Male Reproductive Patterns of Hibernating Korean Greater Horseshoe Bat, *Rhinolophus ferrumequinum korai*: I. Annual Cycle of the Seminiferous Epithelium and Morphological Changes of the Testes

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Abstract

**Background:** Morphological changes of testes and seminiferous epithelium cycle were observed by optical and transmission electron microscopy to determine male reproductive patterns of the hibernating Korean greater horseshoe bat, *Rhinolophus ferrumequinum korai*.

**Materials and methods:** In this study, 40 male *Rhinolophus ferrumequinum korai* were collected from abandoned mines in Gyeongnam and Jeonnam provinces of South Korea from January 2014-December 2015. Differentiation process and cytological traits of seminiferous epithelium following monthly changes were examined with electron microscopic techniques and observed by optical and electron microscopy.

**Results and conclusion:** Male reproductive pattern of hibernating Korean *R. ferrumequinum korai* consists of three main stages. The first is the spermatogenesis stage (from April-September), including spermatocytogenesis (which appears from April-May) and spermiogenesis (from June-September). The activity of spermatogenesis was the highest in August. The lumen of seminiferous tubules was open from mid-April to mid-October. It was closed from November to March of the following year. The second is the phagocytosis stage (from mid-October-mid-November) that is a purification process to prepare for new spermatogenesis the following year. This period is called the cleansing period. The third is the dormant stage that is a state of holding only spermatogonia and Sertoli cells. It is an adaptation strategy to store energy for long periods of hibernation. Compared to previous studies [1], spermatogenesis period preceded one month earlier. This suggests that temperature increase can impact reproductive development and spermatogenesis.

**Keywords:** Annual cycle of the seminiferous epithelium; Male reproductive pattern; *Rhinolophus ferrumequinum korai*; Phagocytosis; Spermatogenesis; Cleansing period

**Introduction**

Mammalian species have various breeding methods. Animals in temperate zones are known to limit their reproductive activities to specific periods in order to maximize progeny survival [2]. In particular, changes in daylight duration are main factors that affect the starting of the breeding season. Many other environmental factors such as estrogen levels, neuropeptides, kisspeptin, gonadotropin releasing hormone/ luteinizing hormone (GnRH/LH), thyroid, food, water, housing, space, and climate availability also affect the onset of reproduction [3].

The breeding period of hibernating bats is interrupted by hibernation. It only occurs at specific times. Breeding types of hibernating bats differ greatly from those of general mammals. Hibernation is a physiological adaptation for long-term survival [1,4-6]. It has significant impact on fertilization [7]. Characteristics of fertilization and early embryogenesis are different among species. Since breeding is done under low-temperature environmental conditions, adaptation strategies such as efficient energy use during breeding are needed [8].

A comprehensive description of male reproduction patterns in hibernating bats is needed because their reproduction events and patterns during hibernation are significantly different from those of other mammals. Their male reproductive cycles can be divided into different patterns based on the timing and duration of major events during their annual reproductive cycle [4,5,9]. Male reproductive patterns of bats have been classified into three types (‘*Pipistrellus* pattern,’ ‘*Myotis* pattern,’ and ‘*Miniopterus* pattern’) considering their spermatogenesis process, Leydig cell, and associated organ changes [10]. In general, bats that live in temperate and tropical regions show seasonal breeding pattern, although some species may not show such pattern [11-13].

Recently, several studies have reported the periodicity of the seminiferous epithelium in bats, including spermatogenesis [14-28]. Major events are similar in most species of bats. However, variations in the process between families and taxa are often observed [15,29-38]. In particular, there has been no report of extinction of immature spermatids due to phagocytosis of Sertoli cells during the annual cycle of the seminiferous epithelium except for the male reproductive type of *R. ferrumequinum korai* [1]. Therefore, the objective of this study was to investigate the relationship between annual and monthly temperature changes in the differentiation of seminiferous tubules’ spermatogenic cells and review male reproductive patterns of *R. ferrumequinum korai*.

**Materials and Methods**

Experimental animals were collected and examined under the...
guidelines of the Kyungnam University Institutional Animal Care and Use Committee (KUIAC).

