Effect of the introduction of rams during the anoestrous season on the pulsatile secretion of LH in ovariectomized ewes

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Summary. Introduction of rams to ovariectomized ewes treated with oestradiol implants ($N = 10$) increased the frequency of LH pulses from 4.8 to 10.6 pulses per 12 h. This effect was reflected by increases in mean levels of LH and the basal levels upon which the pulses were superimposed. In ewes that had not been treated with oestradiol ($N = 5$), there was no significant increase in pulse frequency but mean and basal levels of LH increased slightly after the introduction of rams. In a second experiment, similar effects of the introduction of rams were seen in ovariectomized ewes treated with oestradiol or oestradiol + androstenedione ($N = 16$), but no significant effects of the rams were observed in untreated ewes ($N = 8$) or ewes treated only with androstenedione ($N = 7$). No preovulatory surges of LH were observed in the 30-h period after the introduction of rams.

It was concluded that the ram stimulus probably evokes the increase in pulse frequency by inhibiting the negative feedback action of oestradiol, and that the surge normally observed in entire ewes is dependent on the ovarian response to these pulses. However, the observation of responses in some ewes not treated with oestradiol also raises the possibility that the ram stimulus can act directly on the hypothalamic neurones that control the secretion of LH, and that this effect is enhanced in the presence of oestrogen.

Introduction

The frequency of pulses of luteinizing hormone (LH) in the entire ewe is lower during anoestrus than during the breeding season (Scaramuzzi & Martensz, 1975; Jackson & Davis, 1979). This is due to increased sensitivity of the hypothalamus to negative feedback by oestradiol which interrupts the sequence of endocrine events that normally leads to ovulation (Legan & Karsch, 1979). However, the introduction of rams will induce ovulation in these ewes provided they have been conditioned by a period of isolation from rams (Underwood, Shier & Davenport, 1944; Schinckel, 1954; Oldham, Martin & Knight, 1978). It has been proposed that the ram stimulus accomplishes this by overcoming the inhibitory actions of oestradiol, since the initial response of seasonally anovulatory ewes to the introduction of rams is an immediate increase in the frequency of the LH pulses (Martin, Oldham & Lindsay, 1980a; Martin *et al.*, 1980b; Poindron *et al.*, 1980). It is unlikely that negative feedback by progesterone is an important component in the effect, because the levels of this hormone are low in anovulatory ewes due to the absence of corpora lutea.

Ovariectomized ewes also undergo seasonal changes in hypothalamic sensitivity to oestradiol (Legan, Karsch & Foster, 1977) and oestradiol alone maintains a low frequency of LH pulses during anoestrus (Wright, Findlay & Anderson, 1981; Goodman & Karsch, 1981). Therefore, if the ram stimulus inhibits the effect of oestradiol in the entire ewe, it should also inhibit the effect of oestradiol in 'anoestrous' ovariectomized ewes bearing oestradiol implants. On the other hand, if the ram stimulus has a direct effect on the hypothalamo-hypophysial system, it should also evoke an increase in the secretion of LH in ovariectomized ewes in the absence of oestrogen.

Androstenedione is also secreted by the ovaries of the ewe during the anoestrous and breeding seasons (Scaramuzzi, Baird, Land & Wheeler, 1974; Baird & Scaramuzzi, 1976; Scaramuzzi & Baird, 1977) and has been thought to have a role in negative feedback on the secretion of LH (Scaramuzzi & Martensz, 1975). It may also be involved, therefore, in the response in LH secretion to the introduction of rams.

In addition to an increase in the basal levels of LH, entire anoestrous ewes experience a preovulatory surge 6–52 h after the introduction of rams (Oldham *et al.*, 1978; Knight, Peterson & Payne, 1979; Martin *et al.*, 1980b; Poindron *et al.*, 1980). How this is brought about is not known but it has been suggested that the surge may be induced by a direct action of the ram stimulus on the hypothalamic mechanisms controlling LH secretion, so bypassing the normal system of positive feedback by oestrogen (Oldham *et al.*, 1978; Knight *et al.*, 1979). Such surges should therefore be induced in ovariectomized ewes, in which there will presumably be insufficient oestrogen to exert positive feedback, and the present experiments were designed to test the effects of the introduction of rams on the secretion of LH in ovariectomized ewes.

