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Menstrual cycle in four New World primates: Poeppig's woolly monkey (*Lagothrix poeppigii*), red uakari (*Cacajao calvus*), large-headed capuchin (*Sapajus macrocephalus*) and nocturnal monkey (*Aotus nancymaae*)

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ABSTRACT

Genital organs from 33 nocturnal monkeys *Aotus namcymaae*, 29 Poeppig's woolly monkeys (*Lagothrix poeppigii*), 21 red uakaris (*Cacajao calvus*) and 11 large-headed capuchins (*Sapajus macrocephalus*) were histologically analyzed in order to describe the endometrial changes related to the ovarian cycle. *A. nancymaae* and *S. macrocephalus* showed histological evidence of menstrual cycle with the detachment of the most superficial endometrium and the subepithelial reabsorption of the endometrial functional layer, explaining the extensive presence of both hemosiderin and fibrin clusters in the early follicular stages. In L. *poeppigii,* despite the presence of fibrin clusters promoting the remodeling of the endometrium, we did not observe the detachment of the functional layer of the endometrium, suggesting that this species presents a non-menstruating cycle. Finally, *C. calvus* showed no histological sign of menstrual phase. This reproductive information is useful to improve assisted reproductive techniques in non-human primates, and give us opportunity for comparative studies on the evolution of animal reproductive biology, including humans.

1. Introduction

During the sexual cycle of mammalian females, the endometrium undergoes cyclic and periodic changes, known as estrous cycle, to prepare the uterus to host an embryo and carry out a pregnancy [1,2]. The estrous cycle includes menstruation when, in the absence of fertilization, the detachment of the functional layer of the endometrium occurs along with blood discharge [3] due to the fall of progesterone concentrations [4–7]. Menstruation has only been observed in great primates (monkeys, apes and humans), bats (*Molossus ater, Glossophaga soricina, Carollia perspicillata, Desmondus rotundus*) and macroscelids (Macroscelidea). Considering its convergent evolution, menstruation is suggested to present certain, but still unknown, adaptive advantage [7].

Among Neotropical primates, the large-headed capuchin (*Sapajus macrocephalus*) is the only species known to present menstrual cycles, a feature that makes this species an ideal study model for non-human primates (NHP) [8]. Nevertheless, there is still a scarce information about the menstrual cycle in other NHPs. These conceptual gaps on the endometrial changes throughout the sexual cycle [9] results in a low success of assisted reproduction techniques for NHPs, probably due to different biological and

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physiological characteristics among species that are yet to be uncovered [9,10].

This study aims to describe the endometrial changes in four New World primate species (*Lagothrix poeppigii*, *Cacajao calvus*, *Sapajus macrocephalus* and *Aotus nancymaae*). This reproductive information provides important physiologic information for the development of management strategies and conservation programs for endangered species, and the improvement of reproductive biotechnologies and assisted reproductive techniques in non-human primates.

2. Materials and methods

2.1. Study areas

This study was conducted in two areas of the Peruvian Amazon rainforest. The first one was the Yavarí-Mirín River (YMR, S 04°19.53; W 71°57.33), which spans 107,000 ha of continuous forest, predominantly non-flooding *terra firme* forest. The second area was the Primate Center of the Instituto Veterinario de Investigaciones Tropicales y de Altura (IVITA, Iquitos, Peru). The climate in the region is typically equatorial with an annual temperature of 22–36 °C, a relative humidity ranging from 80% to 100%, and an annual rainfall ranging from 1500 to 3000 mm. Seasons are defined as dry (January–February and July–September) and wet (March–June and October–December).

2.2. Animal collection and histological methods

Between 2004 and 2010, hunters living in the YMR collected all abdominal organs from all hunted preys as part of an ongoing participatory conservation program that involves local hunters in implementing community-based wildlife management [11]. From the collected biological samples, we selected genital organs from 61 non-pregnant adult female primates: 29 Poeppig's woolly monkeys (*L. poeppigii*), 21 red uakaris (*C. calvus*) and 11 large-headed capuchins (*S. macrocephalus*). Hunters were not payed and donated voluntarily the biological samples. This methodology assured that no animal was killed other than those harvested as part of local hunter's normal activities. The research protocol was approved by the Research Ethics Committee for Experimentation in Wildlife at the Dirección General de Flora y Fauna Silvestre from Peru (License 350-2012-AG-DGFFS-DGEFFS). Samples were sent to UAB, Barcelona, Spain, through the export license CITES (No 001954 and ES-BB-00011/12I).

In parallel, we collected the ovaries and uterus from 33 deceased non-pregnant adult female owl monkeys (*A. nancymaae*), that had been kept in captivity at the IVITA. Subjects were maintained under natural light and temperature conditions. The females were fed commercial food with a caloric value of 411.6 kcal and a protein level of 23%. Water was available on an *ad libitum* basis. A post-mortem examination was conducted to verify that none of the individuals died due to any reproductive disorder. All research was conducted in compliance with the American Society of Primatologists' guidelines for the ethical treatment of NHP and adhered to the legal requirements of the IVITA (Protocol No 006/2014). No animal was killed specifically for this research.

Genital organs were maintained in buffered 4% formaldehyde solution (v/v). Samples of the ovaries and uterus were dehydrated and embedded in paraffin wax. The ovaries were serially sectioned at 3-μm sections along the longitudinal axis, and three sections from the uterus of each female were made. Sections were mounted onto a glass microscope slide, and routinely stained with haematoxylin and eosin. Periodic Acid-Schiff (PAS) stain was used to stain connective tissues, mucus and basal laminae, and Masson's trichrome and phosphotungstic acid haematoxylin stains were used to distinguish collagen, keratine and muscle fibres. Sections were examined under a light microscope at 100 ×, 400 × and 1000 ×.

Non-pregnant adult animals with ovaries containing active corpora lutea (CL) were described as being in the luteal phase of the estrous cycle, while females with ovaries bearing large antral follicles and lacking CL were considered to be in the follicular phase of the estrous cycle. In the absence of either large antral follicles or CL, the ovaries were considered inactive. The diameter of the largest antral follicle and CL were recorded. Corpora lutea were considered active after a pregnancy was established or as indicated by luteal cell morphology. Ovarian follicles and luteal tissue were classified according to Mayor et al. [12].

Microscopic features and measurements of the tunica mucosa (endometrium) and tunica muscularis (myometrium) were recorded from the uterine samples. The density of endometrial glands, hemosiderin and fibrin was measured and ranked from 0 to 5, according to the average number of target structures counted in random 1-mm^2 fields at x100 magnification. Significance scoring of density of endometrial glands was 0 (0–2 glands/mm^2), 1 (2–5 glands/mm^2), 2 (5–10 glands/mm^2), 3 (10–15 glands/mm^2), 4 (15–20 glands/mm^2), and 5 (20–25 glands/mm^2). Scoring of density of hemosiderin in the endometrium was 0 (0% of the field with target tissue/mm^2), 1 (1–5%/mm^2), 2 (5–10%/mm^2), 3 (11–20%/mm^2), 4 (21–30%/mm^2), and 5 (>30%/mm^2). Scoring of density of fibrin in the uterine stroma was 0 (0% of the field with target tissue/mm^2), 2 (1–2%/mm^2), 2 (2–5%/mm^2), 3 (6–10%/mm^2), 4 (11–15%/mm^2), and 5 (>16%/mm^2). All variables were determined in five randomly selected fields.

