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# Seasonal Changes in Gonadotropin-Releasing Hormone Secretion in the Ewe: Alteration in Response to the Negative Feedback Action of Estradiol[1]

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## ABSTRACT

Two experiments were performed to test the hypothesis that there is a seasonal change in the negative feedback effect of estradiol on episodic secretion of GnRH in the ewe. The first experiment identified a specific estradiol treatment (delivered by s.c. Silastic implant) that produced a 50% decrease in the frequency of pulsatile secretion of LH in ovariectomized ewes during the anestrous season. In the second experiment, this estradiol treatment was administered to ovariectomized ewes during the mid-breeding and anestrous seasons. Separate groups of ovariectomized ewes not treated with estradiol were included during each season to test for a seasonal difference in the effect of estradiol on episodic GnRH and LH secretion. Samples of hypophyseal portal blood (for GnRH) and jugular blood (for LH) were obtained at 5-min intervals approximately one month after placement of the estradiol implants. During the breeding season, no effect of estradiol was observed on either the frequency or size of GnRH and LH pulses. During anestrus, however, estradiol produced a profound suppression of the frequency of GnRH and LH pulses, and an increase in GnRH pulse size. No significant seasonal change was observed in the characteristics of GnRH and LH pulses in ovariectomized ewes in the absence of estradiol treatment. These findings lead to the conclusion that there is a marked seasonal change in the negative feedback effect of estradiol on episodic GnRH secretion in the ewe, with the steroid being maximally effective during anestrus.

## INTRODUCTION

Seasonal reproduction is the consequence of profound changes in reproductive neuroendocrine activity, and, in a number of species, these changes are associated with a marked shift in responsiveness to the negative feedback action of gonadal steroids on gonadotropin secretion [1–4]. In female sheep, for example, there is strong evidence that the seasonal onset and cessation of the estrous cycle are the consequence of alterations in the negative feedback effect of estradiol [5–7]. During anestrus, a physiological concentration of circulating estradiol can evoke a powerful feedback inhibition of LH and FSH secretion, whereas in the breeding season, the same amount of estradiol is far less effective in this regard. This change in response to estradiol negative feedback is expressed through the neuroendocrine mechanisms that generate the pulsatile secretion of gonadotropic hormones. Specifically, during the anestrous season, physiological circulating concentrations of estradiol produce a marked suppression of the frequency of LH pulses, but in the breeding season, comparable amounts of estradiol are not inhibitory and, if anything, stimulate LH pulse frequency [7, 8].

It has generally been assumed that the seasonal change in the negative feedback effect of estradiol on pulsatile LH secretion reflects a corresponding change in the action of estradiol on episodic GnRH release [4]. This assumption, however, has not been tested directly, and the possibility remains that there is a crucial change in some other aspect of reproductive neuroendocrine function, such as GnRH pulse amplitude or pituitary responsiveness to GnRH. Previous studies provide evidence that a seasonal change in episodic GnRH secretion exists in the ovary-intact ewe [9, 10], but this almost certainly is influenced by marked seasonal differences in secretion of gonadal steroids. In the present study, we tested the hypothesis that there is a seasonal change in the inhibitory effect of estradiol on episodic GnRH secretion in the ewe.

## MATERIALS AND METHODS

### *Animals*

Two experiments were performed on sexually mature Suffolk ewes maintained under natural environmental conditions at the Sheep Research Facility in Ann Arbor, MI (42°18′N). The breeding season in our flock of Suffolk ewes has been determined to occur from mid-September to mid-February [11, 12]. Animals were maintained on pasture supplemented with hay and had free access to mineral licks and water. The ewes were ovariectomized and treated, as described below, with s.c. Silastic implants containing estradiol (implants soaked in water for 12–24 h prior to insertion to prevent an initial peak of steroid release). In experiment 1, peripheral blood (3 ml) was sampled by jugular

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venipuncture. In experiment 2, pituitary portal and jugular blood were collected from conscious undisturbed sheep by use of a remote automated sampling system, modified [13] from the technique of Caraty and Locatelli [14]. The apparatus for portal blood collection was surgically installed 1 wk before the collection. Surgical procedures were performed aseptically under general anesthesia. All procedures were approved by the Committee for the Use and Care of Animals at the University of Michigan.

