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Wild Fulvous Fruit Bats (*Rousettus leschenaulti*) Exhibit Human-Like Menstrual Cycle[1]

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ABSTRACT

We investigated the menstrual cycle of wild fulvous fruit bats (*Rousettus leschenaulti*), focusing on changes in the endometrial and ovarian structure and pituitary and steroid hormones. The menstrual cycle lasts for 33 days in bats studied in their natural habitat and in captivity. Vaginal bleeding was restricted to a single day (Day 1). A preovulatory follicle was found in the ovary on Day 18 when the levels of LH and FSH reached their maxima, accompanied by a thickened endometrium. On Day 24, serum levels of progesterone and estradiol-17 were also maximal, and uterine glands increased in size. After that, the levels of progesterone dropped precipitously, leading to menstrual bleeding. Both the morphologic and hormonal changes observed in fulvous fruit bats during the menstrual cycle resemble similar changes in humans. Fulvous fruit bats may be useful nonprimate laboratory models to study menstruation and menstrual dysfunction.

endometrium, fulvous fruit bat, hormone, menstrual cycle, ovary

INTRODUCTION

Menstruation in humans involves shedding of the endometrium, accompanied by bleeding. It normally occurs if an embryo does not implant at the end of each ovarian cycle [1, 2]. The human menstrual cycle consists of a proliferative phase, a secretory phase, and a menstrual phase. The endometrium undergoes corresponding growth, differentiation, and programmed breakdown. These morphologic changes in the endometrium during the menstrual cycle are driven by changing levels of estrogen and progesterone (P_4) [3, 4]. During the proliferative phase, the endometrial stroma thickens, and the glands increase both in size and number. During the secretory phase, the glandular secretory activities increase, and the endometrium prepares for implantation. Endometrial tissues are shed together with menstrual blood during the menstrual phase in response to P_4 withdrawal [5–7].

In addition to humans, overt menstruation has been recorded in many other species of primates, most of which are Old World monkeys, apes, and a few species of New World monkeys [8]. If the customary definition of menstruation is broadened to include the microscopic presence of red blood cells in the uterine lumen associated with reduced P_4 levels, some nonprimate species can be included [2, 8], such as flying lemurs (*Cynocephalus variegatus*), insectivores (*Erinaceus europaeus* and *Elephantulus myurus*), tree shrews (*Tana tana, Tupaia belangeri,* and *Tupaia minor*), bats (*Carollia perspicillata, Desmodus rotundus, Glossophaga soricina,* and *Molossus ater*), and marsupials (*Dasyurus viverrinus*) [9, 10].

The order Chiroptera is the second largest order of mammals and is composed of the suborders Megachiroptera and Microchiroptera. At present, there are 1116 recognized species of bats worldwide, accounting for almost one quarter of all known mammalian species [11].

Menstruation has been described only in microchiropteran bats [12–15] and is thought to occur only after coitus. Captive short-tailed fruit bats (*C. perspicillata*) menstruate between Days 1 and 5 post coitus (*p.c.*; the first day when spermatoza were identified in vaginal smears was designated Day 1 *p.c.*) [12]. Red-mastiff bats (*M. ater*) menstruate between Days 4 and 10 *p.c.* [14]. Significant growth of the endometrium occurs between Days 5 and 16 *p.c.* in short-tailed fruit bats [12]. It is thought that microchiropteran bats menstruate after coitus because of fertilization failure or early embryo loss [15]. The occurrence of menstruation gives those microchiropteran female bats another opportunity to establish pregnancy during the same breeding season [12].

Therefore, it is thought that there are two main differences in menstruation between bats and humans. 1) Endometrial mitotic activity in humans occurs during the preovulatory phase and reduces soon after ovulation, while most endometrial growth in microchiropteran bats is postovulatory [12]. 2) Microchiropteran bats menstruate only after coitus [12, 14], whereas human menstruation is controlled by pituitary and ovarian hormones and is not dependent on coitus [1, 5].

However, previous studies have shown menstruation only in microchiropteran bats in captivity [12–15], and it is not known whether megachiropteran bats also menstruate. Furthermore, previous studies on menstruation in bats have not included hormone analysis to ascertain whether histologic changes are correlated with endocrine changes, as in primate menstruation [16].

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FIG. 1. Observation of the vaginal bleeding of the fulvous fruit bat. The body weight of this one is 92.50 g, the full length from head to tail is 10.15 cm, and the forearm length is 8.30 cm. Magnified vaginal orifice during menstruation is on the lower left quarter.

