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Reproductive physiology of *Hipposideros terasensis* in Taiwan

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Abstract. Changes in habitat and resource availability have induced bats to develop strategies for reproductive delay, including the processes of insemination, embryo implantation, and fetal development. *Hipposideros terasensis* used the strategy of delayed embryonic development. Bats mated in autumn. Fertilized eggs implanted during the blastula stage, but remained dormant for as long as five months before developing. In male bats, concentrations of testosterone increased from baseline to peak (54.3 ± 4.7 ng/ml) in August, corresponding with spermatogenesis. For female bats, concentrations of progesterone increased to peak levels (27.2 ± 6.8 ng/ml) between February and April, before declining back to baseline after birth in June. Increase and decline coincided, respectively, with resumption of embryo development (March to May) and birth (May to June). Compared to other hibernating species or species with similar reproductive patterns, *H. terasensis* had similar reproductive quiescence and changes and regulation of reproductive hormones.

Key words: delayed embryonic development, *Hipposideros terasensis*, progesterone, Taiwan, testosterone.

There are three well-known types of reproductive delay in bats: delayed fertilization (sperm storage), delayed implantation, and delayed embryonic development (Bradshaw 1962; Fleming 1971; Oxberry 1979; Kimura and Uchida 1983; Heideman 1989; Altringham 1996). For any pattern of delayed reproduction, the hormonal change plays an important role in regulating behavior.

In *Miniopterus shreibersii natalensis* in Africa, concentration of plasma progesterone shows two peaks: one during delayed implantation (Van der Merwe and Van Aarde 1989) and one 216–222 days after fertilization. The second peak coincided with the increase in placental weight. In *Tadarida brasiliensis mexicana*, which shows no hibernation or delayed reproduction, the concentration of plasma progesterone steadily increased until mid-pregnancy (Jerrett 1979). There were two peaks in progesterone concentration in the annual reproductive cycle of *Macrotus californicus* which includes delayed embryonic development (Burns and Easley 1977). The lower peak coincided with retarded embryonic growth rate. The larger peak corresponded with accelerated embryonic and placental development. *Miniopterus scheribersii fuliginosus*, which lives in temperate Japan, hibernates immediately after mating in November. This bat has a 60-day delay until mid-December and early January when the blastocyst begins to implant (Kimura et al. 1987). There is a second delay in fetal development from early January to March. During this time, plasma progesterone concentration does not increase, even though it is lower than the pre-implantation period during hibernation. Fetal development progresses rapidly after bats wake from hibernation (Kimura et al. 1987). Kimura et al. (1987) also discovered that concentrations of plasma progesterone are closely related to volume changes in corpus luteum during pregnancy. Meenakumari et al. (2009) found two parturitions in *Cynopterus sphinx*, with the first pregnancy having delayed embryonic development. Plasma progesterone levels were stable during the two-month delay, before increasing steeply to a peak in the third month.

The main functions of progesterone are to promote endometrial thickening and inhibit uterine contractions, thus maintaining a normal pregnancy (Vander et al. 1990). In bats, the two primary sources for progesterone are the corpus luteum and the placenta. Changes in progesterone are closely related to embryonic implantation and development.

In male reproductive activities with no sperm storage,

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Macrotus californicus shows a synchronous pattern of plasma testosterone concentration changes which coincided with spermatogenesis and development of the accessory genital glands, whereas when *Rhinolophus capensis* has stored sperm, plasma testosterone concentration increases with increasing spermatogenic activity, but, as the concentration declines, the accessory genital glands increase in size (Martin and Bernard 2000). Testosterone, synthesized by stimulating the lutenizing hormone receptor in Leydig cells, primarily acts to induce development of male accessory genital glands.

