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REGULAR ARTICLES

Efficiency of different hormonal treatments for estrus synchronization in tropical Santa Inês sheep

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Abstract The aim of this study was to evaluate the effect of (i) the duration of hormone treatment with progestogen sponges during the seasonal anestrus and (ii) the administration of two doses of prostaglandin at 7 days apart during the breeding season on reproductive parameters of Santa Inês ewes. In experiment 1, 32 ewes received intravaginal MAP sponges for 6 ($G_{6\ days}$), 9 ($G_{9\ days}$), or 12 (G_{12days}) days and 75 μg D-cloprostenol i.m. and 300 IU eCG i.m. 1 day before sponge removal. In experiment 2, 23 ewes received two doses of 0.48-mg sodium cloprostenol i.m. 7 days apart. Ovarian follicular dynamic was assessed through transrectal ultrasonography. Blood samples were collected daily to determine progesterone concentrations. In experiment 1, estrus and ovulation rates did not differ ($P > 0.05$) among protocols and between cyclic and acyclic ewes at the beginning of the experiment. The $G_{9\ days}$ treatment showed a lower dispersion of ovulations in relation to onset of estrus when compared to $G_{6\ days}$ and $G_{12\ days}$. In experiment 2, all ewes exhibit estrus and ovulated after the second dose of prostaglandin, although ewes that were in diestrus at D0 showed subluteal concentrations of progesterone during the follicle development stage of the treatment. In conclusion, the use of progestogen device during 9 days promotes lower dispersion of ovulation when compared to its use for 6 or 12 days, and the protocol of two doses of prostaglandin 7 days apart synchronizes estrus efficiently but results in follicular development under low progesterone concentrations.

Keywords Cyclicity · Ovine · Native breed · Progesterone · Prostaglandin

Introduction

Sheep farming has experienced an outstanding growth in developing countries. In Brazil Southeast region, the proximity with consumers of high demand combined with low offer points out a great potential for this activity. Among the sheep breeds raised in Brazil, Santa Inês stands out because of its adaptability to tropical conditions, maternal ability, prolificacy, and sexual precocity (Balaro et al. 2014). It is well known that depending on latitude and breed, ewes are seasonally polyestrous animals, presenting regular ovulatory activity only in the breeding season (Bartlewski et al. 2011). The reproductive seasonality sets out a huge challenge to the sheep producer that tries to obtain maximal productive efficiency and is an obstacle to regularly offer sheep products throughout the year. Reproductive biotechnologies such as artificial insemination (AI) and multiple ovulation and embryo transfer (MOET) are also benefited with the use of estrus synchronization or induction treatments (Abecia et al. 2012).

Recent reports demonstrated that Santa Inês breed has a variable degree of reproductive seasonality in Southeast Brazil at 21° S of latitude (Balaro et al. 2014), since 70 % of the ewes had short-to-medium seasonal anestrus. Considering that approximately 87 % of corpora lutea (CL) are detected at 24 h after ovulation by Doppler ultrasound in this breed (Figueira et al. 2015), a quick previous evaluation may be reliable to identify if ewes are cyclic or in anestrus. Therefore, it is essential to establish efficient treatments for

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both cases, estrus synchronization for cyclic ewes and estrus induction for anestrous ewes of Santa Inês breed.

In the breeding season, prostaglandin administration either in a single dose or in two doses may be used to synchronize cyclic ewes. Traditionally, extended intervals between the two doses (10 to 12 days) were used and protocols using shorter intervals (7 to 9 days) were recently developed and applied in Corriedale (Olivera-Muzante et al. 2011a; Fierro et al. 2011, 2013) and Merino sheep (Olivera-Muzante et al. 2011b). Hormonal treatments based on progestogens associated with gonadotropins have been adopted when the aim is to induce estrus in the non-breeding season. The duration of progestogen treatment is not unanimous among researchers, and different lengths have been utilized with relative success (Ozyurtlu et al. 2010; Silva et al. 2010). Some researchers reported advantage for short-term (6 days) over long-term protocols (12 to 14 days) (Viñoles et al. 2001; Letelier et al. 2008).

