

## Ultrasonographic and Endocrine Characterization of Follicular Waves in Llamas with a Special Reference to the Overlapping Phenomenon During Successive Waves

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### Contents

Ovarian follicular growth in non-mated llamas occurs in successive waves that generally superimpose their origin on the regression of the preceding wave (overlapping), originating prolonged sexual receptivity in the species. The aim of this study was to perform an ultrasonographic and endocrine characterization of individual and successive waves in non-mated llamas with a special interest on the overlapping phenomenon. Twelve llamas were examined daily by transrectal ultrasonography for at least two consecutive waves. In six females, blood samples were collected daily at the end of each examination. The development of the largest follicle (F) showed a wavelike pattern with a mean duration of 25 days. All waves evaluated were partially overlapped on the preceding wave and emerged at a mean interval of  $15.8 \pm 0.5$  days. This interwave interval determines a mean overlapping degree of 32% of the wave length. Similarly, mean plasma oestradiol-17 $\beta$  (E<sub>2</sub>) concentrations followed a wavelike pattern. However, E<sub>2</sub> concentrations started to decline before the structural regression of the F was observed. Mean basal E<sub>2</sub> concentrations remain higher than  $10.9 \pm 0.6$  pmol/l. In conclusion, follicular activity in non-mated llamas is characterized by continuous emergence of successive waves that always overlap the preceding wave with variable degrees. E<sub>2</sub> production during the follicular wave is shorter in duration than the morphological development of the F. Finally, the overlapping phenomenon maintains increased plasma E<sub>2</sub> concentrations persistently and this could explain the prolonged periods of sexual receptivity registered in llamas.

### Introduction

Llamas (*Lama glama*) are induced ovulators. In non-mated females, follicular activity occurs in successive waves of follicular growth and regression (Fernández-Baca et al. 1970; Adams et al. 1990; Bravo et al. 1990). Ovulation occurs only if an appropriate stimulus induces pituitary LH secretion in the presence of an ovulatory follicle (Bravo et al. 1991; Ratto et al. 2005, 2011). In llamas, the follicle becomes ovulatory after a diameter of 7 mm is reached (Bravo et al. 1990). Thus, during each follicular wave, one follicle becomes dominant, grows to maturity and, if the female is non-mated, regresses without ovulation (Bravo and Sumar 1989; Adams et al. 1990; Bravo et al. 1990). In addition, the occurrence of codominant follicles larger than 7 mm has been reported to occur in 5–45% of follicular waves in camelids (Vaughan et al., 2004, Vaughan 2010; Fernández-Baca et al. 1970; Riveros et al. 2010; Manjunatha et al. 2012).

The time required to complete a wave in llamas varies between studies. Initial studies (Bravo et al. 1990) reported that the time required to complete a wave was on average 13.8 days: growth phase  $4.8 \pm 1.5$  days,

plateau phase  $5.0 \pm 1.6$  days and regression phase  $4.0 \pm 1.1$  days. In accordance with that study, Aller and Alberio (1996) reported a 17-day wave duration. Conversely, other researchers observed a growth and regression profile of the dominant follicle averaging between 20 and 25 days in non-pregnant llamas (Adams et al. 1990; Aba et al. 1995; Chaves et al. 2002). Moreover, Chaves et al. (2002) registered a mean duration of follicular waves of  $22.6 \pm 2.5$  days: growing phase  $9.2 \pm 2.8$  days, plateau phase  $5.2 \pm 1.4$  days and regression phase  $8.2 \pm 2.2$  days. Considering previous reports, the interval between the emergence of successive waves in llamas is between 11 and 20 days (Adams et al. 1990; Bravo et al. 1990; Chaves et al. 2002). In addition, Bravo et al. (1990) reported that follicular activity regularly alternates between ovaries (81%), while other authors observed an alternacy of approximately 50% (Adams et al. 1990; Aller and Alberio 1996).

As induced ovulators, non-mated llamas lack regular luteal phases. Thus, follicular activity develops in an environment of low concentrations of progesterone (P<sub>4</sub>; <3 nmol/l). Follicular diameter and oestradiol-17 $\beta$  (E<sub>2</sub>) concentrations have been reported to be closely correlated during follicular development (Bravo et al. 1990; Chaves et al. 2002; Vaughan and Tibary 2006). Thus, Chaves et al. (2002) reported that E<sub>2</sub> concentrations in anovulatory llamas increase during the growing phase of the dominant follicle, remain high during the plateau phase and decline afterwards. In this latter study, peak E<sub>2</sub> concentrations were attained when the follicle reached its maximum diameter.

