BIOLOGY OF REPRODUCTION **77,** 252–262 (2007)
Published online before print 11 April 2007.
DOI 10.1095/biolreprod.106.058842

Differential Effects of Various Estradiol-17beta Treatments on Follicle-Stimulating Hormone Peaks, Luteinizing Hormone Pulses, Basal Gonadotropin Concentrations, and Antral Follicle and Luteal Development in Cyclic Ewes[1]

David M.W. Barrett, Rajesha Duggavathi,[3] Kate L. Davies,[4] Pawel M. Bartlewski,[5] Edward T. Bagu, and Norman C. Rawlings[2]

Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5B4

ABSTRACT

In a previous study, 10-day estradiol implant treatment truncated the FSH peaks that precede follicular waves in sheep, but subsequent ovine FSH (oFSH) injection reinitiated wave emergence. The present study's objectives were to examine the effects of a 20-day estradiol and progesterone treatment on FSH peaks, follicle waves, and responsiveness to oFSH injection. Also, different estradiol doses were given to see whether a model that differentially suppressed FSH peaks, LH pulses, or basal gonadotropin secretion could be produced in order to study effects of these changes on follicular dynamics. Mean estradiol concentrations were 11.8 ± 0.4 pg/ml, FSH peaks were truncated, wave emergence was halted, and the number of small follicles (2–3 mm in diameter) was reduced ($P < 0.05$) in cyclic ewes given estradiol and progesterone implants (experiment 1). On Day 15 of treatment, oFSH injection failed to induce wave emergence. With three different estradiol implant sizes (experiment 2), estradiol concentrations were 5.2, 19.0, 27.5, and 34.8 (±4.6) pg/ml in control and treated ewes, respectively. All estradiol treatments truncated FSH peaks, except those that created the highest estradiol concentrations. Experiment 2-treated ewes had significantly reduced mean and basal FSH concentrations and LH pulse amplitude and frequency. We concluded that 20-day estradiol treatment truncated FSH peaks, blocking wave emergence, and reduced the small-follicle pool, rendering the ovary unresponsive to oFSH injection in terms of wave emergence. Varying the steroid treatment created differential FSH peak regulation compared with other gonadotropin secretory parameters. This provides a useful model for future studies of the endocrine regulation of ovine antral follicular dynamics.

corpus luteum, estradiol, follicle-stimulating hormone, follicular development, luteinizing hormone

INTRODUCTION

Prior to the use of ultrasonography, earlier studies on the endocrine regulation of the growth of ovarian antral follicles (2–3 mm or more in diameter) in sheep led to the conclusion that these follicles were gonadotropin dependent [1–4]. It was generally concluded that growth of these follicles was largely FSH dependent, but some level of basal LH secretion appeared essential, and if pulsatile LH secretion was present, final growth and maturation could become LH dependent [1–5].

With the use of ultrasonography, it was found that during the ovine estrous cycle, one to three antral follicles emerge or grow from a pool of small antral follicles (1–3 mm in diameter) every 4 to 5 days [6–9]. These follicles grow to a diameter of 5 mm or more in diameter before regression or ovulation [6–9]. Each wave of follicle growth is preceded by a transient peak of FSH secretion [6–10]. The role of basal FSH secretion (peaks and pulses excluded) in ovine antral follicle wave dynamics is unknown, as is the precise role of basal or pulsed LH secretion. The frequency of LH secretory pulses changes across the estrous cycle, largely regulated by the pattern of progesterone secretion during the genesis and regression of the corpus luteum (CL) [1, 11–17], but changes in LH pulse frequency do not appear to be correlated with or functionally related to specific phases of the growth or regression of follicle waves [10, 18]. This is interesting, as there are changes in LH pulse frequency around the time of follicular deviation in a follicular wave in cattle [19, 20]. Suppression of LH pulse frequency decreases growth of the dominant follicle after deviation, but deviation does not require this increase in pulsed LH secretion [21]. Dominance is the process in which one antral follicle growing in a wave exceeds others in growth rate and size and suppresses the growth of other subordinate follicles, probably by way of suppressive follicular secretory products [22]. During each follicular wave in sheep, serum concentrations of estradiol peak at the end of the growth phase of the largest follicle of the wave [7, 23]. Inhibin tends to be produced by a wide size range of antral follicles [24–28]. However, ovarian secretory products (i.e., ovarian steroids and inhibin) may not regulate the peaks in serum concentrations of FSH that precede antral follicular waves, and follicular dominance would not appear to be as marked in the ewe as in cattle [9, 29, 30].

It would be useful to further develop specific experimental models in which we could examine the individual roles of basal gonadotropin secretion, FSH peaks, and pulsatile LH secretion in governing follicular wave development. The GNRH1 agonists and antagonists affect both LH and FSH secretion [31, 32]. Although inhibin specifically suppresses FSH secretion, it is not clear whether this is an effect on basal secretion, peaks, or both. In addition, the maintenance of a constant inhibitory affect of inhibin would be experimentally challenging. Steroid-releasing implants have been widely used

[1]Supported by the Natural Sciences and Engineering Research Council, Canada (N.C.R.). D.M.W.B., K.L.D., and E.T.B. were recipients of University of Saskatchewan Graduate Student Scholarships.
[2]Correspondence: FAX: 306 966 7376;
e-mail: norman.rawlings@usask.ca
[3]Current address: Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch 67404, France.
[4]Current address: Department of Animal and Poultry Science, College of Agriculture, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5A8.
[5]Current address: Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada N1G 2W1.

Received: 9 November 2006.
First decision: 12 December 2006.
Accepted: 13 March 2007.

ISSN: 0006-3363. http://www.biolreprod.org

in ovariectomized ewes to study estradiol and progesterone feedback regulation of gonadotropin secretion [33–35]. The effects of steroids on LH secretion were dose dependent, but on FSH secretion they were variable; the possibility of differential effects on basal gonadotropin secretion versus LH pulses and FSH peaks has not been fully addressed.

In a recent study from our research group [36], we created an experimental model in which only the amplitude and peak concentration of the periodic peaks in mean serum FSH concentrations that precede follicular waves were altered using 10-cm-long estradiol-releasing implants left in place for 10 days in cyclic ewes. Pulsed LH secretion was not affected. The 10-cm estradiol implants had little effect on mean and basal serum FSH concentrations. Supraphysiologic concentrations of estradiol-17β suppress follicle wave development by truncating FSH peaks in cyclic ewes; in addition, injection of physiologic concentrations of ovine FSH (oFSH) reinitiates follicle emergence [36]. Maintenance of the 2- to 3-mm follicle pool does not appear to require regular secretion of FSH peaks.

For the current studies, we hypothesized that: 1) a longer-term treatment (20 days) of cyclic ewes with progesterone and estradiol would maintain suppression of follicle wave emergence and FSH peaks but would not affect the pool of small antral follicles (2–3 mm in diameter), and exogenous oFSH treatment of ewes with this treatment would induce follicle wave emergence; and 2) varying the dose of estradiol released from subcutaneous implants in cyclic ewes in the presence of progesterone from the CL could result in differential regulation of LH and FSH secretion, leading to differential effects on ovarian function. This approach may also allow targeted manipulation of basal gonadotropin secretion, LH pulses, and/or FSH peaks.

The objective of the first experiment was to see: 1) whether longer-term treatment of cyclic ewes with progesterone and estradiol would maintain suppression of follicle wave emergence and FSH peaks and 2) what effect this would have on the remaining small-follicle pool and its responsiveness to oFSH. Exogenous oFSH was given to induce an FSH peak similar in height to endogenous FSH peaks; such a treatment has been shown to result in follicular wave emergence within 12 to 24 h in both cyclic and seasonally anestrous ewes experiencing normal follicular waves [9]. The purpose of the second experiment was to see whether different doses of exogenous estradiol administered during the luteal phase in the ewe would differentially affect pulsatile LH secretion, FSH peaks, or baseline serum gonadotropin concentrations.

MATERIALS AND METHODS

Animals

Care and handling of experimental animals were done according to the Canadian Council on Animal Care's published guidelines. Sexually mature, clinically healthy, cyclic Western White Face (WWF) ewes were kept outdoors in sheltered paddocks. Ewes were fed a maintenance diet of hay, and cobalt iodized saltlicks and water were freely available. The WWF is a cross between the Columbia and Rambouillet breeds. The mean ovulation rate of WWF ewes during the midbreeding season is 1.8 ± 0.2 [7].

Ultrasound Technique

Ovarian antral follicular dynamics were monitored in all ewes by transrectal ovarian ultrasonography (scanning) using a 7.5-MHz transducer stiffened with a hollow plastic rod and connected to a B-mode, real-time echo camera (Aloka SSD-900; Overseas Monitor, Richmond, BC, Canada). This technique has been validated for monitoring ovarian follicular dynamics and CL detection in sheep [37–39]. All images were viewed at a 1.5× magnification with constant gain and focal point settings. Ovarian images were recorded (Panasonic AG 1978; Matsushita Electric, Mississauga, ON, Canada) on high-grade videotapes (Fuji S-VHS, ST-120 N; Fujifilm, Tokyo, Japan) for later examination. The relative positions and dimensions of follicles and luteal structures were also sketched on ovarian charts.