Materials and Methods

Experiment 1

Fifteen mature Merino ewes were ovariectomized in May 1980, and 10 were given subcutaneous oestrogen implants which were 'large' (10 mm × 3.18 mm o.d., Group C) and released 7.4 µg oestradiol/day, or 'small' (5 mm × 2.41 mm o.d., Group B) and released 3.5 µg/day. Five ewes were given empty implants (Group A). In September the implants were renewed. The implants used were similar to those described by Karsch *et al.* (1973), being made from Silastic Medical Grade tubing (Dow Corning) packed with steroid powder (Sigma). The release rates were estimated, after 3 days of preincubation in 0.15 M-NaCl at 37°C, by measuring the release at 37°C over 24 h of [1,2,6,7-³H]oestradiol-17β (New England Nuclear) from implants containing a known amount of the labelled hormone into serum from ovariectomized ewes. The implants were not preincubated before insertion into the ewes but were washed with 70% ethanol to sterilize them and remove any excess steroid. The ewes were maintained under natural lighting and in the absence of rams until early November. Jugular cannulae were inserted and on the following day blood was sampled every 15 min for 6 h, with the ewes standing in single file in a race. Seven mature Merino rams were then placed on either side of the race, and after a delay of 15 min sampling was continued for another 6 h. Plasma was obtained from the blood samples for measurement of LH concentrations.

Statistical analysis. The basal levels of LH, before and after the introduction of the rams, were calculated as the mean of the 10 lowest points in the profile. The mean levels for an individual ewe were calculated from the 10 samples immediately before or immediately after the introduction of rams. There was also a direct comparison of the single sample immediately before the introduction of rams with the one following. Except when paired comparisons were used to test the effects of the introduction of rams, the concentration data were transformed logarithmically before analysis of variance. The effects of steroid treatments, before and after the introduction of rams, were tested by analysis of variance. The effects of the introduction of rams were similarly tested, but using paired comparisons (Sokal & Rohlf, 1969).

Differences for the number of pulses/h between groups and the effects of treatments on this variable were tested for significance by analysis of variance. Mean pulse intervals were estimated as the reciprocal of pulse frequency but this variable is not suitable for analysis when there are <2 pulses during the observation period.

Not all entire anoestrous ewes that are introduced to rams display responses in LH secretion (Martin *et al.*, 1980a). It was therefore necessary to define a 'responder' so that she could be distinguished from a ewe showing normal fluctuations in LH levels. Whether a ewe 'responded' was initially determined subjectively from the LH profiles—the rams evoke an increase in LH secretion at the first sample immediately after they are introduced (Text-figs 1 & 2), as in the entire ewe (Martin *et al.*, 1980a). There was often a 3- or 4-fold increase in the frequency of pulses and the basal levels of LH rose as a result. In most cases, this subjective assessment was relatively simple. However, in ewes with very frequent pulses, or with irregular patterns of LH, the rams were often introduced at the start of a spontaneous, naturally occurring pulse. If these ewes did not 'respond' to the introduction of rams, there was no increase in basal or mean levels, or the frequency of pulses. These ewes were classed as 'non-responders'. With some ewes, no decision could be made and they were excluded from this analysis. The effects of treatments on the proportion of ewes 'responding' were tested by χ^2 analysis.

