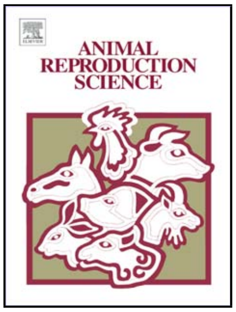
http://dx.doi.org/10.1016/j.anireprosci.2009.03.01...

Celi, P., Miller, D.W., Blache, D. and Martin, G.B. (2010) Interactions between nutritional and opioidergic pathways in the control of LH secretion in male sheep. Animal Reproduction Science, 117 (1-2). pp. 67-73.

https://researchrepository.murdoch.edu.au/id/eprint/6448



Title: Interactions between nutritional and opioidergic pathways in the control of LH secretion in male sheep

Authors: Pietro Celi, David W. Miller, Dominique Blache, Graeme B. Martin

PII: S0378-4320(09)00085-2
DOI: doi:10.1016/j.anireprosci.2009.03.011
Reference: ANIREP 3817

To appear in: *Animal Reproduction Science*

Received date: 9-11-2008
Revised date: 13-2-2009
Accepted date: 25-3-2009

Please cite this article as: Celi, P., Miller, D.W., Blache, D., Martin, G.B., Interactions between nutritional and opioidergic pathways in the control of LH secretion in male sheep, *Animal Reproduction Science* (2008), doi:10.1016/j.anireprosci.2009.03.011

Interactions between nutritional and opioidergic pathways in the control of LH secretion in male sheep

Pietro Celi[1,3*], David W. Miller[2,3], Dominique Blache[3] and Graeme B. Martin[3]

1 Faculty of Veterinary Science, University of Sydney, PMB 3, 425 Werombi Rd, Camden, NSW 2570, Australia.
2 School of Veterinary & Biomedical Sciences, Murdoch University, Murdoch, WA 6150, Australia.
3 UWA Institute of Agriculture, The University of Western Australia, Crawley, WA 6009, Australia.

* *Corresponding author:* Telephone: Int + 61 2 9351 1782; Facsimile: Int + 61 2 9351 1693
Email: pietroc@camden.usyd.edu.au

Abstract

Our aim was to determine the role of opiodergic processses in the effects of nutrition on the secretion of LH pulses in the mature male sheep. In the first of three experiments, adult Merino rams were acclimatised to a maintenance diet and then allocated to one of three dietary groups (n = 5): continuation of the maintenance diet (Group M); reduction to half of the maintenance allocation (Group HM); or supplementation of the maintenance diet with lupin grain (Group HD). An initial administration of naloxone (2 mg/kg body weight, i.v.) was followed at 40-min intervals by 3 further administrations (1 mg/kg). Blood was sampled every 20 min for 12 h before the initial naloxone administration and then for a further 6 h. LH pulse frequency after naloxone treatment was significantly higher in Group HD than in Group HM ($P < 0.05$). The second study tested whether the response to naloxone depended on calcium status. We used 22 adult Merino rams in two consecutive experiments, one in which the rams were fed a maintenance diet, and one in which the rams were fed with the maintenance diet plus 1 kg lupin grain for 5 weeks. In both experiments, rams were allocated to groups that received one of the following treatments: a) 0.02 g/kg calcium borogluconate + 0.2 mg/kg naloxone hydrochloride (Nal + Ca^{2+}; n = 6); b) 0.2 mg/kg naloxone hydrochloride (Nal; n = 6); c) 0.02 g/kg calcium borogluconate (Ca^{2+}; n = 5); d) 0.1 ml/kg NaCl 0.9% (Saline; n = 5). All treatments were given as a single i.v. administration daily for 5 days. Blood was sampled every 20 min for 24 h during the acclimatization period (Day 0) and on the last day (Day 5) of treatment. In the first study (under maintenance), none of the treatments affected LH pulse frequency. In the second study (the lupin-supplemented rams), LH pulse frequency was significantly increased ($P < 0.05$) by the administration of naloxone + Ca^{2+}, naloxone alone and Ca^{2+} alone. Overall, rams on a low plane of nutrition showed the smallest response to naloxone, suggesting that an opioidergic mechanism is not involved in the suppressive effect of restricted nutrition on the gonadotrophic axis. Rather, because testosterone secretion was increased on the high plane of nutrition, the LH responses to naloxone are better explained by the effects of testosterone on opioidergic mechanisms. Finally, we failed to observe any interaction between opioids and calcium in the control of LH secretion.

Extra Key words: naloxone; calcium; testosterone; LH.

1. Introduction

In male sheep, when genetic and photoperiodic influences are permissive, the reproductive centres in the preoptic-hypothalamic continuum are strongly and consistently affected by nutrition (Blache et al. 2006). This is evidenced by changes in the frequency of pulses of luteinizing hormone (LH), reflecting pulses of gonadotrophin-releasing hormone (GnRH), within a few hours after the feeding of a supplement of energy and protein (Zhang et al. 2004). This response stabilizes after 5–7 days and, interestingly, diminishes and finally disappears after 3-4 weeks, around the time that gains in body weight and fat can be detected (Blache et al. 2006).

The signalling pathways that link dietary status and the activity of the neurons that produce the GnRH signal are thought to be partly hormonal in nature (Woods et al. 2000) and may reflect the amount of body reserves (Blache et al. 2006). Among the possible signals are endogenous opioid peptides (EOPs) that are known to inhibit the activity of GnRH neurons. In sheep, EOPs play roles in gonadotrophin responses to gonadal steroids and photoperiod (Tortonese, 1999) and they have been suggested by Ebling et al. (1990) to be involved in the metabolic/nutritional control of gonadotrophin secretion. In other species, the inhibition of LH secretion by EOPs is affected by alterations in metabolic state (Ishizuka et al. 1984; Gregg et al. 1986; Kryzanowska and Czekalski 1992). For example, the opioid antagonist, naloxone, can reverse the inhibitory effects of fasting on LH secretion in rats (Briski 1984; Dyer et al. 1985) and alleviate the metabolic suppression of LH secretion in postpartum dairy and beef cows (Whisnant et al. 1986; Canfield and Butler, 1991). In sheep, low doses of naloxone can stimulate LH secretion (Lincoln et al., 1987), reverse lactational anoestrus (Minoia et al., 1995), facilitate stimulation of LH secretion by exogenous melatonin (Misztal and Romanowicz, 2005) and facilitate the display of oestrous behaviour (Fuentes, 1989). We therefore used naloxone to test whether opioidergic mechanisms are involved in the acute effects of nutrition on LH secretion in the intact Merino ram.

