Temporal variation in LH and testosterone responses of rams after the introduction of oestrous females during the breeding season

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Summary. The response of sexually experienced Ile-de-France rams to the presentation of oestrous females in October at sunrise (Subgroup S) or at 11:00 h (Subgroup N) was studied and compared with unstimulated controls (Subgroup C). Animals (12 per group) were bled for 7 h at 20-min intervals, starting 3 h before stimulation by oestrous females (3 per group). Eight rams from Subgroup S showed an increase of LH pulse frequency and only 3 in Subgroup N ($P < 0.03$). In Subgroup S the introduction of females led to 2- and 3-fold increases in LH pulse frequency during the stimulation period compared with values in Subgroup C or before the stimulation period (3, 1.6 and 1 peaks/rams/6 h respectively; $P < 0.05$). The presence of females also led to an increase in mean testosterone concentrations, and small increases in basal and mean LH values. No differences were found in LH peak amplitudes. In Subgroup N only inconsistent evidence of increases in mean LH and testosterone values was found. No differences between Subgroups S and N in behavioural patterns during stimulation were detected. We conclude that the presence of females affects LH pulse frequency at sunrise but not at noon during the breeding season and this effect is at least partly independent of sexual behaviour. These results suggest a possible circadian variation in CNS sensitivity involving the hypothalamic regulation of LH secretion in response to the presence of oestrous females.

Keywords: temporal variations; LH; testosterone; ram; female effect; teasing

Introduction

It has been demonstrated in several mammalian species that the sudden introduction of oestrous females in a group of males induces certain behavioural and endocrinological changes in these males. Sniffing, nudging, Flehmen, mounting and ejaculation are commonly involved in the male sexual courtship. Some reports have shown that sexual activity or proximity of oestrous females also increased plasma concentrations of LH and testosterone in the mouse (Coquelin & Bronson, 1980), rat (Kamel & Frankel, 1978), hamster (Macrides *et al.*, 1974), rabbit (Agmo, 1976), monkey (Herndon *et al.*, 1981), boar (Liptrap & Raeside, 1978), bull (Katongole *et al.*, 1971) and ram (Sanford *et al.*, 1974a; Moore *et al.*, 1978).

Illius *et al.* (1976) reported that mature rams kept for 6 months in close proximity with cycling (oestrus induced) ewes had larger testes, higher testosterone concentrations and greater sexual and aggressive activity compared with those kept isolated from females. Other authors have found acute LH and testosterone elevations after introduction of females, but this phenomenon has so far been demonstrated only during the non-breeding season, while no response was found in the breeding season (Yarney & Sanford, 1983; Schanbacher *et al.*, 1987).

It has also been established that rams show different LH pulsatile patterns in relation to seasonal daylength variations, with the highest frequency observed during the summer–autumn

breeding season (Sanford *et al.*, 1974b; Schanbacher & Ford, 1976; Pelletier *et al.*, 1982). Although there appear to be no diurnal or circadian rhythms in LH secretion patterns in sheep, there is consistent evidence suggesting that pulse frequency is not a daily randomly distributed phenomenon. Fabre-Nys *et al.* (1984) observed that, in ewes, LH preovulatory surges are more frequent during the night than during the day, and Lincoln & Peet (1977) found that the lowest level of LH pulsatility in rams was after dawn and a maximum 4 h later. In a large scale experiment, Ortavant *et al.* (1982) found that, regardless of the season, the lowest frequency of LH and testosterone pulses is just after dawn, and the highest 3–4 h later.

Therefore a normal high pulse rate could be the reason for the absence of response of rams to stimulation by females during the breeding season. To investigate this possibility, we have compared the female-induced response at times of low or high LH pulsatility at this period of the year.

We have chosen to study the effects of stimulation at sunrise and 4 h later, which in the unstimulated ram are the periods with the greatest contrast in LH pulse frequency (Lincoln & Peet, 1977; Ortavant *et al.*, 1982). We studied also the relationship between male sexual behaviour and LH pulse frequency, since an important role of ejaculation in the LH response during the non-breeding season has also been reported (Yarney & Sanford, 1983), while other studies suggest that LH release may not be totally dependent on sexual behaviour (P. Orgeur & J. P. Signoret, unpublished).

Materials and Methods

Animals and housing conditions. Mature sexually experienced Ile-de-France rams were used. They had been isolated from contact with females for 3 months before the onset of the experiment. Oestrous females used as the stimulus were ovariectomized ewes treated with intravaginal progestagen sponges for 5 days, injected with oestradiol benzoate (50 µg, i.m.) on the 7th day, and used the 8th day. All animals were kept permanently indoors and fed dehydrated lucerne and maize, plus straw, mineral supplement and water *ad libitum*.

