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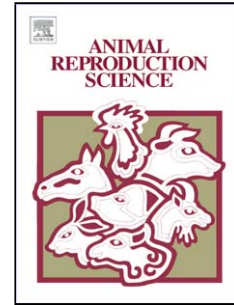
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# 1 **Interactions between nutritional and opioidergic pathways in** 2 **the control of LH secretion in male sheep**

3  
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## 15 16 **Abstract**

17 Our aim was to determine the role of opioidergic processes in the effects of nutrition on the  
18 secretion of LH pulses in the mature male sheep. In the first of three experiments, adult Merino  
19 rams were acclimatised to a maintenance diet and then allocated to one of three dietary groups (n =  
20 5): continuation of the maintenance diet (Group M); reduction to half of the maintenance allocation  
21 (Group HM); or supplementation of the maintenance diet with lupin grain (Group HD). An initial  
22 administration of naloxone (2 mg/kg body weight, i.v.) was followed at 40-min intervals by 3  
23 further administrations (1 mg/kg). Blood was sampled every 20 min for 12 h before the initial  
24 naloxone administration and then for a further 6 h. LH pulse frequency after naloxone treatment  
25 was significantly higher in Group HD than in Group HM ( $P < 0.05$ ). The second study tested

26 whether the response to naloxone depended on calcium status. We used 22 adult Merino rams in  
27 two consecutive experiments, one in which the rams were fed a maintenance diet, and one in which  
28 the rams were fed with the maintenance diet plus 1 kg lupin grain for 5 weeks. In both experiments,  
29 rams were allocated to groups that received one of the following treatments: a) 0.02 g/kg calcium  
30 borogluconate + 0.2 mg/kg naloxone hydrochloride (Nal + Ca<sup>2+</sup>; n = 6); b) 0.2 mg/kg naloxone  
31 hydrochloride (Nal; n = 6); c) 0.02 g/kg calcium borogluconate (Ca<sup>2+</sup>; n = 5); d) 0.1 ml/kg NaCl  
32 0.9% (Saline; n = 5). All treatments were given as a single i.v. administration daily for 5 days.  
33 Blood was sampled every 20 min for 24 h during the acclimatization period (Day 0) and on the last  
34 day (Day 5) of treatment. In the first study (under maintenance), none of the treatments affected LH  
35 pulse frequency. In the second study (the lupin-supplemented rams), LH pulse frequency was  
36 significantly increased ( $P < 0.05$ ) by the administration of naloxone + Ca<sup>2+</sup>, naloxone alone and  
37 Ca<sup>2+</sup> alone. Overall, rams on a low plane of nutrition showed the smallest response to naloxone,  
38 suggesting that an opioidergic mechanism is not involved in the suppressive effect of restricted  
39 nutrition on the gonadotrophic axis. Rather, because testosterone secretion was increased on the  
40 high plane of nutrition, the LH responses to naloxone are better explained by the effects of  
41 testosterone on opioidergic mechanisms. Finally, we failed to observe any interaction between  
42 opioids and calcium in the control of LH secretion.

43

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

45

## 46 **1. Introduction**

47 In male sheep, when genetic and photoperiodic influences are permissive, the reproductive  
48 centres in the preoptic-hypothalamic continuum are strongly and consistently affected by nutrition  
49 (Blache et al. 2006). This is evidenced by changes in the frequency of pulses of luteinizing  
50 hormone (LH), reflecting pulses of gonadotrophin-releasing hormone (GnRH), within a few hours  
51 after the feeding of a supplement of energy and protein (Zhang et al. 2004). This response

52 stabilizes after 5–7 days and, interestingly, diminishes and finally disappears after 3–4 weeks,  
53 around the time that gains in body weight and fat can be detected (Blache et al. 2006).

