0.16	0.029	*	0.57	0.95
C14:0	1.25	0.85	0.70	0.75	0.082	**	0.67	0.17
C15:0	0.90	0.57	0.55	0.53	0.044	**	0.14	0.21
C16:0	19.98	14.85	14.59	14.24	0.574	**	0.64	0.50
C16:1	0.12	0.09	0.14	0.11	0.009	*	**	0.57
C17:0	0.55	0.32	0.36	0.33	0.018	**	0.43	0.13
C18:0	50.56	42.02	34.28	35.11	2.298	**	*	0.79
trans-9 C18:1	0.72	1.78	1.38	1.48	0.099	**	*	0.47
trans-11 C18:1 (VA)	6.07	22.47	24.35	23.71	1.557	**	0.33	0.87
cis-9 C18:1	8.58	9.43	14.38	11.71	1.206	***	*	0.89
cis-9, 12 CLA	3.89	2.06	4.39	2.92	0.679	0.32	***	0.62
C20:0	0.73	0.59	0.52	0.58	0.043	*	0.35	0.27
C18:3 (ALA)	0.53	0.63	0.96	0.85	0.169	***	0.25	0.54
C20:1	0.05	0.11	0.11	0.13	0.012	**	0.80	0.31
cis-9, *trans*-11 CLA	1.09	1.32	2.65	2.52	0.396	***	***	0.27
trans-10, *cis*-12 CLA	0.23	0.21	0.15	0.14	0.022	***	***	0.06
C22:0	0.36	0.30	0.27	0.28	0.020	**	0.36	0.87
C23:0	3.39	2.67	3.04	4.14	0.762	0.56	0.77	0.52
cis-13 C22:1	0.09	0.06	ND	ND	0.003	**	**	1.00
C24:0	0.24	0.13	ND	ND	0.009	**	**	1.00
C22:2	0.35	0.24	0.22	0.22	0.012	**	0.10	0.31
Summations[4]								
SFA	77.33	61.95	52.57	56.18	1.948	**	*	0.69
UFA	22.61	38.05	47.39	43.75	1.948	**	*	0.70
MUFA	15.62	33.94	39.11	37.12	1.629	**	***	0.79
PUFA	6.07	4.11	7.24	6.63	0.682	0.73	***	0.32

RO = rubber seed oil; FO = flaxseed oil; FA = fatty acids; VA = vaccenic acid; CLA = conjugated linoleic acid; ALA = α-linolenic acid; ND = not detected; SFA = saturated fatty acids; UFA = unsaturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.
[1,2,3] For further description, check footnotes of Table 2.
[4] SFA, sum of C8:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0 and C23:0; UFA, total unsaturated FA reported; MUFA, sum of C16:1, *trans*-9 C18:1, *trans*-11 C18:1 (VA), *cis*-9 C18:1, C20:1 and *cis*-13 C22:1; PUFA, sum of *cis*-9, 12 CLA, *trans*-10, *cis*-12 CLA, *cis*-9, *trans*-11 CLA, C18:3 (ALA) and C22:2.
$^{*}P < 0.01$, $^{**}P < 0.001$, $^{***}P < 0.05$.

et al., 2017). In consistent with these studies, we observed that FO and RO supplementation in dietary at 4% of DM also could increase the digestibility of DM and NDF in this study. Conversely, studies noted a decrease in DM, OM and NDF digestibility using flaxseed oil in dairy cows dietary at relative higher levels (5.8% of DM) (Martin *et al.*, 2008), highlighting that the effect of oil supplementation depends on the level of oil being fed. In addition, compared with FO group, RO group significantly decreased the digestibility of NDF, DM, EE and OM. Considering the levels of these oil supplementation in dietary were same and the main FA composition (ALA) were difference between FO (39%) and RO (18%) (Pi *et al.*, 2016). Thus, these differences in nutrients digestibility between FO and RO group may be due to the differential FA composition. On the other hand, a previous study showed that diet addition with roughage (cassava hay) could ameliorate the negative influence on apparent digestibility of DM and OM when supplementing with 4% of rice bran oil (Lunsin *et al.*, 2012a). In this study, the ratio of forage to concentrate was 50:50, indicating that the higher percentage of forage in the base diet may ameliorate the negative influence on nutrient digestion by oil supplementation due to the ability of forage to promote normal rumen function for maximum biohydrogenation. Ueda *et al.* (2003) also observed that increasing the forage portion of the dairy cow diet decreases the negative effects of flaxseed oil compared with increasing the proportion of concentrates in the diet.

Rubber seed oil and flaxseed oil shifted the ruminal fermentation pattern

In this study, RO and FO supplementation had no effects on rumen pH value and ammonia-N. It was consistent with previous research reported that diet supplementation of plant oil had no effects of rumen pH value and ammonia-N level of dairy cows (Lunsin *et al.*, 2012b). However, when feeding supplemental lipid, the molar proportion of ruminal acetate decreased and propionate increased; concomitantly, the ratio of acetate:propionate decreased (Onetti *et al.*, 2001). We also observed that RO and FO supplementation have the same effects on acetate and propionate composition in

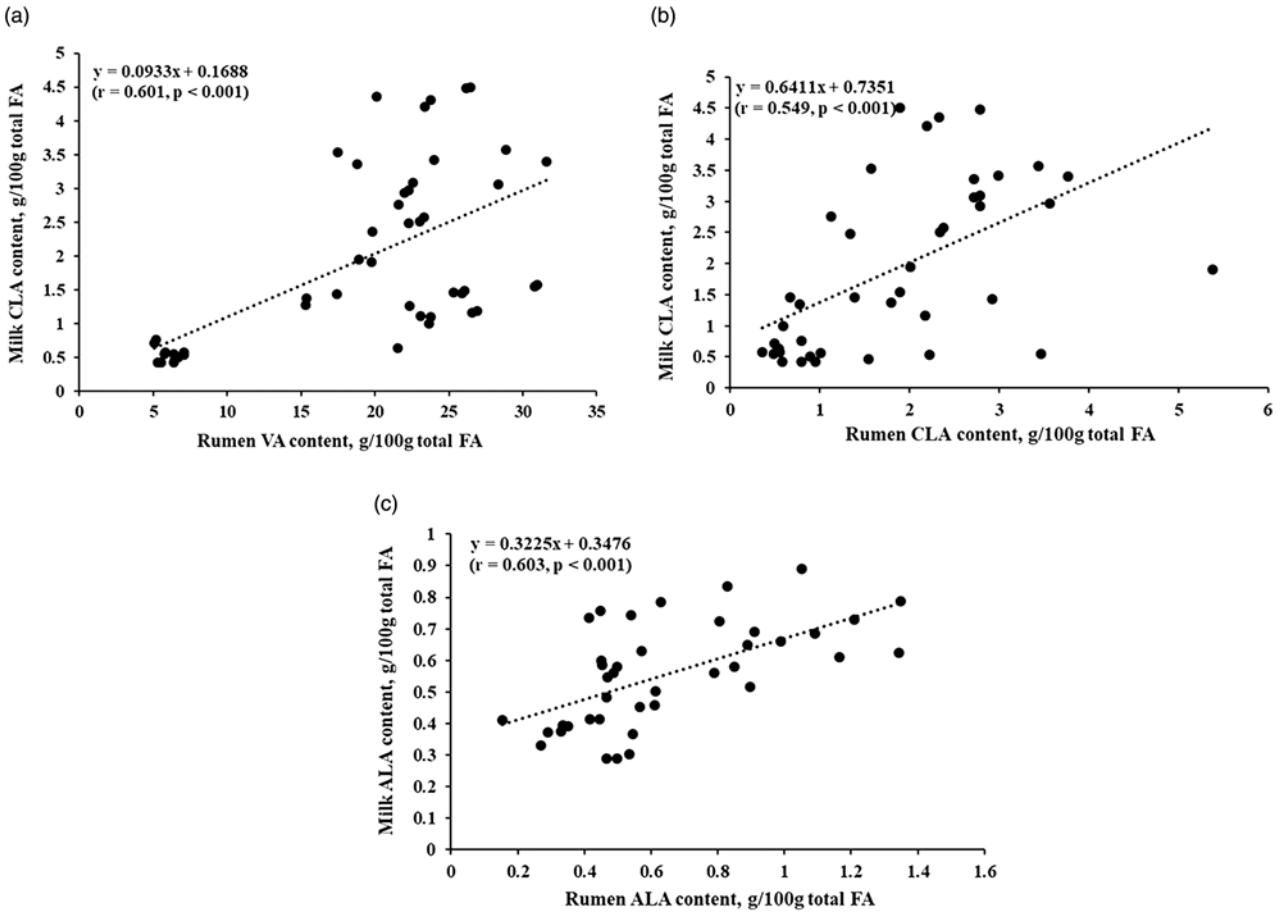

Figure 1 (a) Correlation between proportion of VA in rumen and proportion of *cis*-9, *trans*-11 CLA in milk of dairy cows. (b) Correlation between proportion of *cis*-9, *trans*-11 CLA in rumen and proportion of *cis*-9, *trans*-11 CLA in milk of dairy cows. (c) Correlation between proportion of ALA in rumen and proportion of ALA in milk of dairy cows. CLA = conjugated linoleic acid; FA = fatty acid; VA = vaccenic acid; ALA = α-linolenic acid.

rumen. Studies showed that dietary high non-fibre carbohydrate (**NFC**) or starch could increase the ruminal propionate production and decrease acetate production (Ma *et al.*, 2015; Philippeau *et al.*, 2017) or have no effect on individual VFA (Wei *et al.*, 2018). However, in this study oil groups had the similar content of NFC in the dietary compared with the CON group (37.59% *v.* 35.11%). Thus, dietary NFC or starch may have limited impact on the rumen VFA composition in this study. In addition, one *in vitro* study showed that rapeseed oil, sunflower oil, flaxseed oil or rubber seed oil supplementation could enhance the rumen fermentation pattern with a higher molar proportion of propionate and a smaller acetate to propionate ratio (Jalc and Ceresnakova, 2002; Shi, 2014). Rice bran oil supplementation in dietary at 4% of DM (Lunsin *et al.*, 2012a) or flaxseed oil supplementation in dietary at 2.4% of DM (Kholif *et al.*, 2018) also increased the proportion of propionate whilst the acetate proportion was decreased *in vivo*. Previous studies reported that the ruminal VFA concentration depends on feed digestion and activity of ruminal microflora and feed additives (Morsy *et al.*, 2015; Kholif *et al.*, 2018). Thus, the increased ruminal propionate concentration may be due to the enhanced DM and NDF digestibility with dietary oil supplementation in this study. Moreover, a larger propionate production reflects a higher conversion of glycerol because of the hydrolysis of dietary triacylglycerol into propionate (Kholif *et al.*, 2018). Hence, the increased propionate level from RO and FO is probably due to channelling of excess reduced nicotinamide adenine dinucleotide to propionate production, owing to increased accumulation of hydrogen resulting from inhibition of methanogens by PUFA in the rumen (Patra and Yu, 2012), thereby favouring the succinate pathway that converts carbohydrate to propionate (Van Houtert, 1993) and increasing the alternative flow of hydrogen to produce propionate (Xie *et al.*, 2018).

Volatile fatty acids not only provide energy for ruminants but also maintain the homeostasis of rumen environment. Propionate originating from the rumen can be used as a precursor to synthesize milk lactose (Huhtanen *et al.*, 1998). In this study, milk lactose yield was greatly increased by oil supplementation (8.53%, 7.75% and 6.20% in RO, FO or RFO groups, respectively) compared with CON (Pi *et al.*, 2016), with the propionate proportion increased by 24.28%, 28.13% and 25.43% in RO, FO or RFO groups, respectively, compared with the CON in the rumen. Similarly, a previous study showed that dietary supplementation with fish oil enhanced the milk lactose yield of dairy cows (Keady *et al.*, 2000). In addition, studies showed that dietary supplementation with flaxseed oil increased rumen

propionate production in parallel with increased milk lactose production from dairy cows (Kholif *et al.*, 2018) or lactating goats (Kholif *et al.*, 2016).

We also found that RO and FO supplementation decreased the calculated methane levels. Research has shown that flaxseed oil supplementation reduces the number of ruminal protozoa (Yang *et al.*, 2009) and methanogens (Guyader *et al.*, 2015), that is, the main methane producers, which were competed for hydrogen availability due to the ruminal biohydrogenation of UFA (Toprak, 2015). However, in other trials, protozoa and methanogen numbers remained unchanged after dietary flaxseed oil supplementation (Popova *et al.*, 2017; Popova *et al.*, 2019). Although the population of methanogens and protozoa was not measured in this experiment, our *in vitro* study found that both the population of methanogens and the production of methane were greatly reduced after 4% RO supplementation (Shi, 2014), indicating that RO inhibited the rumen methane production by reducing the population of ruminal methanogens which need further investigation.

Rubber seed oil and flaxseed oil altered the rumen fatty acid composition

Dietary PUFA can be bio-hydrogenated by microorganisms after the PUFA enter the rumen and the final product is mainly C18:0 (Wahle *et al.*, 2004). However, the incompleteness of the hydrogenation process produces a series of intermediate products such as C18:1, CLA and their isomers (Wahle *et al.*, 2004). In this study, diet supplementation of RO and FO, either alone or in combination, increased the levels of VA, *cis*-9, *trans*-11 CLA, ALA, total UFA and total MUFA in rumen, while markedly decreasing the content of total SFA ($P < 0.05$). These results are consistent with a previous *in vitro* study with RO (Shi, 2014) and FO (Wang *et al.*, 2002), which showed that RO and FO supplementation greatly increased the levels of VA, *cis*-9, *trans*-11 CLA and total UFA in a rumen fermentation system.

The effects of oil supplementation on the fatty acid profile and BH of ruminal digesta depend on the type and amount of oil added and on the diet to which the oil is incorporated (Shingfield *et al.*, 2012). The addition of RO at an appropriate dose could, in theory, contribute to the production of healthier products from ruminant livestock (Pi *et al.*, 2016). Milk fat CLA originates both from ruminal BH of dietary PUFA and from endogenous conversion of VA by Δ^9-desaturase in the mammary gland of dairy cows (Griinari *et al.*, 2000). In support of this, supplementation with RO and FO increased the contents of VA in rumen, which was positively correlated with the contents of *cis*-9, *trans*-11 CLA in milk (Figure 1a). FAs from the rumen are an important source for the milk fat synthesis. The levels of VA, *cis*-9, *trans*-11 CLA and ALA in the rumen were significantly increased by oil treatment, and these FAs can form the basis for increasing their content in milk fat as shown in our previous research (Pi *et al.*, 2016). Indeed, we observed that the profiles of CLA and ALA in the rumen were positively correlated with the profiles of those FAs in milk in this study (Figure 1b and c), suggesting that nutritional strategies intervene the rumen FA profile could improve milk fat quality.

Bauman and Griinari (2001) proposed the BH theory, stating that diets causing milk fat depression (**MFD**) alter ruminal lipid metabolism resulting in the formation of specific BH intermediates that directly inhibit milk fat synthesis. However, some CLA isomers, such as *trans*-10, *cis*-12 CLA (Baumgard *et al.*, 2000) and *trans*-10 octadecenoic acid (Shingfield *et al.*, 2009) were shown to cause MFD. Our previous study showed that dietary supplementation with rubber seed oil and flaxseed oil decreases the level of milk fat (Pi *et al.*, 2016). However, the proportion of *trans*-10, *cis*-12 CLA present in the rumen 3 h after feeding significantly decreased with oil supplementation, pointing that other factors may contribute to the decreases of milk fat, for example, the negative effects of dietary oils on ruminal acetate concentration in this study (Onetti *et al.*, 2001), as the precursor of milk fat synthesis. In addition, lower mammary lipogenesis associated with supplementing the diets of animals with PUFA-rich oil may be another reason (Chilliard *et al.*, 2001), which requires further investigation.

Conclusions

Dietary supplementation with 4% rubber seed oil and flaxseed oil improves total tract apparent digestibility of nutrients (dry matter, neutral detergent fibre and ether extracts) and shifts the rumen fermentation pattern by increasing the proportion of propionate. In addition, cows offered rubber seed oil and flaxseed oil showed altered rumen fatty acid composition with enhanced vaccenic acid, *cis*-9, *trans*-11 conjugated linoleic acid and α-linolenic acid. These findings provide a theoretical basis for the application of rubber seed oil in livestock production.

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Declaration of interest

The authors declare no conflicts of interest.

Ethics statement

The experimental design and procedures were approved by the Institutional Animal Care and Use Committee of the Chinese Academy of Agricultural Sciences following the requirements of the Regulations for the Administration of Affairs Concerning Experimental Animals.

Software and data repository resources

Data are not deposited in an official repository.

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animal

Digestibility contributes to between-animal variation in feed efficiency in beef cows

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*Residual feed intake (**RFI**) is an alternative measure of feed efficiency (**FE**) and is calculated as the difference between actual and expected feed intake. The biological mechanisms underlying animal-to-animal variation in FE are not well understood. The aim of this study was to investigate the digestive ability of beef cows selected for RFI divergence as heifers, using two contrasted diets. Fifteen 4-year-old beef cows were selected from a total of 69 heifers based on their RFI following the feedlot test. The selected heifers were ranked into high-RFI (+ 1.02 ± 0.28,* n = *8) and low-RFI (−0.73 ± 0.28,* n = *7), and a digestibility trial was performed after their first lactation. Both RFI groups were offered two different diets: 100% hay or a fattening diet which consisted of a DM basis of 67% whole-plant maize silage and 33% high starch concentrates over four experimental periods (two per diet). A diet effect was observed on feed intake and apparent digestibility, whereas no diet × RFI interaction was detected (*P > *0.05). Intake and apparent digestibility were higher in cows fed the fattening diet than in those fed the hay diet (*P < *0.0001). DM intake (**DMI**) and organic matter apparent digestibility (**OMd**) were repeatable and positively correlated between the two subsequent periods of measurements. For the hay and fattening diets, the repeatability between periods was* r = *0.71 and* r = *0.73 for DMI and* r = *0.87 and* r = *0.48 for OMd, respectively. Moreover, both intake (*r = *0.55) and OMd (*r = *0.54) were positively correlated (*P < *0.05) between the hay and fattening diets. Significant differences between beef cows selected for divergence in RFI as heifers were observed for digestive traits (*P < *0.05), DM and organic matter (**OM**) apparent digestibility being higher for low-RFI cows. Overall, this study showed that apparent digestibility contributes to between-animal variation in FE in beef cows.*

Keywords: digestibility, residual feed intake, beef cows, animal variability, efficiency

Implications

Improvement in feed efficiency is of growing interest in the beef industry due to its potential to increase producer profitability and lower the environmental footprint of beef production. As a complex multifaceted trait under the control of many biological processes, the importance of animal variability in feed efficiency traits is a relevant question that needs to be addressed. In the present study, apparent digestibility contributes to animal variation in feed efficiency. Further research is warranted to identify other biological mechanisms involved in feed efficiency, so as to improve animal selection in multitrait breeding programs.

Introduction

Feed inputs are a major determinant of profitability and represent the largest variable cost in beef production (Nielsen *et al*., 2013). Selection of feed-efficient animals is a way to improve profitability. Traditionally, feed efficiency (**FE**) is expressed as a gain to feed ratio. However, the selection of a high gain to feed ratio resulted in an increase in growth rate and mature cow size (Schenkel *et al*., 2004), impacting in turn on the intake of the cow herd. An alternative measure of FE is the residual feed intake (**RFI**), proposed by Koch *et al*. (1963) and extensively studied over the last decade in both monogastrics (Gilbert *et al*., 2017) and ruminants (Lawrence *et al*., 2011; Xi *et al*., 2016). RFI is calculated as the difference between actual and expected feed intake required to support maintenance and production. RFI allows the evaluation of FE for each animal with regard to its counterparts. Efficient animals have negative RFI (they consume less feed than expected) and are classified as low RFI.

Several biological mechanisms have been suggested to account for differences in RFI. In a review for finishing beef steers, Herd and Arthur (2009) estimated that variation in RFI

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was due to protein turnover and metabolism (37%), digestibility (10%), activity (10%), heat increment of feeding (9%), body composition (5%), feeding patterns (2%) and nearly 30% to other undefined metabolic processes. According to this study and among various biological mechanisms proposed, the contribution of the one relative to the digestive processes (i.e. digestibility and feeding behaviour) could be of importance, but the results are conflicting. Indeed, some studies have shown no relation between RFI of beef or dairy heifers and whole-tract digestibility of dry matter (Lawrence *et al*., 2011; Rius *et al*., 2012), whereas other studies report that diet dry matter digestibility (**DMd**) is negatively correlated with RFI (Nkrumah *et al*., 2006, Krueger *et al*., 2009a). It is unclear, however, whether apparently improved digestive ability of feed-efficient animals is inherent or simply due to a slower passage of digesta through the rumen in relation to lower dry matter intake (**DMI** (Kenny *et al*., 2018)). In some instances, the absence of differences in DMd between cattle of varying RFI phenotype may be related to the nature of the diets offered, as the effect of feed intake on digestion is lower with forage than with concentrate-based diets. The results from recent studies show that the proportion of the different microbial populations in rumen fluid differed between high and low RFI cattle, but such differences appeared to be modulated by the nature of the diet offered. Carberry *et al*. (2012) reported a stronger relationship between RFI classification and rumen microbial diversity when animals were fed a high forage diet (100%) in comparison to a low forage diet (30%). In contrast, Hernandez-Sanabria *et al*. (2012) observed differences in rumen microbial populations between low and high RFI cattle only when the animals were fed a high concentrate diet (100%). Collectively, these studies suggested that FE, reflected by RFI classification, may be partly explained by the digestive ability of the animal, but with a strong interaction with the type of diet consumed. However, this interaction has yet to be investigated in more depth. Therefore, the objective of this study was to investigate the digestive ability of 15 beef cows selected for divergence in RFI as heifers using two contrasted diets: a high forage diet (100% hay of permanent grassland) and a fattening diet (whole-plant maize silage diet/concentrate, 67/33). For that purpose, and assuming that RFI is a repeatable trait across time and stage of production (Kenny *et al*., 2018), the effects of RFI phenotypes on feed intake and apparent whole-tract digestibility and the behaviour of rumen fermentation variables were studied according to diet.

Material and methods

Animals, diets and experimental design

The experiment was performed at the National Institute for Agricultural Research (INRA) at the Saint-Genès-Champanelle (France) experimental farm in full compliance with national legislation on animal care (authorisation to experiments on living animals, no. C6334517, Ministry of Agriculture, France).

This experiment was conducted in association with a larger study designed to evaluate, *inter alia*, the relationship between FE and methane emission of 153 Charolais beef heifers (Renand *et al*., 2016). Briefly, in the trial performed during the winter of 2013 to 2014, two batches of 22 and 47 non-bred heifers aged 22 months were tested in November–December and February–March. The mean age and weight at the beginning of the test period were 675 days (s.d. = 9.3) and 494 kg (s.d. 50.3), respectively. Heifers were accommodated in pens equipped with individual troughs and automatic gates (American Calan Inc., Northwood, NH, USA) and floor covered with wood shavings. After an adaptation and training period of 4 weeks, they were offered *ad libitum* access to a grass silage diet, without supplementary concentrates, over an 8-week test period. The dry matter content of offered silage and refusal samples was measured, and the daily DMI was averaged over the whole test period. The diet distributed to the two batches had a DM content of 20.2 and 21.8 g/kg, respectively, and an estimated metabolisable energy concentration of 9.01 and 9.13 MJ/kg DM (INRA, 2007). Heifers were weighed every two weeks. A regression of weight on the test day was performed individually. The slope of the regression was used to calculate the average daily gain (**ADG**), and the predicted weight after 28 testing days was used as the mid-test weight (**MW**). The residual feed intake of heifers was calculated as the residual of a multiple regression of DMI on mid-test metabolic weight ($\mathbf{MW^{0.75}}$) and ADG in a model fitted for the batch contemporary group. The 69 heifers were inseminated. Among the females that calved at 3 years of age, eight females with the highest (inefficient heifer, high-RFI) or the lowest RFI (efficient heifer, low-RFI) values were not bred and were kept for the present study. After their first lactation (46 months on average), 15 of these 16 non-pregnant and non-lactating cows could enter the digestibility measurement barn (7 low-RFI and 8 high-RFI). Growth and efficiency traits (RFI, DMI and ADG) and body weight (**BW**) of the low-RFI and high-RFI heifers selected are presented in Supplementary Material Table S1. No differences between high- and low-RFI heifers in ADG and BW were observed. RFI averaged −0.73 and 1.02 kg DM/d in low-RFI and high-RFI heifers, respectively.

The 15 cows were housed in individual stalls and fed *ad libitum* a hay diet during a 2-week adjustment period. After that, both RFI groups were offered successively two different diets (hay or fattening diet) over four experimental periods (P1 to P4) each of 3-week duration. For each experimental period, cows were fed *ad libitum* during the first two weeks of adaptation and at 95% of individual *ad libitum* intake during week 3, to reduce the experimental errors when digestibility measurements were performed. The experimental scheme was designed to measure the between-animal variation of intake and apparent digestibility using two contrasted diets. These measurements were performed during two consecutive periods for each diet. During P1 and P2, cows received a hay diet consisting of 100% hay from permanent grassland distributed three times a day to minimise waste.

Table 1 *Ingredients and chemical composition of cows' diets*

Measurements	Hay	WPMS[1]	Concentrate
Chemical composition (g/kg DM)			
CP	111	90	178
NDF	516	394	279
ADF	283	204	134
Starch	–	314	289
Feed value[2] (/kg DM)			
NE_L[3] (MJ/kg DM)	5.26	6.69	7.64
PDIE[4] (g/kg DM)	84	72	125
PDIN[5] (g/kg DM)	74	57	123

[1] WPMS corresponds to whole-plant maize silage.
[2] Calculated according to INRA (2007).
[3] NE_L corresponds to the net energy of lactation.
[4] PDIE corresponds to 'protéines digestibles dans l'intestin permises par l'Energie'.
[5] PDIN corresponds to 'protéines digestibles dans l'intestin permises par l'Azote'.

After 1 week of transition, cows were fed twice a day during P3 and P4 with the fattening diet, which consisted of 67% of whole-plant maize silage (**WPMS**) and 33% of high starch concentrates on a DM basis. Water and salt block were available *ad libitum* and all cows received 250 g/d of mineral-vitamin supplement (Ca:P:Mg:Na = 20:2.5:4.5:3.5%, Galaphos Midi Duo Granule, CCPA, 15006 Aurillac, France). The chemical and nutrient compositions of diets are presented in Table 1.

Measurements and sampling

Body weight and condition score. Cows were weighed at the start of week 2 and at the end of week 3 in each experimental period (P1 to P4). BW changes (final BW minus initial BW in kilograms) over both diets were calculated considering the BW measured in week 2 of P1 or P3 as initial BW and BW measured in week 3 of P2 or P4 as final BW. The body condition was assessed two times during the experiment (in week 2 of periods 1 and 3) by the same two experienced assessors on a 0 to 5 scale (Agabriel *et al.*, 1986).

Intake. For each experimental period, intake and refusals were individually recorded every day. The total daily dry matter intake (DMI) was computed as daily DM offered minus DM refused for each animal. The DM content (24 h in 103°C forced-air dry oven) of feeds was measured once a week for the pelleted concentrate and twice a week (weeks 1 and 2) or every day (week 3) for hay and corn silage offered and refused. The DMI of WPMS was corrected for losses of volatile compounds (ethanol, NH_3 and acetic and lactic acids, Dulphy *et al.*, 1975). For chemical composition analysis, 100 g of each ingredient of the diet was collected daily during week 3 of each period, then pooled per period and stored at −20°C for corn silage and at room temperature for hay. In week 3 of each experimental period, 10% of refusals were sampled daily, pooled per animal × period and stored until analysis.

Total-tract digestibility and rumen fermentation traits. Total-tract apparent digestibility of DM, organic matter (**OM**), neutral detergent fibre (**NDF**) and acid detergent fibre (ADF) was determined by collecting total faeces and urine over 6 days in week 3 of each period. The fresh weight of excreta (faeces and urine mixture) and DM content were determined daily at 09.00. The total daily excretion of each cow was mixed, and a 1% aliquot was dried at 103°C for 24 h to determine the DM content. Another 6% aliquot was immediately dried at 60°C for 72 h and then pooled per animal and period to determine chemical composition (OM, NDF and ADF). The rumen fermentation traits were determined according to the description reported in Supplementary Material S1.

Chemical analysis. Diet ingredients, refusals and faeces were analysed for DM (103°C for 24 h) and ash (550°C for 6 h). NDF using α-amylase and ADF (Van Soest *et al.*, 1991) were analysed on samples dried at 60°C for 72 h and ground through a 1-mm screen.

Measurements of feeding and physical behaviours. The Rumiwatch® system (RWS, Itin + Hoch, Liestal Switzerland) was used to record continuously both feeding (eating and ruminating time) and locomotion (lying and standing time) activities (Zehner *et al.*, 2012). The details of implementation are reported in Supplementary Material S2.

Statistical analysis

Data were analysed by ANOVA using the mixed procedure of SAS software, version 9.3 (SAS Institute Inc., Cary, NC, USA). Data recorded over only one period per diet (body condition score, all fermentation parameters, feeding and locomotion activities) were analysed using a mixed model that included RFI group (low-RFI and high-RFI), diet (hay and fattening diet) and RFI × diet interaction as fixed effects and the cow as a random effect. Data recorded over two periods per diet (feed intake and apparent whole-tract digestibility) were analysed using a mixed model that included RFI group (low-RFI and high-RFI), period (P1 to P4) and their interaction as fixed effects, period as a repeated effect and the cow as a random effect. The specific effect of diet (hay v. fattening diet) was tested by contrast (P1 and P2 v. P3 and P4, respectively). Main effects were considered significant at $P < 0.05$. Differences were localised *post hoc* by the Tukey *t* test. For feed intake and nutrient digestibility, Pearson correlation coefficients between periods within and between diets were determined using the CORR procedure of SAS.

Results

Age, body weight and body condition score

Initial and final BW, mean metabolic BW and mean body condition score (**BCS**) according to the RFI group and the diet are presented in Table 2. There was no effect of RFI group and RFI × diet interaction on these variables. A significant effect of the diet type was observed ($P < 0.0003$): WPMS v. hay diet

Table 2 *Body weight and body condition of cows as a function of residual feed intake (RFI) phenotype and diet*

	Hay		WPMS[1]			*P*-values		
Measurements	Low-RFI[2] ($n = 7$)	High-RFI[3] ($n = 8$)	Low-RFI[2] ($n = 7$)	High-RFI[3] ($n = 8$)	SEM	RFI	Diet	RFI × diet
Initial BW[4] (kg)	660	666	709	710	28.5	0.93	<0.0001	0.56
Final BW[5] (kg)	673	677	751	753	28.6	0.94	<0.0001	0.88
$BW^{0.75}$ [6] (kg)	131	131	140	140	1.2	0.93	<0.0001	0.65
BCS[7] (0 to 5)	2.71	2.97	3.21	3.31	0.22	0.56	0.0003	0.39

[1] WPMS corresponds to whole-plant maize silage.
[2] Low-RFI is efficient.
[3] High-RFI is inefficient.
[4] Initial BW corresponds to the BW measured at the beginning of week 2 in period 1 for the hay diet and period 3 for the WPMS diet.
[5] Final BW corresponds to the BW measured at the end of week 3 in period 2 for the hay diet and period 4 for the WPMS diet.
[6] $BW^{0.75}$ corresponds to the mean of metabolic body weight measured during periods 1 and 2 for the hay diet and periods 3 and 4 for the whole-plant maize silage diet.
[7] BCS corresponds to the average body condition score in the hay and WPMS diets and was measured by two experienced assessors according to the method of Agabriel *et al.* (1986) using a 0 to 5 scale.

Table 3 *Effects of residual feed intake (RFI) phenotype and diet on feed intake and apparent whole-tract digestibility of cows*

	Hay		WPMS[1]			*P*-values		
Measurements	Low-RFI[2] ($n = 7$)	High-RFI[3] ($n = 8$)	Low-RFI[2] ($n = 7$)	High-RFI[3] ($n = 8$)	SEM	RFI	Diet	RFI × diet
Feed intake								
DMI (kg/d)	9.05	9.17	12.3	12.8	0.38	0.54	<0.0001	0.35
DMI (g/kg $BW^{0.75}$)	68.9	69.5	87.6	90.7	2.14	0.50	<0.0001	0.36
OMI (kg/d)	8.2	8.3	11.3	11.8	0.35	0.53	<0.0001	0.33
OMI (g/kg $BW^{0.75}$)	62.4	62.9	80.8	83.3	1.99	0.55	<0.0001	0.42
Digestibility (%)								
DMd	61.1	60.0	70.0	68.3	0.56	0.05	<0.0001	0.46
OMd	65.5	64.2	74.1	72.4	0.55	0.03	<0.0001	0.62
NDFd	62.1	60.0	62.3	60.3	1.02	0.12	0.67	0.89
ADFd	61.3	58.3	63.3	61.2	1.09	0.07	0.0005	0.47

DMI = DM intake; OMI = organic matter intake; DMd = DM digestibility; OMd = organic matter digestibility; NDFd = NDF digestibility; ADFd = ADF digestibility.
[1] WPMS corresponds to whole-plant maize silage.
[2] Low-RFI is efficient.
[3] High-RFI is inefficient.

resulted in a higher initial (710 v. 663 kg) and final (752 v.675 kg) BW, a higher metabolic BW (140 v. 131 kg $BW^{0.75}$) and a higher BCS (3.3 v. 2.8).

Feed intake and apparent whole-tract digestibility

The effect of diet and RFI group on feed intake and apparent whole-tract digestibility coefficients is presented in Table 3. A significant effect of diet type was observed on all these parameters except NDF digestibility (**NDFd**). The DMI expressed in kg per day or in g per kg of metabolic weight and per day was higher ($P < 0.0001$) in cows fed the WPMS diet than in those fed the hay diet (38 and 29%, respectively). Similarly, the OM intake, regardless of the units used, was higher ($P < 0.0001$) when animals received the WPMS diet (40 and 31%, respectively). The total-tract apparent digestibility of DM, OM and ADF was also subject to an effect of the diet. The digestibility coefficients were higher ($P < 0.0005$) when offered the WPMS diet than when cows consumed the hay diet and were 8.5, 8.3 and 2.4 higher on average, respectively. The NDF digestibility coefficients were similar whatever the type of diet consumed by the cows or the RFI class.

A significant effect of RFI class was observed for DM ($P = 0.05$) and OM apparent digestibility ($P = 0.03$). These digestibility coefficients were on average 1.8 g/100 g and 2.3 g/100 g higher in low- than in high-RFI cows fed hay and WPMS, respectively. No effects of RFI group and RFI × diet interaction on DMI, organic matter intake and NDF digestibility parameters were observed. A trend ($P = 0.07$) was only observed for ADF digestibility, which was slightly higher in the low-RFI group.

The repeatability of DMI and organic matter apparent digestibility (**OMd**) measurements between periods and within diets is illustrated in Figure 1a and b. For each diet, DMI and OMd measured in the first period were positively correlated with measurements performed in the second period. These within-diet correlation coefficients ranged from 0.71 to 0.87 for DMI and from 0.48 to 0.73 for OMd. The relationship between hay and WPMS diet measurements (mean/animal/diet) is illustrated in Figure 2 for both DMI (a)

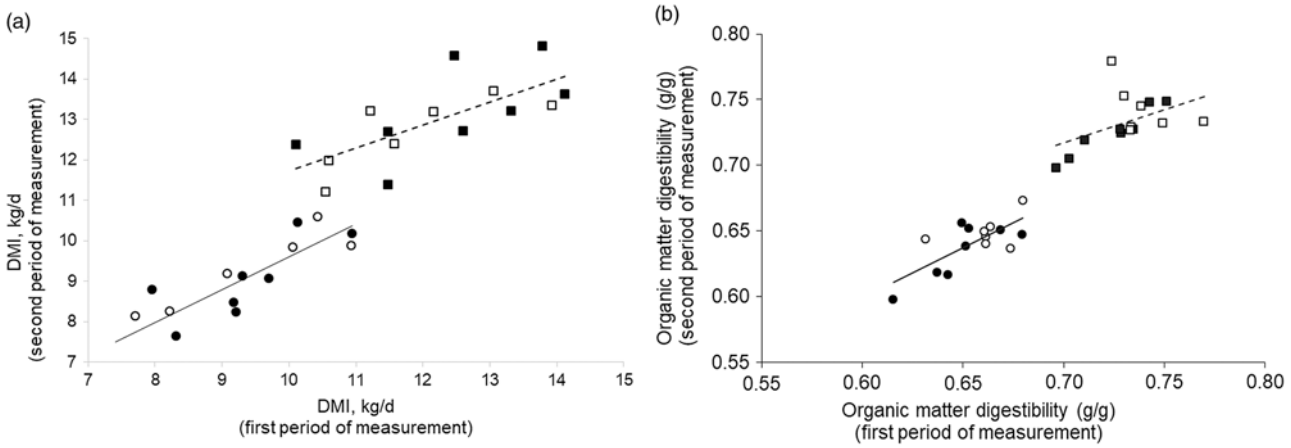

Figure 1 Relationship between the two subsequent periods of measurements of dry matter intake (DMI, kg/d) (a) and of organic matter digestibility (OMd, g/g) (b) for hay and whole-plant maize silage (WPMS) diets measured in divergent residual feed intake (RFI) cows. Hay diet (circles and solid line): DMI_P2 = 1.50 (± 1.18) + 0.811 (± 0.127) × DMI_P1; $n = 15$; $P = 0.0001$; RMSE = 0.540; $r = 0.87$. WPMS diet (squares and dashed line): DMI_P4 = 6.07 (± 1.88) + 0.66 (± 0.154) × DMI_P3; $n = 15$; $P = 0.003$; RMSE = 0.745; $r = 0.71$. Low-RFI cows are represented by open symbols, and high-RFI cows are represented by closed symbols Hay diet (circles and solid line): OMd_P2 = 0.143 (± 0.127) + 0.761 (± 0.193) × OMd_P1; $n = 15$; $P = 0.002$; RMSE = 0.013; $r = 0.73$. WPMS diet (squares and dashed line): OMd_P4 = 0.364 (± 0.188) + 0.504 (± 0.257) × OMd_P3; $n = 15$; $P = 0.072$; RMSE = 0.018; $r = 0.48$. Low-RFI cows are represented by open symbols, and high-RFI cows are represented by closed symbols.

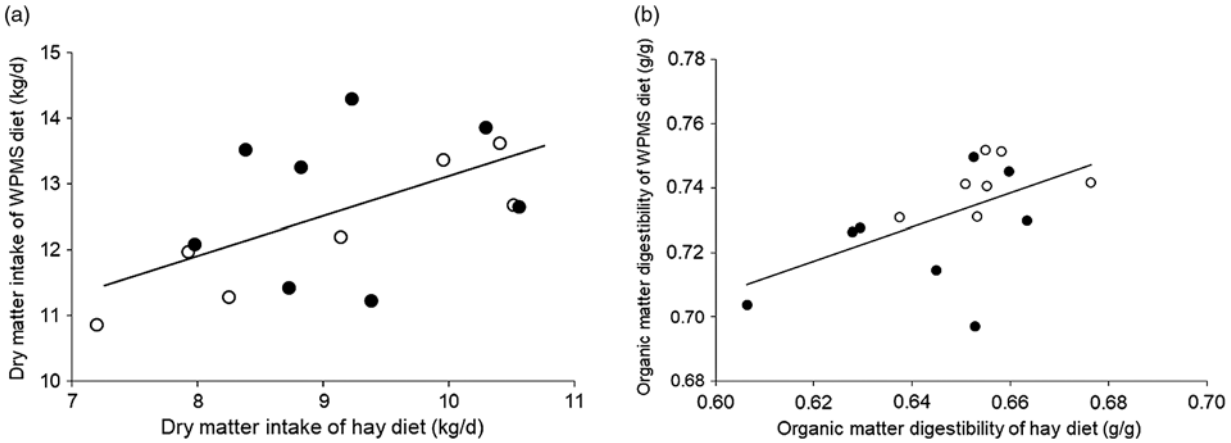

Figure 2 Relationship between measurements of dry matter intake (DMI, kg/d) (a) and between measurements of organic matter digestibility (OMd, g/g) (b) when divergent residual feed intake (RFI) cows were fed the hay and whole-plant maize silage (WPMS) diets (mean of the data of the two subsequent periods). DMI_WPMS = 7.48 (± 2.15) + 0.558 (± 0.234) × DMI_hay; $n = 15$; $P = 0.033$; RMSE = 0.930; $r = 0.55$. Low-RFI cows are represented by open symbols, and high-RFI cows are represented by closed symbols. OMd_WPMS = 0.389 (± 0.147) + 0.529 (± 0.226) × OMd_hay; $P = 0.036$; $n = 15$; RMSE = 0.014, $r = 0.54$. Low-RFI cows are represented by open symbols, and high-RFI cows are represented by closed symbols.

and OMd (b). A positive and significant correlation was observed for both intake ($r = 0.55$, $P = 0.033$) and OMd ($r = 0.54$, $P = 0.03$).

Ruminal fermentations

The effects of diet and RFI group on rumen pH, ammonia, total volatile fatty acid (**VFA**) and molar proportions of fermentation acids are presented in Supplementary Material Table S2. There was an effect of the diet on N-NH_3 and total VFA concentrations. The N-NH_3 concentration was higher in cows fed the WPMS diet than in those fed hay ($P < 0.002$), whereas the concentration of total VFA was 1.3 times higher in the hay diet than in the WPMS diet ($P < 0.0017$). There were no effects of RFI group and RFI × diet interaction ($P > 0.05$) on pH, N-NH_3 and total VFA concentrations.

Proportions (mol/100 mol of total VFA) of acetate, iso-butyrate, iso-valerate and the acetate:propionate ratio were significantly different between the two types of diets ($P < 0.009$). The molar proportions of iso-butyrate and iso-valerate were greatest in the WPMS diet. In contrast, the molar proportion of acetate and the acetate:propionate ratio were higher in cows fed the hay diet than in those fed the WPMS diet. A significant interaction ($P = 0.05$) between RFI phenotype and diet was observed for proportion of valerate: its concentration was 1.8 times higher in high-RFI cows fed the WPMS diet than in low-RFI cows fed the hay diet. The VFA profiles did not differ between the RFI groups.

Budget time of feeding and locomotion behaviours

The effects of RFI phenotype and diet on feeding and locomotion behaviours are reported in Supplementary Material

Table S3. There were no effects of RFI group and RFI × diet interaction on feeding and locomotion behaviours; only a significant effect of the type of diet was observed. Cows fed the WPMS diet spent ($P < 0.01$) less time eating and ruminating in comparison with cows fed the hay diet. Concerning the locomotion activities, cows fed the hay diet spent more time per day in a standing position than those fed WPMS (633 v. 525 min/d, respectively, $P < 0.005$). Conversely, time spent lying was greater in cows fed the WPMS diet than in those receiving the hay diet (914 v. 806 min/day, respectively, $P < 0.005$).

Discussion

In this study, the digestive ability of 15 cows selected for divergence in RFI as heifers was investigated. The average difference in RFI detected between high- and low-RFI heifers was 1.75 kg DM/day of grass silage. These values were within the range of those reported by other authors who studied RFI and animal performances including digestibility measurements in animals differing in breed, gender, diet and age (1.470 and 2.03 kg/d for red angus steers in growing and finishing phase, respectively (McGee *et al.*, 2014); 1.59 kg/d for dairy cows (Xi *et al.*, 2016)). In these studies, the RFI measurement period just pre-dated the digestibility trials, which is not the case in our work since the digestibility trial was performed in dried-off cows, whereas RFI was measured when they were heifers. However, as suggested by Kenny *et al.* 2018, it was assumed that the RFI index is repeatable across time and stage of production. Moreover, the duration of the digestibility trial is not sufficient to calculate an RFI index since each diet feed intake was recorded during 6 weeks, including 2 weeks when cows were fed at 95% of individual *ad libitum* intake.

Feed intake and residual feed intake classification

RFI is gaining widespread acceptance as the most appropriate measure of FE for beef cattle (McDonnell *et al.*, 2016) and is characterised by a significant difference in ingestion between animals for a given level of production. In our work, dry matter intake of both diets did not differ across RFI groups, expressed in kg DM/d, g DM/kg $BW^{0.75}$ or per 100 kg BW (data not shown). This result may seem surprising, but the calculation of RFI was done when the animals were heifers, whereas the digestibility trial was performed in the same animals two years later after their first lactation. This delay could explain, at least in part, the lack of significance between these two variables. Indeed, RFI is a moderately repeatable trait across time (maturity), stage of production and type of diet (Kenny *et al.*, 2018) and a decrease in RFI divergence evaluated in the same animals during growth and lactation has previously been reported (MacDonald, 2014). However, under our conditions, low-RFI cows consumed 0.12 kg DM/d less hay than high-RFI cows, while the BW gain was comparable in both groups: 13 and 11 kg, respectively. Although RFI could not be measured in these short testing periods, these results suggest that cows characterised as low-RFI heifers were more efficient during the experiment than those characterised as high-RFI heifers. Similar results were observed when cows were fed the WPMS diet, low-RFI cows consuming 0.5 kg DM/d less, while the BW gain was similar to that of high-RFI cows (42 kg on average). In this study, measurements were performed for each diet over two consecutive periods of three weeks and the results observed between weeks of measurements within diet were similar. The repeatability of these measurements is high for both the WPMS diet ($r = 0.71$) and the hay diet ($r = 0.87$). When the overall data were used, a positive correlation ($r = 0.55$, $P < 0.04$) between DMI of hay diet and DMI of WPMS diet was observed, showing that the cows that eat the most hay are those that eat the most WPMS diet.

Cows were restricted to 95% *ad libitum* during the digestibility measurements. Under these experimental conditions, no correlation between the average DMI measured during the two periods of hay diet and phenotypic RFI was detected ($r = 0.07$, $P = 0.76$). However, when the cows were offered the WPMS diet, the relation between the average DMI measured during P3 and P4 and the phenotypic RFI was better, albeit not significant ($r = 0.23$, $P = 0.40$). These results are consistent with previous results (Lawrence *et al.*, 2011; McDonnell *et al.*, 2016) reporting a positive correlation between RFI and DMI when cows were fed total mixed rations ($r = 0.50$, $P < 0.01$), but not when they were fed grass silage ($r = 0.07$). Indeed, compared with feeding diets based on high levels of concentrate, feeding high forage diets may limit voluntary intake and thus reduce the expression of inherent DMI potential (Forbes 2005).

Nutrient digestibility

Nkrumah *et al.* (2006) reported a negative correlation between RFI and apparent digestibility of DM ($r = -0.33$, $P < 0.10$) and showed that differences, measured over a 5-day total collection of faeces, accounted for 5% between high- and low-RFI crossbreed steers ($P < 0.05$). Similar results have been obtained by Rius *et al.* (2012) who reported a trend ($P < 0.1$) for greater DM and OM digestibility in low-RFI compared with high-RFI lactating cows. A recent study (Potts *et al.*, 2017) in lactating Holstein cows yielded comparable results. These authors observed a negative correlation between DM digestibility and RFI ($r = -0.30$, $P < 0.01$) when cows were fed a low-starch diet (14% starch and 40% NDF), but not when they received a high-starch diet (30% starch and 27% NDF). In contrast, other authors, using either beef steers or heifers, failed to establish such a relation between these two traits (Lawrence *et al.*, 2011). However, these studies relied on internal markers (lignin and acid-insoluble ash) to estimate the digestibility from faecal grab samples. Systematic and random errors can increase markedly when using markers which limit the ability to detect differences in digestibility between individuals (Rius *et al.*, 2012; Fischer *et al.*, 2016). In our experiment, where the total faeces collection was used to measure apparent whole-tract digestibility, beef cows classified as low- or high-RFI when they were heifers differed significantly ($P = 0.03$) in their

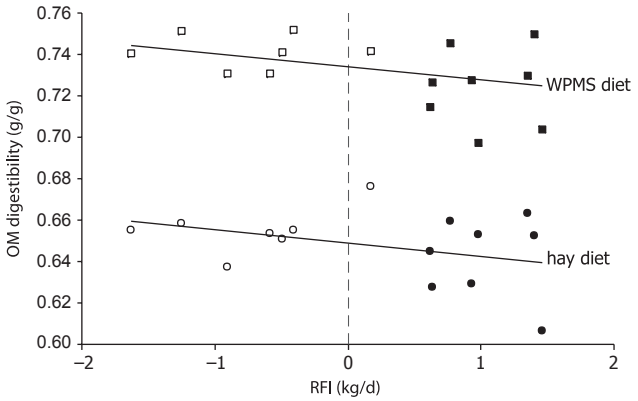

Figure 3 Relationship between the residual feed intake (RFI) measured in heifers and the organic matter apparent digestibility (OMd, g/g) of the whole-plant maize silage diet (WPMS, squares) and the hay diet (circles) measured in the same animals when they become cows. Each point indicates the average digestibility measured in each diet. High-RFI cows are represented by closed squares and circles; low-RFI cows are represented by open squares and circles. OMd = $a - 0.0063$ (± 0.003) $\times$ RFI, with $a = 0.649$ (± 0.003) with hay diet, or 0.734 (± 0.003) with WPMS diet; $n = 30$; n diet = 2, $P = 0.044$; $r = -0.94$. One point corresponds to the average value of two periods of digestibility measured per diet and per cow.

ability to digest OM by about 2 to 2.3 g/100 g OM when they were fed hay or the WPMS diet, respectively (Table 3). Similar results were obtained with DMd ($P = 0.05$). Taken together, a negative within-diet relationship between RFI values and the apparent digestibility of OM ($P = 0.044$, Figure 3) and DM ($P = 0.070$, not shown) was detected. In addition, no interaction between RFI classes and nature of diet was observed, suggesting that the nature of the diet did not modulate the ranking of cows in their ability to digest nutrients, contrary to some published results (Nkrumah *et al.*, 2006; Rius *et al.*, 2012; Potts *et al.*, 2017). Besides the method of sampling and analysis of digestibility, discrepancies in the relationships between RFI and nutrient digestibility among studies could be partially explained by differences in the level of feeding. In a recent review, Cantalapiedra-Hijar *et al.* (2018) conducted a meta-analysis of 15 published studies and found between individuals and within diet a negative correlation between DM digestibility and DM intake. In our experiment, such a relationship was not observed, which is not surprising since intake was limited to 95% *ad libitum* during the digestibility measurement.

Rumen fermentation parameters

VFAs are the major end-products of ruminal fermentation and are largely determined by the diet type, which modulates microbial populations. In our study, there was no relationship between RFI phenotype and rumen VFA concentrations or molar proportions except for the concentration of valerate, since an interaction between RFI phenotype and diet was observed ($P < 0.04$). These results are in contrast to other reports suggesting that inter-animal variations in VFA concentrations are related to the variation in RFI, but the results are highly variable among studies. For example, Krueger *et al.* (2009a) observed that low-RFI heifers consuming a high-forage diet had a higher ruminal acetate:propionate ratio and a lower propionate concentration than their high-RFI contemporaries. In contrast, Lawrence *et al.* (2011) reported a greater concentration of propionate in low-RFI heifers fed a pasture silage plus concentrate diet. In another study, lower concentrations of butyrate and isovalerate were reported in low-RFI steers fed a high-grain ration (Hernandez-Sanabria *et al.*, 2012), whereas Krueger *et al.* (2009b) did not observe any differences in ruminal pH and VFA concentration among divergent RFI cattle fed a high-corn diet. In this study, as there was no relationship within diet between RFI and VFA concentration, it is unlikely that changes in VFA concentration contribute to the inter-animal variation in RFI.

Posture activities and feeding behaviour

As cows were tied up in individual stalls, the detection of only lying and standing behaviours was performed. In our study, the times spent lying (between 53 and 63%) and standing (between 37 and 47%) are generally within the range of those reported by Mialon *et al.* (2008) in young bulls. These authors reported that the lying time ranged from 59.5% to 62.3% in bulls fed the hay-concentrate (44/56) and maize silage-concentrate (57/43), respectively. Besides the effect of the nature of diet on postural behaviours, no differences in time spent standing or lying were observed between the two RFI divergent groups of cows. These results are similar to those reported in dairy heifers (Lawrence *et al.*, 2011). The feeding behaviours (ruminating and eating activities) were assessed by using a nose sensor-based system recently validated in dairy cows (Ruuska *et al.*, 2016; Zehner *et al.*, 2017). In our study, no differences in eating and rumination time or in eating rate were observed between the two RFI groups. These results differ from reports that there is a positive phenotypic or genetic correlation between RFI and daily feeding durations in growing cattle (Nkrumah *et al.*, 2007) and in lactating cows (Xi *et al.*, 2016). However, in these studies, differences in feeding behaviour were also associated with intake differences. Those results were confirmed in a recent meta-analysis by Kenny *et al.* (2018), in which high-RFI cattle receiving an energy-dense high-concentrate diet spent 12% more time eating than their low-RFI contemporaries. At the same time, high-RFI animals exhibited a 17% higher DM intake than low-RFI animals, implying a faster eating rate. Due to the diversity of methods of measuring feeding behaviour (observations, electronic gate, automatic weighing, portable device, etc.) and to the contradictory results reported in the literature, it is difficult to conclude how the feeding pattern helps to explain part of the differences in RFI among animals.

Conclusions

The experimental design and the sample collection and analyses adopted during this study appear to detect

consistently small differences in apparent digestibility in beef cows selected for divergence in RFI as heifers. The correlation between intake and RFI, even though not statistically significant, and between apparent digestibility of OM and RFI, supports the fact that low-RFI cows ingest slightly less and digest slightly more efficiently than high-RFI cows. The lack and/or the weakness of significance on intake could be explained firstly by a restriction of intake to 95% *ad libitum* during periods of measurements and secondly by a possible re-ranking of RFI between the period during which RFI was measured (20 to 22 months) and the period of the digestibility experiment (36 months), due to a decrease in RFI divergence with age and/or physiological stage.

In addition, our results suggested that there was no interaction between RFI phenotypes and the nature of the diet in terms of the measured ingestive and digestive traits. These results suggest that the nature of the diet did not modulate the ranking of cows in their ability to ingest and digest nutrients. Further research is warranted to clearly establish the underlying metabolic and physiological processes that contribute to the divergence of RFI among individuals.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731119001137

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Declaration of interest

None.

Ethics statement

The experiment was approved by the Ethics Committee of the Auvergne-Rhône-Alpes region and the French Ministry of Higher Education, Research and Innovation (ref APAFIS#812-2015061108596805v2).

Software and data repository resources

None of the data were deposited in an official repository.

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animal

Evolution of pig intestinal stem cells from birth to weaning

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*Pig intestinal epithelium undergoes a complete renewal every 2 to 3 days that is driven by intestinal stem cells (**ISCs**) located at the crypt base in their niche. Intestinal stem cells generate a pool of highly proliferative transit-amplifying cells, which either migrate up the villus and differentiate into enterocytes and secretory cells or migrate towards the base of the crypt where they differentiate into Paneth cells that secrete antimicrobial peptides. The balance between ISCs' self-renewal and differentiation controls intestinal epithelial homeostasis; therefore, ISCs are essential for ensuring intestinal epithelial integrity. Detailed knowledge of these mechanisms in pig and other domestic species is very limited. Therefore, the aim of this work was to characterize ISC from birth to weaning. We analysed the duodenum, jejunum and colon of six piglets at birth, 6-day-old nursing piglets and 28-day-old weanlings, one week after weaning. We immunolocalized homeobox only protein$^+$ (**HOPX**) and sex-determining region Y-box 9$^+$ (**SOX9**) cells that identify quiescent and active ISC, respectively. The volume of ISCs was quantified with stereological methods and was compared to that of mitotic cells expressing proliferating cell nuclear antigen and apoptotic cells identified by the presence of cleaved caspase-3. Furthermore, we compared all these values with crypts and villi measurements and their ratio. Our results indicated that both quiescent and active ISCs are present in pig intestine from birth to weaning and are localized in the crypts of the small and large intestine. However, both markers were also observed along the villi and on the colon luminal epithelium, suggesting that at these stages, pig mucosa is still immature. Weaning induced a dramatic reduction of both HOPX$^+$ and SOX9$^+$ cells, but SOX9$^+$ cells underwent a significantly greater reduction in the small intestine than in the colon. This suggests that the two ISC types are differentially regulated along the intestinal tracts. Overall, the pig ISC complex has many similarities with its murine counterpart, but also has some differences. These include active ISC not showing the typical columnar base morphology as well as the absence of bona fide Paneth cells. This is the first description of ISC dynamics during pig's early life and provides useful reference data for future studies, aimed at targeting ISC for the development of efficient alternatives to in-feed antibiotics for preserving intestinal integrity.*

Keywords: epithelium, growth, intestine, renewal, repair

Implications

The pig industry requires that piglets are weaned as early as possible, causing distress and negative health repercussions that are mitigated by an extensive use of antibiotics. The risk of expanding antibiotic resistance led the European Union to ban the use of antibiotics as growth promoters in 2006. The healing process in response to epithelial injury is promoted by intestinal stem cells that, in pig, have been described and characterized only recently. The description of how intestinal stem cells evolve between birth and weaning is important for the development of innovative strategies to promote intestinal epithelium integrity without the use of antibiotics.

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Introduction

The intestinal epithelium displays the highest turnover of all solid organs, undergoing complete renewal every 2 to 3 days in pig (Gelberg, 2014). This is necessary because it must maintain a constant and effective barrier function in a harsh intestinal environment and, at the same time, ensure the absorption of all the necessary nutritional principles (Barker *et al.*, 2012).

The continuous replacement of the intestinal epithelium is driven by the ISCs located at the crypt base in their niche (Meran *et al.*, 2017). Intestinal stem cells generate a pool of highly proliferative transit-amplifying cells, which in most cases, migrate up the villus and differentiate into two major cell lineages: enterocytes for food absorption and secretory cells for mucus and hormone secretion (Meran *et al.*, 2017).

Paneth cells, on the contrary, are the only known cell type to migrate toward the base of the crypt where they secrete antimicrobial peptides and play an essential role in regulating ISC proliferation and differentiation (Clevers and Bevins, 2013).

The two ISC types coexist in the intestinal crypts, both in the small and large intestine (Li *et al.*, 2010). The best-characterized cells are the leucine-rich-repeat-containing G-protein-coupled receptor 5-expressing (**LGR5**$^{+}$) stem cells, which divide approximately every 24 h and, in mouse, are interspersed between the terminally differentiated Paneth cells. The LGR5^{+} cells are commonly named crypt base columnar (**CBC**) cells, as they are slender and reside at the bottom of the crypt (Clevers, 2013). The second type of stem cells are located at the +4 position of the intestinal crypt and are also known as label-retaining cells (**LRCs**), as they show long-term label retention upon irradiation damage and pulse labelling with bromodeoxyuridine and, differently from CBC cells, they are quiescent and act as a reserve population that can replace all intestinal cell lineages after tissue damage (Beumer and Clevers, 2016).

At birth, pigs have relatively mature muscular and nervous systems but their gastrointestinal (**GI**) tract is relatively immature, making them highly dependent on maternal milk for completing development and maturation (Sangild *et al.*, 2013). Naturally, this process would take a couple of months; however, current swine husbandry practices commonly anticipate weaning at 3 or 4 weeks of age (Davis *et al.*, 2006). This makes weaning the main critical period of the pig's production cycle because it is accompanied by multiple stressors that induce transient anorexia, intestinal inflammation and unbalanced gut microbiota. Such circumstances lead to GI infections that cause the death of around 17% of piglets born in Europe (Lallès *et al.*, 2007). For this reason, antimicrobials are still massively used in the swine industry for therapeutic purposes, but are also used as prophylactic or metaphylactic treatments to prevent GI infections (Gresse *et al.*, 2017), despite the ban on antibiotic growth promoters in the European Union since 2006. To comply with this ban, different feed ingredients, either alone or in combination with feed additives that are effective in reducing the incidence and severity of digestive problems, have been and are being actively developed (Pluske, 2013). In this regard, a better understanding of how ISCs regulate the rapid renewal of the intestinal epithelium would be useful for the development of innovative strategies to promote epithelium integrity without the use of antibiotics.

At present, little is known of the characteristics of pig ISC as most studies have been performed on mice and humans, but one study described in some detail the two stem cell populations present in the intestinal mucosa of this species (Gonzalez *et al.*, 2013). The authors provided a detailed description of several cell types in the pig intestinal mucosa, including stem cells and transit amplifying population, in 6- to 8-week-old individuals, but no quantitative assessment was provided.

The aim of this work was thus to describe the quantitative changes of the two main stem cell populations found in pig intestinal epithelium between birth and weaning. The changes were related to the villus and crypt measurements as well as to the amount of intestinal epithelial cell proliferation and apoptosis occurring during the early stages of life. These data will be useful to understand the physiological mechanisms that regulate intestinal epithelium growth during lactation, and its remodelling at the time of weaning.

Materials and methods

Animals and samples collection

A total of 18 healthy crossbred pigs derived from PIC (Large White × Landrace) females and Goland C21 males were obtained from a local farm. Six piglets were randomly selected from different litters, either immediately after parturition (unsuckling newborns), 6 days later (suckling piglets) or one week after weaning, at 28 days of age with body weight ranging from 7.6 to 8.4 kg. From day 10 after birth, while still with their own mother, piglets were exposed to a prestarter diet (Performer PLA, Ferrero Mangimi SpA, Farigliano, CN, Italy), based on cereals, milk powder, fish meal, vitamins and trace elements. At 21 days of age and at a weight ranging between 5.8 and 6.3 kg, piglets were separated from their mother, litters were mixed, and animals were fed *ad libitum* with the same prestarter diet. Animals were euthanized at the farm by intracardiac lethal injection under total anaesthesia according to current Italian regulation (law number 26/2014, attachment IV).

Immediately after sacrifice, in order to be consistent across individuals and across ages, unopened intestinal pieces of approximately 0.5 to 1 cm in length were taken from the central duodenum, jejunum and colon. Samples for histology were promptly fixed in 10% buffered formalin for 24 h at room temperature, dehydrated in graded alcohols, cleared with xylene and embedded in paraffin. Another adjoining fragment of approximately 5 cm was collected from each tract for measuring the organ volume as detailed below.

Histology and immunohistochemistry

After dewaxing and re-hydration, serial microtome sections (4 μm thick) of all samples were stained with haematoxylin–eosin (**HE**) to evaluate the structural aspects of the organs and measure villi and crypts. Other sections were stained with Periodic acid–Schiff to clearly identify goblet cells. Further sections were stained with Landrum's phloxine-tartrazine staining, specific for acidophilic secretory granules that characterize Paneth cells.

Images were acquired using a NanoZoomer S60 Digital slide scanner (C-13210-01, Hamamatsu) and observed at HAMAMATSU PHOTONICS ITALIA S.r.l., Arese, MI (Italy) continuous magnifications between 20 and 800×.

Villi height (from the apex of the villus to the villus–crypt junction), villi width (width of the villus at the middle of the villus height), crypt depth (from villus–crypt junction to the base of the crypt) and the villus:crypt ratio were measured on HE-stained sections with the NDP.view 2 software (Hamamatsu). The reported mean values were based on the measurements of 20 villi and 20 crypts per sample.

We identified different cell populations by immunohistochemistry using antibodies specific for the following four antigens, as previously described (Gonzalez *et al.*, 2013). Homeobox only protein$^+$ (**HOPX**), a marker of +4 cells identifies the slow-cycling, LRC population distinct from the proliferating LGR5$^+$ cell population (Takeda *et al.*, 2011). Sex-determining region Y-box 9$^+$ (SOX9), a transcription factor expressed in mouse ISCs located at the bottom of the crypt and in the transient amplifying population (Shi *et al.*, 2013). Cleaved caspase-3 (**CASP3**), the most well-characterized effector caspase, is normally present in the cell as an inactive proenzyme. It is activated by a proteolytic process at conserved aspartic residues to produce the active enzyme that irreversibly triggers apoptosis (Parrish *et al.*, 2013). Proliferating cell nuclear antigen (**PCNA**), a specific marker of proliferating cells, has been well characterized also in pig intestine (Domeneghini *et al.*, 2004). The antibody details and working concentrations are summarized in Supplementary Table S1.

Antigen detection was performed by indirect immunohistochemistry using the avidin–biotin complex method (VECTASTAIN® Elite ABC, Vector Laboratories, USA) Burlingame, CA (USA) following the manufacturer instructions. Briefly, slides were brought to boiling point in 10 mM sodium citrate buffer and 0.05% Tween20 (pH 6) in a pressure cooker for 1 min for antigen retrieval. After cooling at room temperature for 15 min, sections were rinsed in phosphate-buffered saline (**PBS**, pH 7.4) and were then immersed in a freshly prepared 3% H_2O_2 solution in methanol for 15 min followed by HCl 0.3N in distilled water for 15 min to block the endogenous peroxidase activity. The aspecific binding was prevented by incubating sections using Normal Blocking Serum VECTASTAIN Elite ABC kit at room temperature for 30 min. Sections were incubated with one of the primary antibodies, listed in Supplementary Table S1, and diluted in 4% Bovine serum albumin (BSA) and PBS with 0.05% Tween20 for 1 h at room temperature in a humidified chamber. Sections were then incubated with appropriate biotinylated secondary antibody for 30 min at room temperature in a humidified chamber, followed by staining with the avidin–biotin complex (VECTASTAIN Elite ABC kit) for another 30 min. Finally, sections were incubated in 15% 3,3'-diaminobenzidine (DAB) peroxidase substrate solution until the desired stain intensity was obtained. Sections were briefly counterstained with Mayer's haematoxylin, dehydrated and permanently mounted with an aqueous mounting media (Biomount®, Bio-Optica, Milan, Italy). Secondary antibody controls were performed following the same staining protocol but omitting the primary antibody (Supplementary Figures S1 and S2).

Quantitative analysis

Systematic sampling and quantitative measurements were performed as described in detail previously (Albl *et al.*, 2016, Supplementary Material). Briefly, to determine the volume of each intestinal sample, we used the Archimedes' principle. We accurately determined the length of the intestinal samples collected at the time of sacrifice and performed the volume measurements using a 25 mL graduated cylinder, with a 0.5 mL scale filled with saline solution. The volume, expressed in µl, was then divided for the length of the intestinal segment, expressed in cm, to calculate the volume of 1 cm of each tract. This value was used to measure the volume of the cells positive to each antibody in each tract. We cut 4 µm sections perpendicular to the longitudinal axis of each organ (vertical sections) and collected at systematic random positions. Selected sections were stained either with HE or immunostained with specific antibodies as described above.

We used the HE-stained vertical sections to estimate the epithelium volume density (**Vv**) and the immunostained sections to estimate the Vv of cells positive for HOPX, SOX9, PCNA and CASP3. In accordance with the Delesse principle, a point-count stereologic grid with equally distant test points was used. The magnification range was chosen to allow the relevant portion of the intestinal wall to be contained in each field of vision (100× to 400×).

Vv was expressed as percentage and calculated as follows:

$$\mathrm{Vv}_{(\text{analyzed compartment, reference compartment})} = \left[\Sigma \mathrm{P}_{(\text{analyzed compartment})} / \Sigma \mathrm{P}_{(\text{reference compartment})}\right] \times 100$$

where $\Sigma P_{(\text{analyzed compartment})}$ is the number of points hitting the compartment under study and $\Sigma P_{(\text{reference compartment})}$ is the number of points hitting the relevant structure (i.e., intestinal wall or epithelium).

Furthermore, we used the Vv of HOPX$^+$, SOX9$^+$, PCNA$^+$ and cleaved CASP3$^+$cells to calculate their absolute volume (**Av**) as follows:

$$\begin{aligned}\text{Av of positive cells } (\mu\text{l/cm}) = {} & \mathrm{Vv}_{(\text{epithelium/intestinal wall})} \\ & \times \mathrm{Vv}_{(\text{positive cells/intestinal epithelium})} \\ & \times \text{volume of intestinal segment}\end{aligned}$$

Statistical analysis

The proportional values of each intestinal tract were analysed by Chi-square test across the three age groups (n=18). Quantitative data were expressed as mean ± SD. Results were analysed by using one-way ANOVA followed by all-pairwise multiple comparison test with Holm–Sidak method comparing values of each intestinal tract across the three age groups and comparing values of each age group across each section of the intestinal tract (n=18). Differences were considered statistically significant if $P<0.05$.

Results

Mucosa morphology

In the small intestine, villi height increased from birth to milk feeding and then decreased again at weaning. At the same time, villi's shape changed from digit to tongue-like. Crypts depth decreased at weaning and the villus:crypt ratio

Table 1 *Small and large intestine histometry in the newborn (day 0), suckling (day 6) and weaned piglets (day 28)*

Stages of life[1]	Duodenum				Jejunum			Colon
	Villus length (µm)	Villus width (µm)	Crypts depth (µm)	Villus:crypt ratio	Villus length (µm)	Villus width (µm)	Crypts depth (µm)	Villus:crypt ratio	Crypts depth (µm)
Newborn	$346.83^{a}\pm27.99$	$62.58^{a}\pm4.88$	$114.98^{a}\pm8.99$	$3.07^{a}\pm0.26$	$627.40^{a}\pm48.03$	$58.51^{a}\pm1.65$	$54.24^{a}\pm5.35$	$11.84^{a}\pm1.59$	$132.97^{a}\pm9.07$
Suckling	$568.35^{b}\pm50.13$	$88.11^{ab}\pm15.66$	$122.05^{a}\pm7.54$	$4.90^{b}\pm0.61$	$897.96^{b}\pm7.29$	$87.77^{a}\pm10.90$	$59.76^{a}\pm9.14$	$15.23^{b}\pm2.06$	$193.33^{b}\pm34.38$
Weaned	$278.10^{a}\pm72.44$	$110.19^{b}\pm18.15$	$192.08^{b}\pm25.60$	$1.54^{c}\pm0.22$	$201.76^{c}\pm18.13$	$73.39^{ab}\pm11.56$	$100.24^{b}\pm6.54$	$2.01^{c}\pm0.05$	$250.36^{c}\pm19.17$

Values are expressed as means ± SD.
[a–c] Different superscripts in the same column indicate significant differences ($P<0.05$).
[1] $n=6$ for each stage of life.

increased from birth to milk feeding and sharply decreased at weaning. In the colon, crypt depth increased significantly at each step, from birth to weaning (Table 1). Furthermore, we observed that at birth and during milk feeding, there were more branched crypts than at weaning (Supplementary Figure S3).

At all ages, we did not observe the presence of cells showing the typical Paneth cell morphology, neither in the duodenum nor in the jejunum (Supplementary Figure S4). However, goblet cells were present, not only along the villi but also along the entire crypt depth (Supplementary Figure S5). In newborns' small and large intestine, we observed that the enterocyte nucleus was not in the commonly observed basal position, but was in the apical part of the cytoplasm (Supplementary Figure S6). The measure of enterocyte height indicated that this was not due to a different cell size or shape, but due to a true change of the nucleus position (Supplementary Table S2). We did not observe this peculiar nuclear location in any other age.

The quantitative assessment of the intestinal epithelium Vv measured the ratio between the intestinal surface and the wall thickness of each tract and the different stages of life. We observed that the ratio remained constant from birth to weaning in the small intestine but, at weaning, it was significantly reduced in the colon (Figure 1).

Homeobox only protein

We observed HOPX^{+} cells in the crypts of all tracts at all ages. Positive cells showed both nuclear and cytoplasmic location of the signal and were mostly located in the +4 to +6 positions from the bottom of the crypt, consistent with what has been previously observed in older pigs (Gonzalez *et al.*, 2013) and in mice (Figure 2b, d and f). However, a few HOPX^{+} cells were also observed along the villi in the duodenum and the luminal epithelium in the colon of newborn and suckling animals, whereas in the jejunum, these could also be found after weaning (Figure 2a, c and e).

Stereological quantification revealed that Av of HOPX^{+} cells increased significantly between birth and milk feeding in the small intestine and sharply decreased at the time of weaning in all intestinal tracts (Figure 3a). At birth and during milk feeding, HOPX^{+} cells were several times more abundant in the colon than in the small intestine. However, such a large significant difference largely disappeared at weaning (Supplementary Figure S7A).

Sex-determining region Y-box 9

We observed SOX9^{+} cells at the bottom of all crypts of all tracts at all ages. These cells were mostly round in shape and the SOX9 signal was in the nucleus. We did not determine SOX9^{+} elongated columnar cells, nor did we observe cells that could be recognized as typical Paneth cells with small apical granules (Figure 4a d and f); however, all cells at the bottom of the crypts were positive. SOX9^{+} cells were also observed in the upper parts of the crypts, along the villi and on the colon luminal surface. Typically, in these locations, the signal was also in the cytoplasm (Figure 4a, c and e).

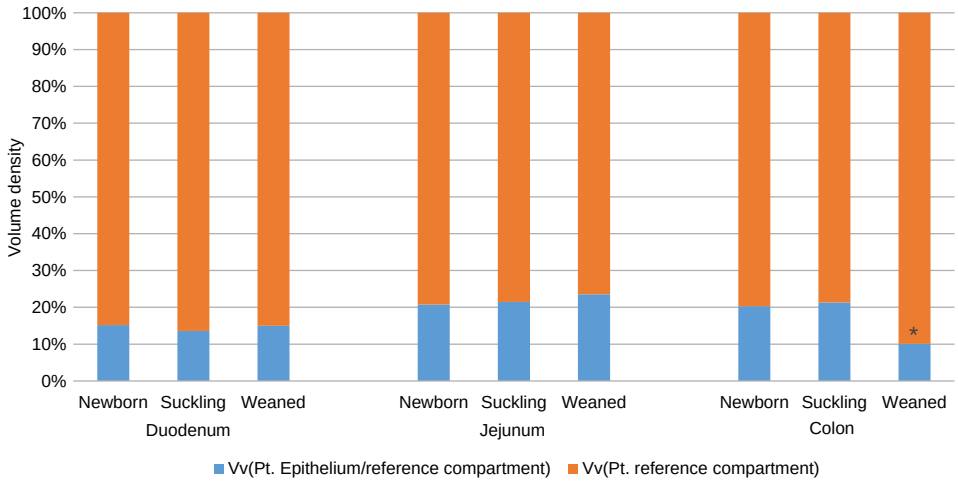

Figure 1 (colour online) Volume density (Vv) of piglets' intestinal epithelium in duodenum, jejunum and colon at different stages of life. Data are expressed as the percentage of the whole intestinal wall. *$P<0.05$. Pt: point.

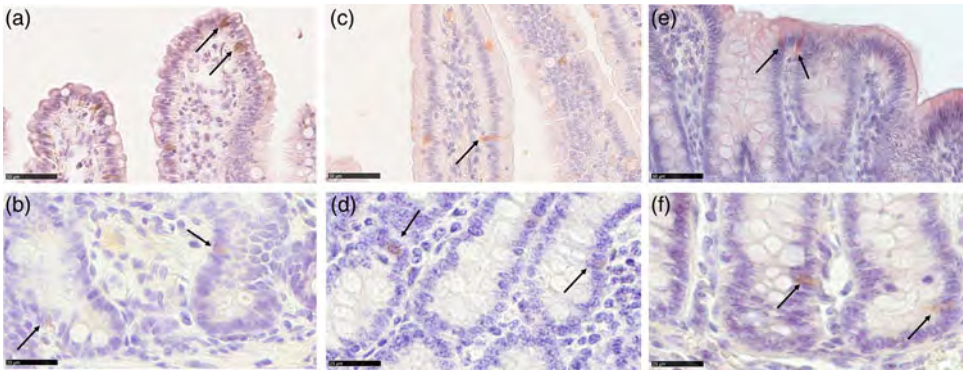

Figure 2 (colour online) Homeobox only protein signal identifies the quiescent stem cell population in piglets' small intestine and colon from birth to weaning. HOPX$^+$ cells (arrows) were observed in the +4 to +6 positions from the bottom of the crypt in the small and large intestine at all ages (b): jejunum weaned; (d): duodenum newborn; (f): colon suckling; scale bar=25 μm). Furthermore, HOPX$^+$ cells were also observed along the villi in the small intestine and on the colon luminal epithelium in newborn and suckling piglets. In the jejunum, HOPX positive cells were also found in weaned piglets (a): jejunum weaned; (c): duodenum suckling; (e): colon newborn; scale bar=50 μm). HOPX$^+$=Homeobox only protein.

Irrespectively of the location within the mucosa, the Av of SOX9$^+$ cells varied in the different tracts and along the development. In most cases, the signal increased between the birth and milk feeding and decreased at weaning within each tract (Figure 3b). However, at all ages, we measured a significantly larger SOX9$^+$cells volume in the colon than in the small intestine (Supplementary Figure S7B).

As expected, the ratio between SOX9$^+$ and HOPX$^+$ cells Av was always in favour of the former. This ratio significantly increased at weaning in the jejunum and colon but not in the duodenum (Figure 3c). However, the SOX9:HOPX volume ratio at birth was significantly lower than at weaning in the small intestine, but did not vary in the colon (Supplementary Figure S7C).

Proliferating cell nuclear antigen

We identified proliferating cells using PCNA immunostaining. In the crypts of all tracts at all ages, most, although not all, cells were proliferative. PCNA$^+$ cells were also present, although at low frequency, along the villi and on the luminal surface of the colon. In all locations, PCNA was specifically localized in the nucleus (Figure 5). Stereological quantification showed that the Av of proliferating cells statistically decreased at weaning, as expected (Figure 6a). Furthermore, the Av of proliferating cells was significantly larger in the colon than in the small intestine at birth and during lactation, whereas, at weaning, the Av dropped considerably, becoming almost similar in all tracts (Supplementary Figure S8A).

Cleaved caspase-3

We used an antibody specific for CASP3 cleaved at Asp-175, the biologically active form of CASP3, to identify cells actively undergoing the process of apoptosis. Cleaved caspase-3 was identified in cells at the apex of the villi and along the luminal colon epithelium, whereas no specific signal was observed in the crypts (Figure 7). Overall, the Av of apoptotic cells was small from birth to weaning and remained constant, except in the jejunum where we observed a gradual and significant increase in CASP3$^+$ cells along the growing phases we studied (Figure 6b). At all ages, apoptotic cells were rare;

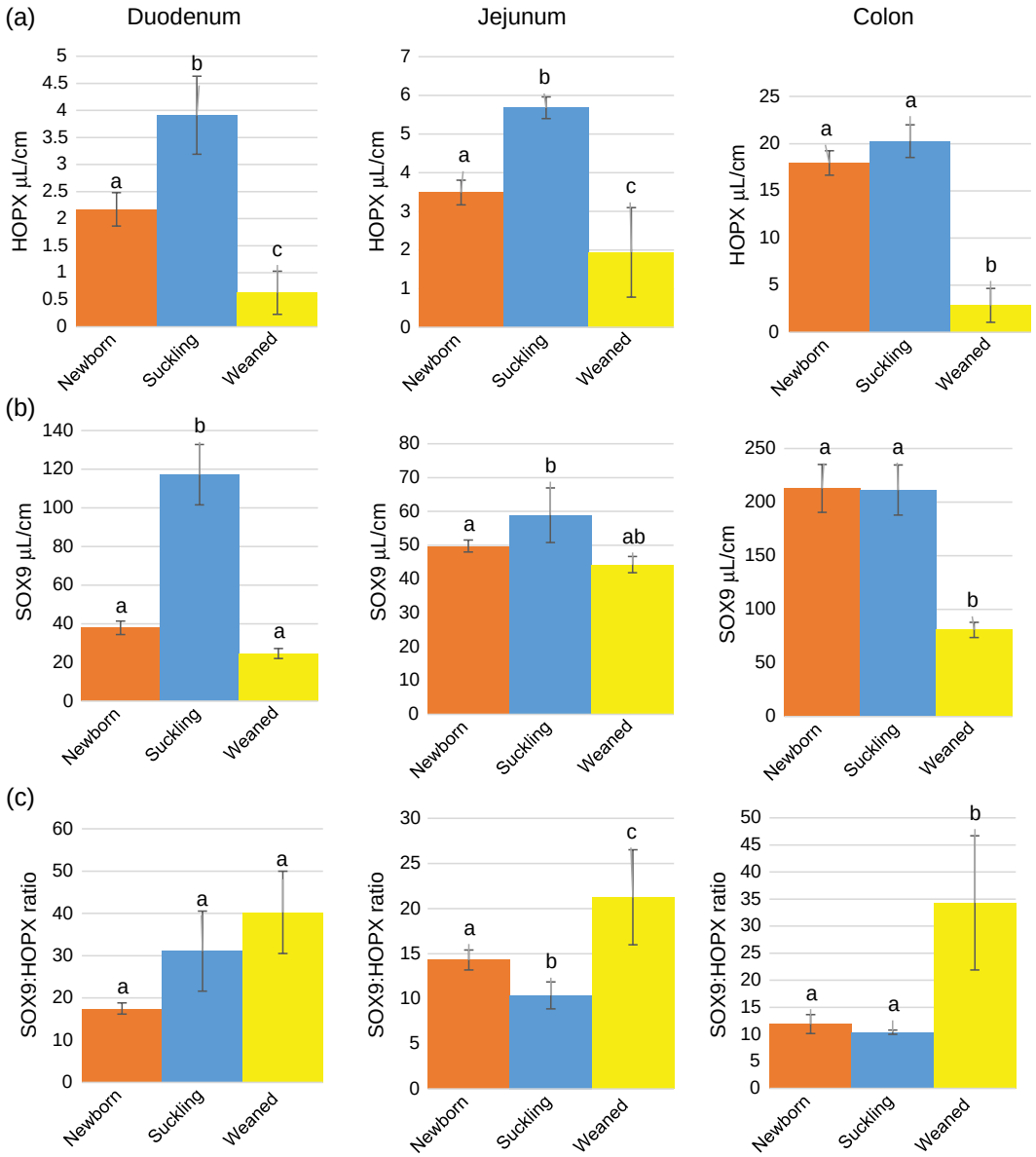

Figure 3 (colour online) Absolute volume of HOPX$^+$ (a) and SOX9$^+$ (sex-determining region Y-box 9) (b) cells and SOX9 : HOPX ratio (c) along the piglets' intestine. HOPX$^+$= homeobox only protein; SOX9$^+$=sex-determining region Y-box 9. $^{a–c}$Different superscripts within the same histogram indicate significant differences (P<0.05).

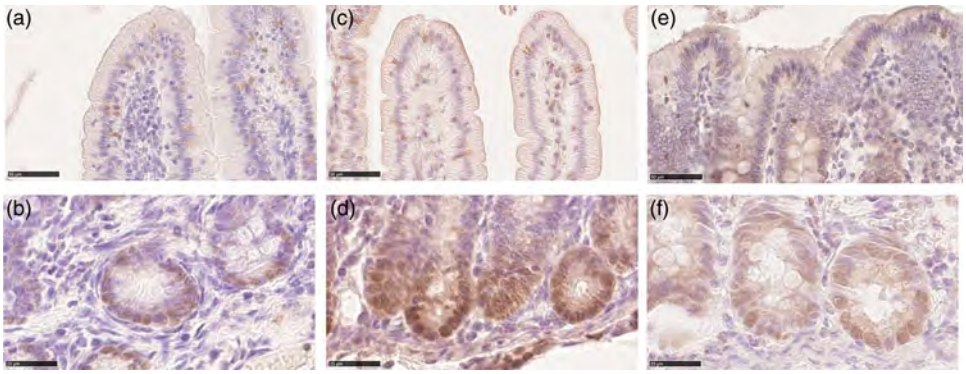

Figure 4 (colour online) Sex-determining region Y-box 9 immunostaining was localized in the nucleus of the cells within the crypt base of both the piglets' small intestine and colon at all ages (b): duodenum newborn; (d): jejunum suckling; (f): colon weaned; scale bar=25 µm). Both cytoplasmatic and nuclear SOX9 signals were observed along the villi in small intestine and colon luminal epithelium at all ages (a): duodenum weaned; (c): jejunum suckling; (e): colon newborn; scale bar=50 µm). SOX9=sex-determining region Y-box 9.

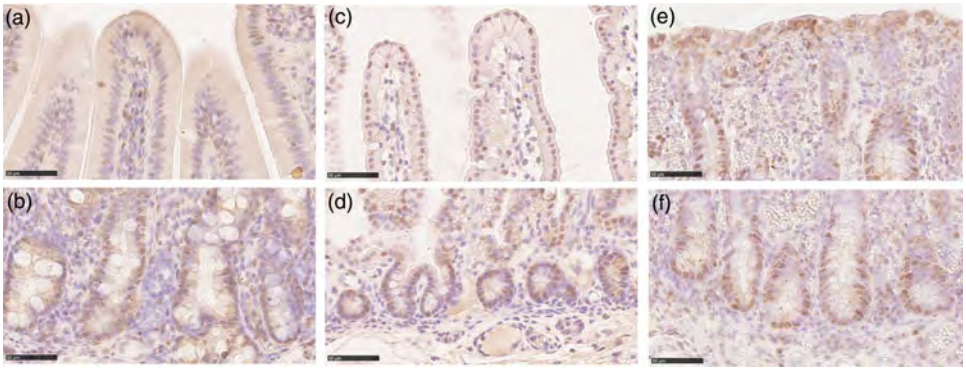

Figure 5 (colour online) Proliferating cell nuclear antigen immunolocalization was observed in the piglets' crypts in all tracts and at all ages (b): duodenum suckling; (d): jejunum newborn; (f): colon weaned; scale bar=50 μm). PCNA^{+} cells were also found along the villi in small intestine and colon luminal epithelium at all stages (a): duodenum suckling; (c): jejunum newborn; (e): colon weaned; scale bar=50 μm). PCNA = proliferating cell nuclear antigen.

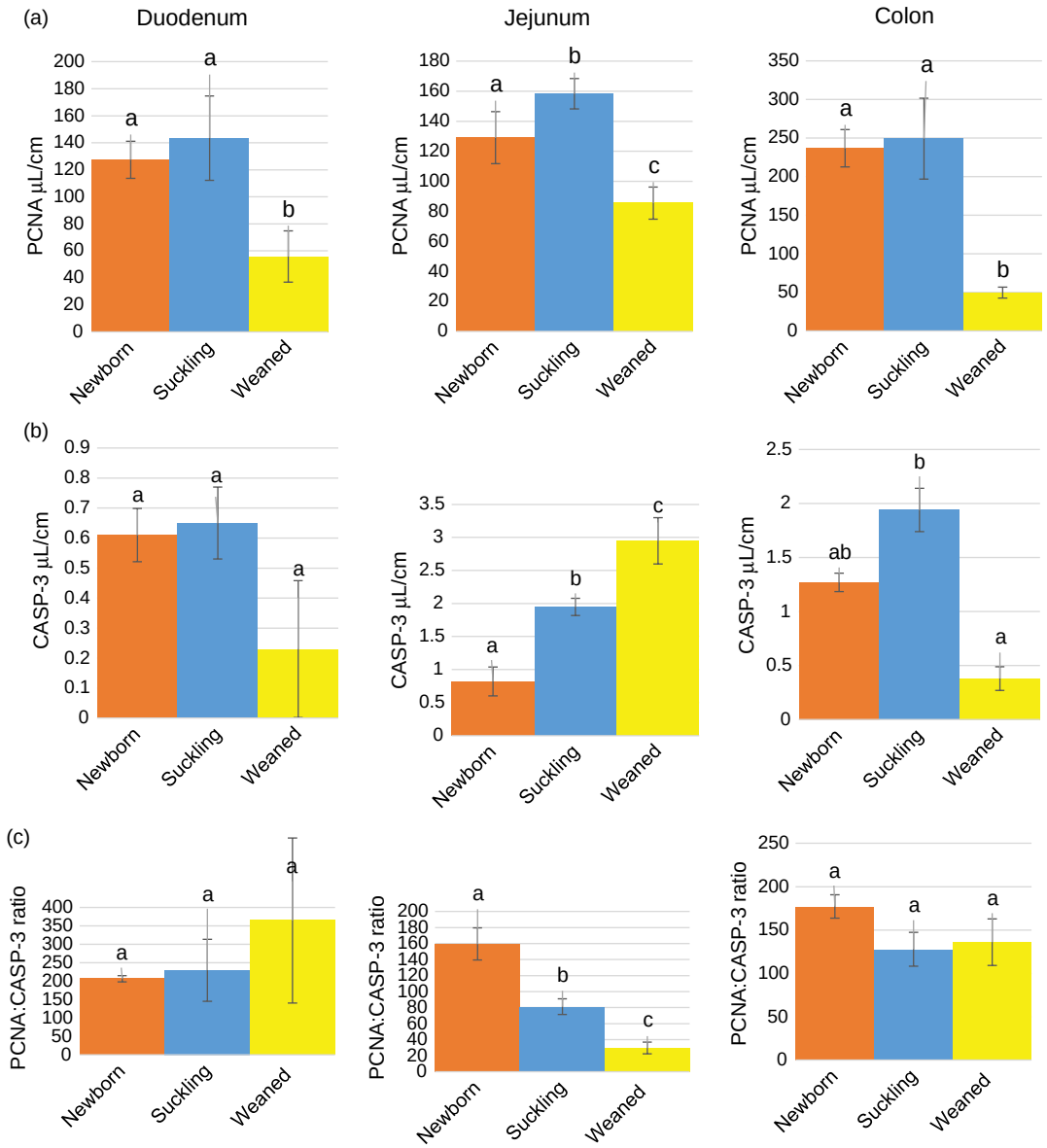

Figure 6 (colour online) Absolute volume of PCNA^{+} (proliferating cell nuclear antigen) (a) and CASP3^{+} (cleaved caspase-3) (b) cells and PCNA : CASP3 ratio (c) along the piglets' intestine. PCNA = proliferating cell nuclear antigen; CASP3=cleaved caspase-3. $^{a-c}$Different superscripts within the same histogram indicate significant differences ($P<0.05$).

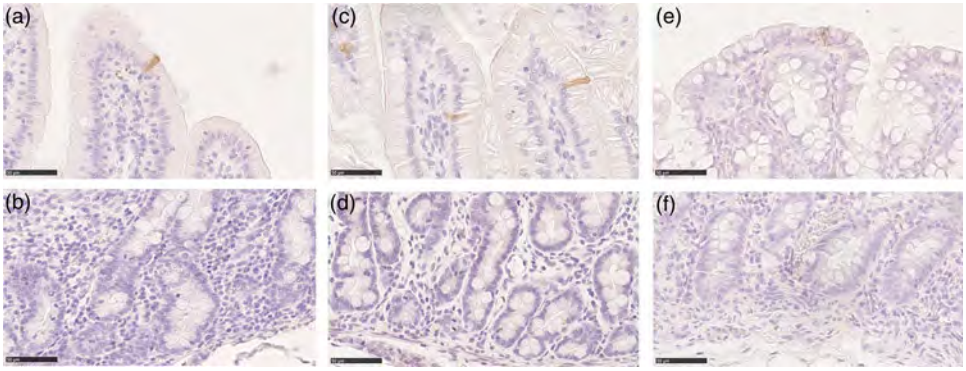

Figure 7 (colour online) Cleaved caspase-3 positive cells were found in the piglets' villi and on the colon luminal epithelium at all ages (a): duodenum weaned; (c): jejunum suckling; (e): colon newborn; scale bar=50 μm). No cleaved caspase-3^{+} cells were found in the crypts (b): duodenum weaned; (d): jejunum suckling; (f): colon newborn; scale bar=50 μm).

however, in most cases located in the colon at birth and during milk feeding, in this tract, apoptotic cells sharply decreased at weaning compared to that during milk feeding (Supplementary Figure S8B). The PCNA:CASP3 ratio showed a balance strongly in the favour of cell division from birth to weaning in the duodenum and colon, whereas in the jejunum, the ratio significantly decreased at each phase, reflecting the significant increase of apoptotic cells described above (Figure 6c). In fact, the jejunum at weaning was the only tract where apoptosis was in equilibrium with proliferation, as expected in an adult animal (Supplementary Figure S8C).

Discussion

In this study, we examined the evolution of ISCs from birth to weaning and correlated it with villi height and shape, crypt depth and villus:crypt ratio, the classical morphological features used to describe the tunica mucosa. We also applied a stereological method for the estimation of the intestinal wall volume in the different tracts, and the Vv of the tunica mucosa epithelial component of ISC as well as proliferating and apoptotic cells.

Our measurements of the tunica mucosa features are largely in agreement with data available in the literature (Godlewski *et al.*, 2005; Skrzypek *et al.*, 2018), confirming that the animals used in this study were healthy and normal. During the first 3 days after birth, the intestine doubles its weight and increases in length by 30% (Li *et al.*, 2014) and by the second week of life, the small intestine reaches its maximum length (Skrzypek *et al.*, 2018). According to a previous study, this results in an increase of the tunica mucosa Vv in the small intestine between foetal stage and birth, while it remains constant until weaning (Van Ginneken *et al.*, 2002). Our measurements of the mucosal epithelium Vv, including crypts, confirmed that it remained constant from birth to weaning in the small intestine. However, in the colon, we observed that mucosal epithelium Vv was significantly reduced at weaning, when we also observed a significant increase in crypt depth, that in mouse is considered a typical sign of inflammation (Erben *et al.*, 2014). The reduction of the colon epithelium Vv in combination with a significant increase in crypt depth suggests that, at this stage, colon epithelium is actively proliferating but it has been unable to fully recover from the regression induced by the weaning process. Indeed, the colon undergoes morphological changes around the time of weaning, similar to what has been described in the small intestine (Brunsgaard, 1998), whose morphological changes have been studied more intensively. In weaned pigs, we also observed a decrease in branched crypts, which is consistent with the notion that pig gut expansion occurs by a process of crypt multiplication in which crypts reproduce themselves by a fission mechanism (Brunsgaard, 1997).

Intestinal epithelial cell proliferation is very active and the whole population is entirely renewed every 2 to 3 days. Such a rapid turnover is driven by a small number of stem cells that are formed by two populations: the reserve ISCs, also known as +4 cells, characterized by a quiescent or slow cell cycle, under homeostatic conditions and the actively proliferating stem cells, also called CBC cells, which are major contributors to epithelial cells renewal.

The reserve ISCs do not possess a typical morphology and can be identified through a number of markers, among which HOPX has been validated in adult pigs (Gonzalez *et al.*, 2013). Our results indicate that HOPX^{+} cells are present in pig's intestinal mucosa from birth to weaning, in both the small and large intestinal tracts. The volume of positive cells, as expected, was quite low and these cells were located around the +4 position. We observed that their volume changed significantly throughout the first weeks of life, following the same pattern of proliferating cells. Although +4 cells are quiescent in the homeostatic conditions found in healthy adult animals, these variations are consistent both with the very rapid development of the whole intestine occurring in the first week of life and the well-known sharp decrease of epithelial proliferation associated with weaning (Heo *et al.*, 2013). We also observed that the volume of HOPX^{+} cells in the colon was significantly higher than in the small intestine at all stages, except at weaning. This is consistent

with the previous observation that colon epithelium Vv is the only one to be affected by the weaning process.

In mouse, typical CBC cells are located at the bottom of the small intestine crypts between two Paneth cells, as well as in the colon crypts, and are characterized by LGR5 expression (Barker *et al.*, 2007). However, in pigs, this marker has proved to be ineffective, whereas SOX9 was localized in the regions where both stem and progenitor cells are expected (Gonzalez *et al.*, 2013). Unfortunately, this leaves only transmission electron microscopy as a way to identify columnar base cells and discriminate them from transient amplifying population (Gonzalez *et al.*, 2013), thus making accurate quantification very difficult.

We also observed that, while all cells at the bottom of the crypts are SOX9 positive (Figure 4), not all of them express PCNA (Figure 5). This suggests that at the bottom of the crypts, there are cells with a labelling pattern ($SOX9^+$ and $PCNA^-$) compatible with the properties of Paneth cells (Bastide *et al.*, 2007; Mori-Akiyama *et al.*, 2007). However, the lack of the typical acidophilic apical granules prevents their description as *bona fidae* Paneth cells rather we are observing Paneth-like cells, as also previously suggested by Gonzalez *et al.* (2013). Irrespectively, these cells are likely to have functional relevance in the light of the recent data indicating that Paneth cells do not only have a well-characterized bacteriostatic function, but also play a fundamental role in the regulation of CBC cells (Clevers and Bevins, 2013).

Interestingly, cells located at the bottom of the crypts showed a distinct nuclear location of the SOX9 signal, whereas, along the villi and on the colon luminal epithelium, the signal was diffused into the cytoplasm as well. This could be explained by the observation that SOX9 is expressed either at low or high steady-state levels in SOX9-enhanced green fluorescent protein ($SOX9^{EGFP}$) transgenic mice (Formeister *et al.*, 2009). In these animals, high levels of $SOX9^{EGFP}$ were accompanied by the expression of chromogranin A and substance P but not by the proliferation marker Ki67, suggesting that SOX9 could also be expressed by post-mitotic enteroendocrine cells.

The SOX9:HOPX ratio was significantly higher in jejunum and colon at weaning, indicating that pig ISCs are likely to follow a functional model where the quiescent, reserve population of stem cells ($HOPX^+$) is specifically activated upon injury and might convert into progeny or active stem cells, which are both $SOX9^+$ (Beumer and Clevers, 2016). Overall, our results confirm and expand the findings of Gonzalez *et al.* (2013) who described $HOPX^+$ and $SOX9^+$ cells in the same locations in animals a few weeks older than those we examined. More recently, LGR5 and BMI1, another marker for +4 cells, have both been cloned in pig (Li *et al.*, 2014, 2018), opening the way for the generation of pig-specific reagents.

The presence of the activated form of caspase (CASP3) was very rare at all ages and in all intestinal tracts that we examined, indicating a very low rate of apoptosis. This is consistent with the high mitosis or apoptosis ratio, typical of the growing phase of these animals also described by previous studies (Widdowson *et al.*, 1976; Skrzypek *et al.*, 2005). In our samples, apoptotic cells were visible only at the apex of the villi or in the colon luminal epithelium. This is in contrast with previous observations in neonatal piglets, which described apoptotic cells along the entire length of the villi, including the lower half and noted that dying cells were eliminated into the lumen as single cells or groups of cells (Godlewski *et al.*, 2005). A possible explanation for such discrepancy is that we used an antibody specific for the cleaved and active form of caspase, whereas the previous studies used an antibody that was described as specific for caspase-3 and may have also recognized the uncleaved precursor. This would have led to the staining of cells still not actively undergoing the process of apoptosis. However, the presence of a small number of CASP3 positive cells was also described in older (6 to 8 weeks) pigs (Gonzalez *et al.*, 2013). This suggests that caspase-3, an essential element of both intrinsic and extrinsic apoptotic pathways, may be activated after initiation of cell shedding (anoikis), consistent with findings in mice that caspase-3 was undetectable until after other features of cell shedding were evident (Marchiando *et al.*, 2011).

Conclusions

We can conclude that the ISC structure described in mouse largely overlaps with our current observations in pig, with some limitations including the lack of an adequate antibody for LGR5 that prevents an unequivocal identification of the so-called active stem cells. However, the recent observation that LGR5 and BMI1 are overexpressed in a pig intestinal cell line (Li *et al.*, 2018) further supports the idea that pig intestinal epithelium proliferation is regulated through mechanisms similar to that of the mouse.

The lack of some typical morphological characteristics associated with *bona fidae* Paneth cells still makes their indisputable identification problematic, even if functionally equivalent cells are likely to be present.

The accurate cell quantification suggests that, at weaning, $HOPX^+$ cells may convert into $SOX9^+$ cells; however, this hypothesis cannot be tested without performing lineage tracing experiments that are not currently possible in pigs. Nevertheless, it will be interesting to verify if similar observations will also be reported in other stressful circumstances like intestinal infections.

Overall, these results are useful to better understand the cellular mechanisms regulating cell proliferation at weaning or other stress and how to possibly harness them to preserve epithelial integrity.

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Declaration of interest

The authors declare no conflict of interest

Ethics statement

The experimental procedures were approved by the University of Milan Ethics Committee (Decision n. OPBA_82_2017).

Software and data repository resources

None of the data was deposited in an official repository.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731119001319

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Conjugated linoleic acid and betaine affect lipolysis in pig adipose tissue explants

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*Consumers' demand of leaner meat products is a challenge. Although betaine and conjugated linoleic acid (**CLA**) have the potential to decrease porcine adipose tissue, their mode of action is poorly understood. The aim of the study was to determine the lipolytic effect of betaine and CLA in the adipose tissue of Iberian pigs. Adipose tissue explants from five pigs (38 kg BW) were prepared from dorsal subcutaneous adipose tissue samples and cultivated for 2 h (acute experiments) or 72 h (chronic experiments). Treatments included 100 μM linoleic acid (control), 100 μM trans-10, cis-12 CLA, 100 μM linoleic acid + 1 mM betaine and 100 μM trans-10, cis-12 CLA + 1 mM betaine (**CLABET**). To examine the ability of betaine or CLA to inhibit insulin's suppression of isoproterenol-stimulated lipolysis, test medium was amended with 1 μM isoproterenol ±10 nM insulin. Media glycerol was measured at the end of the incubations. Acute lipolysis (2 h) was increased by CLA and CLABET (85% to 121%; $P < 0.05$) under basal conditions. When lipolysis was stimulated with isoproterenol (1090%), acute exposure to betaine tended to increase (13%; $P = 0.071$), while CLA and CLABET increased (14% to 18%; $P < 0.05$) isoproterenol-stimulated lipolysis compared with control. When insulin was added to isoproterenol-stimulated explants, lipolytic rate was decreased by 50% ($P < 0.001$). However, supplementation of betaine to the insulin + isoproterenol-containing medium tended to increase ($P = 0.07$), while CLABET increased (45%; $P < 0.05$) lipolysis, partly counteracting insulin inhibition. When culture was extended for 72 h, CLA decreased lipolysis under basal conditions (18%; $P < 0.05$) with no effect of betaine and CLABET ($P > 0.10$). When lipolysis was stimulated by isoproterenol (125% increase in rate compared with basal), CLA and CLABET decreased glycerol release (27%; $P < 0.001$) compared with control (isoproterenol alone). When insulin was added to isoproterenol-stimulated explants, isoproterenol stimulation of lipolysis was completely blunted and neither betaine nor CLA altered the inhibitory effect of insulin on lipolysis. Isoproterenol, and especially isoproterenol + insulin, stimulated leptin secretion compared with basal conditions (68% and 464%, respectively; $P < 0.001$), with no effect of CLA or betaine ($P > 0.10$). CLA decreased leptin release (25%; $P < 0.001$) when insulin was present in the media, partially inhibiting insulin stimulation of leptin release. In conclusion, betaine and CLA produced a biphasic response regarding lipolysis so that glycerol release was increased in acute conditions, while CLA decreased glycerol release and betaine had no effect in chronic conditions. Furthermore, CLA and CLABET indirectly increased lipolysis by reducing insulin-mediated inhibition of lipolysis during acute conditions.*

Keywords: fat, lipids, metabolic modifiers, metabolism, sus scrofa

Implications

Betaine and conjugated linoleic acid (**CLA**) have been reported to decrease carcass fat in different animal models, but the mechanisms are not clearly elucidated. We have shown that acute lipolysis was increased directly by CLA, especially betaine + CLA. These data also demonstrate that they function to promote the partitioning of energy away from lipid accretion within porcine adipose tissue, in part by reducing insulin-mediated inhibition of lipolysis. These effects have favourable consequences for the consumers interested in low-fat pork meat.

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Introduction

Betaine and CLA have the potential to alter growth and body composition in swine, although their mode of action is not well understood. Dietary betaine decreases fat deposition

while increasing carcass lean in pigs (Fernandez-Figares *et al.*, 2002). Dietary CLA may reduce body fat in pigs (Dugan *et al.*, 1997) and a reduction in fat accretion by CLA is also supported by cell culture studies, where the expression of genes regulating lipid accretion were decreased in cells isolated from subcutaneous adipose tissue in response to trans-10, cis-12 CLA (Zhou *et al.*, 2007). Betaine or CLA alone did not produce significant changes in growth performance and body composition in Iberian pigs, an obese breed distributed in Spain and Portugal, but the addition of both promoted a synergistic effect on growth, carcass protein deposition (Fernandez-Figares *et al.*, 2008) and yield of lean cuts (Rojas-Cano *et al.*, 2011). Biochemical and hormone profiles may partially explain the nutrient partitioning effect and increased lean deposition in pigs fed with betaine + CLA diets (Fernandez-Figares *et al.*, 2011). For example, serum triacylglycerol was increased in Iberian pigs fed with CLA and betaine + CLA supplemented diets. Betaine and CLA decreased portal-drained viscera heat production in Iberian pigs, increasing energy availability for other tissues, although the effects on growth performance depend on the energy available for growth and how it is partitioned between protein and fat (Rojas-Cano *et al.*, 2017). Leptin, a polypeptide hormone secreted primarily by adipose tissue, appears to play a major role in feed intake and energy metabolism (Friedman and Halaas, 1998), and it has direct autocrine or paracrine effects on adipocytes (Fruhbeck *et al.*, 1997), which in turn may contribute to the fat-reducing effect of leptin at the central nervous system level. The mechanisms underlying the decrease in fat content reported in pigs fed with betaine or CLA are poorly understood, but could involve the inhibition of lipogenesis (Zhou *et al.*, 2007) `and stimulation of lipolysis. The potential role of betaine and CLA on pig adipose tissue lipolytic response is unknown. We hypothesised that changes in fat accretion elicited by betaine and CLA in pigs could partially be explained by an increased lipolytic response in adipose tissue. In order to demonstrate that betaine and CLA act directly on adipocytes, it is necessary to exclude the involvement of hormonal and neural control by conducting *in vitro* studies. It was, therefore, the objective of the present study to assess the effects of acute (2 h) and prolonged (72 h) culture of betaine and CLA on lipolysis in pig adipose tissue.

Materials and methods

Tissue isolation and culture

For tissue isolation, five Iberian (Silvela strain) barrows (*Sus scrofa mediterraneus*; approximately 38 kg BW), supplied by Sánchez Romero Carvajal (Jabugo S.A., Puerto de Santa María, Spain), were housed in a controlled environment room (21 ± 1.5°C) and offered *ad libitum* water and a barley–soybean meal diet (101 g CP/kg DM, 14.7 MJ ME/kg DM). Adipose tissue samples were acquired following slaughter by electrical stunning and exsanguination.

The isolation and culture procedures were according to Ramsay and Richards (2004). Dorsal subcutaneous adipose tissue samples from between the shoulder blades were acquired following slaughter, diced into strips (1 × 4 cm), placed in Hanks buffer (37°C, pH 7.4) in screw-capped polypropylene Erlenmeyer flasks and transported to the laboratory. In the laboratory, adipose tissue strips were placed in fresh Hanks buffer (37°C, pH 7.4) and dissected clean of any extraneous muscle tissue and further separated into 1 cm cubes in a laminar flow hood. Adipose tissue explants (approximately 100 mg) were prepared by slicing tissue cubes with a Stadie-Riggs microtome. Tissue slices (400 μm thick) were rinsed twice with fresh Hanks buffer (37°C, pH 7.4), blotted free of excess liquid, weighed, transferred to 6-well tissue culture plates with 2 ml of basal medium per well (DMEM/F12 (50 : 50), 0.5% bovine serum albumin, 25 mM HEPES, gentamycin, amphotericin B and penicillin-streptomycin and incubated (5% CO2, 37ºC) for 1 h to wash glycerol and fatty acids. Triplicate tissue slices were then incubated in test mediums (basal medium amended with 100 μM linoleic acid (control), 100 μM trans-10, cis-12 CLA, 100 μM linoleic acid + 1 mM Bet (Aldrich #21,906-1, Tres Cantos, Madrid, Spain) and 100 μM trans-10, cis-12 CLA + 1 mM betaine). The first assay estimated the direct effects of CLA and betaine on acute and chronic lipolysis and incubations were performed for 2 and 72 h, respectively, prior to collection of medium for glycerol analysis.

The second assay examined the ability for betaine and CLA to inhibit acute or chronic insulin suppression of isoproterenol-stimulated lipolysis using tissue of the same pigs than in the first assay. Isoproterenol was used because it is a pure β-agonist, and as such it elicits the greatest lipolytic response in pig adipose tissue (Mersmann, 1984). Tissue slices were incubated in the test incubation buffers, described earlier, with 1 μM isoproterenol ± 10 nM insulin in the presence of betaine and CLA for 2 h (acute) or 72 h (chronic) prior to medium collection for glycerol analysis. Glycerol is released during the hydrolysis of triglycerides correlating with free fatty acids release.

Lipolysis was terminated in medium samples (1 mL) by adding 0.1 mL 30% HClO4, followed by centrifugation at 13,000 × *g* for 5 min. Samples of the supernatant were neutralised with 1 N KOH and frozen for later analysis of glycerol content. Isoproterenol (#I2760, Sigma-Aldrich, St. Louis, MO) was solubilised in milli-Q water and sterile filtered through a 0.22 μm membrane. Bovine insulin (#I1882, Sigma-Aldrich, St. Louis, MO) was solubilised in 0.01N HCl. Control incubations in basal medium included a similar amount of vehicle to exclude the vehicle as a variable.

In order to solubilise fatty acids, linoleic acid and CLA, were bound to fatty acid-free albumin (#A7030, Sigma-Aldrich, St. Louis, MO), according to Svedberg *et al.* (1990). The fatty acid:albumin molar ratio was kept at less than three to ensure that the fatty acids were bound to fatty acid-free albumin. Linoleic acid, cis-9 cis-12 linoleic acid (#10-1802-13) and trans-10 cis-12 linoleic acid (#10-1826-90-13) were provided by Larodan (Malmoe, Sweden).

Media glycerol concentration was measured at the end of the incubations using a commercially available glycerol assay

kit, following manufacturer directions (#MAK117, Sigma-Aldrich, St. Louis, MO). In brief, glycerol concentration was determined by a coupled enzyme assay involving glycerol kinase and glycerol phosphate oxidase, resulting in a colorimetric (570 nm) product, proportional to the glycerol present. Samples were run in duplicate.

Leptin was analysed using a multispecies leptin RIA kit (catalogue no. XL-85K; Millipore Corporation, Saint Charles, MO, USA), following manufacturer directions. The Millipore Multi-Species Leptin assay utilises^{125} I-labeled human leptin and a multispecies leptin antiserum to determine the level of leptin in serum, plasma or tissue culture media by the double antibody/polyethylene glycol technique. Slopes of the standards curves prepared with medium and water were parallel. The intra- and inter-assay average coefficients of variation of concentrations were 8.6% and 11.4%, respectively.

Cell lysis was quantified based on the measurement of lactate dehydrogenase (LDH) activity, as LDH is released from the cytosol of damaged cells into the supernatant. We used a colorimetric assay (Cytotoxicity Detection KitPLUS(LDH); Roche, cat. No 04 744 926 001) based on the generation of nicotinamide adenine dinucleotide (NAD+) and lactate from pyruvate and NADH (Neilands, 1955). LLDH from rabbit muscle (Sigma cat. LLDH-RO Roche) was used as standard. A Victor ™X5 Perkin Elmer 2030 Multilabel reader (Turku, Finland) was used to measure absorbance at 490 nm. Triplicate instrumental analyses were carried out.

Statistical analysis

Data were analysed using a mixed-model ANOVA (PROC MIXED, SAS Institute Inc., Cary, NC, USA), which included the fixed effects of culture conditions (basal, isoproterenol and isoproterenol + insulin), treatments (Control, BET, CLA, CLABET) and the random effect of each pig. The error term used for testing treatment effect was defined by the interaction pig X treatment. Values from each pig were considered as the experimental unit of all response variables. Five separate cell preparations, each from a different pig, constituted an experiment. Results are expressed as least square means ± s.e. Statistical significance was assessed using Fisher's least significant difference test to determine differences between treatment groups. The level of significance was set to 5%.

Results

No differences on tissue viability were found between explants cultivated for 2 and 72 h (27.7 compared with 30.7 mU/ml for 2 and 72 h of culture, respectively, $P > 0.10$).

Acute lipolysis

Acute lipolysis (Figure 1) was increased by CLA and CLABET (81% to 98%; $P < 0.05$) and tended to increase in the presence of BET (27%) under basal conditions. Isoproterenol stimulated lipolysis ($P < 0.001$) by 1090%, relative to basal

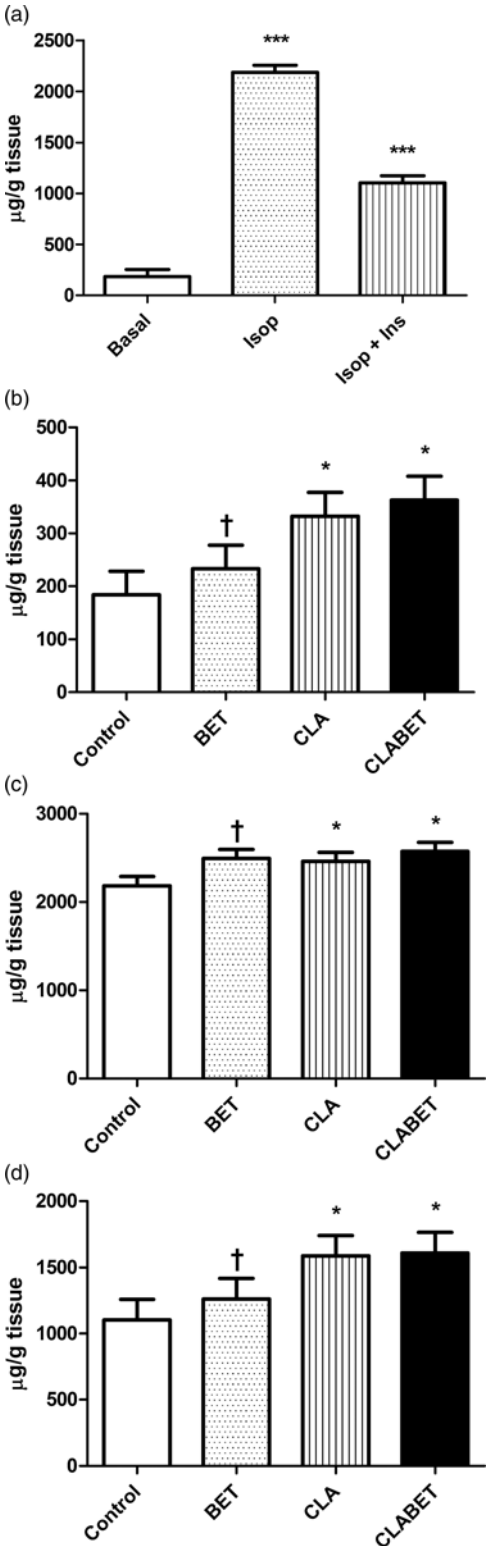

Figure 1 Glycerol release after acute exposure (2 h) to basal media, isoproterenol (Isop; 1 µM;) and isoproterenol + insulin (Isop.+ Ins; 10 nM;) (a). Glycerol release after acute exposure (2 h) to betaine (BET; 1 mM), conjugated linoleic acid (CLA; 0.1 mM) and CLA + BET under basal lipolysis (b), isoproterenol-stimulated lipolysis (c) and isoproterenol + insulin condition (d), respectively. Pig subcutaneous adipose tissue explants were incubated for 2 h in the absence (control) or presence of BET, CLA or CLA + BET, followed by analysis of glycerol content of the medium. Data are expressed as µg/g tissue. † Different from control $0.10 < P < 0.05$; *Different from control $P < 0.05$; *** Different from control $P < 0.001$. $n = 5$.

medium. Acute exposure to BET tended to increase (13%; $P = 0.071$) while CLA and CLABET increased (14% to 18%; $P < 0.05$) isoproterenol-stimulated lipolysis compared with control. Addition of insulin (10 nM) to the medium reduced the isoproterenol-stimulated lipolytic response by 50% ($P < 0.001$). Supplementation of betaine to the insulin-containing medium tended to increase ($P = 0.07$) while CLA and CLABET increased lipolysis (45% on average; $P < 0.05$), partly counteracting insulin inhibition. No interaction was found between factors.

Chronic lipolysis

After 72 h of culture, CLA decreased (18%; $P < 0.05$) lipolysis (Figure 2) while BET and CLABET did not affect it ($P > 0.10$) under basal conditions. Isoproterenol (1 μM) produced a 125% increase in lipolytic response compared with basal conditions ($P < 0.001$) and CLA and CLABET partially decreased isoproterenol-stimulated lipolysis (25% to 29%; $P < 0.001$ relative to control). Insulin reduced the isoproterenol-stimulated lipolysis (60%; $P < 0.001$) back to basal conditions. However, chronic exposure to betaine and/or CLA did not affect insulin inhibition of isoproterenol-stimulated lipolysis. No interaction was found between factors.

Leptin release

No effect of betaine on leptin secretion was found under basal or isoproterenol-stimulated conditions (Figure 3). Nevertheless, CLA increased leptin secretion under basal conditions (27%; $P < 0.05$). Addition of isoproterenol-increased leptin release (68%; $P < 0.001$) with no effect of CLA or betaine exposure ($P > 0.10$). Addition of insulin to the medium further increased isoproterenol-stimulated leptin release compared with basal conditions (464%; $P < 0.001$) but CLA decreased leptin secretion (25%; $P < 0.001$) compared with control, partly counteracting insulin stimulation of leptin release.

Discussion

Although betaine and CLA have an impact on adipose tissue mass when supplemented together to the diet of growing Iberian pigs (Fernandez-Figares *et al.*, 2008), the mechanism by which they reduce body fat accretion is not elucidated. Effects could involve *de novo* lipogenesis, use of preformed fatty acids for lipid synthesis, rates of lipolysis or some combination of these. We have focused our research on the possible effect of betaine and CLA on lipolysis using an *in vitro* approach. Several studies have demonstrated that CLA and betaine can alter insulin sensitivity and thus adipocyte metabolism. *In vivo* studies with Iberian pigs showed increased serum insulin with no change in glucose in CLA and betaine + CLA-fed pigs (Fernandez-Figares *et al.*, 2011), although no changes on indices of insulin resistance were observed.

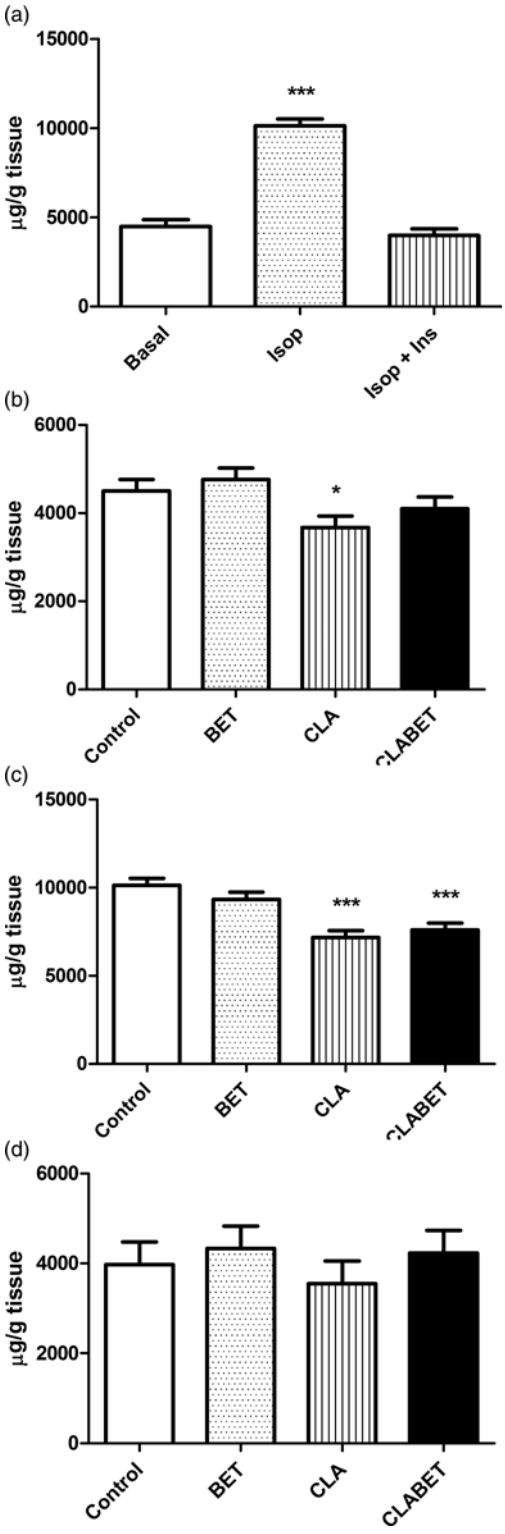

Figure 2 Glycerol release after chronic exposure (72 h) to basal media, isoproterenol (Isop; 1 μM;) and isoproterenol + insulin (Isop.+ Ins; 10 nM;) (a). Glycerol release after chronic exposure (72 h) to betaine (BET; 1 mM), conjugated linoleic acid (CLA; 0.1 mM) and CLA + BET under basal lipolysis (b), isoproterenol-stimulated lipolysis (c) and isoproterenol + insulin condition (d), respectively. Pig subcutaneous adipose tissue explants were incubated for 72 h in the absence (control) or presence of BET, CLA or CLA + BET, followed by analysis of glycerol content of the medium. Data are expressed as μg/g tissue. *Different from control $P < 0.05$; ***Different from control $P < 0.001$. $n = 5$.