Experimental groups and schedule. The experiment was conducted on two separate occasions 6 days apart. The 24 rams were allocated to two groups at random. On the first day (2 October) 12 rams (Group I) were penned in a 10 m² pen, and 3 oestrous females were introduced in the Group at 07:00 h (sunrise time provided by Direction de la Meteorologie Nationale, Tours, France; Subgroup S). The 12 other males (Group II) were kept in another pen 5 m away, without visual communication, and served as controls (Subgroup C). Preliminary studies made in this laboratory have shown that a 30-cm separation from oestrous females did not affect LH frequency in rams. On the second day of the experiment (8 October), the 12 males that had been stimulated served as controls (Subgroup C), while the other 12 were stimulated by 3 oestrous females at 11:00 h (Subgroup N). On both days animals from Subgroups S and N were bled by jugular venepuncture every 20 min for 7 h, starting 3 h before the introduction of females (Period B). Females remained with the males for the next 4 h (Period D). Rams in each Subgroup C were bled every 20 min for only 4 h in parallel with Period D. During this period the sexual behaviour of the rams was recorded by an observer situated 2 m above the ground. Anogenital 'sniffing' (perineal nosing), 'nudging' (lateral approach and foreleg extension, with or without extension and retraction of the tongue and low-pitched vocalizations), 'Flehmen' (typical upper-lip curl back that reveals toothless portion of the upper jaw), 'mounting' (the ram straddles the rear quarters of the ewe with his forelegs, with or without vaginal intromission), and 'ejaculation' (typical deep thrust followed by dismounting) (Banks, 1964) were recorded between each blood sampling. The ram-ram interactions were also recorded as 'fighting' (butts with the head in the flank, and active positional fighting to remain near females) and 'mounting' activity. Animals were identified by numbers attached to their backs. Pre-dawn bleedings were done using individual dim red-light torches.

Hormonal assays. Blood samples were kept at 4°C before centrifugation, and thereafter plasma was stored at –15°C. LH and testosterone concentrations were measured by direct RIA methods (Pelletier *et al.*, 1982; Garnier *et al.*, 1978) on duplicate serum samples. The intra-assay coefficients of variation were 7.2 and 5.4% for LH and testosterone respectively. The sensitivities were 100 pg LH/ml and 200 pg testosterone/ml. The LH peak identification was carried out through utilization of Pulsar algorithm methods (Merriman & Wachter, 1982). The cut-off Gn values were computed for each individual ram and the smoothing window chosen was 200 min (10 points). The efficacy of this method was checked by its correlation with testosterone peaks; in all cases an LH (Pulsar-detected) peak was followed by a testosterone elevation. Hormonal parameters were (1) mean concentration, (2) basal concentration, (3) peak amplitude as peak value and Δ value (difference between peak height and basal level), (4) peak LH frequency; and (5) mean concentration for testosterone values.

Statistical analysis. Comparisons between Period B and the first 3 h of Period D were made with specific non-parametric tests for dependent variables, i.e. the Wilcoxon test (Siegel, 1956) for peak frequency values, and ANOVA paired comparison tests (Sokal & Rohlf, 1981). The beginning of Period D for the testosterone values was delayed by 40 min to take into account the lag of gonadal response towards an LH increase (Terqui *et al.*, 1980).

For comparisons of Period D and Subgroup C values the Mann-Whitney test was used for peak frequency and behavioural observations (Siegel, 1956) and ANOVA for basal concentration, peak amplitude and mean concentration values (Sokal & Rohlf, 1981). A positive response to the introduction of females was considered to have occurred when a significant increase in LH peak frequency was found when comparing values in Period B and the first 3 h of Period D.

Fisher's exact test (Sokal & Rohlf, 1981) was carried out to determine the significance of differences between the proportions of responding rams in Subgroups S and N. A Spearman's rank correlation test was performed between each individual behaviour and LH pulse number values (Sokal & Rohlf, 1981). The level of significance accepted was $P < 0.05$.

Results

Frequency of LH pulses

Before the introduction of females the mean number of LH pulses/animal/6 h did not differ significantly between Subgroups S and N (Table 1). By contrast, during stimulation, LH pulses were more frequent in Subgroup S than in Subgroup N. Also the pulse frequencies were different in Subgroup S between Periods B and D as well as between Period D and the equivalent period in Subgroup C. On the other hand none of these factors (Periods B and D, and Subgroup C values) differed significantly in Subgroup N (see Table 1). Overall, the number of males which showed an increase in the number of LH pulses during the first 3 h of stimulation relative to the number of pulses found during the 3 preceding hours was significantly higher in Subgroup S (8/12) than in Subgroup N (3/12, $P < 0.03$).

