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Revised

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, based on data collected from serial ultrasonographic examination and serum progesterone (P4) 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 beginning of estrus to 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 to multiple ovulators (18.05±1.16 mm). The overall maximum follicular diameter before ovulation was smaller in multiple ovulatory cycles than 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 prior and after 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.

Keywords: donkey, reproduction, body condition score, ovulation, follicle.

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1. Introduction

The *Asinina de Miranda* is an endangered breed of donkey originating from the far north east of Portugal. It is characterized by having a long bay coat, a height of over 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 studbook [1]; the average foaling rate over the past 10 years has been close to 50 foals a year, though this number has increased to around 70 foals a year in the last 2 years [2]. Average age at 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 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 (AEPGA) 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 is 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*;

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however, productive optimization requires greater knowledge of the donkey's reproductive physiology in order to increase foaling rates [4]. Detailed knowledge of the characteristics of the estrous cycle in *Asinina de Miranda* jennies is vital in order to improve the reproductive management of the breed and increase reproductive efficiency. Furthermore, knowledge into how follicles develop as well as their size at specific moments in this process (such as at deviation or at ovulation) is fundamental to pharmacological manipulation of the cycle or to exogenous induction of ovulation.

Jennies are similar to mares in many reproductive aspects but tend to have longer breeding seasons [5, 6], as well as 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 into 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*. In order 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].

ACCEPTED MANUSCRIPT

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 prior to 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 BCS were also tested.

2. Material and methods

2.1. Animals, management and sample collection

This study used 14 non-pregnant clinically healthy jennies of the Portuguese *Asinina de Miranda* breed of donkey. The jennies were aged from 3 to 18 years: 6 young jennies aged between 3 and 5 years, 6 adult females aged between 6 to 8 years and 2 older females aged over 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 body condition scores ranging from 4 to 7 on a 9 point scale (5.7 points on average). BCS was regularly evaluated during the breeding season, at 5-week intervals [18].

Data was collected during two breeding seasons, from April to late September, using a group of seven different animals each year. The regular estrous cycles for both 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 5 females, two from the first year's group and three from the second, recorded 3 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 exam.

The animals were housed in Vila Real, Portugal (41º17'N 7º44'W), in the University facilities, and kept under natural photoperiod. All the animals were routinely vaccinated for equine influenza and tetanus (Proteq-Flu TE^{TM}, Merial S.A.S., Lyon, France) and dewormed every 6 months with 200µg Ivermectin (Noromectin Oral Paste, Norbrook Laboratories, Northamptonshire, UK) per kg of bodyweight. The jennies were kept in a 2,500 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 5–7 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 1kg 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 behaviour 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 *labiae* with exposure of the clitoris) and urinating; raising the tail; and posturing. In contrast, non-receptivity behaviour 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 behaviour.

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

For progesterone measurements, blood samples were collected by venipuncture of the jugular into serum-gel tubes (S-Monovette®, Sarstedt, Nümbrecht, Germany), preceding the ultrasound session, and placed in ice. Samples were centrifuged after collection at 2500 X g for 10 min; serum was harvested and stored at -20ºC until assayed. Serum progesterone concentrations were determined by chemiluminescent immunoassay (IMMULITE 1000®; Siemen's Medical Solutions Diagnostics, Los Angeles, CA, USA), using a commercial progesterone kit (Siemens Immulite® Progesterone Kit) and commercially available reagents (all from Siemens Healthcare Diagnostics, Amadora, Portugal). Interassay coefficient of variance for the controls (respectively CON4, CON5 and CON6 for low, intermediate and high controls - 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 progesterone kit for donkeys, serial dilutions in 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 eight to twelve-hour intervals in estrus by transrectal palpation

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164 followed by ultrasound (US) examination of the genital tracts using a linear-array US
165 scanner equipped with a 5 MHz linear transducer (Shenzhen Veterinary US
166 scanner), according to the procedures described by Ginther [15]. The scanner was
167 connected to a video camera (DCRHC96E, Sony) and all US scans were recorded
168 for subsequent analysis.

