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Characterization of the estrous cycle of *Asinina de Miranda* jennies (*Equus asinus*)

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ABSTRACT

This study aims to characterize the estrous cycle of *Asinina de Miranda* jennies in the breeding season, on the basis of data collected from serial ultrasonographic examination and serum progesterone determinations in 14 females during a total of 33 cycles. The length of the interovulatory interval was 23.8 ± 0.55 days, the diestrus and estrus lasting 17.9 ± 0.46 days and 6.65 ± 0.30 days, respectively. Age and body condition score (BCS) affected the length of the interovulatory intervals; BCS also influenced the diestrus length and the time in heat after ovulation ($P > 0.05$). The incidence of single, double, and triple ovulations was 57.58%, 36.36%, and 6.06%, respectively. Multiple ovulations affected neither the length of the interovulatory interval nor the individual cycle stages ($P > 0.05$) but lengthened the interval from the beginning of estrus to the last ovulation ($P = 0.01$). When combined with age, higher BCS affected the ovulation rate ($P = 0.001$). Deviation of the dominant follicle occurred around Day 8.7 (Day 0 = ovulation) when both single and multiple ovulations were considered. The dominant follicle was larger at divergence in single ovulators (19.18 ± 0.97 mm) compared with that in multiple ovulators (18.05 ± 1.16 mm). The overall maximum follicular diameter before ovulation was smaller in multiple ovulatory cycles than that in single ovulatory cycles (37.2 ± 0.83 mm vs. 40.2 ± 1.41 mm, respectively; $P = 0.03$). The daily growth rate of dominant follicles was independent of the ovulation rate ($P > 0.05$) for the intervals before and after the estrus onset. The dominant follicle size and the follicle growth rates were independent of BCS ($P > 0.05$). Data collected in this study revealed resemblances between Mirandese and other Iberian and Brazilian breeds with regard to estrous cycle characteristics.

1. Introduction

The *Asinina de Miranda* is an endangered breed of donkey originating from the far northeast of Portugal. It is characterized by having a long bay coat, a height of greater than 130 cm, and a calm temperament, which makes it especially suited to agricultural work, milk production, and leisure activities such as asinotherapy. With around 500 females available for reproduction, the breed exhibits very low rates of reproduction. Less than a quarter of existing *Asinina de Miranda* jennies have had foals registered in the stud book [1]; the average foaling rate over the past 10 years has been close to 50 foals a year, although this number has increased to around 70 foals a year in the past 2 years [2]. Average age at the first foaling has increased in recent decades, and for a large proportion of females, introduction into reproduction has been postponed until a later age, when fertility tends to decline and reproductive

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disorders become more prevalent [3]. The fact that most traditional owners show little interest in breeding seems to be the reason for this late entry into reproduction, together with the lack of any need to replace working animals [1,2]. More recently, under the guidance of the National Breeding Association (Associação Para o Estudo e Proteção do Gado Asinino) and following new trends such as breeding females for milk production, a growing number of young jennies are now being put into breeding [1,2].

The *Asinina de Miranda* is an endangered breed, despite attempts to raise interest in it by identifying alternative uses for these animals. It has been suggested that the number of females breeding each year be increased and that jennies should enter into reproduction earlier than currently usual in an effort to increase foaling [1,2]. As there is such a limited amount of available information, this confirms the need for greater research into the reproductive physiology of the species. Milk production is one potential use of donkeys that has recently been exploited and could provide the means of preventing extinction for many breeds, including the *Asinina de Miranda*; however, productive optimization requires greater knowledge of the donkey's reproductive physiology to increase foaling rates [4]. Detailed knowledge of the characteristics of the estrous cycle in *Asinina de Miranda* jennies is vital to improve the reproductive management of the breed and increase the reproductive efficiency. Furthermore, knowledge on how follicles develop and on their size at specific moments in this process (such as at deviation or ovulation) is fundamental to pharmacologic manipulation of the cycle or exogenous induction of ovulation.

Jennies are similar to mares in many reproductive aspects but tend to have longer breeding seasons [5,6] and longer diestrus phases [7]. Consequently, donkeys have longer interestrous intervals [5,8,9], similar to those reported for ponies [7]. The estrus length is similar among jennies, ponies, and mares [7,10,11], ovulation usually occurring 1 to 2 days before the end of estrous behavior [6,9,10], as is also found in mares [12].

There are a few studies available on the characteristics of the estrous cycle for other European donkey breeds [7,11,13,14]. However, there have only been a limited number of comparative studies on differences in donkey breeds, like those known to exist in breeds of horse [15], surveying characteristics such as the rate of ovulation and the prevalence of multiple ovulations, the size of the ovulatory follicle, and the moment of ovulation within the follicular stage. Such characteristics are yet to be determined in *Asinina de Miranda*. To implement conservation programs aimed at rescuing the breed, it is crucial that assisted reproduction is considered, with particular regard to follicular development and the putative factors that may affect it [16,17].

