



## Embryo yield and quality are associated with progestogen treatment during superovulation protocol in lactating Lacaune ewes

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### ABSTRACT

This study assessed the effect of progestogen treatment length on ovarian parameters and embryo yield in superovulated Lacaune ewes collected by nonsurgical embryo recovery. Twenty-three lactating ewes were superovulated 30 d apart using a cross-over design. All ewes received 60 mg of MAP intravaginal sponges for 6 (G-6 group) or 9 (G-9 group) d. A total dose of 133 mg pFSH was given in six decreasing doses (twice a day) starting at 60 h before device removal. Ultrasound examination of the ovaries was performed at the first pFSH injection and one day before embryo recovery, which was performed 6–7 d after the onset of estrus. Embryo recovery was conducted only in ewes that expressed estrus and were mated. There was no difference ( $P > 0.05$ ) in the total number of follicles between G-6 ( $15.7 \pm 1.0$ ) and G-9 ( $15.6 \pm 0.8$ ) at the time of the first pFSH treatment. The percentage of responding donors with  $\geq 3$  corpora lutea (CL; 78.2% [18/23] vs 69.5% [16/23]), mean ( $\pm$ SEM) CL number ( $7.0 \pm 1.2$  vs  $8.1 \pm 1.6$ ), transcervical passage rate (94.4% [17/18] vs 83.3% [15/18]), and ova/embryo recovery rate (54.5% [60/110] vs 68.0% [83/122]) were not different ( $P > 0.05$ ) between the G-6 and G-9 groups. However, the mean number of viable embryos was lower ( $P < 0.05$ ) in the G-6 group ( $1.8 \pm 0.7$ ) than in the G-9 group ( $3.5 \pm 1.1$ ). In conclusion, treatment with an intravaginal MAP sponge for 9 d during a superovulation protocol is beneficial for viable embryo yield in Lacaune ewes out of the breeding season.

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### 1. Introduction

With the increasing effectiveness of nonsurgical embryo recovery in Brazil [1], Brazilian dairy sheep farmers have become interested in assigning Lacaune sheep to multiple ovulation and embryo transfer (MOET) programs. These programs contribute to increased genetic improvement in sheep [2]. However, the

variability of the ovarian response to superovulatory treatments limits the wide application of MOET programs in commercial settings [3], and breeds have been identified as an intrinsic factor responsible for variations [4,5], emphasizing the need of a specific superovulatory treatment for each breed [6]. Despite the world-wide importance of the Lacaune breed for the dairy sheep industry, only a few studies applying MOET have been reported for this breed [4,7,8].

The nonsurgical embryo recovery technique has been improved in recent years as an alternative to surgical procedures [9]. Recently, encouraging results of transcervical passage rates have been reported, with 73–81% in Santa Inês [10,11] and 95% in Lacaune ewes [12], using hormonal treatments to induce cervical dilation, combining d-cloprostenol, estradiol benzoate, and oxytocin. However, these studies were carried out in non-superovulated ewes.

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Therefore, the suitability of this protocol for transcervical embryo recovery in different breeds, across seasons of the year, and in superovulated ewes remains to be known [9].

Traditional superovulatory protocols with progesterone priming over 12–14 d (long-term) have been established due to the length of the luteal phase. However, the circulation progestin concentrations induced by long-term progestin treatments are considered potentially responsible for the variability in superovulatory responses in ewes [13]. Short-term (5 to 7-d) progestin treatments have enabled comparable performance to long-term (12–14-d) treatments in Merino sheep. This also offers some advantages, such as the use of a single sponge instead of two, as is the case in long-term treatments [2]. In a previous study, we observed differences in preovulatory follicular dynamics in progestogen-based estrus induction protocols for 6 or 9 d in Lacaune sheep [12]. Interestingly, we found that the higher frequency of ovulatory follicles from different waves in the 9-d progestin treatment associated with 400 IU of eCG was in some cases associated with multiple ovulations, i.e., <5. Recently, the antral follicular count and anti-Müllerian hormone dosage were considered suitable for the selection of ewes with the greatest potential to respond to superovulation [14]. The ovulatory response after a single eCG treatment was shown to have a high correlation with further superovulatory responses to FSH treatments [15] and can be used as a preselection test for donors. Thus, we hypothesize that the putative low dominance effect observed in the 9-d progestogen treatment may be beneficial for ovarian superstimulation in Lacaune sheep.

The objective of the present study is to evaluate the effect of progestin treatment length (6 vs. 9 d) on the superovulatory response and embryo yield in lactating Lacaune ewes treated with decreasing doses of pFSH followed by nonsurgical embryo recovery.

