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Sperm Storage, Delayed Ovulation, and Menstruation of the Female Rickett's Big-Footed Bat (*Myotis ricketti*)

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Zhe Wang, Bing Liang, Paul A. Racey, Yan-Ling Wang, and Shu-Yi Zhang (2008) Sperm storage, delayed ovulation, and menstruation of the female Rickett's big-footed bat (*Myotis ricketti*). *Zoological Studies* **47**(2): 215-221. We studied the reproductive cycle of female *Myotis ricketti*, a piscivorous bat widely distributed throughout Asia. Copulation occurred from Oct. to Apr., sperm was stored by females, and ovulation was delayed until arousal from hibernation (in May and June). Menstruation was observed during the preovulatory period; this is the 1st report of this phenomenon in the Vespertilionidae. The serum concentration of estradiol (E_2) dramatically increased at menstruation. Concentrations of progesterone (P_4) in non-pregnant bats did not significantly change. Experiments to explore the effect of hormones on sperm storage and delayed ovulation showed that females continued to store spermatozoa 1 wk after an ovariectomy; all oocytes were in meiosis I in Nov.; and the single graafian follicle developed by the ovary could not be induced to ovulate by 10 IU human chorionic gonadotropin in Dec. http://zoolstud.sinica.edu.tw/Journals/47.2/215.pdf

Key words: Histological section, Ovariectomy, Estradiol, Progesterone.

Sperm storage by female animals involves the retention of viable spermatozoa within the reproductive tract for prolonged periods (Neubaum and Wolfner 1999, Crichton 2000). Delayed ovulation by female bats means that a species-specific number of specialized vesicular follicles persists throughout hibernation, and ovulation is postponed until spring (Oxberry 1979). Menstruation in mammals is associated with damage to the uterine epithelium, interstitium, endothelium, and extracellular matrix (ECM), resulting in controlled bleeding, and tissue dissolution and repair (Bulletti el al. 1998).

Although sperm storage by females is widespread in insects (Baer et al. 2006, Collins et al. 2006, Adams and Wolfner 2007), reptiles (Gist and Congdon 1998, Yamanouye 2004), and birds (Das et al. 2006, Zhang 2006), in mammals it is almost entirely restricted to bats that can store sperm for months compared to a few days in other taxa (Racey 1975). At present, the mechanism of sperm storage has yet to be determined (Neubaum and Wolfner 1999). Studies on delayed ovulation of bats are limited and restricted to *Scotophilus heathi* (Abhilasha and Krishna 1996, Singh and Krishna 1996, Abhilasha and Krishna 1998, Krishna and Abhilasha 2000, Chanda et al. 2003). Menstruation has previously been documented in some primates, elephant shrews (Dixson 1998), and 2 families of bats: the Molossidae and Phyllostomidae (Rasweiler 1991, Rasweiler and de Bonilla 1992, Rasweiler and Badwaik 2000).

The piscivorous vespertilionid *Myotis ricketti* is widely distributed in China (Ma et al. 2003) where it was first described in 1894 (Thomas 1984). Our studies first described female sperm storage in *M. ricketti* in 2004 (Wang et al. 2005). This report extends observations to the entire

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year, provides the 1st description of menstruation in the Vespertilionidae, and explores the effects of hormones on sperm storage and delayed ovulation.

MATERIALS AND METHODS

Sample collection

One to 3 adult female *M. ricketti* were collected monthly (Feb. 2004 to Feb. 2006) from a cave (39°42′N, 115°43′E) at Fangshan in Beijing. In addition, a single female *M. ricketti* was obtained in July from the temple of Du-le Si in Tianjin Municipality (40°03′N, 117°24′E). Because of the IUCN conservation status of this endemic bat as lower risk/near threatened (Hutson et al. 2001), minimum numbers were used in our study. Bats were caught by hand during hibernation and by mist net when active, and environmental temperature and body weight were measured at that time.

Vaginal smears

Bats were immediately brought to the laboratory after capture and were killed by decapitation. Vaginal smears were obtained by aspirating distilled water introduced into the vagina with micropipettes, and the aspirate was examined in a phase-contrast microscope.

