


## Ovarian cycle of southern brown howler monkey (*Alouatta guariba clamitans*) through fecal progesterone measurement

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**Abstract** The ovarian cycle in howler monkeys (genus *Alouatta*) has been investigated through several biological parameters (ranging between 16.3 and 29.5 days); however, no data exist concerning the ovarian activity of the southern brown howler monkey (*Alouatta guariba clamitans*). This study aimed to describe the ovarian cycle of *A. g. clamitans* by profiling fecal progesterone concentrations. Over 20 weeks, fecal samples of eight captive adult females of *A. g. clamitans* were collected. The collections were made at dawn, 5 days a week, and the samples were frozen immediately following collection. Next, they were dried, pulverized and hormonal metabolites were extracted to determine progesterone concentrations by enzyme immunoassay. Of the 758 samples tested, the mean concentration of fecal progesterone was  $2866.40 \pm 470.03$  ng/g of dry feces, while the mean concentration at baseline was  $814.47 \pm 164.36$  ng/g of dry feces. Among the eight females, one showed no ovarian cyclicity and three presented periods of probable absence of cyclicity and low progesterone concentrations. A mean duration of  $16 \pm 0.52$  days was observed for the 35 cycles studied. The interluteal phase lasted  $4 \pm 0.37$  days on average,

with a mean concentration of fecal progesterone of  $467.98 \pm 29.12$  ng/g of dry feces, while the luteal phase lasted  $11 \pm 0.50$  days, with a mean concentration of  $4283.27 \pm 193.31$  ng/g of dry feces. Besides describing the characteristics of the ovarian cycle, possible causes for the low concentrations of fecal progesterone and periods of absence of cyclicity are also discussed.

**Keywords** Howler · Fecal progesterone · Reproduction · Noninvasive method · Ovulatory period

### Introduction

The ovarian cycle in howler monkeys has been investigated through their sexual behavior, morphological changes and vaginal cytology and ranges from 16 to 20 days (Colillas and Coppo 1978; Glander 1980; Crockett and Sekulic 1982; Kugelmeier et al. 2011). However, the concentration of fecal metabolites of the sex steroids has established itself as an effective tool in monitoring and as a clearer mode of understanding the reproductive physiology in both captive and free-living primates, precisely because it permits assessment of physiological parameters in a noninvasive manner (Heistermann et al. 2001; Brown 2008; Van Belle et al. 2009; Kugelmeier et al. 2011; Van Belle 2015).

Recently, Herrick et al. (2000) estimated the ovarian cycle of *A. seniculus* as 29 days by determining urinary metabolite concentrations. Fecal metabolite concentrations were also used to estimate the cycles of *A. caraya*, at around 19 days (Kugelmeier et al. 2011), and *A. pigra*, at around 18 days (Van Belle et al. 2009). The data available for both fecal and urinary metabolites indicate that the follicular phase for the genus corresponds to half the length

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of the ovarian cycle (Herrick et al. 2000; Kugelmeier et al. 2011).

Currently, no data exist concerning the ovarian activity of the southern brown howler monkey (*Alouatta guariba clamitans*). This information enables future projections about the viability of primate populations (Kleiman and Rylands 2008) and are relevant to both in situ and ex situ conservation and management proposals (e.g. fertility studies, assisted reproduction, mating and demographic strategies). This study represents the first report aiming to validate the measurement of fecal progestogens for reproductive monitoring of the species and describe the ovarian cycle of southern brown howler monkeys by profiling fecal progestin concentrations.

## Methods

### Study site and captive conditions

The research was conducted between February 8, 2011 and June 25, 2011 in the municipality of Indaial (26°54'S, 49°23'W), Santa Catarina, Brazil, specifically at the Indaial Biological Research Center (*Centro de Pesquisas Biológicas de Indaial*, CEPESBI)—Bugio Project. The center maintains a scientific maintenance program of wild animals (IBAMA registration no. 1/42/98/000708-90), where 41 specimens of *A. g. clamitans* are housed.

Feeding was distributed in six daily meals consisting of domestic cultivar fruits and vegetables, maintenance dog ration, *Cecropia glaziovii* (embaúba) and *Sechium edule* (chayote) leaves collected in native forest, and water provided ad libitum. The howler monkeys were exposed to natural fluctuations in the photoperiod while maintained in open enclosures measuring 3.0 × 3.0 × 2.6 m (width × length × height) that included a covered area of containment/isolation and was enriched with wide strips of water hose, stumps, food platforms and food.