## 2. Materials and methods

### 2.1. Ethics and animal care

The Animal Care Committee of Embrapa Dairy Cattle approved the study design (Protocol number # 2512100516/2016), which was conducted under the principles of the Brazilian Society of Laboratory Animal Science.

### 2.2. Local, animal, and experimental conditions

This study was conducted during the non-breeding season (October to December), on a commercial farm located in Soledade de Minas (latitude 22°3'S, longitude 45°2'W and an altitude of 938 m above mean sea level) in Minas Gerais State, Brazil.

Twenty-three lactating, clinically healthy Lacaune ewes (119.9 ± 6.7 d in milk and producing 1.0 ± 0.1 kg of milk/day/ewe, mean ± SEM) were used. These ewes were primiparous (n = 12) and multiparous (n = 11) and ranged from 16 to 95 months in age, with a bodyweight (BW) of 69.4 ± 2.5 kg and a body condition score (BCS; scale 0–5, with 0 = emaciated and 5 = too fat) of 3.6 ± 0.1 [16].

The animals were kept in collective pens and fed corn silage. A balanced concentrated supplement was offered twice a day to complete their nutritional requirements [17]. Mineralized salt (DeHeus®, Rio Claro, Brazil) and drinking water were available *ad libitum*.

### 2.3. Experimental design

This study was performed in a crossover experimental design with an interval of 30 d between replicates. In the first replicate, the ewes were randomly allocated into two different groups and

received intravaginal sponges containing medroxyprogesterone acetate (MAP; 60 mg; Progespon®, Zoetis, Campinas, Brazil) for either 6 (G-6) or 9 (G-9) d. A single treatment with 37.5 µg d-cloprostenol (Sincrocio®, OuroFino, Cravinhos, Brazil) was administered intramuscularly (i.m.) 24 h before device removal. Ewes were treated with pFSH (133 mg; Folltropin®, Bioniche, Belleville, ON, Canada) i.m. twice daily (12 h intervals) in six decreasing doses (25, 25, 15, 15, 10 and 10%). To achieve this dosing schedule, 10 mL of saline solution was added to 20 mL of the diluents. The pFSH treatment started 60 h (2.5 d) before device removal (i.e., in G-6 and G-9 group the superovulatory treatment began 3.5 d and 6.5 d after the sponge insertion, respectively).

Estrous behavior was recorded over 4 d after device removal using healthy and fertile rams (maximum ratio of 1 ram to 4 ewes) twice a day (morning – 08:00 and evening – 18:00). Rams were rotated in both treatments to minimize male fertility variations. Ewes remained with a ram for at least 30 min in each observation period if no mounting acceptance occurred. Estrus onset was defined as the time when the ewe first stood to be mounted by a ram. Mating was repeated every 12 h until there was no mounting acceptance.

### 2.4. Ovarian ultrasonography

Ovarian ultrasonography (US) was conducted at the time of the first pFSH treatment in all ewes by the same experienced operator using a B-mode ultrasonographic scanner (Mindray®, M5Vet, Shenzhen, China) equipped with a linear transrectal transducer (8.0 MHz) fitted to a plastic rod that allowed its manipulation. These examinations were aimed at determining the number of follicles and corpora lutea (CL). On the sixth and seventh days after device removal, a second ultrasound scanning was performed using the Color-Doppler mode to facilitate the CL count and luteal blood flow assessment to ensure high accuracy in counting luteal structures (CL and luteinized follicles) [18,19].

### 2.5. Nonsurgical embryo recovery and embryo yield evaluation

Ewes displaying estrus and that had mated were subjected to nonsurgical embryo recovery 6–7 d after the onset of estrus (6.8 ± 0.9 d) using the nonsurgical embryo recovery technique [9]. Hormone treatment was administered to induce cervical dilation and to facilitate cervical passage and embryo collection, as described by Figueira et al. [12]. This treatment consisted of 1 mg of estradiol benzoate (Sincrodiol®, Ourofino, Cravinhos, Brazil) i.m. and 37.5 µg of d-cloprostenol (Prolise®, Tecnopec, São Paulo, Brazil) administered laterovulvarly (l.v.) [20] 16 h before nonsurgical embryo recovery. Intravenous treatment with 50 IU oxytocin (Ocitocina forte®, UCB, São Paulo, Brazil) was administered 20 min before embryo collection (Fig. 1). Following oxytocin administration, a combination of 40 mg hyoscine-N-butylbromide and 5 g sodium dipyrone (Buscofin®, Agener Union, Embu-Guaçu, Brazil) was administered both i.v. and i.m. in equal parts. In addition, acepromazine maleate (Acepran 1%®, Vetnil, Louveira, Brazil) was administered i.m. at a dose of 0.1 mg/kg of body weight 20 min prior to cervical manipulation, as previously described [21]. Sedation continued for 45 min, which was considered sufficient time to perform the embryo collection procedure. Ewes in a standing position were restrained in a cart and received 2 mL of 2% lidocaine epidural block (S5–C1) (Lidovet®, Bravet, Rio de Janeiro, Brazil). Cervical immobilization, uterine access, and embryo recovery were performed by the same experienced technician, as described by Figueira et al. [12]. Ultrasound assessment allowed for determining the direction of the first uterine flushing ipsilateral to the ovary with greater CL count.

