

## Circadian misalignment imposed by nocturnal feeding tends to increase fat deposition in pigs

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### Abstract

Misalignment of day/night and feeding rhythms has been shown to increase fat deposition and the risk for metabolic disorders in humans and rodents. In most studies, however, food intake and intake patterns are not controlled. We studied the effects of circadian misalignment on energy expenditure in pigs while controlling for food intake as well as intake patterns. Twelve groups of five male pigs were housed in respiration chambers and fed either during the day (10.00–18.00 hours; DF) or night (22.00–06.00 hours; NF), bihourly the same sequential meals, representing 15, 10, 25, 30 and 20 % of the daily allowance. Paired feeding was applied to ensure equal gross energy intake between treatments. Apparent total tract digestibility, energy balances and heat partitioning were measured and analysed using a mixed linear model. Apparent total tract energy and DM digestibility tended to be lower for NF-pigs than DF-pigs ( $P < 0.10$ ). Heat production was 3 % lower for NF-pigs than DF-pigs ( $P < 0.026$ ), increasing fat retention by 7 % in NF-pigs ( $P = 0.050$ ). NF-pigs were less active than DF-pigs during the feeding period, but more active during the fasting period. RMR was greater for DF-pigs than NF-pigs during the fasting period. Methane production was 30 % greater in NF-pigs than DF-pigs ( $P < 0.001$ ). In conclusion, circadian misalignment has little effect on nutrient digestion, but alters nutrient partitioning, ultimately increasing fat deposition. The causality of the association between circadian misalignment and methane production rates remains to be investigated.

**Key words:** Feed intake patterns: Energy metabolism: Heat production: Methane production: Circadian clock

The timing of food intake in humans and animals is triggered by energy deficiency signals and simultaneously constrained by the endogenous circadian clock<sup>(1)</sup>. The endogenous circadian clock is entrained by environmental factors, so-called zeitgebers, of which the most important zeitgeber is the light/dark cycle<sup>(2)</sup>. In humans who deviate from their normal circadian rhythm due to, for example, shift work, (social) jet lag or night eating syndrome, the circadian eating pattern does not align with the endogenous circadian clock. This can be referred to as a form of circadian misalignment. Recurrent exposure to this phenomenon increases the risk for metabolic disorders like obesity<sup>(3–6)</sup>, reduced insulin resistance<sup>(7)</sup>, type 2 diabetes<sup>(8,9)</sup> or cardiometabolic diseases<sup>(10,11)</sup>.

Like humans, rodents, which are nocturnal animals, gain more weight<sup>(12–14)</sup> and abdominal fat<sup>(12,14)</sup> when fed during the day (non-active period) instead of the night. Increased body weights (BW) in humans and rodents that are exposed to

circadian misalignment may be explained by a greater energetic intake<sup>(15)</sup> or reduced heat loss<sup>(13,16)</sup>. Also, disruptions in the adipocyte-specific circadian clock may play a role<sup>(17)</sup>. In addition, humans have a greater preference for fat-rich food at dinner time than at breakfast time<sup>(18)</sup>, which might be related to obesity associated with late-night feeding<sup>(19)</sup>. However, in most of these studies that investigate the consequences of circadian misalignment, food intake and food intake patterns are not controlled. In addition, pigs are suggested to be a more suitable model for digestive strategies in humans than rodents<sup>(20)</sup> because the dietary habits of pigs more closely resemble those of humans; meal eaters (pigs and humans) compared with nibblers (rats)<sup>(21)</sup>. Besides the similarities in anatomy, physiology and metabolism between pigs and humans, the microbiome of pigs is also more similar to humans than that of rodents<sup>(20)</sup>. In addition, in common pig production systems, pigs, like humans, deviate from their circadian feeding pattern under certain circumstances, for

**Abbreviations:** BW, body weight; CHO, carbohydrate; DF, diurnal feeding; RQ, respiratory quotient; NF, nocturnal feeding; TEF, thermic effect of feeding.

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example, when exposed to high temperatures<sup>(22,23)</sup> or when forced by increased feeding competition between animals housed in the same pen<sup>(24)</sup>.

To disentangle the effects of dietary intake preferences and energetic intake from the effects of circadian misalignment, we investigated the effect of circadian misalignment induced by nocturnal feeding (NF) on energy metabolism in pigs using respiration chambers. Based on earlier results in humans and rodents, we hypothesise that asynchronisation of the endogenous circadian clock and the timing of feed intake stimulate fat deposition in growing pigs.

## Materials and methods

The experiment was conducted under the Dutch Law on Animal Experiments in accordance with EU Directive 2010/63. The experimental protocol was approved by the Animal Care and Use Committee of Wageningen University; the experiment was performed at the experimental facilities of Wageningen University, Wageningen, the Netherlands.

