

## Response of anestrus ewes to the ram effect after follicular wave synchronization with a single dose of estradiol-17 $\beta$

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**Abstract** – Anestrus ewes respond to the introduction of rams with either an ovulation within 2–3 days that may be followed by luteal phases of normal or short length, with delayed ovulations (5–6 days later), or with the luteinization of follicles. The aim of this work was to study the relationship between the growth status of the largest follicle present when rams are introduced and the type of ovarian response in non-treated ewes and in ewes treated with estradiol-17 $\beta$  before ram introduction. Thirteen anestrus Corriedale ewes were divided into 2 groups: E2 ( $n = 7$ ) and C ( $n = 6$ ). The E2 ewes received a single dose of 50  $\mu\text{g}$  estradiol-17 $\beta$  5 days before the introduction of the rams to synchronize the onset of their follicle waves, while C ewes remained untreated. When the rams were introduced, all E2 ewes had the largest follicle in a growing stage in contrast with the C ewes (3 out of 6;  $P < 0.05$ ). Five C and 4 E2 ewes ovulated after the introduction of the rams (Day  $3.4 \pm 0.4$  for C vs.  $4.8 \pm 0.3$  for E2 ewes, respectively,  $P < 0.05$ ). Only one ewe from each group developed a normal luteal phase; 4 C and 3 E2 ewes had short luteal phases. One C ewe and 2 E2 ewes had short luteal phases originating from follicles that did not ovulate. After the first luteal phase, all ewes returned to anestrus without a second ovulation or luteal phase. The remaining E2 ewe did not ovulate or show any changes in progesterone serum concentrations. We conclude that the growth status of the largest follicle alone does not determine the ovarian responding pattern of anestrus ewes to the ram effect.

**follicular dynamics / ultrasound / ram stimulus / ovulation / non-breeding season**

### 1. INTRODUCTION

The reproductive response of anestrus ewes to the introduction of rams (the ram effect) has been known since the 1940s [1]. In brief, if anestrus ewes are preconditioned by a period of isolation, the introduction of rams induces changes in their

reproductive physiology: LH pulsatility is increased, and ovulation is induced in many of the ewes (for a review see [2]). This ovulation is not associated with estrous behavior. In some of the ewes, the first estrus appears in conjunction with the second ovulation 17 to 20 days after ram introduction. In others, there is at first a short luteal phase

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(4–5 days), then a second ovulation also without signs of estrus, followed by a luteal phase of normal duration. Thereafter, another ovulation associated with estrus occurs. The existence of delayed ovulations was originally suggested by Fulkerson et al. [3] and confirmed with laparoscopic [4] and ultrasonographic [5] observations. In our previous experiment [5], we also observed that, in some ewes, luteal concentrations of progesterone are associated with the development of luteinized anovulatory follicles, as was previously reported by Knight et al. [6]. These different patterns of ovarian response lead to a highly characteristic bimodal pattern in the display of estrus after ram exposure and in which there are 2 peaks of estrus in the flock, 17 to 20 and 21 to 25 days later [2].

As in cows [7], follicular growth in ewes occurs in a wave pattern (for review see [8]). The follicular waves emerge approximately every 5 days during the estrous cycle in the breeding season [9]. This wave pattern continues during the non-breeding season in anestrus ewes [10, 11]. As in cattle (for review see [12]), follicle waves are preceded by a transient increase in FSH concentrations that stimulates follicle wave emergence during the breeding [13–15] and non-breeding seasons [16]. It has been shown that the ovarian response to a GnRH challenge is related to the specific growth phase of the largest follicle present in the ovaries during the breeding and the non-breeding seasons [17, 18]. However, no attempts have been made to test whether a similar relationship between follicular status and ovarian response occurs when the ewe's pituitary-hypothalamus axis is challenged with the ram effect.

In cattle, the administration of estradiol suppresses follicular growth and results in the emergence of a new follicular wave at a consistent interval (~ 4 days; for review see [19]). However, there is little information about the effects of estradiol on follicular dynamics in ewes. Administration of estradiol-17 $\beta$  to anestrus ewes induces an

LH surge (for a review see [20]) and causes regression of the dominant follicle and the emergence of a new follicular wave approximately 3 days later [21]. The aim of this work was to study the relationship between the growth status of the largest follicle present when rams are introduced and the pattern of ovarian response in non-treated ewes and in ewes treated with estradiol-17 $\beta$  before ram introduction.