In the absence of ovulation, female llamas develop a follicular phase of long duration during which the beginning of a wave is generally superimposed with the ending of the preceding wave (overlapping phenomenon). It has been suggested that overlapping between successive follicular waves would induce persistent high plasma E<sub>2</sub> concentrations, responsible for the prolonged sexual receptivity registered in female camelids (Bravo and Sumar 1989; Adams et al. 1990). Hence, many studies have reported that anovulatory females could remain receptive to the male for periods of at least 30 days (San-Martín et al. 1968; Fernández-Baca et al. 1970; Novoa 1970; England et al. 1971; Adams et al. 1990). Conversely, other studies have reported that plasma E<sub>2</sub> concentrations are not uniformly high, but register pulses of 4–5 days separated for periods of 1–10 days where E<sub>2</sub> concentrations were at basal levels (Bravo et al. 1990; Brown 2000). However, in those studies, an evaluation of the overlapping phenomenon during the development of successive follicular waves was not included. In addition, the absence of a daily

frequency of ultrasonographic examinations and plasma  $E_2$  concentrations measurement could have made difficult the characterization of the relationship between both parameters during the evaluation of follicular activity.

So far, most studies reporting ultrasonographic and endocrine monitoring of follicular activity have focused on individual follicular waves (Bravo et al. 1990, 1991; Chaves et al. 2002), not considering the overlapping phenomenon during the development of successive follicular waves. This information becomes relevant in order to understand follicular activity and sexual behaviour in the species.

The aim of the present study was to perform an ultrasonographic and endocrine (oestradiol-17 $\beta$  and progesterone) characterization of the follicular wave and successive waves in non-mated llamas with a special interest on the overlapping phenomenon.

## Material and Methods

### Animals

The group of llamas used in the present study ( $n = 12$ ) was selected from a herd of parous females aged 4–8 years. Females were selected considering that they were non-pregnant, non-lactating, clinically healthy and with an adequate size for transrectal palpation. The experimental design and animal care were in compliance with regulations set by the Animal Welfare Committee at the Faculty of Veterinary Sciences, UNCPBA, Tandil, Argentina. All females were kept isolated from males, were fed pasture hay and had free access to water during the entire experimental period.

During the study period, ovarian peculiarities were observed in two females. Thus, before analysis, their data were excluded from the study. The peculiarities included a spontaneous ovulation and a case of codominant follicles.

### Follicular activity characterization and definitions

Follicular activity was evaluated daily using transrectal ultrasonography for at least two complete consecutive follicular waves. Ultrasonographic examinations were performed by a single operator using a Mindray, DP 6600 Vet, with a 5.0-/7.5-MHz variable transducer probe. Each examination procedure was similar to that described by Bravo et al. (1990), and the following parameters were registered: diameter of the largest follicle (F), maximum diameter of the second largest follicle (Fs) and the presence or absence of a corpus luteum. Individual charts were used to register the parameters evaluated every day for each female. The diameter of each follicle was measured 3 times, and the averaged diameters were considered the follicular diameter of that follicle. The F was considered dominant when it grew to at least 7 mm and exceeded the diameter of all other follicles (Adams et al. 1990). The day of follicular emergence, retrospectively determined, was defined as the last time when the F had a diameter  $>3$  mm at the beginning of the wave.

The characteristics of the mean follicular wave and the profile of successive waves were evaluated. The

parameters registered during the mean follicular wave included the following: length of mean follicular wave; number of days taken by the follicle to develop from emergence until the last time it had 3 mm at the end of its regression. To generate the day-to-day mean diameter profile of the dominant follicle during a follicular wave, the day of emergence of each wave was used as a common starting point (day 0); length of the phases in which the wave was divided (growth, plateau and regression): the growth phase was defined as the period between the day on which the follicle emerged until the follicle stabilized its growth around the maximum diameter at the start of the plateau phase during which the follicle has oscillations in diameter  $\leq 0.5$  mm. This phase ended at the beginning of the regression phase that started the first time the follicle had two consecutive decreasing measurements and finished when the follicle reached 3 mm at the end of the wave; growth/Regression rate: millimetres per day of increase or decrease in follicular diameter in each phase; maximum diameter of the largest follicle (F): maximum diameter reached by the dominant follicle during the plateau phase; maximum diameter of the second largest (subordinate) follicle (Fs): maximum diameter reached by the second largest follicle until it began to regress.

The parameters registered during the evaluation of successive follicular waves included the following: percentage of overlapping waves: percentage of waves that superimposed their beginning with the ending of the preceding wave; degree of overlapping: percentage of the whole wave that overlapped their origin to the regression phase of the preceding wave; interwave interval: days between the emergence of two consecutive overlapped or separated follicular waves; interval between successive 7-mm follicles: days between the F of two successive, overlapped or separated, waves reach the diameter considered ovulatory in the species (7 mm); follicular alternacy: follicular alternacy was considered to occur when the new wave develops always in the contralateral ovary of that of the preceding wave. Conversely, it was considered to be no alternacy when waves develop at random between ovaries.

### Blood sampling

Blood samples (5 ml) were collected into heparinized (Heparin Sodium, SOBRIUS<sup>®</sup>, Fada Pharma, Buenos Aires, Argentina) tubes at the end of every ultrasonographic examination from a group of six llamas randomly selected from the twelve females in the study. Venipuncture was performed alternately at high, medium and low positions on the left and right sides of the neck in order to minimize damage to the jugular veins due to the high-frequency sampling protocol (Aba et al. 1999). Within 1 h after extraction, plasma samples were separated by centrifugation and stored at  $-20^\circ\text{C}$  until analysed.