Bats in Taiwan with known reproductive cycles were histologically investigated, to find that only *Hipposideros terasensis* had delayed embryonic development. The reproductive cycles in this species were strongly synchronized between females and males (Chen 1998). Spermatogenesis stopped at the end of August. No sperm was left in the epididymis. Testis and epididymis atrophied in September. Ovaries developed corpus lutea near the end of August, when an unimplanted morula was seen in sections of the uterus, greatly suggesting a high degree of synchronization in reproduction of both males and females. After fertilization, the embryo was implanted immediately, but remained in the gastrula stage until the end of February of the next year. It quickly developed in March, with the mother giving birth in late May. This species has a six-month period of delayed embryonic development.

Although *H. terasensis* still shows a pattern of reproductive delay, in comparison to other types of delayed reproduction in other regions, there is a slight difference in the duration of pregnancy. Moreover, there is no existing study on the control of reproduction by hormonal changes of genus *Hipposideros* in East Asia. The objective of this study is to examine the annual changes in reproductive hormones of *H. terasensis*: progesterone and testosterone. We examined progesterone for its regulation of the different stages of embryonic development. We examined testosterone because its role in spermatogenesis can demonstrate synchrony in male and female *H. terasensis* reproductive cycles.

Study site and method

Study site and sample collection

The sampling took place in an abandoned tunnel in Chungliao, Nantou County, Taiwan (120°44'24.93"E, 23°53'17.63"N). Because of the strong reproductive synchronization of both sexes (Chen 1998), sample size was minimized to three adult bats of each sex each month from March 2006 to February 2007. During daylight, when bats were still in the cave, we captured bats with a net. Each month, we limited the number of intrusions into the cave to two. Age of male bats was determined by swelling of the frontal sac. Age of female bats was determined by swelling of nipples or false nipples. Color was also used to estimate age because darker colors indicate younger bats and brownish colors indicate adult bats (Cheng 2004). To minimize the duration of disturbance, we left the cave area as soon as we had collected the samples.

Measurement and analysis of reproductive hormones

Within four hours after capture and anesthetization by ether, bat external features were recorded: sex, weight, reproductive status, and lengths of forearms, tail, thumbs (with the claws), ears, hind feet, and tibia. Blood was extracted by cardiac puncture. Serum was isolated and stored at –20°C after separation centrifuging at 3,000 rpm for 10 minutes at 4°C. Serum concentrations of testosterone and progesterone were measured by enzyme-linked immunosorbent assay (R&D Systems, Inc, USA). Optical density was determined at 450 nm using a μQuant microplate reader (Bio-Tek, Winooski, USA), and we set the blank well as zero. The limit of progesterone detection was 0.08 ng/ml. The intra- and inter-assay coefficients of variation were less than 0.06%. The limit of testosterone detection was 0.03 ng/ml. The intra- and inter-assay coefficients of variation were 0.09 and 0.12%, respectively.

Histological analysis

Reproductive organs (testis and epididymis for males and uterus and ovaries for females) were dissected and fixed in a solution of formalin, ethylalcohol, and acetic acid which was replaced with different concentrations of t-butanol acid (Willig 1985). These samples were embedded in paraffin and sectioned into 7 μm thick slides. The slides were stained with haematoxylin and eosin (Preece 1978). The collected bats were sent to the National Museum of Natural Science, Taichung, Taiwan, to be stored as skeletal, fur, or fluid-preserved specimens.

Results

Weight

As for the monthly weight changes in male bats, the highest weight (74.2 ± 10.2 g) was observed in July and