2.3. Statistical analysis

All variables were tested for normal distribution using the Kolmogorov-Smirnov test and we performed logarithmic transformations to fit the assumptions of normality and homoscedasticity. Differences between means were tested using the Tukey-Kramer multiple comparisons test. Multiple linear and non-linear regression curves were tested to determine the best fit to the relationship between the endometrial thickness and density of endometrial glands, hemosiderin and fibrin. The maximum follicular diameter was also related to the endometrial thickness and the density of endometrial glands, hemosiderin and fibrin. For this latter analysis, we forced linear regressions to origin and only considered functions with starting point at zero.

Statistical analyses were performed using R-Studio version 0.98.1062 2009–2013 (RStudio, Inc. with lme4 Package and Deducer JRG version 1.7–9, 2003–2011 RoSuDa, Univ. Augsburg). Differences with a probability value of 0.05 or less were considered significant. For normally distributed data, we presented the mean ± standard deviation (SD), whilst for data not meeting this criterion we presented the median and the range.

3. Results

Table 1 shows the main ovarian structures and the studied endometrial features in the four Neotropical primates. While menstrual females were observed for *A. nancymaae* ($N = 4$, 12.5%) and *S. macrocephalus* ($N = 2$, 20%), no *L. poeppigii* and *C. calvus* female was found in the menstrual phase. Females in the luteal and the follicular phase were observed in the four primate species.

3.1. Ovarian structures

The ovaries of all studied females presented antral follicles. The diameter of the most developed antral follicle was observed in

Table 1
Different ovarian and uterine features observed in four New World primate species (*Aotus nancymaae* N = 32, *Lagothrix poeppigii* N = 29, *Cacajao calvus* N = 20, and *Sapajus macrocephalus* N = 10).

Species	Sexual phase	n	Nº follicles	Diameter maximum follicle (μm)	Nº active CL	Endometrial thickness (μm)	Density of endometrial glands (0–5)	Secretion of endometrial glands (0–5)	Density of hemosiderin (0–5)	Density of fibrin (0–5)
Aotus nancymaae	Luteal phase	14	5.0 (0.0–20.0)[a]	912 (0–1300)	2 (1–2)[a]	675 (500–1450)[a]	4 (1.5–5.0)[a]	1.25 (0.0–4.0)[a]	0.0 (0.0–1.5)[a]	0.0 (1.0)[a]
	Follicular phase	14	6.5 (1.0-15-0)[a]	812 (575–3450)	0 (0)[b]	282 (125–763)[b]	1.0 (0.0–3.5)[b]	0.0 (0.0–1.0)[b]	1.5 (0.0–5.0)[b]	3.0 (0.0–5.0)[b]
	Menstruation	4	0.5 (0.0–3.0)[b]	325 (0–763)	1.5 (1–2)[a]	187 (0–500)[c]	1.5 (0.0–5.0)[b]	2.0 (0.0–5.0)[a]	1.5 (0.0–2.0)[ab]	0.0 (0.0–0.5)[a]
			$F_{2,29} = 7.11$, P = 0.02857	$F_{2,29} = 5.01$, P = 0.0817	$F_{2,29} = 256.72$, P < 0.0001	$F_{2,29} = 26.32$, P < 0.0001	$F_{2,29} = 17.65$, P = 0.0001	$F_{2,29} = 27.94$, P < 0.0001	$F_{2,29} = 8.77$, P = 0.0125	$F_{2,29} = 40.72$, P < 0.0001
Lagothrix poeppiggi	Luteal phase	10	6.0 (0.0–15.0)[a]	1712 (0–2875)[a]	2 (2–4)[a]	2217 (1410–3650)[a]	4.75 (3.0–5.0)[a]	2.0 (1.0–3.5)[a]	0.0 (0.0)	0.0 (0.0)[a]
	Follicular phase	19	18.0 (7.0–47.0)[b]	2570 (1075–9580)[b]	0 (0)[b]	420 (120–1944)[b]	2.0 (0.5–5.0)[b]	0.0 (0.0)[b]	0.0 (0.0)	1.5 (0.0–5.0)[b]
	Menstruation	0								
			$t_{27} = 25.77$, P < 0.0001	$t_{27} = 4.84$, P = 0.028	$t_{27} = 647.81$, P < 0.0001	$t_{27} = 24.28$, P < 0.0001	$t_{27} = 13.64$, P < 0.0005	$t_{27} = 250.54$, P < 0.0001		$t_{27} = 11.03$, P < 0.0001
Cacajao calvus	Luteal phase	7	6 (1–14)	1190 (960–1920)	2 (1–2)[a]	2025 (1125–3250)[a]	4.5 (4.0–5.0)[a]	3.0 (2.0–4.0)[a]	0.0 (0.0)	0.0 (0.0)[a]
	Follicular phase	13	6 (2–35)	1220 (690–8060)	0 (0)[b]	342 (113–3350)[b]	1.25 0.0–4.5)[b]	0.0 (0.0)[b]	0.0 (0.0)	0.25 (0.0–5.0)[b]
	Menstruation	0								
			$t_{18} = 1.72$, P = 0.1894	$t_{18} = 0.006$, P = 0.9376	$t_{18} = 248.56$, P < 0.0001	$t_{18} = 11.40$, P < 0.0001	$t_{18} = 9.623$, P = 0.0019	$t_{18} = 767.59$, P < 0.0001		$t_{18} = 5.501$, P = 0.019
Sapajus macrocephalus	Luteal phase	6	4 (1–9)	1125 (1125–1238)	2 (1–2)[a]	2326 (1738–2850)[a]	4.0 (3.0–5.0)	4.0 (2.0–5.0)[a]	0.0 (0.0)	0.0 (0.0)[a]
	Follicular phase	2	3 (1–5)	1135 (250–1220)	0 (0)b	1309 (143–2475)[ab]	1.8 (0.5–3.0)	1.0 (0.0–2.0)[b]	1.5 (0.0–3.0)	1.0 (0.0–2.0)[b]
	Menstruation	2	12.5 (5–20)	1406 (1263–1550)	1 (0–2)[ab]	487 (0–975)[b]	2.0 (0.0–4.0)	0.0 (0.0)[b]	2.0 (0.0–4.0)	2.5 (2.0–3.0)[c]
			$F_{2,8} = 2.837$, P = 0.2421	$F_{2,8} = 1.59$, P = 0.4518	$F_{2,8} = 15.06$, P < 0.001	$F_{2,8} = 8.367$, P = 0.01525	$F_{2,8} = 5.208$, P = 0.0736	$F_{2,8} = 24.595$, P < 0.0001	$F_{2,8} = 7.11$, P = 0.0778	$F_{2,8} = 31.305$, P < 0.0001

a,b,c Values appearing in columns with different superscripts are significantly different (P < 0.05) within species. Differences in microscopic measurements of the uterine tube were tested using 1-way ANOVA and Tukey–Kramer multiple comparisons test.

decreasing order in *L. poeppigii* (9.60 mm), *C. calvus* (8.10 mm), *A. nancymaae* (3.45 mm) and *S. macrocephalus* (1.50 mm). Only in *L. poeppigii,* the most developed antral follicle was larger in the follicular phase compared to females in the luteal phase ($P = 0.028$).