This study aims to characterize the estrous cycle of *Asinina de Miranda* jennies during the breeding season, including the lengths of the interovulatory interval and of the estrus and diestrus stages; the ovulation rate (number of ovulation per estrus) and the prevalence of multiple ovulations; the maximum follicular size before ovulation, considering both single and multiple ovulations; the pattern of final follicular growth, from the beginning of estrus detection up to ovulation; the laterality of ovulation; and the length of time in heat after ovulation. Furthermore, the putative influences of endogenous factors such as age and body condition score (BCS) were also tested.

2. Materials and methods

2.1. Animals, management, and sample collection

This study used 14 nonpregnant clinically healthy jennies of the Portuguese *Asinina de Miranda* breed of donkey. The jennies were aged from 3 to 18 years: six young jennies aged between 3 and 5 years, six adult females aged between 6 and 8 years, and two older females aged greater than 15 years. This distribution attempts to reflect the age pyramid seen in breeding females [2]; the small number of females in the older group was due to exclusion because of ovarian diseases. All the jennies had BCS ranging from four to seven on a nine-point scale (5.7 points on average). The body condition score was regularly evaluated during the breeding season, at 5-week intervals [18].

Data were collected during two breeding seasons, from April to the late September, using a group of seven different animals each year. The regular estrous cycles for both the groups were studied from April to June, producing a total of 33 estrous cycles. Most jennies were found to have two successive estrous cycles; only five females, two from the first year's group and three from the second, recorded three cycles. Complete previous reproductive histories were generally unknown in respect to previous pregnancies, but none of the females had foaled in the preceding breeding season. The existence of regular estrous cycles was confirmed before the onset of the study. All the jennies were considered potentially fertile after a breeding soundness examination.

The animals were housed in Vila Real, Portugal (41°17′N 7°44′W), in the university facilities, and kept under natural photoperiod. All the animals were routinely vaccinated for equine influenza and tetanus (ProteqFlu-Te; Merial S.A.S., Lyon, France) and dewormed every 6 months with 200-μg ivermectin (Noromectin Oral Paste; Norbrook Laboratories, Northamptonshire, UK) per kg of body weight. The jennies were kept in a 2500-m^2 paddock, with a 50-m^2 shelter offering year-round protection from rain, sun, and wind. The animals were fed according to accepted protocols [19], consisting of 5 to 7 kg of hay and straw per jenny twice daily, which corresponded to a dry matter intake of between 1.5% and 2% of body weight, supplemented with 1 kg of concentrate, divided into two daily portions. Clean fresh water was always available. The animals were handled in accordance with EU Directive 2010/63/EU for animal experiments.

The females were group teased daily by a male with a good libido, and their estrous behavior was classified as follows [7,20]: The female was considered to be in estrus or receptive if she exhibited mouth clapping together with at least one of the following signs during the teasing period: winking (rhythmic eversion of the vulvar *labia* with exposure of the clitoris) and urinating, raising the tail, and posturing. In contrast, nonreceptivity behavior included (1)

tail down (holding tail down between hind legs when mounted), (2) lack of interest (no positive or negative responses to the presence or teasing of the jack), and (3) refusing the jack by moving away or kicking. Clapping alone or combined with kicking or moving was considered to indicate a transitional stage into or out of estrus but not recorded as receptive behavior.

For teasing, the male was placed in a paddock adjacent to the jennies, separated by a wire fence. The behavior of the females was observed and recorded for 30 minutes; thereafter, the jack was removed to a box within a closed building, 400 m away from the females.

For progesterone (P4) measurements, blood samples were collected by venipuncture of the jugular into serum gel tubes (S-Monovette; Sarstedt, Nümbrecht, Germany), preceding the ultrasound (US) session and placed in ice. Samples were centrifuged after collection at × 2500 *g* for 10 minutes; serum was harvested and stored at −20 °C until assayed. Serum P4 concentrations were determined by chemiluminescent immunoassay (IMMULITE 1000; Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA), using a commercial P4 kit (Siemens IMMULITE Progesterone Kit) and commercially available reagents (all from Siemens Healthcare Diagnostics, Amadora, Portugal). Interassay coefficient of variance for the controls (CON4, CON5, and CON6 for low, intermediate, and high controls, respectively; Multivalent Control Module; Siemens) ranged from 1.3% and 1.5% for the lower and intermediate controls to 4.6% for the high control. To validate the P4 kit for donkeys, serial dilutions in the buffer of a blood sample obtained from a 40-day pregnant jenny were made. The coefficients of regression obtained were 96%.

2.2. Ultrasound assessment of reproductive activity

During the trials, the jennies' estrous activity was routinely surveyed every other day in diestrus and at 8- to 12-hour intervals in estrus by transrectal palpation followed by US examination of the genital tracts using a linear-array US scanner equipped with a 5-MHz linear transducer (Shenzhen Veterinary US scanner), according to the procedures described by Ginther [15]. The scanner was connected to a video camera (DCR-HC96E; Sony), and all US scans were recorded for subsequent analysis.

