Plasma ghrelin is positively associated with body fat, liver fat and milk fat content but not with feed intake of dairy cows after parturition

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Abstract

Ghrelin is a gastrointestinal peptide hormone that is present in blood mostly in a non-posttranslationally modified form, with a minor proportion acylated at Ser³. Both ghrelin forms were initially assigned a role in the control of food intake but there is accumulating evidence for their involvement in fat allocation and utilization. We investigated changes in the ghrelin system in dairy cows, exhibiting differences in body fat mobilization and fatty liver, from late pregnancy to early lactation. Sixteen dairy cows underwent liver biopsy and were retrospectively grouped based on high (H) or low (L) liver fat content *post-partum*. Both groups had a comparable feed intake in week –6 (before parturition) and week 2 (after parturition). Only before parturition was preprandial total ghrelin concentration higher in L than in H cows and only after parturition was the basal plasma concentration of non-esterified fatty acids higher in H than in L cows. Both before and after parturition, H cows had higher preprandial plasma concentrations of acyl ghrelin, a higher acyl:total ghrelin ratio, lower plasma triacylglyceride concentrations and a lower respiratory quotient compared with L cows. These group differences could not be attributed to an allelic variant of the acyl ghrelin receptor. Rather, the ratio of acyl:total ghrelin correlated with several aspects of fat metabolism and with respiratory quotient but not with feed intake. These results show that endogenous ghrelin forms are associated with fat allocation, fatty liver, and utilization of fat during the periparturient period.

Key Words

- ▶ ghrelin
- ▶ respiratory quotient
- ▶ parturition
- ▶ dairy cows

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Introduction

Ghrelin is a 28 amino acid peptide in monogastric species and a 27 amino acid peptide in ruminants (Dickin *et al.* 2004). It is primarily produced by oxyntic cells in the stomach or abomasum respectively with smaller amounts produced by the intestine and pancreas (Hayashida *et al.* 2001, Hosoda *et al.* 2006). A small proportion of ghrelin is acylated (mostly octanoylated) by ghrelin O-acyl-transferase (GOAT) at Ser³ (Yang *et al.* 2008), resulting in the secretion of both acyl and non-acyl ghrelin into the blood stream (Hosoda *et al.* 2000, ThidarMyint *et al.* 2006). Both forms are involved in the maintenance of energy homeostasis (ThidarMyint *et al.* 2006), but only the acyl form of ghrelin exhibits endocrine activity by activating GH secretagogue receptor (GHSR) signalling and GH release (Itoh *et al.* 2005, Iqbal *et al.* 2006, Bradford & Allen 2008). In ruminants, plasma concentrations of acyl ghrelin increase before meals, in response to fasting, and with decreasing rumen fill (Sugino *et al.* 2002a, Wertz-Lutz *et al.* 2006, Gregorini *et al.* 2009), whereas feeding suppresses the ghrelin surge (Wertz-Lutz *et al.* 2006). Thus, acyl ghrelin concentration is influenced by nutritional state in situations of positive energy balance (EB). Wertz-Lutz *et al.* (2006) found a weak increase in feed intake upon acyl ghrelin injection; however, neither s.c. (Roche *et al.* 2008) nor centrally infused acyl ghrelin (Iqbal *et al.* 2006) leads to a significant increase in feed intake, so an orexigenic role of acyl ghrelin in ruminant species appears questionable. Acyl ghrelin is elevated during early lactation (Itoh *et al.* 2005, Bradford & Allen 2008), a period characterized by high body fat mobilization. This suggests that, in addition to its role in preventing starvation, ghrelin exerts a prominent role in the regulation of fat metabolism. For example, feeding rumen-protected long-chain fatty acids increases the prandial acyl ghrelin surge in cows (Fukumori *et al.* 2012) and sheep with high levels of adiposity have higher ghrelin levels compared with lean animals after overnight fasting (Kurose *et al.* 2005), indicating that the ghrelin system is triggered by lipids. On the other hand, infusion of acyl ghrelin increases plasma non-esterified fatty acid (NEFA) concentrations in cows (ThidarMyint *et al.* 2006, Roche *et al.* 2008), although these circulating fatty acids do not appear to be released from adipose tissue (Grala *et al.* 2010). Studies on rodents and humans suggest that the lipolytic effect of acyl ghrelin may occur in muscle tissue (Barazzoni *et al.* 2005, Vestergaard *et al.* 2011). In different adipose tissues and in the liver, acyl ghrelin reduces lipid export and increases the expression of lipogenic enzymes, thereby favouring hepatic triacylglyceride (TG) deposition

(Barazzoni *et al.* 2005, Davies *et al.* 2009, Grala *et al.* 2010). These studies indicate that acyl ghrelin contributes to the regulation of whole-body fat distribution (Barazzoni *et al.* 2005), while desacyl ghrelin promotes fatty acid uptake and lipid accumulation in adipose tissue (Miegeue *et al.* 2011).

