

Daily patterns of plasma leptin in sheep: effects of photoperiod and food intake

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Abstract

Circulating concentrations of leptin in sheep correlate with body fatness and are affected by level of food intake and photoperiod. The present objective was to elucidate the short-term dynamics of leptin secretion. Frequent blood samples were taken over 48 h from 12 Soay rams after 16 weeks in short-day photoperiod (SD, 16 h darkness:8 h light) with freely available food, and then after 16 weeks in long days (16 h light:8 h darkness) with food freely available (LD) or restricted to 90% maintenance (LDR) ($n=6$ /group). During the second 24 h of sampling, half were food deprived ($n=6$, SD and LD) and half had their meal times shifted ($n=6$, SD and LDR). A homologous RIA was developed, using antibodies raised in chicken against recombinant ovine leptin, to measure plasma concentrations. Simultaneous 24 h profiles of plasma insulin, glucose and non-esterified fatty acids (NEFA) were measured. Plasma leptin was higher in LD than SD, and in

LD than LDR, associated with higher food intake, liveweight and body condition score (adiposity), but tended to be lower in LDR than SD, associated with lower food intake, liveweight and body condition score. There was no evidence for a circadian rhythm of plasma leptin, but clear evidence for post-prandial peaks of low amplitude (15–36%) 2–8 h after meals given at normal and shifted times. Complete food deprivation caused a dramatic fall in plasma leptin to basal levels within 24 h. There was a positive association of plasma leptin with plasma insulin, and negative association with NEFA, both between meals and during fasting. Thus, plasma leptin concentrations in sheep are sensitive to short-term changes in energy balance, as well as to long-term photoperiod-driven changes in food intake and adiposity.

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Introduction

The adipose tissue hormone leptin provides feedback on peripheral energetic status to regulatory systems in the brain. Studies in laboratory rodents have revealed two distinct forms of leptin feedback, one encoding the level of body fat storage and the second, perhaps more important, signalling short-term or diurnal changes in food intake (Ahima & Flier 2000). Daily patterns of leptin secretion in sheep have not previously been reported. Furthermore, given the contrasting nature of the ruminant digestive system, it is important to test the hypothesis that leptin in a ruminant species may be less sensitive in the short-term to the intake of food than in monogastric species.

Plasma concentrations of leptin correlate with body fatness in all species studied to date, including humans (Considine *et al.* 1996), rodents (Maffei *et al.* 1995), sheep (Blache *et al.* 2000, Delavaud *et al.* 2000), and cow (Ehrhardt *et al.* 2000). The level of food intake can also have major influence; for example, plasma leptin is

reduced in sheep fed below liveweight-maintenance requirements (Bocquier *et al.* 1998, Delavaud *et al.* 2000) and in cattle subjected to a 48 h fast (Amstalden *et al.* 2000). It has also been shown that leptin gene expression in ovine adipose tissue (and by inference leptin biosynthesis) is reduced by underfeeding (Bocquier *et al.* 1998) and by a 48 h fast (Kumar *et al.* 1998). However, the dynamics of the leptin response to fasting in the ruminant and the factors that modulate leptin secretion in the short-term remain unclear.

A diurnal pattern of plasma leptin concentrations has been described in man (Sinha *et al.* 1996, Langendonk *et al.* 1998), but this is clearly entrained to the pattern of intake of meals rather than to a circadian oscillator (Schoeller *et al.* 1997). Leptin concentrations rise some time after ingestion of food and are at their nadir in the early morning before a meal has been taken (Schoeller *et al.* 1997). Similarly, there is a diurnal pattern of circulating leptin, associated with the pattern of feeding and characterised by a nocturnal peak, in rats (Saladin *et al.*

1995) and mice (Ahren 2000). In contrast, Blache *et al.* (2000) found no diurnal or meal-related fluctuations in plasma leptin in rams, attributing the apparent species difference to differences in nutritional physiology between monogastrics and ruminants.

Although photoperiod has been reported to influence plasma leptin in sheep (Bocquier *et al.* 1998), there are no data comparing diurnal patterns of leptin concentrations between long and short daylengths.

In rats, the intake of food raises circulating leptin by transiently increasing leptin gene expression in adipose tissue (Saladin *et al.* 1995). This effect is mimicked by a single insulin injection, with or without concomitant hypoglycaemia, suggesting that the increase in leptin mRNA following ingestion of a meal may be mediated by direct actions of insulin on the adipocyte (Saladin *et al.* 1995). Insulin administration increased plasma leptin levels in rats within 4 h (Hardie *et al.* 1996), but not in sheep within 2 h (Kauter *et al.* 2000). However, the interrelationships between endogenous leptin, insulin and ingestion of food remain unresolved for any ruminant species.

In the present study we aimed to elucidate diurnal patterns of plasma leptin concentrations in the ruminant and how these are modulated by nutritional and photoperiodic status. Since validated assays for ovine leptin were unavailable at the outset of this study (Blache *et al.* 2000, Delavaud *et al.* 2000, Ehrhardt *et al.* 2000), we first developed an RIA specific to ovine leptin for use in our laboratory. In seasonal species like sheep, photoperiod influences appetite and body weight, and thereby interacts with imposed nutritional treatments. We therefore aimed to compare, firstly, sheep fed freely in short daylength (SD) with both freely fed sheep in long daylength (LD) and sheep in LD given a daily intake of food restricted to that normally freely consumed in SD (LDR). The second comparison, between LD and LDR sheep, examined the effect of food restriction. Further, we examined the effects on diurnal leptin profiles of either a 24 h fast or a shift in the timing of meals within both photoperiods. Throughout the study, we compared and contrasted with leptin profiles the simultaneous diurnal patterns of plasma insulin, glucose and non-esterified fatty acids (NEFA), since these are recognised short-term indicators of metabolic state.

