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Monitoring sexual steroids and cortisol at different stages of the ovarian cycle from two capuchin monkey species: use of non- or less invasive methods than blood sampling

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ABSTRACT

Endocrine monitoring of non-human primates (NHP) via faecal metabolites of steroid hormones appears as a useful non-invasive alternative to evaluate the reproductive status of free living NHP, as well as of those kept in captivity but of difficult handling. However, validation is needed with plasma values before its application in the field. The aim of the present study was to monitor the different phases of the menstrual cycle from the new world NHP *Sapajus apella* and *S. libidinosus*. For this, hormonal and faecal plasma levels of E_2, P_4 and cortisol were assessed during different days of the menstrual cycle, together with colpocitology. The mean duration of the menstrual cycle according colpocitology was of 21.7 and 21.0 days for *S. apella* and *S. libidinosus,* respectively. These values were similar to those observed via plasma analysis, i.e. 22.7 and 20.3 days for *S. apella* and *S. libidinosus,* respectively. The day of plasmatic E_2 peak was set as Day -1 and the estimated day of ovulation was set as Day 0 and occurred two days earlier in *S. libidinosus* than in *S. apella* females. In both species, it was observed a delay in faecal E_2 peak of six days for *S. apella* and of 11 days for *S. libidinosus* when compared with the plasma peak. A maximum P_4 plasma concentration was observed in the middle of luteal phase in *S. apella* and in *S. libidinosus,* both at around day 5. However, faecal P_4 peaks were detected at days 9 and 8 in *S. apella* and *S. libidinosus*, respectively. Mean plasma and faecal cortisol levels were variable during all ovulatory cycle of *S. apella* and *S. libidinosus* females. Although no exact correlation was observed between plasmatic and faecal profile of steroid hormone, faecal samples were able to indicate ovarian cycle phase, being important to assess the reproductive status of the females applying a non-invasive method.

1. Introduction

Hormonal monitoring via faecal samples is a non-invasive method that avoids multiple catching of animals, does not need animal sedation, and circumvents possible incidents during blood sampling. This technique is applied especially in pregnant females or new-born animals [1, 2], wild animals, or those of small size [3]. However, data on sexual steroids in faecal samples must be validated before application, since metabolism and excretion of these hormones are species-specific [1, 2]. The menstrual phase in Neotropical primates is not macroscopically visible as in old world primates [4]. Furthermore, there is no specific period of male acceptance and mating defining a peri-ovulatory phase. Hence, a proper evaluation and validation of reproductive cycle monitoring by endocrine dosage techniques is essential. This can be done concomitantly with the use of other parameters such as the colpocitology, which is a less invasive method of easy execution, low cost, and defines the menstrual phase as landmark.

The endocrine monitoring of the reproductive activity can contribute with the application of assisted reproduction techniques (ARTs) for Neotropical primates, especially when determination of the day of ovulation, as well as the phase of the cycle are limiting factors [4]. Regarding *Sapajus apella* (*Cebus apella*), the length of the reproductive cycle, cyclicity of the vaginal epithelium during menstrual cycle [5], plasma levels of ovarian steroid hormones [6, 7], and the expected day

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of ovulation based on the E_2 peak [7] were previously described. However, when such studies were conducted, both *S. apella* and *S. libidinosus* (*C. apella libidinosus*) were considered to be part of the same taxon [4]. Therefore, it is unknown if there are differences in the reproductive cycle between these two species. Based on this, we aimed i) to determine the profiles of 17β-oestradiol, progesterone and cortisol in the plasma, as well as their metabolites in faecal samples from *S. apella* and *S. libidinosus* females, ii) to compare the obtained data with vaginal colpocitology, and iii) to assess possible differences between these two species.

2. Materials and methods

2.1. Study site

We conducted our study at National Primate Center (CENP), Ananindeua, Brazil (1°22′57″S and 48°22′52″W). The climate is humid tropical, with an average annual temperature of 28 °C.