Histological studies

Each month, 1 or 2 female bats were selected for histological studies. After the bats were killed, the uteri and ovaries were removed and freed of extraneous fat and connective tissues. The reproductive tracts were weighed, measured, and flash-frozen in liquid nitrogen.

All of the frozen tissues were then fixed in 4% paraformaldehyde (PFA) for 12 h at 4°C, embedded in paraffin wax, serially sectioned at 6 μm, and stained with hematoxylin and eosin.

Measurement of serum estradiol and progesterone

Serum concentrations of estradiol-17 (E_2) and progesterone (P_4) were determined using a radioimmunoassay kit designed for detecting human hormones. Serum samples from all of the killed bats were sent to Beijing University Hospital for the detection of E_2 and P_4. The intra-assay variations were less than 10% for E_2 and P_4. The detection limit of sensitivity was 5 pg/ml for E_2 and 0.2 ng/ml for P_4. All values are presented as the mean ± standard error of the mean (SEM).

Ovariectomy

Five hibernating female bats were collected on 15 Jan. 2005. One day after capture, 4 females were anesthetized with ether, and 3 were given an ovariectomy, while the 4th underwent a sham operation. The 5th female served as a control with no surgery. Females 1-3 were respectively killed at 1, 4, and 7 d after the ovariectomy, and females 4 and 5 were killed on day 7. During the experiment, the bats were housed in a dark cage at 12-17°C.

Oocyte collection and culture

A female captured on 28 Oct. 2005 was kept at room temperature (12-17°C) and killed 6 d later. Oocytes were removed from the its ovaries and cultured in M2 (Sigma, St. Louis, MO, USA) medium at 37°C in a humidified atmosphere of 5% CO_2.

Inducing ovulation by human chorionic gonadotropin (hCG)

Three females were captured on 20 Nov. 2005 and housed at 12-17°C in the dark. Bats were provided with water but no food, to simulate natural environmental conditions during winter. On 5 Dec. 2005 they were each injected with 10 IU hCG (Sigma) intraperitoneally. Twenty-four hours after the injection, they were killed and the ovaries and reproductive tracts were removed.

RESULTS

Observations in nature

Although females of *M. ricketti* were seen in the cave year round, they were less frequently encountered in summer months (May-Aug.), and especially in July when no female was found. Most bats were present from Sept. to Nov., when numbers in the cave increased to at least 2000, and the bats actively emerged to forage at dusk.

Bats hibernated from late Nov. to early Apr., with occasional periods of arousal. The winter temperature in the cave ranged from 8.4 to 12.3°C, whereas in summer it rose to 20.7°C. Hundreds of lactating female *M. ricketti* and a few associated males were found in the temple of Du-le Si (40°03′N, 117°24′E) in summer and early autumn (June-Sept.-), but were not be seen there in winter or spring.

The body weight of female *M. ricketti* was highest in Oct. when food was abundant and copulation had just begun (Table 1). Body weight declined during winter and spring.

Vaginal smears

From late Sept. to mid-Nov., vaginal smears from 6 animals examined contained 70%-99% keratinized epithelial cells. Many spermatozoa were present in the smears of 3 females captured on 17 Nov. 2004, but none was found in other months. The incidence of keratinized epithelial cells, epithelial cells, and leucocytes varied in other months.

Histological studies

The uteri and ovaries of non-pregnant bats were similar in appearance (Fig. 1). The uterus of *M. ricketti* is bicornuate and weighed 10.1 ± 0.13 mg (*n* = 12), while the uterine cornua are 1.5-3 mm in length. The ovary is spherical with a diameter of 1-1.5 mm. Oviducts surround the ovary along with some fat. The ovaries were similar in appearance and each weighed approximately 2.2 ± 0.10 mg (*n* = 12). No vaginal plug was observed.

A single graafian follicle was observed in the ovary from Oct. to June except in May (Fig. 2). During this period, sperm were present in the uterotubal junction (Fig. 2).