## 2. MATERIALS AND METHODS

### 2.1. Animals and animal management

The experiment was conducted within the Department of Physiology, Faculty of Veterinary Science, Montevideo, Uruguay (35° S) during November (mid-seasonal anestrus). Fourteen adult multiparous Corriedale ewes weighing  $48.6 \pm 2.1$  kg and with a body condition of  $3.5 \pm 0.2$  (scale 1–5) (mean  $\pm$  SE) were used. The ewes were moved to the Department of Physiology and isolated (sight, sound, smell) from the rams for one month. The animals received a diet meeting INRA sheep maintenance standards: water and alfalfa hay (8.4% CP and 58.1% crude fiber on the basis of DM) ad libitum, plus 500 g of pellets made from oats, barley, and wheat (12% CP). They were kept outdoors with natural daylight (14L/10D) in sheltered pens (40 m  $\times$  40 m), and indoor box stalls (3 m  $\times$  3 m) were used when required for handling. All animal experimentation was performed in compliance with the regulations set by the National Board for Laboratory Animals in Sweden (Swedish University of Agricultural Sciences, Uppsala, Sweden).

After verification of anestrus status (ultrasonography and progesterone concentrations), 1 ewe was removed because it had a luteinized follicle. The remaining 13 ewes were divided into 2 homogeneous groups according to weight and body condition. Seven ewes (E2 group) received an im injection of 50  $\mu$ g estradiol-17 $\beta$  (Sigma,

E8875, St. Louis, MO, USA) in 0.5 mL of corn oil on Day -5 (Day 0 = introduction of the rams); the other 6 ewes remained without treatment and served as a control group (group C). On Day 0, 3 adult, sexually experienced Corriedale rams provided with markers were introduced to the ewes. Because anestrus Corriedale ewes subjected to the ram effect express maximum reproductive response when estrous ewes are introduced together with the rams [22], another 8 ewes were brought into estrus by an im injection of 350 IU of eCG (Novormón, Syntex, Buenos Aires, Argentina) after a 6-day priming with intravaginal sponges containing 60 mg of medroxyprogesterone acetate (Syntex, Buenos Aires, Argentina), and were joined at the same time as the rams. Estrus was checked daily from Day 0 until Day 23. Although specific behavioral tests were not performed, the rams showed very low libido, with very few matings seen with ewes in hormonally induced estrus.

## 2.2. Blood collection

Blood was collected from the jugular vein of all animals daily from Day -9 to -5; every 4 h from 1 h before estradiol-17 $\beta$  was administered (12:00) for 52 h; daily on Days -2 and -1; every 4 h from the introduction of the rams (12:00) for 56 h; every 12 h from Days 3 to 8; and then daily until Day 23. The samples were allowed to clot for 1 h at room temperature before being centrifuged for 10 to 20 min. The serum was stored at -20 °C until analysis for hormone content.

## 2.3. Ultrasonography

A single operator performed transrectal ultrasonography daily between Day -9 and Day 15 to monitor the follicles and to characterize the ovarian status. The scanner was an Aloka 500 (Tokyo, Japan), real-time, B mode machine with a 7.5 MHz linear array probe, which was adapted for ovines [17, 18]. During each examination, a sketch of

both ovaries was made to record the diameter and position of follicles over 2 mm in diameter. The observations were also recorded on video using individual videocassettes to verify and correct real-time data. After the locations had been recorded, the sketch was compared with that of the previous day. The growth and regression histories (diameters) of each follicle identified were tabulated daily. Ovulation was detected by the collapse of a follicle over 5 mm. Time of ovulation was defined as the moment of the first observation in which the ovulatory follicle was not present. A follicular wave was defined as the growth of a follicle to  $\geq 4.5$  mm, singularly or as part of a cohort; the day of emergency of the follicular wave was the day before the follicle was 3 mm in diameter, with an increase the following day. Follicle growth was calculated by determining the increase of the diameter over 2 consecutive days. A follicle was considered to be in a growing status when it grew  $\geq 0.5$  mm the previous day.

