

Environmental effects and repeatability of the follicular diameter in mares

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ABSTRACT - The objective of this study was to evaluate the effect of environment on follicular diameter and repeatability of the size of preovulatory follicles of mares. Temperature, rainfall, and photoperiod were measured, and their effects were evaluated on follicular dynamics. Data were studied by analysis of variance of follicular size and environment traits. During the five years, 7% double ovulations and 159 anovulatory follicles were recorded. Mean preovulatory follicle diameters of left and right ovaries were 39.3 ± 3.8 and 39.2 ± 3.5 mm respectively. There was no effect of evaluation year on follicular diameter. Reproduction season affected the right preovulatory follicle diameter. There was no effect of temperature and rainfall on follicular diameter. Repeatability values of the preovulatory follicular diameter were low. In the subtropical climate studied, temperature and rainfall caused no effect on follicular dynamics, and photoperiod had only a small effect on dominant preovulatory follicular diameter. Repeatability of follicular diameter was low, so this characteristic should be evaluated daily to predict ovulation and the optimal time to inseminate mares.

Keywords: female, horse, reproduction



Introduction

The widespread use of reproductive biotechnologies for horse breeding is a reality and is increasing worldwide with the need to produce animals with superior genetics to compete in the market (Stroud and Callesen, 2012). With this need for breeding improvement, the use of semen of stallions with superior genetic traits is of great importance, and knowledge of the mare reproductive characteristics is necessary for good embryo recovery during a given breeding season.

Follicular dynamics and preovulatory diameter are essential characteristics to accurately predict the ovulation moment. This can be observed, but it takes lengthy evaluation time during the reproduction management in horse embryo transfer centers, in most cases making it unfeasible (Melo et al., 2012).

Therefore, the use of a standard to classify a follicle as preovulatory is an alternative to predict the most favorable moment to inseminate embryo donor mares. However, there are individual differences and effects of environment on reproductive characteristics, such as age of mare, year of observation, season, and climate. Therefore, evaluation of repeatability would be a useful tool to estimate if this characteristic tends to repeat over observations. Repeatability is a genetic parameter that can estimate the genotypic value of a trait that can be spread to other generations (Pereira, 2012). Therefore, this type of evaluation could help reproduction management of mares and improve breeding regarding the selection of donor mares (Sitzenstock et al., 2013).

The objective of this study was to evaluate the preovulatory follicular diameter, daily growth of follicular diameter, and environmental factors that can influence the preovulatory follicular diameter and to estimate the repeatability of preovulatory follicle diameter of embryo donor mares in an embryo reproduction center.

Material and Methods

This experiment was performed with the approval of the local Committee on Ethical Use of Animals (CEUA) in accordance with the Brazilian Society of Laboratory Animal Science/Brazilian College of Animal Experimentation (SBCAL/COBEA) under case number 307.

A total of 195 embryo donor mares of the Mangalarga Marchador breed, aged from 10 to 23 years of age and with body condition from 7 to 8, were evaluated during five years – from 2012 to 2016. The mares were in an embryo transfer center located in northern Rio de Janeiro, Brazil (latitude: 21°45'15", longitude: 41°19'28", altitude: 13 m). All mares were evaluated throughout the year, so they did not enter into seasonal anestrus in winter. They were evaluated in different reproductive seasons (RS). The period from October to April is considered the breeding season (BS); May is considered the autumnal transition out of breeding season (ATBS); from June to August is considered the seasonal anestrus or non-breeding season (nBS); and September is considered the vernal transition into breeding season (VTBS) in the Brazilian tropical climate.

The animals were kept in an open shelter and outdoor paddock with access to pasture and supplemented twice a day with hay and 2.0 kg of pellet feed (Equitech, Brazil) containing 12% crude protein and 2,700 kcal of digestible energy, with amino acid supplementation of 25 mL/day (Glicopan Energy®, Vetnil, São Paulo, Brazil). Trace-mineralized salt and water were available *ad libitum*.