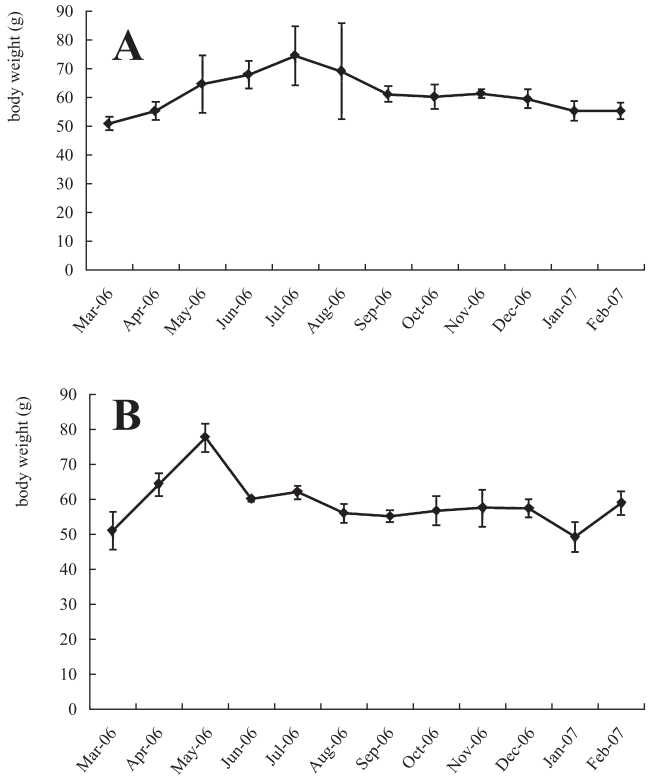

Fig. 1. Monthly changes of body weight of male (A) and female (B) *Hipposideros terasensis* collected from March 2006 to February 2007. Vertical lines indicate standard deviation from a sample size of three.

the lowest (50.7 ± 2.34 g) in March. The minimum average weight of female bats was seen between January and March (49.1 ± 4.3 g), and it gradually increased afterward, until it peaked in May (77.3 ± 4.0 g) (Fig. 1A and B). From the monthly trend of weight change, it was concluded that bats had the lowest weight during winter. Then, it significantly increased during spring, until it plateaued during summer and autumn.

Reproductive cycle of male bats

Histological examination

During the non-reproductive season, it is difficult to observe testis externally. Only when the testis swells during the reproductive season can it be directly palpated.

Histology of testis from males collected in May 2006 revealed mitosis in primary spermatocytes of seminiferous tubules, indicating early spermatogenesis (Fig. 2A). In August, the process continued (Fig. 2B). By September, the tubules were empty and the process terminated (Fig. 2C). Ductus epididymis from bats collected in March 2006 were rather skinny. They started to swell in April, becoming hollow (Fig. 3A). In May, there was still no sign of sperm. By June, they were filled with