54 The signalling pathways that link dietary status and the activity of the neurons that produce the  
55 GnRH signal are thought to be partly hormonal in nature (Woods et al. 2000) and may reflect the  
56 amount of body reserves (Blache et al. 2006). Among the possible signals are endogenous opioid  
57 peptides (EOPs) that are known to inhibit the activity of GnRH neurons. In sheep, EOPs play  
58 roles in gonadotrophin responses to gonadal steroids and photoperiod (Tortonese, 1999) and they  
59 have been suggested by Ebling et al. (1990) to be involved in the metabolic/nutritional control of  
60 gonadotrophin secretion. In other species, the inhibition of LH secretion by EOPs is affected by  
61 alterations in metabolic state (Ishizuka et al. 1984; Gregg et al. 1986; Kryzanowska and Czekalski  
62 1992). For example, the opioid antagonist, naloxone, can reverse the inhibitory effects of fasting  
63 on LH secretion in rats (Briski 1984; Dyer et al. 1985) and alleviate the metabolic suppression of  
64 LH secretion in postpartum dairy and beef cows (Whisnant et al. 1986; Canfield and Butler,  
65 1991). In sheep, low doses of naloxone can stimulate LH secretion (Lincoln et al., 1987), reverse  
66 lactational anoestrus (Minoia et al., 1995), facilitate stimulation of LH secretion by exogenous  
67 melatonin (Misztal and Romanowicz, 2005) and facilitate the display of oestrous behaviour  
68 (Fuentes, 1989). We therefore used naloxone to test whether opioidergic mechanisms are  
69 involved in the acute effects of nutrition on LH secretion in the intact Merino ram.

70 We explored the potential role of calcium in the response to naloxone (Sciorsci et al., 2000;  
71 Minoia and Sciorsci, 2001). Calcium-mediated activity controls GnRH secretion at the  
72 hypothalamic level (Kalra et al., 1993; Ghosh et al., 1996) and, at pituitary level, extracellular  
73 calcium plays an important role in the effect of GnRH on LH secretion (Bourne and Baldwin,  
74 1980; Conn et al., 1981). Studies with rat pituitary cells *in vitro* have shown that the ability of  
75 GnRH to stimulate LH release is reduced in a low-calcium environment (Conn, 1986; Ramey et  
76 al., 1987). Importantly,  $\mu$  opioid receptors seem to be involved in the control of LH secretion  
77 (Panerai et al., 1985). When they bind to  $\mu$ -receptors, opioids inhibit calcium-dependent

78 neurotransmitter release by inhibiting voltage-dependent calcium channels, and a  $\mu$ -receptor  
79 antagonist can prevent this response (Schroeder et al., 1991). We therefore tested whether the  
80 effect of naloxone on LH secretion depends on calcium status in rams on high and low planes of  
81 nutrition.

82

## 83 **2. Materials and methods**

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

87

### 88 *2.1 Animals and experimental protocol*

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

91

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

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

103 weighed before the administration of the treatments to calculate the dose of opioid antagonist to  
104 administer.

105 On the basis of previous studies with sheep (Caraty et al. 1987; Ebling et al. 1990), an  
106 appropriate treatment regime with naloxone hydrochloride (Sigma-Aldrich Pty Ltd, NSW,  
107 Australia) for disinhibiting pulsatile LH secretion is to administer an initial administration (i.v.) of 2  
108 mg naloxone/kg body weight, followed at 40 minute intervals by a further three administrations of 1  
109 mg/kg. Beginning at 06:20 h, blood was sampled via indwelling jugular catheters every 20 min for  
110 12 h prior to the initial naloxone administration and then for a further 6 hours. Plasma was  
111 separated, stored and assayed for LH. Pools made from hourly samples taken prior to naloxone  
112 administration were assayed for testosterone.