The diameters of the ovarian dominant follicles were obtained retrospectively from the average of the narrowest and widest dimensions in selected US scan images, considering only the follicular antrum. One single operator established follicular size measurements, using *ImageJ* software (http://imagej.nih.gov/ij/index.html) on fixed-frame images. A dominant follicle was defined as the one deviating from the other growing follicles, and becoming the largest in the ovary, whether or not it ovulated [21]. The dominant follicle, or follicles in the case of multiple ovulations, was considered ovulatory if it reached ovulation. Day 0 of the cycle was set as the day of ovulation or, in the case of multiple ovulation, as the day of the last ovulation.

The interovulatory interval was defined as the interval (in days) between estrus-associated ovulation in successive cycles or as the period between the last ovulation of each cycle in the case of multiple ovulations. Sequential US records were used to establish the moment of ovulation as the midtime between two US scans when a dominant follicle ceased to be observed during estrus. The beginning of estrus was set at the moment when the female first showed signs of receptivity to the male, with P4 levels below 1 ng/mL, whereas the end of estrus was considered to be the moment when the jenny refused the jack. Diestrus corresponded to the period when serum P4 levels remained above 1 ng/mL and the female refused the jack [22]. The ovulation rate was defined as the number of ovulated follicles, on the basis of US observation of the collapse of the preovulatory follicle(s) and loss of greater than 90% of fluid by the time of the next examination [23].

2.3. Statistical analysis

Statistical analyses were performed using the IBM SPSS Statistics 20 software for Windows. The estrous cycles were normalized to the day of ovulation (Day 0); in the case of multiple ovulations, Day 0 was set at the day of the last ovulation. For graphical representations, the normalized period was defined as the 12 days after ovulation (Day 0). Data for the lengths of the interovulatory interval, diestrus, and estrus; the size of dominant follicles; the time in estrus after ovulation; and the final follicular growth rate are presented as mean ± standard error.

An ANOVA test was conducted, followed by a Bonferroni *post hoc* test for means comparison, to analyze the effect of BCS and age on the length of each cycle stage, the ovulation rate, the growth rate and follicle size, the total level of P4, and the time in heat after ovulation. Total secretion of P4 during diestrus was assessed by estimating the area under curve, applying the trapezoidal rule, i.e., calculating the $\Delta X \times (Y1 + Y2)/2$, using Microsoft Excel 2010 for Windows. Furthermore, a covariance analysis was used to explore the effect of BCS (main effect) and age (covariable) on the ovulation rate. Possible correlations between the ovulation rate and the length of the cycle phases, the follicular growth rate and follicle size, the total P4 level, and the time in heat after ovulation were analyzed by the Pearson chi-square test. Differences and correlations were regarded as significant at a P value of less than 0.05.

Proportional differences were calculated to determine whether the differences in the proportions of multiple ovulations in individual animals were significant.

3. Results

3.1. Estrous behavior

During this study, all jennies in estrus showed homotypical signs of estrous behavior (i.e., characteristic for the species) such as mouth clapping, clitoral *winking*, posturing, or showing increased interest toward the male. Often, the jennies also exhibited heterotypical behavior (i.e., signs of estrous behavior shared among different species), such as the flehmen response, sniffing, chasing other females, or standing to be mounted. Mouth clapping

was the first suggestive sign of the approach of estrus, and it was also the last sign to disappear after ovulation.

3.2. Length of the estrous cycle stages

The present study surveyed a total of 33 estrous cycles. Cases of anovulatory estrus or of split estrus were not observed. The length of the interovulatory interval was 23.8 ± 0.55 days, ranging from 17.6 to 34.7 days. The lengths of diestrus and estrus were 17.9 ± 0.46 days (11.6–27 days) and 6.65 ± 0.30 days (3.15–9.71 days), respectively (Table 1). No significant effect of age on the lengths of estrus (P = 0.682) or diestrus (P = 0.101) was found, although longer interovulatory intervals (P = 0.032) were reported in older jennies when compared with those in younger females. Higher BCS led to longer interovulatory intervals (P = 0.022) and diestrus (P = 0.003) but did not affect the length of estrus (P = 0.944). During the period surveyed, no individual variations were observed neither in the length of interovulatory intervals nor in the length of any phases of the cycle. The ovulation rate did not correlate with the length of the interovulatory interval (P = 0.990) or diestrus (P = 0.169). However, the estrus was longer in multiple ovulators than that in single ovulators (5.22 ± 0.40 vs. 6.96 ± 0.33, respectively; P = 0.03). In addition, the ovulation rate correlated positively with the period from the beginning of estrus to the last ovulation (P = 0.01).