3.2. Uterine features

In terms of the relationship between the endometrial thickness and the density and secretion of endometrial glands, the endometrium was more developed in females in the luteal phase compared to females in the follicular phase in *A. nancymaae* ($P < 0.0001$), *L. poeppigii* ($P < 0.0001$) and *C. calvus* ($P < 0.002$) (Table 1, Fig. 1). Females of *S. macrocephalus* in the luteal phase showed a similar density of endometrial glands respect to females in the follicular phase; nevertheless, endometrial thickness and secretion of endometrial glands was greater in females in the luteal phase ($P < 0.02$).

Table 2 shows the statistics and formulas for the associations between the studied uterine features and the maximum follicular diameter and endometrial thickness in all species. The endometrium thickness and the density of endometrial glands showed a progressive growth related to the diameter of the maximum antral follicle ($R^2 = 0.14$, $P < 0.01$ and $R^2 = 0.31$, $P < 0.01$, respectively; Fig. 2), presenting its maximum growth during the luteal phase ($P < 0.0001$). The endometrium thickness was associated to the density and secretion of endometrial glands ($R^2 = 0.44–0.81$, $P < 0.01$, Fig. 3, and $R^2 = 0.36–0.50$, $P < 0.01$, Fig. 4, respectively).

Most females in the follicular phase presented non-secreting endometrial glands (92.9% *A. nancymaae,* 100% *L. poeppigii,* 100% *C. calvus,* 50% *S. macrocephalus*). Contrary, most females in the luteal phase had highly secreting endometrial glands (85.7% *A. nancymaae,* 100% *L. poeppigii,* 100% *C. calvus,* 100% *S. macrocephalus*).

During the luteal phase, the endometrial thickness and density and the secretion of endometrial glands were less developed in *A. nancymaae* compared to the other primate species ($F_{3,34} = 8.678$, $P = 0.0339$ and $F_{3,34} = 105.05$, $P > 0.0001$, $F_{3,34} = 15.057$, $P = 0.00177$, respectively). No uterine difference was found among *L. poeppigii, C. calvus* and *S. macrocephalus.*

Hemosiderin was observed only in *A. nancymaae* and *S. macrocephalus* during the early follicular growth (Figs. 5 and 6). In *A. nancymaae*, the greater density of hemosiderin was associated with a lower endometrial thickness ($R^2 = 0.19$, $P < 0.01$, Fig. 5) and was higher in females in the follicular phase ($F_{2,29} = 8.77$, $P = 0.0125$). In addition, hemosiderin was observed in *A. nancymaae* females in the follicular phase with a maximum antral follicle <1.2 mm. No analysis was conducted for *S. macrocephalus* due to low sample size. Only three *A. nancymaae* (21.4%) and none *S. macrocephalus* (0.0%) females in the luteal phase presented hemosiderin in the endometrium. *C. calvus* and *L. poeppigii* lacked hemosiderin.

Fibrin clots were observed in the four species during the early follicular growth (<1.2 mm of maximum antral follicle in *A. nancymaae,* <2.6 mm in *L. poeppigii,* <1.3 mm in *C. calvus*; $R^2 = 0.24$, $P < 0.01$). *S. macrocephalus* was excluded from this analysis due to low sample size. Higher density of fibrin clots was associated to a lower endometrial thickness ($R^2 = 0.45–0.79$, $P < 0.01$) (Figs. 7 and 8). Females in the luteal phase had no fibrin clots, except for one *A. nancymaae* female (7.1%) which presented a low density of fibrin clots.

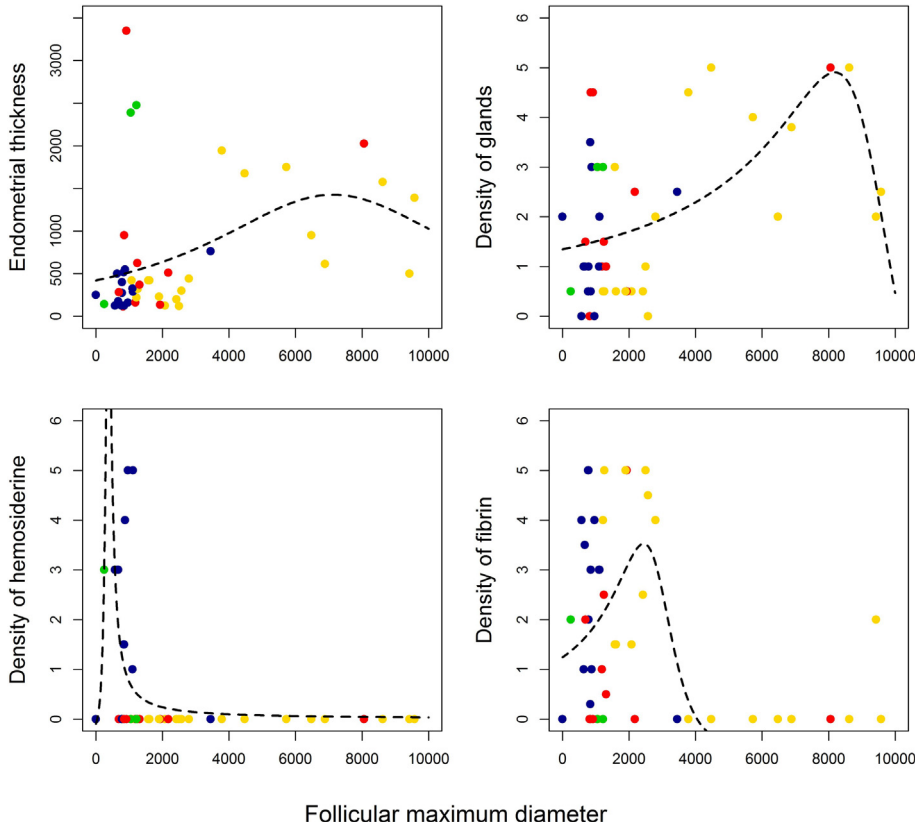

Fig. 1. Relationship between the studied uterine characteristics and the maximum follicular diameter in the *Aotus nancymaae* ($N = 32$, blue dots), *Lagothrix poeppigii* ($N = 29$, yellow dots), *Cacajao calvus* ($N = 20$, red dots) and *Sapajus macrocephalus* ($N = 10$, green dots).

3.3. Menstruating females

Menstruating females were observed only in *A. nancymaae* ($N = 4$) and *S. macrocephalus* ($N = 2$). Fig. 9 includes images of the menstrual cycle in *A. nancymaae*.