We thus undertook the present study to investigate whether the fulvous fruit bat (*Rousettus leschenaulti*), a megachiropteran, menstruates in the wild. In China, this species is a pest of cultivated fruits. Like some other species in Asia [17, 18], adult females have two pregnancies per year and exhibit reproductive synchrony within a population [19]. A single young is delivered after a gestation period of about 125 days [19]. Births are significantly clustered in biannual peaks, and many females in the wild lactate simultaneously. We combined fieldwork to observe vaginal bleeding with laboratory investigations to study histologic and hormonal changes. We report for the first time, to our knowledge, that a megachiropteran, the fulvous fruit bat, exhibits true menstruation. Significantly, we demonstrate that fulvous fruit bats menstruate independently of coitus.

MATERIALS AND METHODS

Reagents

We used the following antibodies and reagents in this study: rabbit anti-P_4 receptor (PGR) antibody (Santa Cruz Biotech, Santa Cruz, CA), mouse anti-estrogen receptor 1 (ESR1) (Abcam Ltd., Cambridge, U.K.), rabbit anti-actin and mouse anti-cytokeratin (Santa Cruz Biotech), horse radish peroxidase (HRP)-goat anti-mouse immunoglobulin G (IgG) and HRP-goat anti-rabbit IgG (Zymed Laboratories, South San Francisco, CA), RNA*later* RNA stabilization reagent (Qiagen Corp., Hilden, Germany), streptomycin avidin-peroxidase (SP) Kit (Zhongshan Corp., Beijing, China), diaminobenzidine (DAB) (Santa Cruz Biotech), and Histomount reagent (Zymed).

Animals

All procedures involving animals were carried out in accordance with the Policy on the Care and Use of Animals, approved by the Ethical Committee, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. The fulvous fruit bats used in this study lived in an old, deserted house in Haikou (20°02′21.71″N, 110°20′37.13″E), China, and their identity was confirmed [20]. There were about 10 000 fulvous fruit bats in this colony.

The body weight and forearm length of each bat were measured to distinguish between adult and immature bats [21]. At least six mature female bats were randomly trapped alive with hand and mist nets from the colony every day from 11 November 2005. Vaginal smears and visual observations determined whether menstruation was occurring. The bats were released into their colony, and at least six more were caught each day until menstrual bleeding was observed (Day 1). From that day until the next menstrual bleeding, at least six adult female bats were captured for histology and hormone measurements every 3 days. In addition, seven female bats captured on Day 1 were housed in a laboratory in Haikou, China, for observation of the subsequent menstruation. Vaginal smears were taken daily to test whether menstrual bleeding had occurred.

Sample Collection

Blood (2–5 ml) was collected, and serum was prepared and stored at −20°C until hormones were assayed. The uteri and ovaries were collected from the

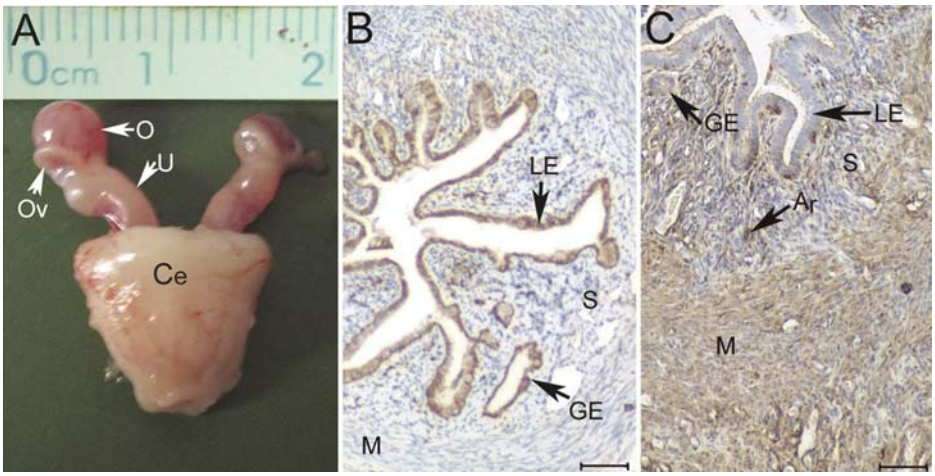

FIG. 2. Morphology of the fulvous fruit bat uterus and the histologic structure by cytokeratin and actin staining. **A**) The uterus of the fulvous fruit bat. Thin sections of the bat uterus stained with antibodies against cytokeratin (**B**) or actin (**C**), counterstained with hematoxylin. Bar = 50 μm. Ce, Cervix; U, uterine horn; O, ovary; Ov, oviduct; LE, luminal epithelium; GE, glandular epithelium; Ar, arteriole; S, stroma; M, myometrium.