We explored the potential role of calcium in the response to naloxone (Sciorsci et al., 2000; Minoia and Sciorsci, 2001). Calcium-mediated activity controls GnRH secretion at the hypothalamic level (Kalra et al., 1993; Ghosh et al., 1996) and, at pituitary level, extracellular calcium plays an important role in the effect of GnRH on LH secretion (Bourne and Baldwin, 1980; Conn et al., 1981). Studies with rat pituitary cells *in vitro* have shown that the ability of GnRH to stimulate LH release is reduced in a low-calcium environment (Conn, 1986; Ramey et al., 1987). Importantly, μ opioid receptors seem to be involved in the control of LH secretion (Panerai et al., 1985). When they bind to μ-receptors, opioids inhibit calcium-dependent neurotransmitter release by inhibiting voltage-dependent calcium channels, and a μ-receptor antagonist can prevent this response (Schroeder et al., 1991). We therefore tested whether the effect of naloxone on LH secretion depends on calcium status in rams on high and low planes of nutrition.

2. Materials and methods

All experimental protocols conformed to the Code of Practice formulated by the National Health & Medical Research Council of Australia and implemented by the Animal Ethics Committee of The University of Western Australia (AAA61/96/96).

2.1 Animals and experimental protocol

The animals were housed indoors in individual pens under natural photoperiod at The University of Western Australia (32° S, 115° E). Between experiments, they were kept outdoors.

Experiment 1: Interactions between nutrition and naloxone on LH secretion in the male sheep

Fifteen adult Merino rams were housed indoors in individual pens under natural photoperiod and acclimatised to the maintenance diet (MD) of 680 g oaten chaff + 170 g lupins/head daily for 3 weeks, in early November (late non-breeding season, southern hemisphere). They were then allocated to one of three nutritional treatments (n=5): continuation of the maintenance diet (Group MD); restriction to half of the maintenance allocation (Group HMD; initially 480 g chaff + 120 g lupins per day, later reduced to 320 g chaff + 80 g lupins per day); or fed the maintenance diet plus a supplement of lupin grain (Group HD; High Diet; initially 680 g chaff + 920 g lupins, and increased in increments over 5 days to 960 g chaff + 1240 g lupins). The animals were kept on the treatment diets for 6 weeks before taking part in the naloxone study at the end of January (beginning of the breeding season). Animals consumed all feed that was provided daily and were weighed before the administration of the treatments to calculate the dose of opioid antagonist to administer.

On the basis of previous studies with sheep (Caraty et al. 1987; Ebling et al. 1990), an appropriate treatment regime with naloxone hydrochloride (Sigma-Aldrich Pty Ltd, NSW, Australia) for disinhibiting pulsatile LH secretion is to administer an initial administration (i.v.) of 2 mg naloxone/kg body weight, followed at 40 minute intervals by a further three administrations of 1 mg/kg. Beginning at 06:20 h, blood was sampled via indwelling jugular catheters every 20 min for 12 h prior to the initial naloxone administration and then for a further 6 hours. Plasma was separated, stored and assayed for LH. Pools made from hourly samples taken prior to naloxone administration were assayed for testosterone.

Experiments 2a and 2b: Opioid-calcium interactions in the effect of nutrition on gonadotrophin secretion in Merino rams

In June (end of breeding season), 22 adult Merino rams were used in two consecutive experiments. During *Experiment 2a*, the rams were fed a maintenance diet (MD) comprising 1 kg oaten chaff plus 10% lupin grain and a complete mineral mix (Siromin; Narrogin Mineral Stockmix, Narrogin, WA, Australia), a regime designed to maintain constant body mass by providing about 8.4 MJ day^{-1} of metabolizable energy and 50 g protein day^{-1} (details of the diets can be found in Bouhkliq et al., 1997). Water was provided *ad libitum*. During *Experiment 2b*, the rams were fed the same maintenance diet plus 1 kg lupin grain (HD) for 6 weeks. Animals consumed all feed that was provided daily and were weighed before the administration of the treatments to calculate the dose of opioid antagonist to administer. During both experiments, each rams received one administration (i.v. at 7.00 h): a) Calcium borogluconate 0.02 g/kg + naloxone hydrochloride 0.2 mg/kg (Nal + Ca^{2+}; n=6); b) Naloxone hydrochloride 0.2 mg/kg (Nal; n=6); c) Calcium borogluconate 0.02 g/kg (Ca^{2+}; n=5); d) 0.1 ml/kg NaCl 0.9% (Saline; n=5) for five days.

For these two experiments, the doses were chosen on the basis of a previous study (Celi et al., 2007) and on the premise that, in the presence of calcium, much lower doses of naloxone would be effective (Sciorsci et al., 2000; Minoia and Sciorsci, 2001). Blood was sampled via an indwelling jugular venous catheter every 20 min for 24 h during the day before the beginning of the treatment period (Day 0) and then on the last day (Day 5) of the treatment period. During *Experiment 2a* we did not observe any effect of the treatments on LH secretion before treatment, so, for *Experiment 2b*, blood was sampled on the first and on the last days of the treatment period (Days 0 and 4, rather than Days -1 and 4) in the possibility that any effects of the treatments would be apparent on the first day of the treatment period.

2.2 Hormone assays

Plasma was separated and stored until assay. All plasma samples were assayed for LH and pooled samples were used to measure testosterone. The LH assay was a double-antibody RIA (Martin, et al. 1980) based on preparation CNRS-M3 of ovine LH (biopotency 1.8 IU NIH-LH-S1/mg) that was used for iodination and standards and had been kindly supplied by M Jutisz (College de France, Paris, France). The limit of detection was 0.24 ± 0.05 ng/mL (mean ± s.e.m). The intra-assay coefficient of variation (%) was estimated in each assay using 6 replicates of 3 control samples containing 0.28 (7%), 1.6 (10%) and 8.2 (11%) ng/mL. The interassay coefficients of variation were 9.5%, 9.2% and 14.3%.

Testosterone was assayed using a non-extraction radioimmunoassay with 1, 2, 6, 7-^{3}H-testosterone (Amersham, Sydney, NSW, Australia) as tracer and an antibody that had been raised in our laboratory against testosterone-3-CMO-HSA (Hötzel et al., 1995). Cross-reactions were 100% with testosterone, 70% with dihydrotestosterone, 3.7% with androstenedione, and less than 0.05% with progesterone, oestradiol-17ß, oestrone and oestriol. The limit of detection of the assay was 0.15 ng/mL and the intra-assay coefficients of variation were 8%, 9%and 5% for quality controls containing 1.4, 3.7 and 15.3 ng/mL. The interassay coefficients of variation were 14.7%, 14.6% and 12.3%.