### Experimental design

Sixty male pigs (42 (SEM 1.8) kg; TOPIGS TEMPO × TOPIGS70) were randomly assigned to one of the two treatments, receiving feed during the day (diurnal feeding, DF) or night (NF). A group of five pigs housed within one chamber was considered as the experimental unit. Group housing allowed for normal circadian oscillations in hormone levels to occur, like, for example, cortisol<sup>(25)</sup> that plays an important role in the regulation of circadian feeding rhythms of pigs<sup>(26)</sup>. Due to the simultaneous availability of four climate-controlled respiration chambers, the experiment was carried out in three consecutive repartitions, with each repartition consisting of a 7-d (NF-group) or 8-d (DF-group) period wherein pigs were allowed to adapt to housing conditions and experimental treatments, followed by a 7-d experimental period. Paired feeding was applied to ensure equal energy intake between treatments: DF-pigs were fed the same amount of feed as was consumed by NF-pigs the previous night. Consequently, the experimental period started 1 d later for DF-pigs than for NF-pigs. Each chamber<sup>(27)</sup> contained one pen of 1.75 × 2.85 m with 40% slatted floor. Temperature was maintained at 20°C and relative humidity at 65%. During the adaptation and experimental periods, pigs were exposed to 12 h of light (from 08.00 to 20.00 hours) and 12 h of darkness. All windows were covered to prevent exposure to light from outside. Dim light (3 lux) was placed above the feeding trough to ensure visibility of feed at night (NF-pigs). Health and welfare were assessed visually twice a day during feeding.

### Diets and feeding

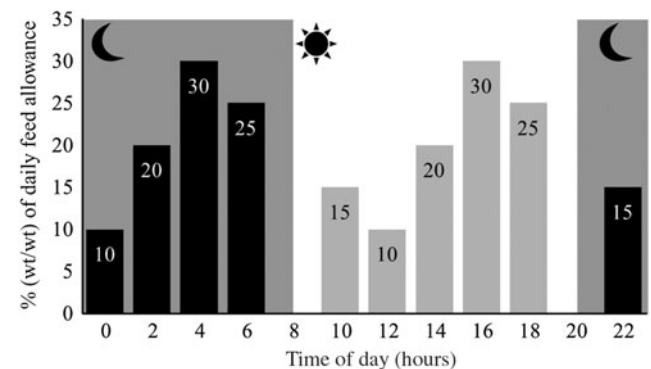
Pigs of both treatments were fed the same pelleted diet (Table 1), representative for standard commercial practice, which was formulated to meet or exceed the nutrient requirements for growing pigs<sup>(28)</sup>. Titanium dioxide (2 g/kg DM) was added as an indigestible marker to measure nutrient digestibility. Each group of DF-pigs was paired with a group of NF-pigs and fed the same amount of feed per kg BW<sup>0.6(29)</sup> as consumed by their paired

**Table 1.** Ingredient composition and analysed chemical composition of experimental diets

Ingredients (g/kg)	
Wheat	577
Soyabean meal	180
Barley	138
Wheat bran	48.5
Soyabean oil	23.4
Premix*	12.5
Calcium carbonate	9.46
Sodium chloride	2.70
Mono-calcium phosphate	2.62
L-Lysine	1.17
Choline chloride	1.09
Phytase	0.41
D,L-Methionine	0.31
All-rac- $\alpha$ -tocopheryl acetate	0.26
L-Valine	0.20
Sodium bicarbonate	0.16
L-Threonine	0.10
L-Tryptophan	0.02
Titanium dioxide	2.00
Analysed chemical composition	
DM (g/kg)	884
Crude protein (g/kg)	173
Energy (MJ/kg)	16.6

\* Supplied per kg of feed: citric acid, 111 mg; propyl gallate, 69 mg; butylhydroxytoluene, 151 mg; sepiolite, 158 g; retinyl acetate, 2400  $\mu$ g; cholecalciferol, 40  $\mu$ g; all-rac- $\alpha$ -tocopheryl acetate, 7.5 mg; menadione nicotinamide bisulphite, 160 mg; thiamine mononitrate, 80 mg; riboflavin, 400 mg; calcium-D-pantothenate, 1.3 g; choline chloride, 12 g; niacinamide, 1.6 g; pyridoxine hydrochloride, 120 mg; folic acid, 120 mg; cyanocobalamin, 1.6 mg; biotin, 12 mg; betaine hydrochloride, 7.9 mg; iron(II)sulphate, 8 g; calcium iodate, 80 mg; copper(II) sulphate, 12 g; manganese(II)oxide, 2.4 g; zinc oxide, 8 g and sodium selenite, 24 mg.