In the initial stages of menstruation, the lamina propria of the endometrium showed dilated blood vessels and multiple microthrombosis (Fig. 10). Blood, fibrinous material and necrotic

Table 2
Statistics and formulas for the association of the studied uterine features and the maximum follicular diameter and endometrial thickness in four New World primate species (*Aotus nancymaae* $N = 32$, *Lagothrix poeppigii* $N = 29$, *Cacajao calvus* $N = 20$, and *Sapajus macrocephalus* $N = 10$).

Species	Associations	Model	Equation	R	R^2	P
Aotus nancymaae	Density of glands ~ Endometrial thickness	Linear model (no intercept)	$y = 0.0044x$	0.76	0.58	<0.01
	Glandular secretion ~ Endometrial thickness	Power model	$y = 1.08e\text{-}04x^{1.45}$	0.60	0.36	<0.01
	Hemosiderin ~ Endometrial thickness	Rational model	$y = (0.25 + 8.46e\text{-}03x)/(1-6.79e\text{-}03x+2.85e\text{-}05x^2)$	0.44	0.19	<0.01
	Fibrin ~ Endometrial thickness	Farazdaghi-Harris model	$y = x/(21.02 + 3.66e\text{-}06x^{3.08})$	0.79	0.62	<0.01
Lagothrix poeppigii	Density of glands ~ Endometrial thickness	Modified Exponential model	$y = 5.68e^{-498.98/x}$	0.90	0.81	<0.01
	Glandular secretion ~ Endometrial thickness	Power model	$y = 1.01e\text{-}05x^{1.55}$	0.71	0.50	<0.01
	Fibrin ~ Endometrial thickness	Rational model	$y = (1.50-1.73e\text{-}03x)/(1-5.37e\text{-}03x+9.20e\text{-}06x^2)$	0.89	0.79	<0.01
Cacajao calvus	Density of glands ~ Endometrial thickness	Modified Exponential model	$y = 5.71e^{-491.63/x}$	0.96	0.92	<0.01
	Glandular secretion ~ Endometrial thickness	Rational model	$y = (-2.57e\text{-}01 + 8.78e\text{-}04x)/(1-6.20e\text{-}04x+2.17e\text{-}07x^2)$	0.67	0.45	<0.01
	Fibrin ~ Endometrial thickness	Farazdaghi-Harris model	$y = x/(59.72 + 6.10e\text{-}07x^{3.27})$	0.67	0.45	<0.01
Sapajus macrocephalus	Density of glands ~ Endometrial thickness	Linear model (no intercept)	$y = 0.0016x$	0.84	0.71	<0.01
	Glandular secretion ~ Endometrial thickness	Power model	$y = -1.51e\text{-}05x^{0.19}$	0.66	0.44	<0.01
All species pooled[a]	Endometrial thickness ~ Follicular diameter	Reciprocal Quadratic model	$y = 1/(2.37e\text{-}03\text{-}4.69e\text{-}07x+3.29e\text{-}11x^2)$	0.38	0.14	<0.01
	Density of glands ~ Follicular diameter	Rational model	$y = (1.35-1.33e\text{-}04x)/(1-2.04e\text{-}04x+1.08e\text{-}08x^2)$	0.55	0.31	<0.01
	Hemosiderin ~ Follicular diameter	Rational model	$y = (-9.24e\text{-}02 + 2.39e\text{-}03x)/(1-5.13e\text{-}03x+7.20e\text{-}06x^2)$	0.54	0.29	<0.01
	Fibrin ~ Follicular diameter	Rational model	$y = (1.25-3.08e\text{-}04x)/(1-6.18e\text{-}04x+1.09e\text{-}07x^2)$	0.49	0.24	<0.01

[a] Models were computed using females at follicular phase only.

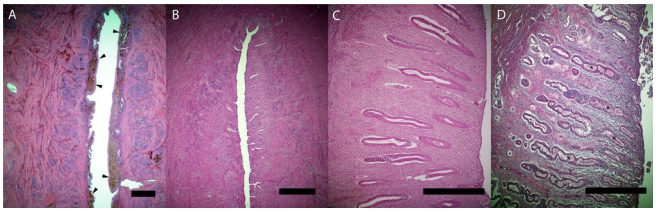

Fig. 2. Sections of the uterine body of non-pregnant females: (A) *Aotus nancymaae*, with an early endometrial growth and presence of hemosiderin (arrowhead), (B) *Cacajao calvus*, with an early endometrial growth and absence of hemosiderin, (C) *Sapajus macrocephalus*, with an intermediate endometrial growth and high density of non-secreting endometrial glands, and (D) *Cacajao calvus*, with a thick endometrium and high density of secreting endometrial glands (*). A: H&E (bar: 0.25 mm), B, C, D: H&E (bar: 0.5 mm).

endometrial cells desquamated with a few neutrophils were frequently observed in the superficial connective tissue.

In the mid menstrual phase, a mass of detached tissue was observed in the light of the uterus composed by abundant desquamated endometrial cells and large quantities of erythrocytes due to the rupture of superficial vessels. There was an increase in macrophages and lymphocytes in the conjunctiva of the lamina propria,.

In the advanced menstrual phase, the loss of endometrium of variable thickness was observed, although the level of endometrium released could not be differentiated. The glands were observed with a very open light, suggesting that this was the deepest part of them. In the sub-epithelial zone of the remaining endometrium accumulations of pyocytes, hemosiderin, and acidophilic materials compatible with fibrin clots were observed.

4. Discusion

The reproductive physiology provides important information for the development of management strategies and conservation programs for endangered species. Nevertheless, especially for New World primates, there are still conceptual gaps that difficult the understanding of the endometrial changes throughout the sexual cycle [11], resulting in a low success of assisted reproduction techniques [12]. Accordingly, the present study pretends to fill part of these gaps by uncovering the process of endometrial changes in four New World primate species.

During the sexual cycle of mammalian females, the endometrium undergoes periodic changes to prepare the uterus to nest an embryo. The proliferative phase of the endometrium coincides with the follicular development regulated by the production of estrogens [1,2], inducing the reconstruction of the connective tissue, the endometrial re-epithelialization and the lengthening of the vessels, which in this phase do not reach the endometrial surface [1,13]. In the four species studied, females at the early proliferative phase had a thin endometrium with few non-secreting glands. As follicular growth progresses, the endometrium increases and harbors a higher density of endometrial non-secreting glands. Only *A. nancymaae* and *S. macrocephalus* presented hemosiderin clusters in the endometrial subepithelium. Hemosiderin is an accumulation of insoluble aggregate of iron deposited in phagocytic cells of different tissues, usually a result of erythrocyte destruction [14], blood extravasations [15,16] or venous stasis [17]. The subepithelial presence of hemosiderin during the proliferative phase in both species could be an indication of menstruation or at least an important blood reabsorption [18,19].

