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Histological and immunocytochemical study of deferens ducts in the Chinese rat snake (*Zaocys dhumnades*)

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Abstract: To investigate the relationship between structure and function of the deferens ducts in the Chinese rat snake (*Zaocys dhumnades*), morphological changes within an annual cycle were observed by routine histological techniques. Also, the correlation of androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR) and aromatase (Ar) expressions in the vas deferens and testis were studied immunohistochemically. To confirm that the sperm and the spherical structure existed in deferens ducts, we also used routine histological technique observed deferens ducts in the Striped-tailed rat-snake (*Elaphe taeniura*), Red-banded snake (*Dinodon rufozonatum*), and Tiger-spotted neck-troughed snake (*Rhabdophis tigrina lateralis*). The results showed that the deferens ducts of the Chinese Rat Snake were composed of efferent duct, epididymal duct and vas deferens. Efferent duct contained sperm from August-October, and the sperm were observed in the epididymal duct from August-the following January. Throughout the year (except July) a large number of sperm were present in the vas deferens where a previously unreported spherical structure formed by spermatids was observed, which showed no significant differences in the IOD values of AR-, ER-, PR- and Ar-immunoreactivities. Since the spermatids in the spherical structure were undergoing spermatogenesis and this phenomenon also existed in the Striped-tailed rat-snake and Red-banded snake, the term, seminiferous spherule, was named for this spherical structure This study demonstrated that the testis was the main site for snake spermiogenesis, and the seminiferous spherule in vas deferens was the other Both the epididymis and vas deferens stored sperm; however, the vas deferens was the main organ for sperm storage.

Key words: Snakes; Deferens ducts; Histology; Spermiogenesis; Sperm storage

乌梢蛇输精管道的组织学与免疫细胞化学观察

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摘要：为探讨乌梢蛇（*Zaocys dhumnades*）输精管道结构与其功能之间的关系,该研究用一般光镜技术观察了乌梢蛇输精管道的显微结构及其年周期变化, 并结合免疫细胞化学方法研究了雄激素受体（AR）、雌激素受体（ER）、孕激素受体（PR）和芳香化酶（Ar）在输精管和精巢中精子细胞表达的相关性。为验证该文在乌梢蛇输精管中观察到的大量精子和圆球状结构，用一般光镜技术还观察了黑眉锦蛇（*Elaphe taeniura*）、赤链蛇（*Dinodon rufozonatum*）与虎斑颈槽蛇（*Rhabdophis tigrina lateralis*）的输精管道。结果表明, 乌梢蛇的输精管道主要由输出小管、附睾管与输精管构成; 8—10 月输出小管中有精子, 8 月—翌年 1 月附睾管中有精子, 全年（除 7 月外）输精管中有大量精子; 在输精管内首次观察到由多个精子细胞构成的圆球状结构, 该结构与精巢中精子细胞的 AR、ER、PR 和 Ar 累计光密度值之间分别无显著差异。由于在乌梢蛇、黑眉锦蛇及赤链蛇的输精管内圆球状结构均可见精子细胞变态形成精子。因此，建议将蛇类输精管内圆球状结构命名为生精小球（seminiferous spherule）。该文认为, 蛇类精巢是精子形成的主要部位, 而输精管内的生精小球是精子形成的另一个部位; 附睾与输精管均可以储存精子, 但输精管是精子储存的主要器官。

关键词：蛇; 输精管道; 组织学; 精子形成; 精子储存
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The Chinese rat snake (*Zaocys dhumnades*) (CRS) is a large non-venomous species endemic to the plains, hills and foothills of 21 provinces in China. They feed on frogs and small mammals, thus playing an important role in maintaining ecosystem balance. To date, research on the reproductive biology of the CRS has mainly focused on captive breeding (Ye et al, 2005), correlation of female body size, egg size and number (Ji et al, 2000), the periodic change of testis, ovary and granulose cells at the microstructure level (Liang et al, 2008, 2009; Zhao et al, 2010), and the correlation of the fine structure in Leydig cells with serum testosterone (Chang et al, 2011). Previous studies have indicated that reptilian deferens ducts, especially the vas deferens, is one of their main sperm storage sites (Gist & Jones, 1987; Gist & Fischer, 1993; Sever et al, 2002; Sever & Hopkins, 2004; Akbarsha et al, 2005, 2006; Han et al, 2008). However, whether the deferens ducts can store sperm and whether the vas deferens has seminiferous spherules in the CRS, and how their structure and annual cycle can change have not yet been reported. In the present study, the morphology and changes of the deferens ducts in an annual cycle of adult male CRSs were investigated by routine histological techniques. In addition, the correlation of androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR) and aromatase (Ar) expressions in the spermatids of the vas deferens and testis were demonstrated immunocytochemically. This research aims to investigate the relationship between morphology and function of deferens ducts, which may provide data for snake reproductive biology, captive breeding and wild resource protection.