group during the night before. At the start of the experiment, level of feed supplied per d was set at 1.6 times the energy requirements of pigs for maintenance (750 kJ net energy/kg BW<sup>0.6(30)</sup>) and, within each pair, this was increased with 75 kJ net energy/kg BW<sup>0.6</sup> per d when no feed residues were collected 2 h after the last meal. For both treatments, feed allowance was adjusted daily, based on BW and expected daily gain that was calculated by dividing daily feed intake by a fixed feed-gain ratio of 1:2.3 g/g, which was based on feed efficiency in pigs of 50 kg BW<sup>(31)</sup>. From day 1 onwards, nocturnal-fed pigs were fed bihourly between 22.00 and 06.00 hours resulting in five successive meals (Fig. 1). Meals had a respective size of 15, 10, 25, 30



**Fig. 1.** Feeding schedule of growing pigs fed five meals bihourly during the day (10.00–18.00 hours; diurnal feeding (DF)) or night (22.00–06.00 hours; nocturnal feeding (NF)). The dark area represents the period where the lights were switched off. ■, DF; □, NF.

and 20% (w/w) of the daily feed allowance, which was based on the bimodal rhythm in feeding patterns of domestic pigs that have *ad libitum* access to feed<sup>(32)</sup>, which corresponds to activity patterns of wild boars in nature<sup>(33,34)</sup>. Diurnal-fed pigs were fed according to the same feeding schedule exactly 12 h later than NF-pigs between 10.00 and 18.00 hours (Fig. 1). Regardless of treatment, meals were consumed 15–30 min after supplementation, which was checked daily using camera recordings. Water was available *ad libitum* throughout the entire study period.

### Measurements

Pigs were weighed before and after the experimental period. During the experimental period, energy and N balances were measured per group of pigs. Feed was sampled daily. At the end of the experimental period, each room was thoroughly cleaned and faeces, urine and cleaning water were quantitatively collected, homogenised, sampled and stored at  $-20^{\circ}\text{C}$ . To avoid N volatilisation, 25% sulphuric acid (0.8/100 ml) was added to the mixture during sampling.  $\text{NH}_3$  in excurrent air was quantitatively trapped in water condensed on the heat exchanger or in 25% sulphuric acid solution. Exchange of  $\text{O}_2$ ,  $\text{CO}_2$  and  $\text{CH}_4$  was recorded in 9 min intervals. The equipment that was used to perform the gas exchange measurements is in detail described by Heetkamp *et al.*<sup>(27)</sup>. A  $\text{CO}_2$  recovery test was performed as a full system check, prior to the start of the experiment<sup>(27)</sup>. In the four chambers, 100.3, 100.2, 100.3 and 99.9% of  $\text{CO}_2$  released were recovered. Per group of pigs, physical activity was recorded continuously by a Doppler radar device. At days 4, 5 and 6 of the experimental week, faecal grab samples, not visually contaminated with urine, were collected at 08.00 and 20.00 hours, weighed and stored at  $4^{\circ}\text{C}$ . At the end of the week, grab samples were homogenised, pooled and stored at  $-20^{\circ}\text{C}$ . Samples of collected mixed faeces and urine and grab faeces were freeze-dried and ground to pass a 1 mm screen (Retsch ZM200). Feed samples were pooled per group of pigs and ground to pass a 1 mm screen (Retsch ZM200). DM was analysed in feed, mixed faeces and urine, grab faeces, freeze-dried mixed faeces and urine and freeze-dried grab faeces<sup>(35)</sup>. Gross energy was analysed in feed, freeze-dried mixed faeces and urine and freeze-dried grab faeces using bomb calorimetry<sup>(36)</sup>. N was analysed in feed, mixed faeces and urine, grab faeces, water condensate and 25% sulphuric acid solution using the Kjeldahl method<sup>(37)</sup>. Ti was analysed in feed and freeze-dried grab faeces<sup>(38)</sup>. All analyses were performed in duplicate.

### Calculations

Energy intake was calculated by multiplying daily feed intake by its gross energy content. Metabolisable energy was calculated by subtracting energy losses in mixed faeces and urine, grab faeces and methane, from gross energy intake. Heat production was calculated with Brouwer's equation without protein coefficients<sup>(39)</sup>. Energy deposition was calculated by subtracting heat production from metabolisable energy intake. N deposition was calculated as N intake – N losses via mixed faeces and urine, grab faeces and  $\text{NH}_3$  in exhaust air captured in sulphuric acid

