

Effects of nutritional status and gonadal steroids on expression of appetite-regulatory genes in the hypothalamic arcuate nucleus of sheep

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Abstract

Sheep exhibit photoperiod-driven seasonal changes in appetite and body weight so that nutritional status increases in long days (LD) and decreases in short days (SD); additionally, they are reproductively active in SD and inactive in LD. We addressed the hypothesis that appetite-regulatory genes in the hypothalamus respond differently to changes in nutritional feedback induced by photoperiod as opposed to food restriction, and that responses would be influenced by gonadal steroid status. Castrated oestradiol-implanted male sheep were kept in SD (8 h light/day) or LD (16 h light/day) for 11 weeks, with *ad libitum* or restricted food (experiment 1; $n=8$ /group). Rams were kept in SD or LD for 12 weeks with *ad libitum* or restricted food (experiment 2; $n=6$ /group). Gene expression (by *in situ* hybridisation) in the hypothalamic arcuate nucleus for leptin receptor (OB-Rb), neuropeptide Y (NPY), pro-opiomelanocortin (POMC) and agouti-related peptide (AGRP) was unaffected by

photoperiod treatment, but food restriction increased NPY and AGRP mRNAs, in experiment 1. In experiment 2, mRNAs for POMC and cocaine- and amphetamine-regulated transcript (CART) were up-regulated and AGRP down-regulated in SD, while food restriction increased OB-Rb mRNA, increased NPY and AGRP mRNAs only in LD and decreased POMC mRNA only in SD. Thus, gene expression responded differently to photoperiod and food restriction, and the melanocortin pathway was up-regulated in SD in reproductively activated rams but not in oestradiol-implanted castrates. These data support the hypothesis that hypothalamic appetite-regulatory pathways respond differently to changes in nutritional feedback induced by photoperiod as opposed to food restriction, with gonadal steroid feedback additionally influencing the responses.

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Introduction

Recent advances in our understanding of nutritional signalling to the brain in the regulation of appetite and energy balance have recognised the importance of circulating leptin and its primary target pathways in the hypothalamic arcuate nucleus (ARC) (Ahima *et al.* 2000). The majority of studies have employed an imposed dietary or energetic challenge that elicits a 'compensatory' response in the animal in order to correct the energy imbalance. However, seasonal animals like sheep also exhibit programmed 'anticipatory' changes in voluntary food intake (VFI) and body weight (BW) in response to annual changes in photoperiod, without any imposed energetic perturbation (Mercer *et al.* 2000a). Thus appetite and BW increase in sheep kept in long-day photoperiod and decrease in sheep kept in short-day photoperiod

(Lincoln & Richardson 1998, Adam 2000). The reproductive neuroendocrine axis is also responsive to annual changes in photoperiod, with activation in short days and inhibition in long days (Lincoln & Short 1980), and it is inhibited during food or growth restriction (I'Anson *et al.* 1991). Thus, both the appetite and reproductive axes are regulated within the hypothalamus where both are sensitive to nutritional as well as to photoperiodic feedback. However, since photoperiod itself drives changes in nutritional feedback, there is clearly important interaction between these two hypothalamic inputs. Indeed, seasonal changes in the sensitivity of the appetite and reproductive neuroendocrine axes to the anorexigenic hormone, leptin, administered into the third cerebral ventricle have been reported (Clarke *et al.* 2001, Miller *et al.* 2002).

Localisation and sensitivity of gene expression to imposed changes in energy balance have been demonstrated

in sheep for critical hypothalamic receptors and appetite-regulating neuropeptides targeted by leptin (Henry *et al.* 2000, 2001, Adam *et al.* 2002, Archer *et al.* 2002). These are notably the genes encoding the leptin receptor (OB-Rb), neuropeptide Y (NPY) and agouti-related peptide (AGRP), which are up-regulated in the ARC during negative energy balance, and pro-opiomelanocortin (POMC) and cocaine- and amphetamine-related transcript (CART), which show no significant change. However, their sensitivity to a voluntary photoperiod-induced reduction in nutritional status as opposed to imposed negative energy balance has yet to be fully resolved. A recent study has reported higher ARC levels of OB-Rb, NPY and AGRP mRNAs, and lower amounts of POMC mRNA, in long-day hyperphagic rams than in short-day hypophagic rams, indicating photoperiodic regulation of these genes (Clarke *et al.* 2003). Here, we used photoperiod as a tool to alter the nutritional status of our sheep and included comparative food restriction treatments within the same study. The data further complement those of Clarke *et al.* (2003) by virtue of differences in experimental design: i.e. a shorter duration of photoperiod exposure, examining responses in both rams and steroid-clamped castrates and increasing the number of ARC neuropeptide mRNAs studied.

In this study, nutritional status was adjusted by subjecting sheep to long-day or short-day photoperiod, with *ad libitum* (AL) or restricted (R) food. We assessed the level of nutritional feedback from endocrine metabolic status, measuring circulating leptin and other putative nutritional messengers such as insulin. We assessed the hypothalamic response to nutritional feedback in terms of VFI (where appropriate), pulsatile luteinizing hormone (LH) secretion (gonadotrophin-releasing hormone (GnRH) by inference, Clarke & Cummins 1982) and expression of nutritionally sensitive hypothalamic genes. Sheep of the Soay breed were used since their responses to photoperiod are especially pronounced. Typically, appetite and gonadotrophin responses to an abrupt change in photoperiod emerge after 4 weeks and full expression of the divergent photoperiodic phenotypes is seen after 8–12 weeks (Lincoln & Richardson 1998, Adam 2000); we therefore used periods of 11–12 weeks of photoperiod exposure in the present experiments.