1 Materials and Methods

From November 2004-October 2005, a total of 20 adult male CRSs, (362.47±92.02) g body weight, (162±11) cm total length, were collected three times from the Qinling Mountains, China (N32°30′−33°20′, E108°00′−108°50′). Two CRSs were used for experiments on the tenth day of each month except from December 2004-early March 2005 when the snakes were hibernating. To confirm that sperm and spherical structures existed in the deferens ducts, other snakes species collected in May 2008 in the Qinling Mountains (N32°30′−33°10′, E108°00′−108°30′), including the Striped-tailed rat-snake (*Elaphe taeniura*) (*n*=2, average 611.2 g body weight, 154 cm total length)(STRS), the Red-banded snake (*Dinodon rufozonatum*) (*n*=2, average 161.3 g body weight, 98 cm total length)(RBS), and the Tiger-spotted neck-troughed snake (*Rhabdophis tigrina lateralis*) (*n*=2, average 68.6 g body weight, 61 cm total length)(TNTS) were also used for the study.

After the CRSs were anaesthetized with ether, their body weight and total length were measured, and the epididymis, vas deferens and testis were removed, cut into pieces and fixed in Bouin's solution, embed in paraffin and sections of 6 μm were cut on a microtome. The epididymis sections and part of the vas deferens were stained with hematoxylin and eosin, the testis sections and the other part of the vas deferens were used for immunocytochemical analysis for AR, ER, PR and Ar. Routine immunocytochemical techniques were used in the experiment. Briefly, the paraffin sections were dewaxed, hydrated and incubated in 3% H_2O_2 for 10 min to remove endogenous peroxidase at room temperature. After three heat treatments by citrate buffer, the sections were washed three times in PBS (pH 6.0) before incubation at 4 °C for 24 h in the primary antibodies (1:100, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.). The sections were then sequentially incubated with goat anti-rabbit IgG (1:100, Wuhan Boster Biological Technology Co., Ltd. and Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.), and Strept Actividin-Biotin Complex (1:200, Wuhan Boster Biological Technology Co., Ltd.) for 30 min at room temperature. Between the steps, the sections were rinsed with PBS. Visualization of the antigen was accomplished with diaminobenzidine reaction solution for 5−20 min. For negative control, the procedures were the same as above except that PBS was used to replace the primary antibodies; no positive signals were observed in the control experiment. In addition, the epididymis and the vas deferens were taken from another three snake species to do routine paraffin section and HE staining.

All the sections were examined by an Olympus BX41 and photographed by a Leica DFC320 light microscope. The immunocytochemical results of AR, ER, PR and Ar in the vas deferens and testis of the CRS were analyzed using IPP (Image-Pro Plus). Immunoreactive intensity was represented by an IOD (Integrated option density) value, and the statistical data were further analyzed by one-way ANOVA, Mean ± *SE*.

2 Results

2.1 Morphology and change of the deferens ducts in an annual cycle

The deferens ducts of the CRS were mainly composed of the epididymis and vas deferens. The paired

epididymides were slender, milk white pipes exiting the anteromedial part of testis. Each epididymal pipe started with a small diameter and coursed caudally to gradually become the larger diameter vas deferens. The paired vas deferens were slender, convoluted, muscular pipes of milk white color and a diameter slightly larger than that of the epididymal pipes, running caudally and accompanying the kidney and ureter to open into the cloaca.