2.3 LH pulse analysis

The LH data were analysed for pulses with a modified version of the "Pulsar" algorithm developed by Merriam and Wachter (1982) and modified for the Apple Macintosh computer ("Munro", Zaristow Software, West Morham, Haddington, East Lothian, UK). The “G” parameters (the number of standard deviations by which a peak must exceed the baseline in order to be accepted) were set at 3.98, 2.4, 1.68, 1.24, and 0.93 for G_1-G_5, these being the requirements for pulses composed of 1 to 5 successive samples that exceed the baseline, respectively. The Baxter parameters describing the parabolic relationship between the concentration of a hormone in a sample and the standard deviation (assay variation) about that concentration were 0.30853 (b_1, the y intercept), 0.00213 (b_2, the x coefficient) and 0.00268 (b_3, x^2 coefficient). The pulse frequency, mean pulse amplitude (the difference between pulse peak and preceding nadir) and mean concentration of LH were calculated for each profile.

2.4 Statistical analysis

Repeated measures analysis of variance was applied to all variables. When main effects or interactions were significant, one-way analysis of variance was applied and Fisher’s protected LSD was used for comparison between treatment groups.

3. Results

Experiment 1: Interactions between nutrition and naloxone on LH secretion in the male sheep

Before naloxone treatment, there was a significant effect of diet on LH secretion with LH pulse frequencies higher in the HD and MD groups than in the HMD group ($P < 0.05$; Table 1). There were no effects of diet on LH pulse amplitude, inter-pulse nadir or mean LH concentrations. There was no interaction between the plane of nutrition and the effect of naloxone treatment for any of the LH pulse variables. Overall, there was a tendency for naloxone treatment to increase LH pulse frequency ($P = 0.10$), but not the other LH pulse variables. However, after naloxone treatment, LH pulse frequency in Group HD was higher than in Group HMD ($P < 0.05$). The inter-pulse nadir and mean concentrations of LH were increased by naloxone treatment in Group HD ($P < 0.05$) but not in the other groups.

Examination of individual pulse profiles, especially in Group HD, indicated that pulses tended to merge with each other making identification of individual pulses difficult and thus error-prone. A better representation of the effect of naloxone was found by calculating a moving average of nine consecutive samples of LH concentration (Fig. 1). Repeated measures analysis of these moving averages indicated that, before and after naloxone treatment, there was no effect of plane of nutrition on LH concentrations, but an effect of time ($P < 0.001$). After naloxone treatment, there was no effect of plane of nutrition but an effect of time ($P < 0.001$) and a tendency for an interaction between plane of nutrition and time ($P = 0.10$) on LH concentrations.

Immediately prior to naloxone treatment, mean plasma concentration of testosterone in the HD group was about 50% higher than in Group MD ($P < 0.05$, Fig. 2).

Experiment 2: Naloxone-calcium interactions in the effect of nutrition on LH secretion

When the rams were fed to maintenance, LH pulse frequency on Day -1 was similar in all groups and was not affected by daily administrations of naloxone and/or calcium (Fig. 3). After 6 weeks on the HD diet, LH pulse frequency was increased ($P < 0.05$) by the administration of naloxone alone, naloxone + Ca^{2+}, or Ca^{2+} alone, on both days of observation (Fig. 3). There was no significant difference between the stimulatory effects of naloxone alone, naloxone + Ca^{2+}, or Ca^{2+} alone on LH pulse frequency after 6 weeks on the HD diet, and there was no effect of treatments on pulse amplitude or inter-pulse nadir (data not shown), or mean LH concentration (Fig. 4), but mean

LH concentration was higher when the rams were fed with HD than MD (Fig. 5; $P < 0.001$). Overall, there was no effect of treatment with Ca^{2+} or naloxone on plasma concentrations of testosterone (data not shown), although concentrations were higher in the animals fed with HD than MD (Fig. 5; $P < 0.001$).

4. Discussion

The main hypothesis of this study, that rams on a low plane of nutrition would be more responsive to a challenge with naloxone than rams on a high plane of nutrition, was rejected. On the contrary, LH pulse frequency was increased by naloxone only in the rams on the high plane of nutrition. Therefore, it can be concluded that an opioidergic mechanism is not involved in the suppressive effect of restricted nutrition on LH secretion. On the other hand, it appears that opioidergic mechanisms are brought into play by the increases in the concentration of testosterone in the rams on the high plane of nutrition. Therefore, in rams, it appears that the opioidergic interactions with nutrition are similar to those with photoperiod (Lincoln et al. 1987).

There is considerable evidence for gonadal steroids exerting negative feedback on LH secretion through an opioidergic mechanism (Schanbacher 1985; Brooks et al. 1986a; Caraty et al. 1987; Lincoln et al. 1987) and this is supported by studies with sheep showing that naloxone can increase LH pulse frequency when gonadal steroid concentrations are high, but not when they are low (Ebling and Lincoln 1985; Brooks et al. 1986b). Our observations agree with this because the response to naloxone was increased by lupin supplementation, a treatment doubled the mean concentration of testosterone. Indeed, steroid hormone receptors are co-expressed in EOP-containing neurones (Simerly et al., 1996). It has to be noted, however, that naloxone can stimulate GnRH release in absence of testosterone, although it is far less effective in these circumstances (Jackson et al., 2000). Moreover, the dose of naloxone used by Jackson et al. (2000) might have not been able to overcome the inhibitory effect of testosterone on LH interpulse interval, suggesting that other pathways than EOPs mediate the negative feedback action of testosterone (Tortonese, 1999). An alternative possibility is that other EOPs, such as dynorphin and orphanin FQ (OQF), acting through the κ opioid receptor and opioid like receptor-1 (OLR-1) respectively, mediate steroid negative feedback on GnRH neurones (Foradori et al., 2006; Foradori et al., 2007). We cannot rule out the possibility thus, that other members and receptors of the EOPs family are involved in this process.

The lack of effect of naloxone in the rams on the low plane of nutrition also indicates that the low frequencies of LH pulses under these circumstances are not due to inhibitory opioidergic mechanisms activated by undernutrition. It could be argued that EOP inhibitory pathways are active during acute changes in nutritional status such as fasting (Dyer et al., 1985) or energy restriction (Canfield et al., 1988), but other inhibitory pathways may be involved in the inhibition of GnRH/LH secretion during chronic undernutrition. For example, neuropeptide Y (NPY) neurones could be activated during chronic undernutrition and this could be accompanied by a suppression of the EOP pathways (McShane et al., 1993).

The size of the LH response to naloxone was not improved by co-administration with calcium, an outcome that agrees with our previous study (Celi et al., 2007). In fact, the low dose of naloxone was able to stimulate LH secretion in well-fed rams, showing that the dose used in this study was sufficient to stimulate the opioid receptors that regulate the GnRH centres but there was no indication at all of an interaction with calcium. We are thus led to reject the hypothesis that calcium and naloxone interact synergistically. An alternative possibility is that another type of opioid receptor, naloxone-independent, is involved. For example, OQF (also known as nociceptin) is a member of the EOP family and it acts through opioid like receptor-1 (OLR-1) to mediate steroid negative feedback on GnRH neurones (Foradori et al., 2007). This needs to be investigated in the context of nutrition-induced changes in LH secretion.