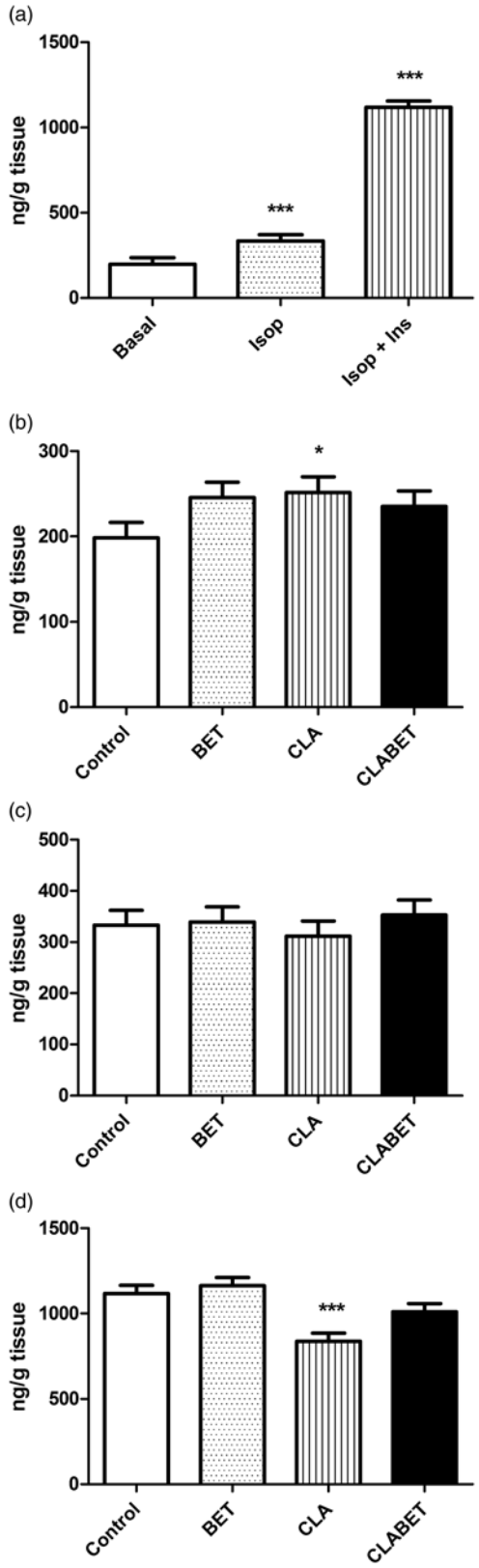

Figure 3 Effect of betaine- (BET; 1 mM), conjugated linoleic acid (CLA; 0.1 mM) and CLA + BET on leptin release under basal (a), isoproterenol (Isop.; 1 μM, b) and isoproterenol + insulin (Isop.+ Ins; 10 nM) conditions (c) in explants of pig subcutaneous adipose tissue cultivated for 72 h. Data are expressed as ng/g tissue. *Different from control $P < 0.05$; ***Different from control $P < 0.001$. $n = 5$.

Lipolysis

The present study provides new insight into the effects of betaine and CLA on body fat in obese breeds. Although cis-9, trans-11 CLA and trans-10, cis-12 CLA are the most biologically active isomers that occur naturally in food (Chin *et al.*, 1992), the latter exhibits the greatest anti-obesity effect (Park *et al.*, 1999).

The effect of CLA on lipolysis was biphasic increasing glycerol release under acute conditions and decreasing it under chronic conditions in our experiments. We expected increased lipolysis in both conditions that would, in part, explain the carcass fat-lowering effect of CLA in pigs. We have found no information in the literature about short-term effect of CLA on lipolysis. The short time of exposure (2 h) would suggest that there was a direct lipolytic effect of CLA.

The decreased glycerol release in our experiments under chronic exposure to CLA was unexpected, but would concur with reduced glycerol production (Yeganeh *et al.*, 2016) under a long-term (8 days) treatment with t10-c12-CLA in differentiating 3T3-L1 cells. On the contrary, 3T3-L1 adipocytes treated with trans-10, cis-12 CLA for 6 days increased basal but not isoproterenol-stimulated lipolysis in relation to the control (Evans *et al.*, 2002). Other authors have reported no changes in lipolysis elicited by trans-10, cis-12 CLA in long-term (48 h) adipose tissue explant cultures of pigs (José *et al.*, 2008) and mature 3T3-L1 cells (Yeganeh *et al.*, 2016). The contrasting responses found in the literature regarding CLA effects on lipolysis could be explained by the differences in lipid metabolism among species, time of culture and culture conditions.

Betaine inhibited fat synthesis by reducing fatty acid synthase activities and increasing hormone-sensitive lipase (**HSL**) activity in adipose tissue of finishing pigs (Huang *et al.*, 2006). In the present study, betaine tended to increase isoproterenol-stimulated lipolysis under acute conditions, most likely through altering HSL activity. Betaine supplementation increased intramuscular fat in Alentejano pig (a sibling of Iberian pig) muscles without affecting expression of genes related to lipolysis (Albuquerque *et al.*, 2017), which indicates that mechanisms regulating fat accretion in intramuscular fat and subcutaneous adipose tissue may be different. Betaine has been reported to decrease homocysteine levels (Craig, 2004), which could increase lipolysis through inactivation of AMPK pathway, as demonstrated in 3T3-L1 adipocytes with basal and isoproterenol-activated lipolysis (Wang *et al.*, 2011). Nevertheless, we have not detected differences in adipose AMPKα1 or AMPKα2 expression between control and dietary betaine supplemented pigs (unpublished results).

Growth hormone (**GH**) can play a role in fat accretion, as it directly signals adipocyte lipolysis (Dietz and Schwartz, 1991). Nevertheless, betaine effects on GH secretion are not consistent in pigs. Interestingly, 50 kg Iberian pigs fed with betaine- or CLA-supplemented diets had decreased serum GH level (Fernandez-Figares *et al.*, 2011), indicating that the mechanism by which betaine (or CLA) decrease carcass fat in Iberian pigs (Fernandez-Figares *et al.*, 2008) is not via the somatotrophic axis.

There is currently little information on what controls leptin secretion in pigs. Several studies have shown that circulating levels of leptin are closely correlated with body fat mass and fat cell size (Friedman and Halaas, 1998), although it has become clear that factors other than changes in adipose tissue mass must be involved (Boden *et al.*, 1997). CLA-decreased lipolysis stimulated or not with isoproterenol (chronic conditions, 72 h) might not be congruent with increased leptin secretion (72 h), as leptin stimulates lipolysis in pig adipose tissue directly (Ramsay and Richards, 2004) and indirectly reducing insulin-mediated inhibition of lipolysis (Ramsay, 2001). Chronic (7 days) exposure to insulin elevated leptin mRNA levels (Ramsay and White, 2000). In the presence of isoproterenol and insulin (Figure 3c), CLA decreased leptin release in line with decreased lipolysis. We speculate that insulin and isoproterenol interact with CLA regarding leptin release, although the mechanism is unknown. Furthermore, trans-10, cis-12 CLA reduced fat accumulation and leptin secretion in 3T3-L1 adipocytes (Ahn, 2006). In contrast with *in vitro* data, serum leptin concentration in growing Iberian pigs fed with CLA supplemented diets (Fernández-Fígares *et al.*, 2011) remained unaltered when compared with control pigs. We have found no effect of betaine on leptin release in agreement with *in vivo* data (Fernandez-Figares *et al.*, 2011), although betaine reduced serum leptin release in dietary-induced obese mice (Jang *et al.*, 2014). Insulin greatly increased leptin release in the presence of isoproterenol. Interestingly, short-term (up to 3 h) exposure to isoproterenol decreased leptin release in human adipocytes and antagonised insulin-stimulated leptin release in rat adipocytes (Ricci *et al.*, 2005) and chronic treatment of human adipose tissue (up to 2 days in culture) with insulin increased relative rates of leptin biosynthesis without affecting leptin mRNA levels (Lee *et al.*, 2007). There is strong evidence in favour of direct effects of insulin on leptin expression (Cusin *et al.*, 1995) in rats. Furthermore, Ramsay and White (2000) showed that insulin can stimulate leptin expression after 72 to 96 h of incubation (but not after 1 to 24 h of incubation) in pig adipocytes. Moreover, insulin stimulates leptin secretion from human and rodent adipocytes at concentrations as low as 0.16 nM (Mueller *et al.*, 1998). The present study replicated these results with a concentration of 10 nM insulin. Difference in sensitivity may be the consequence of species variation due to the relative insulin resistance of the pig adipocyte compared with human or rodent adipocyte (Mersmann, 1989). Our results in pig adipocytes clearly indicate that isoproterenol, and especially isoproterenol + insulin, increase leptin release.

Leptin receptor mRNA has been detected in a variety of porcine tissues, including adipose tissue (Lin *et al.*, 2000), suggesting that leptin can interact with the adipocyte to alter metabolic activity. For example, leptin alters glucose and fatty acid metabolism in pig adipocytes (Ramsay, 2004). It is therefore possible that some of the effects of CLA and betaine on lipolysis may be mediated by leptin.

In contrast, with *in vivo* growth and body composition parameters (Fernández-Fígares *et al.*, 2008), no synergistic effect was found between betaine and CLA in the present study regarding lipolysis and leptin release. In conclusion, betaine and CLA had a biphasic response regarding lipolysis so that glycerol release was increased in acute conditions while CLA decreased and betaine had no effect in chronic conditions.

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Declaration of interest

No conflict of interest exists.

Ethics statement

The study protocol was approved by the Bioethical Committee of the Spanish National Research Council (CSIC), Spain.

Software and data repository resources

None of the data were deposited in an official repository.

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(-)-Epigallocatechin-3-gallate and hydroxytyrosol improved antioxidative and anti-inflammatory responses in bovine mammary epithelial cells

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*(-)-Epigallocatechin-3-gallate (**EGCG**), the major phenolic compound of green tea, and hydroxytyrosol (**HTyr**), a phenol found in olive oil, have received attention due to their wide-ranging health benefits. To date, there are no studies that report their effect in bovine mammary gland. Therefore, the aim of this study was to evaluate the anti-oxidative and anti-inflammatory effects of EGCG and HTyr in bovine mammary epithelial cell line (**BME-UV1**) and to compare their antioxidant and anti-inflammatory* in vitro *efficacy. Sample of EGCG was obtained from a commercially available green tea extract while pure HTyr was synthetized in our laboratories. The mammary oxidative stress and inflammatory responses were assessed by measuring the oxidative stress biomarkers and the gene expression of inflammatory cytokines. To evaluate the cellular antioxidant response, glutathione (**GSH**/**GSSH**), γ-glutamylcysteine ligase activity, reactive oxygen species and malondialdehyde (**MDA**) production were measured after 48-h incubation of 50 μM EGCG or 50 μM of HTyr. Reactive oxygen species production after 3 h of hydrogen peroxide (50 μM **H_2O_2**) or lipopolysaccharide (20 μM **LPS**) exposure was quantified to evaluate and to compare the potential protection of EGCG and HTyr against H_2O_2-induced oxidative stress and LPS-induced inflammation. The anti-inflammatory activity of EGCG and HTyr was investigated by the evaluation of pro and anti-inflammatory interleukins (tumor necrosis factor (**TNF**)-α, interleukin (**IL)-1β**, **IL-6** and **IL-10**) messenger RNA abundance after treatment of cells for 3 h with 20 μM of LPS. Data were analyzed by one-way ANOVA. (-)-Epigallocatechin-3-gallate or HTyr treatments induced higher concentrations of intracellular GSH compared to control cells, matched by an increase of γ-glutamylcysteine ligase activity mainly in cells treated with HTyr. Interestingly, EGCG and HTyr prevented oxidative lipid damage in the BME-UV1 cells by a reduction of intracellular MDA levels. (-)-Epigallocatechin-3-gallate and HTyr were able to enhance cell resistance against H_2O_2-induced oxidative stress. It was found that EGCG and HTyr elicited a reduction of the three inflammatory cytokines TNF-α, IL-1β, IL-6 and an increase of the anti-inflammatory cytokine IL-10. Hydroxytyrosol has proved to be a strong antioxidant compound, and EGCG has shown mainly an anti-inflammatory profile. These results indicated that EGCG and HTyr may provide dual protection because they were able to attenuate oxidative stress and inflammatory responses, suggesting that these phenolic compounds are potential natural alternatives to be used in dairy cattle as feed supplement for reducing the development of oxidative and inflammatory processes related to parturition or as topical treatments for the control of bovine intramammary inflammation.*

Keywords: phenolic compounds, mammary gland, oxidative stress, inflammation, cell protection

Implications

Treating bovine mammary epithelial cell line (**BME-UV1**) with (-)-epigallocatechin-3-gallate (**EGCG**) or hydroxytyrosol (**HTyr**) exerted anti-oxidative and anti-inflammatory effects in bovine mammary epithelial cells. The results of this study emphasized that these polyphenols provide dual protection in bovine cells. In particular, HTyr was able to reduce oxidative stress by inhibiting reactive oxygen species production, whereas EGCG was able to attenuate inflammatory responses by decreasing the expression of pro-inflammatory genes, suggesting that these compounds might be a potential natural alternative to be used in dairy cattle as a

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supplement for reducing the development of oxidative and inflammatory processes related to parturition or as topical treatments for the control of bovine mammary gland inflammation.

Introduction

Polyphenols are a group of secondary metabolites widely found in different plant organs such as fruits and leaves and their related processing products. Many studies have demonstrated that daily consumption of polyphenols-rich beverages and food such as green tea and olive oil is associated with a low incidence of several diseases including cancer, diabetes, obesity, leukemia, Parkinson's and cardiovascular diseases (Visioli *et al.*, 2002; Mota *et al.*, 2015). The beneficial health properties of green tea and olive oil consumption are related mainly to EGCG and HTyr, respectively, two phenolic compounds characterized by a wide range of biological activities (Visioli *et al.*, 2002; Mota *et al.*, 2015). (-)-Epigallocatechin-3-gallate is the most abundant catechin phenolic compound found in *Camellia sinensis* L. leaves; and it is chemically characterized by the presence of two pyrogallol moieties (Figure S1). (-)-Epigallocatechin-3-gallate is responsible for much of the health-promoting properties of green tea including the anti-oxidative, anti-inflammatory, anti-obesity, anti-diabetic and cardio-protective effects (Chen *et al.*, 2015; Mota *et al.*, 2015). Recently, green tea extracts rich in EGCG have gained great attention as supplements and pharmaceutics to prevent serious diseases including cancer and neurodegenerative diseases (Tomas-Barberan and Andres-Lacueva, 2012). Hydroxytyrosol is a low-molecular weight phenol characterized by a catechol moiety (Figure S1). It is found in olive oil products, for example, extra-virgin olive oil and table olives, which are important components of the Mediterranean diet (Bernini *et al.*, 2015). It originates mainly from the enzymatic hydrolysis of oleuropein during the maturation of the olives, processing and storage of olive oil (Gambacorta *et al.*, 2007). During olive oil processing, a considerable amount of HTyr converges in olive oil by-products such as olive oil wastewaters, which represents a valuable source of HTyr (Bernini *et al.*, 2015). Studies have demonstrated that HTyr exhibits antimicrobial, antitumoral, antioxidant and anti-inflammatory activities together to beneficial effects on the cardiovascular system (Bernini *et al.*, 2013; Hu *et al.*, 2014).

Oxidative stress plays a key role in the onset or progression of numerous human and animal diseases. High-yielding dairy cows undergo this deleterious process mainly during the peripartum period (Bernabucci *et al.*, 2005; Castillo *et al.*, 2006). For dairy cows, the transition period is characterized by important physiological changes that affect the health and production performance of the animals. Among tissues and organs, mammary gland, liver, muscles and adipose tissue are the most involved and more susceptible to oxidative stress (Abuelo *et al.*, 2015). Substantial evidence confirms that increased oxidative stress and inflammatory response during the pre-partum and early lactation period may contribute to several metabolic and infectious diseases (retained placenta, ruminal acidosis, laminitis, ketosis, fatty liver, metritis, etc.) in dairy cattle (Contreras and Sordillo, 2011; Abuelo *et al.*, 2015), in particular, oxidative stress contribute to mastitis pathogenesis during the transition period of dairy cows (Aitken *et al.*, 2009). Recently, scientific attention has been oriented toward the use of naturally occurring products. Feed supplementation of antioxidants and anti-inflammatory compounds could potentially improve the health status and performance of animals. However, the therapies used have not achieved this consistently.

Despite the great number of studies describing the antioxidant and anti-inflammatory activities of both EGCG and HTyr in humans (Bernini *et al.*, 2013) and monogastric animals (Mota *et al.*, 2015; Hu *et al.*, 2014), there are no studies, to the best of our knowledge, in the published literature that investigate EGCG and HTyr effects in ruminants. The finding of natural products to be used in dairy cattle, particularly during the peripartum period, as a supplement or as topical treatments could be useful in helping to reduce risk for diseases. On the basis of this lack of literature, the aim of this study was to evaluate both the anti-oxidative and anti-inflammatory effects of EGCG and HTyr in bovine mammary epithelial cell line and to compare their antioxidant and anti-inflammatory *in vitro* efficacy.

Material and Methods

Chemicals

Reagents and solvents of high purity were supplied by Sigma-Aldrich (Milan, Italy). Green tea leaves extract (Teavigo®) was manufactured by Taiyo Green Power under licence from Taiyo Kagaku Co., Ltd., and Swiss-based DSM Nutritional Products. Silica gel (200-300 mesh) and silica gel F254 plates were purchased from Merck (Milan, Italy). Hydroxytyrosol was synthesized according to a patented procedure optimized in our laboratories and purified by flash column chromatography (Bernini *et al.*, 2008).

Chemical Characterization of Teavigo® and Hydroxytyrosol

A pure sample of EGCG was found in a green tea extract commercially available (Teavigo®) while fresh HTyr was synthetized in our laboratories by an ecofriendly and efficient procedure based on the selective oxidation of tyrosol with 2-iodobenzoic acid, followed by a reduction with sodium dithionite (Figure S2; Bernini *et al.*, 2008). Finally, HTyr was purified by flash column chromatography on silica gel. Commercial tea extract Teavigo® and synthetic HTyr were characterized by nuclear magnetic resonance spectra (^{1}H-NMR and ^{13}C-NMR**)** using a spectrometer Avance III 400 MHz Bruker (Germany) dissolving 10 mg of each sample in 0.5 ml of dimethylsulfoxide-d_6 and acetone-d_6, respectively. Chemical shifts were expressed in parts per million (δ scale) and coupling constants in Hertz. Nuclear magnetic resonance spectra (Figure S3) evidenced the high purity both

of EGCG found in Teavigo® (Imperatori *et al.*, 2018) and synthetic HTyr (Bernini *et al.*, 2008).

Cell culture

For this present study, a clonal bovine mammary epithelial cell (BME-UV1, RRID:CVCL_W716), obtained from a pregnant, lactating cow's mammary gland and established by stable transfection with a plasmid encoding the thermolabile large T-antigen (Zavizion *et al.*, 1996), has been chosen as a valid model to study the bovine mammary epithelial metabolism. The BME-UV1 cells respond to epidermal growth factor and the insulin-like growth factor I, which are associated to growth and development of the mammary gland and show a morphology typical of luminal epithelial cells, in a single layer and of polygonal shape (Arévalo Turrubiarte *et al.*, 2016). On the basis of their similarity with bovine lactating mammary epithelial cells, the BME-UV1 have been widely used as *in vitro* models to investigate milk production in dairy cows (Arévalo Turrubiarte *et al.*, 2016).

Bovine mammary epithelial cell lines used in this study were kindly provided by Professor Antonella Baldi (Department of Health, Animal Science and Food Safety, University of Milan, Italy). The cells were incubated in a humidified air with 5% CO_2, at 37°C, until 80% confluence. Cells were cultured in 75 cm^2 tissue culture flasks (Costar, Corning, NY, USA), in a culture medium of DMEM-F12, RPMI-1640 and NCTC-135 (5:3:2 by volume), complemented with 10% fetal bovine serum, 0.1% lactose, 0.1% lactalbumin hydrolysate, 1.2 mM glutathione, 1 μg/ml insulin, 5 μg/ml transferrin, 1 μg/ml hydrocortisone, 0.5 μg/ml progesterone, 10 μg/ml L-ascorbic acid and antibiotics (penicillin 100 IU/ml; streptomycin 100 μg/ml). Culture medium and supplements were purchased from Sigma-Aldrich (Milano, Italy). For this study, BME-UV1 cells at passage number between 39 and 41 were used (Dipasquale *et al.*, 2018). The dissociation of the cell monolayer and subcultures was carried out every 2 or 3 days.

Experimental design

In order to test the antioxidant activities of EGCG or HTyr on bovine mammary gland, BME-UV1 cells were re-suspended in complete culture medium to a concentration of $5x10^5$ cells/ml and dispensed in culture flasks and 96-wells tissue culture plates. After 24 h, the medium was removed and replaced with routine culture medium and 50 μM EGCG or HTyr for 48 h. As a control to each, experiment cells not exposed to EGCG or HTyr were used.

(-)-Epigallocatechin-3-gallate and HTyr were first dissolved into aqueous buffer (culture medium) and from the stock solution followed other dilutions in complete culture medium prior to perform biological experiments. The concentration of EGCG and HTyr used in the experiments was established after screening tests and on the base of observations on BME-UV1, which showed little cytotoxicity toward EGCG or HTyr using concentrations higher than 50 μM after 24-h treatment (data not shown) and of observations on other cells (Peng *et al.*, 2015; Karamese *et al.*, 2016). Among the tested concentrations, 50 μM for both EGCG and HTyr has been chosen being the upper limit dose that did not cause cytotoxicity (data not shown). Cell viability after 48 h from addition of EGCG or HTyr was determined. Also, the content of reduced glutathione (**GSH**) and oxidized form of glutathione (**GSSH**) γ-glutamylcysteine ligase activity (**γGCL**) and reactive oxygen species (**ROS**) and malondialdehyde (**MDA**) concentration were determined. Reduced glutathione and oxidized form of glutathione ratio was calculated.

To test the potential protection of EGCG or HTyr against hydrogen peroxide (H_2O_2)-induced oxidative stress, cells were treated with EGCG or HTyr at non-toxic concentrations (50 μM) for 48 h as described above, followed by incubation at 37°C for 3 h with a single dose application (50 μM) of H_2O_2 and the content of ROS concentration were determined.

To test the preventive efficacy of EGCG or HTyr against induced inflammation and indirect oxidative stress by lipopolysaccharides (**LPS**; from *Escherichia coli* 055:B5, Sigma-Aldrich), cells were pretreated with compounds of interest as described above followed by incubation at 37°C for 3 h with a single dose application (20 μM) of LPS, to match acute inflammation. Gene expression of pro- and anti-inflammatory cytokines was determined. Reactive oxygen species were also determined in cells. The concentration 50 μM of H_2O_2 and 20 μM of LPS were selected on a previous preliminary cytotoxicity study and on results from other published studies (Basiricò *et al.*, 2017; Dipasquale *et al.*, 2018; Mastrogiovanni *et al.*, 2018).

For each experiment, at least three replicates were performed and were repeated at least twice.

Cell viability

For cell viability assay, cells were seeded into 96-wells microplates at an optimal density ($5x10^5$ cells/ml) and were grown with EGCG or HTyr for 48 h. Cell viability was assayed using the Cell Proliferation Kit II (XTT test: sodium 30-[1-(phenylaminocarbonyl) 4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate; Roche Applied Science, Indianapolis, IL, USA) according to the manufacturer's instructions. Briefly, for the XTT test, to each well, after 48 h of EGCG or HTyr exposure, was added 50 μl of a mixture of two reagents, XTT labeling reagent and electron-coupling reagent (50:1). Cells are then placed back into the incubator for 24 h at 37°C. At the end of the incubation period, the absorbance was measured on a plate reader (Tecan Sunrise™) at 450 nm. Results were expressed as optical density.

Thiol redox status

For assessing the thiol redox status of BME-UV1 cells after EGCG or HTyr treatment, the GSH, the GSSH and the activity of γ-glutamate cysteine ligase were determined (Basiricò *et al.*, 2015). Briefly, for the determination of GSH and GSSG, adherent cells were detached using trypsin/EDTA solution and centrifuged at 4 500 × g at 4°C for 5 min. The pellet was re-suspended in 200 μl of PBS and lysed by two cycles of sonication (100 W for 30 s), the samples were centrifuged

(15 000 × g for 5 min at 4°C) and stored at -80°C until analysis. In cell extracts, GSH/GSSH ratio was determined by colorimetric assays (BioAssay Systems, Hayward, CA, USA). Optical density was measured by a spectrophotometer (Tecan Sunrise™) at 405 for GSH/GSSG.

The γGCL activity was determined by a fluorescence assay as described by Chen *et al.* (2015). Briefly, BME-UV1 cells were detached and centrifuged as described above. The cells were re-suspended in TES/SB buffer (wt/vol, 1/4), and were sonicated (100 W for 60 s), centrifuged (15 000 × g for 5 min at 4°C) and supernatants were collected. The supernatants were centrifuged again at 15 000 × g at 4°C for 20 min and the protein concentrations were determined using a BCA Protein Assay Kit from Pierce (Rockford, IL, USA), with bovine serum albumin as standard. For the γGCL activity assay, aliquot of supernatants (30 µl) were mixed with 30 µl of γGCL reaction cocktail and incubated at 37°C for 5 min. Cysteine solution was then added, and the mixtures were incubated at 37°C for 13 min and stopped. After placing on ice for 20 min, the mixtures were centrifuged at 2000 × g at 4°C for 10 min. Aliquot of each supernatant containing γ-glutamylcysteine (***γ*GC**) was added to a 96-well plate designed for fluorescence detection. For each assay, 20 µl of γGC standards was added to generate a standard curve. Next, 180 µl of 2,3-naphthalenedicarboxyaldehyde (**NDA**) was added to each well. Following incubation, the formation of NDA-γGC was measured (472 nm excitation/528 nm emission) using a fluorescent plate reader (Multimode Detector DTX 880, Beckman Coulter Inc., Fullerton, CA). The production of γGC in each sample was calculated using the standard curve. Values were expressed in µM/min/µg of total proteins.

Measurement of reactive oxygen species production

After treatments, cells were washed twice with phosphate buffer saline (Lonza, Swiss) and incubated with 20 µM 2′,7′-dichlorodihydrofluorescin diacetate probe (D6883 Sigma-Aldrich) in PBS at 37°C for 40 min as reported by Basiricò *et al.* (2015). Fluorescence was measured at 485 nm (excitation) and 535 nm (emission) wavelengths on a microplate reader (Multimode Detector DTX 880, Beckman Coulter Inc., USA).

Measurement of malondialdehyde production

For the determination of MDA, lipid peroxidation indicator, after treatments adherent cells were detached using trypsin/EDTA solution and centrifuged at 4500 × g for 5 min at 4°C. The pellet was re-suspended in 200 µl of PBS and lysed by two cycles of sonication (100 W/ 30 s), centrifugation at 15 000 × g for 5 min at 4°C, and samples stored at -80°C until analysis. In cell extracts, MDA concentrations were determined by colorimetric assays (Abcam, Cambridge, UK), CA). Optical density was measured by a spectrophotometer at 540 nm (Basiricò *et al.*, 2015).

RNA isolation and real-time PCR

The gene expression of bovine pro (tumor necrosis factor (**TNF**)-α, interleukin (IL)-1β and IL-6) and anti-inflammatory IL-10, cytokines that may play important roles in the mammary inflammatory response, were assayed in BME-UV1 cultured under the conditions described above and were carried out by real-time-PCR. All primers and probes sequences used, were previously reported by Dipasquale *et al.* (2018).

In order to isolate total RNA, BME-UV1 cells were seeded in cell culture flasks at the concentration of $5x10^5$ cells/ml in complete medium (Thermo Fisher Scientific, Waltham, USA) and treated as described above. Total RNA was isolated by QIAzol Lysis Reagent (79306 Qiagen, Hilden, Germany), according to the manufacturer's instructions and stored at –80°C. RNA was quantified using Quant-iT RNA assay Kit (Invitrogen, Carlsbad, CA, USA) and fluorescence was measured at excitation/emission of 644/673 nm. One microgram of total RNA was reverse transcribed using a Quantitect reverse transcription kit (Qiagen, Hilden, Germany) in a total volume of 20 µl on a PCR Express thermal cycler (Hybaid, Ashford, UK). Quantitative probes real-time PCRs were performed following the manufacturer's recommendations using LightCycler® 2.0 (Roche, Roche Applied Science, Indianapolis, IL, USA). To account for possible variation related to complementary DNA input or the presence of PCR inhibitors, the endogenous reference gene glyceraldehyde 3-phosphate dehydrogenase was simultaneously quantified for each sample, and data were normalized accordingly. In each PCR run, the cDNA samples were amplified in triplicate. Table 1 shows the specific characteristics of primers used for the real-time PCR. The relative quantification of the mRNAs was performed using the ΔΔCT method.

Statistical analysis

All data of the experiment are presented as least-squares means and SEM. The data were analyzed with ANOVA, using a general linear model (GLM procedure of Statistica-7 Software package, Stat Soft, Inc., USA). The model included the fixed effect of treatment (control, EGCG and HTyr), replicates as random effect and the error term. The significance of the differences was assessed by the Fisher's Least significant difference (LSD) test and significance was declared at $P < 0.05$.

Results

Effect of epigallocatechin-3-gallate and hydroxytyrosol on cell viability

As shown in Figure S4, cells were still 100% viable at the concentration used (50 µM) at the end of the incubation period as determined by the XTT assay and no differences were observed between treatments. This assay indicated that the exposure to the different compounds did not show any cytotoxic effect.

Effect of epigallocatechin-3-gallate and hydroxytyrosol on thiol redox status

Oxidized and reduced glutathione: Figure 1 shows changes of non-enzymatic antioxidants like GSH. Compared to the control, an increase of reduced GSH was observed in cells treated

Table 1 *Deoxyribonucleic acid sequences of bovine sense and antisense primers and probes used for real-time PCR analysis*

Gene	Primers and probes	Temperature of annealing (°C)
TNF-α F	TCTTCTCAAGCCTCAAGTAACAAGT	60
TNF-α R	CCATGAGGGCATTGGCATAC	
TNF-α P	FAM-AGCCCACGTTGTAGCCGACATCAACTCC-TAMRA	
IL-1β F	TCCACCTCCTCTCACAGGAAA	58
IL-1β R	CTCTCCTTGCACAAAGCTCATG	
IL-1β P	FAM-CACCACTTCTCGGTTCA-MGB	
IL-6 F	GGGCTCCCATGATTGTGGTA	60
IL-6 R	GTGTGCCCAGTGGACAGGTT	
IL-6 P	FAM-TTCCTGGGCATTCCCTCCTCTGGT-TAMRA	
IL-10 F	CTTGTCGGAAATGATCCAGTTTT	60
IL-10 R	TTCACGTGCTCCTTGATGTCA	
IL-10 P	FAM-CCACAGGCTGAGAACCACGGGC-TAMRA	
GAPDH F	GCATCGTGGAGGAGGGACTTATGA	60
GAPDH R	GGGCCATCCACAGTCTTCTG	
GAPDH P	FAM-CACTGTCCACGCCATCACTGCCA-TAMRA	

TNF-α = tumor necrosis factor-α; *IL-1β* = interleukin-1β; *IL-6* = interleukin-6; *IL-10* = interleukin-10; *GAPDH* = glyceraldehyde 3-phosphate dehydrogenase; F = forward; R = reverse; P = probe.

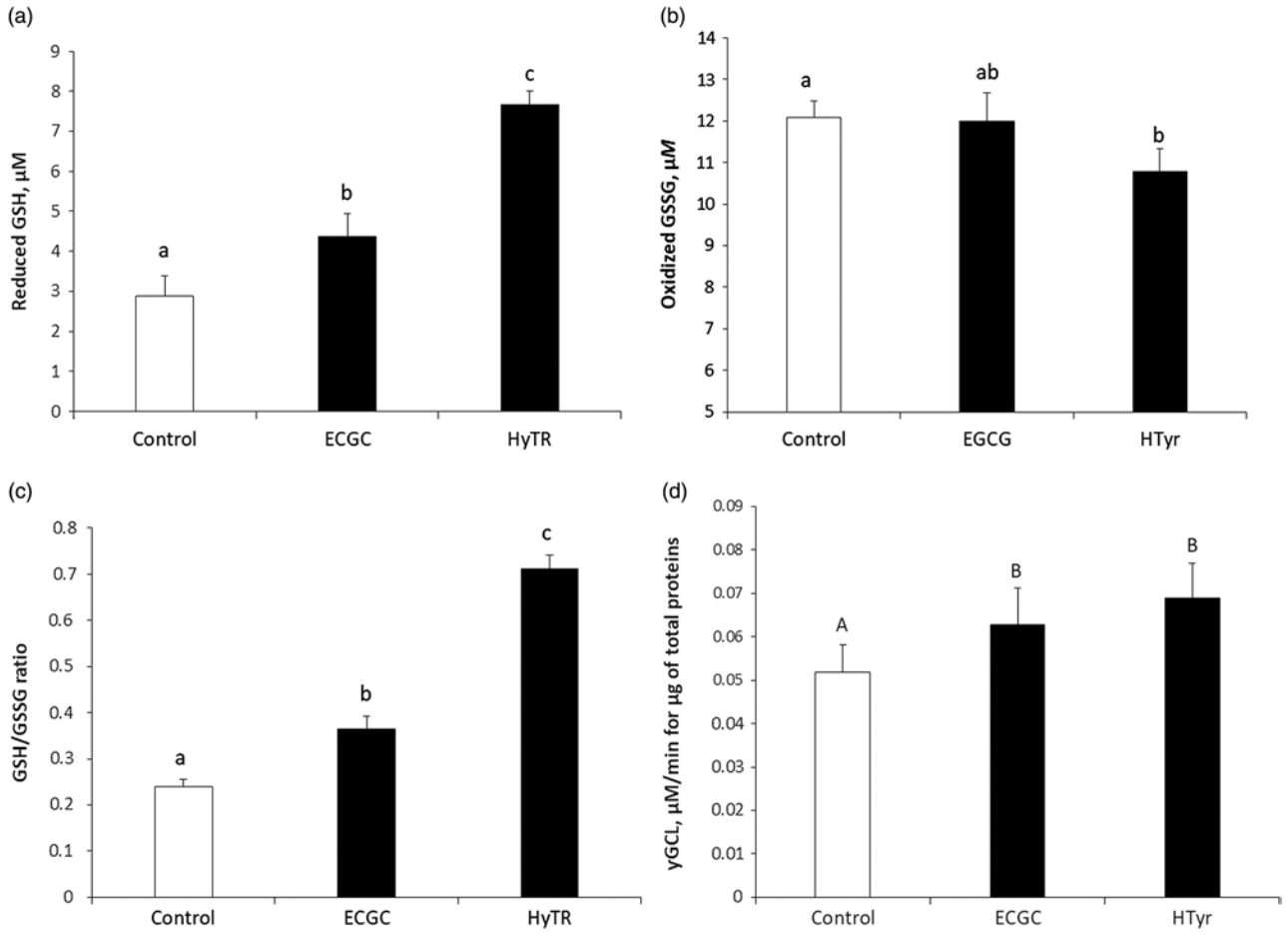

Figure 1 Intracellular concentration of reduced glutathione (GSH; a), oxidized glutathione (GSSG; b), and GSH-to-GSSG ratio (c) and γ-glutamate cysteine ligase (d) activity in bovine mammary epithelial cells after 48 h of exposure to 50 µM (-)-epigallocatechin-3-gallate (EGCG) or hydroxytyrosol (HTyr). The transparent bar represents the control (cells not treated) and the black bars represent cells treated with EGCG or HTyr. Data were analyzed by one-way ANOVA test and are reported as least squares means ± SEM ($n = 6$). Significant differences between treatments are represented by different letters (a, b = $P < 0.05$; A, B = $P < 0.01$). Fisher's LSD test has been used to evaluate differences between treatments.

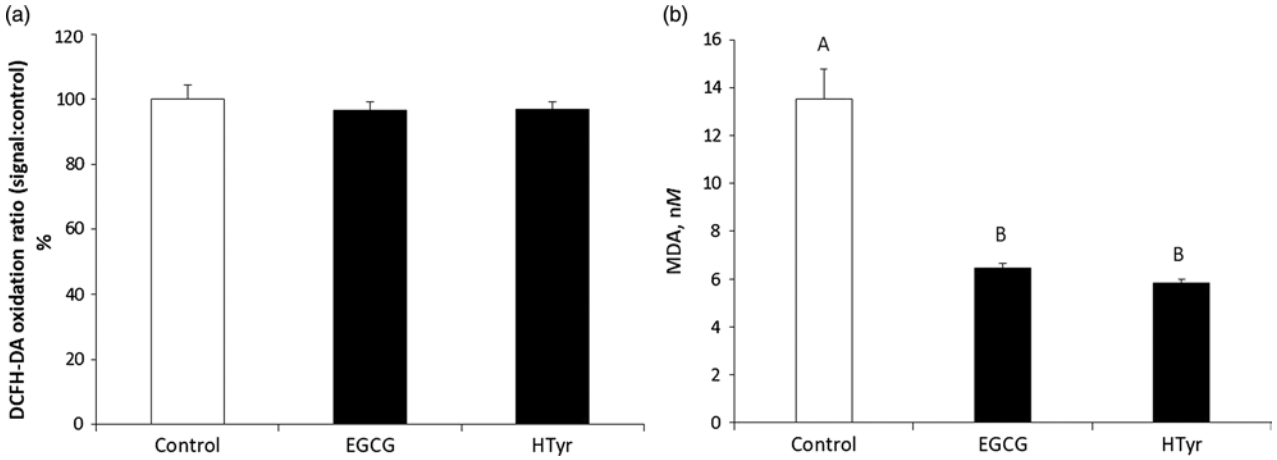

Figure 2 Intracellular production of reactive oxygen species by 2′,7′-dichlorodihydrofluorescin diacetate probe (DCFH-DA) (a) and malondialdehyde (MDA; b) in bovine mammary epithelial cells after 48 h of 50 μM (-)-epigallocatechin-3-gallate (EGCG) or hydroxytyrosol (HTyr) exposure. The transparent bar represents the control (cells not treated) and the black bars represent cells treated with EGCG or HTyr. Data were analyzed by one-way ANOVA test and are reported as least squares means ± SEM ($n = 6$). Significant differences between treatments are represented by different letters (A, B = $P < 0.01$). Fisher's LSD test has been used to evaluate differences between treatments.

with 50 μM EGCG ($P < 0.05$) or with HTyr ($P < 0.01$) (Figure 1a). Among treatments, values of reduced GSH were significantly higher ($P < 0.05$) in cells treated with HTyr compared to EGCG. Oxidized GSH level of cells treated with EGCG was not significantly different from that of control cultures, but it was higher than that observed in HTyr-treated cells (Figure 1b). The ratio GSH/GSSG showed the same trend of reduced GSH; in particular, HTyr showed an increase ($P < 0.05$) of GSH/GSSG ratio compared with EGCG (Figure 1c).

γ-Glutamyl-cysteine ligase activity: After the 48 h, EGCG or HTyr treatments induced a strong antioxidant response by increasing ($P < 0.01$) the intracellular γGCL activity compared with the control (Figure 1d). However, no differences were observed between the two treatments (Figure 1d).

Intracellular reactive oxygen species and malondialdehyde production

Intracellular ROS production as established by dichlorofluorescein fluorescence flow cytometry analysis was measured in cells supplemented with EGCG or with HTyr (Figure 2a). No differences of ROS production were observed between the treatments and the control.

Cell concentrations of MDA, lipid peroxidation indicator, are shown in Figure 2b. The treatment of cells with EGCG or with HTyr reduced ($P < 0.01$) the MDA level compared with untreated cells. No differences of MDA concentrations were observed between the two treatments.

Intracellular reactive oxygen species production after hydrogen peroxide and lipopolysaccharide challenge

The potential of EGCG and HTyr to protect against H_2O_2-induced oxidative stress and indirect oxidative stress promoted by an inflammatory response elicited by LPS stimulation, was assessed by ROS test after exposure for 3 h to H_2O_2 or LPS. As shown in Figure 3a, cells supplemented with EGCG or HTyr and stimulated with H_2O_2 showed a decreased ROS production ($P < 0.01$) compared to the control. After pre-treatment with EGCG or HTyr and stimulation with LPS, BME-UV1 showed a ROS production similar to the control (Figure 3b). (-)-Epigallocatechin-3-gallate and HTyr were able to enhance cell resistance against induced H_2O_2 oxidative stress at 48 h.

Quantification of messenger RNA expression of pro- and anti-inflammatory cytokines

In Figure 4 mRNA expression of TNF-α, IL-1β, IL-6 and IL-10 (A, B, C and 336 D, respectively) are shown. Pro-inflammatory cytokines mRNA was lower ($P < 0.01$) in cells treated with EGCG or HTyr compared with the control. No differences were observed between treatments (Figure 4a, b and c). On the other hand, gene expression of the anti-inflammatory cytokine (Figure 4d) was markedly higher in cells treated with EGCG or HTyr compared to the control. Overall, IL-10 gene expression showed greater levels ($P < 0.01$) in cells treated with EGCG compared with HTyr.

Discussion

Solid scientific evidences clearly demonstrate the healthy bioactivity properties of polyphenols (Tomas-Barberan and Andres-Lacueva, 2012). Thus, attention in phenolic compounds has increased greatly and the main interest is focused on finding naturally occurring antioxidants and anti-inflammatory compounds for nutraceuticals uses to reduce or, if possible, replace current pharmacological treatments.

(-)-Epigallocatechin-3-gallate and HTyr are two of the major simple phenols present in green tea leaves and in olive oils, respectively. Many studies in animals and humans

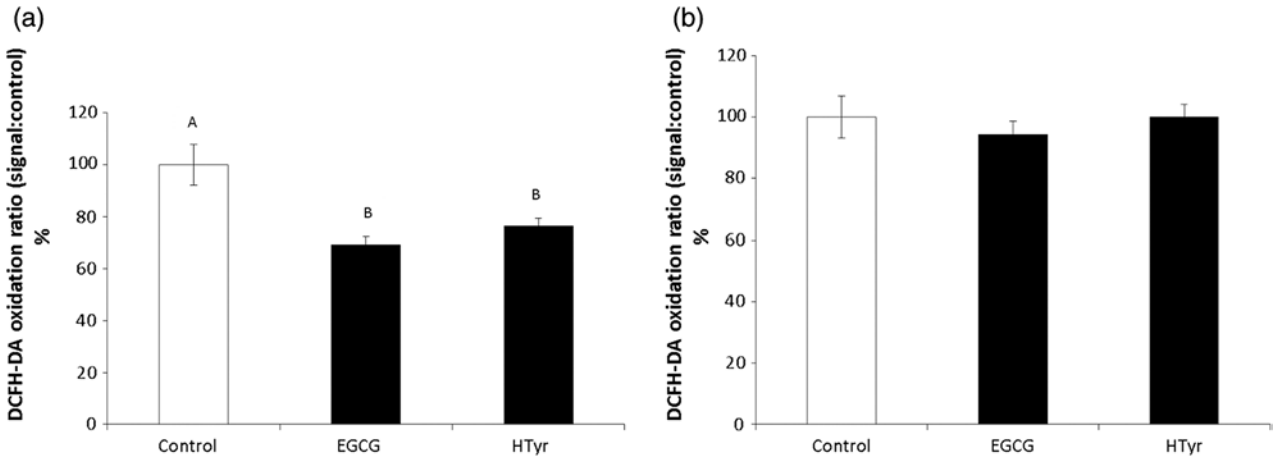

Figure 3 Intracellular production of reactive oxygen species by 2′,7′-dichlorodihydrofluorescin diacetate probe (DCFH-DA) in bovine mammary epithelial cells after 48 h of 50 μM (-)-epigallocatechin-3-gallate (EGCG) or hydroxytyrosol (HTyr) supplementation and 3 h of 50 μM H_2O_2 (a) and lipopolysaccharide (20 μM) treatment (b). The transparent bar represents the control (cells not treated) and the black bars represent cells treated with EGCG or HTyr. Data were analyzed by one-way ANOVA test and are reported as least squares means ± SEM ($n = 6$). Significant differences between treatments are represented by different letters (A, B = $P < 0.01$). Fisher's LSD test has been used to evaluate differences between treatments.

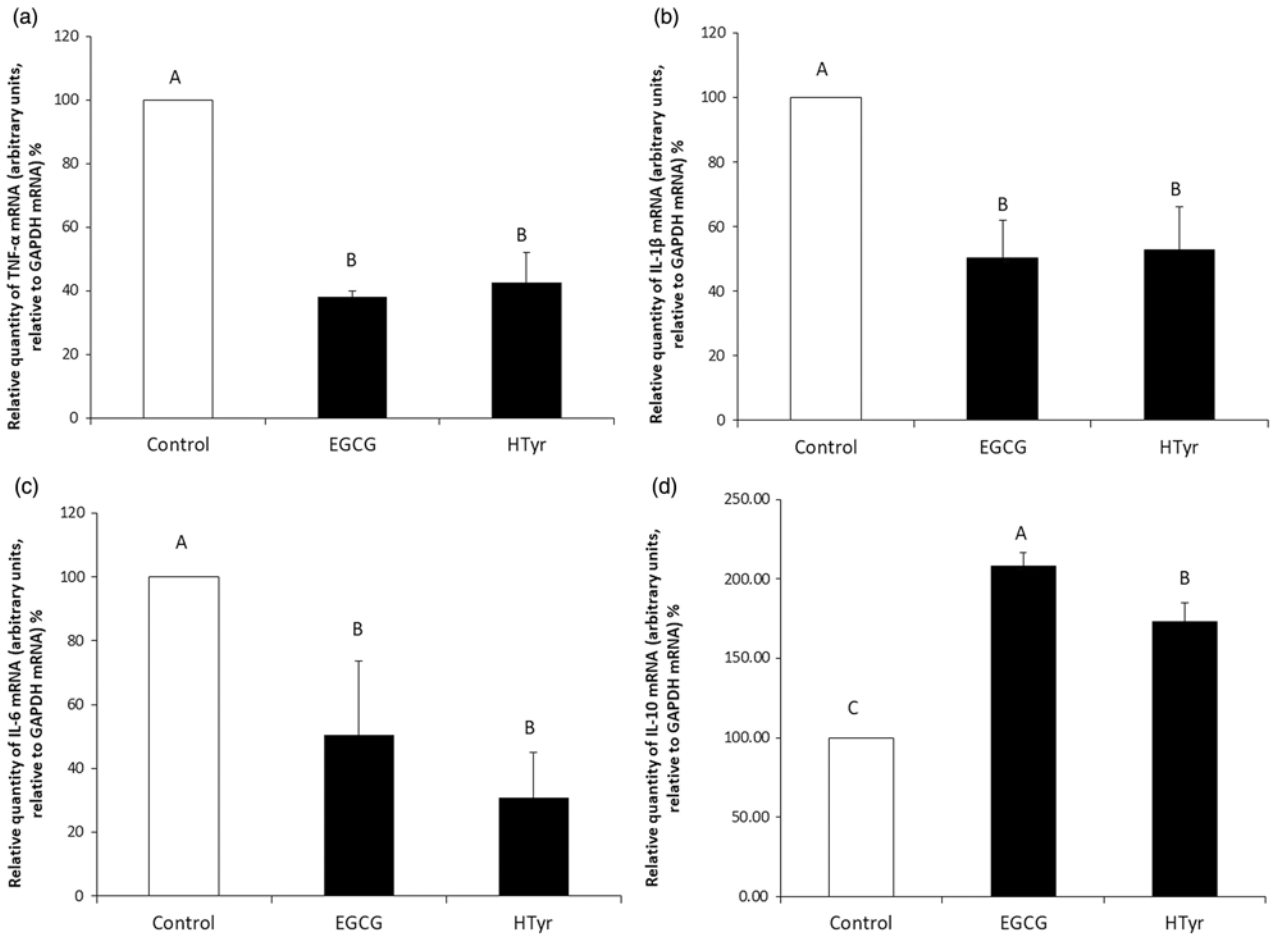

Figure 4 Messenger RNA abundance of tumor necrosis factor-α (TNF-α; a), interleukin-1 (IL-1β; (b), IL-6 (c) and IL-10 (d) in bovine mammary epithelial cells after 48 h of 50 μM (-)-epigallocatechin-3-gallate (EGCG) or hydroxytyrosol (HTyr) supplementation, and after 3 h of lipopolysaccharide treatment (20 μM). The transparent bar represents the control (cells not treated) and the black bars represent cells treated with EGCG or HTyr. Data were analyzed by one-way ANOVA test and are reported as least squares means ± SEM ($n = 6$). Significant differences between treatments are represented by different letters (A, C = $P < 0.01$). Fisher's LSD test has been used to evaluate differences between treatments. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

demonstrated that the bioavailability of phenols after ingestion is dose-dependent (Visioli *et al.*, 2000; Vilaplana-Pérez *et al.*, 2014) and that when circulating in plasma they exert systemically their biological effects (Bernini *et al.*, 2013; Kim *et al.*, 2014).

In dairy cows, Wein *et al.* (2016) showed that intraduodenal administration (10, 20 and 30 mg/kg BW) of green tea extract resulted in increased plasma concentrations of epicatechin, epigallocatechin, epigallocatechin gallate in a dose-dependent manner. In contrast, after intraruminal application, almost none of the catechins contained in the green tea extract were detected in plasma samples.

The goal of this study was to evaluate both the antioxidant and anti-inflammatory effects of EGCG or HTyr in BME-UV1 using chemical and cellular assays, and to compare their different efficacies in mammary tissue.

As reported by Vilaplana-Pérez *et al.* (2014), there is no scientific evidence relating to the physiological concentrations of HTyr after olive oil ingestion by humans, but some authors have suggested that it could be between 10 and 100 μM (Warleta *et al.*, 2011). In this experiment, the concentration of EGCG and HTyr tested was in the range of their possible physiological plasma concentrations. In our experimental conditions, treating BME-UV1 with 50 μM of EGCG or HTyr did not affect cell viability, in relation to the exposure times. These results agree with those achieved in human mammary epithelial cells MCF10A, breast cancer cells MDA-MB-231 and MCF7 (Warleta *et al.*, 2011) and PC12 rat adrenal pheochromocytoma cell line (Peng *et al.*, 2015) in which a 1 to 100 μM treatment of HTyr did not alter the cell cycle or induce apoptosis.

In this study, the individual exposure to 50 μM of each of the two polyphenolic compounds for 48 h strengthened the cellular defences against oxidative damage compared with control cells. Our results showed that cell supplementation with EGCG or HTyr increased the concentration of reduced GSH and the activity of γGCL while decreasing the MDA level. In particular, GSH levels were upregulated differently by HTyr, both with an induction of γGCL and with a reduction of GSSG production as demonstrated by the GSH/GSSH ratio. These results pointed out a differential antioxidant activity of the two compounds in BME-UV1 cells and showed that HTyr acts as a more efficient free radical scavenger than EGCG. Indeed Kim *et al.* (2014) reported that individual polyphenols have distinct specific molecular targets in various tissues with different efficacies and bioavailability. Interestingly, to date, for the first time the cytoprotective effects of EGCG and HTyr in bovine mammary gland were observed, and this is in agreement with literature data showing the antioxidant potential property of polyphenols compound in other species/experimental models (Bernini *et al.*, 2013; Kim *et al.*, 2014; Vilaplana-Perez *et al.*, 2014; Karamerse *et al.*, 2016). Similar results were obtained also by Warleta *et al.* (2011) in an *in vitro* study on human breast cells. Moreover, HTyr protects retinal pigment epithelial cells against this acrolein-induced oxidative stress by the induction of phase II detoxifying enzymes as the γ-glutamyl cysteine ligase (γ GCL) (Liu *et al.*, 2007). Phase II enzymes perform a variety of vital cellular functions important for protecting against oxidative damage. Above all, γ-GCL controls the production of GSH, the major endogenous antioxidant thiol, capable of neutralizing reactive oxygen and nitrogen species that are constant dangers to the integrity of mammalian DNA and lipids (Ramos-Gomez *et al.*, 2001). Chen *et al.* (2015) in an *in vivo* study reported that in mammary gland of rats, the treatment of EGCG (100 or 25 mg kg^{-1} day^{-1}) decreased MDA concentration compared to the untreated model group. Studies in ARPE-19 human retinal pigment epithelial cells have shown that HTyr protects cells from oxidative damage induced by acrolein and endogenous end-product of lipid oxidation in age-related macular lesions (Zhu *et al.*, 2010). Tuzcu *et al.* (2008) reported that EGCG is a powerful antioxidant against lipid peroxidation and observed in thermo-neutral and heat stressed birds (Japanese quail) that MDA concentrations in serum and liver decreased in both groups as dietary EGCG supplementation increased. Therefore, the increased level of reduced GSH, the induction of γGCL and reduced MDA concentration indicate that EGCG or HTyr supplementation improved the antioxidant status of bovine mammary cells.

Measurement of basal and H_2O_2-induced ROS production was used as an additional test to evaluate and compare the potential protection of two different phenolic compounds against oxidative stress, which indeed were able to reduce intracellular ROS concentration in bovine mammary epithelial cells after H_2O_2-induced ROS production. Our findings pointed out a protective effect of HTyr and EGCG against H_2O_2-induced ROS production. These results agree with those achieved by HTyr in human mammary epithelial (MCF10A) cells (Warleta *et al.*, 2011). Moreover, Peng *et al.* (2015) reported that HTyr efficiently scavenges free radicals *in vitro* and displays cytoprotection against oxidative stress-induced damage in PC12 cells. The ROS concentration after EGCG or HTyr treatment on LPS-exposed cells did not differ from the control cells in terms of oxidative stress. This might be explained by LPS incubation time, probably not enough to disturb intracellular redox balance and excessive ROS accumulation.

Moreover, the study examines the role of some pro- and anti-inflammatory markers to verify the effect of EGCG or HTyr on the inflammatory response of BME-UV1 cells. Under our experimental conditions, the exposure to 50 μM of EGCG or HTyr for 48 h induced a cell protection by inhibiting the trigger of the inflammatory process induced by LPS, known as a potent endotoxin able to induce inflammatory responses and to promote the synthesis and secretion of a variety of inflammatory cytokines (Li *et al.*, 2013; Zhang *et al.*, 2014). Among these, TNF-α, IL-1β and IL-6 are known to be important inflammatory mediators involved in the initiation and development of acute mammary inflammation (Fu *et al.*, 2014). Additionally, these inflammatory cytokines cause the production of ROS (Chen *et al.*, 2015). Karamese *et al.* (2016) suggest a close relationship between oxidative stress and inflammation. TNF-α and IL-1β are considered

primary cytokines due to their role in initiating and stimulating the downstream cascade reaction of other inflammatory mediators, such as IL-6. Tumor necrosis factor-α has the functions of promoting inflammatory cell infiltration, injuring vascular endothelial cells and stimulating the generation of ROS (Li *et al.*, 2013). Tumor necrosis factor-α is essential to the immune system but when synthesized in excess can be detrimental to the animal's health. Interleukin-1β is one of the most powerful pro-inflammatory cytokines with the activity of stimulating the acute phase response (Arango Duque and Descoteaux, 2014). Furthermore, IL-1β can accelerate the intracellular accumulation of ROS and destroy antioxidant defence mechanisms by suppressing the activities of superoxide dismutase and glutathione peroxidase activities (Harijith *et al.*, 2014). Interleukine-6 stimulates the production of cytotoxic T-cells and affects several biological activities from immunity to tissue repair (Fu *et al.*, 2014).

The results of this study showed that EGCG or HTyr decreased gene expression of the main pro-inflammatory cytokines and increased the anti-inflammatory cytokine IL-10. In particular, a significant increase in gene expression of IL-10 in EGCG-treated cells was observed. Data presented herein are consistent with other findings indicating an improvement of the inflammatory status in cells treated with EGCG or HTyr (Vilaplana-Pérez *et al.*, 2014). Consequently, suggesting a close relationship between oxidative stress and inflammation. Decreases in oxidative stress can reduce the production of inflammatory cytokines and, in turn, a decrease in inflammatory cytokines can reduce the production of free radicals. Most related studies have proven this relationship. For example, Chen *et al.* (2015) reported that EGCG inhibits the LPS-induced inflammatory response and normalizes anti-oxidant enzyme levels in rats' mammary epithelial cells under mastitis condition. Another study claimed that EGCG led to a decrease in both inflammatory cytokine levels and antioxidant enzyme levels in Hep3B human hepatoma cells (Karamese *et al.*, 2016). A similar study (Zhong *et al.*, 2012) showed that some EGCG byproducts suppress the LPS-induced production of nitric oxide and pro-inflammatory cytokines in macrophages.

In conclusion, to the best of our knowledge this is the first time that anti-oxidant and anti-inflammatory effects of the phenolic compounds EGCG and HTyr on BME-UV1 are evaluated. Moreover, the approach of simultaneously testing these compounds in direct comparison using several assays is indeed novel. In bovine mammary cells, the two phenolic compounds showed a different *in vitro* efficacy. Hydroxytyrosol has proved to be a strong ROS production inhibitor and EGCG has shown mainly an anti-inflammatory activity. These results indicated that EGCG and HTyr provide a dual protection against oxidation and inflammation as they were able to attenuate oxidative stress and inflammatory responses, suggesting that these compounds are potential natural alternatives to be used in dairy cattle as a supplement for reducing the incidence of oxidative stress and related diseases in early lactation or as topical treatments for the control of bovine intramammary inflammation such as mastitis. However, further *in vivo* studies, using molecules protected against microbial degradation, are necessary to fully understand the potential role of these phenolic compounds on the antioxidant and anti-inflammatory capabilities and to test potential health-promoting effects of EGCG and HTyr in transition dairy cow.

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Software and data repository resources

Data or models are not deposited in an official repository.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Ethics statement

None. Not applicable since the study is an *in vitro* model.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731119001356.

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animal

Light intensity preferences of broiler chickens: implications for welfare

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There is considerable debate as to the optimal light intensities for growing chickens. This is influencing regulations and industry practices. The present study examines the preference of broiler chickens for light intensity. A choice system was developed to allow determination of the preferences of broiler chickens for light intensity. This system had three light proof pens each with feeders or waterers but different light intensities. There was a connecting transit pen with a light intensity of 1 to 2 lux. This allowed birds access to the pens each with feeders or waterers. There were markedly more chickens observed in the pens each with feeders or waterers and a light intensity of 20 lux than 5 lux. Moreover, more feed was consumed in the 20 lux pens than 5 pens. There were also high numbers of chickens in the transit compartment with its low light intensity (1 to 2 lux) and no feeders or waterers. Broiler chickens exhibited a preference for 20 lux light intensity for feeding compared to 5 lux light intensity. The present study supports the view that there should be a light intensity of at least 20 lux for the areas around the feeders and also suggests that light intensity may be reduced in other areas for resting and other activities.

Keywords: Illumination, level, choice, meat fowl

Implications

In a choice system, meat-type chickens exhibited a preference to be present and to consume feed under a light intensity of 20 lux compared to pens with light intensities with 5 and 10 lux. However, broiler chickens also congregated in an area with low light intensity (1 to 2 lux) and neither feed nor water. It is suggested that regulations requiring uniform light intensities for broiler chicken production might be reconsidered based on bird's behaviour allowing opportunity to retreat between bouts of eating and drinking to areas with low light intensity.

Introduction

Light intensity influences other aspects of the physiology and behaviour of birds. For instance, young chickens exhibited more resting with longer periods and greater frequency, during the photophase with a light intensity of 5 lux compared to either 50 or 200 lux (Alvino *et al.*, 2009). Turkeys reared under 1 lux showed lower aggression as indicated by injurious pecking than those at 10 lux (Lewis *et al.*, 1998). Locomotory activity is also affected by light intensity with the level of activity of young meat-type chickens being lower when exposed to a light intensity of 5 lux compared to those at either 50 or 200 lux (Blatchford *et al.*, 2009). Similarly, the activity of growing chicks was lower when exposed to dim red light compared to moderate red light (Prayitno *et al.*, 1997).

Environmental conditions employed for raising broiler chickens attempt to maximise production. There is evidence that performance of meat-type chickens is not optimal at light intensities below 1 lux and, hence, these are not employed by commercial poultry producers. For instance, broiler chickens at 0.1 lux exhibited greater mortality together with lower BW and feed intakes at day 14 compared to those raised 1, 5 or 10 lux (Deep *et al.*, 2013). Moreover, feed intake between days 14 and 35 was lower in birds on 0.5 lux compared to 1, 5 or 10 lux (Deep *et al.*, 2013). Similarly, greater growth rates in meat-type chickens have been reported in birds reared under 5 lux than 0.2 lux (Deep *et al.*, 2010; 2013). In contrast, increased wing weights were reported for chickens reared under 0.5 or 1 lux compared to 10 lux (Deep *et al.*, 2010; 2013). There are also reports that growth rates in meat-type chickens are lower in birds raised under light intensities of 25 lux than 5 lux (Olanrewaju *et al.*, 2011). However, in other studies, there

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were no differences in either growth rate or immune functioning of meat-type chickens raised in chambers at 5, 50 or 200 lux (Blatchford *et al.*, 2009) or between 0.1 and 100 lux (Newberry *et al.*, 1986). Moreover, there was no difference in the growth rate or of an indicator of stress, the heterophil: lymphocyte ratio, in chickens raised at either 1 or 10 lux (Lien *et al.*, 2007).

There have been few studies where chickens have the choice of light intensities. Two-week-old chickens spent more time in a 200-lux environment while 6-week-old birds exhibited a preference for 6 lux when given a choice of environments with different light intensities (6, 20, 60 and 200 lux) (Davis *et al.*, 1999). However, the study employed the physiologically anomalous system of continuous lighting. Another report considered preferred light intensity for feeding in laying hens. When the birds had the choice of eating under <1 or 6 or 20 or 200 lux, they disproportionately favoured the high light intensity and consumed the least under the lowest light intensity (Prescott and Wathes, 2002).

Lighting intensity can be a welfare issue in poultry. Continuous high-intensity light has been associated with eye problems including eye damage (Oishi and Murakami, 1985) and increased intra-ocular pressure and blindness in chickens (Chui *et al.*, 1975). Conversely, eye weights are increased at low light intensities being greater at 5 lux compared to 50 and 100 lux (Blatchford *et al.*, 2009) and at 1 lux compared to 10, 20 and 40 lux (Deep *et al.*, 2010). However, it is not clear whether eye weights indicate a welfare issue or a physiological accommodation to low light intensity. Welfare issues have influenced regulations. The light intensity requirements for broiler (meat) production in the European Union are the following 'All buildings shall have lighting with an intensity of at least 20 lux during the lighting periods, measured at bird eye level and illuminating at least 80% of the useable area' (European Union, 2007). This directive uses the information available in a report of the European Commission (European Commission, 2000) in 2000. In the United Kingdom, there is an identical regulation (UK Government, 2007; DEFRA, 2018).

Despite the importance of light intensity to the well-being of broiler (meat-type) chickens and to regulatory and other controls, there is limited information of preferences of birds for different light intensities (Buyse *et al.*, 1996). For instance, it is not known whether young growing broiler chickens exhibit any preferences for light intensities used in the poultry industry. The present study employed a novel choice-type system to examine whether broiler chickens prefer environments with a light intensity of 5 or 10 or 20 lux.

Materials and methods

Study design

The study employed a choice-type design to examine whether broiler chickens prefer an environment/pen with a light intensity of 5 or 10 or 20 lux provided by incandescent lighting [25 w incandescent bulbs (Osram Sylvania, St. Mary, PA 15857)]. The 5 and 10 lux light intensities were achieved, in an iterative manner, by spraying the bulbs with black paint and measuring the resultant light intensities. The transit pens were illuminated by ambient lighting in the house but with the light intensity at bird level being 1 to 2 lux due reduction of light access by light-proofing materials around the pens.

Pen complex for choice study

Each pen was 1.22 m x 1.22 m (area – 1.48 m^2) and contained a hanging feeder (area 0.05 m^2) and automated waterer. Three light-proofed pens were located side by side (see Figure 1) with heavy duty black plastic sheets (BARRACADE 6 – mil Plastic Sheeting,) attached to the sides but the tops open. The connecting corridor was 0.61-m wide and 3.66-m long (area – 2.23 m^2) (see Figure 1). It was also surrounded by black plastic sheeting on the sides leaving the top open. The connecting corridor had doors (of hanging plastic sheeting) such that birds were free to move into the pens and, thus, between the pens. It did not contain either feeders or waterers. No light leakage was observed visually from the pens into the transit compartment. Overall the experimental (pen and connecting corridor) complex was 1.83 m by 3.66 m with an area of complex (6.70 m^2). The floors of the pens and connecting corridor were deep litter composed of pine shavings.

The complexes were placed in the middle of a commercial poultry house. The temperature at litter level in the absence of birds was the same in each compartment of the pen complex. It is unlikely that there were difference in the ventilation in the pens or connecting area as both were surrounded by the same plastic sheeting.

Chickens

Cobb 500 broiler chicks were raised on fresh deep litter with a photoperiod of 18 h of light per day (18L:6 D) from placement (day 1) and access to a commercial feed and water available *ad libitum* prior to and throughout experimentation.

Experimentation

The two replicate choice studies examined the preference of broiler chickens to different light intensities and the data from these were combined in a single analysis. Figure 1 summarises the system employed in the choice study. The three pens (A, B, C) had light intensities of, respectively, 5, 10 and 20 lux while the light intensity of transit pen was 1 to 2 lux. Chicks (30 – 15 female, 15 male) were randomly placed in six choice systems at 2 days old and raised to 39 days old with a final average weight of 2.73 kg. To eliminate potential bias, the following arrangements were used: 5 (pen A – see Figure 1), 10 (pen B), 20 lux (pen C) for two choice systems, 10 (pen A), 20 (pen B), 5 lux (pen C) for two choice systems and 20 (pen A), 5 (pen B), 10 lux (pen C) for two choice systems. The experimental design and execution were approved by the University of Arkansas Animal Care and Use Committee.

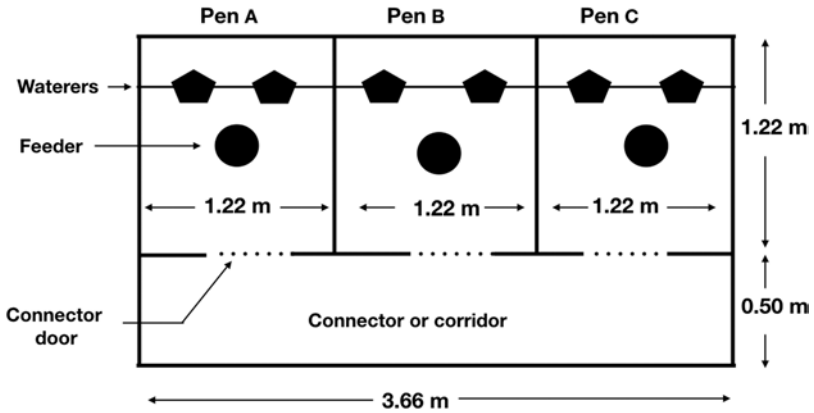

Figure 1 System for choice studies with four light-proof pens. Pens A, B and C included both a feeder and a waterer. There were flaps that could be opened by the chickens between the connecting compartment and each of pen A, pen B and pen C.There were no doors between pen A and B or between pens B and C. There was no feeder or waterer in the connecting compartment.

Measures

Images of the pens showing the number of chicks present in pens A, B and C were taken using a GoProHERO 6 video camera (San Mateo, CA, USA) in time lapse mode with one frame per second at 38 days of age. The camera was manually placed and secured above the pens at the beginning of the photophase. The cameras were positioned such that birds in each pen and the transit compartment could be readily recorded. The number of birds in each pen was determined using 12 images at intervals of 5 min in each of the first and last hours of the photophase (respectively, periods 1 and 3) and 18 times randomly between hours 2 and 17 of the photophase (period 2). Numbers of birds were determined from the images by manual counting. Feed consumption was determined.

Statistical analysis

Numerical data from the six choice complexes were the units for analysis employing the mean numbers of chicks per pen over the specified time or bird density (number of birds per pen over the specified time per square metre. Data on feed consumption per day were analysed by two-way ANOVA [light intensity × trial] with means separated by the Tukey's test. χ^2 analysis was employed to verify that data were independent. However, data on chicken numbers were not independent between the pens with three light intensities and the transit compartment, there being a fixed number of birds. To address this, data were analysed in separate ANOVA comparing 1. bird number at the three light intensities and 2. bird number in the 20 lux pen and the transit compartment. In both cases, data were analysed by three ways ((pens/compartments) × trial with time of day as repeated measures). χ^2 analysis was employed to verify that data in the three light treatments and between the 20 lux pen and the transit area were independent. Differences between time of day within a light intensity treatment were analysed by repeated measures ANOVA. Means were separated by the Tukey's test. Regression analyses were performed with number of chickens per pen regressed against either light intensity and feed consumption per pen *v.* either light intensity.

Results

If broiler chickens had no preferences for light intensity, it would be predicted that they would distribute randomly between pens. However, this was not the case. There were more ($P < 0.05$) chickens in 20 lux pens compared to 10 lux and more ($P < 0.05$) in the 10 lux pens than the 5 lux pens (Table 1). The differences were evident at all time periods (Table 1). There was a positive relationship between bird number per and light intensity (adjusted $R^2 = 0.693$, $P = 0.0033$). There were more ($P < 0.05$) chickens in the 20 lux pens during period 1 than either period 2 or 3 and, as a corollary, fewer ($P < 0.05$) birds in the transit area. The "transit" area was well populated in all periods (Table 1).

It might be expected that few birds would be present in the "transit" area due to the absence of either feeder or waterer. Instead, it was well populated with more birds than in the 20 lux pens during the three time periods examined (Table 1). However, the transit compartment was larger than the individual pens. When the number of birds was expressed per square metre, bird density in the transit area remained greater ($P < 0.05$) than in the 20 lux pens during periods 2 and 3 (Table 2). It might be argued that the area taken up by the feeders impeded chickens remaining in the pens. However, the area of the feeders is only 3.5% of the pen and, thus, their effect is minimal.

There were differences in feed consumption in the choice pen systems with different light intensities (Table 1). Feed consumption was greatest ($P < 0.001$) in the 20 lux compartment and lowest in the 5 lux pen in both studies 1 and 2 (Table 1). There was a positive relationship between feed consumption per day and light intensity (adjusted $R^2 = 0.655$, $P = 0.0318$).

Table 1 *Distribution of young chickens in pens with different light intensities in a choice study*

Comparison of number of birds and feed consumption in pens with different light intensities											
Time during photophase[Δ]	Number of birds in pens with different light intensities			SEM	Effect			Interaction			
	5 lux	10 lux	20 lux		Treatment (pen)	Trial	Time of day	Treatment × trial	Treatment × time of day	Trial × time of day	Treatment × trial × time of day
Period 1 (Early)	3.8[a]	5.8[b]	8.3[cy]	0.48	$P<0.001$	NS	$P<0.01$	NS	NS	NS	NS
Period 2 (Interim)	3.1[a]	5.2[b]	6.6[bx]	0.47							
Period 3 (Late)	3.1[a]	5.9[b]	6.5[bx]	0.49							
Feed intake[#]	1.25[a]	1.41[b]	1.71[c]	0.048	$P<0.001$	NS	NA	$P<0.01$	NA	NA	NA

Comparison of number of birds in pens with 20 lux light intensity with transit compartment with 1 to 2 lux light intensity										
Time during photophase[Δ]	Number of birds		SEM	Effect			Interaction			
	Pen with 20 lux	Transit compartment (1 to 2 lux)		Treatment (transit compartment *v.* 20 lux pen)	Trial	Time of day	Treatment × trial	Treatment × time of day	Trial × time of day	Treatment × trial × time of day
Period 1 (Early)	8.3[py]	12.1[qx]	0.48	$P<0.001$	NS	NS	NS	$P<0.001$	NS	NS
Period 2 (Interim)	6.6[px]	14.9[qy]	0.47							
Period 3 (Late)	6.5[px]	14.4[qy]	0.49							

NS = non-significant; NA = not applicable.
Data show mean number of birds in each pen (two trials each with $n=6$ units consisting of three pens and a connecting corridor/transit area).
[a,b,c] Values within a row (pens with different light intensities) with different superscripts differ $P<0.05$.
[p,q] Values within a row (comparing bird numbers in the 20 lux pen and the transit compartment) with different superscripts differ $P<0.05$.
[x,y] Values within a column(at different times of day) with different superscripts differ $P<0.05$.
[#] kg/pen per day.
[Δ] Period 1 was the first hour of the photophase, period 2 was at random times in hours 2 to 17 of the day and period 3 was the last hour of the photophase.

Table 2 *Density of young chickens (mean number per square) in pens with different light intensities in a choice- study*

Comparison of density of birds in pens with different light intensities											
Time during photophase[Δ]	Number of birds in pens with different light intensities			SEM	Effect			Interaction			
	5 lux	10 lux	20 lux		Treatment (pen)	Trial	Time of day	Treatment × trial	Treatment × time of day	Trial × time of day	Treatment × trial × time of day
Period 1 (Early)	2.5[a]	4.1[b]	5.5[cy]	0.30	$P<0.001$	NS	$P<0.01$	NS	NS	NS	NS
Period 2 (Interim)	2.1[a]	3.5[b]	4.5[b]	0.30							
Period 3 (Late)	2.5[a]	4.0[b]	4.3[bx]	0.30							

Comparison of density of birds in pens with 20 lux light intensity with transit compartment with 1 to 2 lux light intensity										
Time during photophase[Δ]	Number of birds		SEM	Effect			Interaction			
	Pen with 20 lux	Transit compartment (1 to 2 lux)		Treatment (transit compartment *v.* 20 lux pen)	Trial	Time of day	Treatment × trial	Treatment × time of day	Trial × time of day	Treatment × trial × time of day
Period 1 (Early)	5.5[y]	6.0[x]	0.30	$P<0.001$	NS	NS	$P<0.01$	$P<0.01$	NS	NS
Period 2 (Interim)	4.5[px]	7.5[qy]	0.30							
Period 3 (Late)	4.3[px]	7.2[qy]	0.30							

NS = non-significant.
Data shows mean number of birds in each pen (two trials each with $n=6$ units consisting of three pens with different light intensities and a connecting corridor/transit area).
[a,b,c] Values within a row (pens with different light intensities) with different superscripts differ $P<0.05$.
[p,q] Values within a row (comparing bird numbers in the 20 lux pen and the transit compartment) with different superscripts differ $P<0.05$.
[x,y] Values within a column (at different times of day) with different superscripts differ $P<0.05$.
[Δ] Period 1 was the first hour of the photophase, period 2 was at random times in hours 2 to 17 of the day and period 3 was the last hour of the photophase.

Discussion

If there were no preference for light intensities, young chickens would be expected to distribute themselves randomly between the environments with feed and water available but different light intensities. In the present study, there was clearly a preference by the chickens for eating in the pens with the higher light intensity (20 lux) than at either 5 or 10 lux light intensities. This preference is not only supported by greater number and greater density of birds in the 20 lux pen (Tables 1 and 2) but also the elevated feed consumption in the 20 lux pens (Table 1). This is the first report that there is preference by broiler chickens for a higher light intensity, at least for expression of feeding behaviour. The present study was conducted at a single age. Further research is needed to determine whether this applies to other ages and whether broiler chickens prefer to drink at 20 lux. Similarly, laying hens favour eating at the high light intensities (Prescott and Wathes, 2002).

Buyse and colleagues (1996) concluded the literature on light intensity and chickens was 'inconsistent'. These present data suggest that light intensity is a stronger 'driving force' for chicken distribution than stocking density. There have been few consistent preference effects reported in choice studies in chickens (e.g. Senaratna *et al.*, 2012; 2014). Two-week-old birds spent more time in the chamber with high illumination but only during the subjective night (e.g. Senaratna *et al.*, 2014). In contrast, 4-week-old chicks spent more time under low intensity during the photophase (Senaratna *et al.*, 2014).

It was initially thought that chickens would spend little time in the transit compartment due to the absence of either feed or water. Instead, it was considered probable that the chickens would be observed close to the feeders and waterers. However, this was not seen. What was unexpected was that young chickens seemed to congregate in the dimly lit transit compartment (1 to 2 lux) (see Tables 1 and 2). It might be suggested that chickens may move away from the areas where they feed/drink migrating to an area of low light intensity to rest. The present data of higher densities of birds in the transit area are consistent reports of behaviours of adult chickens; birds being farthest apart when walking and closest together when standing and preening (Keeling and Duncan, 1991; Keeling, 1994). Similarly, laying hens favoured eating at the high light intensities (Prescott and Wathes, 2002). The assumption that broiler chickens would locate close to feeders may be anthropomorphism or reflect an exaggerated view of how much time these birds spend eating. Broiler chickens were reported to spend only 4.7% of their time engaged in eating (Weeks *et al.*, 2000).

The higher densities of birds in the 20 lux pen and the transit compartment may reflect flocking and synchronisation behaviours. Adult bantam chickens spontaneously form cohesive flocks after mixing (Keeling and Duncan, 1991). Moreover, synchronisation of feeding was reported in laying hens housed in pairs (Webster and Hurnik, 1994). Similarly, in broiler chickens, there is evidence for synchronisation of feeding activities (Hughes, 1971; Collins and Sumpter, 2007) and of dust bathing (Lundberg and Keeling, 2003). This has been interpreted as social facilitation (Collins and Sumpter, 2007).

The density of birds in the 5 lux pen was markedly (84%) lower than in 1 to 2 lux transit compartment (Table 2). This again is unexpected were bird density to be a major 'driving force' for the distribution of young chickens. What appears to be occurring is that there is preference for eating at 20 lux (compared to 5 and 10 lux). This is consistent with a study where chicks were raised in an environment with graded light intensity and temperatures; they congregated in an area reported to be between 1 and 21 lux (Alsam and Wathes, 1991). Moreover, based on the results of another choice study, it was concluded that in the study of 6-week-old chickens, chickens 'prefer to spend much of their time in a light environment of < 10 lux intensity' (Appleby *et al.*, 1983/1984). The preference for a higher light intensity (20 lux) compared to lower light intensities for feeding (Tables 1 and 2) parallels the results of earlier work.

There was a marked increase in the number of chickens in the 20 lux pens at the beginning of the photophase (Table 1). This early photophase increase in feed intake is consistent with a previous report determining changes in feed intake during the day (Buyse *et al.*, 1993). There was very low feed intake during the scotophase followed by a large rebound increase in feed intake during the early photophase (Buyse *et al.*, 1993).

Conclusions

The study provided evidence that the preference of meat-type chickens is for 20 lux (compared to 5 and 10 lux) light intensity for feeding and that feeding at 5 lux light intensity is the least preferred. In contrast, the meat-type chickens congregated at high densities away from feed and at low light intensities. It is argued that the requirements for resting and feeding are more complex than a simple minimum light intensity and density as set forth in regulations. This is analogous with the demonstration that the multiple aspects of broiler management are important to the welfare of the birds than does stocking density or overcrowding (Dawkins *et al.*, 2004). It is interesting to note that no discernible effects of stocking density were observed on the incidence of behaviours in young broiler chickens (Febrer *et al.*, 2006).

The present results support a recommendation of at least 20 lux for the areas around the feeders and waterers and, further, suggest that light intensity may be reduced in other areas for resting and other activities.

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Declaration of interest

The authors had no conflicts of interests.

Ethics statement

Conceived and designed the experiments: MR, YVT, KC, DJA, CGS. Performed the experiments: MR, YVT, DJA, KC; analysed the data: MR, YVT, KC, DJA, CGS; wrote the paper: CGS, YVT, MR, DJA, KC. The experimental design and execution were approved by the University of Arkansas Animal Care and Use Committee.

Software and data repository resources

None of the data were deposited in an official repository.

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animal

'Would it sell more pork?' Pig farmers' perceptions of Real Welfare, the welfare outcome component of their farm assurance scheme

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*In the UK, the pig industry is leading the way in the adoption of welfare outcome measures as part of their farm assurance scheme. The welfare outcome assessment (**WOA**), known as Real Welfare, is conducted by the farmers' own veterinary surgeon. For the first time, this has allowed the pig industry to evaluate welfare by directly assessing the animal itself and to document the welfare of the UK pig industry as a whole. Farmer perspectives of the addition of a welfare outcome assessment to their farm assurance scheme have yet to be explored. Here, we investigate how the introduction of the Real Welfare protocol has been perceived by the farmers involved, what value it has (if any), whether any practical changes on farm have been a direct consequence of Real Welfare and ultimately whether they consider that the welfare of their pigs has been improved by the introduction of the Real Welfare protocol. Semi-structured interviews with 15 English pig farmers were conducted to explore their perceptions and experiences of the Real Welfare process. Our findings fall into three key areas: the lived experience of Real Welfare, on-farm changes resulting from Real Welfare and suggested improvements to the Real Welfare process as it currently stands. In all the three areas, the value farmers placed on the addition of WOA appeared to reflect their veterinary surgeon's attitude towards the Real Welfare protocol. If the vet was engaged in the process and actively included the farmer, for example through discussion of their findings, the farmers interviewed had a greater appreciation of the benefits of Real Welfare themselves. It is recommended that future similar schemes should work with veterinary surgeons to ensure their understanding and engagement with the process, as well as identifying and promoting how the scheme will practically benefit individual farmers rather than assuming that they will be motivated to engage for the good of the industry alone. Retailers should be encouraged to use Real Welfare as a marketing tool for pig products to enhance the perceived commercial value of this protocol to farmers.*

Keywords: animal-based measures, animal welfare, farmer perspectives, pig, welfare assessment

Implications

There is an increasing move towards the inclusion of welfare outcome assessment within farm assurance schemes. The practical implications and value of these additions for pig farmers are largely unknown. Our findings suggest that the attitude of the vet who conducts the welfare outcome assessment is highly influential on how farmers value these additions to their farm assurance schemes. The benefit to individual farmers was perceived as negligible, but this could be improved substantially by using compliance with the welfare outcome assessment commercially to promote and market pork products.

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Introduction

One response to the growing public criticism of production animal husbandry systems has been the introduction of farm assurance schemes (**FAS**) (Main *et al.*, 2001; Liu *et al.*, 2018). Participation in these schemes is usually voluntary, with membership denoting that the farmer keeps their animals to a certain standard which then enables them to access certain markets and sell their products to certain retailers (van Dijk *et al.*, 2018). Originally such schemes focused on measuring resource or environmental parameters. FAS are now increasingly moving towards assessing animal-based parameters or welfare outcomes (**WO**) instead of, or as well as, resource-based parameters. This shift in thinking, advocated by Farm Animal Welfare Committee (FAWC) and other bodies (Main *et al.*, 2014), demonstrates an increasing recognition that simply ensuring that an animal's environment is of a

sufficient standard does not guarantee that the animal will have good welfare. WO, such as an animal's behaviour and physical condition, provide a more direct insight into an animal's welfare than can be gained by assessing resource-based parameters alone (Mullan *et al.*, 2009a).

In the UK, the pig industry is leading the way in the adoption of WO measures as part of their FAS. Pig production is one of the most intensive livestock industries (Bock and van Huik, 2007) and consequently has been the recipient of some negative attitudes, often influenced by how pig farms are portrayed in the media (Ngapo *et al.*, 2004; Bergstra *et al.*, 2017). FAS for UK pig producers are nominally voluntary; however, in reality joining an FAS is a necessity if farmers are to be able to sell their produce through major slaughterhouses and retailers (Carmen *et al.*, 2007; Hubbard, 2012). Almost all finisher pig herds in the UK are members of the Red Tractor FAS (Agriculture and Horticulture Development Board, (**AHDB**), 2017). In 2013, five WO measures were added to the existing Red Tractor FAS assessment for finisher herds under the name 'Real Welfare'. The WO measures are assessed in a representative sample of pens by each farms own vet, who must be a member of the UK's Pig Veterinary Society (AHDB, 2017). The total number of pigs assessed is dependent on the number of finishing pigs the farm has (AHDB, 2017). The WO measures are taken on three of the four quarterly veterinary inspections that the farm must receive under the terms of their FAS when the vet inspects the farm and pigs to update the farm's Veterinary Health Plan (AHDB, 2017). This creates a rolling average reflecting a more accurate farm status by reducing the effect of the particular situation on each individual visit day, for example with regard to weather or disease (Mullan *et al.*, 2009a; AHDB, 2017). The five WO measures are the number of pigs requiring hospitalisation, the number of lame pigs, the number of pigs with severe, mild or no visible tail damage, the number of pigs with severe, mild or no body marks visible and the number of pigs interacting with environmental enrichment or pen mates and other pen features. Pandolfi *et al.* (2017b) provide further detailed description of the WO measures and the sampling protocol.

The Real Welfare data are not audited by the FAS, but should any issue be identified by the vet, the actions jointly agreed by the vet and the producer to address this issue are included in the herd health plan which is audited (AHDB, 2017; Pandolfi *et al.*, 2017b). Therefore, in practice some dialogue between vet and farmer regarding the WO assessment is expected.

Real Welfare not only allows the pig industry to evaluate welfare by directly assessing the animal for the first time, but has also enabled them to document the welfare of the UK pig industry as a whole something which may be seen as equally as important (Brandt *et al.*, 2017). From an animal welfare perspective, WO assessments (**WOA**) such as Real Welfare provide a mechanism by which animal welfare can be recorded and improvements made within an existing FAS (Main *et al.*, 2012).

The data generated in the first 3 years of Real Welfare have been described and analysed elsewhere (AHDB, 2017; Pandolfi *et al.*, 2017a and 2017b). Real Welfare has been reported a success, with results from 2013 to 2016 demonstrating a reduction in the prevalence of WO measures representing welfare concerns (Pandolfi *et al.*, 2017b). But the impact of Real Welfare from a farmer perspective has yet to be explored.

Here, we aim to investigate how the introduction of the Real Welfare protocol has been perceived by the farmers involved, what value it has (if any), whether any practical changes on farm have been a direct consequence of Real Welfare and ultimately whether they consider that the welfare of their pigs has been improved by the introduction of the protocol.

Materials and methods

Semi-structured interviews with 15 English pig farmers were conducted to explore their perceptions and experiences of the Real Welfare process. The process and interview schedule were approved by the University of Bristol Faculty of Health Sciences Research Ethics Committee. The interviews took place in March and April 2017.

Recruitment of participants

Pig farmers were recruited to participate in the study via an Internet directory (Yell.com) and existing contact lists held by the Bristol Veterinary School. Initial contact was made by telephone. Prospective participants were given information about the study and the possibility of audio recording the interview was discussed, along with data confidentiality procedures and the request for informed consent regarding the use of the data generated. A date and time for the interview was arranged if the prospective participant agreed, and a participant information sheet and consent form were emailed, if this was possible, prior to the interview. Information sheets and consent forms were also taken to the interview to ensure that these had been read and the terms agreed to before the interview commenced.

Farm characteristic data reported by Pandolfi *et al.* (2017b) were used as a guide to ensure that the sample recruited reflected the characteristics of the Real Welfare 2013–16 sample in terms of the system (indoors/outdoors), pen size and feed type. UK pig production is predominantly situated in England (Hubbard *et al.*, 2007) and consequently our sampling strategy focused on three geographic regions of England. Table 1 summarises the characteristics of the participants' pig production enterprises.

The interview process

Interviews were conducted face-to-face ($n = 14$) apart from one interview that was conducted over the telephone at the participants request after an initial interview date required rearranging. The interview schedule consisted of

Table 1 *Pig production enterprise characteristics of the study sample*

Farmer code	Region	Production	System	Substrate	Routinely dock tails	Number finished/year	Pen size at finishing	Assurance scheme	Vet practice
PF1	1	Farrow to finish	Indoors	Straw	Yes	8700	Varies	RT; Retailer 1	1
PF2	1	Finish only	Indoors	Sawdust	Yes	8000	10	RT; Retailer 2	2
PF3	1	Farrow to finish: multisite	Indoors	Slats	Yes	>50 000	Varies	RT; Retailer 1	–
PF4	1	Farrow to finish	Indoors	Slats	Yes	–	28	RT	3
PF5	1	Finish only	Indoors	Straw	Yes	2160	50	RT; RSPCA Assured	3
PF6	2	Farrow to finish	Indoors	Straw	Yes	15 000	Varies	RT	4
PF7	2	Farrow to finish	Indoors	Slats	Yes	5000	10	RT	4
PF8	2	Farrow to finish: multisite	Indoors	Slats	Yes	–	Varies	RT	5
PF9	2	Farrow to finish	Indoors	Straw	No	–	Varies	RT	6
PF10	2	Farrow to finish	Outdoors	Outdoors	No	1440	Varies	None	4
PF11	2	Farrow to finish: multisite	Indoors	Slats	Yes	70 000	Varies	RT	4 and 7
PF12	3	Farrow to finish	Indoors	Slats	Yes	–	34	RT	8
PF13	3	Farrow to finish	Indoors	Straw	Yes	4800	25	RT	4
PF14	3	Farrow to finish: multisite	Indoors	Slats	Yes	27 000 to 28 000	Varies	RT; Retailer 3	4
PF15	3	Farrow to finish: multisite	Indoors	Slats	Yes	9000	25	RT	3

PF = participating farmer; RT = Red Tractor; RSPCA = Royal Society for the Prevention of Cruelty to Animals.

33 predominantly short-answer questions divided into four sections covering details of the farm, the FAS they were part of and the inspection process, Real Welfare in practice and the implications of Real Welfare. An additional section with a further three questions was included for participants who were members of Royal Society for the Prevention of Cruelty to Animals (RSPCA) Assured or the Soil Association FAS. The interview schedule can be found in the Supplementary Material S1. Once the interviewer opened with the short series of questions about the participants' farm (e.g. farm type, size of unit), the interviews typically took the form of a discussion. The schedule was used predominantly as a guide to make sure key topics were discussed rather than rigidly adhered to.

As many of the farmers had limited time available, interviews were kept relatively brief. The mean interview duration was 38 min, with the shortest being 22 min and the longest 1 h 15 min.

Data analysis

All participants gave their consent for the interview to be audio recorded. The recordings were transcribed verbatim, and the resulting transcripts were analysed manually by the first author (JH). An inductive analysis approach was taken to identify common themes between transcripts. Points of variance between participants' responses were also identified and noted. The transcripts were independently analysed by the third author (SM), and the resultant themes subsequently discussed with the first author in light of their analysis. The inductive analysis showed good agreement between the authors of the dominant themes identified, validating the analysis process.

Results and discussion

The pig farmers forming our sample were comparable to the wider population reported in the Real Welfare data (Pandolfi *et al.*, 2017b), with the majority of systems raising their pigs indoors and routinely tail docking.

Despite the relatively small sample size, it is important to note that by the final interviews we reached saturation with no new themes emerging. While a number of interesting themes emerged from the analysis (see Table 2), only those related to Real Welfare and the concept of welfare outcome assessment are reported here.

The lived experience of real welfare

Incorporating WOA without extending the FAS visit by longer than 30 min has been identified as an important criteria for conducting these assessments in practice (Main *et al.*, 2012) and the general impression gained from the farmer interviews was that Real Welfare had been added almost seamlessly to the quarterly vet inspections required by their FAS. Certainly, the extra time added to the visit seemed relatively minimal, 'in our particular case it does not take us a great deal longer' (PF14), 'it's just an extra 20 minutes on her visit really' (PF7), 'it didn't seem to take any longer than a normal ... and it was part, it flowed' (PF13).

Table 2 *Themes that emerged from inductive analysis of the transcripts from the pig farmer interviews*

Theme	Topics encompassed within each theme
The lived experience of Real Welfare	The value of benchmarking, how the assessment process works in practice
Perceived positives and negatives of Real Welfare	The role of the vet, recognition of good practice, the shift in focus to animal-based measures, the financial cost, the assessment protocol
On-farm changes resulting from Real Welfare	Tail docking, environmental enrichment, the hospitalization and treatment of ill pigs
Suggested improvements to Real Welfare	Promotion of Red Tractor, inclusion criteria and compliance with the scheme, suggested improvements to the Real Welfare assessment protocol
The value of written records	Time consuming, value of abattoir reports, not consulted regularly/at all, health plans
Examples of innovation in practice	Alterations to feeding practices, antibiotic provision, environmental enrichment ideas
Value of 'Pig Clubs'/farmer groups	Keeping up to date, sharing ideas, discussing experiences, isolation, loss of local pig farming community
Problem posed by 'animals rights' activists	Undercover footage, leaving the industry, bad publicity, industry image, security

The majority of farmers did not discuss their Real Welfare results with their vet unless there was a problem. Rather it was felt that Real Welfare was something that the vet did, and the farmer was only party to it if something came up:

> Only if there's a problem. If there's not a problem, I don't hear from him no. I just sign the paper and it goes on. (PF1)
>
> I think the Real Welfare thing it happens, he does what he has to do with it. Do I ever look at the results? No. Does it influence over how I manage the unit? No because if we had a problem of that nature we would have addressed it. (PF8)

In this respect, Real Welfare was perceived by some farmers as just another part of the assurance process, and consequently, it was seen as somewhat removed from them and the management of their production system. The farmer quoted above went on to discuss this further, emphasising the lack of unity between those who want to use the Real Welfare data and those whose animals are the data:

> Quite how the Real Welfare data is being accepted in the processor/retailer world I have got no idea. I hope that it's what they were looking for and is ticking the box for them, even if we struggle with the meaning at the coal face. (PF8)

This lack of farmer buy-in to the Real Welfare process is at odds with the industry belief that farmers would use the data generated by the assessment as a benchmarking tool (AHDB, 2017; Pandolfi *et al.*, 2017b). When benchmarking was mentioned by the interviewer, it was often interpreted as a way of trying to better other pig farmers rather than as a useful tool for self-improvement. Many were adamant that pig farmers should all be on the same side, that 'it's not a competition' (PF2). However, when farmers were probed further on this issue many spoke of the benefit of having a 'pig savvy vet' (PF4) who inspected pigs at a number of sites as they were able to make suggestions based on the good results another farm had had with the same changes/intervention. This vet mediated form of benchmarking was seen as a good thing and interestingly a common aspect that was raised by participants in relation to this was environmental enrichment, one of the areas covered by Real Welfare.

The fast turnaround of animals in the pig industry means that the majority of farmers interviewed benchmark their current herd against the performance of their previous herds rather than against other producers. However, some farmers did see a value in being able to benchmark themselves against other producers in the industry:

> I do think it is always interesting to know where you lie in the national herd and [how] are you doing, then you know whether you have gotta, where you ought to be improving or just trying to maintain where you are. (PF13)

Benchmarking is not a panacea for all farmers in every context. Opinions regarding the value of benchmarking can be conflicting, with perceived value relating to the attributes of the benchmarking tool itself as well as the personal goals of individual farmers (Ronan and Cleary, 2000; de Snoo *et al.*, 2010; Phythian *et al.*, 2014). However, engaging in a benchmarking programme has been associated with improved outcomes in other sectors, for example in dairy calves (Atkinson *et al.*, 2017; Sumner *et al.*, 2018). Promoting benchmarking more widely within the pig industry may lead to similar management changes and welfare improvements. Many of the farmers interviewed were or had been members of regional pig farmer groups and found these a useful platform to share ideas and compare practices. Unfortunately, changes within the UK pig industry have resulted in fewer pig farms operating in some regions and the subsequent closure of their associated pig farmer groups. In light of this, promoting the benchmarking opportunities provided by Real Welfare, and even expanding on them, would provide pig farmers with a new platform to connect with their peers and share ideas and best practice as well as demonstrating a practical value of the assessment for the individual farmers.

The perceived positives and negatives of Real Welfare

The perceived positives. The overriding opinion of the farmers we spoke to was that the value of Real Welfare lies with the vet who conducts the assessment. Most farms were

assessed by their regular vet, who is typically seen as 'a valuable part of the business' (PF13) and 'part of the management team' (PF14). The requirements of Real Welfare provided them with an opportunity to spend more time with their vet, and 'pick her brains' (PF7) while they were on farm, which also helped some farmers feel that they were getting their money's worth from the process:

> We walk around with them as they are doing the welfare assessment and they'll say well there's slightly more bite marks on those pigs, you know why would that be? . . . We pay the vets a lot of money and we also have to pay for this Real Welfare for this time that they spend doing it, so we feel that by actually being involved in what they are doing, what they're assessing and what they, you know what they see, sometimes it's quite useful to you have a different set of eyes on your pigs. (PF4)

The value of WOA in enhancing discussion between producers and vets has also been identified in other studies (e.g. Mullan *et al*., 2011a; van Dijk *et al*., 2018). The opportunity that Real Welfare provides for facilitating vet–farmer dialogue could be used to help promote the benefits of this scheme for the individual farmers involved, instead of predominantly focusing on the benefits at an industry level.

Farmers who differed from this opinion were those who were dissatisfied with their current vet and/or practice due to their lack of pig expertise:

> We used to have vets in the past who weren't specialist pig vets and it was a complete waste of time, they just didn't understand anything about managing pigs. (PF4)
>
> I really could do with a vet that is a bit more pig orientated, because it would be more useful. (PF12)

In a broader context, some farmers welcomed the shift in focus that Real Welfare represents:

> The concept of Real Welfare I'm a big fan of and I'm not saying that I am not a fan of assurance, but I like the concept of let's assess the pigs rather than the pens. (PF11)

Real Welfare was also flagged up by some farmers as a way 'to show the public that we are trying our best' (PF1). The success, or otherwise, of Real Welfare in this context is something that will be explored further below.

Finally, some farmers said they found Real Welfare a positive experience because,

> I like being inspected if I am honest. I like at the end of it that you, it's kind of a reward for doing your job right. (PF12)

Recognition of your efforts and successes from external sources can be an important motivator for participation and engagement (Hars and Ou, 2002; Hansen *et al*., 2002). The intrinsic value of feeling that your work is acknowledged, and successes recognised should not be underestimated as a motivating factor for active engagement with FAS.

The perceived negatives. As with the positive perspectives of Real Welfare, some of the negatives were also greatly influenced by the individual vet who conducted the assessment. One farmer, who is part of a multisite operation, reported that the vet practice he uses sends the senior vet to inspect the sows on the breeding unit while 'junior' (PF3) vets conduct the Real Welfare assessments on the finishing pigs. This means that he never consistently has the same people assessing the pigs, something that was perceived to add value by those farmers who had the same vet at each visit. It was not surprising that this farmer did not see any value in Real Welfare, dismissing it as 'pretty much a tick box exercise' (PF3).

As the example above shows, the perceived attitude of the vet towards the Real Welfare process made a significant difference to how it was perceived by the interviewed farmers. The farmer above was given the impression that his vet practice prioritises the welfare of the sows over the finishing pigs when allocating veterinary expertise. Sometimes the attitude of vets towards Real Welfare was expressed more explicitly to the farmers, for example verbally rather than simply through their behaviour.

> He [the vet] can't understand it and he says as much, we are paying for somebody else's data collection really. (PF15)

Understandably, this farmer also saw no value in the Real Welfare process.

In a study preceding the inclusion of WOA in pig FAS, Mullan *et al*. (2011c) identified the need for assessors to accept the measures being included for the assessment to work outside of research projects. Our findings here suggest that further to this, the acceptance of the assessors to the scheme in its entirety is also critical for its success. Given the critical importance of the vet to the success of Real Welfare and the buy-in of the farmers, priority should be given to improve communication with veterinary practices to enhance their understanding of the value it has for individual farmers and the wider industry.

The issue raised by farmer PF15 also reflects the conflict between collecting data that are useful on a farm level and collecting data that support an industry or FAS (van Dijk *et al*., 2018). This can be a difficult balance in practice but for true farmer engagement, tangible benefits for individual farms must be realised as well as for the industry.

Much of the negativity surrounding the Real Welfare assessment was associated with the cost to the farmer of paying the vet to complete the assessment when they felt that they received 'nothing at all' (PF3) in return:

> We've had nothing back from that [Real Welfare] whatsoever so apart from £100 a bill extra from the vet. (PF3)
>
> For me it's had absolutely no impact, other than my wallet is a little thinner now. Probably best not to think by how much to be honest. (PF15)

The cost of mandatory farm assessments has been raised as an issue by farmers in other studies (Alberto, 2007; Hubbard *et al*., 2007). This was particularly related to the fact that costs to the farmer were not reflected in the price they got for their produce (Alberto, 2007).

It was not cost alone that troubled the farmers. Many were unhappy that they had to pay their vet to assess aspects of their pigs' welfare that they believed they assessed themselves on a daily basis:

> I could have told him [the vet] what he was gonna find before he went into the pen. There is the odd lame pig that's maybe slipped the radar and he will pick that up but aside from the odd little bit that's been missed, I could write his Real Welfare for him in here before he ever goes down there. (PF8)

The perception that external inspections to check animal welfare are a waste of time is not isolated to pig farmers (van Dijk *et al.*, 2018). Similar sentiments were reported by sheep farmers responding to Liu *et al.*'s (2018) self-administered questionnaire with 41% of the farmers indicating that external inspections were not important for maintaining animal welfare standards (Liu *et al.*, 2018). One solution to this view might be to introduce some form of self-assessment into the process (van Dijk *et al.*, 2018); however, this is likely to lead to criticism from outside the industry. The accuracy and reliability of animal welfare measures assessed by owners or caretakers can be questionable even with a framework in place to help guide assessments, for example body condition scoring in pet dogs (White *et al.*, 2011) and horses (Stephenson *et al.*, 2011). In a consultation with the pig industry, prior to Real Welfare being incorporated into the Red Tractor FAS, 67% of producers reported to be quite willing to do some self-assessment of welfare as part of their farm assurance scheme (Mullan *et al.*, 2010). This suggests that self-assessment might be an appropriate direction for Real Welfare to take from the perspective of the producers involved; however, more research is needed to evaluate whether WOA can be effectively conducted by the farmers themselves.

The last theme that emerged when farmers considered the negative aspects of Real Welfare was the practicalities of the assessment process itself. Many of the farmers questioned the appropriateness of conducting behavioural observations when pigs are so reactive to the presence of unfamiliar humans.

> . . . it's awfully difficult to judge the behaviour because you are breaking a pattern of behaviour that would normally apply merely by your presence. (PF14)
>
> . . . even if you walk into a room it affects the behaviour of the pigs so it's quite difficult to actually get a genuine assessment of how the pigs are behaving . . . In an ideal world you need to creep into a room and stand there for 10 minutes for them all to settle back down again and then do your assessment, but life's too short for that isn't it? (PF4)

Some vets reportedly conduct the Real Welfare component on a second walk through of the unit with the farmer explaining that 'the pigs tend not to sort of take as much notice on the second walk through' (PF5). Concerns regarding the feasibility of animal-based monitoring in commercial settings have been raised elsewhere (Bracke, 2007). Balancing restrictions on time and money with the need to collect animal-based data means that compromises have to be made in the assessment protocol. It is perhaps worth communicating these decisions to the farmers, so that they do not assume that those developing assessment protocols are unaware of the limitations.

Incorporating WOA into an FAS without incurring too great a time or financial cost for the farmer restricts the number of animals that can practically be assessed at each inspection (Mullan *et al.*, 2009a; Main *et al.*, 2012), as well as assessment procedures that are used. It is partially for this reason that Real Welfare utilises a rolling average (Mullan *et al.*, 2009a); however, some of the farmers voiced concerns about how the Real Welfare sampling strategy worked in practice:

> . . . if they go into the building and there's the first pen that they come to that's tail biting in that pen but that isn't one of the pens they've actually decided that they are going to assess . . . then as far as the welfare result is concerned we have no tail biting, whereas she can see, physically see there's pigs in that pen that are tail biting. (PF4)

Our findings support those of previous studies that suggest that better communication with farmers and vets over the decisions made in the development of WOA, for example why certain measures were included and sampling strategies adopted, would be beneficial so that farmers can appreciate why certain procedures are in place rather than being asked to simply accept them however suboptimal they may seem (van Dijk *et al.*, 2018).

As professionals, vets are typically held in high esteem due to their knowledge and expertise, positioning them as trusted individuals within their clients' information network and the primary go-to source of information and advice on animal health (Osborne, 2002; Carbone, 2010; Hockenhull *et al.*, 2014). Here, the value and expertise of the vet spanned the perceived positive and negative opinions of Real Welfare given by the interviewees, forming an overarching theme throughout our interviews. Our findings demonstrate the impact that an individual vet can have on farmer attitude, acceptance and ultimately behaviour change within the context of FAS. The changing role of the veterinary professional from reactive treatment provider to proactive member of a farm management advisory team and knowledge provider (Atkinson, 2010; Ruston *et al.*, 2016) delivers a valuable opportunity to facilitate practical change and animal welfare improvement. Greater recognition of the value of enhanced communication skills for effective knowledge transfer and facilitation of human behaviour change is now evident, both in the veterinary research literature (Jansen and Lam, 2012; Bard *et al.*, 2017) and in the veterinary curriculums training the vets of the future (e.g. Mossop *et al.*, 2015). Our findings suggest that such increased focus on communication is likely to have wider benefits for the acceptance of WOA in FAS and potentially other animal welfare interventions.

On-farm changes resulting from Real Welfare

Three key areas where Real Welfare had potential implications for practice were raised by farmers during the

interviews; tail docking, environmental enrichment and the hospitalisation of ill pigs. These areas are discussed in turn below, highlighting any changes in management practices influenced by Real Welfare.

Tail docking. Of the 15 farmers we spoke to only 2 (13%) did not routinely dock the tails of their pigs, although most made it clear that this was a practice that was regularly reviewed by themselves and their vet rather than being entrenched in their management regime.

> We tried not docking tails and that was an unmitigated disaster, but it was something we needed to do because we hadn't done for a very long time, so we have proved that its better with the docking. (PF15)

Most of the farmers felt that tail docking was preferable to an outbreak of tail biting, both economically and in terms of welfare:

> If you don't tail dock and you end up getting an incident of tail biting then the consequences of it are absolutely diabolical and you get huge condemnations you get you know atrocious growth rate, poor food conversion it is just devastating financially and for the welfare of the pigs (PF4)
>
> And so the theory as well is that you shouldn't tail dock the pigs unless there's a problem, if I've already got 700 pigs here and suddenly something goes wrong and they're tail biting I've got potentially 700 pigs chucked in the bin ... so it's one of those pre-emptive things that we do, well now the RSPCA has big questions about it as an unnecessary procedure but ... (PF5)

The farmers we spoke to were pro-tail docking purely as a means of reducing the likelihood and subsequent impact of a tail biting outbreak. As voiced in other studies, the farmers felt that tail docking was perceived as a welfare concern by the public simply because they lack the knowledge and understanding of how and why it is conducted, and the consequences if it is not (Lassen *et al.*, 2006). Interviews with farmers and key stakeholders in previous studies have also raised whether tail docking itself is a welfare issue given the scale of the problem it is intended to ameliorate; rather the underlying causal factors are seen as the true welfare concern (Mullan *et al.*, 2011a). Mutilations, such as tail docking, are often seen as a necessity to adapt an animal to the constraints of their housing condition, and while they may be aversive or painful for the individual, they are considered beneficial for the group (Nordquist *et al.*, 2017). Fears that regulations intended to promote welfare in FAS may fail to improve welfare and in fact may actually endanger it have been voiced by other pig producers (Bock and van Huik, 2007; Hubbard *et al.*, 2007). While this sentiment was not expressed explicitly by farmers in this study, many felt that FAS had done nothing to either help them resolve the need to tail dock or to change the practices they already had in place to minimise tail biting occurring:

> Tail biting is an economic loss to us anyway so we are always going to try and do something to stop it, so from that point of view, I don't think farm assurance has helped us one little bit. (PF15)

Overall, the farmers we spoke to unanimously felt that Real Welfare had had a negligible effect on whether or not they tail docked their pigs and the management actions they take to the reduce the chance of a tail biting outbreak. However, it was perhaps not that clear-cut as discussed below.

Environmental enrichment. Environmental enrichment is one means by which the risk of a tail biting outbreak can be minimised (Beattie *et al.*, 1995; van de Weerd *et al.*, 2006; van de Weerd and Day, 2009) and whether enrichment is provided for the pigs is included in the Real Welfare assessment protocol (AHDB, 2017). When it came to discussing enrichment provision rather than management changes to reduce the risk of tail biting, farmers' answers changed:

> No we've not changed a thing, no. No. No. We've put in a few more toys maybe ... but that's mainly to make sure that the toys we put in at the last visit are still there. (PF1)

In one respect, Real Welfare was seen to have improved on-farm practice when it comes to the provision of enrichment:

> For things like pigs' toys if the audits weren't there you might get a little complacent and think yeah that'll do - but if you know you have got an audit coming up why leave everything to the last minute, where you've got to find 20 pig toys ... Just replace the pig toy as it breaks or it gets worn away. Yeah you can always improve, especially on pig toys. (PF2)

Main *et al.* (2014) speculated that excessively prescriptive standards in FAS may inhibit farmer innovation. The provision of environmental enrichment appears to be one area where this holds true. Although most farmers recognised the value of providing enrichment, some felt that there was no need to go beyond the criteria required by the FAS, even if this was not optimal practice for the pigs:

> If you are gonna do a proper job with toys and manipulable material ... you would have to change them every day. Pigs are not dissimilar to humans in that the novelty wears off and they don't use them anymore, so if you are going to keep a pig entertained you have got to change those every day and that's not within the scheme, it's very time consuming. It's not something I am gonna do unless I am forced to, so in terms of toys, I have to tick a box on an assurance scheme and that's what we do. (PF8)

But other farmers were more innovative and went above and beyond the requirements of the FAS, providing additional items that were not considered as manipulable material by the scheme (and that they would consequently receive no recognition for providing), but that they stated their pigs enjoyed playing with, for example balls, chains, old welly boots and plastic drums. Some even discussed rolling out the provision of extra enrichment items to their sows even though they are not covered by the Real Welfare scheme.

Real Welfare seems to have had a positive impact on the provision of environmental enrichment from the perspective of the farmers interviewed. Even if what is provided is simply aimed at meeting the minimum requirements, at least it means that the pigs are being routinely provided with an improvement to their environment that they may not otherwise receive. It was interesting to note that although English and European legislation specifies that all pigs should have permanent access to sufficient material '. . . to enable proper investigation and manipulation activities' (Her Majesty's Stationery Office, 2003; European Union, 2008), none of the farmers said that they provided enrichment to meet legal standards, rather they stated that enrichment was provided and maintained to meet the requirements of Real Welfare. Consequently, it appears that the Real Welfare has provided farmers with an incentive to not only provided enrichment but also to ensure it is maintained and renewed as required.

Hospitalisation and treatment of ill pigs. The hospitalisation and, more specifically, the treatment of ill pigs was one of the aspects of Real Welfare that the farmers appeared to find most controversial. There seemed to be a divide between the farmers as reflected in the quotes below:

> We've got a zero tolerance of casualties . . . It's not fair for the pig to suffer. (PF2)
>
> I think we are more wasteful now . . . Anything we're at all unsure of we shoot rather than give it a chance just because we might get told off for keeping alive an animal that shouldn't be. (PF3)
>
> . . . welfare really isn't helping when you are euthanizing animals, to me that isn't welfare. (PF12)

It is interesting to note that the one reported case of FAS non-compliance that came to light during our interviews concerned a farm where a pig was observed in a hospital pen that the assessing vet believed should have been euthanised. Mullan *et al.* (2011b) reported biases between assessors as a result of their attitude towards farm animal welfare, with those believing in the mental state of a pig more likely to identify pigs as needing hospitalisation, possibly as they are more likely to attribute a negative mental state to a sick or injured pig. For ill pigs, a certain amount of subjectivity is required when assessing whether they should be hospitalised, treated or euthanised, particularly in borderline cases. If farmers have the attitude that they want to give a pig 'a chance' (PF3), they may be more likely to keep the pig alive for treatment than to immediately euthanise it. Whether you are giving an animal a chance or allowing it to suffer is a very narrow and subjective line to tread and perhaps this is an area that requires greater explanation and quantification within FAS protocols.

Suggested improvements to Real Welfare

Three key areas where the Real Welfare scheme would benefit from improvements were identified by the farmers.

Red Tractor should promote Real Welfare to the consumers. Previous studies have documented that many UK pig farmers consider FAS to be a 'necessary evil', something they essentially have to join to remain in business but otherwise see no additional benefit from (Hubbard *et al.*, 2007). The findings of our study support this perspective and take it further, highlighting the negligible return to the farmer and lack of awareness outside the industry that the Real Welfare process even takes place:

> Well from a personal point of view I would scrap the Real Welfare side of things because I have seen nothing in return for the extra time or cost to the farmer. (PF3)
>
> It's [Real Welfare] crept in somewhere and yeah I don't know if people outside of AHDB and the farmers are aware that it even happens I mean our, I don't think consumers know. (PF5)

Some farmers even expressed doubts that many members of the public had heard of farm assurance at all:

> . . . it just doesn't seem to have the power at the other end. You get inspected for a reason, so there is an advertisement mark for your pigs and I think that is where it falls down. The public aren't aware of what we have to do to produce a pig and the Red Tractor doesn't kind of advertise that fact. (PF12)

Aside from discussing the lack of consumer awareness about the welfare standards of Red Tractor-assured pig products, there was a notable absence of discussion about retailer requirements despite farmers who supplied three different major retailers being included in the sample along with a number who sold their products directly to local retailers. None of the farmers explicitly mentioned the role of retailers and the wider market in driving change and welfare improvement; rather FAS were viewed primarily as a means of accessing slaughterhouse facilities and the wider supply chain.

On the whole, the farmers who we spoke to appreciated the need, not only to maintain good pig welfare on farm but also to document it (Brandt *et al.*, 2017). However, from their perspective, Red Tractor needed to go further, to use the Real Welfare initiative to promote the welfare standards of UK pigs to the public to help to sell their product in a highly competitive market rather than just sitting on the Real Welfare data as a safeguard should the public raise concerns.

> . . . where does anyone see what's happening? The shopper and that aren't gonna see anything . . . I don't see how it's made any help for the industry. (PF13)

Essentially many farmers felt that Real Welfare was a reactive rather than a proactive strategy, a concern that has been voiced by pig farmers regarding FAS in other studies (e.g. Thorslund *et al.*, 2017). Raising consumer awareness of FAS requirements has been suggested by farmers previously as a means of benefiting from the welfare standards they are required to meet to gain higher prices for their product (Hubbard *et al.*, 2007; Mullan *et al.*, 2010; Thorslund *et al.*, 2017). Using their farm assurance scheme to gain a

competitive advantage over foreign imports, as raised in the quote below, would be taking this one step further. But to achieve this, consumers need to be aware what standards UK pig farmers are required to meet and to be able to identify UK-produced product at the point of sale through better labelling than currently exists (Hubbard *et al*., 2007).

> . . . the only situation where possibly we could argue that benchmarking would have a use is when we're actually comparing our pig units with other countries . . . and that's probably a very, very good reason for actually using Real Welfare because you can actually say well we've been doing this scheme for so many years . . . so therefore if you don't have that scheme you don't have the right to sell your pigs in our country until you have that kind of a scheme to say that your pigs come up to the same welfare as our pigs do in our country. (PF4)

It is widely accepted that farmers and the public have differing perceptions of animal welfare (Te Velde *et al*., 2002; Vanhonacker *et al*., 2008; Sørensen and Fraser, 2010; Cornish *et al*., 2016). Farmers tend to define welfare as health and productivity and consequently see it as the basis of their job (Mullan *et al*., 2010). Consumers are often perceived by farmers as well intentioned but ill-informed with little understanding of farming (Hubbard *et al*., 2007), although it should be recognised that views are far from uniform even within each stakeholder group (Sørensen and Fraser, 2010). The public's lack of knowledge about farming was also seen as an area that needed to be addressed:

> I appreciate the need to demonstrate to the public that what we do is, for want of a better word, kosher. I think that we should spend a lot more time educating people as to what's involved in producing their food. (PF15)

But the farmers in this study, similar to those of other studies (e.g. Hubbard *et al*., 2007), doubted whether consumers were really willing to pay for the welfare improvements they advocated even if they really understood what happened on farm:

> In theory farm assurance is there for our customers. Is it giving them reassurance? I am not convinced that it is because people aren't seeing the Red Tractor logo and saying I must go and buy that because I know that that's been well looked after. What they are looking at is the bit that's got the pound symbol in front of it . . . (PF15)

One farmer encapsulated this very concisely 'would it sell more pork? This is the question' (PF1). His feeling was that until consumers were made aware of the welfare standards UK pig farms had to adhere to and were prepared to pay a higher price for pig products produced in the UK, Real Welfare and FAS in general have little financial benefit for the farmer.

Consumer willingness to pay for higher welfare products is often overestimated in the published literature, thought to be largely due to publication bias with studies reporting non-significant findings or low willingness to pay either not being submitted or otherwise excluded from publication (Clark *et al*., 2017). Consequently, farmer opinion may be a more accurate reflection of consumer willingness to pay than the published literature which as a whole considerably overestimates how prepared consumers are to pay for highly priced products on welfare grounds alone.

We have to recognise that one of the main incentives for farmers to participate in FAS is financial, including improved market access and better prices (Bock and van Huik, 2007). One possible way of achieving this would be to use engagement with Real Welfare as a selling point for UK pig products rather than purely as a data source for the industry. Although it was positive to note that none of the farmers interviewed raised the concerns voiced in other studies that an industry-level data set had relatively little value and may actually be used against farmers rather than for them (van Dijk *et al*., 2018). However, to utilise participation in Real Welfare to provide a marketing advantage for pork products requires further action by the industry and the retailers. Consumer understanding of the current labelling schemes for animal products that are produced by production systems with differing welfare standards is known to be poor (Ellis *et al*., 2009). Alongside this is the growing awareness that current labelling schemes do not go far enough to distinguish between products produced under FAS with differing welfare standards to enable informed consumer choice, which has led to calls for improved labelling standards (e.g. the 'Labelling Matters' coalition comprised of Compassion in World Farming, Eurogroup for Animals, the RSPCA and the Soil Association formed in 2011and Compassion in World Farming's 'Honest Labelling' campaign launched in 2018). A combination of consumer education and better product labelling is required before farmers are likely to receive any financial recompense for their participation in a scheme driven by public concern but seemingly unsupported by consumer purchasing behaviour.

Inclusion criteria and compliance. Farm assurance schemes need to negotiate a fine balance whereby their standards are stringent enough not to allow all farmers entry, yet are achievable (Main *et al*., 2001). Many of the farmers we spoke to openly questioned the value of the Red Tractor FAS, and within it Real Welfare, given that farmers rarely heard of anyone being suspended or asked to leave the scheme due to non-compliance and poor welfare:

> I don't believe that almost anybody has ever been thrown out of Red Tractor or they are very very few and I think that there are some systems, some sites that maybe don't reach the standards but it actually makes it tougher to defend the industry while we have an assurance scheme that there's not enough people being thrown out of. (PF11)

While some farmers mentioned that anyone can stay on the scheme consequently devaluing it, others had experienced the consequences of non-compliance, or had heard about farmers who had. One farmer who had had their

licence suspended voiced serious concerns about the way the process was handled.

> [It is] really archaic and draconian the way that they enforce that 30 day thing . . . there needs to be a bit more understanding, understanding between the farm assurance body and the farm and the abattoir and you know a bit of give and take there really. (PF4)

Farmers also felt that FAS should be more than just a means of setting a minimum standard and should go further to encourage and recognise the 'good' farmers:

> It's about us belonging to these bodies, which I really question what they are actually doing over and above policing the bad ones and helping to raise those standards. They're not genuinely helping the good ones. (PF10)

Just how this could be achieved in practice was uncertain, particularly regarding welfare. While the possibility of a grading system within each FAS was raised by some farmers, others felt that welfare is not an area that could or should be graded:

> All things deserve good welfare it's not some should have better than others. (PF11)

The Real Welfare assessment procedure. As reflected in the discussion above there seemed to be a degree of confusion about what the Real Welfare scheme was actually intended to do:

> . . . what message are we trying to get across? . . . it's a complete failure on the part of farm assurance schemes and people who are asking for them, to explain what they are after. (PF15)

There seemed to be a lack of clarity on whether the scheme was intended to benefit the farmer, the industry or both parties. A significant body of work went into developing Real Welfare and identifying the most appropriate informative measures to include (e.g. Mullan *et al.*, 2009a, 2009b, 2010). However, there is typically a trade-off between the original design and intention of a WOA protocol and the changes that have to be made to make it feasible to roll out on farm at an industry level (van Dijk *et al.*, 2018) and the results of this trade-off may compromise how the value of the assessment is perceived:

> I think that the problem with the Real Welfare has been they have ended up half arsing it so to speak. In its full incantation as it was meant or originally designed, it was gonna be very very time consuming and I think that's why it got watered down and I think that it's been watered down so far that it's more or less not fit for purpose. (PF8)

Some farmers took a more positive view than PF8, considering that while Real Welfare as it stands is 'far from the finished article' (PF11), it is something that can be developed and refined.