Experiment 2

Thirty-one ewes, ovariectomized and given subcutaneous implants on the same day in August 1979, were isolated from rams and maintained under natural lighting from September 1979 until the experiment in November (late spring, anoestrus). At ovariectomy the treatment groups were established as: Group D, N = 8, control; Group E, N = 7, androstenedione implant (10 mm × 3.18 mm o.d.); Group F, N = 8, oestradiol implant (5 mm × 2.41 mm o.d.); and Group G, N = 8, oestradiol implant + androstenedione implant. The implants were constructed and tested as in Exp. 1, and released 3.5 µg oestradiol/day or 14.9 µg androstenedione/day (*in vitro*). To facilitate the sampling procedure, 16 of the ewes were chosen at random and subjected to the experimental protocol in early November 1979, and the protocol was repeated for the remainder of the ewes 2 weeks later. Between these times the rams were kept away from the experimental area, which was thoroughly ventilated. On each occasion, the ewes were cannulated the day before being introduced to 16 rams and blood samples were collected at 15-min intervals from 2 h immediately before until 10 h immediately after the introduction of rams at 08:15 h. The rams remained in close contact throughout this period and were then moved to pens across the aisle of the work area, where they still had visual, auditory and probably olfactory contact with the ewes. Blood samples were then taken from the ewes at 4-h intervals for a further 30 h. All samples were assayed for LH.

Due to changes in the experimental design, there were some variations in the methods of analysis of the LH profiles. No estimates of the effects on basal LH could be made because too few samples were taken before the introduction of the rams. The mean levels of LH were estimated as the mean of 9 samples taken just before or just after the introduction of rams. In one ewe from each of Groups D, E and F, the LH profiles were irregular and the pulses could not be defined. The data for pulse frequency in these ewes were omitted from the analysis but their other data were retained. A preovulatory surge of LH was arbitrarily defined as a sustained increase in the secretion of LH, with the levels remaining >20 ng/ml for at least 6 h.

Radioimmunoassay of LH

The method has been described in detail elsewhere (Martin *et al.*, 1980a). The limit of detection of the standard curve was 27 ± 7 pg/tube (mean $\pm$ s.e.m.) and non-specific binding was always <5%. Six replicates of 3 pooled samples containing 1.04 ± 0.08 ng/ml, 2.9 ± 0.2 ng/ml and 6.3 ± 0.2 ng/ml were included in each of the 9 assays used in this study and were used to estimate

variation within and between assays. The coefficients of variation within assays were $15.0 \pm 1.8\%$, $9.6 \pm 3.4\%$ and $6.3 \pm 0.9\%$, and between assays were 17.6% , 9.3% and 4.8% for the three pools respectively. The antiserum (UWA-3B) exhibited minor cross-reactions with NIH-FSH-S12 (0.9%), NIH-GSH-S8 (4.6%), NIH-P-S12 (0.2%) and NIH-GH-S11 (1.3%).

Results

Experiment 1

Before the introduction of rams, the basal levels, mean levels and frequency of pulses were all lower in ewes with oestradiol implants than in the control ewes (Table 1). The effect of implant size on these variables was not significant ($P > 0.25$).

After the introduction of rams, 8/10 of the ewes treated with oestradiol 'responded' (Text-fig. 1), compared to 0/5 in the control group ($P = 0.018$). The frequency of LH pulses increased only in the ewes treated with oestradiol ($P = 0.039$). Two oestrogen-treated ewes did not 'respond', and both had very frequent pulses before the introduction of rams, i.e. the oestrogen from the implant did not exert any negative feedback. The mean levels, based on the 10 samples taken before and after the introduction of rams, showed a similar response but there was also a significant increase in the control ewes (Table 1). There was a significant interaction between the treatments on the levels of LH in single samples taken before and after the introduction of rams but the effect in the control group was not statistically significant. Basal levels increased after the introduction of rams but the interaction was not significant ($P = 0.23$), indicating that there was an increase in all three groups (Text-fig. 1).