### Animals and sample collection

Over a 20-week period, fecal samples of eight adult females were collected (Table 1). Females 6 and 7 were originally housed in another enclosure belonging to the Bugio Project and were transferred to the CEPESBI in early March, when they became part of this experiment, such that their fecal samples were only collected for 15 weeks. The enclosures disposal was linear, alternating male and female (Table 1). Their boundary were composed of fences with large spaces that allowed contact between howlers.

Fecal sample collection was performed once a day, 5 days a week, at dawn. In order to avoid the risk of

collecting samples from the previous day and/or the monkeys defecating on the samples, the enclosures were cleaned regularly and any feces accumulated during the evening period were removed at dusk, prior to the early morning collections. In the case of dyads, the individual howlers were separated (led by food stimulus) at the end of each day to ensure individualization of the samples at the time of collection. Samples were not collected when the day dawned under heavy rain, when they appeared to include diarrhea or it was necessary to separate one of the monkeys for a clinical intervention.

### Storage of fecal samples and hormonal extractions

Following collection, the samples were placed in plastic Ziploc bags and immediately placed in a freezer at −20 °C in the CEPESBI. Every month the samples were transported to the city of Curitiba, Paraná, in a cooler containing ice and again stored at −20 °C in the Department of Zoology of the Federal University of Paraná (UFPR). The samples were removed from the freezer and placed in a heater at 80 °C for 72 h for drying. After being dried, they were pulverized inside plastic bags using a rubber mallet and a pistil, and then homogenized. The resulting powder was placed in small plastic bottles and again stored at −20 °C.

The extractions were performed at the Deer Research and Conservation Center (*Núcleo de Pesquisa e Conservação de Cervídeos*, NUPECCE) of the School of Agricultural and Veterinary Sciences of São Paulo State University (FCAV-UNESP), in the city of Jaboticabal, São Paulo, Brazil. To extract the hormone metabolites, 0.5 g of each sample was diluted in 5 mL of 80 % methanol pro analysis. Next, they were vortexed for 30 s in stirring tubes and placed on a homogenizer for 12 h. Later, they were vortexed again for 10 s and centrifuged at 377g for 20 min (Graham et al. 2001). Next, the supernatant extract was separated from the solid phase and placed in plastic tubes, which were preserved at −20 °C until the concentration was determined.

### Progestin assay

Progestin concentrations were determined by enzyme immunoassay (EIA) (Brown 2008). To achieve this, the CL425 antibody (C. Munro, University of California, Davis, CA, USA) was used. The cross-reactivity of the CL425 antibody has been described by Graham et al. (2001). Technical validation of hormonal concentrations was obtained: (1) by observing the parallel arrangement between the standard curve and the curve formed by the pool of fecal extracts prepared by serial dilution in dilution buffer ( $R^2 = 0.97$ , working dilution 1:1000); (2) by

**Table 1** Captive conditions, registration data and reproductive history of the eight adult captive female *Alouatta guariba clamitans* in CEPESBI who constituted the experimental group

Individual	Enclosure condition	Right neighbor	Left neighbor	Estimated age (years)	Sex-age class at admission	Reproductive history	Months in current enclosure
Female 1	Dyad with F2	Male 1	Male 2	4.5	Infant	Nulliparous	3
Female 2	Dyad with F1	Male 1	Male 2	>6	Adult	Unknown*	3
Female 3	Solitary	Male 2	Male 3	>7	Juvenile	Nulliparous	3
Female 4	Solitary	Male 4	Male 5	>11	Juvenile	Nulliparous	1
Female 5	Dyad with a juvenile male	Male 5	Male 6	>13	Adult	Unknown*	1
Female 6	Dyad with F7	Male 6	Male 7	>5	Juvenile	Nulliparous	0
Female 7	Dyad with F6	Male 6	Male 7	>11	Adult	Unknown*	0
Female 8	Solitary	Male 7	Male 8	>6	Adult	Unknown*	0

<sup>a</sup> Has never given birth in captivity

significant recovery of exogenous hormones added to appropriately diluted fecal samples ( $y = 1.010x + 0.571$ ,  $R^2 = 0.98$ ); and (3) by the physiological relevance of the results obtained when the different stages of the reproductive cycle were compared.

