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Research article

Characterization of the estrous cycle in the Amazon spiny rat (*Proechimys guyannensis*)

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ABSTRACT

Males of *Proechimys guyannensis*, a rodent living in the Amazon rainforest are studied in biomedical research because of their antiepileptogenic mechanism. Females are usually taken from experimental designs, because of limited data of this sex. This study aimed to characterize the estrous cycle to include females together with males in research in a more balanced approach. The estrous cycle of *P. guyannensis* based through exfoliative cytology, determination of the vaginal occlusion membrane state, and hormonal analysis. In this study, cytological analyses of vaginal smears were performed for three months, three times a day. The observed length of the estrous cycle was 247 ± 81 h (mean ± SD) with a reproductive phase of 27.08 ± 17.39 h (estrus stage). We observed a frequent presence of both the open and closed states of the vaginal membrane in the estrus stage (fertile period) although only the open stage is a prerequisite for successful copulation. High levels of progesterone and estradiol were detected in proestrus. Levels of follicle-stimulating hormone peaked at the estrus stage. These data will establish the parameters and subsidies to set the grounds for future research either for investigating the biology of this species or to use *P. guyannensis* in research that previously excluded females. Information regarding female *Proechimys* is relevant to not only describe the species but also explain the interaction between sex hormones and physiological responses. Moreover, the present results will enhance rigor and reproducibility in preclinical studies. In conclusion, *P. guyannensis* reproductive cycles can occur spontaneously and cyclically independent of mating stimulation and the high levels of FSH in the estrus stage, suggest that ovulation occurs in the late phase of the estrus.

1. Introduction

The estrous cycle is a cyclical pattern of ovarian activity, essential for reproductive health and is divided into four stages: proestrus, estrus, metestrus, and diestrus (Cora et al., 2015). The classification of each stage reflects the uterine events in response to hormonal oscillations (Marcondes et al., 2002). Through vaginal smear cytology, stages of the estrous cycle can be determined to establish the cycle length (Byers et al., 2012). Likewise, stages of the estrous cycle are linked to the distribution of three different types of cells, keratinized cells, nucleated cells, and leukocytes, as well as to the levels of specific hormones including estradiol, progesterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) (Marcondes et al., 2002). The term "estrous cycle" refers to regular cyclic periods observed in female mammals and differs among species (Reece, 2009; Smith, 2010).

In traditional laboratory rat strains, such as Wistar, the estrous cycle is around 4–5 days (Long and Evans, 1922), with the proestrus stage lasting 12–14 h, estrus 25–27 h, metestrus 6–8 h, and diestrus 55–57 h (Antunes et al., 2016). During the proestrus stage, LH and FSH display a high pulse in these rodents, there is a predominance of nucleated cells as a result of the increased level of estradiol (Suckow et al., 2006; Paccola et al., 2013). The predominant function of LH is the induction of ovulation and formation of the corpora lutea and stimulation of ovarian steroid hormone production, while FSH stimulates estradiol secretion (Suckow et al., 2006). The primary hormone influencing alterations in the vaginal mucus is estradiol (Gal et al., 2014), and during the proestrus stage,

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estradiol is present at high levels, thus stimulating gonadotropin release, triggering ovulation (Graham and Daher, 2015). Estradiol acts on the vaginal mucosa, yielding a stratified epithelium that becomes keratinized (Gupta et al., 1989). The declining in estradiol levels leads to an increase in desquamation of the mucosal epithelium into the vaginal lumen (Gupta et al., 1989).

During the estrus stage, females are receptive to males and ovulation occurs spontaneously (WestWood, 2008; Antunes et al., 2016). In this stage, estradiol level decreases, and non-nucleated keratinized cells are predominant; the vaginal epithelium is thicker, and the layer of keratinized non-nucleated cells is present on the surface (Gronroos and Kauppila, 1959). In the metestrus stage, keratinized epithelial cells and leukocytes are present in the vaginal smears (Gronross and Kauppila, 1959). In this phase, the corpus luteum is formed and produces progesterone (Antunes et al., 2016); however, when fertilization does not occur, the corpus luteum stops progesterone reflecting in a minor surge in estradiol (Byers et al., 2012). In the diestrus stage, after regression of the corpus luteum, progesterone levels sharply decline (Byers et al., 2012) and the predominant cells in the vaginal smears are leukocytes (Antunes et al., 2016).

