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Reproductive Patterns in the Non-Breeding Season in *Asinina de Miranda* Jennies

M Quaresma^{1,2,3}, SR Silva² and R Payan-Carreira²

¹Hospital Veterinário, Escola de Ciências Agrárias e Veterinárias, Universidade de Trás-os-Montes e Alto Douro (UTAD), Vila Real, Portugal; ²Centro de Ciência Animal e Veterinária, UTAD, Vila Real, Portugal; ³Associação Para o Estudo e Proteção do Gado Asinino (AEPGA), Atenor, Portugal

Contents

This study aims to characterize the reproductive patterns in *Asinina de Miranda* jennies during the non-breeding season. Reproductive activity was surveyed in 12 females, aged between 3 and 18 years old, using ultrasound and teasing with a jack. The animals were monitored from September to April, six in each consecutive year. Of these 12 females, nine showed disruption to the normal pattern of ovarian activity during the non-breeding season. Loss of normal cyclicality included anoestrus (41.7%), silent ovulatory oestrus (25%), and persistence of *corpus luteum* (8.3%). Only three females maintained a regular cyclic pattern with oestrous behaviour during the non-breeding season. Anoestrus began in early November and lasted for an average of 147 ± 28 days (113–191 days), ending near to the spring equinox. Onset of silent oestrous cycles began more erratically, between October and February. In both groups the first behavioural ovulation of the year occurred around the time of the spring equinox. Disrupted reproductive activity was preceded by a shorter oestrous cycle only in females entering anoestrus. The mean follicle size in the first ovulation of the year was larger than in the reproductive season (44.7 ± 2.45 mm vs 39.2 ± 3.60 mm) in anoestrous jennies with protracted oestrus. Though age and body condition score (BCS) were associated, changes in BCS below a threshold of four points (for anoestrus) and five points (for silent oestrus) contributed greatly to disruption of reproductive cycles. BCS in females with regular oestrous cycles during the winter season remained unchanged or exceeded five points prior to the winter solstice.

Introduction

Most donkey breeds, such as the *Asinina de Miranda*, are threatened with extinction (F.A.O. 2013; Quaresma et al. 2013b). A more detailed understanding of the species' reproductive cycle is needed for conservation. Moreover, less attention has been drawn to the reproductive cycle of female donkeys compared to horses, and information on the subject remains quite limited. Most species living in temperate zones display a seasonal reproductive pattern, mainly timed by annual changes in photoperiod, the season of birth matching the optimal time of the year for offspring survival (Fitzgerald and McManus 2000; Nagy et al. 2000). Horses are seasonal long-day breeders; 85–95% of mares cease ovarian cyclicality around the autumn equinox, often displaying irregular periods of oestrous behaviour in the transitional periods (Ginther 1992). Jennies, on the other hand, display a more irregular seasonal pattern, possibly related to climate and latitude

variations, but also affected by factors such as nutritional management, age and parity (Ginther et al. 1987; Henry et al. 1987; Lemma et al. 2006).

The seasonal reproductive activity of mares is regulated mainly by the photoperiod, despite the proven influence of additional modulators with an important impact on ovarian activity (Aurich 2011). These factors include body condition (Fitzgerald and McManus 2000; Godoi et al. 2002), age (Morel et al. 2010) and parity (Sharma et al. 2010). Body adiposity and nutritional status have profound effects on the occurrence, depth and length of seasonal anoestrus in mares (Henneke et al. 1983; Ginther 1992; Gentry et al. 2002); females with high body condition score may display regular oestrous cycles or display considerable follicular activity during the winter (Waller et al. 2006). The first ovulation after the non-breeding season might be affected by several parameters, but it usually occurs from April to May, even in mares with low BCS (Henneke et al. 1983; Gentry et al. 2002).

The influence of body condition score on ovarian activity during the nonbreeding season, as described in horses, has not yet been clarified in donkeys. The reproductive characteristics of the breed *Asinina de Miranda* in the reproductive season have already been described (Quaresma and Payan-Carreira 2015) but a deeper understanding of its seasonal variations is needed. The *Asinina the Miranda* breed is a small donkey population with around 60 foaling annually, during the past 5 years. To avoid extinction in 30–50 years, conservation programs have recently set out plans to increase the number of females bred each year and to reduce the age of first offspring production together with a reduction in the mortality rate (Quaresma et al. 2014). The jennies of the *Asinina de Miranda* breed are now being increasingly used for milk production (Quaresma et al. 2014); the ability to breed year-round would contribute to a decrease in the seasonal variation in milk availability and would be of great economic value.

Generalizations in the mare's reproductive cycle and in the effects of particular modulatory factors on the occurrence of anoestrus in winter may not be directly applicable to the donkey. Therefore, this study aims: (i) to characterize the reproductive activity patterns in a group of *Asinina de Miranda* jennies during the non-breeding season, when daylight is significantly shorter in Portugal, through assessment of the length of the

follicular and luteal phases, the maximum size of the dominant follicle during the cycle, as well as the moments of disruption in the cycle and the first oestrus of the year; (ii) to estimate the influences of BCS variations, age and parity on changes in the reproductive pattern.

