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# Intravaginal progesterone device (1.9g) and estradiol benzoate for follicular control in the mare during spring and summer

[*Implante intravaginal de progesterona (1,9g) e benzoato de estradiol no controle folicular de éguas durante a primavera e o verão*]

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## ABSTRACT

The objective of this study was to evaluate follicular growth and ovulatory rates in mares treated with an intravaginal progesterone device (P4) during the 10-day period, associated with the use of estradiol benzoate (EB). The results were compared during the transition period (ET) in the spring and the breeding season in the summer (ER). The variables were submitted to ANOVA (Tukey's test), considering P<0.05. No ovulation occurred during the permanence of the P4 implant in both experimental periods. The ovulatory rate in the ER was 100% (n = 8) and in the ET 62.5% (n = 5; P = 0.0547). Significant differences were observed (<0.001), in both periods, comparing follicular growth rates during the permanence of P4 device (ER: 1.33 ± 0.89*mm*/d; ET: 1.00 ± 0.81mm/d) to the period without P4 (ER: 3.63 ± 1.33 mm/d; ET: 3.31 ± 1.66 mm/d). The present study demonstrated applicability and efficiency of a hormonal protocol using P4 intravaginal device and EB for follicular control in mares, both during ET and ER.

Keywords: progesterone implant, ovulation rate, seasonality, mares

## RESUMO

*O objetivo deste trabalho foi avaliar a taxa de crescimento folicular e a taxa ovulatória em éguas tratadas com dispositivo intravaginal de progesterona (P4) durante o período de 10 dias, associado à utilização de benzoato de estradiol (BE). Os resultados foram comparados durante o período de transição (ET) da primavera com a época de reprodução no verão (ER). As variáveis foram submetidas à ANOVA (teste de Tukey), considerando-se P<0,05. Nenhuma ovulação ocorreu durante a permanência do dispositivo de P4 em ambos os períodos experimentais. A taxa ovulatória na ER foi de 100% (n = 8) e na ET, de 62,5% (n=5; P=0,0547). Diferença significativas (<0,001) foram observadas, em ambos os períodos experimentais, comparando as taxas de crescimento folicular durante a permanência da P4 (ER: 1,33 ± 0,89mm/d; ET: 1,00 ± 0,81mm/d) com o período sem P4 (ER: 3,63 ± 1,33mm/d; ET: 3,31 ± 1,66mm/d). O presente estudo demonstrou aplicabilidade e eficiência do protocolo hormonal utilizando dispositivo intravaginal de P4 e BE para controle folicular de éguas, tanto na ET quanto na ER.*

*Palavras-chave: implante de progesterona, taxa de ovulação, sazonalidade, éguas*

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## INTRODUCTION

Equine breeding in Brazil is constantly developing and improving, with important economic and social relevance in the country, generating significant incomes and employments in this industry. Mares are seasonal polyestrous animals presenting multiple estrous cycles annually, during the months with increased daily light. Hence, the period of reproductive season occurrs in spring and mainly in the summer (Faria and Gradela, 2010), although cyclicality can also be affected by nutrition, temperature, and health status (Adams and Bosu, 1988). On the other hand, suboptimal functioning of the hypothalamus-hypophysis-gonadal axis (HHGA) and low FSH and LH peaks is observed during seasonal anestrus of the mare (Ginther, 1982; Alexander and Irvine, 1991; Nagy *et al*., 2000; Handler *et al*., 2006). Thus, it can be stated that increased luminosity has a positive effect on GnRH hypothalamic pulses and gonadotropins secretion in the mare (Ginther, 1982, 1992; Satué and Gordon, 2013) and the breeding activity is mainly influenced by photoperiod and environmental factors (Samper *et al*., 2002).