All the ova/embryos recovered were counted and the embryos were transferred to a holding medium (Holding Plus®, Cultilab, Campinas, Brazil). Embryo evaluation was performed under a stereomicroscope with magnification  $\times 40$  (Nova®, model XTD-20, Piracicaba, Brazil) and following International Embryo Transfer Society recommendations [22]. The embryo quality score was Grade 1 (excellent or good); Grade 2 (fair); Grade 3 (poor); or Grade 4 (dead or degenerated). Embryos of Grades 1, 2 and 3 were considered viable. The following variables were considered: transcervical penetration rate = number of ewes in which the cervix was completely transposed for uterine flushing/number of ewes in which cervical penetration was attempted  $\times 100$ ; successful recovery rate = number of ewes with at least one ovum or embryo recovered/number of ewes in which the cervix was completely transposed for uterine flushing  $\times 100$ ; ova/embryo recovery rate = total number of ova/embryos (unfertilized ova, embryos of different developmental stages)/number of CL  $\times 100$ ; viable embryo rate = number of viable embryos/total number of recovered ova/embryos  $\times 100$ ; and the unfertilized rate-number of unfertilized ova/total number of ova/embryos recovered  $\times 100$ .

## 2.6. Statistical analyses

Statistical analyses were performed using SAS® software version 9.3 (SAS Inst., Inc., Cary, USA). The statistical models used to study the variables were similar to those proposed by Kaps and Lamberson [23]. The models included order (applied treatments sequence), treatment, and replicate as fixed effects and ewe nested in order as a random effect.

The quantitative data, namely estrus length, interval from device removal to estrus, time of cervical penetration, and total time of procedure, were analyzed using generalized linear models, with Gamma distribution and log-link function using the GLIMMIX procedure. Follicular counts grouped by diameter ( $\leq 3.0$  mm, 3.1–4.0 mm, 4.1–5.0 mm, and  $>5.0$  mm) and the numbers of CL, non-vascularized CL, recovered ova/embryo, unfertilized ova, luteinized anovulatory follicles, and viable embryos were also analyzed using the GLIMMIX procedure, with Poisson distribution and log-link function. The relationships between the variables were assessed by calculating Pearson's correlation coefficient.

The qualitative data, namely estrus rate, percentage of

responding donors rate ( $\geq 3$  CL), percentage of ewes with follicles  $>5.0$  mm and ewes bearing CL at the beginning of the pFSH treatment, transcervical passage rate, successful recovery rate, ova/embryo recovery rate, viable embryo rate, and unfertilized rate, were analyzed using the GLIMMIX procedure, considering binomial distribution and logit link function. The results are shown as means  $\pm$  standard error of the means (MEANS  $\pm$  SEM). Differences were considered significant when  $P < 0.05$ .

## 3. Results

### 3.1. Ovarian population at the beginning of the pFSH treatment

The mean ( $\pm$ SEM) numbers of follicles and ewes with large follicles and CL at the beginning of the superovulatory treatment are summarized in Table 1. No differences ( $P > 0.05$ ) were found between the groups (G-6 and G-9) and replicates (first or second) in the studied follicular diameter categories, percentage of ewes with large follicles ( $>5.0$  mm), and CL at the time of the first pFSH injection.

### 3.2. Reproductive behavior and ovarian response

Estrus rate, interval from device removal to estrus, and estrus length did not differ ( $P > 0.05$ ) between the G-6 and G-9 groups (Table 2). Five ewes (21.7%) in each treatment group did not respond to estrus synchronization. There was greater ( $P < 0.05$ ) estrus length in the second replicate compared to the first replicate. Percentage of responding donors ( $\geq 3$  CL), number of CL, vascularized CL, and non-vascularized CL did not differ between the treatment groups ( $P > 0.05$ ; Table 2). The number of luteinized anovulatory follicles was numerically higher ( $P < 0.10$ ) in the G-6 group than in the G-9 group. There was a higher ( $P < 0.05$ ) number of CL and vascularized CL in the second replicate than in the first replicate.