In general, ovulation occurred less than 15 hours before the end of the estrus, but jennies maintained estrous behavior for a variable period after ovulation (Table 1). In 21 of the 33 cycles studied, estrus lasted between 4 and 53 hours after ovulation, for an average period of 26.3 ± 3.27 hours. In the other 12 cycles, the interval between ovulation and the end of estrous behavior was either shorter than 12 hours (n = 10) or it occurred before the last ovulation was detected, as in the case of a double ovulation that displayed an interval of 45.5 hours between ovulations or in a triple ovulation recording an interval of 114 hours between the first and the last ovulation. No significant differences (P = 0.508) were found for time in heat after the last ovulation between single (23.7 ± 5.06 hours) and multiple ovulations (26.25 ± 3.27 hours). Although animals displaying higher BCS tended to cease estrous behavior sooner after ovulation, the BCS did not significantly affect the number of hours in heat after ovulation (P = 0.05). Nevertheless, longer estruses were linked to a lower number of hours in estrus after ovulation (P = 0.028).

3.3. Prevalence of multiple ovulations

Of the 33 cycles analyzed, 57.58% (n = 19) were single ovulators and 42.42% (n = 14) multiple ovulators, of which 12 (36.36%) were double ovulations and 2 (6.06%) were triple ovulations. The number of cycles with multiple ovulations was significantly higher (P = 0.02) in four jennies, together producing 64.3% (9 of 14) of the multiple ovulations recorded in this study. These animals were evenly distributed between young and mature groups, and their BCS ranged from 4 to 5.5 points at the moment of multiple ovulations. For these females, the prevalence of multiple ovulatory cycles was significantly higher than that in the other multiple-ovulating jennies (81.8% vs. 40%,

Table 1
Characteristics of the estrous cycle in *Asinina de Miranda* jennies in the breeding season.

Type of ovulation	n	Parameter (days)	Mean ± standard error	Minimum, maximum
Single	19	Length		
		Interovulatory interval	23.8 ± 0.78	19.5, 34.10
		Estrus	5.97 ± 0.37[a]	3.15, 8.89
		Diestrus	18.6 ± 0.65	15.0, 27.00
		Intervals		
		Onset of estrus to ovulation	5.22 ± 0.40[a]	3.15, 8.89
		Ovulation to the end of estrus	0.74 ± 0.17	0.00, 1.87
Double	12	Length		
		Interovulatory interval	23.8 ± 0.45	20.5, 26.10
		Estrus	7.30 ± 0.44[a]	4.34, 9.71
		Diestrus	17.0 ± 0.32	14.2, 18.60
		Intervals		
		Onset of estrus to ovulation	6.80 ± 0.27[a]	5.58, 8.72
		Ovulation to the end of estrus	0.496 ± 0.35	−2.56, 2.23
Triple	2	Length		
		Interovulatory interval	24.10 ± 6.60	17.60, 30.70
		Estrus	7.82 ± 1.50	6.32, 9.32
		Diestrus	16.20 ± 4.62	11.60, 20.90
		Intervals		
		Onset of estrus to ovulation	7.91 ± 1.94	5.97, 9.85
		Ovulation to the end of estrus	−0.09 ± 0.44	−0.53, 0.35
Overall	33	Length		
		Interovulatory interval	23.80 ± 0.55	17.60, 34.70
		Estrus	6.56 ± 0.30	3.15, 9.71
		Diestrus	17.90 ± 0.46	11.60, 27.00
		Intervals		
		Onset of estrus to ovulation	5.96 ± 0.31	1.70, 9.85
		Ovulation to the end of estrus	0.60 ± 0.16	−2.56, 2.23

[a] Differences were considered significant at a P value of less than 0.05 level.

respectively). No influences were found for age or BCS in the prevalence of multiple ovulations in these four animals.

In single ovulators, a nonsignificantly higher frequency of ovulations occurred from the right ovary (57.9%; n = 11) compared with the left ovary (42.1%; n = 8). In double ovulators (n = 12), ovulation occurred from a single ovary on four and three occasions, respectively for the left and right ovaries, whereas it occurred from both the ovaries on five occasions. In triple ovulators (n = 2), two of the follicles ovulated from the right ovary and the remainder from the left.

Of the 14 multiple ovulations (12 double and 2 triple ovulations), seven were considered synchronous, with an interval of less than 24 hours between each ovulation. For the remainder, the mean interval between ovulations was 47.7 ± 7.8 hours (41.8 ± 7.85 for double ovulations and 59.4 ± 17.9 for triple ovulations). An unbalanced distribution of ovulations was observed over the length of a day: a higher number of ovulations occurred during daytime (63.3%; n = 31) compared with 36.7% (n = 18) of ovulations occurred during the night; the distribution was similar between single and multiple ovulations (P = 0.612). Higher BCS affected the occurrence of triple ovulations (P < 0.001), but generally, it did not affect the occurrence of multiple ovulations (P = 0.410). When combined with age, higher BCS correlated with a higher ovulation rate (P = 0.001).