A menstrual bat was captured and killed on 4 June 2004. The cavity of the uterine was larger than that of non-menstrual bats and contained

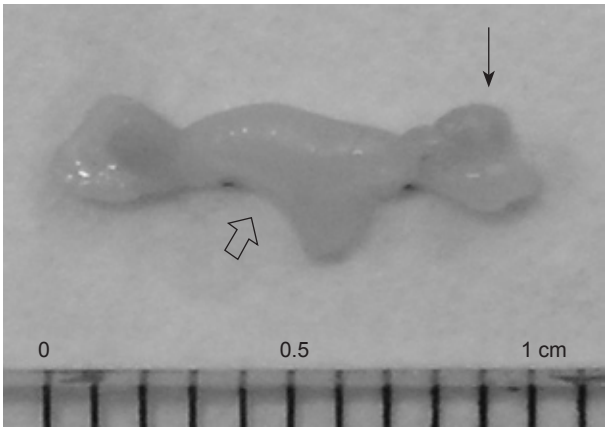

Fig. 1. Bicornuate uterus and ovaries of a female *M. ricketti* killed on 22 Feb. 2006. The hollow arrowhead indicates the right uterine corn, and the solid arrowhead indicates the left ovary.

Table 1. Body weights and blood serum concentrations of estradiol (E_2) and progesterone (P_4) of the female *Myotis ricketti*

Item	Copulation, sperm storage, and delayed ovulation						
Month	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.
BW (g) mean ± SD	33.0 ± 1.9 (13)	25.9 ± 2.1 (3)	25.4 ± 0.3 (3)	24.6 ± 2.1 (6)	23.5 ± 2.6 (11)	23.4 ± 2.2 (6)	
E_2 (pg/ml) mean ± SEM	1 ± 1 to 36 ± 8 (13)						
P_4 (ng/ml) mean ± SEM	2.1 ± 0.1 to 56.4 ± 0.9 (12)						

Item	Menstruation, ovulation, and pregnancy		Pregnancy and lactation		
Month	May	June	July	Aug.	Sept.
BW (g) mean ± SD		22.4 ± 0.5 (2)		23.6 ± 1.4 (6)	25.7 ± 2.2 (9)
E_2 (pg/ml) mean ± SEM	270 ± 22 (1)[a]		10 ± 3 to 60 ± 10 (5)b		
P_4 (ng/ml) mean ± SEM	32.8 ± 0.7 (1)[a]		8.3 ± 0.4 to 18.7 ± 0.6 (3)[b]		

Numbers of bats for each test are given in parentheses. BW, body weight; SD, standard deviation; SEM, standard error of the mean; E_2, estradiol; P_4, progesterone. [a]a menstruating bat; [b]non-pregnant bats.

a number of spermatozoa (Figs. 3A, B). The decidualized endometrial tissue and uterine glands were necrotic and had sloughed off due to bleeding. A preovulatory follicle was found in the ovary (Fig. 3C). Another female killed on the same day had a graafian follicle (Fig. 2J) in the ovary, and its endometrial tissue and uterine glands in the uterine corpus were degenerating (Fig. 3D).

Concentrations of estradiol (E_2) and progesterone (P_4)

Serum concentrations of E_2 were much higher during preovulatory menstruation than during copulation and lactation (Table 1). Concentrations of P_4 in non-pregnant bats varied within a range which differed little from month to month (Table 1).

Ovariectomy

Spermatozoa were still being stored (mainly in the uterotubal junction) 7 d after the ovariectomy in the 3 bats and also in the 2 controls. Histology revealed no apparent difference between sperm storage in intact and ovariectomized bats.