Material and Methods

Animals, management and sample collection

This study used 12 non-pregnant jennies of the *Asinina de Miranda* breed, with ages ranging from 3 to 18 years old (mean 6.17 ± 4.20 years): five maiden jennies aged under 5 years; six adult females aged between 5 and 8 years; and one older female aged over 15. The small number of females in the older group was due to exclusion because of ovarian diseases. Except for the maiden jennets, all the others were non-lactating females that had foaled at least once in their lifetime; none of the females had foaled in the breeding season preceding this study. All jennies involved in the experiments were cycling at the onset of the study, and were considered healthy after a breeding soundness examination. With the exception of the older jenny, which was not mated, all the other females were bred during the reproductive season and became pregnant after the end of the study, proving their fertility.

The study was carried out in two consecutive years, from September to April, using six different animals each year; the animals were randomly assigned to each year. Animals were housed in Vila Real, Portugal (41°17'N, 7°44'W; 431 m above sea level), in the university facilities, and kept under natural photoperiod, with 9 h of daylight at the winter solstice and 15 h at the summer solstice. Both years showed similar climatic conditions in terms of temperature, humidity, rainfall and daylight. The animals were routinely vaccinated for equine influenza and tetanus (Proteq-Flu TETM; 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 bodyweight.

The jennies were maintained under the same management conditions during the entire trial, in a 2500 m² paddock with a 50 m² shelter for protection from rain, sun and wind. Animals were fed according to accepted protocols (Smith and Wood 2008), consisting of an average of 5–7 kg of hay and straw per jenny, which corresponded to a dry matter intake of between 1.5% and 2% of body weight, divided into two feedings a day. During winter, 200–400 g of concentrate per jenny was also distributed twice daily. Clean fresh water was available *ad libitum*.

The animals were handled in accordance to the European Council Guidelines (Directive 2010/63/EU) for protection of animals used for experimental purposes and, as described in Quaresma and Payan-Carreira (2015): the jennies were group teased once a

day, for 30 min, by a male with good libido, placed in a paddock adjacent to that of the jennies, separated by a wire fence. After each teasing session, the male was removed to an indoor paddock in another facility, located 400 m away. The criteria assessed for positive oestrous behaviour and receptivity during teasing was the exhibition of mouth clapping together with at least one of the following signs: winking (rhythmic eversion of the *vulvar labiae* with exposure of the clitoris) and urinating; raising the tail; and posturing. Clapping alone, or combined with kicking or moving was considered to indicate a transitional stage into or out of oestrus, but not recorded as a receptive behaviour. Non-receptive behaviour included: (i) tail down (holding tail down between hind legs when mounted); (ii) lack of interest (no positive or negative responses to the presence of the teasing jack); and (iii) refusing the jack by moving away or kicking.

For progesterone (P4) measurements, blood samples were collected and processed as previously described by Quaresma and Payan-Carreira (2015). In short, the samples were obtained by venipuncture of the jugular into serum-gel tubes (S-Monovette®; Sarstedt, Nümbrecht, Germany). Harvested serum was stored at –20°C until assayed, using a chemiluminescent immunoassay (IMMULITE 1000®; Siemen's Medical Solutions Diagnostics, Los Angeles, CA, USA), and a commercial progesterone kit validated for donkeys (Quaresma and Payan-Carreira 2015). Interassay coefficient of variance for the controls (Multivalent Control Module; Siemens) ranged from 1.3 to 1.5% for the lower and intermediate controls, to 4.6% for the high control.

BCS was evaluated every 6 weeks by two independent operators, by visual appraisal and palpation, as described by Quaresma et al. (2013a), using a nine point scale. Body adiposity was confirmed using ultrasonography (Aloka SSD 500 V real time scanner equipped with a 7.5 MHz linear probe – UST-5512U-7.5, 38 mm). For estimation of subcutaneous fat, ultrasound images were collected on two spots, on the animal's left side, and were used to measure the subcutaneous fat depths at withers (SFW) and the tissue depth between ribs 12 and 13 (TDBRib13).

Seasons were defined according to Blanchard et al. (2003) and adjusted for horses in Portugal (Atayde and Rocha 2011): the non-breeding (winter) season was set between 1st December and 28th February; the autumn transition was set from 1st October to 30th November and the vernal or spring transition from 1st March to 8th April. The data representing the reproductive season was collected for five consecutive weeks, beginning on May 1st and for 4 weeks beginning on September 5th, using the same animals and procedures described herein.

Assessment of the reproductive activity

During the trials, the jennies' ovarian activity was routinely surveyed every other day during dioestrus or anoestrus, and at 8 to 12-h intervals during oestrus, by

transrectal palpation and ultrasound (US) scanning. The US was performed with a 5 MHz linear-array (Shenzhen Veterinary US scanner), following the procedures described by Ginther (1992). The scanner was connected to a video camera (DCRHC96E; Sony, Tokyo, Japan) and all US scans were recorded for posterior analysis. The following parameters were analysed: the maximum follicle diameter before ovulation or regression and the presence of *corpus luteum* (CL) and its lifespan. The diameters of dominant follicles were obtained retrospectively by using fixed frame images to measure the average of the narrowest and widest dimensions in selected US scans, focusing solely on the follicular antrum. In the absence of dominant follicles (anoestrous females), the dimensions of the two largest follicles were determined. Follicular measurements were established by one single operator, using IMAGEJ[®] software (<http://imagej.nih.gov/ij/index.html>).