Dairy cows adapt to early lactation by mobilizing enormous amounts of adipose tissue (up to 10% of body weight, Komaragiri *et al.* (1998)), which results in increased plasma NEFA concentrations and liver TG accumulation (Bobe *et al.* 2004, Vernon 2005, Ingvarsen 2006). Notably, cows with higher plasma NEFA concentrations develop a higher liver fat content (LFC) *post-partum* (pp) compared with cows exhibiting a lower NEFA level (Hammon *et al.* 2009). Thus, we hypothesized that the ghrelin system plays a pivotal role in fat allocation and the development of fatty liver in cows entering the early lactation period. Following this idea, we took liver biopsies throughout the periparturient period and grouped the cows with the lowest and highest LFC pp. The results of the study show that animals with a higher LFC have higher preprandial plasma concentrations of acyl ghrelin, higher acyl:total ghrelin ratios, lower plasma TG concentrations and a lower respiratory quotient, both before and after parturition. The ratio of acyl:total ghrelin correlated with several aspects of fat metabolism and with the respiratory quotient but not with feed intake.

Materials and methods

Animal experiment

All experimental procedures were approved by an ethics committee of the State Government in Mecklenburg-West Pommern (Registration No. LALLF M-V/TSD/7221.3-2.1-021/09). Sixteen of 20 multiparous high-yielding dairy cows (at the end of their second to fourth lactation, each > 10,000 kg milk/305d in an earlier lactation) were bought from one commercial dairy farm and kept in tie stalls in the institutional facility. To reduce genetically based variation in fat metabolism, all cows were selected to be heterozygous at a polymorphic locus in acyl-CoA: diacylglycerol-acyltransferase 1 gene (*DGAT1* K232A: amino acid lysine or alanine at position 232), which is known to affect body fat (Thaller *et al.* 2003) and milk fat (Kühn *et al.* 2007) content. Animals were not milked within the 7 weeks before the expected calving date. They received a total mixed ration twice daily (0700 and 1500 h) during weeks 6 to 4 before parturition consisting of grass silage,

corn silage, barley straw, hay, concentrate and mineral feed (net energy lactation (NEL)=5.9 MJ/kg of dry matter (DM), utilizable protein=128 g/kg of DM) according to the recommendations of the German Society of Nutrition Physiology (2001). During lactation, animals received a total mixed ration consisting of grass silage, corn silage, barley straw, hay, concentrate, extracted rapeseed meal, molassed sugar beet pulp, mineral feed and propylene glycol (NEL=7.1 MJ/kg of DM, utilizable protein=163 g/kg of DM). Mineral salt blocks and water were available *ad libitum*. After parturition, animals were milked twice daily (0630 and 1630 h). Liver biopsies were conducted using a custom-made biopsy needle (diameter 6 mm) on day -34 and day +18 relative to parturition (week -5 and +2 respectively) and liver tissue was frozen at -80 °C before analysis of LFC. Based on LFC on day 18 pp, cows were retrospectively grouped ($n=8$ in each group) according to low (L) or high (H) LFC (for L: LFC<31%; for H: LFC>32% total fat/g DM).

On days -40 and +9 relative to parturition, body weight was determined and back fat thickness was measured weekly using ultrasound (Aloka SSK-500, PPG Hellige GmbH, Freiburg, Germany). On day -39 (week -6) before parturition, and again on day 10 (week 2) after parturition, cows were equipped with an indwelling jugular catheter and kept in respiratory chambers (15 °C, dark-light cycle 0600 to 1900 h; Derno *et al.* (2009)) to which they had been well adapted before the 3-day trial (habituation at least three times: eating, ruminating and lying down). O₂ consumption and the production of CO₂ and CH₄ were measured at intervals of 6 min and the respiratory quotient was calculated as described previously (Derno *et al.* 2009). On the first day of the trial, starting at 0630 h (± 0 h), cows were fed *ad libitum* for 24 h. On the second day of the trial, animals were deprived of feed for 10 h starting at 0630 h. At 1630 h on the second day of the trial, animals were re-fed *ad libitum* until 0630 h of the third day of the trial (to determine the 1- and 14-h compensatory feed intake). Feed intake was determined every 6 min by feed disappearance measured by a scale connected to an electronic registration device. Cows were milked at 0630 and 1630 h on all 3 days of trial. Milk yield was recorded and milk samples were taken for analysis (see below).