The amplitude of photoperiod-induced appetite cycles is greater in rams than in castrates (Kay 1985). Since testosterone concentrations show photoperiodic variation in line with seasonal breeding (Lincoln & Davidson 1977), this indicates that changes in gonadal steroid concentrations may influence hypothalamic appetite drive. Changes in gonadal steroid feedback also influence nutritional feedback effects on hypothalamic GnRH output, given that the suppression of LH in sheep by chronic food restriction is amplified in the presence of oestradiol (Foster & Olster 1985, Beckett *et al.* 1997). We therefore assessed the contribution of

concurrent alterations in gonadal steroid status to the observed responses to alterations in nutritional status in the present study. Gonadal steroid feedback was either clamped at a constant physiological level (using oestradiol-implanted castrates) in the first experiment, or allowed to free-run and change with the photoperiods by using rams in the second experiment.

These experiments addressed the hypothesis that the ovine hypothalamus responds differently to photoperiod-induced changes in nutritional feedback as opposed to experimentally imposed food restriction in terms of gene expression for primary leptin targets in the hypothalamus, and that these responses are influenced by gonadal steroid feedback.

Materials and Methods

All procedures were licensed under the Animals (Scientific Procedures) Act 1986 and received prior approval from the relevant local Ethical Review Committee.

Animals and treatments

Experiment 1 Castrated adult male sheep of the Soay breed, initial BW 33.3 ± 0.80 kg and body condition score (BCS) 2.3 ± 0.02 (scale: 0 = emaciated to 5 = obese; Russel *et al.* 1969), were housed individually at Glensnaugh Research Station (57°N) in October 1997, i.e. from a background of shortening natural photoperiod in the autumn. Two weeks before the start of experiment 1 each sheep received subcutaneously two Silastic oestradiol-containing implants made from Silastic tubing with an external diameter of 4.8 mm (Osteotec Ltd, Christchurch, Dorset, UK) and each containing a 15 mm packed column of 17β -oestradiol (Sigma UK, Poole, Dorset, UK) (after Adam & Findlay 1998); this raised circulating oestradiol concentrations to a steady 4.0 ± 0.20 pg/ml (measured by the RIA of Mann *et al.* 1995), as designed to be equivalent to physiological values seen in ewes in the luteal phase of the oestrous cycle (Goodman 1994). For 11 weeks sheep were exposed to either short-day (SD; 8 h light:16 h darkness) or long-day (LD; 16 h light:8 h darkness) artificial photoperiods ($n=16$ per photoperiod). Daily at 0830 h they were given a complete diet ('Soay mix'; North Eastern Farmers, Bannermill, Aberdeen, UK, containing 40% bruised barley and 35% dried grass pellets as the main ingredients, and 11.6 MJ metabolizable energy (ME) kg/dry matter (DM)). Within each photoperiod, half were fed AL, with a refusal margin of at least 10%, and half were fed an amount restricted (R) to 50% requirements for BW maintenance (0.42 MJ ME/kg BW^{0.75}; Robinson 1983). BW and BCS were measured weekly and VFI was recorded on 5 days of each week (Monday to Friday) by weighing residues before introducing fresh food.

Experiment 2 Adult rams of the Soay breed, initial BW 27.0 ± 1.01 kg, were housed individually at Duthie Experimental Farm (57°N) in May 2000. For 16 weeks prior to the experiment they were exposed to either SD or LD ($n=12$ per photoperiod) and fed AL. The photoperiods were then reversed for the 12-week experiment. By virtue of the preceding photoperiod, initial BW at this stage was 34.6 ± 1.19 kg in SD and 31.0 ± 0.89 kg in LD; half in each photoperiod were fed AL with a minimum refusal margin of 10% and half were fed an amount restricted (R) to 70–100% requirements for BW maintenance. The maintenance requirement was calculated for the actual or predicted SD asymptotic BW for each sheep, equating to 100% maintenance for the rams transferred from SD to LD, and 70% for the rams transferred from LD to SD (effectively increasing to 100% as these sheep lost BW). They were fed twice daily at 0800 h and 1600 h a complete diet (containing 50% chopped hay and 30% bruised barley as the main ingredients, and 10 MJ ME kg/DM). BW and BCS were measured weekly and VFI was recorded daily.

Tissue collection

Blood samples were collected by jugular venipuncture twice weekly before feeding (experiment 1) or weekly at 2 h after feeding (experiment 2). Plasma was stored at -20°C prior to analysis for insulin, leptin, glucose and non-esterified fatty acids (NEFA). On the last or penultimate day before they were killed, blood samples were collected via temporary jugular catheters every 15 min for 8 h, starting at lights-on, and plasma was stored at -20°C for subsequent LH analysis.

The sheep were killed by a lethal i.v. dose of sodium pentobarbitone (Euthatal; Rhone Merieux Ltd, Harlow, Essex, UK) between 0830 h and 1630 h on 2 consecutive days in both experiments. One animal was killed from each group in turn in order to minimise any time of day effect on mean post-mortem results. The brain was excised, flash frozen in isopentane over dry ice and stored at -80°C . In addition, testes were removed and weighed in experiment 2.

Plasma analyses

Plasma concentrations of glucose and NEFA were determined by automated analysis, with sensitivity and intra- and interassay coefficients of variation (CV) of 0.04 mmol/l, 2.0% and 4.0% respectively for NEFA, and 0.34 mmol/l, 0.35% and 2.3% respectively for glucose. Leptin concentrations were determined by homologous RIA (Marie *et al.* 2001) with sensitivity 0.5 ng/ml, intra-assay CV 12.0% and interassay CV 16%. The RIA for insulin (MacRae *et al.* 1991) had sensitivity 0.2 $\mu\text{IU/ml}$, intra-assay CV 4.7% and interassay CV 4.9%. Finally, LH was measured

by RIA (McNeilly *et al.* 1986) with sensitivity 0.05 ng/ml and intra- and interassay CV 7.9% and 9.3% respectively.