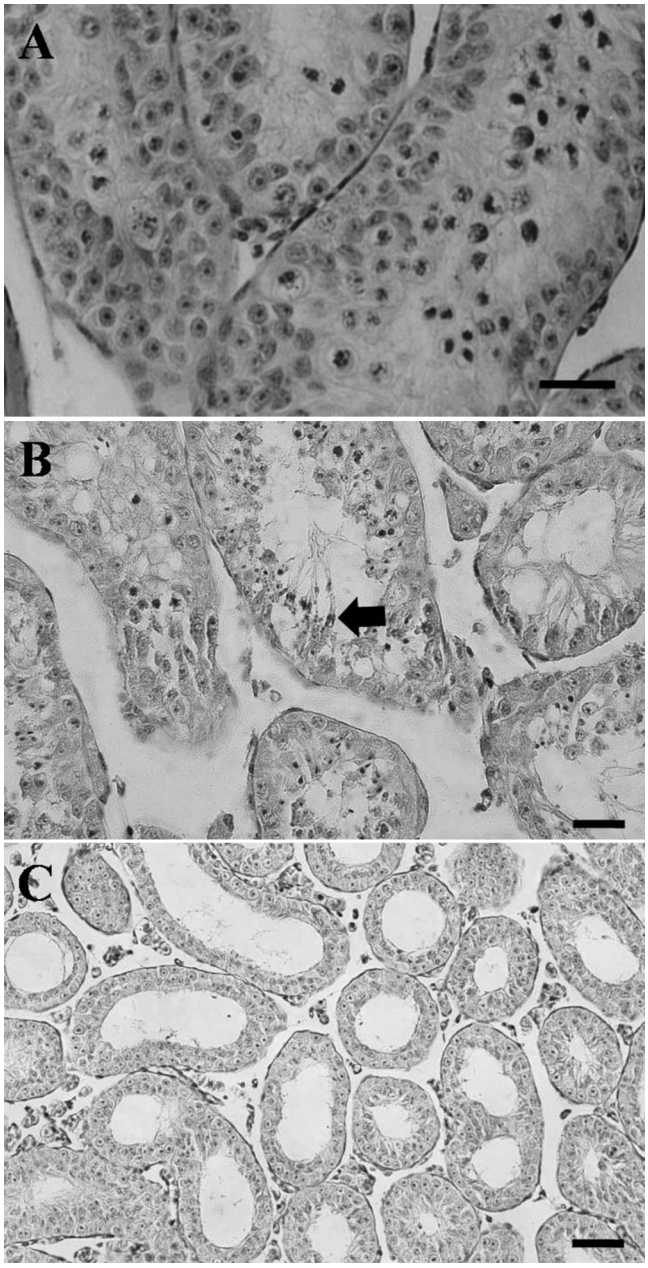

Fig. 2. Testicular changes of adult *Hipposideros terasensis* in central Taiwan: A) early stage spermatogenesis in May; B) lumen with spermatozoa in August; and C) regressed seminiferous tubules and empty lumen in September. Scale bars represent 160 μm. The arrow indicates active spermatocyte.

sperm (Fig. 3B). By September, ducts were hollow again (Fig. 3C). Ductus epididymis began to shrink in August.

In summary, the histological observation of changes in the testis and epididymis of the adult males reveals that the process of spermatogenesis of the testis occurred in May and June, while the appearance and the storage of sperms in the epididymis occurred between June and August.