Table 1. LH and testosterone mean values from Subgroup S (stimulated at sunrise) and Subgroup N (stimulated at noon) rams (10 observations/ram in each period and in the control subgroup)

		Treated group			Pooled standard deviation
	Sub-group	Before stimulation (Period B)	During stimulation (Period D)	Control Subgroup	
Peak LH frequency (no./ram/6 h)	S	1.0 ^a	3.0 ^b	1.3 ^a	0.18
	N	1.1 ^a	1.3 ^a	0.8 ^a	
Basal LH conc. (ng/ml)	S	0.39 ^a	0.43 ^b	0.30 ^c	0.23
	N	0.37 ^a	0.38 ^a	0.40 ^a	
Peak LH height (ng/ml)	S	0.84 ^a	0.74 ^a	0.80 ^a	0.20
	N	0.66 ^a	0.72 ^a	0.78 ^a	
Δ LH value (ng/ml)*	S	0.39 ^a	0.27 ^a	0.46 ^a	0.14
	N	0.23 ^a	0.31 ^a	0.32 ^a	
Mean LH conc. (ng/ml)	S	0.42 ^a	0.50 ^b	0.36 ^a	1.73
	N	0.38 ^c	0.43 ^d	0.43 ^d	
Mean testosterone conc. (ng/ml)	S	13.4 ^a	18.6 ^b	14.3 ^a	10.8 ^b
	N	14.1 ^a	15.2 ^a	10.8 ^b	

Different superscripts in the same rows and for a given variable in the same columns, are statistically significant differences ($P < 0.05$). Control data were compared only with that during Period D of the corresponding experimental group.

*Difference between peak height (peak value) and basal concentration.

Basal concentrations of LH

Slight increases were found during Period D in Subgroup S compared with values in Period D of Subgroup N, Period B in Subgroup S, or in Subgroup C. By contrast no changes were observed in Subgroup N (see Table 1).

Table 2. Behaviour observations for 12 rams expressed as means ($\pm$ standard error) for Period D (4 h)

	Subgroup S*	Subgroup N†
Ram–ewe interactions		
Sniffing	15.1 $\pm$ 3.8	26.2 $\pm$ 8.4
Flehmen	2.5 $\pm$ 0.3	3.9 $\pm$ 1.1 (2)
Nudging	10.9 $\pm$ 5.0 (4)	11.8 $\pm$ 7.8 (5)
Mounting	7.3 $\pm$ 3.2 (5)	7.1 $\pm$ 3.7 (6)
Ejaculation	2.3 $\pm$ 0.8 (6)	1.3 $\pm$ 0.5 (5)
Ram–ram interactions		
Fighting	4.7 $\pm$ 1.2 (8)	4.8 $\pm$ 1.5 (6)
Mounting	6.0 $\pm$ 0.8 (8)	4.3 $\pm$ 0.8 (9)

Numbers in parentheses are no. of rams that did not show the behaviour.
*Stimulated at sunrise.
†Stimulated at 11:00 h.

Peak amplitude values of LH

No significant inter- or intra-group variations were found in peak values or Δ values of LH (see Table 1).

Mean LH concentrations

Intra-group comparisons revealed an increase between Periods B and D in Subgroups S and N. However, Period D values in Subgroup N did not differ from those of Subgroup C. Values in Period D were significantly higher in Subgroup S than in Subgroup N.

Mean testosterone concentrations

A significant increase in testosterone was noted between Periods B and D in Subgroup S. Values in Period D were significantly higher in Subgroups S and N than in Subgroup C (Table 1).

Sexual behaviour

No significant differences were detected in rams between Subgroups S and N (see Table 2). Similar results were obtained after a Spearman’s rank correlation test performed between each observed behaviour and LH pulse number within groups.

After analysis of individual relations between sexual behaviour and LH response we found that, in Subgroup N, for the 3 rams which showed an increase of LH pulses following stimulation by females, all had an ejaculation before an LH peak. By contrast, in Subgroup S such a relation was observed only in 3 rams, 2 animals showed an LH response at least 20 min before ejaculation, and 3 others showed an LH response but without any mounting or ejaculatory activity (see Fig. 1).

Discussion

Our results clearly indicate that (1) rams can respond to the introduction of oestrous females in the breeding season by increased secretion of LH and testosterone; (2) the endocrine response depends on the time of the day at which stimulation by females occurs; and (3) this physiological response does not depend strictly on ejaculation.

Fig. 1. LH patterns in 3 individual rams showing LH pulse frequency increase after ejaculation (Ram A), LH pulse frequency increase before ejaculation (Ram B); and several LH pulses without any mounting or ejaculatory activity (Ram C). All rams are from Subgroup S (stimulated at sunrise). *LH peak detected by Pulsar. Blood samples were taken every 20 min.