Blood samples were taken every 60 min (via extension tubing, i.d. 1 mm, 4 m) from the outside of the chamber starting at 0920 h (± 3 h) until 1420 h (± 8 h) on day 1 (*ad libitum* feeding) and from 0630 h (± 0 h) until re-feeding at 1630 h (± 10 h) on day 2 of the trial. On the third day (14-h re-feeding period), one blood sample was taken at 0630 h (± 0 h). Blood samples for acyl and total ghrelin

were dispensed into 9 ml EDTA-containing monovettes (Sarstedt, Nürnberg, Germany) supplemented with 50 µl protease inhibitor (Trasylol, Bayer) and were immediately placed on ice. Samples for NEFA and TGs were drawn into 2.7 ml fluoride/EDTA-monovettes (Sarstedt). Blood samples were centrifuged at 1565 g for 20 min at 4 °C and plasma stored at -80 °C before analysis.

Milk analyses

Milk samples were analysed for fat, protein and lactose contents (Landeskontrollverband für Leistungs- und Qualitätsprüfung Mecklenburg-Vorpommern E.V.). Energy-corrected milk yield ($((0.038 \times \text{g crude fat} + 0.024 \times \text{g crude protein} + 0.017 \times \text{g lactose}) \times \text{kg milk} / 3.14)$) was calculated in kg according to Reist *et al.* (2002). EB was calculated ($\text{EB} = \text{NEL intake} - (\text{energy-corrected milk yield} \times 3.14 + 0.293 \times \text{kg body weight}^{0.75})$) (Reist *et al.* 2002) and expressed in MJ/day.

Analyses of liver fat, glycogen and protein content

Liver tissue (100 mg) was dried in a muffle-type furnace at 105 °C for 3 h to determine the DM content. Dried samples underwent elementary carbon and nitrogen analysis (CNS-2000; LECO Instrumente GmbH, Mönchengladbach, Germany). Fat and protein content was calculated according to the methods described previously (Kuhla *et al.* 2004). Liver glycogen content was determined using a commercially available photometric test based on amyloglucosidase-catalysed release of glucose (no. 10207748035, Boehringer Mannheim).

Analyses of plasma hormones and metabolites

Plasma NEFA and TG concentrations were determined by an automatic analyzer (Abx Pentra 400; Horiba, Montpellier, France, Clinic for Cattle, Stiftung Tierärztliche Hochschule Hannover, Hannover, Germany) using NEFA kit #600-215S (Wako Chemicals distributed by mti diagnostics, Neuss, Germany) and TG kit A11A01640 (ABX-Horiba). Acyl ghrelin was measured as described previously by Relling *et al.* (2010) using an RIA kit (#GHRA-88HK; Linco Research, St Charles, Billerica, MO, USA). To this end, 200 µl plasma was acidified with 2 µl of 1 mol/l HCl during thawing. Total ghrelin (acyl+desacyl ghrelin) was measured in 400 µl freeze-dried plasma using the RIA method described previously (ThidarMyint *et al.* 2006). Due to limited amounts of plasma, this was not done on the sample obtained at 0630 h on the second day of the trial.

Comparative sequencing of the GHSR gene

Several single nucleotide polymorphisms (SNPs) and two different alternative transcript variants (*GHSR1a* and *GHSR1b*) have been described for the bovine *GHSR* gene (Colinet *et al.* 2009, Komatsu *et al.* 2010). Primers for PCR amplification and re-sequencing of the *GHSR* gene were designed based on the reference mRNA sequence (NM_001143736.1) and genomic sequences (AM931584.1 and NW_003103817.1). Amplicons covered the complete protein coding region and the 3' and 5' UTRs of the *GHSR* gene (Supplementary Table 1, see section on supplementary data given at the end of this article). Comparative sequencing of the targeted gene regions (1365 bp) was performed on genomic DNA and cDNA was prepared from liver samples. Total RNA was extracted using the NucleoSpin II RNA kit (Macherey & Nagel, Düren, Germany). cDNA was synthesized by RT using Superscript III RNase H-reverse transcriptase (Invitrogen) with gene-specific primers (GHSR_R1, GHS_R4; Supplementary Table 1) according to the manufacturer's instructions. The PCR amplicons were isolated from agarose gels using the NucleoSpin Extract II kit (Macherey & Nagel) and sequenced with PCR primers on a capillary MEGABACE sequencer (GE Healthcare, Freiburg, Germany). To identify variable DNA positions, the sequences were analysed by visual inspection of sequencing profiles and sequence alignment to the respective bovine reference sequences.

Statistical analyses

One cow from each period (*ante-partum* (ap) and *post-partum* (pp) respectively) had to be excluded from the analysis due to illness. Descriptive statistics were calculated and tests for

normality conducted (using the UNIVARIATE procedure of Base SAS software). The response variables were approximately normal. Data were evaluated by repeated measures ANOVA (using the MIXED procedure of SAS/STAT software: SAS Institute, Inc. 2009. SAS/STAT 9.2 User's Guide, Second Edition, Cary, NC, USA: SAS Institute, Inc.).

