

Ghrelin secretion is more closely aligned to energy balance than with feeding behaviour in the grower pig

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Abstract

Secretory characteristics of the ghrelin profile for the pig are still unknown. Our objective was to clarify the mechanisms that influence ghrelin secretion during differing feeding patterns. Pigs were initially fed a commercial pelleted diet offered *ad libitum* and blood samples collected for 24 h at intervals of 1 h. The pigs were then entrained for 17 days to a twice daily interval feeding regimen (0900–1000 and 1600–1700 h) and blood samples were collected for 12 h (0800–2000 h). This was followed by a similar interval feeding and blood sampling regimen with the 0900–1000 h feeding period being replaced by a sham feed where pigs were shown their usual feed but none offered. During the *ad libitum* feeding regimen, there was no preprandial rise or postprandial fall in circulating plasma total ghrelin concentration, which

remained constant throughout the sampling period. In addition, no preprandial rise or postprandial fall in ghrelin concentrations was observed when pigs were fed either twice or once daily; however, plasma ghrelin concentration rose gradually over the 12-h sampling period during the twice daily feeding regimen and increased further when pigs were fed once per day. This increase in ghrelin levels coincided with an increase in plasma GH and non-esterified fatty acid concentrations and was not associated with either plasma glucose or insulin concentrations. These results suggest that circulating total plasma ghrelin concentrations in the pig appear to be influenced by chronic changes in energy balance rather than the feeding pattern *per se*.

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Introduction

In most mammals, ghrelin is mainly produced in the mucosa of the stomach fundus, where it is abundant in the oxyntic, pyloric and cardiac glands (Date *et al.* 2000, Tomasetto *et al.* 2000, Dornonville de la Cour *et al.* 2001, Hayashida *et al.* 2001, Rindi *et al.* 2002, Govoni *et al.* 2005). A recent study (Govoni *et al.* 2005) in pigs has shown that ghrelin is most abundant in the oxyntic and cardiac glands and less numerous in the pyloric glands. Ghrelin has also been discovered in the intestines (decreasing from the duodenum to the colon), pancreas, lungs, kidneys, testis, placenta, immune cells, hypothalamus and pituitary (Peeters 2005). Two-thirds of circulating plasma ghrelin is derived from the stomach and the remaining one-third from the small intestine (Peeters 2005).

Ghrelin has been shown to have numerous physiological functions that affect energy balance (Horvath *et al.* 2001, Govoni *et al.* 2005), gastrointestinal motility and secretion (Masuda *et al.* 2000) and feeding behaviour (Wren *et al.* 2000). In humans, plasma ghrelin concentrations increase 1–2 h before the onset of each meal and decrease within 1 h of re-feeding (Cummings *et al.* 2001). Obese humans tend to have lower circulating levels of ghrelin compared with individuals who are under weight (Tschöp *et al.* 2001, Shiya

et al. 2002, Erdmann *et al.* 2003) while weight loss in obese individuals increases circulating ghrelin status: by contrast, weight gain in humans with anorexia nervosa reduces plasma ghrelin concentrations (Ariyasu *et al.* 2001, Otto *et al.* 2001, Erdmann *et al.* 2003).

A similar finding has also been reported for a ruminant species, the sheep in which the fluctuations in circulating glucose status are buffered by the rumen. In spite of this, Sugino *et al.* (2002) observed a transient surge in plasma ghrelin levels prior to feeding in restrictively fed sheep, which then declined. In rodents, stomach ghrelin mRNA levels are increased following an extended fast, while re-feeding restores stomach expression values (Lee *et al.* 2002). These studies have led to the suggestion that ghrelin functions as a neuropeptide signal that initiates the onset of feeding. However, it is also plausible that the preprandial rise in plasma ghrelin may be a consequence of an anticipatory response to the entrainment to a specific feeding pattern (Drazen *et al.* 2006). In their study, both fasted rats not anticipating a meal and those fed *ad libitum* showed no preprandial rise in ghrelin. A similar result has also been observed for *ad libitum* fed sheep (Sugino *et al.* 2002).

Ghrelin influences energy homeostasis by providing a peripheral signal that regulates feeding behaviour through the

activation of neuropeptide Y/Agouti-related peptide neurones in the arcuate nucleus of the hypothalamus (Kamegai *et al.* 2000, Nakazato *et al.* 2001, Shintani *et al.* 2001). The exogenous administration of ghrelin to rats has both orexigenic and adipogenic effects, as it stimulates appetite and reduces fat utilisation (Tschöp *et al.* 2000). The regulation of the orexigenic nature of ghrelin is subject to both the animal's nutritional status (Ariyasu *et al.* 2001, Otto *et al.* 2001, Erdmann *et al.* 2003) and to the type of macronutrient ingested (Erdmann *et al.* 2003). Meals rich in carbohydrates consumed by human subjects have a greater suppressive effect on ghrelin levels than meals rich in fats (Erdmann *et al.* 2003). This effect may be species dependent as no such relationships have been found in the rat (Gomez *et al.* 2004). Studies using rodents fed *ad libitum* have shown that ghrelin is expressed in a diurnal pattern with two peaks, one at 1500 h (during the light period) and the other at 0600 h (at the end of the dark period). The physiological significance of these two peaks is unknown, although they coincide with the timing of the lowest and the greatest gastric emptying and filling activity, when gastric acid secretion and gastric motility are most apparent (Masuda *et al.* 2000).

Although ghrelin secretion has been studied in humans, sheep and rodents, only little is known about the effect of feeding behaviour on plasma ghrelin concentration in growing pigs. Porcine ghrelin status responds to changes in energy balance with concentrations increasing in response to fasting (Salfen *et al.* 2003, Govoni *et al.* 2005, Inoue *et al.* 2005). Moreover, exogenous ghrelin given chronically to weanling pigs increases body weight suggesting that ghrelin may also have an influence on feeding behaviour (Salfen *et al.* 2004). However, the basal secretory characteristics for ghrelin are still unknown for the pig. The objective of this study was to clarify the association between ghrelin secretion, feeding behaviour and changes in energy balance. We tested this association with pigs that were given either voluntary access to feed or entrained to a twice daily feeding regimen and compared the effects of these differing feeding patterns on circulating ghrelin, growth hormone (GH), insulin, glucose and non-esterified fatty acids (NEFA).