The aim of the present study was to evaluate the effect of (i) the duration of hormone treatment (6, 9, or 12 days) with progestogen sponges during the seasonal anestrus on the ovulatory dynamics, sexual behavior, and fertility (experiment 1) and (ii) the administration of two doses of prostaglandin at 7 days apart on reproductive parameters of Santa Inês ewes (experiment 2).

Material and methods

Experimental conditions

This study was approved by the Animal Care Committee of Fluminense Federal University (protocol number 452), and it was conducted under the principles of the Brazilian Society of Laboratory Animal Science. The study was carried out in the rural area of Cachoeiras de Macacu located in the state of Rio de Janeiro (latitude 22° 27′ S, longitude 43° 39′ W, 577 m of altitude). According to Köppen, the climate is a tropical hot-humid type, with temperatures throughout the year ranging from 15 to 30 °C and an annual rainfall ranging from 2.000 to 2.600 mm^3 concentrated mostly in the summer.

In both experiments, the ewes were maintained in a semi-intensive system under natural photoperiods with access to pasture and shelter. Chopped elephant grass (*Pennisetum purpureum*) was offered twice daily, and 250 g per animal of a concentrate with 18 % crude protein was offered once a day, with water and mineralized salt (Ovinofos®, Tortuga, São Paulo, Brazil) ad libitum.

Experiment 1

This experiment was conducted during the months of August and September—transition from winter to spring—characterized as non-breeding season. A total of 32 nulliparous or pluriparous Santa Inês ewes were randomly allocated into three experimental treatments according to their body weight (BW) and body condition score (BCS), respectively: $G_{6\ days}$ $n=11$, 44.97 ± 8.62 and 3.07 ± 0.36 kg; $G_{9\ days}$ $n=11$, 43.49 ± 7.93 and 2.98 ± 0.26 kg; and $G_{12\ days}$ $n=10$, 45.54 ± 7.06 and 2.93 ± 0.17 kg. In a random day of estrous cycle, established as D0, all ewes received intravaginal sponges impregnated with 60 mg of medroxyprogesterone acetate (MAP—Progespon®, MSD Animal Health, São Paulo, Brazil). The sponges were maintained for 6 ($G_{6\ days}$), 9 ($G_{9\ days}$), or 12 ($G_{12\ days}$) days. One day before its removal, 75 μg D-cloprostenol i.m. (Prolise®, Tecnopec, São Paulo, Brazil) and 300 IU eCG i.m. (Novormon®, MSD Animal Health, São Paulo, Brazil) were administered.

Ultrasound assessments were performed every 24 h after sponge insertion and every 12 h after its removal in all ewes. The ewes were subjected to a teaser male after sponge removal, twice a day (7:00 a.m. and 6:00 p.m.), for 3 days until they showed no more estrus signs. The ewes were inseminated at 55.05 ± 0.8 h after sponge removal. After insemination, the females were subjected to a fertile male to promote heterospermy and thus to enhance pregnancy rate. Thirty days after insemination, the pregnancy was diagnosed by ultrasonography. Plasma progesterone concentration was measured in all ewes among the groups from 6 days before sponge insertion to 4 days after sponge removal. Ewes with progesterone concentration ≤1 ng/mL in the five first days of treatment were determined as in anestrus (Minton et al. 1991).

Experiment 2

This experiment was conducted during the month of May (middle of autumn), which is considered as a breeding season in this region. A total of 23 nulliparous and pluriparous Santa Inês ewes with mean BW of 51.63 ± 6.7 kg received two doses of 0.48 mg sodium cloprostenol i.m. (Estron®, Agener, São Paulo, Brazil) 7 days apart in a random day of the estrous cycle, established as D0 and D7.