### Hormone determinations

Oestradiol-17 $\beta$  ( $E_2$ ) plasma concentrations were measured using a radioimmunoassay (RIA) Kit (Siemens

Medical Solutions Diagnostics, Los Angeles, CA, USA) reported for use with bovine plasma (Sirois and Fortune 1990) and validated for use with llama plasma after minor modifications (Aba et al. 1995). Plasma and standards were previously extracted using diethyl ether (Merck, Buenos Aires, Argentina). The interassay coefficient of variation (CV), calculated from the precision profiles of three standard curves, and the intra-assay CV were below 7% and 11% for concentrations between 5.6 and 180 pmol/l, respectively. The lowest amount of E<sub>2</sub> detectable was 5.6 pmol/l. Plasma progesterone (P<sub>4</sub>) concentrations were measured using a RIA Kit (Siemens Medical Solutions Diagnostics) previously validated for use with llama plasma (Bianchi et al. 2007). The sensitivity of the assay was 0.3 nmol/l, and the intra-assay CV was below 13% for concentrations between 0.4 and 128 nmol/l. All samples were measured in duplicate. Hormone concentrations are expressed in SI units. To convert from pmol/l to pg/ml and from nmol/l to ng/ml, a factor of 3.7 for E<sub>2</sub> and 3.2 for P<sub>4</sub> should be used.

#### Endocrine characterization of follicular activity

Plasma E<sub>2</sub> and P<sub>4</sub> concentrations during follicular development were measured in six of twelve llamas in which follicular activity was monitored by ultrasonography, allowing the endocrine (E<sub>2</sub> and P<sub>4</sub>) characterization of 12 complete follicular waves and 18 interwave intervals in those females. As specified for the evaluation of the mean follicular wave, to generate the day-to-day profile of E<sub>2</sub> and P<sub>4</sub> production during follicular development, plasma hormone concentrations were normalized using the day of wave emergence as a common reference starting point (day 0). Then, profiles were processed to obtain the following endocrine parameters: plasma E<sub>2</sub> concentrations during the mean follicular wave: mean plasma E<sub>2</sub> concentration registered between the day of wave emergence (day 0) and the last declining concentration was recorded. To generate the mean E<sub>2</sub> profile during the mean follicular wave, those plasma E<sub>2</sub> concentrations that started to increase, due to the emergence of a new wave, were removed; plasma E<sub>2</sub> and P<sub>4</sub> concentrations during successive follicular waves: mean plasma E<sub>2</sub> and P<sub>4</sub> concentrations registered between the day of wave emergence (day 0) and the end of the period of successive waves were evaluated; mean basal E<sub>2</sub> concentrations: average of the lowest plasma E<sub>2</sub> concentrations recorded during the period of successive follicular waves.

#### Structural and endocrine relationships during follicular activity

For an ultrasonographic (follicular diameter) and endocrine (E<sub>2</sub> and P<sub>4</sub>) characterization of follicular activity, values obtained for each variable were normalized as previously described. In addition, for the analysis of the mean profile of successive waves, the emergence of the new wave was normalized considering the mean interwave interval.

#### Analysis of data

Results are expressed as mean  $\pm$  SEM. Follicular waves' characteristics were analysed by descriptive statistics. Single data points (maximum follicular diameter, interwave interval and interval between successive 7-mm follicles) were compared by Student's *t*-test. Mean follicular growth rates were analysed using analysis of variance (ANOVA) followed by a Fisher's least-significant difference test (LSD) to determine differences between means. The relationship between wave length and maximum follicular diameter and between length of the growing phase of the F and the interwave interval was evaluated by Pearson's correlation. In order to study the alternacy and occurrence phenomenon, the values of complete (20) and incomplete (18) follicular waves were considered using a chi-square analysis for these parameters. Statistical analyses were carried out using the Statistica/W, release 4.0 software package (Statsoft Inc., Tulsa, OK, USA). Statistical significance was set at  $p < 0.05$ .

## Results

#### Ultrasonographic characterization of follicular activity

##### *Mean follicular wave*

In all females, development of the largest follicle (F) showed a wavelike pattern that was clearly divisible into three phases: growth, plateau and regression. Mean follicular wave length was  $25.0 \pm 0.8$  days ( $n = 20$ ), as shown in Fig. 1a. The F grew from emergence at a mean growth rate of  $0.7 \pm 0.04$  mm/day during  $10.1 \pm 0.5$  days and reached the plateau phase during which there were mean oscillations in measurements of  $0.2 \pm 0.02$  mm/day during  $5.6 \pm 0.3$  days. Mean maximum follicular diameter averaged  $10.9 \pm 0.5$  mm with variability between waves (range, 8–16 mm) and was attained 13 days after emergence. Finally, F regressed at a mean rate of  $0.8 \pm 0.05$  mm/day in  $9.3 \pm 0.5$  days.