Materials and Methods

Animals and treatments

Adult Soay rams were individually housed in artificial light-controlled rooms, allowing contact by smell, touch and vision. They were exposed for 16 weeks to short-day photoperiod (SD, 8 h light:16 h darkness) followed by 16 weeks to long-day photoperiod (LD, 16 h light:8 h darkness). In SD, all were fed freely ($n=12$). In LD, half of the sheep continued on free feeding (LD, $n=6$) while the remainder received restricted feeding (approximately 90%

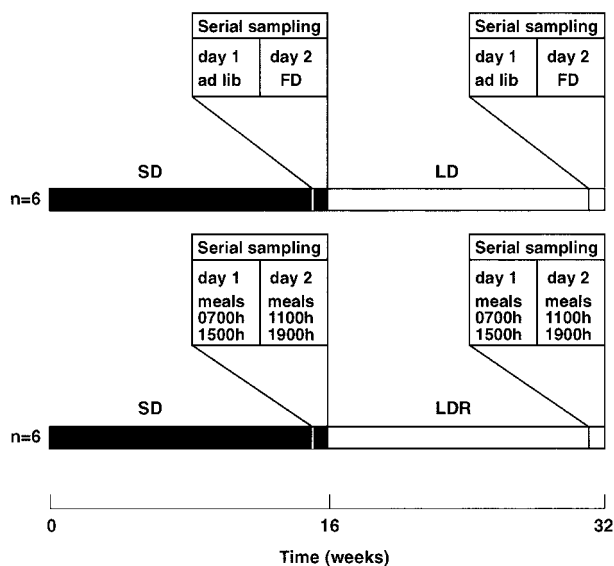


Figure 1 Experimental design. Sheep were kept for 16 weeks in short days (SD, 8 h light:16 h darkness; solid bar) followed by 16 weeks in long days (LD, 16 h light:8 h darkness; open bar). All were fed freely (ad lib) in SD. In LD, one group received food freely and the other group was given restricted food (LDR). Food was given in two meals daily. Serial blood samples were taken over 48 h during which SD/LD sheep had freely available food on day 1 but were food deprived (FD) on day 2, and SD/LDR sheep had meal times shifted on day 2.

liveweight maintenance; LDR, $n=6$) (Fig. 1). The food (complete diet comprising 50% chopped hay, 30% rolled barley, 9% white fishmeal, and containing approximately 10 MJ metabolisable energy/kg dry matter (DM)) was given in two equal amounts at 0700 h and 1500 h (GMT). Sheep on free feeding had a 10% refusal margin, and intakes were measured daily. Liveweight and body condition score (BCS, scale 1 (thin)–5 (fat), Russel *et al.* (1969)) were measured once a fortnight.

Forty-eight hour sampling protocol

During the final week of each photoperiod, i.e. during weeks 16 and 32 of the experiment, blood was taken from an indwelling jugular catheter at intervals of 30–60 min over 48 h, starting at 0500 h (2 h before lights-on) on day 1. On day 1, all sheep received their food in two feeds as usual at 0700 h and 1500 h. The group receiving food freely in both photoperiods had all food removed at 2300 h (i.e. after 18 h of sampling) and received no meals thereafter on day 2 ($n=6$). The sheep in the other group, freely receiving food in SD and restricted food in LD (LDR), had their two meal times shifted by 4 h to 1100 h and 1900 h on day 2 ($n=6$) (Fig. 1).

During the hours of darkness, dim red light was used to facilitate sampling without disturbing the light–darkness

cycle. Blood was collected into heparinised tubes and plasma stored at -20°C until assayed.

All experimental procedures were licensed under the UK Animals (Scientific Procedures) Act of 1986 and received ethical approval from the Rowett Research Institute's Ethical Review Committee.

Leptin assay

Recombinant ovine leptin (Gertler *et al.* 1998) was used for antibody preparation, tracer and standard curve. The antibody (Genosys, Cambridge, UK) was raised in chicken using six fortnightly injections giving a total dose of 1 mg ovine leptin in complete, followed by incomplete, Freund's adjuvant. Eggs produced 77 to 101 days following primary immunisation were collected, immunoglobulins extracted from the yolk using the EGGstract method (Promega, Southampton, UK) and reconstituted in equal volume of buffer (0.01 M potassium phosphate, 0.1 M NaCl, 50 mg/l gentamicin, pH 7.4). Ovine leptin (5 $\mu\text{g}/5\ \mu\text{l}$) was mixed with 5 μl phosphate buffer (0.5 M, pH 7.5) and 0.5 mCi (5 μl) of Na^{125}I solution (ICN Biomedicals Ltd, Thame, Oxon, UK), and allowed to react for 30 s with 25 μg (5 μl) chloramine T (Hunter & Greenwood 1962). The reaction was halted by the addition of 200 μl sodium metabisulphite (12 mg in 10 ml 0.05 M phosphate buffer) and 100 μl potassium iodide (10 mg in 10 ml 0.05 M phosphate buffer). The iodination products were eluted with 0.05 M phosphate buffer containing 0.5% BSA on a Sephadex G25 column and 150 μl fractions were collected. A typical iodination gave a specific activity of 63 $\mu\text{Ci}/\mu\text{g}$. Fractions corresponding to the first part of the first peak were kept and binding to excess first antibody (1/125 dilution) was 82.6%.