The reproductive tract (uterus and ovaries) was examined by transrectal ultrasonography (Mindray® DP-2200 system, Shenzhen, China) with a 5-MHz linear transducer to determine the estrous cycle stage, presence and measurement of follicles, uterine edema, and intrauterine fluid. The evaluations were performed every day from the moment when a follicle having 30 mm diameter was detected. Each mare was evaluated repeatedly during the year.

During five years, the data on average monthly temperature and rainfall were collected, along with daily photoperiod, to calculate the respective reproductive seasonal averages.

The rainfall data were separated into classes, whereby 6 to 100 mm³ was considered "R1" and 101 to 200 mm³ was considered "R2". The same was done with average temperature, whereby 21 to 24 °C was considered "T1" and 24.1 to 28 °C was considered "T2". Finally, the photoperiod was separated into two classes, one with 10:39 to 11:39 h of daylight, considered "P1", and the other with 11:40 to 13:18 h of daylight, considered "P2".

We evaluated the mean preovulatory follicle diameter one day before ovulation (D-1) and the daily growth from five days before ovulation (D-5) until the day of ovulation (D0). We also evaluated the effect of year of evaluation, RS, temperature, rainfall, and photoperiod on preovulatory follicular diameter (PFD). Finally, we calculated the repeatability of preovulatory follicle diameter.

Descriptive statistics were calculated from all collected data. The environmental effect (temperature, rainfall, and photoperiod) and follicular dynamics (dominant follicular diameter, daily dominant follicular growth, and preovulatory follicular diameter) were arranged in contingency tables and analyzed by the PROC MIXED routine, at 5% probability of SAS (Statistical Analysis System, version 9.2).

The repeatability (*t*) of preovulatory follicle diameter was calculated (by year and season) from the estimated variance (VAR) with the REML method of the VARCOM procedure of SAS. Repeatability of preovulatory follicular diameter was calculated using PROC VARCOMP-REML of SAS with:

$$t = \frac{\sigma_B^2}{\sigma_B^2 + \sigma_w^2},$$

in which σ_B^2 = variance among individuals and σ_w^2 = variance within individuals.

Results

The total number of ovulations was 2,124 during all years of evaluation. There were 1975 single ovulations (83%) and 149 double ovulations (7%), of them 38 in the right ovary (RO) and 45 in the left ovary (LO), plus 66 double ovulations, in which one dominant follicle grows in each ovary simultaneously and both follicles ovulate (from left and right ovaries). Finally, there were three triple ovulations, and during the five years, 159 anovulatory follicles were recorded.

The mean diameter of the preovulatory follicles (D-1) was similar in the left and right ovaries (39.3 ± 3.8 and 39.2 ± 3.5 mm, respectively).

There was a progressive increase of follicle diameter until one day before ovulation. This increase was higher from day -4 to day -3, with mean of 2.8 mm for dominant follicular growth, and growth decelerated from day -1 onward. Growth was similar in the left and right ovaries (Table 1).

Evaluating environmental effects (temperature, rainfall, and photoperiod) by month, we observed that there was difference on temperature and photoperiod ($P < 0.05$); however, rainfall did not vary among months ($P > 0.05$) (Table 2).

There was no difference in preovulatory follicular diameter among the years ($P > 0.05$). The same result was observed in both ovaries (Table 3).

Regarding reproductive season, there was an effect only on the diameter of preovulatory follicle in the right ovary ($P < 0.05$), with the highest mean recorded in the ATBS (Table 3).

There was no difference in preovulatory follicular diameter with different temperature (T1 and T2) and rainfall intervals (R1 and R2) ($P > 0.05$) (Table 3).