The statistical model for the zootechnical data contained the fixed effects LFC, period (ap and pp) and their interaction. Repeated measures on the same animal were taken into account (by the repeated statement in proc mixed using a compound symmetry residual covariance structure). The statistical model for the plasma data contained the fixed effects LFC, time (hours) and their interaction. Repeated measures on the same cow were taken into account in the same way as described earlier using an autoregressive block diagonal structure of the residual covariance matrix. Least square means (LSM) and S.E.M. were calculated and pairwise tested for each effect in each model using the Tukey–Kramer procedure for pairwise multiple comparisons. $P < 0.05$ was considered significant. Pearson correlation coefficients between ghrelin forms and dry matter intake (DMI), and between ghrelin forms and parameters reflecting fat metabolism and EB, were calculated and tested (using the CORR procedure of Base SAS software).

Results

Before parturition, the groups did not differ in body weight or EB but H cows had significantly greater back fat thickness than L cows (Table 1), which further increased until parturition (data not shown). Animals in both

Table 1 Zootechnical data before and after parturition from cows with a high (H) and a low (L) liver fat content pp (LSM $\pm$ S.E.M.; P , Tukey–Kramer test)

	Ante-partum (ap)			Post-partum (pp)			P (ap vs pp)	
	L	H	P	L	H	P	L	H
Energy balance (MJ/day)	20.0 $\pm$ 4.8	18.0 $\pm$ 4.8	0.99	−63.4 $\pm$ 5.1	−85.7 $\pm$ 4.8	0.03	<0.01	<0.01
Body weight (kg)	709 $\pm$ 18.2	768 $\pm$ 18.2	0.15	669 $\pm$ 18.5	713 $\pm$ 18.2	0.35	<0.01	<0.01
Ad libitum DMI (kg)	10.7 $\pm$ 0.8	10.6 $\pm$ 0.8	1	17.0 $\pm$ 0.9	15.9 $\pm$ 0.8	0.79	<0.01	<0.01
1-h compensatory DMI (kg)	3.1 $\pm$ 0.4	2.9 $\pm$ 0.3	0.97	4.4 $\pm$ 0.4	5.2 $\pm$ 0.3	0.39	0.03	<0.01
14-h compensatory DMI (kg)	9.2 $\pm$ 0.9	9.9 $\pm$ 0.9	0.94	14.0 $\pm$ 0.9	13.7 $\pm$ 0.9	1	<0.01	<0.01
Back fat thickness (mm) ^a	12.1 $\pm$ 1.8	21.1 $\pm$ 1.8	0.01	13.0 $\pm$ 1.8	23.5 $\pm$ 1.8	<0.01	<0.01	0.28
Energy-corrected milk yield (kg) ^b				46.0 $\pm$ 2.1	49.5 $\pm$ 2	0.25		
Milk fat content (%)				4.0 $\pm$ 0.4	5.5 $\pm$ 0.35	0.02		

DMI, dry matter intake.

^aNote: back fat thickness still increased during ap period until parturition (data not shown) above pp values presented herein.

^bMean between day 1 (*ad libitum* feeding) and 2 (10-h food deprivation).

groups were in negative EB 10 days after parturition, with a significant difference between H and L cows ($P < 0.05$; Table 1). Although H cows had a significantly higher milk fat content ($P = 0.02$) compared with L cows on both days, both groups had a comparable energy-corrected milk yield (Table 1) and comparable *ad libitum* and 14 h compensatory feed intake (Table 1) in week -6 before parturition and week 2 after parturition. Also, liver fat, protein and glycogen contents determined in the ap period did not differ between groups (Fig. 1). From pregnancy to early lactation, LFC increased threefold in H cows ($P < 0.01$) and twofold in L cows ($P < 0.01$; Fig. 1A), while hepatic glycogen decreased in both groups: sevenfold in H cows ($P < 0.01$) and twofold in L animals ($P = 0.04$; Fig. 1B). Moreover, liver protein content decreased after parturition only in cows developing a high LFC ($P < 0.01$; Fig. 1C).

Because plasma metabolite and hormone concentrations may vary periprandially, we took hourly blood samples during the *ad libitum* period as well as after feed deprivation. During both the ap and the pp periods, plasma NEFA concentrations increased with the duration of feed deprivation and reduced after re-feeding ($P < 0.01$). In the ap period, plasma NEFA concentrations did not differ between groups, neither during *ad libitum* feeding nor in response to feed deprivation (Fig. 2A). By contrast, in the pp period, plasma NEFA concentrations were significantly increased in both groups when compared with ap *ad libitum* feeding or the ap feed deprivation respectively ($P < 0.01$). Furthermore, H cows had significantly higher plasma NEFA concentrations than L cows ($P < 0.01$), but these differences were not evident during feed deprivation (Fig. 2B).