The second largest follicle (Fs) reached a mean maximum diameter of  $5.2 \pm 0.2$  mm at 4.8 days after wave emergence. The maximum Fs diameter never exceeded 7 mm during the study (range, 4–6.7 mm). When Fs reached its maximum diameter, the mean diameter of the F was  $6.4 \pm 0.2$  mm with a range of 5.4–8 mm (Fig. 1a).

The mean follicular wave was composed of waves of variable duration, with a range of 18–31 days. Considering arbitrarily the mean duration of the follicular wave (25 days), it was observed that 60% (12/20) of waves were equal to or longer than 25 days and 40% (8/20) lasted <25 days.

The mean wave length was closely correlated with the mean maximum follicular diameter reached by F during the plateau phase ( $r = 0.71$ ;  $p = 0.0005$ ). The mean maximum follicular diameter was  $11.8 \pm 0.7$  mm for waves of longer duration ( $\geq 25$  days) and  $9.6 \pm 0.6$  mm for waves of shorter duration ( $< 25$  days;  $p = 0.037$ ). However, the growth rate of the F was constant ( $0.7 \pm 0.04$  mm/day) until day 7 after emergence independently of follicular wave length ( $p > 0.05$ ). Thus, the F attained the ovulatory size (7 mm) by day 8 after

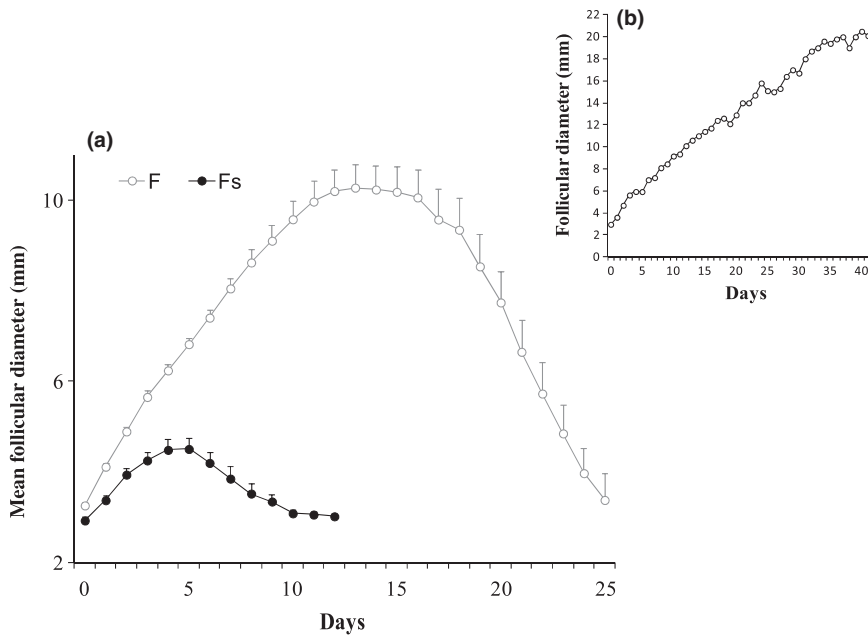


Fig. 1. (a) Mean follicular diameter (mm) of the largest (F) and second largest (Fs) follicle during follicular waves. (b) Daily diameter (mm) of the largest follicle in a llama that developed a cystic or haemorrhagic follicle

emergence in all cases (day 6: 90%; day 7: 95%; and day 8: 100%).

The development of cystic or haemorrhagic follicles was observed in two llamas. In those females, the F reached a maximum diameter of 35.6 mm and 20.5 mm that persisted for more than 20 days. Figure 1b shows the daily diameter of the F in a llama that developed a persistent structure.

#### Successive follicular waves

All follicular waves evaluated were superimposed on the preceding wave. Waves emerged at mean intervals of  $15.8 \pm 0.5$  days with a range of 12–20 days. Similarly, the mean interval between successive 7-mm follicles was  $16.1 \pm 0.6$  days with a range of 12–20 days ( $p = 0.63$ ). The length of the growing phase of the follicular wave was positively correlated with the interwave interval ( $r = 0.56$ ;  $p = 0.01$ ). The interwave interval determined a mean overlapping degree of the whole wave on the preceding wave of  $32.0 \pm 1.8\%$ , corresponding to a mean overlapping of  $8.1 \pm 0.5$  days. The mean degree of overlapping between successive waves was variable with a range of 19–52% (4–13 days). The mean diameter of the growing F at the moment of intersection between successive waves was  $6.5 \pm 0.2$  mm. Ovulatory follicles ( $\geq 7$  mm) developed 48.5% times in the left ovary and 51.5% in the right ovary ( $p > 0.05$ ). The sequential development of ovulatory follicles did not alternate between ovaries (44% ipsilateral and 56% contralateral;  $p < 0.01$ ). Figure 2 shows daily follicular diameters of the F during successive waves in 4 llamas.