Based on measurements of serum progesterone together with ultrasonographic surveillance, the reproductive activity of the jennies was classified according to the following characterizations: (i) Jennies were considered cyclic when displaying a luteal phase with P4 > 1 ng/ml in at least four consecutive blood samples taken 2 days apart, following an oestrus-associated ovulation; (ii) Jennies were defined as having silent oestrus when they displayed regular ovarian follicular development with ovulation in the absence of any behavioural signs of oestrus; (iii) They were considered to be anoestrus when P4 levels registered persistently below 1 ng/ml for more than two consecutive weeks, in the absence of a large follicle and without any signs of behavioural oestrus; (iv) Jennies with anovulation were defined as those that developed a large dominant follicle of over 35 mm, without achieving ovulation, while exhibiting signs of oestrus; (v) Persistence of *corpus luteum* was defined as the presence of a CL while P4 levels remained steadily above 2 ng/ml for more than 40 days.

The interovulatory interval was defined as the interval (in days) between oestrus-associated ovulation in successive cycles, or as the period between the last ovulation of each cycle in the case of multiple ovulations. The moment of ovulation was established as the mid-point between two sequential US scans when a dominant follicle ceased to be observed during oestrus. The beginning of oestrus was set as the point when the female first showed signs of receptivity to the male, with progesterone levels below 1 ng/ml, while the end of the oestrus was considered to be the moment when the jenny stopped displaying these signs in the presence of the male. Whenever the development of large follicles was followed by ovulation in the absence of oestrous behaviour, serum progesterone levels (<1 ng/ml) were used to delimit oestrus. The dioestrus/luteal phase corresponded to the period when serum progesterone levels remained above 1 ng/ml and the female refused the jack (Contri et al. 2014). These criteria were used across all the seasons of the year.

Statistical analysis

Data were analysed using IBM SPSS Statistics 20 software (IBM; Armonk, NY, USA). The lengths of the interovulatory interval, dioestrus, oestrus and the follicle size are presented in terms of mean \pm standard deviation. For statistical analysis, the animals were retrospectively grouped according to the pattern of reproductive activity exhibited during the winter season, as follows: GrA – Females exhibiting anoestrus; GrC – Females displaying regular ovulatory oestrus cycles and oestrus behaviour; GrSE – Females displaying silent ovulatory oestrus. The older jenny (number 12) was excluded from statistical analysis, being the only member of the group with extended *corpus luteum* activity (P4 > 1 ng/ml). Data from this animal was used only for description purposes.

A one-way ANOVA test was conducted, followed by the Bonferroni *post hoc* test for mean comparisons, to estimate correlations between BCS, age and parity and the length of each cycle phase or follicular size. The effects of BCS, age (in years, and according to the subdivisions <5 years and \geq 5 years) and parity on the pattern of ovarian cycles in the non-reproductive season were analysed using a covariance analysis. Furthermore, a general linear model for univariate analysis was used to analyse the influence of BCS on the disruption of the reproductive pattern (globally scored as normal/cyclic and disrupted) and on the category of change (GrC, GrA and GrSE). Differences and correlations were regarded as significant at $p < 0.05$.

Total secretion of progesterone during dioestrus and anoestrus was assessed by estimating the area under curve, applying the trapezoidal rule; that is, calculating the $\Delta X \times (Y1 + Y2)/2$, using Microsoft Excel 2010 for Windows. One-way ANOVA and the Bonferroni *post hoc* for mean comparisons were used to compare the total amount of progesterone secretion between each group of jennies (GrC, GrA and GrSE) and the seasons (autumn transition, non-reproductive, spring transition and reproductive season).

Results

Ovarian activity and cyclicity

Cyclic reproductive activity was shown to have been disrupted in nine jennies (75%) during the non-breeding season. Loss of normal cyclicity included anoestrus ($n = 5$), silent ovulatory oestrus ($n = 3$) and persistence of *corpus luteum* ($n = 1$, jenny number 16). Regular reproductive activity during the non-breeding season was observed in only three females, displaying alternating follicular and luteal phases, and oestrus behaviour (Fig. 1).