The estrous cycle involves a follicular phase in which a 25 to 30mm follicle develops in the ovarie, consistent with manifestations of estrous behavior. In healthy mares, when this follicle reaches 35 to 45mm in diameter, ovulation occurs (Pierson, 1993) usually 24 to 48 hours before the end of the estrus (Lindeberg *et al*., 1992). Estrus synchronization in equine specie represents an important tool for reducing the number of natural breeding, or even for assisted artificial insemination to be carried out at a more precise moment. Thus, pharmacological induction of ovulation is routinely used and can present several advantages as maximizing the use of each ejaculate and reducing breeding expenses and the incidence of reproductive problems in the mares (Klug and Jochle, 2012). Therefore, several hormonal treatments have been used to induce and/or synchronize ovulation since it facilitates equine reproductive management, reduces animal handling, and increases the successfull application of reproductive biotechniques (Mccue, 2003; Teixeira *et al*., 2020).

Progesterone (P4) by various routes of administration is used for estrus synchronization in several domestic species (Squires *et al*., 1979; Rensis *et al*., 2005; Handler *et al*., 2006; Claro Júnior *et al*., 2010; Oliveira *et al*., 2016) being administered alone or followed by prostaglandin F2 alfa (PGF2α) or its analogs. In the mare, P4 treatment accelerates the beginning of the breeding season, improves reproductive performance during spring transition period and facilitates reproductive management during all seasons of the year (Squires *et al*., 1979; Arbeiter *et al*., 1994; Gastal *et al*., 1999; Cuervo-Arango and Clark, 2010; Hanlon and Firth, 2012). Moreover, administration of P4 and estradiol (E2) injections in cyclic mares has been shown to increase the pharmacological control of ovulation compared to P4 alone, because estradiol-17β in the presence of P4 can suppress follicle development (Pinto *et al*., 2004).

Additionally, the administration of estradiol benzoate (EB; an ester of the natural estradiol-17β) followed by long-acting P4 injection was appropriate to treat noncyclic recipient mares. (Kaercher *et al*., 2013; Botelho *et al*., 2015; Silva *et al*., 2016). However, few reports are found in the literature regarding to the efficacy and the effects of using intravaginal P4 device with EB for estrus synchronization and follicular control in mares. Hence, the present work aimed to evaluate follicular growth and ovulatory rates in mares treated with an intravaginal P4 device for ten days and EB injection on the first day of the protocol, and to compare the results of the present hormonal protocol during the spring transition period with the summer breeding season.

## MATERIAL AND METHODS

The experiment was performed in the experimental farm of PUC Minas, located in Esmeraldas, Minas Gerais, Brazil (latitude 19°45'46" S, longitude 106 44°18'47" W). Females were kept on the same pasture of *Cynodon dactylon* with water *ad libitum* (CEUA protocols: CEUA UFMG 57/2018 and CEUA PUC Minas 017/2018). Eight crossbred mares (aging from 4 to 8 years) had their estrus synchronized during spring transition period (september/2018) and repeated in summer season (january/2019). Hence, a total of sixteen ovulatory cycles were assessed. All animals selected for the study were healthy and had no vaginal discharge or uterine alterations at the

beginning of the experimente, during both experimental periods. The same hormonal protocol was performed twice on each animal (once during the seasonal transition period and once during the breeding season; n=16). So, each mare received the same treatment during the spring (transition period; n=8) and then during the summer (reproductive period; n=8), as described below.

The protocol started (Day O; D0) with mares receiving an intravaginal P4 releasing device (1,9 g; CIDR®, Zoetis, São Paulo, Brazil), 5mg of Prostaglandin F2α (PGF2α; 1mL; i.m.; Dinoprost tromethamine; Lutalyse®, Zoetis, São Paulo, Brazil) and 4.0mg of EB (4mL, i.m.; Gonadiol®, Zoetis, São Paulo, Brazil). The P4 device remained for 10 days (D0 to D10). Upon removal of the device on D10, the mares received another dose of PGF2α (5mg; 1mL; i.m.; Dinoprost tromethamine; Lutalyse®, Zoetis, São Paulo, Brazil). When an ovulatory follicle (OF) ≥35mm was detected, the mare received 1.750 UI of hCG (0.7 mL; i.v.; Vetecor®, Hertape, Juatuba, Brazil). This moment was considered the day of ovulation induction with hCG (DhCG). During the period of P4 device insertion (D0 to D10), all animals were evaluated using ultrasonography (Aloka SSD-500; 5 MHz probe; Aloka Inc., Tokyo, Japan) every 24 hours (once a day; always in the same hour of the day; in the morning) to measure the dominant follicle (DF) diameter.

