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Review

Advances in understanding mechanisms of long-term sperm storage-the soft-shelled turtle model

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Summary. Long-term sperm storage is a special reproductive strategy, which can extend the time window between mating and fertilization in some animal species. Spermatozoa of the soft-shelled turtle, *Pelodiscus sinensis*, can be stored in the epididymis and oviduct for at least six months and one year, respectively. How spermatozoa can be stored *in vivo* for such a prolonged period is yet to be explained. We analyze the mechanisms that contribute to long-term sperm storage in *P. sinensis*, and compare them with other species from three different perspectives: the spermatozoon itself, the storage microenvironment and the interaction between the spermatozoon and microenvironment. Characteristics of soft-shelled turtle spermatozoa itself, such as the huge cytoplasmic droplet with its content of several large lipid droplets (LDs) and onion-like mitochondira, facilitate long-term sperm storage. The microenvironment of reproductive tract, involving in the secretions, structural barriers, exosomes, androgen receptors, Toll-like receptors and survival factor Bcl-2, are important for the maintenance of spermatozoa long-term storage. Sperm heads are always embedded among the oviductal cilia and even intercalate into the apical hollowness of the ciliated cells, indicating that the ciliated cells support the stored spermatozoa. RNA seq is firstly used to detect the molecular mechanism of sperm storage, which shows that autophagy, apoptosis and immune take part in the long-term sperm storage in this species.

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Introduction

Fertilization occurs internally in all reptiles, birds and mammals, and in some fishes (e.g. the *chondrichthyes*) and amphibians (Jones, 1999). The requirement for internal fertilization sometimes results in a delay between mating and fertilization, and a need for spermatozoa to remain alive long enough to fertilize eggs (Birkhead and Møller, 1993). In fact, spermatozoa of animals with internal fertilization survive and are stored for variable periods of time before mating inside the male or after mating inside the female. This affects their life histories, mating system evolution and sexual selection systems (Orr and Zuk, 2014). Sperm storage, the extended maintenance of viable spermatozoa, probably occurs to some extent in most internally fertilizing animals (Uller et al., 2013). Because sperm storage increases the time window between sperm production, mating and fertilization, it provides increased opportunity for postcopulatory sexual selection (Sandell, 1990). Sperm storage can be adaptive in ecologically diverse habitats and can result from different selective forces acting on females and/or males, sometimes resulting in coevolution (Orr and Brennan, 2015).

A wide array of animals store spermatozoa, including: earthworms; arthropods such as insects and spiders; birds, such as falcons, quail, finches and geese; reptiles, including soft-shelled turtles, tortoises, lizards

and snakes; and a few mammals, for example bats, hares, horses and dogs (Orr and Zuk, 2012). In these taxa, spermatozoa may be stored for a few hours to several years. However, many of the species that store spermatozoa are threatened and protected by law (Holt and Lloyd, 2010). This poses practical and ethical difficulties for researchers who wish to study sperm storage *in vivo*. The soft-shelled turtle, *Pelodiscus sinensis*, belonging to the ectothermic amniotic reptiles, has high nutritional and pharmaceutical value and is widely raised in freshwater lakes of Asian countries such as China, Japan and Korea (Somfai-Relle et al., 2005). Spermatogenesis in this turtle is seasonal, with spermiation occurring in October (late autumn in Nanjing, China) (Zhang et al., 2008). Next, immature spermatozoa are transferred into the epididymis, where they are stored over hibernation (from November to the following April) until the following mating season (from June to August) (Bian et al., 2013a,b). After mating, the spermatozoa are stored in the oviduct of females until they are used to fertilize eggs. Thus, the soft-shelled turtle is a pratical, easily sources and ethically acceptable model species for uncovering the mechanism behind the long-term sperm storage phenomenon (Chen et al., 2015).

Here, we structure our paper firstly by presenting a list of possible reasons that contribute to long-term sperm storage in soft-shelled turtles (Table 1), and then proceeding to discuss from different perspectives which would help to improve our understanding of the mechanism of long-term sperm storage *in vivo*.

Location and term (duration) of sperm storage

Spermatogenesis in soft-shelled turtle is a seasonally dependent event, and spermiation takes place only in the later autumn (October) (Zhang et al., 2007). Spermatozoa are then transferred into the epididymis and stored there until the following May (Bian et al., 2013a,b), indicating that spermatozoa can be stored in the epididymis of this species for at least 6 months (Fig. 1). After mating during the period from June to August, the spermatozoa go to, and are stored, in the oviduct (Fig. 2), waiting to fertilize eggs. Lots of spermatozoa are found in the oviduct throughout the year. Active spermatozoa are still found in the oviduct after the females have been separated from the male for 12 months, showing that sperm storage in this turtle can be maintained for more than 1 year. Stored spermatozoa have been identified in the isthmus, uterus, and vagina of the oviduct (Han et al., 2008; Chen et al., 2015). Compared with other reptile species (Gist and Jones, 1987; Gist and Congdon, 1998), *P. sinensis* has a greater sperm storage capacity and thus can contain more spermatozoa from multiple males or greater ejaculate masses in a single copulation (Chen et al., 2015).

Fine structural constituents in the spermatozoon are propitious to long-term sperm storage

The spermatozoa develop distinctive morphological features with noticeable differences along the reproductive tracts of male and female *P. sinensis* (Fig. 3), which have important roles for the survival of spermatozoa during long-term storage.

A huge cytoplasmic droplet (CD) with its content of several large lipid droplets (LD) may be the transient organelle serving as the energy source essential for long-lived stored spermatozoa in the epididymis

Most of the spermatid cytoplasm in vertebrates is removed and phagocytosed by Sertoli cells during sperm formation in the testis (O'Donnell et al., 2011). However, a small portion of cytoplasm is generally retained as a CD on the flagellum of spermatozoa during sperm transit through the epididymis (Hermo et al., 2010). Contrary to earlier hypotheses, the CD is not a useless residual cytoplasmic body on the spermatozoa, as it has been reported that CDs play a role in osmoadaptation by allowing water to enter or exit in the cell (Hermo et al., 2010). Moreover, several classes of enzymes have been identified in CDs, including lysosomal hydrolases, glycolytic enzymes and intermediate metabolic enzymes (Dott and Dingle, 1968; Garbers et al., 1970; Harrison and White, 1972; Noland et al., 1983; Oko et al., 1993). Yuan and his/her colleagues perified CDs from murine epididymal spermatozoa and conducted proteomic analyses on proteins highly enriched in CDs (Yuan et al., 2013). They have suggested that CDs can serve as an energy source, providing the energy (i.e., ATPs) required for the continued maturation of epididymal spermatozoa.