The stimulatory effect of exogenous calcium alone on LH secretion in lupin-supplemented rams was not expected. Calcium plays a major role in the mechanism of action of GnRH on the synthesis and release of LH (Barbarino et al., 1982; Bates and Conn, 1984; Kalra et al., 1993). In humans, *in* *vivo* studies have shown that calcium infusion can stimulate LH secretion, although only when the subjects were challenged with GnRH (Veldhuis et al., 1984). In agreement with this observation are the findings that LH release is decreased by calcium influx blockers in men (Barbarino and De Marinis, 1980; Barbarino et al., 1982; Struthers et al., 1983). To our knowledge, there are no reports of interactions between nutritional status and stimulation of LH secretion by calcium, but it could be speculated that an increase in nutrition increases the sensitivity of the pituitary gland to calcium. Interestingly, the size of the response was the same as that seen with the high dose of naloxone, suggesting the activation of an important mechanism. The response to calcium requires confirmation.

5. Conclusion

Overall, EOPs do not play a major, direct role in the inhibitory effect of undernutrition on GnRH/LH secretion, but support previous studies showing that gonadal steroids exert their negative feedback effects on GnRH/LH secretion through opioidergic mechanisms. In addition, there seems to be no interaction between opioids and calcium in the control of LH secretion. Changes in plane of nutrition induce changes in metabolic status reflected in circulating nutrients and metabolic hormones (Blache et al., 2006) and these, not EOPs, seem to be responsible for conveying information about nutritional status to the reproductive centres of the brain.

Acknowledgments

The animal experiments described here would not have been possible without help willingly provided by everyone in the Animal Science Group, especially Margaret Blackberry. This work was supported by the National Health & Medical Research Council of Australia. Pietro Celi was supported by an International Postgraduate Research Scholarship and David Miller by a University Postgraduate Award, both from UWA.

References

Barbarino, A., De Marinis L., 1980. Calcium antagonists and hormone release. II. Effects of verapamil on basal, gonadotropin-releasing hormone- and thyrotropin-releasing hormone-induced pituitary hormone release in normal subjects. J. Clin. Endocrinol. Metabol. 51, 749-753.

Barbarino, A., De Marinis, L., Mancini, A., Makhoul, O., 1982. Calcium antagonists and hormone release. III. Role of calcium in the biphasic release of luteinizing hormone in response to gonadotrophin-releasing hormone in vivo. Acta Endocrinol. (Copenh.) 101, 5-9.

Bates, M.D., Conn P.M., 1984. Calcium mobilization in the pituitary gonadotrope: relative roles of intra- and extracellular sources. Endocrinol. 115, 1380-1385.

Blache, D., Zhang, S., Martin G.B., 2006. Dynamic and integrative aspects of the regulation of reproduction by metabolic status in male sheep. Reprod. Nutr. Develop. 46, 379-390.

Bourne, G.A., Baldwin D.M., 1980. Extracellular Ca^{++}-indipendent and dependent components on the biphasic release of LH in response to luteinizing hormone-releasing hormone *in vitro*. Endocrinol. 107, 780-788.

Boukhliq, R., Martin, G.B., White, C.L., Blackberry, M.A., Murray, P., 1997. Role of glucose, fatty acids and protein in regulation of testicular growth and secretion of gonadotrophin, prolactin, somatotrophin and insulin in the mature ram. Reprod. Fertil. Dev. 9, 515-524.

Briski, K.P., Quigley, K., Meites, J., 1984. Endogenous opiate involvement in acute and chronic stress-induced changes in plasma LH concentrations in the male rat. Life Sci. 34, 2485-2493.

Brooks, A.N., G.E., L., Lees, P.D., Haynes, N.B., 1986a. Opioid modulation of LH secretion in the ewe. J. Reprod. Fert. 76, 693-708.

Brooks, A.N., Haynes, N.B., Yang, K., Lamming, G.E., 1986b. Ovarian steroid involment in endogenous opioid modulation of LH secretion in seasonally anoestrus mature ewes. J. Reprod. Fert. 76, 709-715.

Canfield, R.W., Ralinsky, M.G., Butler, W.R., 1988. Modulation of LH pulse secretion by opioids during negative energy balance in ewes. J. Anim. Sci 66 (Suppl. 1): 410.

Canfield, R.W., Butler, W.R., 1991. Energy balance, first ovulation and the effects of naloxone on LH secretion in early postpartum dairy cows. J. Anim Sci. 69: 740-746.

Caraty, A., Locatelli, A., Schanbacher, B.D., 1987. Augmentation par la naloxone de la fréquence et de l'amplitude des pulses de LH-RH dans le sang porte hypothalamo-hypophysaire chez le bélier castré. Comptes Rendus hebdomadaires des Séances, Academie des Sciences, (Paris) 305 (III): 369-374.

Celi, P., Walkden-Brown, S.W., Blache, D., Széll, A.Z., Wilkinson, H.M., Martin, G.B., 2007. Twin efficiency for reproductive variables in monozygotic twin sheep. Theriogenology 68, 663-672.

Conn, P.M., 1986. The molecular basis of gonadotrophin-releasing hormone action. Endocr. Rev. 7, 3-10.

Conn, P.M., Marian, J., McMillan, N., Stern, J., Rogers, D., Hamby, M., Penna, A., Grant, E., 1981. Gonadotropin-releasing hormone action in the pituitary: a three step mechanism. Endocr. Rev. 2, 174-185.

Dyer, R. G., Mansfiel, S., Corbet, H., Dean, A.D.P., 1985. Fasting impairs LH secretion in female rats by activating an inhibitory opioid pathway. J. Endocrinol. 107, 341-353.

Ebling, F.J., Lincoln G.A., 1985. Endogenous opioids and the control of the seasonal LH secretion in Soay rams. J. Endocrinol. 107, 341-353.

Ebling, F.J.P., Wood, R.I., Karsch, F.J., Vannerson, L.A., Suttie, J.M., Bucholtz, D.C., Schall, R.E., Foster, D.L., 1990. Metabolic interfaces between growth and reproduction. III: Central mechanisms controlling pulsatile luteinizing hormone secretion in the nutritionally growth-limited female lamb. Endocrinol. 126 (5), 2719-2727.

Foradori, C.D., Amstalden, M., Goodman, R.L., Lehman, M.N., 2006. Colocalisation of Dynorphin A and Neurokinin B Immunoreactivity in the Arcuate Nucleus and Median Eminence of the Sheep. J. Neuroendocrinol. 18, 534-541.

Foradori, C.D., Amstalden, M., Coolen, L.M., Singh, S.R., McManus, C.J., Handa, R.J., Goodman, R.L., Lehman, M.N., 2007. Orphanin FQ: Evidence for a Role in the Control of the Reproductive Neuroendocrine System. Endocrinol. 148, 4993-5001.

Fuentes, V.O. 1989. Effect of naloxone, nalbuphine, progesterone and pregnant mare's serum gonadotrophin on the sexual behaviour of ewes. Vet. Rec. 14, 274-276.