### 3.3. Correlations of follicular populations at the time of the first pFSH with superovulatory response and embryo yield

The number of CL in superovulated ewes was positively correlated ( $P < 0.05$ ) with the total number of antral follicles ( $r = 0.36$ ) as

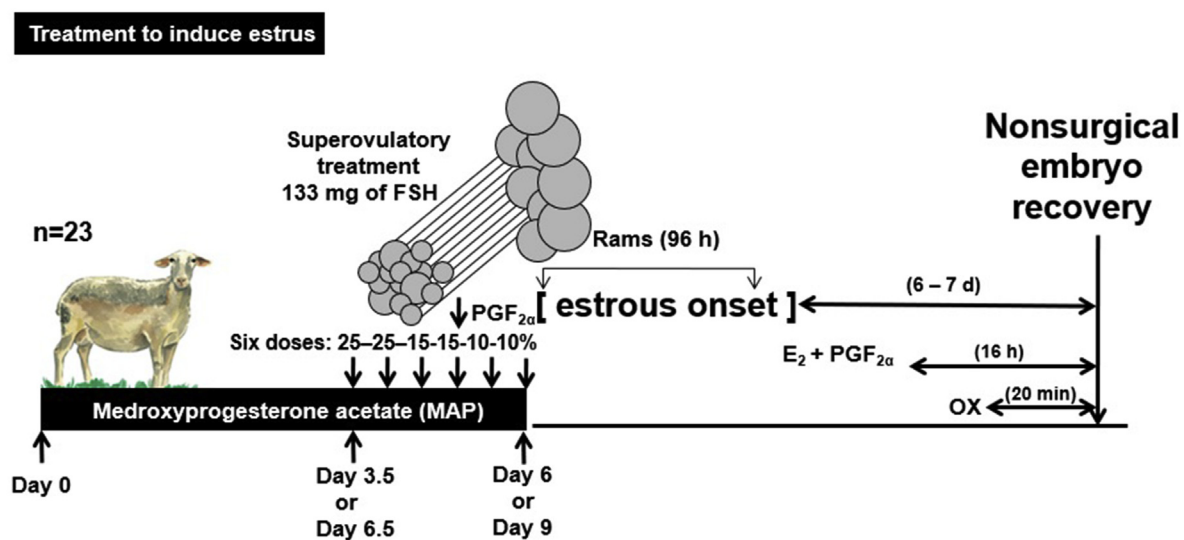


Fig. 1. Schematic representation of treatments used to induce estrus, to promote superovulation, and to induce cervical dilation for nonsurgical embryo recovery in lactating Lacaune ewes. pFSH: 133 mg of porcine follicle-stimulating hormone, PGF<sub>2α</sub>: 37.5  $\mu$ g of d-cloprostenol, E<sub>2</sub>: 1 mg of estradiol benzoate, OX: 50 IU of oxytocin.

well as the numbers of follicles  $\leq 3.0$  mm ( $r = 0.42$ ) and  $>5.0$  mm ( $r = 0.38$ ), and it was negatively correlated ( $P < 0.05$ ) with the number of 4.1–5.0 mm follicles ( $r = -0.33$ ). The number of follicles  $>5.0$  mm was also positively correlated ( $r = 0.37$ ) with the number of ova/embryos recovered. Moreover, the numbers of follicles  $\leq 3.0$  mm and  $>5.0$  mm were positively correlated with the number of unfertilized ova ( $r = 0.34$  and  $0.38$ , respectively). Neither the number of categorized follicles nor the total follicle count was correlated ( $P > 0.05$ ) with the number of viable embryos.

#### 3.4. Transcervical penetration and embryo recovery

The data related to embryo recovery and yield are summarized in Table 2. No difference ( $P > 0.05$ ) was found between the treatment groups or replicates (Table 2) at the time of transcervical penetration and uterine flushing. There was a high fluid recovery efficiency (%): 99% in G-6 and 97.2% in G-9. The numbers of ova/embryos and viable embryos were higher ( $P < 0.05$ ) in the G-9 group than in the G-6 group. In the second replicate, there was a higher ( $P < 0.05$ ) number and rate of viable embryos and a lower ( $P < 0.05$ ) number and rate of unfertilized ova compared to the first replicate.