A large CD, 2.5×4 μm, is attached along the entire midpiece and posterior head of most epididymal

Table 1. Overview of the possible specializations that contributes to long-term sperm storage of the soft-shelled turtle.

Male	Female
The huge cytoplasmic droplet with its content of several larger lipid droplets; Exosomes secreted by epididymal epithelia; Toll-like receptors in epididymal spermatozoa; The structural barrier formed by cell junctions; The epididymal microenvironment maintenance	Several lipoprotein membrane layers of the onion-like mitochondrion; Exosomes secreted by oviductal epithelia; Epithelial and gland cell secretions; Toll-like receptors in the oviduct epithelia; Interaction between spermatozoa and oviduct; The survival factor Bcl-2 of oviductal epithelia; Androgen, Autophagy, Apoptosis and Immune involved in oviductal sperm storage

spermatozoa in *P. sinensis*. There are several lipid droplets (LDs) inside the CDs, and each of the LDs has a diameter of approximately 0.5 μm (Zhang et al., 2015). From later autumn to later spring, corresponding to the early stage and later stage of sperm storage in the epididymis, the average number of LDs decreases, while the number of vacuoles inside the CDs increases. Some CDs just contain several vacuoles instead of LDs in later spring (Chen et al., 2018). The CD attachment site is retained along the midpiece and posterior head of the spermatozoon, which does not migrate down along the principal piece of the tail, from the caput to the cauda

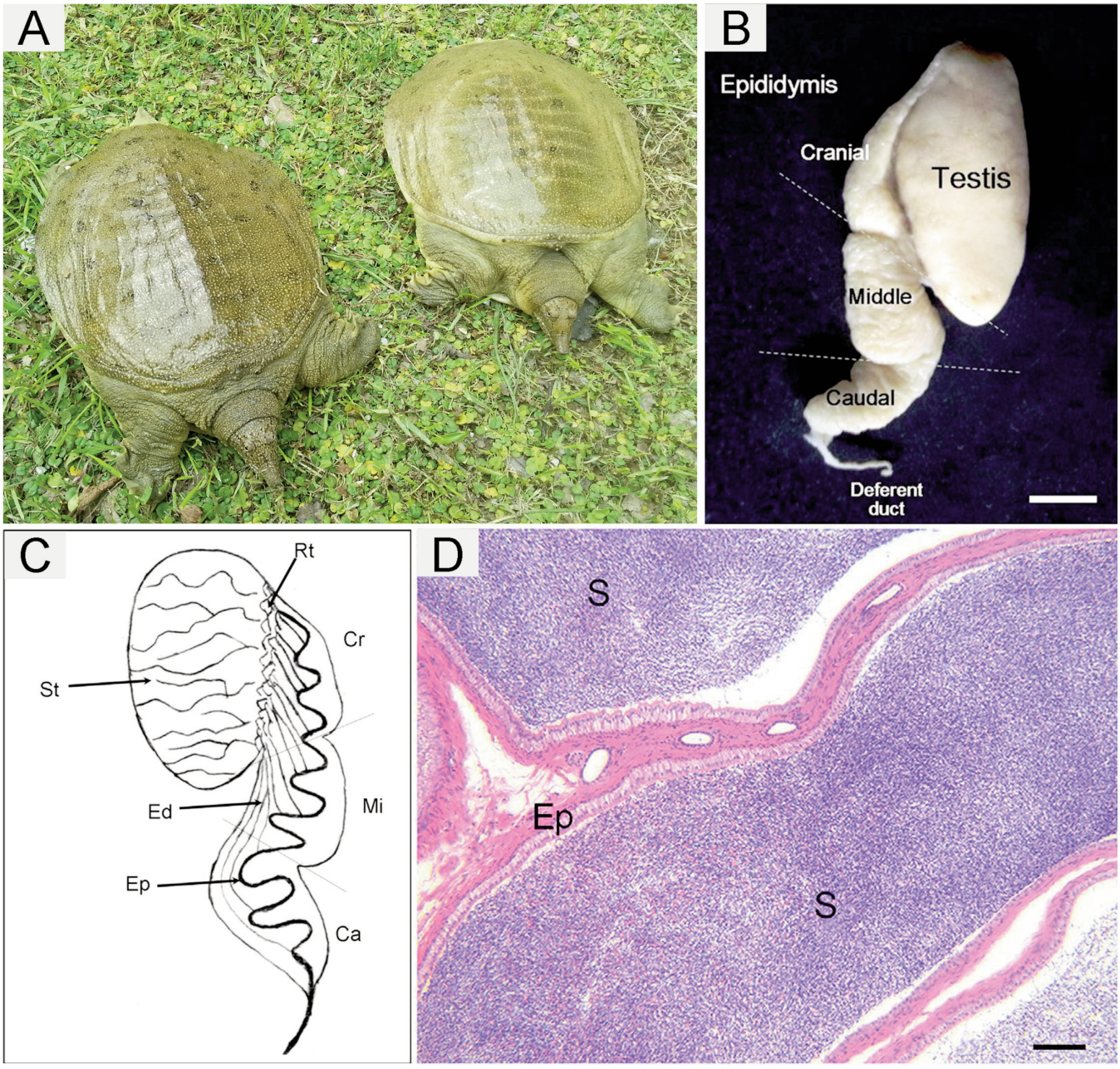

Fig. 1. Spermatozoa stored in the epididymis of *Pelodiscus sinensis*. Adapted from (Bian et al., 2013b). **A.** The Chinese soft-shelled turtle, Pelodiscus sinensis. **B.** Gross anatomic view of *P. sinensis* epididymis showing the three main regions: cranial, middle and caudal. **C.** Diagrammatic representation of the testis and its connections with the epididymis in *P. sinensis*. Note: 22-28 efferent ducts (Ed) branch from the rete testis (Rt) and towards the cranial (Cr), middle (Mi) and caudal (Ca) epididymis (Ep). St: seminiferous tubule. **D.** Numerous spermatozoa are stored in the epididymis of *P. sinensis*. Ep: epithelium, S: spermatozoa; Scale bars: B, 1 cm; D, 100 μm.

epididymidis (Zhang et al., 2015). However, in most vertebrate species, the CD migrates down along the sperm midpiece during epididymal transit (Cooper, 2011). The above ultrastructural and dynamic characteristics imply that CDs may be endogenous energy sources that can sustain spermatozoa longevity during epididymal transit of *P. sinensis*.