2.2. Study animals

All experimental protocols applied in the present study were approved by the Ethical Committee in Animal Research from the Evandro Chagas Institute (IEC/CEAU no. 004/2016) and followed the guidelines of the National Council of Animal Experimentation Control of Brazil. Seven healthy and sexually mature *Sapajus* females (four *S. apella* and three *S. libidinosus*) born in captivity, were selected for our study. The females were individually housed (cages of 80 × 90 × 80 cm) under a natural photoperiod. Their daily diet consisted of fresh fruits and commercial pellet chow (FOXY Junior Supreme, 28% crude protein; São José dos Pinhais, PR, Brazil). Milk, vitamins, minerals, and eggs were supplied once a week. Tap water was available *ad libitum*. Conditioning was performed by cooperation [8], in which the allowance to receive an injection of anaesthetic was rewarded with grape fruits. Each animal was anaesthetized with ketamine hydrochloride (12 mg/kg; im; Vetanarcol®, König S.A., Avellaneda, Argentina) and xylazine hydrochloride (1 mg/kg; im; Sedomin®, König S.A., Avellaneda, Argentina) before vaginal cytology and blood collection.

2.3. Sampling

Colpocitology was daily to monitoring the different stages of the menstrual cycle, which were defined as follicular, luteal or menstrual based on the epithelial cells characterization in vaginal smears collected in each phase, the presence of erythrocytes, leukocytes and the cervical mucus.

For blood collection, each female was sampled during three cycles, resulting in 60–65 samples per *S. libidinosus* female and 70 samples per *S. apella* female. When menstruation took place, blood samples (1 mL per female) were collected every morning under sedation. The femoral vein was punctured with hypodermic needles (20 mm × 0.55 mm; 24 G × ¾") coupled to a 3 mL syringe, and blood was transferred to tubes containing the anticoagulating EDTA. Samples were centrifuged (3000 rpm for 5 minutes) (Modelo Combate, Celm, Brazil) and the obtained plasma was frozen (-70 °C) for hormonal dosage. The day of plasmatic E_2 peak was set as Day -1 and the estimated day of ovulation was set as Day 0 [7].

Faecal samples were collected for metabolites extraction, as previously described [9], and kept frozen (-70 °C) for further analysis. For this, samples were thawed after 20 minutes exposure at room temperature (21 °C), weighed, and aliquots of 500 mg of faeces were separately transferred into glass tubes. Samples were mixed with distilled water (1 mL) and methanol (4 mL) overnight. Subsequently, they were centrifuged (3000 rpm) for 15 minutes. Supernatant was stored at -70 °C for hormonal dosage.

2.4. Hormonal assays

Plasmatic and faecal concentrations of 17β-estradiol (pg/mL), progesterone (ng/mL) and cortisol (nmol/L) were measured by electrochemiluminescence assay using an immunoassay system (Elecsys®2010, Roche Diagnostics, Indianapolis, United States of America). All the protocols were applied according to the manufacturer.

2.5. Statistical analysis

All data were analysed for normal distribution and homogeneity with the tests Kolmogorov-Smirnov (test KS) and Bartlett. Therefore, mean (±SEM) colpocytology data were compared with the non-parametric Kruskal-Wallis followed by unpaired t test, while mean (±SEM) plasmatic and faecal concentrations of 17β-estradiol, progesterone and cortisol were compared with repeated measure ANOVA and tukey as post-hoc test. Furthermore, a correlation analysis between plasmatic and faecal hormonal levels was performed with Spearman test. Statistical analysis was performed the StatView software (SAS Institute Inc., Cary, NC, USA). Differences were considered significant when $P < 0.05$.

3. Results

It was possible to detect the onset of the menstrual phase, in both species, by colpocytology. Table 1 depicts the percentages of each evaluated cell types in both *S. apella* and *S. libidinosus* at different phases of their cycle, i.e. follicular, luteal and menstrual. In both species the small and large intermediate cells had the largest ($P < 0.05$) frequency, regardless the evaluated phase, except for the menstrual phase of *S. libidinosus*, which presented similar distribution of small and large intermediate cells and parabasal ones.