## 2.4. Hormone assays

Progesterone was measured in samples taken from Day -9 to -5; in 1 sample every 12 h during the first intensive bleeding period (Day -5 to Day -3); on Days -2 and -1; every 12 h from Day 0 to Day 7; and in all samples until Day 23. During the second intensive bleeding period (0 to 56 h), the 2 previous and consecutive samples were measured when the concentrations in one sample were above  $0.5 \text{ nmol}\cdot\text{L}^{-1}$  (see luteal phase definition). Progesterone was measured with a direct solid-phase  $^{125}\text{I}$  RIA method (Count-A-Count TKPG, Diagnostic Products Corporation, Los Angeles, CA, USA). The sensitivity of the assay was  $0.1 \text{ nmol}\cdot\text{L}^{-1}$ . Estradiol-17 $\beta$ , LH, and FSH were measured in all samples. Estradiol-17 $\beta$  was measured with a direct solid-phase  $^{125}\text{I}$  RIA method (Count-A-Count TKPG, Diagnostic Products Corporation, Los Angeles, CA, USA) as described by Meikle [21]. The sensitivity of the assay was  $2 \text{ pmol}\cdot\text{L}^{-1}$ . LH was measured with a

liquid-phase RIA previously validated for ovine serum [23]. The detection limit of the assay was  $0.6 \mu\text{g}\cdot\text{L}^{-1}$ . FSH concentration was analyzed in all samples with a liquid-phase RIA previously validated for ovine serum [21]. The sensitivity of the assay was  $0.5 \mu\text{g}\cdot\text{L}^{-1}$ . The inter-assay and intra-assay coefficients were below 10% for all assays.

## 2.5. Definitions

An LH surge was defined as being at least 8 times higher than the basal levels (mean value of all samples). Luteal activity was defined as the presence of progesterone concentrations above  $1.5 \text{ nmol}\cdot\text{L}^{-1}$  in 4 or more consecutive samples when concentrations were measured every 12 h, or above  $0.9 \text{ nmol}\cdot\text{L}^{-1}$  in at least 5 consecutive samples when the values were measured every 4 h. A normal luteal phase was defined as the presence of luteal activity for at least 10 days; a short luteal phase was defined as luteal activity for no more than 4 days.

## 2.6. Statistical analysis

The size of the largest follicle and days of follicular wave emergence were compared with ANOVA, variances were compared with the Bartlett test, and frequencies were compared with the chi-square test. Hormonal profiles were analyzed with the general linear model procedure of the Statistical Analysis System (1999/2000, [24]) using an analysis of variance for repeated measures after log transformation.

## 3. RESULTS

### 3.1. Response to estradiol-17 $\beta$

Group ( $P < 0.0001$ ), time ( $P < 0.0001$ ), and the interaction between group and time ( $P < 0.0001$ ), influenced estradiol-17 $\beta$  concentrations. Estradiol-17 $\beta$  reached maximum concentration in E2 ( $131 \pm 18 \text{ pmol}\cdot\text{L}^{-1}$ ) 4 h after administration