In situ hybridisation

Coronal cryostat sections (20 μm) of hypothalamic tissue were thaw-mounted onto slides double-coated with gelatin and poly-L-lysine, and stored at -80°C . Gene expression for OB-Rb, NPY, AGRP, POMC and CART was measured by *in situ* hybridisation, using techniques described in detail and fully validated elsewhere (Mercer *et al.* 1995, Adam *et al.* 1997). A 308 bp riboprobe complementary to fragments of the intracellular domain of OB-Rb was generated from a cloned sheep cDNA as described previously (Mercer *et al.* 1998). The NPY probe was generated from a 500 bp fragment of rat prepro-NPY cDNA and has been validated for use on sheep tissues (Adam *et al.* 1997). The CART probe (used only in experiment 2) was generated from a cloned sheep 299 bp cDNA as described previously (Barrett *et al.* 2001), and AGRP and POMC probes were generated from cloned Siberian hamster cDNA fragments of 229 bp and 344 bp respectively (Mercer *et al.* 2000b) and have been validated in sheep brain (Adam *et al.* 2002). Briefly, sections were fixed, acetylated and hybridised overnight at 58°C using ^{35}S -labelled cRNA probes ($1-1.5 \times 10^7$ c.p.m./ml). They were then treated with RNase A, desalted, with a final high stringency wash (30 min) in $0.5 \times \text{SSC}$ at 60°C , dried and apposed to Hyperfilm β -max (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Bucks, UK). Intensity and total area of hybridisation over the ARC were quantified on autoradiographic images, using the Image-Pro Plus system (Media Cybernetics, Silver Spring, MD, USA). The integrated intensity of the hybridisation signal was then computed using standard curves generated from ^{14}C autoradiographic micro-scales (Amersham Pharmacia Biotech). For each probe, three to six sections from the medial hypothalamus (approximately 1.5–1.75 mm rostral to the opening of the third ventricle at the base of the hypothalamus just in front of the mamillary body) were analysed for each brain and the results averaged to give a single value for each animal. Within each experiment, all sections for a single probe were processed together and placed against the same sheet of autoradiographic film.

All reagents were obtained from Sigma unless otherwise stated.

Statistical analysis

LH secretory parameters were determined using Pulsefit software (supplied by R. Kushler and M. Brown, University of Michigan, Rochester, MI, USA). Groups were compared by two-way analysis of variance (using SigmaStat, experiment 1, or Minitab, experiment 2). Results are presented as means \pm S.E.M. and differences were considered significant at $P < 0.05$.

Results

Food intake, BW, BCS and plasma metabolic status

Food intake, BW, BCS and plasma concentration measurements in the final week before the animals were killed were averaged to provide terminal values that were representative and temporally aligned with the hypothalamic neuropeptide gene expression analysis.

Experiment 1 (oestradiol-implanted castrates) Mean VFI was significantly higher in LDAL than SDAL ($P<0.05$) and lower in R than AL groups ($P<0.001$) (Fig. 1a). Mean terminal BW was greater in AL than R sheep within each photoperiod ($P<0.001$) but was greater in SD than LD sheep ($P<0.001$) (Fig. 1a). Mean BCS remained in the range 2–2.25 for all sheep and terminal values were not significantly different between the groups (data not shown).

Mean plasma leptin concentrations were higher in AL than R animals ($P<0.01$), but not significantly different between SD and LD. Plasma insulin concentrations were higher in AL than R sheep ($P<0.01$) and higher in SD than in LD ($P<0.01$) (Fig. 1a). There were no differences between photoperiod or intake groups in mean plasma glucose concentrations; mean plasma NEFA concentrations were higher in R than AL sheep within each photoperiod ($P<0.05$) and higher in SD than LD ($P<0.05$) (Fig. 1a).

Experiment 2 (rams) Mean VFI was twice as high in LDAL than in SDAL rams ($P<0.001$) and no different between SDR, SDAL and LDR groups (Fig. 1b). BW was initially higher in SD than LD groups following 'priming' in the opposite photoperiod. BW decreased in SDAL and SDR rams and increased in LDAL rams to reach similar terminal values across these three groups. BW of LDR sheep decreased during the experiment and was lower terminally than that of the other groups ($P<0.05$) (Fig. 1b). Terminal BCS was similar between SDAL and LDAL groups, but higher in SDAL than SDR (mean values 2.62 ± 0.085 vs 2.37 ± 0.085 , $P<0.05$) and in LDAL than LDR rams (2.50 ± 0.064 vs 2.25 ± 0.000 , $P<0.05$).

Plasma leptin concentrations were higher in LDAL than all of the other groups ($P<0.01$); insulin concentrations were lower in LDR rams than in all of the other groups ($P<0.001$; Fig. 1b). Both hormones were reduced by R in LD (leptin, $P<0.01$; insulin, $P<0.001$) but not in SD (Fig. 1b). Plasma glucose was lower in SD than LD ($P<0.001$), with no effect of R; NEFA concentrations were elevated by R in both photoperiods ($P<0.001$), and were also elevated in SDAL compared with LDAL rams ($P<0.001$) (Fig. 1b).