113

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

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

128 For these two experiments, the doses were chosen on the basis of a previous study (Celi et al.,  
129 2007) and on the premise that, in the presence of calcium, much lower doses of naloxone would be  
130 effective (Sciorsci et al., 2000; Minoia and Sciorsci, 2001). Blood was sampled via an indwelling  
131 jugular venous catheter every 20 min for 24 h during the day before the beginning of the treatment  
132 period (Day 0) and then on the last day (Day 5) of the treatment period. During *Experiment 2a* we  
133 did not observe any effect of the treatments on LH secretion before treatment, so, for *Experiment*  
134 *2b*, blood was sampled on the first and on the last days of the treatment period (Days 0 and 4, rather  
135 than Days -1 and 4) in the possibility that any effects of the treatments would be apparent on the  
136 first day of the treatment period.

137

## 138 2.2 Hormone assays

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

147 Testosterone was assayed using a non-extraction radioimmunoassay with 1, 2, 6, 7-<sup>3</sup>H-  
148 testosterone (Amersham, Sydney, NSW, Australia) as tracer and an antibody that had been raised in  
149 our laboratory against testosterone-3-CMO-HSA (Hötzel et al., 1995). Cross-reactions were 100%  
150 with testosterone, 70% with dihydrotestosterone, 3.7% with androstenedione, and less than 0.05%  
151 with progesterone, oestradiol-17 $\beta$ , oestrone and oestriol. The limit of detection of the assay was  
152 0.15 ng/mL and the intra-assay coefficients of variation were 8%, 9% and 5% for quality controls



153 containing 1.4, 3.7 and 15.3 ng/mL. The interassay coefficients of variation were 14.7%, 14.6% and  
154 12.3%.

155

### 156 *2.3 LH pulse analysis*

157 The LH data were analysed for pulses with a modified version of the "Pulsar" algorithm  
158 developed by Merriam and Wachter (1982) and modified for the Apple Macintosh computer  
159 ("Munro", Zaristow Software, West Morham, Haddington, East Lothian, UK). The "G" parameters  
160 (the number of standard deviations by which a peak must exceed the baseline in order to be  
161 accepted) were set at 3.98, 2.4, 1.68, 1.24, and 0.93 for G<sub>1</sub>-G<sub>5</sub>, these being the requirements for  
162 pulses composed of 1 to 5 successive samples that exceed the baseline, respectively. The Baxter  
163 parameters describing the parabolic relationship between the concentration of a hormone in a  
164 sample and the standard deviation (assay variation) about that concentration were 0.30853 (b<sub>1</sub>, the y  
165 intercept), 0.00213 (b<sub>2</sub>, the x coefficient) and 0.00268 (b<sub>3</sub>, x<sup>2</sup> coefficient). The pulse frequency,  
166 mean pulse amplitude (the difference between pulse peak and preceding nadir) and mean  
167 concentration of LH were calculated for each profile.

168

### 169 *2.4 Statistical analysis*

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

173

## 174 **3. Results**

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

176 Before naloxone treatment, there was a significant effect of diet on LH secretion with LH pulse  
177 frequencies higher in the HD and MD groups than in the HMD group ( $P < 0.05$ ; Table 1). There

178 were no effects of diet on LH pulse amplitude, inter-pulse nadir or mean LH concentrations. There  
179 was no interaction between the plane of nutrition and the effect of naloxone treatment for any of the  
180 LH pulse variables. Overall, there was a tendency for naloxone treatment to increase LH pulse  
181 frequency ( $P = 0.10$ ), but not the other LH pulse variables. However, after naloxone treatment, LH  
182 pulse frequency in Group HD was higher than in Group HMD ( $P < 0.05$ ). The inter-pulse nadir and  
183 mean concentrations of LH were increased by naloxone treatment in Group HD ( $P < 0.05$ ) but not  
184 in the other groups.