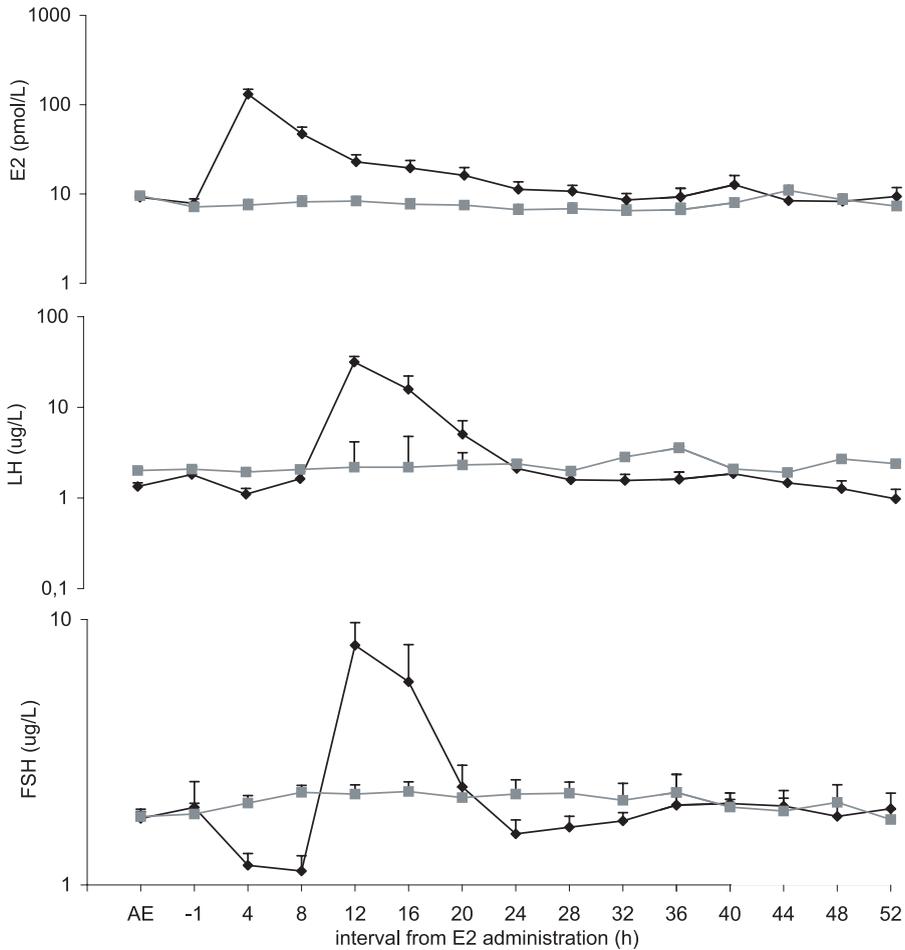
( $P < 0.0001$ ), declined over 12 h ( $47 \pm 9 \text{ pmol}\cdot\text{L}^{-1}$ ;  $P < 0.0001$ ), and reached concentrations similar to the initial concentrations 16 h after administration (Fig. 1A).

The concentrations of LH differed between groups ( $P < 0.0001$ ), and an interaction between group and time was also observed ( $P < 0.0001$ ). An LH surge ( $31.6 \pm 4.8 \mu\text{g}\cdot\text{L}^{-1}$ ) was observed in all E2 ewes 12 h after estradiol-17 $\beta$  administration ( $P < 0.0001$ ), maintaining concentrations higher than basal at 16 ( $15.8 \pm 6.4 \mu\text{g}\cdot\text{L}^{-1}$ ;  $P < 0.0001$ ) and 20 h ( $5.0 \pm 2.1 \mu\text{g}\cdot\text{L}^{-1}$ ;  $P < 0.005$ ; Fig. 1B), respectively. Between 12 and 20 h after estradiol-17 $\beta$  administration, the concentrations were higher in the E2 ewes than in the C ewes ( $P < 0.0001$ ).

The concentrations of FSH differed between groups ( $P < 0.0001$ ), and an interaction between group and time was also observed ( $P < 0.0001$ ). FSH concentrations tended to decrease in E2 ewes in conjunction with maximum estradiol-17 $\beta$  concentrations (8 h;  $1.1 \pm 0.1 \mu\text{g}\cdot\text{L}^{-1}$ ;  $P = 0.06$ ), increasing quickly again 12 ( $8.0 \pm 1.7 \mu\text{g}\cdot\text{L}^{-1}$ ;  $P < 0.0001$ ) and 16 h ( $5.8 \pm 2.2 \mu\text{g}\cdot\text{L}^{-1}$ ;  $P < 0.0001$ ), and returning to basal concentrations 20 h after estradiol-17 $\beta$  administration ( $2.3 \pm 0.5 \mu\text{g}\cdot\text{L}^{-1}$ ; Fig. 1C). Twelve and 16 h after estradiol-17 $\beta$  administration, the concentrations were higher in E2 ewes than in C ewes ( $P < 0.0001$ ).