#### 4. Discussion

This study is a step towards understanding the factors influencing the superovulatory response during the non-breeding season in Lacaune ewes under tropical conditions. Additionally, it confirms the repeatability and efficiency of a hormone protocol to induce cervical dilation for nonsurgical embryo recovery in superovulated lactating Lacaune ewes.

In the current study, there were no significant differences in the number of follicles or the percentage of ewes with CL between the groups at the first FSH injection, and there was a similar superovulatory response in both protocols. However, there was a significantly lower number of viable embryos in the G-6 group than in the G-9 group. Meanwhile, no differences were observed in superovulatory and embryo yield responses when comparing different progesterone exposure times (ranging from 5 to 14 d) in superovulation protocols in the Merino breed [2]. In the G-6 group, there was double the number of luteinized anovulatory follicles, suggesting failures in the ovulatory process and insufficient progesterone priming. It is known that ovarian and endocrine changes after superovulatory treatments can alter follicular development and fertilization processes, decreasing the number and quality of embryos [24–26]. The high number of viable embryos in the G-9 group represents an additional benefit of long exposure to high progesterone concentrations for fertilization and embryo quality, which suggests an important role in the preovulatory follicular development under superovulatory treatment [27]. Progesterone priming also affects the expression of angiogenic factors in large

preovulatory follicles, ensuring adequate luteal development and function [28]. It was demonstrated that Lacaune ewes benefit from increased length of progestogen exposure when comparing estrus synchronization protocols of 6 vs 12 d [29]. Luteal function was improved in the 12-d protocol, based on progesterone concentrations at 14 d after device removal. Therefore, the 9-d protocol seems to provide a better hormonal milieu for *in vivo* embryo production in Lacaune ewes than the short-term (6-d) protocol.

The lower embryo yield in the G-6 group can be partially explained by the fact that 74% of ewes presented large ovarian antral follicles at the beginning of the pFSH treatment, almost 50% more than in the G-9 group, and similar to the 75–80% observed in traditional protocols (long-term, 12–14 d) [30,31]. It is known that follicle dominance may impair the superovulatory response in small ruminants [32]. Despite the controversy regarding the absence of a dominance effect of large follicles during the non-breeding season [33], there was a moderate (~39%) percentage of animals bearing CL at the beginning of the first FSH treatment in both groups and replicates. The dominance effect may have contributed to the observed responses in the non-breeding season. In a previous study with Lacaune ewes synchronized to estrus, we observed a higher number of ovulations in those ewes that received progesterone treatment for 9 d compared 6 d, suggesting a lower dominance effect in the 9-d protocol [12]. If Lacaune ewes present a pattern similar to that of the Santa Inês breed, the emergence of the first and second antral follicular waves occurs approximately on Days 2.0 and 5.9 in relation to the progestin insertion (Day 0), respectively [34]. This may indicate that the G-6 and G-9 ewes had follicle ovulation from the first and second antral follicular waves, respectively. In goats, there is evidence of a lower intensity of the follicular dominance effect in intermediate waves of the same estrous cycle [35]. Therefore, the data suggest that the dominance effect occurs and impacts the embryo yield according to the length of the progesterone treatment. The crossover experimental design brings far more statistical power for a comparison of novel ovarian stimulation treatments, controlling the individual effect.

There was a moderate positive correlation between the number of small follicles ( $\leq 3.0$  mm in diameter) at the first pFSH administration and the superovulatory response, similar to what was reported by other authors [36,37]. This follicular class is representative of a follicular population potentially responsive to FSH and capable of growing to ovulatory size [3]. Interestingly, follicles larger than 5 mm were also positively correlated with the superovulatory response, and follicles with a diameter equal to or smaller than 3.0 mm and greater than 5.0 mm were positively correlated with unfertilized ova. The lack of correlation between the number of small follicles and viable embryos may be due to the fact that follicles of 2 mm in diameter are not sufficiently mature to develop into viable embryos [30]. The recruitment of these smaller follicles might require additional time to complete maturation and support an adequate oocyte development prior to exposure to a

**Table 1**

Ovarian follicle parameters (means  $\pm$  SEM) and number of ewes with CL and large follicles (%) observed at the beginning of a pFSH<sup>a</sup> treatment during a superovulation protocol in lactating Lacaune ewes treated with medroxyprogesterone sponges for 6 (G-6) or 9 (G-9) d in a crossover design of two replicates 30 d apart.