Plasma TG concentrations were not different between L and H cows during *ad libitum* feeding, neither in the ap nor in the pp period (Fig. 2C and D). However, in both groups, plasma TG concentrations were significantly lower after parturition compared with before parturition ($P < 0.05$). Before parturition, feed deprivation provoked no changes in plasma TG concentrations (Fig. 2C), while after parturition, feed deprivation led to increasing plasma TG concentrations in both groups ($P < 0.01$) and to a greater extent in L than in H cows ($P < 0.01$; Fig. 2D).

Because H and L cows differed markedly in their fat metabolism during the pp period, we next examined whether the ghrelin system was involved in the differences in fat allocation and development of fatty liver. Plasma concentrations of acyl ghrelin were comparable between groups during both ap and pp *ad libitum* feeding (Fig. 3A and B). However, the pp *ad libitum* acyl ghrelin concentrations tended to be higher in H than in L cows ($P = 0.07$).

Figure 1

Ante-partum (5 weeks before parturition) and post-partum (18 day after parturition) liver fat (A), glycogen (B) and protein (C) content of cows grouped according to their LFC at day 18 pp in H (closed square) and L (open square) cows. Liver fat, glycogen, and protein content are expressed on a DM basis. Interactions between group $\times$ time were $P < 0.01$ for LFC, $P = 0.42$ for liver glycogen content and $P < 0.01$ for liver protein content. Data are presented as LSM $\pm$ s.e.m. * < 0.05 , ** < 0.01 , *** < 0.001 (Tukey-Kramer test).

Acyl ghrelin decreased from the ap to the pp period in H ($P=0.03$) but not in L animals ($P<0.13$). During times of feed deprivation, acyl ghrelin concentrations continuously increased in both groups ap and pp ($P<0.01$). Moreover, feed-deprived H cows secreted significantly more acyl ghrelin into the circulation than L cows ($P<0.02$), whereas the extent in each group was higher in the pp than in the ap period ($P<0.01$; Fig. 3A and B).

To examine whether the higher acyl ghrelin concentrations in H cows observed after feed deprivation may be due to greater production of total (acyl and desacyl) ghrelin, we next measured the concentration of total ghrelin in plasma. During *ad libitum* feeding, total ghrelin concentrations did not differ between groups ap (Fig. 3C and D) but increased after parturition in H ($P=0.03$) but not in L cows. Also, total ghrelin continuously increased with duration of feed deprivation in both groups, both before and after parturition ($P<0.02$). Interestingly, L cows responded to feed deprivation in the ap period with a significantly higher total ghrelin release compared with H cows ($P=0.03$; Fig. 3C), while this effect disappeared during the pp period (Fig. 3D).

During *ad libitum* feeding, the ratio between acyl and total ghrelin did not differ between groups, nor between the ap and the pp period. However, during feed deprivation, the ratio decreased from ap to pp in H ($P<0.01$) but not in L cows. Also, H cows had a significantly higher acyl:total

ghrelin ratio during feed deprivation than L cows, before ($P=0.02$) as well as after ($P<0.01$) parturition (Fig. 4A and B).

To determine whether differences in L and H phenotypes can be attributed to genetic variation in the ghrelin receptor gene, *GHSR*, we sequenced the complete coding region as well as the 5' and 3' UTRs of the *GHSR* gene. Comparative sequence analysis revealed that the known polymorphisms leading to amino acid variation in the GHSR protein (L24V, D194N and DelR242) displayed no variability in the cows included in our study. Two variable SNPs (c.-7 A>C and c.-128 T>G) located in the 5' UTR of the *GHSR* gene were identified compared with the reference sequences (NM_001143736.1, AM931584.1 and NW_003103817.1, data not shown). However, a clear relationship between allelic variants of these SNPs and cow phenotypes could not be observed.

Group differences in the release of both ghrelin forms may be associated with the type of preferred nutrient utilization, which can be assessed by indirect calorimetry. The respiratory quotient was significantly lower in H compared with L cows ($P<0.05$) both before and after parturition, as well as during *ad libitum* feeding and feed deprivation (Fig. 5), although there were no differences in *ad libitum* or compensatory feed intake between groups (Table 1).

In order to examine whether the plasma ghrelin system plays a role in feed intake control or the regulation

Figure 2

Ad libitum, feed deprived and re-fed plasma concentrations of NEFAs (A and B) and triacylglycerides (C and D) each –6 weeks before (A and C) and 2 weeks after (B and D) parturition in H (closed square) and L (open circle) cows. At day 1, cows were fed *ad libitum* for 24 h, whereas at day 2 animals were feed deprived for 10 h (grey-shaded region) followed by a

14-h re-feeding period until day 3. Time=0 h indicates 0630 h at each day. Blood samples were withdrawn in hourly intervals for the analysis of metabolites as indicated. Data are presented as $\text{LSM} \pm \text{s.e.m.}$ and P values for each, the *ad libitum*- and feed-deprived period are given in the graph (ANOVA).