P. guyannensis (É. Geoffroy, 1803), an Amazon rodent of suborder Hystricomorpha (Simpson, 1945; Reig et al., 1979), is of interest to biomedical researches because of its physiological responses; it is not infected by several pathogens (Lainson and Shaw, 1975) and exhibits an endogenous antiepileptogenic mechanism that can ensures that this rodent can be employed as a model for studying epilepsy (Arida et al., 2005). Most of the studies on *Proechimys* have been performed in males rather than in females (Fabene et al., 2001; Arida et al., 2005; Passos et al., 2015), perhaps to avoid irregular responses found in females because of hormonal interaction. Despite this variability, knowledge about females *Proechimys* is relevant, not just to describe the species but also to explain the interaction between sex hormones and physiological responses. Moreover, this understanding is fundamental for enhancing rigor and reproducibility in preclinical research (Clayton and Collins, 2014). Therefore, to include females in researches, basic knowledge about aspects such as the estrous cycle is necessary. Thus, the aim of this study was to characterize the estrous cycle of the Amazon rodent *P. guyannensis* through exfoliative cytology, determination of the vaginal occlusion membrane state, and hormonal analysis.

2. Material and methods

2.1. Animals

Adult virgin *P. guyannensis* females (1 year old, weight range 210–280 g, n = 29) were housed under controlled conditions of light/dark cycles (12/12 h), temperature 21 ± 2 °C, food and water *ad libitum*. The diet consisted of NUVILABCR1 mouse pellets (Nuvital Nutrients S.A., Colombo/ PR, Brazil). The rodents were obtained from a colony established at the Universidade Federal de São Paulo. Experiments were performed with the approval of the Ministry of the Environment (IBAMA n° 1561643) and the Ethics Committee for Animal Experimentation of the Universidade Federal de São Paulo (CEUA n° 5594280316). Efforts were made to minimize animal suffering, according to the International Ethical Guidelines for Biomedical Research (CIOMS/OMS, 1985).

2.2. Characterization of the estrous cycle

Determination of the estrous cycle was based on exfoliative cytology in 13 females. Vaginal secretions were analyzed daily three times a day, with the first collection between 7:00 and 9:00 a.m., second between 1:00 and 3:00 p.m., and third between 7:00 and 9:00 p.m., for three months. Cell type identification was performed to characterize the length of the estrous cycle and the duration of each phase of the cycle. A single estrous cycle was considered from proestrus stage to another proestrus stage.

Vaginal smears were collected with a plastic pipette and filled with 0.2 mL of saline solution (NaCl 0.9 %), inserting the tip carefully into the vagina of *P. guyannensis*, to avoid cervical stimulation. Three drops of vaginal contents were placed on a slide and fixed with 95% ethanol. Papanicolaou tests (Reny laboratory, Minas Gerais, Brazil) were performed to identify nucleated, keratinized, and leukocyte cells. A previously described estrous cycle identification tool was used as a reference to determine each estrous stage (Byers et al., 2012). Images were taken using an OLYMPUS BX60 optical light microscope with a 20x objective lens, and cell counting was performed with ImageJ software (National Institute of Health, USA).

Blood samples were collected at each stage of the cycle for hormone analysis (three females, for each phase). Briefly, the animals were decapitated for the collection of whole blood. Thirty minutes after collection, the blood samples were centrifuged at 239 $\times g$ (15 min, 24 °C), followed by serum collection and storage at -80 °C until analysis. The measurement of follicle-stimulating hormone (FSH), progesterone, and estradiol was outsourced to a clinical laboratory that used the chemiluminescence method (Beckman Coulter, Unicel DXI-800, Brea, CA) for their determination. LH levels were not determined owing to the low sensitivity of the equipment.