#### Endocrine characterization of follicular activity

##### Plasma $E_2$ concentrations during mean follicular wave

Mean plasma  $E_2$  concentrations described a wavelike pattern similar to that reported for the development of the F, with increasing concentrations during its growing

phase. However, the  $E_2$  wave length averaged  $18.3 \pm 0.6$  days. This wave was shorter than the mean follicular wave ( $p = 0.0001$ ; Fig. 3). Thus, as observed in Fig. 3, plasma  $E_2$  concentrations started to decline before the structural regression of the follicle. Mean maximum  $E_2$  concentration of  $36.9 \pm 2.7$  pmol/l was attained  $11.2 \pm 0.4$  days after emergence. The mean diameter of the F at the day of mean maximum  $E_2$  concentration was  $10.6 \pm 0.6$  mm. Mean plasma  $E_2$  concentration when F reached the ovulatory diameter ( $\geq 7$  mm) was  $18.5 \pm 1.1$  pmol/l.

##### Plasma $E_2$ and $P_4$ concentrations during successive follicular waves

Plasma  $E_2$  concentrations during the development of successive follicular waves followed a cyclic pattern. During the development of successive follicular waves, plasma  $E_2$  concentrations from the preceding wave always started to decline before a new wave emerged. Emergence of a new wave was registered on average  $4.8 \pm 0.7$  days after the beginning of decline in plasma  $E_2$  concentrations. The growth of the new F induced an increase in plasma  $E_2$  concentrations, establishing a cyclic pattern on  $E_2$  concentrations with a mean basal concentration of  $10.9 \pm 0.6$  pmol/l. Mean follicular diameter registered in relation to basal  $E_2$  concentrations was  $4.7 \pm 0.2$  mm (Fig. 4). The variability in interwave intervals during the emergence of successive follicular waves determined a similar variation in plasma  $E_2$  concentrations during follicular activity. Plasma  $P_4$  concentrations remained below 3 nmol/l throughout the experimental period.

#### Discussion

This report provides a detailed ultrasonographic (follicular diameter) and endocrine ( $E_2$  y  $P_4$ ) characterization of follicular activity during the development of

