Monitoring ovarian cycles, pregnancy and post-partum in captive marsh deer (*Blastocerus dichotomus*) by measuring fecal steroids

Bruna Furlan Polegato†, Eveline dos Santos Zanetti*,† and José Maurício Barbanti Duarte

Deer Research and Conservation Center (NUPECCE—Núcleo de Pesquisa e Conservação de Cervídeos), Departamento de Zootecnia, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Via de Acesso Professor Paulo Donato Castellane s/n, Jaboticabal, SP 14884-900, Brazil

*Corresponding author: Deer Research and Conservation Center (NUPECCE—Núcleo de Pesquisa e Conservação de Cervídeos), Departamento de Zootecnia, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Via de Acesso Professor Paulo Donato Castellane s/n, Jaboticabal, SP 14884-900, Brazil. Email: eveline_zanetti@yahoo.com.br

The marsh deer is an endangered species from the marshlands of central South America. This study aimed to characterize certain aspects of the reproductive physiology of marsh deer hinds, including the duration and fecal progestins profile of the estrous cycle, pregnancy and post-partum periods, and evaluate the effect of cloprostenol administration on this species. The experimental group consisted of six females and one fertile male marsh deer. During monitoring of the estrous cycle, the fresh fecal samples were collected daily and, during pregnancy, they were collected twice weekly. The hormonal profile obtained from daily fecal samples indicated that the mean duration of the estrous cycle was 21.3 ± 1.3 days (6.4 days inter-luteal phase and 14.8 days luteal phase; n = 16 estrous cycles). The mean concentration of fecal progestins in the inter-luteal phase was 834 ± 311 ng g⁻¹, in the luteal phase was 3979 ± 1611 ng g⁻¹, value between them was 1457 ng g⁻¹. No significant difference in fecal estrogen concentrations was determined during the estrous cycle. The corpora luteum was not responsive to cloprostenol until Day 6 of the estrous cycle, the period previously described as the inter-luteal phase. Half the females became pregnant following treatment with cloprostenol and two others were fertilized in their natural estrous cycle. Four females delivered fawns, and the mean duration of pregnancy was 253 ± 4 days. Fecal progestin concentrations were similar to those of the estrous cycle during the first 11 weeks of pregnancy and increased significantly (> 15250 ng g⁻¹) thereafter, providing a presumptive diagnosis guideline. Within 60 days of post-partum analyses, 75% of the deer exhibited behavioural estrus and/or ovarian activity. This study generated a broader understanding of the marsh deer species concerning the production of consistent data related to its reproduction. This knowledge can be used to assist the reproductive management of this species and, consequently, to promote its conservation.

Key words: Neotropical deer, fecal estrogen, fecal progestins, enzyme immunoassay, cloprostenol

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†These authors contributed equally to this work and are considered co-first authors.

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Introduction

The marsh deer (Blastocerus dichotomus) is the largest Neotropical deer and shows high specificity for humid environments (Duarte, 1996; Pinder, 1996; Piovezan et al., 2010). Its original geographical distribution has been drastically reduced due to the expansion of human activity and ~65% of the areas once occupied by the species have been lost over the past 40 years (Weber and González, 2003; Márquez et al., 2006). Currently, the marsh deer is either threatened with extinction or is extinct in certain areas and is classified as vulnerable (Duarte et al., 2016). It is also categorized as Appendix I according to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), primarily due to habitat loss.

Knowledge regarding this species is scarce and lacunae exist, particularly concerning its reproduction, making it more susceptible in cases of disaster or imminent threats. Thus, the adoption of more intensive conservation measures is recommended.

In 1998, a program was created to conserve this species in captivity, aimed at maintaining a genetic stock for use in future reintroductions (Figueira et al., 2005). The success of the program depends on management to ensure gene flow between the institutions involved and maximization of the genetic diversity of the population (Zanetti and Duarte, 2008; Duarte, 2010; Piovezan et al., 2010). It is known that in small populations, the loss of genetic variability is mainly due to genetic drift and inbreeding and that the use of assisted reproduction techniques can potentize reproduction, guaranteeing the formation of updated genomic banks and facilitating reproductive management, thus avoiding such losses (Wildt et al., 1997; Wildt and Wemmer, 1999).