When comparing cycle phases within each species, it was observed that in *S. apella* that the highest ($P < 0.05$) percentage of small intermediate cells were observed during luteal and menstrual phase, while the follicular phase presented the highest ($P < 0.05$) percentage of large intermediate cells. Differently, *S. libidinosus* presented the highest ($P < 0.05$) percentage of small intermediate cells were observed only during the luteal phase, while the follicular phase presented the highest ($P < 0.05$) percentage of large intermediate cells (Table 1).

Based on vaginal cytology, mean duration of the ovarian cycle in *S. apella* and *S. libidinosus* females were 21.7 and 21.0 days, respectively, and ovulatory cycles ranged of 19–26 days for *S. apella* and of 11–23 days for *S. libidinosus*. In both species, the luteal phase was the longest when compared to follicular and menstrual phases (Table 2).

According to plasma hormonal dosage, the ovarian cycle of *S. apella* and *S. libidinosus* had duration of 22.7 and 20.3 days, respectively, which was similar to the vaginal cytology findings. Also, as observed by vaginal cytology, luteal phase was the longest phase in the ovarian cycle (Table 2). Vaginal cytology and plasmatic hormonal data were similar for *S. apella*. However, for *S. libidinosus* this similarity was observed only during the menstrual phase, while vaginal cytology indicated significant longer follicular and shorter luteal periods in comparison with hormonal data.

Faecal sampling gave a completely different picture when compared with vaginal cytology and hormonal analysis in the plasma, where for both studied species faecal E_2 levels indicated a longer follicular period in comparison with luteal and menstrual phases.

The mean peaks of plasmatic E_2 in *S. libidinosus* females occurred two days earlier than in *S. apella*. Thus, the beginning of the menstrual phase was considered at different days for *S. apella* (-9 to -7) and for *S. libidinosus* (-7 to -5 days). The individual plasmatic concentrations of E_2 during these described periods ranged of 0.8–4.6 ng mL in *S. apella* and of 0.9–3.1 ng mL in *S. libidinosus*. The maximum plasmatic concentrations of E_2 in *S. apella* and *S. libidinosus* females occurred at day -1, thus so-called pre-ovulatory day. Levels of E_2 ranged of 1.8–15.8 ng mL for *S. apella* and of 1.1–35.4 ng mL for *S. libidinosus*. After the peaks, a gradual decrease in

Table 1
Vaginal epithelial cells distribution during different menstrual cycle phases obtained from *Sapajus apella* and *Sapajus libidinosus*.

Epithelial cell types	Cycle Phases					
	Follicular		Luteal		Menstrual	
	S. apella	*S. libidinosus*	*S. apella*	*S. libidinosus*	*S. apella*	*S. libidinosus*
Basal	1 ± 1^{A}	0^{A}	3 ± 1^{A}	0^{A}	3 ± 1^{A}	1 ± 1^{A}
Parabasal	2 ± 1^{A}	0^{aA}	5 ± 2^{A}	1 ± 1^{aA}	3 ± 1^{A}	33 ± 2^{bB}
Small Intermediate	63 ± 4^{aD}	44 ± 7^{aC}	76 ± 3^{bC}	71 ± 2^{bD}	83 ± 2^{bC}	37 ± 6^{aB}
Large Intermediate	27 ± 4^{aC}	40 ± 5^{aC}	12 ± 2^{bB}	22 ± 2^{bC}	9 ± 2^{bB}	25 ± 7^{bB}
Superficial	5 ± 1^{aB}	13 ± 2^{aB}	2 ± 1^{bA}	4 ± 1^{bB}	2 ± 1^{bA}	3 ± 1^{bA}
Squamous	2 ± 1^{A}	2 ± 1^{A}	3 ± 1^{A}	1 ± 1^{A}	1 ± 1^{A}	0^{A}

[a-c]Different lower-case letters indicate significant differences among cycle phases within each cell type and animal species ($P < 0.05$).
[A–D]Different upper-case letters indicate significant differences among cell types within each cycle phase and animal species ($P < 0.05$).