Materials and Methods

Animals and experimental procedures

Animal care and procedures were approved by the Animal Ethics Committee of the Elizabeth Macarthur Agricultural Institute (EMAI). Ten entire male pigs (large white × landrace), with a live weight of 57.5 ± 2.9 kg (mean \pm S.E.M.), were used for this trial. The pigs were transported from the University of Sydney to the controlled environment facility at EMAI, weighed and allocated to individual pens in the same room and maintained at 22 ± 1 °C and a 12 h (0600–1800 h) light regimen. The pigs were habituated to

their new environment for 6 days prior to the onset of the trial. During the habituation period, all pigs were fed a pelleted commercial grower diet estimated to contain 13 MJ digestible energy and 6.2 g available lysine per kg. Feed was offered *ad libitum* (regimen A) and water was provided using nipple drinkers. Residual feed was collected and recorded each day. On day 7, a catheter was placed into the external jugular vein of each pig via the ear vein (Anderson & Elsley 1969). On day 8, blood samples (3 ml) were collected from each pig at intervals of 1 h for 24 h and activity for each pig was monitored using near infrared surveillance cameras. The video records were examined to measure time spent lying, standing, sitting and feeding for each pig. On day 9, all pigs were entrained to feed at two feeding periods (0900–1000 h and 1600–1700 h) per day (regimen B), a total of 95% of that consumed in regimen A (Table 1). On day 26, blood samples were collected for 12 h (0800–2000 h) at intervals of 1 h and the pig activity was monitored. On day 27, a similar experimental regimen was used but the first feeding period (0900–1000 h) was replaced by a sham feed where pigs were shown their usual feed but none was offered (referred to subsequently as 'modified regimen B: fed once only').

Feed intake and body weights

Estimates of daily feed intake for experimental days 3, 4, 5, 6 and 8 were obtained for regimen A by subtracting the daily residue weight from the weight of the feed offered the previous day. Similarly, feed residues collected at 1000 and 1700 h were subtracted from feed offered at 0900 and 1600 h respectively during feeding regimen B. To account for any feeding expectation induced by human presence, specific feeding and blood sampling personnel were employed over the duration of the experiment. To minimise any feeding response resulting from familiarity with clothing worn by the feeder, those feeding and those sampling wore different coloured attire. Body weights of the pigs were recorded on days 1, 11, 19 and 33.

Hormone and metabolite analyses

Ghrelin peptide was extracted from porcine plasma using octadecyl C₁₈ mini columns (Amersham Biosciences) prior to determination by RIA. Total plasma ghrelin concentrations were determined in duplicate using a commercial double-antibody RIA with a primary antibody raised against porcine ghrelin and ¹²⁵I-labelled ghrelin as the tracer (kit RK-031-52; Phoenix Pharmaceuticals, Belmont, CA, USA). Plasma insulin and GH concentrations were determined by RIA (Downing *et al.* 1995). The insulin RIA was a heterologous assay using an anti-ovine insulin antibody raised in guinea pigs, which was used in the assay at a final dilution of 1:120 000. The antibody cross reacted with porcine insulin (56%) and pro-insulin (32%) relative to ovine insulin, but not with C peptide or glucagon. The reference standards were prepared from purified porcine insulin (Lot #49110830

Table 1 Mean (\pm S.E.M.) plasma concentrations of glucose, NEFA, ghrelin, GH and insulin for pigs fed *ad libitum* (regimen A), twice daily (regimen B) and once daily (modified regimen B)