The assay buffer was 0.05 M phosphate, 0.15 M NaCl, 5 g/l BSA, 100 $\mu\text{l}/\text{l}$ Tween-20, 0.025% sodium azide, pH 7.4. One hundred microlitre samples, standard (5–0.0195 ng/tube in nine serial dilutions in assay buffer) or assay buffer (zero binding (B_0)) and non-specific binding (NSB) tubes) were pre-incubated for 48 h at room temperature with 500 μl first antibody diluted at 1/2000 in assay buffer. Iodinated leptin (8000 c.p.m.) in 100 μl buffer was added and incubated for 24 h at 4°C . Then, 500 μl buffer containing the second antibody (sheep anti-chicken; ref. S253–205, Diagnostics Scotland, Carlisle, Lanarkshire, UK) at 1/100 dilution and 6 μg normal chicken immunoglobulin were added and incubated at 4°C for 3 days. One millilitre polyethylene glycol (4% PEG 8000) was added and the tubes centrifuged at 4°C for 30 min at 2250 g. Supernatants were discarded by decantation and ^{125}I in the pellets was counted for 2 min.

A typical standard curve is shown in Fig. 2a. The NSB count was 2.2% of the total count, and the absolute binding for B_0 was 12.3%. Sensitivity was 45 pg/tube (0.45 ng/ml). Parallelism of the standard curve with serial dilutions of ovine, caprine and bovine plasma samples

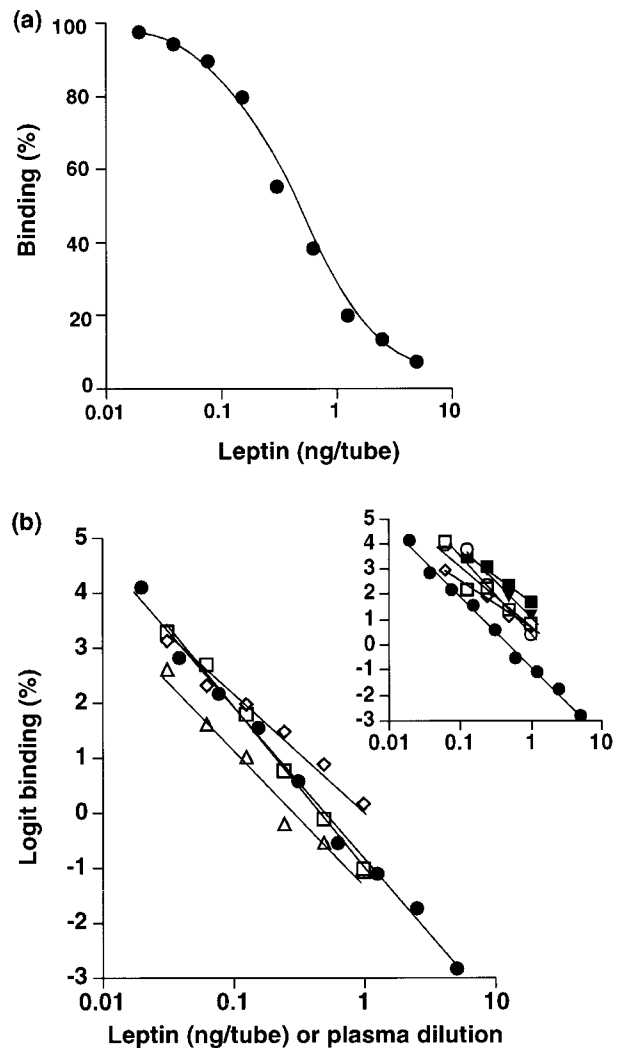


Figure 2 (a) Typical standard curve for leptin assay; (b) linear logit representation of recombinant ovine leptin standard curve (solid symbols) with parallelism shown by serial dilutions of three ovine plasma samples (open symbols), and (insert) three caprine (open symbols) and two bovine (solid symbols) plasma samples.

was demonstrated (Fig. 2b). The coefficient of variation (CV) observed for six ovine plasma samples with leptin concentrations from 3.4 to 16.1 ng/ml was 12% (6.7–13.3%) for intra-assay and 16% (13.3–21.3%) for inter-assay repeatability.

All chemicals and reagents were obtained from Sigma UK Ltd (Poole, Dorset, UK) unless indicated otherwise.

Insulin, glucose and NEFA determinations

Insulin in plasma was measured using the RIA described by MacRae *et al.* (1991). Inter- and intra-assay CV values were 9.2 and 5.2% respectively, and assay sensitivity was 2 $\mu\text{IU}/\text{ml}$.