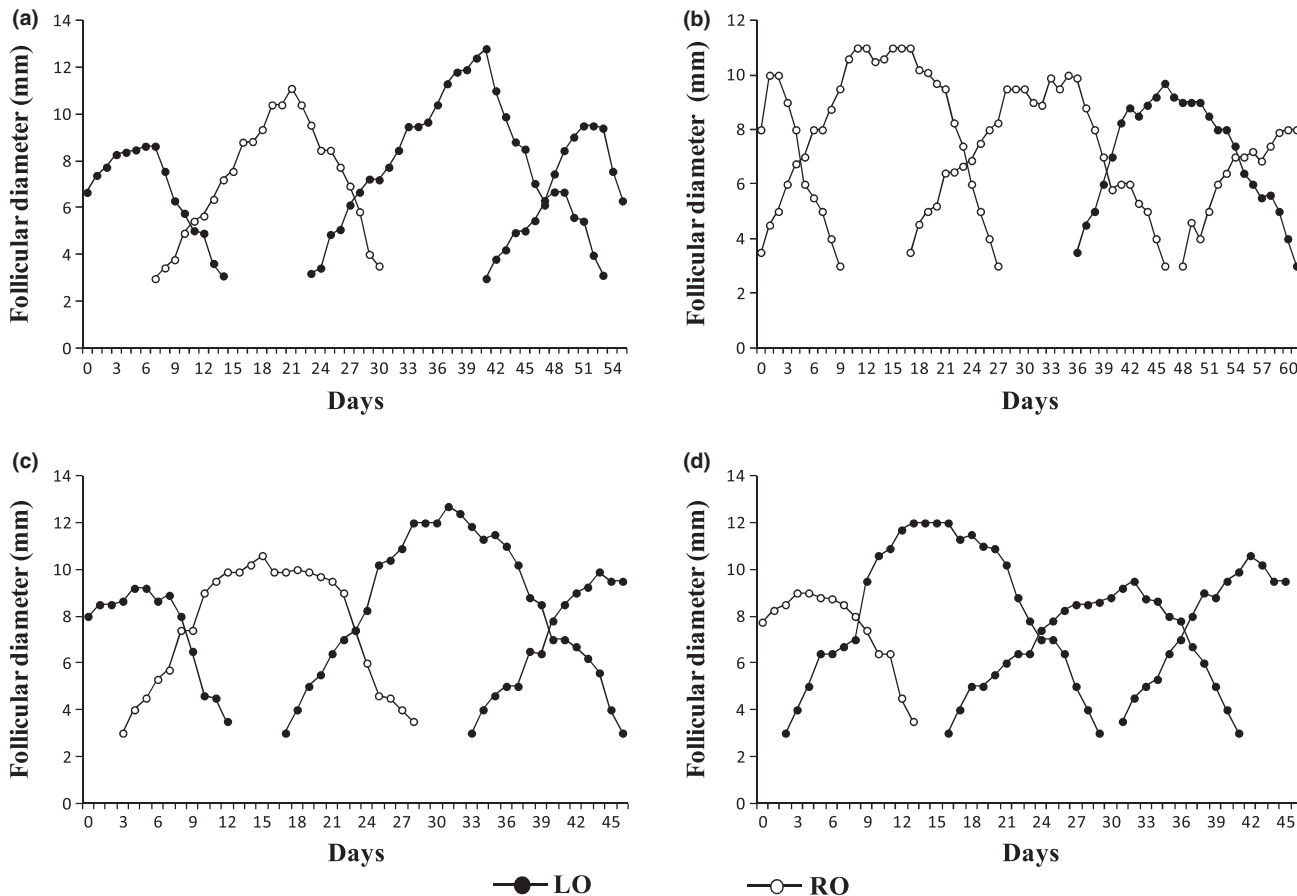


Fig. 2. Daily mean follicular diameter (mm) during successive follicular waves in anovulatory llamas. Panel (a): llama 15; Panel (b): llama 10; Panel (c): llama 22; Panel (d): llama 18. LO: left ovary; RO: right ovary

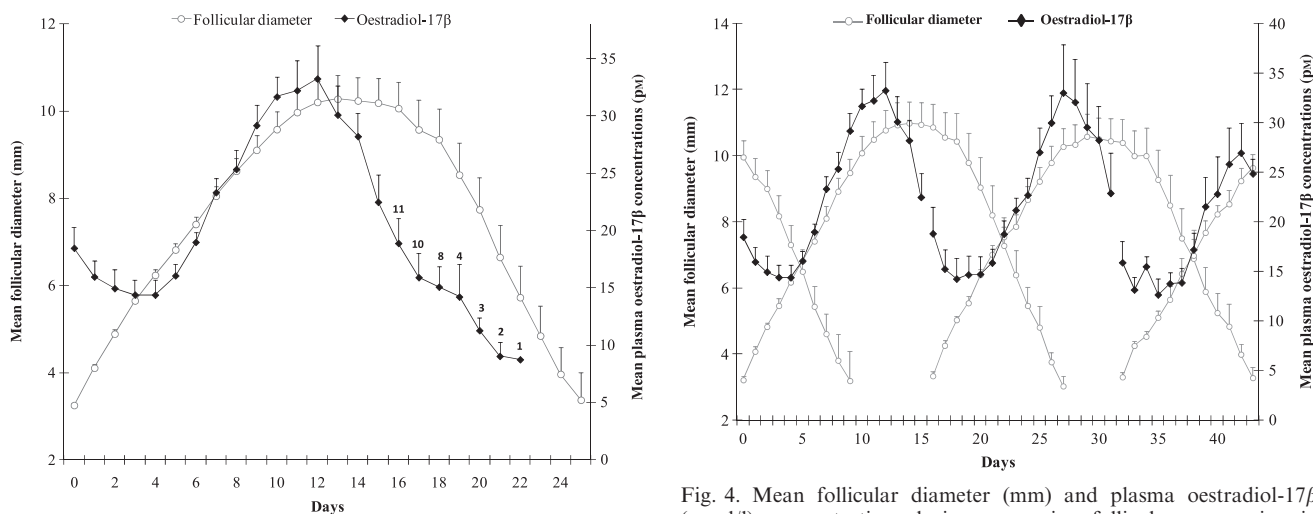


Fig. 3. Mean follicular diameter (mm) and plasma oestradiol-17β (pmol/l; E<sub>2</sub>) concentration during the follicular wave in anovulatory llamas. Day 0: follicular wave emergence. Numbers indicate the E<sub>2</sub> concentrations that remain after eliminating those concentrations that started to increase due to a new wave emergence

Fig. 4. Mean follicular diameter (mm) and plasma oestradiol-17β (pmol/l) concentration during successive follicular waves in six anovulatory llamas. The variables follicular diameter and plasma oestradiol-17β concentrations were normalized using the day of wave emergence as a common reference point (day 0). In addition, for successive waves, the mean interwave was considered the reference starting point (day 0) for those waves (waves 2 and 3). Thus, days 0, 16 and 32 correspond to the day of emergence of waves 1, 2 and 3, respectively

individual and successive waves in non-mated llamas with a special interest on the phenomenon of overlapping. In agreement with previous reports, the development of the largest follicle (F) showed a wavelike pattern clearly divisible into three phases: growth,

plateau and regression (Adams et al. 1990; Bravo et al. 1990; Aller and Alberio 1996; Chaves et al. 2002). The length of the mean follicular wave and their phases resulted longer than those reported by other authors:

13.8 days (Bravo et al. 1990) and 17 days (Aller and Alberio 1996), but were consistent with those registered by Adams et al. (1990) and Chaves et al. (2002): 20–25 and 22.6 days, respectively. The wavelike pattern of follicular development was similar to that described for spontaneous ovulator species in which, unlike camelids, follicular activity occurs in the presence of luteal concentrations of progesterone (cows and mares: Ginther 2000; ewes: Evans 2003; goats: Rubianes and Menchaca 2003). Previous ultrasonographic studies in llamas reported F growth rates of 0.9–1.1 mm/day (Aller and Alberio 1996; Chaves et al. 2002) that were higher than those registered in the present study. However, the F growth rate hereby registered was similar to that reported by Adams et al. (1989, 1990). There is no clear explanation for the discrepancy observed between data, but it could be suggested that genetic, nutritional or environmental factors between different studies could have accounted for the divergences.