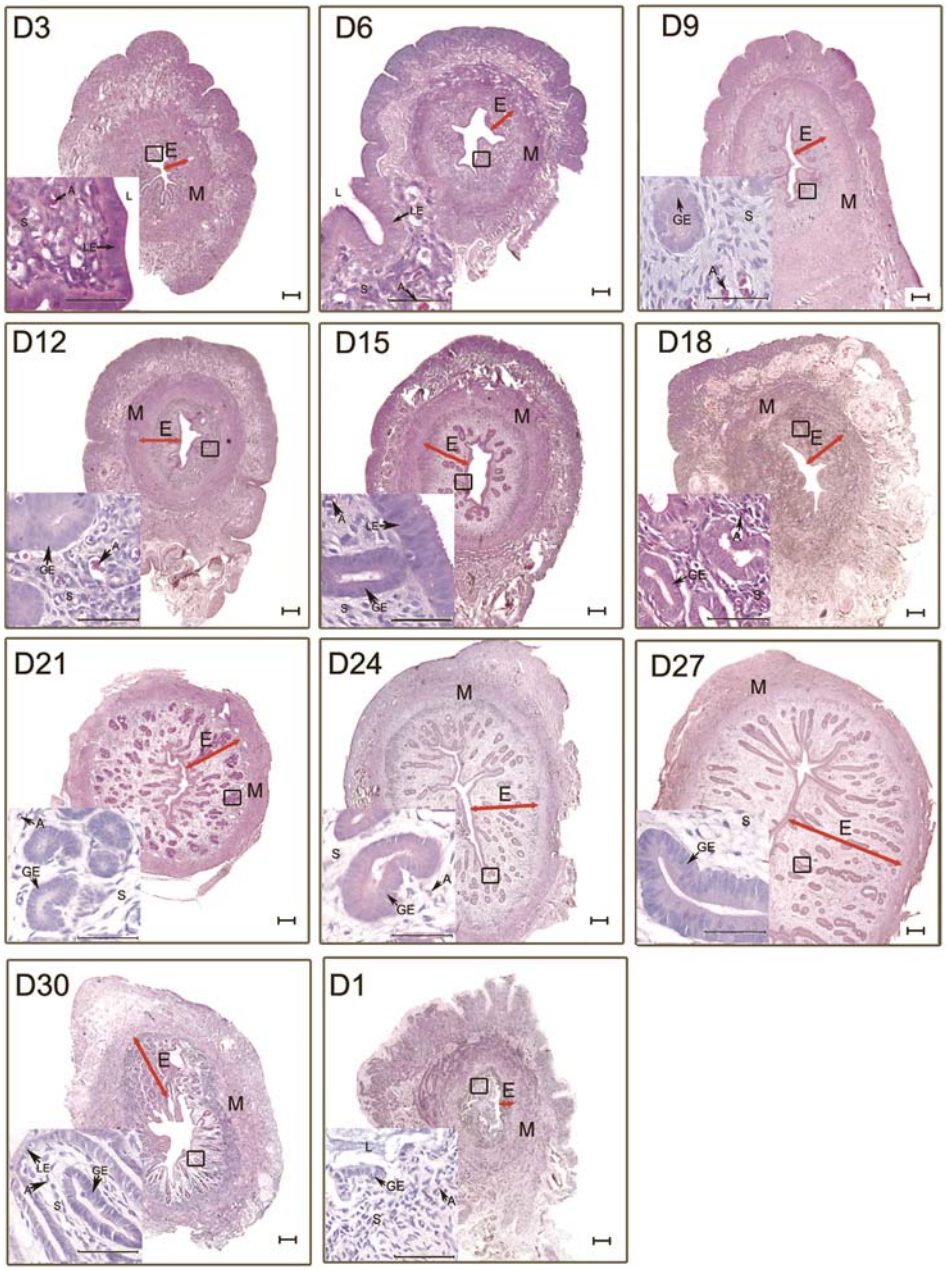

FIG. 3. Representative endometrial histologic structure of fulvous fruit bats at the indicated time of the menstrual cycle (menstruation occurred on Day 1 [D1]). The sections were stained with hematoxylin and eosin. The red line in each picture marks the thickness of the endometrium. Magnifying inserts showing LE, GE, arterioles, and stroma are on the lower left quarter of each picture. Bar = 50 μm. LE, Luminal epithelium; L, lumen of the uterus; GE, glandular epithelium; A, arteriole; S, stroma; E, endometrium; M, myometrium.

fulvous fruit bats under ethyl ether anesthesia. The uterine horn was cut into two parts. One part was stored in RNA*later* RNA stabilization reagent at −20°C for RNA and protein extraction. The other was fixed overnight in 4% paraformaldehyde solution at 4°C, dehydrated with graded ethanol solution (50%, 70%, 95%, and 100%), and then processed for paraffin embedding.

