Effects of breed, ovarian steroids and season on the pulsatile secretion of LH in ovariectomized ewes

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Summary. The effects of season and of oestradiol and progesterone on the tonic secretion of LH were studied in ovariectomized Merino and Suffolk ewes, two breeds which differ markedly in the seasonal pattern of their reproductive activity. In the absence of exogenous steroids, the frequency of LH pulses was lower and the amplitude of the pulses was higher in anoestrus than in the breeding season for Merino and Suffolk ewes 30 days after ovariectomy. In long-term (190 days) ovariectomized ewes, this seasonal change in LH secretion was observed in Suffolk ewes only. During seasonal anoestrus, treatment of ewes with subcutaneous oestradiol-17 β implants (3, 6 or 12 mm in length) decreased the frequency of LH pulses in a dose-dependent manner, with Suffolk ewes being far more sensitive to the inhibitory effects of oestradiol than Merino ewes. The lowest dose of oestradiol (3 mm) had no effect on the secretion of LH in Merino ewes, but reduced secretion in Suffolk ewes. Treatment of ewes with the highest dose of oestradiol (12 mm) completely abolished LH pulses in Suffolk ewes, whereas infrequent pulses remained evident in Merino ewes. During the breeding season, oestradiol alone had no effect on the pulsatile release of LH in either breed, but in combination with progesterone there was a significant reduction in LH pulse frequency. Progesterone effectively decreased LH secretion in both breeds in both seasons. It was concluded that differences between breeds in the 'depth' of anoestrus could be related to differences in the sensitivity of the hypothalamus to both negative feedback by oestradiol and the direct effects of photoperiod.

Keywords: oestradiol; negative feedback; progesterone; sheep

Introduction

The tonic secretion of luteinizing hormone (LH) in sheep is controlled by the inhibitory actions of the ovarian steroids, oestradiol and progesterone. During the breeding season, the pulsatile release of LH is primarily controlled by progesterone (Goodman & Karsch, 1980), although oestradiol may also be required for progesterone to exert its full effect (Karsch *et al.*, 1980; Goodman *et al.*, 1981; Martin *et al.*, 1983). During seasonal anoestrus, the negative feedback actions of oestradiol increase dramatically and this steroid now can act independently of progesterone to inhibit LH release (Legan *et al.*, 1977; Goodman *et al.*, 1982; Martin *et al.*,

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1983). Karsch *et al.* (1987) have demonstrated that both oestradiol and progesterone exert their effects by acting directly on the hypothalamic pulse generator to block the secretion of gonadotrophin-releasing hormone into hypophyseal portal blood. In addition to these steroid-dependent changes in LH release, seasonal differences in LH release in the absence of steroids have also been demonstrated (Goodman *et al.*, 1982; Montgomery *et al.*, 1985; Robinson *et al.*, 1985).

The majority of experiments used to establish these concepts in sheep have used highly seasonal breeds as the predominant experimental animal (e.g. Suffolk). It is well established that breeds vary in the timing and duration of the breeding season, and in the proportion of ewes within a flock that exhibit anoestrus, factors which reflect the 'depth' of anoestrus (Marshall, 1903; Yeates, 1949; Hafez, 1952). It therefore may not be possible to extrapolate the principles established in one breed to others. For instance, in one study using ovariectomized Merino ewes, Martin *et al.* (1983) was unable to demonstrate any negative feedback effects of progesterone in the absence of oestradiol or any direct effects of season on the tonic secretion of LH. This suggests that Merinos, with a less distinct pattern of seasonal breeding, may be less sensitive or less responsive to the effects of steroids and/or photoperiod than are sheep of more seasonal breeds, e.g. Suffolks. Alternatively, differences in experimental design such as the dose of steroid used, time from ovariectomy, nutrition, climate and social environment, may have contributed to the results.

Clearly, a controlled study in which these breeds are compared is required to resolve these differences (see Martin, 1984). In this study we tested whether the Suffolk ewe was more sensitive and responsive to the inhibitory effects of season and the ovarian steroids than was the Merino ewe.