solution and water condensate. Energy deposited as protein was calculated as N deposition  $\times$  6.25 and multiplied by 23.7 kJ/g to calculate energy deposited as protein. Energy deposited as fat was calculated as the difference between energy deposited and energy deposited as protein. Values are expressed as kJ/kg  $\text{BW}^{0.6}$  per d<sup>(29)</sup>. Net carbohydrate (CHO) and fatty acid oxidation were calculated from gas exchange measurements, without protein and methane coefficients<sup>(40)</sup>. RQ was calculated as  $\text{CO}_2$  produced divided by  $\text{O}_2$  consumed. RMR and heat production related to activity were estimated from total heat production and activity data using penalised b-spline regression procedures<sup>(41)</sup>. Activity counts measured by one of the radar devices, which was used in one of the chambers of each batch (2 NF; 1 DF), were cut off to a value of 100 before activity heat was estimated. Apparent total tract digestibility of nutrients was calculated using the following equation<sup>(42)</sup>:

$$\text{Nutrient disappearance (\% of intake)} = \left( 1 - \left( \frac{\text{Nutrient}_{\text{faeces}}}{\text{Ti}_{\text{faeces}}} / \frac{\text{Nutrient}_{\text{feed}}}{\text{Ti}_{\text{feed}}} \right) \right) \times 100 \quad (1)$$

where  $\text{Nutrient}_{\text{faeces}}$  is the nutrient concentration in grab faeces (g/kg DM),  $\text{Ti}_{\text{faeces}}$  is the Ti concentration in grab faeces (g/kg DM),  $\text{Nutrient}_{\text{feed}}$  is the nutrient concentration in the feed (g/kg DM) and  $\text{Ti}_{\text{feed}}$  is the Ti concentration in the feed (g/kg DM).

### Statistics

For all statistical analyses, SAS 9.4 for Windows (SAS Institute) was used. Using a statistical power of 0.8, the minimal number of experimental units (group of five pigs) required for analysis was six per treatment for the response in fat deposition (minimal detectable effect size: 50; SD 25). Data from energy balance, apparent total tract digestibility, heat production, RQ and net CHO and fat oxidation were analysed using a general linear mixed model (equation 2).

$$Y_{ijk} = \mu + T_i + B_j + P_k + e_{ijkl} \quad (2)$$

where  $Y$  = dependent variable;  $\mu$  = overall mean;  $T_i$  = fixed effect of DF or NF;  $B_j$  = block effect batch 1, 2 or 3;  $P_k$  = random effect of pair and  $e_{ijkl}$  = residual error. Interaction between fixed effects  $T_i$  and  $B_j$  was checked and deleted from the model when not significant ( $P < 0.05$ ). Diurnal patterns of total heat production, activity heat, RQ and net CHO and fat oxidation were analysed by using the same general linear mixed model by hour. Diurnal patterns were aligned per feeding time. In this way, the responses to each meal bear the same sign, which makes it possible to compare them. Homogeneity of variances and normality of model residuals were checked visually using the UNIVARIATE procedure. Differences were considered significant if  $P < 0.05$ . Data are reported as LS mean values with their standard errors.

## Results

### General

Throughout the study, two pigs were removed before the start of the experimental week and one pig was removed during the balance week; all three pigs were removed because of fever (body temperature > 40°C). Weight gain during the experimental period of NF-pigs was greater than DF-pigs (790 (SEM 40.5) g/d *v.* 660 (SEM 40.5) g/d;  $P = 0.007$ ).

### Energy balance and nutrient digestibility

Energy intake did not differ between treatments, indicating that paired feeding was executed successfully. Apparent total tract digestibility of DM and energy tended to be greater for DF-pigs than NF-pigs ( $P < 0.100$ ; Table 2). Energy losses with mixed faeces and urine were similar, whereas methane production was 2.06 kJ/kg BW<sup>0.6</sup> smaller for DF-pigs than NF-pigs ( $P < 0.001$ ). The difference in methane production remained constant during 24 hours (Fig. 2). Metabolisable energy intake was similar for both treatments. Heat production was 39 kJ/kg BW<sup>0.6</sup> smaller for DF-pigs than NF-pigs ( $P = 0.026$ ). Energy deposition tended to be smaller for DF-pigs than NF-pigs (38 kJ/kg BW<sup>0.6</sup>,  $P = 0.066$ ). Energy deposited as fat was 35 kJ/kg BW<sup>0.6</sup> smaller for DF-pigs than NF-pigs ( $P = 0.050$ ), whereas energy deposited as protein was similar between treatments. Energy efficiency tended to be 1.6% greater for NF-pigs than DF-pigs ( $P = 0.061$ ).