Figure 3

Plasma concentrations of acyl ghrelin (A and B) and total (acyl+desacyl) ghrelin (C and D) each –6 weeks before (A and C) and 2 weeks after (B and D) parturition in H (closed square) and L (open circle) cows. At day 1, cows were fed *ad libitum* for 24 h, whereas at day 2 animals were feed deprived for 10 h (grey-shaded region) followed by a

14-h re-feeding period until day 3. Time=0 h indicates 0630 h at each day. Plasma samples were obtained in 60-min intervals as indicated. Data are presented as $\text{LSM} \pm \text{s.e.m.}$ and P values for the *ad libitum* and feed-deprived period are given in the graph (ANOVA).

of fat metabolism, we performed Pearson correlation studies. To this end, we used the pp ghrelin peak during feed deprivation as a model for the preprandial ghrelin surge. We found no correlation between *ad libitum* nor between 1- or 14-h compensatory DMI and acyl ghrelin, total ghrelin or the ratio between both forms (Fig. 6A and B). Also, LFC did not correlate with acyl or total ghrelin (data not shown), while we observed a significant positive correlation between the ratio of acyl:total ghrelin and LFC (Fig. 6C). In addition to this, the ratio between acyl and total ghrelin correlated with milk fat content (Fig. 6D), but again no correlations were found between acyl or total ghrelin and milk fat content. Moreover, we found a negative correlation between the acyl:total ghrelin ratio and respiratory quotient (Fig. 6E), as well as

a positive correlation between the acyl:total ghrelin ratio and back fat thickness respectively (Fig. 6F) but not with plasma NEFA concentrations.

Discussion

Acyl ghrelin and feed intake

The transition from pregnancy to lactation is characterized by an increase in feed intake and the allocation of mobilized body fat reserves to the mammary gland to meet the energy requirements of milk production. It has been suggested that the ghrelin system is involved in both the control of feed intake and the regulation of fat metabolism, which led us to examine whether ghrelin is

Figure 4

Ratio between plasma concentrations of acyl ghrelin and total (acyl+desacyl) ghrelin –6 weeks before (A) and 2 weeks after (B) parturition in H (closed square) and L (open circle) cows. At day 1, cows were fed *ad libitum* for 24 h, whereas at day 2 animals were feed deprived for 10 h (grey-shaded region) followed by a 14-h re-feeding

period until day 3. Time=0 h indicates 0630 h at each day. Plasma samples were obtained in 60-min intervals as indicated. Data are presented as $\text{LSM} \pm \text{s.e.m.}$ and P values calculated for the *ad libitum* and feed deprived period are given in the graph (ANOVA).

Figure 5

Mean respiratory quotient determined ante-partum (–6 weeks before parturition) and post-partum (2 weeks after parturition) each during *ad libitum* feeding (24 h) (A) and during 10-h feed deprivation (B) in H (closed square) or L (open square) cows. A lower respiratory quotient indicates greater fat oxidation. Group $\times$ time interaction was $P = 0.67$ for *ad libitum* feeding and $P = 0.44$ for total feed restriction. Data are presented as least squares means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ (Tukey–Kramer test).

involved in the adaptation to lactation in dairy cows. Pioneer studies suggested that the desacyl ghrelin form depressed feed intake (Asakawa *et al.* 2005) and diminished lipolysis (Muccioli *et al.* 2004, Thompson *et al.* 2004, Theander-Carrillo *et al.* 2006), while acyl ghrelin exerted the opposite effect on food intake (Tschöp *et al.* 2000). However, these results are currently the subject of controversial debate (Wortley *et al.* 2004, Barazzoni *et al.* 2007). In ruminants, studies have shown that plasma acyl ghrelin concentrations decrease after feeding and increase before meals and in response to fasting (Sugino *et al.*