Table 1 Mean (\pm S.E.M.) liveweight (LW), BCS, food intake and plasma concentrations of leptin, insulin, glucose and NEFA (averaged over day 1 of intensive sampling) for sheep after 16 weeks with freely available food in short days (SD), and the same sheep after 16 weeks with freely available food in long days (LD) or restricted food (90% liveweight maintenance) in LDs (LDR) ($n=6$ /group). ANOVA was used to compare photoperiods within groups using animals as blocks (SD vs LD; SD vs LDR) and to compare groups within photoperiod (LD vs LDR)

	SD	LD	SD vs LD	SD	LDR	SD vs LDR	LD vs LDR
Intake (g DM/day)	754 \pm 86.3	1517 \pm 86.2	***	731 \pm 26.4	660 \pm 0.0	*	***
LW (kg)	38.0 \pm 3.16	48.5 \pm 3.72	**	37.6 \pm 2.47	32.3 \pm 1.61	**	**
BCS	2.58 \pm 0.05	2.98 \pm 0.06	***	2.63 \pm 0.06	1.87 \pm 0.06	***	***
Leptin (ng/ml)	2.75 \pm 0.37	7.73 \pm 2.02	*	2.58 \pm 0.39	2.08 \pm 0.09	NS	*
Insulin (μ U/ml)	176.2 \pm 48.3	164.7 \pm 14.8	NS	79.3 \pm 12.6	43.5 \pm 3.3	*	***
Glucose (mmol/l)	3.95 \pm 0.05	4.10 \pm 0.13	NS	4.05 \pm 0.05	3.63 \pm 0.12	*	*
NEFA (mmol/l)	0.285 \pm 0.047	0.106 \pm 0.012	**	0.461 \pm 0.056	0.310 \pm 0.031	NS	***

*** $P<0.001$, ** $P<0.01$, * $P<0.05$, NS non-significant.

Glucose and NEFA in plasma were determined by fully automated KONE Analyser methods of Peterson & Young (1968) and Matsubara *et al.* (1983) respectively. The sensitivity of the glucose assay was 0.34 mmol/l and the intra- and inter-assay CV values were 0.35 and 2.3% respectively. The sensitivity of the NEFA assay was 0.04 mmol/l and the intra- and inter-assay CV values were 4.0 and 2.0% respectively.

Statistical analysis

Group mean comparisons (for liveweight, BCS, food intake and plasma concentrations, as shown in Table 1) were analysed by ANOVA, comparing the two groups of different sheep within LD photoperiod (LD vs LDR), and comparing photoperiods within groups of the same animals (SD vs LD and SD vs LDR) using animals as blocks (Genstat 5, Version 4.1) (Genstat 5 Committee 1993). NEFA, insulin, and leptin values in LD were log-transformed in order to normalise residuals. ANOVA was used to compare average leptin values over three defined time periods within 24 h in each photoperiod, again using animals as blocks (Genstat 5, Version 4.1) (Table 2a). Cosinor analysis was undertaken to explore further any diurnal rhythmicity in plasma leptin concentrations, fitting the function $[M+A \times \sin(0.2618 \times t+P)]$, where M is the 24 h mean value (ng/ml), A is the amplitude (ng/ml), t is the time (h) and P is the phase (radians); the significance of this model for each group/day was assessed by a Rayleigh test (Mardia 1972) (Table 2b).

Time-course data (Figs 3 and 4) were analysed by the residual maximum likelihood (REML) method, using Genstat 5 Version 4.1, and under guidance from Biomathematics & Statistics Scotland (BioSS, RRI Group, Aberdeen, UK). In order to take into account correlations between successive measures done on each individual, covariance was modelled by an auto-regressive procedure (Littell *et al.* 1998). The effect of time was tested by a Wald test, using a Chi square statistic; differences between

means were compared by a *t*-test to standard errors of differences specific for each compared pair of means. In this way, the timings of significant increases and decreases in concentrations were detected for each metabolite and hormone during the 48 h sampling periods.

Results

Effect of photoperiod and food restriction

Voluntary food intake doubled in LD compared with SD and this was associated with an increase in liveweight and BCS (Table 1). Plasma leptin increased by 180% and plasma NEFA concentrations decreased whereas plasma insulin and glucose were not changed (Table 1).

Food intake of LDR sheep was 90% of that consumed in SD and they experienced a significant loss of weight and BCS (Table 1). Plasma insulin and glucose were reduced in LDR compared with SD; furthermore, leptin concentrations tended to be reduced whereas NEFA tended to be increased, although these did not reach significance (Table 1).

Compared with LD sheep, LDR animals had lower food intake, liveweight and BCS, and reduced plasma leptin, insulin and glucose but higher plasma NEFA concentrations (Table 1).

Short-term patterns of circulating leptin, insulin, glucose and NEFA

Effect of meals and time of day Statistical (REML) analysis of the patterns of plasma concentrations during day 1 of intensive sampling were undertaken in both SD groups together ($n=12$), and LD and LDR groups ($n=6$ /group). The timing of a significant increase/decrease in concentrations identified by REML analysis was defined at the first significantly different value in a series, and significant concentration peaks and nadirs were detected.

In addition, 24 h leptin concentrations for each individual were both averaged over three 8 h periods, then group means compared by ANOVA to detect overall day–night differences, and subjected to cosinor analysis to detect rhythmicity, giving amplitude and time of zenith (acrophase).

Metabolic parameters followed a pattern clearly related to meal intake (Figs 3 and 4). NEFA levels decreased during the first 30–60 min after each meal in all groups, but less so in LD when the basal levels were lower. Thereafter, plasma NEFA increased 4 h (in LDR), 6–10 h (in SD) or 12–14 h (in LD) after meals. A post-prandial increase of insulin was observed in the first 30–60 min. This was followed by a decrease starting as soon as the second hour after the meal, and plasma glucose was subsequently reduced in proportion to the insulin increases.