Experimental Design

Experiment 1. Twelve randomly selected ewes were monitored daily for estrus with vasectomized, crayon marker-harnessed rams. All ewes received subcutaneous silastic rubber implants containing 10% estradiol-17β (w/w; 10 × 0.33 cm; Sigma Chemical Co., St. Louis, MO) 4 days after ovulation (Day 0 = day of estradiol implant insertion) and 10% progesterone (w/w; 22 × 0.48 cm; Sigma-Aldrich, Oakville, ON, Canada) 6 days after ovulation. The purpose of this progesterone treatment was to maintain progesterone concentrations above baseline to facilitate and maintain estradiol feedback effects on FSH secretion for the duration of estradiol treatment [40]. To make the implants, liquid silastic rubber (A-101 medical grade silicone elastomer; Factor II Inc., Lakeside, AZ) was mixed with the steroid, and a curing catalyst was added (Catalyst; Factor II Inc.). The estradiol mixture was injected into silastic tubing (Silastic laboratory grade tubing; 0.34 cm i.d. × 0.47 cm o.d.; Dow Corning, Midland, MI), and the progesterone mixture was injected into Tygon tubing moulds (0.48 cm i.d.; Norton Plastics, Akron, OH). Once cured, the progesterone implants were removed from the moulds, and all steroid implants were cut into the desired lengths. Implants were soaked in sterile 0.9% (w/v) saline for 48 h at room temperature (estradiol implants) or at 37°C in a water bath (progesterone implants) before insertion. Lidocaine hydrochloride (2%; Xylocaine; AstraZeneca Canada Inc., Mississauga, ON, Canada) was used as a local anesthetic. A 1.5-cm incision was made in the axillary region with a scalpel, the implant was inserted using a trochar, and the incision was closed with wound clips (9-mm MikRon AUTOCLIP; Becton Dickinson Primary Care Diagnostics, Sparks, MD). On Day 15 after implant insertion (19 days after ovulation), 6 of 12 ewes were injected with oFSH (0.5 μg/kg s.c.; NIDDK-oFSH-18; 1 mg has a biologic FSH potency equal to 65.6× NIH-oFSH-S1 or 1640 IU and a biologic LH potency equal to 0.1× NIH-oLH-S1 or 106 IU) prepared in saline with 0.05% bovine serum albumin (w/v; Sigma Chemical) and 50% polyvinylpyrrolidone (w/v; Sigma); control ewes received only the vehicle. The same dose of oFSH was injected 8 h after the initial injection. Ewes were bled every 6 h for 36 h after the first oFSH injection. All implants were removed from all ewes on Day 20 (24 days after ovulation). To determine the secretory pattern of LH, blood samples were collected every 12 min for 6 h (intensive sampling) on Days 9 and 14 from all ewes. For both groups, daily scanning and blood sampling started on the day of estrus at the beginning of the ovulatory cycle in which treatments were given and continued until ovulations were detected after treatment.

Experiment 2. Sixteen ewes were monitored daily for estrus with vasectomized, crayon marker-harnessed rams. At the beginning of the ovulatory cycle in which treatments were given, daily scanning started on the day of estrus and ended when the associated ovulations were detected. Twelve ewes received subcutaneous silastic rubber implants containing 10% estradiol-17β (w/w; Sigma Chemical Co.) on Day 4 after ovulation (Day 0 = day of estradiol implant insertion); separate groups of four ewes received 10-cm (10 × 0.34 cm), 15-cm (15 × 0.34 cm), or 20-cm (20 × 0.34 cm) implants. Four sham-operated control ewes received no implants. Implant handling and insertion procedures were the same as in experiment 1. All implants were removed from all ewes on Day 10 after estradiol implant insertion, after the second period of intensive blood sampling. To determine the secretory pattern of LH, blood samples were collected every 12 min for 6 h (intensive sampling) on Days 6 and 10 from all ewes. Daily blood samples were taken from all ewes starting on the day of estrus at the beginning of the ovulatory cycle in which treatments were given and continuing until ovulations were detected after treatment. Daily scanning was done on all ewes from implant removal until ovulations were detected after treatment.

Follicular Data Analyses

A follicular wave was defined as one or more Graafian follicles that 1) increased in size from 2 or 3 mm in diameter to 5 mm or more in diameter and 2) emerged from the pool of 2- to 3-mm follicles within a maximum period of 48 h [41]. The growth, static, and regression phases of a follicular wave have been defined previously [41]. Ovulation was detected with ultrasonography as the collapse of a large follicle (≥5 mm in diameter). Follicular data were integrated for both ovaries of each animal.

Blood Sampling and Hormone Analysis

Blood samples (10 ml) taken daily and every 6 h were collected by jugular venipuncture into vacutainers (Becton Dickinson, Franklin Lakes, NJ). For intensive sampling, blood was collected via indwelling jugular catheters (5 ml per sample; vinyl tubing, 1.0 mm i.d. × 1.5 mm o.d.; SV70; Critchley Electrical

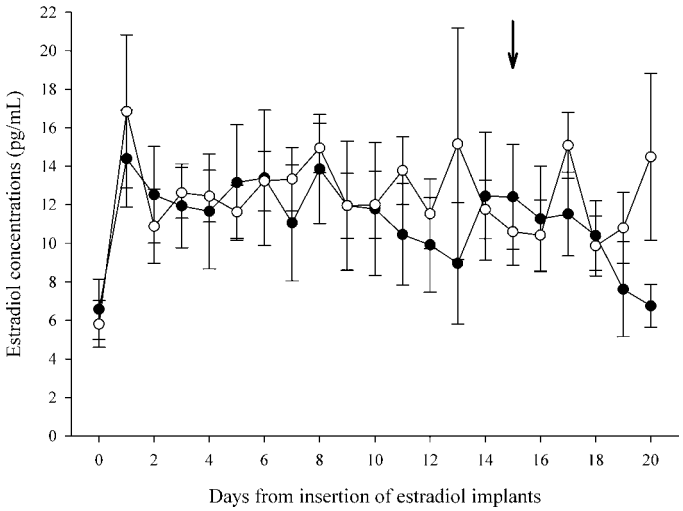

FIG. 1. Mean ± SEM daily serum estradiol concentrations during the implant period in cyclic WWF ewes treated with estradiol and progesterone releasing silastic rubber implants (subcutaneously) with (open circles; n = 6) or without (closed circles; n = 5) an oFSH treatment (indicated by the arrow) on Day 15 after estradiol implant insertion (Day 19 after ovulation). Implants containing 10% estradiol-17β (10 × 0.34 cm) or progesterone (22 × 0.48 cm) were in the ewes for 20 days (Days 4 to 24 after ovulation) or 18 days (Days 6 to 24 after ovulation), respectively. Data were normalized to the day of estradiol implant insertion (Day 0) in all ewes.

Products Ltd., Auburn, Australia). All samples were permitted to clot at room temperature for 18–24 h. These samples were then centrifuged for 10 min at 1500 × *g*, and serum was removed and kept at −20°C until assayed.

Progesterone [40], estradiol [42], FSH [43], and LH [44] concentrations were measured in serum samples by validated radioimmunoassay procedures. Gonadotropin concentrations are expressed in terms of NIAMDD-oFSH-1 and NIAMDD-oLH-24. The assay sensitivities (defined as the lowest concentration of a hormone capable of significantly displacing radiolabeled hormone from the antibody) were 0.03 ng/ml (progesterone), 1.0 pg/ml (estradiol), and 0.1 ng/ml

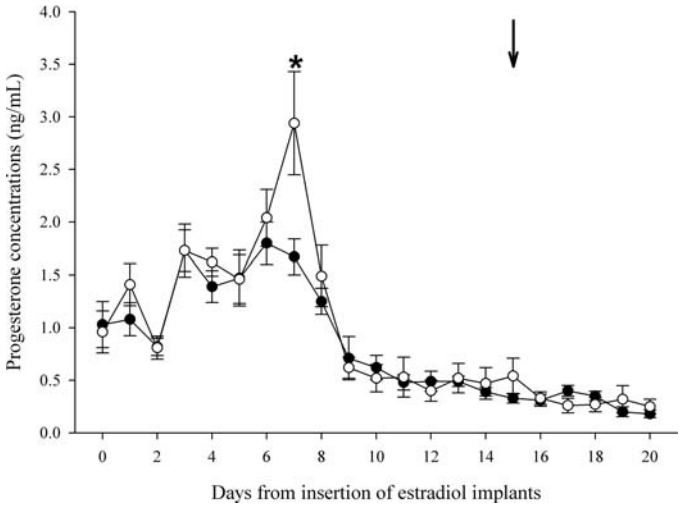

FIG. 2. Mean ± SEM daily serum progesterone concentrations during the implant period in cyclic WWF ewes treated with estradiol and progesterone releasing silastic rubber implants (subcutaneously) with (open circles; n = 6) or without (closed circles; n = 5) an oFSH treatment (indicated by the arrow) on Day 15 after estradiol implant insertion (Day 19 after ovulation). Implants containing 10% estradiol-17β (10 × 0.34 cm) or progesterone (22 × 0.48 cm) were in the ewes for 20 days (Days 4 to 24 after ovulation) or 18 days (Days 6 to 24 after ovulation), respectively. Data were normalized to the day of estradiol implant insertion (Day 0) in all ewes. Asterisks (*) indicate differences between groups ($P < 0.05$).

(FSH and LH). The ranges of standards were 0.1–10 ng/ml, 1.0–100 pg/ml, 0.12–16.0 ng/ml, and 0.06–8.0 ng/ml in the progesterone, estradiol, FSH, and LH assays, respectively. A concentration equivalent to the sensitivity of the assay was assigned to serum samples with hormone concentrations lower than the assay sensitivity. Serum samples were collected daily both prior to and during the period implants were in place and were analyzed for concentrations of estradiol, FSH, and progesterone. All serum samples collected every 12 min were analyzed for concentrations of FSH and LH. All serum samples collected every 6 h were analyzed for concentrations of FSH.

For experiment 1, the intraassay and interassay coefficients of variation (CVs) were 7.4% and 13.5% or 6.1% and 14.9% for reference sera with mean estradiol concentrations of 10.0 or 24.4 pg/ml, respectively. For experiment 1, the intraassay and interassay CVs were 10.7% and 17.8% or 6.6% and 13.1% for reference sera with mean FSH concentrations of 0.24 or 1.28 ng/ml, respectively. For experiment 1, the intraassay and interassay CVs were 6.8% and 14.8% or 8.6% and 11.2% for reference sera with mean progesterone concentrations of 0.12 or 0.66 ng/m, respectively. For experiment 1, the intraassay and interassay CVs were 10.7% and 15.3% or 8.1% and 11.9% for reference sera with mean LH concentrations of 0.11 or 0.73 ng/ml, respectively.

For experiment 2, the intraassay and interassay CVs were 8.5% and 14.8% or 6.7% and 12.7% for reference sera with mean estradiol concentrations of 5.5 or 25.0 pg/ml, respectively. For experiment 2, the intraassay CVs were 3.6% or 9.2% for reference sera with mean FSH concentrations of 0.76 or 3.06 ng/ml, respectively. For experiment 2, the intraassay and interassay CVs were 10.3% and 15.2% or 7.0% and 8.5% for reference sera with mean progesterone concentrations of 0.27 or 1.04 ng/ml, respectively. For experiment 2, the intraassay and interassay CVs were 7.9% and 9.2% or 7.3% and 15.0% for reference sera with mean LH concentrations of 0.40 or 2.68 ng/ml, respectively.

The PC-PULSAR program [45] was used to assess mean and basal serum LH concentrations as well as LH pulse frequency and amplitude in blood samples collected every 12 min for 6 h.