Among the five females that entered anoestrus (GrA), disruption of ovarian cyclicity was observed to begin between November 3rd (at the midpoint between the Autumnal equinox and the Winter solstice) and December 12th. These anoestrous jennies were aged 3 years old ($n = 4$) and 6 years old ($n = 1$). The mean duration of anoestrus was 147 ± 28 days, ranging from 113 to

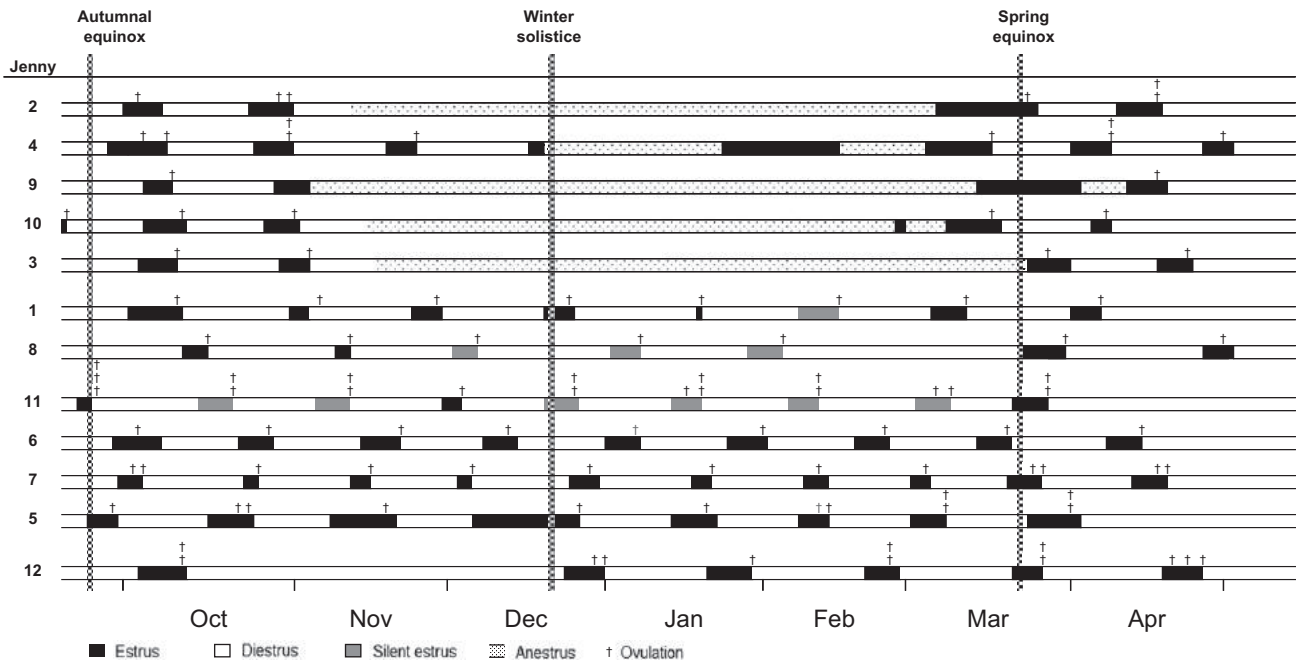


Fig. 1. Reproductive activity pattern of *Asinina de Miranda* jennies from mid September to early May. Oestrus is signalled by solid black bars; silent oestrus is signalled by solid grey bars; point bars signal anoestrus and the blank bars signal dioestrus. Ovulations are represented by †

191 days. During anoestrus, progesterone levels remained within basal levels (below 0.2 ng/ml) while the maximum follicular diameter observed remained below 20 mm. The anoestrus group registered a considerably protracted ($p < 0.001$) interval between two consecutive ovulations (147 ± 28 days), compared to that observed in GrC (24.0 ± 4.3 days) or GrSE (24.0 ± 1.7 days).

Among the females displaying anoestrus, three (jennies number 2, 3 and 10) entered the anovulatory period directly, following a shorter dioestrus than that observed in the jennies that continued cycling as normal (15.6 ± 0.3 days vs 17.0 ± 0.5 days; Fig. 1). Thereafter, successive waves of small follicles were observed, but the developing follicles regressed before attaining 20 mm. The two other females in the group (jennies number 4 and 9) entered anoestrus after a behavioural oestrus (Fig. 1), following the development of a large anovulatory follicle (39.6 ± 6.4 mm). Overall, the last oestrus prior to onset of anoestrus was slightly shorter compared to previous cycles (5.2 ± 0.9 vs 6.3 ± 0.6 days; $p = 0.042$).

For most females in GrA, anoestrus extended until March, close to the spring equinox (Fig. 1); resumption of regular oestrous cycles with ovulation occurred after an elongated oestrus compared to those in the reproductive season (11.9 ± 7.8 days vs 5.6 ± 1.6 days) ($p = 0.024$). Moreover, an increase was detected in the average pre-ovulatory follicle size in the first ovulation of the year (44.7 ± 2.45 mm), compared to that recorded in subsequent cycles during the reproductive season (39.2 ± 3.60 mm). No differences were found in the lengths of the first luteal phase after anoestrus and

the subsequent luteal phases in GrA (17.25 ± 2.46 days vs 17.08 ± 1.07 days) ($p = 0.826$). Similarly, no differences were observed between the lengths of the first and subsequent luteal phases for the jennies in the other groups (17.6 ± 1.09 and 17.5 ± 0.36 days for GrC, $p = 0.928$; and 19.5 ± 1.71 and 19.40 ± 1.76 for GrSE, $p = 0.983$, respectively).