Reproductive status

Experiment 1 (oestradiol-implanted castrates) Mean plasma LH concentration was higher in SD than LD sheep

Table 1 Paired testes weights (g, means \pm S.E.M.) in rams kept for 12 weeks in LD or SD photoperiod with AL or R food (experiment 2, $n=6$ /group)

	AL	R
Photoperiod		
LD	220.3 \pm 13.34	184.5 \pm 7.95
SD	368.0 \pm 7.75	348.0 \pm 7.93

($P<0.05$) but similar between intake groups. Mean LH pulse frequency was unaffected by photoperiod but higher in AL than R sheep ($P<0.05$). Mean LH pulse amplitude was not significantly affected by either photoperiod or intake, but baseline LH concentration was higher in SD than LD ($P<0.05$) (Fig. 2a). There was no interaction between photoperiod and intake on LH secretory parameters.

Experiment 2 (rams) Mean plasma LH concentration was similar between photoperiod and intake groups. Mean LH pulse frequency was higher in SD than LD ($P<0.001$) but similar between intake groups within each photoperiod. Mean LH pulse amplitude and baseline were higher in LD than SD ($P<0.05$ and $P<0.01$ respectively) but similar between intake groups (Fig. 2b). Mean weight of testes when the animals were killed was higher in SD than LD rams ($P<0.001$) with no significant effect of intake (Table 1).

Hypothalamic ARC gene expression

Experiment 1 (oestradiol-implanted castrates) OB-Rb gene expression levels were highly variable and were not significantly different between the groups (Fig. 3a). R increased amounts of NPY ($P<0.01$) and AGRP gene expression ($P<0.05$), with no effect of photoperiod (Fig. 3a). POMC gene expression was not different between photoperiods or intake groups (Fig. 3a).

Experiment 2 (rams) OB-Rb gene expression in the ARC was increased by R ($P<0.001$), with no effect of photoperiod (Fig. 3b). NPY and AGRP mRNAs were increased by R in LD ($P<0.001$) but not in SD; there was no effect of photoperiod alone on NPY gene expression, but AGRP gene expression was higher in LD than SD ($P<0.01$) (Figs 3b and 4). POMC gene expression was higher in SDAL than LDAL ($P<0.05$), and was decreased by R in SD but not in LD ($P<0.05$). CART gene expression in the ARC was higher in SD than LD ($P<0.05$), and there was no effect of R (Figs 3b and 4).

Discussion

Photoperiod treatment alone induced changes in appetite, with VFI higher in LD and lower in SD in both rams and

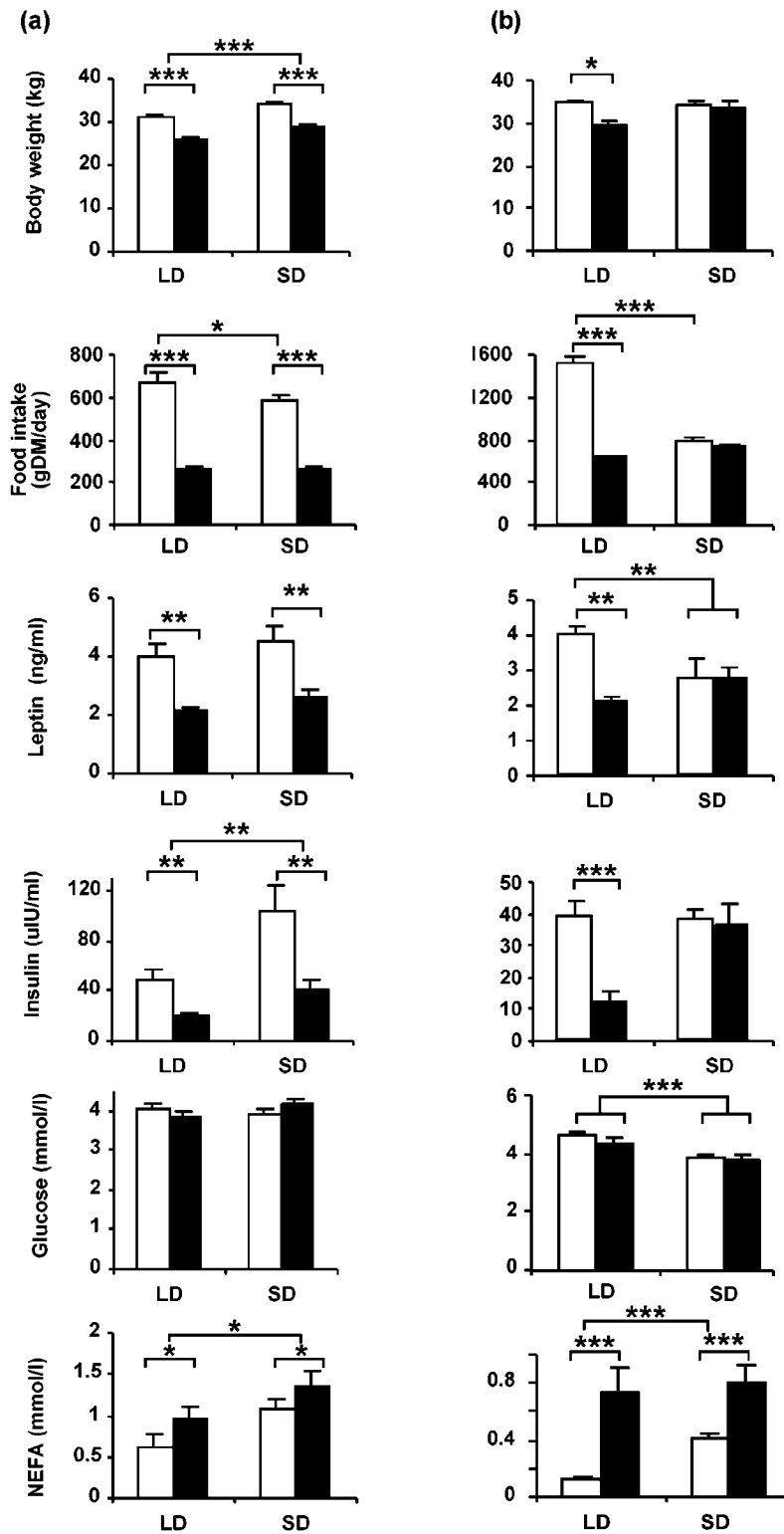


Figure 1 BW, food intake and plasma concentration of leptin, insulin, glucose and NEFA in sheep kept in LD or SD photoperiod with AL (open bars) or R (solid bars) food: (a) oestradiol-implanted castrates (11 weeks, experiment 1) and (b) rams (12 weeks, experiment 2). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Values are means \pm S.E.M.