**Table 2.** Energy partitioning and efficiency, nitrogen efficiency, substrate oxidation and apparent total tract digestibility of DM, nitrogen and energy in pigs fed bihourly five successive meals either during the day (10.00–18.00 hours; diurnal feeding (DF)) or night (22.00–06.00 hours; nocturnal feeding (NF))\*† (Least square means with their standard errors)

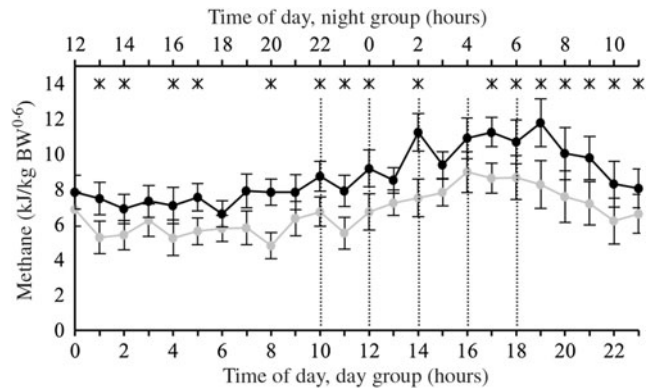
	DF	NF	SEM	<i>P</i> ‡ Diet
Energy balance (kJ/kg BW <sup>0.6</sup> per d)				
Energy intake	2447	2449	63.3	0.878
Faeces + urine	377	378	15.9	0.906
Methane	6.68	8.74	0.92	<0.001
ME intake	2063	2063	49.0	0.967
Heat production	1269	1230	19.0	0.026
RMR	1056	1032	16.1	0.216
Activity heat	213	198	16.1	0.440
Energy deposition	795	833	31.7	0.066
As protein	290	293	9.72	0.557
As fat	505	540	22.5	0.050
Energy efficiency (%)	32.4	34.0	0.52	0.061
N efficiency (%)	47.8	48.3	0.55	0.550
Net substrate oxidation				
Respiratory quotient	1.062	1.065	0.003	0.326
Carbohydrate (g/kg BW <sup>0.6</sup> per d)	86.4	84.6	1.88	0.006
Fat (g/kg BW <sup>0.6</sup> per d)	-6.31	-6.49	0.40	0.427
Apparent total tract nutrient digestibility (%)				
DM	87.1	86.6	0.19	0.068
Energy	86.2	85.6	0.23	0.072
N	84.0	83.2	0.39	0.224

BW, body weight; ME, metabolisable energy.

\* Values are presented as least square means and pooled standard errors of the mean, *n* 6, with each experimental unit being a group of five male pigs.

† Subsequent meals had sizes of 15, 10, 25, 30 and 20% of the daily feed allowance.

‡ No significant effect of batch was observed.



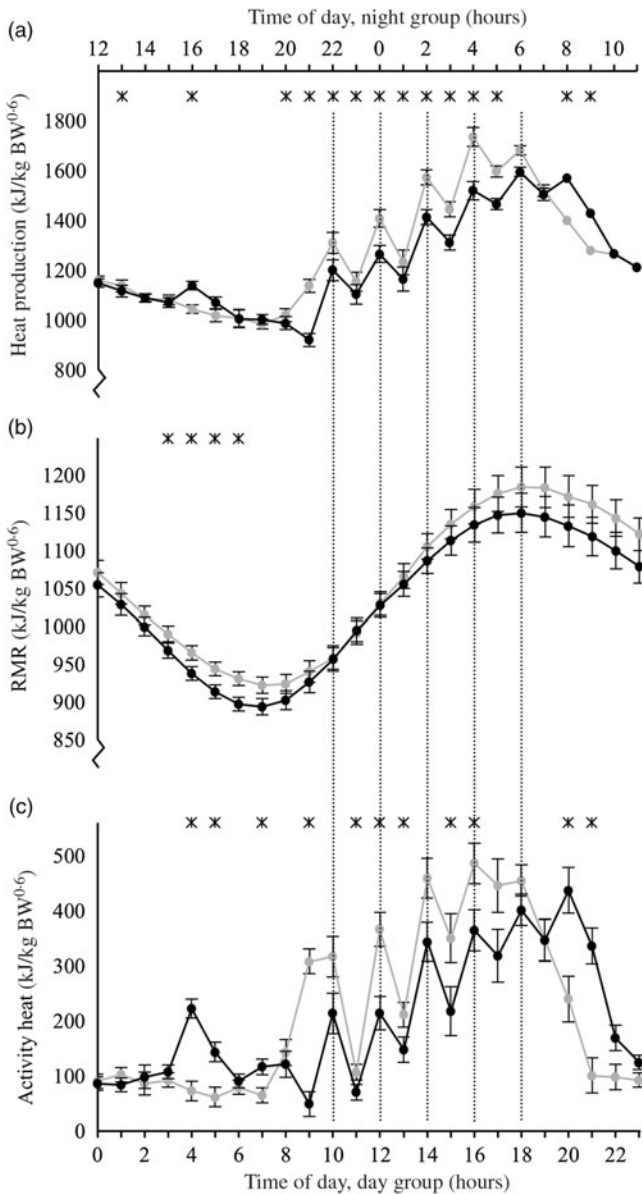
**Fig. 2.** Circadian pattern of energy losses through methane production in male pigs (body weight (BW) 42 (SEM 1.8) kg) when feeding five meals bihourly during the day (10.00–18.00 hours; bottom X-axis; diurnal fed (DF)) or night (22.00–06.00 hours; top X-axis; nocturnal fed (NF)). Dotted lines represent feeding times. Values are presented as least square means and pooled standard errors of the mean, *n* 6 with each experimental unit being a group of five male pigs. \*  $P < 0.05$ . —○—, DF; —●—, NF.