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

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

195

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

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

204 LH concentration was higher when the rams were fed with HD than MD (Fig. 5;  $P < 0.001$ ).

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

208

#### 209 4. Discussion

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

218 There is considerable evidence for gonadal steroids exerting negative feedback on LH secretion  
219 through an opioidergic mechanism (Schanbacher 1985; Brooks et al. 1986a; Caraty et al. 1987;  
220 Lincoln et al. 1987) and this is supported by studies with sheep showing that naloxone can increase  
221 LH pulse frequency when gonadal steroid concentrations are high, but not when they are low  
222 (Ebling and Lincoln 1985; Brooks et al. 1986b). Our observations agree with this because the  
223 response to naloxone was increased by lupin supplementation, a treatment doubled the mean  
224 concentration of testosterone. Indeed, steroid hormone receptors are co-expressed in EOP-  
225 containing neurones (Simerly et al., 1996). It has to be noted, however, that naloxone can stimulate  
226 GnRH release in absence of testosterone, although it is far less effective in these circumstances  
227 (Jackson et al., 2000). Moreover, the dose of naloxone used by Jackson et al. (2000) might have not  
228 been able to overcome the inhibitory effect of testosterone on LH interpulse interval, suggesting  
229 that other pathways than EOPs mediate the negative feedback action of testosterone (Tortones,

230 1999). An alternative possibility is that other EOPs, such as dynorphin and orphanin FQ (OQF),  
231 acting through the  $\kappa$  opioid receptor and opioid like receptor-1 (OLR-1) respectively, mediate  
232 steroid negative feedback on GnRH neurones (Foradori et al., 2006; Foradori et al., 2007). We  
233 cannot rule out the possibility thus, that other members and receptors of the EOPs family are  
234 involved in this process.

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

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

253 The stimulatory effect of exogenous calcium alone on LH secretion in lupin-supplemented rams  
254 was not expected. Calcium plays a major role in the mechanism of action of GnRH on the synthesis  
255 and release of LH (Barbarino et al., 1982; Bates and Conn, 1984; Kalra et al., 1993). In humans, *in*

256 *vivo* studies have shown that calcium infusion can stimulate LH secretion, although only when the  
257 subjects were challenged with GnRH (Veldhuis et al., 1984). In agreement with this observation are  
258 the findings that LH release is decreased by calcium influx blockers in men (Barbarino and De  
259 Marinis, 1980; Barbarino et al., 1982; Struthers et al., 1983). To our knowledge, there are no reports  
260 of interactions between nutritional status and stimulation of LH secretion by calcium, but it could  
261 be speculated that an increase in nutrition increases the sensitivity of the pituitary gland to calcium.  
262 Interestingly, the size of the response was the same as that seen with the high dose of naloxone,  
263 suggesting the activation of an important mechanism. The response to calcium requires  
264 confirmation.

265

## 266 **5. Conclusion**

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

274

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281

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420 **Figure captions**

421

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

426

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

430

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

435

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

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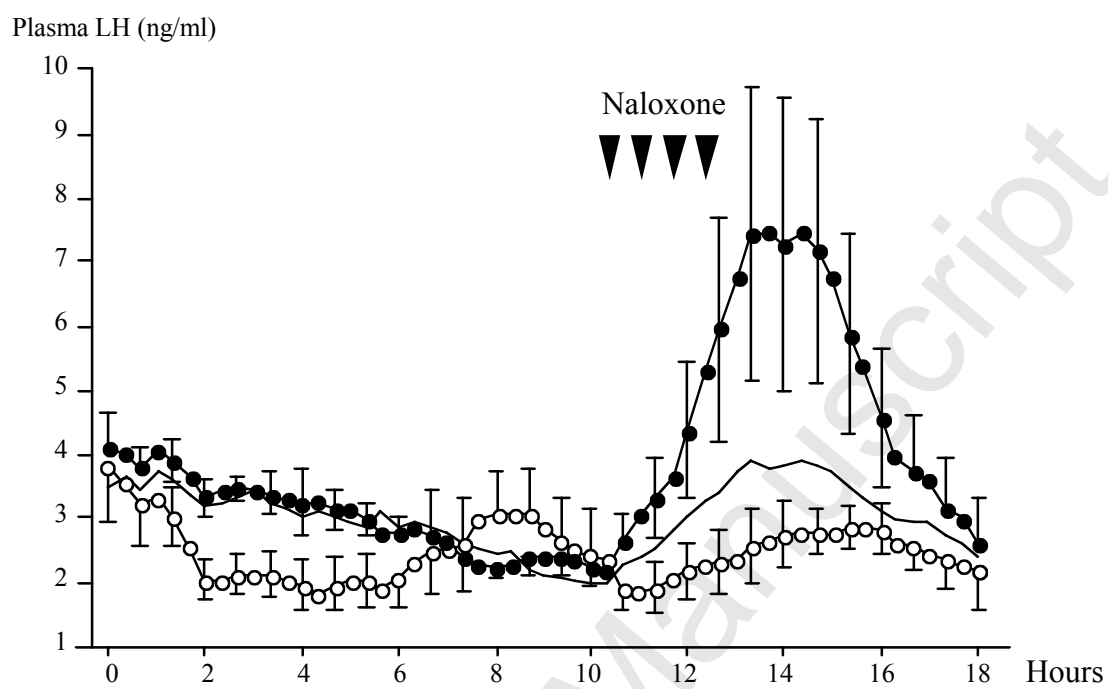
441 **Figure 5.** Effect Low Diet (Experiment 2a; □ ; n = 22) and High Diet (Experiment 2b; ■ ; n = 22)  
442 on mean LH and testosterone concentration in Merino rams. Values are expressed as means  $\pm$  s.e.m.  
443 Different letters indicate statistical difference at  $P < 0.001$ .