Suggested improvements to the scheme included closer working relationships between FAS and vets in the development and setting the requirements of FAS. Vets play a fundamental role in the promotion and safeguarding of animal welfare (Cornish *et al.*, 2016) and as discussed above, vet buy-in is critical for farmers to engage with, and value, FAS.

Another suggestion raised was the possibility of reducing the rate of Real Welfare inspections now that the baseline data have been collected and analysed:

> It creates cost and I am not sure having established the results that we need to maintain it at the same level. I think periodic with welfare inclusion at a veterinary visit would be fine, but I am not at all sure that it needs to be done with such regularity, especially on those farms where standards are high. (PF14)

Whether high welfare standards can be maintained in the absence of regular external inspections is something that needs to be evidenced.

Conclusions

Pig farmer perspectives of Real Welfare, the welfare outcome component of their farm assurance scheme, revealed a number of positive aspects alongside areas where improvement is required. For the majority of farmers, the value of the Real Welfare process lies with the vet who conducts the assessment. Farmers whose vet valued the process engaged them in the assessments and discussed results with them felt that they had benefitted from Real Welfare; farmers whose vets did not see the value of Real Welfare did not perceive any benefit of the process themselves. Improving the commitment of vets to the scheme is likely to also increase engagement of their farmer clients and part of this move could be to give the vets a greater role in the future refinement of the Real Welfare assessment protocol. Our findings highlight the extent of disconnect and paucity of communication between the various stakeholders involved. This is manifested at numerous levels – between vets and farmers, the FAS and vets, the FAS and farmers, the FAS and retailers, farmers and consumers, consumers and retailers. Overcoming this high level of disconnect is critical for greater acceptance of the scheme and realisation of practical and financial benefits. Improved communication at each of these stages is key to achieving this. In particular, enhanced communication and dialogue between FAS stakeholders, vets and farmers would benefit vet and farmer buy-in to the scheme as they would be able to understand the underlying decision-making process rather than dismiss the resultant assessment protocol requirements as ill-informed. While the benefits of Real Welfare for the industry were acknowledged by some farmers, more tangible benefits need to be realised for farmers at the level of the individual. This could be achieved in part through raising awareness of the welfare standards of UK pig production outside of the industry, to consumers and the general public, hopefully leading to greater appreciation of UK pig production standards and resulting in an increase in financial reward for farmers.

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Declaration of interest

The authors declare no conflicts of interest in this article.

Ethics committee

The study was approved by the University of Bristol Faculty of Health Sciences Faculty Research Ethics Committee in February 2017.

Software and data repository resources

None of the data were deposited in an official repository.

Supplementary material

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animal

The effect of calf jacket usage on performance, behaviour and physiological responses of group-housed dairy calves

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*Poor performance and ill-health of calves in the pre-wean period can affect future productivity. Increasing numbers of producers are opting to use calf jackets as a means of mitigating the potential negative effects of low ambient temperatures, wind speed and precipitation on growth and health. This study aimed to use a range of noninvasive monitoring technologies to investigate the effects of using calf jackets in the first 3 weeks of life on calf performance and behavioural and physiological parameters. Ninety Holstein-Friesian calves were allocated to one of the two treatments: (i) Jacketed until 21 days of age and (**J**; n = 44) ii. Nonjacketed (**NJ**; n = 46). Calves were group housed and fed milk replacer (**MR**) and concentrate solid feed via automatic feeders. Calves were weaned at day 56, and the experiment was completed at day 63. Health assessments were conducted on a daily basis throughout the experiment using predefined faecal and respiratory scoring protocols. A range of novel, noninvasive monitoring technologies were used to examine the activity, heart rate and thermal profiles of calves on an individual basis throughout the experimental period. There were no differences in calf live weight (**LWT**), average daily gain (**ADG**) or feed conversion efficiency (**FCE**) in J and NJ calves between days 5 to 20. However, NJ calves consumed more MR and had more unrewarded visits to the milk feeder than J calves during this period. Although calf LWT was comparable across treatments in the week following jacket removal (days 21 to 28), both ADG and FCE tended to be greater in NJ calves. There were no treatment differences in calf LWT at the end of the study (d63). When measured over a period of 24 h and at a mean ambient temperature of 7.7°C, skin surface temperature was 6.37°C higher in J calves. Core body temperature was higher in J calves between days 5 to 20; however, there were no differences in IR eye or IR rectal temperature. No differences in lying behaviour occurred, with calves spending 18 and 17 h/day lying between days 5 to 20 and days 21 to 28, respectively. Under the climatic and management conditions described, no significant benefits to calf performance were found as a result of the provision of calf jackets to group-housed calves in the first 3 weeks of life. The higher frequency of unrewarded visits to the milk feeder in NJ calves during the first 3 weeks of life could be suggestive of a lack of satiety in these calves.*

Keywords: early calfhood, lower critical temperature, thermoregulation, monitoring technologies, thermo neutral zone

Implications

In low ambient temperatures, neonatal dairy calves must expend increased energy to maintain core body temperature, which may impact on performance and health. Calf jackets have been suggested as a method of reducing heat loss and mitigating the effects of fluctuations in environmental conditions. Under the climatic and management conditions in this study, provision of calf jackets in the first 3 weeks of life provided no major benefits to calf performance. However, calf growth and feed conversion efficiency was reduced in the week following jacket removal, suggesting that producers should leave jackets on beyond the third week of life.

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Introduction

Calves are born with functional yet underdeveloped thermoregulatory mechanisms, with rhythmicity of body temperature developing and stabilising during the first two months of life (Piccione *et al.*, 2003; Roland *et al.*, 2016). Due to a relatively high surface/mass-ratio and poor tissue insulation, newborn dairy calves are particularly prone to heat loss and susceptible to the effects of cold ambient temperatures (Olson *et al.*, 1980b). Calves exposed to cold ambient temperatures at birth have shown a reduced absorptive ability of colostral immunoglobulins (Olson *et al.*, 1980a), which can have a direct impact on the development of immunocompetence. The thermo neutral zone (**TNZ**) is defined as

the range of ambient temperatures at which temperature regulation is achieved only by control of sensible heat loss, that is, without regulatory changes in metabolic heat production or evaporative loss (IUPS Thermal Commission, 2001). In ambient temperatures below the lower critical temperature (**LCT**) of the TNZ, calves must increase metabolic heat production to maintain thermal balance (Carstens, 1994). In calves, the LCT has been reported as ranging from +13° C at birth to +8°C at 20 days of age (Gonzalez-Jimenez and Blaxter, 1962); however, this is dependent on calf breed, age, weight, nutrient intake, posture and prevailing environmental conditions. When maintained in ambient temperatures considered to be below the LCT, calves have been shown to increase feed intake (e.g. Nonnecke *et al.*, 2009) and alter lying duration and position (e.g. Hepola *et al.*, 2006). Furthermore, changes in physiological processes such as vasoconstriction and tissue insulation have been reported in perinatal calves bedded on substrates of differing thermal properties (Sutherland *et al.*, 2013) and in those maintained in sub-zero temperatures (Rawson *et al.*, 1989a). Thermic stress as a result of temperatures above or below that of the TNZ can result in economic losses due to increased morbidity and mortality, and negative impacts on calf performance (Roland *et al.*, 2016). Mitigating the effects of environmental factors such as ambient temperatures below the LCT, wind speed and precipitation are therefore of prime concern to producers. To limit the effects of cold ambient temperatures, reducing the potential for external heat loss is extremely important. Early work involving Holstein calves housed in sub-zero temperatures in the first 14 days of life indicated that provision of insulated jackets contributed 52% of the total insulation effect (Rawson *et al.*, 1989a). This highlights the potential of calf jackets as a means of providing a physical barrier to the effects of heat loss. Recently, anecdotal evidence has suggested that an increasing number of producers are opting to incorporate the use of calf jackets into their youngstock management system, with expected potential benefits to both calf performance and health. Although Earley *et al.* (2004) examined the effects of using calf jackets on performance and health, they have been the subject of very little controlled research, especially in terms of the potential impact on behavioural and physiological parameters. Additionally, the calves used by Earley *et al.* (2004) were already over two weeks of age when jackets were provided, as such information regarding their effectiveness when used from the first days of life is lacking. Developments in on-farm monitoring technologies are providing increased opportunities to provide robust and reliable behavioural measures of individual animals in a remote, noninvasive manner (Rushen *et al.*, 2012). It has also been suggested that measuring multiple behavioural and physiological aspects of individual animals could provide a more reliable indicator of overall animal well-being than just a singular measure (Theurer *et al.*, 2013). The aims of this study, therefore, were to (a) investigate the impact of providing calves with jackets in the first 3 weeks of life on behavioural and physiological parameters using a range of novel remote-sensing technologies and (b) determine the efficacy of calf jackets in mitigating the impact of low ambient temperatures on growth and health parameters.

Materials and methods

The study was conducted at the Agri-Food and Bioscience Institute (**AFBI**) research farm in Hillsborough, located in County Down, in Northern Ireland (latitude 52°27′, longitude 6°4′). All procedures and treatments within this study were conducted under the United Kingdom Animals (Scientific Procedures) Act 1986.

Animals

Ninety Holstein Friesian calves (46 females and 44 males) born between 16 September and 21 November 2016 were allocated to the study following weighing at ≤12 h of age, which was used as the birth weight. All calves received 3 l colostrum via oesophageal feeding tube or treated bottle before transfer to the calf-rearing unit.

Treatments and experimental design

On entering the rearing accommodation at ≤12 h of age, calves were individually penned and allocated one of two treatments: (i) Jacketed until 21 days of age and (J; $n = 44$) and (ii) Nonjacketed (**NJ**; $n = 46$). Jackets were breathable and water repellent, with an outer shell constructed with 600 denier Oxford fabric, a 200 g filling and a 210 denier lining (Cosy Calf, Dorset, UK). Calves were balanced across treatments for sex, and average birth weight was 40.4 (SD ± 4.7) and 40.6 kg (SD ± 3.8) for J and NJ calves, respectively. The study was completed when calves reached 63 days of age.

Housing and diet

Calves were fed 2 l colostrum twice daily via a treated bucket on days 1 and 2. At 4 days of age, calves were provided with two feeds, each of 2 l, consisting of a mix of half colostrum/half milk replacer (**MR**) after which all feeds were of a 26% CP, 17% fat MR (Volac International Ltd, Hertfordshire, UK). At 5 days of age, calves were introduced to one of six replicate straw bedded pens of 15 calves. Each group pen was balanced for calf sex, birth weight and treatment. Calves were fed via automatic milk and concentrate feeders (Forster Technik Vario, Engen, Germany). Each pen had one milk feeding station equipped with one teat and one automatic concentrate feeder equipped with one feeding station. Milk replacer was offered at a rate of 150 g/l using a step-up/step-down program, with MR volume increasing from 4 to 5.1 l/day between days 5 and 9 and reducing from 5.1 to 2 l/day between days 33 and 55 with weaning at 56 days of age. Potential metabolisable energy (**ME**) intake from MR in calves consuming 5.1 l/day was 14.42 MJ/day. Calves had *ad libitum* access to concentrate starter feed via automatic concentrate feeders, *ad libitum* straw from racks and free access to fresh water.

Table 1 *Chemical composition of MR, concentrate and straw offered to J and NJ calves throughout the experimental period*

	Milk replacer	Concentrate	Straw
DM (g/kg)	948	954	931
Nitrogen (g/kg DM)	40.5	32.2	5.8
NDF (g/kg DM)	–	273	875
ADF (g/kg DM)	–	131	537
Ash (g/kg DM)	74.6	65.5	39.0
Ether extract (g/kg DM)	170	32	12
Gross energy (MJ/kg DM)	20.9	18	18.8
ME (MJ/kg DM)[1]	18.86	–	–

MR = milk replacer; J = jacketed; NJ = nonjacketed; ME = metabolisable energy.
[1] ME estimated based on MR containing 26% CP, 17% fat and 7.5% ash. On this basis, calves in the present study consuming maximum allowance of 5.1 l/day at the rate of 150 g/l would receive 14.42 MJ/day from MR alone.

Data collection

Ambient temperature. A calibrated EBI 20-TH data logger (ebro Electronic, Ingolstadt, Germany) was situated within each group pen, suspended at approximately 1.5 m above pen floor height for the duration of the experimental period. Ambient temperature was recorded automatically every 15 min throughout the experimental period. Wind speed was not recorded; however, the rearing accommodation was such that it was mechanically ventilated using a fan and duct system, and the side walls were solid, this minimising the potential for drafts at calf height.

Feed nutrient composition, feed intake and calf performance. Samples of MR, starter ration and fresh straw bedding were collected on a daily basis and bulked for each 2-week period throughout the experiment, with samples analysed using the methods as described by Cushnahan and Gordon (1995). Chemical composition of feedstuffs is presented in Table 1. Individual daily milk and concentrate intake was recorded via automatic feeder between 5 and 62 days of age. Individual drinking speed and number of rewarded (MR received) and unrewarded (no MR received) visits were recorded via automatic milk feeder. In addition to this, individual calf feeding behaviour in terms of duration and timing of visits were recorded by both the automatic milk and concentrate feeders. Calf live weight (**LWT**) was recorded automatically on a daily basis via the half BW scales linked to the feed station of the automatic milk feeder. Live weight was also recorded using a manually operated calibrated weigh bridge (Tru-Test Eziweigh 5, Auckland, New Zealand) at birth, days 56 and 63.

Calf health. Faecal consistency was qualitatively scored throughout the study on a daily basis during morning feeding time using the scale of 1 = normal consistency, 2 = slightly liquid consistency, 3 = moderately liquid and 4 = primarily liquid consistency (Quigley *et al.*, 2006). A calf was recorded as having diarrhoea when the score was greater than 2 (Quigley *et al.*, 2006). Respiratory disease scoring, with the exception of rectal temperature, was carried out on a daily basis using the University of Wisconsin-Madison method (McGuirk and Peek, 2014). This involved scoring calves on three visual aspects including eyes, ears and nasal discharge and on the presence or absence of a cough. Each aspect received a score from 0 to 3, with 0 representing normal and 3 the most severely affected. The overall respiratory score was derived from the cumulative score of each aspect. Faecal and respiratory scoring were carried out by the same trained technician. Cases of calf ill health were assessed on an individual basis and treatment was administered and recorded according to predefined protocols provided by a veterinarian.

Calf activity. IceRobotics® IceQube® automatic activity sensors (IceRobotics Ltd., Edinburgh, Scotland, UK) were fitted to the right rear leg of 18 calves per treatment ($n = 36$) between 5 and 60 days of age using the methodology as described by Finney *et al.* (2018). A filter that removed sensor recordings lasting ≤8 seconds was applied to data prior to analysis (Finney *et al.*, 2018). For the purposes of this study, only data recorded between days 5 and 28 will be reported.

Thermal imaging. Thermal images of the same 36 calves that were fitted with activity meters were taken at approximately 1000 h on 5 days/week between 5 and 60 days of age. Images were taken by a trained operator using a calibrated FLIR E8 camera (FLIR Systems UK, Kent, UK) and were of the right eye (plus a 1-cm area surrounding the eye) and the anus (plus a 1-cm area surrounding the anus). Images were taken at a consistent distance (~0.5 m) and angle (~90°) while the calf was standing and prior to introduction of any potential stressors such as weighing. Core body temperature of each of these calves was taken on a daily basis using a rectal thermometer immediately following image capture. Images were processed with FLIR® software (FLIR Systems UK, Kent, UK) using the methodology as described by Scoley *et al.* (2018).

Heart rate monitors. Heart rate (**HR**) monitors (Polar Equine RS800CX Science, Polar Electro UK Ltd, Heathcote Way, Warwick, UK) were fitted to 16 J and 15 NJ calves on days 15 to 20. Calves were selected from those that had also been fitted with activity sensors to ensure that HR data obtained was from a period in which the calves were at rest. Approximately 1 h after ensuring that all calves had visited the automatic milk feeder, selected calves were thermal imaged and then moved to the corner of their group housing pen and penned in with hurdles. On the first day of measurements, each calf had a 5-cm wide strip shaved to skin level on the left-hand side directly behind the shoulder to help improve contact between the calf and monitor. Electrode gel (Spectra 360 Electrode Gel, Parker Laboratories Inc., New Jersey, USA) was applied to the electrode belt prior to each use, this again to ensure contact between the calf and monitor (Clapp *et al.*, 2014). Once fitted, monitors remained on calves for a minimum of 1 h. Recordings of 5-to-10-min duration were processed using both the

Artiifact and Polar software (Kaufmann *et al.*, 2011). Preliminary error correction of the data set was conducted using the Polar software settings as described by Clapp *et al.* (2014) with any data set requiring over 5% error correction rejected (Stewart *et al.*, 2009). Following error correction, the data set was further processed using Artiifact software with resultant HR used in the analysis.

Skin temperature. In a small-scale prospective pilot study, data loggers (Thermochron iButton, model DS1921H-F5#, range 15.0ºC to 46.0ºC, accuracy: ±1ºC, Maxim Integrated, CA, USA) were affixed to nine calves per treatment ($n = 18$) at an average of 18.9 (SD ± 10.9) days of age to record continuous measurement of the skin surface temperature over a 24-h period. To attach the data logger, a methodology based on that described by Sutherland *et al.* (2013) was employed. A small area of hair (~3 cm^2) approximately 2 cm to the right-hand side of the spine at the level of the last rib was clipped to the skin level. This anatomical location was chosen as it was deemed to be the area that would be least affected by the lying position and represent the area most exposed to ambient temperature. The data logger was then placed on the area of skin and covered with a 5 cm^2 of breathable, elastic adhesive bandage (Elastoplast®, Beiersdorf UK, Birmingham, UK). The outer edges of the bandage were further secured to the unclipped hair using glue (KAMAR®, Kamar Products, Inc., Zionsville, IN, USA). The data loggers were set to record temperatures every 10 min during the 24-h period. A data logger (Thermochron iButton, model DS1921G-F5#, Maxim Integrated, CA, USA) was affixed to the outer wall of the group pen in order and was set to record temperature every 10 min over the same 24-h period in which skin temperature was recorded for each of the 18 calves. In addition to this temperature and relative humidity data loggers (iButton Hygrochron DS1923-F5#, temperature range −10ºC to +65ºC, accuracy ±0.5ºC, relative humidity range 0% to 100%, resolution 0.6%, Maxim Integrated, CA, USA) were fitted to a further four calves from the J treatment in the 3 days preceding and following jacket removal. These data loggers were affixed using the same method and with the same recording parameters as previously described.

Statistical analysis. All data were analysed using GenStat® (version 16.2, VSN International Ltd). All statistical models included birth weight as a covariate and housing block as a random term unless otherwise stated. Adequacy of statistical models was evaluated using visual assessment of residual plots. Means predicted from the mixed effects model are presented. Where presented as days 5 to 20 (Period 1), the time during which jackets were worn, and days 21 to 28 (Period 2), the week following jacket removal, data was analysed as two separate periods with a covariate derived from the average value of the last 5 days of Period 1 included in the analysis of Period 2 data.

Calf LWT, daily intakes, proportion of daily MR allowance consumed, drinking speed, measures of temperature and daily lying time were fitted to a repeated measures residual maximum likelihood estimation (**REML**) model with effects of sex, age, treatment and the interaction of treatment x age included. To assess lying duration and occupation of the concentrate and milk feeders per hour, data were averaged on an hourly basis for each calf over each period. Data were fitted to a repeated measures REML model with effects of sex, age, treatment and the interaction of treatment x day or hour of day included. Heart rate data was averaged for each calf over days 15 to 20 and fitted to a mixed-effects model with fixed effects of sex and treatment. Live weight gain, total concentrate intake, total MR intake, time spent per day in the feeders, average faecal and respiratory scores were analysed using a mixed-effects model with fixed effects of sex and treatment. Fixed effects were assessed by comparing the *F*-statistic against an appropriate *F* distribution.

Visits to the automatic milk feeder with and without reward and number of lying bouts were fitted to a generalised linear mixed model (**GLMM**) with poisson distribution with effects of sex, age, treatment and the interaction of treatment x age included. Mathematical equations of the repeated measures REML model and the GLMM are reported in the Supplementary Material as Supplementary Equations S1 and S2, respectively.

Results

Ambient temperature

Mean ambient temperature within the calf rearing accommodation throughout October, November, December and January, respectively, was 11.7°C (5.3°C to 17.4°C), 7.5°C (1.2°C to 15.8°C), 8.9°C (2.8°C to 15.2°C) and 7.2°C (1°C to 12.7°C). During the months of October, November, December and January, respectively, 22.6%, 80.8%, 67.7% and 80.4% of ambient temperature measurements recorded were ≤10°C. A total of 61.4% of ambient temperature measurements recorded across the experimental period were ≤10°C.

Feed intake and calf performance

Between 5 and 20 days of age, NJ calves consumed 92.3% of their daily allowance of MR compared with 88.5% in J calves ($P = 0.005$). Total dry matter intake (**DMI**) was increased by 0.53 kg DM in NJ calves during days 5 and 20 (Table 2; $P = 0.016$); however, there was no difference between days 21 and 28. Total DMI between days 5 and 55 was comparable across treatments, with J and NJ calves consuming 47.9 and 47.7 kg DM, respectively (Table 2; $P = 0.966$). Live weight was comparable across treatments during days 7 to 20 (Table 3; $P = 0.741$) and days 21 to 28 (Table 3; $P = 0.214$). Average daily gain (**ADG**) was 0.48 and 0.49 kg/day, respectively, for J and NJ calves during days 7 to 20 (Table 3; $P = 0.732$); however, NJ calves displayed a 0.12 kg/day advantage over J calves between days 21 to 28 (Table 3; $P = 0.011$). Feed conversion efficiency (**FCE**) in terms of kg gain/kg DMI was also equivalent across

Table 2 *Daily and total intakes of J and NJ calves between days 5 and 55*

	Treatment			
	J	NJ	SED	*P*-value
Daily intake d5-20				
MR (l/day)	4.39	4.58	0.068	0.008
Concentrate (g DM/day)	21.5	25.8	3.19	0.167
Daily intake (days 21 to 28)				
MR (l/day)	5.04	5.04	0.032	0.853
Concentrate (g DM/day)	70.3	83.9	11.35	0.249
Total DMI days 5 to 20 (kg DM)	9.54	10.07	0.213	0.016
Total DMI days 21 to 28 (kg DM)	6.51	6.59	0.121	0.495
Total DMI days 28 to 55 (kg DM)	31.54	30.91	2.177	0.802
Total DMI days 5 to 55 (kg DM)	47.85	47.66	2.332	0.966

J = jacketed; NJ = nonjacketed; SED = standard error of difference; MR = milk replacer; DMI = dry matter intake.

Table 3 *Live weight, ADG and FCE of J and NJ calves throughout the experimental period*

	Treatment			
	J	NJ	SED	*P*-value
LWT (kg)				
Days 7 to 20	45.7	45.8	0.60	0.741
Days 21 to 28	51.2	52.3	0.67	0.214
Days 28 to 55	62.1	63.9	0.87	0.602
Day 56	72.6	74.7	1.19	0.072
Day 63	81.0	83.3	1.41	0.105
ADG (kg/day)				
Days 7 to 20	0.48	0.49	0.046	0.732
Days 21 to 28	0.49	0.61	0.048	0.011
Days 28 to 55	0.67	0.69	0.028	0.441
Days 0 to 56	0.57	0.61	0.041	0.072
Days 56 to 63	1.19	1.22	0.085	0.761
FCE (kg gain/kg DMI)				
Days 7 to 20	0.65	0.65	0.064	0.991
Days 21 to 28	0.55	0.64	0.048	0.069
Days 28 to 55	0.64	0.68	0.045	0.385
Birth to day 56	0.64	0.68	0.030	0.205
Birth to day 63	0.63	0.66	0.030	0.266

ADG = average daily gain; FCE = food conversion efficiency; J = jacketed; NJ = nonjacketed; SED = standard error of difference; LWT = live weight; DMI = dry matter intake.

treatments between days 7 and 20 (Table 3; $P = 0.991$); however, as with the ADG, this tended to be increased in NJ calves during days 21 to 28 (Table 3; $P = 0.069$). Weekly LWT was unaffected by treatment (Figure 1; $P = 0.110$) throughout the experimental period. Weaning weight and ADG between birth and weaning tended to be increased in NJ calves compared with J calves (Table 3; $P = 0.072$); however, there were no differences in LWT at the end of the experiment on day 63 (Table 3; $P = 0.105$) or in ADG between days 56 and 63 (Table 3; $P = 0.761$). There were no differences between treatments in LWT, ADG or FCE between days 28 and 55 (Table 3; $P > 0.1$). There was no

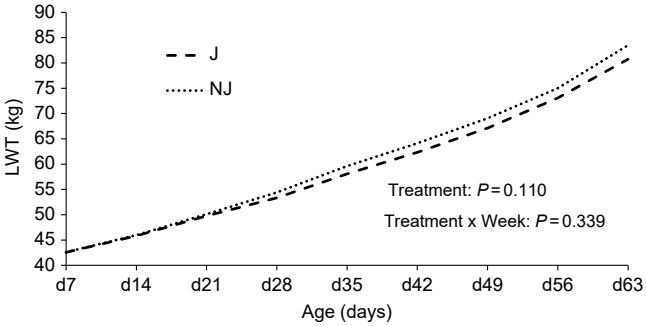

Figure 1 Weekly LWT of J and NJ calves as measured via automatic half BW scales. LWT = live weight; J = jacketed; NJ = nonjacketed.

effect of treatment on FCE between birth to weaning or birth to day 63 (Table 3; $P > 0.1$).

Automatic feeder behaviour

Drinking speed was increased by 57.6 ml/min in NJ calves between days 5 and 20 ($P = 0.017$); however, there was no difference between treatments during days 21 to 28 ($P = 0.270$). Visits to the milk feeder without reward were 19.2% and 11.2% higher in NJ calves than J calves between days 5 and 20 (Figure 2; $P < 0.001$) and days 21 and 28 (Figure 2; $P = 0.006$), respectively. During days 5 to 20, J and NJ calves spent 7.46 and 6.65 min drinking MR, respectively ($P = 0.021$); however, there were no differences in drinking time between days 21 and 28 ($P = 0.281$). During days 21 and 28, J and NJ calves increased time spent per day in the concentrate feeder by 168% and 195%, respectively, when compared with days 5 to 20. There was no difference in total duration of time/day in the concentrate feeder, with values of 16.5 and 17.2 min/day for J and NJ calves ($P = 0.710$).

Lying behaviour

Total daily lying time was unaffected by treatment, with calves spending 18 and 17 h/day lying down during days 5 to 20 and 21 to 28, respectively ($P > 0.1$). There was no effect of treatment x hour on lying time between days 5 and 20; however, there was an effect of hour of day with calves spending less time lying down at 0800 h (Figure 3; $P < 0.001$). Lying time was again affected by hour of the day between days 21 and 28, with decreases in lying time observed between 0600 and 1000 h and 1400 and 1700 h (Figure 3; $P < 0.001$).

Calf health and physiological parameters

There were no treatment differences in either average faecal or respiratory score during the first 4 weeks of life (Table 4; $P > 0.1$). Core body temperature tended to be increased in J calves between days 5 and 20; however, this difference was minimal, representing only a 0.2% increase when compared with NJ calves (Table 5; $P = 0.057$). No treatment differences in either IR eye or rectal temperature were observed during days 5 to 20 or 21 to 28. At an average ambient temperature of 7.7°C (5.6°C to 10.8°C) as measured over

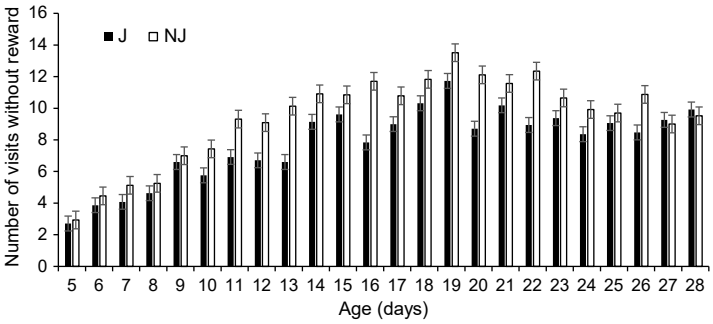

Figure 2 Number of visits to the milk feeder without reward of J and NJ calves between days 5 and 28. Error bars represent SEM. J = jacketed; NJ = nonjacketed.

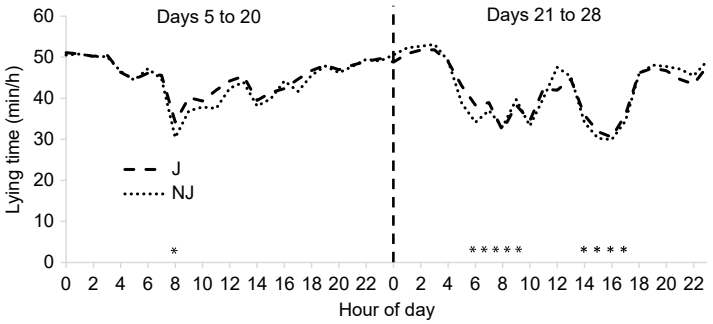

Figure 3 Hourly lying behaviour of J and NJ calves during days 5 and 28. * Represents a significant effect of hour of the day on lying time ($P<0.001$). J = jacketed; NJ = nonjacketed.

a 24-h period from 0600 h to 0550 h, skin temperature of J calves was 6.37°C higher than that of NJ calves, with mean values of 35.32°C and 28.95°C, respectively (Figure 4; $P<0.001$). There was no effect of hour of day on skin temperature (Figure 4; $P = 0.352$), which remained fairly constant throughout the day despite a rise in ambient temperature in the late morning/early afternoon. Removal of jackets had no effect on relative humidity (%) at the skin surface ($P = 0.488$). Mean ambient temperature was consistent in the 3 days prior to and following jacket removal, measuring 8.3°C and 8.5°C, respectively; however, skin surface temperature decreased by 6.14°C the 3 days following jacket removal ($P<0.001$). Heart rate was 98.5 and 95.9 beats per minute (**bpm**) for J and NJ calves, respectively ($P = 0.540$) during the week prior to jacket removal.

Discussion

The LCT of dairy calves is between 8°C and 10°C in the first few weeks of life (Webster *et al.*, 1978); however, this can vary in relation to housing, nutrition and breed (Young, 1981). In the present study, during which 61.4% of ambient temperature measurements were recorded as being ≤10°C, calves of the same breed type, housed in the same environment and offered the same MR level were either provided or not provided with calf jackets for the first 3 weeks of life.

Calf performance and feeding behaviour

Nonnecke *et al.* (2009) reported similar ADG between calves housed in warm or cold environments despite increased grain consumption in cold-housed calves. Similarly, NJ calves in the present study displayed an increased DMI with no resultant improvements in either ADG or FCE (kg gain/kg DMI). Additionally, frequency of unrewarded visits to the milk feeder in the first 3 weeks of life was higher in NJ calves, which is considered to signify that calves have not reached satiety (Byrne *et al.*, 2017). As indicated by the NRC 2001, the maintenance energy requirement for calves ≤3 weeks of age and weighing 40 kg increases from 8.45 to 10.25 MJ/day as the ambient temperature decreases from ambient temperatures of 10°C to 0°C. In the present study, the increase in both feed seeking behaviour and energy intake in NJ calves was likely due to the demands of increased metabolic heat production and maintenance energy requirements as a result of the low ambient temperature.

In the present study, both ADG and FCE were greater in NJ calves between days 21 and 28 when compared with previously J calves. This suggests that on sudden exposure to lower ambient temperatures, energy intake was not sufficient to maintain both adequate heat production and growth in previously J calves (Roland *et al.*, 2016). During this time, all calves were being offered the maximum MR allocation, this representing a potential ME intake of 14.42 MJ/day from MR alone. The only option for increasing energy intake was to increase consumption of solid feed; however, solid feed intake was low across both treatments between days 21 and 28. Jacketed calves, therefore, likely did not increase their energy intake sufficiently to compensate for the drop in temperature of their microclimate following jacket removal. Roland *et al.* (2016) reported that animals

Table 4 *Faecal and respiratory scores of J and NJ calves in the first 4 weeks of life*

	Treatment			
	J	NJ	SED	*P*-value
Average faecal score[1]				
Week 1	1.08	1.03	0.034	0.194
Week 2	1.22	1.22	0.070	0.931
Week 3	1.06	1.08	0.037	0.518
Week 4	1.11	1.05	0.040	0.125
Average respiratory score[2]				
Week 1	0.06	0.07	0.037	0.884
Week 2	0.14	0.14	0.056	0.973
Week 3	0.19	0.13	0.054	0.293
Week 4	0.12	0.09	0.044	0.573

J = jacketed; NJ = nonjacketed; SED = standard error of difference.
[1] Faecal scoring system: 1 = normal consistency, 2 = slightly liquid consistency, 3 = moderately liquid and 4 = primarily liquid consistency.
[2] Respiratory scoring system excluding rectal temperature, whereby: 0 = normal; 3 = most severely affected.

Table 5 *Core body, IR eye and IR rectal temperature of J and NJ calves between 5 and 28 days of age*

	Treatment			
	J	NJ	SED	*P*-value
Days 5 to 20				
Core body (°C)	39.03	38.95	0.045	0.057
IR eye (°C)	38.71	38.78	0.074	0.275
IR rectal (°C)	39.64	39.50	0.106	0.155
Days 21 to 28				
Core body (°C)	38.96	38.87	0.060	0.156
IR eye (°C)	38.58	38.58	0.187	0.884
IR rectal (°C)	39.56	39.47	0.141	0.456

J = jacketed; NJ = nonjacketed; SED = standard error of difference.

continuously maintained under low temperatures show reduced lower and upper critical temperatures. Additionally, Rawson *et al.* (1989a) reported increased tissue insulation values in cold-housed calves when compared with calves fitted with jackets. The findings of the present study could therefore suggest that compared with the NJ calves, calves provided with jackets in the first 3 weeks of life had a higher LCT and potentially lower tissue insulation values, this leading to a more acute response when presented with a sudden reduction in environmental temperature.

Calf health

Thermic stress can impact negatively on calf morbidity and mortality (Roland *et al.*, 2016) with Nonnecke *et al.* (2009) reporting increased respiratory scores and antibiotic costs in calves maintained in cold environments. Furthermore, Hänninen (2003) reported longer lasting diarrhoea outbreaks in calves dairy calves housed in unheated shelters than those housed indoors, which could indicate damage and poorer recovery of intestinal villi as a result of cold stress (Cockram and Rowan, 1989). However, similar to Earley *et al.* (2004), who found no difference in incidence of either respiratory or enteric disease in calves reared outside with or without jackets, there was no difference between treatments in the present study during the first 4 weeks of life. It could be that the ambient temperatures experienced were not low enough to significantly affect health parameters. Further work could examine the effects of calf jacket usage in disease-challenged calves exposed to environmental temperatures below the LCT.

Lying behaviour

Standing increases the metabolic rate of calves (Rawson *et al.*, 1989b) and also increases the LCT, whereby in 6-day-old calves, the LCT is +13.5°C when lying and +17.5°C when standing (Schrama *et al.*, 1993). Previous research has reported increased resting time in calves housed outside compared with those housed inside (Hänninen, 2003). It could have been considered, therefore, that lying duration would have been longer in NJ calves during the first 3 weeks of life when compared with J calves. Following removal of the jackets, it could also have been expected that lying time would increase in the previously J calves. However, similar to Hill *et al.* (2013), no differences in lying behaviour parameters were found between days 5 and 28 despite low ambient temperatures. The results of the present study suggest that ambient temperature and prevailing environmental conditions within the rearing accommodation were such that calves did not have to modify their lying behaviour to conserve energy.

Physiological measurements

When temperatures fall below the LCT, in addition to behavioural responses, calves are required to maintain core body temperature through a number of physiological mechanisms such as an increase of metabolic rate, piloerection and vasoconstriction (Rawson *et al.*, 1989a). Similar to previous research by Rawson *et al.* (1989b) and Scibilia *et al.* (1987) who reported lower rectal temperatures in calves managed in ambient temperatures below the LCT, within the present study NJ calves tended to have a lower rectal temperature during days 5 to 20 when compared with J calves. However, this temperature remained within the upper end of the normal physiological range. Rectal temperature also remained within normal bounds when jackets were removed, with no treatment differences found during days 21 to 28. Additionally, there were no treatment differences in radiated temperature of the eye or rectal area during days 5 to 20 or days 21 to 28. This suggests that NJ calves were able to utilise the additional energy as a result of increased DMI to regulate core body temperature during the first 3 weeks of life.

In environments below the LCT, reduction of heat loss is key. Sutherland *et al.* (2013) reported lower skin surface temperature in calves reared on river stones compared with those reared on sawdust. As changes in skin surface temperature can reflect changes in skin blood flow in response to alterations in environmental temperature, this response was considered to be associated with greater vasoconstriction due to

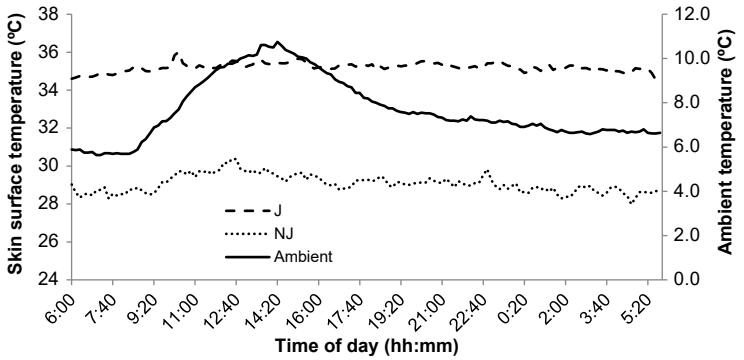

Figure 4 Skin surface temperature of J and NJ calves and ambient temperature over a 24-hour period. J = jacketed; NJ = nonjacketed.

these animals being colder. In the present study, skin surface temperature of NJ calves was 6.37°C lower than that of J calves during days 5 to 20, and skin temperature of J calves fell by 6.14°C in the 3 days following jacket removal. This provides evidence of a calf's ability to thermoregulate through vasoconstriction at the skin surface to maintain core body temperature in response to change in environmental temperatures.

Rawson *et al.* (1989b) reported an increase in average HR of up to 36 bpm in calves housed in cold environments compared with those housed in warm environments. However, in the present study, there were no differences in HR between J and NJ calves during days 15 to 20. Calves in the study by Rawson *et al.* (1989b) were housed in ambient temperatures below 0°C, which suggests that ambient temperature in the present study was potentially not extreme enough so as to produce a response in HR.

Conclusion

Under group housing and the climatic and management conditions described in the present study, provision of jackets in the first 3 weeks of life reduced number of unrewarded visits to the milk feeder; however, no significant benefits to calf performance were observed. However, it must be remembered that calves in the present study were closely monitored and housed in a well-managed system; as such, calf jacket usage may be of more benefit in systems, where calves are exposed to the effects of increased wind speed and precipitation. Average daily gain and FCE were reduced in J calves in the week following jacket removal. This suggests that calves go through a phase of acclimatisation following jacket removal and that it may be beneficial to use jackets past 3 weeks of life when calves are consuming increased amounts of solid feed, this resulting in an overall increase in ME intake. The evidence provided by skin temperature measurements highlights the ability of young calves to thermoregulate through processes such as vasoconstriction. Future research could consider the effects of duration of calf jacket usage on both heat production and energy partitioning in calves provided with varying levels of MR and maintained in low ambient temperatures. Additionally, investigating the age and environmental temperature at which physiological thermoregulatory processes such as vasoconstriction and piloerection occur under various rearing regimes could help to determine best practice guides for calf jacket use and housing conditions.

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Declaration of interest

The authors declare no conflict of interest.

Ethics statement

This project was approved by the Agri-Food and Biosciences Institute Animal Welfare and Ethical Review Body (AWERB).

Software and data repository resources

The data/models regarding the published article are not deposited in any official repository.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/10.1017/S1751731119001071

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animal

Sows' preferences for different forage mixtures offered as fresh or dry forage in relation to botanical and chemical composition

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*Providing forage to feed-restricted pregnant sows may improve their welfare by reducing their high feeding motivation. The aim of this study was to determine sows' preferences for four forage mixtures cultivated in Canada. Forage mixtures were compared when offered either fresh or dry. The four forage mixtures were composed of different proportions and species of legumes (alfalfa (**Alf**) or red clover (**Clo**)) and grasses (tall fescue (**F**) and/or timothy (**T**)): (1) Alf-F, (2) Alf-F-T, (3) Clo-T and (4) Clo-F-T. Voluntary intake was measured, and preference tests were carried out for two experiments: one in spring for fresh forages (*n = *8) and the other in autumn for hays (*n = *8) with different sows housed in individual pens and fed a concentrated diet meeting their nutritional requirements for maintenance and foetal growth. Voluntary intake was measured by offering each forage mixture separately (one forage mixture/day) during 90 min according to a 4 × 4 Latin square design replicated four times. During preference tests, all six combinations of two forage mixtures were offered once (one combination/day) for 45 min to each sow. Individual forage intake was measured, and feeding behaviour was observed. Forages were analysed for botanical and chemical composition. Difference in voluntary intake among the four forage mixtures was determined using a variance analysis followed by Tukey tests for post hoc comparisons. In preference tests, differences between the two forage mixtures offered were determined using a paired Student's* t *test, and the most ingested forage mixture was considered the preferred one. Results from both experiments revealed clear preferences for some forage mixtures when offered either fresh or dry. Forage mixtures with a greater proportion of legumes (AlfT and CloT) were preferred over forage mixtures with a higher proportion of grasses (AlfFT and CloFT). The AlfFT and CloFT forage mixtures contained at least 30% of fescue; therefore, the greater preference for the AlfT and CloT forage mixtures could also be due to the absence of fescue. Sows preferred forages with low DM and NDF concentrations and high CP and non-structural carbohydrates concentrations. Based on results from previous studies, the preferences seen in the present study are most likely due to the greater proportion of legumes, although an effect of tall fescue in preference cannot be excluded. Therefore, offering forages with a high proportion of legumes would be a good strategy to maximise both fresh and dry forage intake in pregnant sows.*

Keywords: *Sus scrofa*, feeding preferences, forage intake, legumes, neutral detergent fibre

Implications

Studies suggest that providing forage to pregnant sows improves their health and welfare. Hence, increasing the intake of forages by sows could improve performance as well. Reliable information is required on the types of forages that should be offered to sows to increase both fresh and dry forage intake, particularly in alternative breeding systems that provide more possibilities of giving access to forages than conventional systems (e.g. fresh forages in pasture-based systems). The results revealed that forages rich in legumes are preferred by sows.

Introduction

Sows are usually feed restricted during gestation to avoid obesity that can alter their reproductive performance due to farrowing and locomotion disorders (Dourmad *et al.*, 1994, Trottier *et al.*, 2015). Sows are commonly fed once or twice a day with a concentrate diet that is rapidly consumed. Stereotypic behaviours and, more specifically, oral stereotypies such as bars biting, chain manipulation

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and "sham" chewing have been observed in feed-restricted sows after feed consumption. The occurrence of those behaviours is negatively correlated with feed allowance (Lawrence and Terlouw, 1993), suggesting a frustration of their feeding motivation because sows did not feel satiated. Feed restriction therefore reduces the welfare of sows due to a prolonged state of hunger (Meunier-Salaün and Bolhuis, 2015).

One solution is to provide a diet with additional fibrous material. High-fibre diets increase the time spent eating and reduce frustration of the feeding motivation in pregnant sows (Bergeron *et al.*, 2000, Meunier-Salaün *et al.*, 2001). Sows fed a very-high-fibre diet spent more time lying down after a meal than sows fed a concentrate diet (Bergeron *et al.*, 2000). This sign of satiety could be explained by the fact that the stomach is full for a longer time because of the water-holding capacity of the fibre (Bindelle *et al.*, 2008).

High-fibre diets also have the potential to improve the health and performance of sows. According to Feyera *et al.* (2017), increasing the dietary fibre supply during the last 2 weeks of gestation reduced the proportion of stillborn piglets and total piglet mortality. Dietary fibres also have the advantage of improving gut health by enhancing beneficial bacteria (Jha and Berrocoso, 2015). Finally, a high-fibre diet can reduce constipation risks during the last stage of gestation and early lactation (Oliviero *et al.*, 2009).

The inclusion of fibres in the diet of pregnant sows is also beneficial during the lactation period. Because energy requirements of lactating sows are high and not covered by the voluntary intake, it is important to maximise feed intake to limit weight loss (Dourmad *et al.*, 1994). Meunier-Salaün *et al.* (2015) showed that feeding pregnant sows with a high-fibre diet increases the feed intake capacity of sows during the lactation period.

To increase the daily fibre intake, a portion of the concentrate diet can be substituted by forages, while maintaining the same energy level. Providing fresh or dry forage to pregnant sows can increase DM feed intake without additional weight gain and improve the welfare, health and performance of sows. Fresh and dry forages are not widely used currently in pig production, and sows' preference for different fresh and dry forages has been poorly investigated. Some studies have shown that sows prefer legumes over grasses offered as fresh forage (Sehested *et al.*, 2004, Rachuonyo *et al.*, 2005). In ruminant species, such as cows, sheep and goats, it is known that the botanical and chemical composition of forages influence preferences and, therefore, forage consumption (Scehovic *et al.*, 1985, Buntinx *et al.*, 1997, Mayland *et al.*, 2000, Burns *et al.*, 2001, Lombardi *et al.*, 2015). For example, the concentrations of NDF and ADF in forages are negatively correlated with preference in different herbivore species (Burns *et al.*, 2001, Horadagoda *et al.*, 2009). To optimize the use of both fresh and dry forages in the feeding of sows, it is necessary to better understand preferences for different types of forage. The objective of the present study was to determine the preference of sows among four different forage mixtures commonly used in Canada offered as dry or fresh forages. The relationship between botanical and chemical composition of forages and this preference by sows was also investigated.

Materials and methods

Animals and treatments

The institutional animal care committee of the Sherbrooke Research and Development Centre of Agriculture and Agri-Food Canada (QC, Canada) reviewed the procedures (authorisation #491) in conformity with the guidelines of the Canadian Council on Animal Care (CCAC, 2009) and the Canadian code of practice (National Farm Animal Care Council, 2014). Two experiments were conducted separately to test the preference of sows for four forage mixtures offered as fresh forage during spring (May–June) and dry forage (hay) during autumn (September–October). For each experiment, eight adult Yorkshire × Landrace sows were used (spring: parity = 2, body weight = 258.9 ± 10.2 kg, back fat = 17.7 ± 3.6 mm; autumn: parity = 2, body weight = 259.4 ± 11.2 kg, back fat = 18.8 ± 4.5 mm; mean ± SD) for a double Latin square design. The sows were between 39 and 78 days of gestation and housed indoors in individual pens (1.5 × 2.5 m) with a plain concrete floor in order to precisely collect forage refusals. Each sow had one neighbour as each pen was adjacent to another with a common side. Each sow had access to two separate troughs that were on the same side of the pen and inaccessible to the neighbouring sow. Sows were fed around 2.7 kg/day of a commercial gestation diet in form of pellets (12.74 MJ/kg ME, 13% CP; Nutreco Canada, Saint-Hyacinthe, QC, Canada) to meet their nutritional requirements for maintenance and foetal growth according to the National Research Council (2012). Sixty percent of the diet was given in the morning at around 0730 h and 40% in the afternoon immediately after the tests at around 1400 h.

Each experiment included a voluntary intake test and a preference test. Voluntary intake was measured by offering each forage mixture separately (one forage mixture per day) according to a 4 × 4 Latin square design. During the preference test, two forage mixtures were offered simultaneously with one combination of two forage mixtures per day.

Four forage mixtures were tested: (1) **AlfFT** with alfalfa (*Medicago sativa* L.), tall fescue (*Festuca arundinacea* Schreb.) and timothy (*Phleum pratense* L.); (2) **CloFT** with red clover (*Trifolium pratense* L.), tall fescue and timothy; (3) **AlfT** with alfalfa and timothy and (4) **CloT** with red clover and timothy. The AlfT and CloT forage mixtures were produced at the Sherbrooke Research and Development Centre of Agriculture and Agri-Food Canada (QC, Canada; 45°36′N, 71°83′W). The AlfFT and CloFT forage mixtures were produced at the Lieutenant farm in Stoke (QC, Canada; 45°49′N, 71°83′W).

Fresh forages were manually harvested using a pole hedge trimmer (model KombiTools; STIHL Limited, London, ON, Canada) in the morning of every test day. Forages for the second experiment with hay were also manually harvested (between 0.5 and 0.8 ha) in the spring (AlfFT, 2 June 2016; CloFT, 15 June 2016; AlfT, 31 May 2016 and CloT,

Table 1 *Experimental design of voluntary intake and preference tests in sows*

Latin square repetition		Days	Sow no. 1	Sow no. 2	Sow no. 3	Sow no. 4	Sow no. 5	Sow no. 6	Sow no. 7	Sow no. 8
1	Adaptation	1	A	B	C	D	A	B	C	D
		2	B	C	D	A	B	C	D	A
		3	C	D	A	B	C	D	A	B
		4	D	A	B	C	D	A	B	C
2	Adaptation	5	A	B	C	D	A	B	C	D
		6	B	C	D	A	B	C	D	A
		7	C	D	A	B	C	D	A	B
		8	D	A	B	C	D	A	B	C
3	Voluntary intake tests	9	A	B	C	D	A	B	C	D
		10	B	C	D	A	B	C	D	A
		11	C	D	A	B	C	D	A	B
		12	D	A	B	C	D	A	B	C
4	Voluntary intake tests	13	A	B	C	D	A	B	C	D
		14	B	C	D	A	B	C	D	A
		15	C	D	A	B	C	D	A	B
		16	D	A	B	C	D	A	B	C
	Preference tests	17	A–B	A–D	B–C	B–D	A–B	A–D	B–C	B–D
		18	A–C	B–D	C–D	A–B	A–C	B–D	C–D	A–B
		19	B–D	A–B	A–C	C–D	B–D	A–B	A–C	C–D
		20	C–D	B–C	A–D	A-C	C–D	B-C	A–D	A–C
		21	A–D	C–D	B–D	B–C	A–D	C–D	B-D	B–C
		22	B–C	A–C	A–B	A–D	B–C	A–C	A–B	A–D
5	Voluntary intake tests	23	A	B	C	D	A	B	C	D
		24	B	C	D	A	B	C	D	A
		25	C	D	A	B	C	D	A	B
		26	D	A	B	C	D	A	B	C
6	Voluntary intake tests	27	A	B	C	D	A	B	C	D
		28	B	C	D	A	B	C	D	A
		29	C	D	A	B	C	D	A	B
		30	D	A	B	C	D	A	B	C

A, B, C and D correspond to the different forage mixtures given to the sows.

14 June 2016), dried and stored in bulk in a barn until being used in autumn. Fresh and dry forages were chopped with knives (fresh) or an electric hedge trimmer (hay) before being offered in order to obtain a particle length of around 10 cm.

Voluntary intake tests

Voluntary intake tests were designed as a double 4 x 4 Latin square with forage mixtures as treatments, sows as columns and days as rows, which was repeated six times (Table 1). The first two repetitions of the double Latin square were considered the adaptation period. The third and fourth repetitions of the Latin square took place consecutively. Then, during the following 6 days, a preference test was performed, and finally, the fifth and sixth repetitions of the Latin square were carried out (Table 1). Data for the analysis of voluntary intake came from the third, fourth, fifth and sixth repetitions of the Latin square only, so that each forage mixture was tested four times on each sow.

For each daily test, the forage was offered *ad libitum* from 1200 to 1330 h. The quantity of forage provided and refusals were measured for each sow. For hay, refusals were dried at 55°C during at least 72 h before being weighed. Sow feeding behaviour was video recorded using specialised viewing software (Omnicast; Genetec Inc., Montréal, QC, Canada). The behavioural elements described in Table 2 were observed continuously for each sow for the third repetition of the double Latin square by three observers blinded to the treatments (with the treatments and sows evenly distributed between

Table 2 *Ethogram with the description of sows behaviour*

Behaviour	Description
Eating forage	The sow is manipulating (oral/nasal contact) or chewing forage (the trough in which the sow is eating forage was noted)
Drinking	The sow has the mouth on the waterer
Resting	The sow is lying (lateral or ventral), not eating forage
Exploration	The sow makes nose-contact with fixtures (walls, bars, floor) or the neighbouring sow, except forage
Other	Any other behaviour that is not mentioned above, whatever the sows' posture

observers). Inter- and intra-observer percentages of agreement were 95% and 97% respectively. The percentage of agreement was calculated based on the beginning and ending time for each behaviour noted by the observers with a tolerance of 4 s maximum. The percentage of agreement was calculated at the beginning of video processing on four videos selected randomly. Ingestion rate was calculated as the DM intake divided by the time spent eating the forage during the 90 min of the test.

Preference tests

Preference tests were conducted over 6 days, with a different pair of forage mixtures tested each day for each pair of neighbouring sows (Table 1). The side of the presentation of forages was balanced between the two neighbouring sows in order to avoid a possible lateral bias. During the 6 days, each sow received once all possible pairwise combinations of two forage mixtures (four forage mixtures tested in pairs, six combinations in total). The sequence of pairs was randomly determined.

Forages were simultaneously offered *ad libitum* from 1300 to 1345 h in two different troughs. The quantity of forage provided and refusals were measured for each forage mixture. The refusals of hay were dried at 55°C during at least 72 h in an oven before being weighed. Sows were video recorded during all the tests using the same equipment as in voluntary intake tests. Behaviours were then observed continuously by one observer (intra-observer percentage of agreement calculated on four videos = 99%). Time spent eating each forage mixture was measured.

Composition of forages

Samples of forages were collected at harvest for botanical composition and just before being offered to sows for chemical composition. For fresh forages, an area of 0.25 m^2 of each forage mixture was harvested on each experimental day ($n = 88$). For hays, six areas of 0.25 m^2 were harvested on the day of mowing each forage mixture ($n = 24$). The botanical composition was determined for each sample after each harvest. Plants of each species were dried separately at 55°C for at least 72 h, and the proportion of each forage species in the mixture was determined on a DM basis. The botanical composition of fresh and dried forages is presented in Tables 3 and 4 respectively.

Overall, 20 samples of hay (one sample per forage mixture per week) and 88 samples of fresh forage (one sample per forage mixture per day) were collected for chemical analysis. These samples were dried at 55°C for at least 72 h in an oven and then ground using a Wiley mill (Thomas Model 4 Wiley®; Thomas Scientific, Swedesboro, NJ, USA) to pass a 1-mm screen. Dried and ground forage samples were scanned by visible near-infrared reflectance spectroscopy (**VNIRS**) using a NIRS DS2500 monochromatic instrument (Foss NIRSystems Inc., Hilleroed, Denmark). A calibration set ($n = 44$) and a validation set ($n = 8$) of samples were selected using WinISI 4 software (version 4.5.0.14017; Infrasoft International, State College, PA, USA) and chemically analysed for concentrations of DM, ash, neutral detergent fibre assayed with a heat-stable α-amylase (**aNDF**), ADF, ether extract (**EE**), water-soluble carbohydrates (**WSC**), starch, total N (**TN**), neutral detergent insoluble N (**NDIN**), P and Ca. The *in vitro* true digestibility (**IVTD**) of DM and the *in vitro* NDF digestibility (**NDFd**) were also determined in these sets of samples. All of these nutritive attributes were determined according to procedures described in Bélanger *et al.* (2018). The chemical composition of fresh and dried forages is presented in Tables 3 and 4 respectively. Crude protein concentration was estimated as CP = TN × 6.25 and the neutral detergent insoluble crude protein (**NDICP**) as neutral detergent insoluble N × 6.25. The concentration of total carbohydrates (**TC**, g/kg DM) was calculated as TC = 1000 − CP − EE − ash, whereas concentration of non-structural carbohydrates (**NSC**, g/kg DM) was calculated as NSC = TC − (aNDF − NDICP). The IVTD (g/kg DM) and NDFd (g/kg aNDF) were calculated as follows:

$$\text{IVTD} = [1 - (\text{post-digestion dry weight following aNDF wash}/\text{pre-digestion dry weight})] \times 1000$$

Table 3 *Chemical and botanical composition of four forage mixtures fed as fresh forage to the sows. Least square means and standard error of the mean (SEM) are presented.*

	Forage mixtures				
	AlfFT	CloFT	AlfT	CloT	SEM
Chemical composition					
CP (g/kg DM)	164[b]	211[a]	193[a]	201[a]	5.18
ADF (g/kg DM)	358[a]	304[b]	351[a]	304[b]	4.17
aNDF (g/kg DM)	534[a]	461[b]	439[b]	395[c]	7.79
IVTD (g/kg DM)	801[b]	852[a]	815[b]	852[a]	6.66
NDFd (g/kg aNDF)	624[b]	688[a]	585[c]	633[b]	9.88
TC (g/kg DM)	648[a]	582[b]	590[b]	582[b]	6.98
NSC (g/kg DM)	136[c]	173[b]	178[b]	231[a]	3.92
WSC (g/kg DM)	41[b]	60[a]	38[b]	61[a]	1.76
P (g/kg DM)	3.5	3.5	3.5	3.4	0.07
Ca (g/kg DM)	7.6[c]	8.9[b]	11.3[a]	11.2[a]	0.33
DM (%)	17.5	14.7	15.7	14.8	0.50
ME (KJ/kg DM)	10.5[b]	11.1[a]	9.6[c]	10.6[b]	0.06
Botanical composition					
Legume (% DM)	28.4[c]	34.9[c]	63.3[b]	75.0[a]	2.91
Timothy (% DM)	26.7	26.8	28.5	24.0	2.93
Tall fescue (% DM)	42.1[a]	31.5[b]	0	0	3.34
Other species (% DM)	2.8[b]	6.9[a]	8.2[a]	1.1[b]	1.05

AlfFT = alfalfa, tall fescue and timothy; CloFT = red clover, tall fescue and timothy; AlfT = alfalfa and timothy; CloT = red clover and timothy; CP = crude protein; ADF = acid detergent fibre; aNDF = neutral detergent fibre assayed with a heat-stable α-amylase; IVTD = *in vitro* true digestibility of DM; NDFd = *in vitro* NDF digestibility; TC = total carbohydrates; NSC = non-structural carbohydrates; WSC = water soluble carbohydrates; ME = metabolisable energy for sows.
[a,b,c] Values within a row with different superscripts differ significantly at $P < 0.05$ (analyses of variance using the MIXED procedure of SAS, $n = 22$ per forage mixture).

Table 4 *Chemical and botanical composition of four forage mixtures fed as hay to the sows. Least square means and standard error of the mean (SEM) from heterogeneous variance analysis are presented.*

	Forage mixtures				SEM			
	AlfFT	CloFT	AlfT	CloT	AlfFT	CloFT	AlfT	CloT
Chemical composition								
CP (g/kg DM)	171[a]	159[a]	167[a]	131[b]	2.2	3.5	1.4	1.8
ADF (g/kg DM)	351[b]	346[b]	368[a]	350[b]	2.6	3.9	1.3	1.1
aNDF (g/kg DM)	518[a]	532[a]	515[a]	488[b]	5.7	10.0	4.4	4.6
IVTD (g/kg DM)	828	814	829	830	5.7	5.4	2.6	3.1
NDFd (g/kg aNDF)	660[b]	654[b]	688[a]	637[b]	8.0	4.5	3.1	5.1
TC (g/kg DM)	645[b]	669[a]	647[b]	686[a]	3.7	6.7	4.0	2.3
NSC (g/kg DM)	151[c]	174[b,c]	183[b]	237[a]	5.2	8.8	2.8	3.3
WSC (g/kg DM)	54[c]	76[b]	56[c]	86[a]	0.5	1.0	0.6	1.1
P (g/kg DM)	3.8[a]	3.1[c]	3.6[b]	3.0[c]	0.02	0.03	0.03	0.04
Ca (g/kg DM)	7.2	7.0	7.8	7.3	0.3	0.4	0.2	0.3
DM (%)	92.5	92.2	92.3	92.3	0.2	0.2	.02	0.2
ME (KJ/kg DM)	10.4[a]	10.7[a]	9.9[b]	10.1[b]	0.06	0.08	0.08	0.04
Botanical composition								
Legume (% DM)	39.7[b,c]	30.9[c]	60.6[a,b]	76.9[a]	8.2	8.7	4.6	5.8
Timothy (% DM)	28.5	18.2	25.6	22.8	4.8	4.9	5.0	5.6
Fescue (% DM)	29.9	43.7	0	0	10.1	6.9	0	0
Other species (% DM)	1.8[b]	7.1[a,b]	13.8[a]	0.3[b]	0.6	3.2	3.1	0.2

AlfFT = alfalfa, tall fescue and timothy; CloFT = red clover, tall fescue and timothy; AlfT = alfalfa and timothy; CloT = red clover and timothy; aNDF = neutral detergent fibre assayed with a heat-stable α-amylase; IVTD = *in vitro* true digestibility of DM; NDFd = *in vitro* NDF digestibility; TC = total carbohydrates; NSC = non-structural carbohydrates; WSC = water soluble carbohydrates; ME = metabolisable energy for sows.
[a,b,c]Values within a row with different superscripts differ significantly at $P < 0.05$ (analyses of heterogeneous variance using the MIXED procedure of SAS; for botanical composition, $n = 6$; and for chemical composition, $n = 5$, for each type of hay).

$$\text{NDFd} = [1 - (\text{post-digestion dry weigh following aNDF wash/pre-digestion dry weight of aNDF})] \times 1000$$

The nutritive attributes described above were thereafter predicted for all forage samples using VNIRS (WinISI 4 software, version 4.5.0.14017; Infrasoft International). The VNIRS predictions were considered successful when the ratio of the standard error of prediction to the standard deviation (**RPD**, ratio of prediction to deviation) was >3 (Nie *et al.*, 2009). The RPD was calculated by dividing the standard deviation of the reference data used in the validation set by the standard error of prediction corrected for bias. The RPD for ADF, aNDF, IVTD, NDFd, TC, NSC and WSC were >3 (ranged from 3.08 to 6.97).

Total N, P and Ca were extracted in all forage samples using a method adapted from Isaac and Johnson (1976). This extract was used to determine total N (method 13-107-06-2-E; Lachat Instruments, 2013) and P (method 13-115-01-1-B, Lachat Instruments, 2013) using a Lachat QuickChem8000 flow injection analysis system (Zellweger Analytics Inc., Lachat Instruments Division, Milwaukee, WI), as well as Ca by inductively coupled plasma optical emission spectrometry (Optima4300DV, Perkin Elmer, Norwalk, CT, USA).

Metabolisable energy (**ME**) was calculated from the chemical composition of the samples of the calibration set previously used for chemical analyses ($n = 44$) using two equations. The first equation estimates ME for growing pigs (**MEg**) as follows:

$$\text{MEg} = 4182 - (9.6 \times \text{Ash}) + (1.1 \times \text{CP}) + (4.1 \times \text{EE}) - (2.4 \times \text{Hemi}) - (4.4 \times \text{ADF})$$

where Hemi is the hemicellulose content calculated as Hemi = NDF − ADF (Noblet and Shi, 1993). The second equation estimates ME for the sows (**MEs**) (Noblet and Perez, 1993) as follows:

$$\text{MEs} = -3.96 + (1.17 \times \text{MEg}) + (0.0132 \times \text{NDF})$$

The values of ME for sows were thereafter predicted for all forage samples using VNIRS (WinISI 4 software, version 4.5.0.14017; Infrasoft International). The RPD was 2.82 which is considered moderately successful (Nie *et al.*, 2009).

Statistical analysis

Statistical analyses were done with SAS® software (Statistical Analysis System, Release 9.4, 2002–2012. SAS Institute Inc., Cary, NC). For voluntary intake tests, the experimental unit was the individual sow at every daily test within each repetition of the double Latin square. The analysis of variance (with heterogeneous variance according to forage mixtures, for fresh forages only) was done using the MIXED procedure of SAS to compare the DM intake between the four forage mixtures with repetition, Latin square within repetition,

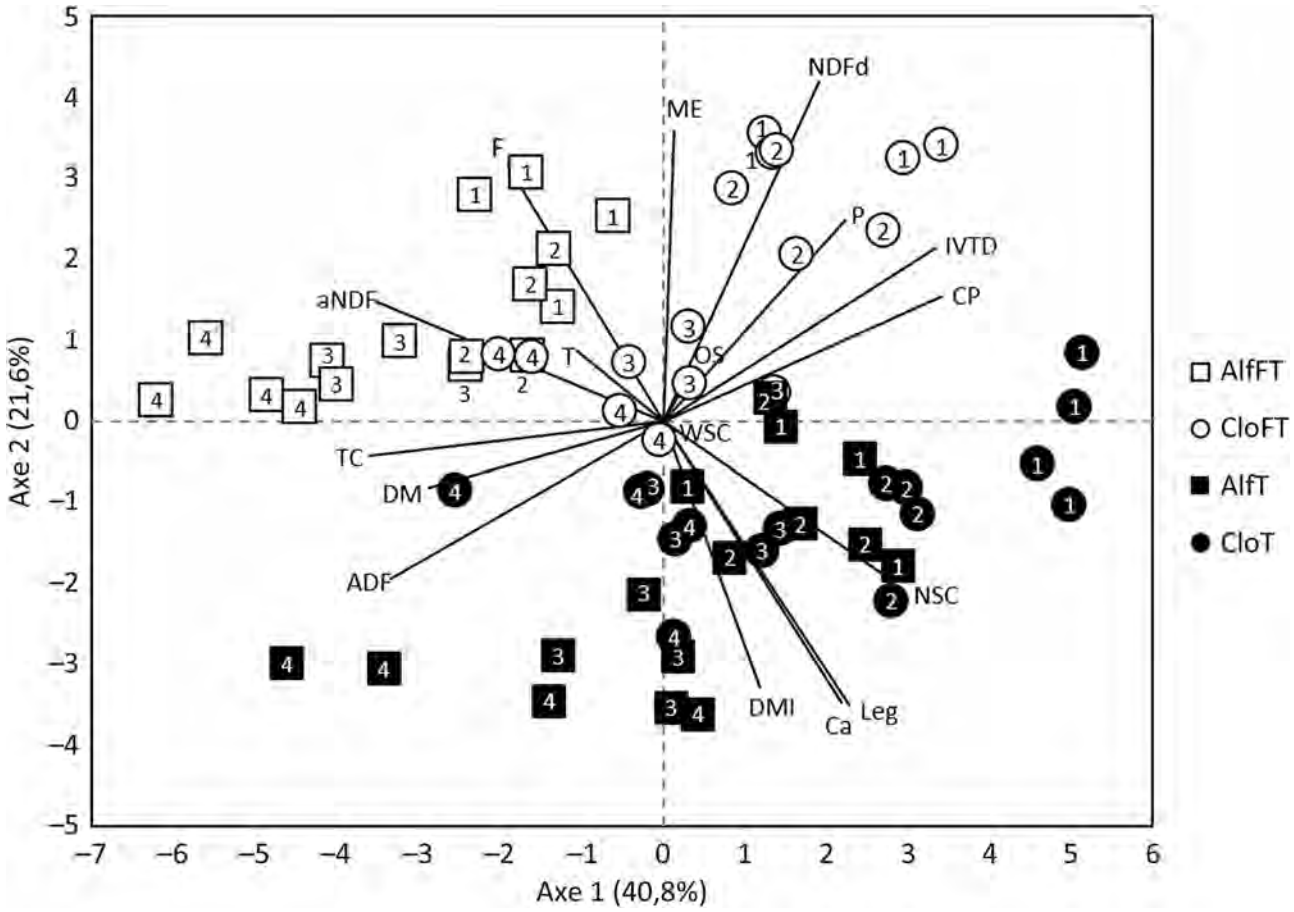

Figure 1 Diagram of the first two principal components of a principal component analysis to illustrate the relationship among forage attributes (aNDF, neutral detergent fibre assayed with a heat-stable α-amylase; F, proportion of tall fescue; ME, metabolisable energy; NDFd, *in vitro* NDF digestibility; IVTD, *in vitro* true digestibility of DM; OS, other species; WSC, water soluble carbohydrate; NSC, non-structural carbohydrate; Leg, legumes; T, timothy; DMI, dry matter intake of forages by sows; TC, total carbohydrate) for four forage mixtures offered as fresh forage in a Latin square design (AlfFT, alfalfa, tall fescue and timothy; CloFT, red clover, tall fescue and timothy; AlfT, alfalfa and timothy; CloT, red clover and timothy). Numbers in symbols correspond to the period of harvest (1, harvest from 28 to 31 May; 2, harvest from 1 to 4 June; 3, harvest from 11 to 14 June; 4, harvest from 15 to 18 June).

animal within repetition and Latin square, days within repetition and Latin square and forage mixture as fixed effects in the model. For the third repetition of the double Latin square only, the MIXED procedure was used to compare the time spent eating forages and the DM ingestion rate between the different forage mixtures.

A principal component analysis (**PCA**) was performed with the PRINCOMP procedure of SAS to explore relationships between the following variables: DM forage intake, botanical composition (percentage of legumes, timothy, tall fescue and other species) and nutritive attributes (DM, ME, ADF, aNDF, IVTD, NDFd, CP, WSC, NSC, TC, P and Ca) for fresh forages during voluntary intake tests. Each data point ($n = 16$ for each forage) corresponds to a forage sample for each day for chemical and botanical composition; forage intake was calculated as the average of DM forage intake of the two sows that had the same forage the same day.

For preference tests, the experimental unit was the sow on each daily test. Paired Student's *t* tests were used to compare the mean of DM intake and time spent eating each of the two forage mixtures for each pair of forage mixtures offered simultaneously. The preference of a forage over another (within each of the six possible pairwise combinations) was determined by comparing the mean of forage intake using a paired *t* test. When the paired *t* test renders a significant result for a pair of forage mixture, then the most consumed forage is considered 'preferred'.

A probability level of $P < 0.05$ was chosen as the limit for statistical significance in all tests, whereas probability levels of $0.05 \leq P < 0.10$ were considered a tendency. Multiple comparisons were performed using Tukey's adjustments of the Student's *t* test. Data provided in tables and figures are least square means with standard error of the mean. In the text, data provided are least square means of the treatments with the common SEM (homogeneous variance) unless stated otherwise.

Results

Fresh forages

The first two components of the PCA accounted for 62.4% of the total inertia (variance). The distribution of the dots indicates variation within and between forage mixtures offered as fresh forage (Figure 1). The first component of the PCA was defined

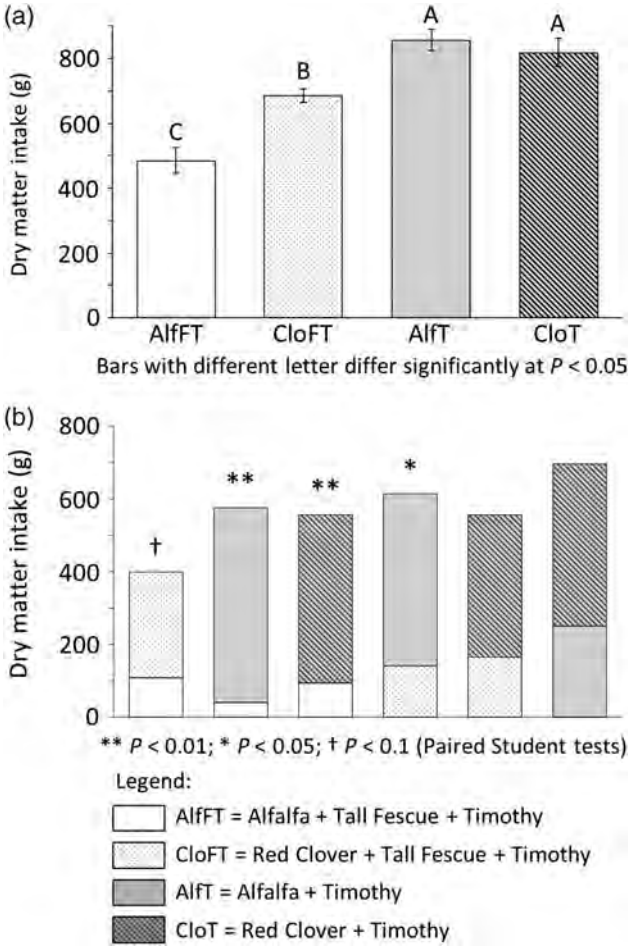

Figure 2 Dry matter intake of fresh forage by sows from (a) four forage mixtures in voluntary intake tests and (b) two forage mixtures for each pair in preference tests.

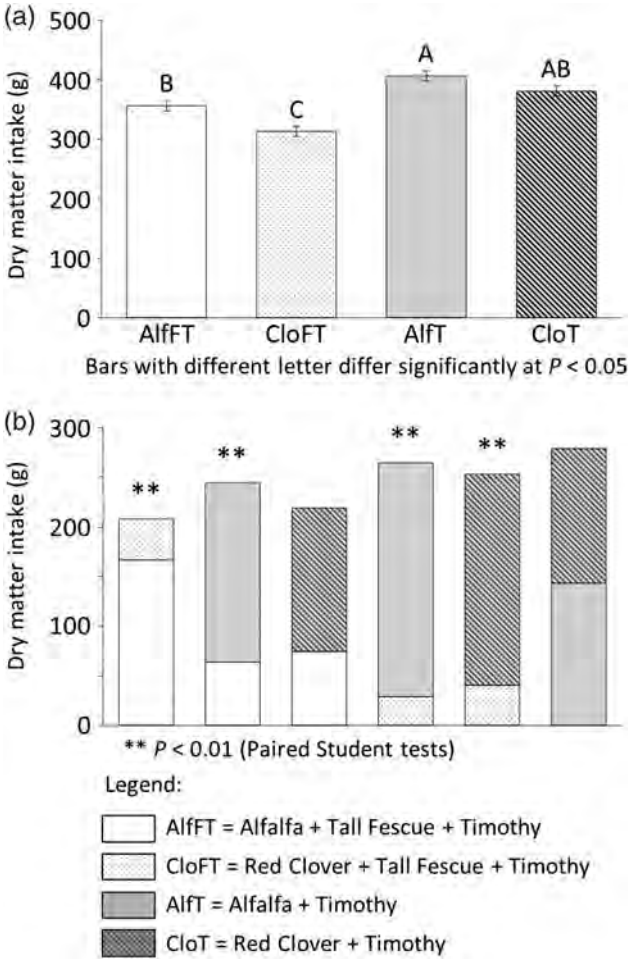

Figure 3 Dry matter intake of hay by sows fed from (a) four forage mixtures in voluntary intake tests and (b) two forage mixtures for each pair in preference tests.

mostly by CP concentration, NSC concentration and IVTD on the positive side and by concentrations of ADF, DM, TC and aNDF on the negative side. Attributes within same group on each side were positively correlated, whereas attributes in opposing groups were negatively correlated. This first component was mostly defined by the days of harvest, and it highlights the variation within mixtures. The same pattern was observed for all forage mixtures with an increase in ADF, DM and TC concentrations from the third to the last repetition of the double Latin square, whereas the CP concentration and the IVTD decreased when advancing in the season. The second component of the PCA was defined mostly by DM intake, the legume proportion and the Ca concentration on the positive side and by NDFd, the proportion of tall fescue, the ME content and the P concentration on the negative side. This second component was mostly defined by forage mixtures, and it confirms the variation among forage mixtures for the botanical and chemical composition.

Voluntary intake. Forage mixtures offered as fresh forage differed in DM intake by sows ($F_{3,69} = 15.9$; $P < 0.001$; Figure 2a). The DM intake of the AlfFT forage mixture was lower than that of the other forage mixtures, while it was lower for the CloFT forage mixture than for the AlfT and CloT forage mixtures. For the third repetition of the double Latin square, the average percentages of time spent eating were 90, 82, 86 and 86 ± 5%, while the average ingestion rates were 5.6, 6.5, 10.5 and 7.8 ± 1.2 g DM/min for AlfFT, CloFT, AlfT and CloT forage mixtures respectively. The forage mixtures did not differ in the percentage of time spent eating ($F_{3,21} = 0.55$; $P = 0.65$), but they differed in their ingestion rate ($F_{3,21} = 3.8$; $P = 0.025$). More specifically, the AlfFT forage mixture was ingested less quickly than the AlfT forage mixture ($P = 0.022$).

The position of the variables in the PCA indicates that forage DM intake was negatively associated with the proportion of tall fescue and, to a lesser extent, to the aNDF concentration and ME content but positively associated with the proportion of legumes and concentrations of NSC and Ca (Figure 1), regardless of the days of harvest associated with the experimental Latin square.

Preference tests. For forage mixtures offered as fresh forage, the DM intake differed significantly between the two forage mixtures offered for three pairs out of the six possible pairs of forage mixtures (Figure 2b). The DM intake was lower for

the AlfFT forage mixture than for the AlfT and CloT forage mixtures, and it tended ($P = 0.088$) to be lower than for the CloFT forage mixture. Moreover, the DM intake was higher for the AlfT forage mixture than for the CloFT forage mixture. Despite these DM intake differences averaged across eight sows, not all sows show the same preference pattern. Individual preference for each pair of forage mixtures was determined by comparing the forage DM intake of each sow for the two forage mixtures offered simultaneously with the preferred forage being the forage eaten for more than 50% of the total forage DM intake of the pair. Six out of eight sows preferred CloT over AlfT, CloT over CloFT and CloFT over AlfFT. The preference for AlfT over AlfFT was observed for all eight sows. The majority of the sows (7/8) preferred AlfT over CloFT and CloT over AlfFT.

For forage mixtures offered as fresh forage, time spent eating also differed between the two forage mixtures offered simultaneously for four pairs of forage mixtures. Sows spent less time eating AlfFT than AlfT ($P < 0.001$; 4 *v.* 40 ± 1 min respectively), AlfFT than CloT ($P = 0.038$; 9 *v.* 36 ± 5 min respectively) and CloFT than AlfT ($P = 0.022$; 14 *v.* 31 ± 3 min respectively). A tendency ($P = 0.07$) for spending less time eating AlfFT than CloFT was also observed (14 *v.* 31 ± 4 min respectively).

Dry forages (hays)

Voluntary intake. Forage mixtures offered as hay differed in DM intake by sows ($F_{3,69} = 21.7$, $P < 0.001$; Figure 3a). The DM intake of the CloFT forage mixture was lower than that of the other forage mixtures (356, 314, 406 and 381 ± 9 g of DM for AlfFT, CloFT, AlfT and CloT respectively), whereas the DM intake of the AlfFT forage mixture was lower than that of the AlfT forage mixture ($P < 0.001$). For the third repetition of the double Latin square, the average percentages of time spent eating were 94, 95, 94 and 93 ± 2% and the average ingestion rates were 3.4, 2.8, 4.1 and 3.8 ± 0.4 g of DM/min for the AlfFT, CloFT, AlfT and CloT forage mixtures respectively. Time spent eating did not differ among forage mixtures ($F_{3,21} = 0.26$; $P = 0.86$), but the ingestion rate differed among mixtures offered as hay ($F_{3,21} = 8.7$; $P < 0.001$). Indeed, the CloFT forage mixture was ingested less quickly than the AlfT ($P < 0.001$) and CloT ($P = 0.005$) forage mixtures.

Preference tests. Forage intake differed between the two forage mixtures offered as hay for four pairs of forage mixtures (Figure 3b). The DM intake of the CloFT forage mixture was always lower than that of other three forage mixtures. The DM intake of the AlfT forage mixture was higher than that of the AlfFT forage mixture.

Despite these differences, individual sows did not show the same preference pattern. The preference of AlfFT, AlfT and CloT over CloFT was observed for all the eight sows. The preference of AlfT over AlfFT was observed for all the eight sows. The majority of the sows (7/8) preferred CloT over AlfFT. Half of the sows (4/8) preferred AlfT over CloT.

Time spent eating differed between the two forage mixtures offered simultaneously as hay for four pairs of forage mixtures. Sows spent less time eating the CloFT forage mixture than the forage mixtures of AlfFT (10 *v.* 34 ± 3 min respectively; $P = 0.004$), AlfT (6 *v.* 38 ± 2 min respectively; $P < 0.001$) and CloT (10 *v.* 35 ± 3 min respectively; $P = 0.003$). Sows also spent less time eating the AlfFT forage mixture than the AlfT forage mixture (16 *v.* 28±2 min respectively; $P = 0.005$).

Discussion

The present study shows that pregnant sows exhibited strong preferences for some forage mixtures over others. Despite variation among individuals, clear preferences were observed at the group level. These preferences were almost similar whether the forage mixtures were offered as fresh forage or hay.

Relationship with botanical composition

Forage mixtures with a great proportion of legumes and without tall fescue, offered either as fresh forage or hay, were preferred by sows over forage mixtures with a high proportion of grasses and including tall fescue offered either as fresh forage or hay. Therefore, the preference of sows seems related to the botanical composition. In previous studies, legumes were also preferred over grasses by sows offered as fresh forage (Sehested *et al.*, 2004; Rachuonyo *et al.*, 2005). According to Rachuonyo *et al.* (2005), the preference for legumes could be due to grasses being more fibrous and harder to graze by non-ruminants. In the present study, the least preferred forage mixtures were those containing tall fescue. Similar results have also been observed in ruminant species. Indeed, tall fescue was the least preferred grass species in cattle among eight different grass species offered as fresh forage (Horadagoda *et al.*, 2009). Tall fescue was also less consumed than other species when offered to sheep as hay (Buntinx *et al.*, 1997). Scehovic *et al.* (1985) reported that tall fescue was less consumed by sheep when compared with ryegrass. These authors similarly observed that ryegrass was also less consumed when sprinkled with tall fescue juice, and they suggested that the low palatability (i.e. palatability of a diet refers to its acceptability features, including taste, smell and texture; Jacela *et al.*, 2010) of tall fescue could be explained by its chemical composition, particularly its richness in sulphur compounds. Therefore, the preference of sows for forage mixtures without tall fescue could be due to the taste of this grass species, but further studies are necessary to investigate the reasons for this poor palatability of tall fescue fed to sows. The botanical composition of forage mixtures also translates into differences in chemical composition. For example, legumes usually contain less fibre than grasses (McLeod and Smith, 1989). Therefore, botanical composition of the forages must be take into account for sow feeding, with a high content of legumes being recommended.

Relationship with chemical composition

The relationship between the chemical composition of forages and voluntary intake has been poorly investigated in swine, whereas it has been well studied in other species, especially ruminants, such as cattle and sheep (Wilman *et al.*, 1996, Buntinx *et al.*, 1997, Horadagoda *et al.*, 2009, Lombardi *et al.*, 2015). As observed in other species, it could be hypothesized that preferences of sows are also related to the chemical composition of the forage. In the present study, AlfFT, the least consumed forage mixture during the voluntary intake test for fresh forages, had a greater aNDF concentration than the other forage mixtures. Moreover, during the preference tests with fresh forages, the most consumed forage mixture always had a lower aNDF concentration than the least consumed forage mixture. The PCA confirmed these results with a clear opposition between forage aNDF concentration and forage intake. These results are in accordance with previous studies in sheep and cattle (Jung and Allen, 1995). For example, Horadagoda *et al.* (2009) found a negative correlation between NDF concentration and cow preference for grass and legume species offered as fresh forage. One explanation to these results could be the structural characteristics of the fibre. Indeed, studies with sows have found that dietary fibre can promote satiety during and immediately following a meal due to the bulkiness properties of the fibre (reviewed by Meunier-Salaün and Bolhuis, 2015). Moreover, some studies have shown a lower feeding rate with the inclusion of fibre in the diet of sows, which could be due to sows spending more time chewing fibrous diets (reviewed by Meunier-Salaün and Bolhuis, 2015). Similarly, in sheep, the rate of DM intake of different fresh plant species was negatively correlated with the NDF concentration of the diet (Wilman *et al.*, 1996). Therefore, the properties of fibre could explain why forage mixtures with a greater NDF concentration were less consumed by sows.

Wilman *et al.* (1996) also studied the relationship between physical and anatomical characteristics of plants and the ingestion rate in sheep. Their results indicated that the ingestion rate was associated with the characteristics of the veins in the plant. A lower rate of intake was associated with large numbers of veins arranged in parallel lines, such as seen in grasses, whereas a higher ingestion rate was associated with broad leaves, such as seen in legumes that have a network of veins in the leaf easier to be broken than the parallel venation of grasses (Wilman *et al.*, 1996). Therefore, the anatomical characteristics of the species could explain why forage mixtures with high proportion of grasses were less preferred by the sows in the present study.

In the present study, AlfFT, the least consumed forage mixture offered as fresh forage during voluntary intake tests, had lower CP, Ca, NSC and WSC concentrations than the other forage mixtures. During the preference tests with fresh forages, the most consumed forage mixtures often had greater CP concentration than the least consumed forage mixture. The effect of forage NSC and WSC concentrations on forage intake has been investigated in cows. The preference of cows was positively related to the forage WSC concentration (Horadagoda *et al.*, 2009). Among different cultivars of tall fescue, cattle preference was positively correlated with the forage NSC concentration (Mayland *et al.*, 2000). Therefore, the lowest intake of the AlfFT forage mixture by sows could also be due to its lower concentration of NSC and WSC. Forage WSC concentration was strongly correlated with palatability in cows (Smit *et al.*, 2006).

It has been shown in cows that the sweet taste increased palatability. Indeed, over four different additives (sweet, sour, bitter and salty), only sucrose increased diet intake in cows (Nombekela *et al.*, 1994). It is also known that weaned piglets like sweetness, as several studies have shown that sweet is preferred over a wide variety of flavours (Jacela *et al.*, 2010). Moreover, a positive correlation between the CP concentration of forages and intake has been observed in sheep and cattle (Heady, 1964), which is in accordance with the present study. Therefore, it seems relevant to favour fresh forages with high level of CP, NSC and WSC for sows feeding.

Following the hypothesis that feedback signals from the gastrointestinal tract would regulate the feed intake according to the energy density of the diet (Li and Patience, 2017), it could be expected that the feed-restricted sows would prefer forages with the highest ME content in order to increase their energy intake. However, the results from the present study suggest that ME content was not positively associated with forage intake as the PCA indicates an opposition between DM intake and ME. Similarly, the least preferred dry forage mixture (CloFT) was the one with the highest ME content. Kyriazakis *et al.* (1991) reported that pigs could adapt their feed intake according to the nutritional composition of the diet only if they had the opportunity to learn about their nutritional consequences. In the present study, sows had access to the forages for 45 to 90 min only and were immediately fed with concentrate pellets afterwards. Therefore, the experimental set up probably limited their possibility to adapt their feed intake to the nutritional composition of the different forage mixtures. Solà-Oriol *et al.* (2009) stipulated that preference could be also regulated by sensorial perception which is most likely the case in the present study. Indeed, preferences observed in the present study are more probably due to short-term sensory factors such as taste and/or the difficulty for chewing fibrous materials. The effect of ME content on forage consumption in sows could be investigated in further experiment with forages *ad libitum* for several days.

In this study, the relationship between the chemical composition of hay and the preferences of sows was not so clear. For hay, during both preference and voluntary intake tests, the CloFT forage mixture was the least preferred of all forage mixtures. However, compared with the other forage mixtures, this forage mixture did not have a greater aNDF concentration or lower WSC, NSC or CP concentrations, which could have explained this lower preference. Further studies are needed to understand the link between hay preferences in sows and forage chemical composition. The least

consumed forage mixture (CloFT) contained the highest proportion of tall fescue and the lowest proportion of legumes. Therefore, the preferences observed for hay could be attributed to some chemical or physical properties of tall fescue that were not measured in the present experiment.

In the present study, for fresh forages, the AlfFT forage mixture had a higher DM concentration (17.5 ± 0.5%) than the other forage mixtures. Therefore, DM concentration of the fresh forages could also influence the preferences observed in sows. Gesshe and Walton (1981) suggested that the preference observed in steers for Russian wild ryegrass was probably due to its high proportion of leaves and high moisture concentration in comparison with the other grass species that were evaluated. In the present study, sows in the experiment with fresh forages consumed, on average, almost double the amount of DM than sows in the experiment with dry forages. This could indicate that the DM concentration of forages could be important to consider, but further studies are needed to investigate the effect of DM concentration on forage preference by sows.

Conclusions

Sows exhibited very strong preferences among forage mixtures, with results suggesting that more than one factor is involved including botanical and chemical composition and/or other characteristics such as DM concentration. Several characteristics of the forage mixtures could explain those preferences, but it is highly probable that preferences resulted from a combination of forage characteristics. Indeed, the multivariate analysis showed that forage characteristics are either negatively associated (e.g. proportion of tall fescue in the mixture and aNDF concentration) or positively associated (e.g. proportion of legumes and NSC concentration) with forage intake. More studies are needed to untangle the relationship between forage characteristics and preference in sows. Based on our results, offering forages with a high proportion of legumes would be a good strategy to maximise both fresh and dry forage intake in pregnant sows.

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Declaration of interest

The authors report no conflicts of interest.

Ethics statement

The Institutional Animal Care Committee of the Sherbrooke Research and Development Centre of Agriculture and Agri-Food Canada (QC, Canada) reviewed the procedures (authorisation #491) in conformity with the guidelines of the Canadian Council on Animal Care (CCAC, 2009) and the Canadian code of practice (NFACC, 2014).

Software and data repository resources

None of the data were deposited in an official repository.

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Survival analysis of mortality in pre-weaning kids of Sirohi goat

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Pre-weaning animals exit a flock through death induced by various reasons, causing significant economic losses to the goat producers. In this study, we investigated the survival from birth to weaning of Sirohi goat kids within framework of the survival analysis. Kid records were accessed from 1997 to 2017, with the information on 4417 pre-weaning animals of farmed Sirohi goat native to the Rajasthan State of India. A multivariable Cox regression was fitted to the data after checking the assumptions of regression. The explanatory variables were sex, type of birth, season of birth, birthweight, doe weight at kidding and year of birth. Model selection eliminated doe weight from the model, and sex, type of birth, season of birth, birthweight and year of birth were retained in the model. With model calibration also, these five covariates were retained in the model. The mortality on the first day after birth was 0.3%, constituting 3.5% of all pre-weaning mortality. The mortality until the end of weaning period was 7.8%. Regression analysis revealed that the higher birthweight at kidding was associated with reduced hazard of death among the kids. Male kids had higher hazards of death compared with female kids. The single-born kids had lower risks of death compared with twin-born kids after accounting for heterogeneity. The winter season had a very high adverse effect on the survival of the kids. With each passing year, risks of death decreased. The results of this study indicate that better survival of kids can be achieved by controlling both environmental and animal-related factors.

Keywords: calibration, discrimination, optimism, censoring, hazards

Implications

The results of the present study suggest that the mortality losses can be minimised by implementing measures to control both environmental and animal-related factors. Since the upper limit of birthweight is still not reached, there is scope for increasing the birthweight through better nutrition of dam, thereby decreasing the kid mortality. A threshold weight of the dam at kidding could be identified vis-à-vis kid mortality, providing another avenue for control of mortality. For generalisability of the model, external validation may be performed on independent datasets of Sirohi goat from other locations.

Introduction

Mortality among the pre-weaning animals is one of the major factors causing significant economic losses to the goat producers. The untimely exits restrict the number of animals available for future replacement and impact the number of animals reaching marketable age. After birth, the kids face a number of physiological, behavioural and immunological challenges affecting the survival of neonates (Dwyer *et al.*, 2016). Numerous studies focusing on the mortality in goats have concluded that generally a higher number of pre-weaning animals die than adults (Sharma *et al.*, 2007; Kumar *et al.*, 2010), and the mortality during the weaning period may reach as high as 37% of the total mortality (Thiruvenkadan and Karunanithi, 2007). The previous studies have concluded that most of the deaths of goat kids during the weaning period are recorded from the first day after birth to the completion of neonatal age. Both environmental and animal-related factors influence the mortality. Past studies have concluded that birthweight, doe weight at kidding, sex, season of birth, type of birth and year of birth affect the survival of the kids (Subramaniyan *et al.*, 2016).

The mortality data constitute a special category of data known as time-to-event data, characterised by whether and when an event occurs during a study period. Further, mortality data are skewed and are subject to 'censoring', which occurs when the information about survival times of some individuals is incomplete. The most common form of censoring is 'right censoring', in which event of interest (e.g. mortality) for the duration of the study is not observed. The survival data can be analysed by linear and logistic regression methods.

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Analysis of survival by linear model ignores censoring and skewness inherent in the mortality data (Ellen *et al*., 2010). Moreover, considering mortality as a binary trait causes severe information loss, because animals dying early or late in the study period cannot be differentiated (Allison, 1997; Sawalha *et al*., 2007). An earlier study has proven the superiority of survival analysis over logistic regression (Southey *et al*., 2001). Survival analysis has been used in studies of lamb survival of Scottish Blackface sheep (Sawalha *et al*., 2007), Lori-Bakhtiari sheep (Vatankhah and Talebi, 2009) and Iranian Kermani sheep (Barazandeh *et al*., 2012). The aim of the study was to identify factors influencing the mortality among Sirohi goat kids within the framework of survival analysis.

Materials and methods

Study population and its management

Sirohi goat is a dual-purpose (meat and milk) animal, adapted to semi-arid and arid climates. The animals were maintained at a breeding farm at ICAR-Central Sheep & Wool Research Institute, Avikanagar, located in the Rajasthan State of India. The goats were housed in sheds provided with open paddocks. Generally, the sheds had chain link fencing to facilitate adequate ventilation. The sheds were roofed over with asbestos sheet to protect the animals from the extremes of temperature and rain. The sides of the sheds were covered in winter to prevent the animals from exposure to cold weather, and as an additional measure of protection from cold, firewoods were burnt in indigenous arrangements inside the sheds during night hours. The animals were reared under the semi-intensive system of management, with about 6 to 7 h of grazing during the day and the supplementary feeding in the form of concentrate in the evening. Selective breeding was practised for improving meat and milk production. Mating was practised in two seasons, characterised as major and minor breeding seasons. The females were mated in the months of May through July in major season. Currently, minor season mating is done in the month of November, but different schedules were followed in the past. Immediately after birth, the new born kids were fed colostrum, navel cord was cut and iodine tincture was applied. Then, the neonatal kids and mothers were transferred to kidding pen, where they were kept for 3 to 4 days so that mother and kid bonded with each other. The kids born dead were classified as 'still born', and they were not given any identification number. Similarly, the kids dying immediately after birth were not given any identification number. Only the kids surviving for 2 to 4 h were given identification number.The kids were offered *ad libitum* creep ration, tree leaves and other fodders starting from about 15 to 20 days of age. The kids were weaned at the age of 90 days. The culling before weaning was generally not allowed, except in the unavoidable circumstances (ricket, tetanus, arthritis, severe stunting, etc.).

Data collection

The data were collected from year 1997 to 2017 containing information on 4428 pre-weaning kids. For each animal identity number, date of birth, sex, type of birth, season of birth, birthweight, doe weight at kidding, year of birth and date of exits were retrieved from the database maintained at the institute. The type of birth had three levels: single-born kids, twin-born kids and triplet kids. Sex had two levels: male and female. Based on the similarities of environmental and pasture conditions, the season of birth was divided into two categories: winter season from November through February and other seasons from March through October. The failure time was calculated from the difference between date of birth and date of exit. The animals that were alive at the end of weaning or culled before weaning were recorded as 'right censored', and time of exits of all such animals was fixed at 90 days (weaning age). The numbers of single-born, twin-born and triplet-born kids in the dataset were 3812, 605 and 11, respectively. The triplets were excluded from further analysis because their number was very small. The dataset consisted of 4417 animals after removal of triplets. There were 2157 males and 2260 females in the edited dataset. Animals born in winter season and other seasons were 1723 and 2694, respectively. The mean birthweights±SD of male and female kids were 3.4±0.6 kg and 3.1±0.5 kg, respectively. At birth, the single-born kids were heavier (3.3±0.5 kg) than twin-born kids (2.8±0.5 kg).

Statistical methods

The survival of the kids was estimated by product-limit method of Kaplan and Meier (1958):

$$S_t = \prod_{t_i \le t} 1 - \frac{d_i}{n_i}$$

where t_i is duration of study at any point i, d_i is mortality up to point i and n_i is number of individuals at risk just prior to t_i (Kaplan and Meier, 1958).

The Cox proportional hazards model was fitted as follows

$$\lambda(t|X) = \lambda_0(t)\exp\{\beta X\}$$

where $\lambda(t \mid Z)$ is the hazard for the failing individual.The hazard is a function of some unspecified 'baseline' hazard $\lambda_o(t)$ and a set of covariates defined by X. β denotes the vector of regression coefficients for different covariates specified in the model. The covariates act to multiply the baseline hazard in a time-independent manner (Cox, 1972). The quantities produced by the model are referred to as 'hazard ratios'. The time-varying coefficient was fitted by extending above basic model as follows

$$\lambda(t|Z(t)) = \lambda_0(t)\ \exp\{\beta x + \gamma X g(t)\}$$

where β and γ are coefficients of time-fixed and time-varying covariates, respectively (Zhang *et al*., 2018).

To model heterogeneity, shared frailty model was fitted as

$$\lambda_{ij}(t) = \lambda_0(t)\ \exp\{\beta x_{ij} + w_i\}$$

where $\lambda_{ij}(t)$ is the hazard function for the j^{th} individual belonging to i^{th} group, $\lambda_0(t)$ is the baseline hazard at time t, x_{ij} is the vector of p covariates and w_i is the random effect for the i^{th} group (Nguti, 2003).

Data analysis

The R package 'survival', based on the R statistical environment (R Core Team, 2018), was used for the survival analysis (Therneau and Grambsch, 2000; Therneau, 2015). Mortality and censoring indicator were coded as 1 and 0, respectively (0 means lack of event including culling). The 'ggplot2' package (Wickham, 2016), 'smoothHR' package (Meira-Machado *et al*., 2013) and base R were used for generation of the plots. The explanatory variables considered for the study were sex, type of birth, season of birth, birthweight, doe weight at kidding and year of birth. Before cox regression, the linearity assumption of birthweight and doe weight was checked, followed by test of proportionality of all the covariates. Proportional hazards assumption of the covariates was assessed by Grambsch-Therneau test and Schoenfeld residual analysis. To deal with time-dependent effect of covariates, linear effect of time was modeled. To estimate heterogeneity in the data, year of birth was modeled as log-normal frailty.

After model selection, the model was internally validated by bootstrapping. The validation of the model was assessed in terms of calibration and discrimination measures. Calibration refers to 'whether the predicted probabilities of the event agree with the observed probabilities' (Steyerberg *et al*., 2001), and it indicates prediction accuracy of a model (Royston and Altman, 2013). Discrimination measures 'the ability of a model to distinguish between individuals who die and those who survive' (Serrano, 2012). It was quantified as Harrel's C statistic (Harrell *et al*.,1996). Model selection and validation were done by invoking appropriate functions of 'rms' package (Harrell, 2018) to perform the bootstrap analysis, specifying 5000 bootstrap samples. Model validation was performed according to Harrell's approach (Harrell *et al*., 1996):

1. Develop the model using all n subjects and whatever stepwise testing is deemed necessary. Let D_{app} denote the apparent D from this model,where D is any statistic.
2. Generate a sample of size n with replacement from the original sample (for both predictors and the response).
3. Fit the full or possibly stepwise model, using the same stopping rule as was used to derive D_{app}.
4. Compute the apparent D for this model on the bootstrap sample with replacement. Call it D_{boot}.
5. 'Freeze' this reduced model, and evaluate its performance on the original dataset. Let D_{orig} denote the D.
6. The optimism in the fit from the bootstrap sample is $D_{boot}-D_{orig}$.
7. Repeat steps 2 to 6 as many times as is specified.
8. Average the optimism estimates to arrive at O.
9. The bootstrap- or optimism-corrected performance of the original stepwise model is $D_{app}-O$. This difference is a nearly unbiased estimate of the expected value of the external predictive discrimination of the process which generated D_{app}. In other words, $D_{app}-O$ is an honest estimate of the internal validity, penalising for over-fitting.

The optimism-corrected C was estimated from the formula $D_{xy} = 2C-1$ (Harrell *et al*., 1996), by plugging in optimism-corrected D_{xy}. D_{xy} and C are Somers' D_{xy} and Harrell's C, respectively.

The R software codes for the above statistical analyses are provided in the Supplementary Material S1.

Results

Kaplan–Meier curve

Out of 4417 animals, a total of 343 animals exited the flock through death until the end of weaning period. The majority of the observations were censored, accounting for 92.2% of the total number of observations. The incidence risk of postnatal mortality on the first day after birth was 0.3%, constituting 3.5% of all pre-weaning mortality. The mortality for the entire weaning period was 7.8%. The Kaplan–Meier curve for mortality is displayed in Figure 1. It shows a steep decline in the first few days after birth of the kids, because 37.3% of the total mortality was recorded within the first week.

Cox regression analysis

The birthweight was found to be non-linear (Supplementary Figure S1). Doe weight was also moderately non-linear (Supplementary Figure S2). Penalised spline term was included in the model for both birthweight and doe weight for correcting non-linearity. The results of the Cox regression of mortality are reported in Table 1, showing estimates of hazard ratios of the covariates along with their Confidence Intervals (CIs) and actual P-values. As a side note, the thresholds for significance levels are arbitrary (Schneider, 2015), and large P-value does not mean no difference and smaller P-value does not necessarily mean more significant (Kim and Bang, 2016). Also, when n is large, the P-value is small (Kim and Bang, 2016). Therefore, as recommended by Schneider (2015), we have provided the results as point estimates of hazard ratios with their 95% CI and actual P-values. Further, we have consciously avoided the dichotomisation of results as 'significant' and 'non-significant'.

Higher birthweight was associated with decreased hazard of death: the hazard of death decreased by 0.78 after adjustment for the effects of other covariates in the model (hazard ratio: 0.22; 95% CI, 0.18 to 0.26). Plotting the log (hazard ratio) against birth weight showed a non-linear downward sloping effect, with lower birthweight associated with higher hazard and higher birthweight having protective effect (Figure 2). From the figure, it is clear that increasing birthweight decreased the hazard of death. Similarly, higher doe weight was also associated with decreased hazard of

Table 1 *Hazard ratios (with 95% lower and upper CIs) from Cox hazards model for mortality data of Sirohi goat kids*

Risk factor	Log (Hazard ratio)	Hazard ratio	95% CI	*P* value
Birthweight	−1.535	0.22	(0.18 to 0.26)	0.00
Doe weight	−0.019	0.98	(0.96 to 1.00)	0.09
Sex (ref: Female)	0.299	1.35	(1.08 to 1.68)	0.01
Type of birth (ref: Single)	−0.257	0.77	(0.51 to 1.17)	0.23
Type of birth*time	0.003	1.00	(0.99 to 1.01)	0.61
Season of birth (ref: Other seasons)	0.790	2.20	(1.77 to 2.75)	0.00
Year	−0.022	0.98	(0.96 to 1.00)	0.03

CI = confidence interval; Time = time to event; Year = year of birth of kids.
Female, Single and Other seasons in parentheses are respective reference categories for the categorical covariates, each having two levels-type of birth (Single and Twin), sex (Male and Female) and season of birth (Winter and Other seasons).

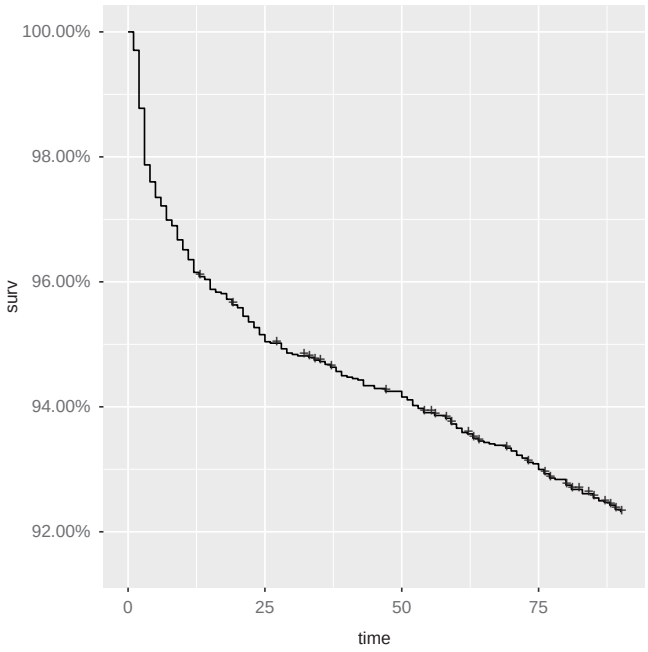

Figure 1 Plot of Kaplan–Meier curve showing survival against time for Sirohi goat kids. The plot shows a steep decline in survival within first few days followed by gradual non-linear decline. Surv is survival probability.

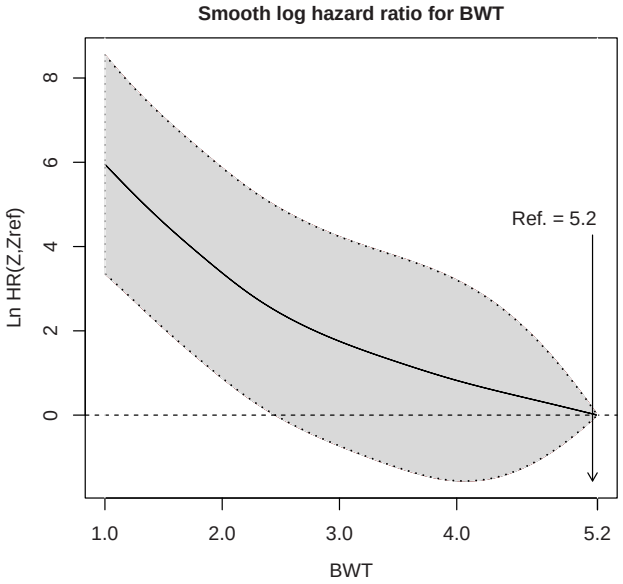

Figure 2 Plot of log (hazard ratio) against birthweight (BWT) of Sirohi goat kids. Birthweight has a downward sloping effect, with lower birthweight associated with higher hazard and higher birthweight having protective effect. The figure was generated using smoothHR package. 5.2 kg is the default reference chosen by the smoothHR package. HR is hazard ratio.

death (hazard ratio: 0.98; 95% CI, 0.96 to 1.00). Figure 3 shows that doe weights up to about 32 kg had higher hazards while doe weights higher than about 45 kg had protective effect. Male kids were associated with higher hazard of death by a factor of 0.35 (hazard ratio: 1.35; 95% CI, 1.08 to 1.68). Type of birth had time-varying effect. The Schoenfeld residual analysis and Grambsch-Therneau test (Supplementary Table S1) suggested evidence of violation of proportional hazards assumption by type of birth. From the visual inspection of the residual plot (Supplementary Figure S3(b)), we can see that the residuals for type of birth are monotonically increasing, and they are crossing the zero-slope line at around day 21. An explanation for this is that twins have better survival until around first 21 days compared with singles, and singles have better survival after day 21. We suspected that unmeasured heterogeneity was responsible for time-varying effect of type of birth. From an examination of the mortality (%) by year (Supplementary Figure S4), we see that there are some years when mortality is unusually high, particularly in the years 1998, 1999, 2003, 2004 and 2011. The survival of single- and twin-born kids was highly heterogeneous over the event time (weaning period) in these years. We found that only 11 twin-born kids died out of total 101 deaths within first 21 days of postnatal life; remaining 90 deaths were from singleton categories. Particularly for years 2003 and 2004, only 3 twin-born kids (out of 73 deaths) died within first 21 days. This explains the earlier observation of better survival of twins until around first 21 days. From the frailty models (with year of birth as the log-normal frailty term) fitted on both complete dataset and truncated dataset (dataset from which data pertaining to the years 1998, 1999, 2003, 2004 and 2011 were removed), the variance for frailty term was 0.29 in complete dataset, which was reduced to 0.09 in the truncated dataset, confirming that there was sufficient heterogeneity in the data due to

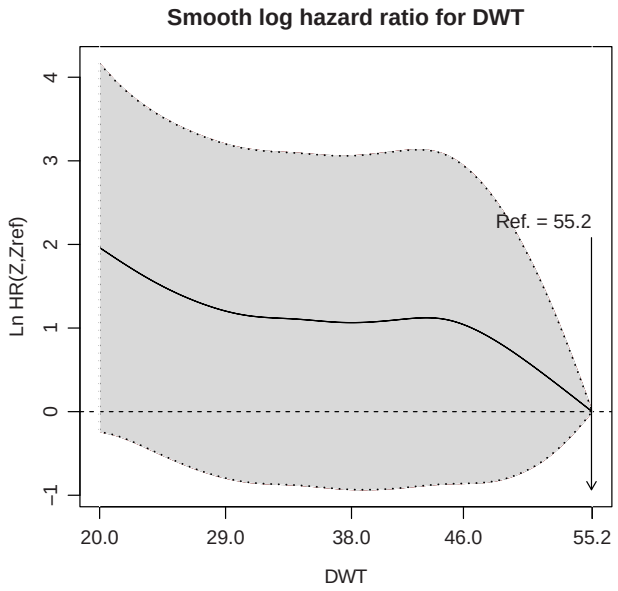

Figure 3 Plot of log (hazard ratio) against doe weight (DWT) of Sirohi goat. Doe weights up to about 32 kg have higher hazards, while doe weights higher than about 45 kg have a protective effect. The figure was generated using smoothHR package. 55.2 kg is the default reference chosen by the smoothHR package. HR is hazard ratio.

these 5 years. The time-varying effect of type of birth disappeared in the truncated dataset (Supplementary Table S2 and Supplementary Figure S5(b)). Moreover, results from analysis of the truncated dataset showed that twins had higher hazard compared with singletons (hazard ratio: 1.06; 95% CI, 0.70 to 1.59). Thus, the time-varying effect of type of birth was due to heterogeneity introduced by these 5 years. Winter season had 2.20 times higher risks of death as compared with other seasons (hazard ratio: 2.20; 95% CI, 1.77 to 2.75). Finally, with each passing year, there was decrease in the risk of death by a factor of 0.2 (hazard ratio: 0.98; 95% CI, 0.96 to 1.00).

Model selection and validation

From the initial six covariates, doe weight was eliminated during resampling validation of the fitted model, and sex, type of birth, season of birth, birthweight and year were retained in the model. The same set of covariates was retained during calibration. The estimates of the optimism from bootstrapping based validation are reported in the Supplementary Table S3, and the estimates are low to very low. The optimism-corrected estimate of the C-statistic (equivalent to Area Under Curve) was 72.3%, showing a reasonably good discrimination. Though doe weight was eliminated during model selection, we have decided to keep it in the final model, keeping in mind its association with kid mortality. So, we considered the final model consisting of birthweight, doe weight, sex, type of birth, season of birth and year of birth.

Discussion

Various estimates of mortality among goat kids during pre-weaning/neonatal period have been reported in the literature. Further, varying patterns of mortality during pre-weaning/neonatal period have been reported by different authors. For example, it has been reported that up to 50% of all pre-weaning mortality among the kids occurs on the first day after birth (Dwyer *et al.*, 2016). By contrast, the kid mortality on the first day of postnatal life was low in the current study (3.5%). Almost half of the total mortality was recorded until the second week of age and mortality until neonatal age, with a figure of 64.4% of the total mortality, had a major share of the total pre-weaning mortality. Singh *et al.* (2008) reported a higher incidence of about 69% until 2 weeks of age in Jamunapari goat kids from India. Casellas *et al.* (2007) concluded that almost 50% of lambs died during first 2 weeks of life, which is in agreement with this study. Fragkou *et al.* (2010) have suggested a stiff target of 3% neonatal lamb mortality in well-managed flock, with an upper acceptable limit of 5%. The estimate of neonatal kid mortality (5.1%) in this study was marginally above the upper limit. Recently, Gowane *et al.* (2018) reported 4.3%, 5.2% and 4.1% mortality estimates in neonatal lambs of Avikalin, Chokla and Malpura sheep, which are the sheep breeds maintained at the same institute where this study was conducted. The estimate of neonatal mortality in this study was almost similar to those reported by Gowane *et al.* (2018). The total mortality in the present study was higher than the average mortality rate of 4.5% reported by Kumar *et al.* (2010) in a field flock of Sirohi goat.

The birthweight is very important determinant of kid mortality. Higher birthweight is associated with better survival. Vigour, udder-seeking and thermoregulatory ability are dysfunctional in low birth weight lambs (Dwyer *et al.*, 2016). Similar reasons could be responsible for high mortality in low birthweight kids of Sirohi goat. Bangar *et al.* (2016) have reported a better survival with higher birthweight in Deccani sheep lamb (hazard ratio: 0.53; 95% CI, 0.40 to 0.69). Subramaniyan *et al.* (2016) have also reported the association of birthweight with kid mortality. In the studies of lamb survival, it has been found that lightest and heaviest lambs had a reduced survival probability (Casellas *et al.*, 2007; Sawalha *et al.*, 2007), but, in this study, the lightest kids had reduced survival probability while the heaviest kids had enhanced survival probability.

Similarly to birthweight, low doe weight was associated with increased incidence of mortality. Colostrum and milk productions are suboptimal in low weight dams resulting in starvation and poor immunity of kids. Low doe weight is also associated with reduced birthweight, which is related to increased incidence of mortality. Due to undernutrition, maternal care and recognition abilities of the mother are compromised, resulting in poor bond between mother and kid (Dwyer *et al.*, 2016). Chowdhury *et al.* (2002) and Subramaniyan *et al.* (2016) have also reported a significant association of doe weight with mortality.

Being male kid was associated with poor survival. Existence of male-linked death has been reported in piglets

(Baxter *et al*., 2012) and human beings (Kraemer, 2000). Impaired thermoregulation in male piglets is one of the factors linked to male-linked death (Baxter *et al*., 2012). At birth, the behaviours like standing, udder seeking and sucking ability are compromised in male lambs and kids compared with female (Dwyer *et al*., 2003). These may be the reasons responsible for greater hazard of death among male kids of Sirohi goat. Similar findings have been reported by other authors in sheep lambs (Southey *et al*., 2003; Sawalha *et al*., 2007), and they have found that male lambs were found to be at a higher risk of death than females. Bangar *et al*. (2016) have reported an almost similar estimate of hazard ratio (hazard ratio for male: 1.37; 95% CI, 1.05 to 1.80) in Deccani sheep lamb. However, Barazandeh *et al*. (2012) have reported that there is no difference between survival of male and female lambs in Iranian Kermani sheep (hazard ratio: 1.00±0.20 SE).

Though the singletons had better survival than twins in this study, we found the contrary in the initial analysis due to heterogeneity in the data. The heterogeneity may be caused due to unmeasured factors, which cannot be modeled in Cox regression. The unmeasured factors may be disease outbreaks, availability of feeds and fodders and condition of pasture. For example, the high kid mortality in the year 2011 was due to pneumonia caused by an unidentified organism. Similarly, Sawalha *et al*. (2007) reported smaller hazard of singletons compared with twins during the postnatal period from 1 to 14 days. Subramaniyan *et al*. (2016) observed better survival in single-born kids of Tellicherry goat. But, Bangar *et al*. (2016) too found that twin had a lower hazard of death by a factor of 0.21 (hazard ratio: 0.79; 95% CI, 0.32 to 1.96).

Besides animal-related factors, mortality is affected by environmental factors like season of birth and year of birth. The winter season had very high hazard of death in this study, and a higher total mortality estimate was noted in the winter compared with other seasons (57.7% *v*. 42.3%). The neonates are sensitive to cold exposure, and a sudden drop in winter temperature causes hypothermia resulting in death. On contrary, Singh *et al*. (2008) reported a higher mortality among kids of Jamunapari goat in the spring season. With each passing year there has been decrease in the risks of death. This could be due to overall improvement in the management in the later years.

Internal validity is a prerequisite for claiming a cause-effect relationship between two variables. In the present study, we found reasonably good estimate of internal validity concluding a good evidence of causality. But, models have the tendency to fit the dataset well on which they have been developed. It is essential to assess the performance of the models through external validation by exposing the models to new data. Thus, to generalise the results of a study, external validity is the next logical step after internal validation. If the study cannot be generalised, then causal effect obtained through internal validation has no meaning at practical levels.

Conclusion

Accurate knowledge about the factors affecting mortality of kids provides guidelines for optimising rearing practices of kids. The Cox regression model developed in this study had reasonably good discrimination ability, and the information generated from the model will definitely help in better management of goat kids. Both environmental and animal-related factors were found to affect the mortality among kids. Kids of low birthweight and male kids were found to have greater hazards of mortality. The singletons had better survival than twins. The winter season was highly unfavourable to the survival of the kids. Special care and management of the low birthweight kids can reduce the mortality losses. The kids need special attention during winter season to protect them from severity of cold weather. The association between the behaviours and mortality among Sirohi goat kids has not been explored till this date. A study is desired in this direction, which may give new insights for better control of mortality.

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Declaration of interest

Authors of the manuscript hereby declare that there is no conflict of interest for this research work.

Ethics statement

Approval of the research work by Institute Ethics Committee was not needed because the study did not involve any experiment on live animals.

Software and data repository resources

The dataset used in this research is part of institute data repository of ICAR-Central Sheep and Wool Research Institute, Avikanagar - 304 501, Rajasthan, India, and is not publicly available.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731119001617

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Influence of diet and manure management on ammonia and greenhouse gas emissions from dairy barns

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*Dairy systems are a source of pollutant emissions, such as greenhouse gases (**GHG**) and NH_3 that are associated with impacts on the environment. Gas emissions in barns are related mainly to diet intake and chemical composition, N excretion and manure management. A reduction in dietary N is known to be an effective way to reduce N excretion and the resulting NH_3 emissions. However, most studies consider manure in liquid form with frequent removal from the barn. In deep litter systems, several processes can occur during the accumulation of solid manure that result in variable gas emissions. The objective of this experiment was to investigate the influence of the interaction between dietary CP (low or high) and manure management (liquid or solid) on gas emissions (NH_3, N_2O, CH_4) at the barn level. Dietary treatments provided either low (**LowN**; 12% CP) or high (**HighN**; 18% CP) degradable protein to modify the amount of total ammonia nitrogen (**TAN**) excreted. The cows were housed for two 8-week periods in two mechanically ventilated rooms equipped to manage manure either in liquid (**LM**; slurry) or solid form (**SM**; deep litter). In the LM treatment, N balance was measured for 4 days. As expected, animals fed the LowN diet ingested 35% less N and excreted 65% less N in their urine, with no reduction in faecal N excretion and N secretion in milk. On the LowN diet, excretion of urea-N and NH_3-N emissions were reduced regardless of the manure management. On the HighN diet, urinary urea-N excretion was three times as high, while NH_3-N emissions were 3.0 and 4.5 times as high in LM and SM, respectively. Manure management strongly influenced CH_4-C emissions, which were 30% higher in SM than in LM, due to the accumulation of litter. Moreover, gas emissions from solid manure increased over the accumulation period, except for NH_3 on the LowN diet. Finally, our results suggest that methods used for national inventories would become more accurate by considering the variability in TAN excretion, which is the primary factor that influences NH_3 emissions.*

Keywords: dairy cow, nitrogen excretion, liquid manure, deep litter, gas emission

Implications

National emission inventories require reliable emission data representative of national practices. In some EU countries, such as France, cattle production systems are very diverse, including deep litters, for which few references exist. This study provides new insight on pollutant gas emissions from liquid and solid manure combined with low or high N excretion levels and recommends some changes to improve consideration of diet and manure management practices in inventory guidelines.

Introduction

Environmental impacts of agriculture in general, and of livestock systems in particular, have been discussed extensively in recent decades. Greenhouse gas (**GHG**) and NH_3 volatilization processes, as well as factors that influence these, are well understood and documented (Monteny and Erisman, 1998; Petersen *et al.*, 2013). In particular, NH_3 emissions from livestock are directly linked to the amount of N the animals excrete, especially in the form of urea (Bussink and Oenema, 1998; Hristov *et al.*, 2011). Urea is rapidly hydrolysed by the enzyme urease to ammonium-N and compounds, referred to as total ammonia nitrogen (**TAN**). A reduction in the dietary protein content appears the most effective strategy to decrease at-source N, and especially TAN, excretion, and related NH_3 emissions (see Hou *et al.*, 2015 for a review). However, most studies consider manure in its liquid form, with daily or more frequent removal from the barn. For deep litter systems, which still represent a large proportion of housing systems in France, the UK and Eastern Europe (Steinfeld *et al.*, 2010), manure is mixed with bedding

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material and accumulates for a few weeks in a thick layer where the oxygen level decreases with depth. This can result in several processes, such as aerobic and/or anaerobic organic matter (**OM**) degradation, urea hydrolysis, nitrification-denitrification and N immobilization (Jeppsson, 1999). These complex interactions among microbial, biochemical and physical processes result in highly variable emissions of NH_3 and GHGs (Webb *et al.*, 2012) that could intensify with high N and TAN excretion levels.