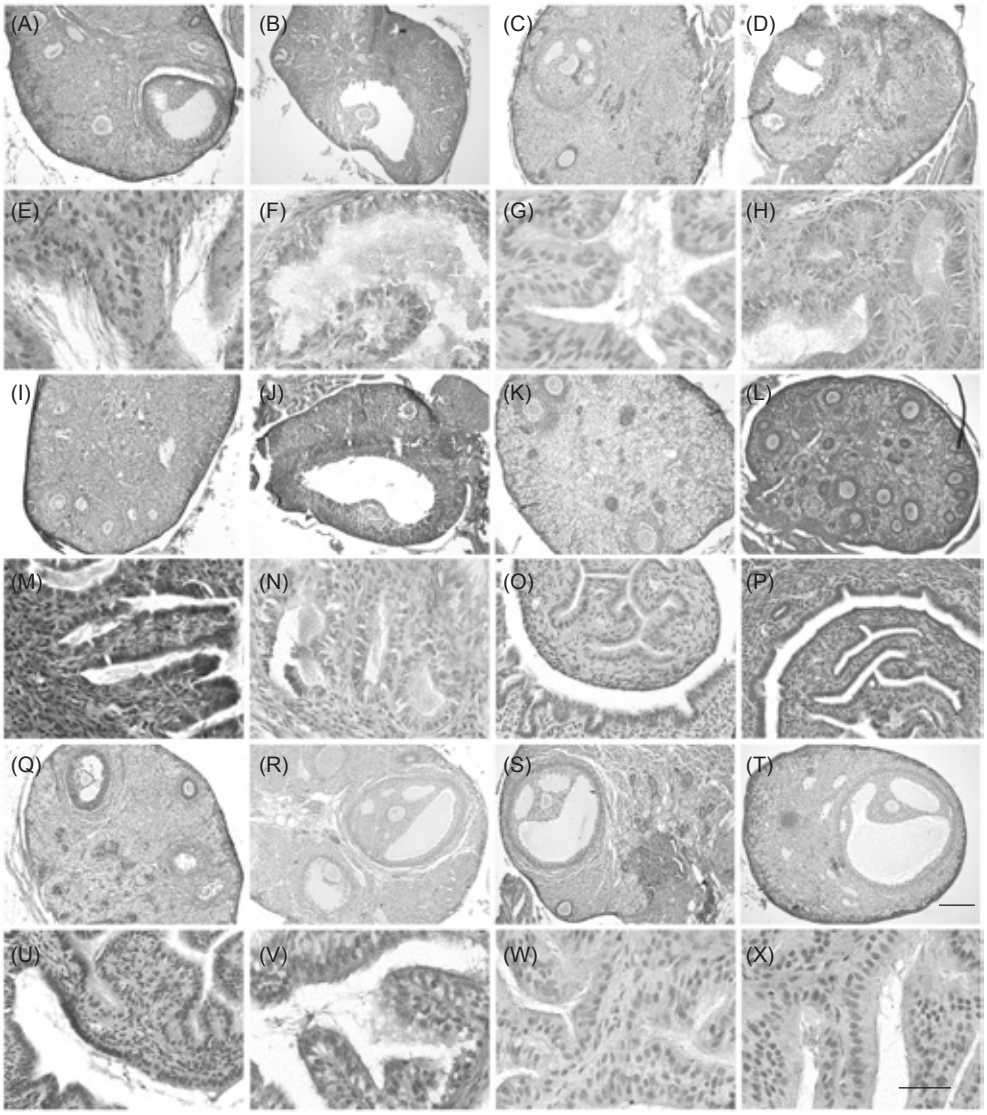

Fig. 2. Sections of ovaries and uterotubal junctions from 12 bats taken during successive months. (A-D, I-L, Q-T) Sections of ovaries. Scale bar = 200 μm. (E-H, M-P, U-X) Sections of uterotubal junctions. Scale bar = 40 μm. Dates: (A, E) 16 Jan. 2005; (B, F) 24 Feb. 2004; (C, G) 30 Mar. 2005; (D, H) 4 Apr. 2004; (I, M) 1 May 2004; (J, N) 4 June 2004; (K, O) 10 July 2004; (L, P) 22 Aug. 2004; (Q, U) 21 Sept. 2004; (R, V) 22 Oct. 2004; (S, W) 17 Nov. 2004; (T, X) 30 Dec. 2004. Hematoxylin and eosin.

Oocyte collection and culture

Twelve oocytes including 2 at the germinal vesicle (GV) stage (Fig. 4) were collected from the pair of ovaries. They were all at stage meiosis (M) I, and became darkened and died after 16 h. The oocytes did not progress to germinal vesicle breakdown (GVBD) or MII as indicated by the presence of the 1st polar body.