Ultrasound assessments were performed every 24 h after the first prostaglandin dose and every 12 h after the second dose. The ewes were subjected to a teaser male every 24 h (7:00 a.m.) after the first dose and twice a day (7:00 a.m. and 6:00 p.m.) after the second dose for 4 days until they showed no more estrus signs. Plasma progesterone concentration was measured in all ewes from D0 until 3 days after the second dose, thus, D10. Animals in diestrus in D0 (progesterone concentration >1 ng/mL) and in another phases of estrous cycle in D0 (P4 <1 ng/ml) were comparatively evaluated.

Ultrasonography

During the experiments, ultrasonography assessments were performed (SonoScape®, S6V, Shenzhen, China) with a transrectal linear probe of 7.5 MHz, coupled with a PVC

structure. The follicular diameter and position and the quantity of follicles and CL were recorded. The time of ovulation was defined as the mean time between the last observation and first no observation of the dominant follicle (>5 mm) after sponge removal. Double ovulation was defined when the second largest follicle at the time of ovulation was greater than 4 mm in diameter.

Artificial insemination and natural mating

The ewes of experiment 1 were subjected to AI with commercial semen (Top in Life®, Jaboticabal, Brazil) through laparoscopy 55.1 ± 0.8 h after sponge removal. Feed and water were restricted for 24 and 12 h before the AI. Fifteen minutes before the procedure, the ewes received the pre-anesthetic treatment using 0.05 mg/kg acepromazine i.v. (Acepran®, Vetnil, São Paulo, Brazil), 0.2 mg/kg diazepam i.v. (Diazepam®, Teuto, Goiás, Brazil), and 0.3 mg/kg morphine i.v. (Dolo Moff®, São Paulo, Brazil). After this procedure, the ewes were positioned using the Trendelenburg position and received local anesthetic at the incision site, using 2 % lidocaine s.c. (Lidovet®, Rio de Janeiro, Brazil) for the insertion of the trocars. The AI was performed by the use of semen at a concentration of 40×10^6/mL, with half of the straw content injected in each uterine horn. After the procedures, ointment (Pearson®, Eurofarma, Rio de Janeiro, Brazil), silver sulfadiazine, and cipermetrin (Bactrovet®, Konig, Avellaneda, Argentina) were topically applied, besides oxytetracycline and hydrocortisone (Terra-cortril®, Pfizer, São Paulo, Brazil). After AI, all ewes were placed together with a ram to elevate heterospermy and thus enhance pregnancy rate.

Plasma progesterone concentrations

Blood samples were daily collected in the morning by jugular venipuncture, into vacuum tubes containing EDTA for progesterone analysis. The samples were immediately chilled at 5 °C and centrifuged (400*g* for 15 min), and then the plasma was separated into microtubes and frozen at −18 °C. The progesterone analysis was performed at the Hormonal Dosage Laboratory, Animal Reproduction Unit, Fluminense Federal University. The progesterone concentrations were determined by commercial solid phase radioimmunoassay (RIA) kit (Immunotech/Beckman Coulter®, Prague, Czech Republic), according to the manufacturer's instructions. The assay sensitivity and intra-assay coefficients of variation were 0.05 ng/mL and 12 %. In addition, all data were within the maximum and minimum points of the curve.

Statistical analysis

For statistical analysis, the software BioEstat® 5.3 was used. The qualitative variables were subjected to Fisher's exact test. The quantitative variables were tested as its normality by the Lilliefors test and then submitted to ANOVA and Tukey's test (normal distribution) or Kruskal-Wallis test and SNK (non-normal distribution). The standard deviation among groups was analyzed through coefficient of variation. The significance level adopted for these analyses was 5 %.

Results

Experiment 1

The overall percentage of ewes detected in anestrus by progesterone analysis at the beginning of the experiment was 28.1 % (9/32), being similar ($P > 0.05$) for the three groups: $G_{6\ days}$ (27.3 %; 3/11), $G_{9\ days}$ (27.3 %; 3/11), and $G_{12\ days}$ (30.0 %; 3/10). These females in anestrus did not present any CL in the ultrasonography until the sponge removal. Conversely, all cyclic ewes at the beginning of the treatment presented CL up to D3.