The intra- and interassay coefficients of variation (CV) were evaluated for two internal controls, with high (C1, with ~35 % binding) and low (C2, with ~70 % binding) hormone concentrations (1:500 dilution and 1:2000 respectively, intra-assay CV: C1 = 20.76 % and C2 = 18.28 %, interassay CV: C1 = 19.99 % and C2 = 20.46 %, standard curve interassay CV < 6.5 %, total of 50 plates).

## Data analysis

The concentrations of progestins were expressed in ng/g of dry feces. Descriptive statistics of the data were performed and always expressed as the mean  $\pm$  standard error of the mean (SEM). Since this study only contemplated data concerning progesterone metabolites, the phases of the ovarian cycle were denominated the interluteal and luteal phases.

The interpretation of hormone concentrations and description of the ovarian cycles were performed individually for each female. First were selected points with lowest progestins values, considered baseline of each cycle. Then the values of mean and standard deviation (SD) of the data set was obtained. The points that were above the mean value + 2SD were considered indicative of luteal phase, and those who were below this threshold were considered interluteal phase (Heistermann et al. 1997).

Since the fecal samples were collected only five times per week, on the days when no collections were performed, the assumed level of progestins was obtained graphically, with a dotted line connecting the two points between the

collection interval. These deduced levels were assumed for all collection intervals and formed part of the calculations of ovarian cycles and its divisions (Fig. 1).

Only complete cycles were considered. The length of the cycles (in days) was defined by the interval between the first day of the interluteal phase and the last of the luteal phase. The likely day of ovulation (day 0) was considered to be the day before the first day of increased concentrations of fecal progestins (early luteal phase), since there is a delay between blood hormone secretion and its subsequent metabolism and excretion in the feces (Shideler et al. 1993; Ziegler et al. 1996, 1997; Campbell et al. 2001).

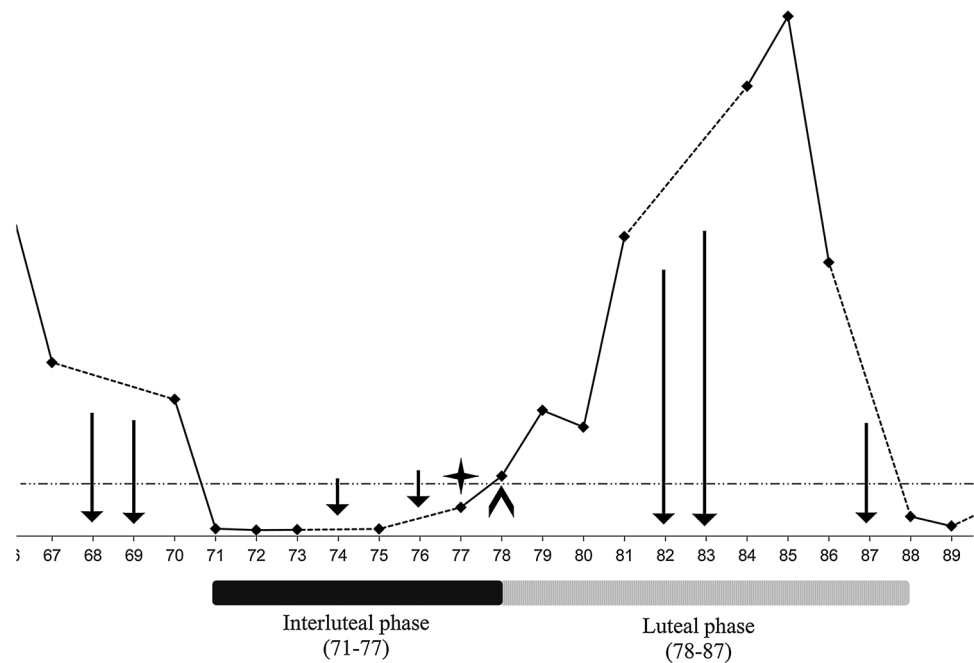
## Results

We analyzed a total of 758 samples. These analyses revealed a wide range of intra and inter-individual concentrations of progestins and in relation to the baseline level obtained for each individual (Table 2).

The profiles presented clearly demonstrated patterns of the rise and fall of progestins, which allowed us to infer the ovarian cyclicity in seven of the eight females studied (Figs. 2, 3). Considering all the females studied, a total of 36 cycles were observed. However, the second cycle of F1 presented an interluteal phase of 20 days, indicating that it may have included a short absence of cyclicity period; therefore, this cycle was disregarded in the analyses.