3.4. Growth pattern of dominant follicles

Deviation of the dominant follicle occurred close to Day 9 before ovulation. In single ovulators, deviation occurred 8.72 ± 0.40 days before ovulation, for a follicle diameter of 19.18 ± 0.97 mm, whereas in multiple ovulators, it occurred on Day 8.92 ± 0.23 before ovulation, regardless of the order of follicle ovulation, for a follicle diameter of 18.05 ± 1.16 mm. The average size of the dominant follicle at the onset of estrus (Table 2) was 25 ± 0.95 mm, differing (P < 0.001) in the case of single and multiple ovulations (29.20 ± 1.41 mm and 22.40 ± 1.02 mm, respectively); no differences were recorded in the average size of the dominant follicle at the onset of estrus between triple (23.30 ± 4.24 mm), double (22.20 ± 0.81 mm; P = 0.130), or single (29.20 ± 1.41 mm) ovulations (P = 0.456).

The overall maximum follicular diameter before ovulation was 38.4 ± 0.68 mm (Table 2). It was smaller in multiple ovulatory cycles (37.20 ± 0.82 mm) than that in single ovulatory cycles (P = 0.03): in single ovulations (n = 19), the mean maximum follicular diameter was 40.20 ± 1.41 mm, contrasting with 36.70 ± 0.86 mm in double ovulations (n = 24) and 38.60 ± 2.39 mm (n = 6) in triple ovulations (Table 2). In the case of multiple ovulations, the maximum follicular diameter did not vary with the order of ovulation, whether double (P = 0.096) or triple ovulations (P = 0.942) were considered, though the second follicle to ovulate was usually smaller. In double ovulations, maximum follicular diameter was 38.30 ± 1.25 and 35.41 ± 1.08 mm for the first and second ovulatory follicles, respectively, whereas in triple ovulations, it was 38.20 ± 7.8, 37.50 ± 1.38, and 40.00 ± 4.39 mm, respectively, for the first, second and third ovulated follicle.

The ovulation rate did not correlate with the daily growth rate of the dominant follicle neither during the period from deviation to the onset of estrus (P = 0.854) nor

Table 2
Follicular development pattern in the breeding season for the *Asinina de Miranda* jennies.

Number of ovulations	n	Parameter (mm)	Mean ± standard error	Minimum–maximum
Single	19	Dominant follicle size		
		At deviation	19.18 ± 0.97	13.20–30.32
		At onset of estrus	29.20 ± 1.41[a]	19.30–46.90
		MFD at ovulation	40.20 ± 1.05[a]	31.80–47.90
		Daily growth rate		
		From deviation to onset of estrus	2.62 ± 0.15	1.60–4.07
		From onset of estrus to ovulation	3.34 ± 0.31	1.66–5.80
Double	24	Dominant follicle size		
		At deviation	17.00 ± 0.95	15.90–25.25
		At onset of estrus	22.20 ± 0.81[a]	15.74–31.79
		MFD at ovulation	36.70 ± 0.86[a]	30.29–44.19
		Daily growth rate		
		From deviation to onset of estrus	2.63 ± 1.86	0.05–8.33
		From onset of estrus to ovulation	3.10 ± 0.25	1.49–6.02
Triple	6	Dominant follicle size		
		At deviation	21.57 ± 4.62	14.09–43.56
		At onset of estrus	23.30 ± 4.24	15.60–43.50
		MFD at ovulation	38.60 ± 2.39	30.36–46.03
		Daily growth rate		
		From deviation to onset of estrus	2.41 ± 0.48	0.05–3.13
		From onset of estrus to ovulation	3.05 ± 0.64	1.57–5.14
Overall	49	Dominant follicle size		
		At deviation	18.46 ± 0.83	15.29–43,50
		At onset of estrus	25.00 ± 0.95	15.60–46.90
		MFD at ovulation	38.40 ± 0.68	30.29–47.86
		Daily growth rate		
		From deviation to onset of estrus	2.60 ± 0.19	0.05–5.83
		From onset of estrus to ovulation	3.18 ± 0.18	1.49–5.80

Abbreviations: MFD, maximum follicular diameter.
[a] Differences were considered significant at a P value less than 0.05 level.

during the period from the beginning of estrus until ovulation (P = 0.955). The daily follicular growth rate during the estrus was significantly higher than that in the period from deviation to the onset of estrus in single (P < 0.001), double (P < 0.001), and triple ovulations (P = 0.004). Higher follicular size at the onset of estrus and higher daily growth rates of the dominant follicle during estrus were associated with ovulation of larger follicles (P = 0.001 and P = 0.027, respectively).