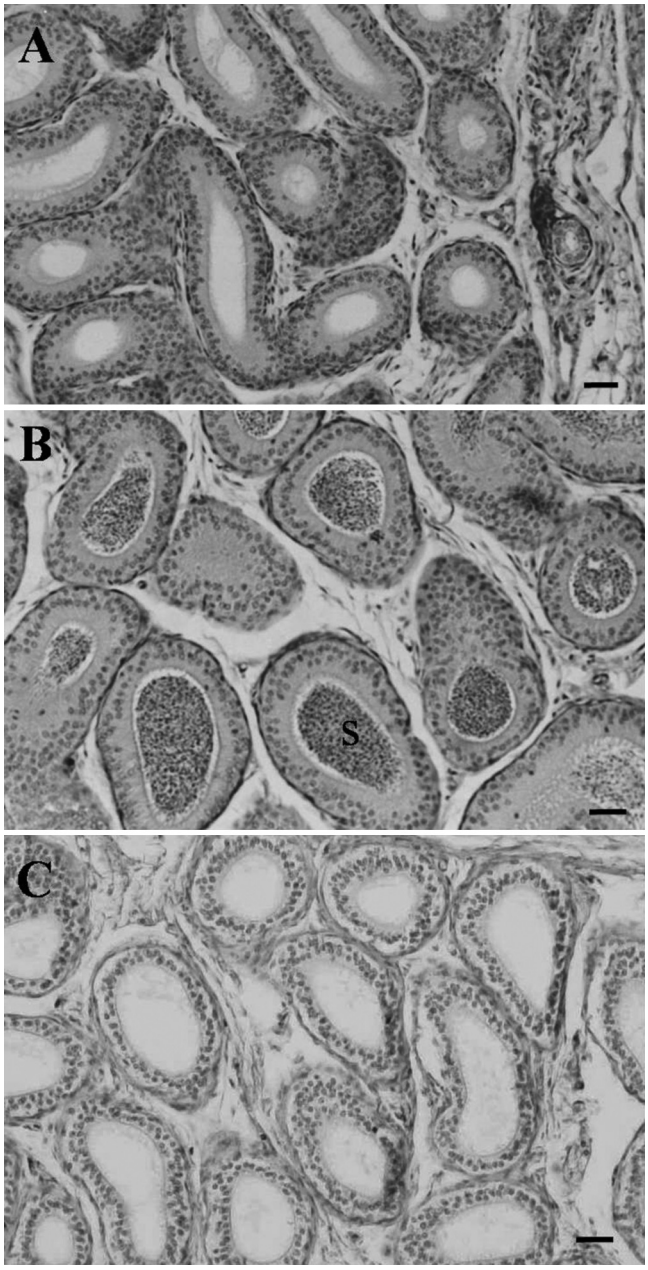

Fig. 3. Changes in cauda epididymidis of adult *Hipposideros terasensis* in central Taiwan: A) lumen enlarged and empty in April; B) lumen enlarged with abundant sperm (s) in June; and D) lumen empty in September. Scale bars represent 160 μm.

Changes in concentration of testosterone

The concentration of testosterone in the serum of *H. terasensis* showed seasonal changes. It was at the lowest level (2.0 ± 0.0 ng/ml) from March to May (time without spermatogenesis) and started to increase from May to June (during the time of spermatogenesis), reaching its peak of 54.3 ± 4.7 ng/ml in August (Fig. 4). By the end of August, the process of spermatogenesis had terminated and the concentration of testosterone also started to decline (Figs. 2A and 4).

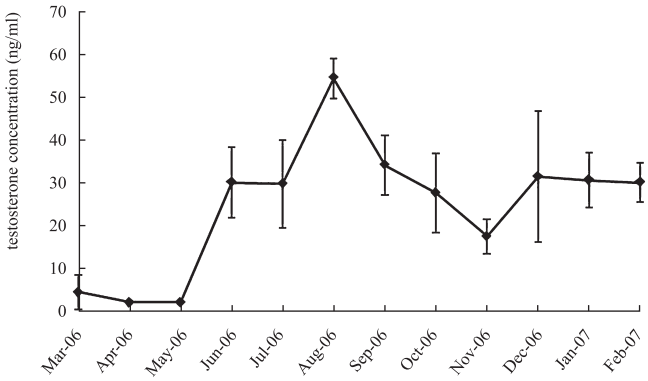

Fig. 4. Changes in serum testosterone concentration in male *Hipposideros terasensis* from March 2006 to February 2007. Vertical lines indicate standard deviations from a sample size of three.

Reproductive cycle of female bats

Histological examination

Morphologically, the female bat has a bicornute uterus and the ovaries are located at the tips of the uterine corners. Direct palpation of specimens collected in April could reveal the female was pregnant.

From February to March 2006, corpus lutea were detected in ovaries (Fig. 5A). In August 2006, Graafian follicles were seen. Corpus lutea were seen again in September 2006, with atretic follicles (Fig. 5B and C). Therefore, ovulation should occur between August and September. In specimens from February 2006, embryos in the gastrula stage were observed in the uterus, which had a developed placental structure (Fig. 6A). In March, the neural plate was in the primitive streak stage (Fig. 6B). On April 7th, fetuses had visibly black eyes, four limbs, and tail bumps. Most female specimens had given birth by early June. No histological slides showed signs of ovulation or fertilization. We observed embryos in the gastrula stage in September (Fig. 6C).

Changes in concentration of progesterone

The concentration of serum progesterone in female bats started to increase from a level of 13.9 ± 11.5 ng/ml in March to the maximum of 27.18 ± 6.8 ng/ml in April 2006. The concentration then dropped to 16.8 ± 1.0 ng/ml after parturition in May and further dropped to the lowest point of 1.9 ± 0.9 ng/ml in June 2006. Between July and September, it then gradually increased to 11.6 ± 2.5 ng/ml before yet another decline. There was a second peak in September 2006 after ovulating, fertilization,