Materials and Methods

Animals and management

Mature Merino ($N = 32$) and Suffolk ($N = 32$) ewes were selected in early January 1984 from a private commercial farm in the northeast of Western Australia. They were transported to the School of Agriculture Field Station in Perth, Australia (32°S), where daylength varies from 14.4 h in mid-summer to 9.8 h in mid-winter. The ewes remained in complete isolation from rams for the duration of the study. The ovaries of the ewes were examined by laparoscopy (Oldham & Lindsay, 1980) at 2-week intervals during January–March 1984 to determine the onset of the breeding season. In mid-March, ewes of both breeds had entered the breeding season (Fig. 1) and 16 ewes from each breed were ovariectomized (Group A) at mid-ventral laparotomy using 700–1000 mg thiopentone sodium (May & Baker, Sydney, Australia) as anaesthetic. The ovaries of the remaining ewes (Controls) were examined at 2-week intervals by laparoscopy throughout the experiment to monitor the seasonal ovarian cycles of each breed. In late October, when some sheep of both breeds had become anoestrous (Fig. 1), a further 8 ewes of each breed were ovariectomized (Group B). Throughout the study all ewes were kept together in one flock in a natural environment with free access to pasture and water. During the summer months they were fed a dietary supplement of oaten hay and lupin grain.

The onset of the breeding and anoestrous seasons was defined as the time at which 50% of the control ewes had begun or ceased ovulatory activity, respectively. All ewes ovariectomized in the breeding season (March, Group A) had corpora lutea. Mean ovulation rates were similar between the two breeds (Merino, 1.27; Suffolk, 1.50). All ewes ovariectomized in anoestrus (October, Group B) were anovulatory.

Experimental design and blood sampling

Seasonal response to ovariectomy. Blood samples were collected from Groups A and B via an indwelling cannula at 10-min intervals for 8 h, 30 days after ovariectomy. This time interval was chosen because the increase in LH pulse frequency after ovariectomy has reached a plateau (Montgomery *et al.*, 1985). The dates corresponded with the breeding season (April: Group A) and anoestrus (November: Group B) of the controls (Fig. 1). Samples were centrifuged at 4°C and the plasma harvested and stored at -20°C until assayed for LH.

Seasonal response to exogenous steroids. On the day after blood sampling of the ewes in Group A, the 16 ewes of each breed were randomly divided into groups of 4 and given either no implant or a s.c. implant packed with 3, 6 or 12 mm oestradiol-17 β (Sigma Chemical Company, St Louis, MO, U.S.A.). The implants were constructed from

Silastic tubing, as described by Karsch *et al.* (1973), and were sealed at each end with 1 cm of Silastic Medical Adhesive (Dow Corning, Midland, MI, U.S.A.). To avoid the transient peak in plasma oestradiol after insertion (Karsch *et al.*, 1973), the implants were preincubated for 4 days in 0.9% (w/v) NaCl (saline). In untreated ovariectomized ewes, plasma oestradiol concentrations were below the level of the assay blank (2.4 ± 0.3 pg/ml) for which no correction was made. The insertion of 3, 6 or 12 mm oestradiol implants increased plasma oestradiol concentrations to 4.7 ± 0.2 , 5.4 ± 0.4 and 6.6 ± 0.5 pg/ml, respectively. The concentrations of oestradiol were linearly related to the length of the implants ($r = 0.71$, $P < 0.01$).

At 10 days after insertion of implants blood samples were collected at 10-min intervals for 8 h. On the following day a controlled intravaginal drug-release (CIDR) progesterone dispenser (Hoechst, Sydney, Australia) was inserted and the ewes were again blood sampled intensively 10 days later. The CIDR was then removed. These time intervals were chosen because the responses to steroid treatment are essentially completed and the concentration of LH has reached a plateau (Goodman *et al.*, 1981). The periods of intensive blood collection (April–May; 42–56 days after ovariectomy) corresponded with the breeding seasons of the controls (Fig. 1). The above experimental and sampling procedures (i.e. with and without progesterone) were repeated on the same ewes in October (190–204 days after ovariectomy), when the controls of both breeds were in anoestrus (Fig. 1). The samples were centrifuged at 4°C and the plasma harvested and stored at -20°C until assayed for LH, progesterone and oestradiol. The insertion of an intravaginal progesterone dispenser increased plasma progesterone concentrations from 0.4 ± 0.1 to 3.3 ± 0.2 ng/ml.