The size and location of the follicles were documented for both ovaries and recorded on individual maps for monitoring. Follicles of 7mm or more in diameter were measured and the diameter was calculated as the average of two linear cross-sectional measurements of the follicular antrum. I.e, the diameter of the largest follicle was determined considering the average of the maximum cross-sectional area of the height and width of a "frozen" image obtained by ultrasound. DF was defined as the one that grew to at least 10mm and exceeded the diameter of all other follicles. From D11 (after the removal of P4 implant), ovarian ultrasonography was performed every 12 hours (always in the same hours in the morning and in the afternoon) until the detection of ovulation. Ovulation was detected by the absence of the previously identified OF and confirmed by the eventual presence of a corpus luteum (CL) in the same ovary. A scheme of the experimental design of the present work is shown in Figure 1.

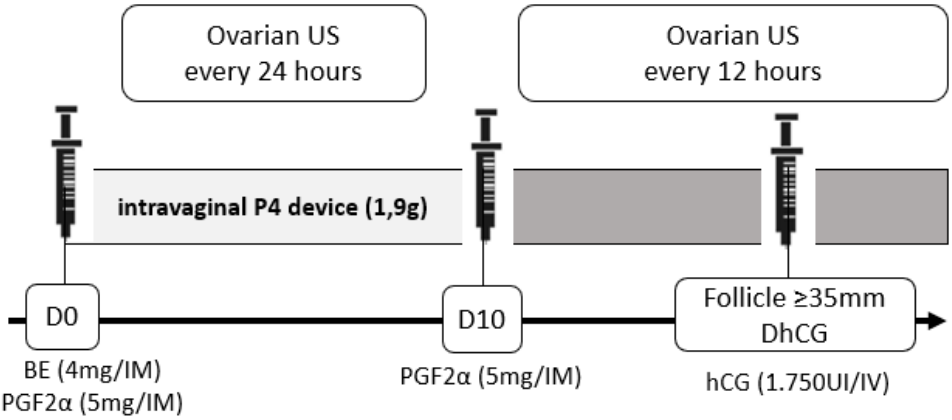


Figure 1. Scheme of experimental design for estrus synchronization in adult mares. US: ultrasonography; BE: estradiol benzoate; P4: progesterone; PGF2α: prostaglandin F2α (Dinoprost); hCG: human chorionic gonadotropin; D0: day 0 of hormonal protocol; D10: day 10 of protocol; DhCG: day of ovulation induction.

As previous stated, when a follicle ≥35mm was detected, ovulation was induced with hCG administration, being considered the zero hour (h0). The time of next ultrasound assessment was performed 12 hours later, being considered hour 12 (h12), and so on consecutively. Thus, the interval from hCG injection to ovulation (which was used to define the moment of ovulation) was considered every 12 hours. When the absence of OF was identified, it was possible to detect a time interval that ovulation occurred (between 12-24 hours, between 24-36 hours, between 36-

48 hours, etc). The mean of this time interval was used for statistical analysis. The follicular growth rate of each mare was determined by measuring the difference in the DF size over 24-hour increments of time. The variables (means ± standard deviation) of the two reproductive periods (spring versus summer) were submitted to ANOVA and compared compared using Tukey's test. Ovulation rate was submitted to Fisher's exact test. Calculations were performed using the software Graphpad Instat 3.06 and $P < 0.05$ was considered significant.