In this study, 40 male Rhinolophus ferrumequinum korai were collected from abandoned mines in Gyeongnam and Jeonnam provinces of South Korea from January 2014 to December 2015 (Table 1) using inhalant anesthesia. Monthly collected materials were immersed in 5 ml of 3% glutaraldehyde aqueous solution (4°C, pH 7.4, Milloning's buffer) and 0.01 ml of RNA stabilization solution (Figure 1). To examine morphological changes of the testes and the stage of differentiation of the seminiferous epithelium according to monthly changes, testes tissues extracted from the epididymis were soaked in 3%-glutaraldehyde aqueous solution (4°C, pH 7.4, Milloning's buffer) for 24 hours. Tunica albuginea was then removed. After that, testicular tissues were cut to 1-1.5 mm³ in size and pre-fixed in 3% -glutaraldehyde aqueous solution (4°C, pH 7.4, Milloning's buffer) for 2 hours. After fixation, tissue slices were washed with the same buffer (4°C, pH 7.4, Milling's buffer) twice (20 min per wash). They were then post-fixed in 1.33% OsO4 aqueous solution (4°C, pH 7.4, Milloning's buffer) for 2 hours. These fixed tissue pieces were washed twice with the same buffer (20 min per wash). After washing, tissue pieces were dehydrated with increasing acetone concentration (65%, 75%, 85%, 90%, 95%, 99%, and 100%) followed by embedding with Epon 812 synthetic resin. These embedded tissues were cut into 400 nm in thickness using an ultramicrotome (MT-1; Sorvall, Dupont) and stained with 0.5% toluidine blue. The differentiation stage of the seminiferous epithelium was observed with an optical microscope. Subsequently, continuous thin sections of 60-70 nm were obtained. They were double-stained with uranyl acetate solution and lead citrate solution followed by observation with a transmission electron microscope (TEM, H-600, Hitachi).

Table 1: Date examined, locality, and individual number of the Korean greater horseshoe bat, Rhinolophus ferrumequinum korai used in this study (all bats were collected from survey sites of abandoned mines).

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<th>Locality</th>
<th>No. of bats</th>
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<td></td>
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Results

To investigate periodic changes of male reproductive cycle of Korean R. ferrumequinum korai, morphological changes of the testes and differentiation pattern of the seminiferous epithelium were observed by optical and electron microscopy. Spermatagonia were observed in all seminiferous tubules from January to December (Figure 2a-2l). The periodic pattern of the male reproductive cycle was divided into active and hibernation periods (Figures 8 and 9). The following results were obtained.

Monthly morphological changes of testis

Morphological changes of the testes of R. ferrumequinum korai were significantly different according to month (Figure 1a-b). The size of testis began to gradually decrease from September (Figure 1i) to March of the following year (Figure 1c). It began to gradually increase from April, the awakening phase (Figure 1d). The testis showed the maximum size in August (Figure 1h).