Autophagy supplies the cell with energy and maintains cellular homeostasis by degrading intracellular organelles and proteins. LDs have been identified as autophagic substrates (Dong and Czaja, 2011), and recent studies in yeast have indicated that the increased lipid abundance may prolong the chronological lifespan of laboratory strains (Handee et al., 2016). Ultrastructural analysis reveals a close relationship between LDs and the autophagic membrane structures over the period of epididymal sperm storage in *P. sinensis* (Chen et al., 2018). Some large LDs are surrounded by membranous vesicles and then seem to be penetrated by membranous structures. These structures result in the formation of smaller LDs or lipophagic vesicles. The number of LDs inside the CD are markedly reduced over the time of sperm storage in epididymis of *P. sinensis*; conversely, the autophagic membrane structures are markedly increased. Simultaneously, LC3-II (Microtubule-associated protein light chain 3, a specific marker for autophagy) expression is enhanced in late (July) compared with early (January) sperm storage (Chen et al., 2018). The LC3-II/LC3-I ratio is considered be an indicator of the intensity of autophagy (Rubinsztein et al., 2009). The above *in vivo* findings indicate that lipophagy is triggered during long-term sperm storage in the epididymis of *P. sinensis*. This concept is further confirmed by *in vitro* experiments. The epididymal spermatozoa of *P. sinensis* (collected in January) have an ability to survive more than 40 days at 4°C. The LDs of the CDs are enveloped and then sequestered by vesicles during the incubation period. Immunofluorescence shows significant LC3-II puncta distributed in the CDs of spermatozoa after 20 days incubation *in vitro*. Specifically, the colocalisation of LC3 and LDs is observed during incubation. Immunoblotting confirms the enhancement of autophagy with increased LC3-II protein levels in conjunction with decreased p62 levels. Pharmacological inhibition of autophagy with 3-methyladenine (3-MA) results in significant fusion of LDs in spermatozoa. These findings first explore the role of autophagy in the degradation of LDs during long-term sperm storage *in vivo* and *in vitro* (Fig. 4) (Chen et al., 2018).

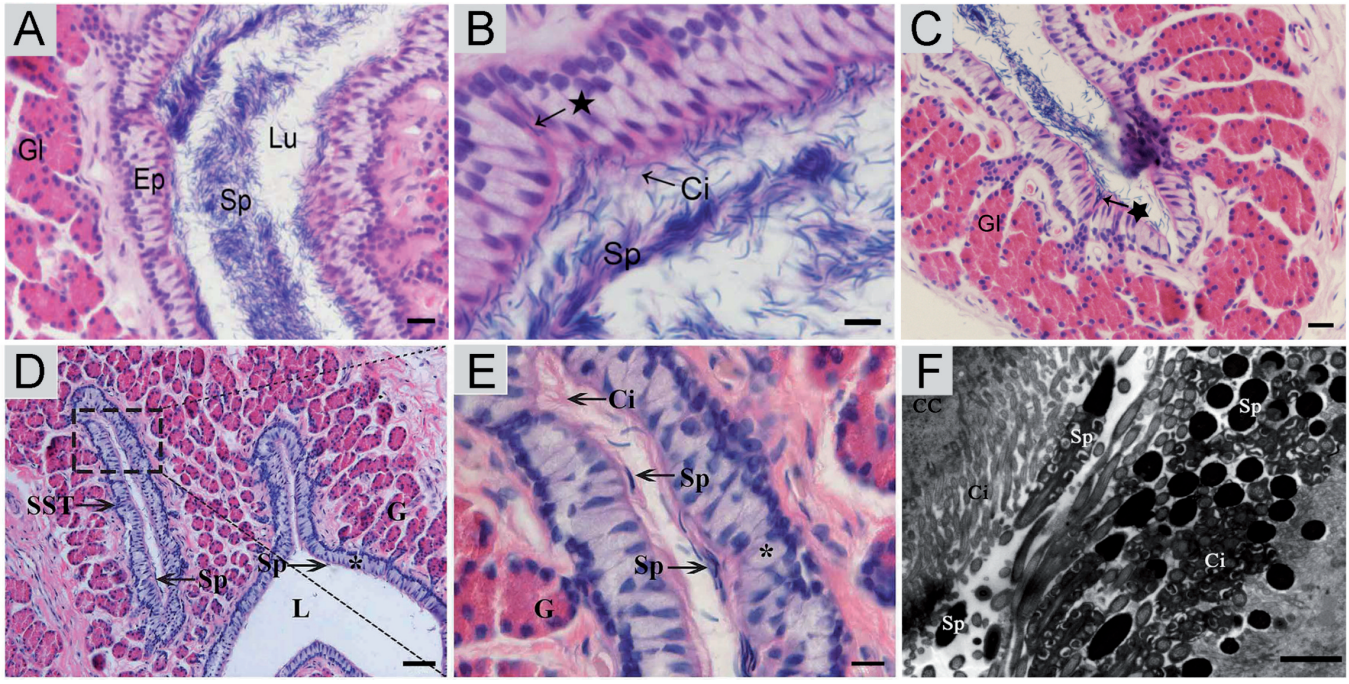

Fig. 2. Large amount of sperm stored in the oviduct of *P. sinensis*. Images A and B quoted from (Le et al., 2015); Images D and E quoted from (Liu et al., 2016b); Image F quoted from (Liu et al., 2016a); Image C: unpublished. **A.** Spermatozoa stored in the lumen of the vagina collected in November. Gl: gland, Ep: epithelia, Sp: spermatozoa, Lu: lumen. **B.** Spermatozoa in contact with epithelial cilia of the vagina collected in November. ★: gland ducts, Ci: cilia; Sp: spermatozoa. **C.** This micrograph shows spermatozoa that have penetrated into gland ducts of uterus collected in April. ★: gland ducts, Sp: spermatozoa. **D.** Spermatozoa stored in the sperm storage tubules (SSTs) of the isthmus collected in January. Sp: spermatozoa; L: lumen. **E.** The higher magnification view of the SSTs indicated by the square. G: gland cell; Ci: cilia; *: epithelium; Sp: spermatozoa. **F.** TEM showed that many spermatozoa were embedded among the cilia collected in January. CC: ciliated cell; Ci: cilia; Sp: spermatozoa; Scale bars: A, C, 20 μm; B, E, 10 μm; D, 50 μm; F, 2 μm.