Radioimmunoassays

Luteinizing hormone. Plasma concentrations were determined in duplicate 200- μl samples, according to the method of Martin *et al.* (1980). Values are expressed in terms of the reference preparation M3-CNRS (M. Jutisz, College de France, Paris; $1.8 \times \text{NIH-LH-S1}$). The limit of detection was 0.30 ± 0.04 ng/ml (mean $\pm$ s.e.m., 18 assays). Six replicates of 3 pooled samples containing 0.84 ± 0.07 , 3.16 ± 0.18 , and 5.40 ± 0.20 ng LH/ml were included in each assay and used to estimate the coefficients of variation (CV) within- ($13.1 \pm 0.9\%$; $6.4 \pm 0.8\%$; $4.9 \pm 0.7\%$) and between- (12.3%, 6.8%, 2.3%) assays.

Progesterone. Samples (100 μl) were assayed in duplicate in 4 assays according to the method of Martin *et al.* (1983). The limit of detection was 0.20 ± 0.06 ng/ml. A pooled plasma sample (3.6 ± 0.4 ng/ml) was used to estimate the within-assay ($11.7 \pm 1.4\%$) and between-assay (11.9%) CV.

Fig. 1. Seasonal changes in photoperiod (a) and ovarian activity in control flocks of 16 untreated Suffolk (b) and Merino (c) ewes. Bars indicate the time of blood sampling in the breeding season (BS) and anoestrus (A).

Oestradiol. Samples were assayed in duplicate for oestradiol-17 β in chromatographed diethyl ether extracts of 2 ml plasma using a previously described technique (Thomas *et al.*, 1988). All samples were assayed in one assay (sensitivity 0.50 ± 0.06 pg/ml). Two pooled samples containing 10.7 ± 0.8 and 15.4 ± 0.8 pg oestradiol/ml were used to measure the within-assay CV (9.4%, 6.6%).

Data analyses

Pulses of LH were defined as described previously (Martin *et al.*, 1983). In brief: (1) both the increase and the subsequent decrease in concentration had to exceed the sum of the assay errors appropriate for the concentration at the onset and the peak of the pulse; (2) the increase had to occupy no more than 2 sampling intervals and the decline had to begin within 2 sampling intervals of attainment of the peak. Pulse amplitude was calculated by subtracting the concentration at the onset of a pulse (nadir) from the maximum concentration. Because the LH pulse amplitude and nadir increased significantly ($P < 0.001$) with time, the effects of steroid treatments on these variables were limited to comparisons within seasons.

The pulses in a profile were counted and the effects of treatments on pulse frequency (pulses/8 h), mean pulse amplitude and mean nadir were analysed by analysis of variance using a repeated measures design (Winer, 1971). This allows for the fact that measures on the same animals are likely to be dependent. To preserve homogeneity of variance of the pulse frequency distributions, the data were transformed by taking the square root of the number of pulses observed in each sampling period for each ewe.

Results

Controls

The mean onset of the breeding season of Merino ewes was 17 January (27 days after the summer solstice; daylength 14.1 h) and the mean onset of anoestrus was 5 October (107 days after the winter solstice; daylength 12.4 h), although 17–25% of the flock continued to ovulate during October and November (Fig. 1). The mean onset of the breeding season of Suffolk ewes was 28 February (69 days after the summer solstice; daylength 12.9 h) and the mean onset of anoestrus was 17 September (89 days after the winter solstice; daylength 11.8 h) (Fig. 1).

Short-term response of LH to ovariectomy

In the Merino and Suffolk ewes the frequency of LH pulses 30 days after ovariectomy was lower ($P < 0.05$) in anoestrus (Group B; daylength 13.4 h) than in the breeding season (Group A; daylength 11.0 h) (Table 1). The amplitude of LH pulses, on the other hand, was 2-fold higher ($P < 0.01$) in anoestrus than in the breeding season (Table 1). There was no significant effect of season on the mean nadir in either breed. During both seasons all measures of LH secretion were greater ($P < 0.01$) in Suffolk ewes than in Merino ewes (Table 1).