The variability in the length of mean follicular wave was positively correlated with the maximum diameter attained by the F during the plateau phase. Thus, waves of longer duration were associated with a greater maximum F diameter. However, the constant mean F growth rate until day 7 after emergence ensured the presence of a follicle  $\geq 7$  mm in the growing or the beginning of the plateau phase on day 8 after emergence in all females independently of follicular wave length. A similar variability on the length of follicular waves was observed in other species of the family Camelidae: bactrian and dromedary camels (Skidmore 2011), alpacas (Vaughan et al. 2004, Vaughan 2010), vicuñas (Miragaya et al. 2004) and guanacos (Riveros et al. 2010). Similarly, Vaughan et al. (2004) reported that in alpacas, follicular growth was similar between F until day 10 after emergence, regardless of different maximum size and interwave intervals.

In cows (Badinga et al. 1992) and sheep (Mann et al. 1992),  $E_2$  is produced primarily by the F of a wave, while subordinate follicles contribute to  $<10\%$  of ovarian  $E_2$  production. Based on the results of the present study, it could be speculated that an analogous situation should occur in llamas. The observation that plasma  $E_2$  concentrations followed a wavelike pattern similar to that observed for the development of the F and a close relationship with follicular diameter during the growing phase is consistent with results reported in llamas (Bravo et al. 1990; Chaves et al. 2002), guanacos (Riveros et al. 2010) and vicuñas (Miragaya et al. 2004). However, in the present study, this relationship was lost during the end of the plateau phase. The early stage of the plateau phase was a transitional period in which the relationship between follicular development and  $E_2$  production was variable. Thus, the decline in plasma  $E_2$  concentrations preceded the structural regression of the F during the follicular wave. Similar observations were registered for cows by Fortune et al. (1991) who found that while the dominant follicle of the first wave is still actively growing, morphological dominance usually coincides with functional dominance, but this congruence is lost during the late plateau phase. In addition, in goats (de Castro et al. 1999) and ewes (Souza et al. 1997), during growth of

the F of the first follicular wave,  $E_2$  concentrations increase, reaching maximal values before maximum diameter is reached, and begin to decline while F is still growing. In sheep (Badinga et al. 1992; Souza et al. 1997), the loss of aromatase activity is one of the early alterations that precedes morphological changes, and this observation could explain the deviation observed in this study between  $E_2$  production and developmental pattern of the F. Maximal  $E_2$  concentrations in the present study were associated with the existence of F at the end of the growing phase or the beginning of the plateau phase. The fact that plasma progesterone ( $P_4$ ) concentrations remained below 3 nM throughout the experimental period clearly indicates the absence of ovulations (Aba et al. 1995).

Follicular activity was characterized by the development of successive follicular waves that always overlapped. However, the degree of overlapping was variable (4–13 days/19–52%). The overlapping feature of follicular activity had been recognized in other induced ovulator species such as ferrets (Lindeberg 2008) and rabbit (Hill and White 1933). Conversely, in female cats, follicular waves do not overlap (Shille et al. 1979). Instead, they are separated by variable intervals. In llamas, Bravo et al. (1990) registered that during follicular development, some waves did not overlap originating variable periods of basal  $E_2$  concentrations. In addition, studies in guanacos (Riveros et al. 2010) reported that only 60% of follicular waves overlapped. Moreover, the degree of overlapping observed in llamas of the present study was greater than that reported by other researchers in anovulatory llamas (1–4 days) and bactrian camels (2–5 days) (Chen and Yuen 1979; Bravo et al. 1990). The greater degree of overlapping registered for llamas in the present study could be related to the longer length of follicular wave compared with that reported by Bravo et al. (1990). Also, as previously mentioned, nutritional, genetic or environmental divergences in the different studies should have been considered.