The secretory phase begins after ovulation, so it is regulated mainly by the action of the CL, consistency all females studied in the

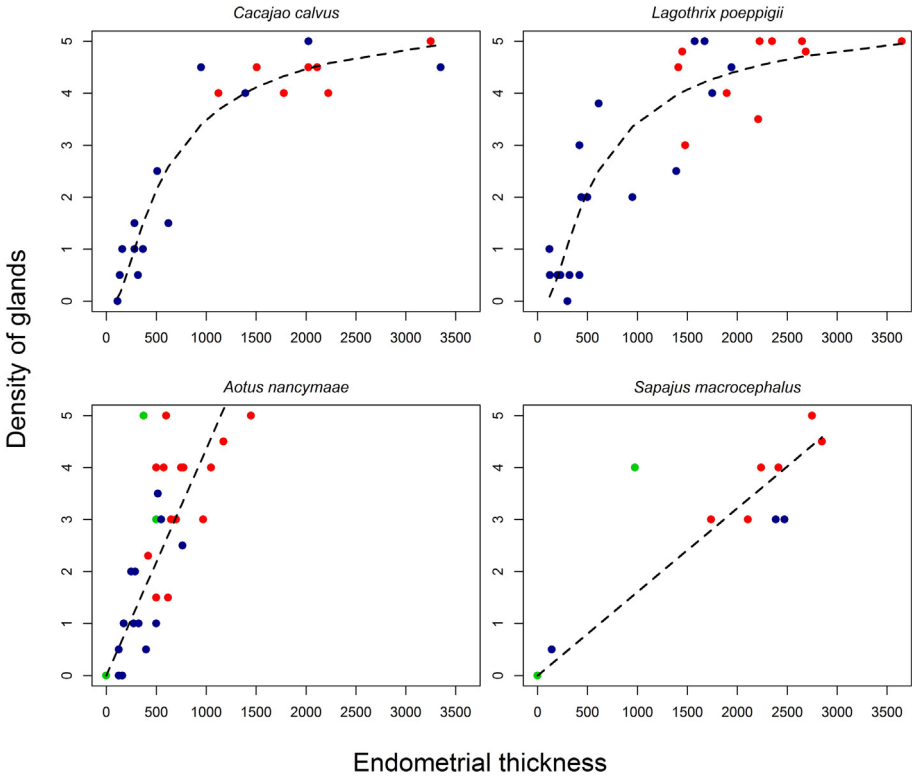

Fig. 3. Relationship between the density of endometrial glands and the endometrial thickness in females in the follicular phase (blue dots), in the luteal phase (red dots) and in menstruating females (green dots) in four New World primate species (*Aotus nancymaae* $N = 32$, *Lagothrix poeppigii* $N = 29$, *Cacajao calvus* $N = 20$, and *Sapajus macrocephalus* $N = 10$).

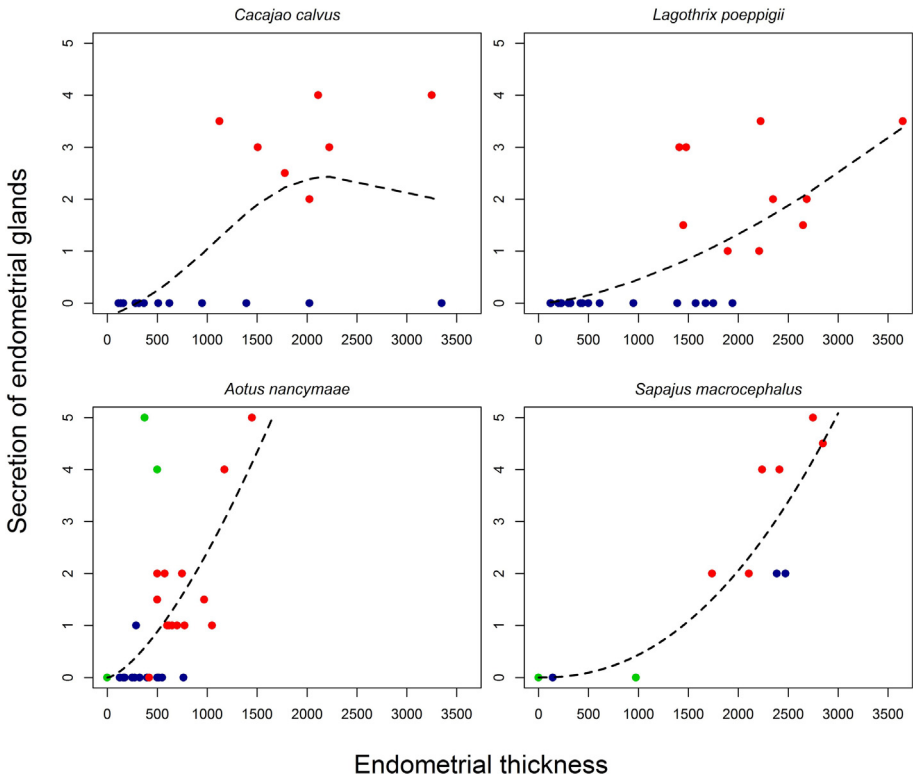

Fig. 4. Relationship between the presence of secretive endometrial glands and the endometrial thickness in females in the follicular phase (blue dots), in the luteal phase (red dots) and in menstruating females (green dots) in four New World primate species (*Aotus nancymaae* $N = 32$, *Lagothrix poeppigii* $N = 29$, *Cacajao calvus* $N = 20$, and *Sapajus macrocephalus* $N = 10$).

secretory phase had at least one active CL. The levels of progesterone and estrogen increase simultaneously during the luteal phase [20], resulting in the increase of the endometrium due to the development of vascularization in the endometrial functional layer, which reaches the surface, and the hypertrophy of the endometrial glands [1,2,13,21]. In the four species, the endometrial thickness varied according to the age of the CL. In the early secretory phase, the hemorrhagic CL has just been formed and is beginning to produce progesterone and estrogen, promoting an endometrial thickness similar to the late proliferative phase. When the CL is completely formed, the endometrium and glands reach their maximum development. Nevertheless, we observed differences in the maximum endometrial development among species, probably related to the uterine size of each species. Finally, in the late secretory phase the CL involutes and degenerates, promoting the decrease in the endometrial thickness and inducing its degradation, preceding the menstrual phase. During the progress of the secretory phase, the endometrial glands increase in number and length, and produce secretion [12,22,23]. In this sexual phase, the absence of hemosiderin and the large presence of macrophages suggest the rapid reabsorption of hemosiderin.

In our study, four females of *A. nancymaae* and two *S. macrocephalus* had deforming and/or detachment of the functional layer of the endometrium, detritus and rupture of superficial vessels, features associated to the desquamation of the epithelial and stromal cells and the release of uterine fluid and blood [1,2,13,21]. In addition, the large presence of macrophages during the menstruation phase suggests an important phagocytic activity at the upper part of the endometrium, or functional layer, which degenerates and detaches [24], with the consequent rupture of arteries, and blood release to the stroma that must be eliminated or reabsorbed. In parallel, fibrin may have a fundamental role in the hemostasis of the menstrual endometrium, regulating the amount of menstrual bleeding [25]. The increase in microvascular permeability in the late menstruation also allows the formation of a fibrin matrix for the migration and proliferation of the post-menstruation endometrium [26]. Thus, the accumulation of fibrin and hemosiderin in the early proliferative phase in *A. nancymaae* and *S. macrocephalus* suggests a previous hemorrhagic endometrial loss.