The last follicular wave observed prior to estradiol-17 $\beta$  administration emerged on Day  $-2.0 \pm 0.6$  and on Day  $-1.8 \pm 0.5$  in the E2 and C groups, respectively (comparison of variances:  $P > 0.1$ ). The emergence of the first follicular wave after estradiol-17 $\beta$  treatment was more homogeneous in E2 ewes (Day  $2.9 \pm 0.1$ ) than in C ewes (Day  $1.7 \pm 0.5$ ) (comparison of variances:  $P = 0.01$ ). No ewe ovulated or showed estrous behavior in the period between estradiol-17 $\beta$  administration and the introduction of the rams.

When the rams were introduced, the growth status was more homogeneous in E2 ewes than in C ewes (comparison of



**Figure 1.** Serum estradiol-17 $\beta$  (A), LH (B) and FSH (C) concentrations in anestrus ewes after the administration of 50  $\mu$ g estradiol-17 $\beta$  (-◆-) at 0 h. Control ewes (-■-) were not injected. AE is the mean concentration of 5 daily samples collected from 5 days before estradiol-17 $\beta$  administration. The Y-axis is in the logarithmic scale.

variances:  $P < 0.01$ ; Tab. I). While the largest follicle was in a growing stage in all E2 ewes, this was the case in only half of the C ewes (7/7 vs. 3/6;  $P < 0.05$ ). The diameter of the largest follicle present in the ovaries when the rams were introduced was larger in the C than in the E2 ewes ( $P < 0.001$ ; Tab. I).

### 3.2. Response to rams

No E2 or C ewes were marked (displayed estrus) during the 23-day experimental period.

Five C and 4 E2 ewes ovulated after the introduction of the rams. Ovulation occurred earlier in group C than in group E2

**Table I.** Characteristics of the largest follicle present when rams were introduced and ovarian responses. NLP: luteal phase of normal length; SLP: short luteal phase; LP: luteal phase.

	C	E2	P
Largest follicle present at ram introduction			
Day of emergence*	1.7 ± 0.5	2.9 ± 0.1	n.s.
Growth the day before (mm)*	0.3 ± 0.3	0.6 ± 0.1	n.s.
Growing phase	3/6	7/7	< 0.05
Diameter when rams were introduced	4.7 ± 0.1	3.7 ± 0.1	< 0.001
Responses to rams			
Ovulated	5/6	4/7	n.s.
Day of ovulation	3.4 ± 0.4	4.8 ± 0.3	< 0.05
Growth the day before (mm)	0.5 ± 0.4	0.7 ± 0.6	n.s.
Diameter at ovulation (mm)	5.5 ± 0.3	5.9 ± 0.7	n.s.
NLP	1/5	1/4	n.s.
SLP	4/5	3/4	n.s.
LP from non-ovulatory follicles	1/6	2/7	n.s.

\* Variances are significantly different:  $P < 0.01$ .

( $P < 0.05$ ; Tab. I). Only one ewe from each group developed a normal luteal phase; 4 C and 3 E2 ewes had short luteal phases. One C ewe and 2 E2 ewes, that did not ovulate had short luteal phases originating from follicles that did not ovulate. After the first luteal phase, all ewes returned to anestrus without a second ovulation or luteal phase. The remaining E2 ewe did not show ovulation or changes in progesterone serum concentrations during the studied period.