In SD, plasma leptin concentrations increased significantly (from 2.43 ng/ml) at 6 h after the first meal to a maximum (2.85 ng/ml) at 8 h, or from 2.55 ng/ml at 4 h to a maximum 3.22 ng/ml at 5 h after the second meal (Figs 3 and 4). Values thereafter decreased during the latter part of the dark phase and the onset of the light phase to a nadir at 2.5 h following the first meal. Thus during the first 8 h of the night (but not during the last 8 h of the night), plasma leptin values were higher than daytime concentrations ($P < 0.01$) (Table 2a). The cosinor model for these data was not significant (Table 2b).

In LDR, plasma leptin increased significantly at 1.5 h (from 1.95 ng/ml) to a maximum (2.53 ng/ml) at 3 h after the first meal, or at 1 h (1.92 ng/ml) to a peak at 2 h (2.62 ng/ml) after the second meal (Fig. 3). Values decreased following the post-prandial peaks to nadirs at the onset of the second meal and at 5–9 h after the second meal (i.e. at the end of light/start of dark phase). Night-time leptin values were therefore less than during the first part ($P < 0.001$) but not the second part of the day in LDR, and the acrophase ($P < 0.001$) was during daytime at 4.8 h after lights-on (Table 2a and b).

In LD, significant increases in plasma leptin were seen at 2.5 h after the first meal, from 6.63 ng/ml to a maximum (7.62 ng/ml) at 6 h, and at 2 h after the second meal (6.58 ng/ml) to a maximum at 3 h (7.53 ng/ml) (Fig. 4). There were no day–night differences in plasma leptin detected in LD, and the cosinor model was not significant (Table 2a and b).

The above data show that the absolute variations of plasma leptin in all three treatments were of low amplitude, 0.72 ± 0.09 ng/ml (range 0.42–1.0 ng/ml), representing increments of $23 \pm 3.7\%$ (14–36%), with these peaks occurring 2–8 h after a meal.

Effect of shifting the meal times For this analysis, plasma concentrations in SD/LDR sheep ($n=6$) were examined by REML during both days of intensive sampling in SD and LDR to reveal whether the metabolic

parameters, including leptin, were affected by the altered timing of meals during day 2. The effects of meals during day 1 are described above. In addition, concentrations were averaged over three periods of 8, 8 and 6 h duration, starting at lights-on, in order to detect overall day–night differences.

As on day 1, plasma NEFA concentrations decreased immediately, insulin increased and there was a subsequent decrease in plasma glucose after each meal on day 2 in both SD and LDR (Fig. 3).

In SD, leptin increased 2 h after the first meal on day 2 ($P < 0.001$) and 1 h after the second meal ($P < 0.001$) to peaks at 4 h after each meal. These increases therefore occurred sooner post-prandially than on day 1. The second leptin peak on day 2 was higher than that on day 1 and was followed by sustained elevated values for the remainder of the sampling period (Fig. 3). Thus, following the meal shift in SD, overall plasma leptin was elevated in the latter part of the night compared with early night- and daytime values ($P < 0.001$, Table 2a). Furthermore, the corresponding cosinor acrophase was shifted to the latter part of the night on day 2 ($P < 0.01$, Table 2b).

In LDR, however, in contrast to day 1, there were no significant changes in plasma leptin over time on day 2 (Fig. 3). There were therefore no significant day–night differences in concentrations and the cosinor model was not significant (Table 2a and b).

Effect of food deprivation Fasting induced a steady decline in plasma leptin concentrations, starting from the peak after the last meal, to basal levels of 1.50–1.75 ng/ml at 26 h after the last meal in SD, and of 2 ng/ml at 33 h after the last meal in LD (Fig. 4). Insulin concentrations decreased similarly to basal values, with the decline initiated within 2 h of the last meal in LD. NEFA remained at high levels during the food-deprivation period. For each individual in LD, insulin, NEFA and leptin patterns on day 2 presented a clear departure from values observed during day 1. Relative to basal values observed before the last meal on day 1, a significant decrease in insulin concentrations was observed at 5.5 ± 0.96 h after the last meal, before the increase in NEFA (at 12.7 ± 1.2 h, $P < 0.001$) and the fall in leptin concentrations (at 15.8 ± 2.3 h, $P < 0.01$). In individual sheep, the NEFA increase preceded or coincided with the initiation of the leptin increase.

Discussion

The present study provides the first evidence for the existence of short-term patterns of plasma leptin concentrations in a ruminant species, superimposed on the long-term regulation of leptin secretion by nutrition and photoperiod.

Photoperiod clearly affected voluntary food intake, live-weight and adiposity (BCS) in the present Soay sheep,