The epididymis of the CRS consisted of efferent duct and epididymal duct. The efferent duct measured about 75 μm in diameter and was thin and lined by a layer of short columnar epithelial cells (11 μm high), displaying aligned nuclei located at middle or lower part of individual cells. The cavity of the efferent duct with flush surface contained acidophilic substance (Fig. 1a). The epididymal duct, 289 μm in diameter, was composed of tall columnar epithelial cells and internal basal cells. The columnar epithelial cell (33 μm high), with acidophilic cytoplasm and oval nucleus located in the center or lower part of the cell, had numerous cilia on the free surface and the base was attached to the basement membrane. The conical basal cells, which were also connected to the basement membrane, were sandwiched by the columnar epithelial cells at the lower position and possessed round nuclei at the basal part of the cells. Transverse slices demonstrated that most columnar cells lining the epididymis duct were the same height, thus rendering the lumen surface flush. However, some columnar cells were different in height, contributing to the irregularity of lumen surface (Fig. 1a).

The wall of the CRS vas deferens was composed medial-laterally of mucosa, muscularis and adventitia. The mucosa included epithelium and lamina propria. The epithelium was a layer of low columnar or cuboidal cells, with nuclei aligned along the middle or lower part of cells and no cilia on the free surface. The muscularis consisting of smooth muscle, and the adventitia, belonging to serosa, was composed of loose connective tissue and simple squamous epithelium. In addition, histology of the vas deferens showed the following characteristics: the mucosa and muscularis in the anterior vas deferens protruded inward and was divided into 2-5 compartments (Fig. 1b). In the posterior vas deferens, only mucosa extended inwardly to form the longitudinal mucosal folds (Fig. 1c). The thickness of muscularis increased gradually from rostral to caudal. Rostrally, only circular smooth muscle existed and showed a maximum thickness of 93 μm. In contrast, the caudal part of the vas deferens, with a maximum thickness of 138 μm, mediolaterally possessed three layers of smooth muscles: longitudinal, circular and longitudinal (Fig. 1b,c).

A large number of sperm and acidophilic substances were observed in the deferens ducts of the CRS, demonstrating a periodic changing pattern during an annual cycle. For the epididymis, no sperm was visualized in the efferent or epididymal ducts; however, both contained an acidophilic substance from April-July. The efferent and epididymal ducts contained more sperm and acidophilic substance in August than in September. In October and November, the sperm and acidophilic substance still existed in the efferent and epididymal ducts, although November possessed fewer sperm in the efferent duct but more sperm in the epididymal duct. From December-the following January, acidophilic substance but no sperm was observed in the efferent duct, contrasting to the existence of both sperm and the acidophilic substance in the epididymal duct, especially sperm in January. With the exception of less acidophilic substance in July, numerous sperm, acidophilic substance and even some spermatids were observed for the whole year in the vas deferens. In January, April, May, June, November and December some spherical structures (25−40 μm) were also observed in the vas deferens. Observation from different sections indicated that the spherical structure was an aggregation of spermatids. The constitutive spermatid, with oval nucleus (4×2 μm) at the cell center, was spindle-shaped and had acidophilic cytoplasm. About 8−12 spermatids were lined up in the longitudinally cut diameter section (Fig. 1d). Collectively, these spermatid nuclei were regularly arranged in the equatorial section of the spherical structure (Fig. 1e). In some spherical structures we observed that the sperm head, just before completion of metamorphosis, was freed from the spherical structure and some residual acidophilic cytoplasm (Fig. 1d,f).

In the STRS, RBS and TNTS deferens ducts, microstructures similar to those of the CRS were also observed. Acidophilic substance but no sperm existed in the efferent duct of the epididymis; however, both acidophilic substance and sperm were observed in the epididymal duct. A large number of sperm were seen in the vas deferens. In addition, spherical structures identical to the ones seen in the CRS were also observed in the STRS and RBS but not in the TNTS (Fig. 1g−i).