The use of assisted reproduction techniques, however, depends on understanding certain features of the species’ reproductive biology, such as the onset of puberty, reproductive seasonality, ovarian cyclicity, luteal function and pregnancy (Duarte and Garcia, 1995). Establishing efficient methods to manipulate the estrous cycle, including techniques for estrus synchronization, are fundamental to achieve artificial insemination and embryo transfer (Pickard et al., 2001), which have great potential to assist conservation programs. The establishment of reliable methods to access reproductive events permits not only the use of reproductive biotechniques, but also monitoring and reproductive management of captive and wild populations (Wasser et al., 1995; Borjesson et al., 1996).

However, to date, knowledge concerning the reproductive biology of this species is scarce and manipulation of the estrous cycle in the marsh deer has yet to be described. Improving current understanding of the reproductive physiology of the species can be partly ascertained by characterizing the profile of reproductive steroid hormones, which is an alternative method of monitoring the phases of the reproductive cycle. However, the use of traditional methods based on containment and periodic blood samples to measure the concentrations of these hormones may be inadequate, since these deer present strong resistance to handling and sensitivity to stress (Nunes and Duarte, 2010; Pereira and Polegato, 2010). When subjected to such conditions, interrupted or failed reproduction, catabolic damage to the deer and increased risk of developing trauma and other injuries can occur (Lasley et al., 1989; Montfort et al., 1990; Morrow and Monfort, 1998; Hamasaki et al., 2001; Monfort, 2002; Schoenecker et al., 2004).

The use of non-invasive methods to monitor reproductive activity in wild species enables studies to be developed on such species. Measuring the metabolites of steroid hormones that are excreted in the feces, urine and saliva is an effective method and the ease afforded by the use of feces make it a safe and practical alternative (Pereira and Polegato, 2010). Thus, the objectives of this study were (i) to validate the measurement of reproductive steroid hormones and their fecal metabolites for reproductive monitoring in the species Blastocerus dichotomus, (ii) characterize certain parameters related to the reproductive physiology of the female, (iii) provide adequate methods to differentiate the phases of the reproductive cycle and (iv) evaluate the effect of cloprostenol (a synthetic analogue of prostaglandin F2α) administration in this species. These measures aim to provide a better understanding of the physiology and dynamics of the corpus luteum (CL) of the species.

Methods

Animals

The experimental group consisted of six adult females and one fertile male marsh deer (Table 1). The group was maintained at the installations of the Deer Research and Conservation Center (NUPECCE) of São Paulo State University (UNESP), Jaboticabal, SP, Brazil. At the onset of the study, the females were isolated from the male for 6 months, ensuring that they were not pregnant during monitoring of the estrous cycle. The deer were also submitted to management conditioning for three months. Management of the diet and feeding were standardized with food offered in individual stalls between 5 and 6 pm. This comprised ~1 kg of concentrated feed (equine feed: Omolene®, Purina Co., Paulinia, SP, Brazil), 2 kg of forage (Glicyne max, Morus Alba or Neonotonia wightii) and water ad libitum.

Monitoring the estrous cycle

During the first 3 months, the females were transferred from individual stalls (4 × 4 m²) to a paddock (30 × 50 m²) every day (between 7 and 9 am), where they remained together. In the afternoon (between 5 and 6 pm), the deer were led back to the stalls, where food was offered. In these periods of handling, the females were observed closely to identify signs of behavioural estrus. These were characterized by the female allowing approximation (even among deer presenting reactive behaviour), evidence of a stop reflex when dorsal pressure was
exerted by the examiner, abundant mucoid vulvar discharge and vulvar hyperemia.

**Manipulation of the estrous cycle and female conception**

To improve the current understanding concerning the estrous cycle in this species, the females were submitted to two treatments to synchronize estrous cycle (n = 3 deer per treatment) consisting of two applications of a synthetic analogue of prostaglandin F\_2\_a, cloprostenol sodium (2 mL, 530 μg i.m.—Ciosin®, Schering Plough Coopers®, Brazil) (Fisher et al., 1994; Asher et al., 1995) at different intervals. The applications were performed with a 12-day interval in treatment 1 and a 6-day interval in treatment 2 (Asher et al., 1995). The day of the estrous cycle at the time of treatment was determined for each female. Following the second application of the drug, the females were placed in contact with a fertile male, twice daily (8 am and 5 pm), and copulation was allowed. The females that did not show apparent estrus following the treatment received an additional dose of cloprostenol (2 mL, 530 μg i.m.) ~15 days after the initial dose, following the same management scheme adopted previously. Females who redisplayed behavioural estrus following the synchronization treatment and copulation were placed in contact with the male again and at least two copulations were observed.