Between the autumnal and spring equinox, grossly corresponding to the transitional and non-reproductive seasons, the three mature jennies from GrSE, aged 6–8 years old, consistently alternated from a follicular phase with ovulation to a luteal phase without exhibiting oestrus behaviour. Two of the females (7 and 8 years old; with 4 and 5.5 BCS points, respectively) started silent oestrous cycles, which numbered between 3 and 6 cycles, during the autumn transition, while the other jenny (6 years old; six points BCS) only exhibited one silent oestrous episode, in February, at the midpoint between the winter solstice and spring equinox (Fig. 1). In one jenny, that recording the highest number of silent oestrous cycles, the pattern of behavioural anoestrus was interrupted once at the beginning of December by the exhibition of a short behavioural oestrus and ovulation. This was preceded by two silent oestruses and followed by four other episodes of silent oestrus. In contrast, during the breeding season no silent oestruses were recorded in any of the 12 jennies surveyed. The lengths of oestrus and dioestrus in the non-breeding season did not significantly differ between GrSE and GrC ($p = 0.442$ and $p = 0.129$, respectively; Table 1).

Jenny number 12 (18 years old) displayed persistence of *corpus luteum*. This female entered the non-breeding season with a persistent CL issuing from an ovulation

Table 1. Characteristics of the reproductive pattern in *Asinina de Miranda* jennies in the non-breeding season: Length (in days) of oestrus, dioestrus and the interval between two consecutive ovulations in females cycling with (GrC) and without signs of oestrus (GrSE) and in anoestrous females (GrA) (Mean \pm SEM)

Group	GrA	GrSE	GrC	p
Oestrus (days)	n.a.	5.61 \pm 2.05	4.99 \pm 1.53	0.442
Dioestrus (days)	n.a.	18.3 \pm 2.58	17.91 \pm 0.94	0.129
Interval between consecutive ovulations (days)	147 \pm 28	24.0 \pm 4.31	24.0 \pm 1.72	0.954

occurring in the autumn transitional season. As expected, the total progesterone produced during the prolonged luteal phase was higher than for the preceding and subsequent luteal phases (538.48 ng/ml vs 289.93 ng/ml and 248.15 ng/ml, respectively). Spontaneous resolution of this luteal structure occurred 69 days post-ovulation and was accompanied by growth of a larger follicle and oestrus 5 days after P4 values had fallen to below 1 ng/ml. This female resumed ovarian cycles around the winter solstice, maintaining regular oestrous cycles thereafter (Fig. 1).

Although three jennies (aged 4–8 years old) retained overt cyclic ovarian activity with oestrus behaviour during the non-breeding season (GrC), non-significant changes in the length of oestrus in the transitional and winter seasons were detected compared to the breeding season (Fig. 1), which were either shorter than in the breeding season (jenny number 10, with 5 years) or increasing in length until December (jenny number 8, with 8 years of age), when the female displayed her longest oestrus (21.8 days), decreasing thereafter. In this group, season did not affect the maximum size of follicles prior to ovulation ($p = 0.652$): the maximum diameter of follicles observed was 39 ± 6.64 mm during the non-breeding season, compared to 37.6 ± 3.33 mm during the breeding season, and 35.42 ± 6.09 mm and 37.3 ± 5.71 mm during autumn and spring transitions, respectively.

The total amount of progesterone produced in each luteal phase in the non-reproductive season did not differ ($p = 0.961$) between jennies with regular ovarian activity and behavioural oestrus (GrC) and those exhibiting silent oestrus (GrSE): 157.5 ± 53.8 ng/ml and 156.18 ± 81.1 ng/ml, respectively. Similarly, the comparison of the amount of progesterone produced in the non-breeding and breeding seasons revealed no seasonal differences in females from GrC (157.5 ± 53.8 ng/ml vs 190.5 ± 81.2 ng/ml in the non-breeding and breeding seasons, respectively; $p = 0.209$) and GrSE (156.18 ± 81.1 ng/ml in the non-breeding season compared to 163.3 ± 76.6 ng/ml in the breeding season; $p = 0.971$).

Multiple ovulations appeared to occur more frequently during the spring and autumn transitions in normally cycling jennies and in anoestrous jennies, in the latter case before and after anoestrus, but were evenly distributed during the entire trial period in jennies displaying silent oestrus in the non-breeding

season; however, this finding was devoid of statistical significance ($p = 0.196$).). In single ovulators, the pattern of reproductive activity in winter affected the average maximum follicular size around the spring equinox, both in the jennies' first ovulation following anoestrus (44.7 ± 2.45 mm) and at the same moment in the reproductive season in jennies with silent oestrus (42.5 ± 5.57 mm). Both of these were larger than those registered in the normally cycling group at the same time (38.7 ± 2.56 mm) ($p = 0.02$). In contrast, regarding the average maximum follicular size in the case of double ovulations, no differences were found at the same time of the year between jennies from GrA and GrC ($p = 0.257$): 40.8 ± 2.93 mm vs 37.32 ± 5.17 mm, respectively.

Effects of body condition, age and parity on the pattern of reproductive activity in the non-breeding season

Age was positively associated with BCS ($p < 0.001$) and with parity ($p = 0.030$), although no relationship was found between parity and BCS ($p = 0.236$). Both parity and age were negatively associated with anoestrus ($p = 0.02$ and $p < 0.001$, respectively); jennies that had never foaled or were younger showed a higher chance of entering anoestrus during the non-breeding season. Age and BCS were observed to have synergic effects on the loss of regular ovarian cycles: younger jennies that also registered a lower BCS score were more likely to undergo anoestrus ($p < 0.001$). Nevertheless, covariance analysis revealed BCS to be the main modulator of anoestrus, with age and parity acting as cofactors.