169 The diameters of the ovarian dominant follicles were obtained retrospectively from
170 the average of the narrowest and widest dimensions in selected US scan images,
171 considering only the follicular antrum. One single operator established follicular size
172 measurements, using *ImageJ* software (http://imagej.nih.gov/ij/index.html) on fixed
173 frame images. A dominant follicle was defined as the one deviating from other
174 growing follicles, and becoming the largest in the ovary, whether or not it ovulated
175 [21]. The dominant follicle, or follicles in the case of multiple ovulations, was
176 considered ovulatory if it reached ovulation. Day 0 of the cycle was set as the day of
177 ovulation or, in the case of multiple ovulation, as the day of last ovulation.

178 The interovulatory interval was defined as the interval (in days) between estrus-
179 associated ovulation in successive cycles, or as the period between the last ovulation
180 of each cycle in the case of multiple ovulations. Sequential US records were used to
181 establish the moment of ovulation as the mid-time between two US scans when a
182 dominant follicle ceased to be observed during estrus. The beginning of estrus was
183 set at the moment when the female first showed signs of receptivity to the male, with
184 progesterone (P4) levels below 1 ng/ml, while the end of estrus was considered to be
185 the moment when the jenny refused the jack. Diestrus corresponded to the period
186 when serum progesterone levels remained above 1 ng/ml and the female refused the
187 jack [22]. The ovulation rate was defined as the number of ovulated follicles, based

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188 on US observation of the collapse of the preovulatory follicle(s) and loss of > 90% of
189 fluid by the time of the next examination [23].

190 *2.3. Statistical analysis*

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

198 An ANOVA test was conducted, followed by a Bonferroni post-hoc test for means
199 comparison, in order to analyse the effect of BCS and age on the length of each
200 cycle stage, the ovulation rate, the growth rate and follicle size, the total level of
201 progesterone and the time in heat after ovulation. Total secretion of progesterone
202 during diestrus was assessed by estimating the area under curve, applying the
203 trapezoidal rule, i.e. calculating the $\Delta X*(Y1+Y2)/2$, using Microsoft® Excell 2010 for
204 Windows. Furthermore, a covariance analysis was used to explore the effect of BCS
205 (main effect) and age (covariable) on the ovulation rate. Possible correlations
206 between the ovulation rate and the length of the cycle phases, the follicular growth
207 rate and follicle size, the total progesterone level and time in heat after ovulation
208 were analyzed by Pearson's chi-square test. Differences and correlations were
209 regarded as significant at $P<0.05$.

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

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3. Results

3.1. Estrous behaviour

During this study, all jennies in estrus showed homotypical signs of estrous behaviour (i.e. characteristic for the species) such as mouth clapping, clitoral winking, posturing or showing increased interest towards the male. Often, the jennies also exhibited heterotypical behaviour (i.e. signs of estrous behaviour 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 to 27 days) and 6.65 ± 0.30 days (3.15 to 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 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 in the length of interovulatory intervals were observed, nor in the length of any phases of the cycle. The ovulation rate did not correlate with the length of the interovulatory

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interval (p=0.990) or diestrus (p=0.169). However, the estrus was longer in multiple ovulators than in single ovulators (5.22 ± 0.40 *vs.* 6.96 ± 0.33; p=0.03). In addition, the ovulation rate correlated positively with the period from the beginning of estrus to last ovulation (p=0.01).

In general, ovulation occurred less than 15 hours before the end of the estrus, but jennies maintained estrous behaviour for a variable period after ovulation (Table 1). In 21 of the 33 cycles studied, estrus lasted between 4 to 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 behaviour 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 last ovulation. No significant differences (p=0.508) were found for time in heat after 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 behaviour sooner after ovulation, 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 post-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%) triple ovulations. The number of cycles with multiple ovulations was significantly higher (p=0.02) in four jennies, together producing 64.3% (nine in fourteen) 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 in the other multiple-ovulating jennies (81.8% *vs* 40%, respectively). No influences were found for age or BCS in the prevalence of multiple ovulations in these four animals.

