Ovarian follicular dynamics during anoestrus in ewes

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The aim of the present study was to document ovarian antral follicle dynamics throughout seasonal anoestrus in sheep. Daily transrectal ultrasonography was performed during four 17 day scanning periods from March to July in Western White-faced crossbred ewes. Blood samples were collected each day with ultrasonographic scanning for measurement of serum concentrations of FSH, oestradiol and progesterone. Blood samples were also taken every 15 min for 6 h, mid-way through each period of ultrasonographic examination, to determine the patterns of secretion of gonadotrophic hormones. Hormonal data were then related to observed changes in follicular populations and the patterns of antral ovarian follicle turnover. Ultrasonography showed that the ovaries of anoestrous ewes remained active and that the largest ovarian antral follicles grew to a periovulatory size (≥ 5 mm in diameter) at all stages of anoestrus. The total number of all ovarian follicles ≥ 3 mm in diameter was lower during early anoestrus compared with at mid-anoestrus because of a significantly smaller number of small (3 mm) and medium (4 mm) ovarian follicles. The largest ovarian follicles (attaining ≥ 5 mm in diameter before regression) exhibited a wave-like pattern of growth; an average of three waves of follicular development were recorded in sheep during each of the four 17 day scanning periods in anoestrus, with follicular waves emerging approximately every 5 days. This rhythmic pattern of follicular emergence was found to be associated with the occurrence of fluctuations in serum FSH concentrations. The growth rate of the largest follicles of the wave increased significantly from early to late anoestrus in sheep. In addition, ovarian follicles not growing beyond 3 mm in diameter showed organized patterns of growth and regression; their numbers tended to be lower (P = 0.09) at 3 days before and on the day of follicular wave emergence. Some ewes were seen to maintain synthesis of progesterone throughout anoestrus. This submaximal progesterone secretion tended to occur at irregular intervals and was not coupled with changes in concentrations or patterns of gonadotrophin release, ovulations or detectable morphological luteinization of ovarian antral follicles. It was concluded that the growth of ovarian antral follicles to an ovulatory size was maintained throughout anoestrus in ewes, with a transient shift in the number of small and medium-sized follicles during mid-anoestrus, and that the periodic emergence of waves of large follicles (≥ 5 mm in diameter) occurred in synchrony with an endogenous rhythm of FSH secretion.

Introduction

Recent ultrasonographic studies conducted in sheep and goats have shown that 3–6 large (growing to ≥ 5 mm in diameter) ovarian antral follicles develop in an orderly succession during the oestrous cycle (sheep: Ravindra et al., 1994; Schrick et al., 1993; Ginther et al., 1995; goats: Ginther and Kot, 1994). A transient increase in serum concentration of FSH accompanies the emergence of these follicles in cyclic sheep (Ginther et al., 1995).

To date, the data describing ovarian follicle dynamics in anoestrous ewes have largely come from postmortem (slaughtered animals) studies of reproductive tracts (Hutchinson and Robertson, 1966; Brand and de Jong, 1973) or experiments using laparoscopy–endoscopy (Smeaton and Robertson, 1971; Noel et al., 1993). The results of these earlier studies showed that ovaries of anoestrous ewes were not inactive. Maximum follicle diameter and mean number of large ovarian follicles did not differ between animals killed during anoestrus and those killed from day 5 of the oestrous cycle onwards (Brand and de Jong, 1973). In other studies, it was reported that there was a tendency for the ovaries of anoestrous ewes to bear significantly greater numbers of small ovarian follicles relative to the ovaries of cyclic ewes (Hutchinson and Robertson, 1966; Ravindra, 1993). In a recent study that examined the ovaries of ewes each day for 18 days in August, November, February and May using laparoscopy (Noel et al., 1993), three distinct groups

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of ovarian antral follicles were seen to grow and regress in an orderly fashion during both the breeding season (August, November, February) and anoestrum (May; Noel et al., 1993). When ultrasonographic observation of the ovaries of anoestrous Western White-faced ewes were made over two 5 day periods, no antral follicles ≥ 6 mm in diameter were recorded and the total numbers of antral follicles did not differ between the two observation periods (Ravindra, 1993).

The purpose of the present study was to use the non-invasive technique of transrectal ultrasonography to describe patterns of ovarian follicular dynamics at different times during anoestrous in ewes. It was investigated whether the orderly emergence of antral follicles, seen during the breeding season, continued throughout anoestrum and if there were changes in follicular dynamics and populations from early to mid- to late anoestrum. The patterns of serum concentration of LH, FSH, oestradiol and progesterone were compared with the pattern of follicle growth and regression to investigate whether peaks in daily follicle concentrations were associated with follicle emergence in anoestrus as they are during the breeding season (Ginther et al., 1995).