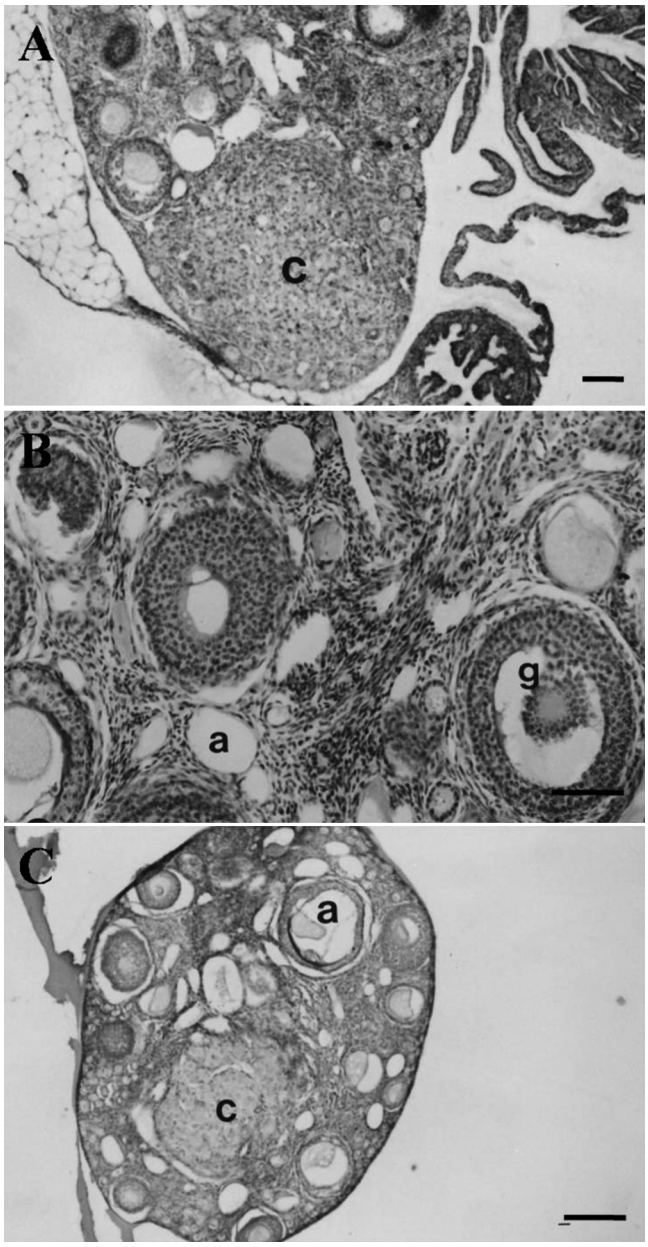

Fig. 5. Ovarian cycle of female *Hipposideros terasensis*: A) single corpus luteum (c) in February; B) Graafian follicles (g) in August; and C) corpus luteum (c) and many artretic follicles (a) in September. Scale bars indicate 320 μm.