End points	G-6 (n = 23)	G-9 (n = 23)	P-value	Replicate 1	Replicate 2	P-value
Number of follicles $\leq 3.0$ mm	12.5 $\pm$ 0.9	11.8 $\pm$ 0.8	0.54	11.3 $\pm$ 0.9	13.0 $\pm$ 0.8	0.13
Number of follicles 3.1–4.0 mm	1.3 $\pm$ 0.2	2.0 $\pm$ 0.5	0.07	1.7 $\pm$ 0.3	1.7 $\pm$ 0.4	0.76
Number of follicles 4.1–5.0 mm	0.7 $\pm$ 0.2	1.0 $\pm$ 0.2	0.22	0.9 $\pm$ 0.2	0.8 $\pm$ 0.2	0.80
Number of follicles $>5.0$ mm	1.2 $\pm$ 0.2	0.7 $\pm$ 0.2	0.17	0.9 $\pm$ 0.2	1.0 $\pm$ 0.2	0.68
Total number of antral follicles	15.7 $\pm$ 1.0	15.6 $\pm$ 0.8	0.99	14.7 $\pm$ 0.9	16.5 $\pm$ 0.9	0.15
Ewes bearing large follicles (%)	73.9 (17/23)	52.2 (12/23)	0.15	60.9 (14/23)	65.2 (15/23)	0.81
Ewes bearing corpus luteum (%)	34.7 (8/23)	43.5 (10/23)	0.52	39.1 (9/23)	39.1 (9/23)	0.98

<sup>a</sup> Six decreasing doses (25, 25, 15, 15, 10 and 10% of total dose) twice daily. The first pFSH administration occurred at 60 h before device removal.

**Table 2**  
Estrous behavior expression, superovulatory response, and embryo recovery variables (means  $\pm$  SEM or %) in Lacaune ewes treated with medroxyprogesterone sponges for 6 (G-6) or 9 (G-9) d in a crossover design of two replicates 30 d apart.

End Points	G-6	G-9	P-value	Replicate 1	Replicate 2	P-value
Ewes (n)	23	23	–	23	23	–
Estrus rate (%)	78.3 (18/23)	78.3 (18/23)	1.00	78.3 (18/23)	78.3 (18/23)	1.00
Interval from sponge removal to estrus (h)	38.1 $\pm$ 5.2 (18)	36.0 $\pm$ 3.1 (18)	0.48	39.3 $\pm$ 3.0 (18)	37.8 $\pm$ 5.2 (18)	0.65
Estrus duration (h)	27.3 $\pm$ 3.7 (18)	29.7 $\pm$ 3.1 (18)	0.65	20.3 $\pm$ 2.7 (18)	36.6 $\pm$ 2.8 (18)	<0.01
Responding donors rate ( $\geq$ 3 CL) (%) <sup>b</sup>	78.2 (18/23)	69.5 (16/23)	0.60	65.2 (15/23)	82.6 (19/23)	0.21
CL count (n)	7.0 $\pm$ 1.1 (23)	6.6 $\pm$ 1.4 (23)	0.52	5.2 $\pm$ 1.2 (23)	8.3 $\pm$ 1.2 (23)	<0.01
Vascularized CL (n)	6.3 $\pm$ 1.1(23)	6.4 $\pm$ 1.4 (23)	0.81	4.9 $\pm$ 1.2 (23)	7.9 $\pm$ 1.2 (23)	<0.01
Non-vascularized CL (n)	0.4 $\pm$ 0.2 (23)	0.3 $\pm$ 0.2 (23)	0.53	0.3 $\pm$ 0.1 (23)	0.3 $\pm$ 0.1 (23)	0.60
Luteinized follicles (n)	1.1 $\pm$ 0.3 (23)	0.5 $\pm$ 0.2 (23)	0.09	0.6 $\pm$ 0.2 (23)	1.0 $\pm$ 0.4 (23)	0.42
Successful cervical passage rate (%) <sup>c</sup>	94.4 (17/18)	83.3 (15/18)	0.30	88.8 (16/18)	88.8 (16/18)	1.00
Duration of cervical passage(min)	5.8 $\pm$ 1.2 (17)	4.6 $\pm$ 0.9 (15)	0.47	6.0 $\pm$ 1.2 (16)	4.5 $\pm$ 1.0 (16)	0.29
Duration of uterine flushing (min)	18.5 $\pm$ 0.8 (17)	19.0 $\pm$ 1.0 (15)	0.64	19.1 $\pm$ 0.8 (16)	18.4 $\pm$ 1.0 (16)	0.53
Ewes with at least one ovum or embryo recovered (%)	82.4 (14/17)	80.0 (12/15)	0.78	75.0 (12/16)	87.5 (14/16)	0.39
CL count in ewes collected (n)	6.5 $\pm$ 1.2(17)	8.1 $\pm$ 1.6 (15)	0.82	6.5 $\pm$ 1.5 (16)	8.0 $\pm$ 1.2 (16)	0.15
Total ova/embryos (n)	3.5 $\pm$ 1.0 (17)	5.5 $\pm$ 1.6 (15)	0.01	4.3 $\pm$ 1.5 (16)	4.7 $\pm$ 1.1 (16)	0.85
Ova/embryo recovery rate (%)	54.5 (60/110)	68.0 (83/122)	0.59	65.4 (68/104)	58.5 (75/128)	0.71
Viable embryos (n)	1.8 $\pm$ 0.7 (17)	3.5 $\pm$ 1.1 (15)	0.03	1.4 $\pm$ 0.5 (16)	3.8 $\pm$ 1.1 (16)	<0.01
Viable embryo rate (%)	50.0 (30/60)	62.7 (52/83)	0.38	32.4 (22/68)	80.0 (60/75)	<0.01
Unfertilized ova (n)	1.6 $\pm$ 0.6 (17)	1.7 $\pm$ 1.2 (15)	0.24	4.5 $\pm$ 1.2 (16)	1.3 $\pm$ 0.3 (16)	<0.01
Unfertilized rate (%)	45.0 (27/60)	30.1 (25/83)	0.06	66.2 (45/68)	9.3 (7/75)	<0.01