The remaining 35 cycles presented a mean duration of  $16 \pm 0.52$  days range 10–22 days). The interluteal phase lasted on average  $4 \pm 0.37$  days (range 1–10 days) with a mean concentration of fecal progestins of  $467.98 \pm 29.12$  ng/g of dry feces. The luteal phase lasted  $11 \pm 0.50$  days (range 7–17 days) with a mean concentration of  $4283.27 \pm 193.31$  ng/g of dry feces (Fig. 4).

**Fig. 1** Example of a description of interluteal and luteal phases, and deductive calculation between sampling intervals based on an observed cycle of Female 1. ✦ day 0 (probable ovulation); ▲ 1° day above estimated basal levels; ↓ days without collect of samples; basal level (1155.11 ng/g dry feces)



**Table 2** Descriptive statistics of the concentration (ng/g dry feces) of fecal progesterins of the eight captive female *Alouatta guariba clamitans* at the CEPESBI, who constituted the experimental group

Individual	Number of samples ( <i>n</i> = 748)	Mean concentration	Standard error	Maximal values	Minimal values	Basal level
F1	103	3812.16	470.03	20,859.81	139.91	1155.11
F2	102	666.13	89.56	4412.12	92.72	193.54
F3	100	3622.33	428.30	19579.19	173.45	942.99
F4	100	3713.96	276.97	12251.48	243.70	1471.30
F5	98	3806.79	371.14	14390.80	113.78	399.99
F6	81	744.29	58.71	2684.44	121.59	–
F7	81	2348.64	191.92	9539.77	192.65	701.59
F8	93	3816.26	335.76	14,621.14	159.45	836.80
Mean values	–	2866.40	123.74	–	–	814.47