**Monitoring pregnancy and the post-partum period**

The females were maintained in the paddock during pregnancy and were brought back to the stalls on the day preceding the collection of fecal samples. About 15 days prior to parturition and during the post-partum period (60 days), the females were maintained exclusively in the stalls to facilitate greater control of the parturition, the handling of fawns and to permit monitoring of post-partum estrus. During this period, the deer were observed closely (at 8 am and 6 pm) to identify signs of behavioural estrus, as described before.

**Table 1: Characteristics of the seven deer of the species Blastocerus dichotomus that composed the experimental group**

| Deer | Age (years) | Weight (kg) | Procedence | Reproductive history | Behaviour |
|------|-------------|-------------|------------|----------------------|-----------|
| F36  | 8 (~03/1998)| 74.9        | Wild⁴      | Pluriparous          | Reactive⁴ |
| F105 | 8 (~05/1998)| 81.2        | Wild       | Pluriparous          | Reactive  |
| F261 | 6 (16/05/2000)| 89.3      | Captive    | Pluriparous          | Reactive  |
| F262 ⁵ | 5 (15/03/2001)| 87.0      | Captive    | Pluriparous          | Non-reactive⁵ |
| F269 | 3 (03/03/2003)| 63.1      | Captive    | Nuliparous           | Very reactive⁶ |
| F270 | 3 (15/10/2003)| 62.5      | Captive    | Nuliparous           | Non-reactive |
| M52  | 12 (~12/1995)| ~110       | Wild       | –                    | Reactive  |

F = female; M = male. ⁴Female in lactation. ⁵Captured in the Rio Paraná basin. ⁶Deer showed strong resistance to being touched (except when in estrus), but tolerated the management procedures well. ⁷Deer tolerated being touched. ⁸Deer showed strong resistance to being touched and only tolerated the management procedures with restrictions.

**Collection and processing of fecal samples**

Fresh fecal samples were always collected in the morning, between 8 and 10 am. During monitoring and manipulation of the estrous cycle the samples were collected daily; during pregnancy (between copulation and parturition), they were collected twice weekly, on Tuesdays and Fridays; and in the post-partum period, on alternate days. Following collection, the samples were stored in plastic bags, identified and frozen at ~20°C. The method described by Graham et al. (2001) was used to extract the metabolites (estrogen and progestin) from the fecal samples. Briefly, 5 ml of 80% methanol was added to 0.5 g of lyophilized and triturated sample material. The mixture was vortexed for 30 s, agitated for 12 h in a horizontal homogenizer, and vortexed again for 15 s. Following centrifugation at 1500g for 20 min, the supernatant was separated and constituted the final extract.

**Determining hormone concentrations**

The concentrations of progestins and estrogens in fecal extracts from the estrous cycle were analysed by enzyme immunoassay (EIA). Only the concentration of fecal progestins was analysed in fecal extracts collected during pregnancy and the post-partum period. The antibodies CL425 and R4972 (University of California, Davis, CA, USA) were used for fecal progestins (P) and fecal estrogens (E2), respectively. These antibodies were chosen because they present high cross-reactivity with metabolites excreted in the feces of B. dichotomus, namely, 5α- and 5β-pregnanes and 17β-estradiol (Poletagay, 2004). All the fecal extracts were diluted in dilution buffer at 1:500 (estrus cycle and early pregnancy), 1:1500 (mid-pregnancy) and 1:2500 (late pregnancy) for P and 1:32 (estrus cycle) for E2. The samples were assayed in duplicate. The validation of hormone concentrations was performed as described by Brown et al. (2004): (i) by the significant recovery of properly diluted exogenous hormones added to fecal samples (y = 1.149x – 2.2556, r² = 0.99 and y = 1.088x + 1.6021, r² = 0.99 for P and E2, respectively); (ii) by comparing a curve parallel to the standard curve formed by the pool of fecal extracts prepared by serial
dilution in dilution buffer \( R^2 = 0.9914 \) and \( R^2 = 0.9744 \), respectively, for fecal P and E2; and (iii) due to the physiological relevance of the results obtained when different phases of the reproductive cycle were compared. The intrassay coefficients of variation were < 10% for all the hormones and controls evaluated. The interassay coefficients of variation were 10.1% (−35% binding, \( n = 57 \) plates) and 12.1% (−75% binding, \( n = 57 \) plates) for P and 7.4% (−20% binding, \( n = 29 \) plates) and 13.9% (−50% binding, \( n = 29 \) plates) for E2. Assay sensitivity was 0.78 ng g\(^{-1}\) (93.7% binding) for P and 1.95 ng g\(^{-1}\) (89.5% binding) for E2. All fecal data are expressed on a dry-weight basis.