Dominant follicles reached 30 mm in diameter 4.1 ± 1.13 days before ovulation, in the case of single ovulations, or 2.9 ± 2.47 days before ovulation in the case of multiple ovulations. The follicular growth rate reported a slowdown of −0.124 ± 0.13 mm/h, as estimated by the difference in diameter between the last two measurements before ovulation; this slowdown did not differ significantly between single or multiple ovulations (P = 0.146; n = 23). Body condition score did not affect the size of the dominant follicle at the onset of estrus (P = 0.688) for this group of *Asinina de Miranda* jennies. Nor did it affect the maximum follicular diameter before ovulation (P = 0.818) or the daily follicle growth rates, during the moments both before (P = 0.729) and after the onset of estrus (P = 0.564).

3.5. Serum progesterone

Mean serum P4 levels at 24 hours after ovulation were 0.48 ± 0.14 ng/mL, rising sharply to values of 5.56 ± 0.86 ng/mL by postovulatory Day 3. Thereafter, and until Day 15, P4 levels rose and remained above 10 ng/mL. Progesterone levels start to drop 2 to 3 days before the onset of estrus, around Days 15 and 16 of the cycle. In estrus, P4 levels remained below 0.2 ng/mL until ovulation (Fig. 1).

The area under the P4 curve, corresponding to the total level of P4 in diestrus, was higher in multiple ovulatory cycles than that in single ovulatory cycles (283.5 ± 18.6 vs. 272.9 ± 21.5, respectively; 95% confidence interval; Fig. 1; P = 0.001).

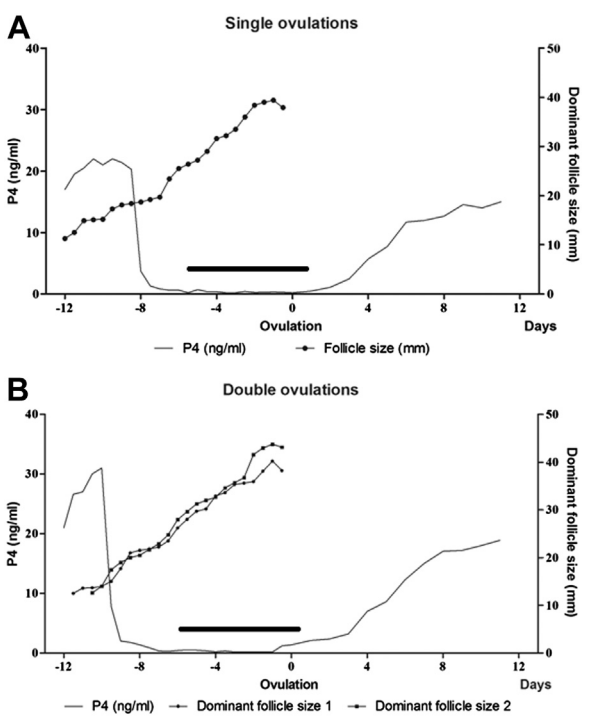

Fig. 1. Serum progesterone and dominant follicle growth during the estrous cycle for the *Asinina de Miranda* jennies (mean ± standard error). The black bar corresponds to the length of estrus. Values are presented in separate for single (A) and double ovulations (B). P4, progesterone.

4. Discussion

Donkeys are often described as displaying longer estrous cycles than horses but similar in length to pony mares [7,13]. This also applies to *Asinina de Miranda* jennies. The present study found that, in spring, the interovulatory interval for this breed was close to 24 days, which is in accordance with similar studies on other breeds. Considerable variation for estimates of the estrous cycle in donkeys can be found in the available literature, which in part might be due to the period surveyed, the age of the jennies, or the methods used to define the cycle stages.

An overall estrous cycle length of 24 to 25 days is currently accepted for the *Catalan* (24.9 days; [13]), the *Anatolian* (25 days; [24]), the Brazilian *Pêga* (24.2 days; [9]) and *Marchador* (23 days; [25]), the *Mammoth* (23.3 days; [6]), the *Martina Franca* (23.6 days; [22]), and the *Baudet de Poitou* (25.8 days; [26]). The present study surveyed estrous cycles mainly during spring (from April to June), but in accordance with studies from other teams, little variation in the length of estrous cycle with season is to be expected in donkeys from spring to autumn [6,9,22].

In this study, the estimated mean estrus length for *Asinina de Miranda* (6.56 ± 0.55 days) was based on basal P4 concentrations combined with the exhibition of typical estrous behavior. This estimate was similar to that reported for other European breeds: 6.7 days for the *Martina Franca* [22], 6.1 ± 2.1 days for the *Zamorano-Leones* [14], and 5.64 ± 0.2 days for the *Catalan* [13]. But, it was shorter than that reported for the *Baudet de Poitou* (7.5 ± 1.2 days) [26] or Brazilian donkeys (7.9 ± 2.5 days) [10].