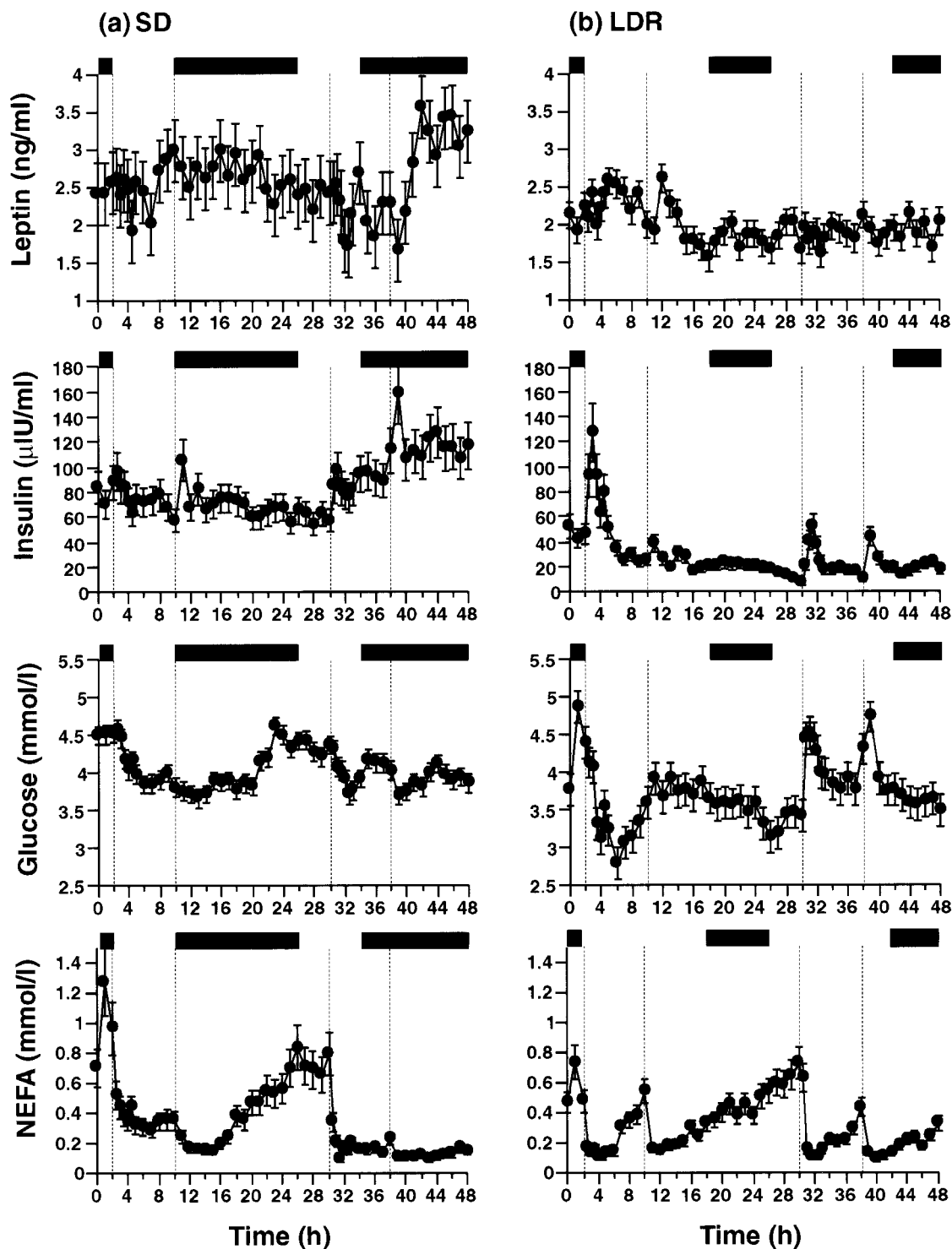


Figure 3 Mean plasma concentrations (\pm S.E.M.) of leptin, insulin, glucose and NEFA over 48 h in sheep ($n=6$) after 16 weeks with freely available food in short days (a) (SD) and after the subsequent 16 weeks with restricted food (90% liveweight maintenance) in long days (b) (LDR). Meal times (vertical lines) were shifted forwards by 4 h on day 2. The solid horizontal bars indicate the periods of darkness.