2002a, Wertz-Lutz *et al.* 2006), using an intervention strategy to model the preprandial acyl ghrelin surge (Bradford & Allen 2008) and synchronize individuals for studying short-term compensatory feed intake. From these observations, it appears that ghrelin might play a role in the control of feed intake in ruminants, as suggested in rodents (Tschöp *et al.* 2000, Nakazato *et al.* 2001). In this study, we measured plasma acyl ghrelin concentrations at hourly intervals during *ad libitum* feeding and feed deprivation. Probably because of different individual feed intakes relative to sampling, we did not observe preprandial acyl ghrelin surges during *ad libitum* feeding, neither before nor after parturition. This is in agreement with findings in sheep (Sugino *et al.* 2002b) and our data also support the observation that acyl ghrelin concentrations increase in response to feed deprivation (Wertz-Lutz *et al.* 2006). The current data are in contrast to the findings of Bradford & Allen (2008), showing that late-lactating cows (positive EB) do not respond with increasing acyl ghrelin concentration after feed withdrawal. However, our cows investigated 6 weeks ap developed only a twofold increase while cows in early lactation responded with a fourfold increase in plasma acyl ghrelin after 9-h feed deprivation. The greater increase in acyl ghrelin in H cows pp compared with ap is in line with earlier findings (Itoh *et al.* 2005, Bradford & Allen 2008), stating that negative EB increases periprandial acyl ghrelin concentrations (Bradford & Allen 2008). During *ad libitum* feeding, only H cows (not L cows) had a significantly higher acyl ghrelin concentration pp when compared with the ap period. Only after feed deprivation did both groups exhibit significantly different plasma acyl ghrelin concentrations, before and after parturition, suggesting that it is not negative EB *per se* (*ad libitum*-fed early-lactating cows are already in negative EB) but rather reduced feed intake in early lactation that increases preprandial acyl ghrelin concentrations. In addition, if the magnitude of the negative EB *per se* accounted for the preprandial acyl ghrelin pulse, large differences in acyl ghrelin between groups would be expected during *ad libitum* feeding because both groups differ significantly in their EB; we did not find such differences, and there was no correlation between preprandial acyl ghrelin peak and negative EB.

There was also no correlation between acyl ghrelin and the amount of DMI during *ad libitum* feeding or after re-feeding. The latter finding leads to doubt regarding the orexigenic characteristics of acyl ghrelin in dairy cows, supporting earlier findings that peripheral (Roche *et al.* 2008) or central (Iqbal *et al.* 2006) infusion of acyl ghrelin failed to significantly increase feed intake in ruminants.

Figure 6

Pearson correlation between the preprandial acyl:total ghrelin ratio (calculated 9 h after feed deprivation 2 weeks pp) and 1-h (A) and 14-h (B) compensatory DMI after re-feeding, liver fat content on day 18 pp (C), milk

fat content at the feed deprived day (D), respiratory quotient (E) and back fat thickness (F), considering each L and H cows. Pearson correlation coefficients (r) and their level of significance (P) are given.

Desacyl ghrelin, the ratio between both ghrelin forms and feed intake

One reason for the proposed non-orexic character of acyl ghrelin in ruminants might be due to a masking effect of desacyl ghrelin, which is not only the dominant ghrelin form but may also exert inverse effects, at least in rodents (Asakawa *et al.* 2005). As already observed for the acyl

form, total ghrelin concentration did not change during *ad libitum* feeding but increased from ap to pp only in H cows; during the pp period, *ad libitum*-fed H cows tended to have higher total ghrelin concentrations than L cows. However, the ratio calculated between *ad libitum* acyl and total ghrelin concentrations revealed no differences between groups or between ap and pp, which is in line

with the comparable DMI of both groups. During deprivation, total ghrelin concentrations increased with time in both groups. Interestingly, rats also respond to fasting with an increase in desacyl ghrelin and no change in acyl ghrelin concentration (Kirchner *et al.* 2009). In fasted compared with fed humans, increased desacyl but decreased acyl ghrelin concentrations have been described while total ghrelin concentrations remained unchanged (Liu *et al.* 2008). Thus, as in monogastric species, ruminants respond to fasting with increased release of desacyl ghrelin. The fasting-induced increase in circulating desacyl ghrelin may be in line with its orexic effect described by Toshinai *et al.* (2006), but the majority of studies performed in rodents report reduced feed intake after central desacyl ghrelin injection (Asakawa *et al.* 2005, Chen *et al.* 2005). To the best of our knowledge, there are no data on the effect of desacyl ghrelin on feed intake in ruminants. However, in rats, it has been suggested to be involved in the regulation of energy homeostasis because of its ability to cross the blood–brain barrier and to induce increased neuronal activity in the hypothalamic arcuate nucleus (Inhoff *et al.* 2009).

In the ap period, total ghrelin was higher in feed-deprived L cows, while at the same time acyl ghrelin was higher in H cows. Accordingly, the acyl:total ghrelin ratio was higher in feed-deprived H than in L cows. The latter finding can be seen in both the ap and the pp periods, suggesting that H cows possess either higher GOAT or lower esterase activity compared with L cows. Moreover, because the ratio did not change in either of the groups with progressive duration of feed deprivation, it may be possible that the granula of the X/A cells of the abomasum (Hayashida *et al.* 2001) store high enough amounts of both ghrelin forms to release them in equal amounts slowly over time.