## RESULTS AND DISCUSSION

The present study demonstrated applicability and efficiency of the hormonal protocol using intravaginal progesterone device (intravaginal P4 device) and estradiol benzoate for follicular control of mares, both in the breeding season (summer; reproductive period), and in the spring transition period. On the first day of the experiment during the spring transition period, none of the mares presented corpus luteum (CL) and on the first day of the breeding season, all the mares presented a CL. In relation to the use of PGF2α to exert its luteolytic effect performed on the first experimental day (D0), it would be necessary for the stage of development of the CL to be responsive to it. In donkeys and horses, the CL becomes responsive from the 3rd and 4th day after ovulation, respectively (Carluccio *et al*., 2008). For this reason, a second dose of PGF2α was performed in our experiment (D10) in both periods, generating similar endocrine patterns in the mares. Even knowing that the mares of the transition period did not have CL, the PGF2α was applied (D0 and D10) also in the spring, so that this would not become a factor of difference between the protocols from the mares in the summer.

Equine is considered a phototropic positive specie in which the increase in luminosity has a positive effect on hypothalamic GnRH pulses and on gonadotropin secretion (Ginther, 1992; Satué and Gardon, 2013). The chances of reproductive activity during the year are directly related to the production of melatonin by the pineal gland, which occurs during hours of absence of light, being considered a decisive factor in the control of seasonal reproduction (Alexander and Irvine, 1991; Ginther, 1922; Aurich, 2011; Mariz, 2008). The increase in the dark period during winter suppresses the HHGA, while more hours of daylight in spring are coordinated with a gradual resumption of GnRH/LH pulsatility and follicular development (Ginther, 1992; Satué and Gardon, 2013). Hence, the spring transition period is characterized by a gradual increase in cyclicality (Donadeu and Ginther, 2002).

As previously mentioned, during the transition period of the present study, no mare had CL at the beginning of the experiment. In a study by Peres *et al*. (2006), when a follicle ≥25mm in diameter is detected during the spring transition, it took the mares 14.9±10.8 days (2-42 days) to present the first preovulatory follicle and 18.00±11.08 days (6-47 days) for the first ovulation to occur, a fact that justifies the absence of CL in this period. On the other hand, the increase in the length of days in the summer stimulates reproductive activity in horses, when the presence of CL was detected in all mares during this reproductive period. However, in the two reproductive periods (summer and spring), ovulations did not occur during the period in which the P4 device was inserted in the animals.

Thus, in both seasons, ovulations were detected only after removal of the P4 device. After P4 removal, the overall ovulation rate was 81.25% (13/16), considering both periods (spring and summer). All the animals (100%; n=8) ovulated in the summer breeding season while 62.5% of animals (n=5) ovulated in the spring transition season (P=0.0547). Therefore, it can be observed a higher ovulation rate for mares in the summer than for mares in the spring. The increased period of darkness suppresses the HHGA while increased daylight hours in spring coordinates with gradual resumption of GnRH/LH pulsatility and follicular development. Thus, irregular reproductive activities occur during the autumn and spring, while regular and frequent reproductive activities is observed during the summer (Ginther, 1992; Bergfelt, 2009; Aurich, 2011; Satué and Gardón, 2013), which justifies the lower ovulation rate for mares in spring comparing with summer (Palmer and Guillaume, 1992; Nagy *et al*., 2000). However, no differences in the time to ovulation occurrrence was detected between the seasons. The results of the time to ovulation are shown in Table 1.

Table 1. Ovulation assessed by ovarian ultrasonography in adult mares synchronized with an intravaginal implant (1.9g) of progesterone (P4) and induced to ovulate with human chorionic gonadotropin (hCG) during spring (transition period) and during the summer (breeding reproductive period)

| | Spring (n=5) | Summer (n=8) |
|---|---|---|
| Days to ovulation after P4 device removal | 4.90±2.10 | 3.81±2.30 |
| Hours to ovulation after hCG induction | 37.20±6.57 | 31.71±8.28 |

According to Table 1, considering the last measurement performed before ovulation, no difference (P=0.1704) was detected in the number of days from P4 removal to ovulation and no difference was detected (P=0.2334) in the time interval from induction with hCG to ovulation. It is worth mentioning that during the summer one mare ovulated before the pharmacological induction with hCG. According to Pierson and Ginther (1985), in mares, when a follicle reaches 30mm diameter in the ovary, ovulation can be detected during few subsequent moments up to seven days after its identification, which agrees with the results here obtained.