The objective of this study was therefore to investigate consequences of the interaction between dietary N (low or high) and manure management (liquid or solid) on NH_3, CH_4 and N_2O emissions at the dairy barn level. Diets provided balanced amounts of energy with degradable protein in deficit (**LowN**) or in excess (**HighN**), which should result in different levels of TAN being excreted. Manure was managed in either liquid form (**LM**) (removed twice a day) or solid form (**SM**) as deep litter that accumulated under the animals for 4 weeks, leading to variable physicochemical processes causing gas emissions. Finally, measured emissions were confronted to estimates using the Tier 2 European Environment Agency (EMEP/EEA; European Environment Agency, 2016) and Intergovernmental Panel on Climate Change (IPCC, 2006) methods to discuss the accuracy of emission factors (**EF**) used for national and international inventories, under the conditions of the study.

Materials and methods

Animals, treatments and experimental design

The experiment was conducted at the INRA dairy experimental farm in Méjusseaume, near Rennes (France), from September 2012 to January 2013. Six Holstein dairy cows in late lactation (242±40 days in milk and 19±3 kg of milk per cow per day) were divided into two homogeneous groups of three cows (initial bodyweight = 617±33 kg). The cows were in their second or third lactation. Each group of cows was housed in a closed and mechanically ventilated experimental room with negative air pressure and a controlled air-conditioning system (temperature maintained at ca. 16°C). Cows were milked in the rooms twice a day (0700 and 1700 h) and were weighed every 4 weeks. For practical reasons, each manure management treatment (LM *v.* SM) was allocated to one room with no changeover during the experiment.

Room A was equipped to manage manure in liquid form (no bedding material). For 14 days, the three cows were tied to measure N balance at the animal level on the last 4 days (**LM_{tied}**); all manure either was collected for 24 h (to quantify N excretion) or fell into a gutter behind the animals and was automatically scraped outside of the room twice a day (0730 and 1630 h). For the following 14 days, the room was transformed to allow the animals to move around in a free-stall barn with a solid floor and cubicles (**LM_{free}**); since the gutter was covered, the solid floor was manually scraped twice a day (0730 and 1630 h) to remove the manure. In room B, animals produced solid manure on a deep litter of straw (SM). Straw was spread homogeneously on the floor each day (80 kg on the first day, 40 kg per day thereafter), and litter accumulated below the animals for 4 weeks. Liquids were collected continuously in the 1% sloping central gutter below the deep litter. The solid manure was removed from the room after 4 weeks. In both rooms, cows moved freely within a 40 m^2 area when not tied (Supplementary Figure S1).

Dietary treatments consisted of total mixed rations offering low (LowN; CP: 120 g/kg DM, 85 g PDIN/kg DM) or high (HighN; CP: 180 g/kg DM, 119 g PDIN/kg DM) dietary degradable protein (**PDIN** – true protein absorbable in the small intestine when rumen-fermentable N is restricted in the rumen; INRA, 2007). The treatments represent extremes of the most commonly used feeding practices in French dairy systems (Pellerin *et al.*, 2013). The rations, composed of an 80 : 20 mixture of maize silage : concentrate, were fed *ad libitum* (refusal maintained at 5% to 10% of feed offered), individually for LM_{tied} and at the group level for LM_{free} and SM twice a day (0800 and 1800 h) with free access to water. Diets were formulated to meet animal protein requirements (Table 1).

The experiment was conducted over two 8-week periods. Each group of cows was assigned to one manure treatment for period 1 and then switched to its alternative for period 2. During each period, the two dietary treatments (LowN *v.*

Table 1 *Diet composition, nutrient content and calculated feeding values of the two experimental diets (LowN, 120 g CP/kg DM; HighN, 180 g CP/kg DM) fed to dairy cows*

Component	LowN	HighN
Ingredient, % DM		
Maize silage	79.0	78.5
Soybean meal mix[1]	5.0	12.8
Soybean meal, formaldehyde-treated	6.0	3.1
Mix concentrate[2]	9.0	3.1
Urea	0.0	1.5
Minerals	1.0	1.0
Nutrients, g/kg DM		
DM	388	389
OM[3]	952	951
CP	121	179
NDF	276	276
ADF	131	128
ADL	14.8	14.2
Feeding value[4]		
UFL/kg DM	0.96	0.95
PDIE, g/kg DM	98.7	100.0
PDIN, g/kg DM	85.5	118.8

[1] Soybean meal mix is composed of 98% soybean meal and 2% molasses.
[2] Mix concentrate is composed of 25% wheat, 25% maize, 25% barley, 20% beet pulp, 3% molasses, 1% vegetable oil and 1% NaCl.
[3] OM=organic matter.
[4] UFL, amount of net energy for milk production contained in 1 kg of a reference barley (87% DM, 2 700 kcal of metabolizable energy); PDIE, true protein absorbable in the small intestine when rumen-fermentable energy is limiting in the rumen; PDIN, true protein absorbable in the small intestine when rumen-fermentable N is limiting in the rumen (INRA, 2007).

Period	1								2[1]							
Sub-period	1				2				1				2			
Week	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Group A	HighN				LowN				LowN				HighN			
	LM tied	LM tied	LM free	LM free	LM tied	LM tied	LM free	LM free	SM	SM	SM	SM	SM	SM	SM	SM[2]
Group B	HighN				LowN				LowN				HighN			
	SM	SM	SM	SM	SM	SM	SM	SM[2]	LM tied	LM tied	LM free	LM free	LM tied	LM tied	LM free	LM free

Figure 1 Description of the experimental design offered to the two groups of three dairy cows (A and B), with two dietary treatments (LowN, 120 g CP/kg DM; HighN, 180 g CP/kg DM) and two manure management systems (LM, white boxes: liquid manure; SM, grey boxes: solid manure, straw-based deep litter). In the LM treatment, for each sub-period, animals were tied for the first 2 weeks (LM_{tied}, hatched boxes) and then allowed to move freely in the free-stall barn with cubicles (LM_{free}, empty boxes) for the last 2 weeks. In the SM treatment, cows were allowed to move freely over the whole area of the deep litter throughout the experience. [1]Two cows, one in group A and one in group B, unexpectedly dried off in the course of period 2. [2]For sub-period 2 of periods 1 and 2, the litter was left alone for 3 days after the cows left the room to measure gas emissions from the litter itself after the accumulation period.

HighN) were offered successively to each group of three cows during two sub-periods of 4 weeks (hereafter referred to as weeks 1 to 4); the order of succession was inverted between the two periods (Figure 1).

Measurements, samples and analyses

Feed sampling and intake. Mechanical water meters were used to monitor daily water intake at the group level in LM_{free} and SM, and at the individual level in LM_{tied}. Offered and refused feed were weighed and sampled daily to determine their DM concentration to calculate cow DM intake. Average samples of concentrate and maize silage offered and refused were taken per sub-period and analysed for OM concentration, total N concentration and fibre content (NDL, ADF and ADL).

Milk yield and composition. Individual milk yield was monitored daily. Morning and evening milk samples were collected 3 days a week to measure protein and fat contents. Daily total N in the milk was measured from morning and evening milk yields and joint samples at the individual level once during week 2 (in LM_{tied}) and once during week 4 (in LM_{free} and SM) of each sub-period.

Faeces and urine. Faeces and urine daily excretion were quantified during the last 4 days and nights of week 2 in the LM_{tied} treatment. Faeces were collected at the individual level in the gutter behind the animals and were weighed daily. Animals were equipped with harnesses to collect urine separately. Urine was immediately acidified with 500 ml of 20% H_2SO_4 to prevent NH_3 volatilization and weighed daily. Samples were collected daily at the individual level (1% of fresh amount for faeces and urine) and averaged per week (four daily samples successively stored in the same container at −20°C) to analyse faecal DM, faecal OM, faecal and urinary total-N and urinary urea-N.

Chemical analyses. Dry matter and organic matter concentrations were obtained by drying of diets and faeces at 80°C for 48 h and ashing at 500°C for 6 h, respectively. Fibre concentration corresponded to ADF, NDF and ADL fractions (van-Soest *et al.*, 1991). Milk protein and fat contents were measured by IR analysis using Milkoscan 605 (Foss Electric, Hillerød, Denmark). Total-N was measured using the Dumas method (Association Française de Normalisation, 2003), while urea-N resulted from a colorimetric enzymatic reaction assessed by a multi-parameter analyser (KONE Instruments 200 Corporation, Espoo, Finland).

N balance. During week 2 of the LM_{tied} treatment, N balance was calculated individually for 4 days based on N intake minus [N in milk plus N excreted (urine and faeces) plus N retained by the animal (based only on its energy balance, as almost no N was retained for growth and gestation in the present study; INRA, 2007)]. The remainder was assumed to be related to losses during N recovery and/or unaccounted-for sources of N excretion.

Litter temperature. In the SM treatment, the temperature of the litter at a 10 cm depth was measured once a week using a stick temperature probe (HI 935005, Hana Instruments, Tanneries, France). The litter surface was virtually divided into six equal zones, in each of which three measurements were taken, resulting in a total of 18 temperature measurements averaged to have one mean temperature data per week (except for week 1 of sub-period 1 of period 2 due to equipment failure).

Gas emission measurements. Gas emissions, as well as temperature and humidity in the rooms, were continuously measured for both LM and SM from weeks 1 to 4 of each sub-period (Supplementary Table S1). Once per period (after sub-period 2), in the SM treatment only, gas emissions were

also measured for 3 days immediately after the cows left the room but before the litter was removed. Gas concentration measurements started 3 days before period 1 to measure basal concentrations in the room without animals. An IR photo-acoustic analyser (INNOVA model 1412; Air Tech Instruments, Ballerup, Denmark) combined with a sampler-doser (INNOVA 1303) and a computer recorded the concentrations. The INNOVA analyser was fitted with six filters, enabling the concentrations of six gases (NH_3, CO_2, CH_4, N_2O, H_2O and C_2H_6O) to be measured. This configuration was chosen to compensate for interference between NH_3 and other volatile molecules (e.g. C_2H_6O; Hassouna *et al.*, 2013). The instrument internally corrected for signal interferences from the gases measured (optical filter/detection limit: NH_3 979/0.5 ppm; CO_2 982/1.5 ppm; CH_4 969/0.4 ppm; N_2O 985/0.03 ppm) and was span-calibrated with known concentrations by the manufacturer before the experiment. Air was sampled in each isolated room at the air inlet and outlet to calculate a gradient. Air samples were extracted from the experimental rooms into the analyser through 3-mm PTFE (Teflon®) sampling lines that were protected with dust filters, insulated and heated to avoid water condensation. The analyser sampled the air and measured gas concentrations at 2-min intervals (1 min for measurement, 1 min for flushing sampling tubes and measurement chamber). Each location (inlet and outlet of each room) was successively analysed for 15 min, the first two measurements being excluded to address potential pollution from one location to the next.

Air was extracted continuously by the extraction duct at a constant rate which did not fluctuate as a function of ambient temperature. The flow rate (Q, in m^3/h per cow) in each experimental room was determined over 1 week at the end of the experiment (not performed during gas concentration measurements because it required another INNOVA analyser with the suitable configuration in terms of filters) using the tracer ratio method (SF_6) and the constant dosing approach (Baptista *et al.*, 1999). Flow rate was calculated as a function of time (t) from the rate of tracer release (ϕT) in m^3/h and the indoor tracer concentration (CT inside) in mg/m^3 after correcting for the background concentration of the tracer (CT outside) (Demmers *et al.*, 2001):

$$Q(t) = (\phi T(t))/(\text{CT inside}\,(t) - \text{CT outside}(t)).$$

Ventilation rate was 702 ± 65 m^3/h per cow for the room with the SM treatment and 763 ± 80 m^3/h per cow for the room with the LM treatment.

Flow rates and gas concentrations were expressed as mean values per hour. Gas emissions were calculated by multiplying the ventilation rate (m^3/h per cow) by gas concentration gradients (corrected from the basal concentrations in the room without animals, in mg/m^3) and were expressed as cumulative gas emissions per cow per day (including for the 3 days without cows at the end of the sub-period in the SM treatment). Emissions were expressed as CH_4-C, NH_3-N and N_2O-N and were validated based on element mass balances (Hassouna and Eglin, 2016).

Emission estimates from national inventory guidelines. NH_3-N emissions were estimated for each treatment based on a default value of TAN excretion or a calculated value (from N balance) and emission factors at the barn level (European Environment Agency, 2016). Enteric CH_4 emissions were estimated from gross energy intake, the CH_4 conversion factor and the energy content of CH_4 (IPCC, 2006). In SM, CH_4 emissions from manure were added based on measured feed digestibility and assuming storage of deep cattle litter for <1 month in temperate conditions. In LM_{free}, as the removal of liquid manure from the room twice a day would have resulted in negligible CH_4-C emissions, the latter were not considered. N_2O-N emissions were estimated from N excretion per head, the fraction of TAN excretion captured in the manure management system, and the relative EF for direct N_2O emissions (IPCC, 2006).

Statistical analyses

In the LM_{tied} treatment, variables related to the N balance were recorded at the individual level and averaged over the 4 days of urine and faeces collection: N intake; N excreted in milk, faeces and urine; retained and unaccounted N; urea-N content in urine, urea-N excreted in urine and N efficiency (N in milk divided by N intake). During period 2, two cows unexpectedly dried off (one per group). Only one was involved in measurements for LM_{tied}. Considering this cow in the N balance calculation could lead to biased interpretation as no more N is exported in milk for this dried cow. All N ingested was therefore excreted in urine and faeces, modifying the repartition of N excretion. To ensure a consistent N balance at the individual level, measurements for this cow were removed from the dataset for all of period 2. The influence of dietary treatment (LowN *v.* HighN) was assessed by the following linear model (SAS, 2013; PROC MIXED):

$$Y_{ijk} = \mu + \text{Nconc}_i + \text{Period}_j + \text{Cow}_k + e_{ijk}$$

where Y_{ijk} is the variable studied; μ is the mean; Nconc_i is the dietary CP concentration (1 df); Period_j is the period of 8 weeks (1 df); Cow_k is a random factor; and e_{ijk} is the error associated with each Y_{ijk}.

In the SM and LM_{free} treatments, only week 4 of the sub-period was considered for analysis. Milk yield, water intake, DM intake and daily gas emissions (CH_4-C, NH_3-N, N_2O-N) were compared at the group level between the two dietary treatments (LowN *v.* HighN), the two manure management systems (liquid *v.* solid) and their interaction. To capture dynamics of gas emissions that are known to be variable between days, daily measurements were used instead of weekly averages. To address temporal correlation among daily measurements of the same groups of cows, linear models for repeated measurements were created using the statistical model (SAS, 2013; PROC MIXED):

$$Y_{ijklm} = \mu + \text{Nconc}_i + \text{Manure Type}_j + \text{Nconc} \times \text{Manure Type}_k + \text{Period}_l + \text{Day (Period)}_{m(l)} + e_{ijklm}$$

where Y_{ijklm} is the variable studied; μ is the mean; Nconc_i is the dietary CP concentration (1 df); ManureType_j is the manure form (liquid or solid, 1 df); $\text{Nconc} \times \text{ManureType}_k$ is the interaction of the two factors (1 df); Period_l is the period of 8 weeks (1 df); Day $(\text{Period})_{m(l)}$ is the day of measurement within the period (12 df); and e_{ijklm} is the error associated with each Y_{ijklm}. The REPEATED statement was added to the SAS procedure (Day (Period)/sub = Group, compound symmetry). The two cows that unexpectedly dried off in period 2 could not be removed from the dataset because they continued to ingest feed and emit gases in the rooms. This can lead to biased intake and emissions results at the group level; however, as both groups were concerned, all treatments were considered to be similarly affected.

In the SM treatment, gas emissions and litter temperature at a 10-cm depth were averaged per week and analysed to determine the influence of dietary treatment (LowN *v.* HighN), the weeks (1 to 4) and their interaction using the statistical model (SAS, 2013; PROC MIXED):

$$Y_{ijkl} = \mu + \text{Nconc}_i + \text{Week}_j + \text{Nconc} \times \text{Week}_k + \text{Group}_l + e_{ijkl}$$

where Y_{ijkl} is the variable studied; μ is the mean; Nconc_i is the dietary CP concentration (1 df); Week_j is the week of measurement for each sub-period (3 df); $\text{Nconc} \times \text{Week}_k$ is the interaction of the two factors (3 df); Group_l is the group of cows (1 df); and e_{ijkl} is the error associated with each Y_{ijkl}. The Period effect was unfortunately confounded with the group of cows and consequently not included in the model. The REPEATED statement was added to the SAS procedure to consider temporal correlation between weeks of the same group of cows' intra-diet treatment (Week/sub = Group(Nconc), compound symmetry).

Regardless of the model, the Room effect could not be considered because it was confounded with the ManureType treatment. The Room effect gathered potential bias from its ventilation system or its orientation to the sun. However, ventilation rates used for emission calculations were assessed for each room individually, and climate conditions were controlled in both rooms by the air conditioner system. Consequences are therefore likely to be very negligible for emission measurements.

Results

Influence of N supply on N balance and excretion

On the HighN diet, N intake increased by 50% ($P<0.01$), N in milk and N in faeces did not differ significantly ($P=0.36$ and 0.49, respectively), but N in urine was almost three times as high ($P<0.01$; Table 2) as that in the LowN diet. Consequently, cows on the LowN diet had significantly higher N efficiency (+12 percentage-units; $P<0.01$). The amount of N the animals retained was similar for both diets (23 to 28 g N per cow per day; Table 2). More N was unaccounted for on the HighN diet than on the LowN diet (70 *v.* 12 g N per cow per day; $P<0.01$). Urinary urea-N was four times as high on the HighN diet ($P<0.01$), with urea-N representing 80% of urinary N on the HighN diet, compared to 53% on the LowN diet ($P<0.01$).

Table 2 *Nitrogen partitioning as a function of dietary N supply (LowN, 120 g CP/kg DM; HighN, 180 g CP/kg DM) offered to dairy cows*

Variable	n[1]	LowN	HighN	SEM	P-value
N partitioning (g per day)					
Intake	10	427	648	17	<0.01
Milk	10	132	122	7	0.36
Faecal	10	162	155	11	0.49
Urinary	10	94	265	9	<0.01
Retained[2]	10	23	28	5	0.36
Unaccounted for	10	12	70	9	<0.01
Urinary urea-N (g per day)	10	50	212	6	<0.01
Urea-N in urinary-N (%)	10	53	80	3	<0.01
N efficiency (%)	10	31	19	1	<0.01

[1] Average of the 4 days of N-balance measurements: five cows (experimental units) × two dietary treatments.
[2] Based on the animals' energy balance (calculated from UFL intake, mean body-weight and fat-corrected milk yields as described by INRA, 2007), using 6 g N retained per UFL (Faverdin and Vérité, 1998). UFL, amount of net energy for milk production contained in 1 kg of a reference barley (87% DM, 2 700 kcal of metabolizable energy; INRA, 2007).

Interaction between N supply and manure management

N supply and manure management did not influence the performance parameters, either alone or in interaction. For emissions, manure management influenced only CH_4-C ($P=0.03$), which were 30% higher in SM than in LM_{free}. In contrast, N supply influenced NH_3-N emissions ($P=0.03$), which increased considerably on the HighN diet. Although the interaction between N supply and manure management was not significant ($P=0.11$; Table 3), the HighN diet resulted in NH_3-N emissions three and four times as high in the LM_{free} and SM treatment, respectively, compared with LowN, reaching 100 gNH_3-N per cow per day in the latter (Table 3). N_2O-N emissions were nearly 0 in almost all situations and therefore close to the INNOVA detection limit (results not shown).

In the SM treatment, CH_4-C and NH_3-N emissions increased every week due to litter accumulation under the animals ($P<0.05$ and <0.01; Table 4). For NH_3-N, however, this increase was observed only on the HighN diet (48 to 99 g NH_3-N per cow per day; $P<0.01$), while emissions remained low on the LowN diet (<22 g NH_3-N per cow per day). Deep litter temperature at a 10-cm depth increased over time, regardless of the diet ($P<0.05$). For CH_4-C and NH_3-N, daily emissions decreased rapidly after the cows left the room (Figure 2). Conversely, N_2O-N emissions in the SM treatment

Table 3 *Performance and gas emissions of groups of dairy cows as a function of dietary N supply (LowN, 120 g CP/kg DM; HighN, 180 g CP/kg DM) and the manure management system for week 4 of each sub-period*

Variable	n[1]	LowN		HighN		SEM	*P*-value		
		LM_{free}	SM	LM_{free}	SM		N	M	N×M
Water intake (L per cow per day)	48	63.2	65.8	72.6	77.3	9.7	0.17	0.43	0.78
DM intake (kg per cow per day)	56	22.2	22.7	23.7	23.9	1.5	0.18	0.54	0.82
Energy-corrected milk yield (kg per cow per day)[2]	56	20.2	19.2	21.0	21.6	1.7	0.17	0.72	0.32
Milk fat (g/kg)	24	40.1	41.6	42.3	39.6	1.8	0.92	0.57	0.21
Milk protein (g/kg)	24	36.4	37.7	37.0	35.4	1.5	0.41	0.81	0.25
Emissions (g per cow per day)									
CH_4-C	56	326	426	321	424	19	0.61	0.03	0.81
NH_3-N	55	21.9	21.6	68.3	98.9	9.9	0.03	0.11	0.11

LM_{free}, liquid manure, free-stall barn with cubicles; SM, solid manure, straw-based deep litter; N, effect of dietary N supply (LowN *v.* HighN); M, effect of manure management system (LM_{free} *v.* SM); N×M, effect of their interaction.
[1] Daily measurements: two groups of cows (experimental unit) × four sub-periods × 7 days (2 days for milk fat and protein); some values can be missing.
[2] Energy-corrected milk yield = milk yield (kg per cow per day) × (0.4 + (0.015 × milk fat (g/kg))) (INRA, 2007).

Table 4 *Gas emissions and litter temperature dynamics in the solid manure management system as a function of dietary N supply (LowN, 120 g CP/kg DM; HighN, 180 g CP/kg DM) offered to dairy cows and the week of measurement (W1–W4)*

Variable	n[1]	LowN				HighN				SEM	*P*-value		
		W1	W2	W3	W4	W1	W2	W3	W4		N	W	N×W
Emissions (g per cow per day)													
CH_4-C	16	340	354	395	426	370	366	398	424	29	0.59	<0.05	0.86
NH_3-N	16	21.1	18.1	18.1	21.6	48.3	75.7	88.0	99.1	5.2	<0.01	<0.01	<0.01
Litter[2]													
Temp. (°C)	16	29.9	36.6	36.0	37.0	29.1	33.2	34.1	35.0	1.5	0.46	<0.05	0.78

N, effect of dietary N supply; W, effect of the week of measurement; N×W, effect of their interaction.
[1] Average per week: two groups of cows (experimental unit) × two dietary treatments × 4 weeks.
[2] Litter temperatures measured at a 10-cm depth, mean values of 18 measurements per week (six zones × three replicates).

started to increase in the final days of the sub-period and increased even more after the cows left the room (Figure 2). This occurred mainly on the HighN diet, with N_2O-N emissions reaching 1.2 g N_2O-N per cow per day at the end of week 4 and 4.5 g N_2O-N per cow per day 3 days after the cows left the room and the litter was left alone.

Gas emission estimates based on national inventory guidelines

Using the standard calculation from EMEP/EEA, estimated NH_3-N emissions were 30 and 50 g per cow per day on the LowN and HighN diets, respectively, regardless of the manure management (Table 5). Using the calculated TAN excretion instead of a default value, NH_3-N was 22 g per cow per day on the LowN diet but increased to nearly 70 g per cow per day on the HighN diet. Measured NH_3-N emissions in LM_{free} (Tables 3 and 4) represented 20% of the calculated TAN excretion, regardless of the diet. In SM, measured NH_3-N emissions from weeks 1 to 4 of litter accumulation represented 17% to 20% of the calculated TAN excretion on the LowN diet and 14% to 30% on the HighN diet (Table 5). CH_4-C emissions from IPCC (enteric for LM; enteric + manure for SM) varied between 360 and 410 g per cow per day. Finally, estimations of N_2O-N emissions were close to 1 to 2 g per cow per day regardless of the manure management (Table 5).

Discussion

Manure management had more influence on methane emissions

Manure management influenced CH_4 emissions, which were 30% higher (+100 g CH_4-C per cow per day) in the SM treatment than in the LM_{free} treatment in week 4. CH_4 emissions from deep litter systems are mainly the result of fermentation processes. CH_4 is formed under warm anaerobic conditions, induced by compaction of the litter, when degradable C is available (Amon *et al.*, 2001). CH_4-C emissions increased over time but decreased rapidly after the animals left the rooms, down to a level close to the difference between the SM and LM_{free} treatments (100 g CH_4-C per cow per day), that is, emissions from the litter itself.

At the room level (animals and manure), dietary N supply did not influence CH_4 emissions. Forage intake and digestibility did not vary between diets (digestible DM: 716 and 724 g/kg DM for LowN and HighN, respectively; SEM = 29 g/kg DM; $P>0.05$) leading to quite similar levels of enteric

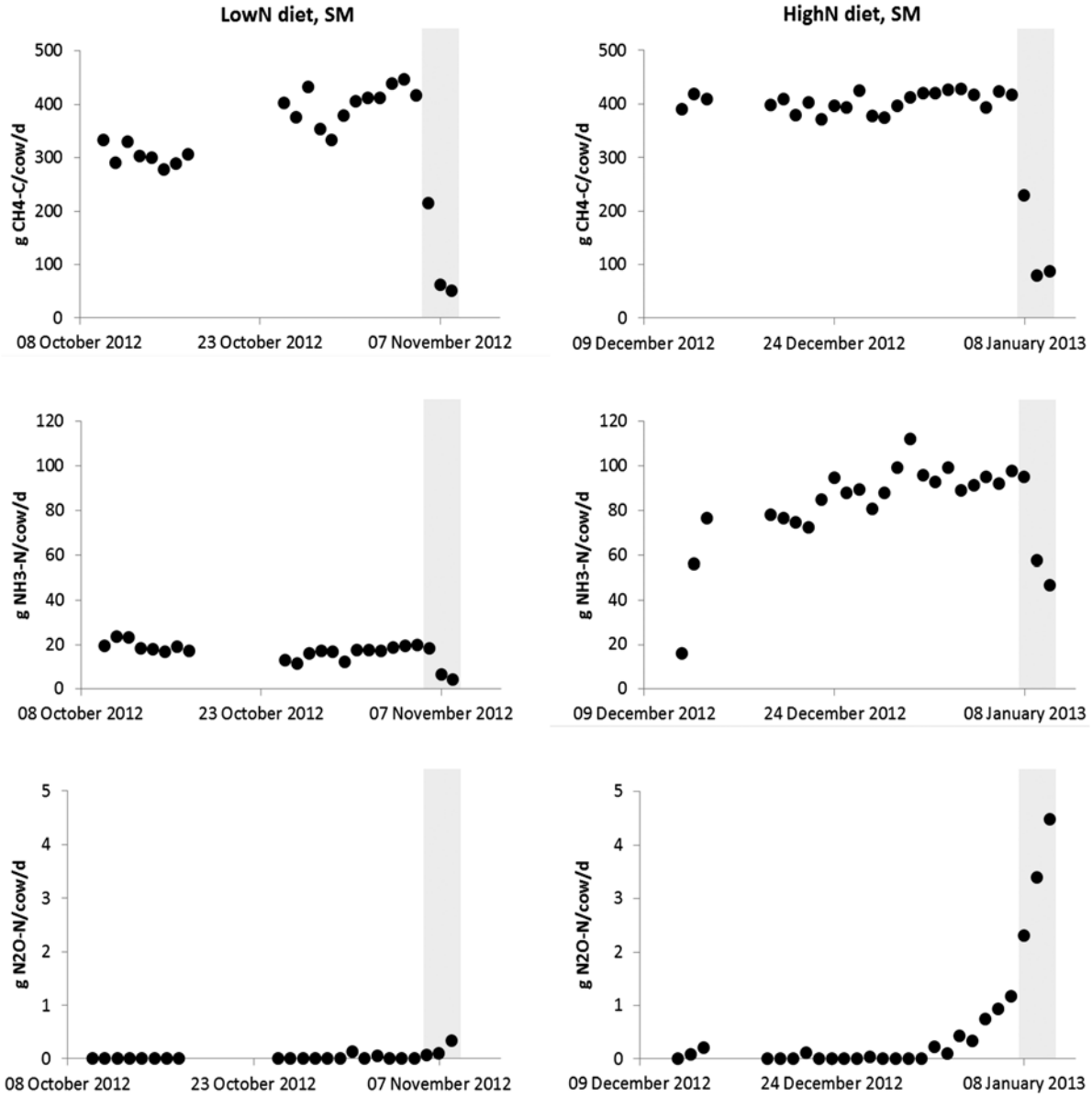

Figure 2 Time-dependent gas emission of CH_4-C, NH_3-N and N_2O-N in the SM treatment offered to dairy cows during sub-period 2 of period 1 (LowN diet, left) and period 2 (HighN diet, right). Gaps indicate days without measurement due to equipment failure. Grey bars represent the 3 days at the end of the sub-periods when the litter was left alone in the room, without cows. SM=solid manure.

methane emissions (Table 5). When expressed in relation to intake, CH_4 emissions from LM_{free} (i.e. enteric methane) were 19.6 and 18.1 g CH_4/kgDMI, respectively, in the range of common observations (Hristov *et al.*, 2013).

Nitrogen-based gas emissions influenced by an interaction between nitrogen supply and manure management

Nitrogen balance and N efficiency measurements are consistent with the literature (Castillo *et al.*, 2000; Cantalapiedra-Hijar *et al.*, 2014; Edouard *et al.*, 2016). The HighN diet resulted in excretions of urea-N in urine and TAN three times as high as those in the LowN diet. The HighN diet also led to more N unaccounted for. These deficits in the balance might be related to substantial TAN losses during manure recovery and/or unaccounted-for sources of excretion, which might be enhanced as the dietary N availability increases (Spanghero and Kowalski, 1997). Accordingly, NH_3-N emissions on the HighN diet were 3.0 and 4.5 times as high as those on the LowN diet in LM_{free} and SM, respectively. This large difference is partly because emissions were observed in week 4 of each sub-period, when NH_3 emissions were at their maximum in SM. These results are consistent with those of studies that estimated NH_3 volatilization from manure following similar dietary manipulations under laboratory conditions (Frank and Swensson, 2002; Burgos *et al.*, 2010) or at the barn level using modelling (Monteny *et al.*, 2002) or *in situ* measurements (Powell *et al.*, 2008). Decreasing the CP content of the diet by modulating the protein balance in the rumen

Table 5 *Estimated total ammonia nitrogen (TAN) excretion and NH_3-N, CH_4-C and N_2O-N emissions using the EMEP/EEA and IPCC Tier 2 methods as a function of dietary N supply (LowN, 120 g CP/kg DM; HighN, 180 g CP/kg DM) and the manure management system offered to dairy cows LM_{free}, liquid manure, free-stall barn with cubicles; SM, solid manure, straw-based deep litter.*

	Variable	LowN		HighN	
		LM_{free}	SM	LM_{free}	SM
EMEP[1]	TAN excreted				
	Default (g per cow per day)[2]	154	154	252	252
	Calculated (g per cow per day)[3]	111	111	345	345
	As percentage of N excreted	43	43	80	80
	NH_3-N emissions (g per cow per day)				
	Default TAN and EF[4]	30.7	29.2	50.4	47.9
	Calculated TAN and EF[5]	22.0	20.9	67.0	63.7
	Estimated EF (% TAN) using measured NH_3-N emissions[6]				
	Week 1		19		14
	Week 2		17		23
	Week 3		17		26
	Week 4	20	20	20	30
IPCC[7]	CH_4-C emissions (g per cow per day)[8]	359	383	391	412
	N_2O-N emissions (g per cow per day)[9]	1.3	1.3	2.2	2.2

[1] EMEP/EEA Tier 2 method (European Environment Agency, 2016).
[2] 60% of N excreted (N in urine + N in faeces, from Table 1).
[3] Equals N ingested – (milk N + faecal N + retained N); see Table 1.
[4] Using TAN = 60% of N excreted and the emission factors $EF_{housing}$ = 20% TAN for liquid manure and 19% TAN for solid manure (Table 3.9 of the EMEP/EEA Tier 2 method).
[5] Using the estimated TAN from the calculation (N ingested – (milk N + faecal N + retained N)) and the emission factors $EF_{housing}$ = 20% TAN for liquid manure and 19% TAN for solid manure (Table 3.9 of the EMEP/EEA Tier 2 method).
[6] Measured NH_3-N emissions from Tables 2 and 3 expressed as a percentage of TAN estimated from the calculation (N ingested – (milk N + faecal N + retained N)).
[7] IPCC Tier 2 method (IPCC, 2006).
[8] Based on enteric CH_4 (equation 10.21), manure CH_4 (equations 10.23 and 10.24) added for SM only.
[9] Direct N_2O emissions (equation 10.25).

[expressed by the ratio (PDIN – PDIE)/UFL; Table 1] is an effective way to reduce NH_3 emissions, as previously discussed by Edouard *et al.* (2016).

In the literature, conflicting results are reported, as solid manure is described to emit less NH_3-N than liquid manure at the barn level (Gac *et al.*, 2007; Schrade and Keck, 2011), or more (Webb *et al.*, 2012). All of these reviews, however, report large intra- and inter- variability in emission measurements due to contrasting environmental conditions, diets, methodological approaches and manure management practices (type and amount of litter, area and depth of the litter bed, removal frequency, frequency of adding fresh litter, etc.). Using the same methodological approach as in the present study, we previously showed that mean daily NH_3-N emissions at the dairy barn level were 25% higher in a straw deep litter system (accumulated over 6 weeks) than in a tying stall under similar conditions (same experimental rooms, 140 g/kg CP in the diet; Edouard *et al.*, 2012).

The originality of the present study was to test the interaction between the dietary N supply and manure management: NH_3-N was higher in the SM treatment when cows were offered the HighN diet, that is, when N and TAN excretions increased. This was most likely due to the substantial accumulation of N, especially urea-N, in the litter and the resulting chemical processes. Urease activity is related to temperature: higher temperatures result in more gaseous NH_3 (Monteny and Erisman, 1998; Webb *et al.*, 2012). The NH_3-N emission dynamics observed were consistent with the litter temperature dynamics. These dynamics were not observed in the SM–LowN treatment combination because the solid manure contained little urea-N. When the cows left the rooms (no new N from fresh urine and faeces), emissions from the solid manure decreased rapidly to a level similar to those of manure storage conditions (around 50 gNH_3-N per cow per day, i.e., 150 mgNH_3-N/h/m^2; Külling *et al.*, 2003; Mosquera *et al.*, 2006; Aguerre *et al.*, 2012).

Conversely, N_2O-N emissions remained nearly 0 for all treatments; they started to increase only in week 4 in the SM–HighN treatment combination and continued to do so after the cows left the rooms. N_2O emissions are linked to incomplete nitrification and denitrification processes associated with oxic and anoxic conditions, respectively. In animal houses that do not use bedding materials, faeces and urine on the floor remain mainly in an aerobic state and are frequently removed from the building. As a result, these systems are likely to emit little or no N_2O, whereas N_2O emissions from deep litter systems are slightly higher (though remaining <2 g N_2O-N per cow per day; Webb *et al.*, 2012). In the SM–HighN treatment combination, compaction by animals and increased moisture of the litter (due to more urine) might have generated partially anoxic conditions that encouraged denitrification and stimulated N_2O emissions (Aguerre *et al.*, 2012; Petersen *et al.*, 2013). These emission dynamics (increase in N_2O emissions after 3 to 4 weeks) are typical

of storage conditions (Külling *et al.*, 2003; Aguerre *et al.*, 2012).

Estimates from national inventory guidelines only partly consistent with measurements

The calculated TAN represented 43% and 80% of N excreted in manure for the LowN and HighN diets, respectively, compared to the default value of 60% use in the EMEP/EEA methodology. This suggests that the recommended value of TAN excretion would be more accurate if determined as a function of dietary CP content, leading to more specific NH_3 emissions. The calculated TAN based on measured excretion and the EMEP/EEA EFs resulted in estimates of NH_3-N emission similar to those measured, especially for liquid manure. Conversely, estimated NH_3 emissions differed greatly from those measured for the SM–HighN treatment combination, suggesting the need to consider emission dynamics related to solid manure management as well when TAN excretion is high.

When compared to measurements in the LM_{free} treatment, enteric CH_4 emissions estimated with the IPCC method were 10% to 20% higher. Nonetheless, given uncertainties in both the measurements and estimates, a difference of ±20% can be considered satisfactory. Conversely, CH_4 emissions from the litter (assuming that they equalled the difference between SM and LM_{free} CH_4 emissions) were three times as high as the IPCC estimate (100 *v.* 30 g CH_4-C per cow per day). Selecting the relevant EF seems critical, because using the EF related to storing deep bedding for >1 month (instead of <1 month) in temperate conditions would have resulted in predicted emissions >600 g CH_4-C per cow per day in the SM treatment, that is, 50% higher.

For N_2O emissions, IPCC estimates were very low (<2 g N_2O-N per cow per day), in agreement with the measurements at the barn level. N_2O emissions could however be a larger issue when deep litter accumulates under the animals for a longer period (increasing emissions in week 4), which the IPCC method might not sufficiently consider.

Conclusions

When combined with a low-N diet, NH_3 emissions from deep litter systems could be maintained at a low level. Conversely, high levels of N in the diet increased NH_3 emissions, which should encourage farmers to be cautious, especially in the case of housing systems that produce solid manure. Moreover, the longer the litter accumulates, the higher gas emissions may be. Comparing measured emissions to those estimated with EMEP/EEA and IPCC guidelines shows that these methods accurately estimate gas emissions under certain conditions but not with diets high in CP or deep litter manure systems. These calculations would in particular require to better take into consideration the variability in TAN excretion, which is the primary factor that influences NH_3 emissions, and the emission dynamics of solid manure. More specific emission factors would finally help estimations at farm level and national inventories to gain precision.

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Declaration of interest

None.

Ethics statement

This work complies with the national legislation applicable at the time of the experiment.

Software and data repository resources

None of the data were deposited in an official repository.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731119001368

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animal

Estimating the impact of clinical mastitis in dairy cows on greenhouse gas emissions using a dynamic stochastic simulation model: a case study

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*The increasing attention for global warming is likely to contribute to the introduction of policies or other incentives to reduce greenhouse gas (**GHG**) emissions related to livestock production, including dairy. The dairy sector is an important contributor to GHG emissions. Clinical mastitis (**CM**), an intramammary infection, results in reduced milk production and fertility, increases culling and mortality of cows and, therefore, has a negative impact on the efficiency (output/input) of milk production. This may increase GHG emissions per unit of product. Our objective was to estimate the impact of CM in dairy cows on GHG emissions of milk production for the Dutch situation. A dynamic stochastic simulation model was developed to simulate the dynamics and losses of CM for individual lactations. Cows receive a parity (1 to 5+), a milk production and a calving interval (**CI**). Based on the parity, cows have a risk of CM, with a maximum of three cases in a lactation. Pathogens causing CM were classified as gram-positive bacteria, gram-negative bacteria, or other. Based on the parity and pathogen combinations, cows had a reduced milk production, discarded milk, prolonged CI and a risk of removal (culling and mortality) that reduce productivity of dairy cows and therefore increase GHG emissions per unit of product. Using life cycle assessment, emissions of GHGs were estimated from cradle to farm gate for processes along the milk production chain that are affected by CM. Processes included were feed production, enteric fermentation, and manure management. Emissions of GHGs were expressed as kg CO_2 equivalents per ton of fat-and-protein-corrected milk (**kg CO_2e/t FPCM**). Emissions of cows with CM increased on average by 57.5 (6.2%) kg CO_2e/t FPCM compared with cows without CM. This increase was caused by removal (39%), discarded milk (38%), reduced milk production (17%) and prolonged CI (6%). The GHG emissions increased by 48 kg CO_2e/t FPCM for cows with one case of CM, by 69 kg CO_2e/t FPCM for cows with two cases of CM and by 92 kg CO_2e/t FPCM for cows with three cases of CM compared with cows without CM. Preventing CM can be an effective strategy for farmers to reduce GHG emissions and can contribute to sustainable development of the dairy sector, because this also can improve the income of farmers and the welfare of cows. The impact of CM on GHG emissions, however, will vary between farms due to environmental conditions and management practices.*

Keywords: disease, health, environmental impact, carbon footprint, modeling

Implications

Dairy production has an impact on global warming. Clinical mastitis, an intramammary infection, reduces productivity of dairy cows. Improvements in the health of cows may benefit production efficiency, and therefore reduce greenhouse gas emissions per unit of product. Greenhouse gas emissions of cows with clinical mastitis increase on average by 6.2% compared with cows without clinical mastitis. This increase is caused by removal of cows (39%), discarded milk (38%), reduced milk production (17%) and prolonged calving interval (6%). Preventing clinical mastitis can reduce greenhouse gas emissions and contribute to sustainable development of the dairy sector.

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Introduction

One of the most urgent environmental issues is global warming, induced by greenhouse gas (**GHG**) emissions (Steffen *et al.*, 2015). The livestock sector is responsible for about 14.5% of the anthropogenic GHG emissions globally (Gerber *et al.*, 2013), of which 30% is emitted by the dairy sector. Important GHGs related to milk production are carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). Emissions of

GHGs are mainly emitted during feed production, enteric fermentation of feed and manure management. The increasing attention for global warming is likely to contribute to the introduction of policies or other incentives to reduce GHG emissions related to livestock production, including dairy. A common method to asses GHG emissions and the impact of mitigation options is life cycle assessment (**LCA**) that takes into account the entire life cycle of a product (Baumann and Tillmann, 2004). Many studies have used LCA to estimate GHG emissions of milk production, by summing emissions of GHGs along the production chain. Several studies investigated mitigation options (e.g. breeding or feeding) to reduce GHG emissions in the dairy sector (Van Middelaar *et al*., 2014a and 2014b). Little attention, however, has been given to the impact of health of dairy cows on GHG emissions. Williams *et al*. (2015) estimated the impact of 10 endemic cattle diseases, Özkan Gülzari *et al*. (2018) the impact of subclinical mastitis (**subCM**), Mostert *et al*. (2018b and 2018c) the impact of subclinical ketosis and foot lesions, and Chen *et al*. (2016) the impact of lameness on GHG emissions. Whereas some strategies to reduce GHG emissions can result in reduced income (Van Middelaar *et al*., 2014a) or reduced welfare (Llonch *et al*., 2017), strategies to improve health of dairy cows might benefit the welfare of the animal, farm profitability (Hogeveen *et al*., 2011) and the environment (Williams *et al*., 2015) simultaneously.

Clinical mastitis (**CM**), an inflammatory process in the mammary gland (IDF, 1987), is one of the most frequent diseases in dairy cows (Seegers *et al*., 2003). Clinical mastitis can reoccur in the same lactation and across lactations due to several pathogens, which can be classified as gram-positive bacteria (e.g. *Streptococcus* spp), gram-negative bacteria (e.g. *E. coli*) or other organisms (Schukken *et al*., 2009). Dairy cows with CM express illness behavior after an infection (Fogsgaard *et al*., 2012) and, therefore, their welfare is considered to be impaired. In addition, CM has a negative impact on production. Clinical mastitis results in reduced milk production (Schukken *et al*., 2009), and fertility (Hertl *et al*., 2014), impairs milk quality, and increases culling and mortality of cows (Hertl *et al*., 2011). These effects depend on the severity and pathogen type causing CM. The impact of CM on production affects farm profitability. Additional costs because of CM are estimated between €61 and €97 on average per cow per year (Hogeveen *et al*., 2011) and $134, $211 and $95 per case of, respectively, gram-positive, gram-negative and other organisms (Cha *et al*., 2011).

Besides the negative impact of CM on farm profitability and on the welfare of cows, CM could also affect GHG emissions of milk production. The negative impact of CM on production reduces, for example, the feed efficiency (kg milk/kg feed intake) of the cow and therefore will increase the GHG emissions per unit of product produced. Hospido and Sonesson (2005) estimated the impact of CM on GHG emissions in Galician dairy herds. This scenario study of Hospido and Sonesson (2005), however, did not simulate the dynamics of CM per parity (e.g. pathogen type, recurrent case of CM and related losses) and did not show the variation in production losses of CM and in emissions of GHGs. Including these aspects will provide a better understanding of the impact of CM on GHG emissions.

Our objective was to estimate the impact of CM on GHG emissions per unit of product, including all processes from cradle to farm gate for the Dutch situation. By using a dynamic stochastic simulation model on cow level that includes several parities, different pathogens and frequency of occurrence of CM in one lactation, and by performing sensitivity analyses, our study can give new insights in the impact of CM on GHG emissions for Dutch dairy farmers.

Material and methods

A dynamic stochastic simulation model was developed in R (R_Core_Team, 2016) and combined with LCA to estimate the impact of CM on GHG emissions per unit of product. First, the model simulated the dynamics and losses of CM for one lactation. Second, GHG emissions were calculated by using an LCA. Input of general production parameters in the first part and the estimation of GHG emissions in the second part were the same as in Mostert *et al*. (2018b and 2018c) that estimated the impact of subclinical ketosis and foot lesions on GHG emissions. The analysis was performed for a predefined Dutch dairy farm (100 dairy cows) applying limited grazing (8 h/day, 170 days/year), which is a common strategy on commercial dairy farms in the Netherlands (Centraal Bureau voor de Statistiek (CBS), 2014). All input values in the model were based on literature (Tables 1 and 2). The model was run with 100 000 iterations.

Dynamics and losses of clinical mastitis

Each cow received a parity (1 to 5+), based on the average herd composition in the Netherlands (International Dutch cattle improvement co-operative (CRV), 2014). Based on the parity, cows received a milk production, BW and a calving interval (**CI**) (Centraal Veevoederbureau (CVB), 2012; CRV, 2014). The CI consisted of a lactation period (350 to 361 days) and a dry period (60 days) (Table 1).

Based on the parity, cows had a risk of CM. After a first case of CM, cows had a risk of a second case, and a risk of a third case in the same lactation (Table 1). Pathogens causing CM were classified as gram-positive, gram-negative or other to estimate the impact of different pathogens (Hertl *et al*., 2011). In total, 39 combinations of pathogen types after one, two and three cases of CM per parity were possible. A case of a specific CM pathogen was assumed to be not protective for a recurrent case of the same pathogen (Cha *et al*., 2016). The day of occurrence of the three cases of CM was fixed in the model and was based on the median of the days of occurrence of CM as found by Hertl *et al*. (2011) (Table 1). A lactation curve was utilized to estimate the average daily milk production per cow based on weekly milk records (Wood, 1967). To estimate the impact of CM on milk production, we estimated the difference between the potential and

Table 1 *Milk production, weight, CI and probabilities of CM of dairy cows per parity*

Input value	Parity 1	Parity 2	Parity 3	Parity 4	Parity 5+	Source
Cows (%)	33	27	17	11	12	CRV (2014)
Milk production (kg/305 days)	7535	8788	9383	9493	9209	CRV (2014)
Milk production advantage CM[1]						Schukken *et al.* (2009)
Gram-positive (kg/day)	0.5	0.6	0.6	0.6	0.6	
Gram-negative (kg/day)	1.1	2.4	2.4	2.4	2.4	
Other (kg/day)	0.9	0.6	0.6	0.6	0.6	
Weight cow (kg)	540	595	650	650	650	CVB (2012)
CI (day)	410	413	415	419	421	CRV (2014)
Mastitis probability						Hertl *et al.* (2011)
First case (%)	16.6	29.9	29.9	29.9	29.9	
Second case (% of first case)	22.3	35.8	35.8	35.8	35.8	
Third case (% of second case)	30.6	40.8	40.8	40.8	40.8	
Days in milk of CM occurrence						Hertl *et al.* (2011)
First case	129	101	101	101	101	
Second case	204	155	155	155	155	
Third case	252	193	193	193	193	
Pathogen type CM						Adapted from Hertl *et al.* (2011)
Gram-positive (%)	37.3	34.3	34.3	34.3	34.3	
Gram-negative (%)	32.4	40.9	40.9	40.9	40.9	
Other (%)	30.3	24.8	24.8	24.8	24.8	

CM=clinical mastitis; CI=calving interval.
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[1] Cows with CM had a higher potential milk production. The potential milk production of cows with CM was estimated by including the milk advantage which depended on the type of pathogen of CM at the first case to the milk production.

Table 2 *Greenhouse gas emissions of feed production and LULuc, enteric methane emissions, and energy and nitrogen content of summer and winter diet of dairy cows*

Input variable	Summer diet	Winter diet
Energy of feed (MJ/kg DM)[1]	6.8	6.5
Nitrogen content (g/kg DM)[1]	30.3	26.8
Feed production (g CO_2e /kg DM)[1,2]	470	468
LULuc (g CO_2e /kg DM)[1]	87	94
Enteric methane (g CH_4/kg DM)[1,2]	20.9	20.5

LULuc=land use and land use change.
Table reprinted from Mostert et al. (2018c) with permission from Elsevier.
[1] Vellinga *et al.* (2013).
[2] CO_2e=carbon dioxide equivalent; CH_4=methane.

actual milk production of a cow with CM. Potential milk production per cow per lactation was based on average milk production of Dutch dairy cows (CRV, 2014). Cows with CM were assumed to have a higher potential milk production (Schukken *et al.*, 2009). Their production, therefore, was adapted by adding the daily higher milk production found by Schukken *et al.* (2009) to the average milk production (Table 1). Reduced milk production resulting from CM was based on the study of Schukken *et al.* (2009) and depended on parity, type of pathogen and number of cases of CM. Reduced milk production and type of pathogen causing CM were based on data from studies of the USA (Schukken *et al.*, 2009; Hertl *et al.*, 2011), because no detailed data about this were available for a Dutch situation. Cows affected by any pathogen type were treated for 3 days with antibiotic, and milk was discarded for 6 days (van Soest *et al.*, 2016). It was assumed that pathogen identification was not performed by the farmer and that the cows received a broad spectrum antibiotic.

Cows with CM and without CM could be culled or could die (Hertl *et al.*, 2011). When a cow is culled, the cow is shipped off the farm and can be slaughtered (the cow has a rest value due to the meat). When a cow dies, the cow dies or is euthanized on the farm (the meat has no rest value). Based on parity and type of pathogen, cows with CM had a monthly increased risk of culling and dying, together called removal, compared to cows without CM (Hertl *et al.*, 2011). Cows removed because of this increased risk were assumed to be removed because of CM, whereas other removals were for other reasons than CM. No differentiations were made between voluntary and involuntary culling, and for other reasons of removal. To avoid a confounding effect of pregnancy status on the culling decision, there was no risk of removal after month 10 (Bar *et al.*, 2008). Removed cows were replaced by an average healthy heifer. To account for the impact on fertility, cows with CM had a prolonged CI of 4 weeks, based on the average impact found by Santos *et al.* (2004) and Wilson *et al.* (2008). Because of data limitations, milk production losses and removal were assumed to be not affected by the severity of CM. The discrete events on getting CM and removal were simulated by drawing random numbers from uniform distributions. The uniform distributions make it possible to assign cows to different categories (e.g. gram-positive, gram-negative, other, or no CM).

Calculation of greenhouse gas emissions

An LCA was performed to estimate the impact of CM on GHG emissions as described in Mostert *et al.* (2018b and 2018c). Emissions of CO_2, CH_4 and N_2O were estimated from cradle to farm gate for processes along the milk production chain that are affected by CM. Processes included were feed production, enteric fermentation and manure management. Emissions related to the production of antibiotics were assumed to be minor and, therefore, were excluded. Emissions of GHGs were estimated for cows with and without CM, and were expressed as kg CO_2 equivalents (**CO_2e**) per ton of fat-and-protein-corrected milk (**FPCM**). Different GHGs were summed up based on their equivalent factor: 1 for CO_2, 28 for biogenic CH_4, 30 for fossil CH_4 and 265 for N_2O (100-year time horizon) (Myhre *et al.*, 2013). The impact of CM on GHG emissions was the difference in emissions per kg FPCM between a cow without (potential production) and with (actual production) CM. To gain insight in the largest contributors, the impact of CM on GHG emissions was analyzed per process (feed production, enteric fermentation, manure management), per parity, per production contributor (reduced milk production, discarded milk, prolonged CI, removal) and per pathogen combination. The last analysis was based on 100 000 cows with CM to increase the number of cows with multiple cases of CM. This reduced the uncertainty to less than 1% on total emissions per combination of pathogens. The estimation of GHG emissions is described in detail in Mostert *et al.* (2018b) and, therefore, only briefly described below.

Based on national statistics of the Netherlands, a summer (170 days/year) and winter diet (195 days/year) were composed (CBS, 2014). Feed ingredients included concentrates and roughage (grass, grass silage, maize silage) (Supplementary Tables S1 and S2). Feed intake (kg DM/cow) was estimated based on the energy content of the diet and the energy requirements of the cow. These energy requirements for maintenance, milk production and pregnancy, and energy requirements for growth (in case of parity 1 and 2) were estimated per day (CVB, 2012). Energy requirement for maintenance was assumed to be 6.7% higher in summer than in winter because of grazing (CVB, 2012). The energy requirements for maintenance were assumed to be the same for cows with or without a disease, while the energy requirement for milk production changed with a change in milk yield. As a result, cows with CM were found to have a lower feed efficiency and a higher energy requirement per ton of FPCM produced.

Emissions of GHGs related to feed production were based on Vellinga *et al.* (2013) (Table 2). Processes included were the production of inputs (e.g. fertilizers, pesticides, machinery, energy), cultivation and harvesting of the crops (including fertilizer application), processing and drying of feed ingredients and transport between processing steps up to the farm gate (Supplementary Tables S2 and S3). In addition to the processes described above, emissions related to land use (**Lu**) and land use change (**Luc**) also were included (Vellinga *et al.*, 2013). Emissions from enteric fermentation were based on Vellinga *et al.* (2013). Emissions from manure were estimated according to IPCC (2006) and emissions factors were based on national inventory reports. Nitrogen (N) excretion, which was used to calculate N emissions from manure management, was estimated based on N intake of the cow, N retention in milk and in growth of the cow and calf. Direct N_2O emissions (De Vries *et al.*, 2011; Vonk *et al.*, 2016), indirect N_2O emissions (i.e. N_2O derived from volatilization of ammonia (NH_3) and nitrogen oxides (NO_x) and from leaching of nitrate (NO_3^-)) (De Vries *et al.*, 2011; Velthof and Mosquera, 2011; Vonk *et al.*, 2016) and CH_4 emissions (De Mol and Hilhorst, 2003) from manure in stables and storage, and from manure deposited during grazing were included. More details about emission factors can be found in Supplementary Table S4.

System expansion was applied to account for the production of meat from calves and cows. Consequences of meat production in terms of GHG emissions were based on the so-called avoided-burden method (Guinée *et al.*, 2002). This method assumes that meat from cows replaces alternative products on the market, and therefore subtracts GHG emissions related to the production of those avoided products from the emissions related to dairy production. The impact of removal of cows and calves on GHG emissions was based on Mostert *et al.* (2018b). Cows that were removed before the end of parity 5 were assumed to be removed too early, resulting in rearing extra heifers. This increases emissions related to non-productive animals. Additional emissions because of removal were estimated as the difference between the emissions of rearing a heifer and the avoided burden of the meat production from that cow. Subsequently, this difference was depreciated over the age of the cow, resulting in a lower impact of removal from older cows (Mostert *et al.*, 2018b).

Sensitivity analysis

Sensitivity analyses were performed to analyze the impact of changes in production parameters and in GHG emission factors as described in Mostert *et al.* (2018b and 2018c). Results of changes were compared with results of the reference situation (Tables 1 and 2). Input parameters were adjusted based on the variation found in literature and analyzed separately. The impact of CM on milk production was doubled (*Increased impact milk production*) (Seegers *et al.*, 2003). The risk of removal resulting from CM was increased by 50% (*Increased risk removal*) (Seegers *et al.*, 2003). The prolonged CI was increased from 28 days to 56 days (*Increased prolonged CI)* (Ahmadzadeh *et al.*, 2009).

The impact of changes in GHG emission factors were analyzed to assess the impact of the GHG emission factors that were used and to assess the potential impact of differences in feeding strategy, feed composition and manure management between farms. Based on variation found in literature, the most important emission factors were changed. Emissions related to the production of feed were changed by ± 25% (*Emissions feed*) (Van Middelaar *et al.*, 2013). Emissions related to enteric fermentation were changed by ± 20%

(*Emissions enteric*) (IPCC, 2006). Emissions related to rearing a heifer were changed by ± 25% (*Emissions heifer*). Emissions of CH_4 and N_2O related to manure storage and grazing were changed by ± 100% (*Emissions manure*) (Olivier *et al.*, 2009).

Results

Impact of clinical mastitis on greenhouse gas emissions

Emissions of cows with CM increased on average by 57.5 (6.2%) kg CO_2e/t FPCM compared with cows without CM, with a range between 17.6 and 173.6 (5 to 95 percentiles) kg CO_2e/t FPCM. Emissions related to feed production and enteric fermentation contributed most to the total emissions (Table 3). With regard to the production parameters, the increase in GHG emissions per case of CM was caused by removal (39%), discarded milk (38%), reduced milk production (17%) and prolonged CI (6%) (Table 4).

The increase in GHG emissions of cows with CM compared with cows without CM varied from 75 (33 to 204) kg CO_2e/t FPCM to 34 (15 to 60) kg CO_2e/t FPCM in parities 1 and 5, respectively. In the months after the first case of CM, 40% of cows with CM were removed, whereas in the same period 17% of cows without CM were removed. Clinical mastitis reduced the milk production of cows that were not removed on average by 4.5%.

The increase in GHG emissions was 48 kg CO_2e/t FPCM for cows with one case of CM, 69 kg CO_2e/t FPCM for cows with two cases of CM and 92 kg CO_2e/t FPCM for cows with three cases of CM compared with cows without CM (Figure 1). The increase in GHG emissions of cows with CM compared with cows without CM varied between the type of pathogens and was highest if the first case of CM was gram-negative (average of dark grey bars: 65 kg CO_2e/t FPCM), followed by gram-positive (average of black bars: 54 kg CO_2e/t FPCM) and other (average of light grey bars: 51 kg CO_2e/t FPCM). The increase in GHG emissions of cows with CM compared with cows without CM was highest when cows died because of CM (322 kg CO_2e/t FPCM) and when cows were culled because of CM (115 kg CO_2e/t FPCM).

Sensitivity analysis

Results of the effect of a change in emission factors and production parameters on the impact of CM on CO_2e/t FPCM are shown in Figure 2, relative to the reference situation (%). The two parameters related to removal had the highest impact. Increasing the removal risk of cows with CM increased the GHG emissions by 19%, whereas increasing the emissions of rearing a heifer increased the emissions by 21%. Increasing prolonged CI increased GHG emissions by 8%, whereas doubling the impact of CM on milk production increased GHG emissions by 4%. A decrease in emissions related to production of feed, enteric fermentation, and manure storage and grazing resulted in a similar but opposite effect.

Table 3 *Average GHG emissions per ton fat-and-protein-corrected milk (kg CO_2e/t FPCM) of dairy cows with no CM, and dairy cows with CM, and the average difference related to feed production, enteric methane, LULuc, manure, avoided burden and rearing extra heifers*

	No CM	CM	Difference
CO_2[1]			
Feed production	192.8	199.3	6.5
LULuc	65.8	68.0	2.2
CH_4[1]			
Feed production	2.7	2.8	0.1
Enteric fermentation	418.3	432.4	14.1
Manure	44.4	45.3	1.0
N_2O[1]			
Feed production	143.7	148.6	4.9
Manure	44.8	46.6	1.8
Total before correction	912.4	943.0	30.6
Avoided burden[2]	−9.6	−12.0	−2.4
Rearing extra heifers[3]	18.6	48.0	29.3
Total after correction	921.5	979.0	57.5

GHG=greenhouse gas; CM=clinical mastitis; LULuc=land use and land use change.
[1] CO_2=carbon dioxide; CH_4=methane; N_2O=nitrous oxide.
[2] Emissions are corrected for meat production of the cows based on system expansion. Meat from cows replaces alternative products on the market, and therefore the GHG emissions of meat from cows is equal to the GHG emissions of the alternative product.
[3] Additional emissions because cows are removed too early resulting in additional GHG emissions for rearing extra replacement heifers.

Table 4 *Average increase of GHG emissions per ton fat-and-protein-corrected milk (kg CO_2e/t FPCM) per parity due to CM (5 to 95 percentiles) in dairy cows related to reduced milk production, discarded milk, prolonged CI, removal and avoided burden*

Contributor	Parity 1	Parity 2	Parity 3	Parity 4	Parity 5+	Average[1]
Reduced milk production	14 (2 to 24)	11 (2 to 28)	9 (1 to 19)	7 (1 to 18)	8 (1 to 19)	10 (1 to 22)
Discarded milk	20 (12 to 39)	24 (11 to 48)	24 (11 to 48)	23 (11 to 47)	23 (11 to 47)	23 (11 to 47)
Prolonged CI	3 (0 to 4)	5 (0 to 8)	4 (0 to 7)	3 (0 to 7)	3 (0 to 7)	4 (0 to 7)
Removal	39 (0 to 187)	20 (0 to 144)	25 (0 to 120)	21 (0 to 85)	5 (−13 to 32)	23 (0 to 141)
Avoided burden[2]	−1 (−3 to 0)	−1 (−3 to 0)	−3 (−15 to 0)	−5 (−14 to 0)	−4 (−14 to 0)	−2 (−14 to 0)
Total	75 (33 to 204)	60 (19 to 169)	58 (18 to 127)	49 (17 to 89)	34 (15 to 60)	58 (18 to 174)

GHG=greenhouse gas; CM=clinical mastitis; CI=calving interval.
[1] A weighted average for all parities was taken which was based on the composition of an average Dutch dairy herd.
[2] Emissions are corrected for meat production of the cows based on system expansion. Meat from cows replaces alternative products on the market, and therefore the GHG emissions of meat from cows are equal to the GHG emissions of the alternative product.

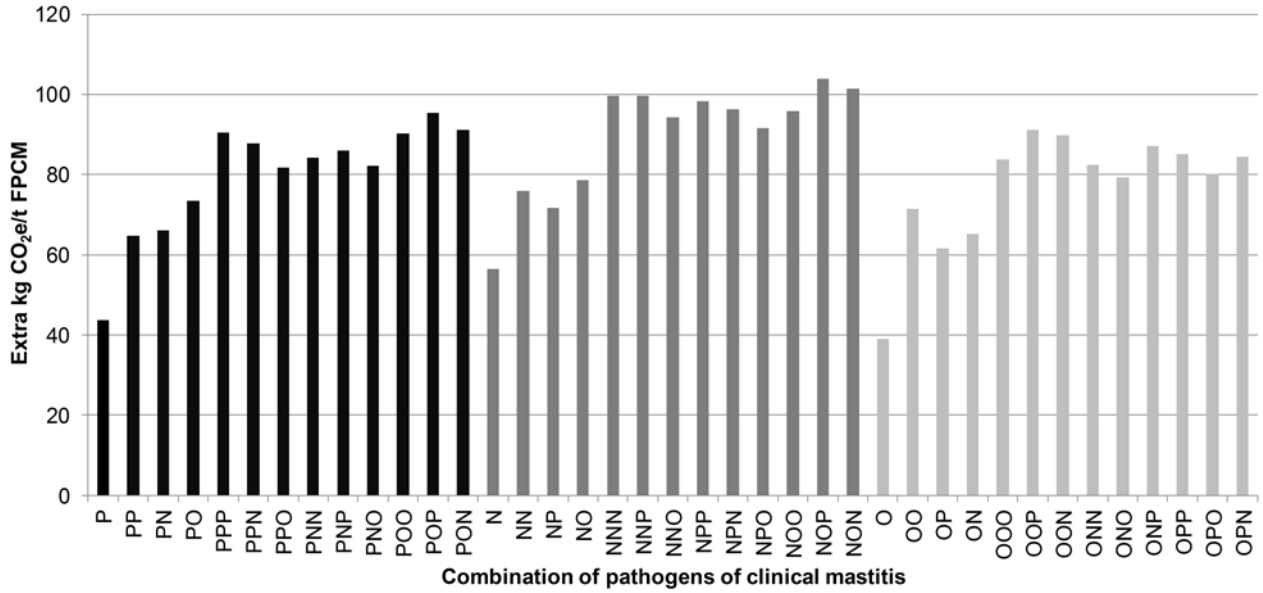

Figure 1 Average increase of GHG emissions per ton fat-and-protein-corrected milk (kg CO_2e/t FPCM) of CM in dairy cows per type of pathogen; gram-positive (P), gram-negative (N) or other (O), and the combinations up to three cases. GHG=greenhouse gas; CM=clinical mastitis.

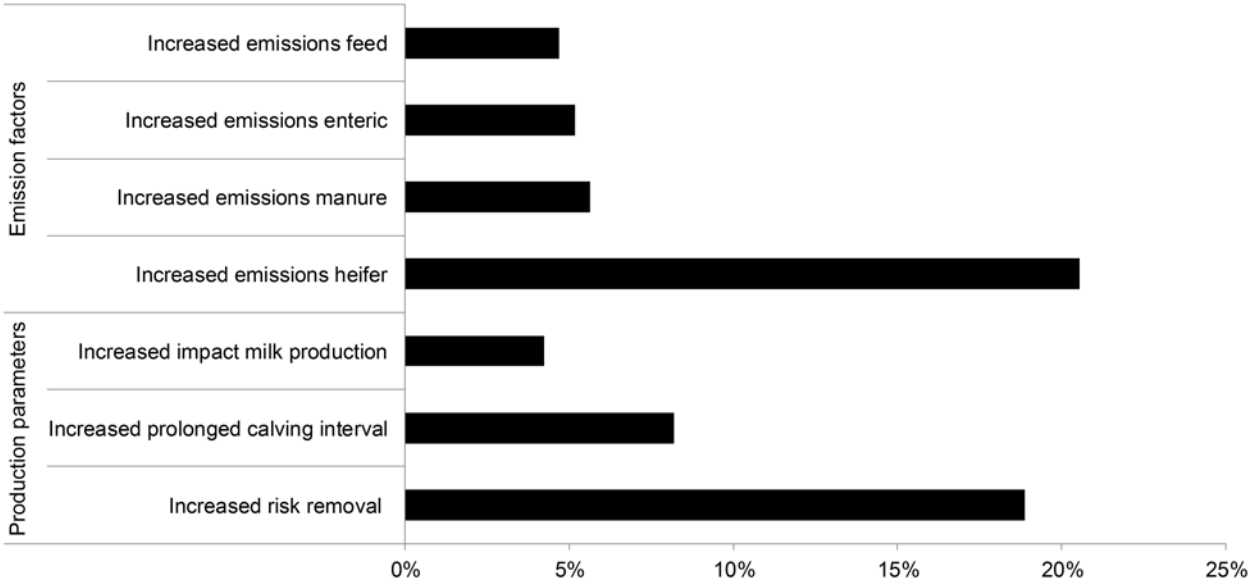

Figure 2 Results from the sensitivity analyses showing the effect (%) of a change in input parameters of emission factors and production parameters on the impact of CM in dairy cows on GHG emissions per ton fat-and-protein-corrected milk (kg CO_2e/t FPCM) compared to the reference situation. GHG=greenhouse gas; CM=clinical mastitis.

Discussion

The relationship between animal health and GHG emissions has been estimated only in a few studies. In the UK, two studies showed that reduced animal health increased the GHG emissions per unit of product (Williams *et al.*, 2015; Bell and Wilson, 2018). Other studies analyzed the impact per type of disease. Mostert *et al.* (2018b and 2018c) estimated that GHG emissions of dairy cows with subclinical ketosis increased by 2.3% and with foot lesions by 1.5% compared with cows without these diseases. Chen *et al.* (2016) showed that lameness in dairy cows can increase GHG emissions at the farm level up to 7.8%, and Özkan Gülzari *et al.* (2018) showed that reducing somatic cell count from 800 000 cells/ml to 50 000 cells/ml potentially can reduce farm GHG emissions intensity by 3.7%. Using a scenario study, Hospido and Sonesson (2005) showed that reducing subCM by 18% and CM by 7% reduced GHG emissions by 2.5% at herd level. In our study, we estimated that preventing a case of CM can reduce GHG emissions of a dairy cow by 6.2%. On herd level in our study, with an incidence of 27% of one case of CM, GHG emissions increased by 1.7% (16 kg CO_2e/t FPCM). Several differences in input and type of model to estimate the impact of (clinical) mastitis on GHG emissions exist between our study and the study of Hospido and

Sonesson (2005). We discuss two major differences. First, we did not include subCM, because no data were available about the relation between CM and subCM. We, therefore, found a lower GHG impact than Hospido and Sonesson (2005). Second, the reduction of subCM and CM in the study of Hospido and Sonesson (2005) resulted in a high increase in milk production. In our study, mastitis resulted in an average milk production loss of 4.5%. Literature showed that CM results in an average milk production loss of 5% (375 kg) over a full lactation (Seegers *et al.*, 2003), whereas in the improved situation (14 cows less affected by CM or subCM) in the study of Hospido and Sonesson (2005) milk production losses were reduced by more than 30 000 kg. The higher milk production losses in the study of Hospido and Sonesson resulted in a higher impact of mastitis on GHG emissions than in our study.

Literature shows differences in losses in milk production, removal and fertility as a result of CM (Seegers *et al.*, 2003). Milk production losses and type of pathogen causing CM were based on data from studies of the USA (Schukken *et al.*, 2009; Hertl *et al.*, 2011). In our model, we assumed that a recurrent case of a pathogen and related milk production losses were the same for the Dutch situation, although these cows have a lower potential milk production. It is unknown if the magnitude of loss depends on the production level of the cow (Seegers *et al.*, 2003). Milk production losses in our study were similar compared with milk production losses (5%) of other modeling studies of CM (Raboisson *et al.*, 2014; van Soest *et al.*, 2016). These studies based their milk production losses of CM on Seegers *et al.* (2003). Therefore, using USA data about milk production losses for the Dutch situation might have had a minor impact on the results.

Removal of cows is farm specific and has varied between literature studies. Removal was the most important contributor (39%) to the impact of CM on GHG emissions. Rearing heifers has a relatively high environmental impact as those animals contribute to GHG emissions for 2 years without producing milk. So far, however, no specific removal models exist to estimate the impact of removal per cow on GHG emissions. Therefore we applied a method to estimate the impact of removal on GHG emissions that is also used in economic estimations. This method of removal and related assumptions are described in Mostert *et al.* (2018b). Sensitivity analyses showed that GHG emissions for rearing a new heifer and removal risk had an important impact on the results. These two aspects can result in high variation between farms.

Prolonged CI was used to estimate the impact of fertility on GHG emissions and this had the lowest contribution to the total impact of CM. In our model, the impact of CM on fertility was simplified, because we did not have the data to include a detailed fertility model. The impact of fertility on, for example, removal was excluded because of lack of data. Including these removals will increase GHG emissions. Garnsworthy (2004) showed that improving UK fertility levels to ideal levels could reduce the proportion of total methane emissions from replacement heifers by 15%. It was assumed that type of pathogen did not influence fertility. Type of pathogen, parity and time of CM occurrence before or after artificial insemination, however, can have an impact on the probability of conception (Hertl *et al.*, 2010). Gram-negative bacteria have in general a higher effect on probability of conception than gram-positive bacteria or other pathogens. The impact of gram-negative combinations, therefore, might have been underestimated. Including a more detailed fertility model can improve the estimation of GHG emissions for specific pathogen combinations.

High variation was found between the impact of different pathogens on GHG emissions. The impact on milk production and removal for different combinations of pathogens was included. Treatment, however, was equal for all pathogens, because it was assumed that farmers, in general, are not aware of the type of pathogen and therefore use a broad spectrum antibiotic. Including a specific treatment per pathogen might reduce disease duration and production losses, and therefore reduce the impact of CM on GHG emissions. Moreover, CM can increase the risk of other diseases and vice versa (Gröhn *et al.*, 2003). No data were available to include the interactions of other diseases with recurrent cases of CM per parity and the impact of this on milk production, removal and fertility. Not being able to include these interactions with other diseases might have led to an over- or underestimation of the results.

Current incentives to reduce CM in dairy cows aim to increase the income of the farmer and to improve animal welfare and will become even more relevant when policies to reduce GHG emissions are implemented for the livestock sector. Targets of maximum GHG emissions can be set per type of dairy farming system (e.g. conventional, organic) for getting a premium. Reducing CM can contribute to a reduction of GHG emissions in the livestock sector. Preventive measures to reduce CM can be management practices (e.g. washing dirty udders and the use of milkers' gloves), treatment of subCM, or vaccination. These preventive measures can be cost-effective (Hogeveen *et al.*, 2011; Kessels *et al.*, 2016), but the impact of these strategies on GHG emissions and other environmental impact categories has not been estimated. Therefore, strategies to reduce CM should also be analyzed from an environmental perspective. Incidence of CM ranges from 2% to 82% on dairy farms in the Netherlands (van Soest *et al.*, 2016). Therefore, results from our study show that farms with a high incidence of CM have a high potential to reduce GHG emissions by reducing CM.

Several other strategies have been investigated to reduce GHG emissions in the dairy sector. Dietary supplements or drugs can reduce enteric CH_4 emissions of dairy cows, but some might have a negative impact on cow welfare (Llonch *et al.*, 2017). Preventing CM, however, can also improve the health and consequently welfare of dairy cows.

Meul *et al.* (2014) showed that the most important contributors (in units per kg FPCM) to GHG emissions of milk production were DM intake of the dairy cow and heifer, application of N from mineral fertilizer and manure, and amount

(kg DM) of purchased concentrates and soybean meal. Preventing CM improves all these three aspects, because the efficiency of milk production increases. Moreover, preventing CM might be more effective for reducing GHG emissions than adopting a feeding strategy, such as dietary supplementation of extruded linseed or nitrate (9 to 32 kg CO_2e/t FPCM) (Van Middelaar *et al.*, 2014a) or increasing milk yield by 698 kg/year per cow and longevity by 270 days per cow (27 and 23 kg CO_2e/t FPCM) (Van Middelaar *et al.*, 2014b). In addition, changing the feeding strategy can reduce GHG emissions of a dairy farm but does not always increase the income of the farmer (Van Middelaar *et al.*, 2014a). Whereas other strategies to reduce GHG emissions might result in a trade-off with another sustainability aspect (i.e. welfare, economics), preventing CM can increase the income of the farmer, can improve the welfare and longevity of the dairy cow and can reduce GHG emissions. Preventing CM, therefore, can be an effective strategy that can contribute to sustainable development of the dairy sector. The impact of CM on GHG emissions and other environmental aspects, however, will vary between farms due to environmental conditions (e.g. climate) and management practices (e.g. milk production, removal, feed and manure management). Therefore, specific farm analyses are needed to estimate the impact of that farm on GHG emissions. Measuring GHG emissions per farm might be difficult, but emission calculations in the model can be improved by using detailed information on farm management practices, including diet and herd composition. Model estimations could be further improved by more detailed data about milk losses, disease incidence, cause of removal, fertility, and the interactions between these factors.

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None.

Declaration of interest

None.

Ethics statement

None.

Software and data repository resources

None of the data were deposited in an official repository.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731119001393.

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Exploration of conservation and development strategies with a limited stakeholder approach for local cattle breeds

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*Many local breeds have become endangered due to their substitution by high-yielding breeds. To conserve local breeds, effective development strategies need to be investigated. The aim of this study was to explore conservation and development strategies based on quantified strengths, weaknesses, opportunities and threats (**SWOT**) for two local cattle breeds from Northern Germany, namely the German Angler (**GA**) and Red Dual-Purpose cattle (**RDP**). The data comprised 158 questionnaires regarding both breeds' SWOT, which were answered by 78 farmers of GA and 80 farmers of RDP. First, data were analysed using the SWOT-Analytic Hierarchy Process (**AHP**) method, which combines the qualitative strategic decision tool of SWOT analysis and the quantitative tool of AHP. Second, prioritised SWOT factors were discussed with stakeholders in order to form final conservation and development strategies at breed level. For GA prioritised strengths were daily gain, meat quality, milk production and the usage of new biotechnologies, weaknesses were genetic gain in milk production and inbreeding, opportunities were organic farming and breed-specific characteristics and threats were milk prices and dependency regarding the dairy business. Consequently, three conservation and development strategies were formed: (1) changing relative weights and the relevant breeding goal to drift from milk to meat, (2) increasing genetic gain and control the rate of inbreeding by the implementation of specific selection programs and (3) selection of unique and breed characteristic components on product level, that is, milk-fat and fine muscle fibers. For RDP defined strengths were robustness, high adaptability for different housing systems and a balanced dual-purpose of milk and meat, weaknesses were inbreeding, breed extinction, genomic selection with young bulls and milk yield, opportunities were organic farming and dual-purpose aspects and threats were milk and decreasing beef cattle prices. Thus, three conservation and development strategies were identified: (1) adjust relative weights and the relevant breeding goal to balance milk and meat yield, (2) increasing genetic gain and avoid extinction by implementing targeted selection programs and (3) selection of unique and breed characteristic traits on breed level, that is, environmental robustness. Quantified SWOT establish a basis for the exploration of conservation and development strategies at breed level. Explored strategies are promising even if the stakeholder approach was limited for small populations regarding a small number of stakeholder groups. The used approach reflects farmers' individual convenience better than existing quantitative strategy decision tools on their own.*

Keywords: analytic hierarchy process, breed level, farmer, red cattle, survey

Implications

This study explores conservation and development strategies for local cattle breeds based on farmer surveys. Quantified strengths, weaknesses, opportunities and threats can be used as an ideal basis to form objective strategies at breed level. This combined approach results in promising strategies even with a limited stakeholder approach. Described approach represents farmers' individual convenience better than existing quantitative strategy decision tools on their own.

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Introduction

Many local breeds have been replaced by high-yielding breeds over the last few centuries, resulting in loss of local breeds (Meuwissen, 2009; FAO, 2010). Due to this breeding history, populations of many local breeds have dangerously decreased and some of them are even threatened by extinction (Fernández *et al.*, 2011). By now two local cattle breeds from Northern Germany are listed as endangered livestock breeds by 'The Society for the Conservation of Old and Endangered Livestock Breeds'. Both breeds are red cattle breeds with a milk-emphasised dual purpose, namely the

German Angler (**GA**) and the Red Dual-Purpose cattle (**RDP**). According to Meuwissen (2009), the best conservation strategies are an increased profitability achieved by genetic improvements and the promotion of breed-specific products. The FAO (2010) suggests a guideline to develop breeding strategies for the sustainable management of animal genetic resources also based on the implementation of effective genetic gain programs. However, Martín-Collado *et al.* (2013) explore conservation and development strategies for local European cattle breeds, which focused production systems and the marketing of new products. Thus, considered aspects in case of effective conservation strategies may be more widespread than just prioritising genetic gain. Furthermore, strategies embracing many breeds should refer to general issues, whereas specific strategies and actions for single breeds should be identified at breed level (Martín-Collado *et al.*, 2013). Investigated actions at breed level may be not only convenient for single breeds, but may also inspire the strategy decision process regarding small endangered livestock populations when done by a limited number of stakeholder groups. The aim of the present study was to explore conservation and development strategies at breed level based on quantified strengths, weaknesses, opportunities and threats (**SWOT**) for two local cattle breeds in Northern Germany.

Material and methods

Data

The data comprised comprehensive questionnaires completed by farmers, who rear one of the two local cattle breeds, namely either the GA or the RDP breed. Both breeds are local in Schleswig-Holstein, which is the most Northern state of Germany. The initial list of possible respondents consisted of 200 questionnaires (100 per breed) and was selected by the breeding organisation RSH (Rinderzucht Schleswig-Holstein e.G., Germany) due to farmers' individual breed activity and engagement. The design and sending of the questionnaires as well as the collection of completed surveys were also done by the breeding organisation. In total, 158 farmers (79%) filled out the questionnaires, thereof 78 participants for GA (78%) and 80 participants for RDP (80%). Each questionnaire consisted of a total of 12 queries, open-ended and multiple-choice questions including information on sub-items, such as farm, herd, expectations, reproduction, traits and difficulties as well as handwritten farmers' personal opinions on the breeds' SWOT (Supplementary Material S1).

Quantified strategy decision tool

To identify strategies using an organised approach, the FAO (2010) suggested employing the SWOT analysis from Weihrich (1982). A SWOT analysis is used to identify SWOT in order to enhance the profitability of an individual production system. This qualitative method has the disadvantage of high subjectivity during its application in decision-making (Hill and Westbrook, 1997; Pesonen *et al.*, 2000; Martín-Collado *et al.*, 2013). Therefore, Kurttila *et al.* (2000) and Saaty and Vargas (2001) developed a combination of the Analytic Hierarchy Process (**AHP**) method, which is a quantitative tool from Saaty (1980), and the SWOT analysis to obtain more complex and reliable decisions. This hybrid method improves the quantitative information basis and forces the decision-maker to think over and analyse the situation more precisely and in more depth (Kurttila *et al.*, 2000). SWOT-AHP methodology has been applied in the fields of forestry (Kangas *et al.*, 2001), silvopasture adoption (Shreshta *et al.*, 2004) and livestock (Wasike *et al.*, 2011).

In the current study, the SWOT-AHP was used to quantify identified SWOT. Statistical analyses and computations for eigenvalues, priority vectors and quality control parameters within the SWOT-AHP methodology were performed by using the R-software (R Core Team, 2018). The SWOT-AHP approach was divided into five steps (Figure 1) and is described below:

(I) Assignment of items
The farmers' responses were allocated as items by an expert team into four single SWOT groups of SWOT. Expert team members were previously identified through the application of certain qualification criteria. These criteria comprised: (1) long-time experiences with both local cattle breeds in practice, (2) deep knowledge of respective production systems and the local environment and (3) profound background in population genetics and management of small populations. In total, four scientists from two German animal breeding departments passed the qualification criteria, and thus, formed the team of experts. Experts discussed their attitudes and valuations as soon as they had reached consensus. The number of items and SWOT factors allocated for each SWOT group by the team of experts exhibited substantial flexibility and was allowed to differ between both breeds.

(II) Defining SWOT factors
The farmers' items were defined as SWOT factors by the expert team (Tables 1 and 2). Generally, the number of SWOT factors within each SWOT group can vary depending on the number of allocated items.

(III) Computation of priority scores for SWOT factors
The computations of the factor priority vector (**FPV**), the group priority vector (**GPV**) and overall priority vector (**OPV**) were performed with AHP rankings by the named expert team above based on the frequency of SWOT factors among farmers. The fundamental scale from Saaty (1980) was used for such AHP rankings (Table 3). This scale represented a transformation from verbal judgements into numerical judgements to determine the relative importance of each element. There were five verbal judgements, which were ranked by their importance from equal to extreme (equal, moderate, strong, very strong and extreme). These judgements were translated into the numeric values of: 1, 3, 5, 7 and 9 with four

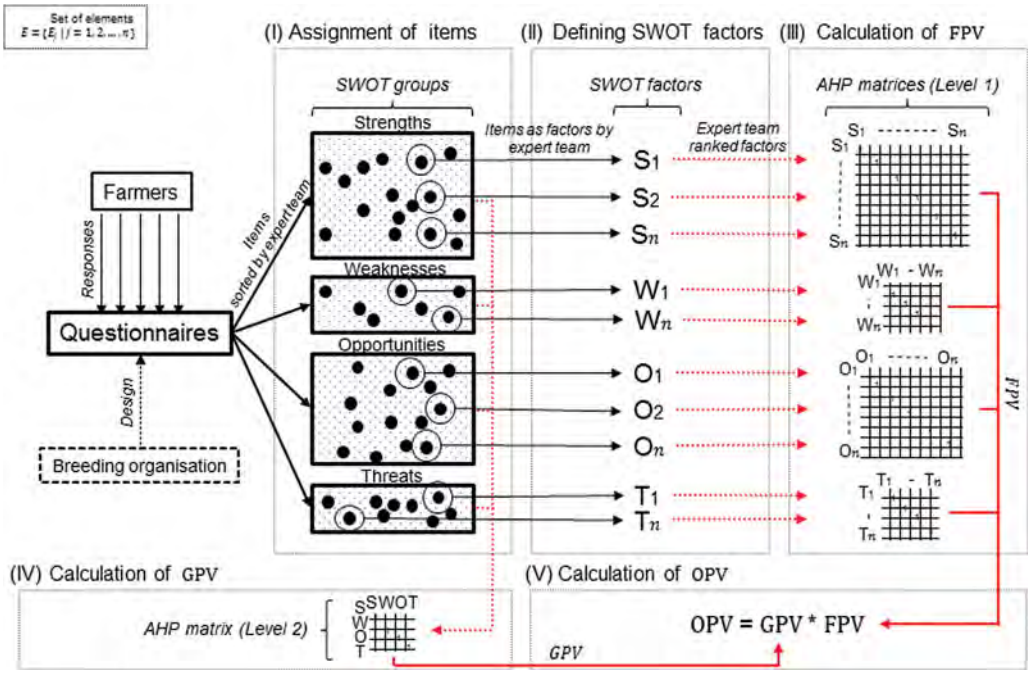

Figure 1 (Colour online) Methodical implementation of AHP based on farmer surveys for local cattle breeds. SWOT=strengths weaknesses opportunities threats; AHP=Analytic Hierarchy Process; GPV=group priority vector; FPV=factor priority vector; OPV=overall priority vector.

intermediate values (2, 4, 6, 8) for compromises in importance. In other words, the scale indicated how important or dominant one element was over another element (Saaty, 2008). The elements for FPV were individual SWOT factors within each SWOT group and for GPV the four single SWOT groups. The elements were ranked within AHP matrices of different levels: Level 1 for the SWOT factors within each SWOT group and Level 2 for the SWOT groups (Figure 1). Kahraman *et al.* (2007), Borajee and Yakchalie (2011) and Görener *et al.* (2012) defined the set of elements as E={E_j | j=1, 2, ..., n}. The results of the n-ranked elements were resumed in an evaluation matrix A (n * n). Each element a_{ij} (i, j= 1, 2, ..., n) was the quotient of the weights of the elements w_{ij} (i, j= 1, 2, ..., n). In this matrix, the element a_{ij}=1, when the weights of elements w_i=w_j. The ranked elements were also expressed via a square and reciprocal matrix:

$$A = (a_{ij}) = \begin{bmatrix} 1 & w_1/w_2 & \dots & w_1/w_n \\ w_2/w_1 & 1 & \cdots & w_2/w_n \\ \vdots & \vdots & \ddots & \vdots \\ w_n/w_1 & w_n/w_2 & \cdots & 1 \end{bmatrix}; a_{ij} = \frac{w_i}{w_j}, a_{ij} \neq 0$$

Each matrix was normalised and their relative weights (A_w) were determined. The relative weights were given by the correct eigenvector (w) corresponding to the largest eigenvalue (λ_{max}), as

$$A_w = \lambda_{max} * w$$

The largest eigenvalue (λ_{max}) was computed by forming the sum of the single normalised eigenvector (w) per row multiplied by the computed priority vector per corresponding column:

$$\lambda_{max} = \sum \left(\sum_{ij} w_{(row)} * \frac{\sum_{ij} w_{(column)}}{\sum \left(\sum_{ij} w_{(column)} \right)} \right)$$

If the pairwise comparisons were completely consistent, the matrix A had rank 1 and $\lambda_{max} = n$. In this case, weights could be obtained by normalising any of the rows or columns of A. The priority vectors of FPV and GPV were computed by dividing the single normalised eigenvectors (w) per column by the sum of all single normalised eigenvectors per column for the single matrices:

$$\text{FPV/GPV} = \frac{\sum_{ij} w_{(column)}}{\sum \left(\sum_{ij} w_{(column)} \right)}$$

It should be noted that the quality of the results was related to the consistency of the comparison judgements. As a quality control, the inconsistency ratio (iCR) of the ranked elements was computed as a ratio between the consistency index (CI) and random index (RI):

$$\text{iCR} = \frac{\text{CI}}{\text{RI}}$$

The iCR provided information on the inconsistency of the ranked elements. An iCR $\leq$ 0.1 was acceptable as a criterion (Kahraman *et al.*, 2007; Borajee and Yakchali,

Table 1 *Priority scores of ranked SWOT factors for GA*

SWOT group	GPV	SWOT factors	FPV	OPV
Strengths	0.375	S_1: GA herd size stay steady for the majority of farmers	0.050	0.018
	0.375	S_2: over 90% of farmers use GA as main source of income	0.053	0.019
	0.375	S_3: the majority of farmers produce their own forage for GA	0.056	0.021
	0.375	S_4: mortality rate of GA calves is low	0.059	0.022
	0.375	S_5: good adaptation of GA to marsh land	0.027	0.010
	0.375	S_6: claws and legs of GA are resilient	0.065	0.024
	0.375	S_7: GA has high resilience to climate	0.037	0.013
	0.375	S_8: GA is a milk-emphasised, dual-purpose breed with good daily gain	0.141	0.053
	0.375	S_9: GA heifers are early-maturing	0.041	0.015
	0.375	S_{10}: GA has good fertility, udder health, foundation, and carcass traits	0.098	0.036
	0.375	S_{11}: the majority of farmers uses artificial insemination for GA reproduction	0.068	0.025
	0.375	S_{12}: the majority of farmers appreciates usage of new biotechnologies for GA	0.101	0.038
	0.375	S_{13}: good meat quality of GA with slight drip losses	0.101	0.038
	0.375	S_{14}: milk of GA contains enhanced ingredients	0.040	0.015
	0.375	S_{15}: milk of GA suits cheese production very well	0.059	0.022
Weaknesses	0.162	W_1: the majority of GA farmers are conventional farmers	0.058	0.009
	0.162	W_2: the majority of GA farmers have no 'old' GA cattle (breeding type)	0.095	0.015
	0.162	W_3: GA farmers do not fatten GA bulls	0.048	0.007
	0.162	W_4: milk yield of GA is not adequate	0.129	0.021
	0.162	W_5: longevity of GA is low	0.128	0.020
	0.162	W_6: low genetic gain and inbreeding of GA	0.316	0.051
	0.162	W_7: milk yield of GA is not competitive with high-yielding breeds	0.226	0.036
Opportunities	0.278	O_1: consumers want beef of high quality	0.086	0.024
	0.278	O_2: local and organic agriculture are important to consumers	0.142	0.039
	0.278	O_3: funding of sustainable organic agriculture	0.078	0.021
	0.278	O_4: funding of local breeds by the government	0.076	0.021
	0.278	O_5: funding moved from conventional to organic farming	0.058	0.016
	0.278	O_6: higher income for farmers with local products	0.100	0.028
	0.278	O_7: enhance the economic ratio between feeding costs and milk yield for GA	0.111	0.031
	0.278	O_8: organic farming produces more benefit for farmers	0.113	0.031
	0.278	O_9: rearing GA in areas with strong climate variabilities	0.040	0.011
	0.278	O_{10}: rearing GA on marsh land	0.026	0.007
	0.278	O_{11}: increase milk protein of GA	0.062	0.017
	0.278	O_{12}: direct marketing of GA products	0.108	0.030
Threats	0.188	T_1: brand awareness of GA breed related to other local breeds is low	0.107	0.020
	0.188	T_2: insufficient local awareness of GA breed	0.107	0.020
	0.188	T_3: strong dependence on milk prices	0.230	0.043
	0.188	T_4: decreasing milk prices	0.170	0.032
	0.188	T_5: monopoly of dairy business	0.132	0.025
	0.188	T_6: establish specialised GA products on market	0.078	0.014
	0.188	T_7: some areas may be too sandy and dour for farmers' own GA forage production	0.046	0.008
	0.188	T_8: consumers' demand for breed-specific products is not high	0.131	0.024

SWOT=strengths weaknesses opportunities threats; GA= German Angler; GPV=group priority vector; FPV=factor priority vector; OPV=overall priority vector.

2011). Thus,the ranking of the elements had to be repeated by the team of experts when the iCR exceeded this threshold. The CI was computed by using the subsequent formula:

$$CI = \frac{\lambda_{max} - n}{n-1}$$

The RI was defined as an expected value of the CI depending on an individual number of ranked elements *n*. These fixed values were obtained from the study of Aguarón and Moreno-Jiménez (2003) for further computations of the CI parameter (Table 4). The authors simulated 100,000 matrices for several sizes of n and calculated standard values for RI.

(IV) Calculation of the GPV
The GPVs were computed for each corresponding SWOT group by the expert team based on the frequency of farmers' items included in each SWOT group. Computation details for the GPV are described in step III computation of priority scores for SWOT factors.

(V) Calculation of the OPV
However, the OPVs were calculated by multiplying the single FPVs of the single SWOT factors with the

Table 2 *Priority scores of ranked SWOT factors for RDP*

SWOT group	GPV	SWOT factors	FPV	OPV
Strengths	0.360	S_1: RDP herd size stay steady for 81% of farmers	0.040	0.014
	0.360	S_2: over 95% of farmers use RDP as main source of income	0.030	0.011
	0.360	S_3: the majority of RDP farmers produce their own forage for RDP	0.050	0.018
	0.360	S_4: the majority of RDP farmers use conventional agriculture systems	0.030	0.011
	0.360	S_5: dual purpose feature of RDP is important for RDP farmers	0.080	0.029
	0.360	S_6: the majority of RDP farmers appreciate the project work	0.060	0.022
	0.360	S_7: longevity of RDP is high	0.060	0.022
	0.360	S_8: carcass weight of RDP is good	0.090	0.032
	0.360	S_9: RDP show high robustness and adaptability to different housing systems	0.120	0.043
	0.360	S_{10}: foundation and udder quality traits of RDP are very good	0.080	0.029
	0.360	S_{11}: RDP show a balanced relation between daily gain and milk yield	0.100	0.036
	0.360	S_{12}: the majority of farmers use artificial insemination for RDP reproduction	0.030	0.011
	0.360	S_{13}: selection of RDP bulls for reproduction are made by farmers themselves	0.010	0.004
	0.360	S_{14}: RDP farmers achieve financial stability due to dual purpose	0.110	0.040
	0.360	S_{15}: carcass and meat quality of RDP is good	0.100	0.036
	0.360	S_{16}: milk of RDP contains enhanced milk protein	0.020	0.007
Weaknesses	0.137	W_1: 19% of RDP farmers will quit their agriculture farms soon	0.050	0.007
	0.137	W_2: the majority of RDP farmers do not want to exchange information	0.105	0.014
	0.137	W_3: milk yield of RDP is too low	0.100	0.014
	0.137	W_4: inbreeding and breed extinction of RDP	0.415	0.057
	0.137	W_5: farmers reject the use of genomic-tested young bulls of RDP	0.175	0.024
	0.137	W_6: milk yield of RDP is not competitive with high-yielding breeds	0.153	0.021
Opportunities	0.308	O_1: consumers want beef of high quality	0.067	0.020
	0.308	O_2: local and organic agriculture are important to consumers	0.096	0.030
	0.308	O_3: higher income for farmers with local products	0.110	0.034
	0.308	O_4: funding of sustainable organic agriculture	0.075	0.023
	0.308	O_5: funding of local breeds by the government	0.043	0.013
	0.308	O_6: high flexibility of dual-purpose breeds on changing markets	0.107	0.033
	0.308	O_7: constant market prices for beef cattle	0.037	0.011
	0.308	O_8: rearing RDP in areas with strong climate variabilities	0.038	0.012
	0.308	O_9: rearing RDP on marsh land	0.023	0.007
	0.308	O_{10}: enhance milk ingredients of RDP	0.057	0.018
	0.308	O_{11}: collaboration of RDP farmers	0.053	0.016
	0.308	O_{12}: search for organic sales markets for RDP products	0.109	0.034
	0.308	O_{13}: improve traits of fertility, udder health, and foundation of RDP	0.090	0.028
	0.308	O_{14}: increase the supply of RDP breeding bulls to the farmers	0.095	0.029
Threats	0.195	T_1: brand awareness of RDP breed related to other local breeds is low	0.055	0.011
	0.195	T_2: insufficient local awareness of RDP breed	0.053	0.010
	0.195	T_3: milk quota disappeared	0.164	0.032
	0.195	T_4: decreasing prices for beef cattle	0.132	0.026
	0.195	T_5: decreasing milk prices	0.146	0.028
	0.195	T_6: decreasing prices for beef cattle due to a massive change from milk to beef cattle production	0.230	0.045
	0.195	T_7: some areas may be too sandy and dour for farmers' own RDP forage production	0.025	0.005
	0.195	T_8: no special market for breed-specific products	0.120	0.023
	0.195	T_9: breeding goals for RDP are not achievable	0.075	0.015

SWOT=strengths weaknesses opportunities threats; RDP=Red Dual-Purpose cattle; GPV=group priority vector; FPV=factor priority vector; OPV=overall priority vector.

corresponding GPVs of each SWOT group:

$$\mathrm{OPV} = \mathrm{GPV} * \mathrm{FPV}$$

The number of computed OPVs should correspond with the number of computed FPVs.

Conservation and development strategies at breed level

In general, SWOT strategies embracing single breeds should be identified at breed level (Martín-Collado *et al.*, 2013) and not just from a selected team of experts. Especially, in small endangered populations the number of experts or stakeholders is limited and a multi-stakeholder approach for single local breeds may be not achievable. Thus, identified strategies can be misleading. Furthermore, the identification of SWOT factors is often unbalanced (Karppi *et al.*, 2001) and

Table 3 *Fundamental scale to determine the relative importance of each element for the German Angler and Red Dual-Purpose cattle by Saaty (1980) and Yüksel and Dağdeviren (2007)*

Intensity of importance	Definition	Explanation
1	Equal importance	Two criteria contribute equally to the objective
3	Moderate importance	Experience and judgement slightly favour one over another
5	Strong importance	Experience and judgement strongly favour one over another
7	Very strong importance	Activity is strongly favoured and its dominance is demonstrated in practice
9	Extreme importance	Importance of one over another affirmed at the highest possible order
2, 4, 6, 8	Intermediate values	Represent compromise between the priorities listed above

Table 4 *Fixed values of RI dependent on the size of matrices to obtain strategy consistency for the German Angler and Red Dual-Purpose cattle by Aguarón and Moreno-Jiménez (2003)*

n	1	2	3	4	5	6	7	8
RI	0.00	0.00	0.525	0.882	1.115	1.252	1.341	1.404
n	9	10	11	12	13	14	15	16
RI	1.452	1.484	1.513	1.535	1.555	1.570	1.583	1.595

n=ranked elements; RI=random index.

a multi-stakeholder approach was preferred in several studies to avoid subjectivity (Impoinvil *et al.*, 2007; Martín-Collado *et al.*, 2013). Quantified SWOT have been identified by applying the quantified strategy decision tool of SWOT-AHP in the step before. To improve the limited amount of stakeholder groups, quantified SWOT factors with the three highest OPVs per SWOT group were comprehensively discussed face-to-face with unbiased representatives of further stakeholder groups, that is, farmers, breeders and staff of the breeding organisation (breeding board). Face-to-face discussions were performed in one group meeting with the following respective representatives: 3 (farmers), 3 (breeders) and 3 (breeding board) per breed. All representatives were equally weighted and discussed their attitudes as soon as they had reached consensus. Then promising conservation and development strategies at breed level were collectively transformed together. This formation process was refereed and protocolled by the expert team.

Results

Quantified strategy decision tool

Farmer surveys were allocated by the expert team and resulted in a total of 87 items, whereby 42 belonged to GA farmers and 45 to RDP farmers. For GA, all 42 items were divided and 15 items were subsequently sorted into the SWOT group of strengths, 7 items into weaknesses, 12 into opportunities and 8 into threats (Table 1 and Figure 2(a)). For RDP, all 45 items were divided and assigned as amounts of 16, 6, 14 and 9 into strengths, weaknesses, opportunities and threats, respectively (Table 2 and Figure 2(a)). The strengths comprised the highest number of items, whereas the weaknesses contained the lowest number of items for both breeds.

The three highest FPVs for the strengths were 0.141 for SWOT factor number 8 (S_8), 0.101 (S_{12}) and 0.101 (S_{13}) for GA (Table 1 and Figure 3(a)) and 0.120 (S_9), 0.110 (S_{14}) and 0.100 (S_{11}) for RDP. The three highest FPVs in weaknesses were 0.316 (W_6), 0.226 (W_7) and 0.129 (W_4) for GA and 0.415 (W_4), 0.175 (W_5) and 0.153 (W_6) for RDP. The three highest FPVs in opportunities were 0.142 (O_2), 0.113 (O_8) and 0.111 (O_7) for GA and 0.110 (O_3), 0.109 (O_{12}) and 0.107 (O_6) for RDP and in threats 0.230 (T_3), 0.170 (T_4) and 0.132 (T_5) for GA and 0.230 (T_6), 0.164 (T_3) and 0.146 (T_5) for RDP (Table 2 and Figure 3(a)). For GA, the computed GPVs were 0.375 for strengths, 0.162 for weaknesses, 0.278 for opportunities and 0.188 for threats (Table 1 and Figure 2(b)). However, for RDP, the calculated GPVs were 0.360 for strengths, 0.137 for weaknesses, 0.308 for opportunities and 0.188 for threats (Table 2 and Figure 2(b)).

The three highest OPVs were 0.053 (S_8), 0.038 (S_{12}) and 0.038 (S_{13}) for GA and 0.043 (S_9), 0.040 (S_{14}) and 0.036 (S_{11}) in strengths for RDP. The three highest OPVs were 0.051 (W_6), 0.036 (W_7) and 0.021 (W_4) for GA and 0.057 (W_4), 0.024 (W_5) and 0.021 (W_6) for RDP in weaknesses, 0.039 (O_2), 0.031 (O_8) and 0.031 (O_9) for GA and 0.034 (O_3), 0.034 (O_{12}) and 0.033 (O_6) for RDP in opportunities, and 0.043 (T_3), 0.032 (T_4) and 0.025 (T_5) for GA and 0.045 (T_6), 0.032 (T_3) and 0.028 (T_5) for RDP in threats (Tables 1 and 2). The three highest OPVs within each SWOT group were highlighted in the graph for each local breed (Figure 3(b)).

The iCR values for GA and RDP are shown in Figure 4. The iCR of the ranked SWOT factors within the four single SWOT groups was 0.080 for GA in strengths (iCRS), 0.066 in weaknesses (iCRW), 0.078 in opportunities (iCRO) and 0.033 in threats (iCRT). The iCR of the ranking between the SWOT groups (iCRG) was 0.001. For RDP, the iCR within each group

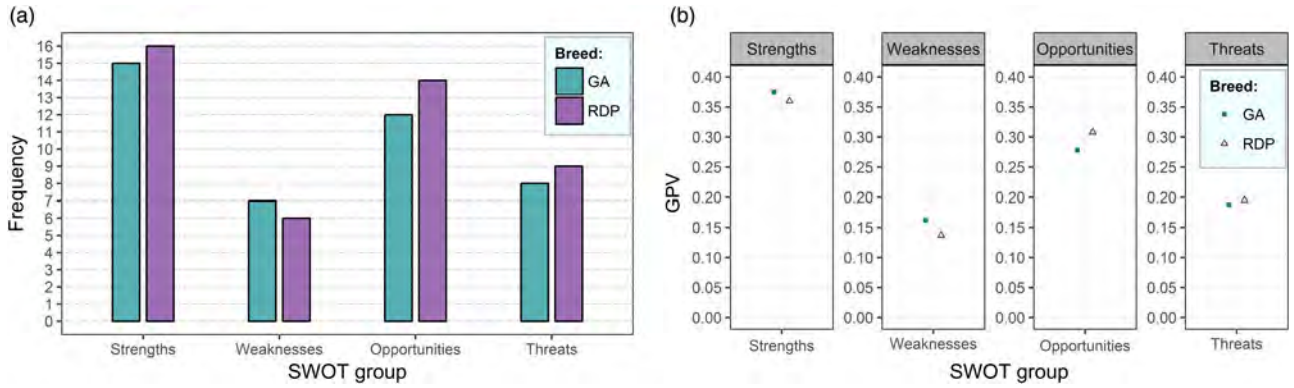

Figure 2 (Colour online) (**a**) Frequency of allocated items and (b) computed GPV via SWOT group for GA and RDP. SWOT=strengths weaknesses opportunities threats; GA=German Angler; RDP=Red Dual-Purpose cattle; GPV=group priority vector.

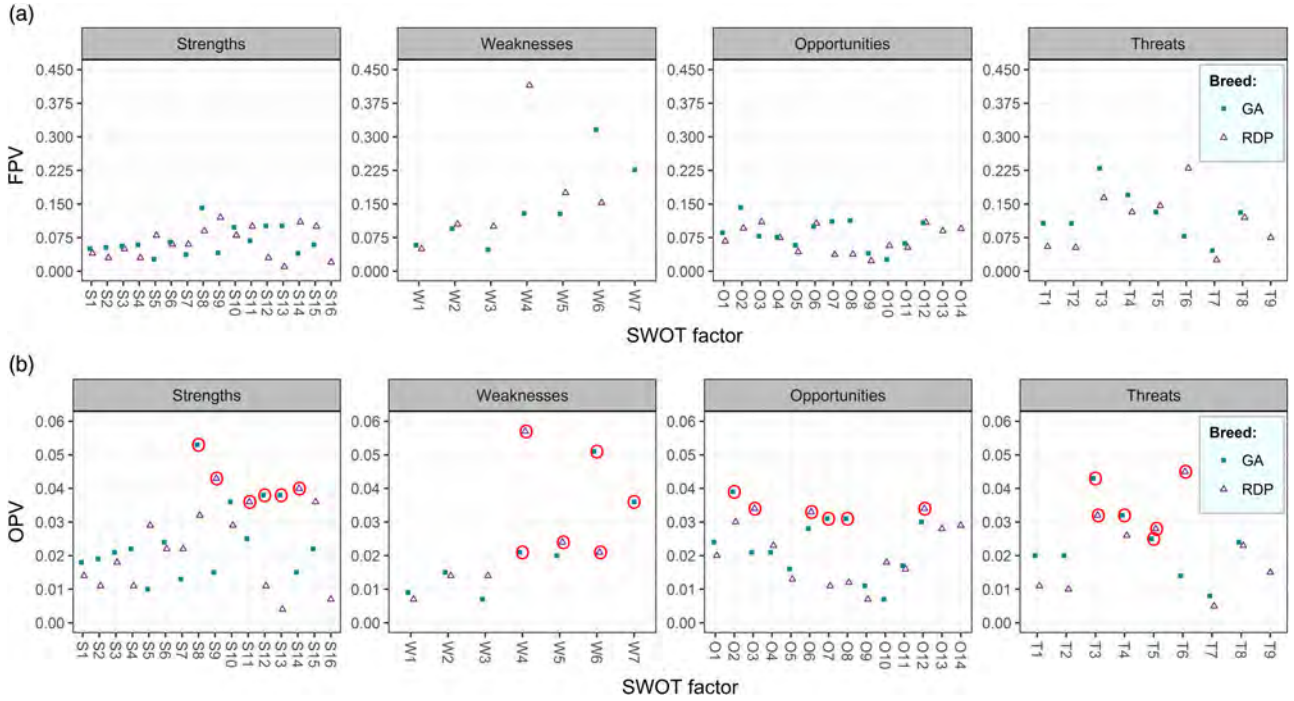

Figure 3 (Colour online) Computed (a) FPV and (b) OPV via SWOT factor for GA and RDP. SWOT factors with three highest OPVs are highlighted (red) for each SWOT group. SWOT=strengths weaknesses opportunities threats; GA=German Angler; RDP=Red Dual-Purpose cattle; FPV=factor priority vector; OPV=overall priority vector.

was 0.095 in strengths (iCRS), 0.043 in weaknesses (iCRW), 0.078 in opportunities (iCRO) and 0.060 in threats (iCRT). The iCR of the ranked four individual SWOT groups (iCRG) was 0.027.

Conservation and development strategies at breed level

Discussions with the respective stakeholder revealed for GA prioritised strengths of daily gain, meat quality, milk production and the usage of new biotechnologies. Furthermore, quantified weaknesses of genetic gain, especially for milk yield, and high rates of inbreeding have been comprehensively considered. In addition, opportunities of organic farming and breed-specific characteristics as well as threats of low milk prices and a high dependency regarding the dairy business were debated in order to explore effective conservation and development strategies at breed level.

Three final conservation and development strategies have been formulated for the GA:

(1) Changing relative weights and the relevant breeding goal to drift from milk to meat,
(2) Implementation of selection programs including genetic gain and the rate of inbreeding,
(3) Selection of unique and breed characteristic components on product level, that is, milk-fat and fine muscle fibers.

However, for RDP defined strengths were robustness, high adaptability for different housing systems and a balanced dual-purpose of milk and meat. Quantified weaknesses were

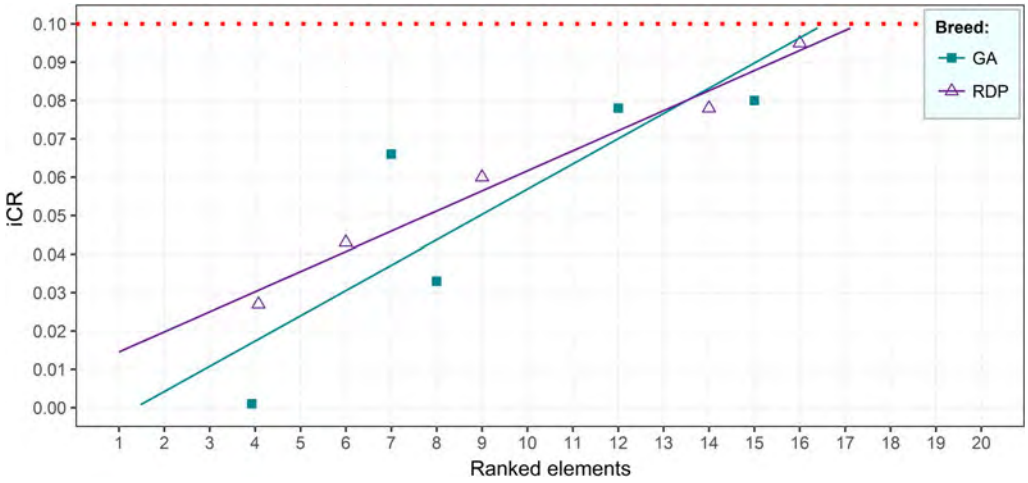

Figure 4 (Colour online) Quality control of comparison judgements via ranked elements for the GA and RDP. The threshold for the iCR is highlighted (red dotted line). GA=German Angler; RDP=Red Dual-Purpose cattle; iCR=inconsistency ratio.

inbreeding, breed extinction, genomic selection with young bulls and milk yield. Additionally, opportunities of organic farming and dual-purpose aspects as well as threats of low milk and decreasing beef cattle prices have been regarded during the strategy developments.

For RDP, three final conservation and development strategies have been formulated:

(1) Adjust relative weights and the relevant breeding goal to balance milk and meat yield,
(2) Increasing genetic gain and avoid extinction by implementing targeted selection programs,
(3) Selection of unique and breed characteristic traits on breed level, that is, environmental robustness.

Discussion

Quantified strategy decision tool

For both breeds, the quality control parameters iCRG, iCRS, iCRW, iCRO and iCRT were ≤ 0.1 (Figure 4). Hence, the quality of all comparison judgements of the ranked elements and the SWOT-AHP analysis in general were consistent. Nevertheless, the ratio between the ranked elements and iCR showed a strong dependency. The iCR was enhanced by an increased number of ranked elements. This suggests that the iCR threshold should also include the number of ranked elements to overcome limitation problems of ranked elements in order to make the AHP method more comprehensive and flexible.

Conservation and development strategies at breed level

Emphasis on meat traits will be enhanced in the breeding goal for both breeds in order to drift from milk to meat in a balanced way. Economic and biological weights as well as correlations between these traits have to be analysed in advance. Especially for the investigation of actual economic weights, product prices have to be comprehensively collected and compared on markets. Additionally, measured additive genetic variance between commercial traits is of special importance in order to assess biological weights carefully. The final identification of relative weights and the conclusive breeding goal can be indicated by using the appropriate contingent valuation method (**CVM**) from Davis (1963). CVM implies a widely used non-market valuation methodology in order to analyse environmental cost-benefits and assess environmental impacts (Cummings *et al.*, 1986; Mitchell and Carson, 1989). This method would be achievable to derive, for example, non-market values in breeding goals (Nielsen *et al.*, 2011) in case of these local breeds.

The adaptation of special breeding approaches, like the traditional or advanced Optimum Contribution Selection (Meuwissen, 1997; Wellmann *et al.* 2012), was prospectively proposed for these local breeds. The Optimum Contribution Selection is a selection method, which enables to increase genetic gain and simultaneously restrict the rate of inbreeding. This is of major importance to maintain small or endangered breeds with a threatened productivity and an enhanced rate of inbreeding (bottleneck population). Consequently, inbreeding depression and breed extinction can be avoided through mating strategies and genetic improvement. Therefore, specific breeding bulls can be suggested for mating at farm level. At this, it has major relevance that farmers follow such suggestions and attend their mating records faithfully.

Focusing unique and breed characteristic components will result in high-quality products, which can be offered on niche markets with higher earnings. Therefore, selection on such specific traits may be intensified by the breeding organisation and could lead to an added value on product level (Verrier *et al.*, 2018; Bernués, 2018; Martín-Collado *et al.*, 2018; Hiemstra *et al.*, 2018). In case of the GA, these added values on product level are milk fat (5.5%) and fine muscle fibers as meat quality trait. However, for RDP the added value will

be not on product level but rather on the production system level following the approach from Verrier (2014) and the study of Schäler *et al.* (2018), where unique traits were investigated within the respective natural production conditions. In case of the RDP, such added value on production system level is robustness in harsh environments. Thus, the RDP may become more efficient regarding pasture feeding and health traits compared to high-yielding breeds (e.g. Holstein Friesian) under the same environmental conditions. Identified added values on product and production level may be also suitable for local or organic agriculture production schemes. Hence, dependency on commercial milk markets and prices is effectively reduced due to the concentration of niche markets, the supply of high quality products and a balanced milk–meat relation.

An implementation of all conservation and development strategies is planned for each breed. According to Gandini and Oldenbroek (2007) and Meuwissen (2009), it can be concluded that such explored strategies, which include the definition of relevant breeding goals, novel marketing products and genetic improvements, are adequate to move further from conservation to utilisation. Explored conservation and development strategies indicate promising and versatile as well as farmer- and consumer-oriented strategies at breed level, even when the number of stakeholder groups was limited. Notice, exploration and design of breeding strategies may be constrained by a biased representation of representatives per stakeholder group. However, if the representation is unbiased a small number of members may well represent a stakeholder group. Thus, it is not the number as such, but whether they are unbiased representatives of the stakeholder group as a whole that matters.

Conclusion

Quantified SWOT establish an ideal basis for the exploration of conservation and development strategies. Therefore, quantified SWOT factors have to be comprehensively discussed with further stakeholder groups and their unbiased representatives at breed level. This integrated approach results in strategies which are realistic, objective and consider individual convenience of the farmers' more than a quantitative strategy decision tool on its own.

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Declaration of interest

All authors declare that there is no conflict of interest.

Ethics statement

Animal Care and Use Committee approval was not obtained for this study because data were obtained from farmer surveys.

Software and data repository resources

None of the data were deposited in an official repository.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S1751731119001447.

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animal

Effects on meat quality and black bone incidence of elevated dietary vitamin levels in broiler diets challenged with aflatoxin

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Vitamins play an essential role in broiler nutrition. They are fundamental for normal metabolic and physiological process, and their requirements for poultry are not fixed and can be affected by multiple factors. In contrast, mycotoxins are a challenging issue because they hinder performance and the immune system. Vitamin supplementation above minimum requirements would permit improvement in productive potential, health, bone and meat quality in a situation of mycotoxin challenge. The objective of this study was to determine the influence of optimum vitamin nutrition in diets contaminated with aflatoxin in broilers from 1 to 44 days of age. A total of 1800 Cobb 500 male chicks were randomized to 15 sets of eight treatment groups, each containing 15 birds using a 2 × 2 × 2 factorial design (commercial vitamin levels and high vitamin levels, two levels of aflatoxin – 0 and 0.5 ppm with binder levels of 0 and 10 000 mg/kg). The mash diets were corn and soybean meal based, formulated according to commercial practices. Feed intake, weight gain and feed conversion were analyzed for birds from 1 to 44 days of age. To determine carcass characteristics (carcass yield, breast yield and leg yield) and black bone syndrome, two birds were slaughtered from each group at 45 days. Other analyses included breast tenderness, water loss by dripping and malonaldehyde concentrations. The results demonstrated that broilers that were fed high levels of vitamins showed better weight gain, feed conversion, carcass yield and breast yield than broilers that were fed diets with commercial vitamin levels ($P < 0.05$); also, broilers that were fed diets containing 0.5 ppm aflatoxin had lower weight gain, carcass yield and breast yield ($P < 0.05$). The use of 10 000 mg/kg of binder improved ($P < 0.05$) feed conversion throughout the rearing period. We conclude that aflatoxin negatively affects performance and carcass yield; however, feeding optimum vitamin nutrition improved these performance traits.

Keywords: binder, carcass yield, malonaldehyde, mycotoxin, performance

Implications

Dietary vitamin supplementation above the established minimum requirements improves health, performance, bone quality and the welfare of poultry. Optimizing vitamin supplies in broilers minimizes poor bone quality and problems relating to feed contamination with mycotoxin.

Introduction

In recent years, the poultry sector has undergone significant transformations, resulting in better production indexes and quality of products purchased by consumers. Improved nutrition has been one of the factors driving these transformations. There is a large amount of data showing the nutritional requirements for proteins, amino acids and minerals, such as calcium and phosphorus. However, few studies have evaluated broiler vitamin requirements.

Vitamins are essential nutrients, involved in over 30 metabolic reactions at the cellular level (Marks, 1975). Among other functions, vitamins are involved in metabolism and act as immunomodulators, improving resistance to infections. In general, nutritionists balance rations based on the minimal necessary levels for performance and profit and add a safety margin based on experience. It is worth noting that, in commercial establishments, vitamin supplementation takes into account the production goals, which must be reached at the lowest possible cost, and a safety margin that allows for potential stress factors. In addition to signs of deficiency and performance, new parameters are being analyzed to determine broiler vitamin requirements. These include immune response, well-being, increased carcass vitamin

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content to improve presentation, shelf life and the nutritional value of meat to consumers.

Higher levels of vitamins in broiler diets compensate for changes in intake, bioavailability, issues that compromise the quality of the food and the level of stress. In general, a significant immune response occurs only when vitamins are supplemented at a level 10 times higher than those recommended by the NRC (1994), or two to three times higher than those commercially used (Leeson, 2007).

According to Felix *et al.* (2009), broiler performance can be improved by higher vitamin levels than those recommended by research centers. However, there is little data and new research is needed to determine the requirements of modern strains of poultry. Since there are many benefits associated with feeding effective levels of vitamins to broilers, the concept of supplying vitamin levels above industry recommendations and nutrition tables has been gaining momentum (Soto-Salanova *et al.*, 2010; Mejia *et al.*, 2014).

One challenging issue faced by the poultry sector is the use of feed ingredients containing aflatoxin. Mycotoxins hinder poultry performance, compromising the immune system and, in extreme cases, causing death. Aflatoxins are absorbed from the gastrointestinal tract, and within 24 h of ingestion of contaminated feed, concentrations of this toxin in the liver, reproductive organs and kidneys are high (Sawhney *et al.*, 1973). Liver damage hampers the metabolism of proteins, carbohydrates and lipids. The use of vitamin levels above those recommended by the NRC can protect against the slowing of animal growth caused by aflatoxins (Santúrio, 2000).

There are a number of commercially available binders that can decrease the effects of grains contaminated with mycotoxins. Binders can physically adhere to aflatoxins and prevent them from being absorbed by the gastrointestinal tract, thus reducing the toxicity to animals (Olver, 1997).

