Animal Reproduction Science 174 (2016) 80–86

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Follicular wave emergence in Santa Inês ewes subjected to long-term, progesterone-based estrous synchronization protocols at different times of the year

M.E.F. Oliveira[a,*], H. Ayres[b], L.G. Oliveira[c], E. Oba[d], R.T. Kridli[e,f], P.M. Bartlewski[e], J.F. Fonseca[g], S.D. Bicudo[d], W.R.R. Vicente[a]

[a] Department of Preventative Veterinary Medicine and Animal Reproduction, College of Agricultural and Veterinary Sciences, São Paulo State University, Jaboticabal, SP, 14.884-900, Brazil
[b] Department of Animal Reproduction, College of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, SP, 05508-270, Brazil
[c] Department of Clinical and Veterinary Surgery, College of Agricultural and Veterinary Sciences, São Paulo State University, Jaboticabal, SP, 14.884-900, Brazil
[d] Department of Animal Reproduction and Veterinary Radiology, Faculty of Veterinary Medicine and Animal Science, São Paulo State University, Botucatu, SP, 18.618-970, Brazil
[e] Department of Biomedical Sciences, University of Guelph, Guelph, ON, Canada
[f] Department of Animal Production, Jordan University of Science & Technology, Irbid, Jordan
[g] Brazilian Agricultural Research Corporation-Embrapa Goats and Sheep, Coronel Pacheco, MG, 36.155-000, Brazil

ARTICLE INFO

Article history:
Received 27 April 2016
Received in revised form 6 September 2016
Accepted 8 September 2016
Available online 9 September 2016

Keywords:
Ewe
Follicular dynamics
Estrus synchronization
Progesterone
Superovulation

ABSTRACT

This study was conducted to document the pattern of antral follicular wave emergence throughout the 14-day, progesterone (P_4)-based estrous synchronization protocol in ewes that were maintained in subtropical conditions, during the period of increasing day lengths (ID), decreasing day lengths (DD), and the transitional period (TP). In addition, the influence of ovarian status (i.e., size of ovarian antral follicles and the presence of corpora lutea) at the outset of P_4 treatment on ensuing ovarian follicular wave development was examined. Sexually mature Santa Inês ewes ($n = 70$) were subjected to one of the two estrous synchronization protocols in the three seasons. On Day 0, the ewes received an i.m. injection of prostaglandin $F_{2\alpha}$ and an intravaginal P_4-releasing device that remained in place for 14 days (G-1CIDR) or was replaced on Day 7 (G-2CIDR). Daily ultrasonography of ovaries was conducted from Days 0 to 15. Mean (±SEM) numbers of follicular waves per ewe were 3.7 ± 0.1 and 3.6 ± 0.1 for G-1CIDR and G-2CIDR ($P > 0.05$). The number of emerging follicular waves was greater ($P < 0.05$) during the ID period than during the TP and DD periods (4.0 ± 0.1, 3.4 ± 0.1 and 3.6 ± 0.1, respectively). The presence of medium-sized antral follicles (4.0 to 5.75 mm) in the absence of corpora lutea at the time of CIDR insertion tended to advance follicular wave emergence. Although the long-term P_4 treatment was not originally designed to synchronize follicular waves, there was a distinctive pattern of follicular wave dynamics during the period of application of CIDRs that was affected mainly by the number of emerging follicular waves and ovarian status at CIDR insertion.

* Corresponding author at: Department of Preventative Veterinary Medicine and Animal Reproduction, College of Agricultural and Veterinary Sciences, São Paulo State University, Jaboticabal, São Paulo, Via de acesso Professor Paulo Donato Castellane, s/n, 14.884-900, Brazil.
E-mail address: m.emiliafraoli@fcav.unesp.br (M.E.F. Oliveira).

http://dx.doi.org/10.1016/j.anireprosci.2016.09.008
0378-4320/

1. Introduction

Multiple ovulation and embryo transfer (MOET) programs in sheep have successfully been implemented in many parts of the world (Menchaca et al., 2010). At present, the imperfections of superovulatory protocols are seemingly the major reason for the great variability in the ovulatory responses and embryo yields; this apparent drawback being arguably one of the primary factors limiting the widespread application of MOET in sheep (Oliveira, 2011). A typical superovulatory treatment regimen begins with the estrous synchronization using a 12 to 14 day treatment with progestagen (Fonseca et al., 2007). In spite of the unquestionable benefits of the hormonally induced estrous synchronization (Sir and Bartlewski, 2009), there are potential undesirable effects of the prolonged progestagen treatment in ewes. During the use of progesterone (P_4)-releasing intravaginal devices, circulating concentrations of the steroid are not sustained and there is a rapid decrease in serum P_4 concentrations before the end of the long-term treatment (between days 10 and 14; Letelier et al., 2009). Varying P_4 concentrations may alter the pattern of antral follicular emergence and growth, which in turn may influence ovulation, fertilization processes and subsequent embryo development (González-Bulnes et al., 2005). One of the strategies employed to circumnavigate these effects of the 14-day estrous synchronization protocols on antral follicular kinetics is to replace the source of P_4, approximately half-way through the period of treatment, to avoid the occurrence of less than normal mid-luteal phase concentrations of the hormone (González-Bulnes et al., 2002; Oliveira et al., 2012).