Immunohistochemistry

Immunohistochemistry was performed with the SP Kit as instructed by the manufacturer. Three slides from each of the six bats at each time point were examined. After being deparaffinized and hydrated, 5-μm sections were boiled in a microwave oven at 92–98°C for 15 min in citrate buffer (10 mmol/L citrate sodium and 10 mmol/L citric acid, pH 6.0) to retrieve antigen. After cooling to room temperature, sections were sequentially incubated at room temperature with 3% H_2O_2 in methanol for 15 min to quench endogenous peroxidase activity, normal blocking serum for 20 min, primary antibody (1 μg/ml) for 3 h, secondary antibody for 15 min, and avidin-conjugated HRP for another 15 min. Intervening PBS washes were necessary after each incubation, except after the normal serum blocking. Immunostaining was visualized with DAB, and the sections were counterstained with hematoxylin. Slides were then rinsed in 95% ethanol for 30 min and mounted with the Histomount reagent. To confirm the antigenic specificity of the antibodies used for immunostaining, Western blotting was performed, and negative controls with normal serum to replace the primary antibody were also included.

Hormone Assays

Serum concentrations of LH, FSH, estradiol-17 (E_2), and P_4 were determined by an RIA kit that was designed for the detection of human hormones. Serum samples from all the killed bats were sent to Beijing North Institute of Biological Technology for the detection of FSH, LH, E_2, and P_4. The intra-assay variation was less than 10% for FSH, LH, E_2, and P_4. The detection limit of sensitivity was 0.2 IU/L for FSH, 0.15 IU/L for LH, 5 pg/ml for E_2, and 0.2 ng/ml for P_4. All values were presented as means ± SEM.

RESULTS

Menstrual Bleeding in Wild-Caught Fulvous Fruit Bats

We began our observation of the fulvous fruit bat colony for menstruation on 11 November 2005. None of the female bats captured from 11 to 15 November showed visible vaginal bleeding. At dawn on 16 November 2005, 15 mature female

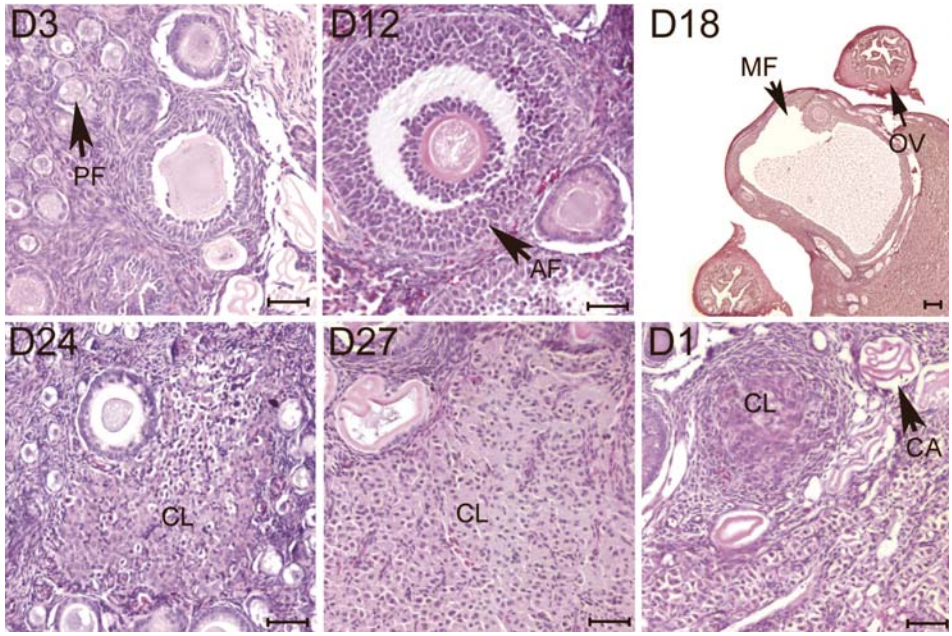

FIG. 4. Ovarian histologic structure of the fulvous fruit bats at the indicated time of the menstrual cycle (menstruation occurred on Day 1 [D1]). Sections were stained by hematoxylin and eosin. Bar = 50 μm. PF, Primordial follicle; AF, antral follicle; MF, mature follicle; CL, corpus luteum; CA, corpus albicans.

bats were caught, and all of them (100%) had vaginal bleeding (Fig. 1). Eight of these bats were killed for histologic and hormonal analyses, and the remaining seven were housed in a laboratory in Haikou, China, for further observation. The next day, six mature female bats were captured, and none exhibited visible vaginal bleeding, suggesting that menstrual bleeding in these bats lasted for only 1 day. We continued to conduct daily observations at the colony and to take at least 6 mature females every 3 days and found no vaginal bleeding until 19 December 2005, when all 10 mature females (100%) captured had vaginal bleeding. On the same day, all seven bats that had been housed in captivity since 16 November, with no male bats present, showed similar vaginal bleeding which lasted for only 1 day. On the basis of these data, we concluded that the menstrual cycle of fulvous fruit bats lasted for 33 days.