In contrast, black bone syndrome (**BBS**) is a condition that seems to be related to redness of the tissue adjacent to the bone, which can blacken during cooking or storage (Baldo *et al.*, 2013). This results in an unappetizing appearance to the meat. For this reason, fast-food suppliers avoid frozen broiler leg portions or use deboned meat instead (Whitehead, 2010). Since vitamin D plays an important role in calcium and phosphorus absorption and, therefore, influences bone quality, the use of higher vitamin levels may help to minimize BBS.

The aim of this study was to analyze the levels of optimum vitamin nutrition (**OVN**) in broiler diets from 1 to 44 days of age, with or without aflatoxin, and their impact on performance, carcass yield, meat quality traits and BBS incidence.

Materials and methods

The trial took place in the experimental poultry facility at the Faculty of Animal Science and Food Engineering of the University of Sao Paulo, Pirassununga campus (Brazil).

Animals, experimental design and diets

A total of 1800 one-day-old male Cobb 500 chicks were randomly allocated to 15 sets of eight different treatment groups of 15 birds, using a factorial $2 \times 2 \times 2$ design (levels of vitamin supplementation: control and OVN; levels of aflatoxins: 0 and 0.5 ppm; levels of binder: 0 and 10 000 mg/kg).

The birds were housed in a temperature-controlled 45×10 m barn, with negative pressure ventilation and cool-cells with insulated asbestos roofing. There were a total of 120 pens, measuring 1.0×1.2 m, and each pen was provided with nipple drinkers, one tube feeder and rice husk litter.

Mashed feed and water were provided *ad libitum* during the whole trial. The barn was heated using an automatic gas heating system, which was activated depending on the internal temperature. The initial temperature was 32°C, and it was gradually decreased according to the housing recommendations for the strain. The lighting system was set according to the Cobb broiler management guide.

The diets were provided in four stages: pre-starter (1 to 7 days), starter (8 to 21 days), grower (22 to 38 days) and finisher (39 to 45 days), formulated according to commercial practices (Table 1). The levels of vitamins used (control and OVN) can be found in Table 2. Control vitamin supplementation considered the current average industry levels in Sao Paulo, Brazil. Deactivated bentonite and yeast-based binder was used in the pre-starter and starter diets only. Aflatoxin used was produced by cultivating the toxic strain *Aspergillus parasiticus* (NRRL 2999) in rice, according to a method developed by Shotweell *et al.* (1966), and was introduced into the formulation by replacing the carrier (sand). Aflatoxin levels in the experimental diets were quantified by HPLC using 1 μg/kg quantification level and recovery coefficient of 85.5%, performed at the LAPEMI laboratory.

Evaluated characteristics

Feed intake, weight gain and feed conversion were analyzed for birds aged 1 to 44 days. At day 45, two birds from each pen were slaughtered to determine the carcass characteristics: carcass yield, breast yield and leg (thigh + drumstick) yield. Breast samples were taken to determine meat quality (tenderness, water loss by dripping and lipid peroxidation), and the tibias was used to verify the incidence of BBS.

To determine tenderness, the breast muscles were placed in cooking parcels (Cryovac/CN-530) and cooked in a water-bath at 82°C. These were then stored for 24 h at 2°C. After cooling, the samples were cut hexagonally $2 \times 1 \times 1$ cm, according to the method described by Froning and Uijtteenboogaart (1988). The samples with fibers positioned perpendicularly to the blades were cut with a texture meter (FTC Texture Test System model TP2) attached to a Warner Braztler with a speed of 20 cm/min and a cell load of 100 kg.

To determine water loss by dripping, the cuts were placed in polyethylene bags, which were labeled and sealed under atmospheric pressure and refrigerated for 48 h at 4°C. After this period, the cuts were weighed again (Bridi *et al.*, 2003),

Table 1 *Nutritional and calculated composition of the basal diet for broilers*

	Pre-starter	Starter	Grower	Finisher
Ingredient (g/kg)	1 to 7 days	8 to 21 days	22 to 38 days	39 to 45 days
Ground corn, 8%	576.20	584.20	644.70	671.20
Soybean meal, 46%	346.00	322.00	272.00	237.00
Meat meal, 43%	44.00	42.00	30.00	26.00
Soybean oil	7.50	25.00	31.50	43.00
Limestone, 38%	4.40	5.00	6.40	7.35
Ground salt	3.85	3.50	2.60	2.65
DL-methionine, 99%	3.10	3.00	2.50	2.10
L-lysine, 98%	2.05	2.30	2.15	2.20
L-threonine	1.10	1.20	0.85	0.70
Sodium bicarbonate	1.00	1.00	2.00	2.00
Vitamin/mineral supplement	5.00	5.00	5.00	5.00
Carrier	5.80	5.80	0.80	0.80
Calculated nutritional levels				
ME, MJ/kg	12.47	12.98	13.40	13.81
CP, g/kg	231.80	220.80	196.10	180.50
Ca, g/kg	10.50	10.10	9.00	8.06
aP, g/kg	5.20	5.00	4.20	3.90
Na, g/kg	2.20	2.10	1.90	1.90
SAA[6], g/kg	10.20	9.80	8.70	7.90
Lysine, g/kg	14.00	13.50	11.80	10.80
Threonine, g/kg	9.50	9.20	8.00	7.30
Analyzed nutritional levels				
CP, g/kg	234.40	221.10	198.70	182.30
Fat, g/kg	44.60	54.00	61.70	67.90
Moisture, g/kg	109.50	108.40	112.60	114.80

ME = metabolizable energy; aP = available phosphorus; SAA = sulfur amino acids.
Mineral supplement per kilogram of diet: copper (100 g), iron (50 g), selenium (200 mg), zinc (50 g), manganese (70 g), iodine (1.2 g).

and the second weight was expressed as a percentage of the initial weight.

Lipid peroxidation was determined based on the amount of malonaldehyde (**MDA**) using the method described by Tarladgis *et al.* (1960). The standard adopted was that of tetraethoxypropane 1.1′3.3″, which releases malonaldehyde during acid hydrolysis at a ratio of 1 mol : 1 mol. The results are expressed in MDA, defined as milligram of malonaldehyde per kilogram of sample.

The tibias were deboned and dried, preserving the periosteum. To determine the prevalence of BBS, bone lightness was analyzed by (L*) test. For this purpose, a Minolta 410R colorimeter was placed on the proximal epiphysis of the growth plate. The tibias received a score based on the light parameters, and their appearance was scored as acceptable (no darkening present in the bone – L* > 40), intermediate (presence of slight darkening in the bone – L* between 40 and 35) and unacceptable (severe darkening in the bone – L* < 35) (adapted from Baldo *et al.* 2013). The results are expressed as percentages of bones classified as acceptable, intermediate and unacceptable in each treatment.

Statistical analysis

Data on performance, carcass yield, meat quality traits and BBS incidence were analyzed using the variance analysis method and the GLM process of the SAS (SAS Institute, Cary, NC, USA). In cases where the differences were significant, the means were compared using the Tukey test at 5% probability. For performance data, the statistical unit was the pen; and for meat quality, carcass yield and BBS incidence, the statistical unit was the animal.

Results

There was an interaction for weight gain ($P < 0.05$) between vitamin levels and aflatoxin, showing that OVN levels in broiler diets helped to improve weight gain, compared with the control. In addition, the inclusion of a binder in the diet significantly improved feed conversion (Table 3).

Regarding carcass traits (Table 4), the treatment did not influence leg (thigh + drumstick) yield. However, OVN levels improved carcass yield compared with the control diet ($P < 0.05$). The presence of aflatoxin decreased carcass and breast yield ($P < 0.05$). There was a relationship between vitamin levels and the presence or absence of aflatoxin with carcass yield ($P < 0.05$). Broilers that were fed diets without aflatoxin had better carcass yield than diets with aflatoxin, and the supplementation of vitamins at higher than recommended (OVN) levels improved carcass yield in broilers that were fed diets with or without aflatoxin. The analysis of interactions between vitamin levels and the presence or absence of aflatoxins demonstrated that broilers that were fed a

Table 2 *Minimum levels of vitamins provided by the control vitamin supplement and OVN supplement for broilers*

Vitamin	Unit	Pre-starter	Starter	Grower	Finisher
Control vitamin supplement					
Vitamin A	IU/ton feed	8 000 000	7 000000	6 000 000	5 000 000
Vitamin D_3	IU/ton feed	2 400 000	2 200 000	2 000 000	1 000 000
Vitamin E	IU/ton feed	12 000	11 000	10 000	8000
Vitamin K_3	mg/ton feed	2000	1600	1600	1600
Vitamin B_1	mg/ton feed	2400	2000	1400	0
Vitamin B_2	mg/ton feed	6000	5000	4000	2000
Vitamin $_{B6}$	mg/ton feed	4000	3000	2000	0
Vitamin B_{12}	mg/ton feed	14	12	10	5
Folic acid	mg/ton feed	1000	800	600	0
Niacin	mg/ton feed	40 000	35 000	30 000	20 000
Pantothenic acid	mg/ton feed	15 000	13 000	11 000	9000
Choline	g/ton feed	346	328	242	128
OVN supplement					
Vitamin A	IU/ton feed	13 000 000	11 250 000	11 250 000	11 250 000
Vitamin D_3	IU/ton feed	4 000 000	4 000 000	4 000 000	4 000 000
Vitamin E	IU/ton feed	225 000	75 000	75 000	75 000
Vitamin K_3	mg/ton feed	3500	3500	3500	3500
Vitamin B_1	mg/ton feed	3500	2500	2500	2500
Vitamin B_2	mg/ton feed	9000	8000	7000	7000
Vitamin B_6	mg/ton feed	5000	5000	5000	5000
Vitamin B_{12}	mg/ton feed	30	25	25	25
Folic acid	mg/ton feed	2250	2250	2250	2250
Niacin	mg/ton feed	70 000	70 000	65 000	65 000
Pantothenic acid	mg/ton feed	17 500	15 000	12 500	12 500
Choline	g/ton feed	550	550	575	575
Vitamin C	mg/ton feed	150 000	150 000	150 000	150 000
HyD^3	mg/ton feed	69	69	69	69
Biotin	mg/ton feed	300	250	250	250

OVN = optimum vitamin nutrition; HyD = hydroxy vitamin D.
The levels of vitamins in premix are values provided by producer.

control diet with aflatoxin had lower carcass yield. Although the birds that were fed a diet with mycotoxin had lower carcass yield, the use of OVN improved carcass yield compared with the birds that were fed a control diet without aflatoxins.

OVN treatment decreased the frequency of unacceptable and intermediate levels of BBS (Table 4) and improved the percentage of acceptable bone lightness ($P < 0.05$). The analysis of breast quality showed that the treatments did not influence water loss by dripping ($P > 0.05$) (Table 4). The addition of aflatoxins in the diet increased MDA levels compared with a mycotoxin-free diet. OVN supplementation of the diet decreased the levels of MDA. Also, meat tenderness significantly decreased when binders were used in the diet. It is worth noting that the presence or absence of aflatoxins and the different vitamin levels had no effect on broiler breast tenderness.

Discussion

The deficiency of one or more vitamins can cause metabolic disorders, leading to reduced productivity, slow growth and an increased incidence of diseases. Without vitamins, metabolic reactions slow and become ineffective (Mavromichalis *et al.*, 1999). Increasing the levels of fat-soluble vitamins can boost immunity (Felix *et al.*, 2009). The benefits of such supplementation were demonstrated in this experiment, since all diets with OVN significantly improved weight gain and feed conversion compared with the control diet, even when challenged by aflatoxin.

Although vitamin supplementation is known to be beneficial, some studies have removed the premix from the diet in the last week of the finishing stage to determine whether this cost-saving measure might affect poultry performance. Broilers that were fed diets without vitamin supplementation, from 28 to 49 days of age, showed decreased weight gain, feed efficiency and breast yield (Deyhim and Teeter, 1993). In addition, broilers that were fed vitamin-free premix diets 1 and 2 weeks prior to slaughter (42 days of age) had lower weight gain, although feed intake and feed conversion were not affected (Christmas *et al.*, 1995). Also, Castaing *et al.* (2003) fed two levels of vitamin supplements to broilers and noted that the higher level (twice the commercial standard) led to a higher weight gain at 38 days (1919 g) compared to the lower level (1878 g). It is clear that adequate

Table 3 *Broiler performance when fed different levels of vitamin in the diet with or without aflatoxin challenge*

	Levels of vitamin supplementation								Probability							
	Control				OVN				Vit	Afla	Bin	Vit × Afla8	Vit × Bin	Afla × Bin	Vit × Afla × Bin	SEM
Afla, mg/kg	0	0	0.5	0.5	0	0	0.5	0.5								
Bin, mg/kg	0	10 000	0	10 000	0	10 000	0	10 000								
1 to 21 days																
FI, g	1159	1159	1153	1153	1161	1161	1155	1155	0.220	0.480	0.944	0.912	0.802	0.127	0.758	0.125
WG, g	804	803	801	799	809	808	806	804	0.005	0.002	0.388	0.004	0.775	0.291	0.137	0.430
FCR, g/g	1.44	1.44	1.44	1.44	1.44	1.44	1.44	1.44	0.943	0.943	0.859	0.836	0.785	0.822	0.921	0.050
1 to 44 days																
FI, g	5256	5242	5247	5234	5220	5206	5211	5199	0.312	0.420	0.878	0.521	0.664	0.234	0.278	0.125
WG, g	3159	3160	3144	3145	3182	3183	3167	3165	0.004	0.004	0.147	0.017	0.235	0.347	0.225	0.430
FCR, g/g	1.67	1.66	1.67	1.67	1.64	1.63	1.65	1.64	0.023	0.143	0.020	0.104	0.247	0.132	0.217	0.050

OVN = optimum vitamin nutrition; Vit = vitamin; Afla = aflatoxin; Bin = binder; FI = feed intake; WG = weight gain; FCR = feed conversion ratio.

Table 4 *Broiler carcass characteristics, incidence of BBS and meat quality of broilers that were fed different levels of vitamin in the diets, with or without aflatoxin challenge, at 45 days of age*

	Levels of vitamin supplementation								Probability							
	Control				OVN				Vit	Afla	Bin	Vit × Afla	Vit × Bin	Afla × Bin	Vit × Afla × Bin	SEM
Afla, mg/kg	0	0	0.5	0.5	0	0	0.5	0.5								
Bin, mg/kg	0	10 000	0	10 000	0	10 000	0	10 000								
Carcass traits, %																
Carcass	69.77	69.94	69.40	69.57	70.00	70.17	69.64	69.81	0.002	0.043	0.211	0.038	0.158	0.254	0.321	0.610
Breast	31.79	31.73	31.63	31.56	31.69	31.63	31.52	31.47	0.025	0.039	0.328	0.425	0.368	0.355	0.271	0.390
Legs	32.97	32.96	33.07	33.06	33.01	33.00	33.11	33.10	0.103	0.303	0.422	0.437	0.670	0.529	0.494	0.720
BBS, %																
Unacceptable (L*>40)	10.67	10.33	12.00	11.66	7.67	7.33	9.00	8.67	0.011	0.458	0.632	0.224	0.355	0.157	0.287	5.160
Intermediate (L*>35 to 40)	19.33	19.67	21.00	21.33	16.33	16.66	18.00	18.33	0.034	0.287	0.189	0.255	0.098	0.152	0.114	7.210
Acceptable (L*<35)	70.00	70.00	67.00	67.00	76.00	76.00	73.00	73.00	<0.001	0.047	0.690	0.871	0.129	0.224	0.199	11.04
Meat quality																
TBARS	2.83	2.85	2.95	2.97	1.49	1.51	1.61	1.63	0.003	0.018	0.348	0.388	0.412	0.255	0.221	3.360
Water loss, %	21.33	21.59	21.38	21.64	20.90	21.16	20.95	21.22	0.117	0.259	0.222	0.133	0.425	0.235	0.367	2.440
Tenderness, kgf	1.41	1.71	1.43	1.73	1.43	1.73	1.45	1.75	0.521	0.456	0.001	0.311	0.244	0.411	0.281	0.630

OVN = optimum vitamin nutrition; Vit = vitamin; Afla = aflatoxin; Bin = binder; BBS = black bone syndrome; TBARS = thiobarbituric acid reactive substances (measured as milligram of malonaldehyde per kilogram of sample). L* indicates values of lightness.

levels of vitamin supplementation must be provided to ensure good performance.

The performance of broilers that were fed diets with aflatoxin was lower than broilers that were fed mycotoxin-free diets. The literature describes several toxic effects caused by mycotoxins in birds, including poor performance, liver diseases, immunosuppression, hemorrhages, poor carcass quality and change in the relative weight of organs (Edds and Bordtell, 1983; Hygino da Cruz, 1996; Moreira, 2000). In addition, mycotoxins can reduce the humoral immune response, facilitating the growth of pathogens and hindering performance (Terrassi *et al.*, 2005). Therefore, the use of additional vitamin supplementation was essential to minimize losses due to the presence of aflatoxin. According to Santúrio (2000), the biggest negative impact was seen when birds were fed aflatoxin during their first 21 days of life, and this impact on weight gain is irreversible until slaughter (42 days), results that were replicated in this study. According to Santúrio (2000), the relationship between aflatoxin and vitamins was not clear.

The addition of binders to the diets did not improve performance, but significantly decreased feed conversion. Binders reduced feed intake, but the weight gain of birds was maintained. Thus, when binders were used, mycotoxins had a reduced negative impact on performance. If mycotoxins are present in the diet, their impact on performance can currently only be mitigated by the use of toxin binders (Diaz *et al.*, 2002; Oguz *et al.*, 2002). The improvement in feed conversion observed in the present study may be explained by the fact of binders adhering to aflatoxin and blocking its absorption from the gastrointestinal tract, making it inert and non-toxic to animals (Batina *et al.*, 2005).

This study showed a significant improvement in carcass yield when birds were fed OVN levels (70.31% OVN vitamin and 69.61% control vitamin levels) and aflatoxin-free diets (70.25% and 0 ppm; and 69.15% and 0.5 ppm). The percentage of breast yield was higher in birds that were fed aflatoxin-free diets (31.87% with 0 ppm and 31.38% with 0.5 ppm).

As expected, this study showed that aflatoxin contamination resulted in a drop in performance and carcass yield; however, higher levels of vitamins in the diet improved broiler performance and carcass yield even when birds were challenged by dietary aflatoxin.

The relative weights of the heart, liver, gizzard and pancreas were also analyzed in this study; however, the treatments had no impact on these characteristics (data not shown). This result contradicts the findings of Giacomini *et al.* (2006), who analyzed the relative weights of organs and carcasses and found that the size of hearts and livers of birds that were fed diets with aflatoxin increased; spleen and gizzard showed no significant difference in relative weight; and the weight of carcasses of birds that were fed diets with aflatoxin significantly reduced. The lack of impact on the relative weight of organs in this study might be explained by the dose of aflatoxin used. It is possible that the amount of aflatoxin in the diet was insufficient to lead to changes in the weights of the analysed viscera, and similar findings were reported by Ortatali *et al.* (2004), who intoxicated broilers for 42 days with 50 and 100 μg aflatoxin per kilogram of feed and found no difference in the relative weight of liver, kidneys, spleen, thymus and bursa of Fabricius. This was hypothesized to be due to a low aflatoxin dosage used.

Regarding the incidence of BBS, the number of acceptable ratings for lightness increased when the birds were fed OVN, whereas the presence of aflatoxin in the diet increased the number of intermediate readings. Aflatoxin can impact liver, kidneys and the organs involved in the biosynthesis of active forms of vitamin D, responsible for transferring calcium and phosphorus from the diet to the blood stream (Hamilton, 1984). Given the toxic effects of aflatoxin and, consequently, a decrease in the amount of active vitamin D, the level of calcium absorbed by the intestine and deposited in the bones decreases (Siloto *et al.*, 2011). This can explain the effects of aflatoxin on bone lightness. In addition, the results show that OVN levels improved bone characteristics in broilers, decreasing the incidence of BBS.

Another important aspect relating to the benefits of feeding vitamins to birds is improved meat quality. As this study shows, the use of OVN in broiler feed decreased lipid peroxidation, a characteristic of much interest to the industry. The oxidation of fats, particularly of unsaturated fatty acids, is the main driver for poor meat quality. Regardless of the source, meats are susceptible to oxidative deterioration, which determines the shelf life of these products. However, several antioxidants, including ascorbic acid and alpha-tocopherol, protect lipids in the membranes from oxidation (Mordenti and Marchetti, 1996). Oxidation occurs in two stages: firstly, the oxidation of phospholipids and, secondly, the oxidation of triglycerides, which is directly related to the degree of unsaturation; the shelf life of meat depends on the oxidation of polyunsaturated fatty acids (Gandemer, 1997). Once lipid oxidation starts, several secondary reactions, such as the formation of free radicals, are triggered. In addition to off-flavor compounds determining the quality of meat, other reactions such as the formation of potentially toxic compounds, such as alcohols, ketones, peroxides and aldehydes, can hinder the safety and stability of meat, leading to nutrient loss and further promoting oxidative reactions (Gray *et al.*, 1996; Nam *et al.*, 1997). The adequate supplementation of antioxidant vitamins improves the stability of membrane structures. As a result, the meat is expected to be more stable. Providing broilers with high levels of natural antioxidants gives the poultry industry a simple method to improve stability against oxidation, sensory quality, shelf life and the acceptability of meats.

Finally, the use of OVN in broiler diets improves performance and carcass characteristics (yield and meat quality) even when aflatoxin is present in the diet, and it can effectively minimize the negative effects caused by mycotoxins in birds regardless of the addition of binders.

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Declaration of interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Ethics statement

The experimental procedures were approved by the University of Sao Paulo Research Ethics Committee.

Software and data repository resources

This article is part of a dissertation, which is deposited in an official repository with open access: http://www.teses.usp.br/teses/disponiveis/10/10135/tde-31012018-151337/pt-br.php

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Effect of dietary chromium supplementation on meat nutritional quality and antioxidant status from broilers fed with Camelina-meal-supplemented diets

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*Poultry meat is a valuable source of nutrients and the enrichment with health-promoting substances such as polyunsaturated fatty acids (**n-3 PUFA**) is an important factor for consumers' choice. Camelina meal (Camelina sativa) is an animal feedstuff used to achieve this goal, but the administration of n-3 PUFA-enriched diets in broiler nutrition can accelerate the oxidative processes in meat leading to a decreased quality of final product. The aim of this study was to investigate the effect of the organic Cr as chromium picolinate (CrPic) on meat quality, fatty acid profile of fat and oxidative stability of meat from broilers fed supplemented dietary Camelina meal. An experiment was conducted on 240 Ross 308 broiler chicken aged 14 days which were assigned to 6 dietary treatments in a randomized complete block design with a 2 × 3 factorial arrangement. Within the treatment arrangement two concentrations of Camelina meal (0% and 3%) and three concentrations of Cr^{3+} (0, 200 and 400 μg/kg) were used. Dietary treatments were: (1) Control diet (C) containing a corn–soybean diet with no added Camelina meal or Cr^{3+}; (2) a C diet containing an additional 200 μg/kg of Cr^{3+} as CrPic; (3) a C diet containing an additional 400 μg/kg of Cr^{3+} as CrPic; (4) a C diet containing an additional 3% Camelina meal; (5) diet 2 containing an additional 3% Camelina meal; (6) diet 3 containing an additional 3% Camelina meal. Chromium supplementation significantly ($P<0.05$) increased the CP concentrations and significantly ($P<0.05$) decreased the crude fat concentrations in breast samples. The Camelina meal groups presented higher values of unsaturated fatty acids, particularly n-3 fatty acids ($P<0.05$). In CrPic groups, increased retention of Zn and Fe ($P < 0.05$) was observed in breast samples, compared to control group, and thiobarbituric acid reactive substances values were significantly ($P<0.05$) smaller. Myoglobin fraction (metmyoglobin and oximyoglobin) concentrations differ significantly ($P<0.05$) from the control group, under the influence of Cr^{3+} supplements. This study found that broilers fed with CrPic supplements showed improved mineral composition and oxidative stability of breast meat, proving an effective protection of lipid molecules from oxidation in PUFA-enriched meat.*

Keywords: chicken, breast, oxidative stability, fatty acids, trace elements

Implications

Chromium improves broilers meat quality, by increasing the protein concentrations and mineral composition. Chromium supplements showed beneficial effects on oxidative stability of meat. The oxidative processes in meat lead to rancidity, drip loss and meat texture and color changes, which are characteristics of an altered product. A higher oxidative stability of the animal product brings economic benefits for producers by increasing shelf life.

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Introduction

Poultry meat is generally appreciated for its polyunsaturated fatty acids (**PUFA**) concentrations, but being low in natural antioxidants it is susceptible to quality deterioration by lipid oxidation during storage or cooking (Aziza, *et al.*, 2010). The oxidative stability of poultry meat is affected by the birds' diet and meat fatty acid composition.

Camelina meal (*Camelina sativa*) is a by-product that remains after oil extraction for biodiesel production that can be used as animal feedstuff beneficial in increasing the n-3 fatty acid content in eggs and it can also affect in a positive manner the meat quality of laying hens and broiler

chickens. Previous studies conducted on the nutrient composition of *Camelina* meal indicated a 30% to 40% CP and 8% to 12% fat of which the α-Linolenic acid (**ALA**) (18:3n-3) fraction constitutes 25% to 30% (Cherian *et al*., 2009; Panaite *et al*., 2016). An important number of studies reported that supplementing layer and broiler diets with *Camelina* meal conducted a significant increase in the n-3 fatty acids in eggs and broiler muscle tissues (Cherian *et al*., 2009; Aziza *et al*., 2010; Panaite *et al*., 2016).

Polyunsaturated fatty acids are a class of molecules readily oxidized by reactive oxygen species (**ROS**) and the administration of *Camelina*-meal-supplemented broiler diet can increase the lipid peroxidation of meat. Antioxidant effects can be provided directly and/or indirectly by a wide number of molecules such as vitamins, lipids, proteins, plant-derived antioxidants, minerals and enzymes (Vertuani *et al*., 2004).

Trivalent chromium (Cr^{3+}) is an essential trace element involved in glucose and insulin metabolism, and has a capacity of counteracting the oxidative stress associated with insulin resistance. Chromium supplements are used in poultry diets due to beneficial effects on broilers' productive parameters, blood parameters (Kroliczewska *et al*., 2004) or nutritive quality of meat (Toghyani *et al*., 2012). Chromium is considered an indirect antioxidant as it prevents auto-oxidation of glucose and activates certain enzymes and stabilization of proteins and nucleic acids (Sahin *et al*., 2002). Studies on chromium effect on nutritive value of broiler meat show that dietary inclusion rate of Cr^{3+} improved meat oxidative stability (Toghyani *et al*., 2012), decreased the muscle cholesterol and improved the muscle protein (Anandhi *et al*., 2006).

The objective of this study was to evaluate the organic chromium capacity to enhance oxidative stability of PUFA-enriched meat.

Material and methods

Experimental design

The experiment was conducted on 240 Ross 308 broiler chicken aged 14 days which were assigned to 6 dietary treatments in a randomized complete block design with a 2 × 3 factorial arrangement. Within the treatment arrangement, two concentrations of *Camelina* meal (0% and 3%) and three concentrations of Cr^{3+} (0, 200 and 400 μg/kg, Sigma Aldrich, Germany) as chromium picolinate (CrPic) were used. *Camelina sativa* was grown in southern Romania and seed material was processed by Marnews Olt, Craiova. The C*amelina* meal used in this study was a pressing residue from the manufacturing process of cold-pressed *Camelina* oil. The chemical composition of *Camelina* meal used in the experiment was published by Panaite *et al*. (2016). Dietary treatments were: (1) Control diet (C) containing a corn–soybean diet with no added *Camelina* meal or Cr^{3+}; (2) a C diet containing an additional 200 μg/kg of Cr^{3+} as CrPic; (3) a C diet containing an additional 400 μg/kg of Cr^{3+} as CrPic; (4) a C diet containing an additional 3% *Camelina* meal; (5) diet 2 containing an additional 3% *Camelina* meal; (6) diet 3 containing an additional 3% *Camelina* meal (Table 1), and fatty acids composition of diets is presented in Table 2.

The animals were housed in digestibility pens in a poultry experimental hall with controlled environmental conditions (average temperature/total period 25.57±0.88^{0}C; humidity 62.97±5.09%; ventilation/broiler 0.64±0.18%; CO_2 level 888.48±101.31 ppm). The light regimen was adequate to the age of the broilers (23-h light/1-h darkness). Each treatment was replicated 4 times with 10 chicks per replicate pen. The birds were fed *ad libitum* and they had free access to the water. At the end of the experiment, 6 chicks aged 42 days from each group were slaughtered and samples of breast were collected for further analysis.

The productive parameters (average daily gain, feed conversion) were calculated from the weekly records of the BWs and daily feed intake.

Chemical analysis

Proximate composition of meat. The CP of the meat samples was determined with Kjeldahl reference method using a semiautomatic Kjeltek auto 1030 – Tecator (FOSS Tecator AB, Höganäs, Sweden) according to SR EN ISO 5983-2:2009 (International Organization for Standardization, 2009). The fat was extracted using an improved version of the classical method by continuous extraction in solvent, followed by fat measurement with Soxhlet after solvent removal (FOSS Tecator AB, Höganäs, Sweden) according to SR ISO 6492: 2001 (International Organization for Standardization, 2001). Dry matter content was measured with a gravimetric method by drying samples at 103°C to constant weight in an oven BMT model ECOCELL Blueline Comfort (Neuremberg, Germany) according to Regulation (CE) nr. 152/2009 and with crude ash by heating at 550°C for 24 h using an ashing furnace Nabertherm Labotherm L15/11/ P320 Comfort (Bremen, Germany) according to Regulation (CE) No. 152/2009.

Trace minerals determinations. Trace minerals' (Cu, Fe, Mn, Zn) concentrations were determined in meat samples using flame atomic absorption spectrometry (**FAAS**) as described by Untea *et al*. (2012) after microwave digestion, by using Thermo Electron – SOLAAR M6 Dual Zeeman Comfort (Cambridge, UK) equipment. For chromium determination, the graphite furnace atomic absorption spectrometry method was used according to Rêczajska *et al*. (2005).

Fatty acids determinations. A gas chromatograph Perkin-Elmer Clarus 500 (Massachusetts, USA), fitted with Flame Ionization Detector (**FID**) and capillary separation column with high polar stationary phase TRACE TR-Fame, (Thermo Electron, Massachusetts, USA), dimensions 60 m X 0.25 mm X 0.25 μm was used in order to determine fatty acids composition of meat samples. Each sample was prepared as described by Panaite *et al*. (2016).

Oxidative stability parameters

Total lipids were extracted using the modified Folch procedure (Folch *et al*., 1957). The minced meat sample (5 g) was homogenized in 30 ml chloroform/methanol mixture (2:1, v/v). The homogenate was filtered in a separation funnel

Table 1 *Ingredients and chemical composition of broilers diets*

	Stage II grower (14 to 28 days)						Stage III finisher (29 to 42 days)					
	Cam 0%			Cam 3%			Cam 0%			Cam 3%		
Diet composition (%)	Cr 0	Cr 200	Cr 400	Cr 0	Cr 200	Cr 400	Cr 0	Cr 200	Cr 400	Cr 0	Cr 200	Cr 400
Maize	34.17	34.17	34.17	34.98	34.98	34.98	39.77	39.77	39.77	39.75	39.75	39.75
Wheat	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00
Rice bran	8.00	8.00	8.00	8.00	8.00	8.00	7.12	7.12	7.12	8.00	8.00	8.00
Soybean meal	22.00	22.00	22.00	21.83	21.83	21.83	16.05	16.05	16.05	15.82	15.82	15.82
Rapeseed meal	8.00	8.00	8.00	5.00	5.00	5.00	9.00	9.00	9.00	6.00	6.00	6.00
Gluten	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Vegetable (soybean) oil	4.20	4.20	4.20	3.50	3.50	3.50	4.90	4.90	4.90	4.20	4.20	4.20
dl-Methionine	0.15	0.15	0.15	0.15	0.15	0.15	0.13	0.13	0.13	0.14	0.14	0.14
Lysine	0.27	0.27	0.27	0.29	0.29	0.29	0.30	0.30	0.30	0.32	0.32	0.32
Calcium carbonate	1.25	1.25	1.25	1.27	1.27	1.27	1.11	1.11	1.11	1.14	1.14	1.14
Calcium phosphate	1.54	1.54	1.54	1.56	1.56	1.56	1.37	1.37	1.37	1.39	1.39	1.39
Sodium chloride	0.37	0.37	0.37	0.37	0.37	0.37	0.20	0.20	0.20	0.20	0.20	0.20
Choline	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Camelina meal	-	-	-	3.00	3.00	3.00	-	-	-	3.00	3.00	3.00
Premix	1.00[a]	1.00[b]	1.00[c]	1.00[a]	1.00[b]	1.00[c]	1.00[a]	1.00[b]	1.00[c]	1.00[a]	1.00[b]	1.00[c]
Total	100	100	100	100	100	100	100	100	100	100	100	100
Calculated analysis, %												
DM	88.02	88.02	88.02	88.26	88.26	88.26	87.96	87.96	87.96	88.23	88.23	88.23
ME poultry, (kcal/kg)	3097	3097	3097	3101	3101	3101	3200	3200	3200	3200	3200	3200
CP	21.53	21.53	21.53	21.50	21.50	21.50	19.50	19.50	19.50	19.50	19.50	19.50
Crude fat	6.15	6.15	6.15	5.78	5.78	5.78	6.86	6.86	6.86	6.51	6.51	6.51
Calcium	0.87	0.87	0.87	0.87	0.87	0.87	0.78	0.78	0.78	0.78	0.78	0.78
Available phosphorous	0.44	0.44	0.44	0.43	0.43	0.43	0.39	0.39	0.39	0.39	0.39	0.39
Lysine	1.20	1.20	1.20	1.20	1.20	1.20	1.08	1.08	1.08	1.08	1.08	1.08
Methionine	0.52	0.52	0.52	0.52	0.52	0.52	0.48	0.48	0.48	0.49	0.49	0.49
Met+Cys	0.92	0.92	0.92	0.91	0.91	0.91	0.85	0.85	0.85	0.85	0.85	0.85
Threonine	0.73	0.73	0.73	0.72	0.72	0.72	0.65	0.65	0.65	0.64	0.64	0.64
Tryptophan	0.21	0.21	0.21	0.21	0.21	0.21	0.18	0.18	0.18	0.18	0.18	0.18
Chemical analysis, %												
Σ Unsaturated acids	51.63	52.12	51.41	52.19	52.11	51.52	49.08	49.99	49.83	50.21	49.25	49.02
Σ n-3	1.40	1.26	1.34	4.05	3.74	4.05	1.54	1.55	1.48	3.45	3.83	3.50
Σ n-6	50.22	50.85	50.07	48.14	48.38	47.47	47.54	48.44	48.35	46.76	45.42	45.52
Ratio n-6/n-3	35.80	40.32	37.34	11.87	12.95	11.72	30.86	31.18	32.57	13.56	11.86	13.02

ME = Metabolizable energy; Cam = Camelina meal; CrPic = Chromium picolinate; Met = Methionine; Cys = Cysteine.
[a] Premix contains: 11×10^5 IU/kg Vit. A; 2×10^5 IU/kg Vit. D3; 2700 IU/kg Vit. E; 300 mg/kg Vit. K; 200 mg/kg Vit. B1; 400 mg/kg Vit. B2; 1485 mg/kg pantothenic acid; 2700 mg/kg nicotinic acid; 300 mg/kg Vit. B6; 4 mg/kg Vit. B7; 100 mg/kg Vit. B9; 1.8 mg/kg Vit. B12; 2000 mg/kg Vit. C; 8000 mg/kg manganese; 8000 mg/kg iron; 500 mg/kg copper; 6000 mg/kg zinc; 37 mg/kg cobalt; 152 mg/kg iodine; 18 mg/kg selenium; 6000 mg/kg antioxidant.
[b] Premix + 200 μg/kg CrPic.
[c] Premix + 400 μg/kg CrPic.

and 7.5 ml of a 0.88% KCl aqueous solution was added. The sample solution was left to rest for 20 h. The lower organic layer was collected and evaporated at room temperature to constant weight. The lipid extract results from the difference between initial and final weight of collector glass.

Primary oxidation products. Peroxide value (**PV**) was measured by ferric thiocyanate method and it was expressed as milliequivalents of oxygen molecule per kg of lipids (Meq O_2/kg) (Pegg, 2005). To the lipid extract sample (0.1 g), 9.9 ml chloroform/methanol (7:3, v/v) solution was added and the mixture was vortexed. After the addition of 50 μl of 10 mmol/l xylenol orange solution and 50 μl $FeCl_2$ solution (1000 mg/kg), the mixture was left at rest for 5 min at room temperature and then, the absorptivity at 560 nm was measured using a V-530 Jasco (Japan Servo Co. Ltd., Japan) spectrophotometer. The value of conjugated dienes (**CD**) and trienes (**CT**) was determined by a spectrophotometric procedure (Pegg, 2005) using a lipid extract sample dissolved in 2,2,4-trimethylpentane (iso-octane) and the absorbance values of sample solution at 233 nm (CD) and 268 nm (CT) were measured using a V-530 Jasco (Japan Servo Co. Ltd., Japan) spectrophotometer.

Secondary oxidation products. P-anisidine value was determined by a method based on the reaction between p-anisidine and aldehydic compounds present in lipid extract samples at acidic conditions (Pegg, 2005). The lipid extract sample was dissolved in iso-octane and the absorbance of the sample solution was measured at 350 nm. The

Table 2 *Fatty acids composition of broilers diets*

	Stage II grower (14 to 28 days)						Stage III finisher (29 to 42 days)					
	Cam 0%			Cam 3%			Cam 0%			Cam 3%		
Diet composition (%)	Cr 0	Cr 200	Cr 400	Cr 0	Cr 200	Cr 400	Cr 0	Cr 200	Cr 400	Cr 0	Cr 200	Cr 400
Myristic acid (C14:0)	0.19	0.26	0.22	0.18	0.19	0.17	0.20	0.20	0.19	0.19	0.21	0.21
Pentadecanoic acid (C15:0)	0.05	0.00	0.06	0.00	0.08	0.11	0.05	0.04	0.04	0.04	0.00	0.00
Palmitic acid (C16:0)	12.45	12.35	12.87	12.34	12.33	12.11	13.90	13.66	13.45	12.89	13.88	14.11
Palmitoleic acid (C16:1)	0.24	0.21	0.23	0.20	0.20	0.20	0.21	0.20	0.21	0.18	0.20	0.20
Stearic acid (C18:0)	2.12	2.13	2.12	2.19	2.22	2.19	1.86	1.88	1.87	1.88	1.91	1.91
Oleic acid (C18:1)	32.01	31.67	31.72	30.31	30.35	30.10	33.27	32.81	33.24	32.20	32.11	32.30
Linoleic acid (C18:2n-6)	49.90	50.55	49.83	47.72	47.96	47.29	47.26	48.17	48.08	46.33	44.99	44.93
Linolenic acid (C18:3n-3)	1.40	1.26	1.34	3.72	3.42	3.45	1.44	1.46	1.38	3.23	3.60	3.50
Arachidic acid (C20:0)	0.32	0.32	0.32	0.43	0.42	0.56	0.37	0.34	0.35	0.43	0.45	0.42
Eicosaenoic acid (C20:1n-9)	0.29	0.30	0.30	1.35	1.27	2.23	0.34	0.31	0.32	1.17	1.31	1.25
Eicosadienoic acid (C20:2n-6)	0.03	0.00	0.00	0.18	0.18	0.20	0.00	0.00	0.00	0.15	0.17	0.16
Behenic acid (C22:0)	0.00	0.00	0.00	0.10	0.09	0.09	0.00	0.00	0.00	0.08	0.09	0.00
Eicosatrienoic acid (C20:3n-6)	0.29	0.30	0.28	0.30	0.30	0.32	0.27	0.26	0.27	0.28	0.27	0.23
Erucic acid (C22: 1n9)	0.07	0.08	0.08	0.09	0.08	0.08	0.09	0.08	0.08	0.08	0.05	0.00
Eicosatrienoic acid (C20:3n-3)	0.00	0.00	0.00	0.26	0.24	0.26	0.10	0.10	0.10	0.22	0.22	0.20
Lignoceric acid (C24:0)	0.26	0.27	0.24	0.25	0.24	0.25	0.28	0.24	0.25	0.25	0.23	0.25
Other fatty acids	0.36	0.31	0.35	0.39	0.39	0.35	0.36	0.23	0.17	0.40	0.31	0.33
Chemical analysis, %												
Σ Unsaturated acids	51.63	52.12	51.41	52.19	52.11	51.52	49.08	49.99	49.83	50.21	49.25	49.02
Σ n-3	1.40	1.26	1.34	4.05	3.74	4.05	1.54	1.55	1.48	3.45	3.83	3.50
Σ n-6	50.22	50.85	50.07	48.14	48.38	47.47	47.54	48.44	48.35	46.76	45.42	45.52
Ratio n-6/n-3	35.80	40.32	37.34	11.87	12.95	11.72	30.86	31.18	32.57	13.56	11.86	13.02

Cam = Camelina meal.

p-anisidine reagent was added to the cuvette, placed in the dark for 10 min and a new spectra was recorded. The values for thiobarbituric acid reactive substances (**TBARS**) were measured according to a method described by Botsoglou *et al.* (1994) using third derivative spectrophotometry with some modifications. Thiobarbituric acid reactive substances were expressed as milligrams of malondialdehyde (**MDA**) per kg of muscle (mg MDA kg^{-1}). The minced meat sample (5 g) was mixed with 10 ml trichloroacetic acid (7.5%) and 5 ml butyrate hydroxytoluene in ethanol (0.8%). The sample solution was centrifuged at 3000×**g** for 3 min. Of the aliquot, 2.5 ml was mixed with 1.5 ml of 0.8% aqueous thiobarbituric acid solution in a test tube and further was incubated at 80°C for 50 min. Following incubation, the sample was cooled under running water and the absorbance was read at 532 nm (sp 0) and 540 nm (sp 3) using a spectrophotometer (Jasco V-530, Japan Servo Co. Ltd., Japan). Thiobarbituric acid reactive substance values were calculated against a standard curve obtained with 1,1,3,3-tetramethoxypropane (**TMP**).

The myoglobin (**Mb**) derivative concentrations were determined by a spectrophotometric procedure. The pigments were extracted according to the method of Viriyarattanasak *et al.* (2011) with some modifications. The frozen sample (2 g) was minced and placed into a 50-ml centrifuge tube. Then, 20 ml cold phosphate buffer (pH 6.8, 40 mM, 4°C) was added. The mixture was centrifuged at 7090×**g** for 30 min at 4°C using a laboratory refrigerated centrifuge (2-16KL, Sigma Laborzentrifugen GmbH, Germany). The supernatant was filtered and the spectra were recorded from 350 to 750 nm (V-530 Jasco, Japan Servo Co. Ltd., Japan) spectrophotometer using phosphate buffer as a blank.

Equations used in determining the relative concentrations of Mb derivatives (metmyoglobin (**MetMb**), deoximyoglobin (**DeoMb**) and oximyoglobin (**OxiMb**)) were proposed by Tang *et al.* (2004):

$$\%\ \text{metMb} = (-0.159R1 - 0.085R2 + 1.262R3 - 0.52)\ \text{x}\ 100$$

$$\%\ \text{deoMb} = (-0.543R1 + 1.594R2 + 0.552R3 - 1.329)\ \text{x}\ 100$$

$$\%\ \text{oxiMb} = (0.722R1 - 1.432R2 - 1.659R3 + 2.599)\ \text{x}\ 100$$

where: $R1 = A_{582}/A_{525}$; $R2 = A_{557}/A_{525}$; $R3 = A_{503}/A_{525}$

Statistics

The comparison for a randomized complete block design with a 2 × 3 factorial arrangement of treatments was performed using the GLM (Minitab version 13; Minitab Inc., State College, PA, USA) followed by Tukey's HSD test ($P < 0.05$).

Table 3 *Effect of supplemental CrPic (μg/kg) and Camelina meal on productive parameters of broilers*

	Cam 0%			Cam 3%				*P*		
	Cr 0	Cr 200	Cr 400	Cr 0	Cr 200	Cr 400	SEM	Cr	Cam	Cr x Cam
BW 14 days (g)	602.20	603.20	605.80	603.30	600.60	605.60	17.44	0.97	0.96	0.99
BW 42 days (g)	1862	1812	1903	1890	1805	1752	39.07	0.22	0.18	0.06
Feed intake (g/day)	123.00	124.30	124.60	119.3	120.90	119.50	13.53	0.99	0.71	0.99
Weight gain (g/day)	57.25	54.93	58.98	58.48	54.75	52.10	1.98	0.29	0.23	0.10
Feed: gain (g:g)	2.13	2.24	2.08	2.03	2.19	2.27	0.19	0.75	0.94	0.71

Cam = Camelina meal; CrPic = Chromium picolinate.

Table 4 *Effect of supplemental CrPic (μg/kg) and Camelina meal on proximate composition of broiler breast*

	Cam 0%			Cam 3%				P		
(%)	Cr 0	Cr 200	Cr 400	Cr 0	Cr 200	Cr 400	SEM	Cr	Cam	Cr x Cam
DM	23.67	23.98	23.92	24.09	23.44	23.80	0.27	0.81	0.72	0.24
CP	20.73^{c}	22.05^{a}	21.33^{b}	21.26^{bc}	21.10^{bc}	20.83^{bc}	0.11	0.02	0.21	0.45
Crude fat	1.38^{a}	1.19^{ab}	1.16^{ab}	1.32^{ab}	1.05^{b}	1.21^{ab}	0.08	0.02	0.45	0.46
Crude ash	1.18	1.21	1.19	1.10	1.25	1.26	0.06	0.32	0.90	0.48

Cam = Camelina meal; CrPic = Chromium picolinate.
Means within a row with no common superscript differ ($P < 0.05$).

Results

The effects of various levels of chromium picolinate and Camelina meal on the productive parameters of broilers

The effect of experimental treatments on broilers' productive parameters is shown in Table 3.

Supplemental CrPic or *Camelina* meal and experimental block were not significant contributors to productive parameters of broilers. During the experiment intervals at 14 and 42 days, the BW, feed intake and weight gain were similar ($P > 0.05$) among treatments.

The effects of various levels of chromium picolinate and Camelina meal on proximate composition of broiler breast

Table 4 shows the effects of dietary CrPic and Camelina meal on proximate composition. Chromium supplementation significantly ($P<0.05$) increased the CP concentrations and significantly ($P<0.05$) decreased the crude fat concentrations in breast samples. Crude ash was not affected by different levels of supplemental CrPic or Camelina meal ($P>0.05$).

The effects of various levels of chromium picolinate and Camelina meal on fatty acids composition of broiler breast

The fatty acids profile of broiler breast samples is shown in Table 5. Broilers fed only CrPic supplements presented higher values of myristic and palmitic acids (C14:0 respectively C16:0) but the results are not statistically sustained. Under *Camelina* meal influence, the breast samples had the lowest stearic and nervonic acids values (C18:0 and C24:1n-9 respectively). The *Camelina* meal groups presented higher values of unsaturated fatty acids. The highest concentrations of oleic acid (C18:1) was registered for *Camelina* meal group only, but the results are not statistically sustained. In breast samples of birds fed CrPic, the levels of monounsaturated fatty acids (**MUFA**) were similar to those fed *Camelina* meal. *Camelina* meal groups showed higher values of ALA (C18:3n-3) compared with other groups.

The results of the present study indicated that the n-3 fatty acids concentrations were significantly ($P<0.05$) increased by dietary *Camelina* meal in breast meat samples. Also, the ratio n-6/n-3 significantly decreased proved that *Camelina* meal supplements led to an improvement of breast broiler meat quality.

The effects of various levels of chromium picolinate and Camelina meal on mineral composition of broiler breast

Trace mineral concentrations (Cr, Cu, Zn, Fe, Mn) in broiler meat are presented in Table 6. Chromium concentrations were significantly higher ($P < 0.0001$) than the control group in CrPic-supplemented groups. In CrPic groups, increased retention of Zn and Fe ($P < 0.05$) was observed in breast samples, compared to control group. The retention of Cu and Mn shows no difference between groups in breast samples.

The effects of various levels of chromium picolinate and Camelina meal on oxidative stability of broiler breast

In Table 7, the oxidative stability parameters for the studied groups are presented. There were significant differences ($P<0.05$) in primary oxidation products between groups, except for CT concentrations. The secondary oxidative products were significantly ($P<0.05$) smaller for all experimental groups (CrPic- and *Camelina*-meal-supplemented groups), in terms of TBARS values.

Myoglobin fractions' concentrations differ significantly ($P<0.05$) from the control group for MetMb and OxiMb, under CrPic supplements' influence.

Table 5 *Effect of supplemental CrPic (μg/kg) and Camelina meal on fatty acids composition of broilers breast samples*

	Cam 0%			Cam 3%				P		
%	Cr 0	Cr 200	Cr 400	Cr 0	Cr 200	Cr 400	SEM	Cr	Cam	Cr x Cam
Capric acid (C10:0)	0.02^{ab}	0.03^{ab}	0.04^{a}	0.04^{a}	0.04^{ab}	0.01^{b}	0.006	0.39	0.81	0.00
Lauric acid (C12:0)	0.04	0.08	0.04	0.03	0.07	0.02	0.014	0.02	0.31	0.78
Myristic acid (C14:0)	0.55	0.55	0.57	0.49	0.54	0.54	0.020	0.24	0.12	0.40
Myristoleic acid (C14:1)	0.28	0.32	0.24	0.25	0.23	0.18	0.050	0.37	0.15	0.84
Pentadecanoic acid (C15:0)	0.35	0.44	0.52	0.47	0.53	0.35	0.050	0.28	0.78	0.01
Pentadecenoic acid (C15:1)	0.45	0.35	0.45	0.49	0.44	0.34	0.071	0.47	0.94	0.36
Palmitic acid (C16:0)	21.61	21.72	22.32	20.73	21.74	21.27	0.489	0.38	0.12	0.51
Palmitoleic acid (C16:1)	2.53	2.63	2.43	2.52	2.42	2.46	0.162	0.84	0.65	0.73
Heptadecanoic acid (C17:0)	0.19	0.17	0.19	0.17	0.17	0.15	0.011	0.77	0.03	0.23
Heptadecenoic acid (C17:1)	0.26	0.16	0.17	0.17	0.15	0.14	0.048	0.34	0.26	0.69
Stearic acid (C18:0)	7.41	7.44	7.84	7.23	7.03	7.10	0.197	0.49	0.01	0.36
Oleic acid (C18:1)	35.65	35.89	35.43	37.02	35.44	36.53	0.670	0.60	0.22	0.35
Linoleic acid (C18:2n-6)	24.87	24.64	24.48	24.41	25.68	25.38	0.971	0.86	0.54	0.70
Arachidic acid (C20:0)	0.17	0.17	0.14	0.13	0.25	0.13	0.044	0.22	0.78	0.38
Eicosanoic acid (C20: 1n9)	0.19^{a}	0.00^{b}	0.00^{b}	0.10^{ab}	0.06^{ab}	0.00^{b}	0.032	0.00	0.69	0.07
Linolenic acid (C18:3n-3)	0.39^{b}	0.49^{b}	0.40^{b}	0.77^{a}	0.77^{a}	0.75^{a}	0.038	0.32	0.00	0.47
Octatetraenoic acid (C18:4n3)	0.13^{ab}	0.08^{ab}	0.10^{ab}	0.09^{ab}	0.15^{a}	0.03^{b}	0.025	0.16	0.48	0.02
Eicosadienoic acid (C20:2n-6)	0.38^{a}	0.19^{bc}	0.19^{bc}	0.28^{ab}	0.17^{bc}	0.13^{c}	0.032	0.00	0.04	0.49
Eicosatrienoic acid (C20:3n-6)	0.32^{c}	0.33^{c}	0.35^{bc}	0.60^{a}	0.60^{a}	0.50^{ab}	0.023	0.51	0.00	0.20
Erucic acid (C22: 1n9)	0.35^{a}	0.25^{ab}	0.27^{ab}	0.26^{ab}	0.15^{b}	0.10^{b}	0.041	0.01	0.00	0.63
Eicosatrienoic acid (C20:3n-3)	0.22^{b}	0.35^{a}	0.43^{a}	0.40^{a}	0.33^{ab}	0.41^{a}	0.028	0.00	0.06	0.00
Arachidonic acid (C20:4n-6)	1.32	1.38	1.13	1.08	1.19	1.20	0.188	0.80	0.41	0.67
Tricosanoic acid (C23:0)	0.28	0.22	0.23	0.22	0.19	0.23	0.031	0.31	0.17	0.65
Docosadienoic acid (C22:2n6)	0.17	0.20	0.20	0.17	0.19	0.14	0.294	0.59	0.31	0.58
Eicosapentaenoic acid (C20:5n3)	0.34	0.35	0.37	0.34	0.31	0.32	0.026	0.80	0.14	0.61
Lignoceric acid (C24:0)	0.39	0.39	0.40	0.39	0.32	0.34	0.046	0.79	0.21	0.69
Nervonic acid (C24:1n9)	0.42	0.39	0.33	0.31	0.13	0.26	0.081	0.42	0.03	0.48
Docosahexaenoic acid (C22:6n3)	0.03	0.11	0.05	0.03	0.06	0.16	0.040	0.16	0.53	0.14
Other fatty acids	0.70	0.70	0.68	0.81	0.69	0.83	0.081	0.70	0.24	0.57
TOTAL										
Σ Saturated acids	31.00^{ab}	31.20^{ab}	32.30^{a}	29.90^{b}	30.86^{ab}	30.15^{ab}	0.055	0.36	0.01	0.27
Σ Unsaturated acids	68.30	68.10	67.01	69.29	68.45	69.03	0.561	0.38	0.02	0.34
Σ n-3	1.10^{b}	1.37^{ab}	1.36^{ab}	1.62^{a}	1.61^{a}	1.67^{a}	0.084	0.16	0.00	0.23
Σ n-6	27.06	26.75	26.35	26.55	27.83	27.34	1.084	0.88	0.56	0.71
Ratio n-6/n-3	24.61^{a}	19.93^{b}	19.58^{b}	16.42^{b}	17.37^{b}	16.51^{b}	0.876	0.02	0.00	0.00

Cam = Camelina meal; CrPic = Chromium picolinate.
Means within a row with no common superscript differ ($P < 0.05$).

Discussion

The effects of various levels of chromium picolinate and Camelina meal on the productive parameters of broilers

The lack of effects on the productive parameters is shown by the statistical analysis and revealed no significant differences and no interaction between CrPic and *Camelina* meal between groups. No impact of dietary *Camelina* meal on BW gain, feed conversion or feed intake of broiler was observed by Aziza *et al*. (2010).

Our findings regarding CrPic effect on productive parameters were similar to Anandhi *et al*. (2006) opinion expressed using 250, 500, 750 organic chromium for a period of 6 weeks. Hossain *et al*. (1998) found that supplementation with CrPic did not affect BW, feed consumption or feed:gain ratio of broilers. Other authors considered that CrPic used in broiler diets had beneficial effect on productive parameters but during the heat stress (Toghyani *et al*., 2012).

The effects of various levels of chromium picolinate and Camelina meal on proximate composition of broiler breast

Our results showed that CP increased and crude fat decreased ($P<0.05$) in breast meat samples under dietary CrPic influence. No interaction was observed between chromium and *Camelina* meal. Similar to our findings, Kim *et al*. (1996) observed lower muscle fat content in broilers fed with 200 and 400 ppb Cr3+ compared to control. They concluded that CrPic supplements have an inhibitory effect on fat synthesis in broiler tissues. Samanta *et al*. (2008) showed that broiler diet supplementation with 500 ppb Cr^{3+} reduced ether extract and improved muscle protein, compared to

Table 6 *Effect of supplemental CrPic (μg/kg) and Camelina meal on mineral composition of broiler meat*

	Cam 0%			Cam 3%				*P*		
	Cr 0	Cr 200	Cr 400	Cr 0	Cr 200	Cr 400	SEM	Cr	Cam	Cr x Cam
Cr (μg kg^{-1})	24.76^{b}	53.61^{a}	53.43^{a}	21.71^{b}	57.88^{a}	53.89^{a}	4.25	0.00	0.87	0.71
Zn (mg kg^{-1})	21.25^{b}	25.43^{a}	23.29^{ab}	22.00^{b}	24.30^{a}	23.65^{ab}	2.62	0.00	0.99	0.60
Cu (mg kg^{-1})	0.32	0.36	0.38	0.40	0.36	0.33	0.08	0.97	0.77	0.15
Fe (mg kg^{-1})	26.12^{b}	32.57^{a}	31.38^{ab}	30.33^{ab}	32.42^{ab}	33.29^{a}	4.13	0.01	0.11	0.35
Mn (mg kg^{-1})	3.00	3.38	3.53	3.39	3.59	3.53	0.39	0.06	0.11	0.43

Cam = Camelina meal; CrPic = Chromium picolinate.
Means within a row with no common superscript differ ($P < 0.05$).

Table 7 *Effect of supplemental CrPic (μg/kg) and Camelina meal on oxidative stability of broilers breast samples*

	Cam 0%			Cam 3%			SEM	*P*		
	Cr 0	Cr 200	Cr 400	Cr 0	Cr 200	Cr 400		Cr	Cam	Cr x Cam
PV (Meq O2/kg)	0.106^{a}	0.064^{b}	0.076^{ab}	0.078^{ab}	0.063^{b}	0.059^{b}	0.008	0.00	0.03	0.23
CD (μmols/g)	2.29^{a}	1.36^{b}	1.98^{ab}	1.81^{ab}	1.39^{b}	1.72^{ab}	0.24	0.02	0.24	0.56
CT (μmols/g)	3.11	2.52	2.97	2.85	2.28	2.46	0.72	0.73	0.58	0.97
P-anisidine	31.00	29.05	32.74	27.82	25.42	28.08	3.59	0.69	0.22	0.98
TBARS (mg/kg)	0.089^{a}	0.050^{c}	0.052^{c}	0.074^{b}	0.057^{c}	0.061^{c}	0.003	0.00	0.89	0.00
Met Mb (%)	29.31^{a}	27.08^{ab}	27.21^{ab}	28.70^{ab}	28.81^{ab}	26.73^{b}	0.372	0.00	0.63	0.06
Deo Mb (%)	47.18	46.75	46.61	47.17	46.82	47.46	0.280	0.61	0.35	0.49
Oxi Mb (%)	8.57^{b}	10.25^{a}	10.24^{a}	8.89^{b}	8.90^{ab}	9.90^{ab}	0.332	0.02	0.23	0.22

Cam = Camelina meal; CrPic = Chromium picolinate; PV = Peroxide value; CD = Conjugated dienes; CT = Conjugated trienes; P anisidine = Para anisidine; TBARS = Thiobarbituric acid reactive substances; Met Mb = Metmyoglobin; Deo Mb = Deoximyoglobin; Oxi Mb = Oximyoglobin.
Means within a row with no common superscript differ ($P < 0.05$).

control. Other researchers did not find significant influence of dietary Cr^{3+} on moisture, CP and crude fat content of broiler meat (Anandhi *et al*., 2006). Sakhari *et al*. (1992) explain that chromium facilitates insulin secretion which enhances protein synthesis and contributes to the transport of amino acids to the site of synthesis. Another theory is that chromodulin (low molecular weight chromium-binding protein) is linked to the action of the insulin receptor, glucose entry is increased into adipocytes, lipogenesis is increased and the net fatty acid release decreases (McNamara and Valdez, 2005).

The effects of various levels of chromium picolinate and Camelina meal on fatty acids composition of broiler breast

The aim of Camelina feeding intake experiments is mainly to enrich the meat in n-3 fatty acids (Cherian, 2012).

Alpha-linolenic acid is the only source of n-3 PUFA, eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**) did not exist in the diets. Linoleic acid (**LNA**) and ALA are the precursors for arachidonic acid (**AA**) and EPA and they are produced by desaturation and elongation, delta 6 desaturase being the rate limiting step in their synthesis (Aziza *et al*., 2010). Between ALA and LNA exists a competitive interaction in which n-3 PUFA suppress the metabolism of n-6 PUFA strongly than reverse, leading to a decreased n-6/n-3 ratio (Cherian *et al*., 2009).

The results of our study showed a strong competition between ALA and LNA, due to an increased level of ALA from *Camelina* meal diets that did not had an inhibitory effect of LNA conversion to AA. Also, even if ALA concentrations significantly increased in breast tissues from Camelina-meal-supplemented groups, no significant differences were noticed for EPA and DHA between groups, the elongation and desaturation processes having a decreased reaction yield. A possible explanation might be that ALA is converted to EPA and DHA slowly, because it is not easy to incorporate ALA to lipid tissues, while LNA is rapidly converted to AA (Coetzee and Hoffman, 2002). Total long chain n-3 PUFA significantly increased ($P<0.05$) in experimental groups, compared to control, except the group supplemented with 3% *Camelina* meal and 200 ppb CrPic, leading to a significant decrease of n-6:n-3 ratio ($P<0.05$).

Similar results were published by Aziza *et al*., (2010) which concluded that *Camelina* meal inclusion rate of 2.5% to 10% in broiler diets didn't produce any significant differences in EPA and DHA concentrations in breast meat. Also, they showed that thigh meat was more efficient in incorporating long chain n-3 fatty acids than breast meat.

The effects of various levels of chromium picolinate and Camelina meal on mineral composition of broiler breast

Significant increased concentrations of Cr, Zn and Fe compared to the control group were noticed under CrPic supplements' influence. Wang and Xu (2004) reported that Cr deposition in tissues increased with the inclusion level of

Cr^{3+} in diet. Sahin and Sahin (2002) showed that CrPic improved the utilization of Zn and Fe and Mn in laying hens reared in low temperature (7°C). Sirirat *et al.* (2013) reported that supplemental CrPic and ascorbic acid improved Zn retention in layer chickens. Supplemental CrPic led to increased Cr and Zn levels in serum, liver, kidney and longissimus muscle of laying hens (Sahin *et al.*, 2002).

Feng *et al.* (2003) reported that iron-binding proteins are involved in chromium binding, transport and storage. Chromium and iron share the same transport mechanism, binding with transferin and they are competitors for the same binding sites. Some authors (Pechova and Pavlata, 2007) showed that Cr may impair Fe metabolism and Cu has a synergetic relation to Fe and can be considered an indirect chromium antagonist.

Sahin and Kucuk (2003) reported that Zn-supplemented birds have reduced lipid peroxidation products. They proposed the zinc's action mechanism to be its capacity to displace transition metals (Fe, Cu) from binding sites. Iron is a redox active metal that can catalyze the propagation step of lipid oxidation. Zago and Oteiza (2001) observed an inverse correlation between the capacity of zinc to prevent iron binding to the membrane and the inhibitory effect of zinc on Fe^{2+}-initiated lipid oxidation.

The effects of various levels of chromium picolinate and Camelina meal on oxidative stability of broiler breast

The oxidative stability of meat is usually established by using three primary oxidation products (PV, CD and CT) and two secondary oxidation products (TBARS, p-anisidine) (Shahidi and Zhong, 2005). The results of oxidative stability of breast meat showed that oxidation occurred slowly depending on the presence and level of CrPic in diet, indicating a dose-dependent impact of chromium on meat oxidative stability (Table 7). From the primary oxidation products, CD concentrations decreased under CrPic influence, and also, the TBARS values (secondary oxidation products) were significantly smaller for CrPic groups. Toghyani *et al.* (2012) showed that the presence of trivalent Cr leads to the activation of insulin receptor kinase and potentiation the actions of insulin. Insulin metabolism influences lipid peroxidation and Cr^{3+}, as an insulin potentiator, acts as an indirect antioxidant.

Myoglobin is considered to be the major catalyst for lipid oxidation in meat. Myoglobin is a monomeric globular heme protein composed of an atom of iron surrounded by an array of pyrole rings. The iron enables Mb to gain (reduction) or lose (oxidation) electrons (Min and Ahn, 2005). There are three types of Mb which give the colour of meat: DeoMb and OxiMb the iron(II) state of Mb and a continuous oxidation of MetMb takes place (iron(III) state) despite to enzymatic reduction of met forms to deoxy forms. *Postmortem* processes inactivate the reductive enzyme systems and stimulate autooxidation of Fe^{2+} to Fe^{3+} (Baron and Andersen, 2002). Lynch and Faustman (2000) showed that the presence of secondary oxidation products increases OxiMb oxidation and decreases enzymatic reduction of metMb, resulting in quality deterioration of meat. In this study, lipid peroxidation was significantly reduced by CrPic supplements. In agreement with observation, a positive correlation between Mb fractions and chromium concentrations was observed (R2 between 0.79 and 0.83). By decreasing the level of MetMb and preventing the oxidation of iron, chromium may act as an indirect antioxidant.

Sahin *et al.* (2010) proposed a mechanism for antioxidant properties of chromium, the potentiation of insulin action, and by this way, reducing lipid peroxidation. Another possible antioxidant mechanism of chromium is by stimulating zinc absorption and its deposition in tissues. Zinc does not directly attack free radicals but is important in prevention of their formation and it can retard oxidative processes (Vertuani *et al.*, 2004). The antioxidant mechanism of zinc (Powell, 2000) may be related to the antagonistic effect of zinc to catalytic properties of redox active transition metals (Fe, Cu). Other researchers showed that zinc can protect membranes from iron-initiated lipid oxidation (Zago and Oteiza, 2001). Our findings showed a retard oxidation of MbFe2+ state (OxiMb) to Mb Fe3+ state (MetMb) in groups with high Zn concentrations in breast (chromium supplements groups).

This study found that broilers fed with CrPic supplements showed improved mineral composition and oxidative stability of breast meat, proving an effective protection of lipid molecules from oxidation. Under *Camelina* meal supplements' influence, the fatty acids profile of breast meat was improved by increasing concentrations of PUFA. In conclusion, future research is needed to establish if CrPic–*Camelina* broiler meat can contribute to a human diet improved in nutritive value.

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Ethics statement

The experiment complied with Directive 2010/63/EU on the protection of animals used for scientific purposes and the experimental procedures were approved by Ethical Commission of National Research and Development Institute for Biology and Animal Nutrition.

Declaration of interest

None of the authors has any potential conflict of interest related to this manuscript.

Software and data repository resources

None of the data were deposited in an official repository.

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animal

Editorial

Evolving technological change in pork production supporting expectations of improved productivity, sustainability and flexibility

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The Australasian Pig Science Association (**APSA**) has a long and storied history of helping to lead thoughtful discussion on important topics affecting pork production. Established in 1987, it has hosted the *Manipulating Pig Production* conference every 2 years, with the 2019 meeting the 17th such event. This conference is viewed globally as an innovative and progressive event. In this regard, the global pork industry is working hard to respond to the many forces affecting its future: management and prevention of diseases, including the debilitating viral disease African swine fever (Sanchez-Cordon *et al.*, 2018), improving productivity to fulfil expectations of demand for product, attention to pork's environmental footprint and satisfying an increasingly demanding and diverse consumer marketplace (Busch and Spiller, 2019). A special issue of *Animal*, including many topics seeking to address the aforementioned issues, is therefore appropriate and timely. The content of this special issue, reflected in its diversity, reveals some of the approaches that are being brought to bear to address these challenges, from precision agriculture to alternative protein sources to improved control of reproduction.

The industry's interest in new technology is perfectly revealed in the report by Boyd *et al.* (2019). They identify innovations which have altered the very trajectory of the industry in North America, and probably globally; this includes the adoption of the tools of molecular genetics to accelerate genetic improvement, molecular-based tools to improve disease diagnosis and control and the tools of near-infrared spectroscopy (NIRS) to improve the precision of feed production (Bjustrom-Kraft *et al.*, 2018; Faba *et al.*, 2019). Some of these advances have truly been game-changers to pork production, but some newer developments may prove to be of equal or greater importance in the future.

One of these developments is precision production ('precision agriculture'), something which agronomists have embraced effectively and now consider it a routine part of their decision-making process. It is now becoming an increasingly important part of animal agriculture (Liebe and White, 2019). Norton (2019) explains that a critical step to improving the precision of pork production is the development and implementation of sound and image analysis to monitor individual pigs. With this ability in place, it will be possible to integrate more robust information which in turn will lead to improved decisions on feeding, housing and management. There are other means of achieving precision in the overall pork production system; NIRS is one of those tools and, as presented by Piotrowski *et al.* (2019), can be used to achieve accuracy in a very specific but nonetheless important aspect of pork production, namely pork carcass and meat quality.

Feed remains the largest single expense in pork production; hence addressing means to optimise its use is always of interest and importance. Tokach *et al.* (2019) provide an extensive review of the recent literature addressing optimised feeding of the hyper-prolific sow. As the authors emphasise, there are two main phases to consider: the periparturient period followed by actual lactation. The former is focused on success in farrowing, expressed by improved birth weights, reduced stillbirths and improved colostrum quantity and quality. The emphasis on feeding the sow during lactation is maximising milk production combined with maintaining body condition in the sow, especially in order to achieve success in re-breeding for the subsequent parity (Theil, 2015).

In interrelated research, Bagnell and Bartol (2019) discuss how maternal programming impacts not just pre-weaning performance but also the adult phenotype. They further present evidence for the lactocrine hypothesis, a mechanism mediated by milk-borne bioactive factors which serve as a vehicle of communication between the dam and her offspring shortly after farrowing. In the end, Tokach *et al.* (2019) and Bagnell and Bartol (2019) both agree on the importance of the periparturient period. Gaining knowledge and improving feeding management around the time of, and shortly after, farrowing could be the key to considerable improvement in

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sow reproductive performance and even post-weaning phenotype (Tokach and Dial, 1992).

Optimising feed costs implies an understanding of ingredient availability, use and cost. A growing area of interest worldwide is the use of insect meal in pig diets, which is reviewed by DiGiacomo *et al.* (2019). The amino acid profile of black solider fly larvae is surprisingly similar to that of soybean meal, laying the foundation for potential successful use. Initial feeding trials with pigs have proven encouraging, but questions remain regarding palatability, nutrient supply and meat quality. One of the characteristics of insect meal is its high fat content, which is a positive with respect to energy intake but may adversely impact ultimate meat quality and feed intake.

While insect meal is being considered for use in pig diets, alternative proteins are also being investigated for human consumption. Warner (2019) provides an interesting overview of cell-based meat and the challenges faced by developers seeking to find a spot in the consumer marketplace. As with many new technologies, cost is a considerable barrier to adoption, but progress is being rapidly made. The greatest competition to cell-based meat may not be the real thing, but rather rapid developments in plant-based products which have also experienced considerable progress in taste and texture. Regulatory and labelling requirements may also be a barrier to finding a place on restaurant menus or grocery shelves.

Water is a conundrum in pig nutrition. It is critical to life and must be provided in sufficient quantity and quality to the pig, but clarity on what this means is elusive (Patience, 2013). Little *et al.* (2019) tackle a related topic, namely the use of water as a vehicle by which medications can be provided to pigs at therapeutic levels. This is a topic of growing importance since there is a widespread desire to reduce the delivery of medication via the feed, and water seems to be a logical alternative. However, the authors express caution due to the very real challenges of delivering the right dose of medication to the full population of pigs. Their data suggest that over- or under-dosing of medications is a real concern that must be addressed.

Control of reproduction remains a topic of active consideration. The issue is not so much one of litter size, but rather control of the initiation and maintenance of reproductive cycles. Lents (2019) reviewed the literature on kisspeptin, a neuropeptide involved in the hypothalamic-pituitary axis and therefore involved in the regulation of gonadotropins and reproduction. Research in swine, as opposed to some other species, has lagged, and the author presents his argument for more attention to this important topic in the future.

Finally, Hutchinson and Terry (2019) take us further afield into territory unfamiliar to many, but perhaps regrettably so. Progress in understanding the connection between the brain and the cells of the immune system is revealing great opportunities in human medicine, especially psychiatry. Similar progress in swine is further down the road, reasons for which are enunciated by the authors.

In summary, the reader can see that APSA has once again developed an outstanding scientific programme for its biennial *Manipulating Pig Production* conference that covers a diverse array of topics highly relevant to the modern pork industry. Meetings of this nature, and associated special issues of the journal, will help to encourage further developments in new technologies, improvements in existing technologies and expanded collaborations in the future.

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Declaration of interest

John Pluske is a member of the APSA Committee.

Ethics statement

Not applicable.

Software and data repository resources

Not applicable.

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animal

Review: innovation through research in the North American pork industry

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*This article involved a broad search of applied sciences for milestone technologies we deem to be the most significant innovations applied by the North American pork industry, during the past 10 to 12 years. Several innovations shifted the trajectory of improvement or resolved significant production limitations. Each is being integrated into practice, with the exception being gene editing technology, which is undergoing the federal approval process. Advances in molecular genomics have been applied to gene editing for control of porcine reproductive and respiratory syndrome and to identify piglet genome contributions from each parent. Post-cervical artificial insemination technology is not novel, but this technology is now used extensively to accelerate the rate of genetic progress. A milestone was achieved with the discovery that dietary essential fatty acids, during lactation, were limiting reproduction. Their provision resulted in a dose-related response for pregnancy, pregnancy maintenance and litter size, especially in maturing sows and ultimately resolved seasonal infertility. The benefit of segregated early weaning (12 to 14 days of age) was realized for specific pathogen removal for genetic nucleus and multiplication. Application was premature for commercial practice, as piglet mortality and morbidity increased. Early weaning impairs intestinal barrier and mucosal innate immune development, which coincides with diminished resilience to pathogens and viability later in life. Two important milestones were achieved to improve precision nutrition for growing pigs. The first involved the updated publication of the National Research Council nutrient requirements for pigs, a collaboration between scientists from America and Canada. Precision nutrition advanced further when ingredient description, for metabolically available amino acids and net energy (by source plant), became a private sector nutrition product. The past decade also led to fortuitous discoveries of health-improving components in ingredients (xylanase, soybeans). Finally, two technologies converged to facilitate timely detection of multiple pathogens in a population: oral fluids sampling and polymerase chain reaction (**PCR**) for pathogen analysis. Most critical diseases in North America are now routinely monitored by oral fluid sampling and prepared for analysis using PCR methods.*

Keywords: genomics, seasonal infertility, amino acids, gut barrier function, functional ingredients

Implications

North American pork production has seen remarkable innovation in the past decade. Genetic improvement rate abruptly achieved a new trajectory, for pigs born late in 2013, because of genomic-enhanced selection. Insufficient intake of essential fatty acids by the mature sow limits litter size and is the primary cause of seasonal infertility. The list of health-promoting ingredients expanded to include xylanase, which improves viability. When oral fluid monitoring for population pathogens is combined with polymerase chain reaction technology, detection is rapid, affordable and the time frame to herd isolation is relatively short (days).

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Introduction

This article identifies the most significant research innovations that, in our opinion, have been applied by the North American pork industry in the past decade. They are primarily the result of leading edge research in North America. Research discoveries were not considered unless they were being integrated into practice, with the exception of gene editing for pathogen control, which is in the approval process.

Innovations that we identified as 'milestone' are diverse. Genome-enhanced breeding values resulted in an abrupt increase in the rate of genetic progress (e.g. piglet genome related to parent genes). Identification of a major gene for porcine reproductive and respiratory syndrome virus (**PRRSv**) and gene editing to ameliorate susceptibility to the virus is the most significant advance in PRRSv control in 25 years. Post-cervical artificial insemination (**PCAI**) is not new, but it has become a means to utilize elite sires to an even greater extent, in accelerating improvement (Safranski, 2008). Structure of the North American genetic industry facilitates the rapid dissemination of genetic improvement.

A remarkable advance in nutrition science resolved the long-standing problem of seasonal infertility for pigs. A direct relationship was observed between lactation fatty acid intake and reproduction (Rosero *et al.*, 2016b), a finding that has been commercially validated in two southern regions of America. We also describe remarkable advances in our understanding of extended maternal influence on the development of neonatal innate immune capability and gut-barrier function, because this formed the scientific basis for a course correction from early weaning (12 to 16 days), for terminal pig production. This is a classic illustration where science was successfully applied to eliminate specific disease pathogens at the genetic nucleus and multiplication levels, but when it was applied to the commercial sector unexpected compromises emerged in piglet viability and growth.

Two important milestones were achieved to improve precision nutrition for growing pigs. The primary advance was the publication of the National Research Council (**NRC**) nutrient requirements for pigs (National Research Council, 2012), a collaboration between scientists from America and Canada. Precision nutrition advanced even further when ingredient description, for metabolically available amino acids and net energy (by source plant), became a private sector nutrition product. The past decade led to fortuitous discoveries of health-improving ingredients for growing pigs to market (xylanase, soybeans). Finally, a remarkable milestone was achieved with the convergence of technologies to detect the presence and to identify disease pathogens, easily and cost-effectively. These involved oral fluid sampling of populations and PCR technology, a means to rapidly identify animal disease to expedite herd isolation, especially African swine fever (**ASF**).

High-impact innovations likely to emerge in the North American pork industry during the next decade were also identified, and these are presented as a refereed supplement to this article (see Supplementary Material S1).

Application of genome science

Genomic advances during the past two decades have been so significant that this field may be justly considered the science of the 21st century. Two important innovations were developed during the past decade that will profoundly impact North American pork production: (a) increasing genomic information to enhance selection accuracy and (b) gene editing for pathogen control. The former allows for more accurately defining parental genetic influence for each piglet, and this has abruptly advanced the rate of genetic improvement. The technology resulted in the first significant breakthrough in the control of PRRSv (Whitworth *et al.*, 2016). This means to pathogen control resulted in the production of pigs that were shown to be completely resistant to PRRSv infection.

Genomic-enhanced breeding values

The first widely utilized genomic test was applied in the 1990s to swine with the development of a genetic test to identify a HAL-1843 mutation of the ryanodine receptor gene (Fujii *et al.*, 1991). This test identified animals that were likely to have an abnormal response to stress (two copies of the mutant allele) and revealed the potential of increased precision in livestock improvement that was possible with emerging technologies. However, this initial genomic tool depended primarily on finding closely linked associations between specific genetic marker and the phenotypic trait of interest (severe stress). The science of genomics continued to advance at a rapid pace through the late 1990s and early 2000s, while the underlying tools used for genomic testing began to dramatically evolve and sophisticate. A remarkable outcome of this evolution was development and commercialization of a new genotyping chip that allowed for rapid description of tens of thousands of genotypes on an individual test candidate (Ramos *et al.*, 2009). This platform was the first application that allowed for a sufficiently expansive description of an animal's genetic makeup using a robust chemistry platform (e.g. few genotyping errors, high call rates, automated platform). When this was combined with dramatically improved computational power, a new generation of technical opportunities emerged for genetic improvement.

Expanding on the development of various statistical approaches to utilize this vast increase in information, Misztal *et al.* (2009) proposed an algorithm that utilized this expanding genomic information to more accurately describe the genomic segments that animals have in common (e.g. how piglet genome relates to each parent). This approach utilized best linear unbiased procedure (**BLUP**) models, but pedigree relationships were genomic-enhanced. From a practical perspective, this estimates the gene segments that any two animals have in common based on the genotype of each. This method is commonly referred to as single-step genomic evaluation or, in practice, relationship-based genomic selection (**RBGS**). Bundling of these technologies, the cumulative additive effects of enhanced computing

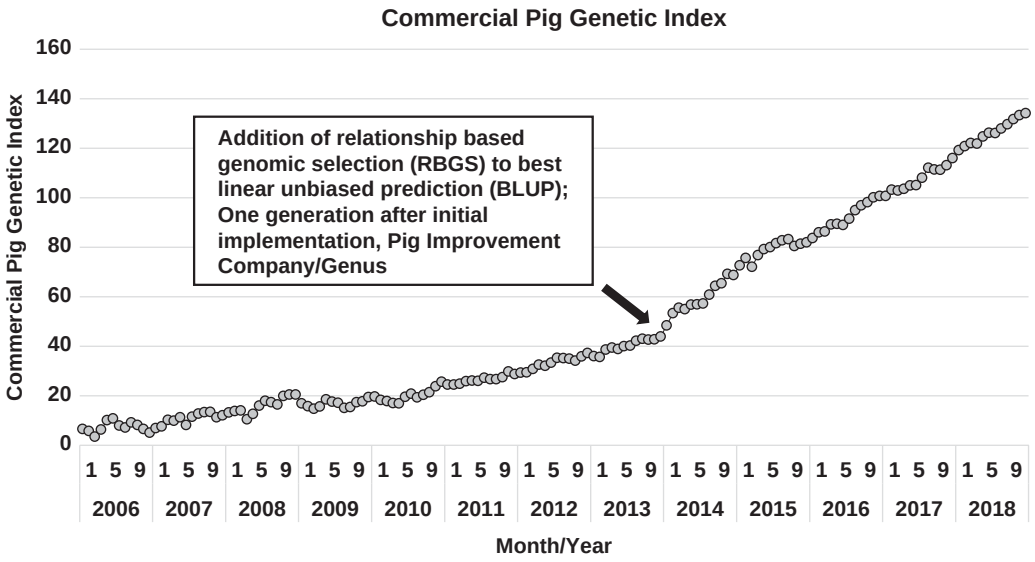

Figure 1 Genetic trend for index value of Pig Improvement Company (**PIC**) terminal sire × PIC F1 Camborough commercial pigs. Index value represents the overall genetic merit of an individual and is calculated as the weighted combination of economically important traits using its record and relatives. The steep increase in the genetic trend at the end of 2013 demonstrates the added value of using relationship based genomic selection (**RBGS**), rather than pedigree-only based relationships to calculate index values.

power, expanded data capture (greater number of pigs captured in more diverse environments) and genomic technology, has abruptly increased the rate of improvement. This innovation bundle was implemented in the North American pork industry in early 2012 and simultaneously improved every trait and animal within the selection program. Delivering marginal improvement across the entire selection landscape enhanced annual progress by over 35% and over 50% for some traits that were either lowly heritable (e.g. pre- and post-wean viability) or sex-linked (e.g. total pigs born). The net result of this is shown in Figure 1, where a dramatic shift in commercial pig genetic index emerged one generation after the implementation of RBGS. In more accurately predicting the genetic index for each animal, the value declined for some individuals and increased for others. The net effect was to increase the range in selection index values.

State-of-the-art genome sequencing technologies have advanced to provide the next level in high-volume access to genomic data with remarkable changes in cost and efficiency. The first human genome draft was published in 2001, at a cost of approximately US$100 million. As with other technologies, evolving adjustments in the technical platform rapidly changed efficiency and cost, so that the cost to sequence the same genome is estimated to be approximately US$1,100 (Wetterstrand, 2019).

Gene editing for pathogen control

The second innovation that emerged in North America is the development and exploration of gene editing as a tool to deliver precise genetic improvement. Gene editing is a method in which DNA is inserted, deleted, modified or replaced in the genome of a living organism. Unlike random insertions of early genetic engineering techniques, current genome editing technologies allow targeting to a specific location and to effect a precise change. Although multiple gene editors exist, identification and development of the clustered regularly interspaced short palindromic repeats (**CRISPR**)-Cas system appears to have enabled a quantum leap in efficiency and precision (Cong *et al.*, 2013). The most significant swine application to date originated in North America with the announcement of pigs, developed at the University of Missouri via gene editing, that were resistant to the catastrophic disease PRRSv (Whitworth *et al.*, 2016). In December 2015, the researchers announced the development of non-transgenic, gene-edited pigs that were resistant to PRRSv, which resulted from a small and precise edit to the *CD163* gene. The pigs, when subsequently challenged with the virus in a controlled challenge study, were no longer able to become infected, seroconvert and circulate the virus. They showed no viremia, antibody response or signs of clinical disease. These results have been replicated at multiple universities, across the globe, which reinforced the technical validity of the results (Burkard *et al.*, 2017; Yang *et al.*, 2018). There is beginning to be a steady stream of results generated in the area of precision gene-edited solutions to enhance the health, well-being and efficiency of the animal (Crispo *et al.*, 2015; Carlson *et al.*, 2016). An alternative to editing the major gene for PRRSv resistance is gene marker-based selection. Although this 'natural' approach is expected to be less effective, it does not require regulatory oversight.

Natural selection for porcine reproductive and respiratory syndrome virus resistance

Genomic techniques to control PRRSv were applied in 2018 for gene editing and natural selection in North America.

Gene edits to remove susceptibility to the PRRSv is the preferred method for disease resistance, but two barriers must be overcome: (a) US Food and Drug Administration clearance is required and demanding and (b) public acceptance is a concern. Genomic selection to identify pigs that have increased resistance is an alternative tactic because there is genetic variation in response to every disease in livestock, including PRRSv (Bishop, 2010). The difference between gene editing and natural selection for PRRSv is that gene edits are expected to result in complete resistance; natural selection for PRRSv would not result in complete resistance (Dunkelberger *et al*., 2017). The latter means that some pigs would still get sick, but they would have increased partial resistance to PRRSv: presumably carrying reduced viral load.

Since vaccinology and medical treatments have proven unsatisfactory in preventing PRRSv infection, a PRRSv host genetic consortium was formed to identify genetic associations to resistance (Lunney *et al*., 2011). They found a major gene, located on chromosome 4, that was associated with resistance to PRRSv. They also observed that, within this major gene, there is a specific genetic marker that could be used for selection. Incorporation of this Wageningen University Resistance gene (WUR) single nucleotide polymorphism genotype into an index scheme is believed to be a means of selecting for PRRSv resistance. If this proves to be true, this is a milestone for pig health because tools such as gilt acclimatization, vaccination and medication have proven ineffective in controlling PRRSv. Sow unit air filtration has evolved to become effective, but this method of reducing PRRSv infection is expensive and is not 100% effective.

Reproductive technology–facilitated genetic improvement

An important milestone was achieved, during the past decade, to significantly increase the use of genetically elite sires. Post-cervical artificial insemination is used extensively in North America for that purpose. This technology is not novel, nor was it primarily advanced by North American scientists; however, recently it has been widely adopted to further accelerate the rate of genetic progress. Post-cervical artificial insemination technology was the outcome of strategic considerations to increase elite sire use. The first is to reduce the number of inseminations required to establish pregnancy. The second is to reduce the number of sperm cells used for each insemination, so that a boar's ejaculate can be used to inseminate more females. The first objective is deliverable using a technology called OvuGel®. This technology is a method of delivering an ovulation synchronizing molecule (vaginal not intramuscular injection). It is commercially available in North America and delivers on single-fixed timed artificial insemination (**SFTAI**) to achieve pregnancy from one insemination. This technology was developed as a novel triptorelin (GnRH agonist) gel formulation, delivered intravaginally to weaned sows to induce ovulation (reviewed by Knox *et al*., 2018).

Post-cervical artificial insemination

In order to reduce the number of sperm cells per insemination dose, sperm needs to be deposited closer to the site of fertilization in the female's reproductive tract. Intrauterine artificial insemination, better known as PCAI, is a process by which semen is deposited beyond the cervix and into the uterine body. Conventional artificial insemination (**AI**) deposits semen in the cervix and requires 3 to 5 billion sperm cells per insemination dose to achieve acceptable fertility. The reason for the large quantity of sperm cells is because the cervix filters sperm, thereby reducing the number of cells that make it to the site of fertilization. Theoretically, PCAI, coupled with a reduction in total sperm dose, is expected to increase the number of piglets born from each boar by 16 000 per year when compared to conventional AI. The advantage of PCAI is estimated to be 23 000 compared to pen mating (Safranski, 2008). This technology is very important to genetic nucleus farms, but a 2007 survey of US sow farms reported that 6% were using PCAI (Knox *et al*., 2013). This increased to more than 40% in 2017 (Stewart K.R., unpublished research). Growth of PCAI in Canada appears to be similar or slightly less, based on PCAI catheter sales.

The adoption of PCAI was initially driven by the technology's ability to facilitate an increase in the number of offspring produced from superior sires. However, as farms began to adopt PCAI, other economic benefits were identified. The process of performing conventional AI is labor intensive; multiple technicians are required, and each insemination takes 3 to 10 min. Post-cervical artificial insemination reduced the amount of time to inseminate each sow to about 1 min or less which reduced the amount of labor required. Large systems utilized this benefit to reduce the number of breeding technicians and to redirect that resource to the farrowing room. Although PCAI insemination has been increasing, it has not been equally accompanied by reductions in sperm content in the insemination dose. Of the 40% of farms that identified as utilizing PCAI in 2017, only 16% reported using low-dose semen technology (Stewart K.R., unpublished research). Adoption of a new technology requires that it perform equal to or better than the current method and PCAI needed to be proven in practice. Research attempting to determine a recommended sperm number for insemination produced equivocal results (Watson and Behan, 2002; Rozeboom *et a*l., 2004; Mezalira *et al*., 2005; Hernandez-Caravaca *et al*., 2012). It appears that PCAI dose concentrations of approximately 1 billion and below tend to decrease fertility.

Deep uterine insemination

In order to further reduce sperm dose to less than 1 billion, deep uterine insemination (**DUI**) may be required; semen is deposited at the anterior end of the uterine horn and closer to the fertilization site in the oviduct. Prior to this, we need to understand the possible economic benefit of further leveraging superior sires. Fast Genetics (Deforest, WI, USA) estimated that DUI, performed with 500 million cells per dose, could produce 21 000 additional pigs per boar per year compared to PCAI with 1 billion cells per dose

(Willenburg and BeVier, 2017). A recent commercial study showed that when SFTAI was combined with DUI, to deliver sperm concentrations ranging from 600 to 75 million (600, 300, 150, 75), pregnancy rates were similar but litter size decreased as sperm number declined below 600 million (Knox *et al*., 2019).

Reproductive nutrition – essential fatty acids and seasonal infertility

A milestone was achieved with the discovery that dietary essential fatty acids (**EFA**), during lactation, are important to subsequent reproduction, exhibiting a dose-related response for achieving and maintaining pregnancy, as well as litter size. The dietary requirement is progressively more important with advancing reproductive cycles. This may be due to a steady depletion of EFA with each reproductive cycle (Rosero *et al*., 2016a and 2016b). A frank deficiency of parent EFA (linoleic acid, α-linolenic) is also more apt to occur during heat stress, when feed intake is reduced, and by feeding diets with no EFA sources added. This nutritional finding has proven successful against the problem of seasonal infertility in pigs in North America. Consequently, this technology is gaining adoption in North America.

Seasonal infertility

Concurrent with the progressive increase in pig output and potential for nutrient deficit is the matter of seasonal infertility. This phenomenon is expressed in various ways, but chief among them is failure to maintain pregnancy. Upon weaning, sows may exhibit slower return to estrus and conceive, but pregnancy is interrupted (Peltoniemi and Virolainen, 2006). In practice, litter size also tends to decline. In North America, seasonal infertility is associated with mating in mid to late summer. While it has been associated with environmental factors, such as photoperiod, compromised reproduction also coincides with extreme heat stress (Ross *et al*., 2017). An initial response to heat stress is the reduction in feed intake. This intake suppression is believed to be a means to reduced fertility. We hypothesized that body EFA reserves become depleted by the reduction in intake combined with marginally deficient lactation diets over successive reproduction cycles, with this being exacerbated by gestation diets that do not recover the prior lactation EFA deficit.

Essential fatty acid intake in lactation and reproduction

The role of EFA (linoleic acid, C18:2n-6; α-linolenic acid, C18:3n-3) in reproduction includes alteration of ovarian follicle and embryonic development, of hormone precursors important to reproduction, and pregnancy recognition and maintenance via cell signals (Thatcher *et al*., 2010). We determined with lactating sows that were nursing 12 pigs during summer heat stress that a practical lactation diet with no added lipid has a profound negative balance of linoleic acid and an apparent deficit in α-linolenic acid. This coincided with reduced sow fertility (farrowing rate < 75%, culling rates > 25% of weaned sows). This reduction in fertility seemed to be increasingly important with advancing sow age, presumably because of a progressive reduction in the body EFA pool over successive lactations (Rosero *et al*., 2016a). We computed the balance of EFA for six published studies, where diet EFA intake and milk EFA output was available (Rosero *et al*., 2015a), and arrived at a comparable imbalance between intake and output (linoleic acid, −25.5 g/day; α-linolenic acid, −2.8 g/day).

Moreover, we conducted a dose-response assay to determine the levels of both EFA (linoleic acid, C18:2n-6; and α-linolenic acid, C18:3n-3) required by the lactating sow for maximum subsequent reproduction (Rosero *et al*., 2016a). Both EFA were studied simultaneously because they have opposing functions, and increasing one EFA alters metabolism and physiological function of the other (Sprecher, 2000). Each EFA benefits reproduction through different modes of action. Provision of 0.45% dietary α-linolenic acid was the most effective dose (0.15%, 0.30%, 0.45%) in eliciting a rapid return to estrus and achieving the highest retention of pregnancy. However, it did not improve litter size. Supplemental linoleic acid improved total pigs born in a linear manner (13.2, 13.8 and 14.0 total pigs born/litter for 2.1%, 2.7% and 3.3% linoleic acid, respectively). Although supplemental linoleic acid improved litter size of first litter sows, the beneficial effects were more evident for older sows (litters 3 to 5).

Based on these findings, and especially for mature sows, a minimum dietary intake of both parent EFA was required to achieve rapid return to estrus, ability to conceive and maintain pregnancy and improved litter size. We concluded that a minimum dietary intake of 10 g of α-linolenic acid/day, simultaneous with a minimum of 125 g of linoleic acid/day should be provided to >98% of the sow population with the level being related to population daily intake. In this study, this equated to approximately 0.45% and 3.1% α-linolenic and linoleic acids, respectively (Rosero *et al*., 2016a). Since linoleic acid intake during lactation exhibited a dose-response increase in litter size and for pregnancy maintenance, we studied this in greater detail by using data from three studies, involving 543 mature sows (parities 3 to 5; Rosero *et al*., 2016b).

Estimating essential fatty acid requirement for lactating sows

Portraying the reproductive data as a continuum from weaning to farrowing was clarifying, in that patterns emerged to improve our understanding of increasing linoleic acid dose (Figure 2). Curves show the cumulative percentage of sows bred and maintaining pregnancy in relation to total sows weaned. Sows fed the reference diet (no added lipid) were more delayed in expressing estrus, and fewer did so. The proportion of weaned sows that were mated and confirmed pregnant was 84.4%, but only 74.4% of weaned sows farrowed. On the other hand, sows that consumed more than 115 g/day of linoleic acid exhibited an improved

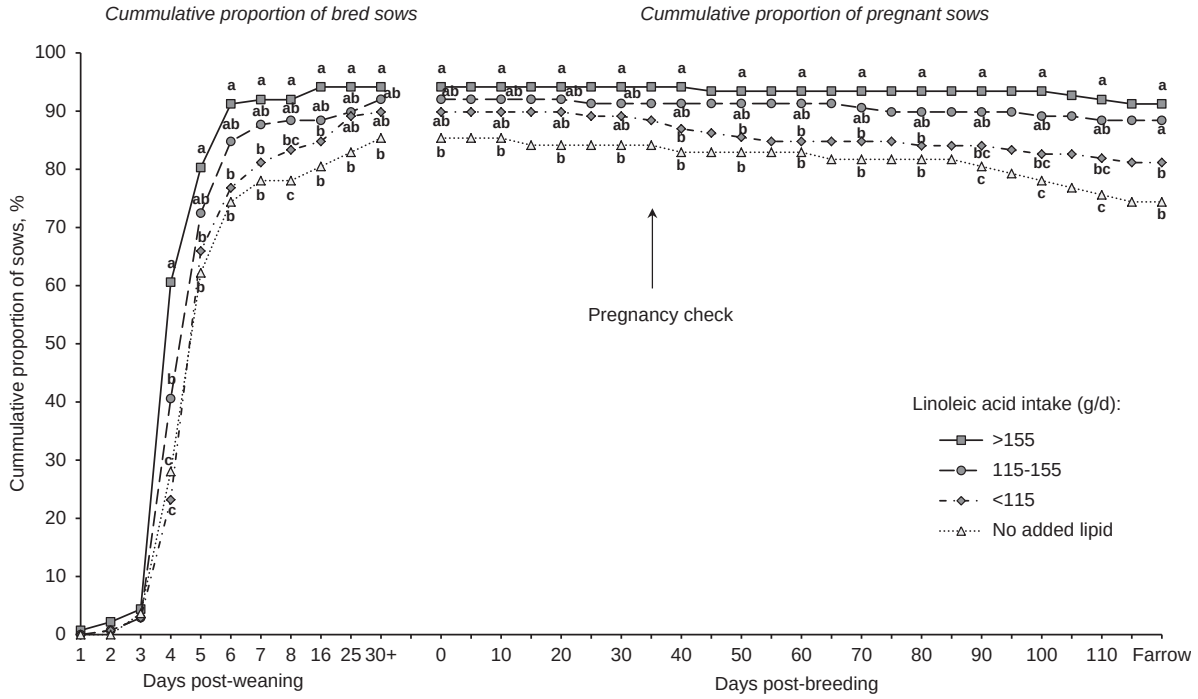

Figure 2 Effect of dietary linoleic acid intake during lactation on subsequent reproduction of sows ($n = 84$ sows fed diets with no added lipids; $n = 152$, 163 and 144 sows for <115, 115 to 155, and >155 g/day of linoleic acid intake, respectively) represents the cumulative proportion bred and pregnant sows relative to the number of sows weaned (SEM = 2.9). Analysis included a total of 543 mature sows (litters 3 to 5) from three studies. Sows fed diets without added lipids consumed 84.4 + 20.3 g/day of linoleic acid. Means represented by symbols without a common letter are different ($P < 0.05$). Figure reproduced from *Journal of Animal Science and Biotechnology*, BioMed Central Publishing, Creative Commons Attribution 4.0 International License (Rosero *et al.*, 2016b).

pattern, with approximately 90% bred (by day 8 post-wean) and maintained as pregnant through to farrowing. Thus, dose-related increases in linoleic acid intake produced dose-responsive improvements in how rapidly and the extent to which mature sows returned to estrus, pregnancy level and the ability to maintain pregnancy. Although consuming more than 155 g/day in lactation did not statistically improve reproduction (*v.* 115 g/day), neither was it deleterious. This pattern of improved reproduction coincided with incrementally reduced rates of culling for reproduction dysfunction (Rosero *et al.*, 2016a).

We employed a measure (pig index) to refine our estimate of the linoleic acid dose response maximum (Figure 3). Pig index is the multiple of farrowing rate and total fully formed pigs born, and it quantifies the total pigs born per 100 sows weaned. The marginal difference when moving from 100 to 125 g/day to 126 to 145 g/day is 54 pigs/100 sows weaned. Moving from the latter to 146 to 170 g/day further improved marginal pigs born by 68. The regression equation in Figure 3 is the basis for computing optimum profit linoleic acid intake, but feeding to deliver less than 125 g/day, for perhaps >98% of the population, is a suggested minimum. This dose-response assay is the first estimate, to our knowledge, of the linoleic acid requirement for reproduction in any species. This estimate will vary with (a) age of sow, being greater for older sows compared to younger sows, and (b) EFA pool recovery in pregnancy. Proper implementation of EFA estimates requires knowledge of seasonal lactation intake and the variance around intake. These findings demonstrate for the first time that summer infertility can be largely mitigated by an important nutritional modification under moderate heat stress.

Extreme and prolonged heat stress and seasonal fertility

We anticipate that seasonal infertility may still manifest in geographical regions with more 'severe' heat stress than encountered during our studies (ca. 36° latitude). Endocrine changes suppress oocyte development and survival, as well as embryo viability (Hansen, 2009); likewise, other physiological processes such as intestinal barrier dysfunction may occur due to localized hypoxia (Ross *et al.*, 2017). Nevertheless, providing the required daily intake (g/day) for each EFA is expected to moderate against the most extreme reduction in reproduction.

Immune development and gut barrier function in weaned pigs

Wean age and gut development

The weaning period has long been considered the most stressful phase of swine production. In contrast to nature where weaning is a gradual process nearing completion between 3 and 4 months of age, weaning in swine production is abrupt and occurs between 2 and 4 weeks of age when many physiological systems, such as the gastrointestinal and immune systems of the piglet, are relatively immature. As a result, the post-weaning period has been associated with

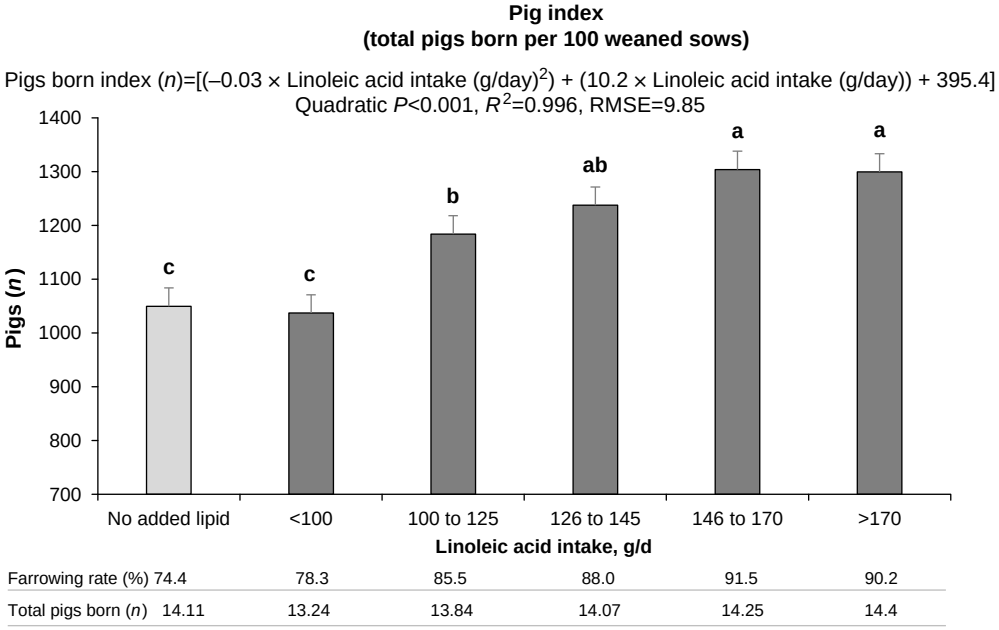

Figure 3 Effect of linoleic acid intake during lactation on pigs born index. This variable represents the total number of fully formed pigs born per 100 weaned sows and was calculated by multiplying subsequent farrowing rate (sows farrowed: weaned) by total number of pigs born/litter. Sows fed diets without added lipids served as control (84.4 + 20.3 g/day of linoleic acid). Means represented by bars without a common letter are different ($P < 0.05$). Figure reproduced from *Journal of Animal Science and Biotechnology*, BioMed Central Publishing, Creative Commons Attribution 4.0 International License (Rosero *et al.*, 2016b).

reduced feed intake and performance concurrent with increased disease susceptibility.

Segregated early weaning: upside and downside

The original work by Alexander *et al.* (1980) proved that very early medicated weaning at 5 to 10 days of age and complete separation from the sow population could reduce the vertical transmission of certain pathogens from the sow to offspring, and thus serve as an elimination strategy for pathogens such as *Actinobacillus pleuropnemoniae*, *Haemophilus parasuis* and transmissible gastroenteritis virus (**TGEv**). This led to the movement toward segregated early weaning (**SEW**) and medicated early weaning (**MEW**) programs in the 1980s into the 1990s. Weaning age in these programs ranged between 10 and 21 days of age (Harris, 1990; Connor, 1992; Wiseman *et al.*, 1992; Fangman and Tubbs, 1997). This pathway to disease elimination resulted in multiple site production, which was formalized as Isowean technology (Harris, 2000): two production sites for complete separation of weaned pigs from the sow herd as a constant, but three site systems were used by some to further separate nursery and finish phases. Unfortunately, this method of disease 'elimination' and minimization was not adequately studied for possible long-term downside on growth and viability.

Unintended consequences of very early weaning (12 to 16 days)

While the immediate benefits of early weaning for specific pathogen elimination were realized, especially for genetic nucleus programs, commercial production suffered with decreased growth and viability (Patience *et al.*, 2000). Commercial pigs easily developed diarrhea, needed increased levels of medication and experienced higher mortality and morbidity. This was confirmed by Main *et al.* (2004), who showed that increasing wean age (from 12 to 21.5 days of age) resulted in linear improvements in wean-to-finish performance and viability. The article by Cabrera *et al.* (2010) also demonstrated that the sow influences the performance of her progeny beyond the colostrum period, and in a profound way. Pigs reared by their mother for 20 days grew more efficiently, had greater loin muscle depth and tended to have fewer pigs die and removed for medical treatment than their 14-day counterparts, even though high health conditions existed.

Field validation of these concepts was conducted by Rosero *et al.* (2016c), where the dynamic of changing health over time and with multiple sets of pigs was used. They studied the impact of wean age in a large population of animals (143 weaned groups; 1139 to 2725 pigs/group) over a 4-year period and showed that incremental increases in wean age (18 to 24 days) improved wean-to-finish performance and reduced mortality. Moreover, this study revealed that the negative impact of early wean age depended on health status of their mothers. This became clear with disease infection (PRRSv, porcine epidemic diarrhea virus (**PEDv**); Figure 4). This is also supported by McLamb *et al.* (2013), where early weaned pigs (16 to 17 days wean age) exhibited more severe growth reductions, diarrhea and intestinal injury when faced with subsequent infectious challenge with F18 *Escherichia coli* later in the nursery period, compared with pigs weaned at 22 days of age. Given the experience of the commercial sector, the industry has moved back to an older wean age, especially to eliminate weaning of extremely young litters (National Pork Board, 2013 and 2018).

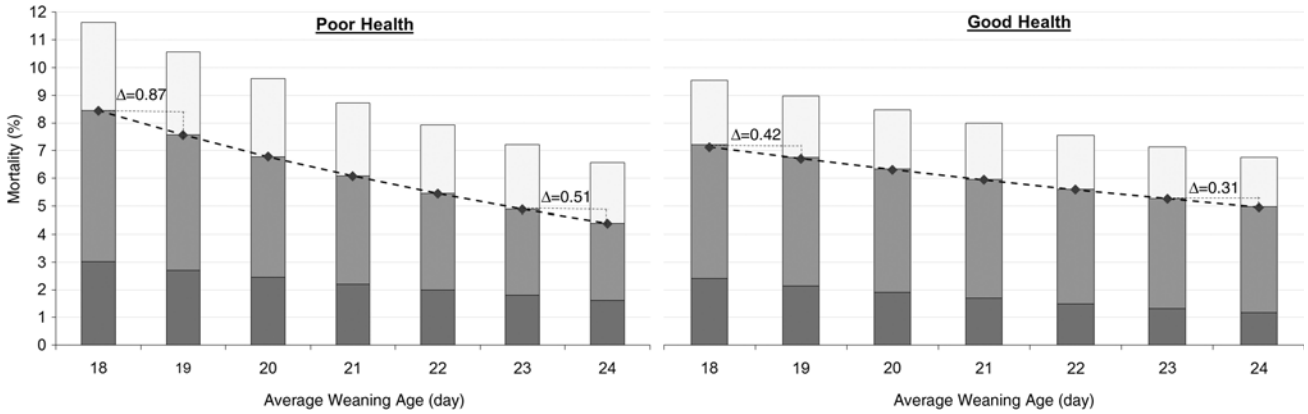

Figure 4 Impact of increasing weaning age from 18 to 24 days on percentage of pigs sold to off-grade market (or cull) and mortality of pigs weaned under (a) poor and (b) good health conditions. Bars and symbols represent estimated means obtained by using nonlinear (Poisson) regressions for mortality and culls. The darkest grey bars represent mortality from weaning to 7 weeks post-weaning (nursery period). The light gray bars represent mortality from 8 weeks post-weaning to marketing (finish period), and the line defines wean-to-finish mortality. The clear bars, above the mortality line, represent cull pigs below minimum full-value weight. The regressions estimate greater impact of increasing weaning age on pigs weaned at younger ages and during poor health conditions. The nonlinear regression for wean-to-finish mortality in poor health is Mortality (%) = Exponential [4.10 + (−0.109 × wean age)], $P = 0.003$, while the regression for good health is Mortality (%) = Exponential [3.05 + (−0.0601 x wean age)], $P = 0.04$ (adapted from Rosero *et al.* 2016c).

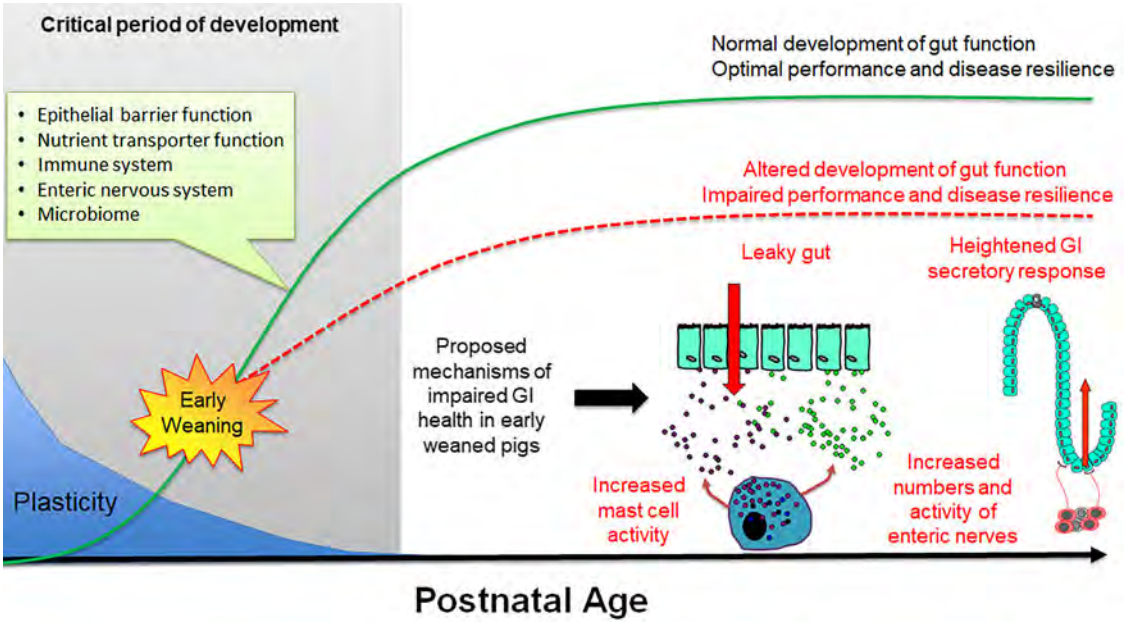

Figure 5 Impact of early weaning on long-term gut development in the pig. The early postnatal period is characterized by extensive development of critical system and gut functions (inset) and high plasticity. Development of gut function during this period shapes long-term gastrointestinal (**GI**) development, function and health (green line). Early weaning is a significant stressor causing intestinal injury (e.g. increased intestinal permeability, inflammation) that alters normal gut development leading to impaired performance and decreased disease resilience (red line). Proposed mechanisms underlying early weaning–induced intestinal dysfunction include increased mast cell activation, intestinal permeability ('leaky gut'), heightened enteric nervous system activity and gut secretory pathways.

Understanding the biological mechanisms of lifetime performance and disease risk associated with early weaning

During the first 3 to 4 months of postnatal life, the gastrointestinal (**GI**) system in the pig undergoes dramatic development including the establishment of the epithelial barrier and digestive functions, microbiome colonization and development of the enteric nervous and immune systems (Pohl *et al.*, 2015). Importantly, the GI system exhibits a high degree of plasticity during this period, and thus environmental factors (e.g. stress, intestinal injury, pathogen challenge) can disrupt the normal development of GI functions leading to long-term changes in gut health and disease resilience (Figure 5). There have been a large number of studies investigating the short-term impacts of weaning on intestinal injury, which is characterized by increased intestinal permeability and a spike in inflammation occurring within the first days of weaning (Bailey *et al.*, 2005; Lalles *et al.*, 2007; Moeser *et al.*, 2007a). Thus, intestinal injury occurs in a peak period of GI

development and programming, but the long-term effect on GI function has only recently been fully studied. Investigations using split-weaned litters allowed a comparison of gut developmental trajectories between early weaned (weaned at 18 days) and their later weaned littermates (weaned at 28 to 30 days). This revealed key insights into the long-term impact of early weaning on gut development, potential disease risk and performance reductions. Early weaning alters the normal development and function of the GI epithelial barrier, enteric nervous system and mucosal innate immune responses (Medland *et al.*, 2016; Moeser *et al.*, 2017; Pohl *et al.*, 2017), which coincides with diminished performance and resiliency to later-life stress or infectious challenges. Disturbance in intestinal barrier function, measured as increased intestinal permeability or 'leaky gut', is significant as increased GI permeability allows an excessive leakage of antigens across the intestinal epithelium, which incites chronic mucosal inflammation and impaired gut function. Loss of barrier function is thought to be a major predisposing factor in a number of human stress-related diseases, including irritable bowel syndrome (**IBS**) and inflammatory bowel disease. Medland *et al.* (2016) showed that early weaned pigs have more numbers of enteric nerves and heightened nerve-mediated secretory activity compared with later weaned pigs. Heightened intestinal permeability and GI secretory function coincided with intermittent, chronic diarrhea in early weaned pigs (Porras *et al.*, 2006; Barbara *et al.*, 2011; Pohl *et al.* 2017). Early weaned pigs exhibited a suppressed mucosal innate immune response and more severe clinical disease and intestinal barrier injury in response to a nursery challenge with F18 *E. coli* (McLamb *et al.*, 2013; Li *et al.*, 2019).

Studies investigating the mechanisms driving these long-term alterations in GI showed that the GI mucosa from early weaned pigs often has a similar morphological appearance (e.g. villus height, inflammatory lesions, crypt depth) compared with later weaned pigs. However, a distinct histological feature reported in early weaned pigs was an increase in the number of intestinal mast cells and level of mast cell mediators such as tryptase (Smith *et al.*, 2010; Pohl *et al.*, 2017). Moreover, administration of the mast cell stabilizing drug, sodium cromolyn, either prior to weaning or in grower pigs, reduced intestinal permeability in early weaned pigs (Moeser *et al.*, 2007b; Smith *et al.*, 2010; Mereu *et al.*, 2015). Together, these studies demonstrate that increased mast cell activity is critical to early and long-term intestinal permeability disturbances in the early weaned pig. The specific GI alterations observed in early weaned pigs are remarkably similar to those of human GI functional disorders such as IBS in which stress, intestinal permeability and heightened mast cell activity are known to be key mechanisms in symptom onset.

Early wean age disturbances may go beyond the GI tract. Furthermore, it is conceivable that early weaning could also impact organ systems other than the GI tract, such as the respiratory system. As discussed earlier, Rosero *et al.* (2016c) showed that performance and mortality of early weaned pigs were more negatively impacted during subsequent natural exposure to PRRSv, a predominantly respiratory pathogen. Epidemiological studies in humans and early life stress models in rodent (e.g. neonatal maternal separation stress) show a link between early life stress/adversity and long-term development of lung immunology, function and respiratory disease risk later in life (Bhan *et al.*, 2014; Hupa *et al.*, 2014; Lee *et al.*, 2017).

Innovations in precision nutrition

Two important milestones were achieved to improve precision in matching diet input to production needs for growing pigs during the past decade, thereby achieving more predictable pig growth and financial outcomes in the barn. These were achieved through public and private sector offerings: (a) detailed ingredient description for metabolically available amino acids (standardized ileal digestibility, **SID**) and net energy (**NE**) to facilitate valuation by ingredient and source plant, and (b) publication of the updated NRC Nutrient Requirements of Swine (2012), both of which enhanced the precision of amino acid and energy nutrition.

Precision in ingredient nutrient description

Near Infrared Spectroscopy (**NIRS**) has proven invaluable in defining the total content of nutrient fractions so that diets could be more precisely composed. Ideally, an ingredient could be allocated to storage in a mill, according to real-time nutrient value. Alternatively, the ingredient source that provides the greatest value would be selected for priority purchase. An important advancement occurred in amino acid nutrition en route to the detail that we have today; amino acids in an ingredient could be determined by direct reading of spectral information (Fontaine *et al.*, 2002). Evonik Nutrition (Essen, Germany) has proven expert, in this regard, having moved from regression equations that indirectly estimate amino acids from protein content.

During the past decade, precision nutrition was advanced even further, and in a major way, when Cargill Animal Nutrition (Minneapolis, MN, USA) allowed market access to its 'Nutrient Reveal' program as a product. This proprietary technology describes nutrient availability for an ingredient in the greatest detail ever afforded nutritionists in North America. Reports include (a) SID amino acids, and (b) NE; the algorithm to compute the latter is more detailed (carbohydrate fraction includes simple sugars) than the NRC (2012) was able to provide. This privately held program is the product of public and private research. Estimation of SID amino acid content is more involved than NIRS analysis, involving NIRS spectral readings related, in a proprietary manner, to an *in vitro* assay that has been aligned with the outcome of pig-based ileal digestion assay (Pilcher C, personal communication). Finally, comparative ingredient value is determined through formula runs.

Publication of nutrient requirements for swine, 2012

Publication of the US Nutrient Requirements of Swine (NRC, 2012) advanced our precision in matching metabolically available amino acids and energy to tissue needs. The North American pork industry underwent considerable changes since the previous 10th edition (NRC 1998), and research in that period contributed a robust amount of new information for many nutrients. This publication was the product of collaboration between North American scientists, with leadership provided by the late Dr Kees de Lange being pivotal to the committee's success. A version of his growth model was adapted for use in estimating SID amino acid and NE requirements for high lean growth pigs. Subsequent empirical data found general agreement with this model (Elsbernd *et al.*, 2017). Another element of their success was that three key advances were available to the committee: (a) a SID lysine curve had been reliably established for high lean growth pigs, through extensive collaboration; (b) extensive ingredient SID amino acid data base existed and the (c) Baker (1997) ideal amino acid pattern (**IAAP**) had been extensively calibrated for five of the most limiting amino acids. The committee used several tactics to create reliability. For example, model prediction of essential amino acid needs was calibrated using a significant body of empirical research to simulate against. During the process of simulation runs to align theoretical estimates with empirical outcomes, efficiencies of absorbed amino acid (SID) for maintenance and growth were adjusted. The rigorous comparison of predicted to actual estimates included competently conducted studies with a meaningful number of pigs, reared in state-of-the-art commercial research facilities. Fortunately, the committee convened at a point in time when the major terminal lines had largely converged to similarity in carcass lean (protein to lipid content). As a result, the lysine curves (SID lysine: Mcal NE) became almost indistinguishable among lean growth genotypes.

Ingredient net energy precision

Equations that were used to predict ingredient NE were an adaptation of those arising from INRA research (Noblet *et al.*, 1994). Resulting estimates were deemed credible for core ingredients, notwithstanding the advantage of more detailed prediction equations (e.g. fractions such as sugars). Although NE is the best descriptor of absorbed energy use, concern about estimate credibility and relative diet simplicity, prior to 2008, precluded the use of the NE system by many North American nutritionists. Privately held empirical validation of ingredient NE estimates is generally not available to the public sector; however, in certain instances, estimates were provided to the committee (Dr de Lange, personal communication). This was the case for animal fat where committee impasse existed as to the accuracy of potential estimates. For the latter, growth assays were conducted, shared with the committee and later reported (Boyd *et al.*, 2014 and 2015). Given the economic value of ingredient energy, we cannot overstate the importance of growth assay to validate dietary NE estimates (Schinckel *et al.*, 2012), as has been routine for validating ideal amino acid patterns.

Table 1 *Comparison of the ideal pattern for essential amino acids (standardized ileal digestibility, SID) in growing pigs, expressed as a ratio to lysine level (let lysine = 1.000), for two points of growth: Baker pattern (1997) v. NRC (2012) model*

	20 kg pig		80 kg pig	
	Baker[1]	NRC[2,3]	Baker[1]	NRC[2,4]
Amino acid				
Lysine	1.000	1.000	1.000	1.000
Threonine	0.650	0.572	0.700	0.608
Methionine + cysteine	0.600	0.561	0.640	0.573
Tryptophan	0.170	0.170	0.190	0.175
Valine	0.680	0.647	0.680	0.659
Isoleucine	0.600	0.521	0.600	0.529
Leucine	1.000	1.006	1.000	1.012
Histidine	0.320	0.344	0.320	0.344
Arginine	0.420	0.457	0.180	0.458
Phenylalanine + tyrosine	0.950	0.936	0.950	0.948

NE = net energy.

[1] Baker patterns published in Biokyowa technical review no. 9 (1997). The ratio of each amino acid to lysine represents the minimum dietary provision required, beyond the amount that is synthesized. The relative decline in the arginine to lysine ratio for the 80 kg pig is based on arginine synthesis completely meeting the amount required for protein synthesis. The arginine ratio for 20 and 80 kg phases was considered generous because arginine is involved in immunocompetency through nitrous oxide generation.

[2] US Nutrient Requirements of Swine (NRC, 2012).