*Experiment 1*

Since anestrous ewes are extremely sensitive to the negative feedback effect of estradiol, we first sought to identify a low-dose estradiol treatment that would produce an unambiguous reduction of LH pulse frequency during the anestrous season without eliminating pulsatile LH secretion altogether. This dose was then used in experiment 2 to examine seasonal differences in the effect of estradiol on episodic GnRH release. Ewes were ovariectomized in anestrus (July 1990) and treated with one of three sizes of estradiol implants: 1 mm, 3 mm, or 10 mm (5 ewes/group). Controls (n = 5) were ovariectomized but not treated with steroid. The estradiol implants were capsules constructed, as described previously [15], of Silastic tubing having inside and outside diameters of 3.35 and 4.65 mm, respectively. Accurate setting of implant length was facilitated by use of a custom-made stainless steel sleeve into which the Silastic capsule was inserted and fixed with Silastic adhesive, such that the estradiol column within the implant extended the desired length beyond the end of the sleeve (Fig. 1). On Day 15 after ovariectomy and placement of estradiol implants, peripheral blood for measurement of LH was sampled at 24-min intervals for 24 h (nighttime samples facilitated by use of a dim red light that produced < 5 lux lateral to the eyes). This sampling interval was previously determined to be sufficient to monitor LH pulse frequency in ovariectomized ewes during the anestrous season [8, 16].

Circulating estradiol was not determined because the concentrations produced by the 1- and 3-mm implants were expected to be well below the limit of detection of our estradiol assay. In this regard, we previously determined that the 10-mm estradiol implant produced a serum estradiol concentration of 1.0 pg/ml (assay sensitivity ~0.5 pg/ml) and virtually eliminated LH pulses in ovariectomized ewes during anestrus [8, 17, 18]. (Serum estradiol concentration in intact anestrous ewes averages ~1 pg/ml [19].) Given the linear relationship between implant length and circulating estradiol concentration [17], the 1- and 3-mm implants are estimated to have produced an increment of serum estradiol of approximately 0.1 and 0.3 pg/ml, respectively.

*Experiment 2*

The objective of this experiment was to determine whether there is a seasonal difference in the effects of estradiol on pulsatile secretion of GnRH. The study was performed during the mid-breeding season (November–December 1990) and the subsequent anestrous season (May–July 1991) on separate groups of ewes that had been ovariectomized 5–6 mo prior to the experiment. Approximately one month before collection of pituitary portal blood, half of the ewes in each season were treated with a 3-mm estradiol implant (OVX+E), the treatment found to produce a 50% reduction in LH pulse frequency in experiment 1. The remaining ewes served as nonsteroid-treated controls (OVX). Thus, there were four groups: breeding season OVX (n = 6); breeding season OVX+E (n = 6); anestrous OVX (n = 8); anestrous OVX+E (n = 8). During anestrus, observations were made at two times on the basis of availability of animals: during May (3 ewes each treatment) and during July (5 ewes each treatment). On the day of sampling, pituitary portal and jugular blood were withdrawn continuously and collected as 5-min fractions for the measurement of GnRH and LH, respectively. Samples were collected for 6 h during the breeding season and 12 h during anestrus; the longer period in anestrus was based on the low pulse frequency observed at that time of year in experiment 1. As described elsewhere [13], a remote collection system was used, and samples were dispensed with the aid of a fraction collector into tubes chilled in an ice bath (tubes contained 0.5 ml of $3 \times 10^{-3}$ M bacitracin for portal blood). Plasma was separated and snap-frozen within 1–1.5 h of collection. After the sampling, the animals were killed by a barbiturate overdose, and the pituitary glands were inspected to determine the location and extent of the portal vasculature that was cut to obtain blood.