	Time	Ghrelin (pg/ml)	NEFA (mEq/l)	Glucose (mmol/l)	Insulin (ng/ml)	GH (ng/ml)
All pigs fed <i>ad libitum</i> (feed remained in trays)	Day 8					
	6:00	129.06 \pm 54.09	0.13 \pm 0.03	6.56 \pm 0.38	0.63 \pm 0.15	0.18 \pm 0.06
	7:00	86.27 \pm 21.46	0.08 \pm 0.01	6.53 \pm 0.40	0.98 \pm 0.14	0.18 \pm 0.04
	8:00	58.22 \pm 7.49	0.08 \pm 0.01	6.30 \pm 0.30	0.90 \pm 0.20	0.15 \pm 0.06
	9:00	56.06 \pm 7.92	0.08 \pm 0.01	6.10 \pm 0.43	0.55 \pm 0.08	0.11 \pm 0.03
	10:00	48.63 \pm 5.77	0.16 \pm 0.02	5.58 \pm 0.17	0.57 \pm 0.09	0.61 \pm 0.07
	11:00	54.27 \pm 1.04	0.20 \pm 0.04	5.32 \pm 0.29	0.51 \pm 0.08	0.28 \pm 0.11
	12:00	58.77 \pm 3.06	0.15 \pm 0.02	5.13 \pm 0.18	0.63 \pm 0.20	0.23 \pm 0.06
	13:00	57.33 \pm 5.85	0.17 \pm 0.05	5.32 \pm 0.30	0.52 \pm 0.11	0.29 \pm 0.02
	14:00	55.98 \pm 2.01	0.08 \pm 0.02	5.65 \pm 0.37	0.49 \pm 0.10	0.35 \pm 0.06
	15:00	57.94 \pm 0.72	0.12 \pm 0.04	5.75 \pm 0.41	0.54 \pm 0.11	0.24 \pm 0.10
	16:00	53.31 \pm 0.65	0.08 \pm 0.01	5.80 \pm 0.23	0.74 \pm 0.11	0.30 \pm 0.11
	17:00	57.98 \pm 1.19	0.07 \pm 0.02	5.93 \pm 0.39	0.78 \pm 0.17	0.70 \pm 0.31
	18:00	61.95 \pm 0.07	0.07 \pm 0.02	6.43 \pm 0.42	0.70 \pm 0.13	0.14 \pm 0.05
	19:00	58.53 \pm 4.86	0.06 \pm 0.01	6.67 \pm 0.56	0.53 \pm 0.08	0.23 \pm 0.05
	20:00	57.84 \pm 4.80	0.08 \pm 0.01	7.47 \pm 0.51	0.84 \pm 0.14	0.16 \pm 0.02
	21:00	58.64 \pm 1.48	0.07 \pm 0.01	10.53 \pm 0.81	0.72 \pm 0.09	0.08 \pm 0.02
	22:00	51.85 \pm 6.13	0.08 \pm 0.02	9.65 \pm 0.46	0.70 \pm 0.11	0.23 \pm 0.09
	23:00	103.97 \pm 39.12	0.08 \pm 0.01	9.15 \pm 0.38	0.65 \pm 0.18	0.04 \pm 0.01
	0:00	160.18 \pm 56.00	0.08 \pm 0.01	8.80 \pm 0.53	0.40 \pm 0.09	0.10 \pm 0.07
	1:00	57.14 \pm 6.48	0.08 \pm 0.02	8.22 \pm 0.54	0.52 \pm 0.11	0.20 \pm 0.07
	2:00	54.89 \pm 6.46	0.08 \pm 0.02	7.96 \pm 0.32	0.62 \pm 0.11	0.12 \pm 0.06
	3:00	59.48 \pm 2.85	0.09 \pm 0.02	7.22 \pm 0.17	0.65 \pm 0.17	0.10 \pm 0.07
	4:00	57.04 \pm 7.54	0.14 \pm 0.05	7.22 \pm 0.22	0.48 \pm 0.11	0.12 \pm 0.05
5:00	56.82 \pm 6.79	0.11 \pm 0.02	6.73 \pm 0.34	0.31 \pm 0.07	0.41 \pm 0.14	
All pigs fed twice daily (feed in trays for 1 h \times 2)	Day 26					
	8:00	143.90 \pm 31.99	0.38 \pm 0.10	5.10 \pm 0.45	0.01 \pm 0.00	1.04 \pm 0.34
	9:00	204.47 \pm 23.12	0.21 \pm 0.02	6.23 \pm 0.67	0.01 \pm 0.00	0.41 \pm 0.13
	10:00	210.87 \pm 15.62	0.17 \pm 0.03	5.43 \pm 0.72	0.98 \pm 0.19	0.26 \pm 0.03
	11:00	184.03 \pm 12.22	0.28 \pm 0.03	5.17 \pm 0.54	0.55 \pm 0.12	0.24 \pm 0.02
	12:00	171.97 \pm 8.73	0.25 \pm 0.05	5.87 \pm 0.54	0.35 \pm 0.14	0.68 \pm 0.14
	13:00	223.87 \pm 26.83	0.18 \pm 0.02	5.30 \pm 0.61	0.32 \pm 0.13	0.22 \pm 0.03
	14:00	221.10 \pm 6.66	0.21 \pm 0.02	6.30 \pm 0.58	0.16 \pm 0.08	0.87 \pm 0.25
	15:00	163.17 \pm 10.56	0.16 \pm 0.01	5.03 \pm 0.16	0.11 \pm 0.08	0.11 \pm 0.05
	16:00	184.90 \pm 20.08	0.14 \pm 0.01	5.15 \pm 0.08	0.03 \pm 0.02	0.06 \pm 0.02
	17:00	182.20 \pm 16.88	0.16 \pm 0.03	5.03 \pm 0.24	0.54 \pm 0.18	0.54 \pm 0.21
18:00	254.40 \pm 12.28	0.15 \pm 0.01	4.57 \pm 0.21	0.16 \pm 0.10	0.39 \pm 0.03	
19:00	260.30 \pm 30.27	0.13 \pm 0.01	4.83 \pm 0.17	0.20 \pm 0.15	0.24 \pm 0.09	
All pigs fed once (feed in trays for 1 h \times 1) morning feed = sham feed	Day 27					
	8:00	153.17 \pm 18.40	0.54 \pm 0.04	4.83 \pm 0.24	0.09 \pm 0.05	0.06 \pm 0.02
	9:00	211.30 \pm 12.34	0.21 \pm 0.04	4.67 \pm 0.09	0.04 \pm 0.03	0.10 \pm 0.04
	10:00	225.07 \pm 9.46	0.21 \pm 0.03	4.60 \pm 0.09	0.02 \pm 0.01	1.25 \pm 0.24
	11:00	253.80 \pm 9.60	0.25 \pm 0.02	4.45 \pm 0.10	0.04 \pm 0.03	1.01 \pm 0.32
	12:00	244.50 \pm 20.21	0.41 \pm 0.05	4.76 \pm 0.31	0.01 \pm 0.00	0.20 \pm 0.04
	13:00	258.63 \pm 15.31	0.41 \pm 0.04	4.51 \pm 0.12	0.01 \pm 0.00	0.11 \pm 0.05
	14:00	265.20 \pm 10.88	0.56 \pm 0.06	4.39 \pm 0.14	0.01 \pm 0.00	0.76 \pm 0.15
	15:00	260.47 \pm 19.95	0.56 \pm 0.05	5.01 \pm 0.21	0.01 \pm 0.00	0.43 \pm 0.14
	16:00	247.87 \pm 18.94	0.68 \pm 0.05	4.90 \pm 0.20	0.03 \pm 0.02	0.20 \pm 0.01
	17:00	284.43 \pm 21.05	0.17 \pm 0.01	7.03 \pm 0.27	0.98 \pm 0.51	0.79 \pm 0.42
18:00	263.67 \pm 18.99	0.18 \pm 0.02	5.45 \pm 0.27	0.91 \pm 0.47	0.30 \pm 0.16	
19:00	282.90 \pm 8.07	0.16 \pm 0.02	5.90 \pm 0.27	0.80 \pm 0.25	0.60 \pm 0.17	

Sigma–Aldrich). Non-specific binding was less than 3%, the assay sensitivity was 0.05 ng/ml and the inter- and intra-assay coefficients of variation were 9.1 and 13.7% respectively. The GH antibody was specific for porcine GH and was supplied by Dr J P McMurtry (Althen & Gerrits 1976). The standard reference used was obtained from the National Institute of Diabetes and Digestive and Kidney Diseases. The assay sensitivity was 1.0 ng/ml and the inter- and intra-assay

coefficients of variation were 12.5 and 8.2% respectively. Circulating NEFA concentrations were determined by the acyl-CoA synthetase/acyl-CoA oxidase method (NEFA C-test; Wako Chemicals USA, Inc. Richmond, VA, USA). Plasma glucose measurements were determined with a commercially available kit (Glucose Unimate 5 Gluc HK, Roche Products Pty Ltd) using a Roche Cobas Mira automatic analyser.