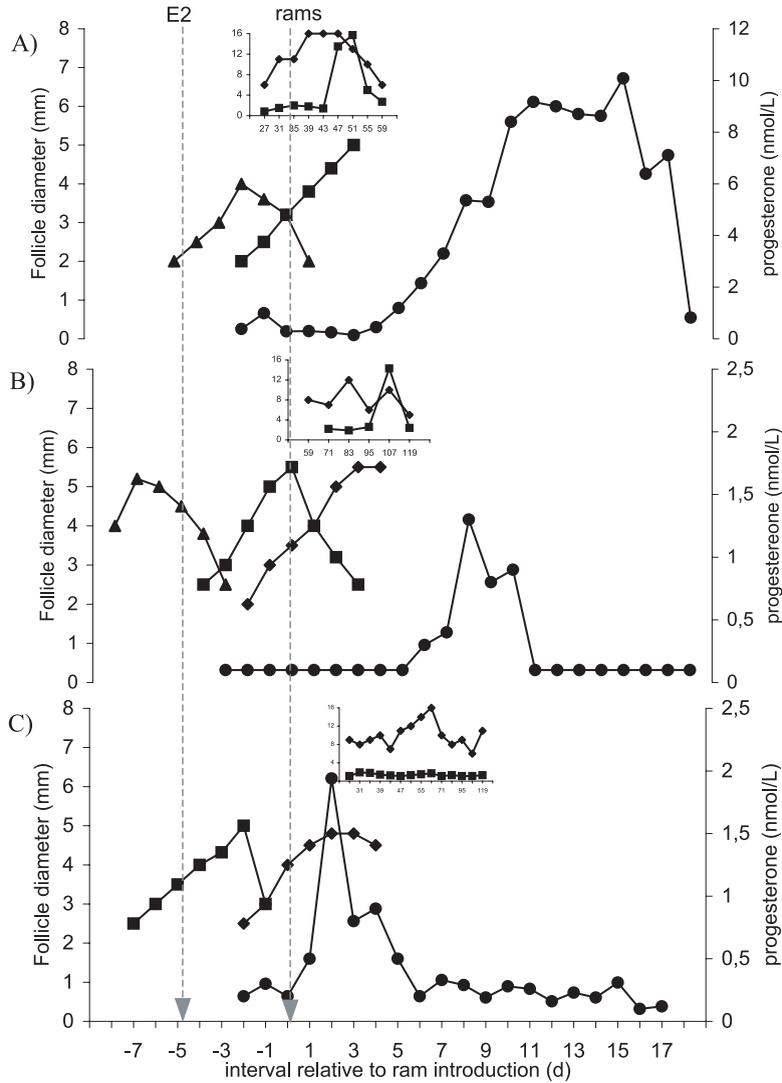
When the ewes were regrouped according to those whose follicles were in the growing stage when the rams were introduced ( $n = 10$ ) or not ( $n = 3$ ), 7 of the ewes that had a growing follicle ovulated; 2 of them had normal luteal phases and 5 had short luteal phases; 2 of them had short luteal phases provoked by follicles that did not ovulate. The 3 ewes that did not have the largest follicles in a growing phase when the rams were introduced had short luteal phases, 2 of them from ovulatory follicles and the other from a non-ovulatory follicle.

The characteristics of the largest follicle present when the rams were introduced were not different between the ewes that

ovulated and those that did not ovulate. The largest follicle grew  $0.4 \pm 0.2$  mm and  $0.4 \pm 0.2$  mm the day before the introduction of the rams, and had a diameter of  $4.1 \pm 0.2$  mm and  $4.1 \pm 0.3$  mm when the rams were introduced (the ewes that ovulated and the ewes that did not ovulate respectively; n.s.).

Estradiol-17 $\beta$  concentrations increased in C ewes that ovulated from  $9 \pm 1$  pmol·L<sup>-1</sup> to a maximum of  $16 \pm 2$  pmol·L<sup>-1</sup> ( $P < 0.001$ ) at  $56.6 \pm 1.6$  h. In the E2 ewes that ovulated, the concentrations increased from  $8 \pm 1$  pmol·L<sup>-1</sup> to a maximum of  $14 \pm 2$  pmol·L<sup>-1</sup> ( $P < 0.05$ ) at  $60.0 \pm 5.3$  h. There were no significant differences in the estradiol-17 $\beta$  profiles between C and E2 ewes ( $P > 0.1$ ).