The consumption of lipoprotein membrane in the onion-like mitochondrion can maintain the long-life of spermatozoa

The exact function of mitochondria in spermatozoa is controversial because they have extensive morphological heterogeneity. Long-term adaptations to various rates of ATP use can be achieved by modifying the number, morphology and location of mitochondria (Meinhardt et al., 1999; Nogueira et al., 2001; Zhang et al., 2015). Mitochondria regulate different aspects of reproductive function, but these aspects are not uniform throughout the animal kingdom (Ramalho-Santos et al., 2009). In insects, the mitochondria fuse to form giant mitochondrial derivatives (Werner and Simmons, 2008), which can drive the elongation of sperm (Perotti, 1973). In mammals, the spermatozoa show considerable metabolic flexibility, and many can function effectively on glycolysis alone without the need for mitochondrial energy production (Hodgson, 2009). The onion-like mitochondria have been observed in certain reptiles, including *Chrysemys picta* (Hess et al., 2010) and *Alligator mississippiensis* (Gribbins et al., 2011). However, there have been very few studies of the function and fate of this type of mitochondria within the female reproductive tract.

The mitochondria of spermatozoa in the epididymis and the early-stage oviduct are typically onion-like in shape with 8-15 layers of closely apposed membranes around a dense substrate centre in *P. sinensis* (Fig. 5). When the spermatozoa are transferred into the oviduct, the number of membrane layers decreases, and gaps gradually emerge between membrane layers in the mitochondria. Finally, the morphology of mitochondria returns to the normal double membrane at the later stage of storage (Zhang et al., 2015). Transformation of mitochondria in spermatozoa from onion-like to normal shape may involved in the different ATP requirements in different storage sites. Froman et al. proposed that spermatozoa stored in the hen oviduct are powered by oxidation of endogenous long-chain fatty acids, perhaps originating from the outer mitochondrial membrane (Froman et al., 2011).

These findings indicate that morphological changes

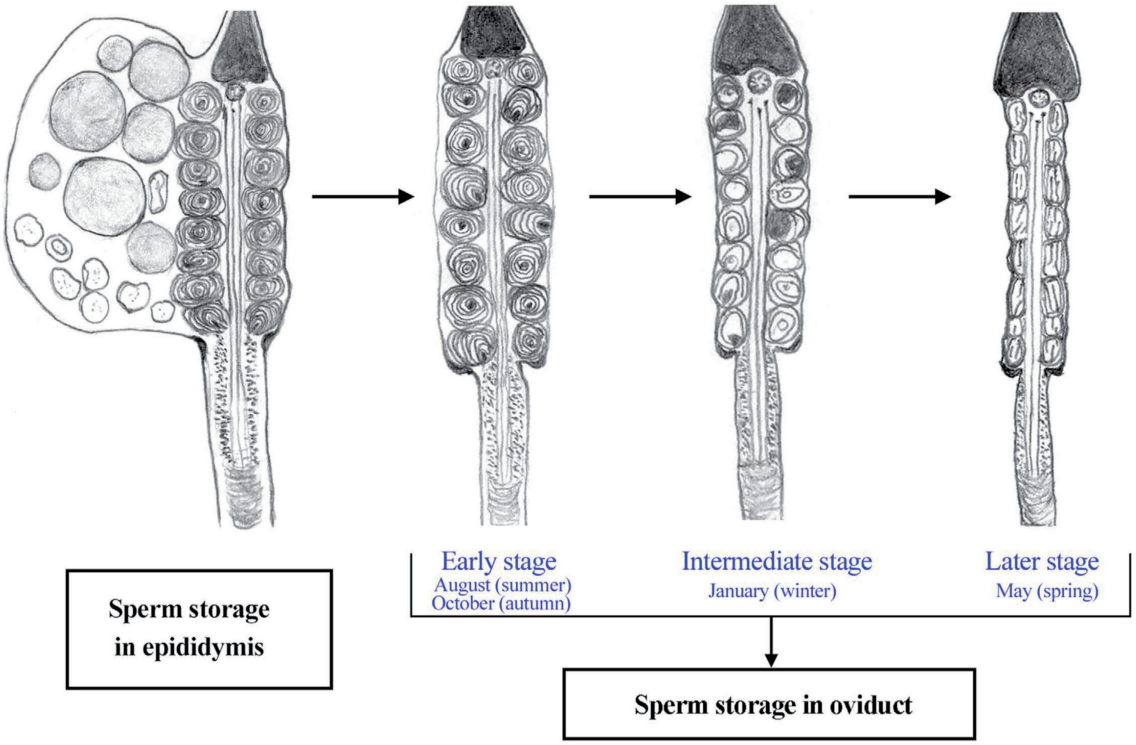

Fig. 3. A schematic organization demonstrating the transformation of spermatozoa in the genital tract of the *P. sinensis*. Quoted from (Zhang et al., 2015). A huge cytoplasmic droplet (CD) with several larger lipid droplets (LDs) is attached along the entire midpiece and posterior head of epididymal spermatozoa. Eight to fifteen concentric laminated membranes aroud a dense substrate core formed typical onion-like mitochondria in the midpiece of epididymal spermatooza. A major consequence of long-term sperm storage in the epididymis is the disappearance of cytoplasmic droplet, suggesting that CD with LDs may be a source of endogenous energy for epididymal spermatozoa. With the long-term sperm storage in the oviduct, the number of concentric lipoprotein membrane layers in the mitochondrion is decreased. The morphology of mitochondrion returns to their normal structure of a double membrane with cristae. Which indicate that the consumption of the mitochondrial lipoprotein membrane may provide an energy/nutrition sustaining long-term sperm storage in the oviduct.

of mitochondria can affect the production of ATP. Recent data suggest a physiological role for ATP synthase oligomerisation in the regulation of the atypical mitochondrial cristae shape observed in human syncytiotrophoblasts (Habersetzer et al., 2013). It is now widely accepted that there is a strong relationship between dimerisation/oligomerisation of ATP synthase and cristae morphology (Strauss et al., 2008; Velours et al., 2009; Davies et al., 2011). In *P. sinensis*, normal mitochondria in early spermatids develop into onion-like mitochondria in later spermatids and immature spermatozoa when spermatogenesis takes place in the testis and epididymis of Chinese soft-shelled turtles (Zhang et al., 2007; Bian et al., 2013a,b; Haseeb et al., 2018). Sperm mitochondria maintain the onion-like structure during sperm storage in the epididymis, but recover the more common cristae configuration during later stages of storage in the oviduct of *P. sinensis*. To our knowledge, this mitochondrial modification is first observed in vertebrate spermatozoa (Zhang et al., 2015). Most mechanistic studies of the relationship between ATP synthase oligomerisation and mitochondrial morphology have been carried out with yeast cells (Habersetzer et al., 2013), because fewer vertebrate cells are available for study. Thus, the turtle spermatozoon, which has typical onion-like mitochondria, can be a useful cell model for mitochondrial research.