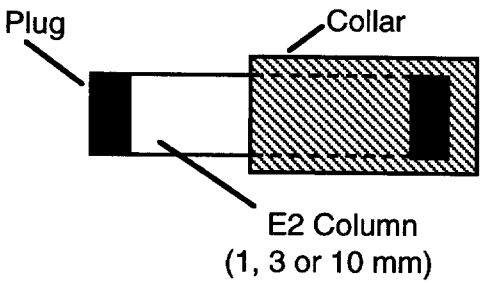


FIG. 1. Diagram illustrating Silastic estradiol (E2) capsule inserted into stainless steel collar, positioned and fixed in place with adhesive to expose either 1, 3 or 10 mm of the estradiol column in the capsule.

*Assays*

GnRH was measured in portal plasma by RIA [13, 20] of methanol extracts of 750-μl aliquots of the portal sample, which contained ~600 μl portal plasma and 150 μl bacitracin. Extracts equivalent to ~240 μl portal plasma per tube were assayed in duplicate, with all samples for each ewe measured in a single assay to minimize the effects of interassay variability. Intraassay variation, as determined by

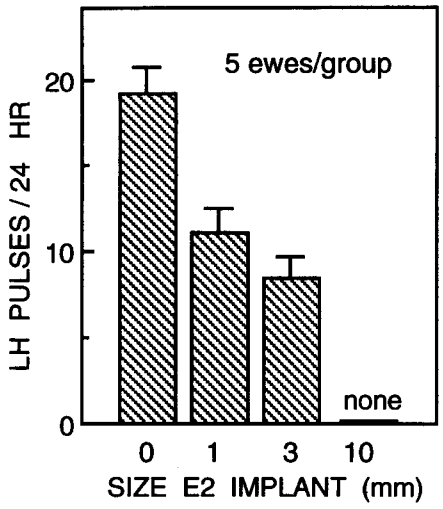


FIG. 2. Mean + SEM number of pulses per 24 h in ovariectomized ewes treated with an estradiol implant of either 1, 3, or 10 mm in experiment 1. Controls were untreated (0 mm).

the median variance ratio of assay replicates [21], averaged 0.036, and assay sensitivity averaged 0.12 pg/tube. LH was quantified by an RIA [22–24] in duplicate 25–200-μl aliquots of jugular serum (experiment 1) or plasma (experiment 2) and is expressed in terms of NIH-LH-S12. Mean inter- and intraassay coefficients of variation averaged 13.5% and 7.6% for pools of serum containing 2.2 and 26.2 ng/ml, and assay sensitivity averaged 0.13 ng/tube.

*Data Analysis*

Values for GnRH are presented as collection rate (pg/min), rather than concentration, to minimize potential error arising from the portal blood collection method; for example, contamination of portal blood with blood from another source within the surgical site [25]. GnRH and LH pulses were identified by the Cluster pulse-detection method of Veldhuis and Johnson [26]. Respective sizes of nadir and peak clusters were 2/1 points for GnRH and 2/2 points for LH. The *t*-statistics for significant increases and decreases were 3.8/3.8 for GnRH and 2.6/2.6 for LH. The size of GnRH pulses was taken as the total amount of GnRH in samples included within a pulse. We analyzed the size of GnRH pulses as a measure of integrated hormone release rather than amplitude (peak minus preceding nadir) because portal blood was continuously sampled and GnRH released during a pulse often spanned more than one 5-min sampling period. An average pulse size was determined in each ewe for the purpose of data analysis. Pulse frequency was defined as the number of pulses per collection period (6 h in the breeding season and 12 h in anestrus). To facilitate seasonal comparisons of frequency, data for the breeding season were converted to number of pulses per 12 h, equivalent to the longer sampling interval during anestrus. Effects of treatment and season on pulse frequency and pulse size were determined by analysis of variance with use of the least significant difference to test for treatment effects.

## RESULTS

*Experiment 1*

As illustrated in Figure 2, estradiol produced a dose-related decrease in the number of LH pulses in the 24-h observation period. Unambiguous LH pulses occurred in each OVX control ewe not treated with estradiol and in each OVX+E ewe receiving 1- or 3-mm estradiol implants (individual patterns not illustrated). In contrast, no pulses were observed in OVX+E ewes treated with 10-mm estradiol implants. Because the 3-mm estradiol implant produced a 50% decrease in frequency, this size was selected for use in experiment 2.