Peaks of FSH in blood samples taken daily were identified using cycle detection software [46]. A fluctuation or cycle was defined as a progressive rise and fall in hormone concentrations that encapsulated a peak concentration (nadir-to-peak-to-nadir [46]). Mean basal FSH concentrations were determined by averaging the lowest points between peaks (nadirs). FSH peak concentration was defined as the concentration of FSH observed at the apex of the FSH peak. FSH peak amplitude was defined as the difference between the FSH peak concentration and the nadir before the peak concentration.

Statistical Analyses

Follicular waves were suppressed in all ewes except one in experiment 1; the latter ewe was dropped from further statistical analysis (implant-alone group). Daily hormone concentrations and numbers of follicles in size classes were normalized to the day of estradiol implant insertion and analyzed from the first estrus to the mean day when ovulations were detected after treatment (Days −5 to 26 [experiment 1] or Days −5 to 19 [experiment 2] from estradiol implant insertion) or were analyzed for the period of implant treatment (Days 0 to 20 [experiment 1] or Days 0 to 10 [experiment 2] from estradiol implant insertion). In experiment 1, daily FSH concentrations were also normalized to the time of the first oFSH injection and were analyzed for 36 h after the first oFSH injection. Two-way repeated-measures ANOVA (SigmaStat7 for Windows Version 2.03, 1997; SPSS Inc., Chicago, IL) was used to assess differences in daily hormone concentrations and numbers of follicles in size classes over time and among the groups of ewes (i.e., experiment 1: with or without exogenous oFSH; experiment 2: treated with 10-cm, 15-cm, 20-cm, or no implants). Two-way ANOVA was used to assess differences in LH secretory characteristics from blood samples collected every 12 min (i.e., mean and basal concentrations, pulse frequency, and pulse amplitude) among treated and control ewes and between intensive sampling days. One-way ANOVA was used to assess differences in characteristics of FSH peaks (see Tables 2 and 4) and ovarian parameters (i.e., interovulatory interval, ovulation rate, interval from implant removal to ovulation, number of follicle waves during the interovulatory interval and period of implant treatment, and interval from implant removal to the emergence of the ovulatory follicle wave) among treated and control ewes. If the main effects or the interactions were significant ($P < 0.05$), Fisher protected least-significant difference was used as a post-ANOVA test to detect differences between individual means. Data are expressed as mean ± SEM.

RESULTS

Experiment 1

Mean daily serum estradiol concentrations. When daily serum estradiol concentrations were normalized to the day of

estradiol implant insertion and analyzed for the period of implant treatment, there was a time effect ($P < 0.05$), but there was no treatment effect ($P > 0.05$), nor was there interaction of treatment by time ($P > 0.05$; Fig. 1). For both treatment groups, serum estradiol concentrations increased from Day 0 to Day 1 after estradiol implant insertion and declined from Day 15 or 17 to Day 18 or 20. There was also a decline in serum estradiol concentrations from Days 1 to 2 in the ewes treated with the implants and oFSH. The mean serum estradiol concentration for the period of implant treatment was 11.8 ± 0.4 pg/ml.

Mean daily serum progesterone concentrations. When daily serum progesterone concentrations were normalized to the day of estradiol implant insertion and analyzed for the period of implant treatment, there was a time effect ($P < 0.001$) and an interaction of treatment by time ($P < 0.05$), but there was no treatment effect ($P > 0.05$; Fig. 2). Serum progesterone concentrations were highest 6 or 7 days after estradiol implant insertion in control and FSH-treated ewes, respectively, and were decreasing after the peak. Comparison of individual means showed that mean serum progesterone concentrations were significantly higher in ewes treated with implants and oFSH compared with ewes treated with the implants only on Day 7 after estradiol implant insertion (Fig. 2).

Ovulations. The ovulation rate before and after implant treatment, mean duration of the interovulatory interval, and the interval from implant removal to ovulation did not differ between treatment groups ($P > 0.05$; Table 1). One animal treated with implants and oFSH ovulated a follicle 2 days after the estradiol implant was inserted and formed a CL that existed for 11 days.

Antral follicle development. One ewe treated with implants and oFSH had an abnormally large follicle (12 mm) at the time of estradiol implant insertion. This follicle grew from 6 to 12 mm in diameter in 3 days. This follicle developed into a luteinized follicle 5 days after estradiol implant insertion and existed for 8 days as a luteinized follicle until regression. The maximum diameter of this follicle was 12 mm.

The number of follicle waves per animal during the period of implant treatment did not differ between treatment groups ($P > 0.05$; Table 1). Nine ewes had no follicle waves emerging between Days 0 and 17 from estradiol implant insertion. Only one ewe from each treatment group had follicle waves emerge between Days 0 and 17 from estradiol implant insertion; waves emerged on Days 8 and 15 in the oFSH-treated ewe and on Day 9 in the ewe treated with vehicle. The interval from the time of the first injection of oFSH to the next follicle wave emergence did not differ between treatment groups (overall average interval: 5.3 ± 0.7 days, range: 0–8 days; $P > 0.05$). Follicle wave emergence around the time of implant removal did not differ between treatment groups (overall average day of emergence from estradiol implant insertion: 21.3 ± 0.4 days; $P > 0.05$). The interval from implant removal to ovulatory wave emergence did not differ between treatment groups ($P > 0.05$; Table 1).

When the number of follicles <2, 2, 3, or 4 mm in diameter was normalized to the day of estradiol implant insertion and analyzed from the first estrus to the mean day when ovulations were detected after treatment, there was a time effect ($P < 0.001$) but no treatment effect ($P > 0.05$; Fig. 3). For both treatment groups, the number of 2- and 3-mm follicles declined from about Day −3 to Day 14 or Day 15 after estradiol implant insertion in control and FSH-treated ewes, respectively. The number of 2-mm follicles subsequently increased from Day 14 or 15, whereas the number of follicles 3 mm in diameter increased from Day 17 or Day 14 in control or oFSH-treated ewes, respectively. The number of follicles 4 mm in diameter decreased markedly from Day 3 or 4 to Day 6 or 7 after estradiol implant insertion and increased from Day 19 or 21 to Day 24 or 22 in oFSH-treated or untreated ewes, respectively.

TABLE 1. Follicle waves and ovulations in cyclic WWF ewes treated with estradiol and progesterone releasing silastic rubber implants with or without an oFSH treatment on Day 15 after estradiol implant insertion (Day 19 after ovulation).

End points	Vehicle (n = 5)*	oFSH (n = 6)*
Ovulation rate (before implant treatment)	1.6 ± 0.2	1.5 ± 0.2
Ovulation rate (after implant treatment)	1.4 ± 0.3	1.5 ± 0.4
Inter-ovulatory interval (day)	30.0 ± 0.8	29.8 ±0.6
Interval from implant removal to ovulation (day)	6.0 ± 0.8	5.8 ± 0.6
No. of follicle waves per ewe during implant treatment period	0.6 ± 0.2	0.7 ± 0.3
Interval from time of implant removal to ovulatory wave emergence (day)	0.8 ± 0.4	1.4 ± 0.7

* Data are presented as mean ± SEM.

When the number of follicles <2, 2, 3, or 4 mm in diameter was normalized to the day of estradiol implant insertion and analyzed from the first estrus to the mean day when ovulations were detected after treatment, there was an interaction of treatment by time for 3-mm follicles ($P < 0.01$; Fig. 3C). On Days −1, 21, and 23 relative to estradiol implant insertion, the number of 3-mm follicles was significantly higher in ewes treated with implants only compared with ewes treated with implants and oFSH, whereas the opposite was observed for the treatment groups on Days 17 and 18 (Fig. 3C).

Characteristics of serum LH concentrations. When mean and basal serum LH concentrations were analyzed for samples taken every 12 min for 6 h, there was no difference between treatment groups during either sampling period ($P > 0.05$). Mean and basal serum LH concentrations were higher during the first intensive sampling period (Day 9 after estradiol implant insertion: mean, 0.31 ± 0.02 ng/ml; basal, 0.29 ± 0.02 ng/ml) compared with the second (Day 14: mean, 0.14 ± 0.02 ng/ml; basal, 0.11 ± 0.02 ng/ml) in ewes treated with or without oFSH ($P < 0.001$). Luteinizing hormone pulse amplitude and frequency did not differ between treatment groups or intensive sampling periods (amplitude overall mean: 0.22 ± 0.01 ng/ml; frequency overall mean: 2.2 ± 0.3 pulses per 6 h; $P > 0.05$).

Characteristics of serum FSH. The mean peak concentration and amplitude for endogenous FSH peaks did not differ between treatment groups ($P > 0.05$). The mean peak concentration for endogenous FSH peaks was higher before implant insertion and after implant removal compared with during the implant period ($P < 0.05$; Table 2). The mean peak amplitude for endogenous FSH peaks was higher after implant removal compared with before implant insertion and during the implant period ($P < 0.005$; Table 2).

TABLE 2. Endogenous FSH peaks before, during, and after implants.

End points (ng/ml)	Before implants*	During implants*	After implants*
Mean peak concentration	2.24 ± 0.31[a]	1.60 ± 0.08[b]	2.43 ± 0.21[a]
Peak amplitude	0.66 ± 0.12[a]	0.56 ± 0.06[a]	1.45 ± 0.16[b]

* Data are presented as mean ± SEM.
[a,b] Different letters within rows denote a significant difference ($P < 0.05$).