Table 1 - Mean and standard deviation of follicular diameter (mm) on days (-5, -4, -3, -2, and -1) before ovulation

Ovary	N	-5	-4	-3	-2	-1
Right	(3050)	30.8±4.7a	32.8±4.1a	35.6±4.0a	37.9±3.6a	39.3±3.8a
Left	(2744)	30.5±5.0a	32.5±4.3a	35.3±3.9a	37.7±3.7a	39.2±3.5a
DFG			(5-4)	(4-3)	(3-2)	(2-1)
Right			2±0.6	2.8±0.1	2.3±0.4	1.5±0.2
Left			2±0.7	2.8±0.4	2.4±0.2	1.5±0.2

N - number of observations; DFG - dominant follicular growth diameter (mm).
Means with a different superscript (a and b) are different ($P < 0.05$).

Table 2 - Means and standard deviations of environmental effects by month of evaluation

Month of observation	Environmental effect		
	Temperature	Rainfal	Photoperiod
1	27.2±1.0a	94.7.5±81.0a	13.1±0.03b
2	27.6±0.5a	35.7±19.2a	12.4±0.03c
3	27.0±0.6a	128.8±53.6a	12.1±0.3d
4	25.7±1.0bc	49.2±37.0a	11.3±0.1f
5	23.2±0.6fg	78.9±64.0a	10.8±0.3h
6	22.6±0.7fg	46.5±29.1a	10.4±0.01j
7	22.1±0.5g	68.1±65.2a	10.6±0.2i
8	22.2±0.5g	39.7.0±27.0a	11.2±0.1g
9	23.5±0.5fe	40.2±30.3a	11.8±0.3e
10	24.3±0.7de	37.6±28.9a	12.4±0.1c
11	25.0±0.9cd	123.2±48.2a	13.1±0.1b
12	26.7±1.2ab	129.1±86.2a	13.3±0.02a

a-j - Means with a different letter in the same column are different ($P < 0.05$).

Table 3 - Effect of year of evaluation, reproduction season, rainfall, and temperature on preovulatory follicular diameter (mm)

Ovary	Year of evaluation				
	2012	2013	2014	2015	2016
N	(387)	(419)	(499)	(131)	(283)
Right	39.4±3.2a	39.4±4.4a	39.4±3.4a	39.1±3.7a	39.3±4.0a
Left	39.5±3.7a	39.5±3.7a	39.4±3.6a	39.2±2.8a	38.6±3.2a
Ovary	Reproduction season				
	BS	ATBS	nBS	VTBS	
N	(899)	(142)	(531)	(192)	
Right	39.0±3.7b	40.7±4.3a	39.5±3.6b	39.4±3.8b	
Left	39.2±3.9a	39.2±3.2a	39.1±2.9a	39.3±3.4a	
Ovary	Temperature (°C)			Rainfall (mm ³)	
	N	T1	T2	R1	R2
Right	(792)	39.4±3.9a	39.3±3.5a	39.4±4.0a	38.9±3.3a
Left	(704)	39.1±3.2a	39.5±3.7a	39.3±3.4a	39.3±3.6a
Ovary	Photoperiod				
	N	P1 (h)	P2 (h)		
Right	(824)	39.6±3.7a	39.1±3.8b		
Left	(743)	39.2±3.0a	39.3±3.9a		

N - number of observations; BS - breeding season; ATBS - autumnal transition out of breeding season; nBS - non-breeding season; VTBS - vernal transition into breeding season; mean temperature ranging from 21 to 24 °C (T1) and from 25 to 28 °C (T2); rainfall ranging from 0 to 100 mm³ (R1) and from 101 to 235 mm³ (R2); day length from 10 to 11:40 h (P1) and from 11:41 to 13:18 h (P2).
a, b - Means with a different letter are different (P<0.05).

The photoperiod had an effect on preovulatory follicular diameter of the right ovary (P<0.05), with greater mean follicular diameter observed when daylight was shorter (P1). However, there was no effect of photoperiod on preovulatory follicular diameter of the left ovary (P>0.05) (Table 3).