2.3. Vaginal membrane

Furthermore, in order to determine whether the vaginal membrane was correlated with the estrous cycle, the vaginal membrane was visually classified into four stages according to Lusty and Seaton (1978): closed, pin-prick, half-open, and open (Lusty and Seaton, 1978). The opening of the membrane was measured using a Vernier caliper (150 × 0.05 mm), from which we obtained the classification: closed, 0.15–0.3 mm; pin-prick, 2–2.3 mm; half–open, 2.7–3 mm; and open, 4–5 mm (Figure 1) (Lusty and Seaton, 1978).

2.4. Isolated females

It has been reported that the presence of males affects hormonal oscillations in other female rodents (Lusty and Seaton, 1978). To determine if the presence of males influences the estrous cycle length in *P. guyannensis*, a subset (n = 4) of our study population was isolated for eight days and separated from the males without visual, auditory, or olfactory access. Next, vaginal smears of these animals were studied as described above.

2.5. Data analysis

The time interval between the three vaginal smears per day, during the three months, was considered to determine the total hours in each estrous phase. The 95% confidence intervals (CI) and mean ± SD were used to establish the duration of the estrous cycle. The interaction between the cycle phase and the vaginal membrane state was determined by two-way ANOVA test followed by Tukey post-hoc test. The states of the vaginal membrane were as the frequency of observation in each estrous stage. Hormonal analysis was reported as mean ± SD. SPSS Statistics software (version 21 from IBM Corp, Armonk, NY, USA) was used for all analyses, and differences were considered significant at $P < 0.05$.

3. Results

3.1. Cytology and hormone profile of the estrous cycle of P. guyannensis

In analyses of the phases of the estrous cycle, we observed many nucleated epithelial cells that displayed small pyknotic nuclei and light-yellow mucus in the proestrus stage (Figure 2A). In the estrus stage, excessive keratinization in the presence of anucleated superficial cells was observed (Figure 2B). In the metestrus stage, keratinized cells and

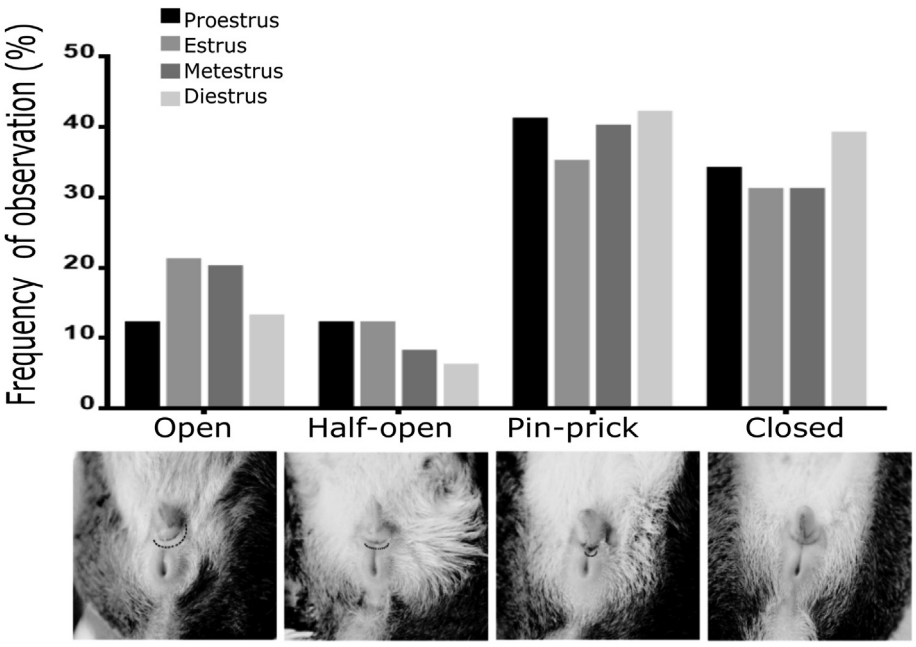

Figure 1. Vaginal membrane stages during the estrous cycle stages in *P. guyannensis.* The line around the vaginal membrane represents the size of the vaginal membrane opening.