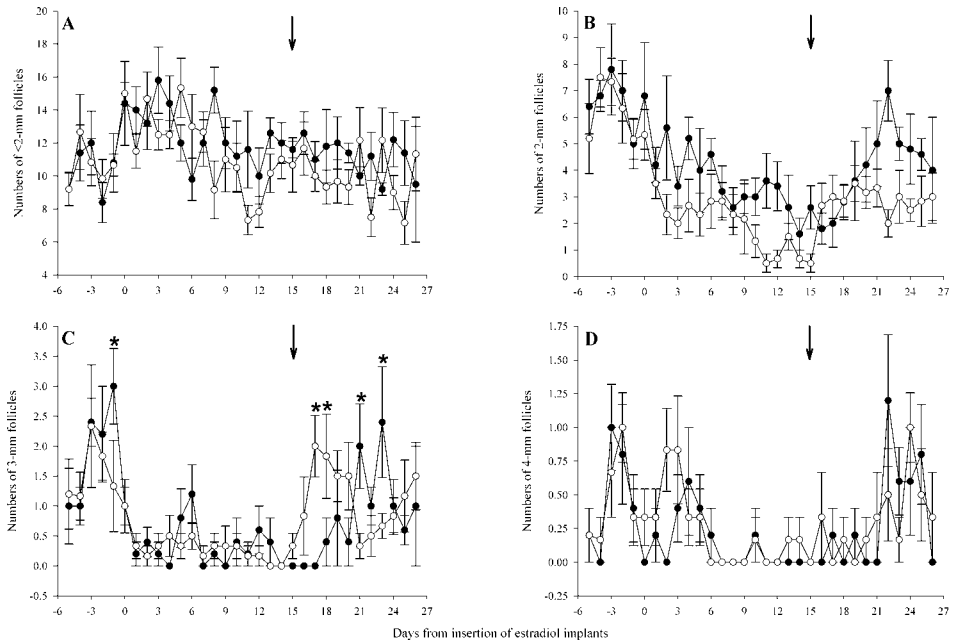

FIG. 3. Mean ± SEM daily numbers of follicles <2 mm (**A**), 2 mm (**B**), 3 mm (**C**), and 4 mm (**D**) in diameter from the first estrus to the mean day when ovulations were detected after treatment in cyclic WWF ewes treated with estradiol and progesterone releasing silastic rubber implants (subcutaneously) with (open circles; n = 6) or without (closed circles; n = 5) an oFSH treatment (indicated by the arrow) on Day 15 after estradiol implant insertion (Day 19 after ovulation). Implants containing 10% estradiol-17β (10 × 0.34 cm) or progesterone (22 × 0.48 cm) were in the ewes for 20 days (Days 4 to 24 after ovulation) or 18 days (Days 6 to 24 after ovulation), respectively. Data were normalized to the day of estradiol implant insertion (Day 0) in all ewes. Asterisks (*) indicate differences between groups ($P < 0.05$).

When mean serum FSH concentrations were normalized to the time of the first oFSH injection and analyzed for 36 h after the first oFSH injection, there was a treatment effect ($P < 0.05$), time effect ($P < 0.05$), and interaction of treatment by time ($P < 0.01$; Fig. 4). Mean serum FSH concentrations were significantly higher in ewes treated with oFSH (2.65 ± 0.20 ng/ml) compared with ewes treated with implants only (1.70 ± 0.22 ng/ml). Serum FSH concentrations were highest at 0.5 days after the first oFSH injection in ewes treated with oFSH. Comparison of individual means showed that serum FSH concentrations from 0.25 to 1.25 days after the first oFSH injection were significantly higher in ewes given oFSH compared with the ewes treated with vehicle (Fig. 4).

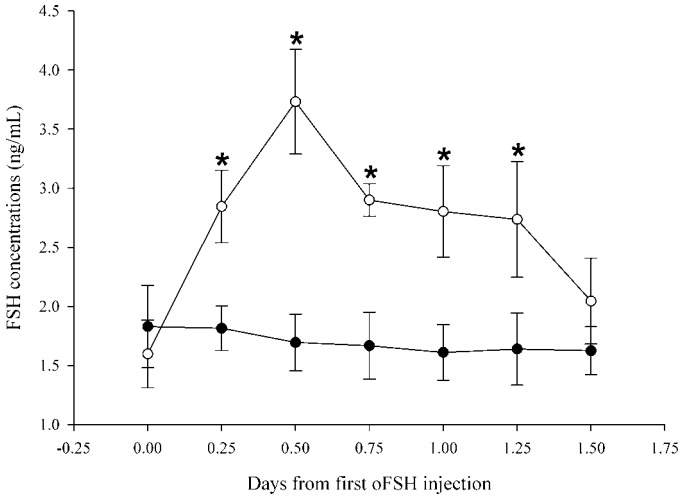

FIG. 4. Mean ± SEM serum FSH concentrations during the 36 h after the first oFSH injection in cyclic WWF ewes treated with estradiol and progesterone releasing silastic rubber implants (subcutaneous) with (open circles; n = 6) or without (closed circles; n = 5) an oFSH treatment on Day 15 after estradiol implant insertion (Day 19 after ovulation). Implants containing 10% estradiol-17β (10 × 0.34 cm) or progesterone (22 × 0.48 cm) were in the ewes for 20 days (Days 4 to 24 after ovulation) or 18 days (Days 6 to 24 after ovulation), respectively. Data were normalized to the time of the first oFSH injection (Day 0) in all ewes. Asterisks (*) indicate differences between groups ($P < 0.05$).

When mean basal serum FSH concentrations were analyzed during the implant treatment period, there was a treatment effect ($P < 0.001$), time effect ($P < 0.01$), and interaction of treatment by time ($P < 0.001$). Mean basal serum FSH concentrations were significantly higher in ewes treated with implants only (1.26 ± 0.02 ng/ml) compared with ewes treated with implants and oFSH (1.01 ± 0.02 ng/ml). Overall, mean basal serum FSH concentrations increased over time.

Experiment 2

Mean daily serum estradiol concentrations. When daily serum estradiol concentrations were normalized to the day of implant insertion and analyzed for the period of implant treatment, there was a treatment effect ($P < 0.005$), time effect ($P < 0.001$), and interaction of treatment by time ($P < 0.001$; Fig. 5). Mean serum estradiol concentrations were significantly higher in ewes treated with 20-cm implants (34.8 ± 4.6 pg/ml) compared with control ewes (5.2 ± 4.6 pg/ml) and ewes treated with 10-cm implants (19.0 ± 4.6 pg/ml). Mean serum estradiol concentrations were significantly higher in ewes treated with 15-cm implants (27.5 ± 4.6 pg/ml) compared with control ewes. Mean serum estradiol concentrations tended to be higher in ewes treated with 10-cm implants compared with control ewes ($P = 0.056$). For the ewes treated with 15-cm and 20-cm implants, mean serum estradiol concentrations increased from Day 0 to Day 1 and declined from Days 1 to 10 after implant insertion. Comparison of individual means showed that mean serum estradiol concentrations were significantly higher in ewes treated with 20-cm implants compared with the control ewes and the ewes treated with 10-cm implants from 0 to 9 days and 1 to 5 days after implant insertion, respectively (Fig. 5). Mean serum estradiol concentrations were significantly higher in ewes treated with 15-cm implants compared with the ewes treated with 10-cm implants on Day 1 and the control ewes from 1 to 5 days and Day 9 after implant insertion (Fig. 5). Mean serum estradiol concentrations were significantly higher in ewes treated with 10-cm implants compared with the control ewes on Days 0, 1, and 7 after implant insertion (Fig. 5).

Mean daily serum progesterone concentrations. When mean daily serum progesterone concentrations were normal-

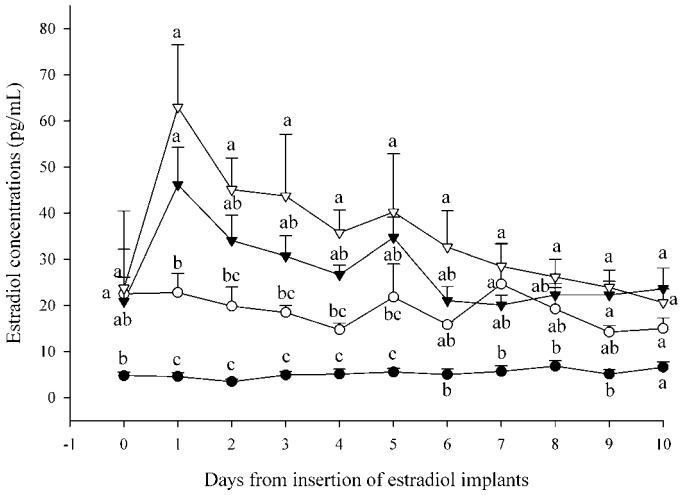

FIG. 5. Mean ± SEM daily serum estradiol concentrations during the implant period in cyclic WWF ewes treated for 10 days (Days 4 to 14 after ovulation) with no implants (control, closed circles; n = 4) or silastic rubber implants (subcutaneously) containing 10% estradiol-17β 10 cm (10 × 0.34 cm; open circles; n = 4), 15 cm (15 × 0.34 cm; closed triangles; n = 4), and 20 cm (20 × 0.34 cm; open triangles; n = 4). Data were normalized to the day of implant insertion (Day 0) in all ewes. Different letters (a–c) within a day denote a significant difference ($P < 0.05$).

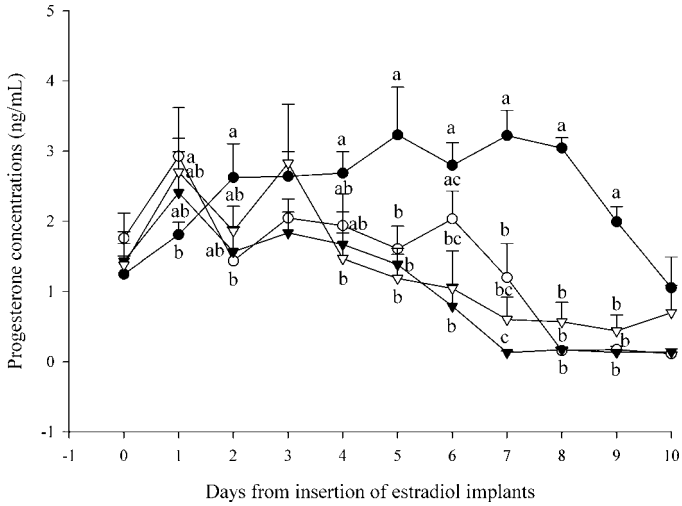

FIG. 6. Mean ± SEM daily serum progesterone concentrations during the implant period in cyclic WWF ewes treated for 10 days (Days 4 to 14 after ovulation) with no implants (control, closed circles; n = 4) or silastic rubber implants (subcutaneously) containing 10% estradiol-17β 10 cm (10 × 0.34 cm; open circles; n = 4), 15 cm (15 × 0.34 cm; closed triangles; n = 4), and 20 cm (20 × 0.34 cm; open triangles; n = 4). Data were normalized to the day of implant insertion (Day 0) in all ewes. Different letters (a–c) within a day denote a significant difference ($P < 0.05$).

ized to the day of implant insertion and analyzed for the period of implant treatment, there was a treatment effect ($P < 0.01$), time effect ($P < 0.001$), and interaction of treatment by time ($P < 0.001$; Fig. 6). Mean serum progesterone concentrations were significantly higher in control ewes (2.39 ± 0.23 ng/ml) compared with ewes treated with 10-cm, 15-cm, or 20-cm implants (1.40 ± 0.23 ng/ml, 1.06 ± 0.23 ng/ml, and 1.34 ± 0.23 ng/ml, respectively). Serum progesterone concentrations were highest at 5, 1, 1, or 3 days after the time of implant insertion and were decreasing by 8, 2, 2, or 4 days in the control ewes and ewes treated with 10-, 15-, and 20-cm implants, respectively. Comparison of individual means showed that mean serum progesterone concentrations were significantly higher in ewes treated with 10-cm implants compared with the control ewes on Day 1 and ewes treated with 15-cm implants on Days 6 and 7 after implant insertion (Fig. 6). Mean serum progesterone concentrations were significantly higher in control ewes compared with ewes treated with 10-cm and 15-cm implants on Days 2, 5, 7, 8, and 9 and Days 5 to 9 after implant insertion, respectively (Fig. 6). Mean serum progesterone concentrations were significantly higher in control ewes compared with ewes treated with 20-cm implants from Days 4 to 9 after implant insertion (Fig. 6).