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1

2 **Figures**

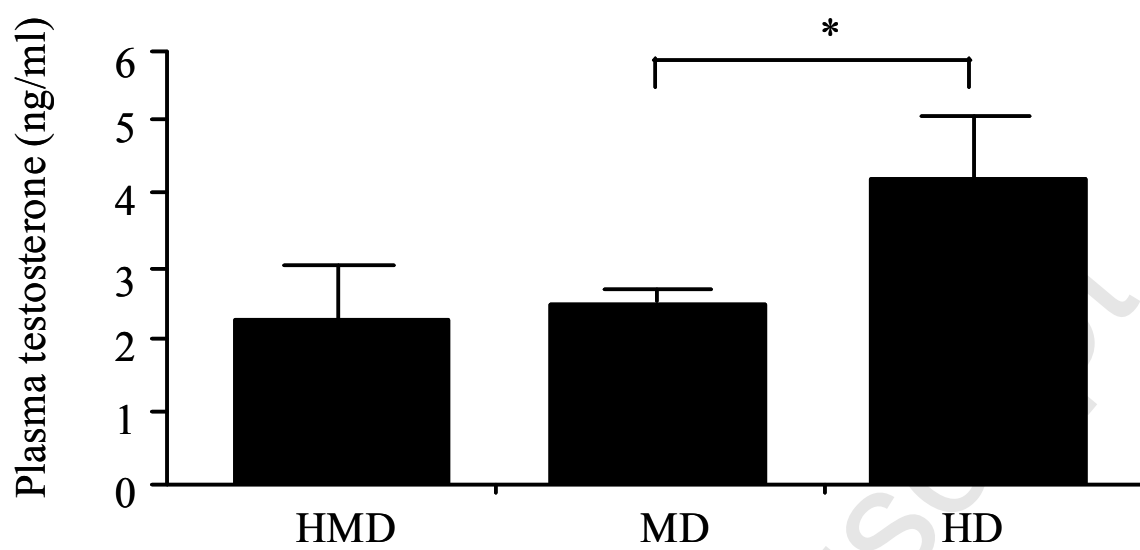
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5 **Figure 1.**