Table 1. Secretion of LH at 30 days after ovariectomy in the breeding season or seasonal anoestrus in Merino and Suffolk ewes

	Merino		Suffolk	
	Breeding season (N = 16)	Anoestrus (N = 8)	Breeding season (N = 16)	Anoestrus (N = 8)
LH pulses per 8 h	8.0 ± 0.4	$5.9 \pm 0.3^*$	9.5 ± 0.4	$8.1 \pm 0.6^*$
Amplitude (ng/ml)	3.2 ± 0.5	$7.6 \pm 0.8^{**}$	4.7 ± 0.5	$10.2 \pm 1.2^{**}$
Nadir (ng/ml)	1.8 ± 0.2	2.6 ± 0.6	3.4 ± 0.2	4.4 ± 0.9

Values given are the mean $\pm$ s.e.m. for the number of ewes given.

* $P < 0.05$, ** $P < 0.01$ compared with breeding season (Student's *t* test).

Fig. 2. Effect of oestradiol in the absence (open bars) and presence (stippled bars) of progesterone on LH pulse frequency in ovariectomized Merino and Suffolk ewes during the breeding season (42–56 days after ovariectomy) and seasonal anoestrus (190–204 days after ovariectomy). Each bar represents the mean $\pm$ s.e.m. of 4 ewes.

Long-term response of LH to ovariectomy

When the patterns of release in Group A ewes (not treated with steroids) at 30 days (i.e. during the breeding season) and at 190 days (i.e. during anoestrus) after ovariectomy were compared, there was a significant increase in both pulse amplitude ($P < 0.01$) and nadir ($P < 0.001$) with time after ovariectomy in both breeds. The frequency of LH pulses in Suffolk ewes was higher ($P < 0.05$) at 30 days after ovariectomy (10.8 ± 0.8 pulses/8 h) than at 190 days ($8.2 \pm 0.5/8$ h), whereas in Merino ewes there was no significant difference ($9.0 \pm 0.7/8$ h at 30 days, $10.2 \pm 0.8/8$ h at 190 days).

Responses to exogenous steroids

Pulse frequency. In ovariectomized Merino ewes, oestradiol alone had no significant effect on the frequency of LH pulses during the breeding season (Figs 2 & 3), although 2 ewes, one treated

(a) Breeding season

(b) Anoestrus

Fig. 4. LH pulse profiles from 2 ovariectomized Merino ewes that appeared to 'respond' to oestradiol during the breeding season. The length of the oestradiol implant is indicated.

with a 6-mm implant and one treated with a 12-mm implant, did appear to show a negative feedback response and had a lower frequency of pulses compared to that of other ewes (Fig. 4). During seasonal anoestrus, the frequency of LH pulses was significantly reduced ($P < 0.01$) in the Merino ewes treated with 6- or 12-mm oestradiol implants (Figs 2 & 3). Insertion of a 3-mm implant did not affect pulse frequency during anoestrus. In the absence of oestradiol, progesterone significantly reduced ($P < 0.001$) the frequency of LH pulses in Merino ewes. This effect was evident in both seasons, with the greatest response being seen during the breeding season (Fig. 2). Ewes receiving a combination of progesterone and oestradiol had the lowest frequency of pulses (Fig. 5), but this effect of synergism was statistically significant ($P < 0.05$) only during the breeding season, and in those ewes treated with a 3-mm oestradiol implant during anoestrus.

Oestradiol had no significant effect on the frequency of LH pulses in ovariectomized Suffolk ewes in the breeding season (Figs 2 & 3). During anoestrus, however, this steroid markedly suppressed ($P < 0.001$) pulse frequency in a dose-dependent manner (Figs 2 & 3). In contrast to the Merino ewes, no LH pulses were observed in the 4 Suffolk ewes treated with 12-mm oestradiol implants (Fig. 3). During the breeding season ($P < 0.001$) and anoestrus ($P < 0.01$) progesterone effectively reduced the frequency of LH pulses in Suffolk ewes (Fig. 2). The interaction between oestradiol and progesterone in the breeding season was significant ($P < 0.05$) (Fig. 5).