These findings are at variance with the conclusions of other workers. Yarney & Sanford (1983) and Schanbacher *et al.* (1987) found no LH response in the breeding season. This difference could be due to the time of stimulation, 09:30 h and 11:00 h in the studies by Yarney & Sanford (1983)

and Schanbacher *et al.* (1987) versus 07:00 h and 11:00 h in our study (good response in Subgroup S and poor in Subgroup N). In ewes, Martin *et al.* (1985) found similar results when testing the 'male effect' at 07:30 h and 19:30 h, i.e. there was a shorter latency to the first LH pulse after introduction and higher ovulation rates in the ewes stimulated in the morning.

This variation of response between sunrise and noon is related to the normal diurnal tendency of LH secretion (Lincoln & Peet, 1977; Ortavant *et al.*, 1982). It may reflect a diurnal ability of the hypothalamic GnRH system to react in the presence of female stimuli. This phenomenon could be explained in several ways, (1) a circadian variation in the feed-back performance; (2) an intrinsic hypothalamic rhythm conditioning a wide variation of circadian threshold, or (3) a combined action of both mechanisms.

There is evidence that an endogenous opioid system plays an important role in GnRH secretion in rams (Malven, 1986, review), especially during the breeding season (Ebling & Lincoln, 1985). Recent studies have shown that gonadal steroids are involved in the action of the opioid system (Schanbacher, 1985). Different levels of sensitivity to negative feed-back of gonadal steroids have been described in relation to annual reproductive changes in sheep: high in the non-breeding season and *vice versa* (Lincoln *et al.*, 1985, review). The spontaneous LH circadian fluctuations observed by Lincoln & Peet (1977) and Ortavant *et al.* (1982) should be influenced in a similar way, with a low sensitivity to negative feed-back at noon, allowing a high frequency of LH pulses; and raised sensitivity at sunrise. In immature female rats, Blank & Mann (1981) found a high LH response in the morning towards injections of naloxone (a potent blocker of opioid receptors) and a low response in the afternoon. Thus a circadian variation in the activity of the endogenous opiate receptors would determine a daily rhythm of negative feed-back sensitivity to gonadal steroids. In such a system the 'female stimulus' would inhibit this feed-back exerted by testosterone, but only when it is strong, i.e. at sunrise, at least during the breeding season. When discussing the 'male effect' mechanisms on LH pulse frequency, Martin *et al.* (1983) suggested that the ram stimulus apparently acts by inhibiting the negative feed-back exerted by oestradiol. Also, in a later study, Martin (1984) proposed that teasing acts probably through a direct effect on the pulsar (hypothetical neuroendocrine central unit that emits the pulsatile signal that releases GnRH). In another seasonal breeder, the Syrian hamster, a circadian variation, parallel to changes in the pineal content of melatonin, in the amount of β -endorphin in the anterior and basal hypothalamus was noted, but this fact has little physiological significance because it was not correlated with a similar variation in the LH response to naloxone injections (Roberts *et al.*, 1985). Despite the importance of melatonin in seasonal reproductive changes (Lincoln & Short, 1980) no correlations with circadian patterns of gonadotrophins have been established so far (Lincoln *et al.*, 1982).

The mechanisms by which natural stimuli such as daylength changes may affect hypothalamic GnRH secretion sensitivity to sensory cues provided by females remain to be established. A diurnal variation in the behavioural cues provided by females cannot be excluded, such an effect was described in the ewe for the onset of oestrus and duration of oestrus by Cahill *et al.* (1975) in a continuous open-field experiment. These authors found an increase of both parameters (in frequency and duration) at sunrise and sunset, and the lowest levels at noon; but the relatively high ambient temperatures observed at noon in the Australian summer (Victoria) may have affected physical activity, i.e. mating activity and, indirectly, the detection of the onset and duration of oestrus. By contrast, no such pattern of behaviour was found by Blockey & Cumming (1970) in a similar study carried out in the autumn. However, Fabre-Nys *et al.* (1984) propose a possible temporal variation rhythm in the onset of oestrus in the ewe, related to the nocturnal preferred period for the onset of the preovulatory LH surge, and the well established link between this surge and the onset of oestrus. In any case, such rhythms of oestrous activity need to be integrated by the CNS.

Our behavioural findings have shown that mounting or ejaculation are not essential to obtain an endocrine response. Yarney & Sanford (1983) reported that ejaculation was very important for the LH response in 4 sexually inexperienced rams. The small number of rams or the lack of sexual experience could explain these differences. The fact that the LH response to the introduction of

females is at least partly independent of mounting suggests that this phenomenon does not depend on tactile stimulation provided by intromission and therefore other cues (visual and/or olfactory) must be involved.

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