Table 2
Mean (±SEM) time interval (days) of each phase (follicular, luteal and menstrual) in *Sapajus apella* (n = 6) and *Sapajus libidinosus* (n = 6) during ovarian cycle. Data were collected based on vaginal cytology and hormonal dosage. Mean (±SEM) plasma concentrations of oestradiol are also presented.

Species	Cycle phases		
	Follicular (days)	Luteal (days)	Menstrual (days)
S. apella			
Vaginal cytology	7.5 ± 0.5^{a}	10.7 ± 0.7^{b}	3.5 ± 0.7^{c}
Hormonal dosage	7.2 ± 0.4^{a}	11.7 ± 0.6^{b}	3.2 ± 0.7^{c}
E_2 (ng/mL)	4.1 ± 1.8	1.1 ± 0.2	1.7 ± 0.5
S. libidinosus			
Vaginal cytology	8.0 ± 0.4^{aA}	10.0 ± 0.7^{bA}	3.0 ± 0.4^{cA}
Hormonal dosage	4.3 ± 0.6^{aB}	13.0 ± 0.6^{bB}	3.0 ± 0.4^{aA}
E_2 (ng/mL)	4.7 ± 2.9	1.3 ± 0.3	2.1 ± 0.5

[a-c]Different lower-case letters indicate significant differences among cycle phases within each analysis method and animal species ($P < 0.05$).
[A–B]Different upper-case letters indicate significant differences between vaginal cytology and hormonal dosage within each cycle phase and animal species ($P < 0.05$).

plasma E_2 was observed and luteal phase started, remaining for a period of 10–14 days in *S. apella* and of 10–15 days in *S. libidinosus*.

Fig. 1 depicts the mean plasmatic and faecal levels of 17β-estradiol (ng mL), progesterone (ng mL) and cortisol (ng/mL) during the ovarian cycle from *S. apella* and *S. libidinosus*.

At the estimated ovulation day (day 0 of cycle), the mean plasmatic concentration of E_2 was 2.2 ± 3.1 ng mL and 2.5 ± 3.3 ng mL for *S. apella* and *S. libidinosus,* respectively. The highest levels of E_2 were observed at follicular phase, where mean values were 4.1 ± 4.4 and 4.4 ± 6.9 for *S. apella* and *S. libidinosus*, respectively.

Plasma concentration of P_4 was relatively low during menstrual and follicular phases in both species, with levels ranging of 8.9–59 ng mL for *S. apella* and of 3.5–37.2 ng mL for *S. libidinosus*. From the day of E_2 peak (day -1) a gradual increase in plasma P_4 was observed in both species. At day of ovulation (day 0 of cycle), the mean plasma P_4 levels in *S. apella* and *S. libidinosus* were 120.6 ± 76.5 ng mL and 132.8 ± 61.6 ng/mL, respectively. From day 6 of the ovulatory cycle (luteal phase), in both species, P_4 levels starts to decline from 300.7 ± 60.1 to 64.8 ± 16.3 ng mL in *S. apella* and from 376.8 ± 62.8 to 21.7 ± 4.3 ng mL in *S. libidinosus.* A maximum P_4 plasma concentration was observed in the middle of luteal phase in *S. apella* (day 5; 631.6 ng mL - range of 83.8 a 631.6 ng mL) as well as in *S. libidinosus* (day 5; 641.4 ng mL - range of 176.2–641.4 ng mL).