Materials and Methods

Animals and management

Twelve sexually mature Western White-faced ewes were kept outdoors in sheltered dry lots and fed a maintenance ration of alfalfa pellets each day. Water, hay and cobalt iodized salt bars were available ad libitum. The Western White Face is largely the result of Rambouillet and Columbia crossbreeding, and the average number of lambs born per ewe is 1.5 ± 0.2 (Rawlings et al., 1987). Ewes were age and weight matched (approximately 3 years old, average body weight 77 ± 8 kg), nulliparous and clinically healthy. They were kept continuously with three vascetomized rams to eliminate any ram effect. During both transition periods (into and out of anoestrous), rams were crayon-harnessed for detection of behavioural oestrus.

Experimental protocol

From 5 January to 1 March, transrectal ultrasonography was performed each day in 12 ewes to confirm the occurrence of the last oestrous cycle of the breeding season. Blood samples were collected each day by jugular venepuncture for estimation of progesterone concentrations. The first eight animals that became anoestrous in a fairly synchronized group were selected for further study. The ewes were then scanned for 17 days (average duration of the oestrous cycle in Western White-faced sheep) commencing 20 March, that is, an average of 46.1 ± 5.0 days after the end of the luteal phase of the last oestrous cycle of the season (the last decrease in serum progesterone to basal concentrations determined in blood samples taken for 7 consecutive days after ultrasonographically detected regression of corpora lutea). Subsequently, ewes were scanned for three further 17 day periods with starting dates 5 weeks apart (second period: 25 April–11 May; third period: 30 May–15 June; fourth period: 5 July–21 July). Blood samples were collected each day by jugular venepuncture, before scanning, and also every 15 min for 6 h via jugular catheters mid-way through each sampling period (29 March, 3 May, 8 June and 13 July). Catheters were inserted 24 h before each intensive bleed. Before the first expected cycle of the breeding season and approximately 2 weeks after the last scanning period above, ultrasonography and blood sampling were restarted and continued each day until oestrous cyclicity resumed. The first significant increase in serum concentrations of progesterone over the previously determined basal concentration (0.13 ± 0.05 ng ml⁻¹) lasting ≥ 6 days (a minimum of 6 days was allowed for possible short luteal phases to occur during the transition to the breeding season) was detected between the 11 August and 15 September, an average 33.3 ± 6.6 days after the last 17 day scanning period in anoestrous.

Ovarian ultrasonography utilized a real-time, B-mode scanner and a rigid 7.5 MHz human prostate transducer (shaft length: 35 cm; shaft diameter: 1.6 cm) and was performed in a barn with a sufficient number of windows to assure natural light during daylight hours. Ewes remained indoors for an average of 2 h day⁻¹, between 13:00 and 15:00 h. Ovarian follicles ≥ 3 mm in diameter were counted and their size and position mapped on ovarian charts. In addition, all images of ovaries were recorded on high-grade video tapes using a compatible video cassette recorder.

Follicular data summary and analysis

Initially, ovarian follicular data (follicles ≥ 3 mm in diameter) were summarized and analysed for each of the four 17 day scanning periods, separately. Follicular data were analysed for period effects using repeated measures analysis of variance (General Linear Model procedures in the Statistical Analysis System SAS/STAT®, version 6; Cary, NC). The following parameters were measured each day: (1) numbers of ovarian follicles in three size classes (small: 3 mm, medium: 4 mm, and large: ≥ 5 mm in diameter); (2) number of all ovarian follicles ≥ 3 mm in diameter (total follicle number); and (3) diameter of the largest follicle (maximum follicle diameter). When the overall analysis was significant (P < 0.05), Duncan's test was used to compare individual means.

A follicular wave was defined as one or more antral follicles growing from 3 to ≥ 5 mm in diameter before regression; the day the follicles were first detected at 3 mm was the day of wave emergence (Ginther et al., 1995). Individual follicles emerging within a maximum of 48 h were regarded as a single follicular wave. For follicular waves in which follicles emerged within a 24 h period, the day of wave emergence was the day on which the first follicle of the group was detected at 3 mm. For follicular waves in which follicles emerged within 48 h, the central day was considered the day of wave emergence. The following characteristics of follicular waves, for each ewe for each scanning period, were determined: (1) mean number of follicular waves; (2) mean number of follicles growing to ≥ 5 mm in diameter per wave; (3) mean maximum diameter attained by the largest follicle of the wave; (4) mean growth rate of the largest follicle of the wave; (5) mean number of days between emergence of adjacent waves (interwave interval); (6) mean lifespan of the largest follicle of the wave; and (7) mean lengths of the growing, static and regressing phases.
of the lifespan of the largest follicle of the wave. Since many follicular waves were not followed from the day of wave emergence until the regression of the largest follicle of the wave, mean follicular lifespan was defined as a sum of mean durations of the growing, static and regressing phases of all individual follicles, regardless of whether the entire lifespan was observed. The ‘growing phase’ was defined as the time taken by a single follicle to grow from 3 mm to its maximum diameter; the ‘regressing phase’ was defined as the time taken by that follicle to regress from its maximum size to 3 mm, and the ‘static phase’ was the time between the end of the growing phase and beginning of the regressing phase. Analyses of variance were performed to compare means among the four scanning periods, for each of these parameters.