Differentiation pattern of the seminiferous epithelium during the active phase

Spermatocytogenesis occurred throughout April (Figures 2d, 4a, 4b, 8 and 9; Table 2) and May (Figures 2e, 8 and 9; Table 2). Spermiogenesis progressed from June (Figures 2f, 5a, 8 and 9; Table 2) to September (Figures 2i, 5b, 8 and 9; Table 2). The activity of spermatogenesis was the highest in mid-August (Figures 2h, 8 and 12). The lumen of the seminiferous tubules was open from April (Figures 2d, 4b (Inset), 8 and 10c; Table 2) to mid-October (Figures 2j, 6a (Inset), 6b (inset), 8 and 11c; Table 2).
Figure 1: Photographs showing the size and morphological changes of testis and epididymis of Korean Rhinolophus ferrumequinum korai according to month. From the middle of October to the middle of March in the following year (the hibernating period), the size of testicles gradually decreased. The size of testis began to gradually increase from April (the awakening period). It showed the largest size in August (the active period). a: January; b: February; c: March; d: April; e: May; f: June; g: July; h: August; i: September; j: October; k: November; l: December; Cp: Caput epididymis; Cr: Corpus epididymis; Cu: Cauda epididymis; T: Testis.
Figure 2: An optical microscope photograph showing the stage of differentiation of the seminiferous epithelium over a one-year cycle. Note that seminiferous tubules from January to March are closed with lumen with only Ad-type spermatogonia and Sertoli cells. Ap-type and In-type spermatogonia as well as primary spermatocytes were observed in the first seminiferous tubules in April. From this period, lumen began to open. In May, a number of primary spermatocytes were observed in the seminiferous tubules. In June, the earliest sperm cells were observed in the seminiferous tubules. In July, numerous spermatocytes and numerous spermatids were observed in the lumen. In August, there were a few primary spermatocytes, a number of early sperm cells and mature sperm cells, and numerous spermatida in the lumen. In September, numerous spermatids were observed in the spermatids and lumen, including many early sperm cells. In October, Ad-type spermatogonia, immature spermatids, and sperm cells were observed. For the first time in this period, many immature spermatids were engulfed as part of the phagocytosis process of Sertoli cells. In November, the pattern of seminiferous tubules was the same as that in October. From this time, the lumen was closed. The lumen of the seminiferous tubules in November and December was also closed. Only Ad-type spermatogonia were observed. Ad, dark type spermatogonium; Ap: Pale type spermatogonium; B: B type spermatogonium; Bl: Basal lamina; D: Diplotene spermatocyte; Es: Elongating spermatid; In: Intermediate spermatogonium; L: Lumen; Lf: Lipofuscin; M: Meta phase; Ms: Mature spermatid; P: Pachytene spermatocyte; PL-L: Pre-leptotene/leptotene spermatocyte; Rs: Round spermatid; S: Sperm; Se: Sertoli cell; St: Spermatid; Z: Zygotene spermatogonium; a: January; b: February; c: March; d: April; e: May; f: June; g: July; h: August; i: September; j: October; k: November; l: December.
Figure 3: Optical and electron micrographs of seminiferous tubules in March. Seminiferous tubules lumen was closed. There were many Lipofuscin granules in the cytoplasm of Ad spermatogonia and Sertoli cell. Ad: Dark type spermatogonium; Bl: Basal lamina; ER: Endoplasmic reticulum; Lf: Lipofuscin; M: Mitochondria; Se: Sertoli cell.

Figure 4: Optical and electron micrographs showing Ap- and In- type of spermatogonia and spermatocytes in the seminiferous tubules in April. Ap-type spermatogonia and somewhat spherical In-type spermatogonia were first seen at this time. Note that primary spermatocytes of zygotene and pachytene stages were surrounded by the cytoplasm of Sertoli cells. Ad: Dark type spermatogonium; Ap: Pale type spermatogonium; In: Intermediate spermatogonium; L: Lumen; M: Mitochondria; N: Nucleus; No: nucleolus; P: Pachytene; rER: Rough endoplasmic reticulum; Se: Sertoli cell; Z: Zygotene.
Figure 5: Electron micrograph showing early Golgi phase (June experiment group) and spermiation phase (September experiment group). In the cytoplasm of spherical sperm cells, there were abundant mitochondria and smooth endoplasmic reticulums (sER), including well-developed Golgi complexes (Gc). In addition, many spermatozoa were observed in the lumen, including sperms in the cytoplasm of Sertoli cells. L: Lumen; N: Nucleus; Rb: Residual body; S: Sperm; Se: Sertoli cell; St: Sperm tail.

Figure 6: Optical and electron micrographs showing the phagocytosis process of Sertoli cells in seminiferous tubules in October. Note that many immature sperm cells were predated as part of the phagocytosis process of Sertoli cells (Figure 6a, Round mark in Inset). In the lumen of the seminiferous tubules in October, there were sperms deviating from Sertoli cells. Some sperms already migrated from the lumen to the epididymis (Figure 6b Inset). In addition, many Lipofuscin were scattered within the cytoplasm of Sertoli cells (Figures 7a and 7b). Ad: Dark type spermatogonium; Bl: Basal lamina; Irs: Immature round spermatid; L: Lumen; Lf: Lipofuscin; S: Sperm; Se: Sertoli cell.
In the seminiferous tubules, a large number of Ad (dark-type), Ap (pale-type) spermatogonia (Figure 2d), intermediate-type spermatogonia (Figures 4a, 4(Inset) and 8), and primary spermatocytes were observed (Figures 2d, 4b, 4b (Inset) and 8) in April. In May, seminiferous tubules were wider than those in April. The number of primary spermatocytes in May was more than that in April (Figure 2e). In June, seminiferous tubules started to show spermatids, including many primary spermatocytes (Figures 2f, 5a and 8). In July, many sperms were observed in the lumens of many elongated spermatids containing primary spermatocytes (Figures 2g and 8). In August, a few primary spermatocytes, a number of early spermatids and mature spermatids, and numerous spermatozoa were present in the lumen (Figure 2h). In September, there were few Ad spermatogonia, several early spermatids (Figures 2i and 5a), abandoned spermatids from Sertoli cell cytoplasm, and numerous sperm in the lumen (Figures 2i and 5b).