The number and size of ovarian antral follicles at the beginning of the super-ovulatory treatment is another factor that can impinge on the ovarian responses in donor ewes. With the use of traditional super-ovulatory protocols, 70% to 85% of donors have large antral follicles on the first day of follicle-stimulating hormone (FSH) administration (Menchaca et al., 2007). The presence of large follicles may alter the timing of the preovulatory luteinizing hormone surge (D'Occhio et al., 1999), ovulatory response as well as embryo yields and quality (Veiga-Lopez et al., 2006; López-Alonso et al., 2005). Alternatively, super-ovulatory treatments initiated at or around the time of follicular wave emergence result in greater and less variable ovulatory responses and embryo yields compared with the treatments begun in the presence of a large, growing antral follicle(s) from the previous wave(s) (Menchaca et al., 2007).

There has been no earlier study of antral follicular dynamics during the long-term CIDR treatments, and no report of photoperiodic and ovarian influences on follicular wave emergence during P_4-based, estrous synchronization in ewes maintained in subtropical conditions. The Santa Inês is a meat breed of wool-less sheep originating in Brazil (Rajab et al., 1992). It was derived from crosses of the Morada Nova, Bergamasca, and native coarse-wool sheep, and is very well adapted to tropical and subtropical climates. There is a paucity of information on the reproductive characteristics of Santa Inês sheep; however, it is possible that some Santa Inês ewes exhibit some degree of sensitivity to photoperiodic changes and undergo anestrus in southeastern Brazil (Balaro et al., 2014).

Therefore, the present study was undertaken to describe and compare the antral follicular wave kinetics during the 14-day period of applying estrous synchronization protocols, with or without CIDR replacement, in ultrasonographically monitored Santa Inês ewes at three distinctive times of the year (a period of decreasing and increasing day lengths as well as the transitional period characterized by relatively stable, long photoperiods, as described by López-Alonso et al., 2005). In addition, the influence of ovarian status (e.g., the size of antral follicles and/or presence of corpora lutea) was examined at the outset of P_4 treatment on the ensuing pattern of follicle wave emergence.

2. Material and methods

2.1. Location, animals and experimental procedures

The present study was conducted in the College of Agricultural and Veterinary Sciences (FCAV) situated in the municipality of Jaboticabal (latitude: 21°15'18"S, longitude 48°19'19"W), São Paulo State, Brazil. In this particular region, there exist three distinctive periods of the year characterized by varying duration of the day length: i. a period from the beginning of winter to the beginning of summer, or between July and November – a period of increasing day lengths; ii. from late summer to early winter, or between March and June – a period of decreasing day lengths; and iii. transitional period (during the summer or between December and February) with relatively consistent long days (López-Alonso et al., 2005).

All experimental procedures were compliant with the guidelines on the Ethics and Animal Welfare, and had been approved by the animal care committee of the College of Agricultural and Veterinary Sciences (FCAV), São Paulo State University "Júlio de Mesquita Filho" (protocol no. 003261-08). Sexually mature ($n = 70$) and clinically healthy Santa Inês ewes (aged between 2 and 3 years, mean (±SEM) body weight of 41.4 ± 2.9 kg) were used in the present study. The ewes were subjected to one of two synchronization protocols at three different times of the year (2 × 3 factorial design; a period of increasing day lengths (ID): G-1CIDR, $n = 12$ and G-2CIDR, $n = 11$; transitional period (TP): G-1CIDR, $n = 12$ and G-2CIDR, $n = 12$; and a period of decreasing day lengths (DD): G-1CIDR, $n = 11$ and G-2CIDR, $n = 12$). Different animals were used during each reproductive phase. Animals were maintained in paddocks with easy access to sheds and were exposed to natural photoperiods and ambient temperatures. Animals were fed corn silage and nutrient balanced feed (200 g/ewe/day) twice daily, and had ad libitum access to water and mineral salt licks.

On Day 0 (random day of the estrous cycle or anovulatory period), all animals were fitted with an intravaginal P_4-releasing device (CIDR™; Pfizer, Austin, New Zealand), which was maintained in place for 14 days (G-1CIDR), and received an i.m. injection of 10 mg of prostaglandin $F_{2\alpha}$ (Lutalyse™; Pfizer, Austin, New Zealand). In the three subsets of animals, the CIDR's were replaced on Day 7

(G-2CIDR); these procedures were repeated in all three periods.

2.2. Ultrasonographic technique

Ovarian follicular wave dynamics and status were assessed by ultrasonography that was performed daily from Days 0 to 15. Transrectal ultrasonography was conducted using a portable B-mode scanner (Aquila; Esaote Group, Pie Medical Imaging, Maastricht, Holland) connected to a stiffened, variable frequency (6–8-MHz) linear-array transducer. One experienced operator performed all examinations. Ewes were examined in a standing position, and the abdominal wall was compressed to facilitate visualization of the uterus and ovaries. The rectum was lubricated with hydro-soluble contact gel prior to insertion of the probe. The transducer was then positioned perpendicular to the abdominal wall and the urinary bladder was identified to determine the location of the uterus. The probe was subsequently rotated laterally to detect both ovaries.

Ovarian follicles were measured using internal electronic calipers and the number, diameter and position of all antral follicles ≥2 mm were sketched on ovarian charts. The mean diameter (average of two dimensions: vertical and horizontal) of each follicle was determined from images containing the largest cross sectional area. A follicular wave was defined as a follicle or a group of follicles 2 to 3 mm in diameter that grew to ≥4.5 mm in size before regression or ovulation. The day of follicular wave emergence was regarded as the day on which the largest follicle of a wave was first detected at 2 or 3 mm (retrospective analysis).