Inducing ovulation by hCG

Treatment with 10 IU hCG did not induce ovulation in *M. ricketti*. All 3 bats treated with hCG still contained a single graafian follicle in the ovary and stored spermatozoa in the reproductive tracts.

DISCUSSION

Sperm storage

The maximum period of sperm storage hitherto reported in bats is 198 d in female *Nyctalus noctula* (Racey 1973). We found spermatozoa in female *M. ricketti* in a female reproductive tract on 22 Oct. and they were last found on 4 June, a period of 225 d. Since sperm are allogeneic antigens in the female reproductive tract, it is remarkable that viable spermatozoa (Racey 1973 1975 1979) would be found in a female reproductive tract over such an extended time. This may be associated with immunologic recognition and immune tolerance.

Sperm storage by female bats is significant for 3 reasons: it allows the animal to wait for maturation of the ovum and not miss an opportunity for fertilization, and it fosters sperm competition. Spermatozoa produced in autumn have to wait for ovulation in spring because of the asynchrony of gamete maturation. If the female does not copulate before or during the ovulatory period, it misses the only chance to become pregnant in the year. As reported in a wide range of bat species (Wilkinson and McCracken 2003), sperm competition (Ginsberg and Huck 1989, Birkhead and Moller 1993, Bernard et al. 1997) may occur in *M. ricketti*, because the testis mass of reproductively active males reached 0.050 ± 0.006 g at a body weight of 25.11 ± 1.90 g (unpubl. data),

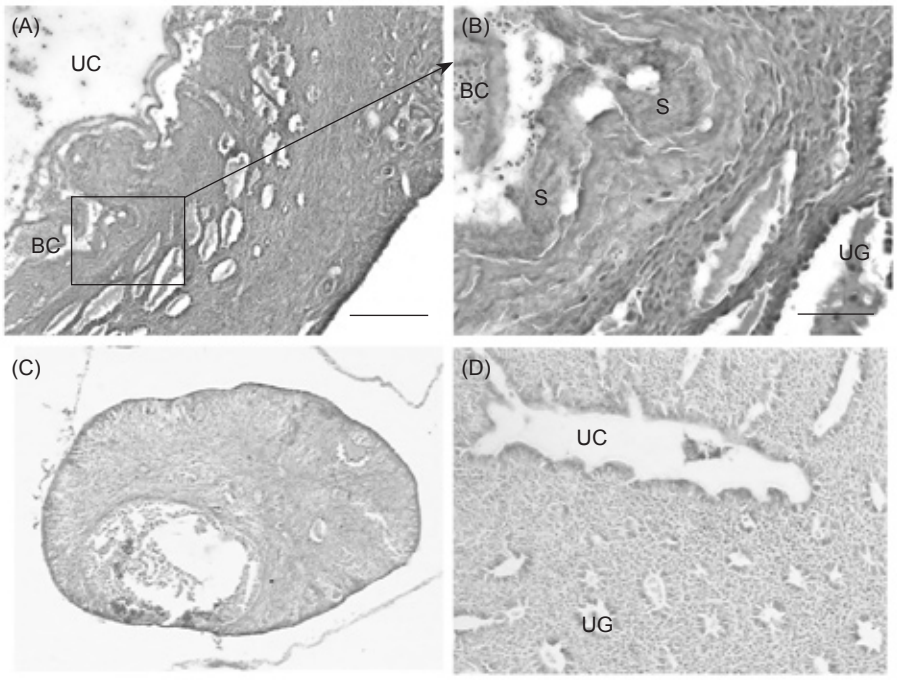

Fig. 3. Sections of uteri and the ovary from 2 *Myotis ricketti* captured on 4 June 2004. (A, B) Sections of the menstrual uterus. B is a magnified area of (A). UC, uterine cavity; UG, uterine gland; BC, blood cells; S, sperm. Scale bar (A) = 200 μm; (B) = 35 μm. (C) Section of the ovary of the same female in A containing a preovulatory graafian follicle. (D) Section of uterus from the other female *M. ricketti* captured the same day. This animal carried the graafian follicle shown in figure 2J. Its endometrial tissue and uterine glands in the uterine corpus were undergoing apoptosis. Magnification of C and D is 81×. Hematoxylin and eosin.

and a high relative testis size is now assumed to be a reliable indicator of sperm competition (Hosken 1997, Tomkins and Simmons 2002, Parker and Ball 2005).