Regarding to the absence of differences in the time to ovulation between both seasons, it can be inferred that mares are expected to show improvement in follicular growth following P4 treatment due to increased LH pulsatility after P4 removal (Arbeiter *et al*., 1994). Previous exposure to P4 stimulates GnRH production and increases the levels of FSH and LH, providing follicular growth after P4 withdrawal (Arbeiter *et al*., 1994; Gastal *et al*., 1999; Hanlon and Firth, 2012). Moreover, P4 supplementation for ten days is effective in accelerating the onset of ovarian activity, in regulating the mares cyclicity and to enhances follicular response to ovulation inducers (hCG) in the transition phase (Gastal *et al*., 1999; Cuervo-Arango and Clark, 2010; Hanlon and Firth, 2012).

The EB was also administered at begining of the protocol (D0). The application of EB together with a source of P4 controls the frequency of FSH release and LH pulses, resulting in regression of FSH/LH-dependent follicles and the beginning of a new follicular wave (Bó *et al.*,1995). In cyclic mares, the follicular development can be suppressed with 17β-estradiol injected one day after ovulation (Pinto *et al*., 2004). Currently, Silva *et al*. (2016) evaluated the administration of different doses of EB in noncyclic embryo recipient mares followed by a single dose of long-acting P4 (1500mg). The highest E2 plasma concentrations were detected at 24 hours after EB administration; which started to decline at 24 hours after EB injection; with no differences among groups. Additionally, it was demonstrated that E2 plasma concentrations were below 0.4ng/mL after the third day post administration of either 2.5mg EB or 5.0mg EB.

Although in the present study the same protocol was used in all animals during both seasons, with the exact same hormone dosages (EB+P4+PGF2α), lower ovulation rates were observed in mares of spring than of summer. It is worth mentioning that no differences in BCS were observed among the experimental groups (summer: 2.81±0.45; spring: 2.75±0.38; P=0.6858). Since the mares of both seasons were benefited with exogenous P4 (from intravaginal device) in the protocol, it is unquestionable that the increased luminosity during tropical summer had offered an extra physiological advantage for equine reproductive performance during the summer, even when those hormones are used. Nonetheless, our data demonstrate that the present protocol allowed satisfactory ovulatory results for mares during the spring transitional season. It is noteworthy that P4 supplementation may have improved the hypophysary reserve of gonadotropins (Samper *et al*., 2002), thus contributing to ovulatory performance of the spring mares.

The size of dominant follicle is an important factor related to fertility in cattle (Pinaffi *et al*., 2015). Larger ovulatory follicles are commonly related to higher probability of pregnancy, since lower pre-ovulatory estradiol concentrations are commomly related to the formation of small CLs and reduced P4 concentrations (Perry *et al*., 2007). Positive correlations were observed among ovulatory follicle diameter, estradiol concentrations and CL area in sheep (Bartlewski *et al*., 1999). In beef cattle, ovulatory follicles lower than 11.3mm generated higher probability of embryonic loss compared to ovulations of larger dominant follicles (greater than 11.3mm in diameter) (Perry *et al*., 2005). In the present

study, the data obtained in the evaluation of dominant follicle sizes from both periods (summer and spring) are demonstrated in Table 2 and Figure 2.

Table 2. Dominant follicle (DF) and ovulatory follicle (OF) diameters assessed by ovarian ultrasonography at different moments of the syncronized estrous cycle, of adult mares receiving an intravaginal implant (1.9g) of progesterone (P4) and induced to ovulate with human chorionic gonadotropin (hCG) during spring (transition period) and summer (breeding reproductive period)

| | Spring (mm) | Summer (mm) |
|---|---|---|
| DF diameter at D0 | 18.69±9.72 | 14.64±3.45 |
| DF diameter at D10 | 23.54±6.03 | 26.46±8.60 |
| Higher DF diameter detected | 37.22±0.55 | 38.37±3.06 |
| OF diameter at the day of ovulation | 33.62±2.17 | 36.56±2.03 |

D0: day 0 of the syncronization protocol (day of P4 device insertion); D10: day 10 of the syncronization protocol (day of P4 device removal); OF: dominant follicle assessed 12 hours before ovulation.