2.3. Hormone assays

Jugular blood samples were collected immediately before each ultrasonographic examination to determine serum concentration of P_4. Blood samples (10 mL) were collected into evacuated tubes without anti-coagulants (Becton Dickinson Diagnostics; São Paulo, Brazil) and labeled with the animal number and collection date. All samples were then centrifuged at 3000 × *g* for 15 min and the sera were separated into aliquots properly marked and stored at −20 °C until the assay for quantitation was conducted. The P_4 concentrations were quantified using a Coat-A-Count RIA kit (Coat-A-Count Progesterone®; Siemens, Washington, DC, USA). The sensitivity of the assay was 0.005 ± 0.001 ng/mL, and the inter- and intra-assay coefficients of variation were 1.0% and 1.0%, respectively.

2.4. Statistical analyses

Statistical analyses were performed using SAS software version 9.2 (2002–2011). Tests for normality of residuals and homogeneity of variances were initially conducted using the SAS Guide Data Analysis. Ovarian and endocrine data were analyzed separately by the logistic regression using Proc GLIMMIX. The input variables included in the model were: treatment group, season, group by season interaction, day of treatment (for serum P_4 concentrations), number of follicular waves during the treatment, and ovarian status on Day 0 (1: presence of corpus luteum/corpora lutea (CL) + large antral follicle(s) (LF: ≥6 mm in diameter); 2: presence of CL + medium-sized antral follicle(s) (MSF: 4.0 to 5.75 mm); 3: presence of CL + small antral follicles (SF: 2.0 to 3.75 mm); 4: absence of CL + LF; 5: absence of CL + MSF; 6: absence of CL + SF). For the analysis of serum P_4 concentration, data were partitioned into two periods (Days 0 to 7 and Days 8 to 15). The variables included in the statistical model were: group, season, days of treatment, and the interaction between group and days of treatment. In a final regression model, non-significant variables were removed based on a criterion of Wald ($P > 0.20$). In the absence of significant interactions, only the main effects were analyzed. The significance level for differences between mean values was set at $P < 0.05$.

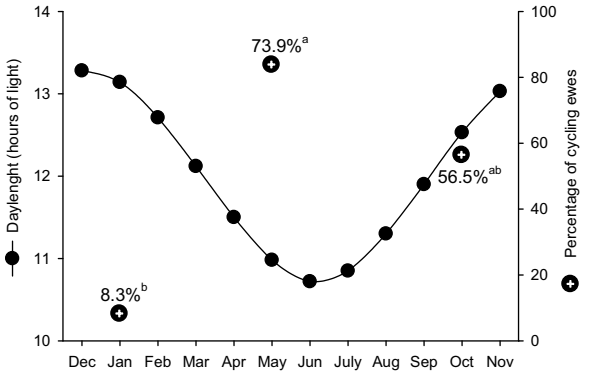

Fig. 1. Average durations of monthly day lengths (day light hours), and the percentage of ewes with P_4 concentrations >1 ng/mL and ultrasonographically detectable luteal structures (i.e., estrous cycling ewes) on the day of insertion of intravaginal controlled drug release (CIDR) devices. Information on annual changes in day lengths was provided by the Agroclimatology of FCAV/UNESP, 2009–2010. $^{ab}P < 0.05$ (χ^2 test).

3. Results

On the basis of the ultrasonographic examinations and serum P_4 measurements, it was determined that the percentage of cycling ewes was 83.9% (17/23) during the period of decreasing day lengths (DD), 56.5% (13/23) during the period of increasing day lengths (ID), and 8.3% (2/24) during the transitional period (TP) ($P < 0.05$; Fig. 1).

Circulating P_4 concentrations were influenced by the time of the year and the type of estrous synchronization protocol (G-1CIDR vs. G-2CIDR groups). Mean serum concentrations of P_4 were generally greater ($P < 0.05$) during the DD period (3.41 ± 0.14 ng/mL) compared with the two other periods (ID: 2.19 ± 0.14 ng/mL; and TP: 1.82 ± 0.14 ng/mL). Concentrations increased 1 day after CIDR insertion and then gradually decreased ($P < 0.05$) in all ewes by Day 7 (Day 0 = first insertion of CIDR); no differences ($P > 0.05$) in serum P_4 concentrations were detected from Days 0 to 7 among the treatment groups or three seasons. In the G-1CIDR group, P_4 concentrations continued to decrease ($P < 0.05$) until CIDR withdrawal on Day 14, while in the G-2CIDR group P_4 concentrations were increased on Day 8 after CIDR replacement on Day 7, followed by a