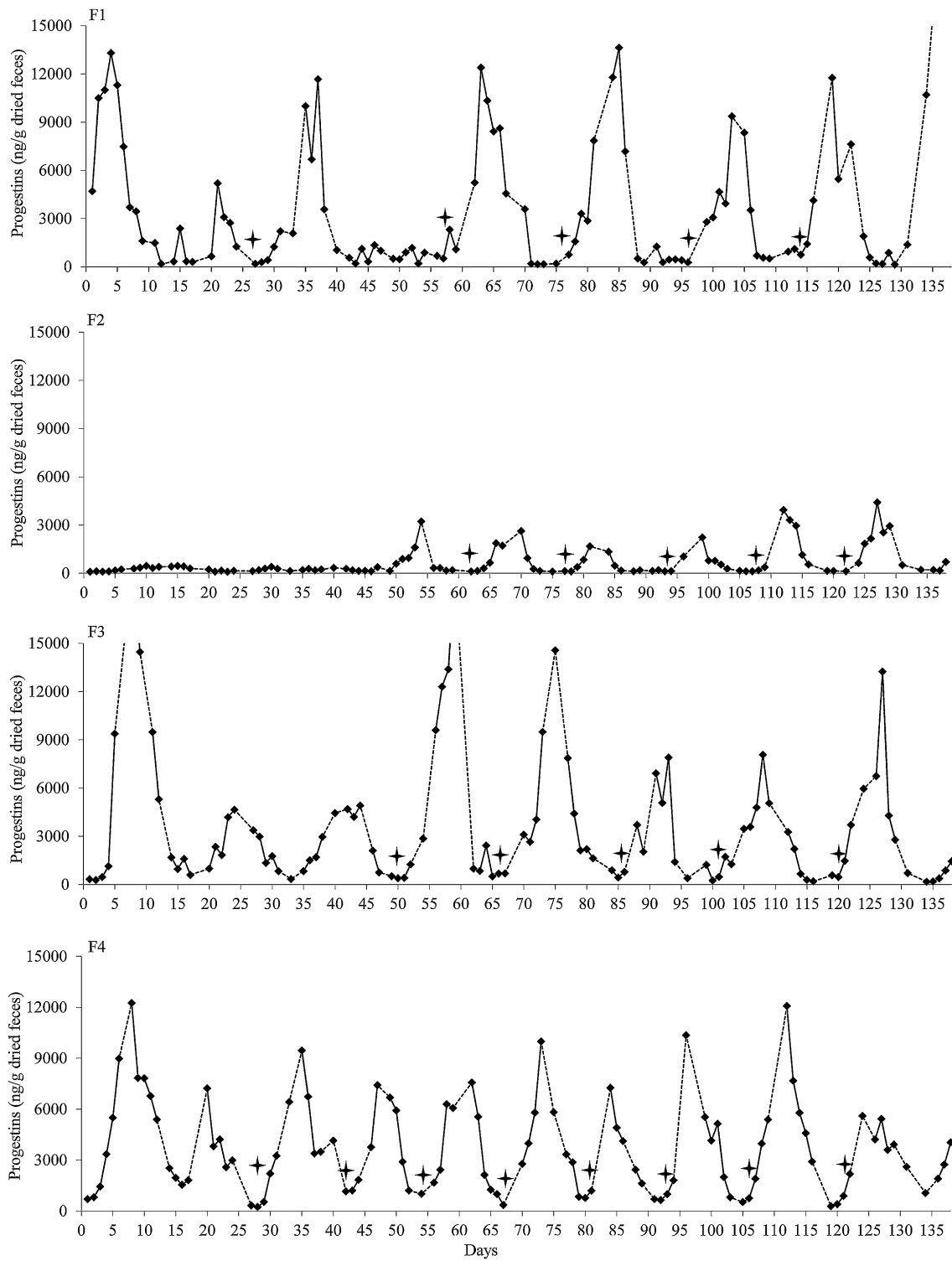
## Discussion

Measurement of fecal progesterins by enzyme immunoassay demonstrated ovarian cyclicity and proved to be a reliable method of achieving this. Fecal metabolites have been measured by this method in wild *Alouatta pigra* (Van Belle et al. 2009) and by radioimmunoassay in captive *A. caraya* (Kugelmeier et al. 2011). In both cases, despite the availability of estrogen profiles, progesterin profiles were considered more reliable for assessing ovarian cyclicity and the likely day of ovulation. These findings affirm that the ovarian cycle can be securely evaluated using only cyclic profiles of progesterone and its metabolites.

The mean length of the ovarian cycle founded in this research is the shortest described for this genus (16 ± 0.52 days), but it is similar to data available for

*Alouatta caraya* and *A. pigra* (Van Belle et al. 2009; Kugelmeier et al. 2011) using sex steroid fecal metabolites. These results also are in agreement with descriptions of ovarian cycles for the genus using other methodologies, as well as reviewed by Van Belle (2015): vaginal cytology in *A. caraya*, 19.7 ± 1 days (Colillas and Coppo 1978), 19.8 ± 0.9 days (Kugelmeier et al. 2011); observation of copulations in *A. seniculus*, 17 days (Crockett and Sekulic 1982); and sexual behavior, together with genital morphological changes in *A. palliata*, 16.3 ± 0.7 days (Glander 1980). The only real discrepancy in the set of data presented for the genus was described by Herrick et al. (2000), who reported a mean ovarian cycle of 29.5 ± 0.5 days for *A. seniculus* using urinary metabolites.