Data for follicles only attaining 3–4 mm in diameter were centralized to each day of wave emergence and summarized for the period 3 days before and after emergence. Parameters determined each day after alignment to the days of emergence were: (1) number of follicles not growing beyond 3 mm, and (2) number of 3 mm follicles that reached a maximum of 4 mm in diameter before regression. For each of these two parameters, a 7 (number of days) × 4 (number of scanning periods) factorial analysis of variance was used to assess the day effect, the period effect and the interaction between the two effects.

Hormone analyses

Blood samples were allowed to clot for 18–24 h and serum was harvested and stored at −20°C until analysis. Samples collected each day were analysed by radioimmunoassay for concentrations of FSH (Currie and Rawlings, 1989), oestradiol (Joseph et al., 1992) and progesterone (Rawlings et al., 1984). Samples collected every 15 min for 6 h were analysed for concentrations of LH (Rawlings et al., 1987) and FSH. Concentrations of LH and FSH are given in terms of NIAMDD-oLH-24 and NIDDK-OFSH-RP1, respectively. The sensitivities of assays were as follows: LH and FSH assays: 0.1 ng ml⁻¹; oestradiol: 1.0 pg ml⁻¹; and progesterone: 0.03 ng ml⁻¹. Serum samples with concentrations of hormones below the assay sensitivity were assigned a concentration equal to the sensitivity of the assay. The intra- and interassay coefficients of variation for ovine reference sera analysed for LH (means: 0.58 or 2.28 ng ml⁻¹) were 6.6 and 11.6% or 5.6 and 6.5%, respectively; for FSH (means: 2.26 or 3.92 ng ml⁻¹) they were 4.1 and 9.5% or 4.8 and 10.7%, respectively; for progesterone (means: 0.58 or 3.50 ng ml⁻¹) they were 12.2 and 9.3% or 14.2 and 10.6%, respectively; and for oestradiol (means: 6.9 or 12.7 pg ml⁻¹) they were 15.0 and 10.8% or 11.1 and 8.7%, respectively. LH and FSH data from intensive bleedings were analysed using the PULSAR program (Gitzen and Ramirez, 1988) to identify and quantify basal and mean hormone concentrations as well as pulse frequency and amplitude. Standard deviation criteria of height and duration (G and Baxter parameters) were used for pulse detection (Merriam and Wachter, 1982). Mean concentrations of hormones for each day as well as parameters determined by the PULSAR program were analysed for period effects using repeated measures analysis of variance.

In samples taken each day, peaks in FSH, oestradiol and progesterone concentration were detected using the cycle detector computer program (or the Threshold Adaptive Method; Clifton and Steiner, 1983). Various parameters of follicular dynamics and hormone concentrations (measured each day and during intensive bleeds) were compared between animals with and without identified fluctuations in serum progesterone concentrations for the four scanning periods in anoestrous.

Associations between days of follicular wave emergence and peaks in serum concentrations of FSH and oestradiol were studied using statistical methods based on those of Ginther et al. (1995). This was not done for progesterone as the cycle-detection analysis showed peaks of progesterone in only some of the ewes studied. FSH peaks that occurred at ≤3 days before the end of the 17 day period of ultrasound scanning as well as the days of wave emergence detected during the first 3 days of each scanning period were withdrawn from the analyses as the corresponding follicle growth wave or FSH peak, respectively, could not always be observed.

Paired Student’s t test was used to compare the mean number of days of follicle wave emergence with the mean number of identified FSH or oestradiol peaks per ewe, per scanning period. Since the mean number of identified peaks of oestradiol concentration and the mean number of emerging follicular waves for all ewes, for all scanning periods were significantly different, oestradiol peaks were not analysed further. The mean interval between adjacent days of emergence (interwave interval) and between adjacent peaks of FSH fluctuations (interpeak interval) were also compared for each period of scanning. In addition, Spearman coefficients of correlation were calculated between the number of days of emergence and the number of FSH peaks. and between the duration of interwave intervals and the duration of interpeak intervals for all ewes, for all four observation periods in anoestrous. Spearman correlations were then calculated between the duration of interpeak intervals (FSH) and the duration of intervals between adjacent days of: (1) the beginning of the static phase; (2) the end of the static phase; and (3) the end of the regressing phase of follicular development (regression to 3 mm in diameter) of the largest ovarian follicles of sequential waves, for all four periods of scanning in anoestrous. Finally, the relative frequency of the occurrence of FSH peaks on the day of wave emergence and on each of the 3 days before or after the day of emergence was calculated for all ewes, for all four scanning periods in anoestrous. For this purpose, each FSH peak was assigned to the emergence of only one follicular wave (the closest wave to the FSH peak in question). The distribution of peaks of FSH fluctuations in relation to days of follicular wave emergence was analysed by chi-squared test.