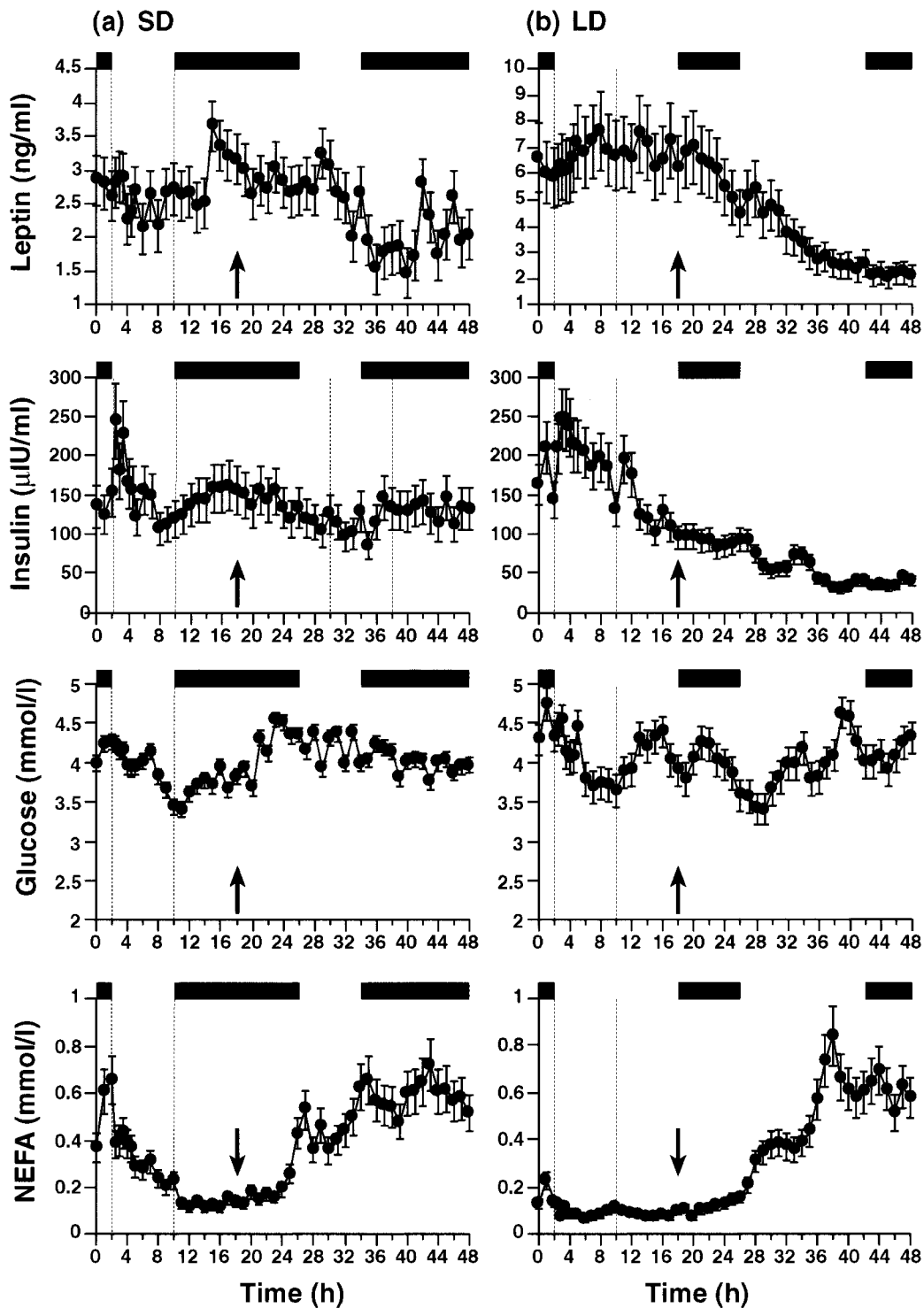


Figure 4 Mean plasma concentrations (\pm S.E.M.) of leptin, insulin, glucose and NEFA over 48 h in sheep ($n=6$) after 16 weeks with freely available food in short days (a) (SD) and after the subsequent 16 weeks with freely available food in long days (b) (LD). Vertical lines indicate meal times on day 1, and all food was removed at 18 h (indicated by arrow) for the duration of day 2. The solid horizontal bars indicate the periods of darkness. Note scale difference for leptin in LD compared with that in SD.

Table 2 (a) Mean plasma concentrations (\pm S.E.M.) of leptin (ng/ml) averaged over three 8 h periods on day 1 of intensive sampling after 16 weeks with freely available food in short days (SD), after 16 weeks with freely available food in long days (LD) or restricted food (90% liveweight maintenance) in LDs (LDR), and over equivalent 6–8 h periods on day 2 following a meal shift in SD and LDR. Meals at 0 h and 8 h relative to lights-on on day 1, 4 h and 12 h on day 2. Dark period 9–24 h in SD, 17–24 h in LD. ANOVA was used to compare periods within photoperiods, using animals as blocks; within rows, a vs b: $P < 0.01$, c vs d: $P < 0.001$. (b) Mean cosinor model parameters (\pm S.E.M.) for the group/days described in (a), and significance of the model: NS, not significant

(a)	Time from lights-on		
	1–8 h	9–16 h	17–24 h
SD, day 1 ($n=12$)	2.51 \pm 0.223 ^a	2.84 \pm 0.320 ^b	2.67 \pm 0.274 ^{ab}
LDR, day 1 ($n=6$)	2.31 \pm 0.127 ^c	1.97 \pm 0.103 ^d	1.81 \pm 0.129 ^d
LD, day 1 ($n=6$)	8.00 \pm 2.241	7.90 \pm 1.927	7.30 \pm 2.186
(a)	Time from lights-on		
	1–8 h	9–16 h	17–22 h
SD, day 2 ($n=6$)	2.28 \pm 0.235 ^c	2.32 \pm 0.318 ^c	3.20 \pm 0.447 ^d
LDR, day 2 ($n=6$)	1.86 \pm 0.113	1.90 \pm 0.106	1.92 \pm 0.097
(b)	Amplitude	Acrophase	Significance
	(ng/ml)	(h)	
SD, day 1 ($n=12$)	0.38 \pm 0.09	12.2 \pm 1.83	NS
LDR, day 1 ($n=6$)	0.34 \pm 0.06	4.8 \pm 0.33	$P < 0.001$
LD, day 1 ($n=6$)	0.87 \pm 0.18	8.8 \pm 2.25	NS
SD, day 2 ($n=6$)	0.56 \pm 0.14	20.1 \pm 0.67	$P < 0.01$
LDR, day 2 ($n=6$)	0.13 \pm 0.02	14.4 \pm 2.09	NS

with all increased in LD as opposed to SD, as is characteristic for this breed (Lincoln & Richardson 1998, Adam 2000). Plasma leptin concentrations followed these photoperiod-induced changes in adiposity, as reported for another seasonal species, the Siberian hamster (Horton *et al.* 2000, Klingenspor *et al.* 2000). However, food restriction in LD to a level of intake similar to that in SD, and the resultant prevention of liveweight gain, prevented the LD increase in plasma leptin. In contrast to the shorter-term (3 week) study of Bocquier *et al.* (1998), the present data suggest that the effect of photoperiod on leptin may be secondary to the photoperiod-driven changes in liveweight and adiposity in sheep rather than a direct effect of photoperiod *per se*.

The effect of food intake restriction, recorded here in LD and not in SD when intake is voluntarily low, was to markedly decrease plasma leptin, in agreement with earlier reports (Bocquier *et al.* 1998, Delavaud *et al.* 2000).