In jennies, characteristic signs of estrous behavior in the presence of the jack include mouth clapping, posturing, tail raising, urinating, and clitoral winking [20]. The main homotypical signs of estrus detected in our study were similar to those described for jennies in other studies and used to delimit the estrus stage [6,7,13]. The present study was able to obtain a more accurate estimate of the duration of estrus by integrating the behavioral signs of group-teased females with individual P4 measurements, thus overcoming the reported weaknesses of group teasing in donkeys [6].

The mean diestrus length for the *Asinina de Miranda* was similar to those reported for *Mammoth* jennies [6] and the *Martina Franca* [22], but it was slightly shorter than those reported for other breeds: 17.9 ± 0.46 days vs. 19.83 ± 0.36 in the *Catalan* [13] or 19.3 ± 0.6 for standard jennies [7].

In the present study, age did not affect the lengths of estrus and diestrus in *Asinina de Miranda* jennies.

Nevertheless, older jennies displayed longer interovulatory intervals, in accordance with those reported for mares [12]. This might be associated with slower growth of the dominant follicle, as argued by Ginther et al. [27]. This finding could not be ascertained in the present study because of a disproportionate distribution of ages, with a predominance of younger jennies. Nonetheless, in the group of females surveyed, the interovulatory interval and the duration of diestrus were significantly affected by BCS: higher BCS lengthened the interovulatory intervals and diestrus in *Asinina de Miranda* jennies. Although changes in BCS or in metabolites and metabolic hormones such as leptin, insulin, or insulinlike growth factor 1 have been associated with follicular activity and mare fertility [28,29], there is a lack of incontrovertible information available on the effect of BCS on conditioned measurements of the duration of each stage of the estrous cycle in cyclic mares [30,31].

Moreover, Fitzgerald and McManus [31] reported similar effects of BCS on the characteristics of the estrous cycle, affirming that the length of diestrus and interovulatory interval was greater in fat mares (BCS $\geq$7) under controlled management than that in mares with moderate BCS. The effect of high BCS on the duration of the estrous stages in different studies may incorporate the effect of other parameters, such as the age and breed of the female, the management (controlled vs. free ranging), the physiological status (postpartum, cyclic), the extent of the period considered (the entire year vs. the breeding season), or the number of consecutive cycles, limiting the scope of this discussion. It is possible that this also occurred in the present study, as older mares tend to display higher BCS levels than younger mares.

Multiple ovulations seem to be higher in donkeys than those in horses [15]; the ovulation rate reported in the available literature varies from around 5% to almost 70% [6,7,10,13]. It has been proposed that one main factor for this variation in donkeys might be the breed [13], although no statistical differences were found among three different Spanish breeds [14]. In the present study, the prevalence of multiple ovulations in *Asinina de Miranda* jennies was 42.42%, of which 36.36% were double ovulations and 6.06% triple. These figures were similar to those reported for the three Spanish breeds, the *Catalan* [13,14], the *Andalusian*, and the *Zamorano-Leonês* [14] but lower than those for *Mammoth* donkeys [6].

As previously reported in mares [15] and *Catalan* jennies [13], multiple ovulations were highly repetitive in *Asinina de Miranda* females. According to Ginther [15], this suggests that it may be a heritable trait. The existence of multiple ovulations did not affect the interovulatory interval in *Asinina de Miranda* jennies, although it extended the estrus and the interval from the beginning of the estrus until ovulation. Our results are supported by comparable descriptions in Spanish donkey breeds [14]. In the present study, the prevalence of multiple ovulations was positively affected by BCS as has also been reported in mares [32]. Information gathered on multiple ovulations in *Asinina de Miranda* jennies, along with the positive effect of BCS on their occurrence, highlights the need to routinely implement an early pregnancy diagnosis service to identify twin pregnancies and minimize their risk to the reproductive efficiency of this breed.

The frequency of ovulation from each ovary registered in this study was similar for the left and the right ovaries, in contrast with that previously reported in horses [15] or donkeys [10]. Yet Taberner et al. [13] also failed to find evidence of statistical differences in the frequency of ovulation from the left or right ovary in *Catalan* jennies. Multiple ovulations may be classified as synchronous, when ovulations occur at intervals less than 24 hours, or asynchronous, if this interval lasts for more than 24 hours. In *Asinina de Miranda* jennies, a similar proportion of synchronous and asynchronous was observed. This contrasts with descriptions of the *Catalan* breed, most of whose ovulations were asynchronous, with intervals ranging from 1 to 9 days [13]. In our study, the maximum interval found between multiple ovulations was 2.48 days, which is longer than that reported for *Pêga* jennies [9] but resembling that reported for Przewalski's mares [33] or standard jennies [5].

When planning assisted reproductive technologies, knowledge regarding development of the dominant follicle, including its size at the onset of estrus, around ovulation and its daily growth rate, is fundamental for manipulating the estrous cycle and inducing ovulation. In *Asinina de Miranda*, these measurements were similar to those reported in other breeds with which the Portuguese breed shares some resemblances in the estrous cycle.