leukocytes along with cervical mucus were found (Figure 2C). In the diestrus stage, we observed the presence of predominant leukocytes and thick white viscous cervical mucus (Figure 2D). The estrous cycle length of *P. guyannensis* was calculated to be approximately 10 days (247 ± 81 h; mean ± SD). The proestrus stage was estimated to last 3 days (ranging from 59 to 79 h), the estrus stage 1 day (24–31 h), metestrus stage 4 days (79–96 h), and diestrus stage 3 days (62–79 h) (Table 1). Thus, nine complete ovarian cycles were observed in female *Proechimys* throughout the duration of this study.

Hormonal analysis revealed that progesterone and estradiol levels were higher in the proestrus stage (Figure 2E). In the metestrus stage, the progesterone and FSH levels were lower, while in the diestrus stage, the estradiol level was the lowest. The FSH level was the highest in the estrus stage (Figure 2E).

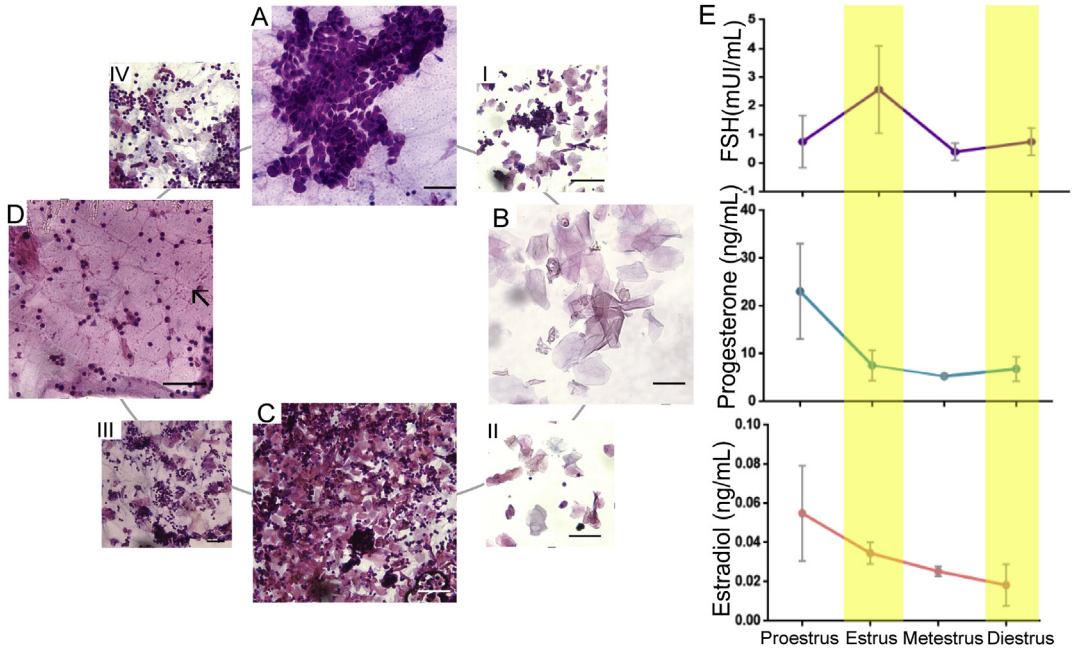

Figure 2. Vaginal smears images of each cycle stage together with hormonal analysis in *P. guyannensis*. A. Proestrus stage showing predominance of nucleated cells, in clusters or alone. B. Estrus stage showing large and irregular keratinized cells. C. Metestrus stage displaying leukocytes and keratinized cells. D. Diestrus stage showing predominance of leukocytes surrounded by a thick cervical mucus, stained with a violet color (arrow). Smaller images represent interfaces among the main stages: I. Proestrus-Estrus interface showing few nucleated cells with keratinized cells; II. Estrus-Metestrus interface displaying few leukocytes at the end of the late estrus stage; III. Metestrus-Diestrus interface showing an increase of leukocytes on late metestrus stage; IV Diestrus-Proestrus interface with nucleated cells and low number of leukocytes. E. FSH, progesterone and estradiol levels in *P. guyannensis* females during the cycle. FSH was measured in mUI/mL and progesterone and estradiol were measured in ng/mL. Scale bar = 50 μm.