Results

The onset of anoestrous occurred between 20 January and 17 February, an average of 46.1 ± 5.0 days before the first 17 day scanning period commenced. Only five of eight ewes were marked by rams at the beginning of the last oestrous cycle of the breeding season. During the last 17 day scanning period of anoestrous, one animal had serum progesterone above the basal concentration for 6 consecutive days, although no luteal structure was detected by ultrasonography. Data for this
ewes were excluded from statistical analyses for this scanning period. In the seven remaining ewes, cyclicity resumed between the 11 August and the 15 September, an average of 33.3 ± 8.6 days after the end of the last 17 day scanning period in anoestrus. An initial increase in progesterone over the basal concentration lasted 9.9 ± 1.7 days (range: 6–12 days). During this initial increase in progesterone secretion, normal corpora lutea (maximum size range: 10–12 mm; duration of accompanying increases in progesterone secretion: 10–12 days) were detected by ultrasonography in three of seven animals (Fig. 1c). In three other ewes, large structures with cavities enveloped by a band of echogenic tissue were seen in the ovaries (Fig. 1d). The occurrence of these ovarian structures was accompanied by a 7–9 day increase in daily serum progesterone concentrations. Oestrus was not detected before formation of the large cavitated ovarian structures but did precede the formation of the corpora lutea. In the ovaries of one ewe, there was no evidence of formation of luteal structures and no ovulation was detected by ultrasonography. Oestrus was not detected and serum progesterone in this ewe was seen to stay above the basal concentration for only 6 days.

Ovarian antral follicle populations during anoestrus

The number of large antral follicles (≥5 mm) and the mean diameter of the largest follicle did not differ between the periods of study (P > 0.05). The number of small (3 mm) and medium (4 mm) ovarian follicles and the mean number of all ovarian follicles ≥3 mm in diameter each day (dTFN) increased from the first to the second scanning period in anoestrus (3 mm follicles: 1.6 ± 0.4, 2.7 ± 0.4, 2.3 ± 0.5 and 2.2 ± 0.5; 4 mm follicles: 0.5 ± 0.1, 0.9 ± 0.2, 0.5 ± 0.2 and 0.7 ± 0.2; dTFN: 2.9 ± 0.5, 4.4 ± 0.7, 3.7 ± 0.7 and 3.4 ± 0.7).
for scanning periods 1, 2, 3 and 4, respectively; \( P < 0.05 \). Follicles are illustrated (Fig. 1a,b).

The number of follicular waves per ewe (2.8 ± 0.1), the number of follicles per wave (1.2 ± 0.1), the mean maximum diameter attained by the largest follicle of the wave (5.9 ± 0.3 mm) and its lifespan (7.7 ± 0.9 days) did not differ among the four periods of study (\( P > 0.05 \)). Mean durations (in days) of the growing (2.8 ± 0.5), static (1.9 ± 0.3) and regressing (2.9 ± 0.5) phases of the largest follicle of the wave did not differ among the four study periods in anoestrous (\( P > 0.05 \)). The growth rates of the largest ovarian follicles of waves increased from the second scanning period (April–May) to the last scanning period (July) in anoestrous ewes (the means for scanning periods 1, 2, 3 and 4 were: 1.0 ± 0.1, 1.0 ± 0.2, 1.2 ± 0.2 and 1.5 ± 0.2 mm day\(^{-1}\), respectively, and the means for periods 1 and 2 differed from those of period 4; \( P < 0.05 \)). The mean interwave interval was 4.6 ± 0.5 days and this did not differ among the scanning periods (\( P > 0.05 \)). Individual follicle profiles (follicles growing to ≥ 5 mm in diameter) are shown for two animals (Fig. 2), for each of the four scanning periods, to illustrate variations in the number of waves per ewe and the maximum size of the largest follicles.

Data for follicles not growing beyond 4 mm in diameter were centralized to the day of wave emergence ± 3 days for all ewes, for each scanning period. When centralized on this basis, the number of 3 mm follicles that grew to a maximum of 4 mm in diameter before regression did not vary by day or by period of scanning (\( P > 0.05 \)). The number of follicles not growing beyond 3 mm in diameter appeared to be lowest 3 days before and on the day of wave emergence (\( P = 0.09 \)), and this number also increased from the first to the second scanning period in anoestrous (1.3 ± 0.3 and 2.5 ± 0.2 for the first and second scanning period of anoestrous, respectively; \( P < 0.001 \)).