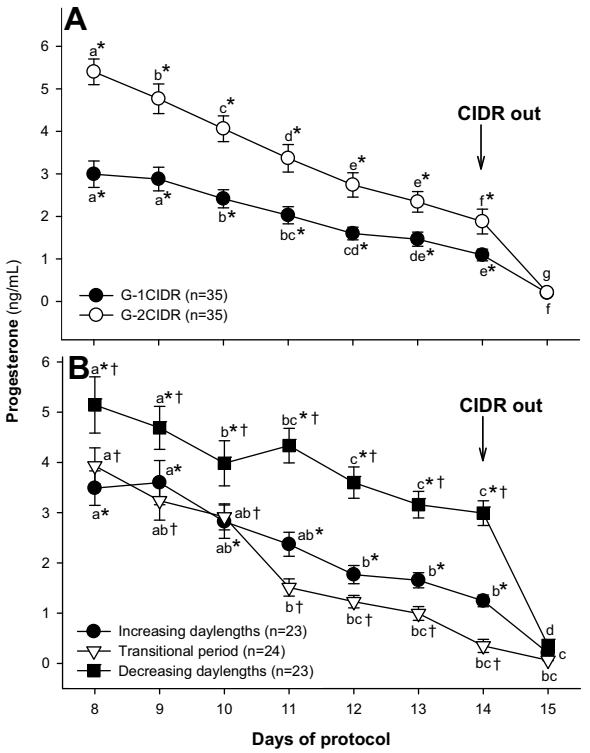

Fig. 2. Mean (±SEM) daily serum concentrations of P_4 in blood samples collected from Days 8 to 14 of the estrous synchronization (G-1CIDR (●): animals subjected to the 14-day estrous synchronization protocol without CIDR replacement and G-2CIDR (○): the protocol with CIDR replacement on Day 7) in Santa Inês ewes during the increasing and decreasing photoperiods, and the transitional period. Day 0 = day of first CIDR insertion in both groups of animals. Different letters denote significant differences between days and mean values denoted by the same symbols (*†) differ between the two groups within days ($P < 0.05$).

gradual decrease ($P < 0.05$) until Day 14. Ewes of the G-2CIDR group had greater ($P < 0.05$) P_4 concentrations as compared with the ewes of the G-1CIDR group from Days 8 to 14. By Day 15 (1 day after CIDR withdrawal), serum P_4 concentrations decreased to a basal or non-detectable concentrations and did not vary ($P > 0.05$) between the G-1CIDR and G-2CIDR groups (Fig. 2).

The ewes of the present study had two to five emerging follicular waves during the treatment period [two waves: 4.3% (3/70); three waves: 34.3% (24/70); four waves: 52.9% (37/70); and five waves: 8.6% (6/70)]. The mean numbers of follicular waves were not affected ($P > 0.05$) by the type of CIDR treatment (3.7 ± 0.1 and 3.6 ± 0.1 for G-1CIDR and G-2CIDR groups, respectively). However, the number of follicular waves during the 14-day estrous synchronization period was greater ($P < 0.05$) during the ID period than during the TP and DD periods (4.0 ± 0.1, 3.4 ± 0.1 and 3.6 ± 0.1, respectively). There was a significant main effect of ovarian status on the day of CIDR removal on the number of emerging follicular waves during the ensuing 14-day period (1: 3.2 ± 0.2^{ab}; 2: 3.9 ± 0.1^{a}; 3: 3 (1 ewe); 4: 3.6 ± 0.2^{ab}; 5: 3.8 ± 0.1^{a} and 6: 3.0 ± 0.3^{b}; different letter superscripts denote significant differences between the subsets of ewes studied).

There was no significant main effect of group or season, and the interaction of these terms was not significant either for the days of follicular wave emergence (Table 1). However, there were significant differences in mean days of follicular wave emergence among ewes varying in the number of emerging waves of follicular development (Table 2) as well as due to ovarian status on Day 0 (day of CIDR insertion or beginning of the estrous synchronization protocol; Table 3). Generally, the first wave of follicular development was observed after CIDR insertion (Wave 1) emerged on average 1 or 2 days later ($P < 0.05$) in animals with 2 or 3 waves of follicular development per 14-day treatment period, and the timing of emergence for Waves 2, 3 and 4 appeared to be inversely related to and differed ($P < 0.05$) among the subsets of ewes varying in the number of emerging waves (Table 2). The emergence of Waves 1, 2 and 3 occurred approximately 2 days later in ewes that only had small antral follicles (ovarian status 6) or a luteal structure and at least one ovulatory-sized follicle (ovarian status 1) compared with the ewes that only had medium-sized ovarian follicles (ovarian status 5) on the first day of the application of the estrous synchronization protocol; with the exception of Wave 2 in the ewes with CL and ostensibly ovulatory-sized follicles there were differences ($P < 0.05$). Moreover, Wave 4 emerged ~1.5 days later in ewes with CL and medium-sized follicles (ovarian status 2) than in animals with medium-sized follicles but no detectable CL on Day 0 (ovarian status 5; $P < 0.05$, Table 3).

The interval between follicular Waves 3 and 4 (i.e., waves emerging on the average between days 9 and 13 of application of the estrous synchronization protocol) was longer in G-1CIDR compared with G-2CIDR groups, and it was longer in anestrous ewes compared with animals studied during the breeding season and transitional period (Table 1). The interval between the first two waves of ovarian follicular development in the study period was greater ($P < 0.05$) in ewes with two waves of follicular development compared with all other animals, and the interval between Waves 2 and 3 was greater ($P < 0.05$) in ewes with three waves compared with animals that had five emerging waves during the estrous synchronization regimen (Table 2). Finally, the interval between Waves 2 and 3 was greater in ewes with ovarian status 1 (CL + large ovarian follicle) compared with in ewes with ovarian status 4 (no CL + large ovarian follicle on the first day of the estrous synchronization protocol).