Morphologic Changes of the Uterus and Ovaries During the Menstrual Cycle

Histologic studies showed that the fulvous fruit bats had a bicornuate uterus and that the two uterine horns were symmetric (Fig. 2A). Each uterine horn measured 0.7 ± 0.02 cm in length. The cervix was broad and was shaped like a hemispheric bulb, 0.96 ± 0.02 cm in width. The ovaries were ellipsoidal, 0.37 ± 0.015 cm in length and 0.16 ± 0.012 cm in width.

To characterize changes in the uterus during the menstrual cycle, we employed antibodies against cytokeratin and actin to discern the epithelium and the blood vessels, respectively [22]. We confirmed by immunoblotting that these antibodies, which are raised against human proteins, recognized the 54-kDa bat cytokeratin and the 43-kDa bat actin, respectively. As expected, antibodies against cytokeratin marked the luminal epithelium (LE) and the glandular epithelium (GE) of the endometrium, whereas those against actin labeled the blood vessels and the myometrium of the uterus (Fig. 2, B and C).

To investigate the morphologic changes in the endometrium of bats during the menstrual cycle, uterine samples were collected at different stages of the menstrual cycle. The cycle phase was determined by endometrial morphology based on hematoxylin and eosin staining (Fig. 3). The uterine wall comprises two functional layers, the endometrium and the myometrium [5]. The endometrium of the fulvous fruit bat was shed (Fig. 3, D1) and regrown (Fig. 3, D3) during the menstrual cycle. On the basis of histologic analyses of numerous uterine sections of bats captured and sacrificed on various days between 16 November (Day 1) and 18 December (Day 33) 2005, we divided these changes into three phases: the proliferative phase (from Days 2 to 18), the secretory phase (from Days 19 to 33), and the menstrual phase (Day 1) (Fig. 3, showing representative photographs).

Proliferative Phase

During the early stage of endometrial proliferation, the endometrial layer was thin, the stroma was compact, and the glands were few in number. Dense capillary blood vessels were evident in the endometrial stroma near the myometrium (Fig. 3, D3 and D6). In the midproliferative phase, the stroma was edematous, and the glands, with long pseudostratified epithelia, increased in number (Fig. 3, D9, D12, and D15). During the late proliferative phase, the edema of the endometrial stroma was reduced (Fig. 3, D18).

Ovarian follicles developed during the proliferative phase (Fig. 4, D3, D12, and D18), and a preovulatory follicle was found in the ovary on Day 18 (Fig. 4, D18).

Secretory Phase

The secretory phase began after ovulation, presumed to be Day 19, and lasted until the menstrual phase of the next cycle. From Days 19 to 30, the endometrium thickened, and the glands had large lumina. The endometrial stromal cells became edematous during the midsecretory stage (Fig. 3, D21, D24, and D27) and decidualized during the late secretory phase (Fig. 3, D30). In the ovary, the corpus luteum gradually grew during the secretory phase (Fig. 4, D24 and D27).

Menstrual Phase

On Day 1 of the menstrual cycle, the upper two thirds of the endometrium were mostly shed along with vaginal bleeding. The endometria became thin, and the glands were sloughed off (Fig. 3, D1). The endometrial shedding was completed within the first day of the menstrual cycle, coinciding with vaginal