During the growth of the F, the development of subordinate follicles was suppressed. Moreover, the emergence of the successive follicular wave was always registered after the end of the growing phase of the F of the preceding wave and after  $E_2$  production from that wave started to decline. Thus, the variability in the interwave interval could have been related to the variable length of the growing phase due to the positive correlation observed between variables. In agreement with the previous finding, it has been reported that the follicular dominance phenomenon is a process through which the F continues to grow faster while the development of subordinate follicles is suppressed (Armstrong and Webb 1997). Furthermore, in ewes, the emergence of a new wave takes place when the F of the preceding wave is at the end of the growing or the plateau phase (Evans et al. 2000; Evans 2003). In cows, the decline in plasma  $E_2$  concentrations allows the occurrence of the FSH surge that precedes a new wave emergence (Adams et al. 1992; Ginther 2000; Fortune et al. 2004). In addition, a similar variation in successive interwave intervals has been reported in alpacas (Vaughan et al. 2004), cats (Shille et al. 1979) and

mares (Ginther and Bergfelt 1992). In cows, there is a relationship between F diameter, length of follicular wave and the interwave interval between 2 or 3 wave cycles (Fortune et al. 1991). Hence, the findings hereby reported could suggest that in llamas, the dominant F suppresses the development of subordinate follicles and the emergence of the successive wave. Moreover, E<sub>2</sub> could be, at least one, of the factors through which the F exerts its suppressive influence.

Oestradiol-17 $\beta$  production from the new growing follicle, during the development of successive waves, increases plasma E<sub>2</sub> concentrations, originating a cyclic pattern in plasma E<sub>2</sub> profile. Thus, mean basal E<sub>2</sub> concentration remained higher than 10.9  $\pm$  0.6 pmol/l. This observation is in agreement with previous reports, suggesting that overlapped waves might maintain E<sub>2</sub> concentrations that might give rise to the prolonged sexual receptivity registered in the species (Brown 2000; Vaughan 2010). Conversely, Bravo et al. (1990) reported that plasma E<sub>2</sub> concentrations were not uniformly elevated, but pulses of 4–5 days separated by variable periods (1–10 days) of basal concentrations were registered. This observation is in agreement with the smaller degree of overlapping registered by Bravo et al. (1990) and even the occurrence of non-overlapping waves that could lead to an earlier and longer decrease in plasma E<sub>2</sub> concentrations. Further studies will be necessary to elucidate whether the mean basal E<sub>2</sub> concentrations registered in the present study are highly enough to induce sexual receptivity.

Both ovaries contribute to ovulatory F in the same proportion. In agreement with the present findings, other authors reported an occurrence of 50% in each ovary (Adams et al. 1990; Bravo et al. 1990; Sumar 1999; Chaves et al. 2002; Vaughan et al. 2004; Vaughan and Tibary 2006). In addition, alternacy in F development between successive waves was not observed in the study hereby reported. Similarly, the absence of follicular alternacy has been observed in cows (Sirois and Fortune 1988), in llamas/alpacas by other groups (San-Martin et al. 1968; Adams et al. 1990; Chaves et al. 2002; Vaughan et al. 2004) and in dromedary camels (Manjunatha et al. 2012). Conversely, research in guanacos (93%; Riveros et al. 2010), vicuñas (77%; Miragaya et al. 2004) and alpacas/llamas (81%; Bravo et al. 1990) has documented the phenomenon of follicular alternacy. However, the lack of standardized criteria between reports and the absence of a clear definition of the alternacy phenomenon in some studies could have accounted for the divergences. The absence of a real alternacy phenomenon in the present study allows suggesting that follicular dominance could be

exerted through a systemic rather than a local mechanism.

The development of cystic or haemorrhagic follicles has been reported in llamas and alpacas with an incidence of 18–21%. These structures are anovulatory follicles of a larger size and longer lifespan than normal follicles (Adams et al. 1990, 1991; Bravo et al. 1993; Bravo 1997). Structures exceeding 12 or 14 mm in alpacas and llamas, respectively, and lasting 25–30 days, have been referred as cystic follicles. It has been suggested that the occurrence of these structures is increased in non-mated females and seems to have high individual repeatability and that their presence is not associated with any ovarian dynamics alteration or abnormality (Adams et al. 1991). A higher incidence of oversized follicles has been observed in the closely related dromedary camel (Tibary and Anouassi 1996).

In conclusion, the present report provides an ultrasonographic and endocrine characterization of individual and successive waves with a special reference to the overlapping phenomenon in non-mated llamas. Follicular activity in anovulatory llamas is characterized by the emergence of successive waves that always overlap with variable degrees. During the development of those waves, the functional ability of the F to produce E<sub>2</sub> is shorter in duration than its morphological development. In addition, the high degree of overlapping between successive waves maintains elevated plasma E<sub>2</sub> concentrations persistently and this could explain the prolonged periods of sexual receptivity reported in llamas.

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### Conflict of interest

None of the authors have any conflict of interests to declare.

### Author contributions

Cavilla, María Verónica has participated in developing the design of the experiment. She has carried out the experiment, collected and analysed the data, and drafted the manuscript. Bianchi, Carolina Paula and Maistruarena, Celeste have contributed to carrying out the experiment and contents of the manuscript. Aba, Marcelo Alfredo as the director of the doctoral project of Miss Cavilla, María Verónica has designed the experiment and has contributed to carrying out the experiment, with the analysis of the data and revising critically the contents of the manuscript. All authors have read and approved the final manuscript.

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