Progesterone plasma levels started to increase at moment of 17β-estradiol peak, with mean values of 68.8 ± 17.2 ng mL in *S. apella*. Differently, in *S. libidinosus*, increase in progesterone plasma levels was observed two days after E_2 peak, at day 1 of the cycle, with mean values of 55.8 ± 18.6 (Fig. 1). The highest E_2 peak obtained from faecal extract from *S. apella* (21.5 ± 5.4 ng/mL) occurred at day 5 from the cycle, with ranging levels of 6.2–46.2 ng mL, while for and *S. libidinosus* this E_2 peak (45.8 ± 15.3 ng/mL) was observed at day 10 from the cycle with ranging levels of 28.6–56.1 ng mL.

In both species, it was observed a delay in faecal E_2 peak of six days for *S. apella* and of 11 days for *S. libidinosus* when compared with the plasma peak. While plasma peak occurred during follicular phase, faecal extract presented a E_2 peak at the end of the ovulatory phase (Fig. 1). Importantly, faecal samples from one of *S. apella* female presented more than one E_2 peak within the same cycle.

The most evident P_4 peaks in faecal extracts occurred at days 9 and 8 in *S. apella* and *S. libidinosus*, respectively. These results differ from plasma P_4 peaks, which occurred at days 4–6 and 5–7 in *S. apella* and *S. libidinosus*, respectively. P_4 plasma levels during luteal phase were 71.2–434.4 ng mL in *S. apella* and of 153.8–385.0 ng mL in *S. libidinosus*. Fig. 1 shows that P_4 was more irregular in *S. apella*, while this hormone presented a clear peak between days 7 and 9 in the ovarian cycle of *S. libidinosus*.

Mean plasma cortisol levels were variable during all ovulatory cycle without a clear peak in *S. apella*, except for the highest mean level observed at day 13 (4080.5 ± 510.1 ng/mL). Mean faecal cortisol levels for this species also did not present a clear peak, sometimes reaching the maximum levels 582.2 and 586.1 ng/mL at days 5 and 7, respectively. On the other hand, for *S. libidinosus* females, plasma cortisol peaks were observed at day -2 with a mean level of 1476.6 ± 520.4 ng mL (ranging of 859–2319 ng mL). As for *S. apella*, no mean faecal cortisol peaks were observed in *S. libidinosus,* with maximum levels observed at day 5 (483.2 ng/mL) (Fig. 1).

4. Discussion

The use of non or less invasive methods to screen the reproductive status of free-living animals remains a challenge. In the present study we evaluated the possibility to replace plasma analysis by colpocitology or faecal sampling from two different NHP species. Colpocitology presented only some slight differences from plasma profiling. For instance, colpocitology indicated that both species presented an ovulatory cycle of 21 days. Plasma profile of *S. apella* indicated a cycle of 22 days, and of 20 days for *S. libidinosus*, with an interval similar to that previously described for *S. apella* (2.1 ± 1.1 days) [6, 7, 10]. Vaginal cytology allowed the assessment of the menstrual phase length, which ranged of 1–5 days in *S. apella.* Similar data was previously reported as ranging of 1–5 days in *S. apella* [6]. However, menstrual phase from *S. libidinosus* was shorter, ranging of 2–4 days. Such differences are explained by species-specific differences. Females from other Neotropical NHP species present differences in their ovulatory cycle. For instance, *Saimiri sciureus* females present an oestrus cycle of 8–9 days, where an increase in plasma E_2 takes 8–10 days between peaks [11]. The *Callithrix jacchus* female does not present cyclic bleeding, hence no definition of oestrus or menstrual cycle is linked to this species. According to Hearn and Lunn [12], they present an ovarian cycle of circa 16 days. However, a cycle of 30 days was also described [13]. *Trachpithecus phayrei crepusculus* females, an old-world primate specie, can present up to 3 cycles until conception.