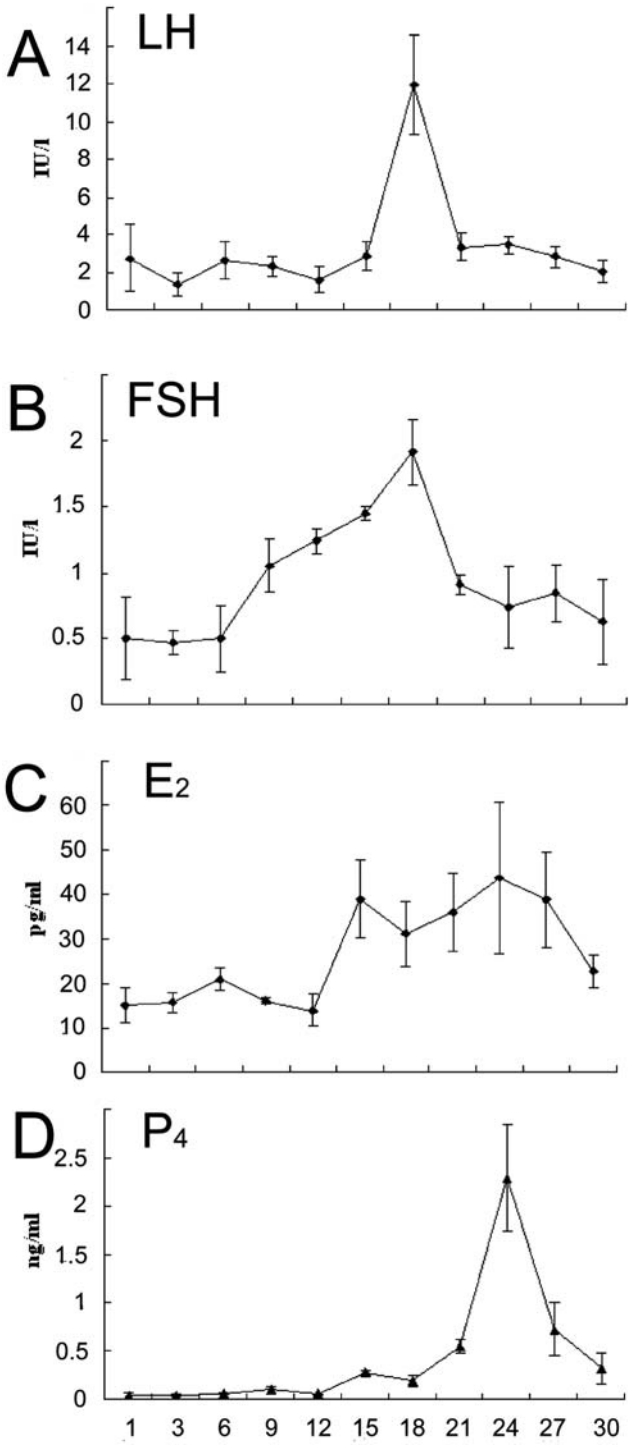

FIG. 5. Hormonal changes during the bat menstrual cycle. Serum levels of the hormones are expressed as international units per liter (**A** and **B**, LH and FSH), picograms per milliliter (**C**, E_2), or nanograms per milliliter (**D**, P_4). Shown are averages (with SEM) of six determinants, each with one female bat.

bleeding, which was evident only on the first day of the cycle. In the ovary, the corpus luteum was regressed on Day 1, which resulted in the formation of a corpus albicans (Fig. 4, D1).

Changes in FSH, LH, E_2, and P_4 During the Menstrual Cycle

To determine the hormonal changes during the menstrual cycle, we examined the serum levels of FSH, LH, E_2, and P_4 (Fig. 5). The FSH levels increased gradually during the proliferative phase (from Days 2 to 18), reaching a maximum on Day 18 (Fig. 5B). In contrast, the LH levels remained low during most of the proliferative phase but exhibited a clear surge on Day 18 (Fig. 5A), coinciding with the peak levels of FSH. Both FSH and LH levels decreased sharply after Day 18 and remained low during the secretory phase (Fig. 5, A and B).

On the one hand, the levels of E_2 rose during the proliferative phase and continued to rise after the LH surge to peak on Day 24 (Fig. 5C). There appeared to be a slight decrease in the E_2 levels between Days 18 and 21, although the decrease was not statistically significant (Fig. 5C).

On the other hand, the P_4 levels exhibited a clear surge around Day 24, which coincided with the maximum levels of E_2 (Fig. 5D). After that, E_2 and particularly P_4 levels rapidly decreased and reached basal levels on Day 30 (Fig. 5, C and D).

Steroid Receptor Protein Expression in the Uteri

We also determined the expression of receptors for estrogen and P_4 during the bat menstrual cycle. ESR1 was found in the stromal cells, GE, and LE. Strong ESR1 signals were seen during the proliferative phase (Fig. 6, D3, D9, and D15) and the early secretory phase (Fig. 6, D21), but low levels of ESR1 were found during the mid- to late secretory phase (Fig. 6, D27 and D30) and during the menstrual phase (Fig. 6, D1). Furthermore, ESR1 was found in both the cytoplasm and the nucleus.

Antibodies against PGR used in this study recognize both PGRA and PGRB. During the early proliferative phase (Fig. 6, D3 and D9), PGR staining was found mainly in the stromal cells around the arterioles. Faint signals were detected in the LE and GE. From the midproliferative phase to the midsecretory phase (Fig. 6, D15, D21, and D27), a strong PGR signal was seen in the LE and the stromal cells, whereas weak signals were found in the GE. During the late secretory phase, the level of PGR proteins increased in the GE and LE but decreased in the stromal cells (Fig. 6, D30). The stromal cells, LE, and GE also expressed detectable levels of PGR during the menstrual phase (Fig. 6, D1). As with ESR1, PGR was found both in the cytoplasm and the nucleus.