During the menstrual phase, the endometrium degenerates and is eliminated through the vagina in the form of bleeding [2]. This is a particular phenomenon in nature, only present in some primates (including humans), bats and macroscelids [7,27]. Most menstruating primate species belongs to the Haplorrhini suborder, and within Haplorrhini, the Old World primates or Catarrhini present an evident menstruation, while in New World primates or Platyrrhini it is a more discreet menstruation and usually only recognizable by microscopic studies [28]. In New World primates, external signs of bleeding have been only observed in *Alouatta*, *Ateles* and *Cebus* [27,29,30]. Our study supports histological evidence of menstruation in *A. nancymaae* and *S. macrocephalus*. Previously, Baer et al. [31] and Bonney et al. [32,33] reported that *Aotus trivirgatus* does not have menstruation because it does not present macroscopic evidence or erythrocytes in vaginal smears. On the other hand, based on vaginal smears Hamlett [34] and Kaiser [28] observed indicators of menstruation in genus *Cebus*.

The lack of evident menstruation or pseudomenstruation in some New World primates may be due to the reduction of the body size [24] and, contrary to women and Old World primates, the absence of spiral arteries in the endometrium [25]. Estrogen regulates variation in blood flow and oxygenation reducing systemic vascular resistance and increasing the cardiac output [35,36]. The

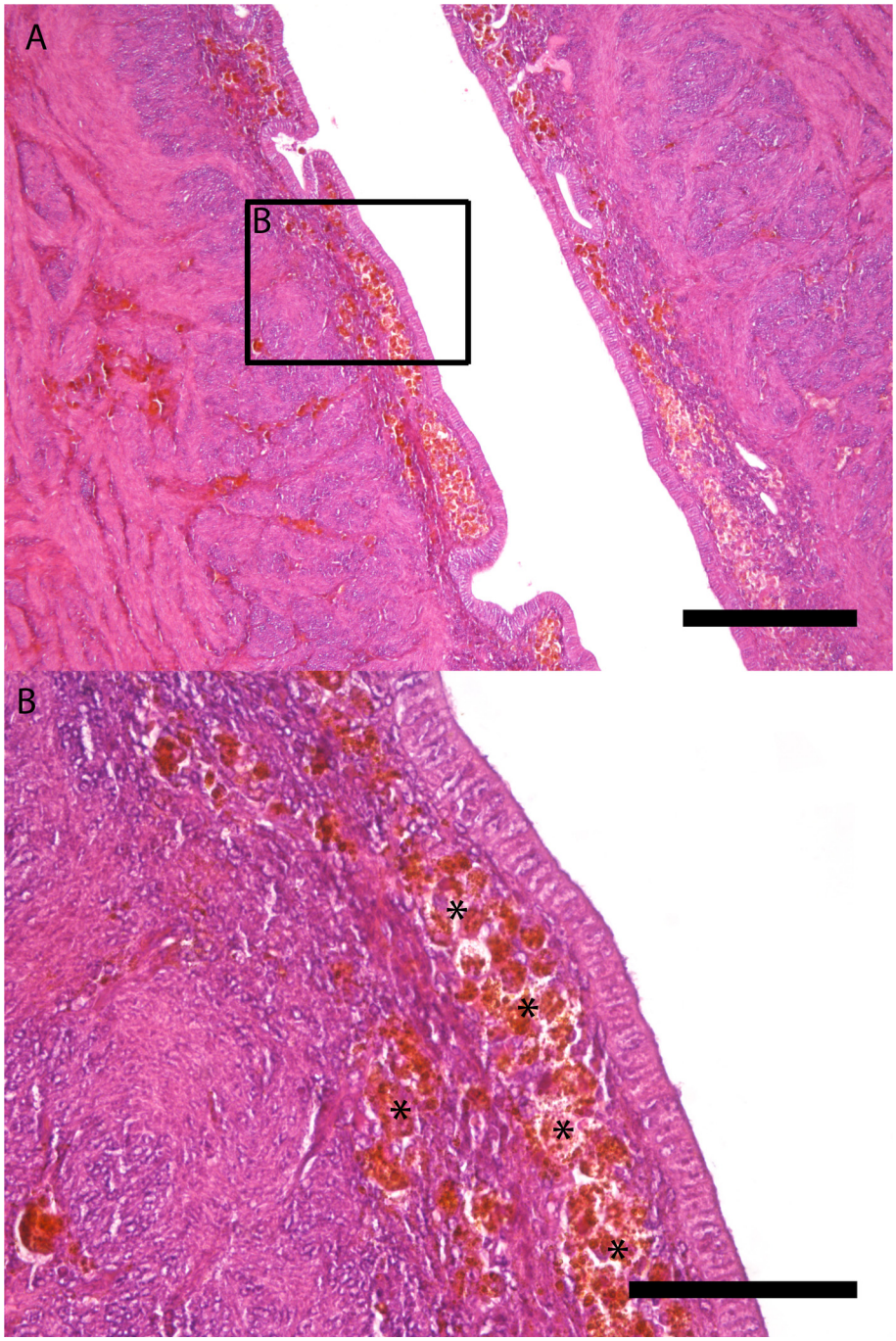

Fig. 5. Sections of the uterine body of a non-pregnant female of *Aotus nancymaae* with an early endometrial growth and presence of hemosiderin (*) beneath the endometrial epithelium. A: H&E (bar: 0.25 mm), B: H&E (bar: 0.15 mm).

fall of estrogen levels at the end of the estrous cycle stimulate the production of prostaglandins, responsible for the contraction of the spiral arterioles [21]. The initial intermittent ischemia and the latterly permanent ischemia 1–2 days before the beginning of the menstrual phase [1,13,21] lead to hypoxia and necrosis of the functional layer. Soon after, the spiral arteries dilate, and break due to their weakening. The released blood removes the degenerated endometrial functional layer, which is detached and expelled as a hemorrhagic exudate [13,21]. Kaiser [25] showed the lack of spiral arterioles in the endometrium of the genus *Cebus*, *Alouatta* and *Ateles*, suggesting that the development of these arterioles could be related to the presence of profuse menstrual bleeding.

There is still a great controversy in the purpose of menstruation. The endometrial degeneration could be a low-cost reproductive strategy and vaginal bleeding might occur when there is too much blood to allow a complete tissue reabsorption [27]. Pseudo-menstruating species might have mechanisms to reabsorb part of the endometrial tissue released, thus avoiding profuse or evident bleeding.