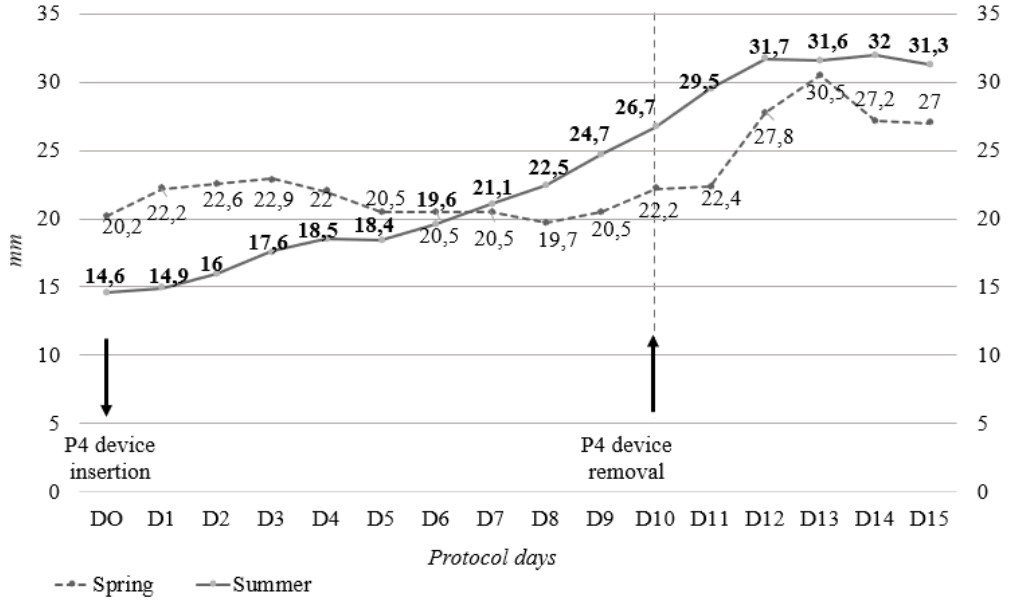


Figure 2. Diameters of the dominant follicle (DF) evaluated by ovarian ultrasound at different times of the estrous cycle in adult mares synchronized with Estradiol Benzoate and intravaginal implant (1.9g) of progesterone (P4 device). D0-D10: days 0 to 10 of syncronization protocol (days of P4 permanence); D10: day of P4 device removal; D11-15: days 11 to 15 of the protocol (days after the removal of the P4 intravaginal device).

Regarding to the follicular size, no statistical differences were observed between the reproductive seasons (summer and spring), either before or after the P4 removal (Figure 2 and Table 2). It also can be observed in Table 2 that no difference was detected between the seasons in the size of the DF at the time of device removal (D10; P=0.4203) which is probably due to the action of PGF2α administered on the first day of the protocol (D0) associated with the ten-day permanence of P4 intravaginal device. Botelho (2012) reported a study using a hormonal combination of P4 and PGF2α in mares. According to the authors, the P4 administration caused a reduction in folliculogenesis while PGF2α stimulated ovarian function, due to an increased pituitary responsiveness.

Considering only the animals that ovulated, also no difference was detected in OF diameter (spring: 33.62±2.17mm, n=5; summer: 36.56±2.03 mm, n=8; P=0.3674). Hence, using the hormonal protocol of the present study it was

possible to observe similar ovulatory follicles in both reproductive periods. Therefore, the use of PGF2α on the first day of the protocol (DO) likely generated a pituitary responsiveness to GnRH in mares of the spring, and an increase in LH production and reserve, which was further benefited by the P4 presence (Randel *et al*., 1996). Therefore, in both groups, it could be observed similar follicular sizes after removal of the device. Additionally, according to Handler *et al*., (2007), the presence of P4 device for 10 days caused a regression of DF and a beginning of new follicular wave in mares, while after P4 removal, occurs a release of HHGA blockage, allowing further follicular development until its ovulation.