Pulse amplitude. Oestradiol had no effect on pulse amplitude during the breeding season of Merino ewes (Fig. 6). In anoestrus, however, pulse amplitude was higher ($P < 0.01$) in those ewes treated with 3- or 6-mm implants of oestradiol (Figs 3 & 6). During the breeding season the amplitude of LH pulses was increased ($P < 0.01$) after treatment with progesterone (Fig. 6).

In Suffolk ewes, the oestradiol implants had no effect on pulse amplitude in either season (Figs 3 & 6). During the breeding season, progesterone significantly increased ($P < 0.01$) pulse amplitude. There was no effect of this steroid in anoestrus, but a combination of progesterone and oestradiol reduced ($P < 0.05$) the amplitude of LH pulses (Figs 5 & 6).

Nadir. In Merino ewes, oestradiol had no effect on nadir during the breeding season, but it was highly effective ($P < 0.01$) in reducing the nadir during seasonal anoestrus (Figs 3 & 6). Progesterone alone had no effect on nadir in either season but, in combination with oestradiol, significantly decreased ($P < 0.05$) it in the breeding season (Fig. 6).

Treatment with oestradiol during the breeding season of Suffolk ewes increased ($P < 0.01$) the nadir, whereas during seasonal anoestrus it caused a profound decrease ($P < 0.001$) (Figs 3 & 6). A combination of oestradiol and progesterone reduced ($P < 0.05$) the nadir in the breeding season.

Fig. 3. Changes in plasma concentrations of LH in ovariectomized Merino and Suffolk ewes treated with oestradiol during the breeding season (a) and during seasonal anoestrus (b). The length of the oestradiol implant is indicated.

(a) Breeding season

MERINO

No implant

3 mm

6 mm

12 mm

SUFFOLK

No implant

3 mm

6 mm

12 mm

(b) Anoestrus

MERINO

No implant

3 mm

6 mm

12 mm

SUFFOLK

No implant

3 mm

6 mm

12 mm

Time (h)

Fig. 6. Effect of oestradiol in the absence (open bars) and presence (stippled bars) of progesterone on LH pulse amplitude and LH pulse nadir in ovariectomized Merino and Suffolk ewes during (a) the breeding season (42–56 days after ovariectomy) and (b) seasonal anoestrus (190–204 days after ovariectomy). Each bar represents the mean $\pm$ s.e.m. of 4 ewes.

Discussion

The study described here shows that during seasonal anoestrus the ovariectomized Merino ewe is less sensitive than the ovariectomized Suffolk ewe to the inhibitory effects of oestradiol, suggesting that quantitative differences exist between these two breeds in the negative feedback mechanism controlling the tonic secretion of LH. Effects of season on the frequency of LH pulses in the absence of steroids were evident in short-term (30 days) and long-term (190 days) ovariectomized Suffolk ewes, but only in short-term ovariectomized Merino ewes. This may suggest that the Suffolk breed is also more sensitive, or responsive for a longer period after ovariectomy, to the 'direct' effects of photoperiod than the Merino breed. Under the same conditions, therefore, the Suffolk ewes are more strongly influenced by both steroid-dependent and steroid-independent effects of season.

Differences between these breeds in the control of tonic LH secretion are also expressed in the ovary-intact ewe. During seasonal anoestrus the frequency of pulses seen in Suffolk ewes (one per

Fig. 5. Changes in plasma concentrations of LH in ovariectomized Merino and Suffolk ewes treated with oestradiol and progesterone during the breeding season (a) and during seasonal anoestrus (b). The length of the oestradiol implant is indicated.

8–12 h: F. J. Karsch, personal communication) is less than that seen in Merino ewes (one per 4–5 h: Scaramuzzi & Baird, 1977). The frequency of pulses of LH control ovarian activity (McNeilly *et al.*, 1982; McLeod *et al.*, 1982; Wright *et al.*, 1984), and so differences between the breeds in the 'depth' of anoestrus are also related to the degree by which the hypothalamo–hypophysial axis is inhibited. Thus, in less seasonal breeds like the Merino the lower sensitivity of the hypothalamus to negative feedback would ensure a higher level of gonadotrophic support for the ovaries throughout anoestrus, compared to the more highly seasonal breeds, such as the Suffolk. The development of follicles that continues during anoestrus (Turnbull *et al.*, 1977) may therefore be expected to be greater in breeds with a 'shallow' anoestrus. This is supported by the observation that during anoestrus (October–November) 17–25% of the control Merino ewe flock continued to ovulate, whereas no control Suffolk ewes ovulated.