CLs that were present before implant removal were maintained after implant removal in three ewes treated with 20-cm implants until their next ovulation(s), which occurred 13–14 days after implant removal. The largest size of these CLs, as determined through ultrasonography after implant removal, was 10 mm in diameter. These ewes had an increase in serum progesterone concentrations from implant removal to a peak concentration at 6 or 8 days after implant removal, followed by a decline.

Ovulations. The ovulation rate, before and after implant treatment, did not differ among the treatment and control groups ($P > 0.05$; Table 3). However, the mean duration of the interovulatory interval and the interval from implant removal to ovulation was longer in the ewes treated with 20-cm implants compared with the control ewes and the ewes treated with 15-cm implants ($P < 0.05$; Table 3). The mean duration of the interovulatory interval and the interval from implant removal to ovulation was longer in the ewes treated with 10-cm implants compared with the control ewes ($P < 0.05$; Table 3).

Antral follicle development. One ewe with a 20-cm implant formed two cystic follicles (CFs). One CF formed 6 days after implant removal and developed into a luteinized follicle (LF) 15 days after implant removal. The other CF formed 9 days after implant removal and developed into an LF 12 days after implant removal. Both follicles existed until the end of the experiment, and the maximum diameter of these CFs/LFs was 13 mm.

The first ovulatory or anovulatory follicle wave to emerge after treatment in all ewes treated with implants had an average day of emergence of 12.7 ± 0.4 days from implant insertion. The interval from the end of treatment to emergence of the ovulatory wave did not differ among the ewes treated with implants ($P > 0.05$; Table 3).

Characteristics of serum LH concentrations. When LH pulse amplitude, frequency, and mean serum LH concentrations were analyzed for samples taken every 12 min for 6 h, control ewes had higher concentrations and more pulses compared with ewes treated with implants ($P < 0.001$; Fig. 7). Mean and basal serum LH concentrations were higher during the second intensive sampling period (Day 10 after estradiol implant insertion) compared with the first (Day 6) in control ewes ($P < 0.01$; Fig. 7). During the first intensive sampling day (Day 6), mean serum LH concentrations were higher in control ewes compared with ewes treated with 10-cm and 15-cm implants ($P < 0.05$; Fig. 7). During the second intensive sampling day (Day 10), control ewes had higher mean serum LH concentrations compared with all ewes treated with implants ($P < 0.001$) and higher basal serum LH concentrations compared with ewes treated with 15-cm and 20-cm implants ($P < 0.01$; Fig. 7).

Characteristics of serum FSH. When mean daily serum FSH concentrations were normalized to the day of implant insertion and analyzed for the period of implant treatment, there was a treatment effect ($P < 0.05$), time effect ($P < 0.001$), and interaction of treatment by time ($P < 0.001$). Mean

TABLE 3. Follicle waves and ovulations in cyclic WWF ewes treated for 10 days (Day 4 to 14 after ovulation) with no implants (control) or silastic rubber implants containing 10% estradiol-17β.

End points	Control (n = 4)*	10 cm (n = 4)*	15 cm (n = 4)*	20 cm (n = 4)*
Ovulation rate (before implant treatment)	1.8 ± 0.3	1.5 ± 0.3	1.8 ± 0.3	1.8 ± 0.3
Ovulation rate (after implant treatment)	1.5 ± 0.3	1.3 ± 0.3	1.5 ± 0.3	1.3 ± 0.3
Inter-ovulatory interval (day)	17.3 ± 0.5[a]	26.0 ± 2.9[bc]	21.5 ± 0.5[ab]	27.7 ± 0.3[c]
Interval from implant removal to ovulation (day)	3.3 ± 0.5[a]	12.0 ± 2.9[bc]	7.5 ± 0.5[ab]	13.7 ± 0.3[c]
Interval from time of implant removal from treatment ewes to ovulatory wave emergence (day)	ND[†]	6.3 ± 3.1	2.8 ± 0.6	6.3 ± 1.8

* Data are presented as mean ± SEM.
[†] ND, not determined. Follicle wave emergence occurred during the implant period and ovarian ultrasonography was not done during this period.
[a–c] Different letters within rows denote a significant difference ($P < 0.05$).

serum FSH concentrations were significantly higher in control ewes (1.04 ± 0.11 ng/ml) compared with ewes treated with 10-cm, 15-cm, and 20-cm implants (0.55 ± 0.11 ng/ml, 0.49 ± 0.11 ng/ml, and 0.63 ± 0.11 ng/ml, respectively). For the control ewes, mean serum FSH concentrations decreased from Days 1 to 9 after implant insertion. For the ewes treated with 10-cm or 15-cm implants, mean serum FSH concentrations decreased from Day 0 to Day 1 or Day 2 after implant insertion, respectively. For the ewes treated with 20-cm implants, mean serum FSH concentrations decreased from Days 0 to 5 after implant insertion, increased from Days 5 to 7, and declined from Days 7 to 10. Comparison of individual means showed that mean serum FSH concentrations were significantly higher in control ewes compared with all ewes treated with implants on Days 1 to 5 after implant insertion (Fig. 8). Mean serum FSH concentrations were significantly higher in control ewes compared with ewes treated with 15-cm implants on Days 6 to 8 after implant insertion (Fig. 8). Mean serum FSH concentrations were significantly higher in control ewes compared with ewes treated with 10-cm implants on Day 8 after implant insertion (Fig. 8). On day 0, mean serum FSH concentration in ewes treated with 20-cm implants were significantly higher compared with control ewes and ewes treated with 10-cm implants; on Day 8, mean serum FSH concentrations in ewes treated with 20-cm implants were significantly higher compared with ewes treated with 15-cm implants. (Fig. 8). Mean basal FSH concentrations were higher in control ewes (0.79 ± 0.07 ng/ml) compared with ewes treated with 10-cm (0.40 ± 0.04 ng/ml), 15-cm (0.29 ± 0.04 ng/ml), or 20-cm (0.42 ± 0.06 ng/ml) implants ($P < 0.001$); however, there was no difference among the ewes treated with implants ($P > 0.05$).

During the implant treatment period and as identified by the cycle detection software, the number of FSH peaks and FSH peak duration did not differ among treatments ($P > 0.05$; Table 4). The interpeak interval was longer in ewes treated with 10-cm implants compared with ewes treated with 15-cm ($P < 0.05$) and 20-cm implants ($P < 0.005$; Table 4). The interpeak interval was longer in control ewes compared with ewes treated with 20-cm implants ($P < 0.05$); however, there was no difference between ewes treated with 10-cm and 15-cm implants compared with control ewes ($P > 0.05$; Table 4). Mean FSH peak concentration was lower in ewes treated with 10-cm and 15-cm implants compared with control ewes and ewes treated with 20-cm implants ($P < 0.005$); however, there was no difference between ewes treated with 20-cm implants and control ewes ($P > 0.05$; Table 4). Mean FSH peak amplitude was lower in ewes treated with 10- and 15-cm

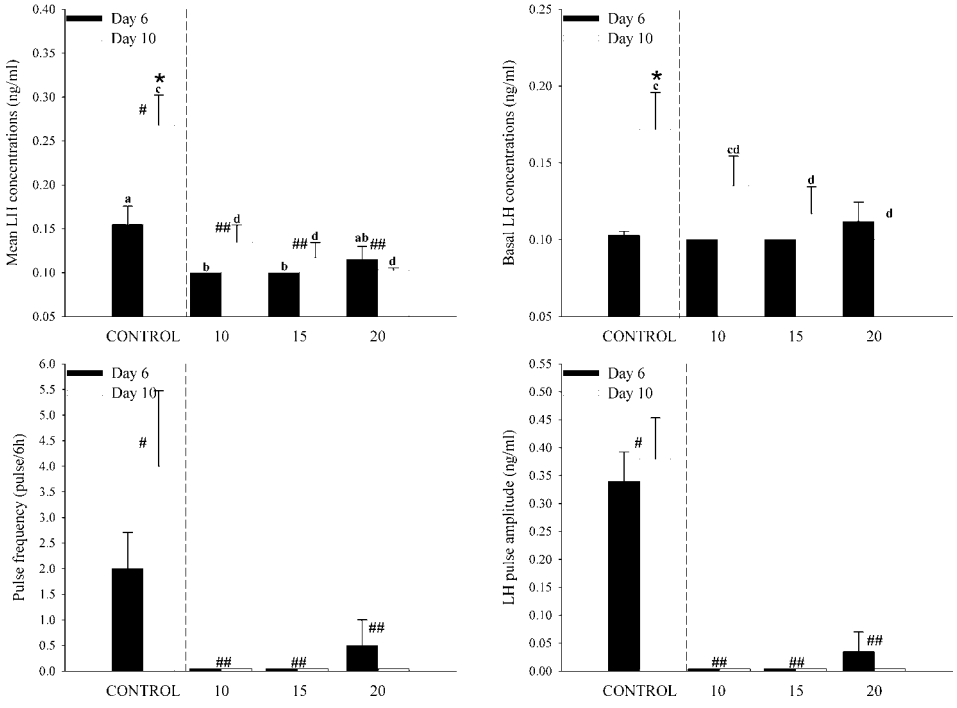

FIG. 7. The characteristics of pulsatile LH secretion (mean and basal serum LH concentrations and LH pulse amplitude and frequency; mean ± SEM) determined from serum samples collected every 12 min for 6 h on Days 6 (black bars; n = 4) and 10 (white bars; n = 4) after estradiol implant insertion (Days 10 and 14 after ovulation) in cyclic WWF ewes treated for 10 days (Days 4 to 14 after ovulation) with no implants (control, n = 4) or silastic rubber implants (subcutaneously) containing 10% estradiol-17β 10 cm (10 × 0.34 cm; n = 4), 15 cm (15 × 0.34 cm; n = 4), and 20 cm (20 × 0.34 cm; n = 4). Pound symbols (#, ##) indicate differences between treatment groups regardless of sampling day ($P < 0.001$). Asterisks (*) indicate differences between sampling days within treatment group ($P < 0.005$). Letters indicate differences between treatment groups within sampling day (a,b: $P < 0.05$; c,d: $P < 0.01$).