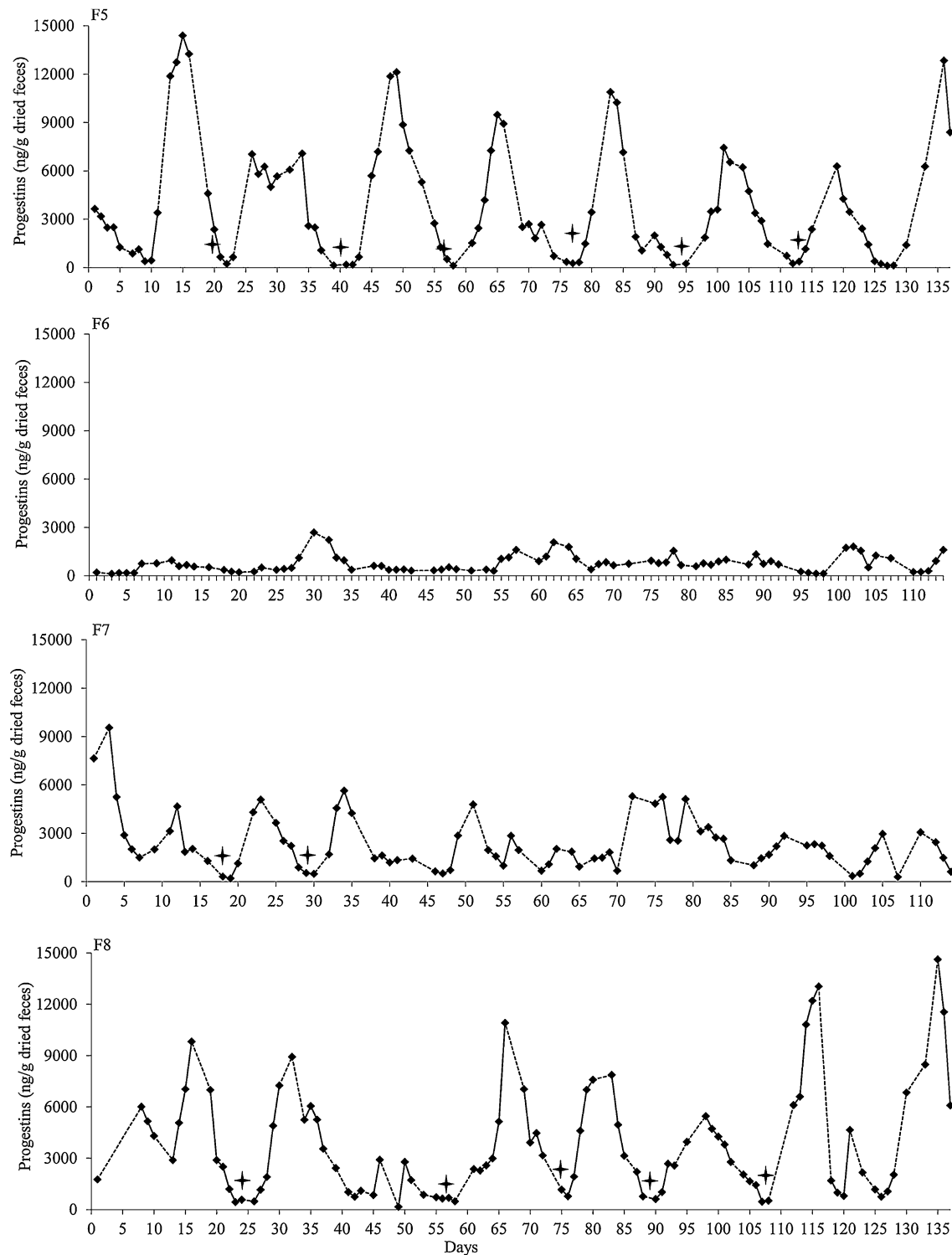
When we consider the phases of the cycle, however, differences emerge. In this study, the interluteal phase



**Fig. 2** Fecal progesterin profiles of four female *Alouatta guariba clamitans* (Females 1, 2, 3 and 4) throughout the study. progesterins samples; —◆ progesterins graphically estimated; + day 0 (probable ovulation)

( $4 \pm 0.37$  days) was much shorter than the luteal phase ( $11 \pm 0.50$  days). Kugelmeier et al. (2011) reported a nonluteal phase ( $9.83 \pm 2.21$  days) similar to the luteal phase ( $9.47 \pm 0.72$  days) for *Alouatta caraya*. It is worth

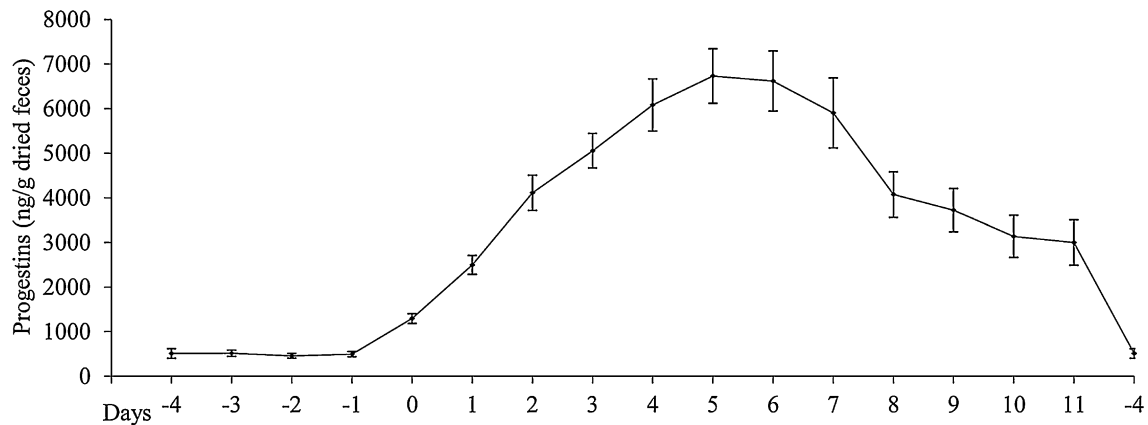
noting that it is precisely the nonluteal phase (corresponding to the interluteal phase in the methods used here) determined by the authors that showed the greatest variability on duration.