### Serum concentrations of gonadotrophins, oestradiol and progesterone during anoestrous

**Serum concentrations of FSH, oestradiol and progesterone measured each day.** Mean serum concentrations of FSH, oestradiol and progesterone measured once a day did not differ among the four 17 day scanning periods in anoestrous (2.2 ± 0.2 ng ml\(^{-1}\), \( 4.3 ± 1.3 \) pg ml\(^{-1}\) and 0.06 ± 0.01 ng ml\(^{-1}\), for FSH, oestradiol and progesterone, respectively; \( P > 0.05 \)). The characteristics of fluctuations in serum FSH, oestradiol and progesterone concentrations in blood samples taken each day during each scanning period and as determined by the cycle-detection computer program (Clifton and Steiner, 1983) are given (Table 1).

Periodic fluctuations in serum FSH concentrations were identified in all animals studied. The number, length and amplitude of peaks measured each day, and the duration of interpeak intervals for serum FSH concentrations did not differ significantly among the four periods of study in anoestrous ewes (Table 1).

Daily fluctuations in serum concentrations of oestradiol were determined in all animals during all periods of study. The mean number, duration and height of detected transient increases in serum oestradiol concentrations and the length of interpeak intervals did not differ significantly among the four study periods in anoestrous (Table 1).

Sporadic fluctuations in serum progesterone concentrations were detected in some ewes during the 17 day scanning periods throughout anoestrous (Table 1). The mean number, length and amplitude of identified progesterone fluctuations, and the duration of interpeak intervals did not differ significantly among the four 17 day periods. Ewes in which progesterone fluctuations were detected had lower mean numbers of medium-sized follicles (4 mm in diameter) than ewes without progesterone fluctuations (0.5 ± 0.1 versus 0.8 ± 0.1, respectively; \( P < 0.05 \)).

**Intensive blood sampling for determination of patterns of gonadotrophin secretion.** The characteristics of pulsatile secretion of LH in anoestrous ewes (mean and basal serum concentrations of LH, LH pulse frequency and amplitude) were not affected by period of ultrasonographic scanning (mean and basal concentration of LH, pulse frequency and amplitude were 0.5 ± 0.05 ng ml\(^{-1}\), 0.2 ± 0.02 ng ml\(^{-1}\), 0.4 ± 0.1 pulse h\(^{-1}\) and 1.5 ± 0.1 ng ml\(^{-1}\), respectively; \( P > 0.05 \)). No FSH pulses were detected in samples from intensive bleedings conducted throughout anoestrous. The mean serum concentration of FSH based on intensive bleed (2.4 ± 0.4 ng ml\(^{-1}\)) did not differ (\( P > 0.05 \)) among the four periods of study nor from the mean concentration of FSH based on one bleed per day.

**Associations between emergence of follicular waves and FSH fluctuations**

Growth and regression profiles of individual ovarian follicles growing from 3 to ≥ 5 mm in size before regression, for four anoestrous ewes during 17 day scanning periods are shown with corresponding daily serum FSH and oestradiol concentrations (Fig. 3). The associations between days of follicular wave emergence and identified FSH peaks are presented (Table 2). The number of emerging follicular waves (days of emergence) and the number of identified FSH peaks per ewe did not differ (\( P > 0.05 \)) and were strongly and positively correlated throughout anoestrous (Spearman correlation: \( r = 0.78 \); \( P < 0.000001 \)). Similarly, the intervals between adjacent days of wave emergence (interwave intervals) did not differ (\( P < 0.05 \)) from the interpeak intervals for serum FSH fluctuations during each of the four scanning periods. These parameters were also positively correlated across anoestrous (\( r = 0.64 \); \( P < 0.00003 \); Fig. 4). The intervals between peaks of FSH fluctuations and the intervals between days of the beginning of follicular static phases were positively correlated throughout anoestrous (\( r = 0.41 \); \( P < 0.02 \)). However, there was no correlation between the interpeak intervals for serum FSH concentrations and the intervals between days on which follicular static phases ended or the days at which follicular regression was complete (\( r = 0.01 \); \( P > 0.95 \) and \( r = -0.06 \); \( P > 0.7 \) for the end of the static phase and the day of regression to 3 mm in diameter, respectively; Fig. 4).

The mean number of days of follicular wave emergence and the mean number of fluctuations in serum oestradiol concentrations per scanning period measured once a day, for all ewes, for all four periods of study in anoestrous, were significantly
Fig. 2. Follicle diameter profiles (•) for two individual ewes (a and b) scanned for 17 days at four different times across anoestrus (first period: 20 March–5 April; second period: 25 April–11 May; third period: 30 May–15 June; fourth period: 5 July–21 July). The arrows indicate the days of wave emergence, defined as the day on which ovarian follicles that grew to ≥ 5 mm in diameter before regression were first detected at 3 mm. Follicles were included in a wave if they emerged within a 48 h period.
Table 1. The characteristics of fluctuations in daily serum FSH, oestradiol and progesterone concentrations determined using the cycle-detection program (Clifton and Steiner, 1983) in blood samples collected each day during four 17 day periods of ultrasonographic examination in anoestrous ewes (first period: 20 March–5 April; second period: 25 April–11 May; third period: 30 May–15 June; fourth period: 5 July–21 July).