In every group, the lowest BCS was observed in January. BCS affected the ability to maintain regular ovarian activity during the non-breeding season ($p < 0.001$). The regular ovarian activity of the animals with lower body condition exhibited some disruption. With the exception of the jennies that kept cycling with behaviour oestrus, all the other females lost BCS before winter (Fig. 2), as follows: BCS fell from 4.50 ± 0.500

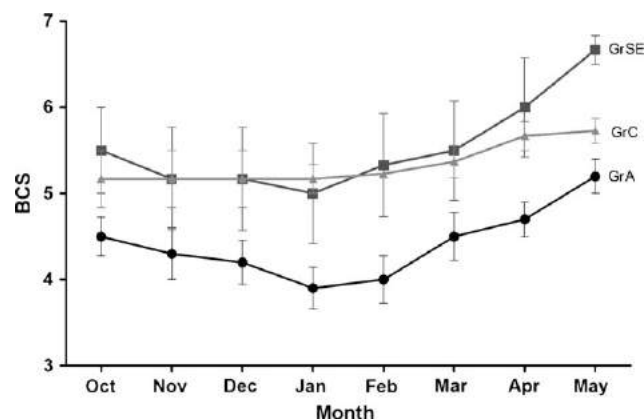


Fig. 2. Evolution of body condition score (mean \pm SD) from October to May in females grouped according to the pattern of ovarian activity in the non-breeding season. Anoestrus – GrA ($n = 5$), silent oestrus – GrSE ($n = 3$) or remaining cyclic – GrC ($n = 3$)

Table 2. Average body condition score (BCS) \pm SD in January (Jan) and its variation from October (Oct) to January and from January to April (Apr), for jennies in anoestrus (GrA), silent oestrus (GrSE) or remaining cyclic (GrC) in the non-breeding season

	GrA (n = 5)	GrSE (n = 3)	GrC (n = 3)	p-value*
BCS (1–9) in Jan	3.95 \pm 0.447 ^(a)	5.17 \pm 0.878 ^(a,b)	5.33 \pm 0.289 ^(b)	0.023
BCS variation				
Oct–Jan	–0.550 \pm 0.371	–0.333 \pm 0.381	0.167 \pm 0.287	0.110
Jan–April	0.750 \pm 0.250	0.833 \pm 0.144	0.267 \pm 0.404	0.092

*Within a row, means (\pm SD) with common superscript do not differ significantly at $p < 0.05$.

to 4.20 ± 0.570 points from October to December and to 4.02 ± 0.634 in February; in the group that showed silent oestrus, BCS decreased from 5.5 ± 0.500 to 5.16 ± 1.04 points between October and January and to 5.25 ± 1.14 up to February. In contrast, BCS remained almost unchanged for the winter season in animals cycling with behaviour oestrus (5.17 ± 0.166 ; 5.17 ± 0.577 and 5.3 ± 0.100 points in October, December and February, respectively).

Jennies in anoestrus (GrA) recorded the lowest BCS during the entire study ($p = 0.01$), which was particularly noticeable from December to February ($p < 0.001$; Fig. 2). In January, BCS was 3.95 ± 0.447 points for animals in anoestrus, 5.16 ± 0.878 points for jennies with silent oestrus and 5.33 ± 0.289 points for cycling jennies (Table 2). Only females with a BCS below five points during autumn and winter entered anoestrus. In fact, anoestrus began in animals whose BCS fell below a threshold of four points in October (Fig. 2). Resumption of regular ovarian activity and oestrus was observed after BCS values had returned to those recorded in October, which occurred between February and March (Fig. 2). Moreover, the length of anoestrus was significantly affected ($p = 0.032$) by BCS between the winter solstice and spring equinox. However, the jennies' age did not affect anoestrous duration ($p = 0.998$).

Data from BCS was confirmed in ultrasound scans of the tissue depth between ribs 12–13 (TDBrib13) and at subcutaneous fat depths at withers (SFW). Fat deposits at TDBrib13 affected ovarian activity during the non-breeding season ($p = 0.006$); in contrast, the SFW depths recorded were not significantly different between

the groups ($p = 0.095$). Overall, failure to display cyclic reproductive activity was positively associated with a lower BCS ($p < 0.001$) or TDBrib13 thickness ($p = 0.006$) (Table 3).

Discussion

Data gathered in this study are essential for a better understanding of the reproductive biology of this species and breed, and also to understand how to maximize reproductive efficiency, which is crucial to enable the conservational programs of breeds at risk of extinction, like the *Asinina de Miranda*. This study revealed disruption to regular reproductive activity in most of the *Asinina de Miranda* jennies during the non-breeding season; all but three failed to exhibit behavioural signs of oestrus reflecting regular ovarian cyclicity in winter. In this population, the disrupted ovarian pattern recorded during winter included anoestrus (5 : 12; 41.7%); silent oestruses (3 : 12; 25%) and prolonged *corpus luteum* (1 : 12; 8.3%). Such changes were not recorded during the breeding season, independently of BCS, age or parity.