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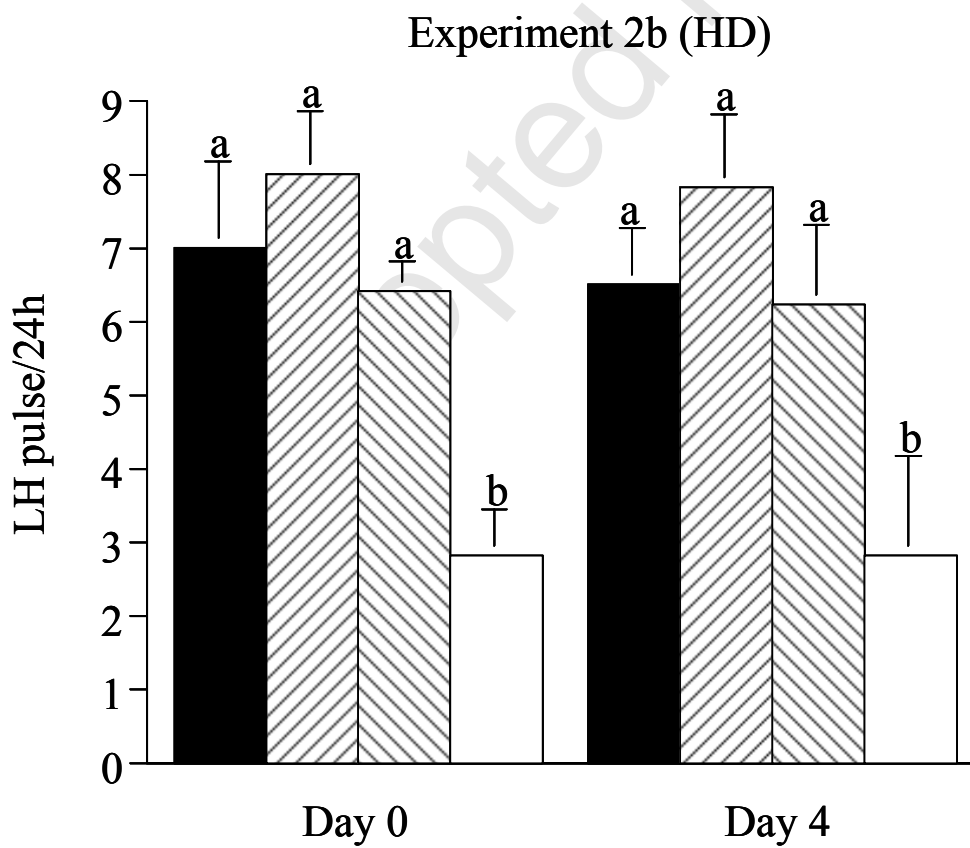
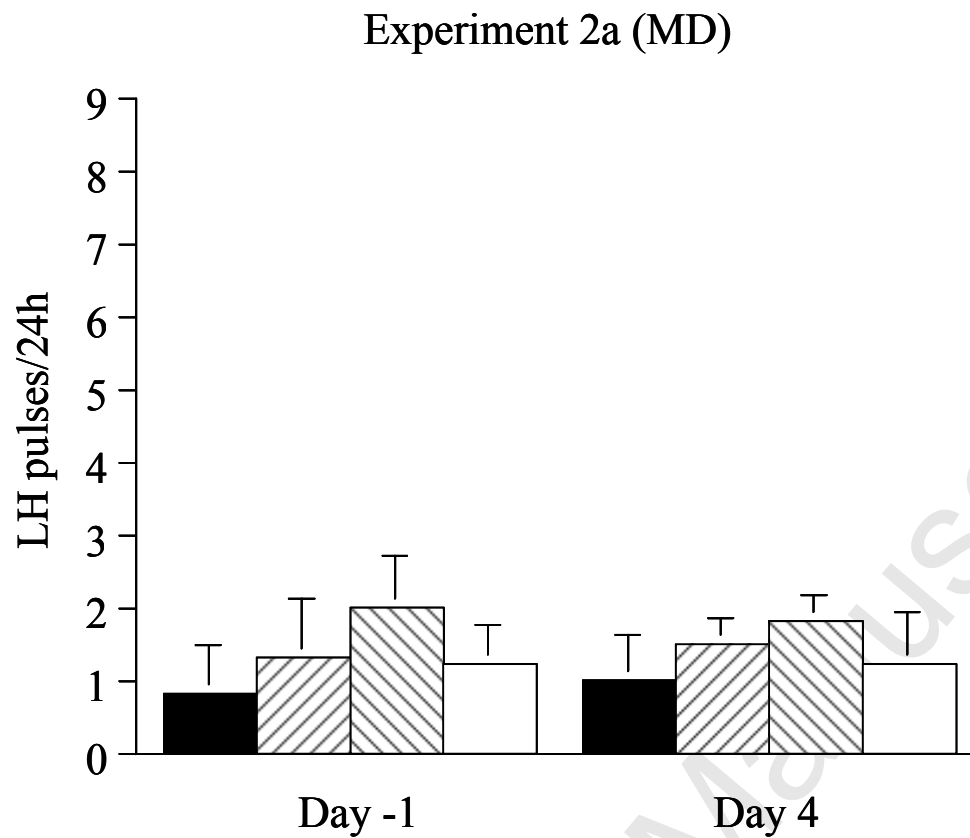
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11 **Figure 2.**