It is now apparent that the above observations masked short-term fluctuations of leptin concentrations during the day. These short-term variations were likely to reflect changes in secretion rather than clearance. Leptin is rapidly cleared from the circulation by renal extraction,

which is a high capacity, non-saturable process (Cumin *et al.* 1997, Zeng *et al.* 1997). Like Blache *et al.* (2000), we found no evidence for a diurnal rhythm of plasma leptin in sheep occurring independently from the timing of meals, in either photoperiod. Although leptin was elevated at night in SD, the timing of the nocturnal peak was shifted forwards when meal times were shifted forwards, and no such nocturnal rise was seen in LD. Serum leptin levels in men and women show a diurnal pattern (Sinha *et al.* 1996, Langendonk *et al.* 1998), but it is equivocal to what extent this is an intrinsic circadian rhythm. The characteristic nocturnal peak occurs during the extended night-time inter-meal interval and during sleep; importantly, the diurnal pattern is modulated by altered patterns of both meal intake (Schoeller *et al.* 1997, Taylor *et al.* 1999) and sleep (Simon *et al.* 1998). Leptin secretion is also highest at night both in rats, associated with night-time feeding (Saladin *et al.* 1995), and in mice, associated with feeding pattern (Ahren 2000). No nocturnal increase in serum leptin is seen in seasonal Siberian hamsters in either long- or short-day photoperiods (Horton *et al.* 2000). Perhaps the most important determinant of the diurnal pattern of circulating leptin in mammals is feeding behaviour and the timing of food intake.

Here we have evidence for post-prandial plasma leptin 'peaks' in sheep, which were delayed (2–8 h) relative to the characteristic post-prandial insulin peaks (0.5–1 h), and of low amplitude (15–36%). Interestingly, the delayed response may not be attributable to the ruminant gut physiology, since meals have been reported to induce a similar leptin response in men (18–26% increase at 6–8 h; Dallongeville *et al.* (1998)). The stimulus to increase leptin secretion post-prandially is open to speculation, but the relative changes in other circulating metabolic parameters may provide some clues. In rats there is good evidence that this stimulus may be provided by direct actions of insulin on the adipocyte (Saladin *et al.* 1995, Hardie *et al.* 1996), and in mice diurnal variations in circulating leptin and insulin are correlated (Ahren 2000). Although an increase in plasma leptin in sheep has not been seen in response to insulin administration (Kauter *et al.* 2000, M Marie, S M Rhind & C L Adam, unpublished observations), these studies may have terminated prematurely (2 and 4 h post-injection respectively) in view of the present findings. The present data are consistent with a positive temporal relationship between plasma insulin and leptin, with increases in insulin preceding by up to 7 h those of leptin (Figs 3 and 4). During inter-meal intervals, in addition to the positive association between plasma concentrations of leptin and insulin, there was a negative association with NEFA concentrations. These relationships were further exemplified during food deprivation. As observed in rats (Dallman *et al.* 1999), the decrease in insulin preceded the increase in NEFA, which occurred shortly before or simultaneously with the decrease in leptin (Fig. 4). The changes in NEFA and leptin may have been responses to

the reduced insulin feedback, or indeed to other signal(s) of negative energy balance. Since both leptin and NEFA are released into the circulation from the same cell type, it is tempting to speculate that leptin secretion is coordinated with triglyceride synthesis within the adipocyte. NEFAs are released when net triglyceride synthesis switches to net breakdown, and this may effectively switch off leptin secretion, since intracellular elevation of free fatty acids in rat adipocytes has been shown to inhibit leptin secretion (Shintani *et al.* 2000). Conversely, increased leptin secretion may be linked with lipogenesis. The basal circulating concentrations of leptin (in starved or LDR sheep) remained above the detection limit of the assay, suggesting that there exists both a basal level of leptin secretion, which is insensitive to proximate fluctuations in food intake, and a higher level of leptin secretion which is sensitive to food intake.

Reduced leptin secretion following 48 h food deprivation has been reported previously for ruminants (sheep: Kumar *et al.* 1998, cattle: Amstalden *et al.* 2000), yet the time course of the decrease has not been studied to date. Here, we report a rapid decline in plasma leptin during food deprivation in sheep, effective from the time of the first missed meal and reaching basal levels within 24 h of the last meal, regardless of adiposity. This is an important demonstration of the 'dual control' of circulating leptin in a ruminant; whereas background concentrations in the fed animal generally reflect adiposity, there is superimposed an acute sensitivity to short-term alterations in food intake.

In conclusion, plasma leptin concentrations in sheep were clearly sensitive to long-term photoperiod-driven changes in body weight, adiposity and food intake, but also to the short-term changes in energy balance that occurred both as a function of meal intake and as a result of food deprivation. There was no evidence for an independent effect of photoperiod or for a diurnal rhythm of plasma leptin in sheep. A strong association between leptin and insulin and NEFA was suggested, but no clear relationship with circulating glucose. However, any putative roles of insulin and NEFA in controlling leptin secretion remain unresolved. Finally, although the magnitude of the daily fluctuations in plasma leptin were small, single samples taken in longitudinal studies should clearly be taken at a standardised time post-prandially to be representative, and not during fasting. These data provide clear evidence of the sensitivity of leptin to short-term alterations in food intake in sheep, contrary to the hypothesis that a ruminant physiology would dampen such effects. Thus sheep, like rodents, apparently experience two distinct forms of leptin feedback, one encoding body fat reserves and the second signalling short-term changes in food intake.

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