Cellular and molecular mechanisms of long-term sperm storage in the epididymis and oviduct

Exosomes in the epididymis and oviduct

Cell-cell communication is a very complicated mechanism for information exchange. Direct membrane contact is the most obvious way for two cells to

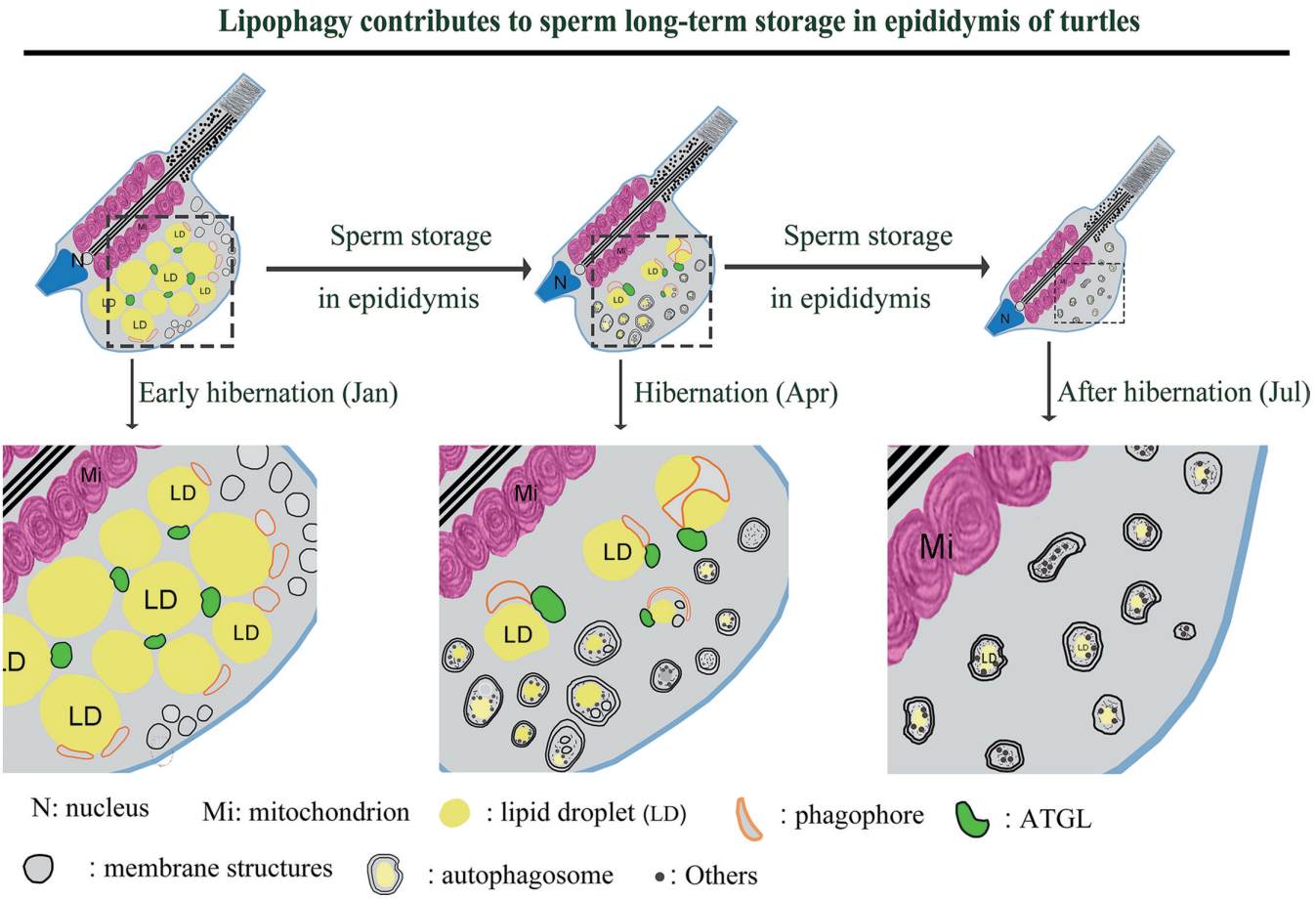

Fig. 4. Schematic representation of the role of lipophagy during sperm long-term storage in the epididymis of the *P. sinensis*. Quoted from (Chen et al., 2018). A large CD with numbers of LDs of spermatozoa serve as an important energy source during long-term storage in epididymis. In the early stages of sperm storage (January), large LDs are distributed within the CD. With the long-term sperm storage in epididymis, Large LDs are penetrated or sequestered by pro-autophagosomes into smaller LDs. Moreover, adipose triglyceride lipase (ATGL) is also involved in the degradation of LDs. This shows that lipophagy and lipolysis are triggered to maximize LDs breakdown sustaining spermatozoa live through hibernation.

communicate (Klohn et al., 2013). However, some soluble secreted molecules have also been shown to be important mediators of information transmission, including cytokines, hormones, and bioactive lipids. An increasing number of studies have shown a third mechanism for cell-cell communication where exosomes, membrane-bound vesicles released by one cell, can transmit essential information to its target cells (Johnstone, 2006). Epididymal exosomes can constitute the microenvironment of the epididymis to allow for long-term sperm storage, which is important for each step of sperm maturation and storage in the epididymis (Belleannee, 2015). Exosome marker CD63 expression is found in each of the three epididymal regions of *P. sinensis* (Chen et al., 2016). By TEM analysis which is considered the gold standard for exosome identification (Gyorgy et al., 2011; da Silveira et al., 2012), the epididymal exosomes in the turtle are round in shape and approximately 50-300 nm in diameter. This is similar to those previously described in mammals (Caballero et al., 2013; Schwarz et al., 2013). Importantly, TEM results provide significant evidence for apocrine secretion of exosomes. Multivesicular bodies (MVB) in the principal cells of the turtle have also been detected. MVB fusion with the plasma membrane leads to the release of the internal vesicles into the extracellular space. There is a clear significant difference between the above two exosome secretion pathways (Chen et al., 2016). Based on these observations, it is hypothesized that the principal cells secrete exosomes into the turtle's epididymal lumen by apocrine secretion and MVB pathways, while only one secretion pathway involved in exosomes has been reported in the mammalian epididymis.