4. Discussion

Serum concentrations of P_4 in the present study were within the ranges observed during the course of CIDR-based estrous synchronization protocols in several previous studies (Oliveira et al., 2016). The replacement of CIDRs resulted in an increase in circulating concentrations of P_4 (>2 ng/mL) until the end of the treatment (Day 14), which eliminated the potential effects of low P_4 concentrations on antral follicular lifespan (Letelier et al., 2009; González-Bulnes et al., 2005). The present experimental protocol involved the administrations of prostaglandin $F_{2\alpha}$ on Day 0; although luteolytic doses of exogenous prostaglandin may occasionally induce premature

Table 1
Mean (±SEM) days of follicular wave emergence during the 14-day estrous synchronization period according to the type of the protocol used and the duration of day lengths. See text for the details of experimental design and statistical comparisons. Day 0 = day of CIDR insertion or beginning of the estrous synchronization protocol. G-1CIDR: protocol without CIDR replacement during the 14-day estrous synchronization regimen and G-2CIDR: protocol with CIDR replacement on Day 7. The numbers of ultrasonographically detected follicular waves are given in parentheses (columns 2–6). [ab]Mean values denoted by different letter superscripts are different ($P < 0.05$). ND: not detected.

Group Time of year	Days of wave emergence					Inter-wave intervals (days)			
	Wave 1	Wave 2	Wave 3	Wave 4	Wave 5	1–2	2–3	3–4	4–5
G-1CIDR (n = 35)	2.0 ± 0.3 (35)	5.9 ± 0.1 (35)	9.1 ± 0.32 (33)	11.9 ± 0.3 (23)	13.0 ± 0.6 (5)	3.9 ± 0.2	3.5 ± 0.2	3.5 ± 0.2[a]	2.6 ± 0.2
G-2CIDR (n = 35)	2.1 ± 0.3 (35)	5.9 ± 0.3 (35)	9.7 ± 0.3 (34)	12.1 ± 0.3 (20)	13.0 (1)	3.8 ± 0.2	3.9 ± 0.2	3.0 ± 0.2[b]	2.0
Increasing day lengths (July–November; n = 23)	2.0 ± 0.3 (23)	5.4 ± 0.3 (23)	8.8 ± 0.4 (23)	12.2 ± 0.3 (19)	13.4 ± 0.2 (5)	3.4 ± 0.2	3.5 ± 0.2	3.8 ± 0.2[a]	2.6 ± 0.2
Transitional period (December–February; n = 24)	2.0 ± 0.4 (24)	6.2 ± 0.4 (24)	9.5 ± 0.4 (22)	11.2 ± 0.4 (10)	11.0 (1)	4.1 ± 0.3	3.7 ± 0.2	2.8 ± 0.3[b]	2.0
Decreasing day lengths (March–June; n = 23)	2.1 ± 0.4 (23)	6.2 ± 0.4 (23)	10.0 ± 0.3 (22)	12.1 ± 0.3 (14)	ND	4.1 ± 0.3	3.9 ± 0.3	2.7 ± 0.2[b]	–

Table 2
Mean (±SEM) days of follicular wave emergence according to the number of follicular waves detected in individual Santa Inês ewes during the entire14-day estrous synchronization protocols at different times of the year. Day 0 = day of CIDR insertion or beginning of the estrous synchronization protocol. Numbers of ewes exhibiting the 2-, 3-, 4- or 5-wave pattern of follicular wave emergence are given in parentheses (column 1). [a–d]Within columns, mean values denoted by different letter superscripts are different ($P < 0.05$).

No. of waves	Day of wave emergence					Inter-wave intervals (days)			
	Wave 1	Wave 2	Wave 3	Wave 4	Wave 5	1–2	2–3	3–4	4–5
2 (*n* = 3)	3.7 ± 0. 7[a]	10.0 ± 0.0[a]	–	–	–	6.3 ± 0.7[a]	–	–	
3 (*n* = 24)	2.6 ± 0.4[a]	6.6 ± 0.3[b]	10.7 ± 0.3[a]	–	–	4.0 ± 0.3[b]	4.1 ± 0.3[a]	–	–
4 (*n* = 37)	1.7 ± 0.2[b]	5.4 ± 0.2[c]	9.0 ± 0.2[b]	12.2 ± 0.2[a]	–	3.8 ± 0.2[b]	3.6 ± 0.2[ab]	3.2 ± 0.2	–
5 (*n* = 6)	1.2 ± 0.4[b]	4.0 ± 0.5[d]	6.8 ± 0.5[c]	10.5 ± 0.4[b]	13.0 ± 0.5	2.8 ± 0.2[b]	2.8 ± 0.4[b]	3.7 ± 0.3	2.5 ± 0.2

Table 3
Mean (±SEM) days of follicular wave emergence during the14-day estrous synchronization period according to the ovarian status on Day 0 (day of CIDR insertion or beginning of the estrous synchronization protocol) in Santa Inês ewes at different times of the year. Day 0 = day of CIDR insertion or beginning of the estrous synchronization protocol. The ovarian status on Day 0 was as follows: (1: presence of corpus luteum/corpora lutea (CL) + large antral follicle(s) (LF: ≥6 mm); 2: presence of CL + medium-sized antral follicle(s) (MSF: 4.0 to 5.75 mm); 3: presence of CL + small antral follicles (SF: 2.0 to 3.75 mm); 4: absence of CL + LF; 5: absence of CL + MSF; 6: absence of CL + SF. The numbers of ultrasonographically detected follicular waves are given in parentheses (columns 2–6). [a–c]Within columns, mean values denoted by different letter superscripts are different ($P < 0.05$). ND: not detected; *Data withdrawn from statistical comparisons.