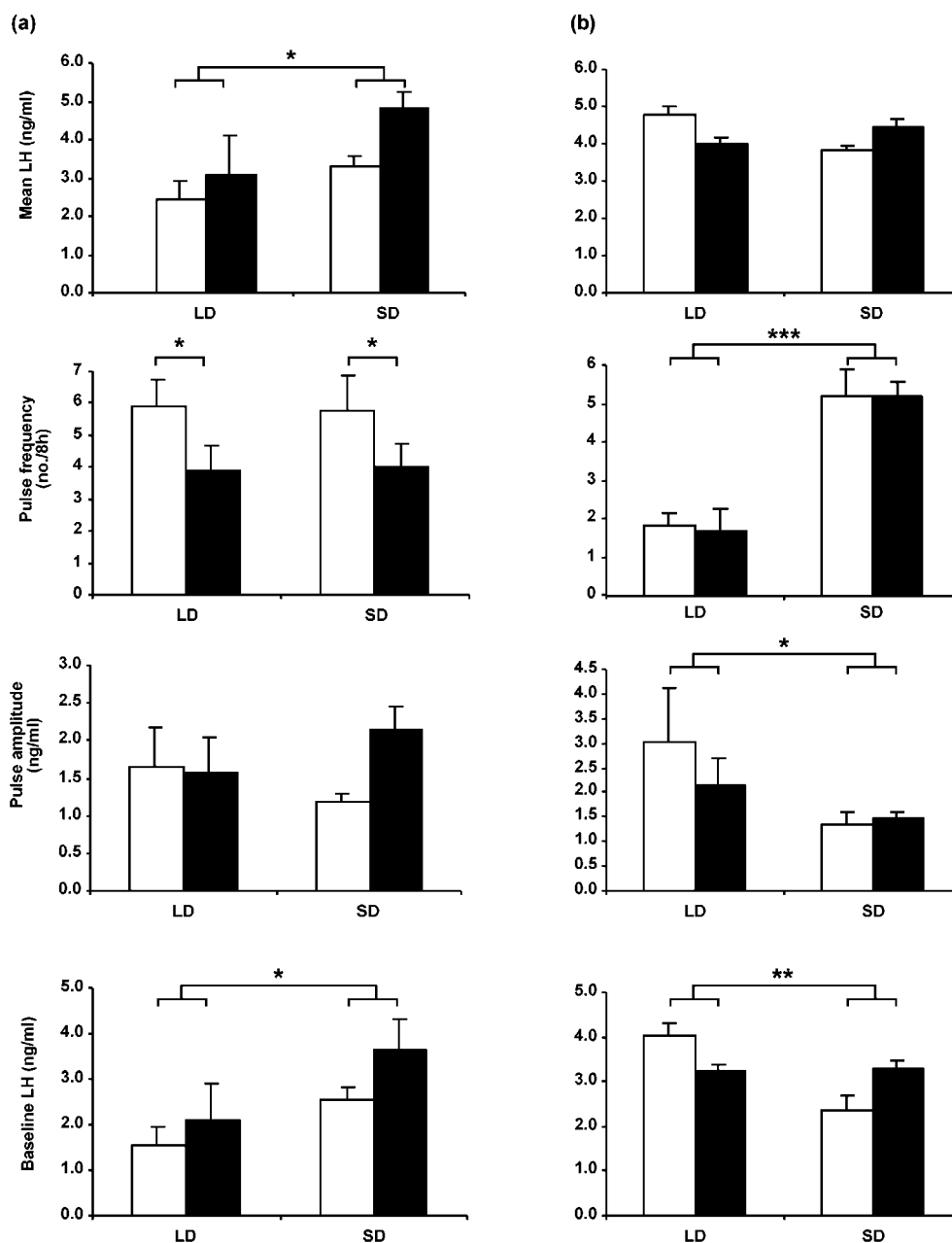


Figure 2 Plasma LH secretory parameters in sheep kept in LD or SD photoperiod with AL (open bars) or R (solid bars) food: (a) oestradiol-implanted castrates (11 weeks, experiment 1) and (b) rams (12 weeks, experiment 2). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Values are means \pm S.E.M.

oestradiol-implanted castrates, as expected (Kay 1985). Importantly, these AL-feeding animals were satiated in both photoperiods and this was generally reflected in their plasma metabolic profiles. In both experiments, plasma NEFA concentrations were lower in LD, indicative of an anabolic state, and higher in SD, indicative of catabolism and mobilisation of fat reserves. Terminal plasma leptin

and glucose concentrations in experiment 2 (rams) were higher in LD than SD, although insulin values and BW were similar in both photoperiods. However, in experiment 1 (oestradiol-implanted castrates), plasma leptin and glucose values were not different between the photoperiods, and both plasma insulin concentrations and BW remained higher in SD than LD. These data indicated that