Table 1. Length of estrous cycle stages in 13 *P guyannensis* females.

	Cell types				Estrous cycle length	
Estrous cycle stages	Nucleated	Keratinized	Leukocytes	Total number of cells	Mean±SD	Days
Proestrus	100%	-	-	133	65.11±38.57	2–3
Estrus	-	100%	-	61	27.08±17.39	1
Metestrus	-	44% (n=115)	56% (n=146)	261	87.08±43.71	3–4
Diestrus	-	-	100%	222	70.59±42.76	2–3

3.2. Vaginal membrane analyses

Different states of the vaginal membrane were observed in each estrous stage; therefore, no correlation between vaginal membrane states and estrous cycle phases was observed. Closed and pin-prick states were observed more frequently than open and half-open states (Figure 1). Surprisingly, even in the estrus stage, the closed and pin-prick states were observed.

3.3. Isolated females

When rodents were isolated to avoid contact with males, no alterations in the cycle and vaginal membrane states were observed. We noted vaginal openings during the experiment with spontaneous and continuous cyclicity.

4. Discussion

The main purpose of the study was to characterize the estrous cycle of *P. guyannensis* based through exfoliative cytology and hormonal analysis. To our knowledge, this is the first study describing the estrous cycle of these rodents. The estrous cycle is ten days in length, during which metestrus is the longest stage (three to four days) and estrus the shortest (one day). We observed elevated progesterone and estradiol levels during proestrus, while FSH levels were higher at the beginning of estrus. We suggest that cycles and vaginal opening can occur spontaneously even without association with a male.

The longer estrus cycle of *Proechimys* is in contrast with the short cycle length of Wistar rats (Figure 3). Female myomorph rodents (i.e. mouse, hamsters, and rats) have an estrous cycle length of approximately five days (Long and Evans, 1922; Mahoney et al., 2011), while *Proechimys guyannensis* requires ten days to complete the cycle. The length of the cycle varies with species; *P. chrysaeolus* has a five to eight days to complete the cycle (Sabogal-Guáqueta et al., 2013), while *P. guairae* has a cycle that lasts 22.5 ± 3.4 days (Weir, 1973). Other histricomorph rodents including *P. guyannensis* have longer estrous cycles, e.g., *Octodon degus* (21.2 ± 0.56 days), *Myocastor coypus* (35.5 ± 10.8 days), and *Galea spixii* (15.6 ± 1.3 days) (Felipe et al., 2001; Mahoney et al., 2011; Santos et al., 2015), thus potentially representing a characteristic of this rodent family.

In the vaginal membrane experiments, we observed closed and pin-prick states even during the estrus stage, which may represent an obstacle to mating even in the fertile period. This fact is inconsistent with the highest spermatogenic efficiency observed in males *P. guyannensis* (Lara et al., 2016). In another species of *Proechimys (P. guairae)* a lower frequency of vaginal opening was also observed during the estrous cycle (Lusty and Seaton, 1978); however, in other Hystricomorph rodents, such as guinea pigs, the vaginal opening is associated with the estrous cycle (Lilley et al., 1997). For *P. guyannensis*, our study confirms that vaginal openings are independent of the estrous cycle even in isolated females. Furthermore, as spontaneous ovulation occurred in isolated females, we confirm that *P. guyannensis* can ovulate in the presence or absence of males. Spontaneous ovulation species such as guinea pigs, primates, and *Octodon degus* have a corpus luteum that secrets progesterone independent of mating stimulation (Mahoney et al., 2011). The estrous cycle in these species is longer (14–35 days) than that in Wistar rats, and the luteal phase typically lasts for 12–13 days (Elder, 1938; Hilliard, 1973; Mahoney et al., 2011).