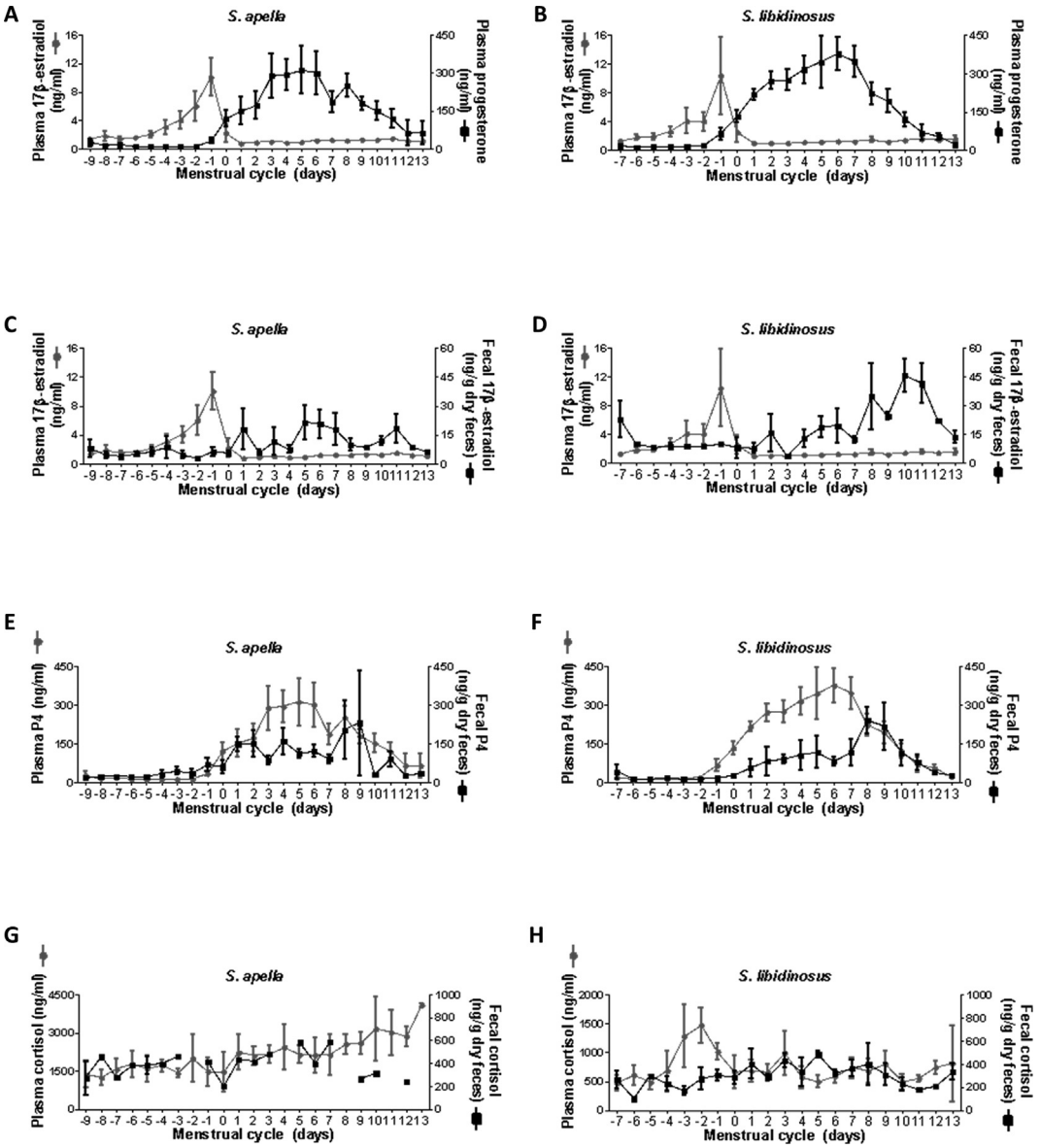

Fig. 1. Plasmatic levels of 17b-oestradiol (ng/mL) and progesterone (ng/mL) during the ovarian cycle from *Sapajus apella* (A) and *Sapajus libidinosus* (B). Comparison of the mean plasma and faecal 17b-oestradiol (ng/mL) levels during the ovarian cycle from *S. apella* (C) and *S. libidinosus* (D). Comparison of the mean plasma and faecal progesterone (ng/mL) levels during the ovarian cycle from *S. apella* (E) and *S. libidinosus* (F). Comparison of the mean plasma and faecal cortisol (ng/mL) levels during the ovarian cycle from *S. apella* (G) and *S. libidinosus* (H).