**Statistical analysis**

Data analyses of the estrous cycle were performed based on the model proposed by Thompson et al. (1998), with modifications. The three lowest values of progestin concentration of each estrous cycle were considered basal and from these, the mean and standard deviation (SD) were calculated. Values greater than the limit (mean + 2SD) were considered indicative of the luteal phase and values below this were considered indicative of the inter-luteal phase. To calculate the duration of the estrous cycle, the day the concentration of fecal progestins reached the value indicative of the inter-luteal phase was considered the day one (D1) of the cycle. To determine the minimum concentration of fecal progestins indicative of pregnancy, the mean of the first month that showed significantly different concentrations of fecal progestins from the estrous cycle was subtracted from the standard error of the mean (SEM).

The data are presented as the mean ± SEM and comparisons between the deer, the estrous cycle phases (luteal and inter-luteal) and the different months of pregnancy and anestrus were performed using repeated-measures analysis of variance (ANOVA), followed by the Scott-Knott test. The fecal hormone concentration values were submitted to analysis of variance following logarithmic transformation of the hormone data (Morrow et al., 1995). Correlation between the variables was determined by Pearson’s correlation test.

The E2:P ratio was calculated for the days on which both hormones were analysed. All the analyses were performed using the SAS software (SAS Institute Inc., Cary, NC, USA) and the significance level for all statistical tests was 5% \( (P < 0.05) \).

**Results**

**Estrous cycle**

A total of 16 complete estrus cycles were evaluated, with a mean duration of 21.3 ± 1.3 days (range: 19–23 days), as determined by the hormonal profiles. The mean duration of the inter-luteal phase of the cycle was 6.4 ± 1.2 days, while the mean duration of the luteal phase was 14.8 ± 1.3 days. These means include data from five of the six females, since F269 remained anestrus from Day 12 of monitoring. No differences \( (P > 0.05) \) were observed in estrous cycle duration among females or different cycles of the same female (Fig. 1 and Table 2). Of the 20 estrous periods observed using the fecal progestin profile, 13 (65%) were also detected by behaviour. Regular detection of behavioural estrus was only possible in two females (F105 and F270; \( n = 8 \) estrous periods) (Fig. 1), and in all cases, these behaviours were correlated with hormonal profiles (Fig. 1). The duration of estrus behaviour ranged from 1 to 2 days \( (n = 13 \) estrous periods).

The mean concentration of fecal progestins for the inter-luteal phase of the estrous cycle was 834 ± 311 ng g\(^{-1}\) (range: 393–1431 ng g\(^{-1}\)) and differ \( (P < 0.05) \) from the luteal phase (3979 ± 1611 ng g\(^{-1}\) (range: 1498–11 364 ng g\(^{-1}\)), as determined by the individual profiles (Table 2). Based on the criteria defined above, a concentration of 1457 ng g\(^{-1}\) constitutes the limit value between the two phases. However, it was not possible to differentiate deer in the inter-luteal phase from anestrus deer. No significant differences in E2 concentrations were verified during the estrous cycle \( (P > 0.05); \) however, differences \( (P < 0.05) \) in the ratio of the concentrations of this hormone \( (E2) \) and P concentrations \( (E2P) \) were observed. A negative correlation was determined between the concentration of P and the E2:P ratio \( (r = −0.39, P < 0.001) \). The peak values obtained for the E2:P ratio coincided with behavioural estrus or occurred one day after behavioural estrus (Fig. 1).

**Manipulation of the estrous cycle**

When used up to Day 6 of the estrous cycle, cloprostenol did not trigger an effective luteolytic response. All the females who responded to the drug exhibited behavioural estrus, which began on average 58 h following administration (range: 40–64 h). One deer (F269) was in anestrus and did not respond to the treatment (Fig. 2).