The ciliated and gland cells in *P. sinensis* oviduct are also involved in the release of exosomes; these cells show positive expression of CD63 in different months (Waqas et al., 2017). By CD63 immunostaining, the quantity of exosomes is low during breeding season. But the expression level is higher during the sperm storage period. During transiting in the female reproductive tract, the mammalian oviduct secretions (oviductosomes) are thought to be responsible for maintaining the fertilizing ability of spermatozoa as they are stored in the reservoir compartment and remain viable until the ovulation occurs (Al-Dossary et al., 2013). Huang et al. reported that CD63 was increased in the sperm storage tubules (SSTs) in which sperm are stored after insemination in chickens (Huang et al., 2017). Electron microscopy and immunohistochemical (IHC) results provide the first experiments *in vivo* to substantiate the apocrine pathway of exosomes in *P. sinensis* (Waqas et al., 2017). In the study by Al-Dossary et al. (2013), the data revealed that the vesicles fuse with the sperm plasma membrane, although the authors did not comment on this. It is likely that such sperm-vesicle union is the mechanism by which Plasma Membrane Ca^{2+}-ATPase (PMCA) 4a is transferred to spermatozoa (Al-Dossary et al., 2013). In *P. sinensis*, some exosome-like particles were in close contact with the sperm membrane and cilia in the oviduct lumen that directly gives clear speculation about the role of these nanoparticles related to different transferring properties and fusogenic characters (Waqas et al., 2017). The

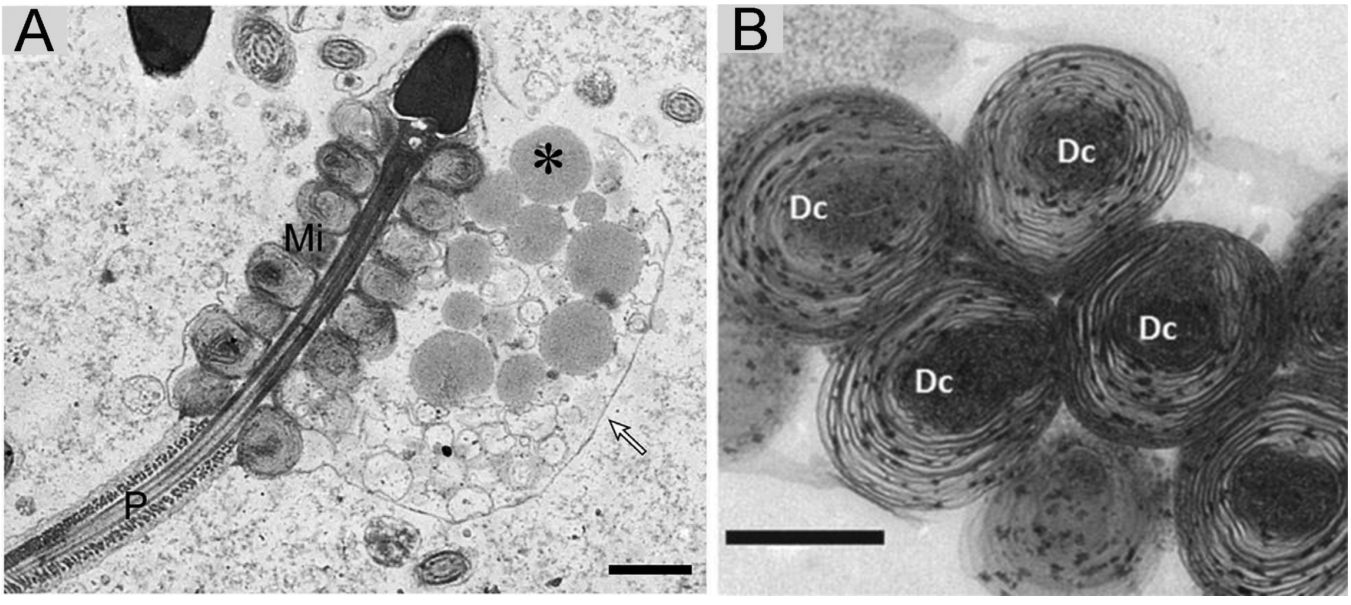

Fig. 5. The onion-like mitochondria of epididymal spermatozoa in *P. sinensis*. Adapted from (Haseeb et al., 2018). **A.** Electron micrograph of epididymal spermatozoa. Mi: mitochondria, P: principal piece of sperm, *: lipid droplets. **B.** Higher magnification image of onion-like mitochondria. Dc: dense core; Scale bars: A, 1 μm; B, 200 nm.

epithelial ciliated cells and gland cells are involved in the release of exosomes directly into lumen and give strong to moderate positive CD63 expression during sperm storage, whereas moderate to weak CD63 expression during breeding period. Intracellular MVBs, intracellular and extracellular exosomes, and their contact with cilia and with the sperm membrane give this turtle a unique secretory morphology, which underlines the usefulness of this model species for understanding cellular communication through exosomes.