The results for estrus behavior, ovulation, and fertility are shown in Table 1. The estrus response rate and ovulation considering only ewes in anestrus at the beginning of the treatment were similar ($P > 0.05$) among groups, respectively: $G_{6\ days}$ (33.3; 66.7 %), $G_{9\ days}$ (66.7; 66.7 %), and $G_{12\ days}$ (66.7; 100 %). No difference was found ($P > 0.05$) in the estrus response rate between anestrus (55.5 %; 5/9) and cyclic ewes (82.6 %; 19/23). Likewise, the ovulation rate was similar ($P > 0.05$) between anestrus (77.8 %; 7/9) and cyclic ewes (82.6 %; 19/23). One female from $G_{6\ days}$ and one from $G_{12\ days}$ treatment ovulated until 84 h after sponge removal without estrous behavior; interestingly, both were observed in anestrus at the beginning of the experiment. The coefficient of variation for $G_{6\ days}$, $G_{9\ days}$, and $G_{12\ days}$ was, respectively, for estrus duration (35.85, 30.54, and 48.29 %), interval from sponge removal to estrus (19.41, 24.81, and 47.33 %), interval from sponge removal to ovulation (15.49, 14.76, and 19.70 %), interval from estrus to ovulation (23.67, 04.15, and 36.45 %), and diameter of the largest follicle (7.78, 10.33, and 4.44 %).

No difference was found ($P > 0.05$) in the plasma progesterone concentrations among the treatments throughout the experiment (Fig. 1). Similarly, no difference was found ($P > 0.05$) among the treatments when compared to D0, the day of prostaglandin and eCG administration (D5, D8, and D11, according to the treatment), and the day of sponge removal.

Experiment 2

Two ewes were removed from ultrasonography assessments due to deep positioning of their uterus and ovaries in the pelvic-abdominal area, with a poor quality image.

Table 1 Reproductive end points from Santa Inês ewes subjected to estrus induction receiving progestagen sponges for 6 ($G_{6\ days}$), 9 ($G_{9\ days}$), or 12 ($G_{12\ days}$) days

End points	Experimental groups		
	$G_{6\ days}$	$G_{9\ days}$	$G_{12\ days}$
Estrus (%)	72.7 (8/11)	72.7 (8/11)	80.0 (8/10)
Ovulation (%)	81.8 (9/11)	72.7 (8/11)	90 (9/10)
Estrus duration (h)	39.0 ± 14.0 (8) [12–60 h]	42.0 ± 12.8 (8) [36–72 h]	42.0 ± 20.3 (8) [12–72 h]
Interval from sponge removal to estrus (h)	46.0 ± 8.9 (8)[a] [29.5–53.5 h]	31.0 ± 7.7 (8)[b] [17.5–41.5 h]	32.5 ± 15.4 (8)[b] [17.5–65.5 h]
Interval from sponge removal to ovulation (h)	70.4 ± 10.9 (9) [56.9–83.2 h]	58.7 ± 8.7 (8) [44.1–70.9 h]	63.5 ± 12.5 (9) [42.9–80.3 h]
Interval from estrus to ovulation (h)	25.9 ± 6.1 (8) [15.4–29.7 h]	27.7 ± 1.1 (8) [26.5–29.4 h]	28.9 ± 10.5 (8) [14.5–49.9 h]
Diameter of the largest follicle (mm)	5.8 ± 0.5 (9)[b]	6.3 ± 0.7 (8) [a]	6.2 ± 0.3 (9)[a]
Diameter of the 2nd largest follicle (mm)	4.4 ± 0.9 (4)	4.4 ± 0.9 (7)	4.9 ± 1.2 (6)
Number of ovulations	1.3 ± 0.5 (9)	1.6 ± 0.5 (8)	1.4 ± 0.5 (9)
Fertility rate (%)	45.5 (5/11)	36.4 (4/11)	20.0 (2/10)

() Number of animals. [ ] Range. Different letters in the same line indicates statistical difference ($P < 0.05$)