Karsch *et al.* (1983) have shown that during the breeding season oestradiol can increase the frequency of LH pulses in ovariectomized Suffolk ewes and they postulated that oestrogen may have a stimulatory role in producing the rise in LH during the follicular phase of the oestrous cycle. In contrast, we observed no effect of this steroid on pulse frequency during the breeding season. However, larger sized implants of oestradiol were used by Karsch *et al.* (1983) than in the present study. In addition, we performed our experiment in the mid-breeding season (25 May), 26 days before the winter solstice, a time when the direct photoperiodic drive on LH would be expected to be high (Robinson *et al.*, 1985) and the frequency of LH pulses may have already been maximal.

Concentrations of progesterone similar to those found during the mid-luteal phase of the oestrous cycle reduced pulse frequency and increased pulse amplitude in ovariectomized Suffolk ewes (Goodman & Karsch, 1980), a result also observed in this study. Further studies, in ovariectomized Suffolk and Merino ewes, have shown that at lower doses a combination of oestradiol and progesterone is required to inhibit LH (Goodman *et al.*, 1981; Martin *et al.*, 1983). In our study, we did observe a significant effect of mid-luteal levels of progesterone in the absence of oestradiol, but oestradiol was needed in both breeds before pulse frequency approached that seen during the luteal phase of the oestrous cycle. In fact, both steroids are secreted during the luteal phase (Baird *et al.*, 1976) and the synergistic interaction between the two steroids has been demonstrated at both high and low concentrations of progesterone (Goodman *et al.*, 1980). Furthermore, we have shown that active immunization against progesterone during the breeding season results in a dramatic increase in the frequency of LH pulses (Thomas *et al.*, 1987). Finally, suppression of follicular activity (and presumably oestradiol secretion) during the luteal phase will increase LH pulse frequency without affecting circulating concentrations of progesterone (Wallace & McNeilly, 1986). Therefore, it appears that while physiological doses of progesterone alone can slow the LH pulse generator in ovariectomized ewes, a combination of both oestradiol and progesterone is required to restore fully normal patterns of LH secretion.

During the breeding season oestradiol had no effect in sheep of either breed on the amplitude of the LH pulses. Martin *et al.* (1983) previously obtained similar results in ovariectomized Merino ewes, but a large reduction in pulse amplitude in ovariectomized Suffolk ewes treated with oestradiol was observed by Goodman & Karsch (1980) and Goodman *et al.* (1981). The reason for the lack of an effect in this study is probably related to the level of oestradiol used, as our steroid implants were 2–10 times smaller than those used by these workers. There was little difference in the peripheral concentrations of oestradiol in the 2 studies, but this is probably due to variations between laboratories in the oestradiol assays. Furthermore, Goodman & Karsch (1980) observed the response in ewes that had been ovariectomized for 10 days, whereas the ewes in this study had been ovariectomized for up to 42 days and the pituitary gland may have become unresponsive to oestradiol. Karsch *et al.* (1977) observed that the response of ewes to negative feedback diminished with time after ovariectomy.

Seasonal changes in the frequency of LH pulses in the absence of gonadal steroids have not been previously reported for ovariectomized Merino ewes. However, these differences were only

detected in ewes that had been ovariectomized for as little as 1 month. In Merino ewes that have been ovariectomized for 6 months or more, in this study and in the study of Martin *et al.* (1983), seasonal changes in frequency were not detectable. Seasonal changes in tonic LH release were still evident in untreated long-term ovariectomized Suffolk ewes, suggesting that sheep of this breed remain responsive for a longer period to the 'direct' effects of photoperiod than do Merino ewes. Montgomery *et al.* (1985) have shown that changes in pulse frequency in the absence of steroids are also lost in long-term ovariectomized Ile-de-France ewes. Since the Suffolk ewe has a deeper anoestrus and also faster pulses of LH during the breeding season than the Merino ewe, this suggests that the effects of season could be stronger in both directions in highly seasonal breeds, such as the Suffolk.

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