[3] NRC inputs: NE, 2.395 Mcal NE/kg; 4.5% fermentable fiber, gender balance, thermoneutral temperature, space not factored, model intake curve. Model protein deposition maximum estimate, 126 g/day.

[4] NRC inputs: NE, 2.501 Mcal NE/kg; 6.5% fermentable fiber, gender, temperature, space as above. Model protein deposition maximum estimate, 144 g/day.

Notwithstanding the NRC NE equation being less descriptive, it was pivotal in completing the shift of North American nutritionists to NE-based formulation. The most limiting element in moving toward a more descriptive algorithm to derive ingredient NE estimates is the analytical detail required. The limitation to the public advancements is the unavoidable complexity with which different energy sources are used for maintenance as well as for lean and lipid gain. This approach considers the differing efficiencies of utilization according to both energy source (fat, protein, starch and fiber) and metabolic outcome of energy in the diet (Birkett and de Lange, 2001a, 2001b and 2001c). This is the basis for the Cargill ingredient energy prediction model.

Empirical derivation of ideal amino acid pattern for growth

The laboratory of David Baker (1997) advanced the application of ideal amino acid pattern (**IAAP**) in North American nutrition practice by deriving the pattern through empirical assay. It proved the error of using the whole-body pattern for lysine, methionine and tryptophan. In addition, the pattern was estimated for three growth phases, thereby accounting for the changing contribution of tissue growth and maintenance to the final pattern (Fuller *et al.*, 1989). Ultimately, the Baker pattern was a guiding force in the design

of amino acid studies, during the lean growth transformation era (1990 through about 2012), and for amino acid research in general. A large number of empirical studies not only refined the proposed pattern for the most limiting amino acids, but they provided estimates for the NRC to simulate against. A comparison of the Baker IAAP with the resulting NRC growth model pattern is shown for two body weights (Table 1). Published data and the NRC model are in relative agreement on IAAP estimates for threonine, total sulfur amino acids and valine; some questions remain about the tryptophan ratio as most nutritionists consider the NRC estimate to be slightly low. Continued pressure to reduce diet cost and protein content of weaned pig diets, beyond the most limiting amino acids (lysine, threonine, methionine, tryptophan, valine), have since led to studies on the IAAP for presumed next limiting amino acids: isoleucine (Clark *et al.*, 2017) and histidine (Cemin *et al.*, 2018). The National Research Council (2012) also introduced an important concept relative to the dynamics of threonine ratio; it confirmed that the IAAP of threonine increases with BW but that it also increases with dietary fermentable fiber level.

Synthetic amino acid use

Synthetic amino acid use for pigs in North America has grown almost exponentially during the past 20 years. During this period, threonine, tryptophan and valine have been commercialized, at a cost-competitive price; isoleucine is likewise emerging for use. Profit maximization was the primary driver behind this development, with protein sources being displaced. It is not uncommon to use up to 0.65% L-lysine hydrochloride in weaned pig diets with no performance effect, provided that threonine, methionine, tryptophan and valine are also added. The North American profit model progressively expanded amino acid use, but the adoption of the NE system expanded their use further. The soybean NE : corn NE ratio does not exceed 0.82 (NRC, 2012), so the combination of corn and amino acids is a means to increasing diet energy.

North America and the European Union (**EU**) use amino acids extensively, but for different reasons. Since the primary North American driver is profit based, we have led the world in calibrating the IAAP. The European Union leads the world in amino acid use (MT of all amino acids/year) for food animal diets, but the primary driver for their growth has been legislation, with diet cost being important, but secondary. Political pressure to reduce nitrogen output led to an early demand for four to five amino acids even though diet cost initially increased. Whereas North America devoted considerable resources to IAAP validation, EU scientists devoted enormous effort to ingredient SID estimates with the results from both regions being important and complimentary.

Global growth of synthetic amino acid use has been remarkable, with the EU leading for all amino acids except for lysine, where China leads all regions. Lysine use for food animals in North America is expected to increase from 300 000 MT annually to 500 000 MT from 2010 to 2020 (Grand View Research, San Francisco, CA, USA). Although the quantity is less for North America, the rate of increase is similar to that for the EU, over the same period (500 000 to 775 000 MT/year).

Health-promoting and -compromising properties of ingredients

The library of health-promoting ingredients expanded during the past decade. The level of peroxidized lipid products that compromise viability in weaned pigs was also shown for the first time. Some ingredients can be either health promoting or compromising; effects to be shown involve gain, efficiency of feed use, viability and medical need.

Soybean ameliorates respiratory immune stress

A precautionary note is raised regarding the significant use of synthetic amino acids, when respiratory infection occurs. We learned that respiratory inflammatory episodes (e.g. influenza, mycoplasma pneumonia) severely impair growth and feed conversion during an infection period, and that soybean meal ameliorates these growth-suppressing effects (Johnston *et al.*, 2010). This was later confirmed in a study with influenza-virus-infected pigs (Gene Gourley and Dean Boyd, unpublished research) and with weaned pigs that were deliberately infected with PRRSv (Rochell *et al.*, 2015; Smith *et al.*, 2019).

Soybeans contain phytochemicals that have proven to be health-promoting, including: (a) isoflavones (anti-inflammatory; Zaheer and Akhtar, 2017), (b) phenols (antioxidants; Shahidi and Ambigaipalan, 2015) and (c) saponins (suppress inflammatory mediators of tumor growth; Lima *et al.*, 2017). On the basis of isoflavone and perhaps phenol contents, soybean displacement is a concern when respiratory health is compromised. It is unclear about growth-promoting aspects of soybeans (rate, composition) under conditions of low immune stress.

Dietary xylanase improves viability

There is growing appreciation that ingredients not only supply essential energy and nutrients, but they also possess functional properties that influence animal health. This has been conclusively shown with soybeans, but the dietary enzyme, xylanase, has recently been shown to improve viability of growing pigs. Gut health improvement has held such promise, and the enzyme xylanase may be a substantive example of a measurable production outcome. While attempting to measure energy release, Zier-Rush *et al.* (2016) discovered that the addition of the carbohydrase xylanase to the diet of wean-to-finish pigs (12 to 140 kg) improved viability (3.99% to 2.39%). Although pigs were healthy, mortality declined in a dose dependent manner and by 40%. Numerous field studies have confirmed this report.

The mode of action has not yet been elucidated, but improvements in gut barrier function and improved gut microbiome profile are potential candidates. Research in poultry, having received dietary xylanase, showed promising developments for selected bacterial groups (beneficial, harmful) in the intestinal tract of broiler chicks (Vahjen

et al., 1998). However, the results were not completely consistent; xylanase reduced enterobacteria in both luminal and GI tissue samples, and *Lactobacillus* species were increased in tissue but not luminal samples. Xylanase also appeared to enhance production of butyric acid, well known as a preferred fuel of colonic epithelial cells. *In vitro* studies have confirmed these findings and extended our understanding of a possible role of xylanase in improving gut health (Ravn *et al.*, 2017).

In newly weaned pigs challenged with F18 Enterotoxigenic *E. coli* (**ETEC**; Li *et al.*, 2019), xylanase was added to a diet containing a highly fermentable fiber. They observed improved gastrointestinal barrier function and reduced inflammatory intermediates, which were all associated with faster rate of gain. An earlier study by Li *et al.* (2018) evaluated a carbohydrase blend, consisting of cellulase, xylanase and β-glucanase, in pigs that were not ETEC-challenged. They reported improved growth associated with improved gut barrier integrity and reduced immune system activation. In this study, a treatment of xylanase alone did not produce the same response as the enzyme blend. Collectively, these data suggest that carbohydrases may enhance animal health and viability mediated by reductions in the permeability of the intestinal barrier, leading to reduced exposure by the pig to immunologically active molecules and toxins.

Diet oxidative stress impairs weaned pig health and viability

Oxidative stress refers to the disproportionate production of free radicals resulting, for example, from excessive lipid peroxidation. Under conditions of normal metabolism, generation of free radicals is balanced by ubiquitous antioxidant defense mechanisms, notably those associated with ascorbic acid, glutathione, protein thiols and various scavenging enzymes (Chakraborthy *et al.*, 1994). Feeding peroxidized fats to weaned pigs impaired function and morphology of the intestinal tract and growth and nutrient digestibility, but in a dose-dependent manner (Rosero *et al.*, 2015b). The finding of a dose-related impairment of gut barrier function led to a field study with 2200 pigs, proving that there was a dose-related impairment of dietary lipid peroxidation on viability, medical treatment and number of excessively small pigs at the end of the nursery phase. Total antioxidant capacity and serum vitamin E decreased linearly with increasing peroxidation, which underscores the importance of lipid quality control to increasing oxidative stress in weaned pigs (Chang *et al.*, 2019).

This illustrates the importance of quality control procedures (initial peroxide value, hexanal, 2, 4-decadienal). We expect that the younger pig is more susceptible to oxidative stress-induced impairment of gut barrier function than older pigs, although growth and efficiency of gain for all ages of growing pigs are impaired (Overholt *et al.*, 2018). Older growing pigs appear more resilient to oxidative stress, since peroxide stress had no impact on gut integrity.

Technologies converge to improve pathogen detection and containment

Disease pathogens are estimated to be responsible for over 20% mortality from birth to harvest in farm animals, including pigs (National Academies of Sciences, Engineering, and Medicine, 2019), which represents a substantial economic loss. If a disease cannot be prevented by vaccine or biosecurity methods, then the next tactic is detection at first infection, that is, in the incubation stage, before the pathogen spreads throughout the population. Surveillance is fundamental to control and eradicate infectious agents; however, an adequate method for early detection in populations was not available until recently. Serum sampling is a good means for detection, but it is not easy to apply, and it represents a small proportion of the herd. Testing a few pigs makes it very difficult to detect disease that resides in 1% to 2% of the population. Confirming disease more rapidly and coordinating responses within regions are major advances in North American disease response and eradication technology.

Polymerase chain reaction methodology for pathogen detection

A milestone was achieved for swine veterinarians in North America when PCR testing of oral fluid samples converged to enable early and rapid detection of disease. Early detection of a pathogen makes it more likely to prevent pathogen movement to other sites, and beyond a geographic area. Oral fluid sampling was found to be a reliable means of PRRSv detection (Prickett *et al.*, 2008). It was determined to be more accurate than serum-derived samples, because more pigs were represented. The combination of oral fluid sampling with PCR analysis was 'game' changing for practicing veterinarians, for three reasons (Donovan T, personal communication): (a) population surveillance (*v.* sentinels), (b) pathogen detection at an affordable price and (c) disease containment. The polymerase chain reaction technology has been refined over the past 20 years to become a rapid and affordable means of replicating a DNA sequence for subsequent assay.

Population sampling using oral fluids collection

Oral fluids contain salivary gland liquids, but also virus particles (nucleic acids), antibodies from oral and tonsillar tissues and from blood capillaries. Introduction of this sampling method to the North American pig veterinary sector was facilitated by prior use in human medicine. Antibodies and pathogens can be detected in oral fluids collected from infected humans and animals. Oral fluid use gained prominence in the mid-1990s as a rapid and reliable means to assessing human immunodeficiency virus infection in human patients (Hodinka *et al.*, 1998).

The first application of this method of population surveillance to pig veterinary practice occurred with successful surveillance of PRRSv and porcine circovirus type 2 (**PCV2**) infections in three commercial populations (Prickett *et al.*, 2008).

Since that time, oral fluid sampling of sow herds has been instrumental in PRRSv eradication, from specific farms, because the virus can be detected even when prevalence is below 1% to 2% of the population. Swine veterinarians in North America applied oral fluid-based testing methodologies for an increasing number of respiratory and enteric disease diagnostic applications (Bjustrom-Kraft *et al.*, 2018).

Polymerase chain reaction technology is routinely used for most of the critical diseases in North America, including PRRSv, PEDv, PCV2, porcine deltacorona virus (**PDCoV**), influenza A virus. This technology is also used for other pathogens (*Mycoplasma hypopneumoniae, Actinobacillus pleuropneumoniae*, transmissible gastroenteritis virus*, Lawsonia intracellularis, Senecavirus A*). Evidence for the rapid employment of oral fluid specimens in the North American pork industry is shown by the total number of oral fluid tests performed for pathogens each year (2010 to 2018), at three major US diagnostic laboratories. Total number of pathogen tests per year increased from approximately 21 000 in 2010 to nearly 400 000 in 2018 (updated from Bjustrom-Kraft *et al.*, 2018). Potential introduction of ASF to North America is an immediate concern. An oral specimen assay has been developed for detection of this pathogen (Grau *et al.*, 2015), but we are not aware that the US Department of Agriculture has accepted it as an official test, to this point.

Central reporting and communication system

Introduction of PDCoV and PEDv into North America (2013) caused veterinarians to coalesce to develop a central reporting and communication system, which will prove invaluable if other foreign diseases are introduced. This status reporting system grew rapidly after 2013, when PEDV and PDCoV viruses infected North American swine herds. This program, known as the Morrison Swine Health Monitoring Program, involves weekly reporting of PCR diagnostic results, with a weekly communiqué returned. This system allowed veterinarians to respond to the PEDV threat in a coordinated manner to limit pathogen spread. Oral fluid assay and a national scheme of communication are critical steps forward. Should ASF infect North America, the system is in place to identify and contain the threat to the farm with the end being to limit the pathogen to a locale or region.

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Declaration of interest

No competing interests.

Ethics statement

This review is a not a presentation of original research. Authors attempted to accurately portray results and conclusions from published research.

Software and data repository resources

None.

Supplementary materials

For supplementary material for this article, please visit https://doi.org/10.1017/S1751731119001915

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animal

Review: Nutrient requirements of the modern high-producing lactating sow, with an emphasis on amino acid requirements

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*Sow productivity improvements continue to increase metabolic demands during lactation. During the peripartum period, energy requirements increase by 60%, and amino acid needs increase by 150%. As litter size has increased, research on peripartum sows has focused on increasing birth weight, shortening farrowing duration to reduce stillbirths and improving colostrum composition and yield. Dietary fibre can provide short-chain fatty acids to serve as an energy source for the uterus prior to farrowing; however, fat and glucose appear to be the main energy sources used by the uterus during farrowing. Colostrum immunoglobulin G concentration can be improved by increasing energy and amino acid availability prior to farrowing; however, the influence of nutrient intake on colostrum yield is unequivocal. As sows transition to the lactation period, nutrient requirements increase with milk production demands to support large, fast-growing litters. The adoption of automated feed delivery systems has increased feed supply and intake of lactating sows; however, sows still cannot consume enough feed to meet energy and amino acid requirements during lactation. Thus, sows typically catabolise body fat and protein to meet the needs for milk production. The addition of energy sources to lactation diets increases energy intake and energy output in milk, leading to a reduction in BW loss and an improvement in litter growth rate. The supply of dietary amino acids and CP close to the requirements improves milk protein output and reduces muscle protein mobilisation. The amino acid requirements of lactating sows are variable as a consequence of the dynamic body tissue mobilisation during lactation; however, lysine (**Lys**) is consistently the first-limiting amino acid. A regression equation using published data on Lys requirement of lactating sows predicted a requirement of 27 g/day of digestible Lys intake for each 1 kg of litter growth, and 13 g/day of Lys mobilisation from body protein reserves. Increases in dietary amino acids reduce protein catabolism, which historically leads to improvements in subsequent reproductive performance. Although the connection between lactation catabolism and subsequent reproduction remains a dogma, recent literature with high-producing sows is not as clear on this response. Many practical aspects of meeting the nutrient requirements of lactating sows have not changed. Sows with large litters should approach farrowing without excess fat reserves (e.g. <18 mm backfat thickness), be fed* ad libitum *from farrowing to weaning, be housed in a thermoneutral environment and have their skin wetted to remove excess heat when exposed to high temperatures.*

Keywords: amino acid, colostrum, energy, litter size, pig

Implication

Sow productivity has increased dramatically in the last decade. With improved productivity, requirements for energy and amino acids increase during lactation. To meet these needs, sows should be in proper body condition before farrowing to encourage high feed intake, and provided full access to feed in the few days before and during lactation. Diets should contain high-energy, low-fibre ingredients to maximise energy intake, and formulated with sufficient amino acid levels to meet the demands for milk production and minimise tissue catabolism. Today's sows are resilient and, with proper nutrient intake, can withstand the rigorous demands of increased productivity.

Introduction

Genetic selection and improvements in health, management and nutrition have led to unprecedented levels of sow productivity. In 2016, pigs weaned per sow per year averaged 25.7 in the United States, with an even higher productivity in the major pork production countries in Europe, ranging

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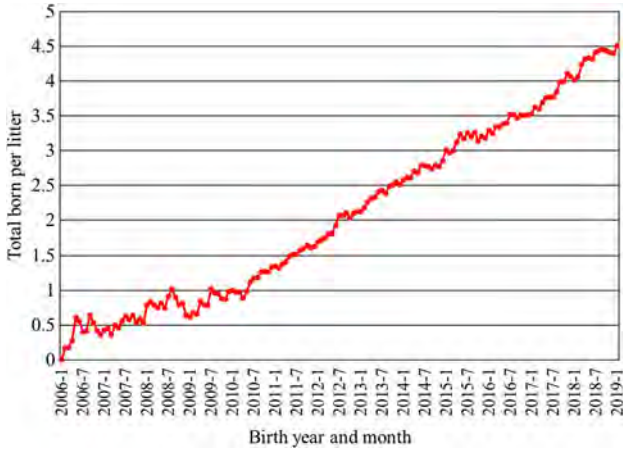

Figure 1 Genetic trend for total pigs born per litter at the nucleus level from Genus PIC (M. Culbertson, personal communications, 12 February 2019).

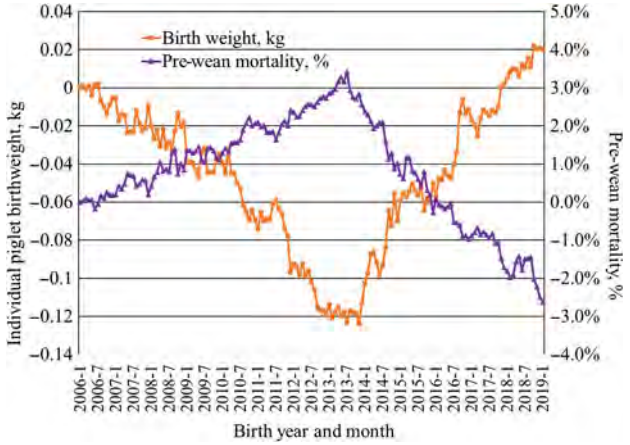

Figure 2 Genetic trend for individual pig birthweight and pre-wean mortality from Genus PIC (M. Culbertson, personal communications, 12 February 2019).

from 27.0 in Spain to 32.1 in Denmark (Agriculture and Horticulture Development Board, 2017).

Much of the increase in pigs weaned per sow has been a result of increased litter size. An increased use of genomics has accelerated the rate of progress in recent years. Data from Genus PIC illustrate the speed of change. From 2006 to 2019, the genetic trend at the nucleus level for total pigs born increased by approximately 0.334 pig per year, or an increase by 4.5 pigs per litter over the 13-year period (Figure 1). Initially, this led to a decrease in individual pig birth weight with average birth weight decreasing by approximately 120 g from 2006 to 2013 with a concomitant increase in pre-weaning mortality (Figure 2). After changing the selection criteria to offset this trend in 2013, the decrease was quickly reversed. Within 6 years, the previous loss in average birth weight was recovered, and in fact, the average birth weight was 20 g greater in 2019 than reported in 2006 while maintaining a steady increase in total born per litter. Because of heavier birth weights, pre-weaning mortality also decreased almost 6 percentage units from the high in 2013.

The improvements in reproductive performance increase metabolic demands on the sow during gestation and lactation. Today's modern genotype females are also faster-growing and have less adipose tissue than their predecessors. In commercial production, it is not uncommon to see gilt tenth rib fat depth at farrowing average 16 mm and parity 2 and older sows having fat depth ranging from 12 to 16 mm (Kim *et al.*, 2015; Thomas *et al.*, 2018). These changes in body composition and reproductive performance alter nutrient requirements during gestation and lactation. Increases in litter size increase total fetal growth in late gestation, farrowing duration, colostrum needs and milk production. In this review, the nutrient demands for these biological processes are discussed, dividing the sections into the peripartum and lactation periods and the unique requirements during each period.

Peripartum transition period

While several studies have been conducted to evaluate changing nutrient requirements in late gestation (day 90 to parturition), few studies have focused on the days immediately prior to parturition. The transition period has been loosely defined as the last 10 days of gestation to the first 10 days of lactation (Theil, 2015). During the peripartum transition period, a rapid shift in nutrient requirements and nutrient partitioning occurs due to an exponential increase in fetal and mammary growth, uterine components and colostrum synthesis (Feyera and Theil, 2017). Typically, sows are limit-fed a gestation diet, then receive a set amount of lactation feed for 2 to 3 days prior to farrowing. The lactation diet is a higher lysine (**Lys**), higher energy diet than the gestation diet. The change from lower Lys limit-fed gestation diet to a nutrient-dense lactation diet can be met with metabolic challenges as the sow has to rapidly adapt to a new diet composition. It is important to minimise this rapid shift in nutrients at the time of parturition to avoid a negative impact on parturition and lactation performance (Martineau *et al.*, 2013). The goal of the transition period should be to meet the changing requirements for fetal and mammary tissue growth, prepare the sow for the upcoming lactation demand and supply nutrients during parturition for maximum piglet survival at birth. Another critical activity in the peripartum transition period is colostrum production, which is estimated to begin 2 to 3 days before the onset of parturition (Devillers *et al.*, 2004).

Transition period feeding and farrowing duration

Parturition is an energy-demanding process. As litter size continues to increase, there is also an increase in farrowing duration. A normal birthing interval is 15 to 20 min, which could lead to a 300-min farrowing duration for a litter of 15 piglets. Several factors have been associated with an increase in farrowing duration, including sow backfat >17 mm at farrowing (Oliviero *et al.*, 2010) and increased litter size (van Dijk *et al.*, 2005). Recently, Feyera *et al.* (2018) observed that farrowing duration is reduced if sows have access to feed and eat at

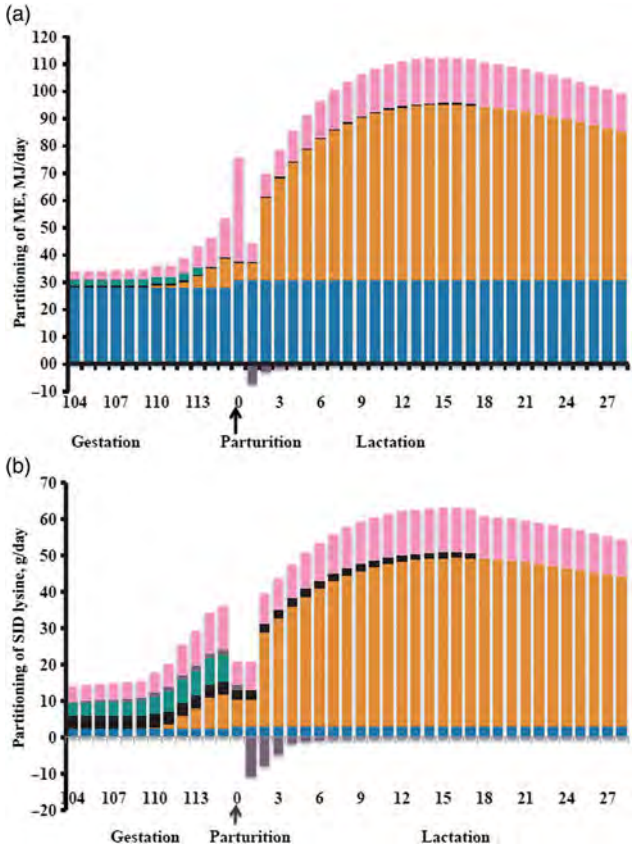

Figure 3 Calculated metabolisable energy (ME; panel a) and standardized ileal digestible (SID) lysine (panel b) requirements for maintenance (blue bars), colostrum/milk production (orange bars), mammary growth (black bars), fetal growth (green bars), uterine components (purple bars) and additional heat loss for energy or oxidation/transamination or amino acids (pink bars) in sows during transition and lactation. (Reprinted from *Livestock Science*, 201, Feyera and Theil, Energy and lysine requirements and balances of sows during transition and lactation: A factorial approach, 50–57, 2017, with permission from Elsevier.)

least 3 h before farrowing, hypothesising that this is due to a greater availability of energy. However, Cools *et al.* (2014) fed a lactation diet *ad libitum* starting on day 105 of gestation and did not affect farrowing duration. This study had fewer total born (11 pigs), which may explain the reason why no differences were observed. Several other nutritional strategies during the transition period have been investigated for their effects on farrowing duration. Reduced farrowing duration was observed with added phytase (Manu *et al.*, 2018) or soluble fibre sources (Theil *et al.*, 2014), but not with creatine (Vallet *et al.*, 2013) or a dietary nitrate supplement (van den Bosch *et al.*, 2019). Interestingly, Feyera *et al.* (2018) observed that during late gestation the uterus partially satisfies its energy demand using acetate and butyrate from dietary fibre inclusion. Conversely, during farrowing, these short-chain fatty acids are not extracted by the uterus, but rather triglycerides and glucose are used as the energy source. Therefore, while short-chain fatty acids may be used by the uterus in late gestation, feeding a diet containing increased triglycerides and glucose a day prior to parturition could supply the readily absorbed energy required by the uterus during parturition, which could positively benefit uterine contractions and reduce farrowing duration and stillbirth rate.

Energy requirements in the peripartum transition period

Dietary energy requirements during gestation are derived from body maintenance, growth of conceptus and maternal demands from the mammary and uterus. These requirements will also depend on sow BW, parity and environmental conditions (Trottier *et al.*, 2014). Of particular interest in the transition period are the requirements to support an exponential growth rate of the fetal, mammary and uterine components. Feyera and Theil (2017) used a factorial approach to model metabolisable energy (**ME**) requirement in the last 12 days of gestation, and estimated a 60% increase in requirement during this time period from 33.9 to 55.6 MJ ME per day (Figure 3). The greatest proportion of required ME (75% to 80%) during the end of gestation is derived from maintenance and depends on sow BW gain (Noblet *et al.*, 1990). Thomas *et al.* (2018) observed gilt-mobilised fat tissue to meet the energy needed in late gestation for fetal growth and colostrum production. Decaluwe *et al.* (2014) observed an increase in backfat loss from day 108 to farrowing when sows were only fed 1.5 *v.* 3.0 kg/day of a transition diet. Similarly, Cools *et al.* (2014) observed that sows fed a lactation diet *ad libitum* from day 105 of gestation had less backfat thickness loss compared with limit-fed sows. Hansen *et al.* (2012) observed that total intake of ME from day 108 to 112 of gestation was negatively correlated with piglet weight gain at peak lactation, indicating that a less negative energy balance around parturition is inhibitory for sow milk yield at peak lactation, likely because of the negative impact on feed intake. While energy supply in the peripartum transition period is important to meet changing tissue needs, it is crucial to supply energy without contributing to excess BW gain and backfat stores that will lead to a negative impact in lactation feed intake, milking ability and litter growth.

Colostrum intake is highly correlated with increasing piglet survivability, with a recommended intake of 200 ml per pig in the first 24 h (Ferrari *et al.*, 2014; Moreira *et al.*, 2017). However, even with the mobilisation of fat reserves before farrowing, sows with low feed intake produced less colostrum and litter weight gain in the first 24 h (Decaluwe *et al.*, 2014). Sows fed a lactation diet starting on day 104 of gestation produced more colostrum compared with sows fed a gestation diet (Garrison *et al.*, 2017). In contrast, no difference in piglet colostrum intake or sow colostrum yield was observed due to supplemental fat type (Theil *et al.*, 2014) or increased Lys and energy (Gourley at al., 2019).

Colostrum quality, as measured by immunoglobulin G concentration, has increased when feeding sows a tall oil fatty acid supplement (conjugated linoleic acid source) starting on day 107 of gestation (Hasan *et al.*, 2018) or high Lys and energy from day 113 of gestation to farrowing (Gourley *et al.*, 2019). Colostrum immunoglobulin G was not increased

with increased dietary fibre (Loisel *et al*., 2013). Thus, increased sow energy or amino acid intake in the few days prior to farrowing, during colsotrogenesis, can be beneficial to the colostrum quality.

Fibre use as an energy source in the peripartum transition period

Several studies have investigated the effects of dietary fibre during the transition period and its influence on colostrum yield, piglet survival and lactation performance. Loisel *et al.* (2013) fed a low- (13.3% total dietary fibre) or high- (23.4% total dietary fibre) fibre diet to pigs from day 106 of gestation until parturition. They observed that low-birth-weight pigs (<900 g) from sows fed high-fibre diets had increased colostrum intake, increased colostrum lipid concentrations and a reduction in pre-weaning mortality (14.7% *v.* 6.2%), but decreased colostrum immunoglobulin A concentrations, and no difference in total sow colostrum yield (3.9 *v.* 3.8 kg). Feyera *et al.* (2017) fed a dietary fibre-rich supplement (22% crude fibre) to pigs from day 102 of gestation to farrowing (280 g/day from day 102 to 108, and 570 g/day from day 109 to farrowing) and observed a reduction in stillbirths (8.8% *v.* 6.6%) and decreased piglet death from low viability (2.8% *v.* 1.5%) compared with sows fed a control diet (4.1% crude fibre). The researchers attributed the decrease in stillbirths to a greater amount of short-chain fatty acids available as energy in the colon, or from a reduction in sow constipation. Oliviero *et al.* (2009) demonstrated that increased fibre feeding pre-farrowing (7% *v.* 3.8% crude fibre) reduced constipation around parturition. Guillemet *et al.* (2010) observed that sows fed a high-fibre diet in gestation (12.8% *v.* 3.5% crude fibre) transitioned more rapidly to a nutrient-dense lactation diet and tended to lose less backfat during the lactation period. However, fibre inclusion during the last 8 to 10 days before farrowing has not been shown to impact birthweight, litter gain, colostrum yield or metabolic criteria of the sow (Loisel *et al*., 2013; Feyera *et al*., 2017). Therefore, added fibre during transition may help transition a sow to a lactation diet and reduce stillbirths, but with limited to no impact on colostrum or litter growth.

Amino acids in the peripartum transition period

Fetal growth (22.7%), mammary growth (16.8%) and colostrum production (16.1%) represent the majority of the total required standardised ileal digestible (**SID**) Lys in late gestation, with the remaining requirement for oxidation/transamination, maintenance and uterine components (Feyera and Theil, 2017). These researchers predicted that relative to day 104 of gestation, the SID Lys requirement increased 149% by day 115 of gestation to approximately 35 g of SID Lys per day (Figure 3). This requirement is a significant increase compared with Lys typically provided in commercial production today. Therefore, the sow is likely in a negative Lys balance in the last few days before parturition. Mammary growth increases rapidly in the 10 days prior to farrowing, and will continue to increase up to day 10 of lactation (Kim *et al*., 1999). The number of pigs determines the amount of Lys and amino acids required, and the sow will mobilise body fat and protein to support litter growth if her feed intake or diet quality is inadequate (Theil, 2015). Recently, it has been demonstrated that birth weight can be increased in gilts by supplying 40 g SID Lys per day beginning on day 107 or 113 of gestation (Gourley *et al*., 2019). Additionally, if fetal growth requirements are met, the female will partition increased nutrient intake towards backfat (Garrison *et al*., 2017; Gourley *et al*., 2019). It is unknown from these studies whether body protein also increased during this period, but it is well understood that a gilt's requirement for maternal body protein is greater compared with older parity females (Trottier *et al*., 2014). Thus, gilts may benefit more from an increase in Lys and amino acids in the transition period due to partitioning towards body protein reserves and fetal growth. There is limited data during the transition period to understand the importance of amino acids besides Lys; however, Kim *et al.* (2009) suggested that in late gestation, the sow requires increased amounts of arginine and leucine for fetal and mammary parenchymal tissues. Therefore, while high dietary Lys can be beneficial during the transition period, more research is needed to understand if additional amino acids will be of benefit for colostrum production and fetal growth.

Lactation

Although lactation represents only 15% to 20% of the productive cycle of a sow, it is undeniably the most metabolically demanding stage of production. The sow's priority in lactation is to sustain milk production for the large and fast-growing litter of piglets, but is often not solely attained by voluntary feed intake. The mobilisation of body fat and protein reserves appears to be critical to support milk production in high-producing sows, although it is unclear whether body mobilisation is an obligatory process in modern sows (Pedersen *et al*., 2019). The typical negative effects of severe catabolism in lactation on the subsequent reproductive performance of sows is well established (Koketsu *et al*., 1996), but modern sows seem to be more resilient to the effects of lactational catabolism (Patterson *et al*., 2011). This distinctive characteristic of the modern sow can be related to changes in biology and body lean composition, although sow resilience over successive parities has not been widely evaluated. Therefore, the main goal of the nutrition program for lactating sows should be to maximise feed intake to sustain milk production, without excessive mobilisation of BW reserves.

Energy requirements in lactation

The energy requirements of the modern lactating sow have increased significantly along with a marked increase in the number of piglets nursed. Milk production represents 65% to 80% of the energy requirements of lactating sows (Figure 4; National Research Council, 2012) and is the reason

Table 1 *Estimated daily milk production and mobilisation of body reserves[1] of lactating sows according to the number of piglets nursed per sow and weight at weaning*

Piglets per litter, *n*	10	12	14	16
Piglet weaning weight, kg	7.0	6.8	6.4	5.8
Milk production, kg/day	8.7	10.3	11.3	11.7
Sow BW gain, g/day	−206	−636	−915	−968
Sow body protein deposition, g/day	−21	−63	−91	−96
Sow body fat deposition, g/day	−103	−316	−455	−482

[1] Estimates derived from the NRC (2012) model assuming a feeding level of 6.5 kg/day of a lactation diet containing 13.8 MJ metabolisable energy per kilogram in a 21-day lactation for multiparous sows. Piglet growth rate estimated from published studies prior to the genetic selection for piglet birth weight (Beaulieu *et al.*, 2010; Huber *et al.*, 2015; Fan *et al.*, 2016; Strathe *et al.*, 2017a; Pedersen *et al.*, 2019), which is expected to increase piglet weaning weight.

Figure 4 Energy requirement estimates for maintenance and milk production and estimated energy intake of lactating sows. Estimates were derived from the NRC (2012) assuming 14 piglets per litter and 6.4 kg piglet weaning weight in a 21-day lactation for multiparous sows.

for an abrupt threefold increase in energy requirement within the first week of farrowing. The energy demand during lactation can impose a metabolic challenge to sows (Pedersen *et al.*, 2019). If energy intake is insufficient, sows prioritise and sustain milk production at the expense of their own body reserves (Table 1). Energy intake is typically lower than lactation requirements, resulting in sows with a negative energy balance during most of lactation (Figure 4; NRC, 2012). This demonstrates the biological inability of lactating sows to consume enough feed to meet the energy requirements and, at the same time, presents an opportunity to develop nutritional strategies to stimulate sows to achieve an optimal level of energy consumption with minimal mobilisation of body reserves.

The energy concentration of lactation diets is an important determinant of energy consumption and is typically modified by the use of fats, oils or fibres in the diet. An increase in dietary energy concentration typically represents an increase in energy intake at the same feed intake until a level at which the dietary energy concentration negatively affects feed intake (Xue *et al.*, 2012). Studies demonstrated that increasing the energy concentration of lactation diets from 12.8 to 13.4 MJ ME/kg improved energy intake and consequently reduced weight loss and increased litter growth rate during lactation (Xue *et al.*, 2012). However, lactation diets with a high energy concentration of 13.8 to 14.2 MJ ME/kg had a negative impact on feed intake (Xue *et al.*, 2012) and, thus, did not further increase energy intake.

Increasing energy density with fats or oils is a nutritional strategy that seems to be particularly important for lactating sows under heat stress conditions (Rosero *et al.*, 2012) and for prolific and high-producing lactating sows (Strathe *et al.*, 2017a). In a literature review, the addition of 2% to 11% fats and oils in lactation diets improved the energy intake of sows by an average of 7% or 4.6 MJ ME per day (Rosero *et al.*, 2016). As sows prioritise lactation needs, the additional energy is preferentially partitioned for milk and converted as milk fat output (Rosero *et al.*, 2015). Consequently, the benefits of greater energy intake are observed as improvements in litter growth rate because of a greater amount of energy provided through the milk (Rosero *et al.*, 2015, 2016). Similarly, lactation diets with high levels of dietary fibre resulted in a reduction in energy intake (Schoenherr *et al.*, 1989). Fibrous diets have low energy and bulk density, which physically restrict a sow's ability to consume the volume of feed necessary to achieve a high energy intake (Schoenherr *et al.*, 1989).

In summary, the addition of high-energy ingredients to lactation diets allows an increase in energy intake and energy output in milk. Consequently, there is a reduction in BW loss and an improvement in litter growth rate during lactation.

Amino acid and protein requirements in lactation

The amino acid requirements of high-producing lactating sows have increased substantially to support the milk production demand of large litters. The number of piglets nursed per sow as well as the litter growth rate during lactation dictate the amino acid requirements of lactating sows (Table 2). The amino acids for milk production represent most of the requirements, as lactating sows utilise as much as 70% of dietary protein for milk protein synthesis (Pedersen *et al.*, 2016). It appears that milk production is hardly changed by lactation diet because sows are able to mobilise body reserves (Noblet and Etienne, 1987). However, the supply of dietary amino acids and CP close to the requirements can improve milk protein output (Strathe *et al.*, 2017b)

Table 2 *Daily lysine requirement estimates[1] (grams of standardised ileal digestible lysine per day) of lactating sows according to the number of piglets nursed per sow and weight at weaning*

	Piglets per litter, *n*			
Piglet weaning weight, kg	10	12	14	16
5.8	43.0	47.5	52.2	57.0
6.0	43.8	48.3	53.2	58.3
6.4	45.3	50.2	55.4	60.7
6.8	46.8	52.0	57.5	63.2
7.0	47.5	53.0	58.6	64.3

[1] Estimates derived from the NRC (2012) model assuming a feeding level of 6.5 kg/day of a lactation diet containing 13.8 MJ metabolisable energy per kilogram in a 21-day lactation for multiparous sows. For primiparous sows, the lysine requirements in grams per day are approximately 5% lower due to lower milk production but approximately 5% higher as a diet percentage due to lower feed intake. Piglet growth rate estimated from published studies prior to the genetic selection for piglet birth weight (Beaulieu *et al.*, 2010; Huber *et al.*, 2015; Fan *et al.*, 2016; Strathe *et al.*, 2017a; Pedersen *et al.*, 2019), which is expected to increase piglet weaning weight.

and reduce muscle protein mobilisation in lactating sows (Gourley *et al.*, 2017; Pedersen *et al.*, 2019). Recent studies underline that a dietary intake of both balanced protein and essential amino acids is mutually important to sow and litter performance during lactation (Strathe *et al.*, 2017b; Huber *et al.*, 2018; Pedersen *et al.*, 2019).

Dietary intake of balanced protein supplies essential amino acids and nitrogen necessary to synthesise non-essential amino acids. The high-producing sow seems to benefit from a balanced protein intake during lactation by improving litter growth rate and reducing BW loss (Strathe *et al.*, 2017b, Pedersen *et al.*, 2019). Studies with high feed-grade amino acids suggested that increasing digestible CP up to 13.5% (approximately 15.5% CP) improved litter growth rate by increasing sow milk protein output (Strathe *et al.*, 2017b). Higher levels of digestible CP of 14.3% (approximately 16.5% CP) seemed to minimise sow BW loss by sparing muscle protein mobilisation for the purpose of milk production (Strathe *et al.*, 2017b). Thus, lactation diets may need a minimum digestible CP content of 13.5% to 14.3%.

Recently, several studies have evaluated amino acid requirements to ensure optimum performance of high-producing lactating sows. In general, the amino acid requirement estimates vary depending on performance criteria and statistical methodology applied in the study. Lysine requirement estimates are the most frequently studied, as models predict a substantial increase in Lys requirements of lactating sows with large, fast-growing litters (Table 2). The literature seems to agree on the effect of increasing dietary Lys intake to reduce BW loss and body protein mobilisation, but is conflicting in terms of the influence of dietary Lys intake on litter growth rate and subsequent reproductive performance (Xue *et al.*, 2012; Shi *et al.*, 2015; Gourley *et al.*, 2017). Studies using a range of 0.50 to 0.81 g SID Lys per MJ ME determined that the Lys requirement estimate to minimise sow BW loss in the lactation period is around 0.72 to 0.79 g SID Lys per MJ ME (Xue *et al.*, 2012; Shi *et al.*, 2015; Gourley *et al.*, 2017). Although the estimates seemed to be within the same range for primiparous and multiparous sows, the BW loss has been reported to be considerably greater in primiparous than multiparous sows, at around 12% (Shi *et al.*, 2015) and 7% (Xue *et al.*, 2012; Gourley *et al.*, 2017), respectively. The reduction in sow BW loss is presumably the consequence of a low mobilisation of muscle protein, as evidenced by a reduction in loin eye depth loss during lactation (Shi *et al.*, 2015; Gourley *et al.*, 2017). Lower concentrations of plasma urea nitrogen and plasma creatinine as a result of increased Lys intake support a reduction in sow body protein utilisation and muscle catabolism (Xue *et al.*, 2012). However, there is no consensus on the effect of dietary Lys on body fat stores (Shi *et al.*, 2015; Gourley *et al.*, 2017). It is proposed that the mobilisation of energy and protein are not completely independent. Thus, the interaction between amino acid and energy requirements is more complex and subject to factors involved in nutrient deficit, including energy and protein intake, energy and protein output in milk, growth rate of the litter and lactation length (Dourmad *et al.*, 2008).

Milk production and milk composition are arguably the most important factors capable of stimulating and supporting an improvement in litter growth rate (Strathe *et al.*, 2017b). However, the influence of dietary Lys intake on milk production and composition is not well understood. In a study with primiparous sows, milk protein content increased with dietary Lys levels up to 0.81 g SID Lys per MJ ME in a range of 0.55 to 0.81 g SID Lys per MJ ME (Shi *et al.*, 2015), but no other recent Lys requirement studies have evaluated sow milk composition (Xue *et al.*, 2012; Gourley *et al.*, 2017). In contrast, an increase in milk protein content is not reflected in an improved growth rate of primiparous litters (Shi *et al.*, 2015). While some studies observed no influence of dietary Lys intake on the growth rate of primiparous litters (Shi *et al.*, 2015; Gourley *et al.*, 2017), others suggested an improvement in litter growth rate up to 0.72 to 0.79 g SID Lys per MJ ME for primiparous and multiparous sows (Xue *et al.*, 2012; Gourley *et al.*, 2017). Estimating Lys requirements for litter growth rate is seemingly complex due to the capacity of sows to maintain milk production and sustain litter growth rate by mobilising body reserves (Noblet and Etienne, 1987). Moreover, the estimation of Lys requirements for litter growth rate probably requires a multifactorial approach by taking into account parity, lactation curve, daily Lys intake,

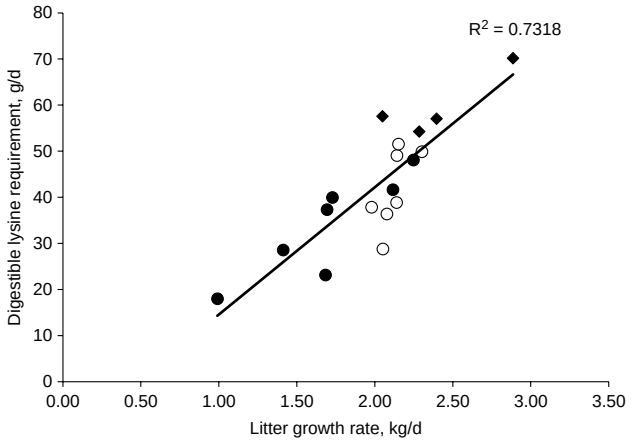

Figure 5 Regression curve to estimate the digestible lysine requirement to optimise litter growth rate from published studies. The regression curve originally derived from published lysine requirement studies from 1972 to 1997 summarised by Pettigrew (1993) in the solid circles and Boyd et al. (2000) in the open circles. The present updated curve contains data from studies published from 1998 to 2017, represented by the diamonds. The updated regression indicates that 27 g of digestible lysine intake per day is needed for each 1 kg of litter growth, and sows are expected to mobilise 13 g of lysine per day from body protein reserves.

growth rate of the litter, milk production and milk composition, as these factors affect how Lys is required and partitioned by lactating sows.

Interestingly, the amount of daily digestible Lys intake per kilogram of litter daily gain is consistent around 24 to 25 g for the recent studies on Lys requirements to improve litter growth rate for lactating sows (Xue *et al.*, 2012; Gourley *et al.*, 2017). Previous reviews conducted by Pettigrew (1993) and Boyd *et al.* (2000) determined a positive correlation between increased Lys requirements and litter growth rate. The regression using published data from 1972 to 1997 indicated that 26 g of total Lys or approximately 22 g of digestible lysine intake per day is needed for each 1 kg of litter growth, and sows are expected to mobilise 8 g of Lys per day from body protein reserves (Boyd *et al.*, 2000). The original equation has been updated (Figure 5) with Lys requirements for optimal litter growth rate from published studies conducted between 1998 and 2017 with primiparous and multiparous sows (Sauber *et al.*, 1998; Yang *et al.*, 2000, Xue *et al.*, 2012; Gourley *et al.*, 2017). The new regression predicted an increase to 27 g per day in the amount of digestible Lys intake required for each 1 kg of litter growth, and also an increase to 13 g per day in the expected mobilisation of Lys from body protein reserves. This predicted increase in the estimates of both Lys requirement and mobilisation of reserves coincides with the expectation for modern sows, which are leaner and higher milk producers than sow genotypes in the past.

It is well recognised that excessive weight loss and mobilisation of body reserves during lactation are associated with a prolonged wean-to-oestrus interval and inferior subsequent reproductive performance in sows (King, 1987; Koketsu *et al.*, 1996). Thus, the attenuation of lactational catabolism with an increase in dietary Lys intake in lactating sows (Xue *et al.*, 2012; Shi *et al.*, 2015; Gourley *et al.*, 2017) has been intuitively related to improvements in subsequent reproduction. Early studies consistently demonstrated the effect of amino acid intake on improving wean-to-oestrus interval and litter size (King, 1987; Touchette *et al.*, 1998), mediated by the release of reproductive and metabolic hormones (King and Martin, 1989; Tokach and Dial, 1992). However, the influence of dietary Lys intake on subsequent reproductive performance of modern sows is not as clear based on recent studies. There is evidence to suggest an improvement in the secretion of estradiol and luteinising hormone in primiparous and multiparous sows around the peak of lactation with dietary Lys levels of 0.72 to 0.79 g SID Lys per MJ ME (Xue *et al.*, 2012). These hormones play an important role in follicular development during lactation and cyclicity return after weaning (Soede *et al.*, 2011). Indeed, the same study demonstrated a short wean-to-oestrus interval with dietary Lys levels of 0.72 to 0.79 g SID Lys per MJ ME (Xue *et al.*, 2012). However, there is no consensus in the literature (Shi *et al.*, 2015; Gourley *et al.*, 2017). For primiparous sows, Gourley *et al.* (2017) fed dietary SID Lys of 0.52 to 0.81 g per MJ ME and observed an improvement in the number bred within 7 days after weaning; however, the effect on wean-to-oestrus interval is not consistent (Xue *et al.*, 2012; Shi *et al.*, 2015; Gourley *et al.*, 2017). The effect of dietary Lys on reproductive hormones during the first lactation was not evident in another recent study (Shi *et al.*, 2015). Likewise, dietary Lys levels during lactation did not seem to have an influence on the conception rate (Shi *et al.*, 2015) or the number of piglets born in the subsequent parturition (Gourley *et al.*, 2017).

The lack of a clear influence of dietary Lys intake during lactation on reproduction in the subsequent cycle seemed to corroborate with the remark that the reproductive performance of modern primiparous sows is increasingly resilient to the negative effects of tissue catabolism during lactation (Patterson *et al.*, 2011). Greater protein reserves of modern sows may provide more reserves to limit the dietary amino acid influence on subsequent reproduction.

The requirements of essential amino acids in milk and mammary gland tissue increase as the number of piglets nursed increases (Kim *et al.*, 2001). The most limiting amino acids for milk production are typically Lys, threonine and valine (Kim *et al.*, 2001; Soltwedel *et al.*, 2006); thus, the requirements of the latter amino acids as a ratio to Lys have been recently re-evaluated for high-producing lactating sows. The threonine requirement estimate to optimise the litter growth rate of lactating sows was approximately 65% of SID Lys with a range of 52% to 84% (Greiner *et al.*, 2018). However, the lack of other threonine requirement studies with modern lactating sows hinders the validation of threonine requirement estimates.

Recent studies did not reach a consensus about the requirement estimates of valine as a ratio to Lys. Valine concentrations above 76% of SID Lys provide no improvement in litter growth rate and sow backfat loss in a valine range of 76% to 97% of SID Lys (Strathe *et al.*, 2016). However, an

Table 3 *Daily phosphorus requirement estimates[1] (grams of standardised total tract digestible phosphorus per day) of lactating sows according to the number of piglets nursed per sow and weight at weaning*

	Piglets per litter, *n*			
Piglet weaning weight, kg	10	12	14	16
5.8	17.5	20.3	23.3	26.3
6.0	18.1	20.9	24.0	27.2
6.4	19.2	22.2	25.5	28.9
6.8	20.2	23.5	27.0	30.7
7.0	20.7	24.2	27.8	31.5

[1] Estimates derived from the NRC (2012) model assuming a feeding level of 6.5 kg/day of a lactation diet containing 13.8 MJ metabolisable energy per kilogram in a 21-day lactation for multiparous sows. For primiparous sows, phosphorus requirements in grams per day are approximately 5% lower due to lower milk production but approximately 5% higher as a diet percentage due to lower feed intake. Total calcium intake is estimated at two times the digestible phosphorus requirement. Piglet growth rate estimated from published studies prior to the genetic selection for piglet birth weight (Beaulieu *et al.*,2010; Huber *et al.*, 2015; Fan *et al.*, 2016; Strathe *et al.*, 2017a; Pedersen *et al.*, 2019), which is expected to increase piglet weaning weight.

improvement in both criteria was evident with very high levels of valine (113% of SID Lys) for litter growth rate and 88% of SID Lys for minimising backfat loss (Xu *et al.*, 2017). The requirement for valine in lactating sow diets seemed to be independent of total branched-chain amino acid concentrations, indicating that leucine and isoleucine do not spare the requirement of valine for sows in lactation (Moser *et al.*, 2000).

The requirement for tryptophan for lactating sows has been estimated to be 22% of SID Lys to maximise feed intake and at 26% of SID Lys to minimise BW loss in primiparous sows, with no effect on multiparous sows (Fan *et al.*, 2016). However, similar to threonine, the lack of other tryptophan requirement studies with modern lactating sows hinders the validation of tryptophan requirement estimates. Furthermore, studies evaluating the requirements of branched-chain amino acids and sulphur-containing amino acids, among others, for high-producing lactating sows are non-existent in recent literature.

The variation in amino acid requirements for lactating sows could be a consequence of the dynamic body tissue mobilisation during lactation (Kim *et al.*, 2009). The ideal dietary amino acid profile for lactating sows is influenced by the amino acid profile in milk and mammary gland tissue, and the amino acid resulting from body tissue mobilisation (Kim *et al.*, 2001). Because of these differences, threonine is a critical amino acid for sows with low lactation feed intake and substantial mobilisation of body reserves during lactation, whereas valine is an important amino acid for sows with high feed intake and limited mobilisation of body reserves during lactation (Kim *et al.*, 2001; Soltwedel *et al.*, 2006). Although the second- and third-limiting amino acids for lactating sows vary according to body tissue mobilisation, Lys is consistently the first-limiting amino acid (Kim *et al.*, 2001; Soltwedel *et al.*, 2006).

In summary, the dietary provision of amino acids close to the requirements of lactating sows allows a reduction in body protein mobilisation and has the potential to improve litter growth rate. The influence of amino acid intake on sow and litter performance seems to be even more complex for primiparous sows, as recent studies failed to report an amino acid-derived improvement in performance during the first lactation.

Calcium and phosphorus requirements in lactation

Calcium and phosphorus requirements for high-producing lactating sows have been currently estimated using a modelling approach (NRC, 2012). A scarcity of recent research prevents the validation of model-derived requirement estimates. The dynamic mobilisation of calcium and phosphorus in catabolic sows during lactation adds complexity to their requirement estimates using empirical studies.

The requirement estimates of calcium and phosphorus for lactating sows are primarily influenced by milk production (NRC, 2012). High-producing lactating sows with large, fast-growing litters have a considerable increase in calcium and phosphorus requirements (Table 3) in order to support their demand in milk production (Table 4). Moreover, calcium and phosphorus requirements are expected to increase throughout the lactation period following the sow milk production curve. The dietary intake of calcium and phosphorus is of great importance for primiparous sows to support their growth and development of bone and muscle tissues (NRC, 2012). Moreover, calcium and phosphorus are likely more critical for primiparous sows that might not have these mineral reserves for mobilisation as a multiparous sow.

Practical considerations in feeding programs

Diet formulation is only one step in developing a feeding program for today's sow. High feed intake is necessary to meet the energy and amino acid requirements of high-producing sows. The feeding system, environment, sow body condition and choice of ingredients will influence daily feed intake during lactation and have as much impact on sow productivity as nutrient levels in the diet.

Advances in feed delivery systems

Producers and researchers have long debated whether feed should be gradually increased during the first week of

Table 4 *Estimated daily calcium and phosphorus output[1] in sow milk according to the number of piglets nursed per sow and weight at weaning*

Piglets per litter, *n*	10	12	14	16
Piglet weaning weight, kg	7.0	6.8	6.4	5.8
Total calcium milk output, g/day	27.4	32.3	35.7	36.9
STTD phosphorus milk output, g/day	13.7	16.2	17.9	18.5

STTD = standardised total tract digestible.
[1] Estimates derived from the NRC (2012). Milk phosphorus is predicted from milk nitrogen output at a ratio between standardised total tract digestible phosphorus and nitrogen of 0.196. Milk calcium is predicted from milk phosphorus output at a ratio between total calcium and standardised total tract digestible phosphorus of 2. Piglet growth rate estimated from published studies prior to the genetic selection for piglet birth weight (Beaulieu *et al*., 2010; Huber *et al*., 2015; Fan *et al*., 2016; Strathe *et al*., 2017a; Pedersen *et al*., 2019), which is expected to increase piglet weaning weight.

lactation or provided *ad libitum* immediately after farrowing. Research in this area is not new, but continually showed that *ad libitum* feeding mostly results in a higher feed intake over the entire lactation phase than step-up programs (Stahly *et al*., 1979; Moser *et al*., 1987). The increased size of swine facilities coupled with advances in equipment design have made *ad libitum* feed delivery a reality in most large production systems.

Environment and sow intake

Sows maintained in the thermoneutral zone will have a higher feed intake than sows experiencing heat stress. McGlone *et al*. (1988) demonstrated that drip coolers were more effective at relieving heat stress than snout coolers or increases in diet energy density. Black *et al*. (1993) summarised that 'increasing heat loss from the sow, particularly through increasing the area of wet skin, has a greater positive effect on animal performance than modifying the diet'. An increased use of evaporative cool cells and drip coolers allows farms in hot climates to greatly increase feed intake compared to not using these technologies.

Gestation body condition

Numerous studies have demonstrated that sows with a higher backfat at farrowing have a lower feed intake during lactation than sows with a lower backfat at farrowing. Dourmad (1993) found that providing high levels of feed intake during gestation decreased lactation feed intake by resulting in smaller meals and shorter feeding duration. Increasing the fibre in gestation diet, while providing the same energy intake, increased meal frequency during lactation, but did not increase feed intake (Guillemet *et al*., 2006). Data from more modern sows (Kim *et al*., 2015) illustrate that lactation feed intake decreases linearly as backfat before farrowing increases, with the greatest decrease in feed intake for sows with >20 mm of backfat at farrowing.

Producers understand the importance of maintaining sows in the correct body condition, but have difficulty achieving the goal in the field. Sows are often over- or underconditioned on individual farms. Although ultrasound is a better tool to assess sow backfat than body condition score (Young *et al*., 2004), it can be too time-consuming and difficult to accomplish in the field. The invention of a sow caliper (Knauer and Baitinger, 2015) provides a fast, unbiased tool for producers to assess body condition.

Phase feeding

The information provided in this review suggests that phase feeding may provide benefits for lactating sows. A peripartum diet fed prior to and immediately after farrowing may be targeted towards reducing stillbirths and encouraging sow feed consumption. A lactation diet, fed for the remainder of lactation, would be designed for optimal milk production and subsequent reproduction. The use of a lower nutrient-dense diet until day 10 after farrowing lowered feed cost, but did not influence the performance of sows in a Danish commercial study (Sorensen, 2007). Similarly, Craig *et al*. (2016) found that feeding a constant energy level during lactation resulted in similar performance to sows that were offered a lower energy diet before day 14 and a higher energy diet after day 14 of lactation. Conversely, Pedersen *et al*. (2016) found that altering the diet to meet the sows' changing requirements as lactation progressed increased sow milk yield and pig weaning weight compared with feeding a single lactation diet; however, the single lactation diet used in the study was below the sow's requirement for amino acids for much of lactation. Thus, more research is needed to determine if providing two different diets during lactation provides any productivity benefits compared with feeding a single lactation diet that more closely meets the sows' requirements.

Conclusion

In summary, the lactating sow has demonstrated remarkable resiliency in the face of rapid improvements in production and nutritional challenges. Many practical aspects of meeting the nutrient requirements of high-producing sows have not changed. With increased milk production, amino acid and energy requirements must be met in order to avoid excessive body tissue catabolism. Future research needs to continue to improve our understanding of sow's requirements during the peripartum transition period to reduce farrowing duration and increase pig survival. Our knowledge of these and other facets of sow management will ultimately improve the welfare of the sow and her offspring.

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Declaration of interest

There are no conflicts of interest to declare.

Ethics committee

All work is within guidelines and requirements of ethics committees.

Software and data repository resources

None of the data were deposited in an official repository.

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Review: Maternal programming of development in the pig and the lactocrine hypothesis

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*Maternal effects on development are profound. Together, genetic and epigenetic maternal effects define the developmental trajectory of progeny and, ultimately, offspring phenotype. Maternally provisioned environmental conditions and signals affect conceptus, fetoplacental and postnatal development from the time of conception until weaning. In the pig, reproductive tract development is completed postnatally. Porcine uterine growth and uterine endometrial development occur in an ovary-independent manner between birth (postnatal day = **PND 0**) and PND 60. Milk-borne bioactive factors (**MbFs**), exemplified by relaxin, communicated from lactating dam to nursing offspring via a lactocrine mechanism, represent an important source of extraovarian uterotrophic support in the neonatal pig. Lactocrine deficiency from birth affects both the neonatal porcine uterine developmental program and trajectory of uterine development, with lasting consequences for endometrial function and uterine capacity in adult female pigs. The potential lactocrine signaling window extends from birth until the time of weaning. However, it is likely that the maternal lactocrine programming window – that period when MbFs communicated to nursing offspring have the greatest potential to affect critical organizational events in the neonate – encompasses a comparatively short period of time within 48 h of birth. Lactocrine deficiency from birth was associated with altered patterns of endometrial gene expression in neonatally lactocrine-deficient adult gilts during a critical period for conceptus–endometrial interaction on pregnancy day 13, and with reduced litter size, estimated at 1.4 pigs per litter, with no effect of parity. Data were interpreted to indicate that reproductive performance of female pigs that do not receive sufficient colostrum from birth is permanently impaired. Observations to date suggest that lactocrine-dependent maternal effects program postnatal development of the porcine uterus, endometrial functionality and uterine capacity. In this context, reproductive management strategies and husbandry guidelines should be refined to ensure that such practices promote environmental conditions that will optimize uterine capacity and fecundity. This will entail careful consideration of factors affecting lactation, the quality and abundance of colostrum/milk, and practices that will afford neonatal pigs with the opportunity to nurse and consume adequate amounts of colostrum.*

Keywords: colostrum, nursing, neonate, uterus, postnatal development

Implications

This review focuses on maternal lactocrine programming of postnatal reproductive tract development in pigs by way of mother's milk. The importance of nursing from birth on reproductive development and performance is emphasized, with data on both short-term effects in the neonate and long-term effects in adults. Data support the lactocrine hypothesis and milk as a conduit for delivery of maternally derived bioactive factors driving postnatal development. Results reinforce the importance of optimizing conditions that ensure adequate consumption of first milk (colostrum) by nursing young through effective reproductive management in swine production systems. Lactocrine programming has broad implications for human health.

Introduction

Maternal effects on development and reproductive efficiency include environmental conditions and signals provided by the dam that affect developmental trajectory and offspring

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phenotype (Bagnell and Bartol, 2019). For economically important domestic animals such as the pig (*Sus scrofa domesticus*), an optimal developmental trajectory is defined by a series of organizational events effecting a developmental program that results in an adult phenotype with potential for maximal fertility and fecundity. Because phenotype is not fixed genetically, but is defined dynamically through the course of development (Bartol *et al.*, 2017), a single genotype can produce more than one phenotype. Practically, these observations reinforce the importance of husbandry in management of animal production environments throughout the life cycle.

In the pig, as in other litter-bearing species, prolificacy – the number of offspring per litter – is a function of ovulation rate, fertilization rate and embryo survival (Kemp *et al.*, 2018). The latter is determined to a significant extent by functional uterine capacity, defined as the maximum number of fetuses that can be carried to term (Bennett and Leymaster, 1989; Vallet *et al.*, 2013a). Clearly, litter size can be no greater than either maternal ovulation rate or uterine capacity (Bennett and Leymaster, 1989). In a mathematical model, changing either ovulation rate or uterine capacity independently did not predict large changes in litter size (Bennett and Leymaster, 1989), indicating that both must change coordinately to achieve a positive effect on prolificacy. To complicate this picture, effective selection for larger litters is associated with lower and more variable piglet birth weights, and increased pre-weaning mortality (Kemp *et al.*, 2018). However, positive selection for uterine capacity did improve fetal survival and lifetime sow productivity (Freking *et al.*, 2016). Thus, conditions that optimize functional uterine capacity should optimize fecundity.

Large litter sizes characteristic of modern swine production challenge the capacity of highly prolific dams to support offspring postnatally (Foxcroft, 2012; Kraeling and Webel, 2015). In such circumstances, marked pre-weaning piglet mortality often reflects insufficient colostrum (first milk) consumption by piglets that, on a within litter basis, typically vary significantly in size and must compete for access to the udder and teat position (Wu *et al.*, 2010; Vallet *et al.*, 2015). Because they are immunologically incompetent at birth, colostrum consumption is essential for piglet survival (Vallet *et al.*, 2013b; Vallet *et al.*, 2015; Poonsuk and Zimmerman, 2018). Transmission of immunoglobulins and other milk-borne bioactive factors (**MbFs**) to nursing piglets in colostrum provides protection against infectious diseases and supports maturation of the gastrointestinal tract as they gain immunological competence during the first month of neonatal life (Poonsuk and Zimmerman, 2018). These observations alone encourage management strategies designed to improve colostrum availability and quality in order to minimize pre-weaning losses and optimize fecundity (Rohrer *et al.*, 2014; Vallet *et al.*, 2015; Farmer, 2018). However, beyond neonatal survival, evidence indicating that colostrum consumption on the day of birth (postnatal day = **PND 0**) has lasting effects on fecundity in adult female pigs (Bartol *et al.*, 2013; Vallet *et al.*, 2015) elevates the importance of colostrum (Vallet *et al.*, 2015) as a maternal factor affecting reproductive development and performance.

Studies of uterine development in the pig and other domestic ungulate species (Bartol *et al.*, 1993; Spencer *et al.*, 2019) established that female reproductive tract tissues, including the uterus, remain organizationally plastic during early neonatal life. Data for the pig (Bartol *et al.*, 1993), indicating that uterine growth and uterine wall development proceed normally prior to PND 60 in gilts ovariectomized at birth, suggested that extraovarian factors support porcine uterine development in the postnatal period. Colostrum was proposed as a potential source of such uterotrophic support (Yan *et al.*, 2006b; Bartol *et al.*, 2008). The term 'lactocrine' was coined to describe a mechanism by which MbFs are communicated from mother to offspring in colostrum/milk by consequence of nursing (Bartol *et al.*, 2008). The 'lactocrine hypothesis' for maternal programming of postnatal development posits that disruption of lactocrine signaling shortly after birth will alter the program and trajectory of development with short-term organizational effects and long-term consequences for adult phenotype. Studies designed to test the lactocrine hypothesis for maternal programming of uterine and reproductive development in the pig are summarized in several recent reviews (Bagnell *et al.*, 2017; Bartol *et al.*, 2017; Bagnell and Bartol, 2019). Here, objectives are to provide an overview of this work from a production point of view in the context of maternal programming of postnatal reproductive development and performance.

Milk as a delivery system for bioactive factors

Milk-borne bioactive factors of environmental origin

Maternal effects on development begin at conception and can be influenced *in utero* during pregnancy, as well as postnatally by environmental exposures that can alter the trajectory of offspring development. These effects can be either positive or negative and ultimately can influence the adult phenotype. There is a large literature in support of environmental effects (i.e., nutrition, climate and chemical or hormonal perturbations) during critical periods affecting the programming in early development of the fetus that led to the concept of developmental origins of health and disease (Barker, 1998). In addition, since development continues postnatally, nursing provides a means of extending maternal influence by delivery of MbFs, including environmental agents that can affect neonatal outcomes.

Maternal exposure to environmentally derived endocrine disrupting agents can have lasting consequences on offspring development into adulthood (Bartol and Bagnell, 2012). For example, bisphenol A (**BPA**), an estrogenic endocrine disrupting agent, was detected in both cow and human milk (Mendonca *et al.*, 2014). Relatively high BPA in maternal serum–breast milk dyad samples suggested high BPA exposure by consequence of nursing. Postnatal BPA exposure was linked to delays in development of the

pituitary–neuroendocrine axis and onset of puberty (Franssen *et al.*, 2016). Toxic environmental agents can also be concentrated in milk. Lipophilic xenobiotics can pass from maternal adipose stores into the maternal circulation and can be concentrated in milk (Lehmann *et al.*, 2014). Thus, in comparison to maternal exposure to environmental toxins, neonatal exposure to milk-borne toxicants may occur at higher levels, over shorter time periods when postnatal development of multiple organ systems is occurring. Since milk intake in neonatal pigs is estimated to be up to 30% of body weight (Coalson and Lecce, 1973), maternal exposure to environmental endocrine disrupting chemicals and/or toxicants could pose a significant exposure risk to nursing young (Bartol and Bagnell, 2012).

Milk-borne bioactive factors of maternal origin

In addition to providing nutritional and immunological support for developing offspring, colostrum/milk provides a means for delivery of a wide variety of maternally derived MbFs in support of neonatal growth and development. These include growth factors as well as steroid and peptide hormones found in higher concentrations in milk than in the maternal circulation. Metabolic hormones including leptin, ghrelin, adiponectin and glucocorticoids are transferred from mother to offspring in milk (Power and Schulkin, 2013) and can affect metabolism, growth and development. Glucocorticoids in milk were linked to more nervous and less confident temperament in both human and non-human primate offspring (Hinde *et al.*, 2015).

The value of maternally derived MbFs in support of postnatal development is evident from loss of function studies showing that the absence of specific MbFs in milk has deleterious effects on development. Increased adiposity and altered hypothalamic gene expression were found in wild-type mice fostered to interleukin-6-null dams, in which milk leptin content was twofold higher than in wild-type dams, suggesting that milk composition has programming effects on adiposity (Lager *et al.*, 2011). This supported earlier studies indicating that neonatal rats cross-fostered to enable nursing of diabetic dams showed hypothalamic changes and altered expression of genes involved in body weight regulation (Fahrenkrog *et al.*, 2004). Likewise, maternal tumor necrosis factor-α (**TNFα**) deficiency led to reduced milk chemokine levels and improved adult spatial memory, suggesting a TNFα-regulated lactocrine pathway programming brain development and memory (Liu *et al.*, 2014). Similarly, peroxisome proliferator-activated receptor-γ (**PPARγ**)-null mice produced a toxic milk, high in inflammatory lipids resulting in hair loss and growth retardation in nursing young, illustrating the importance of lactocrine-active PPARγ in protecting nursing offspring (Wan *et al.*, 2007).

Milk also contains small, non-coding microRNAs (**miRNAs**) that regulate gene expression by blocking translation and/or promoting messenger RNA (**mRNA**) degradation. These miRNAs are enclosed as cargo in milk-borne exosomes that also carry mRNA, protein and lipids and provide another means for lactocrine transmission of information. Exosomes, which protect miRNAs from degradation by heat and acidic conditions, are found in milk of several species (Bartol *et al.*, 2017) including the pig (Gu *et al.*, 2012). These milk-borne miRNAs pass the intestinal barrier and enter the bloodstream to target organ systems in a lactocrine manner. Data for the pig indicate that these milk-borne miRNAs are functionally important for development of the neonatal immune system (Gu *et al.*, 2012).

Maternal somatic cell transfer by way of milk is documented in several species as another means of communication with nursing offspring. Porcine milk contains maternal immune cells (Scharek-Tedin *et al.*, 2015) that, when ingested during nursing, can cross the neonatal intestine, enter the bloodstream and populate neonatal organs (Jain *et al.*, 1989). In humans, breast milk contains mammary stem cells that were reported to colonize neonatal tissues with potential to alter postnatal development by way of microchimerism (Barinaga, 2002). Collectively, these observations establish that milk is more than food (Hinde and German, 2012) and lactocrine transmission evolved as a means of delivering a plethora of non-nutritive, MbFs to nursing offspring (Bartol and Bagnell, 2012).

Relaxin: a prototypical lactocrine-active factor

A series of studies in neonatal gilts established relaxin as a prototypical lactocrine-active factor. Relaxin is a 6-kDa peptide hormone and member of a family of neohormones that evolved to support viviparity and lactation (Ivell and Anand-Ivell, 2017). Well known as a hormone of pregnancy, actions of relaxin also include effects on cervical connective tissue remodeling and growth-promoting effects on the uterus. Evidence for trophic effects of relaxin on the neonatal porcine uterus (Yan *et al.*, 2006a) led to studies designed to identify a source of relaxin in the neonatal pig. Porcine colostrum was identified as this source (Yan *et al.*, 2006b), an observation consistent with detection of relaxin in the milk of other species (Bagnell and Bartol, 2019). Detection of a bioactive factor in milk does not, by itself, constitute evidence for action of that MbF in the neonate. Therefore, criteria for determining the physiological relevance of MbFs in the neonate were defined (Peaker and Neville, 1991). Observations indicating that relaxin meets the criteria for a lactocrine-active MbF in the pig include (1) detection of biologically active prorelaxin in colostrum (Frankshun *et al.*, 2011); (2) immunoreactive relaxin detected in the neonatal circulation only in pigs allowed to nurse (Yan *et al.*, 2006b); (3) relaxin receptor (**RXFP1**) expression in porcine uterine (Yan *et al.*, 2006b) and cervical (Yan *et al.*, 2008) tissues from birth; (4) growth-promoting effects of relaxin administered for 2 days from birth on the neonatal uterus (Yan *et al.*, 2006a) and cervix (Yan *et al.*, 2008). Taken together, these studies established relaxin as a prototypical lactocrine-acting factor and supported the idea that milk is an important conduit for communication of MbFs to nursing offspring (Bagnell and Bartol, 2019).

Lactocrine effects on neonatal reproductive development

Uterus

To test the lactocrine hypothesis, a lactocrine-null condition was imposed during the first 48 h of life, by feeding a porcine milk replacer in lieu of nursing, and effects on uterine development were evaluated on PND 2 and PND 14. There was no effect of replacer feeding from birth on uterine weight (Chen *et al.*, 2011) or endometrial histoarchitecture (Miller *et al.*, 2013) by PND 2. However, in the absence of nursing, uterine glandular and luminal epithelial cell proliferation were reduced and there was a decrease in endometrial stromal estrogen receptor-α (**ESR1**) localization by PND 2 (Miller *et al.*, 2013). Effects of replacer feeding for 2 days from birth were evident by PND 14, when imposition of the lactocrine-null condition reduced both endometrial thickness and uterine gland development (Miller *et al.*, 2013). Antiadenogenic effects, including reduced gland penetration depth, observed at PND 14 in response to replacer feeding for 48 h from birth were similar to those observed in gilts treated daily from birth with the anti-estrogen ICI 182 780 (Tarleton *et al.*, 1999). Notably, returning gilts deprived of colostrum for the first 48 h of life to nursing at the end of PND 2 failed to rescue the uterine phenotype of reduced endometrial thickness and glandular development observed in replacer-fed gilts at PND 14 (Miller *et al.*, 2013) These studies reinforced the importance of lactocrine signaling from birth on uterine endometrial development in the pig.

Colostrum composition fluctuates over the course of porcine lactation. Consumption of colostrum can be delayed if sows fail to initiate lactation, fail to produce enough colostrum for the litter or if access to colostrum consumption is compromised by within litter competition for access to the udder (Vallet *et al.*, 2013b). In pigs, timing of colostrum intake coincides with a period of gut permeability to colostral macromolecules, which are typically present in high concentrations at birth and decline over the next 24 to 48 h in association with the loss of gut permeability, termed gut closure (Poonsuk and Zimmerman, 2018). In the tammar wallaby, timing of milk intake, milk composition and rate of milk production influence growth of pouch young and offspring phenotype dramatically (Trott *et al.*, 2003). Therefore, it was of interest to determine whether timing of colostrum consumption or duration of nursing in pigs affected lactocrine-mediated development of uterine and cervical tissues.

Studies of the neonatal porcine uterine transcriptome indicated that matrix metalloproteinases (**MMPs**) and tissue inhibitors of the MMPs (**TIMPs**) were affected by both age and lactocrine signaling between birth and PND 2 (Rahman *et al.*, 2016). The MMP2 and MMP9 gelatinases remodel the extra cellular matrix and are co-expressed with TIMPs, which regulate MMP activity. Uterine (Chen *et al.*, 2011;Ho *et al.*, 2017) and cervical (Frankshun *et al.*, 2012) proMMP9 (latent) and MMP9 (active) as well as uterine TIMP protein abundance was greater in nursed gilts when compared to replacer-fed animals. However, none of these proteins were detectable in porcine reproductive tissues when nursing was delayed by 12 h (Ho *et al.*, 2017). In addition, duration of nursing is important since extending nursing from 30 min to 12 h from birth, increased active and latent MMP9 proteins in reproductive tissues to levels comparable to those observed for gilts nursed for 2 days from birth (Ho *et al.*, 2017). By contrast, uterine MMP2 levels were detected but unchanged by age at first nursing or duration of nursing, indicating that not all uterine protein production is lactocrine-sensitive. Uterine MMP2 and MMP9 activities, detected by zymography, mirrored immunoblotting data. In other studies, a single feeding of colostrum was effective in supporting cervical (Camp *et al.*, 2014) and endometrial cell proliferation at 12 h postnatal (George *et al.*, 2018). In addition, there was no effect of method of delivery of a single dose of colostrum, either by nursing, bottle feeding or orogastric gavage, on uterine developmental markers at 12 h postnatal (George *et al.*, 2018). Collectively, these data indicate that both age at first nursing and duration of nursing are important in neonatal porcine female reproductive tract development, and that lactocrine effects can be detected in nursing piglets within 12 h of birth (Bagnell and Bartol, 2019).

Nursing and the neonatal uterine transcriptome

Global analysis of neonatal porcine gene expression in response to age and lactocrine signaling from birth to PND 2 was investigated by RNA sequencing (**RNAseq**) (Rahman *et al.*, 2016). With respect to age, more than 3200 uterine genes in nursed gilts and over 4500 genes in lactocrine-null gilts were differentially expressed on PND 2 when compared to uterine gene expression in uterine tissues obtained from gilts at birth. With respect to lactocrine effects, more than 890 differentially expressed genes were identified on PND 2 when nursed and milk replacer-fed gilts were compared. Bioinformatic analyses of biological processes revealed age-sensitive pathways that included ESR1 and hedgehog signaling cascades. Lactocrine-sensitive pathways in the neonatal porcine uterus identified on PND 2 included those involved in response to wounding, cell adhesion, the plasminogen activator network and coagulation (Rahman *et al.*, 2016).

Post-transcriptional regulation of gene expression by miRNAs is a mechanism that could be responsible, in part, for global uterine gene expression differences observed in response to age and nursing. Small non-coding miRNAs target mRNAs and can decrease mRNA stability and block translation. Consequently, effects of age and nursing from birth on the porcine uterine miRNA transcriptome were evaluated in tissues obtained on PND 2 (George *et al.*, 2017) using the same neonatal uterine tissues on which RNAseq analyses were performed (Rahman *et al.*, 2016). Integration of miRNAseq and mRNAseq data enabled target prediction analyses designed to identify potential miRNA–mRNA interactions. Results showed that about 10% of age- and lactocrine-sensitive differences in uterine gene expression could be explained by differential uterine miRNA expression

(George *et al.*, 2017). Biological processes predicted to be affected by age and nursing in uterine tissues on PND 2 as defined by the miRNA–mRNA interactome included cell-to-cell signaling, cell and tissue morphology, and cell growth and proliferation (George *et al.*, 2017). Observations were consistent with morphogenetic activities associated with uterine growth and endometrial development in the early neonatal period (Bartol *et al.*, 1993).

Cervix

Cervical histology of gilts nursed from birth to PND 2 was similar to that of replacer-fed gilts. However, by PND 14, imposition of the lactocrine-null state from birth reduced cervical crypt depth and luminal epithelial height when compared to gilts nursed over the same period (Camp *et al.*, 2014). Cervices from replacer-fed, PND 14 gilts were histologically similar to cervices from newborn pigs. Similar to observations for the uterus (Miller *et al.*, 2013), returning replacer-fed gilts to nursing on PND 2 failed to rescue the PND 14 cervical phenotype in that both cervical crypt and stromal cell proliferation at PND 14 were reduced to levels comparable to those reported for gilts fed milk replacer from birth (Camp *et al.*, 2014).

In other studies designed to evaluate short-term effects of nursing in the cervix and to develop a more efficient bioassay protocol for assessment of lactocrine effects in the neonate, a single feeding of hour 0 colostrum or milk replacer was given at birth, followed by milk replacer feeding through 12 h postnatal. The single feeding of colostrum, but not replacer, increased cervical cell proliferation by 12 h postnatal (Camp *et al.*, 2014). In addition, when delivered orally, IGF1 found naturally in relatively high concentrations in pig milk (Simmen *et al.*, 1988), increased cervical cell proliferation and markers of IGF1 action, including phosphorylated AKT and anti-apoptotic B-cell lymphoma 2(BCL2), by 12 h postnatal in both colostrum and replacer-fed gilts. Taken together, these data showed that nursing supports cervical development in neonatal pigs, and that IGF1 is a potential lactocrine-active factor for reproductive tract development as illustrated in a 12-h bioassay system to identify lactocrine active MbFs.

Testis

Evidence for lactocrine effects on development of the male reproductive system comes from studies on neonatal testicular development in boars (Rahman *et al.*, 2014). Development and proliferation of two major cell types in the pig testis, Sertoli (McCoard *et al.*, 2001) and Leydig cells (Franca *et al.*, 2000), occurs within the first month of neonatal life. In addition, Sertoli cell number, determined before puberty (Franca *et al.*, 2000), influences testicular size and sperm production (McCoard *et al.*, 2001), indicating that the neonatal period is critical for porcine testicular development.

Nursing for 2 days from birth increased Sertoli cell proliferation when compared to boars fed a commercial pig milk replacer over the same period (Rahman *et al.*, 2014). In a similar manner, Sertoli cell number and GATA4 protein abundance were greater in nursed boars on PND 2. There was no effect of age or nursing on Leydig cell-associated testicular protein levels, including the steroidogenic enzyme P450scc or insulin-like factor 3. However, testicular *RXFP1* expression increased from birth to PND 2 in replacer-fed, but not in nursed, boars. This was thought to be due, in part, to the absence of milk-borne relaxin in replacer-fed animals (Yan *et al.*, 2006b). Relaxin is detectable in colostrum and in the circulation of nursed pigs (Yan *et al.*, 2006b) and administration of exogenous relaxin decreased *RXFP1* expression in the neonatal porcine uterus and cervix (Yan *et al.*, 2008). Given that relaxin increased Sertoli cell proliferation *in vitro* (Cardoso *et al.*, 2010), the absence of milk-borne relaxin in replacer-fed boars could remove inhibition of testicular *RXFP1* expression and alter testicular development. Potential for maternal lactocrine programming of testicular function remains to be explored.

Long-term lactocrine effects in the adult

Lactocrine-null conditions, imposed experimentally by feeding milk replacer in lieu of nursing from birth, altered the developmental program in neonatal porcine reproductive tract tissues (Bagnell *et al.*, 2017; Bartol *et al.*, 2017). However, lactocrine deficiency can also occur naturally through maternal (e.g., mastitis and agalactia) as well as neonatal factors (e.g., within litter competition for teat position, birth rank and low birth weight) (Wu *et al.*, 2010). An immunoglobulin immunocrit assay, developed to monitor immunoglobulin transfer from mother to offspring during nursing (Vallet *et al.*, 2013b), was established as an indirect measure of colostrum intake in nursing pigs (Vallet *et al.*, 2015). The lactocrine hypothesis predicts that minimal colostrum consumption on PND 0, indicated by low serum immunocrit and lactocrine deficiency in nursing gilts, will be associated, ultimately, with reduced adult uterine capacity. A retrospective study of 381 gilts showed that low serum immunocrit on the day of birth was linked to reduced lifetime fecundity and live litter size across four parities (Bartol *et al.*, 2013). Subsequently, in a large prospective study, PND 0 immunocrit was obtained from 16 762 piglets and subsets of these gilts were assigned to study a variety of reproductive parameters (Vallet *et al.*, 2015). Results showed that low PND 0 immunocrit was associated with reduced growth and increased age at puberty. In addition, in a group of 799 females, low immunocrit on the day of birth was associated with reduced number of piglets born alive, consistent with the initial report (Bartol *et al.*, 2013). Litter size differences for adult females with low *v.* high PND 0 immunocrit were approximately 1.4 piglets per litter (Vallet *et al.*, 2015). In addition, high PND 0 immunocrit in neonatal gilts was linked to improved lactational performance when these females reached adulthood, suggesting lactocrine effects on programming of mammary gland function.

Disruption of uterine receptivity to implantation during the periattachment period of pregnancy can lead to reduced

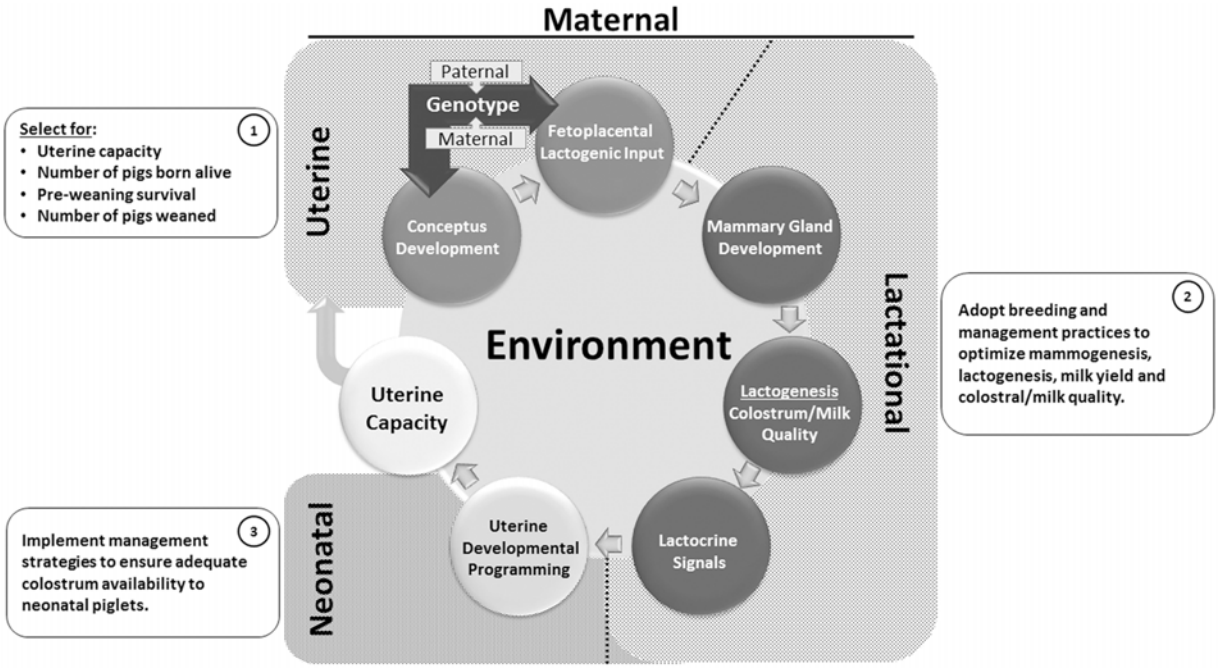

Figure 1. Programming porcine uterine capacity. Uterine capacity is determined by an interaction of genotype with maternally provisioned environmental conditions affecting mammogenesis, lactogenesis and lactocrine programming of postnatal uterine development. With conceptus genotype established, interactions between developing conceptuses and the intrauterine environment determine patterns of conceptus development, survival and fetoplacental lactogenic potential. In turn, endocrine conditions of pregnancy define patterns of mammogenesis and lactogenesis. Nursing ensures lactocrine transmission of MbFs. Lactocrine signaling affects the neonatal uterine developmental program, the trajectory of uterine development and uterine capacity. Reproductive performance of female piglets that do not receive sufficient colostrum is permanently impaired. Therefore, management strategies designed to improve colostrum quality and availability are important for optimization of uterine capacity. Practical actions to optimize uterine capacity and maternal lactocrine programming of postnatal development (boxes 1 to 3) include (1) selection for uterine capacity, number of piglets born alive, pre-weaning survival rate and number of pigs weaned; (2) adoption of breeding and management practices designed to optimize mammogenesis, lactogenesis, milk yield and colostral/milk quality; and (3) implementation of management strategies designed to ensure adequate colostrum availability to neonatal piglets. Adapted with permission from Bartol and Bagnell (2012). MbF = milk-borne bioactive factor.

reproductive performance in pigs (Bazer *et al.*, 2011). On pregnancy day (**PxD**) 13, elongated porcine conceptuses initiate attachment to uterine luminal epithelium. Documented negative effects of neonatal lactocrine deficiency on live litter size in adult female pigs (Bartol *et al.*, 2013; Vallet *et al.*, 2015) prompted study of the impact of lactocrine deficiency on the endometrial transcriptome during the periattachment period of early pregnancy on PxD 13. Global transcriptomic analysis revealed more than 1100 differentially expressed endometrial mRNAs at PxD 13 in high *v.* low immunocrit gilts (George *et al.*, 2019). In addition, in terms of miRNA–mRNA interactions, target prediction analysis revealed 5 differentially expressed miRNAs predicted to target over 60 differentially expressed mRNAs in the endometrium of high *v.* low immunocrit gilts on PxD 13. These endometrial mRNAs and related miRNA–mRNA interactions were associated with lactocrine-sensitive gene families for which predicted functions included solute transport, endometrial receptivity and immune response (George *et al.*, 2019). Taken together, these observations showed that impairment of reproductive performance in lactocrine-deficient, adult female pigs is reflected by alterations in endometrial gene expression in the periattachment period of early pregnancy.

Conclusions

Studies designed to test the lactocrine hypothesis for maternal programming of reproductive development and uterine capacity in the pig indicate that, beyond ensuring postnatal survival through the passive transmission of immune competence from mother to nursing offspring (Poonsuk and Zimmerman, 2018), lactocrine communication via colostrum affects uterine developmental trajectory and, ultimately, determines functional uterine capacity in adults. Genetically, selection for uterine capacity over 11 generations increased live litter size by approximately 1.6 piglets (Freking *et al.*, 2016). This kind of genetic advantage could be effectively negated by failure to ensure adequate colostrum consumption by nursing piglets at birth. Reduction in live litter size for neonatally lactocrine-deficient gilts was estimated at 1.4 piglets per litter with no effect of parity (Vallet *et al.*, 2015). Such permanent impairment of reproductive performance in adult, neonatally lactocrine-deficient female pigs is significant. Observations emphasize the importance of developmentally critical interactions between genotype and the maternally provisioned lactocrine environment in programming uterine capacity and reproductive efficiency. While MbFs responsible for lactocrine programming of

reproductive development remain to be defined, husbandry guidelines aimed at optimization of genetic and environmental conditions affecting porcine uterine capacity can be proposed, as summarized in Figure 1. Evidence for lactocrine programming of uterine development demands studies designed to identify MbFs responsible for lactocrine signaling and related mechanisms regulating organizational processes and events that ultimately determine adult uterine capacity and fecundity in the pig.

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Declaration of interest

Authors declare no conflict of interest.

Ethics statement

None.

Software and data repository resources

None.

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Review: kisspeptin and reproduction in the pig

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The activation of the hypothalamic–pituitary axis is critical for the initiation and maintenance of reproductive cycles in pigs and is influenced by a number of factors, such as nutrition, metabolism and gonadal steroids. Kisspeptin is a neuropeptide that is expressed in discrete regions of the porcine hypothalamus and is positioned to mediate the action of many of these factors. The expression of kisspeptin in the pig hypothalamus does not appear to be regulated by gonadal steroids in the same way as other species. It is unclear if kisspeptin is mediating nutritional or metabolic effects on gonadotropin secretion in pigs as it takes large deficits in feed intake or BW to affect hypothalamic expression of the KISS1 *gene in the porcine hypothalamus. There appears to be little genetic diversity in kisspeptin or its receptor that is useful for improving reproduction in swine. Both peripheral and central injection of kisspeptin strongly stimulates the secretion of gonadotropin hormones, LH and FSH, in gilts. Similarly, synthetic analogues have been developed and showed potential promise as tools to manage reproductive cycles in gilts and sows. Review of the literature nonetheless reveals that research on kisspeptin and its function in controlling reproduction in pigs has lagged that of other livestock species.*

Keywords: swine, gonadotropin, cyclicity, puberty, nutrition

Implications

Kisspeptin has emerged as a key regulator of reproductive function in pigs. It acts within the central nervous system to stimulate the secretion of reproductive hormones from the anterior pituitary gland that promote the initiation and maintenance of reproductive cycles. Kisspeptin holds tremendous promise to provide new methods to control reproduction and fertility in pork production; however, research in swine has fallen behind that of other livestock species. Given the unique differences in the regulation of reproduction between livestock species, pig-specific research is needed to fully capture the benefits that kisspeptin can bring to improving reproduction in pigs.

Introduction

Reproductive failure is the number one reason for culling gilts and sows (Knauer *et al.*, 2006; Tummaruk *et al.*, 2009). Approximately 30% of replacement gilts never farrow (Stancic *et al.*, 2011). A common reason for this is that gilts fail to become cyclic or their cyclicity is delayed beyond acceptable ages (Saito *et al.*, 2011). Initiation of puberty is dependent upon the activation of the hypothalamic–pituitary–gonadal axis. In general, pubertal development culminates with the activation of high-frequency pulses of LH (Diekman *et al.*, 1983; Lutz *et al.*, 1984; Camous *et al.*, 1985) that are driven by cyclic increases in the secretion of gonadotropin-releasing hormone (**GnRH**) from the hypothalamus (Lutz *et al.*, 1985; Kraeling and Barb, 1990). Inadequate gonadotropin secretion also results in pubertal failure and contributes to prolonged wean to oestrus intervals in postpartum sows (Edwards and Foxcroft, 1983). Moreover, insufficient gonadotropin secretion can underlie seasonal dips in cyclicity of gilts and sows (Armstrong *et al.*, 1986, Barb *et al.*, 1991). Even though it has been known for many decades that dysregulation of the hypothalamic–pituitary axis contributes to reproductive inefficiency of pigs that results in considerable financial loss, little progress has been made in understanding the regulation of this key physiological process. In the last decade and a half, kisspeptin has emerged as being critically important in controlling gonadotropin secretion and is central to the effects of nutrition, disease, stress and season on gonadotropin secretion in laboratory and livestock species. The intent of this review is to examine the current state of knowledge regarding kisspeptin and regulation of reproduction in the pig with reference to other species where information in the pig may be lacking.

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Kisspeptin and reproduction

Kisspeptin and central regulation of the reproductive neurosecretory axis

Kisspeptin is the peptide product of the *KISS1* gene (Kotani *et al.*, 2001; Ohtaki *et al.*, 2001). Synthesized as a pre-prohormone, kisspeptin is proteolytically cleaved to produce a series of peptides ranging from 10 to 54 amino acids in length. These kisspeptins share complete homology at the c-terminus and retain full biological activity. The kisspeptin receptor is a G-protein-coupled receptor that was found to be critical for the initiation of puberty in lab animals (Seminara *et al.*, 2003). Kisspeptin and kisspeptin receptor gene expression are hormonally regulated (Navarro *et al.*, 2004), and early studies revealed that central (intracerebroventricular, **ICV**) and peripheral (intravenous, **IV**; subcutaneous, **SC**) treatment with kisspeptin had potent stimulatory effects on the secretion of gonadotropin hormones in laboratory rodents (Matsui *et al.*, 2004; Thompson *et al.*, 2004) and nonhuman primates (Shahab *et al.*, 2005). It was subsequently reported that kisspeptin powerfully stimulates gonadotropin secretion, particularly LH secretion, in livestock species, including sheep (Caraty *et al.*, 2007a), goats (Hashizume *et al.*, 2010), cattle (Kadokawa *et al.*, 2008) and horses (Magee *et al.*, 2009). Prepubertal gilts received one of two doses of kisspeptin (10 or 100 μg) injected in to the lateral ventricles of the brain (Lents *et al.*, 2008). Both kisspeptin treatments produced a robust and immediate surge-like secretion of LH that was sustained for several hours. The 10-μg dose of kisspeptin elevated LH secretion for about 2 h, whereas the 100-μg dose increased LH for the entire 3-h post-injection sampling period. The magnitude of this LH release was one-half to two-thirds that induced by an IV injection of GnRH (100 μg). The 100-μg dose of kisspeptin also stimulated FSH secretion for over 3 h, and this release of FSH was similar in magnitude to that stimulated by GnRH. These results firmly established that kisspeptin is a potent stimulant of LH in the pig. The more potent effect of kisspeptin in stimulating secretion of LH compared with FSH was attributed to the fact that LH secretion is more responsive to GnRH than is FSH. It was concluded that kisspeptin plays a major role in the onset of LH pulses in the pig during puberty.

A substantial body of evidence indicates that the effect of kisspeptin to stimulate LH secretion occurs centrally within the hypothalamus. Kisspeptin receptor is expressed in GnRH neurons of the ovine hypothalamus (Smith *et al.*, 2011), and kisspeptin-stimulated secretion of LH in ewes is accompanied by a concomitant release of GnRH (Messager *et al.*, 2005; Caraty *et al.*, 2007b). Moreover, kisspeptin failed to stimulate LH secretion in ewes treated with neutralizing antibodies to GnRH (Arreguin-Arevalo *et al.*, 2007), and in ewes in which the hypothalamus had been disconnected from the pituitary to eliminate GnRH input to gonadotroph cells (Smith *et al.*, 2008b), demonstrating that kisspeptin stimulates LH secretion in a GnRH-dependent manner. Although similar studies have not been reported for pigs, the spatial distribution of kisspeptin expression within the porcine hypothalamus implies kisspeptin regulation of GnRH neurons in the pig as well.

Structural organization of kisspeptin neurons in the hypothalamus

In the central nervous system of rodents, kisspeptin cells are localized primarily in two discrete regions involved in the regulation of gonadotropin secretion, including the anteroventral periventricular nucleus (**AVPV**) near the preoptic area (**POA**) and the arcuate nucleus (**ARC**; Smith *et al.*, 2005). Livestock do not have a true AVPV similar to that in rodents; rather kisspeptin neurons are organized in the POA and ARC (Franceschini *et al.*, 2006). Immunoreactive kisspeptin was localized in these regions to cell bodies and fibres. In the POA of the ewe, kisspeptin immunostaining was observed to extend from the organum vasculosum of the lamina terminalis to the opening of the preoptic recess into the third ventricle. During sexual maturation in the ewe, messenger RNA (**mRNA**) for kisspeptin is expressed in the discrete region of the periventricular (**PeV**) nucleus (Redmond *et al.*, 2011a). Similarly, kisspeptin gene expression is found in the PeV of sexually mature and developing gilts (Tomikawa *et al.*, 2010; Ieda *et al.*, 2014). This is a similar area as the AVPV in mice and may be important for GnRH secretion controlling ovulation in pigs.

Kisspeptin mRNA is expressed in the medial basal hypothalamus (**MBH**) of gilts (Thorson *et al.*, 2017) within the ARC (Tomikawa *et al.*, 2010). A spatially distinct pattern of kisspeptin expression is seen throughout the ARC of the pig, with the greatest gene expression in the medio-caudal sections (Tomikawa *et al.*, 2010; Thorson *et al.*, 2018). This is similar to the ARC distribution of kisspeptin observed in sheep and cattle (Redmond *et al.*, 2011a; Cardoso *et al.*, 2015). The work on localization of kisspeptin within the porcine hypothalamus has thus far been limited to the expression of mRNA for the kisspeptin gene. Reports identifying the spatial distribution of kisspeptin-expressing cells with immunocytochemistry are lacking for pigs. Preliminary data demonstrate that neuronal cell body as well as nerve fibres can be identified within the porcine ARC (Figure 1). It is thus anticipated that the structural organization of kisspeptin neurons within the porcine ARC is similar to other species. Specifically, kisspeptin neurons in the POA act on GnRH cell bodies, whereas kisspeptin neurons in the ARC regulate GnRH terminal axons in the median eminence.

Oestrogen feedback and expression of kisspeptin

Escape from oestrogen negative feedback with advancing age is critical for increased LH secretion in gilts (Berardinelli *et al.*, 1984; Barb *et al.*, 2000; Barb *et al.*, 2010a), but the mechanisms behind this hormonal regulation of reproduction are not understood. Oestrogen receptor is not expressed in GnRH neurons, suggesting that the feedback effects of oestrogen on the GnRH pulse generator are mediated through other afferent neurons. Kisspeptin neurons in sheep express oestrogen receptor alpha (Franceschini

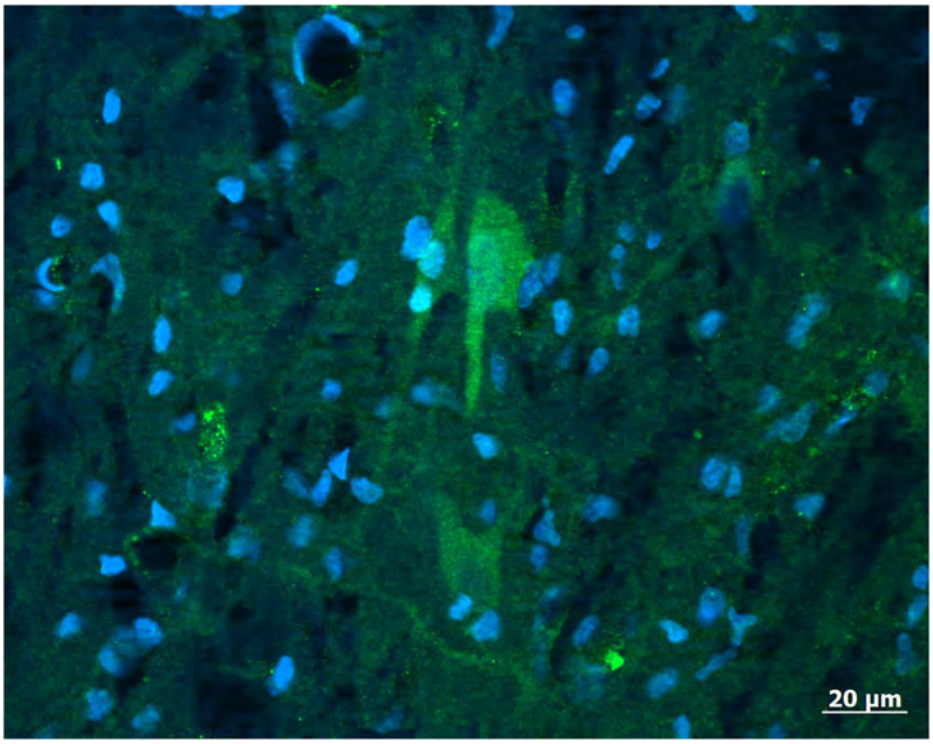

Figure 1 (Colour online) A population of neurons (arrow) positive for kisspeptin (green) and simultaneously counterstained with DAPI (4′,6-diamidino-2-phenylindolel; blue) to reveal neuronal and glia nuclei were found in the arcuate nucleus of the porcine hypothalamus. The antibody labelled cell body as well as nerve fibres. Image was captured with a Zeiss Axioplan 2 imaging photomicroscope (Carl Zeiss Vision, Oberkochen, Germany) equipped with a digital camera (Axio Cam MRc) and appropriate filters. The captured image was evaluated with the Axio Vision 4.6 Imaging system. For the preparation of microscopic illustrations, Corel Graphic Suite 11 was used only to adjust brightness, contrast and sharpness. *Source*: CA Lents, unpublished.

et al., 2006; Bedenbaugh *et al*., 2017), and oestrogen receptor signalling in kisspeptin neurons is critical for the timing of puberty in mice (Mayer *et al*., 2010). Oestrogen was shown to have profound negative effects on the expression of kisspeptin in the ARC of adult ewes (Smith *et al*., 2007), demonstrating that hypothalamic expression of kisspeptin is under the influence of gonadal steroids. In prepubertal ewe lambs, the expression of kisspeptin mRNA in the POA, PeV and ARC areas increased with pubertal maturation (Redmond *et al*., 2011a). The expression of kisspeptin in the ARC was correlated to the age-related increase in LH pulses, suggesting that kisspeptin neurons in this region of the hypothalamus are critical for the pubertal transition in gonadotropin secretion of sheep. This contrasts with the gilt in which the expression of the kisspeptin gene in the PeV region of the hypothalamus was undetectable, by *in situ* hybridization, during pubertal maturation (from 0 to 5 months of age) (Ieda *et al*., 2014). Robust expression of the kisspeptin gene in the ARC of these gilts was observed, but the ARC expression of kisspeptin did not differ with age or puberty status. This finding was corroborated by a recent report that the expression of kisspeptin gene in the MBH of prepubertal, peripubertal or postpubertal luteal phase gilts did not differ (Thorson *et al*., 2017). Thus, it appears that the pubertal decrease in sensitivity to oestrogen negative feedback in the gilt does not involve changing kisspeptin gene expression. However, it is noted that the gene expression for tachykinin 3 (*TAC3*), which encodes neurokinin B, and the tachykinin 3 receptor (*TAC3R*) gene is upregulated in the MBH of peripubertal gilts compared to prepubertal gilts (Thorson *et al*., 2017). Given the important role of neurokinin B, acting through its receptor, to stimulate kisspeptin and LH secretion (Nestor *et al*., 2012; Goodman *et al*., 2013), it is speculated that a reduced sensitivity to oestrogen negative feedback and the upregulation of LH pulse frequency in the gilt involve increased neurokinin B activation of kisspeptin neurons. In this regard, kisspeptin neurons in the ARC are likely critical for pubertal onset in gilts.

Oestrogen has a biphasic effect on LH secretion in pigs. Small developing follicles secrete low levels of oestrogen, which suppresses LH (Kesner *et al*., 1989). As circulating concentrations of oestrogen secreted from large preovulatory follicles increase, oestrogen acts positively to stimulate an ovulatory surge of LH about 72 h later (Kraeling *et al*., 1998). Treating ovariectomized ewes with a kisspeptin receptor antagonist (p-271) abolished the oestrogen-induced surge of LH (Smith *et al*., 2011), suggesting that kisspeptin is essential for the release of GnRH necessary to induce the LH surge. In this regard, the upregulation of kisspeptin expression in the PeV of the gilt may play a critical role in generating oestrogen-induced ovulatory surge of LH in pigs. When sexually mature ovariectomized gilts were treated with a dose of oestradiol that caused an ovulatory surge of LH, the expression of the kisspeptin gene in the PeV was increased compared to control ovariectomized gilts (Tomikawa *et al*., 2010). The expression of kisspeptin mRNA in the ARC,

however, did not differ between oestrogen-treated and control gilts. This implies that discrete subpopulations of kisspeptin neurons in the porcine hypothalamus independently control both surge and tonic secretion of LH.

Kisspeptin receptor gene is expressed in many tissues, including the hypothalamus (Li *et al*., 2008). The expression of the kisspeptin receptor gene in the porcine hypothalamus differs with stage of the oestrous cycle (Li *et al*., 2008). Hypothalamic expression of the kisspeptin receptor gene is increased near the time of puberty in rats and primates (Navarro *et al*., 2004; Shahab *et al*., 2005). These changes are driven by sex steroids from the maturing gonad. How kisspeptin receptor expression changes with sexual maturity or gonadal steroids in the pig is unknown.

Kisspeptin effects on other reproductive organs

The receptor for kisspeptin is expressed in the anterior pituitary gland and the gonad of the pig (Li *et al*., 2008), suggesting a direct effect of kisspeptin on these reproductive tissues. Kisspeptin neural fibres are located in the external zone of the median eminence (**ME**) of the ewe (Pompolo *et al*., 2006), and kisspeptin is secreted into the hypophyseal portal vasculature of sheep (Smith *et al*., 2008b) where it can affect pituitary function. In this regard, kisspeptin stimulated LH secretion from the primary cultures of ovine (Smith *et al*., 2008b) and porcine (Suzuki *et al*., 2008) anterior pituitary cells. In the porcine ovary, both kisspeptin and kisspeptin receptor are expressed in the granulosa cells and oocytes of developed follicles (Basini *et al*., 2018). Kisspeptin may function in the ovary for ovulation and development of the corpus luteum (Peng *et al*., 2013; Laoharatchatathanin *et al*., 2015). It was recently reported that kisspeptin improved *in vitro* development of porcine oocytes (Saadeldin *et al*., 2018). It is becoming increasingly evident that kisspeptin and its receptor can have important effects on peripheral reproductive tissues. What is unclear is the biological relevance this has for pig reproduction. Nonetheless, direct effects at the level of the anterior pituitary gland or ovary could have important implications in the development and use of kisspeptin or kisspeptin analogues for managing reproduction of gilts and sows.

Kisspeptin and nutritional regulation of reproduction

Changing energy balance and hypothalamic expression of kisspeptin

It is well established that the initiation of puberty and postpartum reproductive cycles in pigs are metabolically gated. In general, higher growth rates and backfat are positively associated with earlier cyclicity. Limiting dietary energy and amino acids, or feed restriction, even if supplying metabolizable energy above maintenance requirements, delays sexual maturity in gilts (Beltranena *et al*., 1991; Miller *et al*., 2011; Calderón Díaz *et al*., 2017) and decreases LH pulsatility (Prunier *et al*., 1993; Booth *et al*., 1996). Gilts of modern genotypes typically exhibit more than adequate growth rates (Amaral Filha *et al*., 2009), but these are less fat and are leaner than in previous generations. This may limit their ability to deal with short-term nutritional challenges associated with diet or housing changes. Gilts, in particular, are very sensitive to metabolic shifts, and even short-term energy restrictions (7 to 10 days) are sufficient to suppress LH pulses (Whisnant and Harrell, 2002; Thorson *et al*., 2018), and realimentation of gilts restores LH pulses in as little as 12 h (Booth *et al*., 1996). At issue here is what are the physiological mechanisms that underlie this nutritional regulation of tonic release of LH in gilts?

Zhou *et al*. (2014) restricted feed to cyclic gilts for a prolonged period (100 days), to the point that they ceased cycling. Nutritionally induced acyclicity in pigs results from a complete loss of LH pulsatility (Armstrong and Britt, 1987). Using quantitative PCR, it was shown that kisspeptin, kisspeptin receptor and GnRH mRNA expression were all downregulated in the MBH of nutritionally induced acyclic gilts (Zhou *et al*., 2014). This demonstrates that the negative effects of undernutrition on LH secretion in the pig may be mediated through the suppression of the kisspeptin neuronal system. Long-term undernutrition and prolonged BW loss, however, are not common under typical circumstances. More recently, Thorson *et al*. (2018) used short-term (10 days) negative energy balance to induce a mild loss in bodyweight that would be similar to what might occur under normal circumstances. In this case, ovariectomized feed-restricted gilts showed reduced frequency and increased amplitude of LH pulses. This change in LH pulse pattern is reflective of a late prepubertal or late lactation LH pulse pattern and would be insufficient to support final maturation and ovulation of ovarian follicles. In this study, *in situ* hybridization was used to measure the spatial distribution of kisspeptin mRNA expression throughout the entire hypothalamic ARC. No differences in ARC expression of the kisspeptin gene between feed-restricted and full-fed gilts were observed (Thorson *et al*., 2018). Thus, nutrition-induced changes in LH pulse patterns of gilts can occur without altering the transcription of hypothalamic kisspeptin, which in pigs appears to depend on the duration and magnitude of nutritional restriction.

Feed restriction has been the primary approach to understand nutritional regulation of the reproductive neurosecretory axis in livestock. The converse approach is to feed additional nutrients or energy. In one study, prepubertal gilts were fed either a standard diet formulated to meet the nutritional requirements for gilts (3.22 MCal/kg digestible energy, 19.1% CP) or the standard diet with additional energy in the form of added fat (Zhuo *et al*., 2014). As would be expected, the gilts fed the higher-energy diet exhibited greater BW gain and accumulation of backfat, resulting in their attaining puberty 12 days earlier than gilts fed the standard diet. When gene expression in the MBH was quantified with PCR, no differences in the expression of kisspeptin, kisspeptin receptor or GnRH mRNA were observed in gilts fed standard or high-energy diets. This would be consistent with the observation of Thorson *et al*. (2018) that modest energy restriction

of gilts did not affect ARC expression of kisspeptin. On the other hand, mRNA expressions for kisspeptin and its receptor were upregulated in the hypothalamic tissue containing the caudal POA and PeV of gilts fed a higher-energy diet (Zhuo *et al.*, 2014). The POA of the porcine hypothalamus contains a population of GnRH neurons that are considered critical for the initiation of pubertal cycles (Kineman *et al.*, 1988). This implies that dietary regulation of pulsatile secretion of LH in gilts may depend on hypothalamic subpopulations of kisspeptin neurons that respond differently to nutritional signals in regulating the GnRH pulse generator.

Kisspeptin links leptin with gonadotropin secretion

A nutritional signal that has important impacts on the reproductive axis of the pig is leptin (Barb *et al.*, 2008; Hausman *et al.*, 2012). Leptin is secreted by adipose tissue and is a key regulator of appetite. Age-related increases in the synthesis and secretion of leptin in the pig (Qian *et al.*, 1999) are associated with increased expression of leptin receptor in the hypothalamus (Lin *et al.*, 2000). Both events are related to increased secretion of LH during the pubertal escape from oestrogen negative feedback (Barb *et al.*, 2000; Barb *et al.*, 2010a). Furthermore, leptin stimulates the secretion of GnRH from the hypothalamus and LH from the anterior pituitary gland of prepubertal gilts (Barb *et al.*, 2004a). Consequently, leptin is considered a permissive metabolic signal for the initiation of puberty, and indeed, serum concentrations of leptin are genetically correlated with age at puberty in gilts (Kuehn *et al.*, 2009). Thus, leptin acting at the level of the hypothalamus is clearly important for the pubertal increase in pulsatile LH secretion in pigs.

Importantly, GnRH neurons lack leptin receptor (Quennell *et al.*, 2009; Louis *et al.*, 2011), which implies that leptin must affect GnRH secretion indirectly through second- or third-order neurons. Leptin receptors are expressed throughout the ARC of the pig hypothalamus (Lin *et al.*, 2001; Czaja *et al.*, 2002b), indicating that kisspeptin neurons in this region may be direct targets for leptin. In this regard, leptin stimulated the firing of kisspeptin neurons in hypothalamic slices of the ARC from guinea pigs (Qiu *et al.*, 2011). Although this is strong evidence for a direct effect of leptin on kisspeptin neurons, other studies have called this assumption into question. It was reported that few kisspeptin cells in the hypothalamus of mice or sheep co-localized with STAT3 (Louis *et al.*, 2011; Quennell *et al.*, 2011), the major intracellular signalling molecule induced by leptin acting at its receptor. These and other studies have led to the general conclusion that leptin probably affects the secretion of GnRH through other neuronal systems, such as neuropeptide Y (**NPY**).

Neuropeptide Y is an orexigenic neuropeptide that is negatively regulated by leptin. Leptin receptor is co-localized with NPY neurons in the pig (Czaja *et al.*, 2002a), demonstrating that NPY is a primary target for leptin in the porcine hypothalamus. When pigs received ICV injections of leptin, it suppressed the stimulatory effect of NPY on food intake (Barb *et al.*, 2006). Furthermore, the pulsatile secretion of LH in gilts is strongly suppressed by NPY (Barb *et al.*, 2006). This implies that leptin-induced reduction in NPY is important for increased LH pulses in gilts. Between 30% and 60% of kisspeptin cells in the ARC of sheep and cattle are in close apposition to NPY fibres (Backholer *et al.*, 2010; Alves *et al.*, 2015), suggesting that NPY might directly suppress kisspeptin neurons to affect GnRH secretion. Fibres for NPY are also in close contact with GnRH neurons in the POA and MBH of heifers (Alves *et al.*, 2015). When the heifers were fed a high-energy diet that promoted an early age at puberty, the proportion of GnRH neurons contacted by NPY fibres in the MBH were reduced compared with heifers fed a low-energy diet; however, diet did not alter the number of kisspeptin neurons contacted by NPY dendrites (Alves *et al.*, 2015). These data are interpreted to mean that when circulating concentrations of leptin are low, the expression of NPY is upregulated and suppresses the pulsatile secretion of LH by acting to directly inhibit GnRH release. The suppression of GnRH secretion by NPY may be further amplified by its inhibition of kisspeptin neurons, which attenuates kisspeptin-stimulated release of GnRH. The prepubertal rise of leptin in pigs likely suppresses the negative NPY tone and permits the transition to a higher LH pulse frequency for the initiation of puberty and reproductive cycles (Figure 2).

Increasing leptin suppresses feed intake not only by lessening NPY action but also by upregulating proopiomelanocortin (**POMC**) neurons in the hypothalamus (Barb *et al.*, 2008). The *POMC* gene produces the alpha-melanocyte-stimulating hormone (**α-MSH**), which, acting at melanocortin-3 and -4 receptors, is anorexigenic in pigs (Barb *et al.*, 2010b). The ARC of the porcine hypothalamus contains dense populations of POMC-expressing cells that project neural fibres rostrally to the POA and anterior hypothalamic area (**AHA**) (Kineman *et al.*, 1989) where GnRH neurons are found. This indicates a strong possibility for POMC to affect GnRH release in pigs directly or indirectly through kisspeptin neurons in the ARC. Many kisspeptin cells in the ARC of sheep and cattle are in close contact with POMC neuronal fibres (Backholer *et al.*, 2010; Cardoso *et al.*, 2015). Feeding heifers a high-energy diet, which reduced the age of first oestrus, resulted in greater circulating concentrations of leptin, greater expression of α-MSH in the ARC and more kisspeptin cells being contacted by POMC fibres (Cardoso *et al.*, 2015). These results suggest that increased signalling from POMC to kisspeptin neurons stimulates increased GnRH secretion for puberty in heifers. In support of this, treating ewes in the luteal phase of the oestrous cycle with a melanocortin agonist (MTII) increased POA expression of kisspeptin, which was accompanied by increased LH pulsatility (Backholer *et al.*, 2009). Whether the same relationship between kisspeptin and POMC neurons exists in the pig is unknown. There are distinct structural differences in the distribution of POMC neurons in pigs compared with ruminants. Unlike cattle (Leshin *et al.*, 1988), for example, pigs do not exhibit POMC fibres in the external zone of the ME (Kineman *et al.*, 1989). Treating gilts with a melanocortin antagonist (SHU9119) or a

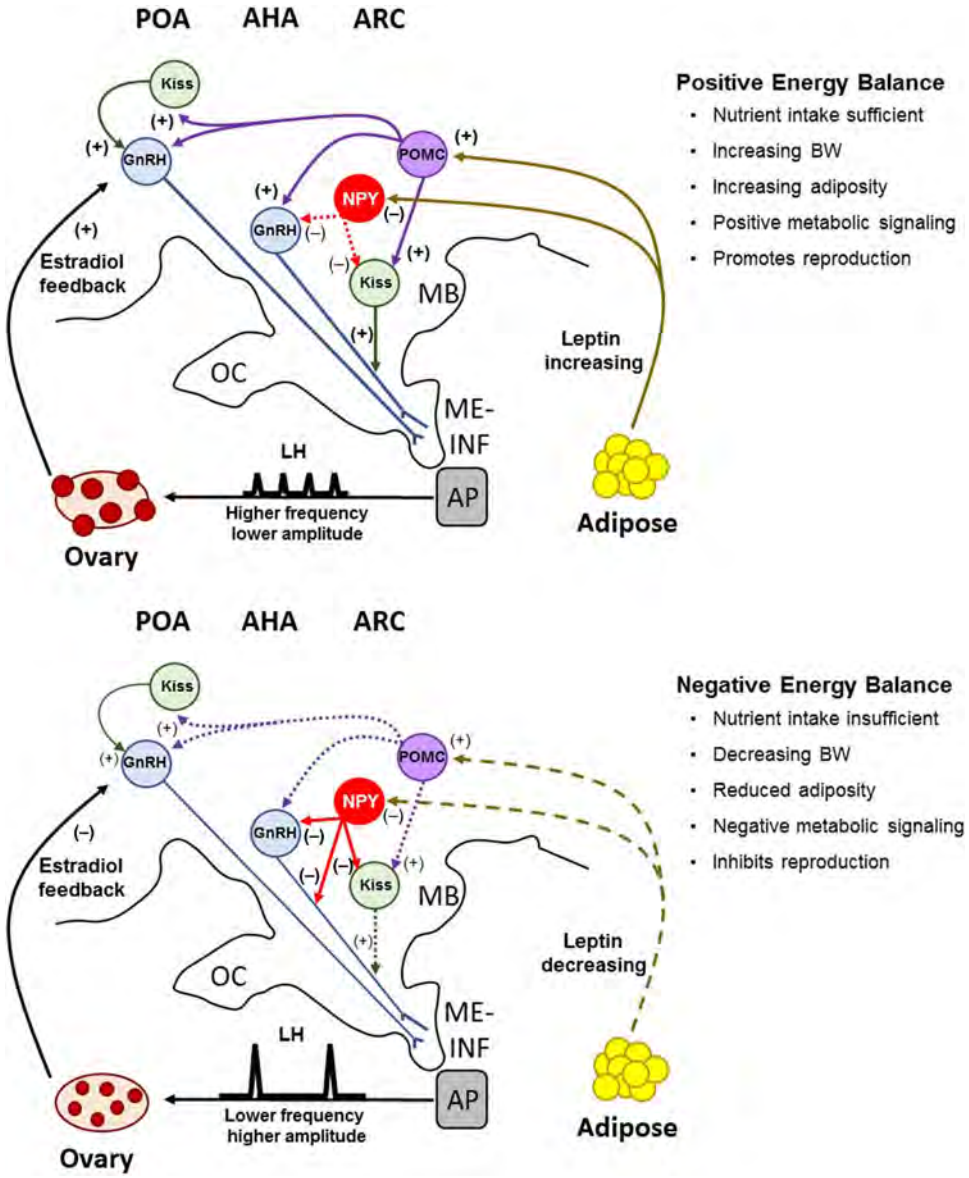

Figure 2 (colour online) A model depicting the proposed role of Kiss, NPY and POMC neuronal pathways and their regulation of GnRH secretion during positive and negative energy balance in the pig. Positive energy balance, characterized by nutrient sufficiency, is signalled to the hypothalamus by NPY and POMC cells in the ARC, which act as metabolic sensors for the activation of GnRH secretion. Increasing leptin inhibits the negative effects of NPY on GnRH and Kiss neurons, and upregulates the activation of POMC cells, which project axonal fibres rostrally to the POA and AHA. These afferent projections are predicted to impact both Kiss and GnRH expression cells in the POA and ARC, thus driving increased secretion of GnRH for high-frequency, low-amplitude pulses that promote ovarian follicular growth and maturation. During negative energy balance, leptin is reduced and lessens the excitatory signal (POMC) and increases the inhibitory signal (NPY). Remodelling of neuronal projections from NPY to GnRH neurons inhibit GnRH release for high-amplitude, low-frequency LH pulses that limit the maturation of ovarian follicles. The inhibitory signal of NPY to GnRH secretion is amplified by its inhibition of Kiss neuronal stimulation of GnRH secretion. These neuronal systems are sensitive to feedback from ovarian oestradiol, which switches from one of positive influence to one of negative influence when leptin concentrations are decreasing. OC=optic chiasm; MB=mammillary body; ME=median eminence; INF=infundibulum; AP=anterior pituitary gland; Kiss=kisspeptin; NPY=neuropeptide Y; POMC=proopiomelanocortin; GnRH=gonadotropin-releasing hormone; ARC=arcuate nucleus; AHA=anterior hypothalamic area; POA=preoptic area.

melanocortin agonist (NDP-MSH) failed to affect LH secretion (Barb *et al.*, 2004b). Caution should be used when interpreting these results because gilts were ovariectomized. Thus, whether POMC signalling at kisspeptin or GnRH neurons in the hypothalamus is related to the pubertal transition in pulsatile secretion of LH in gilts remains to be fully answered.