Ghosh, B.R., Wu, J.C., Miller, W.L., 1996. Gonadotropin-releasing hormone-stimulated calcium mobilization is altered in pituitary cultures from anestrus ewes. Biol. Reprod. 54, 753-760.

Gregg, D.W., Moss, G.E., Hudgens, R.E., Malven, P.V., 1986. Endogenous opioid modulation of luteinizing hormone and prolactin secretion in postpartum ewes and cows. J. Anim. Sci. 63, 838-847.

Hötzel, M.J., Walkden-Brown, S.W., Blackberry, M.A., Martin, G.B., 1995. The effect of nutrition on testicular growth in mature Merino rams involves mechanisms that are independent of changes in GnRH pulse frequency. J. Endocrinol. 147, 75–85.

Ishizuka, B., Quigley, M.E., Yen, S.S.C., 1984. Postpartum hypogonadotrophinism: evidence for increased opioid inhibition. Clin. Endocrinol. 20, 573-578.

Jackson, G.L., Kuehl, D.E., 2000. Interactions of photoperiod, testosterone, and naloxone on GnRH and LH pulse parameters in the male sheep. Dom. Anim. Endocrinol. 18, 97-110.

Kalra, S.P., Sahu, A., Kalra, P.S., 1993. Ageing of neuropeptidergic signals in rats. J. Reprod. Fert. Suppl. 46, 11-19.

Kryzanowska, B., Czekalski, S., 1992. Differences in gonadotropins response to LRH stimulation after naloxone administration in patients with anorexia nervosa. Proc. International Congr Endocrinol., Nice, France 9, 575.

Lincoln, G.A., Ebling, F.J., Martin, G.B., 1987. Endogenous opioid control of pulsatile LH secretion in rams: modulation by photoperiod and gonadal steroids. J. Endocrinol. 115, 425-438.

Martin G.B., Oldham C.M., Lindsay D.R., 1980. Increased plasma LH levels in seasonally anovular Merino ewes following the introduction of rams. Anim. Reprod. Sci. 3, 125-132.

McShane, T.M., Petersen, S.L., McCrone, S., Keisler, D.H., 1993. Influence of food restriction on neuropeptide-Y, proopiomelanocortin, and luteinizing hormone-releasing hormone gene expression in sheep hypothalami. Biol. Reprod. 49, 831-839.

Merriam, G.R., Wachter, K.W., 1982. Algorithms for the study of episodic hormone secretion. Am. J. Physiol. 243, E310–8.

Minoia, P., Sciorsci, R.L., 2001. Metabolic control through L calcium channel, PKC and opioid receptors modulation by an association of naloxone and calcium salts. Curr. Drug Targets: Immune, Endocr. Metab. Disord. 1: 131-137.

Minoia, P., Sciorsci, R.L., Cinone, M., Desiante, D., Celi, P., 1995. Rimozione dell'anaestro stagionale in pecore mediante calcio e naloxone. Proc. XLIX Congresso S.I.S.Vet., Salsomaggiore Terme (PR) - Italy.

Misztal T, Romanowicz K., 2005. Effective stimulation of daily LH secretion by the combined treatment with melatonin and naloxone in luteal-phase ewes. Acta Neurobiol Exp (Wars). 65(1), 1-9.

Panerai, A. E., Petraglia, F., Sacerdote, P., Genazzani, A.N., 1985. Mainly mu-opiate receptors are involved in the luteinizing hormone and prolactin secretion. Endocrinol. 117, 1096-1099.

Ramey, J.W., Krummen, L.A., Wilfinger, W.W., Highsmith, R.F., Baldwin, D.M., 1987. Effects of a low calcium environment on luteinizing hormone biosynthesis in cultured rat anterior pituitary cells. Endocrinol. 120, 1514-1520.

Schanbacher, B.D., 1985. Endogenous opiates and the hypothalamic-pituitary-gonadal axis in male sheep. Dom. Anim. Endocrinol. 2, 67-75.

Schroeder, J.E., Fishbach, P.S., Zheng, D., McCleskey, E.W., 1991. Activation on mu opioid receptors inhibits transient high- and low-threshold Ca2+ currents, but spares a sustained current. Neuron 6, 13-20.

Sciorsci, R.L., Bianchi, P., Minoia, P., 2000. High levels of endorphin and related pathologies of veterinary concern. A review. Immunopharmacol. Immunotoxicol. 22, 575-626.

Simerly, R.B., Youn,g B.J., Carr, A.M., 1996. Co-expression of steroid hormone receptors in opioid peptide-containing neurons correlates with patterns of gene expression during the estrous cycle. Brain. Res. Mol. Brain. Res. 40, 275-284.

Sinclair, K.D., Broadbent, P.J., Hutchinson, S.M., 1995. Naloxone evokes a nutritionally dependent LH response in post-partum beef cows but not in mid-luteal phase maiden heifers. Anim. Sci. 61, 219–230.

Struthers, A.D., Miller, J.A., Beastal, G.H., McIntosh, W.B., Reid, J.L., 1983. Calcium antagonists and hormone release: effect of nifepidine on luteinizing hormone-releasing hormone- and thyrotropin-releasing-hormone-induced pituitary hormone release. J. Clin. Endocrinol. Metab. 56, 401-404.

Tortonese, D.J., 1999. Interaction between hypothalamic dopaminergic and opioidergic systems in the photoperiodic regulation of pulsatile luteinizing hormone secretion in sheep. Endocrinol. 140, 750-757.

Veldhuis, J.J. Borges, Drake, C.R., Rogol, A.D., 1984. Divergent influences of calcium ions on releasing factor-stimulated anterior pituitary hormone secretion in normal man. J. Clin. Endocrinol. Metab. 59, 56-61.

Webb, R., Baxter, G., McBride, D., Nordblom, G.D., Shaw, M.P.K., 1985. The measurement of testosterone and oestradiol-17B using iodinated tracers and incorporating an affinity chromatography extraction procedure. J. Steroid. Biochem. 23, 1043-1051.

Whisnant, C.S., Thompson, F.N., Kiser, T.E., Barb, C.R., 1986. Effect of naloxone on serum luteinizing hormone, cortisol and prolactin concentrations in anestrous beef cows. J. Anim. Sci. 62, 1340-1345.

Whisnant, C.S., Goodman, R.L., 1991. Endogenous opioid suppression of luteinizing hormone pulse frequency and amplitude in the ewe: Hypothalamic sites of action. Neuroendocrinol. 54: 587-593.

Woods, S.C., Schwartz, M.W., Baskin, D.G., Seeley, R.J., 2000. Food intake and the regulation of body weight. Annu. Rev. Psychol. 51:255–277.