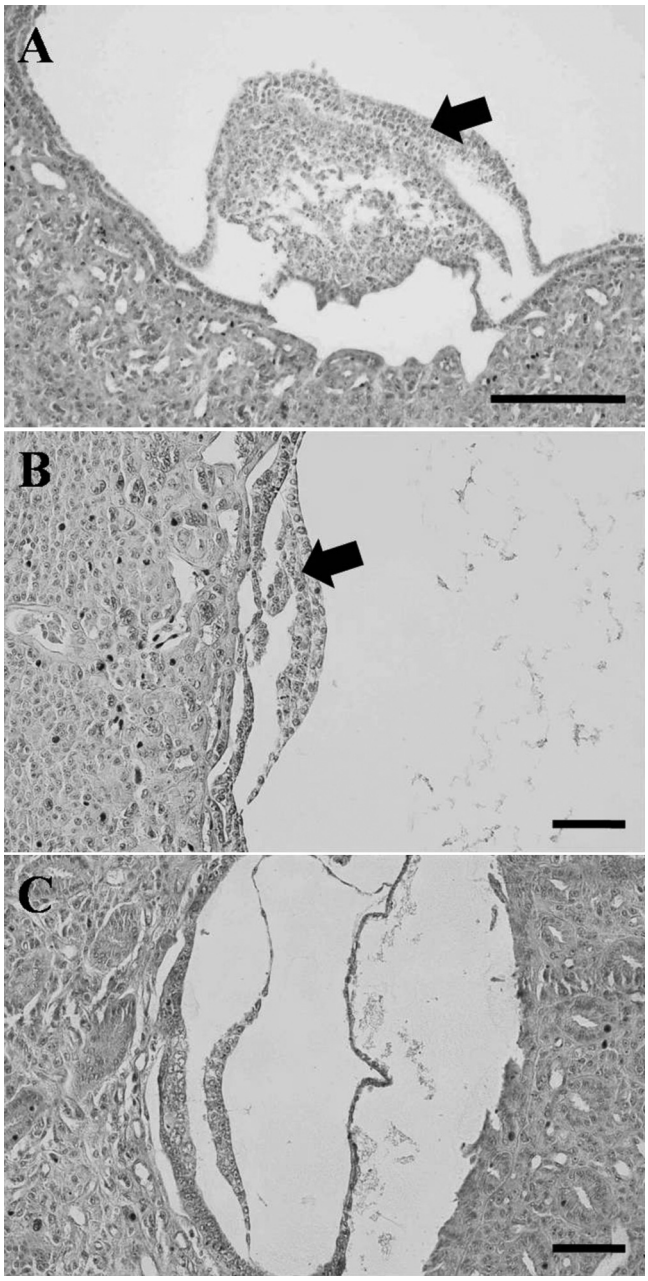

Fig. 6. Changes in the uterus of female *Hipposideros terasensis*: A) embryo at gastrula stage with inner cell mass (arrow) in February; B) embryo in primitive streak stage, showing neural plate (arrow) in March; and C) blastula stage of early implantation in September. Scale bars indicate 320 μm.

and implantation. As shown in Fig. 7, the changes in the concentration of progesterone display two peaks.

Discussion

Delayed embryonic development after implantation is a rare phenomenon in mammals, and even among bats, only few species are reported to exhibit this characteristics (Mead 1993). *Hipposideros terasensis* has delayed embryonic development (Chen 1998, this study). Summing up the histological observation of ovaries and uteri in adult females, it can be concluded that after the appearance of corpus luteum in ovaries during September, they remained unchanged from September to the end of February. Fetuses did not develop from the gastrula stage until April. Then, they grew until birth in late May.

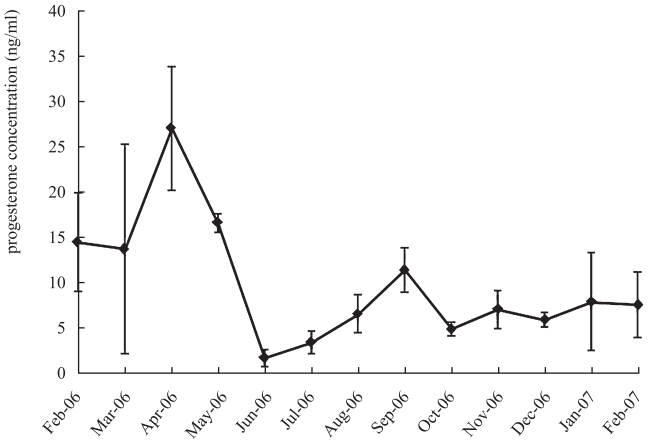

Fig. 7. Changes in serum progesterone concentrations in female *Hipposideros terasensis* from March 2006 to February 2007. Vertical lines indicate standard deviation from a sample size of three.

These results support Chen's (1998) conclusions that *H. terasensis* has delayed embryonic development. That pregnancy lasted about nine months was primarily due to embryonic arrest at the gastrula stage for five months after implantation.