Kisspeptin and genetic control of fertility

A role for kisspeptin in reproduction did not become evident until it was discovered that some patients with hypogonadal hypogonadism (**HH**) harboured mutations in the gene for kisspeptin receptor (de Roux *et al.*, 2003; Seminara *et al.*, 2003; Semple *et al.*, 2005). Cases of HH are characterized by a lack of gonadal development and low levels of

gonadotropin secretion from the anterior pituitary gland. Individuals with HH arising from mutations in the kisspeptin receptor gene failed to transition through puberty. Patients with HH displayed gonadal responsiveness to treatment with exogenous gonadotropin. Moreover, the secretion of endogenous gonadotropins could be restored when individuals were treated with GnRH (Seminara *et al.*, 2003). Mouse models were developed in which the gene for kisspeptin or its receptor were knocked out (Funes *et al.*, 2003; Seminara *et al.*, 2003; d'Anglemont de Tassigny *et al.*, 2007; Lapatto *et al.*, 2007). Indeed, these mice recapitulated the HH phenotype and failed to become pubertal. Males had small testes with no sperm production, reduced sex steroids and lacked the development of secondary sex characteristics. Female kisspeptin receptor-knockout mice failed to have normal ovarian follicular maturation or to become pregnant upon exposure to fertile males. Despite being hypogonadotropic, these mice had normal levels of GnRH in the hypothalamus and a normative response to exogenous GnRH. Other than an infertile phenotype, these mice were completely normal.

Genetically modified large animal models for the study of the kisspeptin system did not exist until 2016 when pigs that contained an edit in the kisspeptin receptor gene were developed (Sonstegard *et al.*, 2016). Boars were produced through somatic cell nuclear transfer using cell lines from White Composite pigs that had been genetically edited with TALen gene editing techniques. The gene edit introduced a premature stop codon in the third exon to create a kisspeptin receptor knockout. The kisspeptin receptor-knockout pig produced a similar hypogonadal phenotype to that observed in mouse knockout models. Histological analysis revealed the formation of seminiferous tubules in the testes; however, no sperm was observed in the lumen of these tubules. The lack of sperm production was presumed to be a consequence of hypogonadotropism as indicated by low circulating concentrations of testosterone (0.20 to 0.25 ng/ml). Results from Sonstegard *et al.* (2016) unequivocally demonstrated that kisspeptin neuronal signalling in the hypothalamus is essential for sexual maturation in the pig.

Kisspeptin receptor-knockout boars were noted as being phenotypically normal with similar behaviour and growth as barrow pigs. Although intact, the low androgen production from kisspeptin receptor-knockout boars indicates that their risk of developing boar taint would be low. The possibility of utilizing pigs that are edited for kisspeptin or its receptor for pork production has been postulated (Sonstegard *et al.*, 2017), and there would be an obvious advantage from an animal welfare standpoint; pigs would not be subjected to castration. Beyond regulatory concerns is the obvious practical challenge of how to produce pigs at a commercial scale from animals with a gene edit that produces an infertile phenotype. When kisspeptin receptor-knockout pigs were treated with either exogenous gonadotropin hormones (both LH and FSH) or GnRH to initiate testicular development, testicular size increased over 200% compared to sham-treated kisspeptin-knockout boars (Sonstegard *et al.*, 2016). Despite histological evidence that testes of hormone-treated knockout boars could potentially support sperm production, no sperm were produced. This illustrates that managing the reproductive phenotype of kisspeptin receptor-knockout pigs will not be straightforward. Does postnatal proliferation of Sertoli cells in these pigs occur to support normal sperm production? Is the spermatogonial stem cell niche in these pigs properly established? These and other important questions will have to be addressed before genetic manipulation of the kisspeptin system for pork production could become practical. Nonetheless, these types of genetically modified pig models hold tremendous potential as a scientific tool to gain new insights into the reproductive development of pigs that could lead to new discoveries to improve pork production.

Genome-wide association studies have been conducted for delayed puberty and age at puberty in White crossbred and composite maternal lines of pigs (Tart *et al.*, 2013; Nonneman *et al.*, 2014 and 2016). Several genomic regions containing genes regulating growth, adiposity and neural function were identified; however, genomic associations for regions containing kisspeptin or its receptor were not identified. Using a genetic resource population developed from White Duroc × Chinese Erhualian crossbred pigs, Li *et al.* (2008) identified a number of polymorphisms in the kisspeptin receptor gene. Many of the alleles were fixed or occurred at a higher frequency in Chinese breeds than in Western breeds. Five different haplotypes for polymorphic sites within the kisspeptin receptor gene with frequencies greater than 0.01 were evaluated for association with age at puberty, but no significant association was detected, despite substantial variance in age at puberty within the population. Phenotypic associations for genetic variants that occur at low allele frequency are not easily detected in typical genomic analyses. Functional variants in kisspeptin receptor with deleterious effects would result in complete or substantial infertility and would not likely increase in allele frequency because gilts and boars harbouring such mutations would be quickly culled from the herd. In the case of the kisspeptin gene, it is small and nucleotide variation in the coding sequence is rare. The few kisspeptin sequence variants that have been found in humans are typically monoallelic, and in rare instances these are associated with a reproductive condition, these are more typically associated with precocious puberty rather than hypogonadotropism (De Guillebon *et al.*, 2011). Thus, it is unlikely that adequate genetic diversity exists in kisspeptin or kisspeptin receptor genes for genetic selection to improve gilt and sow fertility.

Use of kisspeptin and kisspeptin analogues for pharmacological control of reproductive cycles

The potential for kisspeptin to be useful in managing reproductive cycles and ovulation in livestock is obvious. The bulk of the work in this area has been conducted in sheep (Redmond *et al.*, 2011b) with a few studies in cattle (Whitlock *et al.*, 2008; Ezzat Ahmed *et al.*, 2009) and mares

(Magee *et al.*, 2009; Decourt *et al.*, 2014). These studies encompass a wide range of differences in seasonality, presence of gonadal steroid feedback, stage of the ovarian cycle and state of sexual maturation that must be taken into careful consideration when comparing studies. In general, IV injection of kisspeptin at doses ranging from 0.75 to 5 nmol/kg induce a surge-like release of LH in cattle and sheep (Caraty *et al.*, 2007a; Whitlock *et al.*, 2008; Redmond *et al.*, 2011b). Endocrine changes for prepubertal gilts treated with approximately 11.4 to 56.9 nmol/kg (768 to 3839 nmol per gilt) of kisspeptin have been reported (Lents *et al.*, 2008) and are similar to those observed in other species. All doses of kisspeptin induced a surge-like release of LH. The 11.4 nmol/kg dose of kisspeptin is probably maximal in pigs as there was no further increase in LH secretion beyond this dose. Regardless of treatment dose, the pulse of LH was transient, lasting for about 1.5 h before returning to baseline due to the short half-life (<60 s) of kisspeptin in serum. Moreover, FSH secretion in these gilts was not effectively stimulated by IV treatment with kisspeptin. These data were recently corroborated when it was reported that IV treatment of 18-week-old gilts with kisspeptin at even higher doses (3839 and 7678 nmol per gilt) induced similar secretory profiles of LH (Ralph *et al.*, 2017). The secretory profile of gonadotropins in prepubertal gilts treated with a single injection of kisspeptin would not be expected to affect ovarian follicular growth sufficiently to lead to oestrus and ovulation.

To sustain the secretion of gonadotropin hormones, repeated injections of kisspeptin are required. Repeated injections of kisspeptin to ovariectomized (Caraty *et al.*, 2007a) or seasonally anoestrous ewes (Caraty *et al.*, 2013) resulted in repeatable pulses of LH, but no effects on FSH. Similar results were obtained in prepubertal ewe lambs treated every hour for 24 h (Redmond *et al.*, 2011b). An LH surge occurred at about 17 h after the start of treatment in four of the six kisspeptin-treated lambs and resulted in ovulation. These lambs failed to maintain corpora lutea, suggesting that gonadotropin output after cessation of kisspeptin treatment was not adequate for luteotropic support. Sufficient sexual maturation in females must occur such that the GnRH pulse generator can be sustained to make kisspeptin treatment useful in prepubertal animals. The other approach that has been tested is to continuously infuse kisspeptin. In adult ovariectomized (Caraty *et al.*, 2007a) or anoestrous ewes (Caraty *et al.*, 2007a; Sebert *et al.*, 2010), continuous infusion resulted in an initial increase in LH secretion followed by an ovulatory surge of LH between 15 and 30 h later. Approximately 80% of kisspeptin-treated ewes ovulated. The initial kisspeptin-induced increase in LH and FSH was followed subsequently by increasing the concentrations of oestradiol from developing follicles that were critical for the induction of the subsequent LH surge. Similar increases in LH and FSH were seen with kisspeptin treatment in the mare, but kisspeptin failed to stimulate ovulation (Magee *et al.*, 2009; Decourt *et al.*, 2014). Important differences between mares and ewes are that mares are oestrus for a longer duration, the timing of the ovulatory LH surge relative to the start of oestrus is more variable, and the LH surge is more protracted. There have been no studies in pigs evaluating the effect of sustained infusion of kisspeptin on inducing an LH surge or ovulation; however, it is noted that a long duration of oestrus and variable timing of the LH surge relative to the onset of oestrus and ovulation in pigs is similar to that of the mare. This highlights the important need for species-specific studies in kisspeptin research.

Repeated injections or chronic infusion are not practical for the reproductive management of livestock. Some researchers have tried to alter kisspeptin to increase its half-life or potency to overcome this issue. This approach was successful for GnRH but has proven to be less useful for kisspeptin. There are some kisspeptin analogues that have been developed and tested for their ability to control reproduction in livestock (Table 1). The effects of kisspeptin analogue TAK683 (kisspeptin receptor agonist; Ac-[D-Tyr46, D-Trp47,Thr49,azaGly51,Arg(Me)53,Trp54]metastin(46-54)) on LH have been extensively characterized in goats. Small doses (3.5 nmol) given IV or SC to cyclic does after the removal of a progesterone device (CIDR) resulted in elevated concentrations of LH for 10 h (Kanai *et al.*, 2017). The SC treatment resulted in a preovulatory increase in oestradiol and advanced ovulation by 3 days. In ovariectomized does, TAK683 had minimal (Goto *et al.*, 2014) to no effect (Kanai *et al.*, 2017) on LH secretion, which highlights the importance of ovarian steroids in modulating the effects of kisspeptin analogues. Other studies reported that IV injections of 35 nmol of TAK683 induced surges of LH lasting 6 to 12 h in follicular and luteal-phase does (Endo *et al.*, 2015). The timing of oestradiol changes associated with TAK683 injection depended upon the phase of oestrous cycle at the time of treatment, but ovulation was induced in five of five does and four of five does when TAK683 treatment was initiated in the follicular and luteal phase of the oestrous cycle, respectively. The magnitude of LH release induced by TAK683 was greater when does were treated in the follicular phase (Endo *et al.*, 2015) than in the luteal phase (Endo *et al.*, 2015; Rahayu *et al.*, 2017).

Although these data would support the expectation that TAK683 could be useful for inducing or timing ovulation in livestock, there are negative effects. The TAK683 molecule was developed as a kisspeptin receptor antagonist. After the initial increase in LH secretion, TAK683 suppresses LH pulsatility and circulating concentrations of LH within 24 h from the start of treatment (Yamamura *et al.*, 2014). Pulsatile secretion of LH was completely abolished in ovariectomized does treated with a 5-day SC infusion (500 nmol/kg) of TAK683. This effect has intriguing potential for managing reproduction in pigs. Altrenogest is the only approved product available to prevent oestrus and ovulation in pigs and is used extensively to synchronize oestrus. Kisspeptin antagonists could be a useful alternative because pituitary responsiveness to GnRH remains intact. Chronic SC infusion of TAK683 appears to abolish LH pulses in goats without affecting the GnRH pulse generator (Yamamura *et al.*, 2014). However, there may be negative effects on ovarian follicles

Table 1 Effect of kisspeptin analogues on the indices of reproductive function in livestock

Species (sex)	Reproductive status	Compound	Dose	Effects on				References
				LH (duration)	FSH (duration)	Gonadal steroids	Ovulation	
Goat (doe)	Adult OVX	TAK683	5 μg, IV	No effect	Not reported			Kanai *et al.* (2017)
Goat (doe)	Cyclic adult, synchronized	TAK683	5 μg, IV, SC	Increased (10 h)	Not reported	Initial increase	Advanced 3 days	Kanai *et al.* (2017)
Goat (doe)	Adult OVX	TAK683	500 nmol/kg per week, SC	Abolished LH pulses	Not reported			Tanaka *et al.* (2013)
Goat (doe)	Adult OVX	TAK683	35 nmol, IV	Increase (4 h)	Not reported			Goto *et al.* (2014)
Goat (doe)	Adult cyclic, synchronized	TAK683	35 nmol, IV	Increased (6 h)	Increased (6 h)	Immediate suppression	3 of 4	Goto *et al.* (2014)
Goat (doe)	Adult cyclic, synchronized	TAK683	35 nmol, IV	Immediate surge (8 h)	Not reported	Supressed after 6 h	5 of 5	Endo *et al.* (2015)
Goat (doe)	Adult cyclic, day 3 of CIDR	TAK683	35 nmol, IV	Increased at 12 h	Not reported	Increased after 6 h	4 of 5	Endo *et al.* (2015)
Goat (doe)	Adult cyclic, day 5 luteal	TAK683	35 nmol, IV	Increased by 14 h	Not reported	Increased with LH		Rahayu *et al.* (2017)
Goat (doe)	Adult cyclic, day 9 luteal	TAK683	35 nmol, IV	Minimal increase	Not reported	No effect		Rahayu *et al.* (2017)
Sheep (ewe)	Adult noncyclic	FTM080	0.5, 2.5, 5 nmol, IV	Increased <1 h				Whitlock *et al.* (2015)
Sheep (ewe)	Adult noncyclic	C17	15 nmol, IM	Increased (9 h)	Increased (5 h)			Beltramo *et al.* (2015)
Sheep (ewe)	Adult cyclic, synchronized	C6	15 nmol, IM	Increased (10 h)	Increased (5 h), again 24 h later		12 of 12	Decourt *et al.* (2016)
Sheep (ewe)	Adult noncyclic, progestin primed	C6	15 nmol, IM	Increased (12 h)	Not reported		8 of 12	Decourt *et al.* (2016)
Sheep (ram)	Adult	C6	15 nmol, IM	Increased (10 h)	Not reported	Increased >12 h		Beltramo and Decourt (2018)
Cattle (bull)	Prepubertal	C6	20 nmol, IM, daily, 4 days	Day 1, increased (5 h); day 4, subacute	No effect	No effect		Parker *et al.* (2019)
Pig (gilt)	Prepubertal, after PG600 and Matrix	C6	60 nmol, IM	Increased (>16 h)			5 of 7	Ralph *et al.* (2018)

OVX=ovariectomized; CIDR=progesterone-controlled internal drug release; PG600=400 IU pregnant mare serum gonadotropin and 200 IU chorionic gonadotropin; TAK683=Ac-[D-Tyr46,D-Trp47,Thr49,azaGly51,Arg(Me)53,Trp54] metastin(46-54); C17=Palm-γ-Glu-Tyr-Lys[γ(N-Palm-Glu)]-Trp-Asn-Ser-Phe-Glyψ[Tz]Leu-Arg-Tyr-NH2; C6=Palm-γ-Glu-Tyr-Asn-Trp-Asn-Ser-Phe-Glyψ[Tz]Leu-Arg(Me)-Tyr-NH2; FTM080=4-Fluorobenzoyl-Phe-Gly-Leu-Arg-Trp-NH2; IV=intravenous; SC=subcutaneous; IM=intramuscular.

at subsequent ovulations. Goto *et al.* (2014) reported that ovulations induced in cyclic does with TAK683 resulted in lower levels of progesterone during the luteal cycle. Moreover, the emergence of new follicles was delayed in these goats. Consequently, they ovulated smaller, less mature follicles in the subsequent oestrous cycle, which would be expected to reduce fertility. These and other issues will need to be addressed for the effective use of such kisspeptin antagonists in pork production.

Another well-characterized compound is the kisspeptin receptor agonist C6 (Palm-γ-Glu-Tyr-Asn-Trp-Asn-Ser-Phe-Glyψ[Tz]Leu-Arg(Me)-Tyr-NH2). In adult rams, i.m. injections of C6 (15 nmol) resulted in a sustained (10 h) increase in circulating concentrations of LH that were accompanied by elevated concentrations of testosterone (Beltramo and Decourt, 2018). Prepubertal bulls treated with C6 exhibited greater secretion of LH lasting 6 h, but FSH and testosterone were unaffected (Parker *et al.*, 2019). This may indicate that adequate pubertal development must occur in bulls to achieve the expected gonadal response. Moreover, repeated injections of C6 to prepubertal bulls for 4 days resulted in a loss of LH secretory response to treatment, possibly resulting from kisspeptin receptor desensitization. In adult acyclic ewes primed with progesterone or in cyclic ewes, a single IM injection of C6 resulted in an increase in LH secretion lasting 10 to 12 h (Decourt *et al.*, 2016). This treatment in cyclic ewes resulted in an initial surge-like release of FSH followed by a sustained increase in the secretion of FSH, such as would follow ovulation, approximately 24 h after C6 treatment started. Indeed, all 12 of the cyclic ewes treated with C6 ovulated, and 8 of 12 noncyclic ewes ovulated in response to C6 treatment. A preliminary report indicated that a single IM injection of C6 to 18-week-old gilts caused a sustained increase in LH secretion resulting in ovulation in five of seven gilts (Ralph *et al.*, 2018). In this case, gilts had been primed with human chorionic and pregnant mare serum gonadotropin followed by altrenogest such that adequate follicle development was present for ovulation to occur. It is not clear if C6 may be useful for stimulating oestrus and ovulation in non-steroid-primed prepubertal gilts or postpartum sows. Nonetheless, the results point to a real possibility that kisspeptin analogues could be developed for the control of reproduction in pigs.

Is kisspeptin involved in seasonal infertility of swine?

In the northern hemisphere, seasonal decreases in fertility are evident in commercial swine. This seasonal infertility is a decrease in the reproductive performance of pigs during summer and early fall (July, August, September) that manifests as reduced farrowing rates during the winter months (November, December, January). In the southern hemisphere, decreased farrowing rates are observed in late summer to early autumn. Seasonal infertility usually affects 5% to 10% of breeding females, but this can be as high as 25% in some geographical locations or production systems. The summer decrease in reproductive performance of pigs is generally characterized by decreased cyclicity of replacement gilts, increased wean to oestrus intervals of sows, increased loss of pregnancy and reduced litter size, with gilts and first parity sows being most affected (Tummaruk *et al.*, 2007; Bloemhof *et al.*, 2013). It must be noted that seasonal infertility is an issue affecting all geographical regions of pork production and a wide array of management and housing systems. Many risk factors, including heat stress, nutritional stress, immune function, ovarian function, uterine function, and others, have been linked to seasonal infertility of pigs, which illustrates the extremely complex aetiology of the problem. The discussion that follows is highly focused. For a broader perspective, the reader is referred to more comprehensive reviews (Love *et al.*, 1993; Peltoniemi and Virolainen, 2006; Bertoldo *et al.*, 2012; De Rensis *et al.*, 2017).

It is generally concluded that seasonal infertility in swine involves, to some extent, inadequate gonadotropin secretion. Reduced GnRH, LH and FSH in weaned sows has been reported during summer months (Armstrong *et al.*, 1986). The reason for this is not fully understood, but it is speculated to be related to increased sensitivity to oestradiol negative feedback during summer months. The suppressive effect of oestradiol on basal concentrations of LH and LH pulse amplitude of gilts, however, was greater during short-day photoperiod than in long-day photoperiod (see Love *et al.*, 1993). Further, season had little to no effect on the LH secretory response of ovariectomized gilts treated with low or high levels of oestradiol, nor was there a season × oestradiol interaction on the response of gilts to exogenous GnRH (Cox *et al.*, 1987; Smith *et al.*, 1991). Seasonally anoestrus weaned primiparous sows responded normally to pulsatile treatment with GnRH (Armstrong and Britt, 1985), exogenous oestradiol (Cox *et al.*, 1983) and gonadotropin hormones (Britt *et al.*, 1986), suggesting that the hypothalamic–pituitary–ovarian–axis of sows remains fully functional during summer months. These data indicate that any potential seasonal effect on gonadotropin secretion in the pig lies upstream of GnRH, but seasonal responses to oestradiol feedback are yet to be fully resolved.

Seasonal acyclicity exhibited by caprine and ovine species is determined by increases in daylength that result in an increase in sensitivity to oestrogen negative feedback and molecular changes in the expression of kisspeptin in the hypothalamus associated with a reduced frequency of LH pulses (Smith *et al.*, 2007; Smith *et al.*, 2008a). Seasonal infertility of domestic swine coincides with the nonbreeding season of Eurasian wild boar (Mauget, 1982), and it has been proposed that the kisspeptin neuronal system may underlie seasonal differences in gonadotropin secretion of domestic sows (De Rensis *et al.*, 2017). There have been no published studies evaluating the effect of season, photoperiod or melatonin on kisspeptin function in pigs; so this hypothesis remains to be tested. It is difficult to unravel the confounding of seasonal changes in temperature and photoperiod, but these factors can have independent effects on sow fertility (Tantasuparuk *et al.*, 2000; Sevillano *et al.*, 2016) (Auvigne *et al.*, 2010). Regardless, seasonal effects on LH seem to

be largely related to a suppression of feed intake and its associated metabolic state. As discussed above, fluctuations in nutrient intake seem to have little effect on the expression of kisspeptin mRNA unless nutritional restriction is severe and prolonged. Whether differences in feed intake are affecting the transcription and expression of kisspeptin protein, however, remain to be determined. Administering kisspeptin or its analogues to seasonal anoestrous ewes overcomes the seasonal inhibition of LH secretion leading to ovulation (Caraty *et al.*, 2007a; Sebert *et al.*, 2010; Decourt *et al.*, 2016). It is expected that as kisspeptin analogues become developed and optimized for use in swine, these will be useful tools for mitigating seasonal effects on sow fertility.

Conclusion

Strong evidence has accumulated that kisspeptin is a critical component in the regulation of gonadotropin secretion and reproduction in swine. Anatomical distribution of the kisspeptin neuronal system is organized similarly in the pig as other species, but there are clear differences in its regulation, particularly from gonadal steroids. In this regard, research on the role of kisspeptin in regulating reproductive biology of pigs has lagged that of other livestock species, particularly sheep and goats. This is a critical area of need going forward because of the distinct physiological differences between species. Kisspeptin and kisspeptin analogues demonstrate promise for use in managing reproductive cycles and ovulation in pigs. However, pig-specific research is needed to optimize the use of such compounds for a precise control of fertility in pork production.

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None.

Declaration of interest

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Ethics statement

No relevant information to declare for review article.

Software and data repository resources

None of the data were deposited in an official repository.

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Review: What innovations in pain measurement and control might be possible if we could quantify the neuroimmune synapse?

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It has taken more than 40 years for the fields of immunology and neuroscience to capture the potential impact of the mechanistic understanding of how an active immune signalling brain might function. These developments have grown an appreciation for the immunocompetent cells of the central nervous system and their key role in the health and disease of the brain and spinal cord. Moreover, the understanding of the bidirectional communication between the brain and the peripheral immune system has evolved to capture an understanding of how mood can alter immune function and vice versa*. These concepts are rapidly evolving the field of psychiatry and medicine as a whole. However, the advances in human medicine have not been capitalised upon yet in animal husbandry practice. Of specific attention are the implications that these biological systems have for creating and maintaining heightened pain states. This review will outline the key concepts of brain–immune communication and the immediate opportunities targeting this biology can have for husbandry practices, with a specific focus on pain.*

Keywords: pain, glia, biophotonics, biomarker, animal welfare

Implications

The intimate relationship between neuronal processes and glial function is driving a neuroscience revolution. There is a growing appreciation for the active role glia and immune-like signalling within the central nervous system has in changing brain function and hence behaviour of the organism. This is of specific importance to the objective quantification and treatment of pain. Importantly, by targeting the neuroimmune synapse it may be possible to create disease-modifying pain treatments.

Introduction

How do we know we are sick and why is this concept important for quantifying behaviour?

Illness is actively avoided in human and animal population (Yirmiya *et al.*, 2000). All species implement a myriad of physiological and behavioural practices to diminish the likelihood of experiencing illness. In addition, multiple cellular and molecular capacities have evolved to protect and defend the host from pathogens (Yirmiya *et al.*, 2000). But if illness is encountered, how does the individual know it is sick? How does the organism know to adapt at a whole system behavioural level to the presence of a molecular toxin or cellular pathogen? This may seem like a simple question, yet applying the *Descartes* philosophy of the body as a machine, with each anatomical part able to work in isolation. However, this philosophical and experimental approach has not been successfully applied to solve this simple question. Nor does the *Descartes* approach provide the intellectual or mechanistic complexity required to explain such a multisystem-coordinated response.

The ability to quantify illness provides us with a key access point to the inner workings of biological systems. The body 'knows' when it is not in homeostasis and responds accordingly, but not always proportionally. The response to illness intentionally adapts multiple systemic systems and is not a random series of unlinked events. Therefore, being able to quantify this distributed response means a point measurement has the potential to give broad insights into the current physiology of multiple systems and would, therefore, be a critical diagnostic tool.

Recent advances in neuroscience and the convergence of scientific disciplines to create next-generation measurement and imaging technologies provide us with a unique opportunity to intervene and measure illness. This review will

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introduce the principle concepts that underpin key cellular and molecular signals associated with the illness response, and the implications this has for future interventions and measurement approaches in areas of unmet need, such as pain. It will draw upon recent accumulated evidence from other reviews and primary research papers (Hutchinson *et al.*, 2007; Hutchinson *et al.*, 2011; Grace *et al.*, 2014; Nicotra *et al.*, 2014; Hutchinson, 2018a). As will be described here, harnessing the neuroimmune understanding of the interactions between, and within, complex physiological systems can literally allow the creation of windows into the body. Through these new insights, it is possible to measure and modulate disparate physiological systems. Moreover, it is also possible to form hypotheses that begin to explain biological coherence across multiple systems and point to mind–body convergence. These developments have significant implications for future animal husbandry practices. Evolution of the knowledge in this field will provide the tools that will allow the movement from empirical-based practice to precision and individualised interventions (Hutchinson *et al.*, 2018). In some cases, the quantification of these molecular events and the individualisation of husbandry practices may enable the reduction, and in some cases, the elimination of certain interventions.

The neuroimmune interface – the new building block of the central nervous system

In order to capture the full potential of recent developments in neuroscience, new foundational principles will now be introduced in order that the field of veterinary and animal sciences may gain insights into this rapidly developing field. This will allow appreciation of the cellular and molecular origins of the new era of mind–body convergence.

A fundamental dogma in neuroscience that the brain is immune privileged has been surpassed. It is now acknowledged that bidirectional signalling occurs between neuronal and immunocompetent cells (Buchanan *et al.*, 2010). Many immunocompetent cells are present in the central nervous system. These include glia (microglia, astrocytes and oligodendrocytes), endothelial cells, perivascular macrophages and infiltrating peripheral immune cells such as T cells and monocytes/macrophages (Eroglu and Barres, 2010; Grace *et al.*, 2011; Grace *et al.*, 2014). This neuronal and immune interfacing is not mere cellular cohabitation within anatomical compartments. Instead, immunocompetent cells engage intimately at the microscale and molecular level with neuronal processes to maintain brain homeostasis (Grace *et al.*, 2014). These cells form the structures that create and maintain the blood–brain barrier, neuronal myelination and encapsulate synapses (See the Primer of Psychoneuroimmunology Research (2016)).

The fundamental building blocks of the central nervous system should no longer be thought of as just the presynaptic and postsynaptic terminals forming the neuronal synapse. Instead, this basic building block in its simplest form is a tripartite relationship between an astrocyte and the neuronal processes (Grace *et al.*, 2014). In its more complex form, up to five cellular participants can contribute to this orchestrated relationship (Grace *et al.*, 2014). Importantly, at least one of these participants can be derived from the peripheral immune system. These cellular interactions allow for regular surveillance of brain disturbance, damage or infection, and can contribute to drug responses (Liu *et al.*, 2011; Northcutt *et al.*, 2015), stress and depression (Liu *et al.*, 2014) and exaggerated pain states (Hutchinson *et al.*, 2013). Given this profound new view of the brain, an intentional reframing of the brain's immune capacity will follow, including an introduction to the capabilities of these central-nervous-system-resident immunocompetent cells.

Immunocompetence of the brain – breaking the central immunoprivileged dogma

Until recently, glia were dismissed as to potentially contributing to the normal function of the brain and spinal cord (Allen and Barres, 2009). This conclusion was perpetuated owing to the principle dogma that the central nervous system was an immune-privileged organ. To be immune privileged is dictated by the exclusion of classical peripheral immune cells from crossing an anatomical barrier, in this case the blood–brain barrier, and hence preventing normal immune surveillance operations from occurring. However, it is now appreciated the brain and the spinal cord can host resident immunocompetent cells under states of both health and disease. Both glial cells and immune cells from the periphery are now appreciated to modulate neurotransmission and hence complex behavioural responses orchestrated by the central nervous system (Eroglu and Barres, 2010; Jacobsen *et al.*, 2014; Jacobsen *et al.*, 2016a).

The historical view of these cells being passive bystanders in the brain or mere components of the extracellular matrix must be surpassed (Allen and Barres, 2009). Instead, new terminology is needed to encompass the breadth of the molecular engagement, intimate spatial cellular connections, trophic and signalling relationships between the neuronal and immunocompetent cells. This can be best described as the neuroimmune interface (Grace *et al.*, 2014). The neuroimmune interface is not static. It is a dynamic multicellular functional unit which is replicated billions of times throughout the brain and spinal cord. It is comprised of neuronal processes (including both pre- and postsynaptic bodies), astrocytic projections and microglial surveillance (Allen and Barres, 2009). In some cases, chemotaxis facilitates selective migration of peripheral immune cells across the blood–brain barrier. This cellular movement also supports localised immunocompetent and neuronal cellular events

that contribute to behavioural modulation. In sum, these responses protect the whole organism (Buchanan *et al.*, 2010). A description of some of the key cell types that create the neuroimmune interface follows provided with a specific focus on astrocytes and microglia, as the majority of research has focused on their role in brain health and disease.

Astrocytes – the stars of the central nervous system

Named after their star-shaped morphology, astrocytes are the most abundant cell type in the central nervous system (Allen and Barres, 2009). Unlike their neuronal cousins, astrocytes are not linear in their connectivity or signalling capacity. Their star-shaped morphology enables them to simultaneously provide structural support to neuronal systems and enable the formation of the blood–brain barrier. This is a dynamic process and as such astrocytes are critical to regulating cerebral blood flow. Other cellular projections of astrocytes can form the tripartite synapse structure. Via these projections and the molecular transporters which they express specifically within the synaptic cleft, astrocytes can contribute actively to synaptic transmission. Finally, astrocytes provide the neuronal energy supply through critical trophic support and are among the first responders to promote repair of neuronal systems. As has been briefly described here, astrocyte morphology and functions are highly polarised and heterogeneous (Watkins *et al.*, 2005). Changes in astrocyte function can rapidly impact multiple central nervous system outputs. As will be discussed, each of these features of astrocyte biology makes them exquisitely positioned to modify multiple complex cellular systems which can translate to alterations in behaviour (Jacobsen *et al.*, 2016a). If astrocyte phenotype can be harnessed through external interventions, a ready population of cellular mediators to change behaviour is available. In the animal husbandry context, modulation and augmentation of astrocyte function have the translational potential to modify all central nervous system outputs, including sympathetic and parasympathetic responses (Allen and Barres, 2009). Key developments in targeting selective astrocyte populations and discrete anatomical locations will allow the introduction of interventions that can move affective state in a positive direction and broadly control exaggerated pain states (Jacobsen *et al.*, 2016a).

Microglia – the macrophages of the brain (sort of)

Microglia are commonly thought of as the tissue-specific phagocytes of the central nervous system. They are highly reactive in their phenotype and display profound regional heterogeneity throughout the parenchyma, presumably to coordinate diverse responses to insult, or in dynamic response to the microenvironment of their local neuroimmune interface (Graeber and Streit, 2010). Under basal conditions, microglia are found in a surveillance state. Their cytoarchitecture enables them to continuously sample the extracellular space for perturbations in their microenvironment. They are spacially dynamic, with cellular processes reaching out into extracellular voids and making contact with adjacent cell membranes (Watkins and Maier, 2003).

When a signal or event is detected, microglia can rapidly transition to a state of reactive gliosis, resulting in changes in cell number, morphology, phenotype and motility (Hutchinson *et al.*, 2011). But microglia are not only a phagocytic cell. Microglia contribute extensively to the neurokine signalling environment, of the central nervous system (Dodds *et al.*, 2016), through the expression of membrane-bound and intracellular signalling proteins (e.g., mitogen-activated protein kinases), and the release of immunoregulatory products, such as cytokines and chemokines (Watkins *et al.*, 2007).

These sentinel and neurokine signalling roles of microglia make them perfectly positioned to be early reporters of change so that the whole of an organism's physiology can respond (Dodds *et al.*, 2016). Critically, these signalling events can occur during the asymptomatic phase of the pathology, meaning that if these signals can be externalised, it makes microglia and their signalling factors potential biomarkers of health and disease early enough to provide personalised interventions without a broader impact on the herd. However, each of these cellular processes can occur with significant speed and potency, making quantification of these events within specific neuroanatomical compartments challenging (Jacobsen *et al.*, 2016b). As such, we are presented with a similar case for astrocytes, with microglia providing an excellent target to change behaviour (Jalleh *et al.*, 2012; Jacobsen *et al.*, 2016a).

Neurokine signalling – a new signalling language of the central nervous system

Given the intimate relationship between neuronal and immunocompetent cells, the previous disconnect between the molecular signalling languages of the neuronal and immune systems has been overcome. It is now clear that classical neurotransmitters act at receptor and ion channel systems expressed by both neuronal and immune cells (Dodds *et al.*, 2016). Clearly, the functional consequences of these molecular mediators on each cell type are different. However, neurotransmitter release from a neuron can impact neuronal and immune cells alike when they express the given paired receptor (Dodds *et al.*, 2016). Likewise, factors considered immune in nature, such as cytokines and chemokines, can impact neuronal function through their receptors expressed by neuronal systems (Dodds *et al.*, 2016). Therefore, it is clear that naming molecules as neuro or immune has limited our imagination for the potential targets these molecules can have in the central nervous system. In reality, there are a range of factors which are derived from both immunocompetent and neuronal cells alike and can impact a disparate number of cellular targets often independent of their classical cellular assignment they have been given.

However, a key distinction can, however, be drawn between the immune molecular mediators quantified in the periphery, which impact peripheral physiology, and those molecules that change brain function. Significantly, fewer (molar concentration) of the immune molecular mediators are required to change brain function and hence behaviour (Dodds *et al.*, 2016). Classically, a systemic immune response can result in log-fold increases in cytokines and chemokines which are required to coordinate a systemic immune response. In contrast, a doubling of already single-digit levels of immune mediator molecules is sufficient to trigger a behavioural change. An excellent example of the potency of the neurokine signalling is the impact that microglial-derived cytokine expression and release can have on pain processing, with below detection levels of Interluekin-1 beta (**IL-1β**) sufficient to elicit a painful response (Hutchinson *et al.*, 2007). Therefore, the immune signalling within the central nervous system has been termed neurokine, designating its uniqueness from that in the periphery.

How deep these differences may extend is still being determined. For example, the receptor co-localisation and ionic conditions that exist within the central nervous system are very different from the conditions that an immune receptor may operate under peripheral conditions (Hutchinson *et al.*, 2011). As such, co-localisation and tertiary peptide structures of the receptor systems may all be modified and contribute to the potency and efficacy differences.

Biological coherence through the neuroimmune interface

The unique spacial localisation of this constellation of immunocompetent cells within the critical anatomical structures of the central nervous system makes them pivotal to the health and disease of the brain and spinal cord (Hutchinson, 2018a). Complimenting this neuroscience revolution, and the movement away from viewing the brain as an immune-privileged organ, has been the acknowledgement of a key bidirectional communication and macro- and microanatomical colocation between the brain and the peripheral immune system (Grace *et al.*, 2014; Grace *et al.*, 2016). These discoveries have developed from an appreciation that how an organism behaves and interacts with its environment, can modify peripheral immune function (Prossin *et al.*, 2016b) and how the immune system functions can change the way the organism behaves and responds to environmental stimuli (Harrison *et al.*, 2009). In each of these cases, there is a key role for the neuroimmune interface in translating peripheral to central neuronal and immune stimuli. Importantly, this mind–body connectivity means that there is a distributed immune signal in the periphery which can be quantified to determine the status and functioning of the neuroimmune interface (Kwok *et al.*, 2012; Kwok *et al.*, 2013). This capacity to literally create a minimally invasive 'window into the body' provides unprecedented capacities to objectively diagnose and quantify brain and spinal cord states, including those relating to pain and affect (Hutchinson *et al.*, 2018).

So . . . how does an organism know that it is sick?

The apparently simple question of 'how does an organism know it is sick?' can now begin to be unravelled through an exploration of the underappreciated molecular signalling and structural morphology, that is, the micro- and nanoscale realms of the neuroimmune interface (Hutchinson, 2018a). It is the neuromodulatory capacity of these cells that allow the change of behaviour during times of illness (Miller and Raison, 2016). These behavioural adaptations have been linked to changes in cognitive function, mood, depression, anxiety, pain, addiction and reward (Thomas and Hutchinson, 2012; Liu *et al.*, 2014). Several key examples of this bidirectional communication will now be provided which have clear implications for animal husbandry practices and the management and welfare of animals.

The illness response – immune to brain communication

The illness response is a coordinated set of behavioural adaptations which develop during the course of an infection (Dantzer, 2001). During infection, the behaviour of an individual changes to express little motivation to achieve or perform normal daily functions, which include eating and drinking, socialising and they are often fatigued and have trouble maintaining normal sleep rhythms (Kelley *et al.*, 2003). In addition, other sensory disturbances are likely, including increased sensitivity to pain, trouble-performing cognitive tasks and the inability to experience pleasure (altered affective state) (Yirmiya *et al.*, 2000). This state has been replicated experimentally following both central and peripheral administration of bacterial endotoxin or recombinant proinflammatory cytokines, such as IL-1β. It is hypothesised that this innate central motivational state promotes recovery and is mediated by proinflammatory cytokines, such as IL-1β acting indirectly or directly in the brain to change the neuroimmune interface function (Kelley *et al.*, 2003; Dantzer and Kelley, 2007). Importantly, the response is heterogeneous across multiple brain and spinal cord centres, allowing for the presentation of each distinct behavioural outcome outlined above (Hutchinson *et al.*, 2007).

Critically, pharmacological blockade of these immune factors, or attenuation of the glial response, blocks the presentation of these adaptations. The similarities of these illness responses to chronic depressed mood and other psychiatric disturbances have now prompted some to hypothesise that the previously neuronal- and neurotransmitter-focused hypotheses for pathology presentation could actually have a neuroimmune interface origin (Dodds *et al.*, 2016). This suggests that rather than targeting neuronal processes to improve long-term mood, perhaps neuroimmune mechanisms should be considered (Hutchinson *et al.*, 2007).

Moreover, when attempting to quantify these negative affective states, immune measures may prove a useful source of biomarkers (Buchanan *et al.*, 2010).

Brain to immune communication – behaviour can drive immune response

The first demonstration of brain control of immune function remains a key motivator for the growth of the entire neuroimmune interface field. Ader and Cohen (1975) employed Pavlovian conditioning in rats to elicit a conditioned immune response. Here, they paired a sweet-tasting solution with the systemic exposure to Cyclosporin A, a profound immune suppressor. Following repeated pairings and hence conditioning, a challenge of just the sweet-tasting solution was given. The immune response observed in these animals was as though they had just received the Cyclosporin A (Ader and Cohen, 1975). This discovery has subsequently been replicated in human populations (Kirchhof *et al.*, 2018). Therefore, it is anticipated it is conserved across multiple species.

It has taken some time for the scientific field to come to grips with the implications of this discovery. However, it is clear that the brain and therefore, the state of consciousness of the individual, can impact the peripheral immune system function. In the case of Ader and Cohen, the response was a conditioned immune response that took several days to build. Prossin *et al.* (2016a) have demonstrated that in humans the recall of an acutely distressing memory of the death of a loved one was sufficient to elicit a change in immune function. Critically, the change in immune function was proportional to the emotional memory-induced activation of brain function. Hence, connecting the physiological state and immune function, a biological coherence that bridges classically held discipline-based divides, is being observed (Hutchinson, 2018b).

In the case of immune–brain and brain–immune communication, the question should be asked as to where this cycle begins and ends. Clearly, if illness changes brain function to create depressed mood, then this has the ability to feed forward to enable greater peripheral immune modulation. It is also the case that if depressed mood creates a modified or dysregulated immune system that may be more susceptible to infection, which if illness occurs, may drive greater immune susceptibility. Therefore, when striving to maintain an illness-free husbandry environment, if infection control is the only intervention, a critical contributor has been overlooked. Could effective infection control be augmented if the status of the neuroimmune interface was known and modified to facilitate optimal peripheral immune surveillance, tissue repair and pathogen clearance? What else could be improved beyond infection control? What other unwanted outcomes does the neuroimmune interface contribute to that are critical to the animal husbandry field? We will now review the specific implications this approach has for acute and persistent pain in animals.

The pain problem – the pain opportunity

Pain in animals is an experience that we are unable to reliably diagnose or quantify (Grace *et al.*, 2010). Even when animals in pain are identified, verifying the success of interventional treatments is still ineffective (Williams and Page, 2014). These limitations arise from our inability to objectively measure pain (Nightingale, 2012). This is not a problem limited to animals. In human clinical populations, the individual can be questioned and spoken to, world class successful pain treatments are ranked at between 4 and 10 based on numbers of individuals needed to treat to achieve a 50% reduction in their pain score (Katz *et al.*, 2015).

In livestock production, acute pain is experienced due to management procedures, such as castration and tail docking, injuries from fighting or poor housing conditions, diseases such as mastitis or other infections and at birth (Williams and Page, 2014; Ison *et al.*, 2016). These acute injuries can transition into the persistence of pain, which has a profound impact on the wellbeing and resilience of the animal that cause increased costs and reduced productivity (Williams and Page, 2014; Ison *et al.*, 2016).

This transition to persistence of pain serves no benefit to the animal. Therefore, prevention, diagnosis and treatment of this persistence of pain are critical. For example, soon after birth, pain can interfere in mother–young bonding leading to malnutrition, infection or even death of the newborn (Mora-Medina *et al.*, 2016). Persistence of pain throughout life is a chronic stressor for the animal, leading to reduced food intake and hence lower daily average weight gain, producing less volume and lower quality product (Williams and Page, 2014; Ison *et al.*, 2016).

A growing consumer-driven pressure is also changing the markets available for sale of livestock products, which may limit the access of products to premium-priced markets serviced by welfare-sensitive companies. Therefore, there is a pressing need for tools that can objectively diagnose and measure pain in animals, with associated innovations in pain treatment options. Such innovations in pain measurement and treatment will directly benefit the afflicted animals and the industry as a whole by improving product quality.

An example of a potential trigger for the classical presentation of the persistence of pain in animals is amputation (Flor, 2002). While on the decline in livestock, surgical removal practices such as tail docking, castration and dehorning is still widespread. This practice itself causes pain, resulting from the resection of peripheral nerves and the possible formation of traumatic neuromas and causes significant ongoing sensitisation to mechanical stimuli (Castel *et al.*, 2014; Di Giminiani *et al.*, 2017). The parallel of these events in humans is considered to be significantly painful (Cravioto and Battista, 1981). This process causes adaptations in both peripheral and central sites (Flor, 2002) and is associated with the phenomenon of residual stump pain and phantom limb pain. Painful symptomatic neuromas following amputations are observed in up to a quarter of amputees (O'Reilly *et al.*, 2016).

However, the solely neuronal view of persistence of pain has given way to an integrated neuroimmune hypothesis of exaggerated pain (Grace *et al.*, 2014). Glial cells, and peripheral immune cells circulating through the central nervous system, are now understood to be integral to creating and maintaining the neuroexcitatory states that underpin persistent pain (Nicotra *et al.*, 2012). The anatomical distribution of the disturbed neuroimmune interface contributes predictably to the behavioural consequence. That is, if the neuroimmune interface within the somatosensory system is perturbed then modified pain behaviours can be expected. Interestingly, the greater prevalence of exaggerated pain in females also appears to have its origins in this neuroimmune interface involvement, through oestrogenic priming of immune functions (Nicotra *et al.*, 2014). Hence, the persistent pain problem and the neuroimmune contributors are likely to be even more relevant in livestock owing to the predominance of female animals in many production settings such as the breeding herd.

Therefore, it is critical to understand this bidirectional communication between the peripheral immune, spinal immune and brain immune systems which maintain the signalling and neuronal reactivity that are sufficient to create heightened pain states in livestock. Moreover, while neuronal processes are critical for the conduction of heightened pain, it is hypothesised there is an anatomically distributed immune signal that triggers conduction of the exaggerated pain response.

The key role of the brain and spinal immune system in persistent pain

The neuronal mechanisms of persistent pain have been complemented in the last two decades by the heightened appreciation for the impact that central immunocompetent cells, glia, have in persistent pain. Until activated, glia are thought to have little-to-no role in the pain experience (Watkins *et al.*, 1997). However, both astrocytes and microglia in the spinal cord are activated (defined immunohistochemically by increased expression of reactivity markers) in response to inflammation and damage of peripheral tissues, peripheral nerves, spinal nerves and spinal cord (Watkins and Maier, 2003).

Enhanced pain, associated with every relevant chronic animal pain model examined to date, is blocked by disruption of glial activation and spinal cord proinflammatory cytokine actions (Watkins and Maier, 2003), thereby demonstrating the crucial role neuroinflammatory mechanisms play in multiple forms of persistent pain. Proinflammatory cytokines and other mediators like reactive oxygen and nitrogen species increase neuroexcitability in spinal nociceptive pathways by enhancing glutamate release, increasing AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor expression, phosphorylating NMDA (N-methyl-D-aspartate) receptor subunits and downregulating astrocyte glutamate transporters (Dodds *et al.*, 2016). Such mediators can also induce disinhibition of neuronal excitability by reducing the release of GABA (γ-aminobutyric acid) and glycine from interneurons and inhibitory descending projections, while glial-derived BDNF (brain-derived neurotrophic factor) downregulates the potassium-chloride cotransporter KCC2 (potassium-chloride transporter member 5) via TrkB (tropomyosin receptor kinase B) receptors, impairing $GABA_A$ channel hyperpolarisation (reviewed by Grace *et al.* (2014)). Thus, the immunocompetent cells of the central nervous system are perfectly situated to contribute substantially to the initiation and maintenance of multiple pain behaviour heightening mechanisms, but the anatomical location of these brain and spinal cord immune cells keep them hidden from peripheral sampling.

While healthy males and females do not have substantial differences in sensitivity to acute pain, females are significantly more susceptible to experience persistent pain than males (Nicotra *et al.*, 2012 and 2014). One of the reasons for this difference in the experience of persistent pain may be due to a difference in neuroimmune interface contributors (Nicotra *et al.*, 2012 and 2014) and in some cases possible differences in peripheral immune cellular mediators (reviewed by Dodds *et al.* (2016)). While the field is yet to reach consensus on the exact mediators and contributors to this component of female pain persistence, it is clear that neuroimmune interface contributors are an overlooked, yet profound participant in the effects, and raises the possible need for sex-specific pain treatments (Hutchinson *et al.*, 2011). An exploration of the neuroimmune changes and contribution to pain in livestock has not been performed, and therefore these innovations have yet to be capitalised upon in livestock husbandry practices.

The peripheral immune system – a novel hunting ground for biomarkers

Given the recognition that there is bidirectional communication between brain/spinal and peripheral immune cells, and that the reactivity status of peripheral immune responses is shared by their central nervous system counterparts, we have explored this hypothesis that peripheral immune cells which have the capacity to be represented at the neuroimmune interface mirror, what is occurring within spinal cord and brain sites. Hence, tapping into the biological coherence by sampling and assaying the peripheral blood is achievable. In recent breakthroughs in the persistent pain field, this concept has been confirmed in a series of preclinical and clinical proof-of-principle studies (Grace *et al.*, 2010 and 2011; Kwok *et al.*, 2012). It has established that brain and spinal neuroimmune interface pain mechanisms rely on a biological coherence supported by peripheral immune cells (Grace *et al.*, 2010 and 2011), and that the *ex vivo* activity of peripheral immune cells can stratify patients into chronic pain and healthy populations (Kwok *et al.*, 2012). A similar approach has been applied to other illness response and maladaptive illness responses like that occurring in depressed mood. Here, peripheral immune factors and responses have been quantified and linked to distinct disease states and may serve as objective diagnostic biomarkers in the future (see Liu *et al.* (2014) for review).

Quantifying the illness response to guide practice

Two sets of tools are required to capitalise on this new discovery. Firstly, research grade measurement tools and analytical methods which are costly and time-consuming do have a place in this field (Jacobsen *et al.*, 2016a and 2016b). These technologies often have a heightened level of specificity and sensitivity and are, therefore, considered the gold standard for analysis. They are critical for benchmarking all other results. Moreover, these technologies can be used to establish best practice. However, these tools will not be able to inform day-to-day practice alone as they are often bulky and not physically fit for purpose owing to their size and complexity (Jacobsen *et al.*, 2016b; Hutchinson *et al.*, 2018). Instead rapid, rugged, cheap, reproducible and deployable technologies that measure the same or similar biological factors are needed. Moreover, these technologies need to deliver actionable information from analytical grade data.

In order to validate these putative peripheral biomarkers of biological coherence, a greater insight of the working of the neuroimmune interface is required. However, the current tools available to the neuroscience and immunology research fields are insufficient to explore the real-time function of these underappreciated cells of the neuroimmune interface (Jacobsen *et al.*, 2016b; Hutchinson, 2018a). As discussed, the signalling events that occur at the neuroimmune interface engage high potency large peptides, and short-lived reactive species which are not detectable with the quantitative or spacial resolutions needed to understand how this system functions. Therefore, a new generation of technologies is required (Jacobsen *et al.*, 2016b).

Such technological advances cannot be achieved by researchers acting in isolation (Hutchinson, 2018a; Hutchinson *et al.*, 2018). Instead, it is through transdisciplinary communities and through large-scale funding schemes like the Australian Government's Centres of Excellence programme that such challenges can be met. The Australian Research Council Centre of Excellence for Nanoscale BioPhotonics has a key mission to deliver new sensing and measurement tools to open new windows into the neuroimmune interface (Australian Research Council Centre of Excellence for Nanoscale BioPhotonics, 2019). Importantly, the field of biophotonics has the potential to deliver both gold standard research grade measurement technologies and rapid-cheap-deployable tools that enable decision-making in the field (Hutchinson, 2018a; Hutchinson *et al.*, 2018).

Why use light to examine the neuroimmune interface?

Multiple analytical approaches are available to researchers to obtain data across single molecule (nano), through secondary and tertiary biological structures (micro) to subcellular and cellular anatomy (macro). However, few of these have been translated to practical field uses that provide actionable information. Many times, it is the complexity and cost of the technology that limits their broader relevance. In contrast, light-based imaging and sensing tools have significant advantages in capturing the critically needed information in a non-destructively fashion from biological processes over the desired scales (Hutchinson, 2018a; Hutchinson *et al.*, 2018). Importantly, these light-based technologies allow the range of scales to be explored that need to be captured in order to understand the complexity and real-time functioning of the neuroimmune interface.

These multimodal imaging and sensing approaches are beginning to allow for complex biological events to be quantified (Jacobsen *et al.*, 2016b; Li *et al.*, 2018). It is clear that the cellular and molecular mechanisms that underpin biological coherence will require multiple systems to be working in concert. Quantifying each one of these processes in parallel is unlikely to provide the required contextual information to understand the whole system. Therefore, hypothesis-free, sample phenotyping or 'fingerprinting', approaches are key to unravelling the complex biology. A range of such methodologies exploit light-based measurements, such as Raman and tissue autofluorescence acquisition. In each case, the multidimensional data sets acquired using these approaches can provide complex information on hundreds and thousands of molecular features in parallel (Hutchinson, 2018a; Hutchinson *et al.*, 2018). However, alone these approaches may not yield sufficient specificity or sensitivity of actionable information.

Combining the aforementioned phenotyping technologies with specific measurement techniques is required. Historically, this has driven the creation of a great range of synthetic biology and chemically derived spectrally distinct fluorescent and luminescent probes that allow for parallel sensing and imaging (Jacobsen *et al.*, 2016b). However, the ability to separate these probes spectrally, that is, by their colour alone, is limited. As such, new tools that incorporate other forms of multidimensionality and specificity are required. An example of this is the use of the 4th dimension of time encoding, in addition to spectral information, which provides a future where tens of targets could be simultaneously measured with single excitation and detector pairs (Jacobsen *et al.*, 2016b). Here, 4D encoding utilises the 3D spatial information of where the molecule is in space, together with the precisely engineered timed release of photons from nanomaterials (Fan *et al.*, 2018). This 4D imaging approach allows for spectrally identical nanomaterials to be differentiated based on their individualised timed photoemission, providing unprecedented plexing of imaging information (Hutchinson, 2018a; Li *et al.*, 2018). It is through the use of a combination of these tools that measurement advances in the field can be made. But can scalable interventions also be sourced?

Targeting of the neuroimmune interface – an untapped field

The acknowledgement of the neuroimmune interface in a range of pathologies provides a range of new targets for

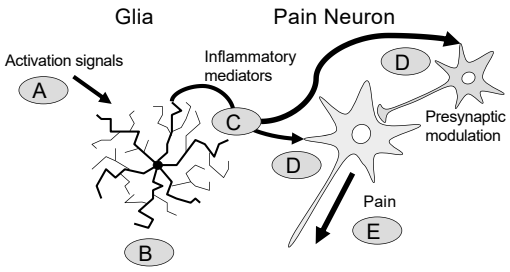

Figure 1 **Step A:** An activation or series of activation signals are required to activate glia often mediated via cell surface pattern recognition receptors that can be antagonised, such as innate immune receptors. These systems have the capacity to detect both endogenous and exogenous signals that allow cross-sensitisation and integration of complex physiological and environment stimuli into behavioural consequences. **Step B:** Glial activation and reactivity is an all-encompassing term used for the state in which glia release proinflammatory mediators and signalling primers. This state can be modulated or attenuated, thereby inhibiting various cellular events that block glial reactivity or its downstream consequences. An anti-inflammatory environment can also be produced, which increases the threshold that an activation signal has to overcome to activate the cells. **Step C:** Immune proinflammatory mediators, such as proinflammatory cytokines and chemokines, can be neutralised prior to reaching their intended receptor target (pre- and/or postsynaptic) using soluble receptors (which exist endogenously), neutralising antibodies, decreasing maturation of cytokines into their active form or increasing the rate of cytokine degradation. In some cases, generation of a second round of immune-derived innate immune pattern recognition signals can also be targeted. **Step D:** The action of many glial inflammatory mediators on neurons (pre- and/or postsynaptic) can also be antagonised at neuronal receptor sites. Importantly, it is also now acknowledged that under some conditions neuronal systems can bypass glial involvement by direct expression of innate immune pattern recognition systems and thereby become directly sensitised. **Step E:** Included here are the myriad of currently employed neuronal-targeted therapies that decrease the neuronal signalling of pain signals (pre- and/or postsynaptic). However, notice that if only Step E is targeted only a portion of the problem is addressed and the neuroimmune interface remains unbalanced. Unfortunately, the standard approaches to the management of human and animal acute and chronic pain continue to employ a Step E methodology likely contributing to the failure to adequately treat pain in humans and animals alike (Nightingale, 2012). Adapted from Hutchinson et al. (2007).

the pharmacological prevention and management of the condition, such as persistent pain. Moreover, these targets may reside within the systems that balance the biological coherence and as such may be highly tractable.

This process builds upon the hypothesised model of pharmacological interventions at the neuroimmune interface. Here the example for persistent pain is used, highlighting theoretical points (Figure 1: Steps A to E) where pharmacological targets can be directed to neuroimmune interface pain contributions.

Conclusions

It is clear that a greater understanding of the illness response and an ability to quantify it will have profound benefits across animal husbandry practices. The field exploring brain–immune communication is rapidly moving and will only make profound steps forward with new technologies and engagement across disciplines. Transdisciplinary collaborations that imagine, design, create and curate these technologies are needed. Therefore, a common language and alignment of expectations within the collaborations is needed. The convergence of human and animal biological sciences with common experimental and measurement technologies will enable a more rapid translation of clinical discoveries across both species, culminating in better health outcomes and productivity overall.

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Declaration of interest

None.

Ethics statement

This work is a review and as such does not have direct ethics approval requirements.

Software and data repository resources

None.

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Review: Precision livestock farming: building 'digital representations' to bring the animals closer to the farmer

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*Economic pressures continue to mount on modern-day livestock farmers, forcing them to increase herds sizes in order to be commercially viable. The natural consequence of this is to drive the farmer and the animal further apart. However, closer attention to the animal not only positively impacts animal welfare and health but can also increase the capacity of the farmer to achieve a more sustainable production. State-of-the-art precision livestock farming (**PLF**) technology is one such means of bringing the animals closer to the farmer in the facing of expanding systems. Contrary to some current opinions, it can offer an alternative philosophy to* 'farming by numbers'*. This review addresses the key technology-oriented approaches to monitor animals and demonstrates how image and sound analyses can be used to build 'digital representations' of animals by giving an overview of some of the core concepts of PLF tool development and value discovery during PLF implementation. The key to developing such a representation is by measuring important behaviours and events in the livestock buildings. The application of image and sound can realise more advanced applications and has enormous potential in the industry. In the end, the importance lies in the accuracy of the developed PLF applications in the commercial farming system as this will also make the farmer embrace the technological development and ensure progress within the PLF field in favour of the livestock animals and their well-being.*

Keywords: livestock production, image analysis, sound analysis, technology

Implications

This work address the main approaches utilised in developing precision livestock farming tools. Precision livestock farming is an approach that enables the farmer with more objective information about the animal to make better choices about the sustainability of their production system. This paper demonstrates some of the key solutions and the approaches taken to develop technologies with sound and image analyses.

Introduction

Economic pressures continue to mount on modern-day livestock farmers. Most farmers now find themselves in a situation where they, in order to maintain their livelihood, must exploit the economies of scale. As a result, those who are surviving often have limited time to interact with their animals. Meanwhile, attention to animal welfare has heightened and stakeholders in the livestock sector are more conscious about managing and slaughtering animals in more welfare-friendly ways (Blokhuis *et al.*, 2019). Society in general is demanding closer attention to the needs of individual animal. Thus, for the case of the most intensive livestock sectors, and especially for poultry and pig production (where a large number of animals per farm is involved), the gap between societal and production demands is widening. However, most of the society do not realise that taking good care of the animals is essential to achieve good productivity, health and welfare. Closer attention to the individual animal's needs does impact not only animal welfare and health but also the capacity of the farmer to achieve sustainability (economic, environmental and societal) targets.

Technology, which is developing at a rapid pace, is enabling a better interaction between animal and farmer despite the challenges faced. Twenty years ago, the ability to carry a personal computer was a novel experience, yet nowadays we have more powerful devices that fit in the pocket. This evolution in the information and communication technologies (**ICTs**) has had a significant impact on the agricultural industry too. Crop and horticultural production have for the last number of years witnessed an explosion of new software systems, monitoring devices and machines that exploit

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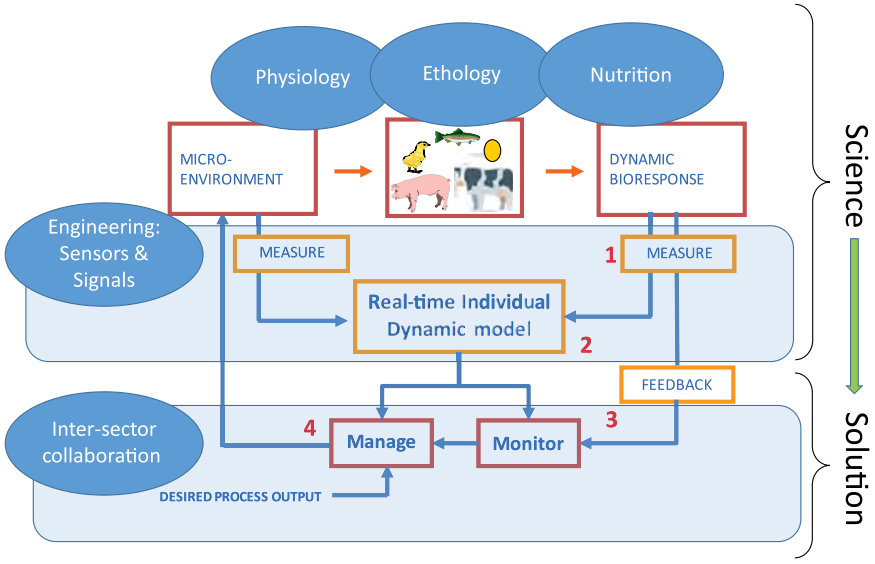

Figure 1 (Colour online) General scheme showing how bio-response monitoring and management of livestock animals can go from science to solutions (Aerts *et al.*, 2019).

the latest capabilities in sensing, communication, processing and power management (van Evert *et al.*, 2017). Since the early 1990s, livestock production has also started to see more research into ICT-supported management of livestock farming systems (Halachmi and Guarino, 2016). Much of the initial developments focused on decision support systems, that is, to simulate many scenarios that affect the economic or sustainability indicators of production. These optimised functions were representative of farm processes and did not seek to interface with the animals themselves. However, the animal is the central part of the process and the technology must support them at every moment to realise a better life for them, as it does for humans.

State-of-the-art technology can bring the animals closer to the farmer. Precision livestock farming (**PLF**) was developed to provide better information to the farmer on the animals by exploiting the known principles of process engineering to provide a level of automation. Indeed, it seems from recent contributions that PLF is the main force behind industrialising farming (Werkheiser, 2018). However, it is in fact much more than that and can instead offer an alternative philosophy to '*farming by numbers*', because technology can support the farmer. Technology can collect relevant information about the animals in a continuous manner and thereby build more in-depth insight into their requirements. Some animal rights proponents go against this perspective and even argue that technology actually fuels the growth of a factory-based farming industry (Stevenson, 2017). However, we must recognise that as science gains further knowledge on the care of animals, we can also build better 'digital representations' of the animals. This empowers farmers to make better choices that are not alone driven on profits but instead on the actual needs of the animals and their care at all times.

In the literature, various studies have aimed at the development of automatic monitoring systems for livestock production. Examples include monitoring drinking behaviour (Domun *et al.*, 2019) or to detect infected coughs by sound analysis (Exadaktylos *et al.*, 2008), assess the thermal comfort (Shao and Xin, 2008) or estimate the live weight (Wu *et al.*, 2004) by means of image processing. Furthermore, during the last couple of years there has been a number of scientific reviews compiling the potential of PLF from the perspective of technology developments addressing key production and environmental challenges (Benjamin and Yik, 2019; Halachmi *et al.*, 2019; Tullo *et al.*, 2019). Other reviews have discussed the ethical concerns of eroding the relationship between the farmer and the animal that PLF could promote (Werkheiser, 2018; Bos *et al.*, 2018). This review will address the key approaches to monitor animals and highlight methods towards building 'digital representations' of them by giving an overview of some of the key concepts related to PLF, tool development within image and sound analyses and value discovery during PLF implementation. This review will mainly focus on the examples within the pig and poultry production.

The principles of precision livestock farming

The primary objective of PLF is to develop livestock management and monitoring systems with technologies to support the farmer (Berckmans, 2014). This includes the use of sensor technology for observing animals (Darr and Epperson, 2009), the application of modern control theory to improve autonomy of the production process (Frost *et al.*, 2004), and the use of advanced data processing methods to synthesise and combine different types of data (Terrasson *et al.*, 2016). Precision livestock farming is based on the interaction between different scientific disciplines and stakeholders in the livestock industry. From Figure 1 it is clear that three distinct conditions within a system need to be fulfilled to

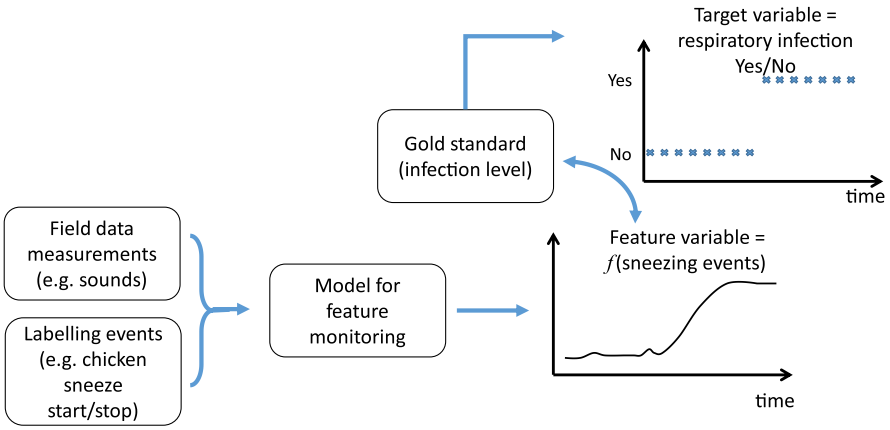

Figure 2 (Colour online) Approach for detection of chicken sneezing events from sound data (adapted from Berckmans (2013)).

achieve sufficient levels of monitoring and management to be considered a PLF system (Berckmans, 2006):

1. Animal variables (i.e. parameters related to the behavioural or physiological state of the animal) need to be measured continuously with accurate and cost-effective sensor technology,
2. A reliable prediction (expectation) must be available on how animal variables will vary or how the animal will respond at every moment, and
3. Predictions and on-line measurements are integrated in an analysing algorithm for automatic monitoring and/or management.

It should be noted that the development of the PLF systems require collaboration among different disciplines. An example of the collaborative PLF methodology (Carpentier *et al.*, 2019) is the development of a system for detection of an occurring chicken respiratory infection (i.e., the target variable), by deriving the amount of coughs over a certain period of time (i.e. the feature variable) from a continuously measured sound signal (i.e. the process output). This methodology is further schematically illustrated in Figure 2. As can be noticed, the scheme also contains the use of gold standards to unambiguously establish the relationship between target and feature variables as well as an audiovisual labelling process. The latter is involved in identifying a number of predefined 'features' (e.g. the exact start and end point of each sneeze) from the audio and video recordings. Thus, the PLF methodology demands a high level of collaboration between multiple research fields including animal scientists (e.g. physiologists, ethologists and nutritionists), laboratory technicians, data scientists and engineers among others.

Monitoring animal variables

As described above, a PLF system includes the monitoring of animal variables, which can be, for example, behavioural or physiological measures. In the following, the review will describe in more detail how such animal variables can be continuously monitored non-invasively using examples from image and sound analyses.

Image analysis

Image analysis has been demonstrated to have potential in monitoring livestock animals since the early 1990s (van der Stuyft *et al.*, 1991). Cameras have the advantage that they do not need to be mounted to the animal itself and, therefore, no extra stress for the animals is induced. Image analysis has been used in the past to measure some important bio-responses regarding health, welfare and growth parameters of the animals including weight estimation (Schofield *et al.*, 1999; Mollah *et al.*, 2010), assessing the gait and lameness of broiler chickens (Aydin *et al.*, 2010, 2013), measuring the water intake in pigs (Kashiha *et al.*, 2013a) and identifying marked pigs in a pen (Kashiha *et al.*, 2013b). In the following, aggression among pigs will be used as an example of PLF research using image analysis.

Aggression among pigs as an example. A key welfare-focused application of image analysis in the PLF research is to analyse the social interactions of animals. An example of such is aggression among pigs. It is widely recognised that aggression is an important problem that threatens the health, welfare and growth of pigs in modern pig industry (D'Eath and Turner, 2009). Usually, aggressive behaviours of pigs are found by direct observation of the producer. However, manual observation is labour-intensive and time-consuming. Detection of such problems with computer vision technologies and image analysis can instead provide advantages of uninterrupted, real-time and continuous monitoring. Using this technology for recognition and control of aggression can help to improve the efficiency of recognition, increase animal welfare and productivity and reduce economic losses of pig farms (Faucitano, 2001; Bracke *et al.*, 2002). Recently, image analysis has been widely used for behaviour analysis of pigs including tripping and stepping behaviour recognition (Gronskyte *et al.*, 2015) and mounting behaviour recognition (Nasirahmadi *et al.*, 2016). However, as the complexity of aggressive behaviours in pigs is very high, the investigation of aggressive behaviours in pigs based on image analysis has been limited. Viazzi *et al.* (2014) focused on the detection of aggressive behaviour among pigs in general, whereas

Oczak *et al.* (2014) and Chen *et al.* (2017) classified the aggressive behaviour into high- and medium-aggression among pigs. However, only using the high and medium intensity for definition and recognition of aggressive behaviours will be subject to greater interference by other behaviours. For instance, chase and play among pigs can also produce similar high intensities (Viazzi *et al.*, 2014). Thus, more work is needed to be able to recognise the unwanted behavioural trait, aggression, from the wanted behavioural trait, play, using image analysis. One approach to achieve this distinction would be the ability to recognise specific aggressive markers from image analysis, including the head-to-head knocking, head-to-body knocking, parallel pressing, inverse parallel pressing, ear biting, neck biting, body biting and tail biting (Oczak *et al.*, 2013).

A PLF tool that recognises specific behavioural patterns, such as the aggressive behaviour markers of pigs, on the individual animal level by image analysis uses several steps and techniques that is described in the following, including target tracking, extraction of animal foreground, extraction of behavioural features in the data and recognition of important behavioural interactions.

Target tracking. To recognise specific behavioural patterns on the individual level using image analysis first demands a technique that can individually recognise each animal from the other animals in the group. This is also referred to as target tracking. However, presently many computer vision systems have the shortcoming that they cannot perform this task continuously without labelling the animals. Even with manual video recordings it is not easy to assign an identity to each animal and keep track (Oczak *et al.*, 2014). Previously, the solution to this problem was either to evaluate the behaviour at the group level (Viazzi *et al.*, 2014) or to mark individuals with artificial symbols (Kashiha *et al.*, 2013b). However, neither of these methods meets the requirements of commercial farms for individual selection of animals. Methods currently being investigated as alternatives to manual labelling include the 5D Gaussian model (Ahrendt *et al.*, 2011) and the Gabor texture feature method (Huang *et al.*, 2018).

Extraction of animal foreground. A second step is to be able to detect the animals from other objects in the environment, also referred to extraction of animal foreground or image segmentation. After such a procedure, the image should only include the animals and the rest as a unified background colour. Some methods can extract animal foreground with high accuracy but cannot separate animals that are close together (target adhesion) and, thus, make it difficult to subsequently locate the feature points on each animal body (e.g. the mixed Gauss model method (Guo *et al.*, 2014)). Other methods can separate animals that are close together, but cannot be used for accurate extraction of animal foreground as the contour of the animal is either rough and incomplete or of a specific shape (e.g. the Otsu-based background subtraction proposed by Nasirahmadi *et al.* (2015) and the method of merging fitted ellipses proposed by Lu *et al.* (2016)). Thus, more work is required on this challenge.

Extraction of behavioural features in the data. A third step is to decide on the behavioural features that should be extracted to recognise the behaviour in question and to develop techniques to extract these chosen behavioural features. This is a part of the image analysis process that needs considerable consideration. In the case of pig aggression, features of mean intensity and occupation index (Viazzi *et al.*, 2014; Oczak *et al.*, 2014) were found to be useful in the past. Later, the acceleration feature (Chen *et al.*, 2017) and motion features with higher discrimination (e.g. kinetic energy (Zhao *et al.*, 2016), displacement, etc.) have been applied with success and can be further developed and combined with the position features of aggressive pigs (e.g. distance between head and head, distance between head and body and distance between head and tail). In the process of behavioural feature extraction, the difficulty lies in the location of individual feature points on the animal body including, for example, the location of the animals head and tail (Kashiha *et al.*, 2013a). Without going into further detail, existing methods for locating feature points include the point distribution model (Cangar *et al.*, 2008) and the kink points method (proposed by Frost *et al.*, 2004). By analysing the motion of these feature points between adjacent image frames, more accurate motion features and position features can be extracted.

Recognition of important behavioural interactions. A fourth and final step is to classify the image frames into the important behavioural interactions in question (such as aggressive behavioural markers among pigs) based on the values of the extracted behavioural features. Methods for such classifications include linear discriminant analysis (Viazzi *et al.*, 2014), neural networks (Oczak *et al.*, 2014) and hierarchical clustering (Chen *et al.*, 2017), among others. The threshold of each behavioural feature can then be used in the recognition rules of, for example, aggressive behaviours. As an example, when two pigs in a frame simultaneously meet at a certain acceleration (motion feature) and with a certain distance (position feature), it can be considered as a frame with head-to-head knocking. When the ratio of such frames in a period of time exceeds a fixed value, also referred to as the minimum recognition unit (Chen *et al.*, 2017), it is considered that an event of head-to-head knocking occurred and, thus, that an aggressive interaction among the pigs in the group occurred. Further work is required to develop better rules (thresholds) to recognise each aggressive behaviour in frames of videos and to choose the accuracy, sensitivity and specificity being suitable for evaluation of the recognition results (Oczak *et al.*, 2014).

To summarise, image analysis do show great potential as a method to recognise specific behavioural patterns, both for the individual animal and for interactions between animals. However, challenges still exist within each step of the image analysis process that needs to be investigated further.

Sound analysis

Sound analysis is a second method for automatic continuous recording of animal variables that is non-invasive to the animal, as the microphones measuring the sound can be mounted within the animal house without causing additional stress to the animal. Within the PLF research field and sound analysis, quite a lot of attention has been given to measuring animal vocalisation. In the following, examples of PLF research within pig and chicken vocalisations will be presented.

Pig vocalisations. Various studies comprise automatic detection of pig coughs, a good indicator for respiratory problems in pigs. This automatic cough detection is not new. In 1999, Van Hirtum *et al.* published on selecting coughs (Van Hirtum *et al.*, 1999). In the years following, the detection algorithm was further improved (Van Hirtum and Berckmans, 2003) and a recent study of Berckmans *et al.* (2015) showed that a detection tool could give warnings up to 2 weeks earlier, compared to a situation where the pigs were observed by the farmer and the veterinarian. In addition to the cough detection, Van Hirtum and Berckmans (2002), Exadaktylos *et al.* (2008) and Ferrari *et al.* (2008) showed that it is possible to decipher between cough from healthy and sick pigs. Van Hirtum and Berckmans (2003) investigated the possibility to use cough sound as a biomarker for three types of aerial pollution (irritating gas, dust and temperature). Thorough analysis of pig vocalisations including duration and amplitude of the vocalisation signal can also be used as a sign of distress in the pig (Cordeiro *et al.*, 2018). Further classification of the pig vocalisations into vocal types including pig screams also showed to contain valuable information in identifying painful and distressful situations such as castration and ear biting (Von Borell *et al.*, 2009; Diana *et al.*, 2019), whereas pig barks may contain valuable information in identifying play behaviour or as alarm signals (Newberry *et al.*, 1988; Chan *et al.*, 2011). Recent research show a potential to differentiate pig vocalisation not only into coughs but also into screams, grunts and squeals based on several sound signal features (Vandermeulen *et al.*, 2015; Diana *et al.*, 2019).

Chicken vocalisations. Over the last years, there has been an increasing interest in the analyses of chicken vocalisations, as this seems to contain a lot of information about these animals. Information that can be of great importance to the farmer, if presented in the correct way. The literature on analysis of chicken vocalisations shows enormous possibilities of this research. Vocalisations of chickens have been researched in relation to welfare (Zimmerman *et al.*, 2000), social separation (Feltenstein *et al.*, 2002), thermal comfort (Moura *et al.*, 2008), feather pecking (Bright, 2008), diseases (Carpentier *et al.*, 2019) and growth (Fontana *et al.*, 2015). However, the potential in the use of microphones is not limited to automatic recording of vocalisations but can, for example, also be used to measure feed uptake by broilers using the pecking sound (Aydin *et al.*, 2014). The unique feature in the latter case is that a sound detection system was defined based on a sound sensor attached to the feeder pan. In this way, it was possible to perform and analyse sound measurements in real time in a fully non-invasive, but still automated, manner during the full growth process of this group of animals.

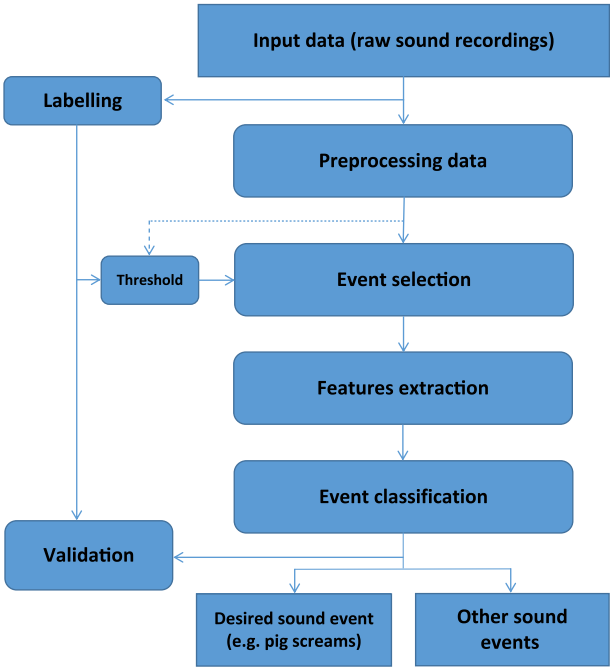

Figure 3 (Colour online) Overall approach for the development of a sound analysis-based precision livestock farming tool for livestock animals.

Applications to localise and monitor the health and well-being of livestock animals are feasible with sound analysis. For each application, the main effort is to develop an algorithm for automatic detection of sound events and then associate these specific sound events to health, distress and abnormal behaviour. As with image analysis, the workflow of such an algorithm within sound analysis takes several steps, as illustrated in Figure 3 and described in the following. These steps include sound recording, pre-processing, event selection, feature extraction and event classification.

Sound recording and pre-processing. The first step in sound analysis is the acquisition of the raw sound data (i.e. the input data). Sound is recorded and saved in blocks of a certain time, which can easily be adapted. For recording the sounds, the sampling frequency can also be adapted and the most optimal position for mounting the microphone should be explored. This includes the height of the microphone and the relative position from the walls and disruptive sound sources (e.g. ventilation). The sound data also need pre-processing, which is a specialised sound analysis procedure to separate the clear sound sources, also called foreground noises (e.g. knocking sounds, animal vocalisations), from specific interfering noises, also called background noises (fan noise, heaters). The goal is to clean up the sound data before moving on to event selection and feature extraction. For this pre-processing, many different approaches are

possible from simple solutions like bandpass filtering to more sophisticated solutions like the ones used in speech processing. In the case of animal vocalisations, a more sophisticated solution is needed as this gives a better filtering where the background is removed as much as possible. Good filtering will make it possible to select and classify sound events with the targeted accuracy and precision and it will facilitate the next steps in the process.

Event selection and feature extraction. The third step in the algorithm workflow is to select events from the pre-processed sound data. An event is a time frame in the audio signal with a certain meaning (e.g. knocking, pecking and animal vocalisations). In this step, an automatic annotation of all relevant acoustic events is made and the events are identified with an onset and offset time. The algorithm will need a threshold or multiple thresholds to decide when an event will start or end. These thresholds are based on the labelling process that occurred during the development of the algorithm and the output of the event selection for a given threshold (i.e. how great a proportion of events were selected). The fourth step is to extract feature values from the selected events to calculate specific audio characteristics of the events including energy (total energy of the event, relation of the energy in different bands in the event), frequency information (peak frequency, mean frequency), spectral centroid, bandwidth, envelop of the event and zero-crossing rate. The main objective is to identify features with physical meaning (e.g. the mean frequency of different vocalisations).

Event classification. The final step in the algorithm workflow is the classification of the sound events. Based on the values of the different features, the events are assigned to different classes (e.g. pig vocalisations into screams and coughs). By assigning a threshold to different features, cut-offs can be made between classes. Decision on the thresholds can be done manually based on the physical meaning of a feature (e.g. a pig cough could never be shorter than 0.1 s or longer than 1.5 s). Another approach is using automatic classification tools like hidden Markov models, Gaussian maximum likelihood estimators and neural networks among others. However, using these automatic tools makes the algorithm prone to overfitting, making it difficult or impossible to see precisely how the classification was done in retrospect. It is worth noting that these algorithms require an excellent feature database for efficient and effective classification such as databases from other audio processing disciplines (e.g. the MPEG-7 database for music processing).

To summarise, the vocalisations of livestock animals including pigs and chickens seem related to several animal health and welfare parameters. Further, sound analysis show great potential as a method to automatically recognise these specific vocalisations of livestock animals as well as to be used for other relevant applications within the livestock production.

Applications of monitoring technologies in the production process

The purpose of developing PLF applications is to monitor the health and welfare of the individual animal (and when not possible, a group of animals) in a continuous, dynamic and real-time manner on-farm to facilitate the farmer in caring for the animals. In the subsequent section, two examples of such applications from the pig and broiler production will be presented to understand the value of PLF implementation. Both application examples highlight the possibility to separate welfare monitoring from retrospective production results, so that animal health and welfare can be managed effectively in the present time.

Water usage in the pig production

During the growing process of animals, behaviours such as drinking and feeding are indicative of their health and well-being. In the production of pigs, drinking behaviour has been considered by many authors as a way to judge the health and welfare of the pigs. Pigs generally have stable diurnal drinking pattern unless influenced by stressors from disease or the environment (Madsen and Kristensen, 2005; Andersen *et al.*, 2014). Andersen *et al.* (2014) found that tracking the dynamic characteristics of drinking behaviour within a day is important as part of health and welfare monitoring. The study by Madsen *et al.* (2005) was the first one to consider the dynamic modelling of water drinking patterns and demonstrated its power in detecting disease outbreaks (Madsen and Kristensen, 2005). One way to measure water intake is to utilise water meters, and multiple studies have found predictive information in this measure when considering unwanted events within the pig production including tail biting, pen fouling and diarrhoea (Jensen *et al.*, 2017; Dominiak *et al.*, 2019; Larsen *et al.*, 2019). Other approaches focus less on the precise water consumption of the animals and instead focus more on the frequency and duration of the visits to the drinker. For example, Kashiha *et al.* (2013a) researched the use of camera-based monitoring of drinking behaviour in pigs. The aim of the experiment was to determine whether half-hourly water volume usage (in litres) in a pig barn could be estimated by analysing the drink nipple visits. Image analysis enabled the dynamics of water visits to be linked with meter measurements over the 13 days of the experiment. However, using water meters and cameras as above do not provide data at the individual animal level. This was enabled using high-frequency radio-frequency identification (**RFID**) systems that Maselyne *et al.* (2016) successfully implemented by comparing RFID-based visits with visual observations and flow meter measurements. All in all, the three techniques presented above provide the possibility to evaluate the time varying drinking behaviour of pigs; and given the potential RFID and camera systems, it is becoming more feasible for farmers to monitor individual water intake patterns.

Flock behaviour in broiler production

Although not at the individual animal level, the behaviour of a broiler flock has recently been correlated to specific welfare problems in the broiler production. Fernández *et al.* (2018) used the commercially available PLF camera system to extract values on the activity and occupation patterns of a broiler flock. They found a positive relation between the deviations in occupation patterns and the footpad lesion scores indicating that birds, which tend to cluster together for long periods, present an increased chance of having higher levels of footpad lesions. They also found a negative relation between the deviations in the activity pattern and the hock burn scores, indicating that a higher activity of the flock would improve the hock burn scores. This can be related with the fact that having less active broiler chickens staying still for longer periods on badly conditioned litter can worsen this kind of lesion (Haslam *et al.*, 2007). Another camera-based monitoring approach has been developed by Dawkins *et al.* (2013). The approach is based on an analysis approach called optical flow. This approach derives the general movement flow patterns of birds from the images, as well as the variance, skew and kurtosis of the flow and have been correlated with health and welfare indicators (Dawkins *et al.*, 2013).

The future potential of precision livestock farming in livestock production

Welfare management is the key challenge today and often relates to both the health and production of the animals. It is not enough for the farmer to be told after the animals have left the herd that the system is not at a suitable welfare level. After this point, the farmer cannot take appropriate action. With PLF, real-time on-farm welfare monitoring and management becomes feasible, and many opportunities exist beside the examples already mentioned. Continuous monitoring of key variables on the farm will make real-time welfare management possible. As the PLF research community grows, we still have to do more work to realise the practical benefits of PLF technologies in the commercial production. Only with accurate systems can we start to convince farmers of the capacities of such technology to link productivity, health and welfare management. Examples from the pig and poultry productions have been used in the current literature review. However, the PLF field of research is most certainly not limited to these two livestock species but is also well established within, for example, the dairy and beef cattle productions. In the following, the opportunities with PLF in an often forgotten livestock production form, aquaculture, will be discussed.

Opportunities in other sectors: aquaculture as an example

Aquaculture is traditionally considered a low-technology sector (Føre *et al.*, 2018). Almost all biological responses of fish are expressed underwater. Given the industry's drive toward larger production cages, there is now an even greater need for technologies that enable farmers to maintain a high level of control over the production processes in fish cages. Fish farmers have found it hard to understand the behavioural repertoire of fish and thereby obtain direct relationship with their animals. In this context, there is a clear need for quantifying fish behaviour and translating this information into a reliable welfare assessment system. Much of the previous research has focused on a qualitative description of behaviour, whereas recent technologies coupled to state-of-the-art computer vision and machine learning techniques open the door to a data-based description of fish behaviour. This approach will allow for a more careful inspection of a species' whole behavioural repertoire, possibly highlighting patterns of behaviour that were previously overlooked by human observers. Previous research efforts aimed at aquaculture have focused on the development and use of technologies for monitoring and estimating animal variables in tanks and cages (e.g. Alfredsen *et al.*, 2008; Føre *et al.*, 2011). Other research has tried to link the biological responses of farmed fish with the underlying mechanisms as a function of external factors such as feeding and management (e.g. Oppedal *et al.*, 2011). While such studies represent essential building blocks for the development of new technology-based methods for intensive aquaculture, the tools for drawing a foundation for decision support by combining monitoring technologies with knowledge on the biological characteristics of fish population has been non-existent (Føre *et al.*, 2018). Hence, an adaptation of the methodology therein to fish farming will build a bridge between technological and biological research results and efforts.

Conclusion

Precision livestock farming can provide the key to a sustainable livestock production in the future. In this review, we have identified some of the key principles of PLF and key ideas behind the development of PLF sound and image tools as an non-invasive method of measuring animal variables. A key point is that the development and operation of PLF applications involve several steps and techniques that need to be investigated further to make the developed systems accurate and implementable in commercial environments. By adopting such tools the PLF field will progress and the farmer will also embrace the value of the technology. Therefore, we must strengthen collaboration between key stakeholders as well as ensure that scientific rigour is enforced through the development and validation of these systems.

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Declaration of interest

The authors declare no conflict of interest.

Ethics statement

Not applicable.

Software and data repository resources

Not applicable, as no data were used and no models were created in the conduction of this literature study.

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animal

Short Communication: The potential of portable near infrared spectroscopy for assuring quality and authenticity in the food chain, using Iberian hams as an example

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*This communication assesses the use of a portable near infrared (**NIR**) instrument to measure quantitative (fatty acid profile) properties and qualitative ('Premium' and 'Non-premium') categories of individual Iberian pork carcasses at the slaughterhouse. Acorn-fed Iberian pigs have more unsaturated fats than pigs fed conventional compound feed. Recent advances in miniaturisation have led to a number of handheld NIR devices being developed, allowing processing decisions to be made earlier, significantly reducing time and costs. The most common methods used for assessing quality and authenticity of Iberian hams are analysis of the fatty acid composition of subcutaneous fat using gas chromatography and DNA analysis. In this study, NIR calibrations for fatty acids and classification as premium or non-premium ham, based on carcass fat measured* in situ*, were developed using a portable NIR spectrometer. The accuracy of the quantitative equations was evaluated through the standard error of cross validation or standard error of prediction of 0.84 for palmitic acid (C16:0), 0.94 for stearic acid (C18:0), 1.47 for oleic acid (C18:1) and 0.58 for linoleic acid (C18:2). Qualitative calibrations provided acceptable results, with up to 98% of samples ($n = 234$) correctly classified with probabilities $\geqslant 0.9$. Results indicated a portable NIR instrument has the potential to be used to measure quality and authenticity of Iberian pork carcasses.*

Keywords: Iberian ham, near infrared spectroscopy, counterfeiting, slaughterhouse, classification

Implications

Iberian hams are labelled according to the pigs' diet and the percentage of the pigs' Iberian ancestry, with an acorn diet and pure-bred Iberians being most desirable. In order to confirm authenticity of a carcass chemical analysis of the fat and genotyping are required from off-site laboratories, adding time to the final verification. There is a clear need for a method of analysis that is rapid, accurate and applied to the carcass online to differentiate the Iberian ham production systems. Using a handheld near infrared machine in the abattoir to accurately classify carcasses based on feeding regimes would markedly improve consumer confidence in the authenticity of the provenance of this premium product.

Introduction

Iberian ham is a dry cured product originating from Spain and is considered a luxury food item. The most highly valued Iberian ham, 'Iberico de bellota' is derived from a purebred black Iberian pig, farmed in free range systems, and fed on acorns and grass during the finishing period to live weights of 150 to 160 kg. Iberian pig meat has high levels of intramuscular fat which is considered a quality trait by consumers and provides the enhanced taste due to aroma development that occurs during the curing process (Muriel *et al.*, 2007). To satisfy the rising demand for Iberian ham, modified production systems have evolved and include crossbreeding, indoor rearing and dietary modifications. These additional farming systems have led to a decrease in the sensory quality of the dry cured products and difficulties in identifying the provenance of the product (Muriel *et al.*, 2004). In 2014, Spain phased in a classification system for Iberian ham that identified the dietary regime and the percentage of Iberian ancestry. This system was implemented to restore confidence in the market place and to prevent mislabelling and fraud.

The most common methods used for assessing quality and authenticity of Iberian hams are analysis of the fatty acid composition of subcutaneous fat using gas chromatography (**GC**) and DNA analysis for verification of genotype. Recently the near infrared spectroscopy (**NIRS**) has been applied to

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accurately predict parameters of interest, markedly reducing analysis times from days to minutes. Many natural products absorb near infrared (**NIR**) radiation at specific wavelengths; in particular N–H, O–H and C–H bonds are strongly absorbed by NIR radiation. A sample's NIR spectrum is a composite of all the absorbances from all the molecular bonds in the sample. Calibrations can be developed using two sets of data, the spectra produced by scanning a set of samples on an NIR machine and the reference data consisting of the chemical analysis of the samples. Research conducted at the University of Cordoba (De Pedro *et al.*, 1995) confirmed the potential of NIRS as a method of identifying carcasses based on the feeding regime. However, benchtop NIR machines are immobile, and their applications in commercial environments are limited. Recent advances in instrumentation have led to a number of portable handheld instruments appearing in the market. While the reduction in size of the NIR instruments allows for portability and application within the commercial environment, the miniaturisation of the machine reduces wavelength range and resolution which may impact the accuracy of some calibrations.

The objective of this research was to compare the accuracy of a handheld portable NIR machine operated within the abattoir to measure fatty acid profile of fat samples with a conventional benchtop machine. Applying NIR technology within the abattoir could provide rapid and accurate assessment on the quality and authenticity of the individual carcasses and markedly enhance customer confidence.

Materials and methods

Adipose tissue samples collected for near infrared scanning and reference analysis

The main data set used to generate models for the MN1700 comprised 495 samples from 45 different producers, collected over 2 years at a commercial slaughterhouse between 2015 and 2017. Samples of subcutaneous adipose tissue were taken from the tail insertion area in the coxal region. Sixty-six samples were collected between 2015 and 2016, and the remaining 429 were analysed in the same way in 2017. Samples were classified as either premium grade (bellota) or non-premium grade. A subsample (50 g) of each adipose tissue sample was analysed by NIR using the following instruments:

1. Benchtop NIR machine used in laboratory: FOSS NIR Systems 6500 (FNS6500) monochromator spectrometer (FOSS-NIR Systems Inc., Silver Spring, MD, USA), equipped with an interactance-reflectance fibre optic and covering the spectral range 400 to 2500 nm, with a spectral interval of 2 nm, and running WINISI 1.5 software (Infrasoft International, State College, PA, USA).
2. Portable handheld NIR machine used in the abattoir: a MicroNIR Onsite Lite (MN1700) produced by Viavi Solutions Inc. (formerly JDSU Corporation, Santa Rosa, CA, USA) was used. The MN1700 covers the range 900 to 1700 nm with an approximate spectral interval of 6.2 nm.

After scanning the samples were then melted in a microwave oven and the fatty acid composition of each sample was determined by GC following the methodology outlined in De Pedro *et al.* (2013).

On the initial 66 samples collected in 2015, two different scanning approaches were taken with the MN1700. One technique involved averaging five scans moving the probe continuously over the sample in a 'W' pattern. The second technique involved averaging 20 spot measurements taken in a predefined pattern across the sample. Spot measurements were 12 times more variable than the continuous movement method. Therefore, the continuous movement technique was used to collect the data for the quantitative and qualitative work.

Improving spectrum quality

The signal to noise ratio (**S/N**) is another important parameter to be considered when aiming to acquire a high-quality spectrum. The signal to noise ratio varies from one spectrometer to another, and system design and software settings can help to maximise this ratio. One solution to improve the S/N ratio is averaging over repeat measurements. Several measurements were made to establish the number of spectra to be averaged for every scan. A compromise between high S/N and a rapid spectral acquisition was achieved by averaging 200 scans for each spectrum. This allows the analysis of every pig carcass even if high processing speeds of 100 or more carcasses per hour are achieved. Therefore, forcing the acquisition of 5×200 spectra to be collected, and averaging these for the final spectrum to be predicted, would increase the accuracy of prediction. Setting the number of scans to average can be done in the Viavi software, while averaging the five spectra was done in the WinISI software.

Quantitative models

The determination of the fatty acid profile has a high relevance for the quality control of Iberian pig meat products. Fatty acid profile of the subcutaneous adipose tissue performed by GC has been traditionally used for classifying and/or authenticating animals in different commercial categories, with acorn-fed Iberian ham having more unsaturated fats than those fed on compound feed. Before the FOSS spectra were used to develop calibrations, they were trimmed to the MN1700 range (908 to 1676 nm) and interpolated using cubic splines to give absorbances at the same 125 wavelength points as the MN1700. Six pre-treatments were investigated: raw absorbance spectra, first derivative, and second derivative, each tried without and with Standard Normal Variate (**SNV**) pre-processing. In the case of two treatments, the SNV was applied after the derivative. The numbers of factors were chosen based on the plot of Root Mean Square Error of Cross-Validation (**RMSECV**) versus number of factors, observing where curve starts to flatten out, giving the best RMSECV for the optimum number of factors.

Table 1 *Numbers of partial least squares (PLS) factors, root mean square error of cross-validation (RMSECV) and ratio of predicted to deviation (RPD) for separate PLS calibrations for four fatty acids developed on Iberian pig adipose tissue*

	Wet chemistry fatty acid data				FNS6500			MN1700		
	Mean (%)	SD (%)	Min (%)	Max (%)	PLS Factors	RMSECV (%)	RPD	PLS Factors	RMSECV (%)	RPD
Palmitic C16	23.4	2.1	18.4	28.9	8	0.63	3.3	14	0.84	2.5
Stearic C18	12.0	2.3	7.7	18.6	6	0.76	3.0	4	0.94	2.4
Oleic C18:1	50.1	3.7	40.9	58.3	8	1.1	3.4	13	1.47	2.5
Linoleic C18:2	8.0	1.1	4.8	11.4	6	0.47	2.3	13	0.58	1.9

Table 2 *Confusion matrices for Linear Discriminant Analysis (LDA), Quadratic Discriminant Analysis (QDA) and Nonparametric Bayes (NPB) using principal components derived from raw spectra of Iberian pig adipose tissue for both calibration (using cross-validation) and validation sets*

		Calibration ($n = 295$)		Validation ($n = 200$)	
		Premium	Non-premium	Premium	Non-premium
True class	Premium	160		105	
	Non-premium		135		95
LDA	Premium	155	5	103	2
	Non-premium	10	125	3	92
QDA	Premium	154	6	102	3
	Non-premium	8	127	3	92
NPB	Premium	156	4	103	2
	Non-premium	5	130	1	94

Qualitative models

The objective with qualitative models is to use the spectral data to make a direct classification of the carcass as either premium or non-premium, without the need for a quantitative prediction of the fatty acids. Given that there will be samples for which the classification is uncertain, it is important to select methods that are able to quantify that uncertainty. Therefore, the initial focus is on algorithms whose output has the form of probabilities of class membership. Of the 495 samples, 265 were premium grade (bellota) and 230 were non-premium grade. Three Bayesian methods have been applied: linear discriminant analysis (**LDA**), quadratic discriminant analysis (**QDA**) and a nonparametric approach, all with the same underlying structure. The principle is to reduce the spectral data, to scores or principal components, with the scores scaled so that each has a variance of one over the training samples. Then, the multivariate distributions of these scores, conditional on class membership, are modelled by fitted probability distributions. The difference between the three methods lies in the probability models used for the within-class distributions of the spectral data. Linear discriminant analysis (McLachlan, 1992) uses two multivariate distributions with different means but a common covariance matrix. Quadratic discriminant analysis also uses two multivariate normal distributions, but now with different covariance matrices (McLachlan, 1992). The third approach, based on the method for quantitative calibrations described in Fearn *et al.* (2010), uses more flexible kernel density estimates to model the within-group distributions of the spectral data. All three methods were programmed in MATLAB, using routines from the partial least squares (**PLS**) Toolbox (Eigenvector Research Manson, WA, USA) to implement pre-treatments. For purposes of validation, the sample set was divided randomly into a calibration set of 295 samples (160 premium, 135 non-premium) and a validation set of 200 samples (105 premium, 95 non-premium). The approaches were tuned on the calibration set by cross-validation, and then the selected model for each approach was evaluated on the validation set.

Results

Quantitative models

The best calibrations used second derivative, calculated by a Savitzky-Golay filter with a second-order polynomial and a widow width of five points, which is around 30 nm with these 125-point spectra, and then SNV. The Root Mean Square Error of Cross-Validation values, using leave-out-one-producer, and numbers of factors were recorded. The same pre-treatments (second derivative + SNV) were used for the MN1700 and the RMSECV and PLS factors were recorded. Table 1 compares outputs from the FSN6500 and MN1700 for this calibration exercise.

Qualitative models

The confusion matrices for LDA, QDA and Nonparametric Bayes (**NPB**) are shown in Table 2. The overall error rates for LDA are 5.0% on the calibration set and 2.5% on the validation set. For QDA the error rates of 4.7% on the calibration set and 3.0% on the validation set are almost identical to those of LDA. Both have 20 errors out of 495, overall. Interestingly, it is not necessarily the same samples that are misclassified. Comparing the two lists of 20 misclassified samples, only 5 appear in both lists. Finally the overall error rates for NPB of 3.1% on the training set and 1.5% on the validation set are like those of LDA and QDA. Nonparametric Bayes gives slightly better classification although all the error numbers are small for all three techniques.

Discussion

Quantitative models

For the quantitative calibrations, comparisons have been made between the FSN6500 and the MN1700 (Table 1). As expected the FSN6500 gave better results in terms of the RMSECV and ratio of predicted to deviation (**RPD**). However, while the MN1700 shows a deterioration in accuracy, the results still show promise. Further work, including investigating different nonlinear approaches, will be needed to improve them.

Qualitative models

For the qualitative approach, the three Bayesian methods all give acceptable results in terms of classification success. To properly compare probabilities will require more samples due to the low error rates overall; comparing errors in probability bins on this small dataset is subject to considerable random error. More samples would also be desirable if more producers could be included. Although 45 producers are represented, many of these only contribute a small number of samples, while some contribute 40 or 50.

Conclusions

This work undertaken as part of the European Food Integrity Network clearly shows the application of NIRS in the food chain, using Iberian hams as an example. The emergence of portable handheld NIR instruments strengthens this potential by allowing *in situ* measurements to be made along the supply chain. The work reported here clearly demonstrates the feasibility of using the MN1700 for on-site classification of carcasses, linked to the quantitative fatty acids' calibration, and provides a tool that can be used in slaughterhouses. More work needs to be undertaken on the portable instrumentation to improve the accuracy and robustness of the calibrations, but the current study provides a strong foundation. Only if the method is adopted commercially will the cost of collecting many more samples be justified.

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Declaration of interest

No potential conflict of interest is reported by the authors.

Ethics statement

This paper was written within the guidelines produced by the ethics committee.

Software and data repository resources

None of the data were deposited in an official repository.

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animal

Review: Insect meal: a future source of protein feed for pigs?

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*Are insects the farm animal of the future? A key agenda for agricultural production systems is the development of sustainable practices whereby food and feed can be produced in an environmentally efficient manner. These goals require novel approaches to complex problems and demand collaboration between scientists, producers, consumers, government and the general population. The provision of feed for animals is a major contributor to land and water use and greenhouse gas (**GHG**) emissions. Further, overfishing and a reduction in available land and water resources on which crops can be grown has led to an increase in price of protein ingredients such as fish meals and oils and soybean meals. Determination of novel solutions to meet the feed protein requirements of production animals is key to the development of sustainable farming practices. The Australian pork industry aims to develop production systems that efficiently use available resources (such as feed and energy) and limit the production of emissions (such as manure waste and GHGs). Invertebrates (insects e.g. black soldier flies) are naturally consumed by monogastric and aquatic species, yet the large-scale production of insects for feed (or food) is yet to be exploited. Most insects are low producers of GHGs and have low land and water requirements. The large-scale production of insects can contribute to a circular economy whereby food and feed waste (and potentially manure) are reduced or ideally eliminated via bioconversion. While the concept of farm-scale production of insects as domestic animal feed has been explored for decades, significant production and replacement of traditional protein sources has yet to be achieved. This review will focus on the potential role of insect-derived protein as a feed source for the Australian pig production industry.*

Keywords: Black soldier fly, alternate protein, sustainability, waste, larvae

Implications

The growth of human populations has increased the demand for animal proteins for human food, creating increased pressure on natural resources. Concurrently, consumers are more concerned with the environmental impact of food production. In response, animal enterprises need to develop novel and sustainable production methods to meet these increased demands. Insects, which can bioconvert organic waste (e.g. vegetable waste and manure) into a novel animal feed protein source are currently under investigation. This review will discuss the current state of mass rearing of insects as a feed source for pigs.

Introduction

The global demand for protein as food and feed is increasing as human population increases and food consumption patterns change in response to economic growth. A key example is the increased buying power of the emerging Asian middle class who are consuming more premium imported products such as meat. Furthermore, consumption of pork has increased domestically in Australia over the last decade (Allen Consulting, 2017). The Australian pork industry, with its existing strong reputation for innovation, is well placed to lead research, development and adoption of novel feed products in response to this increased global demand (Bittner *et al.*, 2017). The production of animal protein for human consumption is frequently scrutinised by the general public for the impact (both real and perceived) upon greenhouse gas (**GHG**) emissions, land and water use, and impacts upon biodiversity (Williams and Price, 2010). Yet, the production of meat (and fibre) continues to be important cultural and economic practices. Australian livestock agriculture industries have been increasing in size over the past two decades and currently account for an estimated 2.7% of gross domestic product, 58% of land use and 59% of water extractions (Jackson *et al.*, 2018). According to the Food and Agriculture Organization of the United Nations, the goal of livestock production is to ensure that the system does not create undue pressure on ecosystems, biodiversity, land and forest resources and water quality. This view is supported by the

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Australian pork industry that is focused on reducing the environmental impact of the industry by improving the use of resources and managing emissions that contribute to global climate change.

The provision of feed protein sources for animal production is responsible for a large portion of the associated environmental impact of livestock industries. As the demand for animal proteins increases, the gap between supply and demand of feed protein will continue to increase. Soybean meal, the major feed plant protein source included in monogastric animal diets, has a large environmental impact due to land and water use to grow soybeans and the large-scale transportation of this commodity (Wiedemann *et al.*, 2016). Soybean meal availability for animal feed can be limited due to its use in human food streams and low productivity; and in Australia most soybean meal is imported from the US and South America (M'Gee, 2011). While other feed protein sources such as meat meals and tallow are balanced sources of amino acids (**AA**s) and are an efficient use of waste products from the animal production industries, they require processing and transport to be suitable for use in monogastric animal diets. Replacement of soybean meal with locally derived protein sources will likely lead to reductions in associated land use, energy use and emissions. A potential alternate feed protein source previously unexploited by large-scale production is insects. With feed conversion ratios approaching 2.0 (van Huis, 2013), the efficiency of production of insects is superior to traditional domestic animal species.

Insects are cold-blooded, rapid growing, protein and lipid rich, require minimal space and contribute to the natural diet of many species. Further, the waste produced by insect production is a valuable source of organic fertiliser that can be utilised in crop production, thus creating a closed circular economy. Insects, such as fly larvae, can bioconvert products such as vegetables and fruits, grain and grain residues, manure and animal remains into proteins (35% to 55% **CP**) and lipids (10% to 40%). The FAO (2011) suggests that a major initiative to reduce food waste would involve cooperation between farmers both to reduce oversupply and to find alternative streams in which waste can be efficiently used. Insects commonly examined for commercial rearing include black soldier flies (**BSF**s; *Hermetia illucens*), common houseflies (*Musca domestica*) and yellow mealworms (*Tenebrio molitor*). Black soldier flies are endemic to Australia, do not bother humans as they do not eat as adults and do not naturally carry pathogens, making them an ideal candidate for large-scale rearing. Fly larvae are found to naturally grow in manure from pigs, cattle and poultry. Fewer substrates have been explored for rearing mealworms. Several recent reviews have discussed the potential use of insects as animal feed (Rumpold and Schlüter, 2013, Makkar *et al.*, 2014, Wang and Shelomi, 2017). This review will discuss the potential for insect-derived protein to contribute to the protein feed sources available to the Australian pig production industry. While this review will not discuss the technical processes of rearing insects on a commercial scale, it is imperative that rearing processes are efficient in terms of output, energy/resource usage and GHG emissions to remain sustainable. For example, rearing temperature can impact the conversion efficiency of insects, and if climate control is required the energy requirements of the system would be greater (Rumpold and Schlüter, 2013). The insect farming industry is in its infancy, with large scale-research and investment required in Australia and worldwide to confirm the safety, reliability, sustainability and potential contribution of insects as an animal feed source.

Insects as feed and food

The most commonly consumed insects globally are beetles, caterpillars and crickets (van Huis *et al.*, 2013). As there are more than 40 insect species that are endemic to Australia (Rumpold and Schlüter, 2013), there is likely an appropriate insect species that can be tailored for individual production industries and types. The large-scale production of insects may contribute to filling the feed protein gap in a more efficient manner, as demonstrated by crickets (up to 80% digestible material) who are two times as efficient as chickens in converting feed to meat; four times as efficient as pigs and 12 times more efficient than cattle (van Huis *et al.*, 2013). Further, to produce a similar quantity of protein, pork or chicken enterprises would need 2 to 3.5 ha land, while insects would require only 1 ha of land to produce equivalent insect (mealworm) protein (van Huis *et al.*, 2013). In a review commissioned nearly a decade ago, the Australian poultry cooperative research centre concluded that while there is potential for insects to be used as a protein feed source for poultry production systems, further assessment of cost-benefits, investigations into feed security and growth responses to insects are required (Khusro *et al.*, 2012).

Life cycle assessments consider emissions throughout the production system and include the emissions from inputs such as animal feed. Replacing the soybean meal content of pig diets with larvae reared on waste is modelled (by attributing life cycle assessment) to result in reduced global warming potential and land use, though a consequential life cycle assessment demonstrated contradictory results (van Zanten *et al.*, 2018). Substitution of feed products with alternate by-products or co-products will not always result in a net reduction of the overall environmental impact, as in complex diet formulations direct substitutions are not always possible and additional ingredients may need to be included to ensure the resultant diets remain balanced. Further, the processing and transportation of insect proteins contribute to the overall emission and energy use which may not always be favourable when compared to traditional protein feeds. While water use by insect rearing processes has not been directly assessed, drought-resistant larvae exist and are predicted to have lower water, and certainly land use, compared to terrestrial animals and crops.

The Australian pork industry is dedicated to producing environmentally sustainable pork that also maintains a competitive pork industry in Australia. It is widely accepted that

the feed costs associated with animal production systems can account for 60% to 70% of the cost of the entire enterprise. The high cost of feed is particularly impactful in pig production systems, with Australia having some of the highest grain prices in the world, leaving Australian producers especially vulnerable. For example, a recent downturn in pork consumption combined with increased feed costs lead to a 1.7% reduction in the number of Australian pigs slaughtered (Australian Pork Limited, 2019). Australian pork industries already contribute to reducing waste by heavily utilising agricultural and food processing by-products (such as yeast, tallow and oil seed pressings) as animal feed. Further, Australian piggeries are located in close proximity to agriculture and food processing facilities where waste is produced, and many piggeries have infrastructure in place to handle by-products. Thus, pig production systems are ideally placed to develop and adopt novel feed sources such as insects. This aligns directly with the seven main reasons for which the pork industry has developed a sustainability strategy (Australian Pork Limited, 2013).

Swill feeding pigs (such as processor and catering waste, non-processed animal products and offal) is illegal in Australia. This waste contributes to the estimated >5 million tonnes of food wasted at the production and pre-consumer stage in Australia each year, with over 75% of this waste sent to landfill (Pickin *et al.*, 2018). It is often cheaper for food producers to dispose of waste rather than to find methods to utilise non-sellable food and feed waste. However, insects can bioconvert waste into a high-protein and high-fat products potentially suitable as animal feed sources. A large variety of insects are suitable for intensive production, meaning an ideal insect candidate is available for most environmental growing conditions and feed sources worldwide. While insects may provide a viable method via which food and feed wastes can be bioconverted, it is unlikely that these waste streams would provide a sufficient volume of substrate to produce a protein source in adequate volumes to totally replace soybean meal and other common protein feed sources. Assuming a production of 5 million tonnes of food waste and a feed conversion rate of 2.0, the contribution of insects reared on feed waste in Australia would only be 2.5 million tonnes, which would be able to only partially replace protein feed imports. However, insects could contribute to the available feed and manure waste management processes of various industries, while concurrently producing a valuable feed product.

The current state of insect production and uptake

Recently, significant investments in insect rearing start-up companies have been made in Australia and worldwide. At the beginning of 2019, at least 42 European companies were involved in the production of insect meal (Mancuso *et al.*, 2019), which according to the International Platform of Insects for Food and Feed (**IPIFF**) produced approximately 6000 tonnes of insect meal per year. In 2017, the insect rearing company Ynsect, a French company specialising in rearing insects for aquaculture with the goal of expanding into pig, poultry and pet foods in the future, raised AUD50 million from private investors (Jasper, 2017). To date, no data are available regarding large-scale commercial rearing of insects, though the IPIFF predicts that insect meal production will increase to 200 000 tonnes in 2020 and 1.2 million tonnes in 2025 (IPIFF, 2019). These volumes are still well below the current production levels of protein feeds and co-products produced, and this industry, which is in its infancy, requires large investment, research and development to grow into a viable competitive commodity. Research into automation processes is required to reduce the current high costs associated with production. Price thus remains a deterrent to larger adoption of insect proteins as current production levels remain low. European prices for BSF larvae range from €2 to 9/kg (approximately. AUD3 to 14) (Mancuso *et al.*, 2019).

The insect protein association of Australia (**IPAA**) was recently established to promote the use and role of insect protein within the Australian food and feed industries. The IPAA currently has 11 Australian member companies listed as openly trading or developing insects as food or feed, though not all currently operating companies are members of IPAA. While the Australian climate is well suited, currently no Australian facilities are capable of mass rearing insects, and no best practices have been developed anywhere worldwide. Mass rearing and breeding of insects will not require complex infrastructure, though the environmental conditions need to be managed to optimise insect growth. The development and adoption of new technologies across agricultural sectors will ensure that Australia remains a world leading producer of high-quality and safe animal protein for human consumption. Australia is uniquely placed to develop a sophisticated zero waste food production system as there is both available space and access to waste products that can be distributed in a safe manner.

A significant barrier to the uptake of insects as human feed is the aversion to eating insects by Western consumers, likely driven by the association of insects with disease (Bartrim, 2017). Insects have historically contributed to the diets and cultural practices of humans in a manner that is often overlooked. For example, the bogong moths (*Euxoa* = *Agrotis infusa*) which migrate to the mountains of New South Wales in populations of the millions, were feasted upon by Indigenous Australian populations for weeks to months each year (McKeown, 1944). Modern descriptions of culinary practices and art of Indigenous Australians underplay and, in most instances, completely overlook the role of insects (Meyer-Rochow and Changkija, 1997), and the modern diet consumed by Indigenous Australians has been heavily influenced by European diets. Despite a rich cultural history of entomophagy throughout Asia, Australia and Africa, consumers from Western societies are more likely to adopt insects as an animal feed rather than as a human food protein source. Marketplace acceptance of insects as food in Western societies is primarily driven by personal attitudes towards food (i.e. acceptance of novel foods), cultural and familial exposure and interest in the environmental

impact and sustainability of the food (Verbeke, 2015). Attitudes towards insects as an animal feed source are thought to be positive, as highlighted by a survey of agricultural stakeholders and farmers in Belgium (Verbeke *et al*., 2015). Further, German dog-owning consumers were willing to accept insects for feeding meat- or egg-producing animals or domestic pets (Von Jeinsen and Heise, 2018).

It is worth considering the environmental and socio-economic benefits of this novel industry, which may surpass the current projections based solely on costs of input *v*. output. Quantifying the environmental benefits of insect production systems is less straightforward, as demonstrated by contrasting responses from life cycle assessments (van Zanten *et al*., 2018). Insects can also be produced as bait for smaller mammals, for their medicinal values and for the provision of fertiliser. These properties are particularly beneficial to the production of small-scale farms in developing countries. These small-scale practices may be less viable economically as stand-alone farms; but when considered as additional income streams or to produce protein sources for use as feed or food, there would be localised economic empowerment benefits. This is particularly true for the development of food and economic security in developing nations. For example, producing insects in orphanages in the Democratic Republic of Congo can generate significant income (approximately US$300/month), while also producing a sustainable and nutritious food and feed source (Franklin *et al*., 2018).

The production of insects on a commercial scale will require the establishment of a new supply chain with contributions from farmers, nutritionists, biochemists, processors and marketers. As summarised by Aarts (2019), the food and feed industries need to work together to promote the advancement and acceptance of the insect production industries by getting the science right, not compromising on food/feed safety, innovating, thinking outside the norm and collaborating. The ethical, legal and safety implications of insect production in Australia must be considered and framework developed as the industry progresses and grows.

Legal implications

The legalities of feeding insects to animals differ worldwide, as summarised for key regions (European Union (**EU**), US, China, Mexico, Australia and Canada) by Lähteenmäki-Uutela *et al*. (2017). In Australia, edible insects are currently classified as 'novel food' for human consumption. According to the Department of Agriculture and Water Resources, in Australia insects are currently fed to fish, poultry, pigs and pets. Animal feed materials in Australia are regulated by the Australian Pesticides and Veterinary Medicine Authority (**AVPMA**). Feed products do not require registration with the AVPMA if they (•) are fed as part of a normal diet; (•) are intended solely for nutritional purposes; (•) do not contain medications or other active ingredients and (•) do not make any health, production or performance claims. Insects are, therefore, not required to be registered. As farmed animals, however, insects are prohibited from being fed manure, catering waste or unprocessed meat products.

As a novel food source, Australian regulations (Food standards Australia New Zealand (**FSANZ**)) dictate that products containing insects undergo pre-market assessment (unless previously approved as are mealworms, crickets and mealworm beetles currently) and be approved by FSANZ before the product can be sold.

In Europe, the IPIFF has requested that European feed legislation be updated to allow the feeding of insects reared on vegetable substrates for pets, aquaculture, poultry and pigs. In July 2017, insect proteins (from BSF, house flies, yellow mealworms, lesser mealworms, house crickets, banded crickets and field crickets) were approved for inclusion in aquaculture diets in the EU (Boloh, 2018). Insect feed for ruminants is currently banned worldwide due to the perceived risk of BSE, though specific research into the transmission of prion (and other) diseases by insects is yet to be completed. China has an established regulatory framework for insect production, which has allowed for the upscaling of insect production. In Australia, regulatory hurdles exist that pose challenges for importing live insects and approval is required to import insect-derived feed products (which must be dead, and heat treated). As global research into feeding different types of livestock, infectious disease transmission, chemical accumulation and allergens are conducted regulatory frameworks are likely to be updated. These processes will, however, take time.

In the EU, it is unlikely that insects reared on animal waste or manure would be permitted to be fed to domestic production animals, as prohibited by the current classification of insects as farmed animals (which cannot consume manure, catering waste or unprocessed former foods containing meat or fish) (Boloh, 2018). However, advancements in understanding the processes and screening to assure product safety may provide scope to change these regulations.

Ethics is an aspect of large-scale insect production that is rarely considered. Do insects feel pain? Does it matter? Insects are comfortably reared in high-density environments likely without impacting animal welfare. However, methods of killing insects are not well researched, with current practices including freezing, cooking and boiling. Australia and other developed nations have stringent ethics regulations that apply to domestically farmed animals; and if insects are to become farm animals in the future, it is likely similar guidelines would need to be defined and adhered to by commercial industries.

Chemical composition of insects

Insect chemical composition and size are determined by the insect species and the feed consumed during the rearing process. For example, one female BSF can produce 400 to 600 larvae, with each larvae estimated to consume 0.5 g organic matter per day. BSF flies do not consume feed as adults and thus accumulate protein and fat stores as growing larvae. Thus, the composition of feed provided to the growing larvae will dictate the final fat and protein content. This is promising as it allows for the manipulation of final products to meet the specific individual needs of different production

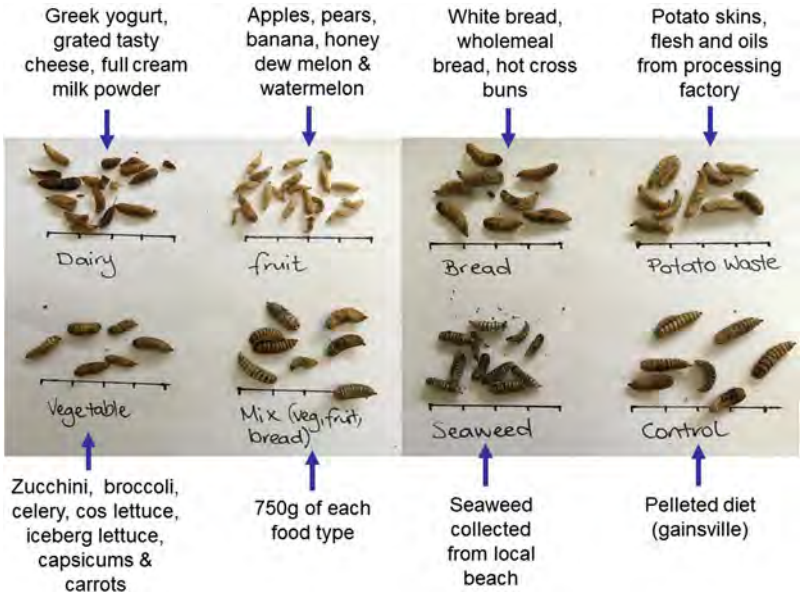

Figure 1 (Colour online) Example of black soldier fly larvae reared on different food waste streams for 7 days.

systems and animal types within a production system (i.e. weaner *v.* grower pigs). Figure 1 demonstrates the range of sizes of larvae reared on various food waste substrates for 7 days. Conversely, Lalander *et al.* (2019) investigated a range of rearing substrates on BSF larvae growth and nutrient content, demonstrating that AA and CP content did not vary greatly, while some sewage sludge were less suitable as rearing substrate than abattoir or fruit and vegetable wastes. Insects commonly investigated as animal feed sources, such as BSF larvae, housefly larvae and mealworms, range from 40% to 60% CP and 10% to 20% fat, and are high in minerals such as calcium (approximately 10% ash). The essential AA index was found to be favourable for BSF larvae as feed for growing pigs and broiler chickens (Veldkamp and Bosch, 2015). Table 1 presents the AA composition of BSF larvae compared to the common pig protein feed sources, demonstrating a favourable composition for inclusion in pig diets.