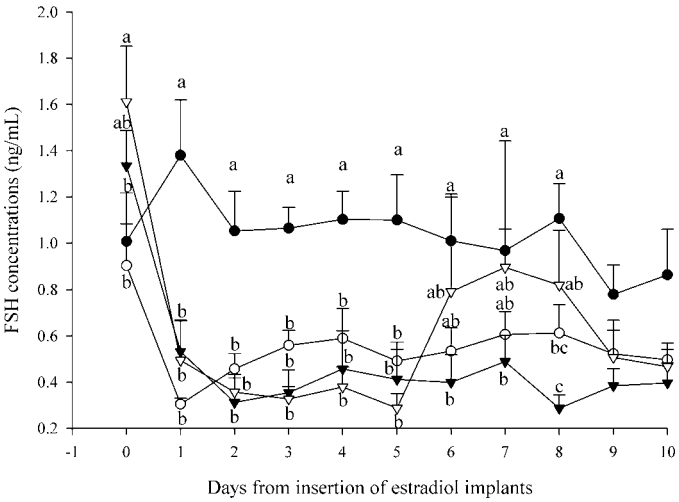

FIG. 8. Mean ± SEM daily serum FSH concentrations during the implant period in cyclic WWF ewes treated for 10 days (Days 4 to 14 after ovulation) with no implants (control, closed circles; n = 4) or silastic rubber implants (subcutaneously) containing 10% estradiol-17β 10 cm (10 × 0.34 cm; open circles; n = 4), 15 cm (15 × 0.34 cm; closed triangles; n = 4), and 20 cm (20 × 0.34 cm; open triangles; n = 4). Data were normalized to the day of implant insertion (Day 0) in all ewes. Different letters (a–c) within a day denote a significant difference ($P < 0.05$).

implants compared with ewes treated with 20-cm implants ($P < 0.005$; Table 4). Mean FSH peak amplitude was lower and tended to be lower in ewes treated with 15-cm ($P < 0.05$) and 10-cm ($P < 0.09$) implants compared with control ewes, respectively (Table 4). In ewes treated with 10-cm and 15-cm implants, there was a 42% and a 36% reduction in mean FSH peak concentration and FSH peak amplitude, respectively, compared with control ewes.

DISCUSSION

The estradiol implants used in experiment 1 produced serum estradiol concentrations that were 2.3-fold higher than in the control ewes of experiment 2. In experiment 2, the animals treated with 10-, 15-, and 20-cm estradiol implants had concentrations of estradiol that were 3.7-, 5.3-, and 6.7-fold higher than the control ewes, respectively. In experiment 1, the animals had mean serum estradiol concentrations that were comparable to those ewes treated with 10-cm estradiol implants in our previous study [36]. The 10-cm estradiol implants used in experiment 2 produced serum estradiol concentrations that were 1.6-fold higher than the 10-cm estradiol implants used in experiment 1. The two studies were done in the same breeding season, but two separate batches of implants were used.

In both of the present studies, FSH peaks were objectively identified by cycle detection software. In experiment 2, the estradiol implants had no effect on the number or the duration of the FSH peaks that preceded follicular waves, but 15- and 20-cm implants reduced the interpeak interval. In experiments 1 and 2, the 10- and 15-cm implants clearly truncated the FSH peaks; both peak concentration and amplitude were reduced in experiment 2, and peak concentration was reduced in experiment 1. Surprisingly, the 20-cm implants in experiment 2 had no effect on the FSH peak concentration or amplitude compared with controls. In our previous study [36], the 10-cm estradiol implants clearly truncated the FSH peaks by reducing peak concentration and amplitude; serum estradiol concentrations were similar to implant-treated ewes in experiment 1 of the present study. In experiment 1, treatment with estradiol implants resulted in increased basal serum FSH concentrations over time during the implant period. However, in experiment 2, with higher serum concentrations of estradiol, the estradiol implants reduced basal and mean serum FSH concentrations. In our previous study in cyclic ewes [36], 10-cm estradiol implants had no significant effect on basal or mean serum FSH concentrations. In experiment 1 of the current study, the 10-cm implants were in place for twice as long as they were in the ewes of our previous study in cyclic ewes [36]. In experiment 2 of the present study, the 10-cm implants produced higher-than-expected serum concentrations of estradiol but were present for a shorter time period than they were in experiment 1. In other studies on the negative feedback regulation of FSH secretion by estradiol, only mean serum FSH concentrations were measured [27, 42, 47]. In these studies, mean serum FSH concentrations were reduced [27, 42] or unaffected by estradiol treatment [47]. Bartlewski et al. [7] showed that Finn ewes have significantly higher mean serum concentrations of estradiol and that the peaks in concentration during follicular waves were greater compared with WWF ewes throughout the estrous cycle. However, the majority of FSH peak characteristics examined in that study did not differ between breeds, with the exception that the FSH peak concentration of the first peak of the cycle was higher in Finn ewes compared with WWF ewes. In the current study, when serum estradiol concentrations were increased in WWF ewes compared with those seen in Finn ewes, FSH peaks were truncated. In experiment 1 of the present study, similar to our previous results [9, 36], treatment with exogenous oFSH created a physiologic FSH peak in every animal. In summary, basal serum FSH concentrations were reduced by very high serum estradiol concentrations (19.0–34.8 pg/ml) or increased over time with long-term (20-day) treatment with moderately high serum estradiol concentrations (11.8 pg/ml). In addition, these various estradiol implant treatments reduced FSH peak concentration, amplitude, and/or interpeak interval.

The estradiol implants used in experiment 2 significantly reduced mean LH concentrations and LH pulse amplitude and frequency. A dose-dependent decline in basal LH concentrations was only observed on Day 10 after implant insertion in

TABLE 4. Endogenous FSH peaks during the implant period in cyclic WWF ewes treated for 10 days (Day 4 to 14 after ovulation) with no implants (control) or with silastic rubber implants containing 10% estradiol-17β.

End points	Control (n = 4)*	10 cm (n = 4)*	15 cm (n = 4)*	20 cm (n = 4)*
Number of peaks per ewe	2.3 ± 0.5	2.0 ± 0.0	1.5 ± 0.5	1.3 ± 0.6
Inter-peak interval (day)	4.0 ± 0.3^{ab}	4.5 ± 0.3^{a}	3.0 ± 1.0^{bc}	2.0 ± 0.0^{c}
Peak duration (day)	4.6 ± 0.5	4.0 ± 0.3	4.0 ± 0.9	3.6 ± 0.9
Mean peak concentration (ng/ml)	1.35 ± 0.11^{a}	0.67 ± 0.07^{b}	0.46 ± 0.10^{b}	1.49 ± 0.34^{a}
Peak amplitude (ng/ml)	0.63 ± 0.12^{ab}	0.27 ± 0.06^{bc}	0.18 ± 0.04^{c}	1.09 ± 0.36^{a}

* Data are presented as mean ± SEM.
$^{a–c}$ Different letters within rows denote a significant difference ($P < 0.05$).

ewes treated with estradiol implants in experiment 2. The lower serum estradiol concentrations created in our previous study (9.9 pg/ml [36]), compared with experiment 2 of the current study (19.0–34.8 pg/ml), had no effect on the endogenous LH secretory patterns. Overall, during the period of implant treatment, the estradiol implants used in experiment 2 abolished LH pulsatility, whereas those used in experiment 1 did not affect the endogenous LH secretory pattern. Therefore, a clear threshold was seen for the suppression of LH secretory pulses by estradiol, whereas a wider range of estradiol concentrations, encompassing the concentrations at which LH pulses were suppressed, truncated the FSH peaks that preceded follicle waves. The various estradiol implant treatments used in the present study differentially regulated LH and FSH secretion—this may allow for targeted manipulation of basal gonadotropin secretion, LH pulses, and/or FSH peaks in future studies.

Cyclic ewes treated daily from Days 3 to 21 after ovulation with injections of estradiol (0.5–2.5 mg/day) had suppressed follicle development [48, 49]. Concentrations of FSH were not measured in these two studies. FSH is required for follicles to develop to an ovulatory size [1, 3–5] and, above physiologic FSH concentrations, both the length of FSH exposure and FSH concentration determine the number of follicles that grow [5]. However, in the studies described here only gross follicle numbers and sizes were recorded, based on postmortem or one-time observations. In experiment 1 of the present study, similarly to the two experiments of our previous study [36], one of the most profound effects of the 10-cm estradiol implant treatment was on follicle development. In these three experiments, the estradiol concentrations created by the 10-cm implants significantly blocked follicular wave emergence. Because the FSH peak concentrations were reduced, follicles present in the 2- to 3-mm follicle pool failed to receive the appropriate FSH signal to stimulate their entry into a follicle wave. In other words, there appears to be a threshold FSH concentration that a FSH peak must reach or exceed to induce the emergence of a follicle wave. However, it is interesting that the 10-cm implants in our previous study [36] had no effect on the number of follicles in the small follicle pool (2–3 mm in diameter), whereas the number of follicles in the small-follicle pool was reduced in experiment 1 of the current study. In the present study, when a physiologic FSH peak was created in ewes with generally suppressed follicle wave emergence, a new follicle wave did not emerge until about 5 days after injection, but there was an increase in the number of 3-mm follicles for about 5 days after injection. In the previous study [36], when a physiologic FSH peak was created in ewes with suppressed follicle wave emergence, a new follicle wave emerged 1.5 days after injection. When a physiologic FSH peak is created during the interwave interval in normal anestrous ewes, a follicle wave emerges within about 24 h after injection [9]. In other words, in experiment 1, injected oFSH did not elicit a follicle wave. In experiment 1 of the present study, perhaps the reduced population of follicles in the small-follicle pool and/or a decreased responsiveness of this pool to oFSH resulted in the failure of the exogenous oFSH to stimulate follicle wave emergence. We did not follow antral follicle development during the implant period of experiment 2. In summary, the estradiol implants of experiment 1 reduced FSH peak concentration to a point where follicle wave emergence was virtually suppressed and the extended period of treatment (20 days), compared with our earlier study (10 days [36]), reduced the small-follicle pool (1- to 3-mm follicles). The injection of a physiologic dose of oFSH failed to stimulate follicle wave emergence. It would appear that over an extended period of time (20 days), periodic peaks in serum concentrations of FSH are required to maintain the pool of small follicles that can respond to a peak in FSH to produce a follicular wave; basal serum concentrations of FSH are not sufficient for maintaining the growth of small antral follicles.