Because *H. terasensis* has no sperm storage and the reproductive cycles of both sexes are highly synchronized (Chen 1998), we decided to reduce the sample size in this study. The appearance of Graafian follicles in ovaries in August was closely followed by the presence of corpus lutea in September. Since there was active spermatogenesis in male bats in June and July and residual sperm in the epididymis at the end of August, we inferred that mating, ovulation, and fertilization occurred before mid-August. Our results were consistent with Chen (1998), even though we had limited sample size to three of each sex each month.

Testosterone levels in male bats gradually increased from May until August. This coincided with spermatogenesis. Testosterone decreased after August when the spermatogenesis also stopped. This pattern is similar in other mammalian species in that all reproductive activities are synchronized (Kawamoto 2003). After copulation spermatogenesis decreases, seminiferous tubules and epididymis regress, and plasma testosterone concentration declines. Spermatogenesis and activity of the accessory genital glands are synchronized but are not clearly linked to plasma testosterone concentration. Martin and Bernard (2000) suggested that another protein, steroid binding globulins, may play an important role, and it is therefore very difficult to explain the data without information on plasma steroid binding globulins levels.

Progesterone levels in female bats remained at low levels during delayed embryonic development from October until March. Then, it started to increase rapidly, corresponding with the period of accelerated fetal development. It peaked in April, decreasing after parturition in May. Fleming (1971) once speculated that the delay in embryonic development in *Artibeus jamaicensis* was because of negligible growth in the corpus luteum, resulting in a low levels of luteotrophic hormones. This would also reduce concentrations of progesterone, also slowing embryonic development.

Hipposiseros terasensis was similar to *Macrotus californicus* in that there were two peaks in the concentration of serum progesterone during pregnancy. The first peak occurred in the first three months, and the second peak occurred half a month before the parturition. Burns and Easley (1977) suggested the first peak is related to formation of the placenta before the delay in embryonic development characterized by decreased concentrations of progesterone. The second peak would be consistent with the rapid embryonic development. Burns and Wallace (1975) pointed out that sudden increase in estrogen and progesterone at the time of implantation was to promote the process of implantation and the development of the chorioallantoic placenta. Banerjee et al. (2009) suggested that the increase in serum melatonin concentration closely coincided with the period of ovarian recrudescence, which may be responsible for delayed embryonic development in *Cynopterus sphinx* by suppressing progesterone synthesis.

Variation in embryonic development after implantation may have been determined by the mothers' body temperature and availability of resources (Bradshaw 1962; Racey 1982). Domesticated *Pipistrellus pipistrellus* increased rate of post-implant development when temperature increased. Decreased temperatures combined with a decreased availability of resources slows development (Racey 1982). This is true for Japanese *Miniopterus schreibersii* (Kimura and Uchida 1983).

From the evolutionary perspective, delayed embryonic development is one way to guarantee the survival of newborns by correlating the start, maintenance, and end of pregnancy with environment, neural endocrine conditions, and condition of ovaries and uterus (Anthony 2000; Heideman 2000; Martin and Bernard 2000; Lopes et al. 2004). Embryonic diapause is the least common type of reproductive delay in mammals and has only been demonstrated in bats (Meenakumari et al. 2009). Regulation at the gene and hormonal level is more

complicated than for delayed fertilization and delayed implantation. The molecular or cytological effects or mechanisms are still unknown, including how hormones influence cell cycle arrest, start of development, mitosis, and cell differentiation and proliferation.

Taiwan is located in the subtropics and has at least 35 species of bats. Three of these species are confirmed to have reproductive delay: *Pipistrellus abramus* has sperm storage (Ko 1995), *Miniopterus schreibersii* has delayed embryonic implantation (Huang 2000), and *Hipposideros terasensis* has delayed embryonic development (Chen 1998). Other than this study, there is no research on the hormonal conditions of reproductive delay in these three species. We documented relationship between the reproduction process revealed through histology with changes in progesterone and testosterone in *H. terasensis*.

Hipposideros terasensis is a common species endemic to Taiwan with large and stable populations. Their long pregnancy and pattern of delayed embryonic development makes them an ideal model for study of evolutionary developmental biology.

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