**Differentiation pattern of seminiferous epithelium during hibernation**

Upon hibernal onset starting in October, spermatozoa of seminiferous tubules were transferred to the seminiferous tubules lumen (Figures 2j and 6a (Inset)). There was no mature sperm (Figures 2j and 6b (Inset)). In some seminiferous tubules, spermatogonia of Ad-type, immature sperm cells, and spermatogonia cells were observed (Figure 6a (Inset)). For the first time in this period, numerous immature sperm cells were predated as part of the phagocytosis process of Sertoli cells (Figure 6a). In addition, numerous lipofuscin granules were scattered within the cytoplasm of Sertoli cells (Figure 6b) and the lumen of the seminiferous tubules was open (Figures 2j, 6a (Inset) and 6b (Inset)). In November, seminiferous tubules were observed in immature sperm cells (Figures 7a and 7b) in the same manner as in October. The lumen of seminiferous tubules was closed for the first time (Figures 2k, 7a (Inset) and 7b (Inset)). These seminiferous tubules were also closed in December (Figure 2l), January (Figure 2a), February (Figure 2b), and March (Figure 2c and Figure 3 (Inset))). Only Ad-type spermatogonia and Sertoli cells were present in these seminiferous tubules (Figures 2l and 2a-2c). Many lipofuscin granules were scattered in the cytoplasm of

![Figure 7](image-url): Optical and electron micrographs showing the phagocytosis process of Sertoli cells in the seminiferous tubules in November. The seminiferous tubules in November, like those in October, were phagocytized to a number of immature spermatids as part of the phagocytosis process of Sertoli cells (Figures 7a and b). From this point on, the lumen was closed (Figures 7a (inset) and 7b (Inset)). Lipofuscin was scattered within the cytoplasm of Sertoli cells (Figures 7a and b). Ad: Dark type spermatogonium; Lf: Lipofuscin; N: Nucleus; Pg: Phagocytosis; Se: Sertoli cell; Spt: Sperm tail.
Due to administrative district reform, Masan City was integrated into Changwon City in July 2010 while Chungmu City was integrated into Tongyeong City in January 1995.


(†) Chungmu Weather Station name has been changed from Chungmu (162) to Tongyeong (162) since January of 1995 due to administrative recomposition.
(‡) Masan Weather Station name has been changed from Masan (155) to Changwon (155) since July of 2010 due to administrative recomposition.
(♣) Suncheon Weather Station number has been changed from 256 to 174 since April of 2011. Cm, Chungmu-si; Cw, Changwon-si; Ms, Masan-si; Nh, Namhae-gun; Sc, Suncheon-si; Ty, Tongyeong-si.

Table 3: Comparison of temperature changes from the nearest meteorological stations at the same survey sites between 1991-1992 and 2014-2015.

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Due to administrative district reform, Masan City was integrated into Changwon City in July 2010 while Chungmu City was integrated into Tongyeong City in January 1995.
Figure 9: Hibernating Korean Rhinolophus ferrumequinum korai showing three major stages of the male reproductive pattern. The first was the spermatogenesis stage (from April to September), including spermatocytogenesis (from April to May) and the spermiogenesis (from June to early October). The second was the phagocytosis stage (from mid-October to mid-November), which was a purification process to prepare for new spermatogenesis in the following year. The third was a dormant stage. It was a state of holding only spermatogonia and Sertoli cells as an adaptation strategy to have efficient use of energy for long hibernation.