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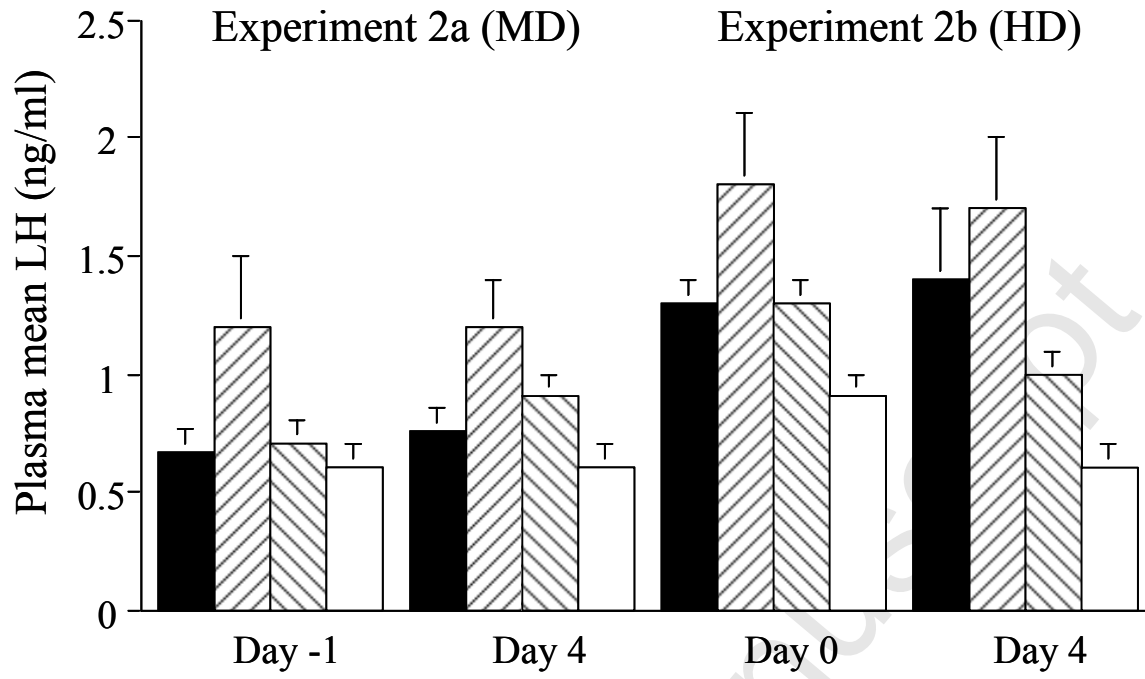
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16 Figure 3.