<table>
<thead>
<tr>
<th></th>
<th>FSH  (ng ml⁻¹)b</th>
<th>Oestradiol  (pg ml⁻¹)b</th>
<th>Progesterone  (ng ml⁻¹)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of fluctuations per ewe per scanning period*</td>
<td>2.20 ± 0.30</td>
<td>3.20 ± 0.30</td>
<td>3.50 ± 0.70</td>
</tr>
<tr>
<td>Length of fluctuation (days)</td>
<td>4.50 ± 0.20</td>
<td>3.60 ± 0.30</td>
<td>2.80 ± 0.30</td>
</tr>
<tr>
<td>Interpeak interval (days)</td>
<td>4.70 ± 0.50</td>
<td>4.20 ± 0.50</td>
<td>3.20 ± 0.40</td>
</tr>
<tr>
<td>Mean amplitude (mean peak concentration)</td>
<td>2.75 ± 0.18</td>
<td>6.37 ± 0.52</td>
<td>0.17 ± 0.02</td>
</tr>
</tbody>
</table>

All values are mean ± SEM.
No significant period effect was obtained for any of the parameters above and the data for each hormone were averaged over the four periods of study in anoestrous.

*Only complete fluctuations (nadir to nadir) determined during all four scanning periods in anoestrous were enumerated and used for calculation of the mean length of fluctuation, interpeak interval and peak concentration.

Fluctuations were detected in all animals studied during all four scanning periods in anoestrous.

Fluctuations were detected in eight, four, five and four animals during the first, second, third and fourth scanning periods, respectively. Means were calculated only for animals in which fluctuations in the concentrations of the hormone measured each day were determined.

Fig. 3. Growth and regression profiles of individual ovarian follicles (●—●) growing from 3 to ≥5 mm in diameter, with corresponding concentrations of FSH and oestradiol, for four ewes (a–d), for different 17 day scanning periods in anoestrous (first period: 20 March–5 April; second period: 25 April–11 May; third period: 30 May–15 June; fourth period: 5 July–21 July). The arrows indicate the days of follicular wave emergence, defined as the day on which follicles of the wave were first detected at 3 mm, and the asterisks denote peaks of FSH–oestradiol fluctuations (dashed vertical lines encapsulating identified peaks) determined using the cycle-detection program (Clifton and Steiner, 1983).

different (2.1 ± 0.3 versus 3.2 ± 0.3; mean number of days of emergence versus mean number of identified oestradiol fluctuations, respectively). A similar comparison was not made for progesterone peaks since they were detected only in some animals throughout anoestrous.

When FSH peaks were assigned to the nearest day of follicle wave emergence, chi-squared analysis of the frequency of the FSH peaks occurring on the day of emergence (day 0) and on each of 3 days before and after emergence revealed that most peaks (72%; P < 0.05) occurred from day –1 to day 1 (Fig. 5).
Table 2. Summary of associations between the emergence of follicular waves and peaks of serum concentrations of FSH recorded each day during four 17 day scanning periods during anoestrus (first period: 20 March–5 April; second period: 25 April–1 May; third period: 30 May–15 June; fourth period: 5 July–21 July).

<table>
<thead>
<tr>
<th>Scanning period</th>
<th>First (n = 8)</th>
<th>Second (n = 8)</th>
<th>Third (n = 8)</th>
<th>Fourth (n = 7)</th>
</tr>
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<tbody>
<tr>
<td>Number of events per ewe</td>
<td>Days of emergence</td>
<td>2.5 ± 0.4</td>
<td>2.0 ± 0.6</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>FSH peaks</td>
<td>2.4 ± 0.4</td>
<td>2.2 ± 0.3</td>
<td>2.0 ± 0.5</td>
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<tr>
<td>Length of interval (days) between</td>
<td>Days of emergence of adjacent follicular waves</td>
<td>4.2 ± 0.9</td>
<td>4.8 ± 1.0</td>
<td>4.2 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Peaks of adjacent FSH fluctuations</td>
<td>4.4 ± 0.7</td>
<td>4.9 ± 1.1</td>
<td>4.4 ± 1.1</td>
</tr>
</tbody>
</table>

All values are mean ± SEM.

Fluctuations of FSH concentrations were determined using the cycle-detection program (Clifton and Steiner, 1983).

* No statistically significant difference between the two events was determined. In addition, the number of days of emergence and number of FSH peaks per ewe were positively correlated (Spearman correlation: r = 0.78; P < 0.000001) for all four scanning periods.

* No statistically significant difference between the two types of interval was determined. The durations of the two intervals were positively correlated (r = 0.64; P < 0.00003) for the four observation periods in anoestrus.