*Experiment 2*

GnRH pulses coincided with LH pulses in all but a very few instances in which sampling began or ended around

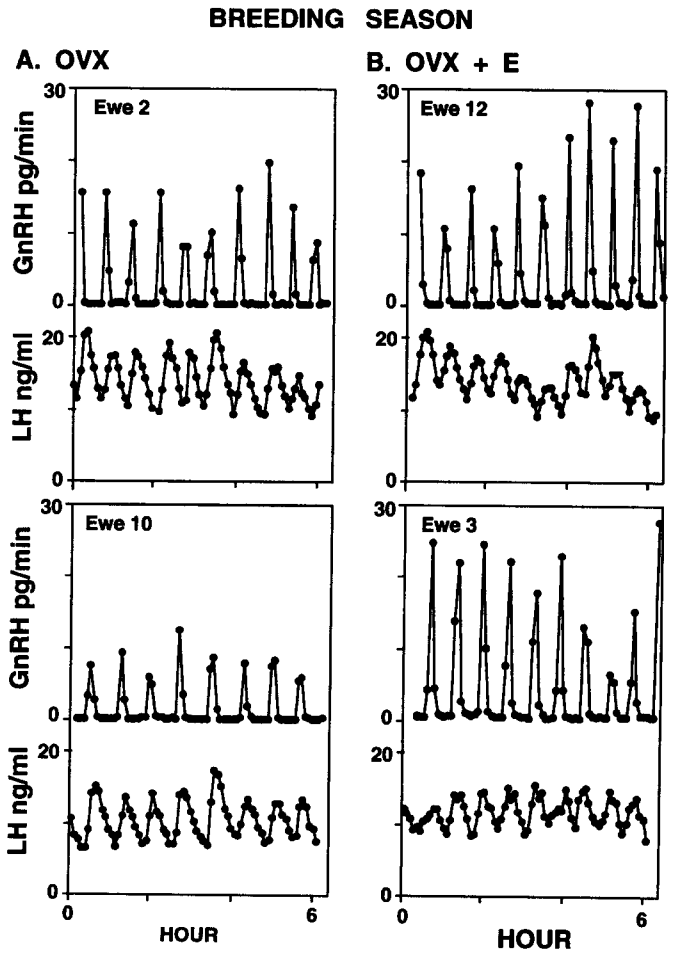


FIG. 3. Representative patterns of GnRH (as pg/min in portal plasma) and LH (as ng/ml jugular plasma) during the breeding season in OVX (panel A) and OVX+E (panel B) ewes in experiment 2. Samples were obtained at 5-min intervals for 6 h.