The concentrations of FSH increased in the C ewes that ovulated from  $1.7 \pm 0.2$  ng·mL<sup>-1</sup> to a maximum of  $2.3 \pm 0.4$   $\mu$ g·L<sup>-1</sup> ( $P < 0.05$ ) at  $61.8 \pm 12.6$  h. In the E2 ewes that ovulated, the FSH concentration was  $2.1 \pm 0.2$   $\mu$ g·L<sup>-1</sup>, increasing to a maximum of  $4.0 \pm 1.3$   $\mu$ g·L<sup>-1</sup> (NS,  $P > 0.1$ ) at  $82.0 \pm 22.7$  h. There were no



**Figure 2.** Follicle profiles (-▲-, -■-, -◆-), and progesterone (-●-) responses in 3 anestrus ewes that were treated with 50 µg estradiol-17β (first arrow) 5 days before the introduction of rams (second arrow). In these ewes, the largest follicle emerged 2 days before ram exposure. This follicle responded with the following: (A) a normal luteal phase, (B) a short luteal phase after an ovulation, and (C) a short luteal phase without ovulation. Estradiol-17β (-◆-) and LH (-■-) surges are shown in the inset graphs when corresponding.

significant differences in FSH profiles between the C and E2 ewes ( $P > 0.1$ ).

There was no relationship between the follicle status and ovarian response. Figure 2

shows the follicle profiles and LH, estradiol-17β, and progesterone patterns in the three E2 ewes in which the largest follicle emerged on Day 2 and responded with a

normal luteal phase (A), a short luteal phase after an ovulation (B), and a short luteal phase without a previous ovulation (C).

#### 4. DISCUSSION

We observed a high synchronization of follicular wave onset approximately 3 days after estradiol-17 $\beta$  administration, confirming previous findings [21]. However, we did not observe a direct relation between the follicular status and the ovarian response to the introduction of the rams. In fact, even the largest follicle was in a growing and homogeneous status in all E2 ewes, the ovarian responses were similar to those observed in C ewes, which, since no attempts were made to manage the follicular growth, presented a random status of their largest follicle when the rams were introduced. The only difference provoked by estradiol-17 $\beta$  was a delay on the ovulation of the E2 ewes, which agrees with earlier observations of Martin [25]. We did not observe any relation between the ovarian response according to the status of the largest follicle present when the rams were introduced, despite the ewes being treated or not with estradiol-17 $\beta$ . In previous experiments, we observed that the largest follicle responded differently to a GnRH challenge according to the growing status [17, 18]. However, our results agree with those of Bartlewski et al. [26], who observed that large follicles of a similar age and a similar growth stage respond with different ovarian patterns to GnRH administration. When the ewes that ovulated were analyzed retrospectively, despite the original group to which they belonged, there were no differences on the follicular status when the rams were introduced. One possible explanation for our results is that while in Rubianes et al. [17, 18] experiments, the ewes received a pharmacological stimulation, in the present experiment the response reflected how the ewe's hypothalamic-pituitary axis responded to the ram stimuli.

Also the possible effects of the LH and FSH surges provoked by estradiol-17 $\beta$  administration on the characteristics of the largest follicle should be considered.

The ewes responded with a high incidence of short luteal phases (overall, 10 from 13 stimulated ewes). This result agrees with Perkins and Fitzgerald [27], who observed a high incidence of short luteal phases after the introduction of low libido rams. It remains to be elucidated whether this is related to an insufficient LH response in the ewes when low libido rams are used. The short luteal phases in E2 ewes originated from follicles ovulating approximately 5 days after the introduction of the rams. Although the occurrence of delayed ovulations followed by normal luteal phases has been previously reported [4, 5], we now report the existence of delayed ovulations followed by short luteal phases.

All of the ewes that show ovarian responses after the introduction of the rams returned to anestrus without a second ovulation or luteal phase, and without displaying estrous behavior during the expected period for ram-stimulated ewes (Days 17–25). We have observed similar outcomes in a previous experiment done under laboratory conditions [5], but we obtained different results when the ewes from the same flock were stimulated under field conditions in the same anestrus period (Exps. 1 and 2 from [28]). Sheep show a stress response after transportation [29], and during adaptation to a new environment [30]. Although maximum attempts were made to minimize the possible negative effects of transportation and the joining of the rams, these may not have been enough to eliminate the problem.

We conclude that the growth status of the largest follicle alone does not determine the ovarian responding pattern of anestrus ewes to the ram effect.

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