Figure 10: Optical microscope photographs comparing the initiation stage of differentiation of seminiferous tubules between 1991 and 2015. In seminiferous tubules 25 years ago (April 1991), only Ad-type spermatogonia was observed. Note that the lumen was closed (Figure 10a). In May 1991, the lumen was open as the beginning of the spermatogenesis process (Figure 10b). In April 2015, spermatogenesis and lumen were open in the experimental group (Figure 10c). In May, seminiferous tubules were more active than those in April (Figure 10d). L: Lumen.
**Figure 11:** Optical micrographs comparing differentiation stages of the seminiferous epithelium of *Rhinolophus ferrumequinum korai* between 1991 and 2015. Numerous spermatozoa were observed in the lumen of seminiferous tubules in October 1991. The lumen was open (Figure 11a). In November, 1991, immature sperm cells were predated by phagocytosis of Sertoli cells while the lumen was closed (Figure 11b). Only a few spermatogonia were present in the seminiferous tubules in October, 2015. Note that all spermatogenesis-completed sperm moved to the epididymis while sperms were not present in the lumen (Figure 3c). Also note that the lumen of the seminiferous tubules was closed in November, 2015 while immature sperm cells were predated by Sertoli cells (Figure 11d). Ad: Dark type spermatogonium; L: Lumen; Pg: Phagosome; S: Sertoli cell; S: Sperm; St: Spermatid.

**Figure 12:** Comparison of male reproductive patterns of four kinds of domestic bat species. Note that only immature sperms were killed by Sertoli cells in October and November, just before and immediately after hibernation of *R. ferrumequinum korai* (the present study). (a) Immature spermatozoa shown in Lee et al. occurred in November; (b) Immature spermatozoa shown in this study occurred from mid-October to mid-November. In particular, spermatogenesis initiation in this study occurred one month earlier than that described in Lee et al. [1].
Correlation between differentiation of seminiferous epithelium and monthly temperature changes

According to this study, the size of testis (Figure 1a-1l) and the stage of differentiation of seminiferous epithelium were remarkably different according to month (Figure 2a-2l). These differences were also evident according to annual and monthly temperature changes (Table 3). The average annual temperatures in Changwon-si, Tongyeong-si, Namhae-gun in Gyeongnam, and Suncheon-si, Jeonnam in 1991 were 14.6°C, 14.7°C, 13.9°C and 12.6°C, respectively. The average temperature in these three regions was 14.4°C. In 2015, average temperatures at Changwon-si, Tongyeong-si, and Namhae-gun were 14.6, 14.8, and 14.7°C, respectively. The average temperature of these three regions was 14.7°C. It was 13.2°C at Suncheon in Jeonnam. When temperatures at Changwon-si, Tongyeong-si, Namhae-gun and Suncheon in April (the spermatogenesis initiation period) were compared between 1991 and 2015, the difference in temperature was about 0.4°C (1991: Changwon, 13.8°C; Tongyeong, 13.1°C; Namhae, 12.8°C; average, 13.2°C; 2015: Changwon, 13.9°C; Tongyeong, 13.2°C; Namhae, 13.7°C; average, 13.6°C). Overall, changes in annual temperature and monthly temperature between 2015 and 1991 were 0.3 to 0.4°C (April for spermatogenesis).

Discussion

Spermatogenesis of mammals is a series of complicated and elaborate process involving maturation and differentiation of diploid (2n) spermatagonia into germ cells of highly differentiated haploid (n) [39-43]. Differentiation processes of spermatogenesis involve many sudden changes in the cell. Such changes have been used for biochemistry and gene regulation studies as well as the identification and classification of ultrastructural changes of sperm [44-46]. In addition, the differentiation process of the seminiferous epithelium affects the reproductive cycle by abiotic factors such as temperature and photoperiod [47,48].

Although information on the duration of the spermatogenic cycle is well known for a large number of bats [16], there has been no report on the extinguishment of immature spermatids by phagocytosis of Sertoli cells during the annual cycle of the seminiferous epithelium except for the male reproductive type of R. ferrumequinum korai [1]. The time and duration of the spermatogenesis process in bats is similar to each other [16-22,49]. However, hibernating bat species has unique reproductive patterns. According to the present study, the size of testis began to gradually increase from April, the beginning of spermatogenesis (Figure 1d). In August, the size of testicles was maximized (Figure 1h). It then gradually decreased from September (Figure 2i) to March, the end of hibernation (Figure 1c). In addition, diameter changes of seminiferous tubules showed a tendency almost the same as those of testis sizes (Figure 2a-2l). Diameter change is consistent with spermatogenesis. The maximum activity of spermatogenesis is known to be related to the diameter of the largest seminiferous tubules and the maximum testicle weight [10,50,51]. Sperm production is directly related to the size of testes [52-54]. Although body temperature does not inhibit sperm maturation in epididymis fluid, it has a significant effect on sperm viability and sperm maturation in storage capacity and cauda epididymis [54]. According to some authors, lower temperature of the epididymis makes sperm maturation and storage easier [55-57].