Statistical analysis

Data on feed intake, body weight, hormone and metabolite concentrations were analysed using linear mixed models via the REML procedure using GenStat Release 9 (www.vsni.co.uk). As these were repeated measures data (either hourly over a 24-h period or daily over an 18-day period), adjustments were made for the serial correlation of random errors (using a power model). Significance of fixed effects was assessed using Wald χ^2 tests. NEFA, insulin, GH and ghrelin concentrations were analysed on a logarithmic scale. All data are presented as mean \pm S.E.M.

Results

Live pig performance

Body weights Body weights for all pigs increased over the experimental period. Body weights (mean \pm S.E.M.) for day 1 were 57.50 \pm 2.91 kg, day 11 63.49 \pm 2.31 kg, day 19 70.30 \pm 2.31 kg and day 33 82.63 \pm 2.86 kg (Fig. 1).

Feed intakes

Ad libitum feeding (regimen A). Daily feed intake for days 1–6 was 3.18 \pm 0.06 kg and did not change (3.17 \pm 0.12 kg) on day 7 when pigs were catheterised (Fig. 1).

Twice daily feeding (regimen B). During the first week of entrainment to regimen B, pigs consumed a greater proportion of feed during the afternoon (1.31 \pm 0.02 kg) when compared with the morning feeding period (1.09 \pm 0.04 kg). After day 7, intakes for both morning and afternoon periods were similar (1.39 \pm 0.04 kg and 1.37 \pm 0.11 kg

respectively). However, feed intakes for this feed regimen were 87% of *ad libitum* feeding (regimen A).

Once daily feeding (modified regimen B). The average feed intake for the 1600–1700 h feeding period was 1.90 \pm 0.14 kg, a difference of 60% when compared with *ad libitum* feeding (regimen A).

The efficiency of feed utilisation The efficiency of feed utilisation expressed as weight of feed consumed (kg) relative to live weight gain (kg) for the same period is presented in Fig. 1.

Mean (\pm S.E.M.) feed: gain (kg/kg) for days 1–11 was 6.26 \pm 2.75, for days 11–19 was 3.23 \pm 0.20 and for days 19–33 was 2.78 \pm 0.07.

Hormones and metabolites

Plasma insulin

Ad libitum feeding (regimen A). Apart from a significant ($P=0.03$) rise in plasma insulin concentration to 0.98 \pm 0.14 ng/ml at 0700 h, there was no significant change throughout the remainder of the 24-h sampling period. However, from 1500 h onwards, the secretory pattern appeared to be cyclical with a periodicity of 6 h and amplitude of \sim 0.2 ng/ml (Fig. 2).

Twice daily feeding (regimen B). During the morning and afternoon feed periods (0900–1000 h and 1600–1700 h), circulating insulin concentrations significantly increased ($P<0.001$) to 0.98 \pm 0.19 ng/ml and 0.54 \pm 0.18 ng/ml respectively.

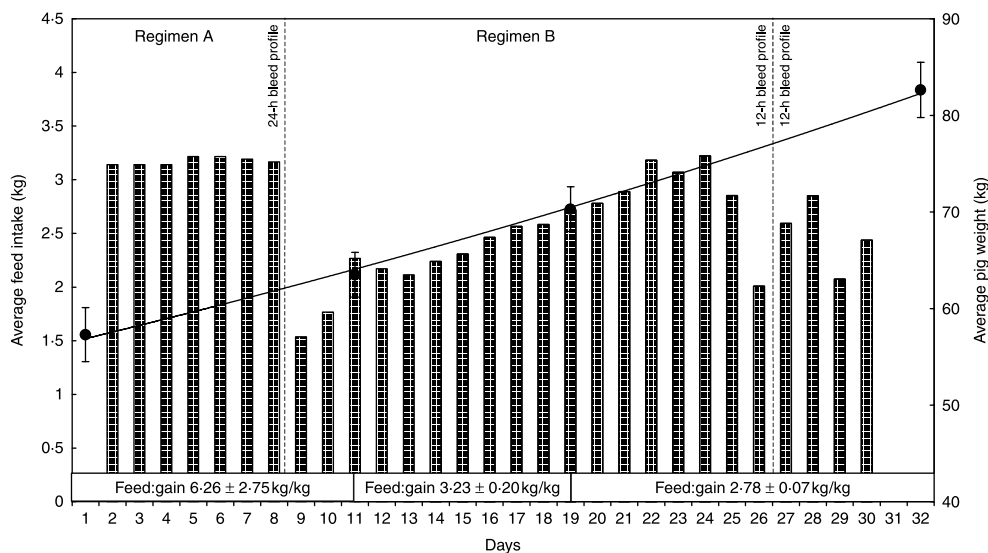


Figure 1 Comparison of mean daily feed intakes, $n=10$ (■), and mean body weights; $n=10$ (●), of pigs recorded during the course of the trial. These were then used to calculate the feed: gain ratio (kg/kg) for days 1–11, 11–19 and 19–33.

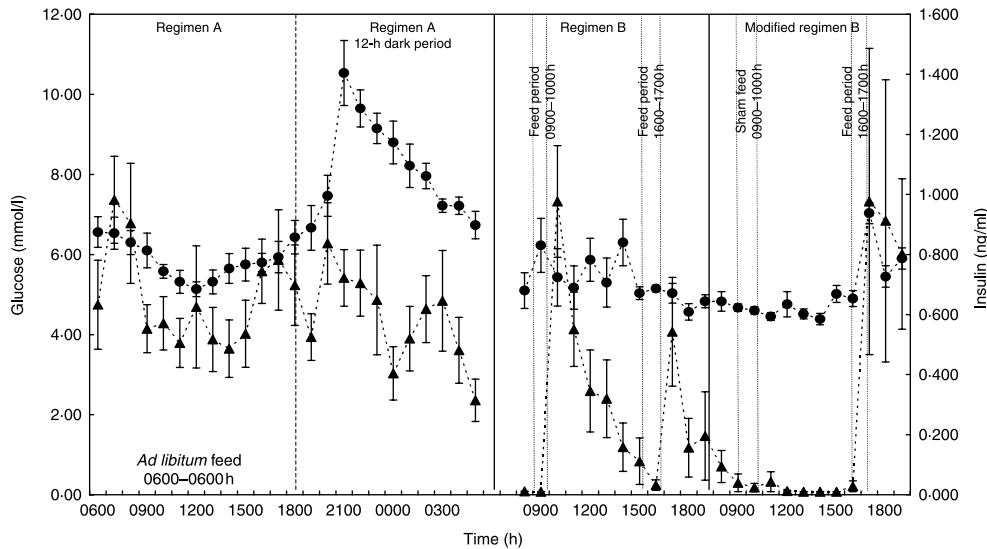


Figure 2 Comparison of circulating plasma glucose, $n=7$ (●), and insulin, $n=7$ (▲), taken hourly on days 8, 26 and 27.