In the present study, the dominant follicle was first detected in the ovary as the fastest growing follicle at about 13 days before ovulation. The mean follicular diameter at the onset of estrus, corresponding to Days 5 to 6 before ovulation in *Asinina de Miranda* jennies, was close to 25 mm, which is in accordance with that of the *Brazilian Marchador* [25] but lower than that reported for the *Martina Franca* (around 31.5 mm; [22]) in the same season.

The dominant follicle reached 30 mm around 2.5 to 4 days before ovulation, for multiple and single ovulations. The average maximum follicular diameter observed in jennies in the present study was 38.4 mm, which is similar to that reported for the *Pêga* [9] and *Marchador* [25] or in standard jennies [7]. But, it was lower than that recorded in *Catalan* (close to 45 mm; [13]) or *Martina Franca* jennies (43.7 mm; [22]). In contrast to the research carried out by Taberner et al. [13], which fails to provide evidence of a link with ovulation type (simple vs. multiple), the maximum follicular diameter was the largest in the cases of single rather than multiple ovulations. Similar observations have also been reported in mares [21] and Brazilian *Marchador* jennies [25].

As expected, the daily follicular growth is higher during estrus than that in the period between deviation and the onset of estrus, as acknowledged in mares [12]. Little information is available for donkeys, as most studies have focused on the follicular growth rate in the 5 days preceding ovulation, which corresponds to estrus. In the present study, the daily growth rate of the dominant follicle was significantly higher after the onset of estrus (3.18 ± 0.18 mm/day) than that in the period before estrus (2.60 ± 0.19 mm/day), independently of the ovulation rate considered. Compared with other studies, the mean daily

follicular growth during estrus for *Asinina de Miranda* jennies was slightly higher than that described for the Brazilian *Marchador*, (2.39 ± 0.37 mm/day [25]) or the mare (2.7 mm/day [15]) but lower than that reported in *Catalan* jennies (3.7 mm/day [13]). As previously reported for Catalonian donkeys [13], there is a slowdown in the daily growth rate of the dominant follicle on the day preceding ovulation in *Asinina de Miranda*. Such knowledge of follicular dynamics is of utmost importance for controlling ovulation in any breed, enabling drug administration schedules and timing of insemination to be personalized.

In the present study, the jennies' BCS affected neither the size of the dominant follicle nor its growth pattern. This seems to contrast with the work of Gastal et al. [16], which reported that in mares, the body condition was positively linked with the maximum diameter of preovulatory follicles for the first ovulations of the breeding season. Moreover, Lemma et al. [17] found that the BCS was positively correlated to the diameter of the preovulatory dominant follicle in Ethiopian jennies. The relatively constant moderate body condition evidenced by the females in the present study, however, might explain the differences between our results and those referred to that mentioned previously.

In general, the cyclic changes in P4 levels in *Asinina de Miranda* resemble those reported in other donkey breeds [9,22,34] and mares [21]. Individual variations are expected both in the onset of P4 peak and in P4 levels, as has also been described in mares with estrous cycles of similar length [35,36]; such variations were associated with differences in the secretory capacity of the CL and the hormonal catabolic rate and appear to be more significant in the first 5 days of the diestrus [36]. Moreover, the existence of multiple ovulations and their frequency of occurrence may also influence the levels of P4 measured. Comparison of P4 levels in diestrus, using the area under the curve, shows that, in our study, they were affected by the number of ovulations, in accordance with data presented by Meira et al. [9] in *Pêga* jennies.

4.1. Conclusions

The present study has enabled identification of the estrous cycle characteristics of *Asinina de Miranda* jennies during the breeding season. Data collected revealed some resemblances with other Mediterranean and Brazilian donkey breeds. It was observed that the BCS was positively linked to multiple ovulations and the length of interovulatory intervals, although the jennies maintained a moderate BCS. Furthermore, jennies with higher BCS appeared to cease estrous behavior after ovulation faster than those with a lower score. The BCS did not affect estrous and diestrous duration *per se* and did not seem to be linked to dominant follicle size and growth rate. The present study also showed that at the onset of estrus, the dominant follicle was about 25 mm in diameter. This study also provides important data on measurements concerning follicular growth for those intending to manipulate the *Asinina de Miranda's* cycles for assisted reproduction.

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Author contributions: M. Quaresma and R. Payan-Carreira conceived the study and participated in its design. M. Quaresma conducted the animal reproductive assessment and the sequential blood collection and ultrasound examinations. M. Quaresma analyzed and collected data from ultrasound films and interpreted the data. In addition, both the authors were responsible for compiling the literature review, drafting, and finalizing the article. Both the authors read and approved the final article. Finally, both the authors studied and addressed the issues raised in the review panel's comments and together revised the article.

Competing interests

None of the authors has any financial or personal relationship that could inappropriately influence or bias the content of the article.

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