This species is characterized by a cycle of 28 days [14].

Plasma levels of ovarian steroids are linked to follicular development and ovulation. Herewith, we observed peaks of E_2 in *S. apella* and in *S. libidinosus*, followed by an increase in plasma P_4. In ovarian folliculogenesis during the menstrual cycle, the oestrogen peak marks the end of the follicular phase, followed by an increase in P_4 synthesis, which plays an important role in ovulation [15]. This event characterizes the beginning of the luteal phase. The mean peaks of plasmatic E_2 in *S. libidinosus* females occurred two days earlier than *S. apella*, due to the difference in cycle length between these two species.

Plasma and faecal levels of E_2, P_4 and cortisol were correlated in the present study. Faecal samples from *S. apella* females indicated an irregular cycle, sometimes with more than one E_2 peak within the same cycle, which was not observed in the plasmatic profile. On the other hand, faecal E_2 peak in *S. libidinosus* occurred 11 days later than that observed with the plasma samples. For *Trachpithecus phayrei crepusculus,* both faecal E_2 e P_4 show clear patterns indicating ovulation and conception, where faecal E_2 levels correlate with menstrual cycle, although P_4 faecal levels were insufficient to determine luteal phase [14]. Muren et al. [16] also demonstrated that faecal E_2 levels can be used to determine the different ovarian cycle periods in captive Sichuan golden monkeys (*Rhinopithecus roxellana*), but without a correlation with the plasmatic profile. Differently, in *S. apella* and *S. libidinosus* females, faecal P_4 profile was sufficient to determine luteal phase.

In *S. apella* and *S. libidinosus* no faecal cortisol peaks were observed. Adults *S. apella* females showed an increase in the plasma cortisol levels with the ovulatory cycle. Differently, in *S. libidinosus,* plasmatic cortisol levels presented a remarkable increase close to the day of ovulation. This might be explained as species-specific differences and, unfortunately, we could not find any other reason for such difference.

The follicular phase of the *S. libidinosus* is shorter than that of *S. apella*, based on the mean peaks of plasmatic E_2 occurring two days earlier in *S. libidinosus* females. In the past, studies were performed without distinguish these two species [4]. Furthermore, based on progesterone plasma levels, it was observed that in *S. apella* the luteinisation of granulosa cells occurs before ovulation, while in *S. libidinosus* such event will

take place after ovulation. It was previously described that in *S. apella*, the luteinized ovarian tissue from previous cycles might be active in subsequent cycles [6, 7], which does not appear to occur with *S. libidinosus*. All these differences are also of primordial importance when developing protocols for oocyte *in vitro* maturation.

The absence of an exact correlation between plasmatic and faecal profile of steroid hormones is not an issue, since such endocrinological monitoring aims to determine long-term patterns such as chronic stress, seasonal patterns, and reproductive status [17]. Furthermore, faecal analysis permits the analysis of the endocrine status of the animals without the stress caused by handling [18]. With the present results, it will be possible to monitor the reproductive cycle of free-living *S. apella* and *S. libidinosus* females. Besides this, it will be possible to combine this information with behaviour of captive females to plan proper conservation actions. However, based on this study, blood sampling remains necessary to determine the ovulation day in these species. Since this was a first study in this species, the routine blood and faecal collection will help to obtain more robust data in the near future.

Declarations

Author contribution statement

M.C.M. Lima: Performed the experiments; Analyzed and interpreted the data.

S.R.R.A. Scalercio: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

C.T.A. Lopes, N.D. Martins: Performed the experiments.

K.G. Oliveira: Analyzed and interpreted the data.

M.C. Caldas-Bussiere: Conceived and designed the experiments.

R.R. Santos, S.F. S. Domingues: Conceived and designed the experiments; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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