Considering cell structures of the seminiferous epithelium according to month (Figures 2-8, 10 and 11) and annual cycle of the spermatogenic epithelium (Table 2; Figures 8, 9, 12 and 13) shown in this study, spermatogenesis began in April and ended in September. Our results confirm that the male reproductive pattern of Korean R. ferrumequinum korai is ‘Pipistrellus pattern’ according to Lee et al. [1] because spermatogenesis does not occur during the mating period or hibernation period. In particular, spermatocytogenesis occurred throughout April and May while spermiogenesis occurred from June to September. Spermatogenesis process activity was the highest in mid-August. In addition, the lumen of seminiferous tubules was open from mid-April to mid-October. It was closed from November to March of the following year. The mating period was from September to the...
beginning of October at the latest. This means that most spermatooza that have undergone spermatogenesis process in August and September might have migrated to the epididymis (Figures 2h and 2i). After mating, bats will soon be in hibernation. There will be no need for sperm production anymore. Therefore, they can have efficient energy use for long hibernation. In particular, from mid-October to mid-November, immature spermatids in seminiferous tubules during hibernation were completely destroyed by phagocytosis of Sertoli cells (Figures 6a, 7a and 7b). In phagocytosis, more than half of differentiating spermatogenic cells are known to undergo cell apoptosis before spermatogenesis in mammals. They are rapidly and selectively cleared by phagocytosis of Sertoli cells [1,58]. Inhibition of phagocytosis in living animals will result in a decrease in the number of epididymal spermatooza, suggesting that phosphatidylserine-mediated phagocytosis of apoptotic cells by Sertoli cells is necessary for efficient production of spermatooza [58]. In hibernating bats, this phagocytosis is a preparation stage for new spermatogenesis in the next year. This is called a cleansing period. Spermatogenesis initiation and duration of some hibernating bats are shown in Table 2 and Figure 12. There were some differences in their spermatogenesis initiation and duration between species (Figure 12). In the case of R. ferrumequinum korai [1] and Miniopterus schreibersi fallaxinus [20] spermatogenesis initiation occurred from May. However, initiation of spermatogenesis in Myotis macrodactylus [22] and R. ferrumequinum korai in this study started in April. Notably, spermatogenesis of Korean R. ferrumequinum korai occurred in May (Figures 10b and 12; Table 2) in a previous study [1]. However, it occurred in April in this study (Figures 2d, 9, 10c and 12; Table 2). The fact that spermatogenesis initiation took place about a month earlier in the present study (Figures 10 and 12; Table 2) suggests that the rising temperature might have affected the timing of spermatogenesis (Table 3). Based on yearly and monthly temperature changes shown in Table 3, the average temperature in 2015 was 0.3 to 0.4°C higher than that in 1991. It has been reported that abiotic factors such as temperature and photoperiod can affect the reproductive cycle of bats [47,48]. There is a concern that many species might become endangered if they do not develop new seasonal strategies to adapt to the rising temperature [59]. Although the reproductive cycle of Sturnira lilium bat might be directly influenced by factors such as temperature and food availability [48,60], the timing of male cycle of tropical sperm-storing bats and onyx hibernate bats seems to be dependent on temperature more than on nutrition. Their spermatogenesis is generally stopped or suppressed in the coldest months [61-64]. Based on the above results, the male reproductive pattern of hibernating Korean R. ferrumequinum korai is ‘ Pipistrellus pattern’. It consists of three main stages. The first is the spermatogenesis stage (from April to September), including spermatocytogenesis (which appears from April to May) and spermiogenesis (from June to September). The second is the phagocytosis stage (from mid-October to mid-November) which is a purification process to prepare for the new spermatogenesis in the following year. The third is the dormant stage which is a state of holding only spermatogonia and Sertoli cells. It is an adaptation strategy to have efficient use of energy for long hibernation. Compared to previous studies [1], their spermatogenesis period preceded one month earlier. This suggests that temperature increase can affect their reproductive development and spermatogenesis.

Compliance with Ethical Standards

Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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Conflict of Interest

The author declares he has no conflict of interest.

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