2.2 Immunoreactivities in the vas deferens and testis

In the spherical structure of the vas deferens and testis of the CRS, AR-, ER-, PR- and Ar-immunoreactivities were observed in the spermatids, andimmunostaining was localized to nucleus and/or

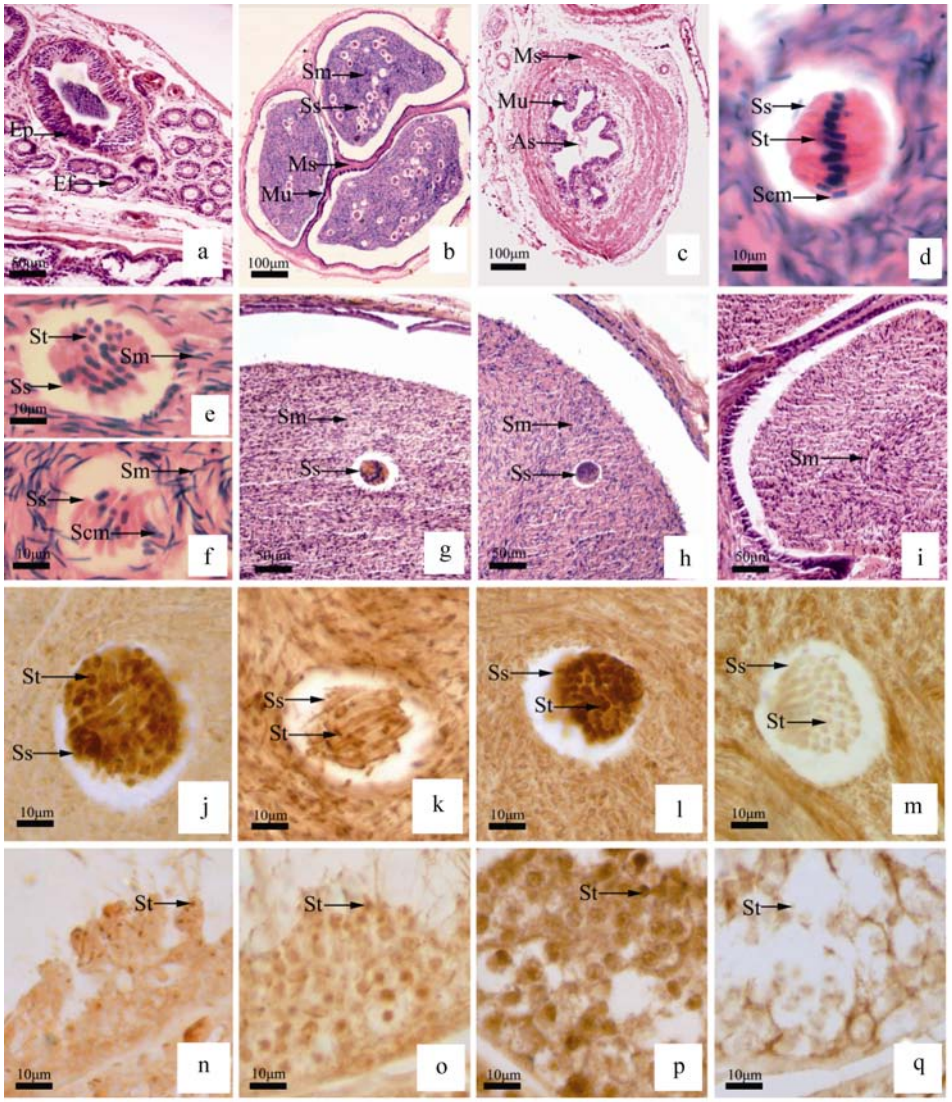

Fig.1 Histological study of the male reproductive organ in four species of snake

a) Epididymis section in the Chinese Rat Snake (CRS) (*Zaocys dhumnades*), ×20; b) The front section of vas deferens in the CRS, showing the seminiferous spherule and a large number of sperm, ×10; c) The back section of vas deferens in the CRS, ×10; d) The seminiferous spherule from the longitudinally cut diameter section in the vas deferens of the CRS, ×100; e) The seminiferous spherule from the equatorial section in the vas deferens of the CRS, ×100; f) Section of the seminiferous spherule in the CRS, showing the sperm closing to completion of metamorphosis, ×100; g) Section of the vas deferens in the Striped-tailed rat-snake (*Elaphe taeniura*), showing the seminiferous spherule and a large number of sperm, ×40; h) Section of the vas deferens in the Red-banded snake (*Dinodon rufozonatum*), showing the seminiferous spherule and a large number of sperm, ×40; i) Section of the vas deferens in the Tiger-spotted neck-troughed snake (*Rhabdophis tigrina lateralis*), showing a large number of sperm but no the seminiferous spherule, ×40; j) Showing strong AR-immunoreactivity of spermatids in the seminiferous spherule of the CRS, ×100; k) Showing strong ER-immunoreactivity of spermatids in the seminiferous spherule of the CRS, ×100; l) Showing strong PR-immunoreactivity of spermatids in the seminiferous spherule of the CRS, ×100; m) Showing weak Ar-immunoreactivity of spermatids in the seminiferous spherule of the CRS, ×100; n) Showing strong AR-immunoreactivity of spermatids in the testis of the CRS, ×100; o) Showing strong ER-immunoreactivity of spermatids in the testis of the CRS, ×100; p) Showing strong PR-immunoreactivity of spermatids in the testis of the CRS ×100; q) Showing weak Ar-immunoreactivity of spermatids in the testis of the CRS, ×100.