5. Conclusions

This study presents histological evidences of menstrual cycle in *A. nancymaae* and *S. macrocephalus*, which present the

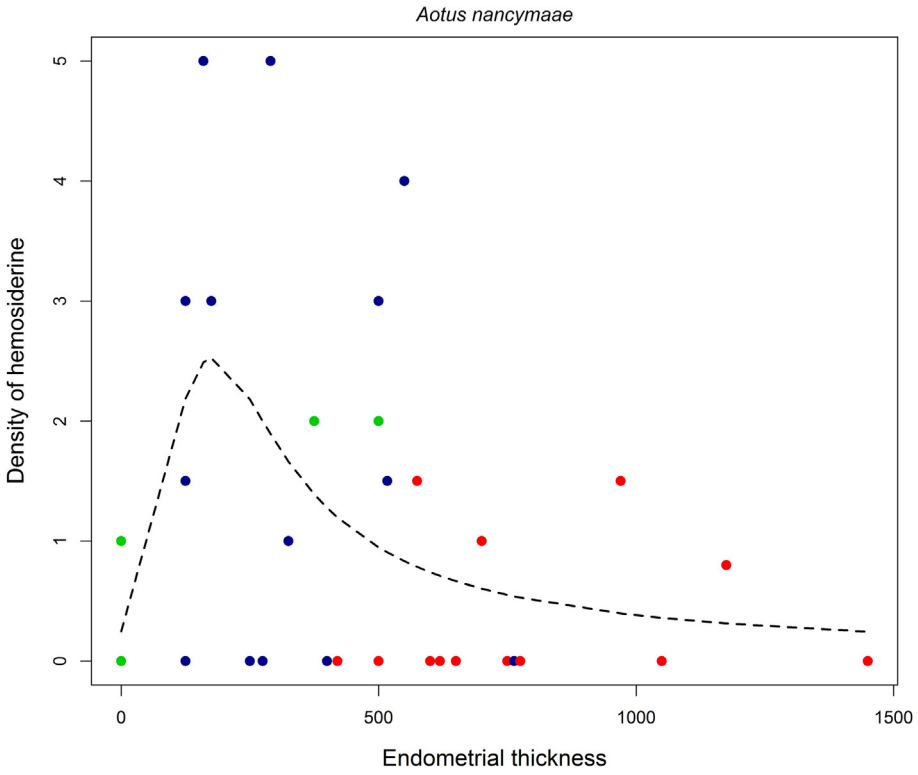

Fig. 6. Relationship between the density of hemosiderin in the sub-epithelial zone of the endometrium and the endometrial thickness in females in the follicular phase (blue dots), in the luteal phase (red dots) and in menstruating females (green dots) in *Aotus nancymaae* N=32).

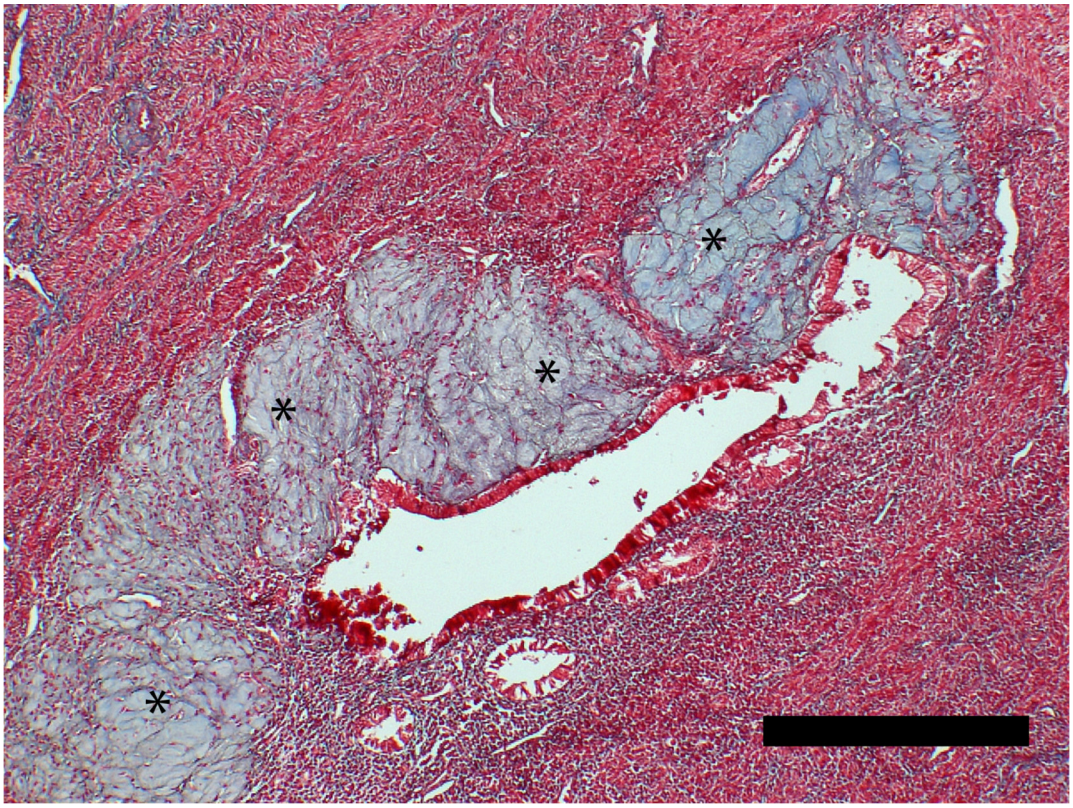

Fig. 7. Section of the uterine body of a non-pregnant *Lagothrix poeppigii* female in the follicular phase with high amount of fibrin clots (*) beneath the endometrial epithelium. Masson's trichrome (bar: 0.5 mm).

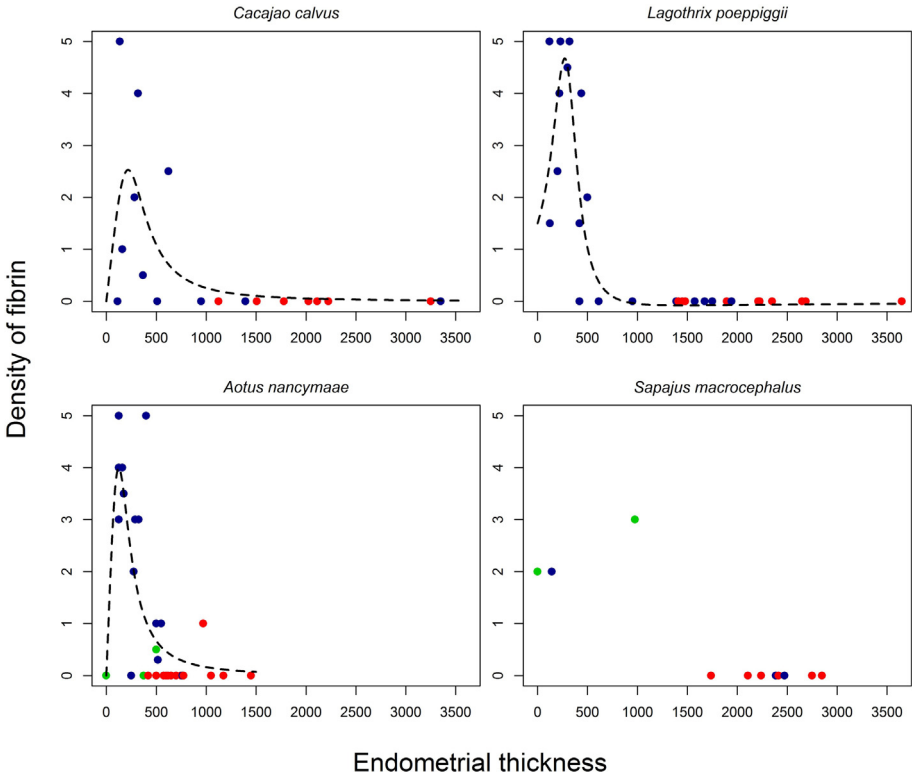

Fig. 8. Relationship between the density of fibrin clots in the endometrium and the endometrial thickness in females in the follicular phase (blue dots), in the luteal phase (red dots) and in menstruating females (green dots) in four New World primate species (*Aotus nancymaae* $N = 32$, *Lagothrix poeppigii* $N = 29$, *Cacajao calvus* $N = 20$, and *Sapajus macrocephalus* $N = 10$).