### Heat production and substrate oxidation

Total heat production was greater for DF-pigs than NF-pigs during the feeding period ( $P < 0.050$ ; Table 2). At 1, 2 and 6 h before the 1st meal and 2 and 3 h after the last meal, heat production was smaller for DF-pigs than NF-pigs (Fig. 3). Activity-related heat production responded to the imposed feeding patterns; DF-pigs were more active than NF-pigs during the feeding period, however, less active 3, 5 and 6 h before the 1st meal, and 3 and 4 h after the last meal ( $P < 0.05$ ). RMR was greater for DF-pigs than NF-pigs during the fasting period reaching statistical significance between 4 and 8 h before the 1st meal ( $P < 0.05$ ). On average, RQ did not differ between treatments; however, at the end of the feeding period, between the 3rd and 5th meal, RQ tended to be greater for NF-pigs than DF-pigs (Fig. 4). For both treatments, RQ exceeded unity continuously and net fatty acid oxidation remained negative throughout the day. Net CHO oxidation was greater for DF-pigs than NF-pigs and reached significance 1 h before the 1st meal was fed until the last meal was fed.

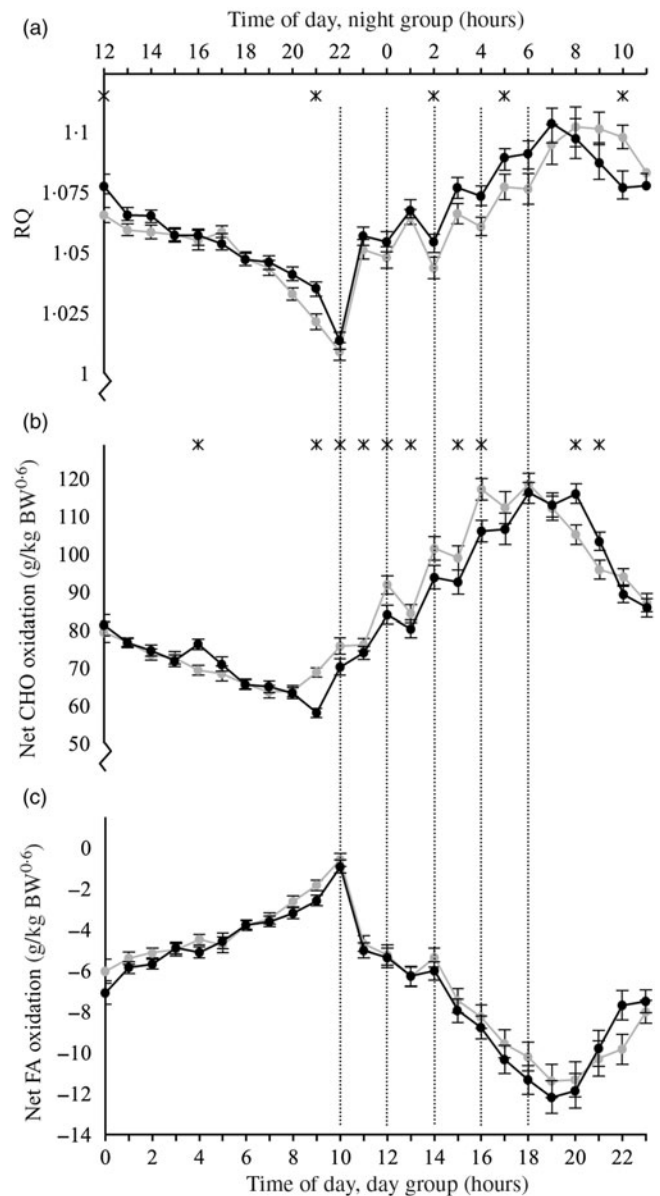
## Discussion

Disruption of circadian feed intake rhythm in humans, for example, due to shift work, (social) jet lag or night eating syndrome, is associated with metabolic disorders. In our study, circadian misalignment in pigs induced by NF seemed to increase the amount of energy deposited compared with DF due to a lower heat production. The surplus of energy in NF-pigs was completely stored as fat confirming our hypothesis that asynchronisation of the endogenous circadian clock and the timing of feed intake increases fat deposition in growing pigs. Protein deposition was not affected by NF, which is in contrast with Malmlöf<sup>(43)</sup> who observed a reduced protein deposition in nocturnal-fed pigs of similar BW (38–54 kg). In the study of Malmlöf<sup>(43)</sup>, however, NF was induced by providing the daily ration in three meals instead of two, whereby only the 3rd meal was supplied at midnight. In addition, different genetics