Once daily feeding (modified regimen B). From 0900 until 1600 h, plasma insulin concentrations remained constant (0.02 ± 0.01 ng/ml) and were significantly ($P=0.02$) lower when compared with concentrations for the same period during the previous day (0.33 ± 0.04 ng/ml). Following re-feeding at 1600 h, insulin concentrations increased significantly ($P<0.001$) to 0.98 ± 0.51 ng/ml and were elevated for a further 2 h after feed was removed at 1700 h.

Plasma glucose

Ad libitum feeding (regimen A). The photoperiod regimen (12 h light: 12 h darkness cycle) that the pigs were exposed to had a profound effect on the plasma glucose profile. During the light period, plasma glucose decreased from 6.56 ± 0.38 mmol/l at 0600 h to 5.13 ± 0.18 mmol/l by 1200 h then gradually increased over the afternoon period to 6.43 ± 0.18 mmol/l by 1800 h. During the dark period, plasma glucose concentrations increased significantly ($P<0.001$) to 10.53 ± 0.81 mmol/l at 2100 h then declined to 6.73 ± 0.34 mmol/l by 0500 h (Fig. 2).

Twice daily feeding (regimen B). Plasma glucose concentrations were relatively constant throughout the sampling period with a mean value of 5.37 ± 0.14 mmol/l, although concentrations tended to be higher and more variable between 0800 to 1400 h (5.63 ± 0.23 mmol/l) compared with the remainder of the sampling period (4.97 ± 0.08 mmol/l).

Once daily feeding (modified regimen B). The plasma glucose profile was relatively constant (4.68 ± 0.06 mmol/l) prior to the onset of re-feeding at 1600 h. Following re-feeding, plasma glucose increased to 5.03 ± 0.24 mmol/l and remained elevated for a further 2 h after feed was removed at 1700 h.

Plasma ghrelin

Ad libitum feeding (regimen A). Plasma ghrelin concentrations remained relatively constant over the 24-h sampling period (67.2 ± 5.5 pg/ml; Figs 3 and 4).

Twice daily feeding (regimen B). Circulating ghrelin concentrations were variable throughout the 12-h sampling period (200.4 ± 10.2 pg/ml) and values were significantly higher ($P<0.05$) when compared with the same 12-h sampling period as the *ad libitum* feeding regimen (56.6 ± 1.0 pg/ml). Three distinct peaks in plasma ghrelin concentrations were observed at 0900 h (204.5 ± 23.1 pg/ml), 1300 h (223.9 ± 26.8 pg/ml) and 1800 h (254.4 ± 12.3 pg/ml) with corresponding troughs at 1200 h (172.0 ± 8.7 pg/ml) and 1500 h (163.2 ± 10.6 pg/ml). These peaks and troughs did not appear to be associated with the time of feeding (0900–1000 and 1600–1700 h).

Once daily feeding (modified regimen B). Mean plasma ghrelin concentrations during the once daily feeding regimen (245.9 ± 10.4 pg/ml) tended to be higher than during the twice daily feeding regimen (200.4 ± 10.2 pg/ml).

Plasma NEFA

Ad libitum feeding (regimen A). Plasma NEFA showed little variation throughout the sampling period with average NEFA concentrations of 0.10 ± 0.01 mEq/l. These increased significantly ($P<0.001$) to 0.20 ± 0.04 mEq/l by 1100 h, returning to baseline values 3 h later, where they remained for the rest of the sampling period (Fig. 3).

Twice daily feeding (regimen B). Mean plasma NEFA concentrations during the twice daily feeding regimen

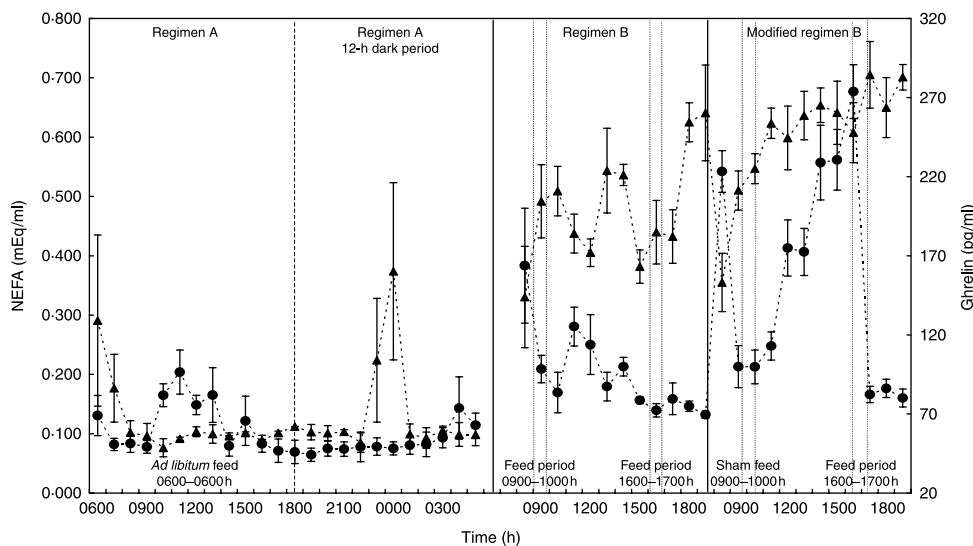


Figure 3 Comparison of circulating plasma NEFA, $n=7$ (●), and ghrelin, $n=7$ (▲), taken hourly on days 8, 26 and 27.

(0.20 ± 0.02 mEq/l) were significantly greater ($P < 0.05$) and more variable than when the pigs were fed *ad libitum* (0.10 ± 0.01 mEq/l). Prior to feeding at 0800 h, NEFA concentrations were elevated (0.38 ± 0.09 mEq/l). During the feeding period (0900–1000 h), concentrations declined to 0.17 ± 0.03 mEq/l and remained relatively constant (0.20 ± 0.02 mEq/l) throughout the sampling period.