**Pregnancy**

Three females became pregnant following treatment with cloprostenol (F105, F262 and F270) and two others were fertilized in subsequent natural estrous periods (F36 and F261). F105 was fertilized, but miscarried in early pregnancy according to a prolonged luteal activity for ~60 days following copulation. Of the four females in which pregnancy went to term, two (F261 and F270) became ill in the final trimester. The mean duration of pregnancy was 253 ± 4 days \( (n = 4) \).

Endocrine characterization of pregnancy showed that the concentrations of fecal progestins remained consistent with the values of the luteal phase of the estrous cycle until about the third month of pregnancy \( (P > 0.05) \), after which they began to gradually increase. In the fourth month of pregnancy, the concentration of progestins nearly doubled compared with previous months \( (P < 0.05) \), reaching a peak in the eighth month, at which point the mean concentrations were 6-fold greater than those obtained during the initial phases \( (P < 0.05) \) (Table 2). Concentrations of fecal progestins \( \geq 15 \) 250 ng g\(^{-1}\) were indicative of pregnancy.
Post-partum period

The concentrations of fecal progestins began to decline in late pregnancy (vary from 1 week to 1 month before parturition) and only achieved basal concentrations following parturition (4–8 days). During analysis of the post-partum period, three (F261, F262 and F270) of the four deer studied presented a cyclic pattern in the excretion of fecal progestins, which demonstrates the resumption of ovarian activity and confirms the existence of post-partum estrus in this species. A common characteristic among these deer was the occurrence of a short cycle, with lower concentrations of fecal progestins preceding normal cycles (Table 3).

Figure 1: Monitoring the estrous cycle by determining the concentrations of fecal progestins (black line) and fecal estrogen:fecal progestin, ratio (gray line) in six females of Blastocerus dichotomus. The arrows indicate the day behavioural estrus was detected.
Table 2: Characteristics of the estrous cycle and pregnancy in five female *Blastocerus dichotomus*