Epithelial and gland cell secretions are important for the nutrition and maintenance of spermatozoa stored in the oviduct

The oviduct is the location of fertilization and sperm storage. Structurally, the oviduct of reptiles consists of five segments: the infundibulum, magnum, isthmus, uterine and vagina. In most reptilian species, the epithelium of the oviduct is primarily composed of two types of cells, ciliated and secretory cells (Girling, 2002). The function of ciliated cells is to maintain

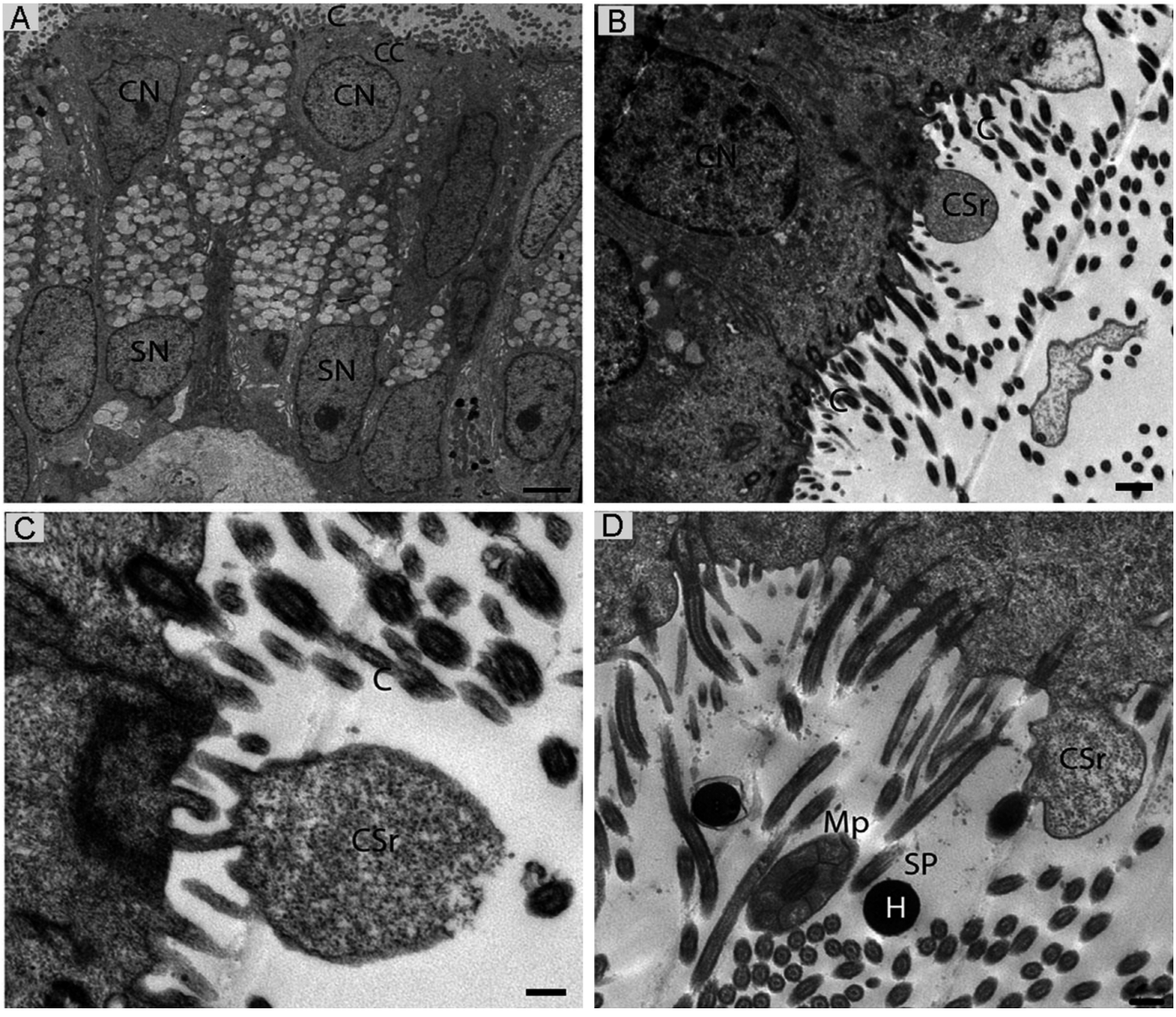

Fig. 6. Electron micrograph of the ciliated cells in the oviduct of *P. sinensis*. Adapted from (Waqas et al., 2015). **A.** Microphotograph of the uterus showing ciliated cell (CC) with apical nuclei (CN) and the presence of cilia (C) on the axial border as well as secretory cells (SC) with basal nuclei (SN). **B-D.** TEM micrographs of the isthmus showing evidence of the involvement of ciliated cells with cilia (C) in the secretions, the secretory bulb (CSr) is releasing into the lumen, where spermatozoa (Sp) with a prominent head (H) and mid-piece (Mp) are stored. C: cilia; CN: nuclei; Scale bars: A, 10 µm; B, D, 2 µm; C, 1 µm.

mucosal movement and remove cellular debris while ciliary movement may be involved in assisting the movement of spermatozoa and ovulated eggs (Palmer and Guillette, 1990). Non-ciliated or secretory cells are involved in producing the lubricating fluid, which contains mucus, maintaining a moist and clean oviduct lumen (Sarkar et al., 2003; Aviles et al., 2010). Several studies have shown that epithelial secretory cells contain putative secretory granules consisting of electron-dense and electron-lucent granules that are primarily released in a merocrine or apocrine fashion. Variations in the morphological features of these granules have been found in different species (Odor and Augustine, 1995). The oviductal epithelium in the lizard Calotes versicolor contains glycosaminoglycans and substances that are nutritive in function and provide carrier matrices for stored spermatozoa (Sever and Hamlett, 2002).

Evidence of the role of carrier matrices related to sperm storage has also been observed in the red-sided garter snake *Thamnophis sirtalis*; in this species, carrier matrices facilitate the transport of sperm from the anterior vagina to the infundibulum in addition to acting as a nutritive store (Halpert et al., 1982). Bakst and Bauchan suggested that oviductal secretions are key factors for sperm storage in birds, as the microvillus blebs on the apical tips of sperm storage tubule (SST) epithelial cells are thought to have a key role for the sustained storage of spermatozoa (Bakst and Bauchan, 2015), supplying metabolic substrates and transport vesicles to support sperm survival in the SST lumen. In the oviduct of hens, the fatty acids secreted from the SSTs may contribute to the sperm survival (Huang et al., 2016). The ultrastructure and cytology of cells in the oviduct of *P. sinensis* are quite similar but sometimes they show markedly variability; the number of glands in the isthmus is found to be variable, and glands are sometimes observed to be missing in the middle of the lamina propria. Secretory cells are commonly identified in all the parts, except the magnum, where these cells contained only dark granules (Waqas et al., 2015). In *P. sinensis*, the storage location of spermatozoa is restricted to the isthmus, uterus and vagina (Le et al., 2015). The epithelium lining of these three segments consists of ciliated and secretory cells. Varying quantities of spermatozoa are observed in the lumen of the isthmus, uterus and vagina, and most of these spermatozoa are alive as they are not stained by eosin (Waqas et al., 2017). The prominent feature of the ciliated cells is the presence of cilia on their apical borders. These cells are found to be involved in the release of huge apocrine secretions into the lumen through secretory vesicles (Fig. 6). The release of these large vesicles ultimately aids in