Once daily feeding (modified regimen B). Mean plasma NEFA concentrations for pigs fed once daily (0.36 ± 0.05 mEq/l) were significantly greater ($P < 0.05$) and more variable when compared with pigs fed *ad libitum* (0.10 ± 0.01 mEq/l)

and twice daily (0.20 ± 0.02 mEq/l). From 0800 to 1100 h, pigs displayed a similar pattern in circulating NEFA concentrations to the twice daily feeding regimen. By 1200 h, NEFA concentrations were elevated significantly (0.41 ± 0.05 mEq/l; $P = 0.011$) and continued to increase to 0.68 ± 0.05 mEq/l until pigs were fed at 1600 h at which time they declined to 0.17 ± 0.01 mEq/l within the hour in which feed was offered.

Plasma GH

Ad libitum feeding (regimen A). Plasma GH concentrations were released in a pulsatile pattern over the 24-h sampling

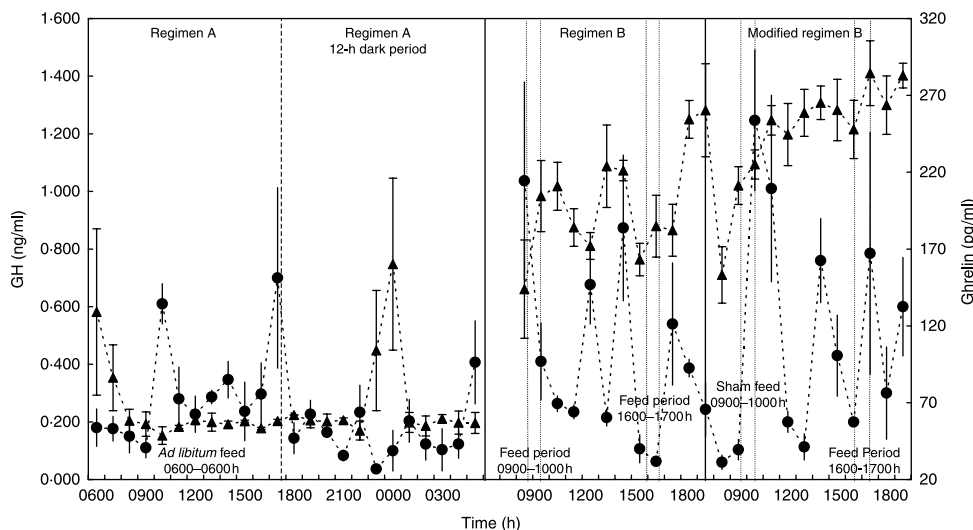


Figure 4 Comparison of circulating plasma GH, $n=7$ (●), and ghrelin, $n=7$ (▲), taken hourly on days 8, 26 and 27.

period with peaks occurring at 1000 h (0.61 ± 0.07 ng/ml), 1700 h (0.70 ± 0.30 ng/ml) and 0500 h (0.41 ± 0.11 ng/ml; Fig. 4).

Twice daily feeding (regimen B). Circulating GH concentrations displayed a similar pattern of secretion over the 12-h sampling period as for *ad libitum* feeding. However, the GH secretory profile was more variable with peaks occurring at 0800 h (1.04 ± 0.34 ng/ml), 1200 h (0.68 ± 0.14 ng/ml), 1400 h (0.87 ± 0.25 ng/ml) and 1700 h (0.54 ± 0.21 ng/ml).

Once daily feeding (modified regimen B). The plasma GH secretory profile was similar to twice daily feeding although the timing for these peaks were ~ 2 h later with GH peaks occurring at 1000 h (1.25 ± 0.24 ng/ml), 1400 h (0.76 ± 0.15 ng/ml), 1700 h (0.79 ± 0.42 ng/ml) and 1900 h (0.60 ± 0.17 ng/ml).

Behavioural observations

Observations of pig behaviour are presented in Fig. 5.

Ad libitum feeding (regimen A) Pigs were observed to be more active during the light period (0600–1800 h) and remained recumbent during the dark period (1800–0600 h). This pattern was not affected by the hourly presence of humans to collect blood samples throughout the 24-h period.

Twice daily feeding (regimen B) The activity of the pig increased prior to the onset of the morning feeding period (0900–1000 h) during which feed was consumed constantly for 45 min until the pigs were satiated and their activity declined until the feed was removed. Between the morning

and afternoon feeding periods, pigs were mainly inactive with activity slightly increasing prior to the onset of the afternoon feeding period. Pigs consumed feed constantly for the hour then reclined again to a state of relative inactivity soon after feed was withdrawn.

Once daily feeding (modified regimen B) The pigs spent more time standing during the 12-h bleed period compared with the twice daily feeding regimen.

Discussion

The main findings from this study were twofold. The first finding was that plasma ghrelin concentrations were not aligned with either feeding activity or to plasma glucose. The observation that ghrelin status was not related to any metabolic factor in animals fed *ad libitum* suggests that the secretion of this hormone is not associated with acute feeding responses. We observed periodic feeding activity mainly within the daylight hours and yet ghrelin status remained unaltered over the duration of the 24-h sampling period.

The second finding was the dissociation between plasma glucose and insulin concentrations at night when pigs were offered voluntary access to feed. Even though pigs were observed not to be feeding plasma glucose rose during the darkness–light cycle with little change in plasma insulin concentration. By contrast, plasma glucose and insulin rose in association with feeding activity when pigs were either fed once or twice daily.

The results from the current study suggest that ghrelin expression differs in pigs when compared with other species,