(...) Number of animals.

<sup>a</sup> Six decreasing administrations (25, 25, 15, 15, 10 and 10% of total dose) twice daily. The first pFSH administration occurred at 60 h before device removal.

<sup>b</sup> Including ewes that did not display estrous behavior.

<sup>c</sup> NSER was performed in all ewes that expressed estrous behavior.

preovulatory LH surge [38].

Despite the low production of embryos in both groups, there was an increase in the superovulatory response, resulting in a higher number and rate of viable embryos in the second replicate, in which the estrus length was longer than in the first one. The estrus evaluation is especially important due to its association with the LH peak and ovulation [39]. In sheep, a minimum amount of LH is essential after the removal of the progestogen to increase the superovulatory response and the number of viable embryos recovered, particularly during the non-breeding season [40,41]. Therefore, the longer estrus length accompanied by a higher number of CL in the second replicate may suggest a better temporal relationship between the onset of estrus and the LH release. Moreover, it is known that the ovulatory follicles from progestogen-treated sheep may have deficiencies in 17 $\beta$ -estradiol secretion (responsible for estrous behavior) during the preovulatory phase and have deleterious effects on oocyte developmental competence [42]. However, since progestogen-based protocols, superovulatory treatments, and animal management did not vary between the replicates, other factors such as nutritional status or stress may be related to the differences observed in the estrous behavior between the replicates. In any event, these data suggest that LH supplementation at the end of the protocols [6] may offer benefits by reducing the frequency of anovulatory follicles.

The repeatability of the superovulatory and embryo yield responses deserves attention. It is known that laparotomy often causes the formation of post-operative adhesions in the uterus, oviducts, and ovaries, thus inducing a reduction in the superovulatory response [43] and embryo recovery rate after repeated surgery [44]. A nonsurgical approach has been proposed primarily to overcome this limitation. The repeatability observed in superovulation and embryo yield responses demonstrates the advantage of nonsurgical embryo recovery over laparotomy [45].

This study used lactating ewes from the perspective of repeated transcervical embryo collections in the accelerated breeding program of a commercial farm without interrupting milk production. Thus, it is necessary for sheep to be submitted to MOET programs when they are in lactation and during the non-reproductive season.

When this approach is employed, the first aspect that deserves attention is that there seems to be an impairment of the superovulatory response in animals of high milk potential [46]. In ewes, the 17 $\beta$ -estradiol metabolism increases due to elevated feed intake [47]. Likewise, the increase in the liver blood flow (LBF) resulting from the elevated feed intake in ewes may increase the steroid metabolism [48]. Therefore, the shorter length of estrus in animals that are fewer days in milk may reflect the specific feeding intake for each stage of lactation. The antagonism between a high potential for milk production and the ability to produce embryos in sheep needs to be elucidated for a better application of MOET programs in dairy sheep.