Drying and processing of larvae will impact the resultant nutrient content and availability due to changes in chemical and physical properties of proteins and lipids. For example, oven-dried larvae (60°C) had a superior AA digestibility compared to microwave-dried larvae (Huang *et al.*, 2019). Conversely, cooking method did not impact CP or fat content of crickets or mealworm larvae, though *in vitro* **DM** digestibility was reduced (Poelaert *et al.*, 2017). Raw mealworms had a greater *in vitro* CP digestibility compared to cooked mealworms, which was comparable to the CP digestibility of plant proteins (Poelaert *et al.*, 2016). However, feeding raw insects may not be feasible for numerous reasons including storage, transportation and product safety. Promisingly, Poelaert *et al.* (2017) conclude that the intestinal fermentation of feeds (insects, meats and plants) in pigs is primarily influenced by the base ingredient composition rather than the cooking methods applied. The quality of insect protein will also be influenced by the age/stage of insect production. This is primarily due to chitin, an indigestible polysaccharide in the cuticle of insects, which is present in greater concentration in adult crickets compared to larvae and leads to a lower *in vitro* digestibility in adults (Poelaert *et al.*, 2016). Chitin has properties that may be beneficial outside of feed production, such as a plant fertiliser, in food processing or as an immune modulator. Insects have also been shown to have antimicrobial properties.

Antimicrobial stewardship is a key focus of agricultural production systems, including pig production systems. Antimicrobial peptides (**AMP**s) are small and are both constitutively expressed and induced in response to infection or fungal challenge in insects (larvae) and can potentially be fed to production animals as an alternative to antibiotics (Li *et al.*, 2012). Insects have been used as treatments for disease in traditional medicine throughout history. More recently, insects have been shown to possess over 150 AMPs that do not lead to the development of resistance, exhibit activity against bacteria, fungi, parasites and viruses and are safe for supplementation into animal diets (see review by Jozefiak and Engberg, 2017). While further research is required to determine the mechanisms of expression, action and methods to exploit these insect AMPs properties, insects potentially have nutraceutical benefits in addition to the provision of dietary protein and fat.

Conversely, as reviewed by Mlcek et al. (2014), insects (particularly their chitin content) have the potential to induce an allergic reaction when consumed. These reactions occur in humans as they would in a typical food allergy whereby a reaction to specific proteins induces a histamine response via the immune system (van der Fels-Klerx *et al.*, 2018a). These allergies can also develop via cross-reactivity when allergies to related proteins (such as crustaceans) can induce a similar allergic response. Humans working with insects

Table 1 *Amino acid content (mg/g) of BSF larvae in comparison with common protein feed sources*

Amino acid (mg/g)	BSF larvae[1]	Soybean meal[2]	Fish meal[2]	Soy concentrate[2]	Whey protein[2]
Alanine	24	15	63	27	49
Arginine	17	26	64	46	21
Aspartic acid	34	40	85	72	108
Cysteine	4	5	9	9	21
Glutamic acid	43	63	128	120	167
Glycine	19	16	99	27	18
Histidine	12	10	20	16	22
Isoleucine	16	17	37	29	58
Leucine	25	27	65	49	102
Lysine	24	22	69	39	96
Methionine	6	5	26	8	19
Phenylalanine	15	18	33	33	33
Proline	20	18	53	33	58
Serine	14	19	48	34	47
Threonine	14	15	39	25	72
Tryptophan	4	5	9	8	21
Tyrosine	18	13	26	23	18
Valine	21	17	45	31	58
Total	321	351	918	630	988

BSF = black soldier fly.
[1]Data based on larvae reared on a pelleted diet containing 89.9% DM; 22.5% CP; and 13.5 MJ/kg DM metabolisable energy.
[2]Values obtained from the INRA CIRAD AFZ feed tables.

(such as insect producers or endpoint feed manufacturers) may also be vulnerable to allergic reactions via the inhalation of dust or dermal contact with insects (FAO, 2013), though these risks can be reduced by basic protective measures. The ingestion of insects may also initiate allergic responses that may not be prevented by subjecting them to treatments, such as heat treatment, though research into these processes in humans and production animals is scarce. It has been demonstrated that the particle size of the chitin will impact the allergic response, with medium particles initiating allergic inflammation and smaller particles reducing inflammatory responses (van der Fels-Klerx *et al.*, 2018a). Further research is required into the possible allergic responses to insects as feed and the potential for processing to eliminate or reduce these responses, particularly in pigs.

Insect meal in pig diets

Feeding BSF to pigs has received renewed attention in recent years, though it is not an entirely new concept. Over four decades ago, wild BSF larvae reared on beef cattle faeces and urine were collected, dried and fed to a small number of growing pigs. These studies demonstrated that while fat content may impact palatability and digestibility, BSF larvae are a suitable ingredient for pig diets (Newton *et al.*, 1977). Table 2 summarises the few publications to date that have examined the feeding of insects to pigs. Neumann *et al.* (2018) compared the complete substitution of soybean meal in piglet and grower pig feed with either BSF larvae (partially defatted) or algae meal (*Spirulina platensis*) and observed no difference in the growth parameters and a superior apparent N digestibility in the BSF diet. Replacing the soybean meal content of growing/finishing pigs diets with either 50 or 75 and 100% BSF meal (partially defatted; 61% CP and 14% lipid) had no negative effect on the resultant pork quality and sensory parameters with improved juiciness in BSF supplemented groups (Altmann *et al.*, 2019). Pigs fed diets supplemented with BSF larvae produced back fat with higher polyunsaturated fatty acid content, likely driven by the high-fat content of the larvae (Altmann *et al.*, 2019). While further larger scale experiments are required to confirm the palatability, inclusion level, growth responses and meat quality of the commercially raised pigs fed insects, the overall initial results are positive.

Safety of insect protein

A key consideration of any new feed product is the safety and acceptability of the product, which needs to be free of contaminants such as pathogens, bacteria, chemicals, toxins and heavy metals. These considerations need to be made specific for each species of insect reared and for each species to be fed the insect diets. Unlike other species, BSFs are not disease vectors as they do not lay their eggs on decaying organic materials and the adults do not eat decayed materials (van Huis *et al.*, 2013). Feeding animal-derived protein sources to livestock has an inherent risk of disease transfer, for instance, transmissible spongiform encephalopathy (BSE in ruminants) and other prion diseases, which has led to the worldwide prohibition of such proteins from most animal production systems (including Australia). To date there has been no evidence to suggest that insects carry prions, though further specific research are required.

Contaminants in insect rearing feed, particularly if the feed is a waste product, need to be eliminated (if possible) or quantified. Some contaminants such as veterinary drugs and prions need to be completely removed from the rearing diets, while others may be removed by processing. In a review of the risks of chemical contaminants when feeding various fly larvae species, Charlton *et al.* (2015) demonstrated that heavy metals posed the greatest risk of accumulation in resultant insects. While of the 1140 compounds measured, only 7 were present in larvae, with Cd posing the greatest risk (Charlton *et al.*, 2015). Similarly, BSF larvae reared on substrates spiked with heavy metals (As, Cd, Pb, Hg, Cr and Ni) accumulated Cd and Pb in significant quantities, while As in larvae was at the same concentration as that found in the rearing material (Cai *et al.*, 2018). Both mealworms and BSF larvae have demonstrated the ability to consume feeds containing mycotoxins and pesticides and remove these toxins, so that the resultant larvae/mealworms do not accumulate the toxins (Cai *et al.*, 2018, Van Der Fels-Klerx *et al.*, 2018b). In addition, pesticides were not accumulated in BSF larvae reared on substrates spiked with pesticides (chlorpyrifos, chlorpyrifos methyl and pirimiphos methyl) (Purschke *et al.*, 2017).

Table 2 Summary of published growth responses in pigs fed diets containing insects

Pig age/number of animals	Insect species	Feed inclusion level	Growth responses	Reference
5-week-old barrows, $n = 6$ (Latin square)	BSF	33%	Increased feed intake ($P < 0.05$); reduced apparent DM digestibility ($P < 0.05$)	(Newton *et al.*, 1977)
Early weaned pigs (*n* not reported)	BSF	0%, 50% or 100% replacement of dried plasma	50% diet improved performance; 100% diet decreased performance	(Newton *et al.*, 2005)
Weaned pigs ($n = 120$)	Mealworm	0%, 1.5%, 3.0%, 4.5% and 6.0% replacement of soybean meal	Linear increase in BW, ADG, ADFI, DM and CP digestibility	(Jin *et al.*, 2016)
Weaned female pigs ($n = 48$)	BSF	0%, 30% and 60% replacement of soybean meal	Linear increase in ADFI No effect on growth	(Biasato *et al.*, 2019)
Barrows ($n = 48$)	BSF	50%, 75% and 100% replacement of soybean meal	No effect on base meat quality measures, increased juiciness ($P < 0.05$); higher back fat PUFA contents ($P < 0.05$)	(Altmann *et al.*, 2019)

BSF = black soldier fly; ADG = average daily gain; ADFI = average daily feed intake; PUFA = polyunsaturated fatty acid.

Rearing insects on manure

Rearing insects on manure is a promising approach for recycling manure waste that can otherwise contribute to environmental issues associated with pollution and GHG emissions. Under the research priorities of the Australian Government, enhanced food production via improved management and use of waste is a key practical challenge for Australia's future. The reduction in manure dry weight when being consumed by BSF larvae ranges from 30% to 50%; while the larvae can also reduce the N content of the manure by 30% to 80% (Newton *et al.*, 2005, Myers *et al.*, 2008, Oonincx *et al.*, 2015). BSF larvae can also reduce the emission of volatile organic compounds from poultry, pig and dairy cattle manure, by greater than 87%, with up to 100% reductions observed (Beskin *et al.*, 2018). The contamination of manure with veterinary medications is one of the greatest risks of the proposition of larvae rearing as animal feed, as demonstrated by contamination of larvae fed manure with a coccidiostat antiprotozoal agent used to treat chickens (Charlton *et al.*, 2015). However, BSF larvae are able to reduce the pathogenic bacterial count in pig manure (Elhag *et al.*, 2018), reduce the *E*scherichia *coli* counts in dairy manure (Liu *et al.*, 2008) and *E. coli* and *Salmonella enterica* in dairy and chicken manure (Erickson *et al.*, 2004), though this response was not observed in slightly acidic pig manure. This important finding indicates that BSF larvae can be used to eliminate pathogen loads from manure prior to their use as fertiliser, while the resultant larvae can then potentially be used as an animal feed source.

Conclusion

Insects pose an opportunity to develop a novel sustainable feed source for pig producers in Australia. Inclusion of insects directly in the diet of Western population is likely to be met with resistance, while the inclusion of insects in the diets of production animals is more likely to be accepted widely. The AA and energy contents of insects such as BSF are ideally suited for inclusion in pig diets as a replacement to soybean meal and other feed protein sources. The potential for insects to produce AMPs provides an exciting opportunity to produce feed for production animals as an alternative to antibiotics. While it is technically possible for insects to be included in the commercial diets of production animals, further research is required in the following areas: (•) rearing, processing and storage methods – practices need to be scalable and energetically and economically sustainable; (•) legal frameworks and regulations need to be developed to ensure product safety and reduce risk; (•) large-scale examination of on-farm inclusion of insects in animal diets to confirm palatability, inclusion levels, growth parameters, product quality and safety; (•) examining the possibility and safety of rearing insects on manure; and (•) measurement of bioactive/nutraceutical properties of insects that may have benefits in addition to the provision of nutrients. Although it is unlikely that the production of insects from feed waste alone would be possible on a large enough scale to eliminate the requirement for additional protein sources such as soybean meal, insects provide a valuable opportunity to contribute to the available feed sources. As a side stream or manure management system for the Australian pig production industry, with adequate research and development, insects such as BSF could contribute to producing a sustainable circular economy. While optimal rearing environments and processes are yet to be determined, it is likely that insect production will reduce GHG production, water consumption and land use compared to the traditional protein sources. Determination of novel solutions to meet the feed protein requirements of production animals is key to the development of sustainable farming practices, and insects are an ideal contributor to this goal in the future.

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Declaration of interest

The authors declare no conflicts of interest.

Ethics statement

Not applicable.

Software and data repository resources

None of the data were deposited in an official repository.

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animal

Review: Water medication of growing pigs: sources of between-animal variability in systemic exposure to antimicrobials

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On many Australian commercial pig farms, groups of growing pigs are mass-medicated through their drinking water with selected antimicrobials for short periods to manage herd health. However, delivery of medication in drinking water cannot be assumed to deliver an equal dose to all animals in a group. There is substantial between-animal variability in systemic exposure to an antimicrobial (i.e. the antimicrobial concentration in plasma), resulting in under-dosing or over-dosing of many pigs. Three sources of this between-animal variability during a water medication dosing event are differences in: (1) concentration of the active constituent of the antimicrobial product in water available to pigs at drinking appliances in each pen over time, (2) medicated water consumption patterns of pigs in each pen over time, and (3) pharmacokinetics (i.e. oral bioavailability, volume of distribution and clearance between pigs and within pigs over time). It is essential that factors operating on each farm that influence the range of systemic exposures of pigs to an antimicrobial are factored into antimicrobial administration regimens to reduce under-dosing and over-dosing.

Keywords: antimicrobial concentration, water consumption, pharmacokinetics, efficacy, antimicrobial resistance

Implications

Antimicrobials provide substantial animal productivity, health and welfare benefits to the Australian pig industry. Mass medication of groups of growing pigs through drinking water offers several advantages over continuous antimicrobial administration in feed, and is a valuable tool for pig producers and veterinarians. However, significant variation between pigs in systemic exposure to an antimicrobial (i.e. the antibiotic concentration in plasma over time) occurs when a group of pigs is administered an antimicrobial through their drinking water. Many pigs in a group may be under-dosed or over-dosed. Under-dosing with an antimicrobial may lead to reduced clinical efficacy and failure to eliminate the target pathogen, and thus may contribute to the development of antimicrobial resistance in pig-specific bacterial pathogens. Over-dosing may increase the risk of toxicity to the animal and the persistence of residues in pigs at slaughter, and unnecessarily increase farm medication costs.

Introduction

On many commercial pig farms, groups of growing pigs are mass-medicated for short periods with antimicrobials through their drinking water to manage herd health. This method of delivery, commonly called 'water medication', may be conducted strategically at regular intervals to achieve metaphylaxis and when necessary to treat clinical disease caused by bacterial pathogens.

A water medication dosing event on a pig farm commonly involves administration of the calculated dose of a selected antimicrobial product to a group of pigs over several hours. On Australian pig farms, dosing events are typically commenced after sunrise, when pigs have begun eating and drinking and regular farm staff members have commenced their working day. A dosing event may be conducted once or repeated on several consecutive days.

Two methods are used on commercial pig farms to mass-medicate pigs through their drinking water: (1) Direct dosing of the water supply by adding medication into a header tank located at or within each shed, or (2) proportional dosing, using a water or electric-powered pump that continuously draws up a concentrated stock solution of medication from a container and injects it into a shed's drinking water supply line at a volumetric ratio that can be set by the operator. A recent survey of Australian pig farms found that the majority had water medication dosing systems. Most farmers used proportional dosing pumps to deliver medications into the drinking water line, while some used direct dosing (Edwards, 2018).

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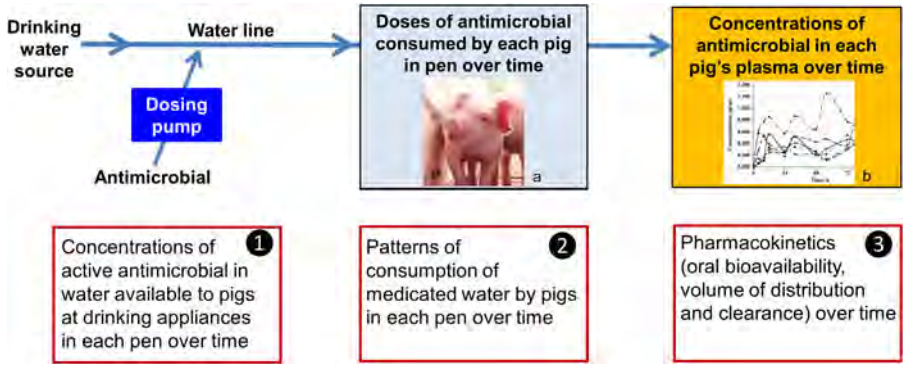

Figure 1 (Colour online) Sources of variability in systemic exposure of pigs to an antimicrobial during a water medication dosing event.
[a] Source: Kanters Special Products BV.
[b] Source: Mason *et al.* (2009).

When a regimen for antimicrobial administration through drinking water or feed *ad libitum* for a group of pigs is determined, the dose is commonly based on a measurement or estimate of the average BW of the group and the pigs' daily water or feed consumption. However, it cannot be assumed that all animals will experience the same level of systemic exposure to the antimicrobial over time. Several studies have assessed the pharmacokinetic (**PK**) parameters of different water-soluble antimicrobials after administration to pigs in drinking water offered *ad libitum* over several days. These studies found significant variation between pigs in systemic exposure to the antimicrobial and time taken from commencement of administration to attainment of a steady-state plasma concentration of antimicrobial.

Prats *et al.* (2005) found that when 20 to 25 kg pigs penned in groups of six were administered doxycycline in water offered *ad libitum* for 5 days, the mean maximum plasma concentration (C_{max}) was 2.2 μg/ml (SD: 1.6 μg/ml), and the mean area under the plasma antimicrobial concentration-time curve (**AUC**) was 138.4 μg·h/ml (SD: 125.5 μg·h/ml). Jensen *et al.* (2006) administered amoxicillin to individually penned weanling pigs in water offered *ad libitum* for 4 h/day (after withholding water for 3 h) for 2 days. They found large variations between pigs' C_{max} and AUC values. This was associated with large variation in the dose of amoxicillin consumed (control pigs, Day 1: median dose ingested: 26 mg/kg (range: 9 to 41 mg/kg); median C_{max}/dose: 0.29 kg/l (range: 0.14 to 1.03 kg/l); median AUC/dose: 2.5 kg·h/l (range: 1.3 to 5.9 kg·h/l)). Mason *et al.* (2009) administered tetracycline to individually penned 16 kg pigs at three concentrations (125, 250 and 500 mg/l) in water offered *ad libitum* for 5 days. The mean AUC_{total} values found were 30.71 μg·h/ml (SD: 6.61 μg·h/ml), 44.93 μg·h/ml (SD: 8.26 μg·h/ml) and 73.74 μg·h/ml (SD: 4.88 μg·h/ml) respectively. Lindquist *et al.* (2014) found that when weanling pigs penned in groups of 10 were administered tetracycline in water offered *ad libitum* for 5 days, their mean plasma tetracycline concentration at 102 h was 1309 mg/l (SD: 492 mg/l).

Several studies have also detected high levels of between-animal variability in plasma concentrations when an antimicrobial was administered to pigs through feed *ad libitum* over several days (Agersø and Friis, 1998a; Anfossi *et al.*, 2002; del Castillo *et al.*, 2006; Reyns *et al.*, 2007; Godoy *et al.*, 2011; Soraci *et al.*, 2014). Soraci *et al.* found that between-animal variability in plasma concentrations was higher when fosfomycin was administered to pigs through feed *ad libitum* than through drinking water *ad libitum*, with CVs of 41% to 61% for feed compared to 19% to 30% for water. If these estimates of variability are accurate, then water medication has an important advantage over in-feed medication.

Understanding the sources of between-animal variability in systemic exposure to an antimicrobial when medicating a group of pigs on a farm through their drinking water is critical. It enables dosage regimens to be designed which not only are effective but also minimise development of antimicrobial resistance (Toutain and Lees, 2006; Bon *et al.*, 2018). These are as follows:

1. Variability in dose applied; that is, the concentration of the active constituent of the antimicrobial product in water available to pigs at drinking appliances in each pen over time,
2. Variability in dose consumed; that is, patterns of consumption of medicated water by pigs in each pen over time, and
3. Variability in PKs; that is, oral bioavailability, volume of distribution and clearance between pigs and within pigs over time (Figure 1).

In this review, we explore these three sources of variability, identifying knowledge gaps, and consider the implications for successfully water medicating pigs at the group level.

Variability in the concentration of active antimicrobial in water over time

Antimicrobial product solubility

Solubility is the most critical factor in the formulation of any antimicrobial product that is administered to pigs through drinking water. After mixing in water, the active constituent must remain in solution at close to the concentration required for clinical efficacy and maximum inhibitory activity against the targeted microbe for the entire dosing period. Solubility contributes not only to the dose of the active constituent consumed by the pig, but also to its absorption and distribution in the animal (Crea *et al.*, 2012).

Table 1 provides details about nine water-soluble antimicrobial products registered in Australia that are commonly

Table 1 *Nine water-soluble antimicrobial products registered in Australia that are commonly administered to pigs*

Product	Active constituent(s)	Strength (proportion of active constituent in product)	Solubility of active constituent in water[ab] (mg/ml)	Concentration of active constituent(s) in water at pigs' drinking appliance as per label directions (mg/ml)	Antibiotic : water ratio by volume for dosing pump above which solubility of active constituent (based on a and b) is exceeded
A	Amoxycillin as amoxycillin trihydrate	870 g amoxycillin/kg	2.7[a] mg amoxycillin trihydrate/ml	0.2 mg/ml (=0.23 mg product/ml × 0.87)	1 : 14
B	Lincomycin as lincomycin hydrochloride	>790 g lincomycin/kg	50[a] mg lincomycin hydrochloride/ml	0.032 mg/ml (=0.041 mg product/ml × 0.79)	1 : 1562
C	Trimethoprim-sulphadiazine	80 g trimethoprim/kg and 400 g sulphadiazine/kg	4.0[b] mg trimethoprim/ml and 6.7[b] mg sulphadiazine/ml	0.053 mg trimethoprim/ml (=0.667 mg product/ml × 0.08) and 0.267 mg sulphadiazine/ml (=0.667 × 0.4)	1 : 25 (sulphadiazine being the more limiting active)
D	Oxytetracycline as oxytetracycline hydrochloride	880 g oxytetracycline/kg	>100[a] mg oxytetracycline hydrochloride/ml	0.293 mg/ml (=0.333 mg product/ml × 0.88)	1 : 341
E	Chlortetracycline as chlortetracycline hydrochloride	950 g chlortetracycline hydrochloride/kg	8.6[a] mg chlortetracycline hydrochloride/ml	0.238 mg/ml (=0.25 mg product/ml × 0.95)	1 : 36
F	Lincomycin as lincomycin monohydrate Spectinomycin as the sulphate tetrahydrate	222 g lincomycin/kg and 445 g spectinomycin/kg	50[a] mg lincomycin hydrochloride/ml and 50a mg Spectinomycin pentahydrate/ml	0.02 mg lincomycin/ml (=0.094 mg product/ml × 0.222) and 0.04 mg spectinomycin/ml (=0.094 mg product/ml × 0.445)	1 : 1190 (spectinomycin being the more limiting active)
G	Tilmicosin as tilmicosin phosphate	250 mg tilmicosin/ml	566[a] mg tilmicosin/ml	0.2 mg/ml (=0.8 ml product/l)	1 : 2830
H	Neomycin as neomycin sulphate	600 g neomycin/kg	50[a] mg neomycin sulphate/ml	0.22 mg/ml (=0.37 mg product/ml × 0.6)	1 : 227
I	Tylosin as tylosin tartrate	800 g tylosin/kg	50[a] mg tylosin tartrate/ml	0.2 mg/ml (=0.25 mg product/ml × 0.8)	1 : 250

[a] *Source*: TOKU-E (2016).
[b] *Source*: Yalkowsky *et al.* (2010).

administered to pigs. It highlights the challenges of using high concentration stock solutions in proportional dosing pumps of water-soluble antimicrobial products containing amoxicillin, trimethoprim-sulphadiazine and chlortetracycline. Even a stock solution with a concentration of 3.5 mg active amoxicillin/ml, intended for injecting into the drinking water supply line at a ratio of 1 : 33 by volume, exceeds the solubility of amoxicillin in water.

Drinking water sources on Australian pig farms may be a municipal water supply, surface water from a nearby river, lake or farm dam or underground water extracted from a bore. Bore water is the most commonly used water source, with surface water the next most common. The quality of the water from both these sources can be highly variable, as most bores in agricultural regions of Australia are relatively shallow, extracting water from upper aquifers (Edwards, 2018; Victorian Government Department of Environment, Land, Water and Planning, 2019).

The solubility of the active antimicrobial may be influenced by the water's pH, concentration of salts and metal ions, and the temperature of the water with which it is mixed (Yalkowsky *et al*., 2010). However, water-soluble antimicrobial products may contain non-active constituents intended to improve solubilisation of the active constituent (Kalepu and Nekkanti, 2015). For example, the solubility of amoxicillin is affected mainly by the pH of the water. Over an intermediate pH range (approximately 3.5 to 6.5), at which the amoxicillin molecule has components with positive and negative charges and an overall charge of zero (i.e. its zwitterionic form), solubility is at its minimum. At very low and very high pH levels, where cations or anions predominate, solubility is substantially increased. The temperature and the salt concentration of the aqueous solution have a lesser effect on the solubility of amoxicillin (Crea *et al*., 2012; Felix *et al*., 2016).

Antimicrobial product stability and homogeneity

The stability of an antimicrobial in solution may be affected over time by many external factors, including water pH, water hardness, water pipe materials, chlorine, metal ions, pH modifiers, other antimicrobials and stability enhancers (Dorr *et al*., 2009; Jerzsele and Nagy, 2009; Acero *et al*., 2010). Amoxicillin degrades much more rapidly over a 24-h period when in contact with metal (galvanised steel) compared to plastic. Amoxicillin degrades more rapidly in water with a neutral to high pH than in water with a low pH. Jerzsele and Nagy (2009) found that after 24 h mixed in aqueous solutions with a pH of 10, 7 and 3, the proportions of initial amoxicillin concentration retained were 68.2%, 72.4% and 79.6% respectively. Sodium carbonate (soda ash), which is commonly added to amoxicillin products in stock solutions to enhance solubility, thus may enhance degradation. To a lesser extent, amoxicillin degrades more rapidly in water that is moderately hard to hard (>100 mg equivalent $CaCO_3$/l) (Jerzsele and Nagy, 2009).

Chlorine reacts with several classes of antimicrobials, including amoxicillin, tetracyclines, trimethoprim and fluoroquinolones (Postigo and Richardson 2014). Amoxicillin is very sensitive to chlorine at concentrations commonly used to disinfect drinking water. Acero *et al*. (2010) found that amoxicillin was very rapidly removed from water with chlorine concentrations between 0.2 and 1 mg/l, as typically used in full-scale drinking water treatments by chlorine. Amoxicillin is not very reactive with other antimicrobials, with a precipitate forming only when it is mixed with chlortetracycline. Interactions between other commonly used antimicrobials may occur, resulting in precipitates (Dorr *et al*., 2009).

Products containing the same active constituent do not necessarily retain >95% of their initial concentration at 24 h after mixing, as shown in a study by Boeren *et al*. (2006) that compared eight water-soluble amoxicillin products. Two of the eight unnamed products assessed only retained 52.3% and 55% of their initial amoxicillin concentration of 4.8 mg/ml at 24 h after mixing. This suggests that only high quality antimicrobial products can be assumed to yield stable stock solutions, and that fresh batches of stock solutions should be prepared daily.

Passage of antimicrobial product through drinking water distribution system

There is a lag between commencement of a water medication dosing event and delivery of medicated water to pigs in a pen within a shed. Consistent with the nature of steady-state fluid flow through pipelines, the lag time is a function of: (1) the distance between the header tank or dosing pump and the pen, and (2) the velocity at which water flows through the drinking water distribution system to the drinking appliances in the pen. The concentration of antimicrobial in water at the drinking appliance over time is then a function of the concentration of antimicrobial in drinking water being discharged from the header tank or in the water supply line just downstream of the dosing pump over time and the velocity at which water flows through the distribution system (Crane Co. Staff, 2011). When a header tank is being used, the inlet valve may be deliberately left open so the tank continuously refills with unmedicated water, and top-up boluses of medication added. If so, then the concentration of antimicrobial in water flowing from the tank along the water line to pigs is likely to fluctuate considerably over time.

To deliver water to pigs through a farm's piped drinking water distribution system, pressure is required. This is provided either by gravity (from an elevated water source or storage facility) or by one or more pumps. The system must provide a minimum pressure during periods of peak demand and acceptable working pressures during average demand periods, so that water flow rates from drinking appliances in pens at the end of the water line are acceptable at all times. Hourly demand may increase if water is being used for other purposes, such as pressure-washing concrete pens in a building or spray cooling in hot weather (Crane Co. Staff, 2011).

Frictional pressure losses occur along every section of the pipe system. They are largely a function of the lengths, diameters and internal surface smoothness of pipes, the number and types of fittings installed along the pipeline and the velocity of the water flow. Excessive numbers of bends

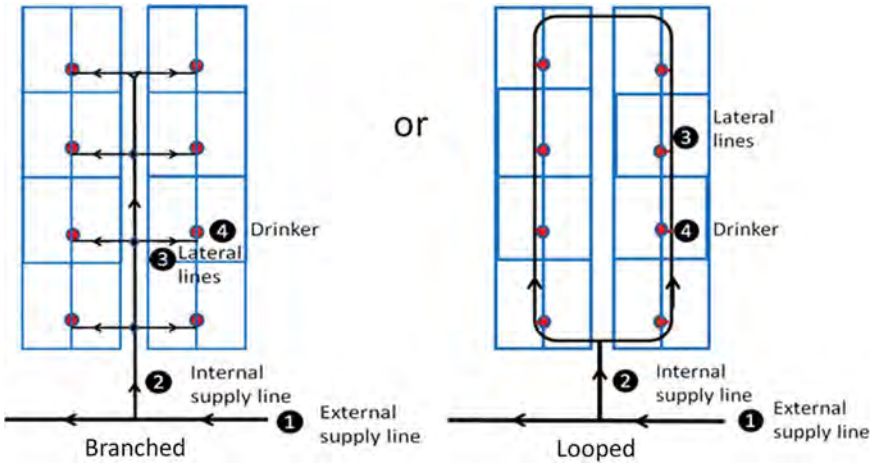

Figure 2 (Colour online) Two alternative configurations of a drinking water distribution system for a conventional, concrete-floored building housing weaner pigs (Adapted from AHDB Pork, 2018).

and changes in the diameters of pipes along the water line (constrictions or expansions) result in pressure losses due to deceleration and flow separation downstream. It is possible that these disturbances in flow may be advantageous during medication dosing events if they provide in-line mixing and reduce sedimentation of insoluble particles. Changes in pressure (head loss) along a water pipeline due to friction can be calculated using the Darcy–Weisbach equation (Crane Co. Staff, 2011).

Water supply lines supplying buildings and pens within buildings may be branched or looped (Figure 2). In a branched system, water velocities and pressure losses are greater than in a looped system, and capacity is reduced, especially during periods of high demand. There may be many dead ends where water is stagnant and sediments may accumulate. A looped system requires greater length of piping and more valves than a branched system and is therefore generally more costly than a branched system to construct, but has several advantages: greater reliability, ease of repair and maintenance, and the ability to provide water flow with a pump of smaller capacity (Watts *et al.*, 2016). A farm's drinking water distribution system may fail to provide every pig in each pen, in each building, with unrestricted access to drinking water throughout the day if it has been poorly designed or modified over time without due regard for the key principles of fluid mechanics, or if sections of pipe have become partially occluded by mineral build-up or biofilms.

Variability in medicated water consumption patterns of pigs over time

Water usage patterns and drinking behaviour

During a water medication dosing event, the patterns of consumption of medicated water by pigs in each pen over time are subject to pigs' diurnal activity rhythm, with most feeding and drinking activity occurring during daylight hours. This diurnal water consumption usage pattern is maintained by pigs even when they are housed in sheds with continuous lighting (Meiszberg *et al.*, 2009). Drinking occurs in frequent, short bouts. Water consumption and drinking patterns are driven by satiety and also influenced by factors including stress, boredom, hunger, environmental temperature, disease, feed type and constituents and water flow rates. Like many animals, pigs are prandial drinkers, with 75% to 85% of their drinking events being related to meals.

Using the data reported by Li *et al.* (2005) in Tables 1 and 3 of their paper from two experiments exploring water intake and wastage with several combinations of water flow rates and drinker heights, we calculated mean daily voluntary water consumptions of growing pigs of between 60 and 117 ml/kg BW. Water consumption over each 24-h period is characterised by one or two distinct peaks, with several studies observing two distinct peaks – one after sunrise and another in the mid-late afternoon (Madsen and Kristensen, 2005; Rousseliere *et al.*, 2016). As pigs grow, and their daily water usage increases with BW, the amplitude of each peak in usage becomes greater. The timing, shape and amplitude of the peaks in water usage may be influenced by environmental conditions within the building, particularly ambient temperature and humidity. Diurnal drinking patterns have been found to vary markedly between winter and summer, presumably as an adaptation to heat stress (Brumm, 2006).

Recent studies that have used automated video or radio-frequency identification (**RFID**)-based systems to measure water usage/consumption patterns of individual animals have found large variations between animals and within animals over time. Andersen *et al.* (2014) used RFID ear tags and readers over drinking appliances coupled with electronic water flow meters to study water usage patterns and drinking behaviour of individual pigs (8 to 9 weeks old) in 8-h periods over 4 days when housed in pen groups of 3 and 10 animals. Mean daily water usages were 4767 and 5212 ml for pigs penned in groups of 3 and 10 respectively, with a large SD of 753. Water usages of individual pigs within each 8-h period were also highly variable. Soraci *et al.* (2014) measured the daily water usage of 30 kg pigs, housed in pen groups of 18, using a video system and water flow meters. Mean water usage was 3.7 l/day (SD: 0.3 l/day). Rousseliere

et al. (2016) used an RFID system and water flow meters to measure daily water usage of individual pigs (7 to 10 weeks old) penned in groups of 19. Mean water usage was 107 ml/kg BW. The mean number of visits by pigs to drinking appliances each day was 27.2 (SD: 12.3), and mean water consumed by each pig on each visit to the drinker was 104 ml (SD: 133 ml). Large between and within-animal variations in water drinking behaviour were observed within each pen group over a 22-day period.

Pigs are very social animals. Social hierarchies may have a substantial impact on between-animal variability in the water consumption and drinking patterns (Soraci *et al*., 2014). When a small pen group of pigs (e.g. 20 pigs) is formed, they quickly establish their social ranks within the group, and each pig's social rank within the group then remains relatively stable. Day to day, intermediate and lowly ranked pigs in the group may be subject to acts of aggression by higher ranked pigs attempting to control and dominate limited resources in the pen (i.e. feed and water). If so, this could contribute substantially to between-animal variability in daily consumption of feed and water, and to feeding and drinking patterns within a pen, and ultimately systemic exposure to an antimicrobial during medication.

The study by Soraci *et al*. (2014) found that in a small pen group, social rank in the group explained up to around one-third of the between-animal variation in systemic exposure to an antimicrobial. It appears that social facilitation (the tendency for animals to synchronise their behaviour) influences drinking behaviour in pigs to some extent (Turner *et al*., 2000). When pigs are housed in a large pen group (greater than about 100 pigs), they do not attempt to control resources such as feeders and drinking appliances, opting instead to adopt a more tolerant and less aggressive social strategy (Samarakone and Gonyou, 2009). This may result in less between-animal variability in daily water consumption and drinking patterns.

Other known factors that may influence water consumption and drinking patterns

Disease. Most studies indicate that pigs suffering from diarrhoea caused by *Salmonella* spp. or *Escherichia coli*, or respiratory disease caused by *Actinobacillus pleuropneumoniae*, consume less water per day, have altered drinking patterns and commonly have reduced feed consumption (Pijpers *et al*., 1991; Krsnik *et al*., 1999; Ahmed *et al*., 2015).

Feed type and constituents. While diets with excessive protein levels may result in increased water consumption, generally dietary factors appear to have minimal impact on water consumption (Shaw *et al*., 2006).

Water flow rate. Provided the number of drinking appliances in a pen is appropriate for the pen group size, older pigs can readily increase their drinking time to compensate for water flow rates from drinking appliances that are lower than those appropriate for their BW. However, younger pigs may not be able to adapt sufficiently, resulting in lower daily water use, feed consumption and growth rates (Neinaber and Hahn, 1984; Barber *et al*., 1989).

Daily water wastage by pigs may range from 9% to 60% of total water usage, depending on many factors, including water flow rates, drinker design and position, room temperature, levels of competition between pigs, diet and water quality (Li *et al*., 2005; Meiszberg *et al*., 2009; Andersen *et al*., 2014, Wang *et al*., 2017). Unfortunately, many published studies investigating the drinking behaviour of pigs have only measured the total water usage/disappearance, without measuring the volume spilled by pigs, and the terms 'water usage/disappearance' and 'water consumption' have often been used interchangeably. Patterns of pigs' water consumption and wastage within each 24-h period under different herd management and environmental conditions have not been documented simultaneously.

Variability in pharmacokinetics

During and after a water medication dosing event, between-animal variability in the time course of plasma concentrations of an antimicrobial may be due to differences in one or more of the following processes: oral bioavailability, the rate of drug absorption, the apparent volume of distribution, clearance from the central compartment or plasma and distribution to peripheral compartments in the animal's body. Even small differences between pigs in these processes, especially oral bioavailability, may have substantial effects on the plasma antimicrobial concentration-time curve, regardless of variability in the dose applied and consumed by pigs over time.

Oral bioavailability of different antimicrobials

The oral bioavailability of different antimicrobials in the pig varies widely. While the oral bioavailability of sulphadiazine/trimethoprim and of enrofloxacin is over 80%, that of oxytetracycline and of chlortetracycline is very low, at less than 10%. The oral bioavailability of amoxicillin is generally low, with reported values varying widely from 11% to 48% (Agersø and Friis, 1998b; Godoy *et al*., 2011). The low bioavailability of amoxicillin after oral administration could be explained by a pre-systemic loss, probably in the intestine.

Oral bioavailability may be influenced by the presence of food in the gastrointestinal tract at the time of antimicrobial administration. Food-related effects on bioavailability have been observed in pigs for lincomycin, spiramycin, enrofloxacin, tetracycline and chlortetracycline, but not for amoxicillin and oxytetracycline (Nielsen and Gyrd-Hansen, 1996; Agersø and Friis, 1998b). Very little is known about between-animal variability in oral bioavailability and other PK parameters for specific antimicrobials. Whether an antimicrobial's oral bioavailability remains constant within a given pig over time, and is similar when the antimicrobial is administered as one or more boluses *v.* through drinking water provided *ad libitum*, is also not known.

Table 2 *Effects of different antimicrobial classes used in pigs*

Inhibitory action	Antibiotic classes	Pharmacokinetic/ pharmacodynamic index
Time-dependent	β-lactams (for efficacy) Older macrolides Lincosamides Sulphonamides	$Time_{24\ h}$>MIC*
Co-dependent	Tetracyclines β-lactams (for resistance) Fluoroquinolones (*v.* anaerobes) New macrolides (tulathromycin)	$AUC_{24\ h}$§/MIC
Concentration-dependent	Aminoglycosides Fluoroquinolones Metronidazole (*v.* anaerobes) Polymixins	C_{max}/MIC‡

Source: Adapted from Turnidge and Paterson (2007), Lees *et al.*, (2015).
* MIC = minimum inhibitory concentration of target pathogen.
§ $AUC_{24\ h}$ = area under plasma antimicrobial concentration-time curve over 24 h following administration.
‡ C_{max}/MIC = maximum plasma concentration divided by MIC.

Inhibitory action of different antimicrobials

The optimal concentration and duration required for inhibiting a targeted bacterial pathogen vary with the different modes of action of antimicrobials. While some classes of antimicrobial have time-dependent effects, others have concentration-dependent effects, and some are dependent on both time and concentration. These different effects determine the PK/pharmacodynamic (**PD**) index that best predicts efficacy (Lees *et al.*, 2015; Hardefeldt *et al.*, 2019) (Table 2 and Figure 3).

For a β-lactam such as amoxicillin, for which efficacy is time-dependent, the targeted minimum inhibitory concentration (**MIC**) should be achieved for ≥40% to 50% of a 24-h period (i.e. 9.6 to 12 h) to achieve a bactericidal effect (Rey *et al.*, 2014). A study on another β-lactam antibiotic, cefotaxime, in mice showed that it had a bacteriostatic effect on *Klebsiella pneumoniae* when plasma levels were above the MIC for 30% to 40% of the dosing interval, but that plasma concentrations needed to be maintained above MIC for 60% to 70% of the dosing period to achieve maximal inhibition (Craig, 1998).

Other factors that may alter pharmacokinetics in pigs

Infectious diseases. Pigs infected with *Salmonella typhimurium* were found to have 54% greater systemic exposure to amoxicillin after intramuscular administration than healthy pigs, with faster absorption and a much longer terminal half-life (Agersø *et al.*, 2000). Amoxicillin has been found to have significantly greater oral bioavailability (44.7% *v.* 14.1%) in pigs with respiratory disease (Godoy *et al.*, 2011). However, in pigs with *E. coli*-induced diarrhoea, the area under the plasma antimicrobial concentration-time curve (AUC) of amoxicillin was decreased by more than 50% on the first day after dosing in water for 4 h, leading the authors to suggest that a higher loading dose may be appropriate (Jensen *et al.*, 2006).

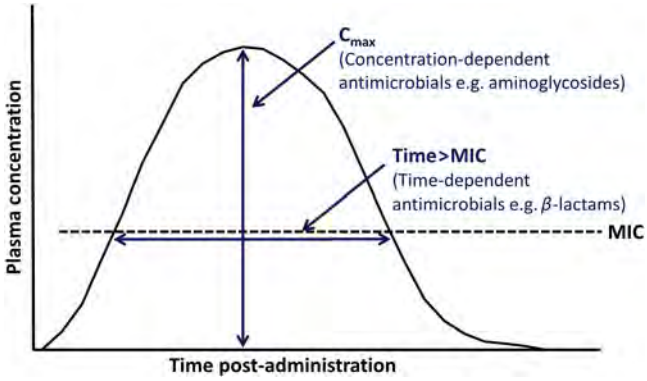

Figure 3 (Colour online) Principal pharmacokinetic–pharmacodynamic (PK–PD) characteristics of antimicrobial drugs in animals (Adapted from Hardefeldt *et al.*, 2019). C_{max} = maximum plasma concentration, MIC = minimum inhibitory concentration of target pathogen.

Aging. The impact of animal aging on the PKs of antimicrobials in pigs is unknown. This possibly reflects the complexity and diversity of animal aging across species and production systems. However, in rats an age-related improvement in the permeability of intestinal mucosa to one type of cephalosporin antimicrobial, cefazolin, was reported but not to another, cephradine (Morita *et al.*, 1992). Changes in the PKs of antimicrobials and other types of drugs in elderly patients have been well described in the human pharmacology literature. For example, absorption, volume of distribution and clearance of amoxicillin may be reduced. Inter-patient variability in PK parameters is also greater in older patients than younger ones (Benson, 2017).

Successful water medication of pigs at the group level

Successful water medication of a group of pigs may be defined not only as an absence of clinical signs in pigs and achievement of maximum growth rates, but also as elimination (or at least a dramatic reduction in numbers) of the pathogen, and prevention (or at least minimisation) of the selection and propagation of resistant pathogens (Toutain and Lees, 2006). Success will depend on the plasma concentration of the antimicrobial and the length of time for which the target concentration is sustained.

When administering a dose of an antimicrobial to a group of pigs, a wide distribution of systemic exposures to the antimicrobial is observed due to the three sources of variability in exposure described in this review. When water medicating a group of

pigs, the key question should therefore be: 'What is the probability that the majority of pigs in the group will attain the systemic exposure to the antimicrobial required to eliminate the target pathogen when using this administration regimen?'

The success of an administration regimen at a population level must therefore be expressed as the proportion of animals in the group that attain a defined target value for the PK/PD index that best predicts maximum efficacy for the antimicrobial based on its inhibitory action. This is denoted as the 'probability of target attainment' (**PTA**). An ideal value for this probability is situation-specific. However, a PTA of 0.9 is commonly chosen. If so, an administration regimen is considered sub-optimal unless at least 90% of the animals in a group attain a target value for the PK/PD index. For example, 50% of $Time_{24h}$>MIC for amoxicillin. The clinical merit of a PTA of 0.9 is yet to be validated in clinical trials (Turnidge and Paterson, 2007; Rey *et al.*, 2014).

The time at which a water medication dosing event is commenced and how quickly the quantity of antimicrobial is ingested by the pigs are critical. If dosing is commenced during a period of low water consumption by pigs, and the total dose of antimicrobial is consumed by pigs over an extended period, for example 12 to 24 h, then the plasma concentration of the antimicrobial in pigs would be expected to rise slowly and the PK/PD index attained may not be high. Conversely, if dosing is commenced during a period when pigs' water consumption is moderate to high, and the total dose of antimicrobial is ingested by pigs over a shorter period, for example 8 h, then the plasma concentration of the antimicrobial should rise more rapidly, leading to a higher PK/PD index being achieved. This could assist in achieving clinical efficacy and minimising development of antimicrobial resistance, as it aligns with the 'mutant selection window' (**MSW**) concept.

The MSW concept states that to minimise development of antimicrobial resistance it is best to administer a high dose of antibiotic over a short time period, to minimise the length of time in which the plasma concentration of the antimicrobial lies between the MIC and the higher 'mutant preventive concentration' (Lees *et al.*, 2015). Violation of the MSW concept is more likely when water medication dosing events are not timed with periods of high water consumption, the dosing event is conducted over an extended period of time and/or a lower dose of antimicrobial per kilogram BW per day is being administered for metaphylaxis (rather than a higher dose for treatment of clinical disease).

Conclusions

Substantial between-animal variability in the systemic exposure of pigs to antimicrobials (i.e. the antimicrobial concentration in plasma over time) can occur during administration in drinking water, resulting in many pigs in a group being under-dosed or over-dosed. Sources of this variability are:

1. Variability in dose applied; that is, the concentration of the active antimicrobial in water available to pigs at drinking appliances in each pen over time,
2. Variability in dose consumed; that is, patterns of consumption of medicated water by pigs in each pen over time, and
3. Variability in PKs; that is, oral bioavailability, volume of distribution and clearance between pigs and within pigs over time.

Standard antimicrobial administration regimens for water medication on farms do not account for these sources of between-animal variability. They are based on the assumption that all animals will consume the same dose and will experience the same level of systemic exposure to the antimicrobial over time. If these sources of between-animal variability are better understood, antimicrobial administration regimens may be optimised for each farm situation to account for factors influencing the range of exposures of pigs in a group to the antimicrobial. Under-dosing and over-dosing may thereby be reduced.

More information is required before it would be possible to quantify the three sources of between-animal variability in systemic exposure to an antimicrobial administered through drinking water and determine their relative contributions to total between-animal variability. This would be very useful for determining where to focus efforts to reduce between-animal variability in systemic exposure to an antimicrobial on each farm and thus optimise the administration regimen.

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Declaration of interest

The authors have no conflicts of interest to disclose.

Ethics statement

Not required for this review.

Software and data repository resources

No new software or database was generated as part of the outcomes of this literature review.

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Review: Analysis of the process and drivers for cellular meat production

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Cell-based meat, also called 'clean', lab, synthetic or in vitro *meat, has attracted much media interest recently. Consumer demand for cellular meat production derives principally from concerns over environment and animal welfare, while secondary considerations include consumer and public health aspects of animal production, and food security. The present limitations to cellular meat production include the identification of immortal cell lines, availability of cost-effective, bovine-serum-free growth medium for cell proliferation and maturation, scaffold materials for cell growth, scaling up to an industrial level, regulatory and labelling issues and at what stage mixing of myo-, adipo- and even fibrocytes can potentially occur. Consumer perceptions that cell-based meat production will result in improvements to animal welfare and the environment have been challenged, with the outcome needing to wait until the processes used in cell-based meat are close to a commercial reality. Challenges for cell-based meat products include the simulation of nutritional attributes, texture, flavour and mouthfeel of animal-derived meat products. There is some question over whether consumers will accept the technology, but likely there will be acceptance of cell-based meat products, in particular market segments. Currently, the cost of growth media, industry scale-up of specific components of the cell culture process, intellectual property sharing issues and regulatory hurdles mean that it will likely require an extended period for cellular meat to be consistently available in high-end restaurants and even longer to be available for the mass market. The progress in plant-based meat analogues is already well achieved, with products such as the Impossible™ Burger and other products already available. These developments may make the development of cellular meat products obsolete. But the challenges remain of mimicking not only the nutritional attributes, flavour, shape and structure of real meat, but also the changes in regulation and labelling.*

Keywords: lab meat, environment, health, consumer, animal welfare

Implications

Cell-based meat, also called 'clean', lab, synthetic or *in vitro* meat, has attracted much interest from the media recently. The drivers for cell-based meat production include food security, environment and sustainability, consumer and public health/safety and animal welfare problems associated with meat production, but not all of these challenges will be met by a move to industrial scale cell-based meat. This review discusses the process of cell-based meat production and summarises the significant challenges for the appearance on retail shelves.

Introduction

The provision of safe, affordable protein is critical to human nutrition and food security (Waughray, 2018). Protein, particularly from animal-sources, has been shown to play a key role in brain development and function in all humans, particularly in the elderly and in children (Mann, 2018). Although many vegetarian protein sources are available as alternatives, meat continues to be very attractive to most consumers (Van Der Weele and Tramper 2014; Piazza *et al.*, 2015).

As a global community, the drivers for the agriculture and processing industry are focused on food security. Figure 1 shows the projected increase in meat production required for the major meat-producing species by 2050 (FAO, 2012). For these reasons, there has been serious consideration of, and advances in, the production of some nutrients from cellular agriculture. At a consumer level, the proponents of cellular meat have focused their communication strategy around the societies' present concerns around meat consumption, including environmental issues, animal welfare, safety and health (Hocquette, 2016). The increase in urbanisation of human beings and associated loss of connection to

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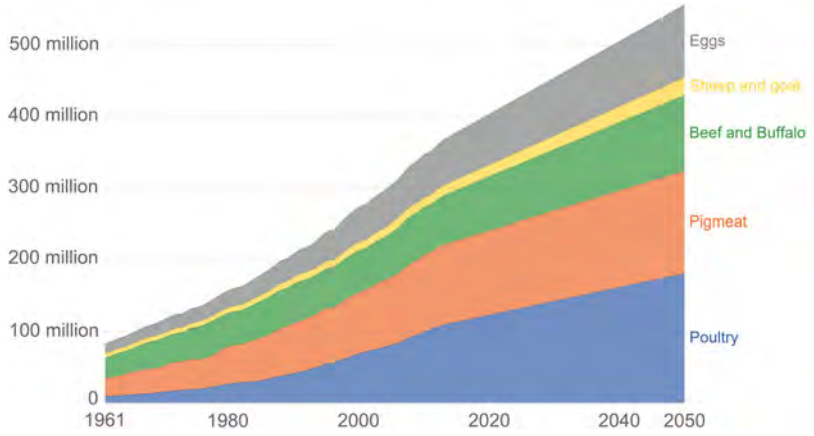

Figure 1 (Colour online) Global meat production (tonnes), based on future population projections and expected impacts of regional and national economic growth trends on meat consumption. Data from 1961 to 2013 are based on published FAO estimates and from 2013 to 2050 are based on FAO projections. Graph from https://www.3fbio.com/hello-world/, with permission, who used data from FAO (2012).

the farm, and how meat is produced, has also led to some consumers' lack of understanding and acceptance of meat production on farms.

This review focuses on the production of meat protein from muscle cells. Reducing the environmental impacts of meat production, and particularly greenhouse gas (**GHG**) emissions, is potentially a significant potential advantage of cultured meat, but reducing the need for animals in meat production is also an important facet of cell-based meat production, particularly for some urban consumers who are concerned about animal welfare (Tuomisto and Teixeira de Mattos, 2011; Post, 2012).

This review describes the process of cellular meat production, including the challenges and constraints. The drivers for cellular meat production are also presented and analysed, and the future is discussed.

Definition and process of cellular meat production

The definition of cellular meat production is meat made from stem cells, which tries to mimic traditional meat (Hocquette, 2015). It is essentially a 'substitute for meat made from animals'. The common terms used for this type of meat production include lab meat, cultured meat, *in vitro* meat, artificial meat, synthetic meat and for those keen to promote the industry, 'clean meat'. Nomenclature for cell-based meat is widely debated, and for an excellent discussion on the various terms and their history and socio-political connotations, see Stephens *et al.* (2019). Producers, investors and advocates of cell-based meat production prefer to use the term 'clean meat' in order to promote consumer acceptance as it has positive connotations (Bryant and Barnett, 2019). Conversely, those arguing against the term 'clean' meat point out that the term implies that conventional meat production is 'dirty'. The term cell-based meat has been selected for use throughout this article due to the linkage with cellular agriculture, although it is recognised that company marketing and brands will choose different nomenclature, which may be dictated by regulators.

The development of cellular meat production from cells involves a number of biological principles regarding how muscle cells are made *in vivo*. Replicating these processes at an industrial scale presents a number of technical challenges (Hocquette, 2016). Hence the development of novel meat and protein products is utilising ground-breaking technologies designed to meet the issues facing the conventional meat industry (Bonny *et al.*, 2017).

Muscle comprises muscle cells (myocytes), fat cells (adipocytes), connective tissue cells (fibroblasts) and vascular tissue (endothelial cells). Extensive research has been conducted on conditions for allowing muscle, fat and connective tissue cell culture *in vitro*, but the aims of these studies have been very diverse. Due to the complexity of conditions involved for culture of each individual cell type, successful and continued co-culture of these cells has not yet been achieved. Different cell types, particularly stem cells, can be used as a source of material to generate cellular meat, and the following section focuses on muscle cell production. The general stages of development of a mature muscle fibre, from a stem cell, including the growth factors required are shown in Figure 2. The stages of production of meat cells, including the critical decision points, are shown in Figure 3.

Source of cells

In order to manufacture cellular meat, cells with a large capacity for multiplication must be identified and sourced. Ideally these cells have a self-renewing capacity and can infinitely continue to divide, wherein lies the first challenge (see decision point 1 in Figure 3). Myogenesis starts after the formation of the embryo, continues through the life of the foetus and is mostly completed at birth (Orzechowski, 2015). At birth, all muscle cells (fibres) in the muscle tissue are fully formed and mature, with the main changes during growth and maturation of the animal being changes in muscle cell width (hypertrophy) and some changes in muscle fibre type. Muscle fibres are multinucleate, being a result of fusion of myoblasts which are uninucleate. In animal skeletal muscle tissue, myosatellite stem cells lie just outside the muscle cell,

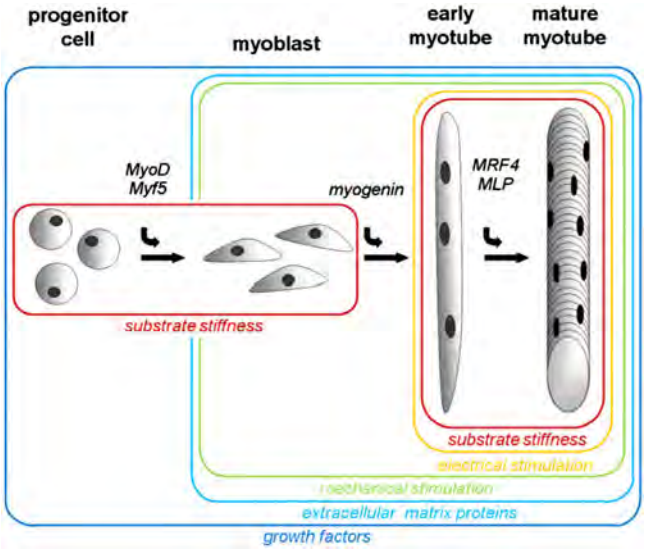

Figure 2 (Colour online) Factors affecting muscle cell proliferation, differentiation and maturation. Substrate stiffness is involved in both the proliferation of myosatellite cells and the maturation of myotubes, whereas mechanical stimulation is important for alignment of myoblasts and maturation of myotubes. Extracellular matrix proteins and growth factors are involved in both differentiation and maturation. Note the myogenic regulatory growth factors indicated at each stage being MyoD (myoblast determination protein 1), Myf5 (myogenic factor 5), myogenin (MyoG, myogenic factor 4), MRF4 (mygenic factor 6 or herculin) and MLP (muscle LIM protein). *Source*: From Langelaan *et al.* (2010).

between the sarcolemma and the basement membrane and are usually in a quiescent state (non-dividing) (Datar and Betti, 2010). When stimulated, for example, when muscle cell damage occurs, these satellite cells can divide and provide new nuclei. These new nuclei, once formed, then are transferred from extracellular, to intracellular within the muscle cell. The majority of myogenesis occurs during the development of the embryo and foetus, but myogenesis can also occur through recruitment of myosatellite cells for muscle regeneration after injury or as an adaptation to workload (Orzechowski, 2015). As an organism ages, the regenerative potential of the myosatellite population rapidly decreases, hence the preference for harvesting myosatellite cells from neonate animals (Datar and Betti, 2010).

A stem cell has a unique ability to develop into many different cell types in the body, and all stem cells can self-renew (make copies of themselves through division) and differentiate (develop into more specialised cells). Stem cells vary widely in what they can and cannot do, and in the circumstances in which they can, and cannot, do certain things. Different types of stem cells, depending on their derivation, have differing longevity and persistency in terms of number of replications *in vivo* (Kadim *et al.*, 2015). The longevity and derivation are of key importance in muscle cell culture, as a short-lived source of stem cells needs to be constantly replenished with new tissue from a living animal. The four types of stem cells relevant to this article are embryonic, tissue-specific, induced pluripotent and myosatellite. Embryonic stem cells are obtained from the inner call mass of the blastocyst that forms 3 to 5 days after an egg is fertilised by sperm. Embryonic stem cells are pluripotent (can give rise to every cell type in a fully formed body), have long-term persistency, and their number of divisions may be limitless (Kadim *et al.*, 2015). Tissue-specific stem cells, also called adult stem cells, can generate cell types specific to the tissue from which they are derived, and divisions are usually limited to 50 to 60 (see Kadim *et al.* (2015) for more detailed description). Induced pluripotent stem cells are cells that have been engineered in the laboratory (*in vitro*) to behave 'like' embryonic stem cells. These have variable, and often unknown, persistence and number of replications. Muscle tissue removed by biopsy from a living mammal can be used to derive 'myosatelite stem cells', which can only differentiate into a muscle cell. For myosatelite stem cells derived from adult animals, the number of divisions can be as low as 20 *in vitro* (Mouly *et al.*, 2005).

The most promising cell type for cellular meat production is the myosatellite cell, as these are the primary adult stem cell for muscle, although the isolation, culturing and maintenance of a high-quality population of myosatellite cells *in vivo* is challenging (Post, 2012). Embryonic stem cells would be an ideal starting source for cellular meat production due to the pluripotent nature, but the proliferation and differentiation can be difficult (Kadim *et al.*, 2015). Mosa Meat in the Netherlands (https://www.mosameat.com/) harvests muscle tissue from a living cow with a biopsy, deriving adult myosatellite stem cells whereas in Israel, BioFood Systems (https://www.biofood-systems.com/) wants to produce beef products using bovine embryonic stem cells.

Growth media for proliferation, merging and maturation

Growth of the cells in culture requires nutrients, similar to cells in living tissue. In the absence of a blood supply providing nutrients (and removing waste), cells are bathed in a culture media which provides the important nutrients and growth factors. The growth factors required for proliferation, differentiation and maturation are usually provided by adding 10% to 20% growth media (Stephens *et al.*, 2018). The nutrients required, such as carbohydrates, lipids, amino acids and vitamins, are relatively straightforward to predict and calculate, but different growth factors (and hormones) are required at different stages, as shown in Figure 2. This is the second series of challenges and decision points in cellular meat production, being how to supply the right growth media for each stage (decision point 2 in Figure 3).

The culture media needs to be formulated to ensure high rates of cell growth with not only the appropriate level of nutrients, but also the appropriate myogenic regulatory growth factors (myoblast determination protein 1 (**MyoD**), myogenic factor 5 (**Myf5**), myogenin factor 4 (**MYOG**), myogenic factor 6 (**MRF4**) and muscle LIM protein (**MLP**); see Figure 2) and hormones (insulin, thyroid hormone, growth hormone). A description of the requirements for growth factors and hormones at different stages for muscle cells from different species is given in Burton *et al.* (2000). All of these are necessary to culture the cells and to allow them

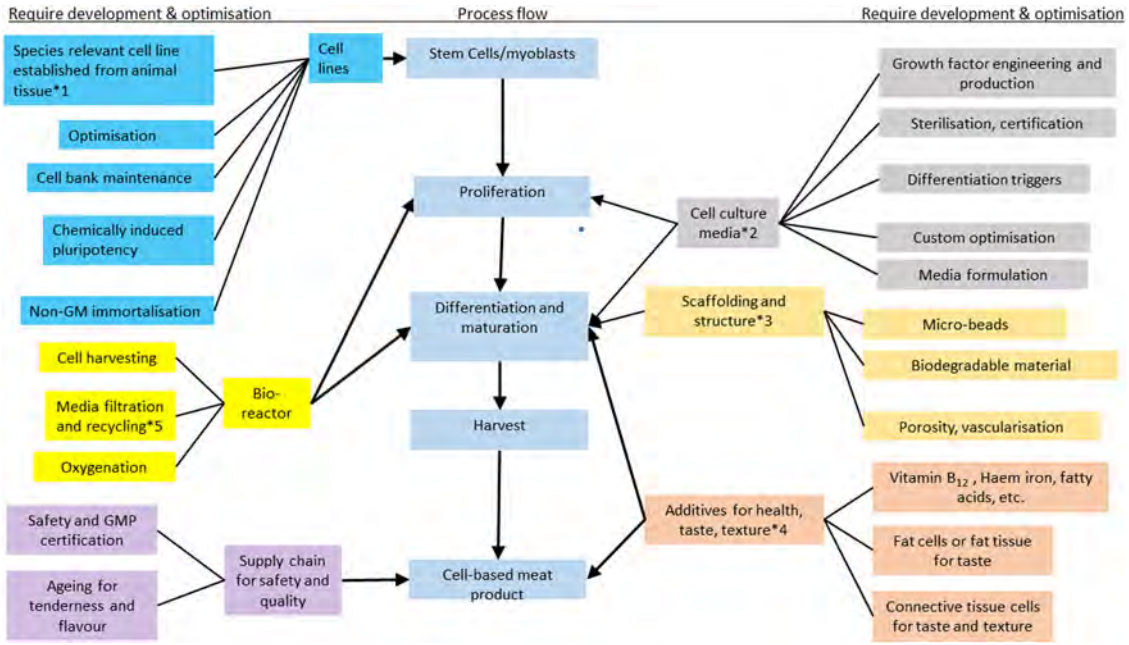

Figure 3 (Colour online) Diagram showing the stages of cellular meat production including the process flow (blue boxes in the centre), the components requiring research, development and optimisation on the extreme left and right and the critical decision points marked by an asterisk (*). Choices which are important decision points for both small scale and industrial production include: (1) source of tissue to derive cells from, (2) growth media to stimulate differentiation, proliferation, formation of myotubes and maturation of adipocytes and muscle cells, (3) scaffold or matrix on which cells can grow, (4) when to add nutrients or fat tissue/cells in order to simulate sensory and nutrient attributes of whole muscle, (5) whether to recycle water and nutrients for growth medium. *Source*: Adapted from Specht *et al.* (2018) and Bhat *et al.* (2019).

to proliferate and differentiate (Bonny *et al.*, 2017). It should be noted that proliferation (increase in number of cells) is possible only at the satellite cell stage and the mononucleate myoblasts stage. Myotubes (multinucleate fused myoblasts) and myofibres (multinucleate mature muscle cells) do not proliferate and hence the importance of extensive and rapid proliferation at the stem cell and myoblast stages (Datar and Betti, 2010). Traditionally stem cells are cultivated in a medium containing nutrients and foetal, or calf, bovine serum, and the precise composition of this serum is not defined (Burton *et al.*, 2000). Attempts have been made to synthesise serum-free growth media from mushrooms, algae and plants, but there has been limited success with these synthetic growth media (Datar and Betti, 2010; Stephens *et al.*, 2018). *In vitro* cell culture is usually conducted in an aseptic environment, due to risk of contamination which can result in bacterial contamination and cell death (Sanders, 2012; Phelan and May, 2015). Cellular meat production requires a preservative, such as sodium benzoate, added to the growth media, to protect the growing cells from yeast and fungus. Sodium benzoate is a common preservative added to processed meat products (Hoang and Vu, 2016). Furthermore, antibiotics are often added to the growth media of cells in long-term culture to prevent any infection from bacteria (Burton *et al.*, 2000, Renzini *et al.*, 2018, Stephens *et al.*, 2018). Patents for industrial production of cell-based meat state that the process will be achieved without antibiotics (or hormones) (Van Eelen, 2007). It remains to be seen whether antibiotics, or anti-bacterials, will be routinely or occasionally required during muscle cell culture.

Biomaterial/scaffold, electrostimulation and mechanical stimulation

Similar to the need for biochemical stimulation by growth factors and hormones described earlier, biomechanical, biophysical and electrical stimulation are also necessary for proliferation, differentiation, maturation and fully functioning skeletal muscle cells.

Application of a biomechanical stimulus to satellite cells can induce the formation of muscle precursor cells (myoblasts, see Figure 2) (Langelaan *et al.*, 2010). Biophysical stimuli are also crucial in the maturation process towards functional muscle cells with a high level of functional sarcomeres (Kosnik *et al.*, 2003). In addition, neuronal activity is required for the development of mature muscle fibres (cells), and this can be simulated through the application of electrical stimulation (Figure 2) (Langelaan *et al.*, 2010).

Mechanical stretch is another biophysical stimulus which can be applied and appears to be crucial in myogenesis, proliferation and differentiation. Mechanical stretch applies to various stages of the muscle cell process and facilitates; alignment of myotubes, fusion of myotubes to form muscle fibres, hypertrophy of both myotubes and muscle fibres, proliferation of myoblasts and activation of satellite cells (Vandenburgh and Karlisch, 1989, Tatsumi *et al.*, 2001).

At the stage of differentiating from stem cells into myotubes, the cells need to be attached either to a mesh scaffold, such as a collagen meshwork, or to a carrier such as microcarrier beads, which can also be made from collagen (Bhat *et al.*, 2015). The myotubes can then fuse into myofibres, given the appropriate cues in the growth media and environment.

In vivo, the cells attach to the insoluble network of extracellular matrix proteins through integrin receptors located in the sarcolemma, which allows transmission of applied force to the cytoskeleton (Langelaan *et al.*, 2010). Hence *in vitro*, this needs to be replicated in order to allow biomechanical and biophysical stimulation. The selection of material for the scaffold is important in cellular meat production and is indicated by decision point 3 in Figure 3. The scaffold-based method is limited to producing a layer of cells only 100 to 200 μm thick, in static culture, due the lack of blood supply and the diffusion limits for the nutrients and growth factors in the growth medium (Datar and Betti, 2010).

Scaling up and industrial production

A major challenge in cell-based meat production is to produce the nutrients, hormones and growth factors in large quantities and compatible with human food (Hocquette, 2016). All these compounds will need to be produced by the chemical industry, and the waste and pollution generated will need to be included in the environmental analysis of the effects of cellular meat production.

Identification of a self-replicating line of satellite cells is important to the process, in order to remove the reliance on animals. As myosatellite stem cells derived from a biopsy are presently the most effective source for cellular meat production, this remains a challenge for the scaling-up process.

Availability of cost-effective growth medium for cell proliferation and growth is a critical component of the scale-up process. Bovine foetal calf serum is the preferred growth media, due to its effectiveness, but is prohibitively expensive (Stephens *et al.*, 2018), and it is derived from animals.

For differentiation, and in particular for growth, a continuous supply of nutrients and growth factors as well as a supply of oxygen and removal of waste products is required in the culture medium. This is proposed to be achieved at large scale by bioreactors having continuous agitation and flow, allowing perfusion of required gas and chemicals into and out of the cells. Certainly it is the lack of a vascular system in the current muscle cell culture systems, for both supply of nutrients and removal of waste, that presently limits the width of the muscle cell tissue to 1 to 2 cells thick, or 100 to 200 um.

Although cellular meat production is generally considered to be most feasible at an industrial scale, some companies (e.g. FM Technologies) are proposing two possible model systems where cellular meat production could occur at a small business or even individual consumer scale. FM Technologies calls this a 'distributive approach to sustainable manufacturing' (https://www.future-meat.com/). It proposes that localised production could be achieved through several models. The first model could be where farmers maintain a population of livestock for periodically deriving a source of myosatellite cells through biopsy. In this model, for example, animals could be kept in backyards, or on animal-friendly urban farms, and serve as living donors (Van Der Weele and Tramper 2014). Alternatively, the model proposed by FM Technologies has centralised stores of stem cells, which are periodically shipped to local communities. The local community would have small-scale bioreactors to feed small villages or regions, and the technological know-how would be provided from a central point. The proposed distributive approach is partly in response to the criticism that cellular meat production is likely to be controlled by multinational corporations. Also, if production occurs centrally, or even only in select countries, environmental costs associated with transport may negate any environmental benefits (Mattick *et al.*, 2015a). These models at this stage are purely theoretical and remain to be tested or proven, and the proposed approach may struggle to be cost-effective, sustainable or efficient without involvement of large corporations and multinationals.

Recycling of growth media has the capacity to ensure industrial scale cellular meat production has a much lower water and nutrient footprint than without recycling, and this is indicated in Figure 3 by decision point 5. The management of metabolic waste by disposal, recycling or upgrading needs to be considered for design and implementation of a hazard and critical control points (**HACCP**) procedure (see the 'Regulation, labelling and hazard and critical control points' section) (Stephens *et al.*, 2018). Cell-based meat factories will need to consider constructing on-site treatment or recycling systems to limit emitted pollution, which may be required by state and federal regulations (Mattick *et al.*, 2015a). If recycling is implemented, the emissions from excess nutrients in the waste water could be similar to, or lower than, a poultry operation (Mattick *et al.*, 2015a). Recycling of culture medium can be enabled through the replenishment of utilised nutrients such as glucose, glutamine and other amino acids and carbohydrates and also through removal of waste products such as lactate and ammonia (Moritz *et al.*, 2015). It is also possible that growth factors and cytokines produced by muscle cells can be reused in subsequent culture to stimulate cell growth. It is proposed that recycling would involve cells staying in the reactor and in-line recycling of media, with removal of waste products through chromatographic purification (Moritz *et al.*, 2015). In addition, it is likely that micro-carrier beads can be recycled. The inclusion of waste water recycling in the life-cycle analysis to determine the impact on environment, emissions and water use is an important consideration.

Harvesting and manufacture of cellular meat products

Obviously the products of muscle cell culture, using collagen scaffold or micro-beads for structure, do not have the same structure as skeletal muscle tissue from animals, and can only be used for processed meat products (Datar and Betti, 2010). In addition, if the scaffold or carriers are edible, they can be included in the cellular meat mix and harvested from the bioreactor without separating cells from scaffold/carrier (Stephens *et al.*, 2018). But this is not always desirable, as the matrix may need to be reused, the matrix may contribute an unacceptable texture, flavour or functionality to the final product or the matrix may be non-edible. In this case, removal of the cells from the matrix can be achieved through

chemical means or through environmental manipulation of pH, temperature and so on (van der Weele *et al*., 2019). Any chemicals used obviously need to pass food safety tests and achieve regulatory approval (see the 'Regulation, labelling and hazard and critical control points' section).

The flavour of meat is derived from more than 750 compounds. Flavour is composed of the volatiles developed during cooking that contribute to the aroma or odour and are detected in the retro-nasal cavity and the taste, which is detected on the tongue by taste receptors (Watkins *et al*., 2013; Frank *et al*., 2016). Meat derived from whole muscle has a mixture of fat cells, fatty acids in both membranes and fat cells, fibroblasts and the protein collagen, muscle and endothelial cells and compounds such as haem-iron, creatine, carnitine glutamate and other compounds which uniquely and together contribute to the overall experience of the flavour of meat. The flavour of meat develops during cooking and some fatty acids and amino acids prevalent in meat (e.g. linoleic acid and inosine monophosphate) contribute to the unique flavour fingerprint. Whether cell-based meat products will be able to replicate the >750 compounds associated with the flavour of whole muscle is a question that remains to be answered, as well as a challenge.

The texture of meat, mouthfeel and breakdown during oral processing contribute to the overall sensory experience. The texture, particularly hardness, of meat changes during cooking, in response to temperature, as proteins denature at different temperatures, causing shrinkage in the muscle at a macro- and micro- level and changes in the gel matrix binding proteins together (Purslow *et al*., 2016). Adipocytes are the main fat deposits in muscle tissue and are a rich source of aroma, taste and juiciness in the consumer's mouth (Orzechowski, 2015). Stroma, or connective tissue, comprises cells called fibroblasts, which extrude collagen fibrils to form the extracellular matrix of muscle. Both stroma and muscle cell protein integrity have important contributions not only to texture and tenderness, but also to particle breakdown and size in the mouth, and thus are pivotal in the consumer's sensory experience. Furthermore, upon prolonged heating in appropriate conditions, collagen breaks down to gelatine, which has a unique and desirable flavour, as well as contains important amino acids for human muscle function and health (e.g. glutamate) (Arihara, 2006; Toldra *et al*., 2012).

The tenderness and flavour of whole muscle develops during the ageing, or maturation, process *postmortem*. During ageing, proteases such as calpains degrade the proteins in the micro-structure, particularly the myofibrils (Koohmaraie *et al*., 1991). Ageing of meat is conducted in an anaerobic environment for a period of days to weeks, usually by packaging the meat in a sealed bag, after removing all air through applying a vacuum, and subsequently storing at temperatures of −1 to 1°C. As cell-based meat products presently rely on formulation of a burger, or pattie, using only cells, the product does not need to be aged to improve tenderness. For example, beef patties are often made from cow beef, which is inherently tough, but once grinding/mincing occurs, this toughness of the whole muscle is no longer relevant. Assuming that there will be success in the future in producing whole muscle products from cells, then attention to ageing for optimal tenderness and flavour will be required.

Hence the manufacture of a product from purely muscle cells, such as cell-based meat, will have a different texture and mouthfeel to a product made from whole muscle derived from the *post-mortem* carcass of a living animal. This is a challenge for manufacture of cell-based meat products and requires addition of binders, such as carrageenan, collagen powder, xanthan gum or mannitol (Post, 2012) to simulate the texture derived from whole muscle.

The production of cell-based meat will be classed as a 'manufactured' meat, as it will involve the addition of ingredients for health, functionality, texture and flavour. This will likely push meat from animals into the premium end of the market, and cell-based meat products could supply the bulk, cheap end of the market if conventional meat products become more expensive and the palatability and versatility of manufactured meats improve (Bonny *et al*., 2017).

Aleph Farms (https://www.aleph-farms.com/), an Israeli start-up launched in 2017, announced in December 2018 that it succeeded in producing a lab-grown 'minute steak' made from bovine cells. According to its website, the tiny steaks are 3 mm wide – roughly the size of a very thin strip of roast beef. In the future, Aleph Farms' aim is to grow a whole steak.

The generation of a whole muscle, complete with blood supply, connective tissue, fat cells, muscle cells and associated structure, has neither been achieved in human biology and medicine nor in cellular meat production. Hence, as pointed out by Hocquette (2016), the process of producing meat products from muscle cells should be called cellular muscle production, as it contains only muscle cells. The cell-based production of a whole steak is technologically far more complex and requires technological break-throughs in providing a vascular supply through a thick layer of muscle tissue as well as determining the right growth media for co-culture of muscle, fat, connective tissue and endothelial cells.

Analysis of drivers for cellular meat production

The main drivers for cellular meat production are food security, environment, animal welfare, health and food safety. In this section, available data are presented and discussed on the likelihood that cellular meat production will satisfy the consumer concerns as well as be produced economically and in sufficient quantity.

Food security and sustainable diets

Food security is defined by the Food and Agriculture Organization (**FAO**) as existing when 'all people at all times have physical and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy lifestyle' (FAO, 1996, 2010;

cited in Nelson *et al.* 2016). Global meat production has increased dramatically since the 1960s to meet the increase in population and also because of the increase in affluence of consumers in some countries (Ritchie and Roser, 2017; Ranganathan, 2016). The global population is estimated to reach 9 billion by the year 2050, and according to this projection, the meat industry would need to increase production by approximately 50% to 100% to maintain per capita demand of the growing populations (Cornish *et al.*, 2016; Bonny *et al.*, 2017). Global meat production in 2018 was 263 million tones and is expected to reach 445 million tonnes in 2050 (Waughray, 2018). Figure 1 shows the predicted increase in the major animal-derived protein foods required by 2050, to meet the increase in demand for animal protein. Food production in general will have to increase by at least 70% to meet both the calorie and nutritional needs of the human population (Hocquette *et al.*, 2015; Cornish *et al.*, 2016). The capacity of meat production, using conventional production practices, is thought to already be at its maximum, and any increase in production would come at too high a cost to the environment (Moritz *et al.*, 2015). Cellular meat production is considered as a hopeful addition to the suite of alternative protein production systems (Van Der Weele and Tramper 2014).

Food systems have the potential to nurture human health and support environmental sustainability; however, according to some, they are currently threatening both (Willett *et al.*, 2019). A global dietary transition, associated with 'perceived' negative effects on health and environment and characterised by an increase of animal-based diets to the detriment of plant-based diets, has occurred in the past few decades (Cliceri *et al.*, 2018). Sustainability has become an integral component in analysis of dietary patterns and guidelines around the world (Jones *et al.*, 2016). A sustainable diet can be defined as one which maintains long-term health while avoiding excessive degradation and consumption of natural resources (Gussow and Clancy, 1986). The most common measure of sustainability is GHG emissions (**GHGE**s) along with life cycle assessment (**LCA**, see next section). Less data are available, but recognised as of equal importance are energy, water consumption and nutrient use as well as, more recently, nutritional quality of diets (Jones *et al.*, 2016).

It is most likely, if cellular meat production proceeds, that it will supplement the availability of meat protein from animal sources. Due to the high demand for meat protein and the potential increased consumption in many poor countries, some predictions even include a scenario where meat-animal production from agriculture is maintained alongside the development of a cellular meat industry (Stephens *et al.*, 2018). Depending on the costs of production, cellular meat products may always be a niche product for consumers who have the income to make purchasing decisions based on their ethics. It is self-evident that for cellular meat production to be part of the solution for feeding the world, it will need to be cheap and ideally be produced locally.

Environment and resource use

Environmental sustainability is the rates of renewable resource harvest, pollution creation and non-renewable resource depletion that can be continued indefinitely (Daly, 1990). If they cannot be continued indefinitely, then they are not sustainable.

Improved GHGE efficiency of production has been proposed as one of the biggest potential advantages of cultured meat over conventional livestock production systems (Lynch and Pierrehumbert, 2019). In fact, meat production, particularly beef production, is considered to be the greatest single contributor to GHGE of any industry (Lynch and Pierrehumbert, 2019). Thus the supporters of cellular meat production propose it is more sustainable as it will produce much lower GHGEs (Tuomisto and Teixeira de Mattos, 2011).

There are still substantial uncertainties in most components of the GHG balance of livestock production systems, method of ranking and LCA methodologies, and these depend on assumptions, approach and criteria selected, for example, GHGEs per unit land area, per kilogram livestock or per unit product. The reader is directed to reviews on the methodological challenges in both LCA (Cederberg *et al.*, 2013; Sala *et al.*, 2017) and in calculation of GHGEs (Crosson *et al.*, 2011) for further detail. In order to compare GHGE across species, the work of one author has been selected to ensure a consistency of assumptions and methods. The GHGEs per kilogram LW (kg CO_2-e/kg LW, excluding land use and direct land use change emissions) from meat production systems in Australia vary from the lowest for pork (2.1 to 4.5) (Wiedemann *et al.*, 2018), grass-finished lamb is intermediate (6.1 to 7.3) (Wiedemann *et al.*, 2016b) and grass finished beef is the highest (10.6 to 12.4) (Wiedemann *et al.*, 2016a). The calculation for grass-fed beef and lamb does not take into account carbon sequestration and storage by permanent pastures, and if this is included, the carbon impact will reduce by 30% to 50% (Soussana *et al.*, 2010). For pork, the GHGEs per kilogram wholesale meat were also estimated and were 6.36 kg CO_2-e/kg of wholesale pork (Wiedemann *et al.*, 2018) and 2.5 to 3.1 for boneless chicken (Wiedemann *et al.*, 2017b).

The resources used and GHGEs from production of 1 kg of ready-to-eat product have been modelled for comparison across dairy-protein, chicken meat, cell-based meat and other forms of protein, including traditional (soya, wheat) and novel forms (insect-protein, myco-protein) (Smetana *et al.*, 2015). Cell-based meat production was predicted to require 2 to 10 times the amount of energy, vastly more tap water and have moderate-to-low requirements for transport (Table 1). The GHGEs from cell-based meat production were predicted to be much higher than for the other protein types by Smetana *et al.* (2015), but others predicted much lower GHGEs from cell-based meat, which were comparable to the levels for other protein production systems. Cell-based meat production also had much higher predicted non-renewable energy usage than the other protein production systems in the modelling of Smetana *et al.* (2015), and in this

Table 1 *(a) Resources used per functional unit (FU, 1 kg of ready-to-eat product) from cradle to grave in the production of different protein-based meals (b) comparison of the greenhouse gas emissions (GHGEs) and non-renewable energy use per 1 kg of ready-to-eat product (FU)*

	Chicken	Dairy	Cellular-based meat	Insect-based	Gluten-based	Soya-based	Myco-protein-based	References
(a) Resources used								
Electricity (MJ)	49.78	12.27	103.5	10.762	8.94	10.002	21.32	(Smetana *et al.*, 2015)
Tap water (kg)	16.3	4.2	420	1.34	0.954	0.73	40	(Smetana *et al.*, 2015)
Transport (km)	850	360	110	128.5	141.1	2791	215.45	(Smetana *et al.*, 2015)
(b) GHGEs and non-renewable energy								
GHG, kg CO_2 eq./kg (FU)[12]	5.2–5.82	4.38–4.95	23.9–24.64	2.83–3.02	3.59–4.03	2.65–2.78	5.55–6.15	(Smetana *et al.*, 2015)
Range in values for other references (no. of references)	1.3–5.5 (*n*=7)	3.8–6.2 (*n*=1)	1.8–10 (*n*=2)	2.7–20 (*n*=2)	1.55 (*n*=2)	0.34–3.72 (*n*=2)	2.4–2.6 (*n*=1)	See 17 references in Smetana *et al.* (2015)
Non-renewable energy use, MJ/kg (FU[1])	51.64–63.4	48.79–59.1	290.7–373	32.0–40.4	39.7–49.2	27.78–36.9	60.07–76.8	(Smetana *et al.*, 2015)
Range in values for other references	1.3–54	55.5	25.2–31 700	34–170	1.4–2500	1.5–3000	38	See 17 references in Smetana *et al.* (2015)

Source*: Derived from Smetana et al.* (2015).
[1] FU=functional unit and is 1 kg chicken, 6 kg skimmed milk.
[2] For comparison to pork in this table, global GHG emissions for pork are relatively similar to chicken meat (6.1 *v.* 5.4 kg CO_2-eq/kg CW respectively; MacLeod *et al.*, 2013) and are much lower than beef and sheep meat (see text and Wiedemann references).

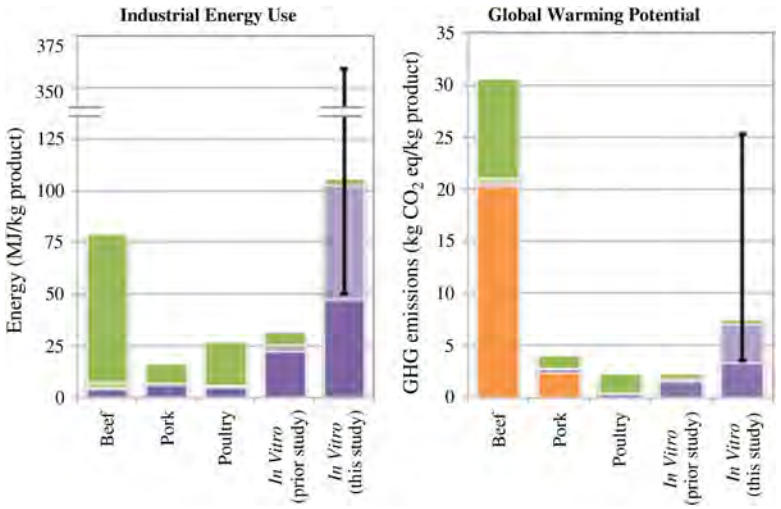

Figure 4 (Colour online) A comparison between beef, pork, poultry and cellular (*in vitro*) meat production for the energy usage and GHGE (greenhouse gas emission) attributed to agricultural production including feedstock processing and transport (green and light purple), on-farm energy use for livestock and biomass cultivation, bioreactor cleaning and facility (dark purple) and animal/carnery waste products for livestock/cellular meat (orange). *Source*: From Mattick *et al.* (2015c).

case, the comparisons across protein types by other authors were extremely variable.

Mattick *et al.* (2015a) compared beef, pork, poultry and cellular meat production (see Figure 4), and their modelling showed that the predicted global warming potential of cellular meat production was approximately equivalent to (previous study), or higher than (current study), that of pork and poultry production, but much lower than that attributed to beef production. The predicted energy usage of cellular meat production was four times higher than that of pork and poultry and beef production, which is consistent with the more recent study of Lynch and Pierrehumbert (2019).

Lynch and Pierrehumbert (2019) recently modelled the potential climate change impacts of four cell-based meat production systems and three beef cattle production systems, over 1000 years (Figure 5). In both scenarios of perpetual

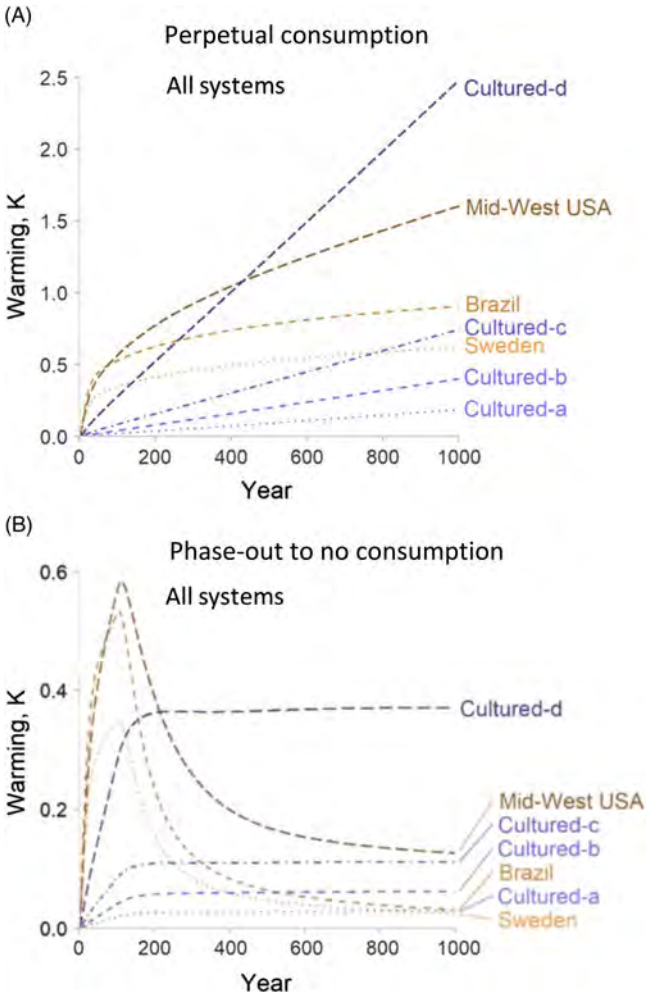

Figure 5 (Colour online) Predicted environmental warming impacts for the production of beef cattle, under three different cattle production systems (Brazil, Sweden, mid-West USA) or cellular meat production (cultured; a- low emissions system, b- and c- moderate emissions systems, d- high emissions system) under these production systems (a, b, c, d) for 1000 years. (A) – assumes perpetual consumption at very high rates (250 Mt per year), (B) – assumes initial consumption at very high rates followed by a decline to zero consumption. *Source*: Derived from Lynch & Pierrehumbert (2019).

consumption of animal protein, and phase-out to no consumption, the warming potential of one cellular meat scenario (cultured-d, high emissions system in Figure 5a and b) would exceed the warming potential of beef production in Brazil, Sweden or USA. The predicted warming potential of cellular meat production 'b' and 'c' (medium emissions system) was similar to that for mid-West USA cattle production. They concluded that cultured meat is not 'prima facie' climatically superior to cattle. The conclusion of Lynch and Pierrehumbert (2019) is that 'replacing cattle systems with cultured meat production before energy generation is sufficiently decarbonised could risk a long-term, negative climate impact'.

In summary, the existing data are consistent in showing that cell-based meat production has similar levels of GHGEs to pork and poultry production but much lower than beef production. The studies also consistently demonstrated

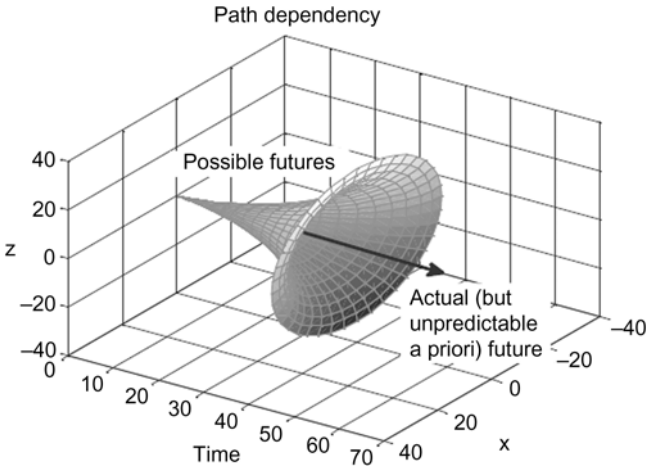

Figure 6 (Colour online) Path dependency and unpredictability inherent in the evolution of complex systems over time. *Source*: From Mattick *et al.* (2015a).

that cell-based meat production will have much higher non-renewable energy usage, and likely higher water usage. Hence the environmental impact of cell-based meat may be comparable, or even worse, than traditional forms of animal-based production systems, especially if compared to pork and poultry. It should be noted that there was considerable variability in the methods used, and the assumptions made, in each study. As cell-based meat production is not yet occurring, a number of assumptions have been made, and will need to be made, regarding an industry scale system. Mattick *et al.* (2015a, 2015b, and 2015c) who have studied the environmental consequences of cell-based meat production, as well as cellular agriculture, state that 'because the cellular agriculture technology largely replaces biological systems with chemical and mechanical ones, it has the potential to increase industrial energy consumption and, consequently, greenhouse gas emissions'. Mattick *et al.* (2015a) have also explained that in the absence of a commercial-scale process on which to base a lifecycle inventory (i.e., the detailed methodology), any analysis of the environmental implications cannot be interpreted as conclusive or definitive. Taking into account Mattick's comments, the need for further research and development on the industrial process (Figure 2) and the unpredictability of the future of a complex system (Figure 6) such as cell-based meat production, the effects on the environment are, in fact, unpredictable. But as the environment is a major driver for the development of cell-based meat production, the modelling should continue and hopefully improve as the process is better defined and commercial production is initiated.

Public and consumer health

Our pre-human ancestors ate meat, and the consumption of meat is thought to support the historical early development of a larger and more elaborate brain which required the diversion of energy from the gastrointestinal tract, resulting in a reduction in the size of the tract relative to other species (Mann, 2018; Willett *et al.*, 2019). Humans cannot digest

plants and grass, and eating meat from herbivores is an efficient way for human beings to valorise grass (Post, 2012).

The effect of cell-based meat production is a topic where there is, in some instances, a big gap between consumer/public perception and reality. Some of the reported consequences of red meat consumption include nutrition-related diseases, foodborne illnesses and antibiotic-resistant pathogens strains, due to overuse of antibiotics. In addition, some of the perceived problems with consumption of animal-derived meat include the use of growth hormones and 'chemicals' in animal production. Hopkins (2015) shows evidence of the overemphasis and overrepresentation of the views of vegetarians in the media, particularly in regard to the reception of cell-based meat among vegetarians.

Nutrient attributes of animal-derived meat. Meat is well known to be a good source of high-quality protein, including essential amino acids, Vitamin B_{12}, iron, zinc and selenium. For example, from 100 g of pork, the consumer derives ~37% of their daily protein requirements, 67% of their daily Vitamin B_{12} requirements and 15% to 16% of their selenium and zinc requirements (McAuliffe *et al*., 2018). Thus in order to compare foods, it is important to compare them not only on a nutrient basis but also on a nutrient bioavailability and gut health basis. The most well-known example is the much lower bioavailablity of non-haem iron from plants, as a source of iron, relative to haem iron from meat sources. Animal-source foods are also the only natural source of Vitamin B_{12}, so deficiency is prevalent when intake of these foods is low due to their high cost, lack of availability or cultural or religious beliefs (WHO, 2008). Meat is known to be the biggest source of protein for human consumption.

Nutrients predicted to be in cell-based meat. In order for cell-based meat products to supplement meat from animals, they will need to either replicate or increase bio-availability and levels of the nutrients referred to earlier. Skeletal muscle cells can make many bio-available proteins, fatty acids, growth factors and cytokines. But Vitamin B_{12} is not produced by muscle cells in culture, but is taken up from the culture media *in vitro* or from the blood *in vivo* (Post and Hocquette, 2017). Cellular meat does not have high levels of iron, unless the culture is grown under a low oxygen environment (Post and Hocquette, 2017). Furthermore, many of the healthy n-3 fatty acids and poly unsaturated fatty acids are generated from the animals diet (McAuliffe *et al*., 2018). As the levels of the healthy n-3 fatty acids in the membranes of muscle cells in culture may be low, these fatty acids could be added to cell-based meat products as a 'health bonus' (Post, 2012). Vitamin B_{12} needs to be added to the culture medium in order for the cell-based meat to contain this vitamin. In general, the nutritional composition of the cellular meat will be dependent on the nutrients added to the growth medium during production, unless these can be added as fortifications to the product (see decision point 4 in Figure 3).

Diet-related diseases. Consumption of meat, particularly red meat, has been related to obesity, atherosclerosis, cardiovascular disease and colon cancer, although some of the constituents related to these diseases may be produced during cooking (e.g. heterocyclic amino acids) (Post, 2012; Orzechowski, 2015), hence it is possible that cell-based meat culture could be tailored to remove specific compounds. In addition, there is evidence that the incidence of these diet-related diseases is due to overconsumption of food, and of meat. Hence the evident solution is for affluent Western countries to reduce intake.

Pathogens and animal transmission of diseases. Foodborne pathogens, such as *Salmonella*, *Campylobacter*, *Listeria* and *Escherichia coli*, are responsible for many cases of illness, and sometimes death, around the world. Over the period 2009 to 2016 in the US, the single source foods that sickened people the most were fish (17%) and dairy (11%) followed by chicken (9%) and beef, pork, vegetables and molluscs being lower (6% to 8%) (https://www.cdc.gov). Epidemiological studies demonstrate that these pathogens and emerging diseases such as avian and swine influenza increase in incidence under intensive production and housing. Also, cell-based meat production will reduce the human-animal interactions and thus is expected to reduce incidences of epidemic zoonoses and other emerging diseases (Datar and Betti, 2010). Interestingly, strains of pigs and poultry which are resistant to S*almonella* are being developed (Bonny *et al*., 2015), and there is potential for technological advances in this area. The high degree of environmental control required for cell-based meat production will likely reduce the risk of foodborne pathogens and may allow improvements in health and safety (Bonny *et al*., 2015).

Anti-microbials and chemicals. The use of sterile environments and antimicrobials during cell-based meat production will likely be used to eliminate pathogens such as *Salmonella* and *E. Coli* from the production process. Historically, the extensive and long-term use of antimicrobials is known to generate public and consumer health and safety issues. In addition, generating the required sterile environment during production is very expensive and is very difficult at an industrial scale (Bonny *et al*., 2017). This is exemplified by the consideration that sterile environments for cell culture can be difficult to maintain long term at lab scale (see 'Growth media for proliferation, merging and maturation' section), let alone at an industrial scale, and hence the likelihood that strong chemicals and anti-microbials will be required in the production process. Conversely, there will be reduced exposure to dangerous chemicals in animal production such as pesticides and fungicides (Post, 2012) which can be injurious not only for humans if ingested but also for wildlife.

Growth hormones. As discussed in the 'Growth media for proliferation, merging and maturation' section, large-scale production of cell-based meat will likely require the addition of natural, or artificially manufactured, growth hormone (Post, 2012). This is not problematic for public or consumer health, as the growth hormone would not remain in the harvested tissue. But one of the big concerns consumers have about animals-based meat production is the use of growth hormones (and steroids) (Gatti, 2019). Hormonal growth promotants (**HGPs**) are naturally occurring hormones such as oestrogen, or synthetic alternatives, and are used on about

40% of Australian cattle to accelerate weight gain (Food-Standards-Code-Australia, 2011). According to research by the European Federation of Animal Health, a single consumer would need to eat more than 77 kg of beef from an HGP-treated beast in one sitting to get the same level of oestrogen hormone found in one egg (Food-Standards-Code-Australia, 2011). Hence on this aspect, the consumer 'perception' of cell-based meat may be far removed from reality.

Cell-based meat can be engineered to be healthier and more functional by either manipulating the culture media to change levels of nutrients, vitamins, fatty acids or fat content (Bhat *et al*., 2015). Levels of these nutrients can also be controlled through addition post-harvest, but of course this can occur in any manufactured meat product and is not unique to cell-based meat.

Animal welfare

Ethical issues are increasingly important in our food choices, and the consequence has been the development of societal concerns. According to Cornish *et al*. (2016) 'the production of food from animals poses many ethical challenges'. These societal concerns are varied and encompass the right to kill animals and to prevent any suffering of animals (Hocquette, 2016). Implicit in the prevention of suffering, often espoused by vegans and vegetarians, is the erroneous perception that all animals raised on farms undergo 'suffering'. In a similar manner, some consumers use anthropomorphic considerations and assume animals in pens, or in intensive livestock system or in feedlots, undergo suffering. It is mostly consumers in industrialised, wealthy, Western cultures who hold these views, whereas consumers in less industrialised, lower wealth economies generally do not hold these views (Cornish *et al*., 2016). If cell-based meat production requires a regular, or irregular, biopsy sample from an animal, it is not clear whether this will allay the ethical concerns some consumers hold around animal production.

A major limitation in cell-based meat production is the need for a supply of bovine serum for the culture medium. About 50 l of bovine serum is required to make one burger (Mark Post cited on https://www.wired.com.uk), and this volume of serum requires blood from 91 to 333 foetuses (3-month-old foetus has 150 ml of blood, near full-term foetus has 550 ml of blood) (Jochems *et al*., 2002). Hence until animal-free serum is available for muscle cell culture at industrial scale, the production of cell-based meat products will not meet consumers' demands for animal welfare. Consideration of animal welfare in traditional animal production, and in cell-based meat production, is likely the most polarising and contentious of the consumer concerns.

Food safety

Safety is a very important issue for animal products (Hocquette, 2016). Many consumers hold misperceptions that antibiotics, growth hormones and steroids/anabolics are used in all forms of animal production for meat. Certainly, and for good reason, the proliferative use of antibiotics for prophylactic reasons, and also as growth promotants (mainly in the poultry industry), needs to undergo dramatic reduction. Hence some argue that cell-based meat will be safer to eat. In addition, promoters of cellular meat argue that it will be free of microorganisms and parasites, as it will be produced without animals. However according to some, the high rate of proliferation required of stem cells will likely produce genetic instability which may result in sporadic cancerous cells (Hocquette, 2016), which will require monitoring in HACCP plans (see later). At this stage it is unclear whether the growth hormones, nutrients and other chemicals (antibiotics, preservatives such as sodium benzoate) added to the growth media are safe in the context of human food, and this will need to be investigated and documented.

A key concern of regulators (see the 'Regulation, labelling and hazard and critical control points' section) will be food safety which requires implementation of auditable procedures in each step of the production chain as part of a HACCP plan for each individual company (see the 'Regulation, labelling and hazard and critical control points' section).

Consumer acceptance

Perhaps the most significant challenge for cell-based meat to overcome is that of consumer acceptance (Bryant and Barnett, 2019). There is a diversity of opinions in the media regarding consumer acceptance and consumer concerns. Unfortunately, much of this is driven by the media, and the media tends to report itself, as well as over-representing the vegan and vegetarian point of view (Hopkins, 2015). The diversity of opinion is well represented by comments on a news release titled 'Lab grown meat could be in restaurants by 2021, cutting down land and water required to produce red meat by more than 90%' (Gatti, 2019). The comments included quite a few stating that 'lab meat will have less chemicals, less pesticides, no antibiotics and no hormones' as well as 'meat grown in a lab is disgusting and we already have too much Frankenfood in the USA' and 'does not sound natural or appetising' with the majority of other comments being around 'how great to cease producing and slaughtering animals'. Although some people also commented 'how sad it will be to no longer see animals and sheep as part of the rural landscape', some of these comments are very ill-informed, as it is likely that cell-based meat will be produced using more chemicals than animal-based meat production. Certainly these comments reflect the urban consumers' 'perceptions' of animal-based meat production. Surveys conducted show that between 16% and 66% of consumers would be prepared to eat cell-based meat products although the concerns around cell-based meat include the perceived unnaturalness, perceived risks to human health and uncertainty regarding price and taste (Bryant and Barnett, 2019). Consumer acceptance can only be theoretical as no product is presently available to display to consumers or to allow taste tests, and the perceived consumer acceptance is highly dependent on how the product is named (and framed). Using the name 'clean' meat or 'animal-free'

meat invokes positive attitudes from consumers compared to using the names 'cultured' meat or 'lab-grown' meat (Bryant and Barnett, 2019).

Many consumers do not think that cellular meat production will be the solution to reducing meat production; rather they prefer to reduce their meat consumption (Hocquette *et al*., 2015). In fact, in a survey of 817 educated people, participants were not convinced that cellular meat production will be tasty, safe or healthy (Hocquette *et al*., 2015). Concerns about cellular meat production as an unnatural and risky product also appear to be a significant barrier to public acceptance of the product (Bhat *et al*., 2017). Generally speaking, people might be ready to taste cell-based meat products but may not want to regularly consume cell-based meat either in restaurants or at home (Verbeke *et al*., 2015).

Regulation, labelling and hazard and critical control points

Before it can reach the shelves, cell-grown meat will be required to go through regulatory hurdles and paperwork. There are presently 44 companies listed on the Australian clean meats website (https://www.cleanmeats.com.au), and of these, 19 are in the US. For the cellular meat industry in the US and other countries to succeed, companies need assurance that their product will be responsibly regulated, in the case of the US, by the United States Department of Agriculture (**USDA**) and the Food and Drug Administration (**FDA**). Although literature has existed on the considerations for regulation of cultured meat since 2013 to 2014 in both the US and the European Union (**EU**), this literature identified the regulations in place at the time were inadequate to deal with cell-based meat production without significant development (Stephens *et al*., 2018). The considerations in the US were that if the production is defined as explant (expanding existing muscle tissue), it would require FDA New Animals and Drugs application whereas if scaffold-based, this should follow FDA food additive regulation. Whereas in EU, cell-based meat production would be subject to novel food regulation via the European Food Safety Authority (**EFSA**) risk assessment. The exception here is that the EFSA novel food regulation excludes genetically modified food, which may not be the case for cell-based meat (Stephens *et al*., 2018).

In November 2018, the USDA and FDA announced an agreement for a joint regulatory framework for the production of cell-cultured food products derived from livestock and poultry. The agreement outlines the point of transfer as follows. 'FDA oversees cell collection, cell banks, and cell growth and differentiation. A transition from FDA to USDA oversight will occur during the cell harvest stage. The United States Department of Agriculture will then oversee the production and labelling of food products derived from the cells of livestock and poultry' (Piper, 2019; Rollins and Rumley, 2019). This agreement was formalised on 7 March 2019 in a memorandum signed by principals from USDA and FDA laying out the delegation of responsibility (Piper, 2019; Rollins and Rumley, 2019); however the details concerning inspection and the labelling process are still to be worked out by the respective agencies.

In some states in the US (e.g. Missouri), legislation has been introduced and sometimes passed, prohibiting cell-based meat companies from using the label 'meat'. The United States Department of Agriculture's labelling authority overrides that of the states; hence the USDA's future ruling on what cell-based meat can be called will, in theory, be final. Cultured meat has already faced resistance from the US meat industry and will probably face a labelling battle once it comes on the market. Hence law suits and fights will likely proceed in court, whatever labelling the product has finally approved by the USDA (Piper, 2019).

In Europe, a Brussels agriculture committee has approved a ban on producers of vegetarian food using nomenclature usually deployed to describe meat (Boffey, 2019). Instead proposed that terms such as 'veggie discs' rather than 'veggie hamburger' be used. The proposed terms will now be voted on by the full parliament after May's European elections, before being put to member states and the European Commission. After the vote in the European Parliament on revisions to a food-labelling regulation, it is likely that any introduction of cellular meat products will face similar restrictions in labelling, particularly as Europe tends to be far stronger in banning any foods associated with increased chemical use during production, which is likely to be the case with cell-based meat. The possibility of cell-based meat being better for animal welfare may sway European regulators to be more lenient, as Europe in general is strongly pro-animal welfare.

In order to develop regulatory guidelines, the procedures for production must be clearly outlined. At this early stage, procedures are not developed; thus regulations will need to follow the development of documented procedures, and there are likely to be several or many pathways for production. Regulations presently being developed in USA and EU will be watched closely for providing guiding principles to regulators in other countries, where a cell-based meat production industry is developing.

Regulatory guidelines, HACCP plans and auditing will need to consider a number of steps and aspects, including governance at local, state and federal levels. The initial collection of samples will be governed by animal welfare acts. A HACCP plan is designed as a preventative food safety system in which every step in the manufacture, storage and distribution of a food product is scientifically analysed for microbiological, physical and chemical hazards. Thus for the production and processing of cell-based meat, a HACCP plan will be required to specify auditing for identification of all possible pathogens, possible physical contamination and the safety of chemicals added, including methods to prevent contamination at each HACCP step that are usually subject to either state or federal government agencies, depending on whether the product will be consumed locally or exported. Auditing procedures for cell-based meat

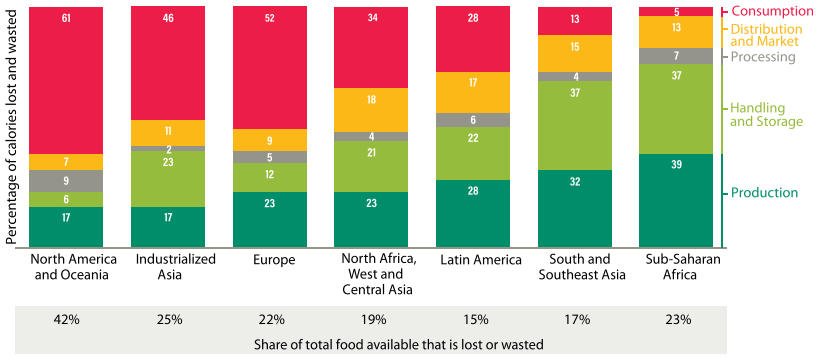

Figure 7 (Colour online) Food lost or wasted by region and stage in the value chain in 2009 (percentage of kilo calories lost and wasted). *Source*: From Lipinksi *et al.* (2013).

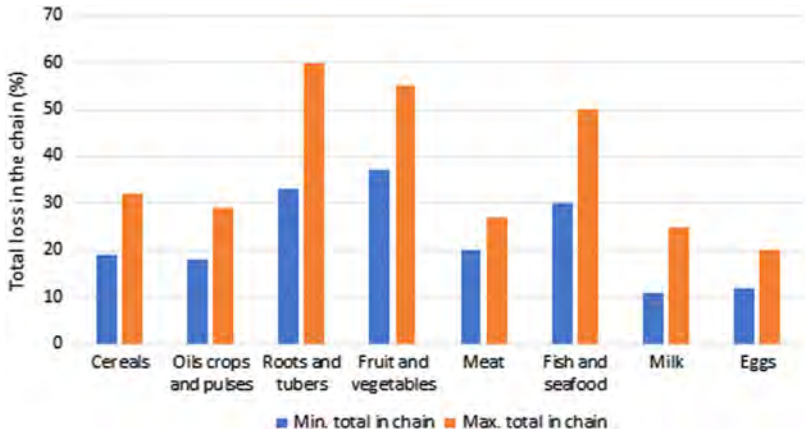

Figure 8 (Colour online) Total losses (%) in the chain for different categories of food. *Source*: Derived from data in FAO (2011).

production will also likely need to include monitoring and quality assurance function at each stage, testing of genetic stability and management of metabolic waste by disposal or recycling (Stephens *et al.*, 2018).

Other possibilities to address consumer concerns and food security

There are other possibilities to address consumer concerns and food security. Some of these are already in progress and have variable implementation in countries around the world; others have been researched and are yet to be implemented, and some possibilities require extensive research. These are discussed here.

Meat substitutes

There has been a rapid increase in plant-based protein products on the supermarket shelves, many of which are presented in a simulated meat form, such as veggie burger and veggie sausages. The mainstream meat substitutes include soy-based products, wheat-based products, pea protein and mycoproteins with a high and increasing value in the world markets (Hocquette, 2015). Protein for meat substitutes can also be derived from algae and insects (see Table 1). Consumers are increasingly aware of the potential health benefits of the meat substitutes; hence the demand is centred around good health and wellness. The global meat substitute market size was estimated at US$3.71 billion in 2016 with projections of expansion by 7.5% every year to 2022 (Grand-View-Research, 2018). Growing preference towards a vegan diet owing to several health benefits and environmental concerns is the major factor driving the market. There are many companies with products in the substitute meat market, and three important companies with high publicity include Impossible Foods (US) that produces a burger that 'bleeds', Beyond Meat whose products are made from pea protein and Quorn Foods Ltd (UK) that have been around for a while. Many consider meat substitutes manufactured from plant proteins and mycoproteins will have a greater potential than cell-based meat in the near future (Bonny *et al.*, 2015).

Reduce food waste or increase utilisation of waste streams from manufacturing

In 2013, it was estimated that approximately one-third of all food produced was wasted (Hocquette, 2016). This varies with region as North America and Oceania show the highest food waste of 42% compared to the lowest of 15% for Latin America (Lipinski *et al.*, 2013) (Figure 7). Hence reductions in food waste will have a direct benefit for increasing food security, while addressing environmental concerns by increasing efficiency. This waste occurs on the farm, during transport, manufacturing, storage, at retail and also in the consumer's home. For North America and Oceania, most of the waste

(61%) occurs during consumption, whereas for sub-Saharan Africa, most of the waste (39%) occurs during production (Lipinski *et al.*, 2013) (Figure 7). The waste also varies significantly with commodity; the total loss in the chain for meat is in the range of 20% to 27% compared to roots and tubers where the total loss is 33% to 60% (Figure 8) (FAO, 2011).

Extraction of protein and macronutrients from all parts of the animal and also from waste streams of animal processing and conversion from non-edible to edible is also important. The yield of the carcass (fat, muscle and bone) expressed as a % of the live weight of an animal is about 50% to 55% for sheep and cattle and 70% to 75% for pigs and poultry. The proportion of carcass meat from the animal is only ~33% for cattle and sheep with ~13%, ~10.5% and 3.5% of the animal comprising organs, skin/hides and blood, respectively (https://meattechnologyblog.blogspot.com/2014/01/carcass-yield.html). Trimmed fat and muscle are retained and utilised for manufacturing, but for example, often blood and also bone end up as blood and bone meal for animal feed. There are many opportunities to extract more edible nutrients from the animal, including from blood, organs, hides and hoofs/trotters (Toldra *et al.*, 2012 and 2016). Westernised countries often 'waste' or underutilise parts of the animal that other cultures prize for the recognised nutritional value. Some religions focus on respect for the life of an animal being slaughtered, including offering a prayer of thanks for every animal slaughtered (Farouk, 2013; Farouk *et al.*, 2016). A component of respecting the life of animals, and their use for human food, should include efficient use of the whole animal, rather than focusing on just the skeletal muscle for human food.

Dietary changes, re-distribution of nutrients

In 2013, the US and Australia were the highest annual consumers of meat (excluding fish and seafood) in the world, being in the range of 115 to 116 kg/person per year compared to the majority of the African continent which has an average annual consumption of 5 to 40 kg/person per year (Ritchie and Roser, 2017). It is well recognised that the high levels of obesity, CVD and other diseases could be reduced in affluent societies, including Australia and US by reducing food consumption, including meat. Malnutrition is an underlying cause of death of 2.6 million children each year, while anaemia, from a lack of iron in the diet, affects 35% of the world's population (https://www.gainhealth.org/about/). Thus it is clear that while affluent societies need to decrease their food, and meat consumption, the poor countries suffering drastically need to increase their food, and also meat consumption.

Many consumers consider animal products to be the most desirable way to access nutrient-rich and tasty protein. Meat is commonly perceived as the core of protein delivery as it has more protein, micronutrients and amino acids per kilogram of all the dietary protein sources (Waughray, 2018). In addition, consuming meat is considered a sign of affluence for the world's emerging middle classes. Alternatives to meat exist, and young people seem to be shifting towards 'non-meat'-based food. It will be interesting to observe how far this shift to plant-based meals occurs as there is a predicted increase in the consumption of all meats (beef, pork, poultry, sheepmeat) across all of the continents and all of the 31 diverse countries included in the FAO (2018).

Considering a strict vegan diet (no milk, eggs, meat, dairy) is associated with a higher risk of nutrient deficiencies, especially in the vulnerable young and elderly (Hocquette, 2016), and this does not seem to be a solution to the concerns around animal production across all consumers in all countries.

Improvements in animal welfare

Alternative ways to consider the animal welfare aspects of animal production are to evaluate the critical points where animal welfare may be compromised and introduce technological innovation and industry transformation in how meat is produced.

Research has been conducted on the stressors encountered between farm and slaughter (Grandin, 1997 and 1998; Ferguson and Warner, 2008; Warner *et al.*, 2010) and the effects on meat quality (Voisinet *et al.*, 1997; Warner *et al.*, 2007; Edwards *et al.*, 2010), and some of the results have been implemented, resulting in improvements in animal welfare. Recently, infra-red video cameras have been used to measure physiological stress responses on the farm and at the slaughter plant (Jorquera-Chavez *et al.*, 2019). These technologies offer promise for continuous monitoring of stress as well as disease throughout the life of the animal, potentially enabling continued improvements in animal welfare. The Danes have developed low-stress handling systems for handling of pigs pre-slaughter which have demonstrated improvements in welfare as well as in meat quality (Aaslyng and Barton-Gade, 2001), hence less wastage.

The European Union–funded Welfare Quality® project was a very large project with significant outcomes for defining and standardising welfare and developing animal-based measures across the EU and also in other countries (Blokhuis *et al.*, 2010). Interestingly, retailers and the food industry, in some countries such as those in Europe, and also Australia, have been very successful in identifying a consumer demographic who responds to issues of animal welfare and food quality (Buller *et al.*, 2018). This demand for higher welfare supply chains has been met by retailers as there is now a commodity value on welfare, in some countries. As a result of both consumer demand and retail recognition of the commodity value of animal welfare, livestock production practices in many countries have recently shown significant improvements through incorporating new concepts to improve animal welfare and reduce animals suffering (Hocquette and Chatellier, 2011).

Mobile slaughter units are being developed around the world, where the slaughtering occurs either on the farm or in a small village or town (Carlsson *et al.*, 2007; Eriksen *et al.*, 2013), and these are predicted to reduce the stressors as well as environmental costs attributed due to transport of animals. These approaches listed earlier will never satisfy the

consumers committed to cessation of all forms of animal production; although this segment is vocal and media-grabbing, they are not the majority of the population.

Changes in livestock production

In the future, the quality of animal products will be defined by the sustainability of the production system (Scollan *et al.*, 2010). There has been continued progress in identifying the components of the beef cattle production system which can be changed, in order to reduce methane emissions in particular. Intensive cattle finishing in feedlots has been shown to reduce GHGEs, compared to pasture-based systems (4.6 to 9.5 *v.* 10.2 to 12.4 kg CO_2-e/kg LW, respectively) (Wiedemann *et al.*, 2016a and 2017a), and the application of lignite to feedlots reduces ammonia emissions by a further 30% (Sun *et al.*, 2016). There are also options being researched for pasture-based systems, including carbon capture by use of permanent pastures (Soussana *et al.*, 2010) and through planting of trees (Doran-Browne *et al.*, 2018). Furthermore, the emissions per unit product can be decreased either by increasing livestock production efficiency or by targeting the source of the emissions, for example, by feeding or using novel technologies to reduce methane (Scollan *et al.*, 2010). For example, *in vitro* ruminal testing of seaweed as a food source for cattle has been shown to reduce methane production by up to 50%, depending on the amount administered (Kinley *et al.*, 2016). Feeding seaweed to cattle to reduce methane emissions shows a lot of promise and obviously requires further investigation. Other feedstuffs are being investigated, and progress will be made in the future. Studies have also shown that methane emission is heritable and thus genetic selection can be used to reduce methane emissions from cattle production (Pickering *et al.*, 2015).

Summary and conclusions

Consumer demand for cellular meat production derives from concerns over environment, animal welfare, consumer and public health aspects of animal production, use of antibiotics in the animal industries and food security.

As a concept, it has been suggested that cultured meat overcomes some of the ethical problems of livestock production but has also been criticised as a problematically technocentric, profit-motivated approach which will be dominated by large corporations (Lynch and Pierrehumbert, 2019).

The present limitations to cellular meat production include the identification of pluripotent cell lines in mammals, availability of cost-effective bovine-serum-free growth medium for cell proliferation and maturation, scaffold materials for cell growth, scaling-up to an industrial level and at what stage mixing of myo-, adipo- and fibrocytes can occur. In addition, consumer perceptions that cell-based meat production will result in improvements to animal welfare and the environment have been challenged, with the outcome needing to wait until cell-based meat is close to a commercial reality. Challenges for cell-based meat products include the simulation of nutritional attributes, texture, flavour and mouthfeel in animal-derived meat products. There is some question over whether consumers will accept the technology, but likely there will be acceptance of cell-based meat products in particular market segments. Currently, the cost of growth media, industry scale-up of specific components of the cell culture process, intellectual property sharing issues and regulatory hurdles mean that it will likely require take an extended period for cellular meat to be consistently available in high-end restaurants and even longer to be available for the mass market (Waughray, 2018).

Cellular meat production will likely be more efficient in utilising agricultural feedstocks than traditional forms of meat production as it may substitute industrial processes for the internal biological work done by animal physiology (Mattick *et al.*, 2015a). Alternatively, maybe the upscaling and costs of production and technology development will not be sufficient for the price to drop substantially, and this high-tech food will remain at best an exclusive gastronomic, molecular cuisine (Banis, 2018).

The plant-based meat analogues are very well developed, with products such as the Impossible™ Burger and other products already available. These developments may make the development of cellular meat products obsolete (Banis, 2018). But the challenges remain of mimicking not only the flavour, shape and structure of real meat, but also the required changes in regulation and labelling.

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Declaration of interest

The author declares no competing interests.

Ethics statement

Not applicable.

Software and data repository sources

None of the data were deposited in an official repository.

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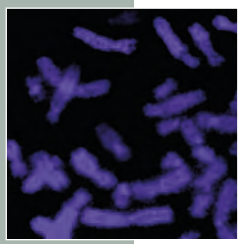

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