It is worth mentioning that, although the largest mean diameter reached by the DF was 37.22±0.55mm in spring and 38.37±3.06mm in the summer (P=0.3870), in both seasons a reduction in the follicular diameter was observed before ovulation in some mares, which explain the numerically inferior size of OF compared to the size of higher DF diameter presented in Table 1. According to Koskinen *et al*. (1989), the pre-ovulatory follicle has an average daily growth of three millimeters until two days before ovulation, when it remains in constant size during approximately two days. At 12 hours before ovulation, a reduction is observed (around two to three millimeters in diameter), and then ovulate. According to Ginther *et al*. (2009), the growth rate of pre-ovulatory follicles observed in the two days before ovulation was 1.2±0.8 mm when measured in young mares, 2.0±0.4 mm for intermediate ages and 1.7±0.7 mm for those who were older. The results of follicular growth rates before and after the P4 device removal of the present study are demonstrated in Table 3.

Table 3. Follicular growth rate (mm/day) assessed by ovarian ultrasonography during the insertion of intravaginal implant (1.9g) of progesterone (P4; from day 0 until day 10) and after P4 implant removal (from day 11 until ovulation) of adult mares induced to ovulate with human chorionic gonadotropin (hCG) during spring (transition period) and during the summer (breeding reproductive period)

| Follicular growth rate | Day 0 to Day 10 (P4 insertion) | D11 to ovulation (after P4 removal) |
|---|---|---|
| Spring | 1.00±0.81mm/day a | 3.31±1.66mm/day b |
| Summer | 1.33±0.89mm/day a | 3.63±1.33mm/day b |
| Overall | 1.15±0.84mm/day a | 3.44±1.25mm/day b |

a,b: lowercase letters on the same line indicate P<0.05

As demonstrated in Table 3, a significant difference (P<0.001) was observed in the overall follicular growth rates during the P4 insertion period (D0 to D10), compared to the period in which the animals no longer had an P4 device insertion (D11 to ovulation). This difference was observed both in the transition season (spring; P=0.0054) and in the reproductive breeding season (summer; P<0.001). The higher follicular growth rates observed after the P4 device removal can be explained by the sudden drop of this hormone at this moment. The elevated levels of P4 promoted by the implant presence possibly caused an increased gonadotrophic LH reserve in the pituitary, generating an increased stimulus for follicular growth after the device removal (Handler *et al*., 2007).

However, it was interesting to note that no difference was observed for follicular growth rates between the seasons, both before (spring: 1.00±0.81 mm/day *vs.* summer: 1.33±0.89 mm/day; P=0.6107) and after the removal of P4 device (spring: 3.31±1.66 *vs.* summer: 3.63±1.33; P=0.5900).

Although purulent vaginal secretion was observed during P4 device insertion in two mares of the transition season and in one mare in the breeding season, these vaginal discharge alterations were spontaneously resoluted within 48 hours after P4 removal, in all animals. Canisso *et al*. (2013) compared the administration of oral P4 and intravaginal P4 device. The authors also observed the incidence of moderate vaginitis, with a small amount of visible exudate in the vulvar area of some mares exposed to the intravaginal device for 48 hours. Additionally, similar to the present study, it was observed that this condition was promptly resolved for up to 2 days after its removal.

## CONCLUSION

It was concluded that, under the conditions of the present study, the intravaginal device containing 1.9g of P4 allowed mares ovulating during the spring transition season to present similar follicular development performance to the mares of the reproductive season. In addition, the P4 + EB protocol used in this study allowed adequate follicular control, as well as satisfactory ovulatory rates for mares in the summer and in the spring. Although no differences in follicular growth rates were detected between seasons, higher ovulatory rates were observed for mares in the summer compared to mares in the spring.

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