There was no significant effect of size of implant on any of the variables measured, including the magnitude of the response to the introduction of rams. The tendency for the ewes with smaller implants to show a larger response was due to 2 ewes in the group which displayed pulses of very

Table 1. Effect of the introduction of rams on LH levels and pulse frequency in ovariectomized ewes

	Treatment			
	Group A (control)	Group B (oestradiol, small)	Group C (oestradiol, large)	Group B + C
No. of ewes	5	5	5	10
Basal LH (ng/ml)				
Before rams	5.80 ± 0.57	2.47 ± 1.02	1.52 ± 0.39	2.00 ± 0.54
After rams ^f	7.53 ± 0.78	5.55 ± 1.91	3.87 ± 1.05	4.71 ± 1.06
Mean LH (ng/ml)				
Before rams	7.03 ± 0.64	4.32 ± 1.35	2.28 ± 0.67	3.31 ± 0.79
After rams	8.89 ± 0.69	8.40 ± 2.84	4.70 ± 1.14	6.55 ± 1.57
Single sample LH (ng/ml)				
Before rams	7.11 ± 0.95	4.50 ± 1.02	2.20 ± 0.82	3.35 ± 0.73
After rams	8.89 ± 1.19	10.29 ± 3.47	4.59 ± 0.95	7.44 ± 1.95
Pulse frequency (no./h)				
Before rams	1.28 ± 0.07	0.40 ± 0.24	0.40 ± 0.21	0.40 ± 0.15
After rams	1.32 ± 0.09	0.90 ± 0.22	0.87 ± 0.18	0.88 ± 0.14
Pulse interval (min)				
Before rams	47 ± 3	150 ± 90	150 ± 79	150 ± 56
After rams	45 ± 3	67 ± 16	69 ± 14	68 ± 11
No. of ewes 'responding'	0	4 ^b	4 ^b	8 ⁱ

Values are mean $\pm$ s.e.m.

Effects of oestradiol: ^a $P < 0.003$; ^b $P < 0.001$.

Effects of introduction of rams: ^c $P = 0.001$; ^d $P = 0.034$; ^e $P = 0.056$; ^f $P = 0.009$; ^g $P = 0.039$.

Compared with Group A, ^h $P = 0.056$; ⁱ $P = 0.018$; ^j $P = 0.003$.

Text-fig. 1. Experiment 1: effects of the introduction of rams (0 h) on the pulsatile secretion of LH in ovariectomized ewes treated with oestradiol. Ewe 1 was given a 10 mm implant, and Ewes 2, 3 and 4 were given 5 mm implants. All were classed as 'responders'. Ewes 5 and 6 were not treated with oestradiol: neither was classed as a 'responder', but in Ewe 6 there was an increase in the basal level of LH after the introduction of rams.

Text-fig. 2. Experiment 2: effects of the introduction of rams on the secretion of LH in ovariectomized ewes. Ewes 7 and 8 were treated with oestradiol alone, but only Ewe 7 'responded' to the rams. Ewe 9 was treated with oestradiol and androstenedione and 'responded' to the ram stimulus.

high amplitude (Ewe 4, Text-fig. 1). Although the secretion of LH increased in the oestradiol-treated ewes after the introduction of rams, the frequency of pulses was still lower than in the control ewes. Mean and basal levels were also lower, but not significantly so ($P = 0.19$, $P = 0.11$). If only the 'responders' are included, this result does not change.

Experiment 2

Before the introduction of rams, oestradiol had lowered the frequency of the LH pulses (Table 2)

Table 2. Effects of oestradiol, androstenedione and the introduction of rams on the frequency of LH pulses in ovariectomized ewes

Group	No. of ewes*	Pulse frequency (no./h)		Pulse interval (min)		Ewes responding
		Before rams	After rams	Before rams	After rams	
D (control)	8	1.50 ± 0.05	1.34 ± 0.05	40 ± 1	45 ± 2	0
E (androstenedione)	6	1.33 ± 0.11	1.33 ± 0.05	45 ± 4	45 ± 2	0
F (oestradiol)	7	$0.50 \pm 0.16^\dagger$	$0.90 \pm 0.23^\ddagger$	120 ± 38	67 ± 17	4§
G (oestradiol + androstenedione)	7	$0.54 \pm 0.21^\dagger$	$0.95 \pm 0.18^\ddagger$	111 ± 43	63 ± 12	5§
D + E	14	1.43 ± 0.06	1.34 ± 0.04	42 ± 2	45 ± 1	0
F + G	14	$0.52 \pm 0.13^\dagger$	$0.93 \pm 0.14^\ddagger$	115 ± 29	65 ± 10	9¶

Values are mean $\pm$ s.e.m.