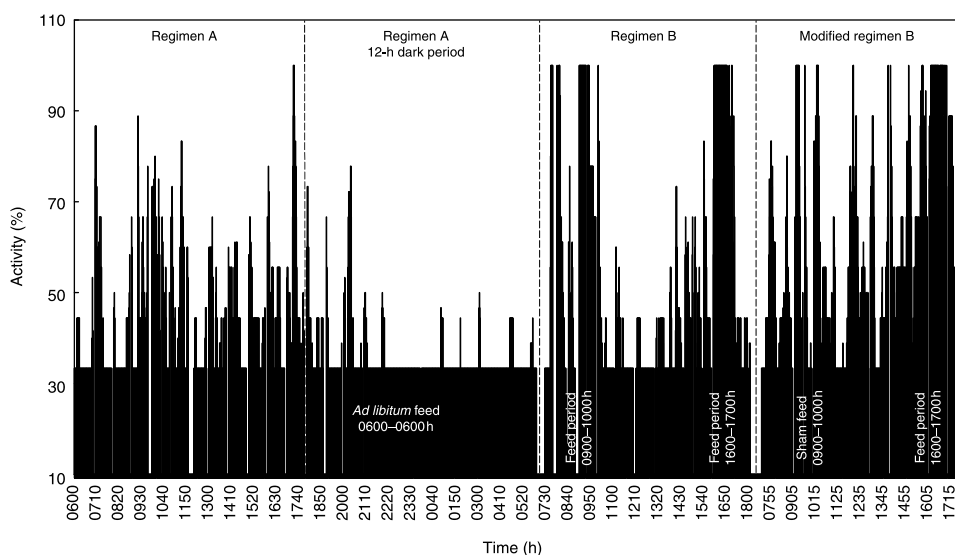


Figure 5 Using video footage, observations of individual pigs lying, sitting and standing were recorded every 30 s for days 8, 26 and 27. The shaded areas of the graph represent the mean pig activity, $n=6$, as a percentage. If all pigs were lying mean activity would equal 33% and if all pigs were standing mean activity would equal 100%.

as no significant preprandial surge or postprandial fall in plasma ghrelin concentrations were shown regardless of the feeding regimen imposed. Studies in humans placed on a fixed feeding regimen of three meals per day show a preprandial rise in plasma ghrelin followed by a postprandial fall at each accompanying meal (Cummings *et al.* 2001). In the current study, there was no alignment between ghrelin secretory pattern and the time of feeding for the twice daily feeding regimen. Similarly, the studies of Sugino *et al.* (2002) have also shown a preprandial rise in ghrelin in Suffolk rams maintained on a once daily feeding regimen. However, these studies have shown that there were no plasma ghrelin surges in animals fed *ad libitum*, a finding consistent with the present study. A proposed function for the preprandial rise in ghrelin is to stimulate hunger and initiate feeding (Cummings *et al.* 2001), although it now appears that this rise may simply be an anticipatory response to a conditioned physiological reflex rather than a feeding signal *per se* (Sugino *et al.* 2002). This notion is supported by the recent findings of Drazen *et al.* (2006) who have demonstrated with rats, that the secretion of ghrelin pre- and postprandially can be entrained by feeding meals at a set time and duration independent of the dietary energy status of the animal. Our study did not show a significant preprandial ghrelin surge or postprandial fall when pigs were fed either *ad libitum* nor when entrained to a periodic feeding regimen. This finding may be a consequence of using growing pigs, as pre-pubertal children are less sensitive to the inhibitory influence of food intake on ghrelin secretion when compared with adults (Bellone *et al.* 2004). It may also be the result of less frequent sampling or simply reflect a species difference. Alternatively, as suggested by Sugino *et al.* (2002), the lack of a ghrelin surge and decline may be attributed to the positive energy balance in which these animals were maintained. However, our behavioural data suggest that the pigs in the current study were not fed to reach satiety, as their increased activity prior to feeding resembled a heightened awareness for feed. This observation is corroborated by our endocrine data, which show metabolite and metabolic hormone patterns consistent with those for fasted gilts (Govoni *et al.* 2005). This was not the case when animals were offered the feed *ad libitum*. Our data suggest that pigs fed *ad libitum* consume feed at regular intervals and mainly throughout the daylight hours when housed in individual pens. However, it should also be noted that the ghrelin RIA used in the current study measured total ghrelin concentrations and that this may not reflect the biologically active form, acylated ghrelin (Paik *et al.* 2006). Therefore, any future studies should also measure acylated ghrelin and this may contribute to a better understanding of the pattern and control of ghrelin secretion in pigs.

The dissociation between feeding frequency, circulating glucose and insulin status in animals fed *ad libitum* is less easily explained. It may be related to fluctuations in insulin sensitivity during the period of darkness, which is also under the influence of suprachiasmatic nucleus (SCN) (La Fleur 2003). La Fleur (2003) has postulated that glucose

metabolism is regulated in a rhythmic fashion over a 24-h light/darkness cycle through the endogenous circadian oscillator located within the SCN and that this rhythm is independent of feeding activity. Studies in humans and rodents have shown an alteration in both plasma glucose and insulin concentrations and variation in sensitivity of tissues to insulin over a 24-h light/darkness period. For example, humans have a higher glucose output and insulin requirement in the early morning hours (Carroll & Nestel 1973, Lee *et al.* 1992), whereas rodents have higher concentrations of glucose and insulin in the dark period compared with the light period (Pauly & Scheving 1967), which may be associated with the initiation of physical activity (La Fleur 2003): it is important to recognise that rodents are nocturnally active and circulating insulin and glucose concentrations are higher during the dark phase (Pauly & Scheving 1967). Similarly, diurnal fluctuations in the actions of the D₂ dopamine receptor agonist bromocriptine on insulin sensitivity have been observed, with increased sensitivity in the morning and suppression in hepatic glucose production in the afternoon (Luo *et al.* 1999). Therefore, during an *ad libitum* feeding regimen, insulin secretion over the 24-h light/darkness cycle may be aligned more to whole body energy balance of the pig rather than to feeding behaviour or circulating glucose concentrations.

By contrast, when pigs were entrained to feed twice daily, we observed the classical insulin response to the consumption of food and the subsequent release of glucose. It is of interest that the insulin response to the 0900–1000 h feeding period was greater than that for the 1600–1700 h feeding period, even though similar quantities of feed were consumed for these two periods. Comparable findings have also been described for pigs entrained to feed twice daily at 12-h intervals where the postprandial insulin concentration was 75% greater for the morning compared with the evening feeding periods (Koopmans *et al.* 2005). Increased morning concentrations of both glucose and insulin have also been observed in humans (Bolli & Gerich 1984, Trumper *et al.* 1995), which has been suggested as an anticipatory response of glucose metabolism to the upcoming period of activity (La Fleur 2003). Therefore, the differences in plasma insulin secretion between the two feeding periods may be the result of a difference in the activity of the pig at these times. Our behavioural data suggest that these pigs tended to be more active during the morning hours than in the afternoon and that this difference in activity may induce changes in both plasma insulin concentrations and/or insulin tissue sensitivity under the influence of the SCN (La Fleur 2003).