Ghrelin and fat metabolism; ghrelin and respiratory quotient

There is increasing evidence from recent literature that acyl ghrelin is more involved in lipid mobilization, fat distribution between tissues and substrate utilization than in the control of feed intake (Wortley *et al.* 2004, Barazzoni *et al.* 2005, Kurose *et al.* 2005, Rigault *et al.* 2007, Huda *et al.* 2011, Vestergaard *et al.* 2011). For example, acyl ghrelin administration into the human femoral artery elevates venous NEFA concentrations, suggesting that ghrelin stimulates lipolysis in skeletal muscle (Vestergaard *et al.* 2011). Similarly, acyl ghrelin infusion into cows leads to an elevation of plasma NEFA concentration

(ThidarMyint *et al.* 2006, Roche *et al.* 2008). These findings are consistent with our results showing that early-lactating H cows with the higher preprandial acyl ghrelin surge have a higher lipolysis rate (as indicated by higher basal plasma NEFA concentrations). On the other hand, long-chain fatty acids fed in a rumen-protected form may trigger the acylation of ghrelin (Fukumori *et al.* 2012). Whether endogenously released fatty acids from adipose tissue contribute to the higher preprandial acyl ghrelin concentration during early lactation remains to be determined.

The different magnitude of increase of both ghrelin forms in response to feed deprivation might also be associated with a different sensitivity of ghrelin receptors. Whereas the receptor for desacyl ghrelin is not yet known, acyl ghrelin binds to GHSR1a (Komatsu *et al.* 2010, 2012). SNPs in the *GHSR* gene were found to be associated with growth and carcass traits in cattle (Zhang *et al.* 2009, Komatsu *et al.* 2011) and with growth, eating behaviour and obesity in humans (Baessler *et al.* 2005, Gueorguiev *et al.* 2009). In the current study, the allele distribution of the identified variable *GHSR* SNPs did not differ between groups, suggesting that the differences in fat distribution and respiratory quotient between H and L cows cannot be attributed to variations in the *GHSR* gene. However, differences in expression of the *GHSR1a* and *GHSR1b* genes, as is found in several tissues and is altered in an age-dependent manner (Komatsu *et al.* 2012), may be associated with the H and L phenotype. This question will be addressed in future studies.

Administration of acyl ghrelin in rats also diminishes the TG content in muscle (Barazzoni *et al.* 2005) and reduces hepatic mitochondrial β -oxidation (Rigault *et al.* 2007), leading to an increase in liver TGs (Barazzoni *et al.* 2005). This tissue partitioning effect may be present in periparturient dairy cows because H cows (with higher preprandial acyl ghrelin concentrations) developed higher liver and milk fat contents in early lactation compared with L cows. This assumption is further strengthened by the fact that H cows exhibited a higher ratio between acyl and total ghrelin. Desacyl ghrelin stimulates fatty acid uptake in cultured adipocytes (Miegueu *et al.* 2011), reduces gastric emptying and thus leads to negative EB in rodents (Asakawa *et al.* 2005). Interestingly, *ad libitum*-fed H cows, which tended to have higher total ghrelin concentrations, were indeed in more negative EB. The association between higher desacyl ghrelin concentration and lower feed passage rate in *ad libitum*-fed H cows was not investigated. However, in obese fasted humans, desacyl ghrelin was lower and acyl ghrelin was elevated compared with non-obese counterparts (Rodríguez *et al.*

2009); this supports our findings in feed-deprived H and L cows before parturition. The ratio of acyl:total ghrelin is also higher in obese than in non-obese humans (Barazzoni *et al.* 2007, Rodríguez *et al.* 2009), suggesting this ratio as a suitable indicator for body fat. Following this idea, we found strong direct correlations between the acyl:total ghrelin ratio and parameters reflecting the extent of body fat mobilization in early lactation, such as liver and milk fat contents and back fat thickness. Additionally, we found a strong indirect correlation between the acyl:total ghrelin ratio and the respiratory quotient, the latter a reciprocal measure of whole-body fat oxidation. Surprisingly, H cows had a lower respiratory quotient not only in early lactation but also in late gestation (when fat mobilization does not occur), indicating a generally greater utilization of fat in H than in L cows, independent of the fatty acid load in early lactation. Consistently, H cows with a higher acyl:total ghrelin ratio and higher back fat thickness had a greater propensity to mobilize and oxidize body fat reserves, which confirms the suggested role of ghrelin in influencing metabolic fuel preference (Wortley *et al.* 2004).

Taking the results of this study together, we conclude that neither acyl nor desacyl ghrelin concentrations refer to the amount of DMI of high-yielding dairy cows in early lactation. By contrast, due to the high correlation of the acyl:total ghrelin ratio with back fat thickness, liver and milk fat contents, but also whole-body fat oxidation, the ghrelin system links mobilized tissue fat distribution, fat retention in the liver and metabolic fuel preference to ensure proper metabolic adaptation to lactation.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-12-0384>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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