Yang, K., Haynes, N.B., Lammings, G.E., Brooks, A.N., 1988. Ovarian steroid hormone involvement in endogenous opioid modulation of LH secretion in mature ewes during the breeding and non-breeding season. J. Reprod. Fert. 83, 129-139.

Zhang, S., Blache, D., Blackberry, M.A., Martin, G.B., 2004. Dynamics of the responses in secretion of LH, leptin and insulin following an acute increase in nutrition in mature male sheep. Reprod. Fertil. Develop. 16, 823-829.

Figure captions

Figure 1. Smoothed plasma LH profiles for the HMD (open circles), MD (plain line) and HD (closed circles) dietary treatments. Each point represents the mean calculated as a moving average over 9 consecutive samples. Values are means ± s.e.m. (N.B. for clarity, standard errors not shown for Group M and only for every second point in the other two groups).

Figure 2: Mean plasma testosterone concentrations in rams fed a Maintenance Diet (MD), half or the Maintenance Diet (HMD) or a High Diet (HD) prior to naloxone administration. Values are means ± s.e.m. * Significant at $P < 0.05$.

Figure 3. Effect of one administration of Ca^{2+} + Nal (■ ; n = 6), Nal (▨ ; n = 6), Ca^{2+} (▩ ; n = 5) and Saline (□ ; n = 5) for five days on LH pulse frequency in Merino rams fed either a Maintenance Diet (Experiment 2a) or a High Diet (Experiment 2b). Values are expressed as means ± s.e.m. Different letters indicate statistical difference at $P < 0.05$.

Figure 4. Effect of one administration of Ca^{2+} + Nal (■ ; n = 6), Nal (▨ ; n = 6), Ca^{2+} (▩ ; n = 5) and Saline (□ ; n = 5) for five days on mean LH concentrations in Merino rams fed either a Maintenance Diet (Experiment 2a) or a High Diet (Experiment 2b). Values are expressed as means ± s.e.m.

Figure 5. Effect Low Diet (Experiment 2a; □ ; n = 22) and High Diet (Experiment 2b; ■ ; n = 22) on mean LH and testosterone concentration in Merino rams. Values are expressed as means ± s.e.m. Different letters indicate statistical difference at $P < 0.001$.

Figures

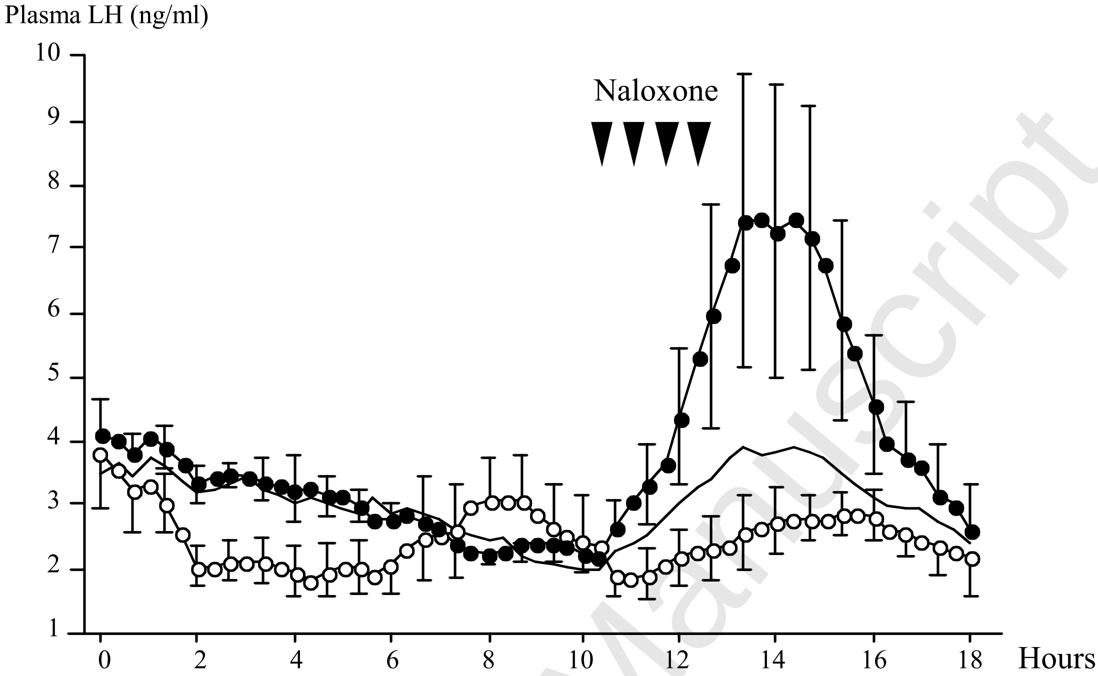


Figure 1.