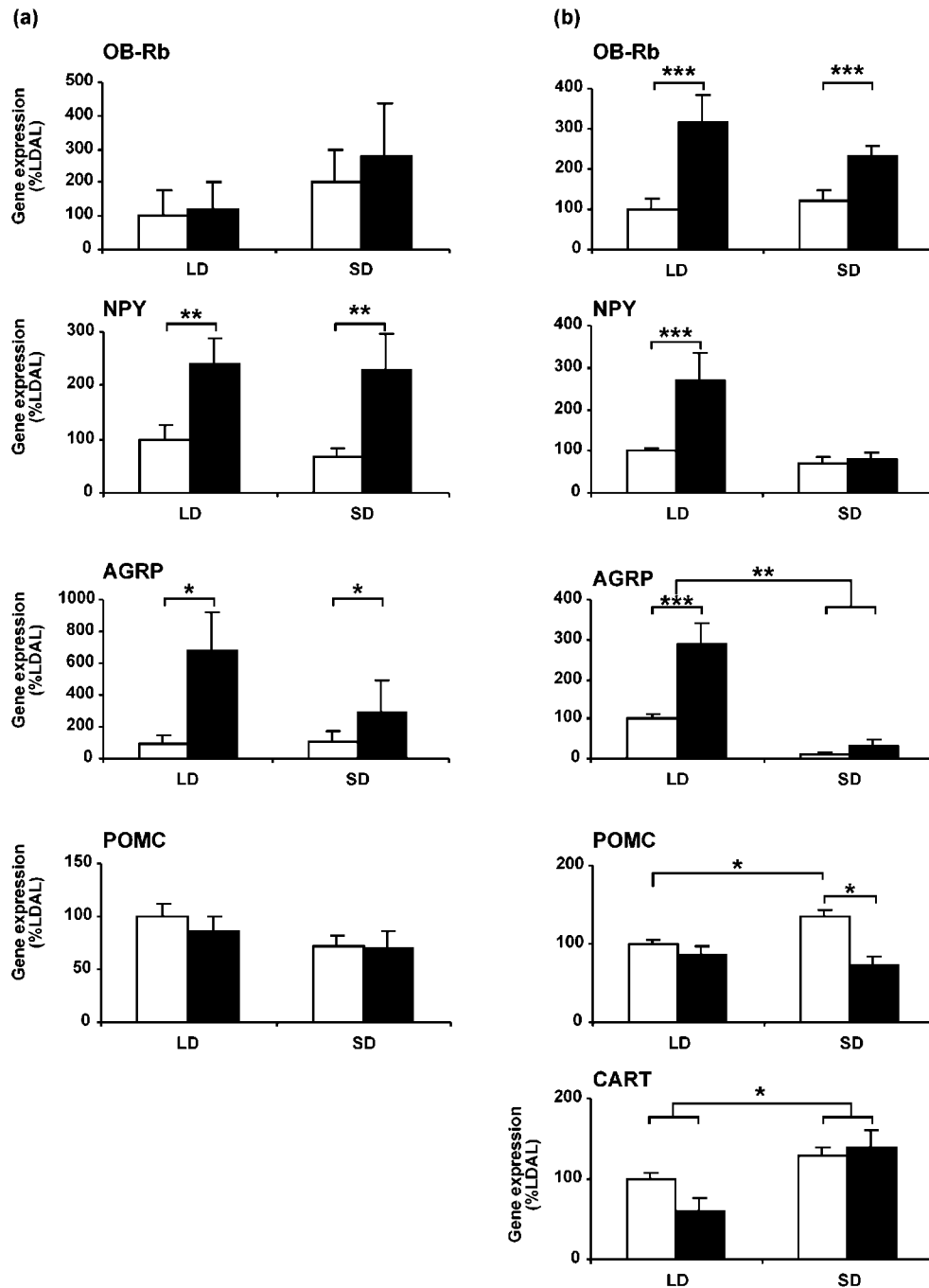
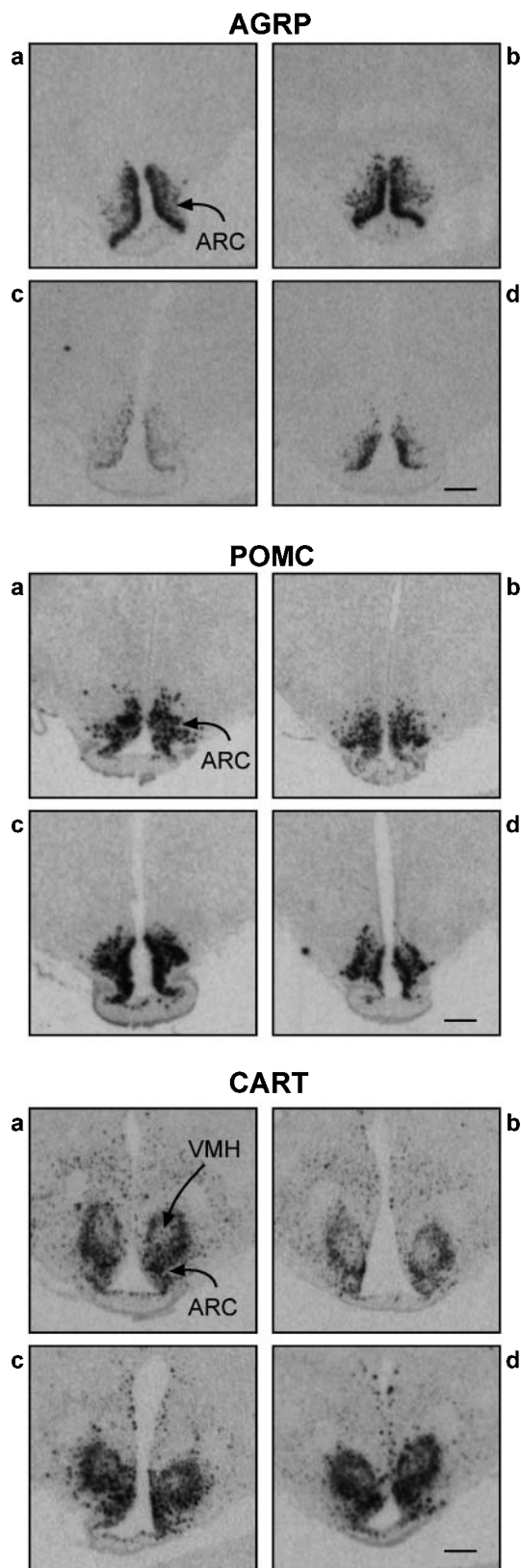