In single ovulators, a non-significantly higher frequency of ovulations occurred from the right ovary (57.9%; n=11) compared to the left ovary (42.1%; n=8). In double ovulators (n=12), ovulation occurred from a single ovary on 4 and 3 occasions respectively for the left and right ovaries, while it occurred from both ovaries on 5 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), 7 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 to 36.7% (n=18) of ovulations occurring 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 prior to ovulation, for a follicle diameter of 19.18 ± 0.97 mm, while 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 prior to ovulation was 38.4 ± 0.68 mm (Table 2). It was smaller in multiple ovulatory cycles (37.20 ± 0.82 mm) than 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 with 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 follicle, respectively, while in triple ovulations it was 38.20 ± 7.8, 37.50 ± 1.38 and 40.00 ± 4.39 mm, respectively, for the 1st, 2nd and 3rd 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 from the beginning of estrus until ovulation ($p=0,955$). The daily follicular growth rate during the estrus was significantly higher than in the period from deviation to 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 showed a slowdown of -0.124 ± 0.13 mm/hour, as estimated by the difference in diameter between the last two measurements prior to ovulation; this slowdown did not differ significantly between single or multiple ovulations ($p=0.146$; $n=23$). BCS 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 prior to 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 progesterone 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 post-ovulatory day 3. Thereafter, and until day 15, progesterone levels rose and remained above 10 ng/ml. Progesterone levels start to drop 2 to 3 days prior to the onset of estrus, around day 15 and 16 of the cycle. In estrus, progesterone levels remained below 0.2 ng/ml until ovulation (Figure 1).

The area under the progesterone curve, corresponding to the total level of progesterone in diestrus, was higher in multiple ovulatory cycles than in single

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ovulatory cycles (283.5 ± 18.6 *vs.* 272.9 ± 21.5; 95% confidence interval; Figure 1) (P=0.001).

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 progesterone concentrations combined with the exhibition of typical estrous behaviour. 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 for Brazilian donkeys (7.9 ± 2.5 days) [10].

In jennies, characteristic signs of estrous behaviour 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 behavioural signs of group teased females with individual progesterone 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 for 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, due to 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 body condition scores 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 IGF-I 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].

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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≥7) under controlled management than 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 (post-partum, cyclic) or 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 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], even though 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 3 Spanish breeds – the *Catalan* [13, 14], the *Andalusian* and the *Zamorano-Leonês* [14], but lower than for *Mammoth* donkeys [6].

As previously reported in mares [15] and in *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 as well as the interval from the beginning of the estrus until ovulation. Our results are supported by comparable descriptions in Spanish donkey breeds [14]. In

411 the present study, prevalence of multiple ovulations was positively affected by BCS,
412 as has also been reported in mares [32]. Information gathered on multiple ovulations
413 in *Asinina de Miranda* jennies, along with the positive effect of BCS on their
414 occurrence, highlights the need to routinely implement an early pregnancy diagnosis
415 service to identify twin pregnancies and to minimize their risk to the reproductive
416 efficiency of this breed.

417 The frequency of ovulation from each ovary registered in this study was similar for
418 the left and the right ovary, in contrast with that previously reported in horses [15] or
419 donkeys [10]. Yet, Taberner et al. [13] also failed to find evidence of statistical
420 differences in the frequency of ovulation from the left or right ovary in *Catalan*
421 jennies. Multiple ovulations may be classified as synchronous, when ovulations occur
422 at intervals less than 24 hours, or asynchronous, if this interval lasts for more than 24
423 hours. In *Asinina de Miranda* jennies, a similar proportion of synchronous and
424 asynchronous was observed. This contrasts with descriptions of the *Catalan* breed,
425 most of whose ovulations were asynchronous, with intervals ranging from 1 to 9 days
426 [13]. In our study the maximum interval found between multiple ovulations was 2.48
427 days, which is longer than that reported for *Pêga* jennies [9], but resembling that
428 reported for Przewalski's mares [32] or for standard jennies [5].

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

435 In the present study, the dominant follicle was first detected in the ovary as the
436 fastest growing follicle at about 13 days prior to 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 25mm, which is in accordance with that of the *Brazilian Marchador* [25], but lower than that reported for the *Martina Franca* (around 31.5mm; [22]) in the same season.

The dominant follicle reached 30mm around 2.5 to 4 days prior to ovulation, for multiple and single ovulations, respectively. 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 45mm; [13]) or in *Martina Franca* jennies (43.7mm; [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 largest in the cases of single rather than multiple ovulations. Similar observations have also been reported in mares [21] and in Brazilian *Marchador* jennies [25].