It is currently accepted that female donkeys reared in temperate zones are subject to seasonal effects in reproductive function. However, in donkeys a clear division of the year into breeding and non-breeding seasons is unusual (Ginther et al. 1987). It is possible that the photoperiod variations may affect donkeys differently compared to horses in terms of magnitude because they evolved as a species closer to the equator (Beja-Pereira et al. 2004). It has been shown that jennies can have regular oestrous cycles throughout the year (Blanchard et al. 1999), although some studies have described seasonal anoestrus and irregularities in ovarian cycles in standard jennies (Ginther et al. 1987; Henry et al. 1987). Seasonal anoestrus has been recorded in a varying proportion of animals, ranging from 33.3% (4 : 12 females; Ginther et al. 1987) to 76.9% (10 : 13 females; Henry et al. 1987); in either case, anoestrus was attributed to photoperiod variations.

Contradictory data exist regarding differences in the length of oestrous cycle or in the duration of each cycle stage in anoestrous mares (Nequin et al. 1990; Ginther 1992; King et al. 1993; Blanchard et al. 2003), which may be associated to a year-effect or to differences in geographic location, the breed or type of females used,

Table 3. Body condition score (BCS), subcutaneous fat depots (mean \pm SD) during the non-breeding season (December to February) for the jennies with disturbed ovarian activity (anoestrus – GrA, silent oestrus – GrSE), or remaining cyclic (GrC) in the non-breeding season

	GrA (n = 5)	GrSE (n = 3)	GrC (n = 3)	p-value*
BCS (1–9)	4.05 \pm 0.157 ^(a)	5.19 \pm 0.203 ^(a,b)	5.26 \pm 0.203 ^(b)	0.001
SFW (mm)	3.95 \pm 1.32	6.52 \pm 4.67	5.56 \pm 2.08	0.095
TDBrib13 (mm)	13.1 \pm 2.15 ^(a)	15.2 \pm 3.51 ^(a,b)	16.3 \pm 2.73 ^(b)	0.006

*Within a row, means (\pm SD) with common superscript do not differ significantly at $p < 0.05$.

SFW – depth at withers; TDBrib 13 – subcutaneous tissue depth at ribs.

the animals' age or their nutritional plan (Ginther et al. 2004). While in mares a shortening of oestrus during the non-breeding season and a protracted oestrous cycle has been reported (Dowsett et al. 1993), in jennies a protraction of ovulatory oestruses in the early and late breeding season has been described by Henry et al. (1987), with dioestrus remaining unaffected. The present study found no differences in the interovulatory interval in jennies that cycled year-round, with or without signs of oestrus, during the non-reproductive season.

In the same way as reported by Henry et al. (1987), the jennies in GrA showed an average reduction of 2 days in the length of the last cycle before entering anoestrus, compared to animals retaining cyclicity in the non-breeding season. Females in GrSE also tended to present shorter oestrus than those in GrC; though the differences between these two groups were non-significant. Furthermore, this study also showed a protracted first oestrus of the year, a particularity already recorded in standard jennies by Ginther et al. (1987).

As has been described for mares (King et al. 1993), onset of anoestrus may occur after three different conditions: following the resolution of a spontaneous CL by normal luteolysis, resolution of a prolonged CL unaccompanied by follicular growth, or an ovulatory oestrus in association with follicular atresia. In our study, anoestrus followed either a shortened dioestrus with spontaneous luteolysis or an ovulatory oestrus. The fact that *corpus luteum* persistence was only detected once in one female limits the possibility of exploring this issue in *Asinina de Miranda* jennies. The sole case of extended luteal function was followed by resumption of ovarian cyclicity, not of anoestrus.

In the present work, onset of anoestrus occurred in November. After anoestrus, the first ovulation occurred in mid-March (around the spring equinox) in all but one anoestrus female, exhibiting the lowest BCS. For this particular female, ovulation was achieved following an increase of BCS to four points, by mid-April. The anoestrus length in the non-breeding season varied among females, as has also been described for mares (Dowsett et al. 1993). In jennies, the average length reported for seasonal anoestrus ranged from 39 to 72 days, for females located in Wisconsin (Ginther et al. 1987), to 166 ± 63 days, in Brazilian standard jennies (Henry et al. 1987). In this study, the mean anoestrus period lasted for 141 days, varying from 113 to 191 days, thus being close to that reported in Brazil. This study also suggests that the seasonal delineation established for horses in Portugal does not apply exactly to donkeys, particularly regarding the vernal transition, which is set for mares between the 1st March and 8th April (Atayde and Rocha 2011). In fact, most jennies in the present study resumed a normal reproductive pattern in mid-March, April already being in the breeding seasons.