Figure 3 Gene expression for OB-Rb, NPY, AGRP, POMC and CART in the ARC of sheep kept in LD or SD photoperiod with AL (open bars) or R (solid bars) food: (a) oestradiol-implanted castrates (experiment 1) and (b) rams (experiment 2). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Values are means \pm S.E.M.

the contrasting anabolic and catabolic photoperiodic phenotypes were less well established in experiment 1 (without priming in the opposite photoperiod) than in experiment 2 (with priming). In contrast to photoperiod

treatment alone, food restriction caused BW loss in both photoperiods in both experiments. The restricted animals were clearly unsatiated, mobilising body reserves (with increased plasma NEFA in all R groups compared with



their respective AL groups) and with reduced circulating insulin and leptin (except in the more mildly restricted experiment 2 SDR rams) but with glucose levels maintained. Thus altogether there were clear differences in nutritional state between the groups of animals against which to compare the activities of nutritionally sensitive, appetite-regulating hypothalamic pathways. In addition, these groups had either a constant low level of oestradiol feedback (experiment 1, oestradiol-implanted castrates) or, as indicated by the differences in testis size (Lincoln & Davidson 1977), high circulating testosterone in SD and low testosterone in LD (experiment 2 rams).

Photoperiod treatment alone had no effect on OB-Rb gene expression in either experiment. This was despite elevated plasma leptin in LD in experiment 2, although it could perhaps be explained in experiment 1 by the lack of significant difference in circulating leptin between the photoperiods. However, the rams of Clarke *et al.* (2003) also showed no differences in plasma leptin between the photoperiods, yet had up-regulated OB-Rb mRNA in LD. In contrast, male Siberian hamsters in LD have both increased circulating leptin and up-regulated OB-Rb mRNA in the ARC (Mercer *et al.* 2001). The foregoing observations suggest that photoperiod can override the effects of systemic leptin concentration on hypothalamic expression of its receptor and that photoperiod may directly influence leptin receptor gene expression. Furthermore, the decrease in plasma leptin as a result of food restriction had no significant effect on OB-Rb mRNA in experiment 1 whereas it was associated with increased OB-Rb gene expression in LD in experiment 2 as expected (Mercer *et al.* 2001). However, the increase in OB-Rb gene expression induced by food restriction in SD in experiment 2 was not associated with decreased plasma leptin, again suggesting dissociation of the ligand-receptor relationship. Nonetheless, it appears from experiment 2 that OB-Rb expression levels are not up-regulated by low circulating leptin brought about by photoperiod-induced change in nutritional status but they are up-regulated when low circulating leptin is induced by food restriction. Overall, the findings support the hypothesis that hypothalamic OB-Rb gene expression responses to leptin feedback are regulated by photoperiod. There was no evidence that OB-Rb gene expression was affected by gonadal steroids.

Photoperiod treatment alone had no effect on NPY gene expression in either oestradiol-implanted castrates

Figure 4 Typical coronal hypothalamic autoradiographs showing gene expression in the ARC for AGRP, POMC and CART in rams kept for 12 weeks in (a and b) LD or (c and d) SD photoperiod with (a and c) AL or (b and d) R food (experiment 2). AGRP gene expression is higher in LD than SD and is increased by R; POMC gene expression is higher in SD than LD and is decreased by R only in SD; CART gene expression is higher in SD than LD, with no effect of R. VMH=ventromedial hypothalamic nucleus. Bar=1mm.

(experiment 1) or rams (experiment 2). Similar results are seen in freely feeding Siberian hamsters (Adam *et al.* 2000, Mercer *et al.* 2001); however, Clarke *et al.* (2003) reported that amounts of NPY mRNA were higher in LD than SD rams. Reasons for the apparently conflicting results from the two sheep studies may include differences in the duration of photoperiod exposure used (18 weeks in Clarke *et al.* (2003) and 11–12 weeks in the present study) and differences in the methodologies employed; i.e. Clarke *et al.* (2003) used perfused brains whereas the present brains were frozen, and the *in situ* hybridisation signal was analysed in photographic emulsion-dipped sections in the former study by counting silver grains at the cellular level whereas we analysed the signal by film autoradiography and optical densitometry measurements at the level of the whole hypothalamic nucleus. Nonetheless, in both photoperiods in experiment 1 and in LD in experiment 2, orexigenic NPY gene expression was up-regulated by imposed food restriction, in agreement with previous findings (Adam *et al.* 1997), and the lack of NPY up-regulation in SDR in experiment 2 probably reflected the lack of severity of the food restriction, which had not altered the circulating leptin concentrations unlike the other restriction treatments. Circulating leptin appeared to be inversely related to corresponding NPY gene expression data in the AL vs R comparison, consistent with leptin feedback regulating NPY (e.g. Henry *et al.* 1999); however, this relationship breaks down when comparing the LDR and SDAL rams in experiment 2. They had similar food intake and plasma leptin and yet NPY gene expression was up-regulated in the presence of reduced leptin caused by the food restriction but not in the presence of the leptin signal of similar magnitude induced by SD. This would indicate that NPY neurones are somehow primed to anticipate and not react to reduced nutritional (or leptin) feedback during SD. Conversely, the difference in NPY gene expression between the photoperiods was not accompanied by differences in circulating leptin in the rams of Clarke *et al.* (2003). Both scenarios are consistent with photoperiod feedback overriding nutritional (leptin) feedback in regulating NPY gene expression. Similarly, the decrease in plasma insulin in the food-restricted animals could also have been causally related to the increase in NPY gene expression (Schwartz *et al.* 1992), yet the decreased plasma insulin seen in the AL-fed LD vs SD oestradiol-implanted castrates was not associated with altered NPY gene expression. Finally, NPY gene expression was not affected by gonadal steroid status in the present animals.