Another interesting observation from experiment 1 that is similar to our previous study [36] is that FSH peaks continued to occur in the absence of follicle wave development in most ewes. These peaks were of reduced concentration, but they nevertheless were identified by the objective cycle detection software. It appears that the FSH peaks may be entrained independently of the growth and regression of previously developing follicular waves in the ewe. If secretory products from follicular waves were important for entraining the FSH peaks, then obviously, based on the present results, estradiol could not be a candidate. The other most obvious candidate would be inhibin [50]. Both inhibin and estradiol do suppress FSH secretion in the ewe, but the effects of inhibin on FSH peaks have not been studied directly. Most inhibin is produced from the larger follicles of a wave (88% by follicles $\geq$4 mm in diameter [26, 51]); however, up to 12% could be produced by follicles $<$4 mm in diameter [26, 51]. Inhibin secretion does increase as the follicles of a wave grow during the breeding season [6, 52], but not during anestrus [53]. The regular follicular waves and antecedent FSH peaks must occur independently of any influence of inhibin in anestrous ewes [53]. With no follicular waves emerging during 18 consecutive days of the implant treatment period in the nine ewes in experiment 1 of the present study, it is difficult to see how changes in secretory products of the follicles of a wave could have entrained the second, third, fourth, and/or fifth reduced FSH peaks observed during the implant period. The present and previous findings do beg the question as to whether some mechanisms other than ovarian follicular feedback govern the timing of the FSH peaks proceeding follicle wave emergence in the ewe. It is interesting that ovariectomized ewes appear to maintain the generation of FSH peaks with a periodicity similar to the intact ewe [30].

In both of the present studies, ovulatory wave emergence appeared to be delayed in ewes treated with implants compared with the control ewes of a previous study [36]. This may be due to the lack of an adequate FSH signal for follicle wave emergence until removal of the implants. Ovulatory wave emergence appeared to occur around the same time in experiment 1 of the present study and in ewes treated with 10-cm estradiol-releasing implants in a previous study [36]. However, in the present study, ovulatory wave emergence appeared to be delayed longer in ewes with higher serum estradiol concentrations (experiment 2) compared with ewes with lower estradiol concentrations (experiment 1). The interval from implant removal to ovulation appeared delayed in experiment 1 compared with the control ewes of experiment 2 of the present study; these intervals are similar to previous observations [36]. In experiment 2 of the present study, the interval from implant removal to ovulation was delayed in treated ewes compared with control ewes—this may have been a reflection of the timing of ovulatory wave emergence. Timing of ovulation delay was longest in ewes treated with 20-cm implants, because the CLs of three of four of these ewes were maintained for a significantly longer period of time than those from other ewes. In both experiments, the ovulation rate did not differ among treatment groups or over time, which is similar to what we observed previously [36]. Cyclic ewes treated from Days 3 to 21 with daily injections of very high estradiol concentrations fail to ovulate [48]. Cyclic ewes treated with estradiol implants have lower ovulation rates [54].

However, Finn ewes have a higher ovulation rate with higher circulating estradiol concentrations compared with WWF ewes [7]. Atkinson et al. [47] found that the ovulation rate of cyclic ewes was unaffected by implants releasing estradiol (0.57 μg/day for 19 days and 1.90 μg/day for 2, 4, and 6 days). In the present studies, the implants delayed ovulatory wave emergence and the timing of ovulation without having a significant affect on ovulation rate.

When serum progesterone concentrations were declining during the implant period in the control ewes of experiment 2, there was an increase in mean and basal serum LH concentrations and LH pulse frequency. In experiment 1, progesterone treatment was designed to maintain progesterone concentrations above baseline to facilitate and maintain estradiol feedback effects on FSH secretion for the duration of estradiol treatment; this was achieved. During the implant period of experiment 2, serum progesterone concentrations were significantly higher and sustained for longer in control ewes compared with ewes treated with implants. This decrease in progesterone production in ewes treated with implants was probably due to the significantly reduced LH support for the CL. LH is required for luteal function and luteal growth [55–58]. Interestingly, when the 20-cm estradiol implants were removed, CLs were rescued from regression and progesterone concentrations increased in three ewes of that treatment group. Perhaps the removal of the very high estradiol concentrations resulted in a significant increase in serum LH concentrations that maintained the CLs. Unfortunately, the LH secretory pattern after implant removal was not determined in experiment 2 of the present study.

In summary, concentrations of estradiol-17β that were 2.3-fold higher than control ewes for 20 days (ewes treated with 10-cm implants in experiment 1) suppressed follicle wave development by truncating FSH peaks in cyclic ewes, and injection of physiologic concentrations of oFSH on Day 15 after estradiol implant insertion failed to induce follicle wave emergence. Concentrations of estradiol that were 2.3-fold higher than control ewes for 20 days (ewes treated with 10-cm implants in experiment 1) reduced the small follicle pool (2–3 mm in diameter). Maintenance of the small-follicle pool over a 20-day period would appear to require regular secretion of FSH peaks. Perhaps the reduced population of follicles in the small-follicle pool and/or a decreased responsiveness of this pool to oFSH resulted in the failure of the exogenous oFSH to stimulate follicle wave emergence. The data presented also bring into question the involvement of secretory products from the follicles of a wave in entraining the FSH peak that initiates the subsequent follicular wave. In experiment 1, basal serum FSH concentrations increased over time in ewes with serum estradiol concentrations that were 2.3-fold higher than control ewes for 20 days (ewes treated with 10-cm implants); pulsed LH secretion was not affected. In experiment 2, basal serum FSH concentrations were reduced, LH pulsatility was abolished, and progesterone production was altered in ewes with serum estradiol concentrations that were 3.7-, 5.3-, and 6.7-fold higher (ewes treated with 10-cm, 15-cm, and 20-cm estradiol implants, respectively) than control ewes. In addition, FSH peak concentration and amplitude were decreased in ewes with serum estradiol concentrations that were 3.7- and 5.3-fold higher (ewes treated with 10-cm and 15-cm estradiol implants, respectively) than control ewes. Ewes with serum estradiol concentrations that were 6.7-fold higher (ewes treated with 20-cm estradiol implants) than control ewes had no change in FSH peak concentration or amplitude compared with control ewes. The interpeak interval of FSH peaks was shortened in ewes with serum estradiol concentrations that were 5.3- and 6.7-fold higher (ewes treated with 15-cm and 20-cm estradiol implants, respectively) than control ewes. The various estradiol implant treatments used in this study differentially regulated LH and FSH secretion, which could allow for the development of useful gonadotropin suppression and replacement models for the further study of the endocrine regulation of ovarian follicular waves in the ewe.

ACKNOWLEDGMENTS

The authors thank Dr. A. F. Parlow of the National Institute of Diabetes & Digestive & Kidney Diseases/National Hormone and Pituitary Program for the provision of reagents for the gonadotropin assays, Ms. Susan J. Cook for her help in blood sampling and radioimmunoassay, and Mr. Tim Hegan for his help in blood sampling and implant surgeries.

REFERENCES

1. Baird DT, Land RB, Scaramuzzi RJ, Wheeler AG. Endocrine changes associated with luteal regression in the ewe; the secretion of ovarian oestradiol, progesterone and androstenedione and uterine prostaglandin F2alpha throughout the oestrous cycle. J Endocrinol 1976; 69:275–286.
2. McNeilly AS, Picton HM, Campbell BK, Baird DT. Gonadotrophic control of follicle growth in the ewe. J. Reprod Fertil Suppl 1991; 43:177–186.
3. Scaramuzzi RJ, Adams NR, Baird DT, Campbell BK, Downing JA, Findlay JK, Henderson KM, Martin GB, McNatty KP, McNeilly AS. A model for follicle selection and the determination of ovulation rate in the ewe. Reprod Fertil Dev 1993; 5:459–478.
4. Campbell BK, Scaramuzzi RJ, Webb R. Control of antral follicle development and selection in sheep and cattle. J Reprod Fertil Suppl 1995; 49:335–350.
5. Picton HM, Tsonis CG, McNeilly AS. FSH causes a time-dependent stimulation of preovulatory follicle growth in the absence of pulsatile LH secretion in ewes chronically treated with gonadotropin-releasing hormone agonist. J Endocrinol 1990; 126:297–308.
6. Souza CJ, Campbell BK, Baird DT. Follicular waves and concentrations of steroids and inhibin A in ovarian venous blood during the luteal phase of the oestrous cycle in ewes with an ovarian autotransplant. J Endocrinol 1998; 156:563–572.
7. Bartlewski PM, Beard AP, Cook SJ, Chandolia RK, Honaramooz A, Rawlings NC. Ovarian antral follicular dynamics and their relationships with endocrine variables throughout the oestrous cycle in breeds of sheep differing in prolificacy. J Reprod Fertil 1999; 115:111–114.
8. Evans AC, Flynn JD, Duffy P, Knight PG, Boland MP. Effects of ovarian follicle ablation on FSH, oestradiol and inhibin A concentrations and growth of other follicles in sheep. Reproduction 2002; 123:59–66.
9. Duggavathi R, Bartlewski PM, Barrett DM, Gratton C, Bagu ET, Rawlings NC. Patterns of antral follicular wave dynamics and accompanying endocrine changes in cyclic and seasonally anestrous ewes treated with exogenous ovine follicle-stimulating hormone during the inter-wave interval. Biol Reprod 2004; 70:821–827.
10. Bartlewski PM, Beard AP, Rawlings NC. An ultrasound-aided study of temporal relationships between the patterns of LH/FSH secretion, development of ovulatory-sized antral follicles and formation of corpora lutea in ewes. Theriogenology 2000; 54:229–245.
11. Hauger RL, Karsch FJ, Foster DL. A new concept for control of the estrous cycle of the ewe based on the temporal relationships between luteinizing hormone, estradiol and progesterone in peripheral serum and evidence that progesterone inhibits tonic LH secretion. Endocrinology 1977; 101:807–817.
12. Baird DT. Pulsatile secretion of LH and ovarian estradiol during the follicular phase of the sheep estrous cycle. Biol Reprod 1978; 18:359–364.
13. Jackson GL, Davis SL. Comparison of luteinizing hormone and prolactin levels in cycling and anestrous ewes. Neuroendocrinology 1979; 28:256–263.
14. Karsch FJ, Foster DL, Legan SJ, Ryan KD, Peter GK. Control of the preovulatory endocrine events in the ewe: interrelationship of estradiol, progesterone, and luteinizing hormone. Endocrinology 1979; 105:421–426.
15. Wheaton JE. Regional brain content of luteinizing hormone-releasing hormone in sheep during the estrus cycle, seasonal anestrus, and after ovariectomy. Endocrinology 1979; 104:839–844.
16. Wheaton JE, Mullett MA, Cornelius SG. Plasma follicle stimulating hormone and luteinizing hormone patterns during the estrous cycle of ewes. Theriogenology 1984; 21:989–999.