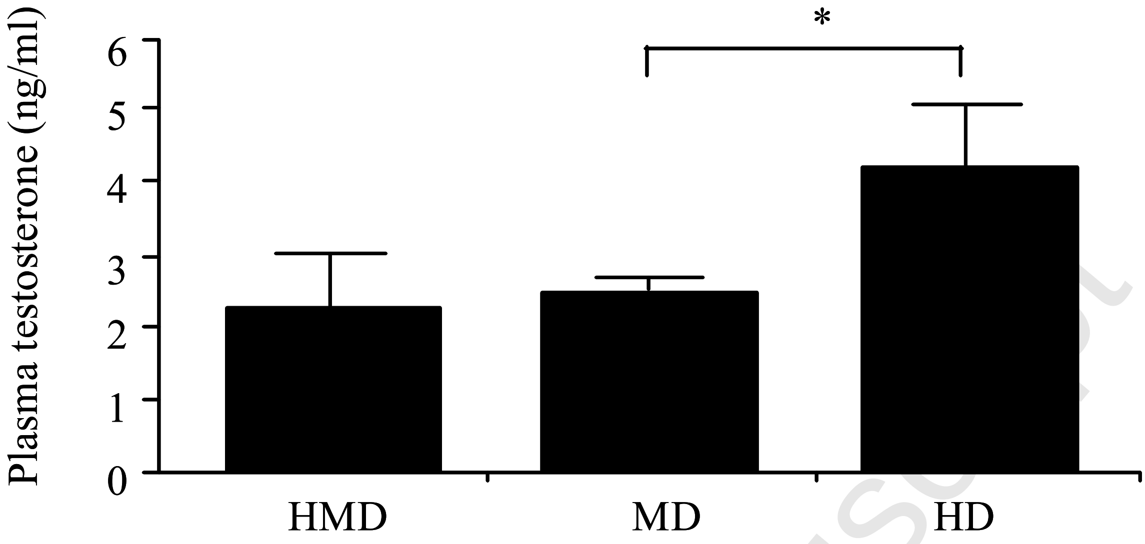
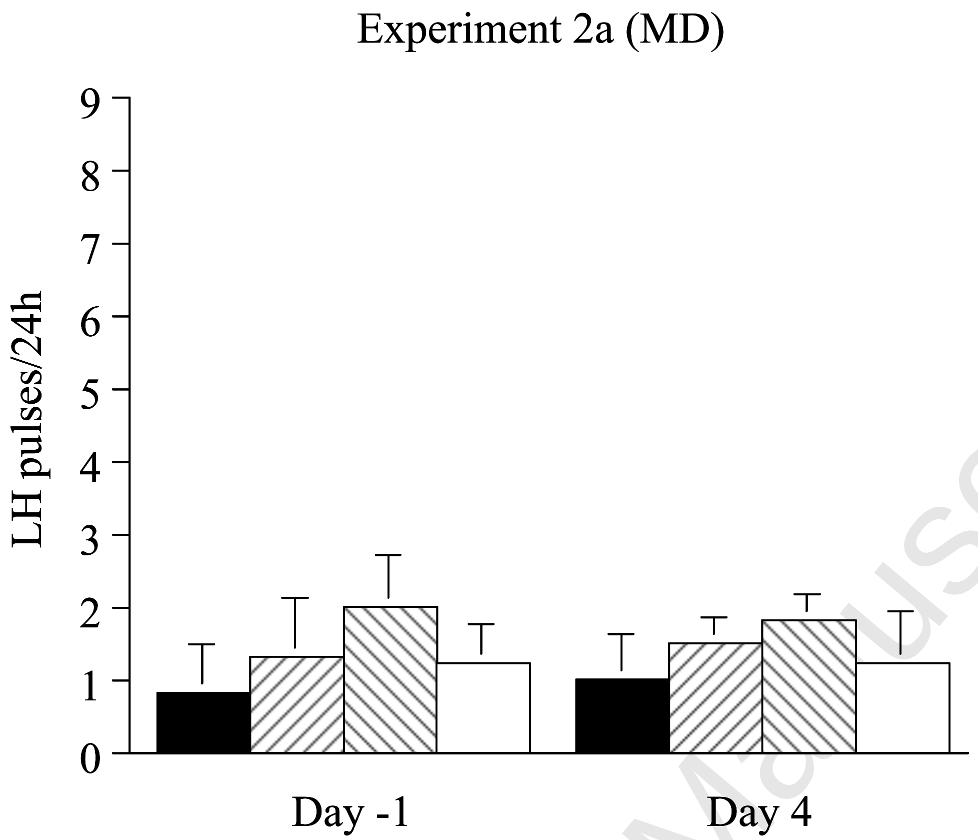


Figure 2.


Experiment 2a (MD)
LH pulses/24h
0
1
2
3
4
5
6
7
8
9
Day -1
Day 4

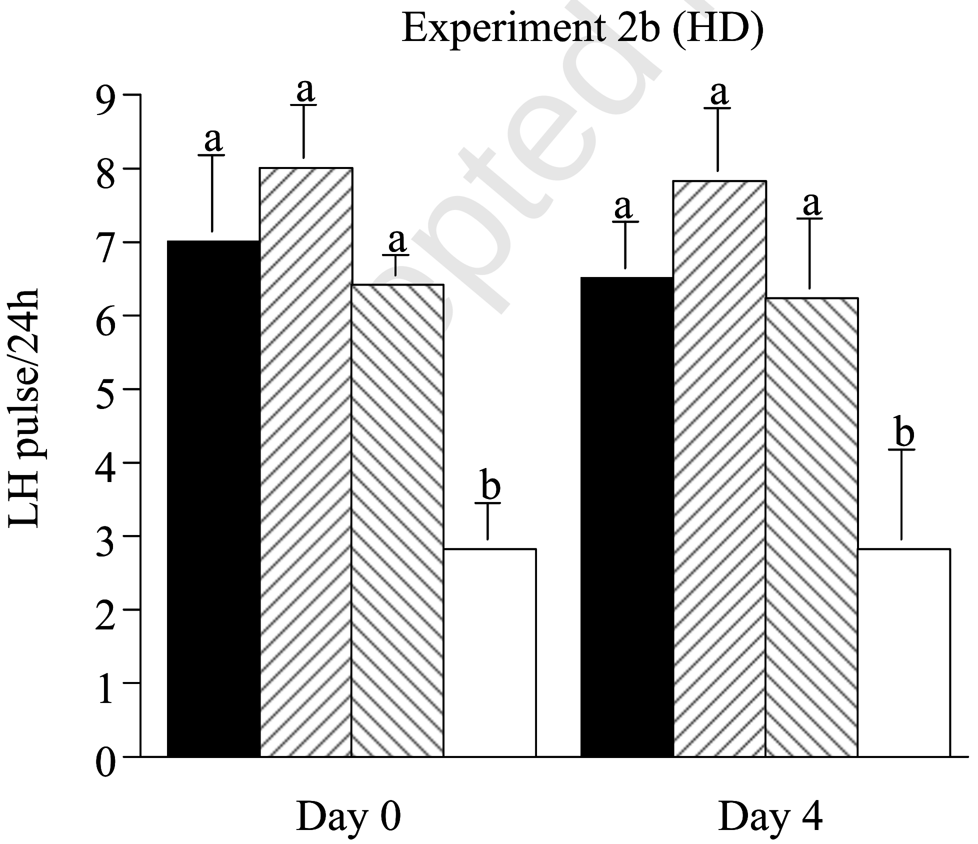

Experiment 2b (HD)
LH pulse/24h
0
1
2
3
4
5
6
7
8
9
a
a
a
b
a
a
a
b
Day 0
Day 4

Figure 3.