**Fig. 3.** Circadian patterns of total heat production (a), RMR (b) and activity-related heat production (c) in pigs (body weight (BW) 42 (SEM 1.8) kg) when feeding five meals bihourly during the day (10.00–18.00 hours; bottom X-axis; diurnal fed (DF)) or night (22.00–06.00 hours; top X-axis; nocturnal fed (NF)). Dotted lines represent feeding times. Values are presented as least square means and pooled standard errors of the mean,  $n$  6 with each experimental unit being a group of five male pigs. \*  $P < 0.05$ . —○—, DF; —●—, NF.

(Sw. Landrace × Yorkshire) were used that may have affect the predisposition for protein and fat deposition<sup>(44)</sup>. In contrast, in our study, the complete daily ration was supplied during the night. Our results are in line with the association between circadian misalignment and increased fat deposition that may lead to obesity in humans<sup>(3–6)</sup>, and with research in rodents, wherein disruption of the circadian rhythm by DF increased BW<sup>(12–14)</sup> and abdominal fat<sup>(12,14)</sup>. In contrast to these studies, our study



**Fig. 4.** Circadian patterns of respiratory quotient (RQ) (a), net carbohydrate (CHO) oxidation (b) and net fatty acids (FA) oxidation (c) in pigs (body weight (BW) 42 (SEM 1.8) kg) when feeding five meals bihourly during the day (10.00–18.00 hours; bottom X-axis; diurnal fed (DF)) or night (22.00–06.00 hours; top X-axis; nocturnal fed (NF)). Dotted lines represent feeding times. Values are presented as least square means and pooled standard errors of the mean,  $n$  6, with each experimental unit being a group of five male pigs. \*  $P < 0.05$ . —○—, DF; —●—, NF.

controlled for energetic intake and measured complete energy balance, enabling quantification of total fat and protein deposition.

### Energy partitioning

When averaged over days, the reduced heat production in NF-pigs was explained by numerical differences in the same

direction for activity heat and RMR. Nocturnal-fed pigs were particularly less active than DF-pigs 1 h before and during the feeding period, but were more active than DF-pigs, particularly 6 h before (16.00 hours) and 2 h (08.00 hours) after the feeding period. The increase in activity by NF-pigs at 08.00 hours coincided with onset of lights; however, no environmental stimulus could explain the increase in activity by NF-pigs at 16.00 hours. Under *ad libitum* feeding conditions, activity<sup>(45)</sup> and feed intake<sup>(32)</sup> of pigs peak at the end of the afternoon (about 16.00 hours), a few hours before the lights are turned off. The remarkable increase in activity at 16.00 hours observed for NF-pigs may therefore be related to foraging behaviour imposed by the endogenous central clock that is entrained by light and regulates the timing of behavioural rhythms<sup>(1,46)</sup>, among which the diurnal rhythm of feed intake<sup>(26)</sup>. In contrast to NF-pigs, DF-pigs were more active prior to the feeding period and remained more active than NF-pigs after each meal, thereby levelling out the difference in activity heat between treatments in the fasting period. Also, the increase in activity prior to the feeding schedule in DF-pigs may be imposed by the endogenous central clock, as pigs often show a small peak of feed intake after the onset of lights under *ad libitum* conditions<sup>(32)</sup>. For both treatments, diurnal CHO oxidation coincided with the circadian activity pattern indicating that either free glucose or glucose mobilised from muscle glycogen storages was used as energy source for activity. RMR was numerically lower for NF-pigs than DF-pigs most of the day, starting during the largest meals (4th and 5th meal) and reaching statistical significance in 4–7 h before the 1st meal. The latter is in line with McHill *et al.*<sup>(16)</sup> who observed a reduced energy expenditure during scheduled sleep in humans exposed to a night shift schedule. Variation in RMR is explained either by differences in BMR, which is associated with maintaining basal life processes, body temperature and organ functioning<sup>(47)</sup>, or by differences in the thermic effect of feeding (TEF). In humans, TEF is greater after consumption of a meal in the morning and afternoon than in the evening and night<sup>(48,49)</sup> and lower in humans that are exposed to circadian misalignment<sup>(48)</sup>. These results are in line with the reduced postprandial RMR observed in NF-pigs. Moreover, circadian misalignment induced by shift work<sup>(9)</sup> and sleep disturbances<sup>(50)</sup> is associated with a reduced glucose tolerance resulting in impaired glucose uptake, which in turn is associated with a lower TEF<sup>(51–53)</sup>. TEF can be split in a short-term component, which represents the within-day variation in RMR that is related to ingestion, digestion and absorption of food, and a long-term component, which is calculated as the difference between resting heat production when fed and fasting heat production (BMR)<sup>(54)</sup>. From our results, short-term component can be calculated as the difference between RMR and the minimum value of RMR measured within a day, which was similar for DF-pigs (136 kJ/kg BW<sup>0.6</sup>) and NF-pigs (138 kJ/kg BW<sup>0.6</sup>). These results suggest that the numerical difference in TEF between treatments is mainly explained by a lower long-term component in NF-pigs than DF-pigs, when assuming that fasting heat production was the same. Our results show that both RMR and activity heat reduced total energy expenditure of NF-pigs, hence explain the greater fat deposition in NF-pigs than DF-pigs.