* Ewes with irregular profiles are omitted.

† Effect of oestradiol—before rams $P < 0.001$; after rams $P \approx 0.009$.

‡ Effect of introduction of rams—all ewes $P \approx 0.08$; Group F + G only $P \approx 0.001$.

§ Compared with Group D, for Group F $P \approx 0.084$, for Group G $P \approx 0.031$.

¶ Compared with Group D + E, $P \approx 0.005$.

and the mean levels of LH (Table 3) relative to untreated ewes or ewes with androstenedione implants. Androstenedione had no significant effect on the secretion of LH, either before or after the introduction of rams.

After the rams were introduced, 9 ewes 'responded' with an increase in LH levels (Text-fig. 2) and all 9 had been treated with oestradiol (Table 2). There was no obvious response in any of the control ewes or ewes treated with androstenedione alone (Text-fig. 2). Among ewes treated with oestradiol, the rams increased the frequency of pulses, and the mean levels. This is supported by observation of the effects of oestradiol on the change in mean levels induced by the rams (Table 3): the size of the change was significantly higher in the ewes treated with oestrogen than in other ewes. The net change in the ewes not treated with oestradiol was often negative because the rams were introduced after the peak of a spontaneous pulse.

A preovulatory surge of LH was not observed in any of the ewes during the 40-h period following the introduction of rams.

Table 3. Effects of oestradiol, androstenedione and the introduction of rams on the levels of LH in ovariectomized ewes

Group	No. of ewes	Mean LH values (ng/ml)			Single sample Δ LH§
		Before rams	After rams	Δ	
D (control)	8	4.44 $\pm$ 0.78	3.91 $\pm$ 0.82	-0.46 $\pm$ 0.26	-0.55 $\pm$ 0.80
E (androstenedione)	7	3.45 $\pm$ 0.39	2.85 $\pm$ 0.33	-0.60 $\pm$ 0.18	+0.0 $\pm$ 0.20
F (oestradiol)	8	0.92 $\pm$ 0.27*	1.35 $\pm$ 0.33†	+0.45 $\pm$ 0.35‡	+0.58 $\pm$ 0.60
G (oestradiol + androstenedione)	8	1.24 $\pm$ 0.41*	1.83 $\pm$ 0.27†	+0.45 $\pm$ 0.35‡	+0.09 $\pm$ 0.27
D + E	15	3.98 $\pm$ 0.46	3.46 $\pm$ 0.52†	-0.52 $\pm$ 0.16	-0.28 $\pm$ 0.43
F + G	16	1.15 $\pm$ 0.23*	1.53 $\pm$ 0.20†	+0.39 $\pm$ 0.20‡	+0.33 $\pm$ 0.32

Values are mean $\pm$ s.e.m.

* Effects of oestradiol, $P < 0.001$.

† Effects of rams (paired comparisons): all ewes—not significant; Group F + G, $P \approx 0.078$.

‡ Effects of oestradiol, $P \approx 0.004$.

§ All comparisons, not significant.