| Deer | Observed cycles | Inter-luteal phase (days) | Luteal phase (days) | Length (days) | [P] Inter-luteal (ng g⁻¹) | Luteal progestins (ng g⁻¹) | Pregnancy length (days) | Pregnancy Weeks |
|------|-----------------|--------------------------|--------------------|--------------|--------------------------|---------------------------|------------------------|------------------|
| F36  | 1st 5 16 21 663 4034 | 249 (F) 4.1 kg*** | 2607 5013 4226 17 850 25 356 31 209 23 225 25 946 22 524 | W1 | M1 (ng g⁻¹) | M2 (ng g⁻¹) | M3 (ng g⁻¹) | M4 (ng g⁻¹) | M5 (ng g⁻¹) | M6 (ng g⁻¹) | M7 (ng g⁻¹) | M8 (ng g⁻¹) | M9 (ng g⁻¹) |
|      | 2nd 6 16 22 733 5311 |   | 6511 8730 6289 11 870 24 109 22 724 29 207 35 295 18 535 | 6720 7312 8410 26 127 28 984 33 216 39 844 30 307 | 24 992 28 463 |   |   |   |   |   |   |   |   |
|      | 3rd 4 16 20 734 3384 |   | 7324 5469 6830 16 038 27 175 14 431 | W3 |   |   |   |   |   |   |   |   |   |   |
|      | 4th – – – – – – |   | 6013 5352 10 841 19 015 30 120 | W4 |   |   |   |   |   |   |   |   |   |
| Mean | 5 a 16 a 21 a 710 a 4243 a |   | Mean 5659 a 6127 a 7469 a 27 072 a 21 316 a 25 990 a 27 368 a 21 195 a | – |   |   |   |   |   |   |   |   |   |
| F105 | 1st 9 13 22 896 4052 | 257 (M) 4.5 kg*** | 1162 8409 7422 18 398 14 421 | W1 |   |   |   |   |   |   |   |   |   |
|      | 2nd 8 13 21 757 3945 |   | 6471 4847 7837 18 144 22 119 34 923 | W2 |   |   |   |   |   |   |   |   |   |
|      | 3rd 7 16 23 821 4815 |   | 6100 6477 9592 18 111 25 027 32 952 | W3 |   |   |   |   |   |   |   |   |   |
|      | 4th – – – – – – |   | 8479 6495 12 383 28 192 | W4 |   |   |   |   |   |   |   |   |   |
| Mean | 8 a 14 a 22 a 824 a 4270 a |   | Mean 5407 a 6569 a 9651 a 17 515 a 29 338 a | – |   |   |   |   |   |   |   |   |   |
| F261 | 1st 8 15 23 773 3448 | 250 (F) 4.9 kg*** | 1737 4164 6206 | W1 |   |   |   |   |   |   |   |   |   |
|      | 2nd 6 16 22 682 4519 |   | 6471 4847 7837 | W2 |   |   |   |   |   |   |   |   |   |
|      | 3rd 7 16 23 671 3919 |   | 6100 6477 9592 | W3 |   |   |   |   |   |   |   |   |   |
|      | 4th – – – – – – |   | 8479 6495 12 383 | W4 |   |   |   |   |   |   |   |   |   |
| Mean | 7 a 15.7 a 22.7 a 708 a 3962 a |   | Mean 5407 a 6569 a 9651 a 17 515 a 29 338 a | – |   |   |   |   |   |   |   |   |   |
| F262 | 1st 5 15 20 851 2869 | 257 (M) 2.4 kg*** | 1737 4164 6206 | W1 |   |   |   |   |   |   |   |   |   |
|      | 2nd 5 15 20 1035 3364 |   | 6471 4847 7837 | W2 |   |   |   |   |   |   |   |   |   |
|      | 3rd 7 15 22 925 4496 |   | 6100 6477 9592 | W3 |   |   |   |   |   |   |   |   |   |
|      | 4th – – – – – – |   | 8479 6495 12 383 | W4 |   |   |   |   |   |   |   |   |   |
| Mean | 5.7 a 14.7 a 20.3 a 937 a 3576 a |   | Mean 4784 a 5565 a 7929 a 15 266 a 32 726 a 42 024 a 58 934 b 59 347 b | – |   |   |   |   |   |   |   |   |   |
| F270 | 1st 5 15 20 907 2913 | 257 (M) 2.4 kg*** | 2265 8370 6206 | W1 |   |   |   |   |   |   |   |   |   |
|      | 2nd 6 15 21 973 4108 |   | 5925 6697 18 509 13 393 | W2 |   |   |   |   |   |   |   |   |   |
|      | 3rd 7 12 19 770 2850 |   | 5718 6465 8469 | W3 |   |   |   |   |   |   |   |   |   |
|      | 4th 6 16 22 718 3007 |   | 6706 4341 9292 | W4 |   |   |   |   |   |   |   |   |   |
| Mean | 6 a 14.5 a 20.5 a 842 a 3219 a |   | Mean 4650 a 8220 a 7562 a 20 662 a | – |   |   |   |   |   |   |   |   |   |

*Female had a miscarriage in early pregnancy.
**Female that were ill during the final trimester of pregnancy.
***Sex (male [M] or female [F]) and birth weight of the fawn.
Means within column with uncommon and lowercase letters (a, b and c) differ (P < 0.05) by the Scott-Knott test; means within row with uncommon and capital letters (A, B, C and D) differ (P < 0.05) by the Scott-Knott test.
Hormonal analyses performed on fecal samples proved to be an efficient alternative for monitoring reproductive events in the marsh deer. The results obtained in this study showed that this technique has the potential to clarify reproductive status in this species, since it characterizes and differentiates the different phases of the reproductive cycle. Given its non-invasive nature, it can be applied when monitoring captive and wild populations, even when dealing with species that are highly sensitive to stress (Nunes and Duarte, 2010).

Table 3: Characteristics of the post-partum period in four female Blastocerus dichotomus

| Deer | [P] Parturition (ng g\(^{-1}\)) | [P] Basal\(^a\) (days) | Anestrus\(^b\) (days) | First luteal phase | Second luteal phase |
|------|-------------------------------|-----------------------|----------------------|-------------------|-------------------|
|      |                               |                       |                      | Duration (days)   | Mean [P] (ng g\(^{-1}\)) | Duration (days) | Mean [P] (ng g\(^{-1}\)) |
| F36  | 16 553                        | 7                     | Over 53              | –                 | –                 | –                | –                |
| F261\(^c\) | 12 412                  | 5                     | 14                   | 4                 | 2052              | 12               | 4640             |
| F262 | 21 230\(^d\)              | 10\(^f\)              | 6                    | 4                 | 1963              | 14               | 2592\(^g\)       |
| F270\(^c,d\) | 9907                      | 3                     | 14                   | 6                 | 2314\(^a\)        | 12               | 4591\(^g\)       |

\([P]\) Fecal progesterin concentration.
\(^a\) Period for [P] to achieve inter-luteal phase concentration.
\(^b\) Considering an inter-luteal phase of 6 days.
\(^c\) Illness during the final trimester of pregnancy.
\(^d\) Lost fawn; deer did not lactate.
\(^e\) [P] 5 days post-partum.
\(^f\) Behavioural estrus 6 days post-partum.
\(^g\) Behavioural estrus before [P] achieved luteal phase concentration.