Day 0 ovarian status	Days of wave emergence					Inter-wave intervals (days)			
	Wave 1	Wave 2	Wave 3	Wave 4	Wave 5	1–2	2–3	3–4	4–5
Status 1 (*n* = 9)	3.0 ± 0.8[a] (9)	6.6 ± 0.8[ab] (9)	10.6 ± 0.8[a] (8)	11.7 ± 0.7[ab] (3)	ND	3.6 ± 0.5	4.5 ± 0.6[a]	3.3 ± 0.3	–
Status 2 (*n* = 13)	2.2 ± 0.3[ab] (13)	6.3 ± 0.3[ab] (13)	10.0 ± 0.4[a] (13)	12.9 ± 0.3[a] (11)	12 (1)	4.2 ± 0.3	3.7 ± 0.2[ab]	3.0 ± 0.3	3.0
Status 3 (*n* = 1)*	0 (1)	3 (1)	11 (1)	ND	ND	3.0	8.0	–	–
Status 4 (*n* = 9)	2.2 ± 0.6[ab] (9)	5.8 ± 0.5[abc] (9)	9.1 ± 0.5[ab] (9)	12.2 ± 0.5[ab] (5)	ND	3. 6 ± 0.3	3.3 ± 0.2[b]	3.8 ± 0.4	–
Status 5 (*n* = 32)	1.5 ± 0.2[b] (32)	5.4 ± 0.3[bc] (32)	8.7 ± 0.3[b] (31)	11.5 ± 0.3[b] (23)	12.8 ± 0.5 (5)	4.0 ± 0.2	3. 5 ± 0.2[ab]	3.3 ± 0.2	2.4 ± 0.2
Status 6 (*n* = 6)	3.3 ± 0.8[a] (6)	7.3 ± 0.7[a] (6)	10.6 ± 0.6[a] (5)	12 (1)	ND	4.0 ± 0.8	3.8 ± 0.2[ab]	2.0	–

follicle rupture (Barrett et al., 2002; Davies et al., 2006), no untimely ovulations were observed in the present study.

The average number of follicular waves emerging during the 14-day P_4 treatment in this study was similar to that during the luteal phase of the ewes' estrous cycle (two to five waves (Evans et al., 2000; Ginther et al., 1995)). Insertion of a new CIDR did not affect the total number of emerging follicular waves but it shortened the interval between waves emerging after CIDR replacement at the mid-treatment period (Waves 3 and 4). It is speculated that CIDR replacement may have shortened the interval between consecutive FSH peaks, thereby advancing the emergence of the next follicular wave (Baby and Bartlewski, 2011a,b). The number of emerging follicular waves was greater during the period when day length was increasing (ID) than during the transitional (TP) and period of decreasing day lengths (DD). In addition, the inter-wave interval between Waves 3 and 4 was longer during the ID period compared with both the TP and DD periods. However, mean serum concentrations of P_4 were greater in

ewes during DD than in ewes during the TP and ID periods, suggesting that seasonal suppression of follicle wave kinetics was not solely controlled by serum P_4concentrations. In sheep maintained in temperate climates Thieíry et al. (2003), found out that serum P_4 concentrations did not differ between varying day lengths following ovariectomy and CIDR treatment. However, in multiparous Sarda ewes (Italy), melatonin deficiency was associated with fewer emerging follicular waves during the induced estrous cycle, mainly because the average lifespan of all waves was longer in pinealectomized ewes compared with the control animals (Manca et al., 2014). Collectively, these observations may be interpreted to suggest that melatonin, in addition to its well-known effects on the secretion of GnRH, may directly influence the growth pattern of ovarian antral follicles.

The number of follicular waves was greater in animals with ovarian status 2 (presence of luteal structures and medium-sized antral follicle(s)) and 5 (absence of CL and presence of medium-sized ovarian follicle(s)) compared with the ewes with ovarian status 6 (absence of CL and presence of small antral follicles) at the outset of applying the estrous synchronization protocol. The emergence of Wave 1 was delayed in ewes with ovarian status 1 (presence of CL and large antral follicle(s)) and 6, while ovarian status 5 was characterized by an advanced emergence of Wave 1 follicles. The delayed wave emergence in the ewes with ovarian status 1 and 6 may be due to the inhibitory action of inhibin on FSH secretion Guthrie et al. (1992); observed a greater secretion of inhibin A by large follicles and small follicles compared with medium-sized follicles. The influence of the functional CL at the time of CIDR insertion and prostaglandin injection on subsequent FSH secretion and follicular wave emergence remains to be elucidated.

Current superovulatory protocols in sheep are associated with great variability in ovulatory responses and embryo yields (Bartlewski et al., 2008) because superovulatory treatments are usually initiated at a random stage of follicular wave lifespan. To achieve optimal results, the first dose of exogenous gonadotropins should be given on the day of follicular wave emergence (Bartlewski et al., 2008). The use of P_4-releasing intravaginal devices for up to 14 days has yielded variable serum P_4 concentrations, which may have altered the lifespan of large antral follicle and the rhythmicity of follicular wave emergence (Letelier et al., 2009; González-Bulnes et al., 2005). However, while the replacement of CIDR shortened the interval between Waves 3 and 4, it did not affect the number of emerging follicular waves or mean days of wave emergence. Thus, the insertion of new CIDR approximately half-way through the 14-day estrous synchronization treatment appears to be an unnecessary extra cost.