DISCUSSION

To our knowledge, this is the most comprehensive study thus far of menstruation in a nonprimate species. For the first time, we have observed two consecutive vaginal bleedings in a bat colony in its natural habitat. In addition, we have demonstrated that fulvous fruit bats exhibit all the characteristics, both morphologic and hormonal, of a true menstruation during the 33-day cycle.

During the proliferative phase (from Days 2 to 18), the high level of FSH (Fig. 5B) was correlated with ovarian follicle development (Fig. 4, D3, D12, and D18). Along with the growing follicles, elevated levels of E_2 were observed during this period. The elevated E_2 levels are consistent with its role in stimulating uterine endometrial proliferation, as indicated by endometrial thickening, the edema of stromal cells, and the increased numbers of glands and developing arterioles (Fig. 3). A clear surge of LH was observed on Day 18, together with the maximum level of FSH (Fig. 5, A and B). Histologically, a large mature follicle was observed in the ovary on the same day (Fig. 4, D18). These observations suggest that fulvous fruit bats ovulate on Day 18 of the menstrual cycle.

Following ovulation, FSH and LH rapidly returned to basal levels (Fig. 5, A and B), and granulosa cells began to luteinize, as indicated by the development of a corpus luteum (Fig. 4,

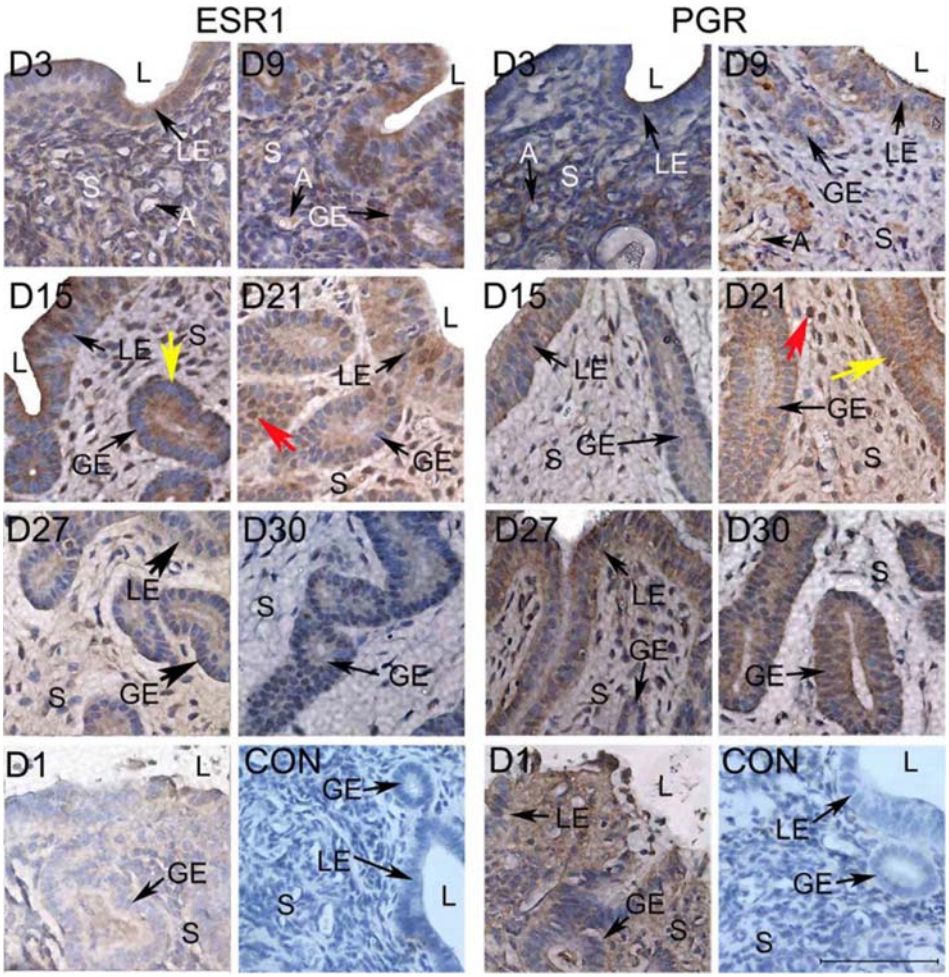

FIG. 6. Expression of ESR1 and PGR in the endometria. Shown are representative sections of the bat uterus at the indicated time of the menstrual cycle (menstruation occurred on Day 1), stained with antibodies to ESR1 (left two columns) or to PGR (right two columns). Both receptors can be seen in the cytoplasm (yellow arrow) and the nucleus (red arrow). Control sections were immunostained with normal serum in place of the primary antibody (CON). Bar = 50 μm. ESR1, Estrogen receptor 1; PGR, progesterone receptor; LE, luminal epithelium; L, lumen of the uterus; GE, glandular epithelium; A, arteriole; S, stroma.