**Fig. 3** Fecal progesterin profiles of four female *Alouatta guariba clamitans* (Females 5, 6, 7 and 8) throughout the study. Progesterins samples; —◆ progesterins graphically estimated; + day 0 (probable ovulation)

Several studies show that the follicular phase, is shorter in Neotropical primates compared with Old World primates (Dixon 1983; Kendrick and Dixon 1983; Heistermann and Hodges 1995; Carosi et al. 1999; De

Vleeschouwer et al. 2000), and can last as little as 2 days in captive female *Ateles geoffroyi* (Campbell et al. 2001). This short length could signify that follicle maturation (ranging from 10 to 17 days in many mammals, including



**Fig. 4** Representation of the mean of the ovarian cycle of *Alouatta guariba clamitans* (mean  $\pm$  standard error). Normalized according Day 0, which is the onset of the luteal phase

Old World primates) occurs throughout the luteal phase. Thus the interval between the end of one luteal phase to the onset of the next could merely represent the final steps of dominant follicle maturation (reviewed in Baird et al. 1975).

Females 2, 6 and 7 showed low concentrations of progesterins compared with the other females studied. This finding cannot be explained of diurnal variability in the excretion of fecal metabolites (Sousa and Ziegler 1998) or as results of a fiber-rich diet (Wasser et al. 1993), due to methodology of sample collection and standardized diet of howlers. Old age can alter ovarian activity and consequently hormone levels (Helvacioğlu et al. 1994; Heistermann et al. 2001; Hernández-López et al. 2010). However this is an improbable explanation for this study, because F5 is the female who has the oldest estimated age (over 13 years-old) and was the only one to present signs of old age, such as cataracts and teeth loss. Despite this, her hormone levels and the regularity of her ovarian activity are similar to that of other females.

The regime of captivity, which involves numerous facets, both biotic and abiotic, can threaten the welfare of animals and cause stress, indicated by an increase in glucocorticoids (Morgan and Tromborg 2007; Rangel-Negrín et al. 2009; Mason 2010). The ill effects of chronic stress include its harmful effects on reproductive function, which can cause a reduction in progesterone levels (Sapolsky et al. 2000; Wingfield and Sapolsky 2003). The F6–F7 dyad were moved to the CEPESBI at the beginning of the study, which may have triggered stress due to physical containment, transportation and readjustment to the new environment. In particular, females of *Alouatta* are highly sensitive to process of handling and translocation (Aguilar-Cucurachi et al. 2010).

Although F6 and F7 were only moved from one enclosure to another, this procedure may nonetheless have

generated chronic stress for most of the study period. Female 7 had two ovarian cycles and few stereotyped behaviors (Dada et al. 2011) while F6 did not present ovarian cyclicity and presented sexual activity only 100 days after they were moved. Another piece of evidence is that she showed severe stress indicative stereotypies, including self-mutilation. In contrast, F2 did not experience ovarian cycles throughout the study period, showed no evidence of severe behavioral stress, nor did she suffer a change in enclosure prior to the study, suggesting that stress was not the main factor of her acyclicity.

Environmental factors between the postnatal development phase up to sexual maturity can be determinants for progesterin levels during adulthood. Women without access to good sanitation, healthcare, stable food resources and who experience great physical stress between birth and menarche possess lower levels of salivary progesterins than women who do not experience such conditions (Ellison 1996; Nuñez-de La Mora et al. 2007). Although the hypothesis of individual variation cannot be discarded, all females at CEPESBI passed through some traumatic event and analysis of their effects are impossible. The measurement of fecal glucocorticoids and longitudinal studies monitoring the behavioral and hormonal ontogeny of the howler monkeys received by the CEPESBI may clarify these unresolved issues.

In conclusion, the novel data for the species in question presented robustness that provides information concerning the reproductive physiology of Neotropical primates, while corroborating the potential of determining the concentrations of fecal progesterins, a noninvasive method that assessed ovarian activity in the monkeys in this group. Moreover, it should offer insights for improving the ex situ management of the genus *Alouatta*, as well as providing support research on wild animals.

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