The estimated variance values for preovulatory follicle diameter were VAR (mare) = 0.042 and VAR (residual + mare) = 0.587, with the result of repeatability $t = 0.07$. The estimated variance values for preovulatory follicle diameter were VAR (season) = 0.035 and VAR (residual + season) = 0.280, with result of repeatability $t = 0.11$.

Discussion

The rate of double ovulation of Mangalarga Marchador mares in this study was low and not significant for an embryo transfer program, because the rate (7%) of double ovulation did not increase the embryo recovery rate considering all the ovulations during the evaluation period. Squires and McCue (2007) also reported that mares of breeds such as Thoroughbreds, Warmbloods, and Draft horses have higher rates of spontaneous double ovulation compared with others, so they have a greater embryo recovery rate per estrous cycle and are more productive in embryo transfer programs. Since double ovulation increases embryo production, the selection of Mangalarga Marchador donor mares with this natural trait can promote the genetic improvement of this breed for reproductive characteristics. It can thus be a criterion for selection of reproductive characteristics, as established in other countries such as Germany (Sitzenstock et al., 2013), associated with the morphofunctional traits that are already used for genetic improvement of horses (Andersson et al., 2012).

Knowledge of the pattern of follicular diameter variation can help make choices in embryo transfer programs by predicting the nearest moment to ovulation. It is unfeasible to evaluate ovulation by ultrasound of all mares during their cycles due the excessive time this would take. Mares are usually evaluated once a day or every other day, so we observed a pattern of daily follicular growth from day -5 until ovulation (Table 1). Another observation that can be a tool to predict ovulation is that as the

moment of ovulation nears, the daily follicular growth decreases, indicating that ovulation is about to happen. Ginther et al. (2008) observed that follicles continue to grow at a steady rate until two days before ovulation, when follicular size reaches a plateau of approximately 40 mm. This decreasing growth can be due to the peak production of inhibin near ovulation (Satué and Gordón, 2013).

Regarding the effect of year of evaluation, there was no influence ($P > 0.05$) on follicular dynamics of mares (Table 3). There were differences of temperature, rainfall, and quality/quantity of pasture, but none of those environmental effects caused changes in ovary traits in this study.

The RS defined in this study into four categories (BS, ATBS, nBS, and VTBS) can be used as a model in tropical regions of the southern hemisphere if climate and photoperiod do not have abrupt changes. The RS did not affect the follicular diameter in most evaluations (Table 3), except in ATBS, wherein the average preovulatory follicular diameter was slightly larger than other RS. This suggests that during this transitional phase, inadequate gonadotropin stimulation, mainly by LH with low circulating concentration, can act to delay ovulation, leading to a greater follicular growth until ovulation. Other authors have observed low concentration of LH in this phase, as well as no follicular diameter growth above 16 mm and no dominant follicle development (Donadeu and Watson, 2007; McKinnon et al., 2011). King et al. (2010) reported that the VTBS is characterized by the resumption of follicular deviation, with one to three anovulatory follicular waves before ovulation, and that during the winter (considered as the nBS), mares enter seasonal anestrus. In northern Rio de Janeiro state, mares continue cycling during the year, probably because the concentration of LH during transitional phases and nBS are sufficient to promote signals for final follicular development and ovulation (Table 3).

Environmental effects such as temperature and rainfall did not influence follicular dynamics (Table 3). This is probably because these two parameters do not have a sufficient variation to influence follicular dynamics, suggesting there are other factors that can affect the dominant follicular diameter. Warriach et al. (2014) reported that mares continue cycling throughout the year in subtropical regions even with lower temperatures than in the present study in winter or nBS. Higher temperatures and humidity during summer cause lower reproductive rates of mares, suggesting that the higher temperature can cause thermal stress, with negative effects on reproductive performance by stimulating release of corticotrophins and affecting the hypothalamus-hypophysis-gonad axis, decreasing the synthesis and release of gonadotropin FSH and LH (Kelley et al., 2011; Warriach et al., 2014). Even with the high summer temperatures, when the mares were evaluated in this study, they responded very well to hormonal treatment to induce ovulation, with small number of anovulatory follicles and usually one ovulatory wave. There was no superovulation protocol; then, multiple ovulation occurred naturally in the studied mares. These observations suggest that the mares studied are adapted to tropical climate, because Mangalarga Marchador is a Brazilian breed (Table 3).