Delayed ovulation

Compared with sperm storage, there have been fewer investigations of delayed ovulation in bats, and the maximum period of delayed ovulation previously reported was up to 5 mo (Oh et al. 1985). In the present study, we observed a single graafian follicle in an ovary of *M. ricketti* for 5-7 mo (late Oct.-early Apr. or early June). Like sperm storage, delayed ovulation is a distinct phenomenon in bats, especially since, in most species, only a single graafian follicle is maintained in the ovary throughout hibernation.

Since ovulation occurs several months after copulation, *M. ricketti* is most likely a spontaneous ovulator. Culture of the oocytes and treatment of intact females with hCG demonstrated that the single graafian follicle was not mature enough to ovulate during hibernation.

Menstruation

Menstruation has been reported in 4 species of Neotropical bats belonging to the Molossidae and Phyllostomidae (Rasweiler 1991, Rasweiler and de Bonilla 1992, Rasweiler and Badwaik 2000), whereas *M. ricketti* is a temperate-zone vespertilionid. Menstruation in *M. ricketti* commenced during the preovulatory period which is similar to other menstruating bats and as with humans, serum levels of E_2 rose dramatically at that time. In addition, the non-menstrual female captured on the same day with the menstrual one may have been about to menstruate, because its endometrial tissue and uterine glands were becoming necrotic.

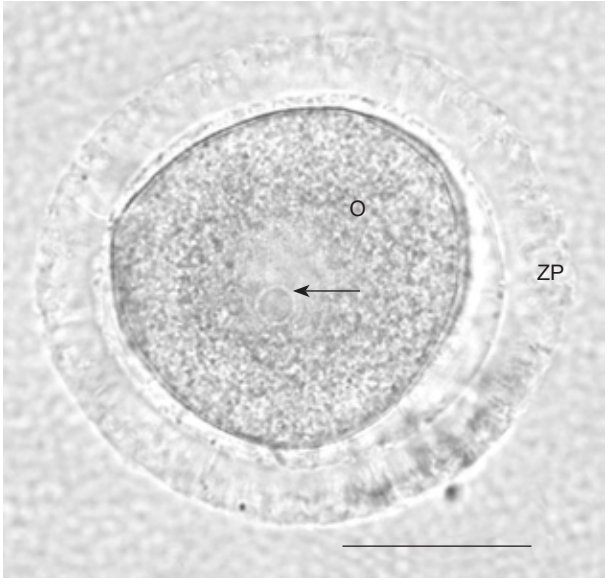

Fig. 4. Germinal vesicle-stage oocyte of *Myotis ricketti* killed on 3 Nov. 2005. The arrowhead indicates the germinal vesicle. O, ovum; ZP, zona pellucida. Scale bar = 40 μm.

Pregnancy and lactation

Since females of *M. ricketti* are seldom seen in the cave from May to Aug. and were not found there during July, it is most likely that pregnant bats move elsewhere during pregnancy and lactation. This was supported by the discovery of a group of predominantly females of *M. ricketti* which were lactating at a temple in Du-le Si (located about 180 km from the cave) from June to Sept. So, *M. ricketti* appears to ovulate and become pregnant from May to early June, when males cease storing sperm (unpubl. data), and the females began to roost separately from males. They give birth from mid-June to July, when newborn bats were first found in Du-le Si, and then lactate until Sept. before copulation.

Future work

Female *M. ricketti* appears to be a suitable animal model for studies of several reproductive phenomena in mammals, such as sperm storage, delayed ovulation, menstruation, and immune tolerance. More in-depth molecular and genetic studies are needed to shed further light on these phenomena.

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