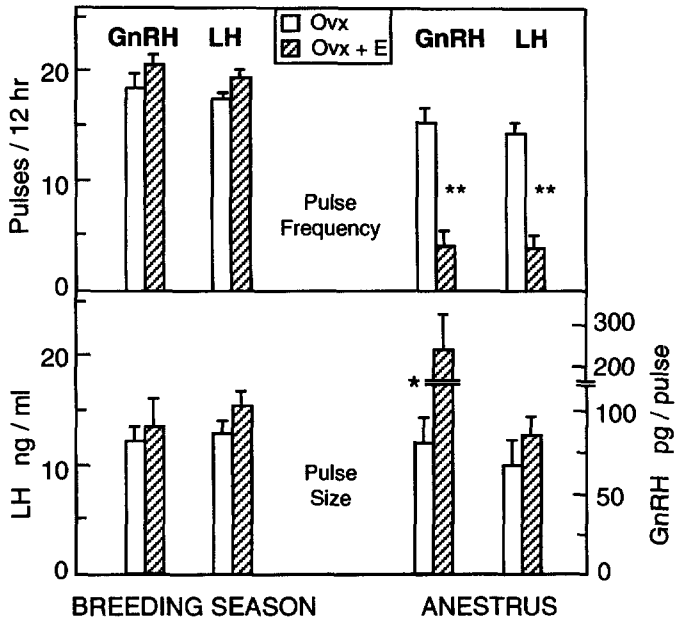


FIG. 4. Mean + SEM frequency (top) and size (bottom) of GnRH and LH pulses in ovariectomized (Ovx, open bars) and estradiol-treated ovariectomized ewes (Ovx+E, cross hatched bars) during either the breeding season (left, n = 6 ewes/group) or anestrus (right, n = 8 ewes/group) in experiment 2. Note break in ordinate for GnRH pulse size to accommodate large mean pulse size during anestrus. $^{**}p < 0.001$ for comparison of frequency of GnRH and LH pulses in Ovx vs. Ovx+E ewes in anestrus. $^{*}p < 0.03$ for comparison of GnRH pulse size in Ovx vs. Ovx+E ewes in anestrus. To facilitate seasonal comparisons, frequencies are expressed as pulses/12 h for both breeding and anestrous seasons.

the time of a pulse, such that an obvious secretory episode of one hormone failed to meet the criteria for pulse identification (e.g., Fig. 3, start of sampling in ewes 2 and 12). During the breeding season, unambiguous and coincident pulses of GnRH and LH were observed in each OVX and OVX+E ewe, and there was no effect of estradiol on either frequency or size of GnRH or LH pulses (representative patterns in Fig. 3, group means in Fig. 4). In marked contrast, estradiol significantly altered secretory patterns during anestrus, decreasing the frequency of both GnRH and LH pulses ($p < 0.001$; Figs. 4 and 5). Estradiol also enhanced the size of GnRH pulses in anestrus ($p < 0.03$), an effect most evident in ewes having the lowest frequencies (e.g., Fig. 5B, ewe 2 vs. ewe 16). Although GnRH pulse size was significantly increased, the size of LH pulses was not (Fig. 4).

No significant seasonal difference was observed in either the frequency or size of GnRH and LH pulses in OVX ewes not treated with estradiol (Fig. 4). There was a tendency ($p < 0.08$) for LH pulses to be more frequent in the breeding season than during anestrus (mean ± SEM; 17.6 ± 0.6 vs. 14.5 ± 0.9 pulses/12 h, respectively). This trend was also apparent for GnRH, but it was less evident ($p > 0.10$) than that for LH because of greater variability (18.6 ± 1.3 vs. 15.4 ± 1.4 pulses/12 h, respectively). The time of sampling during anestrus (i.e., May vs. July) did not influence the patterns of GnRH and LH secretion in either OVX or OVX+E ewes.

## DISCUSSION

The foregoing observations demonstrate that there is a marked seasonal change in the negative feedback effect of estradiol on the episodic secretion of GnRH in the ovariectomized ewe. During the breeding season, the low dose of estradiol used in our study had no discernible inhibitory effect on pulsatile GnRH release, whereas in anestrus, the same treatment produced a profound reduction of GnRH pulse frequency. In light of earlier evidence that a seasonal difference does not exist in circulating estradiol concentrations in ovariectomized ewes treated with constant-release estradiol implants [5, 17], our results lead to the conclusion that there is an increased responsiveness of the GnRH neurosecretory system to the negative feedback action of estradiol during anestrus. This extends the previous conclusion that there is a seasonal change in the negative feedback effect of estradiol on pulsatile LH secretion [7, 8] and supports preliminary evidence [13] that this seasonal change applies to GnRH secretion as well. In addition, our study provides direct evidence that the well-documented seasonal shift in the negative feedback action of estradiol on LH secretion during anestrus is effected via a reduction in frequency of output of the GnRH neurosecretory system, rather than by a decrease in the size of GnRH pulses or a major diminution of pituitary responsiveness to GnRH.

A marked change in frequency was not the only characteristic of episodic GnRH secretion that varied with season in the OVX+E ewe. Of interest, the size of GnRH pulses also changed, but in this case, values increased during anestrus. It is important to stress that a certain degree of caution must be exercised when differences among animals in the size of GnRH pulses are interpreted, because the absolute amount of GnRH quantified by our method can vary with the location and extent of the portal vasculature cut to sample blood [13], which is difficult to standardize among sheep. Nevertheless, it is noteworthy that no group differences were evident in the location and size of the cuts. Further, we previously observed a similar seasonal difference in the size of GnRH pulses in ovary-intact ewes [10], with amplitude also being greater in anestrus than during the breeding season (either the luteal or follicular phase of the estrous cycle). Because this seasonal difference occurs in OVX+E ewes, in which circulating estradiol does not vary seasonally [5, 17], the change in pulse size cannot be attributed to alterations in gonadal steroid milieu and more likely reflects seasonal differences in neuroendocrine function. For example, the increased size of GnRH pulses in anestrus may be secondary to the reduced pulse frequency, allowing more releasable GnRH to accumulate within GnRH neurons or greater quantities of stimulatory neurotransmitters to accumulate