Discussion

Figure 2: Excretion of fecal steroid hormone metabolites of six female Blastocerus dichotomus during two different estrus synchronization treatments using cloprostenol (F36, F262, F269: two applications of cloprostenol at 12-day intervals; and F105, F261, F270: two applications of cloprostenol at 6-day intervals). White arrows indicate the day in the estrous cycle which each female was on the day of treatment administration (A = anestrus) and gray arrows indicate the day behavioural estrus was detected (time, in hours, between treatment administration and detection of behavioural estrus).
The mean duration of the estrous cycle obtained in this study was slightly shorter than that previously observed for this species (24 days; Duarte and Garcia, 1997; Schwarzenberger and Dreben, 1998), and comparable to other cervid species with similar body size (17–21 days for Axis axis (Chapple et al., 1993), 13–22 days for Cervus unicolor and 15–24 days for Cervus elaphus (Asher et al., 1997), 14–23 days for Cervus eldi thamin (Monfort et al., 1990), 17–21 days for Cervus nippon taionamus (Liu et al., 2002)). The difference between the luteal and inter-luteal phases of the estrous cycle was evident when monitoring fecal progestins; however, no difference was verified in fecal estrogen concentrations. It is possible that the failure to detect fecal estrogen peaks could be due to lower concentrations of circulating estrogen or because it is excreted as a urinary metabolite, as reported for other ungulate species (Schwarzenberger et al., 1996). Research indicates that estrogens can induce adult female sheep to express behavioural estrus and that progesterone enhances the role of estrogen in sexual behaviour (Keverne et al., 1983). Thus, the E2:P ratio was more effective at indicating the time of ovulation than isolated analysis of steroid hormones, as suggested previously for Gazella dama mhorr (Pickard et al., 2001), for Mazama gouazoubira (Zanetti et al., 2010) and as described in humans (Lenton et al., 1989). The peak values of the E2:P ratio coincided with the period of behavioural estrus, such that the behavioural data assured the accuracy of endocrine monitoring.

Cloprostenol adequately promoted luteolysis in cyclic female marsh deer, suggesting that it could be an important drug for manipulating the estrous cycle of this species. However, the action of this drug is directly related to the presence of a functional CL (Asher et al., 1993; Whitley and Jackson, 2004). Cloprostenol was unable to promote luteolysis in the marsh deer when treatment was administered while the deer was in anestrus or when applied up to Day 6 of the estrous cycle, during which the CL is absent or hypofunctional. This finding is similar to that observed for C. elaphus (Asher et al., 1995) and for most mares (Pinto, 2013), which proved to be insensitive to the action of prostaglandin F2α up to Day 6 of the estrous cycle and contrasts with that observed for sheep (Rubianes et al., 2003) and some mares (Pinto, 2013), in which luteolysis can be induced from day three of the estrous cycle. This refractory period of the CL coincides with the duration of the inter-luteal phase of the estrous cycle of the species B. dichotomus, in which the CL is still in formation, secreting small quantities of progesterone and basically consists of small luteal cells that are unresponsive to prostaglandin F2α (Berisha and Schams, 2005).

The time until the onset of behavioural estrus following treatment with cloprostenol was similar to that previously reported for other deer species, such as M. gouazoubira (40–69 h) (Zanetti et al., 2010) and Dama dama (42–64 h) (Jabbour et al., 1993) and this variation is related to the follicular stage present at the moment of luteolysis induction (Rubianes et al., 2003; Barros and Ereno, 2004).

All the deer that responded to cloprostenol, i.e. displaying behavioural estrus, had ovulation and formation of the CL, as determined by the concentrations of fecal progestins. The pregnancy rate following synchronization with this drug was 50% (3/6); however, although this is low, it is numerically superior than that reported for Orix dammah (37.5% (Morrow et al., 2000)) and D. dama (40.7% (Jabbour et al., 1993)) using similar treatment protocols, followed by artificial insemination. In this case, the different forms of breeding could have influenced the difference between the studies, as well as the small number of deer that constituted the experimental group herein.