As expected, the daily follicular growth is higher during estrus than in the period between deviation and 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 in the period prior to estrus (2.60 ± 0.19 mm/day), independently of the ovulation rate considered. Compared to 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

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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 did not affect the size of the dominant follicle nor its growth pattern. This seems to contrast with the work of Gastal et al. [16], which showed that in mares the body condition was positively linked with the maximum diameter of pre-ovulatory follicles for the first ovulations of the breeding season. Moreover, Lemma et al. [17] found that BCS was positively correlated to the diameter of the pre-ovulatory 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 above.

In general, the cyclic changes in progesterone levels in *Asinina de Miranda* resemble those reported in other donkey breeds [9, 22, 34], as well as in mares [21]. Individual variations are expected both in the onset of progesterone peak and in progesterone 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 *corpus luteum* 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 progesterone measured. Comparison of progesterone 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.

5. Conclusions

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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 BCS was positively linked to multiple ovulations and the length of interovulatory intervals; although the jennies maintained a moderate body condition score. Furthermore, jennies with higher BCS appeared to cease estrous behaviour after ovulation faster than those with a lower score. BCS did not affect estrous and diestrous duration *per se,* and neither did it 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.

6. Conflict of interest statement

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

7. Authors' participation

MQ and RP-C conceived the study and participated in its design. MQ conducted the animal reproductive assessment as well as the sequential blood collection and ultrasound exams. MQ analysed and collected data from ultrasound films and interpreted the data. In addition, MQ and RP-C were responsible for compiling the literature review, drafting and finalizing the paper. Both authors read and approved the final manuscript. Finally, both authors studied and addressed the issues raised in the Review panel's comments, and together revised the manuscript.

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625

626 **Figure captions**

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

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

Type of ovulation	*n*	Parameter (days)	Mean ± SE	Minimum - Maximum
Single	19	Length		
		Interovulatory interval	**23.8 ± 0.78**	**19.5 - 34.10**
		Estrus	**5.97 ± 0.37***	**3.15 ± 8.89**
		Diestrus	**18.6 ± 0.65**	**15.0 - 27.00**
		Intervals		
		Onset of estrus to ovulation	**5.22 ± 0.40***	3.15 - 8.89
		Ovulation to 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***	4.34 – 9.71
		Diestrus	**17.0 ± 0.32**	**14.2 - 18.60**
		Intervals		
		Onset of estrus to ovulation	**6.80 ± 0.27***	5.58 - 8.72
		Ovulation to 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 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 end of estrus	**0.60 ± 0.16**	**(-)2.56 – 2.23**

*Differences were considered significant at a $P < 0.05$ level.

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

Number of ovulations	*n*	Parameter (mm)	Mean ± SE	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***	**19.30 – 46.90**
		MFD at ovulation	**40.20 ± 1.05***	**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***	15.74 – 31.79
		MFD at ovulation	**36.70 ± 0.86***	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

*Differences were considered significant at a P < 0.05 level; MFD = maximum follicular diameter.