Studies in a number of different species have attempted to identify a metabolic signal that regulates ghrelin secretion. These studies have produced conflicting results, which have complicated our understanding of the metabolic role for ghrelin. For example, studies in humans (Flanagan *et al.* 2003, Broglio *et al.* 2004) and rodents (Ishii *et al.* 2002) have demonstrated either a negative relationship between insulin and ghrelin or no significant relationship (Caixas *et al.* 2002, Espelund *et al.* 2005). Although in the same study, Espelund

et al. (2005) observed a circadian rhythm in plasma ghrelin, which had a strong negative correlation with plasma cortisol concentrations but was unrelated to GH status. Even from the same species, differences have been reported: *Kamegai et al.* (2004) demonstrated that insulin and leptin inhibited ghrelin while glucagon stimulated ghrelin secretion from an isolated perfused rat stomach, in a dose-dependent manner, while the expression of the ghrelin gene in the stomach either increased (*Toshinai et al.* 2001) or was unaffected (*Lee et al.* 2002) following the insulin or leptin treatment in rats *in vivo*.

Results from the current study suggest that ghrelin secretion is not modulated by changes in either insulin or glucose secretion irrespective of the feeding regimen. This result is in contrast to that of *Govoni et al.* (2005) who have shown in gilts a decline in insulin secretion with a corresponding rise in ghrelin levels. The discrepancy between these two studies may be a consequence of the experimental design and in particular the severity of the fasting: *Govoni et al.* (2005) fasted gilts for 72 h then re-fed these same animals, whereas in the current study pigs were allowed to adapt to the new feeding regimen by being habituated to a twice daily feeding pattern over a period of 2 weeks prior to the onset of the sampling. The altered endocrine status of pigs subjected to the fasting period in the *Govoni et al.* (2005) study are similar to the metabolic responses observed for the once daily feeding pattern in the present study with reduced insulin and elevated NEFA concentrations accompanied by increasing ghrelin and GH levels. The elevated NEFA concentrations are indicative of negative energy balance and are usually associated with protracted periods of inappetence or feed withdrawal, as we have reported previously in fallow deer (*Newman et al.* 1998).

Results from our current study indicate that insulin may not play an important role in ghrelin secretion, as the secretory profiles for these hormones were not related irrespective of the feeding regimen. *Sugino et al.* (2002) demonstrated similar findings by showing a negative relationship between plasma ghrelin and the feeding regimen. In their study, ghrelin concentrations increased as the period of feeding decreased; sheep fed twice daily had a significantly higher calculated area under the response curve for plasma ghrelin when compared with animals fed four times daily, which in turn was significantly higher when compared with animals fed *ad libitum*. Similarly, in the current study, there was a negative association between plasma ghrelin concentrations and the corresponding feeding regimen; plasma ghrelin concentrations were reduced with *ad libitum* feeding and elevated in response to once daily feeding. Interestingly, the plasma ghrelin secretory profile also showed distinct differences in response to the three feeding regimens. The ghrelin profile for the twice daily feed pattern showed three distinct peaks over the 12-h period occurring at 1000, 1400 and 1900 h, a pattern not dissimilar to that observed for humans (*Cummings et al.* 2001). Whereas when pigs were fed once daily, the plasma ghrelin profile showed no distinct peaks with concentrations rising over the 12-h sampling period. These data suggest that both circulating ghrelin

concentrations and its secretory profile are responsive to the amount of feed an animal consumes. Therefore, the type of feeding regimen an animal is exposed to, irrespective of species, may influence ghrelin secretion, which in turn reflects the energy status of the animal.

In the current study, the increase in the variability of the secretion of plasma GH coincided with the elevation in plasma ghrelin concentrations in pigs fed once or twice daily. This finding is in keeping with other studies that show the stimulatory nature of ghrelin on GH secretion and has been described for a variety of species including the pig (*Kojima et al.* 1999, *Arvat et al.* 2000, *Sugino et al.* 2002, *Hashizume et al.* 2003). It has been suggested that ghrelin has a modulatory role on GH secretion rather than a direct effect on the physiological system that stimulates the endogenous production and secretion of the GH pulse (*Broglio et al.* 2003, *Cummings & Shannon* 2003). However, our data suggest that ghrelin may influence the pulsatility of GH secretion in the pig as the number of GH pulses increased from two over a 12-h period (0800–2000 h) in *ad libitum* fed pigs to four for both once and twice daily feeding. This difference may simply reflect a species difference as GH plays an important role in the pig for both weight gain and lipid metabolism (*Etherton et al.* 1986) and as such the differences in the patterns of variations of both plasma ghrelin and GH concentrations may signify this difference.

It is conceivable that the increase in the pulsatility of GH secretion in the current study may act to stimulate lipid mobilisation, as NEFA levels were elevated when pigs were fed twice daily and were further elevated when fed once a day compared with those fed *ad libitum*. A relationship between plasma NEFA and ghrelin concentrations in response to a 54-h fast has been reported in grower pigs (*Inoue et al.* 2005) and also in fasted gilts (*Govoni et al.* 2005). Our results also show an association between plasma ghrelin and NEFA concentrations in pigs that were fed once daily, a difference in feed consumption of 60% when compared with *ad libitum* feeding. Therefore, the plasma ghrelin concentrations may simply reflect the long-term energy status of the pig rather than acting as an acute regulator of energy intake: in effect it acts as a negative feedback signal to the satiety centre of the hypothalamus (*Kojima et al.* 1999) to maintain live weight.

Although this study has not been able to clearly identify a physiological role for circulating ghrelin concentrations in the pig, we have shown that porcine ghrelin expression does differ to that in other mammals. There is neither a preprandial rise nor postprandial fall in circulating ghrelin concentrations in response to feeding. Plasma ghrelin does not appear to influence feeding behaviour acutely; however, it does reflect the energy status of the pig.

Our study has also demonstrated that insulin sensitivity is more closely aligned to a twice daily feeding pattern than to an *ad libitum* feeding regimen. In view of its importance in promoting growth processes, it is tempting to speculate that interval feeding may lead to more efficient utilisation of energy substrate for metabolism and growth in grower pigs: this was certainly the case in the present study with an

improvement in the efficiency in feed utilisation between the *ad libitum* and twice daily feeding periods. The practicalities of implementing such a feeding regimen and the improvements in feed utilisation achieved for growth on a commercial scale remain to be investigated in further studies.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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