Nevertheless, these animals were evaluated for estrus detection and their data were utilized. Reproductive end points regarding estrus and those obtained by ultrasonography are summarized in Table 2. Three out of 18 ewes which showed estrus behavior after the first dose were already in estrus in the first estrus detection time (24 h after the first prostaglandin dose), impairing their determination of onset of estrus. The variables of interval of prostaglandin dose to ovulation, interval of onset of estrus to ovulation, and largest and second largest follicle diameters were evaluated in a different manner between the first and second prostaglandin doses; thus, only a descriptive approach was made on these data. There was no difference ($P > 0.05$) in the diameter of the largest follicle after the second prostaglandin administration between the ewes detected in diestrus at D0 (6.4 ± 0.3 mm) and those in other phases of the estrous cycle at D0 (6.3 ± 0.7 mm).

The plasma progesterone concentrations throughout the treatment are shown in Fig. 2. It is important to highlight that all the ewes were cyclic during the assay, with each ewe showing progesterone concentration ≥1 ng/mL at least for 1 day during the experiment. A significant difference in progesterone concentration between the ewes detected in diestrus at D0 and those in other phases of the estrous cycle occurred over time.

Discussion

In the first experiment, the reproductive seasonality degree observed is compatible with that reported by Balaro et al. (2014) in Santa Inês ewes in the Brazilian Southeast. Mode B ultrasonography assessment was reliable since it corroborated with the plasma progesterone concentrations to define the status of cyclicity in the ewes at the start of the experiment. However, it is known that

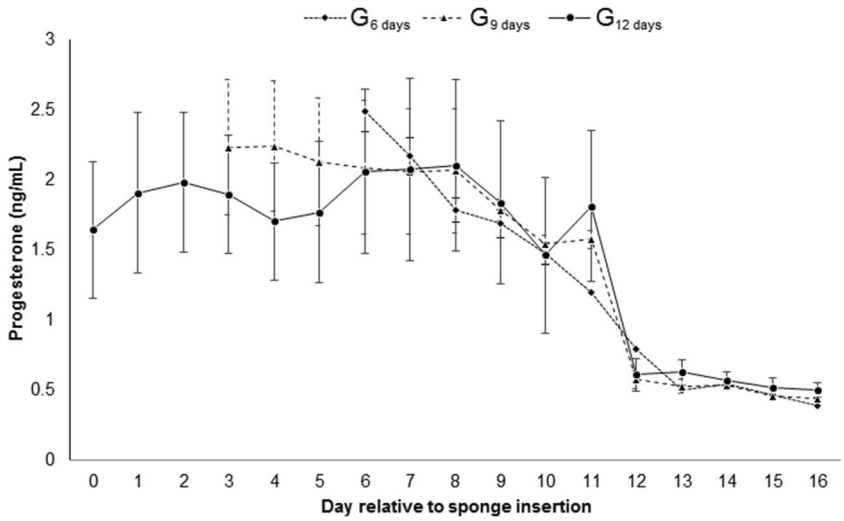

Fig. 1 Plasma progesterone concentrations in Santa Inês ewes submitted to estrus synchronization with MAP-impregnated sponges maintained for 6 (G6 days), 9 (G9 days), or 12 (G12 days) days. ANOVA/Kruskal-Wallis ($P > 0.05$)

Table 2 Reproductive end points of Santa Inês ewes subjected to estrus synchronization after the first or second dose of cloprostenol (PGF2α) administered 7 days apart during the breeding season

End points	Dose of PGF2α	
	First	Second
Estrus response rate (%)	78.3 (18/23)[a]	100 (23/23)[b]
Interval from PGF2α to estrus (h)	39.5 ± 10.6 (15) [26.8–50.8]	36.1 ± 9.5 (23) [26.8–50.8]
Estrus duration (h)	34.4 ± 7.7 (15) [24.0–48.0]	39.1 ± 10.4 (23) [24.0–60.0]
Ovulation rate (%)	85.7 (18/21)	100.0 (21/21)
Interval from PGF2α to ovulation (h)	66.9 ± 24.4 (18) [58.8–85.2 h]	69.6 ± 7.8 (21) [53.1–87.7 h]
Interval from estrus to ovulation (h)	39.0 ± 8.3 (15) [32.1–58.5 h]	33.1 ± 7.3 (21) [25.7–50.7 h]
Diameter of the largest follicle (mm)	5.9 ± 0.7 (18)	6.3 ± 0.5 (21)
Diameter of the 2nd largest follicle (mm)	4.6 ± 0.8 (16)	4.7 ± 0.9 (20)
Number of ovulations	*	1.7 ± 0.5 (21)