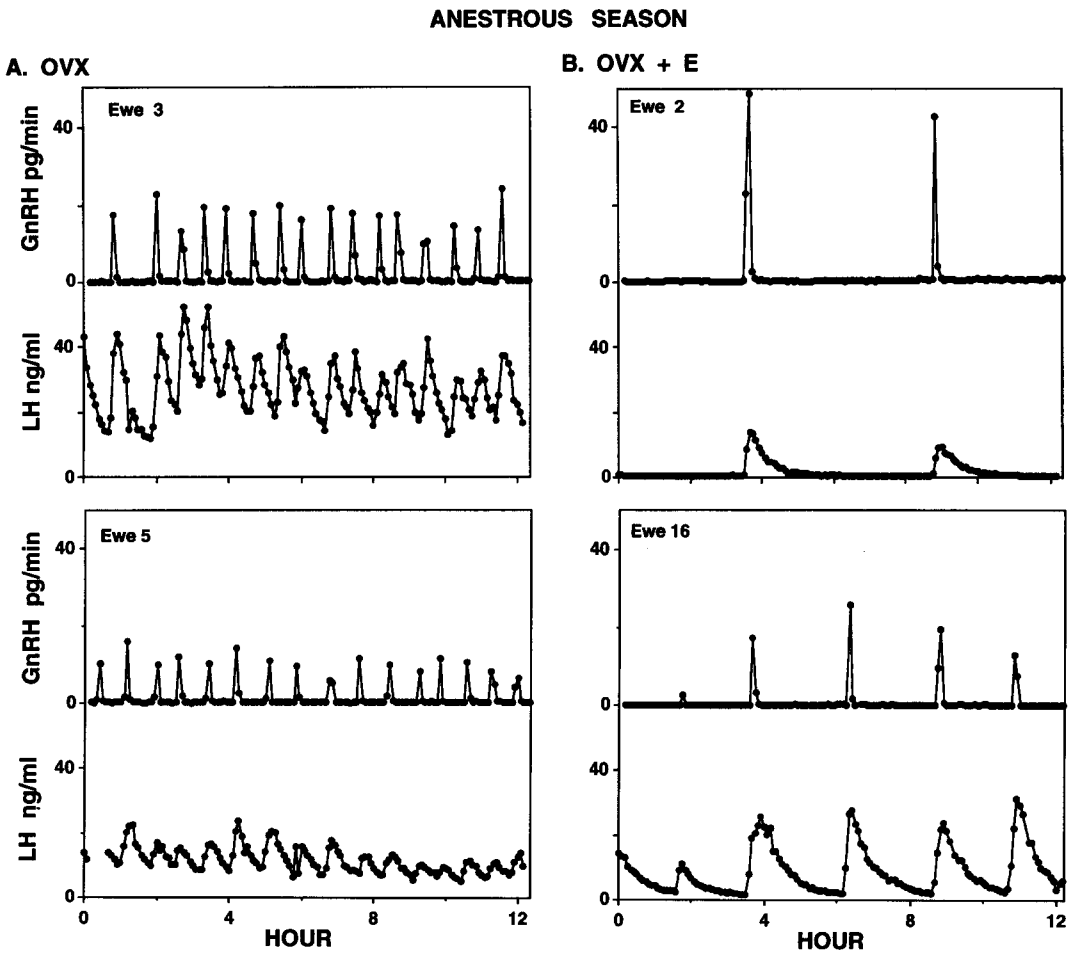


FIG. 5. Representative patterns of GnRH and LH over a 12-h period during anestrus in OVX (panel A) and OVX+E (panel B) ewes in experiment 2. LH values are absent near the start of collection for ewe 5 due to technical problems obtaining jugular blood. Further details in Figure 3 legend.