ARC gene expression for the orexigenic endogenous melanocortin antagonist AGRP was up-regulated by R in both experiments (except in the more mildly restricted SDR group in experiment 2) in agreement with earlier findings (Henry *et al.* 2001). AGRP mRNA was also up-regulated by LD in the rams (experiment 2), in agreement with Clarke *et al.* (2003),

but not in the oestradiol-implanted castrates (experiment 1). This indicated that testosterone, circulating concentrations of which would have been elevated in SD given the increased testis size (Lincoln & Davidson 1977), might be either directly down-regulating AGRP gene expression or indirectly facilitating down-regulation of AGRP by SD. Such an effect of testosterone has not been reported in the literature but it is noteworthy that AGRP gene expression is conversely decreased in male Siberian hamsters in LD when their circulating testosterone is highest (Mercer *et al.* 2000b). Furthermore, AGRP gene expression was decreased in SD hamsters given subcutaneous implants to raise systemic testosterone concentrations (J G Mercer, personal communication) and AGRP gene expression is apparently not regulated by photoperiod in female Siberian hamsters (Adam *et al.* 2000). It is therefore tempting to speculate that testosterone inhibition of orexigenic AGRP may contribute to the greater amplitude of photoperiod-induced appetite cycles seen in rams compared with castrates (Kay 1985). Similarly, there was a clear influence of testosterone (in this case stimulatory) on ARC gene expression for POMC, the precursor for the anorexigenic peptide α -melanocyte-stimulating hormone. POMC mRNA was up-regulated in SD in the reproductively active rams (in agreement with Clarke *et al.* 2003) but not in the oestradiol-implanted castrates; it is also up-regulated in LD in Siberian hamsters when they are reproductively active (Mercer *et al.* 2001). Consistent with these data, Hileman *et al.* (1998) reported ARC POMC mRNA to be down-regulated in LD in castrated sheep but only in those with testosterone replacement, suggesting that testosterone amplifies the POMC response to photoperiod. It is tempting to speculate that the melanocortin pathway may be implicated in driving photoperiod-induced appetite cycles in the ram and/or in mediating the testosterone amplification of these cycles. In addition, the present data indicated that testosterone might also be implicated in amplifying the POMC response to negative energy balance, since POMC mRNA was down-regulated by R compared with AL, in agreement with published sheep data (McShane *et al.* 1993), but this response was seen only in rams and only in SD.

Gene expression for the anorexigenic neuropeptide CART was up-regulated in the ARC of the SD (reproductively active) rams. In our laboratory, up-regulation of CART mRNA is a consistent finding in SD (reproductively inactive) Siberian hamsters of both sexes and at different ages (Adam *et al.* 2000, Mercer *et al.* 2003). It is unlikely therefore that photoperiod-induced changes in CART are affected by changes in gonadal steroids, and it is probable that CART mRNA was also elevated in SD in the castrates of experiment 1. CART gene expression in the ARC was not affected by food restriction in either

photoperiod, and overall the data are consistent with a role for CART in mediating the anorexic response to SD (Mercer *et al.* 2003).

The characteristic photoperiod effects on the reproductive neuroendocrine axis were clearly expressed in rams, with LH pulse frequency (and GnRH by inference) markedly increased in SD, but there was no effect of food restriction in either LD (with low circulating testosterone) or SD (with high testosterone). Although oestradiol-implanted castrates had increased LH output in SD, this was not attributable to an increase in pulse frequency, suggesting a reduced intra-hypothalamic influence of photoperiod on GnRH pulsatile secretion compared with rams. These data are consistent with amplification by the increased concentration of gonadal steroids of SD stimulation of the reproductive neuroendocrine axis in rams, and this axis therefore appears more sensitive to photoperiod in rams than in oestradiol-implanted castrates. Conversely, the data also indicated that sensitivity of the reproductive neuroendocrine axis to nutritional feedback is increased in oestradiol-implanted castrates compared with rams. Food restriction reduced LH pulse frequency in the oestradiol-implanted castrates but not in the rams, irrespective of photoperiod, supporting the hypothesis that oestradiol sensitises the reproductive neuroendocrine axis to altered nutritional feedback (Foster & Olster 1985, Beckett *et al.* 1997). In the more photosensitive rams, decreased nutritional feedback could not override photoperiodic regulation of reproductive neuroendocrine output. Thus, there was no evidence for photoperiodic regulation of the reproductive neuroendocrine response to nutritional feedback, irrespective of gonadal steroid status, but gonadal steroid status clearly influenced photoperiodic and nutritional effects on appetite-regulating hypothalamic pathways.

Overall these data are consistent with a causal role for CART in SD-induced anorexia in seasonal animals (Mercer *et al.* 2003) and with a novel role for testosterone interacting with the melanocortin pathway to amplify SD anorexia in rams. The results support the hypothesis that the sheep hypothalamus responds differently to voluntary photoperiod-induced changes in nutritional feedback as opposed to imposed changes in nutritional feedback induced by food restriction in terms of gene expression for appetite-regulating hypothalamic neuropeptides, and these responses were influenced by gonadal steroid status.

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