As: acidophilic substance; Ef: efferent duct; Ep: epididymis duct; Ms: muscularis; Mu: mucosa; Scm: the sperm closing to completion of metamorphosis; Sm: sperm; Ss: seminiferous spherule; St: spermatids.

cytoplasm. Strong AR-, ER- and PR-immunoreactivities, and weak Ar-immunoreactivity were visualized in the spermatids of the spherical structure and the testis (Fig. 1j−q). The IOD value of AR, ER, PR and Ar showed no significant differences between the spherical structure of the vas deferens and the testis (Tab. 1).

Tab. 1 Integrated option density (IOD) value in the spermatids of Chinese rat snake *Zaocys dhumnades*

Spermatids	AR (androgen receptor)	ER (estrogen receptor)	PR (progesterone receptor)	Ar (aromatase)
In spherical structure	93.70±18.33*	30.57±5.11*	41.87±6.46*	135.50±14.52*
In testis	106.07±22.14	30.16±6.83	40.20±8.73	132.35±19.70

* $P>0.05$ showing no significant difference between the spherical structure of the vas deferens and testis (n=30), (ANOVA-LSD).

3 Discussion

3.1 Seminiferous spherule in vas deferens was the other site for snakes' spermiogenesis

Spermiogenesis is the physiological processes in which the round spermatid becomes tadpole-like sperm through complicated morphological and functional changes. Liang et al (2008) observed the annual variation in testicular microstructure of the CRS and reported that spermatids began metamorphosis in testis in July and reached peak spermiogenesis in August and September. In October and November some spermatids were still becoming sperm. Therefore, the testes were the main site for the CRS spermiogenesis. The present study revealed some spherical structures existed in the vas deferens of the CRS in January, April, May, June, November and December. In some spheres, we observed that the head of sperm, as it neared completion of metamorphosis, was freed from the spherical structure, and some residual acidophilic cytoplasm (Fig. 1d,f). This suggested that spermatids in the spherical structure were undergoing spermiogenesis. Based on these observations, we postulated that the spherical structure in the CRS vas deferens was the other site for spermiogenesis.

Previous studies have indicated that AR, ER, PR and Ar play different roles during spermiogenesis. The AR is essential for the round spermatid to become elongated sperm (Holdcraft & Braun, 2004). Spermiogenesis is a process dependent on estrogen, and estrogen acts directly on the ER of sperm and spermatids to maintain sperm function and prevent spermatid death (Robertson et al, 1999). Progesterone binds directly to PR on germ cells at different developmental stages, participating in testis development and maturation (Witt et al, 1994). The Ar in the sperm can convert androgen to estrogen, and might contribute to sperm transport (Hess et al, 2001). Our results indicated that the spermatids of the spherical structure and the testis in the CRS showed strong immunoreaction for AR, ER and PR and weak Ar-immunoreactivity, and the IOD value of AR-, ER-, PR- and Ar-immunoreactivities demonstrated no significant differences between the spherical structure of the vas deferens and the testis (Tab. 1). These data indicate that the spermatids in the spherical structures of the vas deferens and testes possessed the same function and that the spermatids in the spherical structure were undergoing spermiogenesis. This supports the inference that the spherical structures in the CRS vas deferens were the other site for spermiogenesis.

For the other three snake species in this study, the STRS and RBS showed spherical structures in the vas deferens identical to those observed in the CRS. The head of the sperm, nearing metamorphosis completion, was freed from the spherical structure. These findings also showed that the spherical structures of the vas deferens were the other site for spermiogenesis in the STRS and RBS. However, no spherical structure was observed in the TNTS, which may be because the TNTS does not form the spherical structures during spermiogenesis, or the timing of the TNTS spermatogenesis was different from that of the other three species.