Fig. 4. Spearman correlations between the duration of FSH interpeak intervals and intervals between adjacent days of wave emergence (○), beginning of the static phase (●), end of the static phase (■), and end of the regressing phase (regression to 3 mm in diameter) (■) of the largest ovarian follicles of sequential waves, for all ewes, for all four periods of ultrasonography during anoestrus.

Discussion

The absence of behavioural oestrus ('silent oestrus') in three of eight ewes before the last luteal phase of the breeding season is similar to previous observations in sheep (Smeaton and Robertson, 1971; Rawlings et al., 1977). The restoration of ovarian cycles, as determined by increases in serum progesterone concentrations, was not consistently accompanied by behavioural oestrus or by ovulation and development of normal corpora lutea. Luteinization of unovulated ovarian follicles was observed in cyclic sheep (Schrick et al., 1993). In addition, a phenomenon referred to as 'short-lived' or 'inadequate' corpora lutea has been described in peripubertal ewes, as well as in adult ewes during transition into the breeding season and from the postpartum period (Hunter, 1991). Such structures may not be particularly echogenic and could have been missed in the present study which used ultrasound scanning of ovaries.

The numbers of small (3 mm) and medium (4 mm) but not large (≥5 mm) ovarian antral follicles were affected by the stage of anoestrus. The mean numbers of follicles in the 3 mm and 4 mm size class increased significantly from the first (March–April) to the second (April–May) scanning period of anoestrus. Earlier postmortem studies, using ewes killed during mid-anoestrus, suggested that the number of small and medium-sized ovarian follicles was significantly greater during anoestrus than during the luteal phase of the oestrous cycle (Brand and de Jong, 1973). Earlier studies suggested that the
corpus luteum of pregnancy exerted residual local inhibitory effects that resulted in suppressed follicular growth in post-partum ewes (Bartlekowski et al., 1995). The cause of the increase in follicle numbers from early to mid-anoestrus remains unknown. As ewes move into the breeding season, the number of small ovarian antral follicles (2–3 mm) has been reported to decrease again (Ravindra and Rawlings, 1997).

The present results strongly indicate that growth and atresia of the largest (≥5 mm) ovarian follicles in anoestrous ewes exhibit a wave-like pattern. Similar results were also obtained for ovaries transplanted to the neck in anoestrous Finn × Merino ewes, when the ovaries were examined by ultrasonography for 10 consecutive days during mid-anoestrus (Souza et al., 1996). Previous studies conducted during the oestrous cycle in ewes led to contrasting conclusions regarding the number of phases or waves of follicular growth (continuous growth, no waves: Schrick et al., 1993; 2–3 phases or waves: Ravindra et al., 1994; 3–6 follicular waves: Ginther et al., 1995). This may reflect factors such as breed (fertility), environment and management.

During an ultrasonographic study of ovarian follicle dynamics conducted during two 5 day observation periods in late anoestrus (Ravindra, 1993), no ovarian follicles >5 mm in diameter were detected. The largest ovarian follicles in the present study were seen to grow to a maximum size of 9 mm before regression. The proportion of ewes with ovaries bearing ovarian follicles reaching a maximum size of 5, 6, 7, 8 and 9 mm was 43, 30, 20, 5 and 2%, respectively. Therefore, it was concluded that ovarian follicles present in the ovaries of anoestrous ewes could grow to an ostensibly ovulatory size. In addition, the effect of day on numbers of follicles not growing beyond 3 mm in size, centralized to the day of wave emergence (±3 days), approached significance (P = 0.09), suggesting the existence of a rhythmic pattern of small antral follicle turnover in anoestrous ewes. This finding is in contrast to the conclusions of studies conducted in cyclic Polypay (Ginther et al., 1995) and Western White-faced sheep (Ravindra, 1993) that the development of small and medium antral follicles does not involve a phasic phenomenon. Whether the more organized pattern of small follicle growth found in the present study is restricted to ewes outside of the breeding season (seasonal effects) or is breed-dependent (genetic effects), or both, remains uncertain. Changes in the number of small antral follicles, as seen in the present study, may reflect a synchronized emergence of cohorts of early antral follicles <3 mm in diameter in anoestrous sheep.

The mean growth rate of the largest ovarian follicles of the wave increased significantly from the second (April–May) to the fourth (July) scanning period in anoestrus. The incidence of the faster growth of ovarian follicles to a ≥5 mm range in the last period of study in anoestrous may be the result of changes in the degree of ovarian responsiveness to gonadotrophic stimuli, especially to LH, as anoestrous ewes; diminished ovarian responsiveness has been reported in anoestrous sheep (Legan et al., 1985).

In the present study, the emergence of follicular waves generally overlapped the regression phase of the largest follicle of the previous wave, and the growth of ovarian follicles to 4 mm in diameter during the growing and static phase of a follicular wave was infrequent. However, the marked follicular dominance seen in cattle (Ginther et al., 1996) was not evident in ewes during anoestrus.