17. Rawlings NC, Cook SJ. LH-secretion around the time of the preovulatory gonadotropin surge in the ewe. Anim Reprod Sci 1993; 30:289–299.
18. Duggavathi R, Bartlewski PM, Barrett DMW, Rawlings NC. The temporal relationship between patterns of LH and FSH secretion, and development of ovulatory-sized follicles during the mid- to late-luteal phase of sheep. Theriogenology 2005; 64:393–407.
19. Ginther OJ, Bergfelt DR, Kulick LJ, Kot K. Pulsatility of systemic FSH and LH concentrations during follicular-wave development in cattle. Theriogenology 1998; 50:507–519.
20. Kulick LJ, Kot K, Wiltbank MC, Ginther OJ. Follicular and hormonal dynamics during the first follicular wave in heifers. Theriogenology 1999; 52:913–921.
21. Ginther OJ, Bergfelt DR, Beg MA, Kot K. Follicle selection in cattle: role of luteinizing hormone. Biol Reprod 2001; 64:197–205.
22. Ginther OJ, Wiltbank MC, Fricke PM, Gibbons JR, Kot K. Selection of the dominant follicle in cattle. Biol Reprod 1996; 55:1187–1194.
23. Souza CJ, Campbell BK, Baird DT. Follicular dynamics and ovarian steroid secretion in sheep during the follicular and early luteal phases of the estrous cycle. Biol Reprod 1997; 56:483–488.
24. Mann GE, McNeilly AS, Baird DT. Source of ovarian inhibin secretion during the oestrous cycle of the sheep. J Endocrinol 1989; 123:181–188.
25. Campbell BK, Picton HM, McNeilly AS, Baird DT. Effect of FSH on ovarian inhibin secretion in anoestrous ewes. J Reprod Fertil 1991; 91: 501–509.
26. Mann GE, McNeilly AS, Baird DT. Hormone production in vivo and in vitro from follicles at different stages of the oestrous cycle in the sheep. J Endocrinol 1992; 132:225–234.
27. Mann GE, Campbell BK, McNeilly AS, Baird DT. The role of inhibin and oestradiol in the control of FSH secretion in the sheep. J Endocrinol 1992; 133:381–391.
28. Webb R, Campbell BK, Garverick HA, Gong JG, Gutierrez CG, Armstrong DG. Molecular mechanisms regulating follicular recruitment and selection. J Reprod Fertil Suppl 1999; 54:33–48.
29. Duggavathi R, Bartlewski PM, Barrett DM, Rawlings NC. Use of high-resolution transrectal ultrasonography to assess changes in numbers of small ovarian antral follicles and their relationships to the emergence of follicular waves in cyclic ewes. Theriogenology 2003; 60:495–510.
30. Duggavathi R, Bartlewski PM, Agg E, Flint S, Barrett DM, Rawlings NC. The effect of the manipulation of follicle-stimulating hormone (FSH)-peak characteristics on follicular wave dynamics in sheep: does an ovarian-independent endogenous rhythm in FSH secretion exist? Biol Reprod 2005; 72:1466–1474.
31. Robertson JF, Blamey RW. The use of gonadotrophin-releasing hormone (GnRH) agonists in early and advanced breast cancer in pre- and perimenopausal women. Eur J Cancer 2003; 39:861–869.
32. Coccia ME, Comparetto C, Bracco GL, Scarselli G. GnRH antagonists. Eur J Obstet Gynecol Reprod Biol 2004; 115(suppl 1):S44–S56.
33. Webb R, Baxter G, Preece RD, Land RB, Springbett AJ. Control of gonadotrophin release in Scottish Blackface and Finnish Landrace ewes during seasonal anoestrus. J Reprod Fertil 1985; 73:369–378.
34. Brooks AN, Haynes NB, Yang K, Lamming GE. Ovarian steroid involvement in endogenous opioid modulation of LH secretion in seasonally anoestrous mature ewes. J Reprod Fertil 1986; 76:709–715.
35. Kasa-Vubu JZ, Dahl GE, Evans NP, Thrun LA, Moenter SM, Padmanabhan V, Karsch FJ. Progesterone blocks the estradiol-induced gonadotropin discharge in the ewe by inhibiting the surge of gonadotropin-releasing hormone. Endocrinology 1992; 131:208–212.
36. Barrett DMW, Bartlewski PM, Duggavathi R, Davies KL, Rawlings NC. Suppression of follicle wave emergence in cyclic ewes by supraphysiological concentrations of estradiol-17β and induction with a physiological dose of exogenous ovine follicle-stimulating hormone. Biol Reprod 2006; 75:633–641.
37. Schrick FN, Surface RA, Pritchard JY, Dailey RA, Townsend EC, Inskeep EK. Ovarian structures during the estrous cycle and early pregnancy in ewes. Biol Reprod 1993; 49:1133–1140.
38. Ravindra JP, Rawlings NC, Evans AC, Adams GP. Ultrasonographic study of ovarian follicular dynamics in ewes during the oestrous cycle. J Reprod Fertil 1994; 101:501–509.
39. Duggavathi R, Bartlewski PM, Pierson RA, Rawlings NC. Luteogenesis in cyclic ewes: echotextural, histological, and functional correlates. Biol Reprod 2003; 69:634–639.
40. Rawlings NC, Jeffcoate IA, Rieger DL. The influence of estradiol-17beta and progesterone on peripheral serum concentrations of luteinizing hormone and follicle stimulating hormone in the ovariectomized ewe. Theriogenology 1984; 22:473–488.
41. Bartlewski PM, Beard AP, Cook SJ, Rawlings NC. Ovarian follicular dynamics during anoestrus in ewes. J Reprod Fertil 1998; 113:275–285.
42. Joseph IB, Currie WD, Rawlings NC. Effects of time after ovariectomy, season and oestradiol on luteinizing hormone and follicle-stimulating hormone secretion in ovariectomized ewes. J Reprod Fertil 1992; 94:511–523.
43. Currie WD, Rawlings NC. Fluctuation in responsiveness of LH and lack of responsiveness of FSH to prolonged infusion of morphine and naloxone in the ewe. J Reprod Fertil 1989; 86:359–366.
44. Rawlings NC, Jeffcoate IA, Currie WD, Cook SJ. Control of the surge release of LH and FSH in oestradiol- and progesterone-treated ovariectomized ewes. Can J Anim Sci 1988; 68:1089–1096.
45. Gitzen JF, Ramirez VD. PC-Pulsar—Pulsar for the IBM-PC: Operating Instructions Distributed with the PC-Pulsar program. University of Illinois, IL; 1988.
46. Clifton DK, Steiner RA. Cycle detection: a technique for estimating the frequency and amplitude of episodic fluctuations in blood hormone and substrate concentrations. Endocrinology 1983; 112:1057–1064.
47. Atkinson S, Adams NR, Martin GB. LH, FSH and ovulation rate in ewes treated with exogenous oestradiol. J Reprod Fertil 1989; 86:383–390.
48. Piper EL, Foote WC. Ovulation and corpus luteum maintenance in ewes treated with 17-beta-oestradiol. J Reprod Fertil 1968; 16:253–259.
49. Piper EL, Foote WC. The effect of 17-beta-estradiol on corpus luteum function in sheep. Biol Reprod 1970; 2:48–52.
50. Martin GB, Price CA, Thiery JC, Webb R. Interactions between inhibin, oestradiol, and progesterone in the control of gonadotrophin secretion in the ewe. J Reprod Fertil 1988; 82:319–328.
51. Campbell BK, McNeilly AS, Mann GE, Baird DT. The effect of stage of estrous cycle and follicular maturation on ovarian inhibin production in sheep. Biol Reprod 1991; 44:483–490.
52. Souza CJ, Campbell BK, Webb R, Baird DT. Secretion of inhibin A and follicular dynamics throughout the estrous cycle in the sheep with and without the Booroola gene (Fec^{B}). Endocrinology 1997; 138:5333–5340.
53. Evans ACO, Duffy P, Quinn KM, Knight PG, Boland MP. Follicular waves are associated with transient fluctuations in FSH but not oestradiol or inhibin-A concentrations in anoestrous ewes. Anim Sci 2001; 72:547–554.
54. Adams NR, Atkinson S, Sanders MR. Effects of oestradiol implants on the ovulation rate of the ewe. J Reprod Fertil 1989; 87:169–174.
55. Niswender GD, Farin CE, Gamboni F, Sawyer HR, Nett TM. Role of luteinizing hormone in regulating luteal function in ruminants. J Anim Sci Suppl 1986; 62:1–13.
56. Wiltbank MC, Niswender GD. Functional aspects of differentiation and degeneration of the steroidogenic cells of the corpus luteum in domestic ruminants. Anim Reprod Sci 1992; 28:103–110.
57. Wiltbank MC. Cell types and hormonal mechanisms associated with mid-cycle corpus luteum function. J Anim Sci 1994; 72:1873–1883.
58. Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW. Mechanisms controlling the function and life span of the corpus luteum. Physiol Rev 2000; 80:1–29.