( ) Number of animals. [ ] Range. * Not measured. Different letters in the same line indicates statistical difference ($P < 0.05$)

the identification of corpus luteum is impaired during the period from luteolysis to the conclusion of a new luteinization. Thus, serial ultrasonography assessments should be performed taking into account the pattern of sheep estrous cycle for efficient determination of their cyclic ovarian activity.

Estrus response rate was on average 75 %, with no statistical difference among all treatments. This value is slightly inferior to those reported by other groups (Santos et al. 2010; Silva et al. 2010). The ovulation rate did not differ among the groups (~81 %). The ovulation rate did not differ between ewes in anestrus at the beginning of the experiment and those with cyclic ovarian activity at the same period. These data indicate that all three treatments were equally efficient in synchronizing and inducing estrus and promoting ovulation. Regarding its efficiency for anestrus ewes, it can be explained by the use of eCG, providing a suitable support of gonadotropins needed for final follicular development, and by the use of the progestogen device itself, proportioning a favorable hormonal environment for the follicular development even in a context of low endogenous progesterone concentrations (Levya et al. 1998).

Regarding the distribution of the ovulation time, it is noteworthy that $G_{9\ days}$ showed a lower dispersion of interval from sponge removal to ovulation and interval from estrus to ovulation when compared to the other groups. The coefficient of variation of $G_{9\ days}$ for these two variables was the only one lower than 15 %, with a minimal dispersion in the interval from estrus to ovulation (4.15 %). Considering the use of timed artificial insemination (TAI), the G_{9days} treatment would be suggested, since when using TAI it is extremely desirable that ovulation occurs in a minimal range. Moreover, in reproductive programs where AI is performed only after estrus detection, a higher concentration of AI procedures in a short time interval would also be preferred. Thus, the G_{9days} treatment can be considered the more suitable for both TAI programs and AI after estrus detection.

The greater interval from sponge removal to estrus and the lower follicular diameter detected in $G_{6\ days}$ when compared with those in $G_{9\ days}$ and $G_{12\ days}$ can be attributed to a greater follicular turnover proportionated by increased progestogen concentrations to which the ewes of $G_{6\ days}$ were subjected throughout the treatment. Although no difference has been found in the

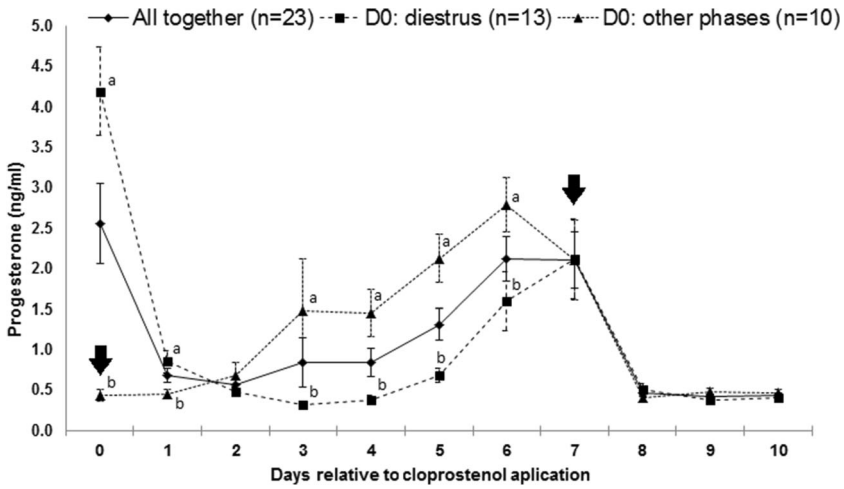

Fig. 2 Plasma progesterone concentrations in Santa Inês ewes submitted to estrus synchronization with two doses of prostaglandin 7 days apart. *Arrows* indicate application of prostaglandin. *Lowercase letter* indicates difference at $P < 0.05$. (ANOVA/Kruskal-Wallis)