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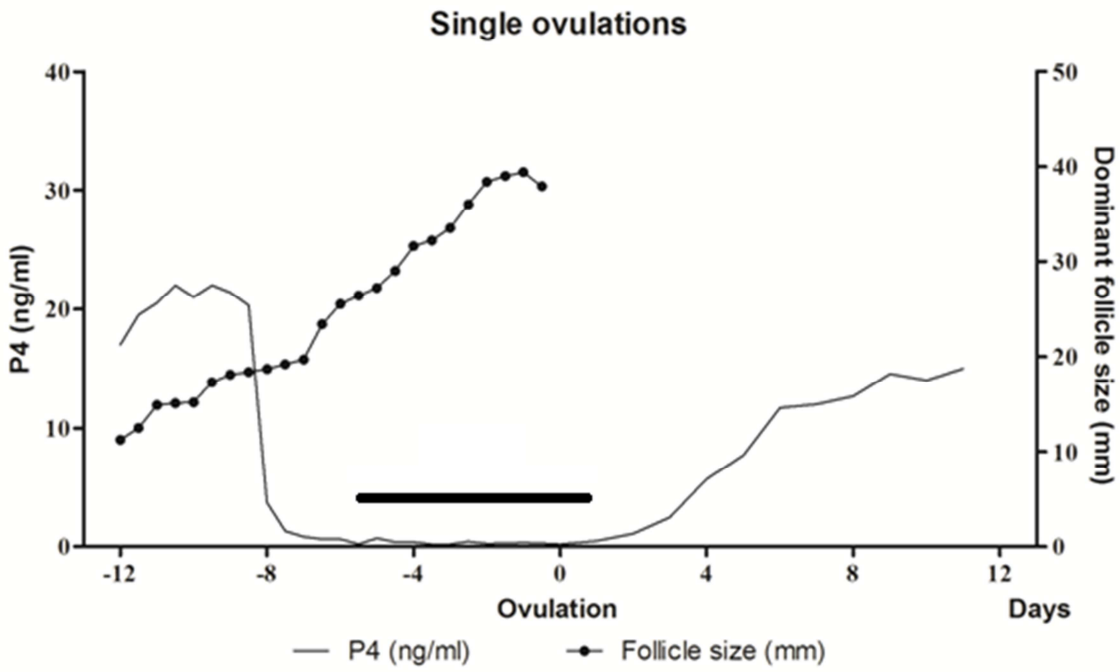
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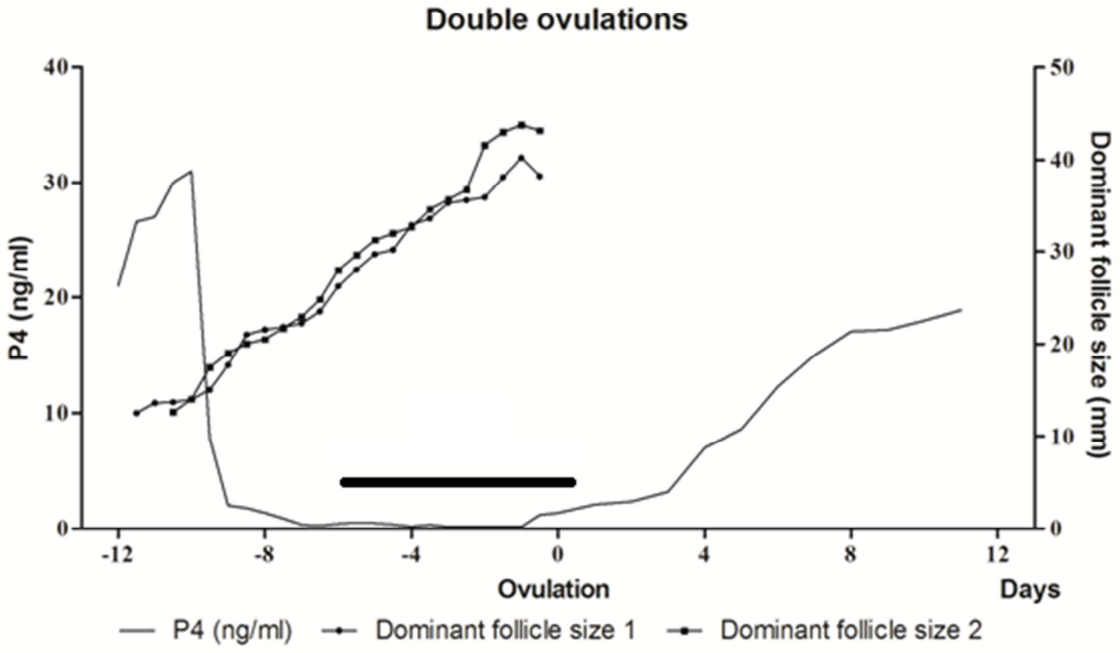
P4 (ng/ml)
Dominant follicle size (mm)
Ovulation
Days
P4 (ng/ml)
Follicle size (mm)


Double ovulations
P4 (ng/ml)
Dominant follicle size (mm)
Ovulation
Days
P4 (ng/ml)
Dominant follicle size 1
Dominant follicle size 2

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Highlights

- We revised the manuscript as requested.
- We reformulated the sentences that were less clear, and an English revision of the final manuscript form was undertaken
- Some concerns regarding the material and methods section were clarified

- This paper presents the lengths of the interestrous interval, estrus and diestrus in the Portuguese donkey breed *Asinina de Miranda*
- We also describe the ovulation rate in the studied population.
- Follicular growth rates and the sizes of the dominant follicle at the onset of estrus and at ovulation are detailed in single and multiple ovulations
- The total progesterone level in diestrus is compared in multiple and single ovulatory cycles.

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