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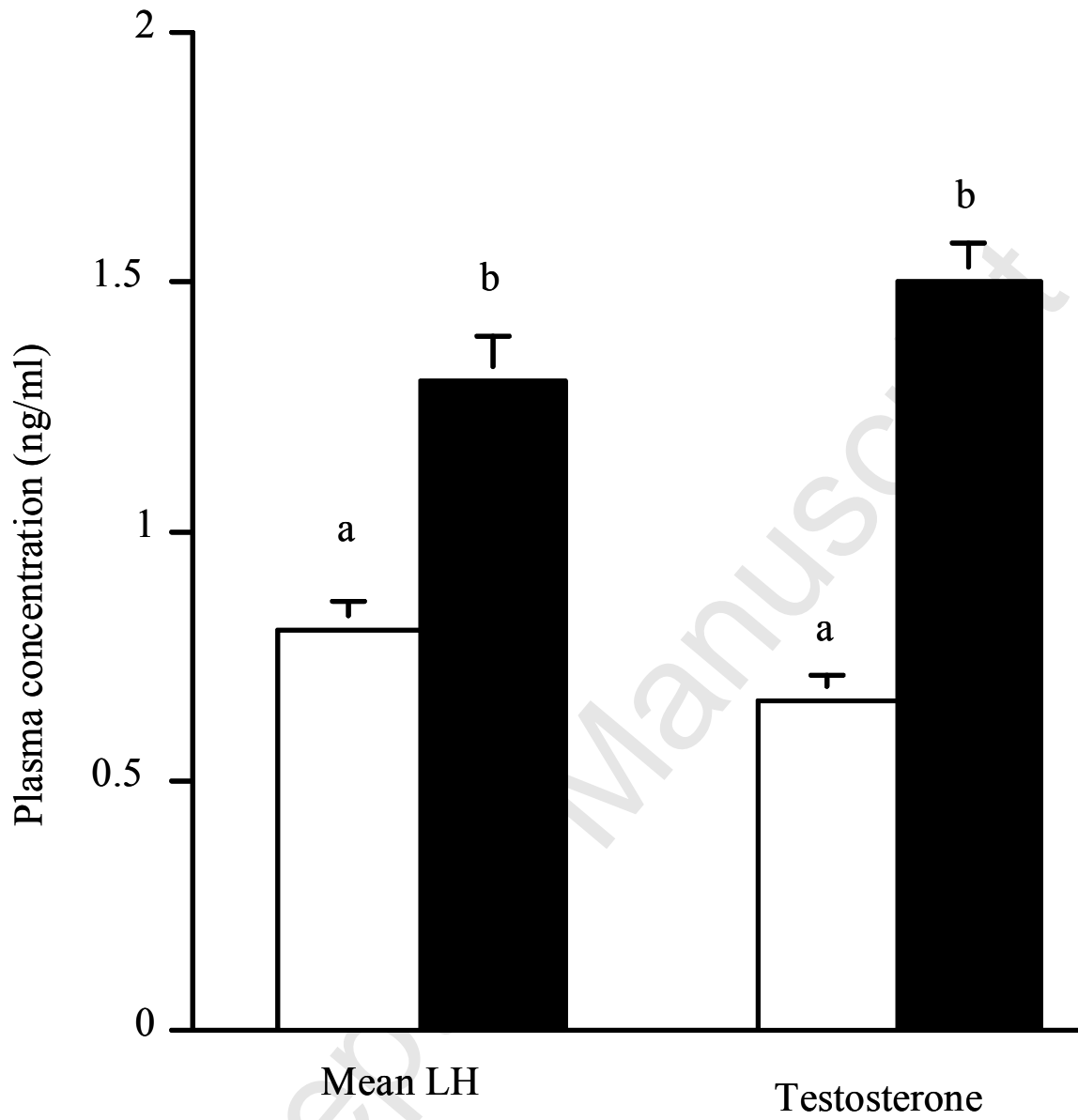
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19 **Figure 4.**

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24 Figure 5.



1 **Tables**

2

3 **Table 1:** LH pulse variables before and after naloxone injection in rams fed a Maintenance Diet4 (MD), half or the Maintenance Diet (HMD) or a High Diet (HD). All figures are means  $\pm$  s.e.m.

5

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

6

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

8