Regarding the effect of photoperiod on follicular diameter (Table 3), we observed that the period when daylight hours was lowest (P1 – 10:39 to 11:39 h) corresponded to nBS and VTBS (winter and spring), and the mean follicular diameter (day –1) was bigger than in P2 (11:40 to 13:18 h of daylight), which corresponds to breeding season and ATBS (summer and autumn). According to Sharp (2011), mares raised in north America achieve seasonal anestrus during winter when daylight is lowed compared with summer. During periods with shorter daylight, the pineal gland is stimulated to synthesize and release more melatonin, and this hormone can suppress the hypothalamus, causing release of GnRH; therefore, there is less stimulus on the hypophysis to release gonadotropin, mainly LH. Therefore, in winter and transitional phases, the concentrations of LH might not be sufficient to promote follicular development and ovulation of a dominant follicle. In the mares of this study, the photoperiod was not sufficient to interrupt ovulation, suggesting there were other factors favorable to ovary dynamics (McKinnon et al., 2011). The nutritional supplementation associated with good body condition of the mares (Aurich, 2011), climate (Oliveira et al., 2015), and genetic adaptations of the Mangalarga Marchador breed to this environment (Sitzenstock et al., 2013) are likely the factors causing mares to continue cycling year-round. However, the observation that the follicle diameter was bigger in shorter photoperiod can be explained by the fact that even though the hours of darkness were not sufficient to suppress follicular development, the dominant follicle spent more time to ovulate and,

consequently, increased in size compared with what happened in the longer photoperiod, probably due to lower concentration of LH (McKinnon et al., 2011).

Regarding repeatability of the preovulatory follicle diameter during the five years of evaluation, we observed a low value of $t = 0.07$, reflecting considerable variation and suggesting that follicle diameter cannot be expected to be the same one day before ovulation in every evaluation. When repeatability was calculated by season, we observed a result of $t = 0.11$, also low. Lefrançois and Bruyas (2016) also observed low repeatability of the preovulatory follicular diameter in mares, and that knowledge of mare history regarding the diameter preceding ovulation would not be useful to estimate the best follicle diameter at which to breed the mare at the optimal time near ovulation. Furthermore, in this study, we evaluated the frequency of the diameter of the preovulatory follicle and observed that preovulatory follicle at 38 to 41 mm was the majority (58%).

It is not possible to be certain that ovulation will occur on the day after observing a follicle with a specific diameter, but follicles at 38-41 mm do indicate that ovulation is near. Therefore, we can suggest that the low repeatability of follicular diameter indicates that the size of the preovulatory follicle is affected more by environmental factors than by mare genetics (Pereira, 2012).

Conclusions

In subtropical climates, photoperiod and temperature do not affect follicular dynamics. The low repeatability of the follicular diameter suggests that this characteristic must be evaluated daily when a follicle with diameter of 30 mm or larger is detected to predict the moment of ovulation and the optimal time to inseminate the mare.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: M.A.S. Rua. Data curation: M.A.S. Rua, C.R. Quirino, A.C.C. Rodrigues and A. Bartholazzi Junior. Formal analysis: M.A.S. Rua and C.R. Quirino. Investigation: M.A.S. Rua, C.R. Quirino, L.F. Matos and A. Bartholazzi Junior. Methodology: M.A.S. Rua and C.R. Quirino. Project administration: M.A.S. Rua and C.R. Quirino. Software: M.A.S. Rua. Supervision: M.A.S. Rua and C.R. Quirino. Validation: M.A.S. Rua. Writing-original draft: M.A.S. Rua and C.R. Quirino. Writing-review & editing: M.A.S. Rua.

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