To sum up, among the four species of snakes investigated, three had the same spherical structures in the vas deferens, accounting for 75% of the total species. Therefore, it was probable that the occurrence of spherical structures in the vas deferens is almost universal across snake species. In addition, the spermatids of the spherical structures were undergoing spermiogenesis, indicating that these spherical structures constituted another site for spermiogenesis. Based on these results, we suggested that the spherical structures be named seminiferous spherules. Spermiogenesis of the CRS in the testes peaks in August and September (Liang et al, 2009). We inferred that the limited lumen of the seminiferous tubule offered inadequate space for the testis spermatids to complete metamorphosis, and that some spermatids were transferred through the epididymis to the vas deferens where they cohered into the seminiferous spherule and gradually changed to sperm. Ultimately, during the reproductive period, sperm reach maximum number to enhance the success rate of reproduction. This might be considered an ingenious reproductive strategy for snake species during evolutionary history.

What are the substances or forces that allowed spermatids to cohere into the seminiferous spherule in the vas deferens and whether the spermatids were an isogenous group? These questions need further study.

3.2 Sperm storage of reptiles and adaptive evolutionary significance

Sperm storage is a physiological phenomenon, in which sperm that has been discharged from the testes must remain in the male or female genital duct for a long time waiting for ovulation to fertilize (Mozafar et al, 2004). Previous studies have shown that sperm storage is universal across reptile species, but whether the sperm is stored in the male deferens ducts or in the female oviduct differed greatly among species. For female reptiles, lizards and snakes store sperm in the front of the vagina and the oviduct infundibulum (Gist & Jones, 1987), while two freshwater turtles (*Sternotherus odoratus* and *Trachemys scripta*) stored sperm in the tubal protein secreting portion (Gist & Congdon, 1998). Sperm of the turtle (*Terrapene carolina*) are stored in the tubal isthmus (Gist & Fischer, 1993; Sever & Hamlett, 2002). The skink (*Scincella laterale holbrook*) generally stores sperm in the distal vagina (Sever & Hopkins, 2004), while the Chinese soft shelled turtle (*Pelodiscus sinensis*) stores sperm in the back of tubal protein secreting portion to the front of uterus (Zhang et al, 2008). For male reptiles, sea turtles such as *Chrysemys picta* and *Trachemys scripta* store a large number of sperm in the epididymis (Gist et al, 2002). The lizard (*Sitana ponticeriana cuvier*) stores sperm in the vas deferens and epididymis, with the vas deferens providing long-term sperm storage (Akbarsha et al, 2005). In the southeast Brazil snake (*Crotalus durissus terrificus*), sperm is stored in the vas deferens (Almeida-Santos et al, 2004). In Central and South American Caiman Crocodiles, a large number of sperm are stored in the vas deferens (Guerrero et al, 2004). The present study indicated that for CRS, sperm were stored in the efferent duct from August−October, in epididymal duct from August-the following January, and a large number in the vas deferens throughout the whole year (except in July). In addition, a large number of sperm were also observed during May in the vas deferens of the STRS, RBS and TNTS.

The present study, together with the previous data mentioned above, demonstrated that whether sperm is stored in female oviducts or male deferens ducts, sperm storage in reptiles is universal. In addition, from previous male Squamata research, although both the epididymis and vas deferens store sperm, the vas deferens serves as the main and long-term storage organ. Universal sperm storage across reptiles species can be explained by the desynchronization of the timing of spermiogenesis, ovulation, and mating (Gist & Congdon, 1998, Almeida-Santos et al, 2004) and as a result, after production and discharge from the testes, sperm require storage either in the epididymis and vas deferens or in the oviduct after mating to complete the process of sperm maturation for fertilization following ovulation. Based on the above analysis, we concluded that reptile sperm storage is an effective and advantageous reproductive strategy, which evolved gradually through phylogeny as an adaptive measure to the surrounding environment and as a necessary stage in their reproductive cycle. By storing sperm, the breeding time of reptiles can be extended, the successful rate of reproduction is enhanced, and species continuation is realized.

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