The combination of 6 or 9 d of progestogen exposure with a reduced dose of pFSH (133 mg) resulted in a low efficiency of the embryo yield in the Lacaune breed. In a study with the same breed [4], greater superovulatory response (~12 CL) and embryo production (~7) were observed after treatment with 16 mg Armour equivalent of pFSH plus 500 IU eCG at the end of a long-term (14-d) progestogen protocol. It is worth noting that this is a simple comparison since the study also indicated that the number of ovulations and the percentage of viable embryos can be reduced outside of the breeding season. Moreover, the use of higher doses of FSH may promote more ovulations, although this may not necessarily ensure a high correlation with viable embryo production [41]. For instance, a greater embryo yield was observed after treatment with 128 mg pFSH than after treatment with 200 mg [49]. Interestingly, a lower number and rate of viable embryos was induced by a dose of 133 pFSH compared to doses of 100 and 200 mg pFSH in Santa Inês ewes that received superovulation treatment protocols combining pFSH with 300 IU eCG [50].

Despite a high fluid recovery efficiency, the ova/embryo recovery rate after nonsurgical embryo recovery showed similar results to the 65% reached in previous studies [51,52]. In those studies, the ova/embryo recovery rates were evaluated by dividing the number of recovered structures (ova and embryos) by the number of CL counted by laparoscopy. Recently, studies have evaluated Color-Doppler ultrasound imaging as a substitute for laparoscopy to count the CL in superovulated sheep, with a very good accuracy in

identifying animals that did not respond adequately and which were not feasible for collection [18,19]. This deserves special attention because there appear to be limitations to achieving high ova/embryo recovery rates for nonsurgical embryo recovery in non-superovulated animals [11,12]. Therefore, Color-Doppler ultrasonography is an excellent tool for donor screening and the evaluation of embryo recovery efficiency with attention to animal welfare and the possibility of successive collections [9]. The recovery rate was slightly lower than the rates reported by laparotomy recovery in the same breed (77%) [4] and in the Santa Inês breed (77%) [53], but it is comparable to what has been observed in other breeds, such as Corriedale (49–68%) [54] and Dorper (57%) [49].

Finally, it should be highlighted that earlier results reported in non-superovulated Lacaune ewes with the same length of progestogen treatment [12] appeared to be a good screening test of donors, as previously proposed [15]. The average total structures and viable embryos recovered (respectively) in non-superovulated Lacaune favored the 9-d progestogen treatment (1.6 and 1.4) when compared to the 6-d (0.6 and 0.4) treatment [12]. The results of the present study confirm this perspective with significant superior total ova/embryos and viable embryos, respectively, recovered in the 9-d progestogen protocol (5.5 and 3.5) compared to the 6-d protocol (3.5 and 1.8) for superovulated lactating Lacaune ewes.

## 5. Conclusions

The Lacaune ewes superovulated in the 9-d progestogen-based estrus induction protocol presented a better embryo yield after nonsurgical embryo recovery than in the 6-d protocol. The mean ova/embryo number and ova/embryo recovery rate after nonsurgical embryo recovery are considered satisfactory. However, taking into account the fact that approximately 20% of donors failed to display estrus, 20–30% did not respond to the superovulatory treatment, and there was a low superovulatory response (e.g., CL count of 7.0 and 6.6 for the 6-d and 9-d MAP treatments, respectively), it appears that 133 mg FSH may not have been sufficient to elicit a greater superovulatory response in lactating Lacaune ewes out of the breeding season. This indicates the importance of additional studies using the 9-d progestogen/progesterone protocol as the basis for investigating different doses of FSH.

## CRedit authorship contribution statement

**Lucas Machado Figueira:** Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Project administration, Writing - original draft, Writing - review & editing. **Nadja Gomes Alves:** Methodology, Supervision, Resources, Data curation, Formal analysis, Writing - review & editing. **Ana Lucia Rosa e Silva Maia:** Investigation, Formal analysis, Writing - review & editing. **Joanna Maria Gonçalves de Souza-Fabjan:** Investigation, Supervision, Resources, Formal analysis, Writing - review & editing. **Ribrio Ivan Tavares Pereira Batista:** Investigation, Writing - review & editing. **Maria Clara da Cruz Morais:** Investigation, Writing - review & editing. **Renato Ribeiro de Lima:** Data curation, Formal analysis. **Maria Emilia Franco Oliveira:** Formal analysis, Writing - review & editing. **Jeferson Ferreira da Fonseca:** Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing - review & editing, Supervision, Funding acquisition, Resources, Project administration.

## Declaration of competing interest

The authors declare that there is no conflict of interests.

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