The results of the present study are in accordance with the conclusions of previous experiments documenting production of gonadotrophic hormones and ovarian steroids in anoestrous ewes. Namely, that the frequency of LH pulses does not differ significantly among different stages of anoestrus (Yuthasastrakosol et al., 1977; Jackson and Davis, 1979; Walton, 1975; Walton et al., 1980; McNatty et al., 1984); that the pattern of FSH secretion in anoestrous ewes is non-pulsatile (Bister and Paquay, 1983); that periodic fluctuations in serum FSH concentrations occur at approximately 5 day intervals in anoestrous ewes (Bister and Paquay, 1983); that progesterone production is suppressed, yet sporadic deviations of serum progesterone concentrations from basal or non-detectable amounts are observed in some animals throughout anoestrus (Thorburn et al., 1969; Yuthasastrakosol et al., 1975; l’Anson, 1983; Ravindra, 1993); and that serum oestradiol concentrations remain relatively low throughout the entire period of the nonovulatory season in ewes (Yuthasastrakosol et al., 1975). It has been reported that serum FSH concentrations tend to be lower in mid-anoestrous sheep compared to early anoestrous animals (Oussaid et al., 1993) but such a relationship was not confirmed in the present study.

It is concluded that sporadic fluctuations in serum progesterone concentrations occurring during anoestrus were random and were not associated with ovulations or luteinization of ovarian structures. Ravindra (1993) reported that serum progesterone concentrations and the total number of antral ovarian follicles ≥2 mm in diameter present in the ovaries of anoestrous ewes were negatively correlated. In the present study, the occurrence of increases in daily serum progesterone concentrations was found to be associated with decreased

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**Fig. 5.** Relative frequency of occurrence of FSH peaks, identified using the cycle detection program (Clifton and Steiner, 1983), and detected within 3 days before and after the day of wave emergence, and the daily diameter of the largest follicle (■) of waves for the day of, and 3 days after, follicular wave emergence. The graph was compiled from the data for 55 emerging follicular waves obtained during 147 day periods of ovarian ultrasonographic examination and blood sampling in anoestrous ewes.
numbers of 4 mm ovarian follicles throughout anoestrus. The source of progesterone secretion in anoestrous ewes remains to be elucidated.

On the basis of the cycle-detection computer program, cyclic fluctuations in oestradiol secretion were identified in all animals studied. It is possible that peak values of oestrogen fluctuations coincide with periods of increased production of oestradiol by the largest follicles which are known to be the main source of oestrogens in cyclic (Cox et al., 1971; Biersing et al., 1972; Baird et al., 1976) and anoestrous sheep (Scaramuzzi and Baird, 1977; Souza et al., 1996). However, in the present study, the number of determined oestradiol fluctuations was significantly greater than the number of emerging follicular waves. In anoestrous ewes, even though follicles grow into the ovariolytic size range, oestrogen production appears to be lower than it is during the breeding season (Yuthasatrakosol et al., 1975). However, when pulses of GnRH were given to anoestrous ewes, oestrogen production appeared to increase as large antral follicles grew in the ovary (Souza et al., 1996). The lower production of oestradiol in anoestrous ewes, coupled with assay variability and sampling once a day, may have precluded the assignment of discrete peaks in serum concentration of oestradiol to particular follicular waves in the present study.

The present study demonstrated a relationship between transient increases in daily serum FSH concentrations and the initial phase of ovarian follicular wave development throughout anoestrous in ewes. The results suggest that rhythmically generated increases in serum FSH concentrations may initiate the emergence of follicular waves in anoestrous ewes (antral follicles growing from 3 to ≥5 mm in diameter before regression). Moreover, as LH output is suppressed during the entire period of anoestrus, it is likely that FSH alone has the potential to stimulate ovarian antral follicle development up to peri-ovariolytic size throughout anoestrous in sheep. This observation is in agreement with earlier suggestions that LH is of importance predominantly for the terminal phase of antral follicular growth and maturation leading to ovulation (Uilenbroek and Richards, 1979). Apparently, the absence of an LH drive prevents ovulatory cycles, yet ovarian antral follicular turnover is not impaired during seasonal anoestrus in ewes and closely resembles that of ewes during the breeding season. In addition, the lack of correlation between later stages of follicular wave development (the end of the static and regressing phases of individual follicles ≥5 mm) and peaks of serum FSH concentrations, suggests that transient increases in FSH secretion trigger the emergence of follicular waves but that later stages of follicular development are independent of the periodicity of FSH release. This conclusion is further supported by the distribution of identified FSH peaks in relation to the day of wave emergence; peaks of FSH fluctuations were seen to occur most frequently within 1 day before and after, and on the day of wave emergence. In summary, an endogenous rhythm of FSH secretion, unchanged throughout the year (Bister and Paquay, 1983), appears to stimulate and synchronize the emergence of waves of follicular growth in sheep during (Ginther et al., 1995) and outside of the breeding season.

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