detachment and bleeding of the endometrium and subepithelial reabsorption of the endometrial functional layer. These features explain the presence of hemosiderin and fibrin clusters in the endometrium of these species and the fact that both species do not present an evident external menstruation. In *L. poeppigii*, despite the presence of fibrin clusters promoting the remodeling of the endometrium, no detachment of the endometrial functional layer or hemorrhage were observed, suggesting a non-menstruating cycle. Finally, *C. calvus* did not show any histological sign of menstrual phase. Further studies on cellular apoptosis in the endometrium or uterine metalloproteinase expression, which are enzymatic proteins responsible for the degradation of the components of the extracellular matrix, should be conducted to confirm this evidence.

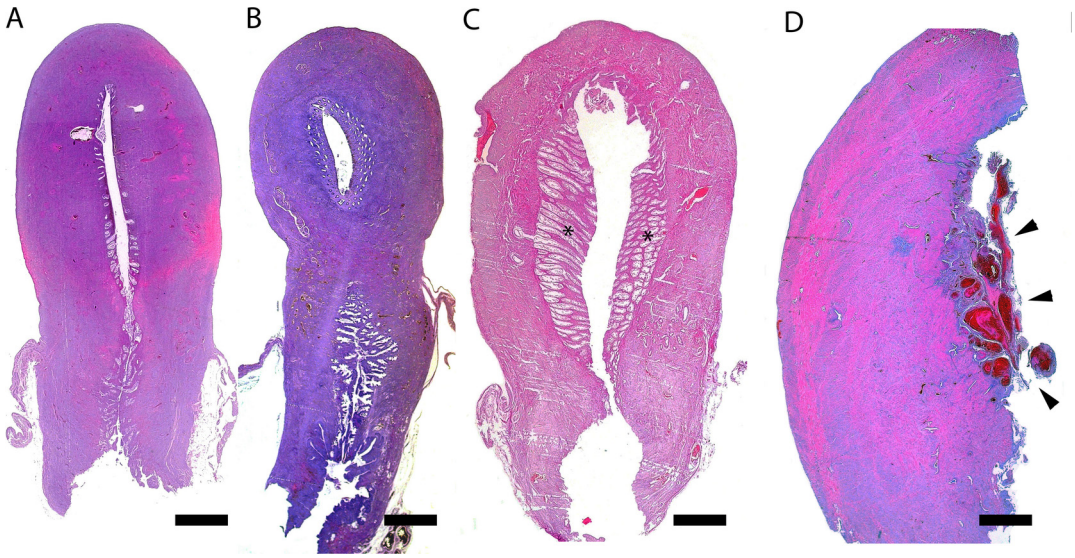

Fig. 9. Sections of the uterine body of non-pregnant *Aotus nancymaae* females (A) in the follicular phase with an early endometrial growth, (B) in the follicular phase with a mid-developed endometrial growth, (C) in the luteal phase with a thick endometrium and high density of secreting endometrial glands (*), and (D) in the initial menstrual phase showing abundant red blood cells (arrowheads). A, B, C, D: H&E (bar: 1 mm).

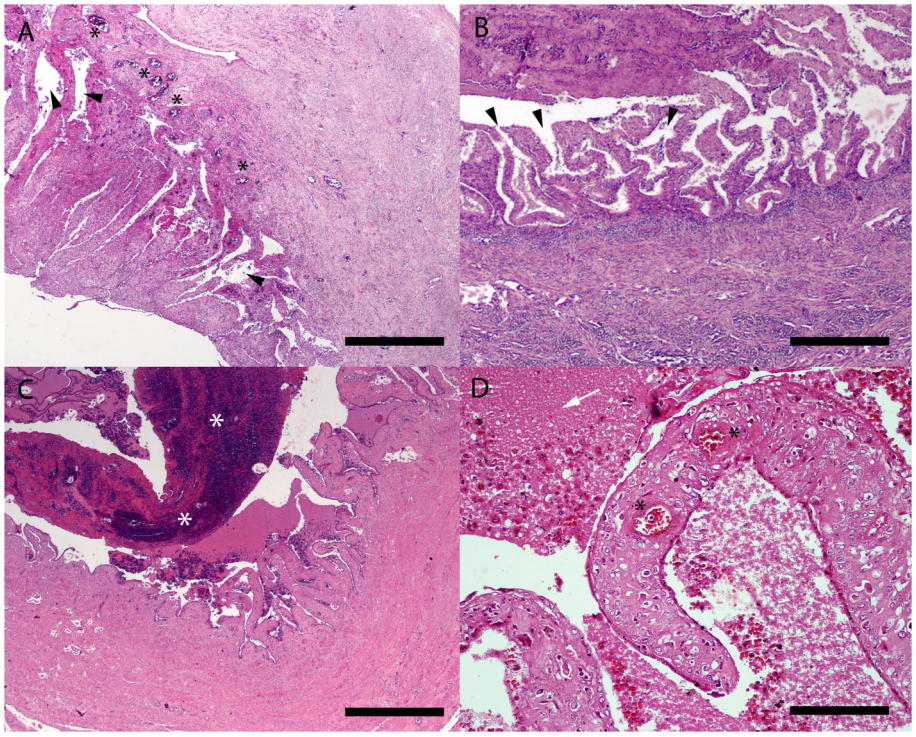

Fig. 10. Sections of the uterine body of non-pregnant females: (A) *Aotus nancymaae* in the early menstruation phase, showing developed endometrial glands with a wide overture (arrowheads) suggesting the detachment of the most superficial part of them, and abundant and dilated blood vessels (*) in the deepest endometrium, (B) *Aotus nancymaae* in the menstruation phase, detailed image of endometrial glands with a wide overture (arrowheads) and the detachment of the most superficial part of them, (C) *Sapajus macrocephalus* in the advanced menstruation phase, with an endometrium showing multiple microthrombosis, abundant red blood cells and fibrinous clots (*) in the uterine lumen, and (D) *Sapajus macrocephalus* in the advanced menstruation phase, detailed image of the endometrium showing abundant and dilated blood vessels (*) in the deepest endometrium and clots of red blood cells (white arrow) in the uterine lumen. A, C; H&E (bar: 0.5 mm), B, H&E (bar: 0.15 mm), D: H&E (bar: 75 μm).

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