The mean gestation period observed for this species was shorter than that described previously (271 days) (Frädrich, 1995) and is compatible with other uniparous cervid species of similar body size (C. elaphus (Asher et al., 2005), C. elaphus namnodes (Stoops et al., 1999), C. eldi thamin (Monfort et al., 1990), C. nippon (Hamasaki et al., 2001), D. dama (Willard et al., 1998), Rangifer tarandus tarandus (Ropstad et al., 2005)). However, the gestation period can be extended if the female suffers severe food restriction and, in some cases, can lead to the birth of fawns with body mass index below normal (Verme, 1965). Due to the correlation between fawn mortality and its body weight at birth, females can significantly ravage their energy reserves to try to ensure that the fawn is born with an adequate body mass (Garcia et al., 2006). This plasticity in the physiological response was observed in the two females that became ill in the final trimester of pregnancy and in which the period of gestation was extended by about a week, apparently as a form of compensation. However, despite presenting a severely diminished body mass and prolonged period of gestation, F270 produced a fawn with low birth weight, which died one day following parturition.

The pattern of fecal progestin excretion during pregnancy was similar for all the deer in the study. Based on the hormonal data, a presumptive diagnosis of pregnancy in B. dichotomus can be determined from the second trimester onward (from the four month of pregnancy). This finding is similar to that reported for other cervid species (Capreolus capreolus (Sempéré, 1977), C. elaphus nelsoni (White et al., 1995; Garrott et al., 1998), C. elaphus namnodes (Stoops et al., 1999), C. eldi thamin (Monfort et al., 1990), D. dama (Willard et al., 1998)) and is due to the fact that the placenta synthesizes progesterone in most deer species during pregnancy. Steroidogenesis is obviously faster in the luteal tissue than in the placenta, thus during early pregnancy, when the placental volume/area is small, the importance of this source of progesterone synthesis and secretion is limited. During mid-pregnancy, the placental volume becomes much greater than the luteal volume and it is likely that the placenta is a physiologically significant source of steroid synthesis from this period onward (Flood et al., 2005).

Post-partum estrus, which has previously been described in this species (Frädrich, 1995), was observed in three of the four
deer in which pregnancy went to term and seems to be related to the abundance of food and habitat stability (Robbins, 1983). This is probably due to the association of a marked decline in progesterone concentrations and a sharp increase in estrogen concentrations observed in some ungulates during parturition. This provides a favourable hormonal environment for the expression of behavioural estrus (Pereira et al., 2006). The resumption of ovarian activity was characterized by the hormonal profile and presented some peculiarities, such as the appearance of a shorter cycle showing a lower concentration of fecal progestin excretion preceding the normal estrous cycles. This cycle could be related to the luteolytic influence of the involuting uterus due to an increased and prolonged release of prostaglandin F2α and an incomplete restoration of LH release leading to insufficient follicular growth and maturation, as previously reported in sheep (Schirar et al., 1989). Thus, as documented in the Mohor gazelle (Pickard et al., 2001), the conception rate in post-partum estrus may be lower than normal, suggesting failure of the reproductive tract, which may be unable to sustain pregnancy (Pereira et al., 2006).

Conclusion

This study validated the measurement of reproductive steroid hormones and their fecal metabolites for reproductive monitoring in the species B. dichotomus, provided adequate methods to differentiate the phases of the reproductive cycle and evaluated the effect of cloprostenol (a synthetic analogue of prostaglandin F2α) administration in this species; generating a broader understanding of the marsh deer species concerning the production of consistent data related to its reproduction. This knowledge can be used to assist the reproductive management of this species and, consequently, to promote its conservation.

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Author contribution

Polegato, Zanetti and Duarte contributed to design the study and analysed the data. Polegato and Zanetti contributed during the experimental phase. All authors have contributed to drafting of article.

Ethics approval and consent to participate

The study was approved by the Animal Ethics and Welfare Committee (Comitê de Ética e Bem-estar Animal, CEBEA) of the Faculty of Agrarian and Veterinary Sciences (Faculdade de Ciências Agrárias e Veterinárias, FCAV) UNESP, Jaboticabal, SP, Brazil.

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