Discussion

The ovariectomized ewe treated with oestrogen and the entire ewe have virtually identical responses to the introduction of rams during the anoestrous season. In both types of ewe there is a rapid increase in the frequency of the LH pulses, and a concomitant increase in mean and basal levels. The ram stimulus is most probably a pheromone (Knight & Lynch, 1980), and presumably influences LH in the ewe through neural pathways connecting the accessory olfactory bulbs and the anterior hypothalamus (Estes, 1972). From there, the response would be mediated by gonadotrophin-releasing hormone (GnRH). The lag between the introduction of the rams and the onset of the first pulse is less than 15 min, and most of this interval would be occupied by the time required for the pheromone to be detected, since neural transmission is rapid and GnRH releases LH within 3 min (Reeves, Arimura & Schally, 1970).

The ram stimulus apparently acts by inhibiting the negative feedback exerted by oestradiol, the principal factor maintaining low levels of LH during anoestrus (Legan *et al.*, 1977). Androstenedione has no role in this system. This effect must last for several weeks, because ewes which ovulate in response to the introduction of rams usually experience several consecutive oestrous cycles (Oldham & Cognie, 1980). However, in previous studies, the effects of the ram stimulus on the frequency of the LH pulses apparently diminished within a few days (Martin *et al.*, 1980b), although the frequency tended to be very high in the first few hours and even after falling

may remain at a frequency exceeding that in anoestrous ewes. Nevertheless, the duration of the effect of the rams on the action of oestradiol needs to be measured, if only to verify the role of negative feedback by oestrogen in the control of seasonal breeding.

In Exp. 1, ovariectomized ewes not treated with exogenous steroids also had an increase in the secretion of LH after the introduction of rams. The increases were small compared to those seen in steroid-treated ewes, and could not be repeated in Exp. 2, in which larger numbers of animals were tested. Part of the response may have been due to statistical error since the paired comparisons used to test for the effects of the ram stimulus do not remove any of the confounding effects of time of day. In Exp. 2, in which there was no response to the rams in the untreated ewes, the rams were introduced earlier in the day (08:00 h). The paired comparisons were used to remove between-ewe variation in the patterns of LH secretion. Sampling error may also have contributed to the apparent responses of the untreated ewes. When the pulses are very frequent, it is quite probable that the rams were introduced during the rising phase of a spontaneous pulse and, in the analysis of single samples, this would be a positive response. This error of interpretation due to chance could also explain the different results seen in Exp. 2.

Despite this, the possibility that the ram stimulus really is able to increase the secretion of LH in ovariectomized ewes must be considered. In the profile for Ewe 6 (Text-fig. 1), for example, the increase in the baseline is far greater than can be explained in terms of assay error and probably too rapid to be explained by changes in metabolism. It is therefore possible that the rams induced an increase in the frequency of the pulses in these ewes which could not be detected in samples taken every 15 min for 6 h. This problem is even more likely to arise in Exp. 2, in which the frequency before ram introduction was assessed over only 2 h.

Increases in the tonic secretion of LH in untreated, gonadectomized rams and ewes have also been observed after the transition from anoestrus to the breeding season (Lincoln & Short, 1980; Goodman & Karsch, 1981) and in ovariectomized ewe lambs around the expected time of puberty (Foster & Ryan, 1979). There are two possible explanations for all of these effects: first, there may be sufficient negative feedback from adrenal steroids for the ram stimulus to operate through this system; second, the ram stimulus may have a stimulatory effect independent of steroid feedback on the hypothalamic centres controlling the secretion of LH. This latter system may, in fact, be the real mechanism behind all of these changes, including puberty, seasonal breeding and the effect of the rams. The changes in sensitivity to negative feedback would therefore accentuate rather than control the changes in secretion of LH that are programmed by the brain in response to the external environment. This concept is important, because it implies that the brain controls the ovary and not the converse.

Surges of LH similar to those seen before ovulation in entire ewes were not observed in ovariectomized ewes, irrespective of steroid treatment or whether they responded to the introduction of rams with an increase in their basal levels of LH. This finding indicates that positive feedback by ovarian oestradiol is essential for the preovulatory surge that follows the introduction of rams. This first ovulation is apparently the result of a series of endocrine events that are virtually identical to those which induce ovulation in spontaneously ovulating ewes during the breeding season.

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