within terminals of neurons that are afferent to GnRH neurons. This explanation is compatible with our finding that, among OVX+E ewes during anestrus, the slower frequencies were associated with larger GnRH pulses. Whatever the explanation, it is of interest that the increased size of GnRH pulses in OVX+E ewes during anestrus was not associated with a significant augmentation of LH pulses, perhaps because the pituitary was maximally stimulated by the smaller GnRH pulses of the breeding season, so this phenomenon may not be of crucial importance to seasonal alterations in gonadotropin secretion.

An unexpected finding was the absence of a significant seasonal difference in episodic GnRH secretion in ovariectomized ewes not treated with estradiol (i.e., there was no steroid-independent seasonal change despite a striking shift in response to estradiol negative feedback). For LH, a relatively small but significant decline in pulse frequency had previously been observed during anestrus [7, 8], especially when individual ovariectomized ewes were monitored throughout the year [16], and we expected our study to disclose a similar change in GnRH pulses. There was, however, no significant seasonal difference in frequency of either GnRH or LH pulses in the ovariectomized ewes in the present study (although trends for a reduced rate in anestrus were apparent). It is pertinent to note that a similar absence of a seasonal difference was described in the only other report comparing episodic GnRH secretion in ovariectomized ewes [25]. Perhaps if observations had been made on more ewes or on the same animals at multiple stages of the breeding and anestrous seasons, a seasonal difference might have become evident.

It is of interest to place our results into a physiological context related to regulation of seasonal reproduction. In the ovary-intact ewe, pulses of GnRH and LH occur during the luteal phase of the estrous cycle, primarily because of the negative feedback effect of the elevated level of circulating progesterone [10, 24, 25, 27–29]. During anestrus, the frequency of episodic GnRH and LH secretion is extremely low despite the lack of corpora lutea and the consequent absence of an elevation in progesterone [9, 10]. Ovarian fol-

licles do develop during anestrus and respond to the infrequent pulses of LH by secreting estradiol, albeit at a rate far lower than during the follicular phase of the estrous cycle [19, 30, 31]. Our finding that GnRH pulse frequency during anestrus can be dramatically reduced by an extremely low circulating concentration of estradiol supports the hypothesis [4, 8] that the amount of estradiol secreted during anestrus is sufficient to hold episodic GnRH secretion in check. In this regard, our findings provide a physiological basis for the marked suppression of reproductive neuroendocrine function under conditions in which the gonadal output of hormones eliciting negative feedback is relatively low.

Finally, it is important to stress that the seasonal shift in responsiveness of the GnRH neurosecretory system to the negative feedback action of estradiol is likely to be of fundamental physiological importance to the seasonal waxing and waning of the estrous cycle of the ewe. After regression of the corpus luteum during the breeding season, the follicular phase of the estrous cycle is ushered in by a sustained volley of high-frequency pulses of GnRH, which, via the stimulation of LH secretion, promotes the remaining steps in the preovulatory sequence: the final stages of follicular maturation, the preovulatory estradiol rise, initiation of the GnRH and LH surges, and estrous behavior [4, 27, 28]. None of these events occurs after the withdrawal of progesterone in anestrus [10, 19]. Importantly, the sustained volley of high frequency pulses of GnRH needed to start the sequence does not occur [10], because the follicle, by virtue of its secretion of estradiol, enforces a profound inhibition of episodic GnRH release. As a result, pulses of GnRH in anestrous ewes occur so infrequently that follicles cannot develop to the preovulatory stage; estrous cycles therefore cease. It is important to point out that the absence of cycles in anestrus is not the consequence of marked alterations in other components of the neuroendocrine mechanisms that generate the estrous cycle, because all of the remaining preovulatory steps can be induced during anestrus by appropriate physiological stimuli [4, 13, 17, 19, 32]. Viewed in this context, the seasonal alteration in the inhibitory effect of estradiol on episodic GnRH secretion is one, and perhaps the major, neuroendocrine determinant of seasonal changes in ovarian cyclicity.

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