In Wistar rodents, during the proestrus stage, the estrogen level increases, and ovarian follicles grow rapidly (Paccola et al., 2013). Consequently, the corpus luteum becomes functional and secretes progesterone that inhibits FSH (Fortune, 1994). On the other hand, a high level of FSH initiates several morphological changes, leading to ovulation and pregnancy if fertilization occurs. Ovulation occurs from proestrus to the end of the estrus stage (Marcondes et al., 2002). In *P. guyannensis,* progesterone level is highest during the proestrus stage and lowest in the metestrus stage. Estradiol level is higher during the proestrus stage and lowest in diestrus stage (Figure 2). FSH levels are higher in the estrus

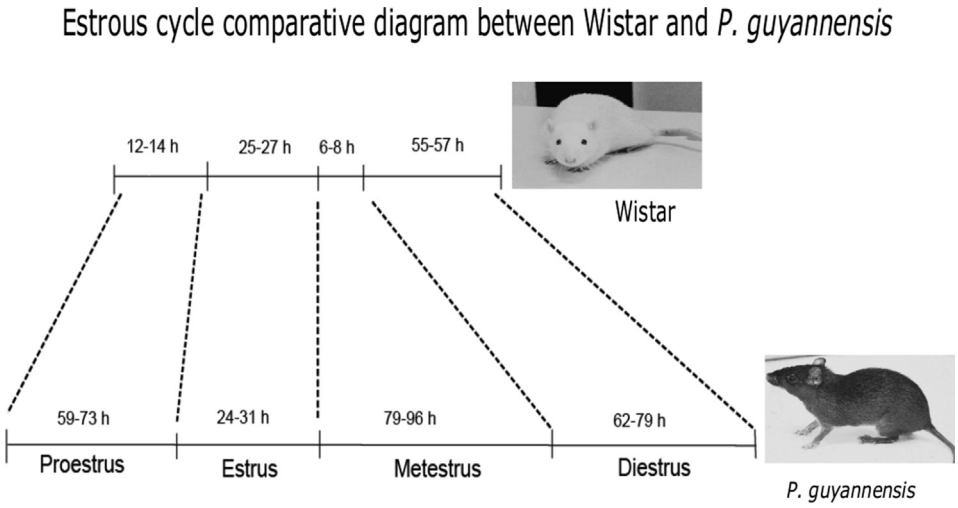

Figure 3. Estrous cycle comparative length diagram between Wistar rats and *P. guyannensis.* Wistar rats estrous cycle length was taken from Antunes et al. (2016).

stage (fertile period), suggesting that ovulation occurs in the late phase of estrus. These observations may assist in experimental design according to the estrous stages.

In summary, *P. guyannensis* displays a longer estrous cycle than other rodents and lacks an association between the vaginal membrane opening and the estrous cycle stages. This study shows that reproductive cycles can occur spontaneously and cyclically in *P. guyannensis* without males. Hormonal assays revealed high levels of FSH in the estrus stage (fertile period), suggesting that ovulation occurs in the late phase of the estrus. The characterization of the estrous cycle helps to understand the reproductive cycles and raises questions regarding the reproductive strategy used among these rodents. A greater understanding of the estrous cycle in female *P. guyannensis* provides insights into the role of hormones in physiological responses and would allow researchers to include females together with males in their research in a more equal approach.

Declarations

Author contribution statement

Viviam Sanabria: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Simone Bittencourt: Conceived and designed the experiments; Wrote the paper.

Tomás de la Rosa: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Jomênica Livramento, Célia Tengan, Carla A. Scorza, Esper Cavalheiro: Contributed reagents, materials, analysis tools or data.

Débora Amado: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; 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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