Based on the present results, future superovulatory treatment modalities should be more tailored to the season, expected number of emerging waves, and the ovarian status of ewes. There were more emerging follicular waves and the interval between Waves 3 and 4 was longer during the ID period compared with other times of the year. However, the photoperiod did not affect the timing of emergence for follicular Wave 2 (between Days 5 and 6 on the average) and Wave 3 (between Days 9 to 10 of the protocol). In spite of the seasonal differences in the total number of follicular waves emerging during the entire period of P_4 treatment, most of the ewes had a follicle wave emerging between Days 9 and 10.5. Therefore, it is proposed that there are advantages in starting an exogenous gonadotropin treatment on Days 9 or 10 after CIDR insertion to synchronize the beginning of the superovulatory treatment with follicular wave emergence in the majority of ewes. The most profound differences in the mean times of Wave 3 and 4 emergence were observed between animals with the ovarian status 5 (absence of CL and presence of medium-sized ovarian follicle(s)) and 6 (absence of CL and presence of small antral follicles). Therefore, superovulatory treatments should commence ~1 day later in ewes with ovarian status 6 as compared with those with ovarian status 5 (i.e., on Days 5 or 9 for ovarian status 5 and on Days 6 or 10 for ewes with ovarian status 6). In the remaining ewes, superovulatory protocols may be initiated on either of the 2 days corresponding to Wave 2 or Wave 3 emergence. Ultrasonographic examinations can be performed immediately prior to CIDR insertion and after the ewes have been classified based on ovarian status, superovulatory treatments could commence on the expected days of follicular wave emergence. Future studies are needed to compare the proposed superovulatory treatment regimens to the current,fully randomized regimens in terms of ovulatory responses and transferrable embryo yields. To recapitulate, although the long-term, P_4-based estrous synchronization protocols were not devised to synchronize follicular wave emergence in ewes, there was a distinctive and predictable pattern of wave emergence throughout the 14-day treatment with CIDRs in Santa Inês ewes. The days of follicular wave emergence were influenced mainly by the number/frequency of follicular waves and ovarian status on the day of CIDR insertion. The present observations may pave a way to developing new superovulatory protocols, which by considering ovarian activity in individual ewes and adjusting the timing of the superovulatory stimulation to synchronize the timing of treatments with follicular wave emergence would improve ovarian responses and embryo production.

Conflict of interest

None to declare.

Acknowledgements

The authors would like to thank the FAPESP (*Fundação de Amparo à Pesquisa do Estado de São Paulo*) for financial support in the form of an operating grant (process no 2008/05503-2) and a PhD scholarship awarded to MEFO (process no 2008/01665-8). MEFO, EO, JFF and WRRV were CNPq fellows. Thanks are also extended to the Jordan University of Science and Technology for funding the sabbatical leave of RTK. The present results were presented, in the preliminary form, at the Ludwig-Fraenkel Symposium in Wrocław, Poland (September 5–6, 2013).

References

Baby, T.E., Bartlewski, P.M., 2011a. Circulating concentrations of ovarian steroids and follicle-stimulating hormone (FSH) in ewes with 3 or 4 waves of antral follicle emergence per estrous cycle. Reprod. Biol. 11, 19–36.

Baby, T.E., Bartlewski, P.M., 2011b. Progesterone as the driving regulatory force behind serum follicle-stimulating hormone (FSH) concentrations and antral follicular growth in cyclic ewes. Reprod. Fertil. Dev. 23, 303–310.

Balaro, M.F.A., Fonseca, J.F., Oba, E., Cardoso, E.C., Brandão, F.Z., 2014. Is the Santa Inês sheep a typical non-seasonal breeder in the Brazilian Southeast? Trop. Anim. Health Prod. 46, 1533–1537.

Barrett, D.M.W., Bartlewski, P.M., Rawlings, N.C., 2002. Ultrasound and endocrine evaluation of the ovarian response to prostaglandin $F_{2\alpha}$ given at different times during the luteal phase in ewes. Theriogenology 58, 1409–1424.

Bartlewski, P.M., Alexander, B.D., Rawlings, N.C., Barrett, D.M.W., King, W.A., 2008. Ovarian responses, hormonal profiles and embryo yields in anoestrous ewes superovulated with Folltropin®-V after pretreatment with medroxyprogesterone acetate (MAP)-releasing vaginal sponges and a single dose of oestradiol-17β (E_2-17β). Reprod. Domest. Anim. 43, 299–307.

D'Occhio, M.J., Jillella, D., Lindsey, B.R., 1999. Factors that influence follicle recruitment, growth and ovulation during ovarian superstimulation in heifers: opportunities to increase ovulation rate and embryo recovery by delaying the exposure of follicles to LH. Theriogenology 51, 9–35.

Davies, K.L., Bartlewski, P.M., Epp, T., Duggavathi, R., Barrett, D.M.W., Bagu, E.T., Cook, S.J., Rawlings, N.C., 2006. Does injection of prostaglandin F2α (PGF2α) cause ovulation in anestrous Western White Face ewes? Theriogenology 66, 251–259.