Although seasonal changes in photoperiod are considered the most important factor for synchronizing

seasonal reproductive activity in equids, several authors also agree that additional factors may modify the intensity of the decrease in reproductive activity, such as the binomial effect of nutrition and BCS (Fitzgerald and McManus 2000; Ferreira-Dias et al. 2005; Galisteo and Perez-Marin 2010). These would interact with neuroendocrine mechanisms controlling GnRH pulse frequency and therefore, with gonadotropin support to the ovary (Fitzgerald and McManus 2000; Ginther et al. 2004). It has been shown that, in the non-breeding season, anoestrus mares display decreased LH secretion (van Niekerk and van Niekerk 1997b) and this decrease, which affects both FSH and LH pulses, is gradually observed for several cycles prior to the onset of anoestrus (Irvine et al. 2000). These changes are reflected in a reduction in ovarian steroid synthesis, which might explain the differences in the length of the oestrous cycles stages or the interovulatory intervals that were recorded during the non-breeding season.

Covariance analysis demonstrated that BCS plays an important role in the pattern of ovarian activity during the non-breeding season, suggesting that body fat is an important modulator of reproductive activity in winter. The majority of females surveyed (75%) showed a disrupted pattern of reproductive activity in the non-breeding season. These changes were rather more observed in females with poor body condition between the autumn equinox and the winter solstice. In addition, only after regaining BCS did the females return to regular ovarian activity, which occurred around the spring equinox for most jennies. Earlier studies in mares showed that body reserves when approaching the non-breeding season might determine the suspension of reproductive activity. Similarly, an increase in BCS before the breeding season would hasten the resumption of ovarian cycles, after the non-breeding season (Vecchi et al. 2010).

Age and parity were used as covariables for BCS effects since in the present study it would not be possible to analyse the individual contributions of each factor. Herein, age was positively associated with BCS and parity. Accordingly, most of the younger animals (4 : 5) were also the ones with lower BCS (between 3.5 and 4.5 points). In the present work, anoestrus females were generally younger than females in GrSE and GrC. A possible explanation is that younger females display increased imbalance in their energy metabolism than middle aged jennies, and a lower amount of body adipose reserves might increase their susceptibility to experience a profound negative energy balance that will exert stronger negative influences on the hypothalamic-pituitary axis.

Similar situations have been described in mares, where mean body weight was lower in younger females than in mature ones. Young mares aged 2–5 years old are believed to have less inherent body fat, which does not allow them to successfully mobilize energy stores to support regular ovarian activity, thereby entering into anoestrus more easily and exhibiting a longer anoestrus

than mature mares (Fitzgerald and McManus 2000; Ginther et al. 2004). Additional studies on a larger population must be undertaken to isolate age influences on ovarian activity in jennies.

In our study, females with the lowest BCS between the autumnal equinox and the winter solstice entered anoestrus, while those maintaining a BCS of five points showed silent ovulations, and females cycling regularly throughout the year were able to keep their BCS continually above five points. This suggests that jennies will maintain regular ovarian cycles if their body condition remains stable or rises above a given threshold around the autumn equinox, as has also been described in mares (Henneke et al. 1983; Fitzgerald and McManus 2000). Moreover, Ginther et al. (2004) demonstrated that the interval from the winter solstice to the first ovulation of the year was wider in mares that lost body condition; moreover, these authors also argue that the greater the loss in body fat, the longer this interval will be.

In females with an unchanging body condition score, residual influences of adiposity on hypothalamic-pituitary-ovarian dynamics will persist, thus enabling regular ovarian activity to be maintained. Females that evidenced small changes in adiposity would display an intermediate situation: although not sufficient to compromise ovulation, disruption to sex steroid production by ovarian structures might interfere with exhibition of oestrous behaviour, thus favouring the occurrence of silent oestrus. Henry et al. (1987) refer to silent oestruses in a small number of jennies during the summer but not during the non-breeding season; however, silent oestruses were not found outside of the non-breeding season in the present study.

To conclude, the results revealed that the majority of the jennies engaged in this study display disrupted ovarian activity during the non-breeding season. Disruption to regular reproductive activity could include anoestrus, silent oestrus or persistence of *corpus luteum*. When it is not possible to follow ovarian activity by the

use of ultrasound, the absence of cyclic evidence of behavioural oestrus in jennies displaying silent oestrus will also be perceived as anoestrus. Of major significance, we found that abnormal cycles and anoestrus appear to be linked to BCS, highlighting the importance of monitoring female BCS around the autumn transition and the need to maintain the BCS of jennies above a given threshold if anoestrus is to be avoided. Further research, with larger BCS categories and BCS variations, in females of a wider age range would be helpful to consolidate these observations and to establish in a more precise way the threshold for body condition to guarantee an early onset of regular ovarian cycles and ovulation.

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Conflict of interest

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

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Author contributions

Quaresma M, Silva S and Payan-Carreira contributed greatly with the conception and design of this study, particularly regarding how data was acquired, analysed and interpreted, as well as participating in drafting the article and critically revising it for significant intellectual content before giving their final approval of the version for publication.

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Author's address (for correspondence): Miguel Quaresma, Hospital Veterinário, Escola de Ciências Agrárias e Veterinárias, Universidade de Trás-os-Montes e Alto Douro (UTAD), Quinta de Prados, 5000-801 Vila Real, Portugal. E-mail: miguelq@utad.pt