### Nutrient digestion

In humans, circadian misalignment is associated with gastrointestinal disorders related to motility<sup>(55)</sup>, inflammation<sup>(56,57)</sup> or cancer<sup>(58,59)</sup> indicating that disruption of the circadian clock may have a negative impact on gastrointestinal tract health and possibly function in the long term. Also on short term, some digestive processes, such as secretion of gastric acid in humans<sup>(60)</sup> and pancreatic juice in rats<sup>(61)</sup>, show a clear circadian rhythm independent of feed intake. Consequently, it could be hypothesised that circadian misalignment may affect nutrient digestion. Short-term effects of circadian misalignment on gastrointestinal function, for example, ATTD of nutrients have not been extensively investigated before. In the present study, NF of pigs tended to decrease apparent total tract digestibility of energy (0.6 %-unit) and DM (0.5 %-unit) compared with DF and numerically decrease total tract N digestibility. Together with results of Jørgensen *et al.* where timing of feed intake (08.00, 16.00 or 24.00 hours) did not affect ileal DM and N digestibility<sup>(62)</sup>, our results indicate that effects of circadian misalignment on nutrient digestibility are minor. NF increased energy losses through methane, a gas mainly produced through microbial fermentation in the large intestine of pigs. Regardless of feeding regimen, methane production increased during the feeding period. Possibly increased colonic motor activity, which increases after feed consumption and with physical activity<sup>(63,64)</sup>, enhanced flatulence, and consequently methane excretion. The greater daily methane production in NF-pigs than DF-pigs indicates that NF may have changed the composition and/or activity of the microbial population. The composition of the gut microbiome is dynamic as it exhibits diurnal fluctuations, which are influenced by time of eating, as was shown in humans and mice<sup>(65,66)</sup>. In mice, these diurnal fluctuations are suppressed by disruption of the host circadian clock thereby changing the faecal microbiota composition<sup>(67)</sup>, which is suggested to influence the host metabolism<sup>(19,68)</sup>. For example, transferring intestinal microbiota of jet-lagged humans (circadian misaligned) to germ-free mice, resulted in obesity and glucose intolerance compared with transfer of intestinal microbiota of non-jet-lagged people<sup>(66)</sup>. These results indicate that the greater fat deposition in NF-pigs than DF-pigs potentially relates to changes in gut microbiota composition, but do not show the relation with methanogens specifically. In humans, a greater methane production is associated with a higher BMI and fat percentage<sup>(69,70)</sup>. It is suggested that methane facilitates and accelerates fermentation through removal of H atoms<sup>(69,71)</sup> consequently increasing microbial SCFA production and thus nutrient supply for the host. In addition, the presence of methane may slow down small intestinal digesta transit<sup>(72)</sup>, thereby increasing nutrient absorption<sup>(70)</sup>. Consequently, the greater fat deposition in NF-pigs may be related to the greater methane production; however, the causality between circadian misalignment and methane production remains unexplained.

### Conclusions

When maintaining energetic intake and identical meal patterns, NF of pigs reduces heat production, which at equal protein gain

resulted in increased fat deposition. In addition, NF altered diurnal activity patterns of pigs, but did not affect the overall level of energy expenditure related to physical activity. The present study provides evidence that circadian misalignment has little effect on nutrient digestion, but alters nutrient partitioning, ultimately leading to an increase in fat deposition coinciding with an increase in methane production. The causality of the association between circadian misalignment and methane remains to be investigated. Our results show that feeding during the night affects growth performance of pigs by increasing fat deposition. Timing of feed intake in relation to performance is particularly important in pigs that are forced to deviate from their normal feeding pattern, for example, when exposed to a high stocking density or high ambient temperatures, but may certainly also be relevant for pigs fed a restricted number of meals per d. Future feeding strategies may be focused at minimising feed supply during the night.

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