endogenous progesterone concentrations among the treatments, it is known that after 6 days the progestogen concentrations fall to subluteal values (Rubianes and Menchaca 2003). An increased progestogen concentration reduces LH pulsatility, resulting in an increased follicular turnover (Levya et al. 1998), leading to a lower follicular diameter and more time needed for determination of estrus behavior by the ovulatory follicle.

Pregnancy rates did not differ among the treatments, but lower rates (~34 %) were obtained in comparison with other studies that have used MAP-synchronized ewes (Santos et al. 2010). Nevertheless, animals subjected to stressing conditions, as maybe has occurred in the present study due to twice daily ultrasonography and manipulation, have cortisol secretion increased which admittedly affects the hypothalamic-pituitary axis secretion of gonadotropins in ruminants (Dobson et al. 2000).

In the second experiment, taking into account the estrus response rate after the second dose of prostaglandin, it can be concluded that 7 days of interval is sufficient to promote the formation of a responsive corpus luteum, thus allowing great synchronization. The season in which the experiment was carried out as well as the full availability of forage with good nutritional content were certainly factors that contributed to the high synchronization rate reached after the second prostaglandin dose. The interval from the second dose to estrus was shorter than that described by some authors, like Silva et al. (2010) and Godfrey et al. (1997) using 9 and 10 days of interval, respectively, and similar to Menchaca et al. (2004), which also used 7 days. These differences can be due to the phase of the estrous cycle and follicular development found during the time of prostaglandin application. This variability observed among several studies in the interval from prostaglandin to estrus is directly reflected in the interval from prostaglandin to ovulation, generating an obstacle for the adoption of TAI in these protocols.

At the time of the first dose of prostaglandin in a group of ewes with cyclic ovarian activity, some animals can be observed in proestrus and estrus, thus reducing the interval to ovulation after the first dose in these animals and increasing the dispersion of the ovulation. Obviously, this fact prevents TAI after a single dose of prostaglandin.

Plasma progesterone concentration fluctuation throughout the treatment is similar to that found by Fierro et al. (2011). Ewes in diestrus at D0 are subjected to a hormonal environment of low progesterone during the phase of follicular development before the second dose of prostaglandin, due to the luteal regression promoted by the first dose. Meanwhile, the ewes that were in other phases of estrous cycles in D0 (low progesterone in D0) showed higher progesterone concentrations during the phase of follicular development before the second dose, getting higher values in 4 of 5 days before the second dose. The low plasma progesterone concentration in D0 can be explained by the phase of estrous cycles in which the ewes were at the time of the first dose (proestrus, estrus, metaestrus).

In a natural estrous cycle, the first follicular wave after the luteal regression develops under a hormonal environment of low progesterone, determining prolonged follicular development, in addition to a greater diameter reached by the largest follicle of this wave when compared to the waves that emerge during diestrus, with the exception of the ovulatory wave (Bartlewski et al. 2011). Thus, in a protocol of two doses of prostaglandin 7 days apart, some ewes will be subjected to subluteal progesterone concentration during the phase of follicular development (ewes at diestrus in D0), and in other ewes the follicular development will be natural, in an environment of low plasma progesterone concentration, which provides prolonged follicular persistence, as occurs with the first follicular wave post-ovulation (ewes in other phases of estrous cycle in D0).

Conclusion

The use of progestogen device for 9 days promotes lower dispersion of ovulation when compared to its use for 6 or 12 days, making this protocol more suitable for TAI and AI after estrus detection. The protocol of two doses of prostaglandin 7 days apart synchronizes estrus efficiently but results in follicular development under low progesterone concentrations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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