Evans, A.C.O., Duffy, P., Hynes, N., Boland, M.P., 2000. Waves of follicle development during the estrous cycle in sheep. Theriogenology 53, 699–715.

Fonseca, J.F., Souza, J.M.G., Bruschi, J.H., 2007. Sincronização de estro e Superovulação em Caprinos e Ovinos. 2° Simpósio de Caprinos e Ovinos, Belo Horizonte/MG-UFMG, Anais, 167–195.

Ginther, O.J., Kot, K., Wiltbank, M.C., 1995. Associations between emergence of follicular waves and fluctuations in FSH concentrations during the estrous cycle in ewes. Theriogenology 43, 689–703.

González-Bulnes, A., Santiago-Moreno, J., Cocero, M.J., Souza, C.J.H., Groome, N.P., Garcia-Garcia, R.M., Lopez-Sebastián, A., Baird, D.T., 2002. Measurement of inhibin A predicts the superovulatory response to exogenous FSH in sheep. Theriogenology 57, 1263–1272.

González-Bulnes, A., Veiga-Lopez, A., Garcia, P., Garcia-Garcia, R.M., Ariznavarreta, C., Sanchez, M.A., 2005. Effects of progestagens and prostaglandin analogues on ovarian function and embryo viability in sheep. Theriogenology 63, 2523–2534.

Guthrie, H.D., Rohan, R.M., Rexroad, C.E., Cooper, B.S., 1992. Changes in concentrations of follicular inhibin alpha and beta A subunit messenger ribonucleic acids and inhibin immunoactivity during preovulatory maturation in the pig. Biol. Reprod. 47, 1018–1025.

López-Alonso, C., Encinas, T., Veiga-López, A., García-García, R.M., Cocero, M.J., Ros, J.M., McNeilly, A.S., González-Bulnes, A., 2005. Follicular growth, endocrine response and embryo yields in sheep superovulated with FSH after pretreatment with a single short-acting dose of GnRH antagonist. Theriogenology 64, 1833–1843.

Letelier, C.A., Contreras-Solis, I., García-Fernández, R.A., Ariznavarreta, C., Tresguerres, J.A.F., Flores, J.M., González-Bulnes, A., 2009. Ovarian follicular dynamics and plasma steroid concentrations are not significantly different in ewes given intravaginal sponges containing either 20 or 40 mg of fluorogestone acetate. Theriogenology 71, 676–682.

Manca, M.E., Manunta, M.L., Spezzigu, A., Torres-Rovira, L., Gonzalez-Bulnes, A., Pasciu, V., Piu, P., Leoni, G.G., Succu, S., Chesnau, D., Naitana, S., Berlinguer, F., 2014. Melatonin deprival modifies follicular and corpus luteal growth dynamics in a sheep model. Reproduction 147, 885–895.

Menchaca, A., Vilariño, M., Crispo, M., Pinczak, A., Rubianes, E., 2007. Day 0 protocol: superstimulatory treatment initiated in the absence of a large follicle improves ovarian response and embryo yield in goats. Theriogenology 68, 1111–1117.

Menchaca, A., Vilariño, M., Crispo, M., Castro, T., Rubianes, E., 2010. New approaches to superovulation and embryo transfer in small ruminants. Reprod. Fertil. Dev. 22, 113–118.

Oliveira, M.E.F., Cordeiro, M.F., Ferreira, R.M., Souza, S.F., Pieroni, J.S.P., Rodrigues, L.F.S., Fonseca, J.F., Vicente, W.R.R., 2012. Does supplemental LH changes rate and time to ovulation and embryo yield in Santa Inês ewes treated for superovulation with FSH plus eCG? Ciência Rural 42, 1077–1082.

Oliveira, M.E.F., Ayres, H., Oliveira, L.G., Barros, F.F.P.C., Oba, E., Bicudo, S.D., Bartlewski, P.M., Fonseca, J.F., Vicente, W.R.R., 2016. Effects of season and ovarian status on the outcome of long-term progesterone-based estrus synchronization protocols and ovulatory follicle development in Santa Inês ewes under subtropical conditions. Theriogenology 85, 452–460.

Oliveira, M.E.F., 2011. State-of-the-art in the superovulation of ewes. Acta Sci. Vet. 39, 65–70.

Rajab, M.H., Cartwright, T.C., Dahm, P.F., Figueiredo, E.A., 1992. Performance of three tropical hair sheep breeds. J. Anim. Sci. 70, 3351–3359.

Sir, C., Bartlewski, P.M., 2009. Analyses of parental and seasonal influences on the synchrony of dexamethasone-induced lambing and lamb characteristics. Livestock Sci. 131, 119–124.

Thieíry, J.C., Robel, P., Canepa, S., Delaleu, B., Gayrard, V., Picard-Hagen, N., Malpaux, B., 2003. Passage of progesterone into the brain changes with photoperiod in the ewe. Eur. J. Neurosci. 18, 895–901.

Veiga-Lopez, A., Gonzalez-Bulnes, A., Tresguerres, J.A.F., Dominguez, V., Ariznavarreta, C., Cocero, M.J., 2006. Causes, characteristics and consequences of anovulatory follicles in superovulated sheep. Domest. Anim. Endocrinol. 30, 76–87.