D24 and D27). The high P_4 levels were consistent with its role in facilitating endometrial differentiation and glandular development (Fig. 3). At midsecretory phase, both stroma decidualization and glandular secretion were evident (Fig. 3, D24 and D27). These changes are consistent with endometrial preparation for implantation. If the ovum did not fertilize or the embryo did not implant, the corpus luteum regressed (Fig. 4, D1), which was accompanied by falling E_2 and P_4 levels (Fig. 5, C and D) during the late secretory phase. When E_2 and P_4 fell to the lowest levels, on Day 1, the upper two thirds of the endometrium sloughed off (Fig. 3, D1), and vaginal bleeding occurred (Fig. 1). In the ovary, the corpus luteum continued to regress and finally became a corpus albicans (Fig. 4, D1).

Because of the relatively large amounts of blood required for hormone determination, we collected blood from each bat only once during the whole cycle. As a result, large numbers of bats were used to derive the hormone profiles; this is partly responsible for the relative large SEMs of these determinations. Therefore, further research is required to determine whether E_2 levels exhibit a "twin-peak" profile. In particular, blood from the same animal on different days should be used to avoid individual differences.

The observation of two consecutive menstruations of a bat species in their natural habitat allows us to conclude that fulvous fruit bats menstruate with a 33-day cycle. The cycle length is further substantiated by the observation of two

TABLE 1. Comparison of menstrual cycle between the human and both suborders of bats.

Parameter	Human	Megachiropteran bats[a]	Microchiropteran bats[b]
Length of menstrual cycle (days)	28–35	33	NA
Day of ovulation	~Day 14	~Day 18	Day 2 *p.c.*[c]
Endometrial proliferative activity	Preovulatory	Preovulatory	Postovulatory
Endometrial secretory activity	Secretory phase	Secretory phase	NA
Stromal cell changes	Stroma decidualization	Stroma decidualization	NA
Hormonal changes			
FSH and LH peak	Day 12–15	Day 18	NA
E_2 peak	Day 11 and Day 22	Day 15 and Day 24	NA
P_4 peak	Day 22	Day 24	NA
Relationship between coitus and menstruation	Coitus-independent	Coitus-independent	Coitus-dependent

[a] Data from current study.
[b] NA, data unavailable.
[c] Data from short-tailed fruit bats.

consecutive vaginal bleedings of the seven bats maintained in captivity and by histologic and hormonal changes characteristic of a true menstrual cycle. In humans, the menstrual cycle lasts between 21 and 35 days, with an average of 28 days [5]. Equally important is our demonstration of true menstruation of 7 of 7 (100%) female fulvous fruit bats housed in the absence of male bats, thus for the first time establishing that fulvous fruit bats menstruate independently of coitus. Our results raise some doubt about previous reports that microchiropteran bats menstruate after coitus [12, 14]. Housing female microchiropteran bats in the absence of male bats, as done in our study of fulvous fruit bats, is required to firmly establish whether they display coitus-dependent menstruation.

Our study showed that the menstrual cycle of the fulvous fruit bat shared many similarities with those of the human menstrual cycle. We summarize the differences and similarities of menstrual cycles between humans and two suborders of bats in Table 1.

Menstrual dysfunctions, including menorrhagia (excessive menstrual blood loss), dysmenorrhea (painful periods), and amenorrhea (absent menstrual periods), are relatively poorly studied human conditions that affect millions of women of reproductive age [7, 23]. There are very few animal models currently used to study such menstrual dysfunction. So far, the best animal model is the baboon, which has been used extensively [24, 25]. An attempt to produce a mouse model of menstruation by P_4 withdrawal has been only partially successful, because although such mice appear to exhibit artificial decidualization in the endometrium, they exhibit few other endocrine and histologic characteristics of menstruating human females [7, 26]. The present study demonstrated that fulvous fruit bats exhibit true menstruation, with histologic and endocrine characteristics very similar to those in human menstruation. Fulvous fruit bats may represent a valuable nonprimate experimental model to study menstruation and menstrual dysfunction in humans.

In addition to the true menstruation as demonstrated in the present study, fulvous fruit bats share many other common reproductive features with humans. These include a functional corpus luteum [27], hemochorial placenta [28], long gestation period (normally about 125 days) with a single embryo (typically) [18], and two mammary glands [29]. These characteristics make the species even more attractive as the first small animal model for studying menstrual mechanisms and menstrual dysfunction.

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