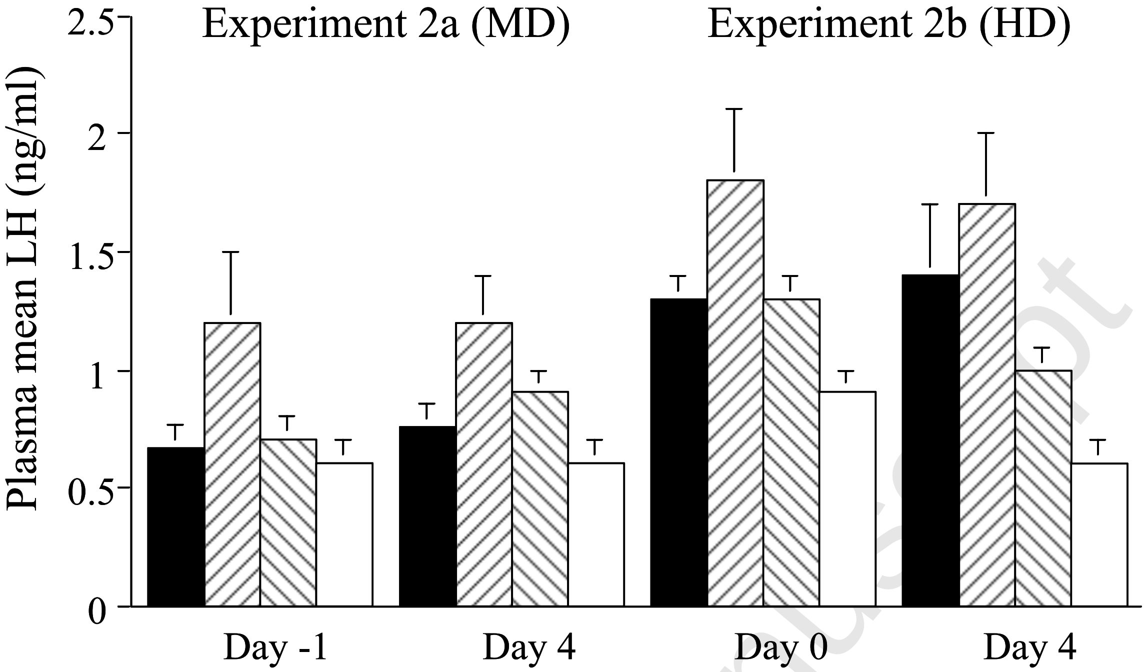

Experiment 2a (MD)
Experiment 2b (HD)
Plasma mean LH (ng/ml)
2.5
2
1.5
1
0.5
0
Day -1
Day 4
Day 0
Day 4

Figure 4.

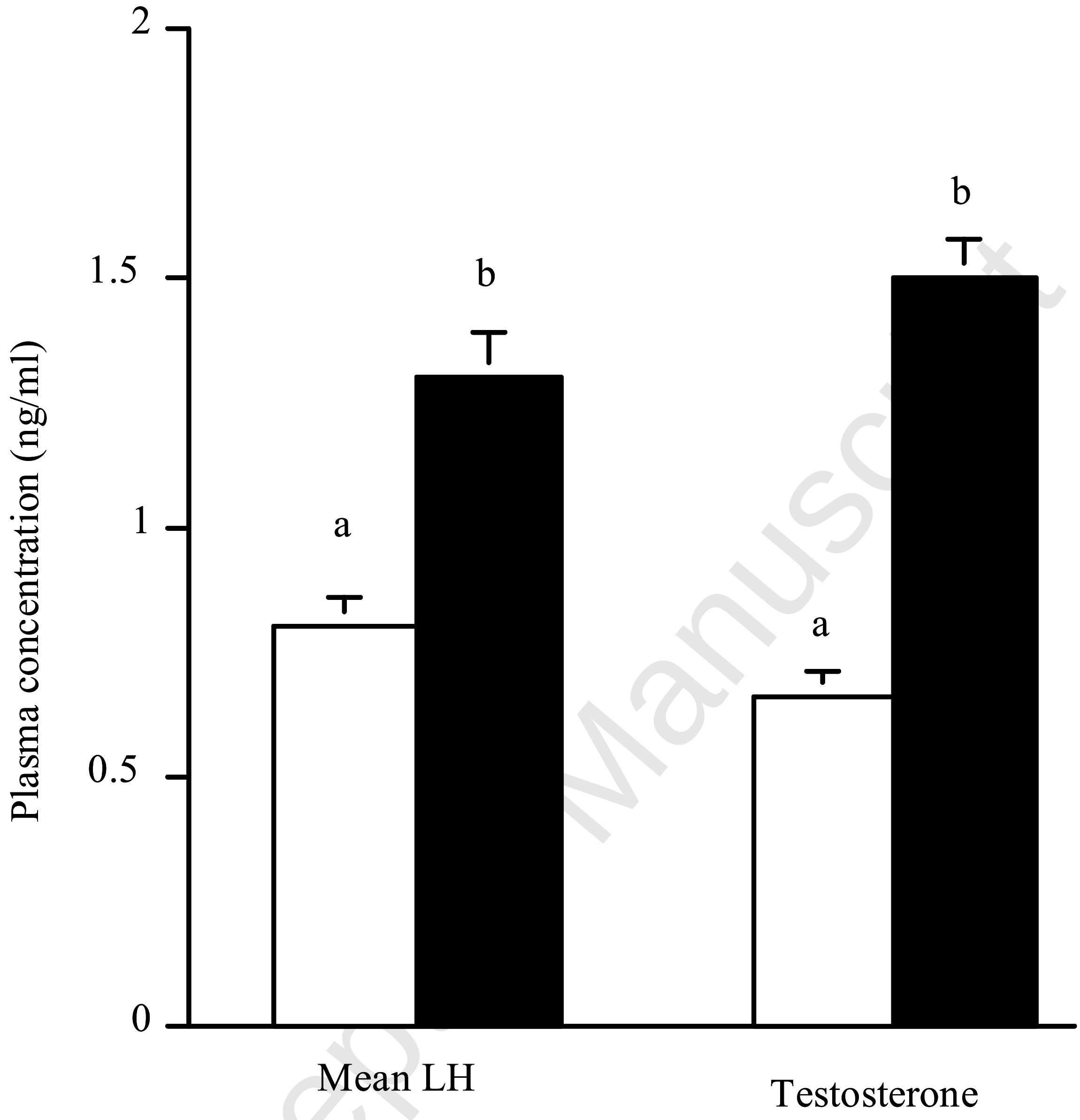

Plasma concentration (ng/ml)
2
1.5
1
0.5
0
a
b
a
b
Mean LH
Testosterone

Figure 5.

Tables

Table 1: LH pulse variables before and after naloxone injection in rams fed a Maintenance Diet (MD), half or the Maintenance Diet (HMD) or a High Diet (HD). All figures are means ± s.e.m.

	Naloxone Treatment	**Group HD**	**Group MD**	**Group HMD**
Mean Pulse Frequency (pulses/6 hr)	Before	3.5 ± 0.28^{a}	3.0 ± 0.16^{a}	1.8 ± 0.28^{b}
	After	3.9 ± 0.70^{a}	3.3 ± 0.49^{ab}	2.7 ± 0.40^{b}
Mean Pulse Amplitude (ng/ml)	Before	3.9 ± 0.58^{a}	2.7 ± 0.14^{a}	3.1 ± 0.27^{a}
	After	3.6 ± 0.47^{a}	2.7 ± 0.50^{a}	2.6 ± 0.62^{a}
Nadir (ng/ml)	Before	1.7 ± 0.13^{a}	2.1 ± 0.26^{a}	1.9 ± 0.30^{a}
	After	3.9 ± 1.17^{b}	2.5 ± 0.61^{ab}	1.6 ± 0.35^{a}
Mean LH concentration (ng/ml)	Before	3.1 ± 0.21^{a}	2.8 ± 0.20^{a}	2.7 ± 0.47^{a}
	After	4.6 ± 1.04^{b}	3.2 ± 0.55^{ab}	2.4 ± 0.31^{a}

Letters exclusively different, across and down columns within variable, significant at $P < 0.05$.