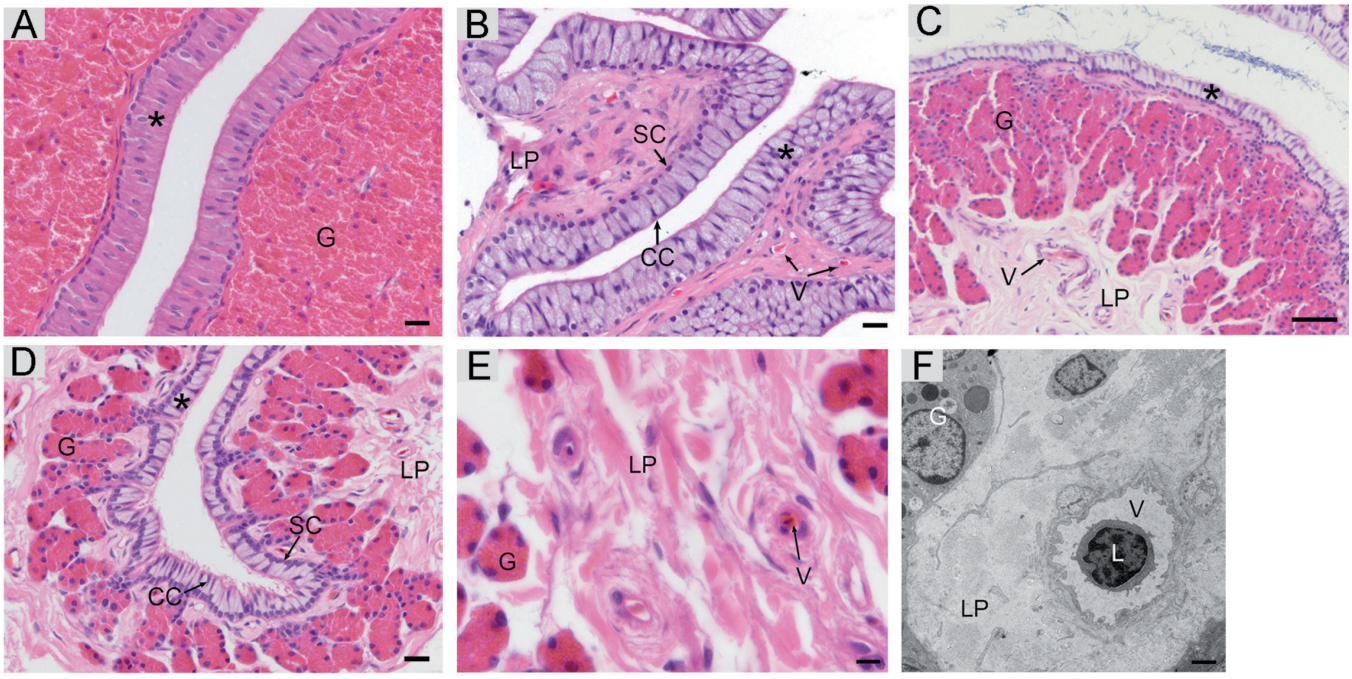

Fig. 7. Immune cells were scarce in the mucosa of the oviduct. Adapted from (Chen et al., 2015). (A) Magnum, *: epithelium; G: gland. **B.** Isthmus, LP: lamina propria; SC: secretory cells; CC: ciliated cells; *: epithelium; V: blood vessel. **C.** Uterine, V: blood vessel; LP: lamina propria; G: gland; *: epithelium. **D.** Vagina, G: gland; *: epithelium; CC: ciliated cells; SC: secretory cells; LP: lamina propria. **E.** Few immune cells occasionally appeared inside the blood vessel of the lamina propria. G: gland; LP: lamina propria; V: blood vessel. **F.** Electron micrograph of lymphocyte inside the blood vessel of the lamina propria in the uterine. G: gland; LP: lamina propria; V: blood vessel; L: lymphocyte; Scale bars: A, B, D, E, 10 µm; C, 50 µm; F, 2 µm.

the mixing of secretions from the epithelium, which are found around the spermatozoa. Secretory cells are attached to the basal lamina and extend to the lumen. The most prominent cellular feature of these cells is the presence of biphasic secretory granules in the supranuclear region. The matrix of the granules exhibits electron-dense and electron-lucent portions, with the electron-dense portion always observed at the periphery. Beneath the surface epithelium, oviductal glands are prominent. The glands are entirely filled with secretory granules and connected to the exterior lumen through a modified gland duct. The granules exhibit a central compact region surrounded by concentric layers. Biphasic secretory granules, the concentric granules, and the electron-dense granules can release the material slowly for a long time in *P. sinensis* oviduct (Waqas et al., 2017). The mucosa of isthmus in *P. sinensis* shows extensive folding and convolutions. Majority of the mucosa is fused into sperm storage tubules (SSTs) orientiated toward the longitudinal axes of the oviduct. Mucosal folds also distributed in the uterine and vagina, but the form of SSTs scarcely shown compared with the isthmus (Han et al., 2008). Periodic acid-Schiff (PAS)-positive materials secreted from the epithelium are also indicated in the SSTs of the oviduct in *P. sinensis*. In the base of the epithelium there is a layer of blood vessels, enabling the exchange of materials. Moreover, the glands distributed in the inner mucosa are open to the lumen of the oviduct. It is possible that the spermatozoa are maintained for their lengthy storage period by secretions of the gland and the epithelium cells of SSTs (Fig. 2D,E).

Immune cells are sparse in the epithelium and lamina propria of oviduct which may be an indication of immune tolerance during sperm storage

The genital tract is a unique immunological environment that must both support the reproductive function and resist infection. A compromise state must be established that will allow selective immune privilege for gametes and the developing fetus within the context of an otherwise immunocompetent female reproductive system (Clark and Schust, 2013). There is a full set of

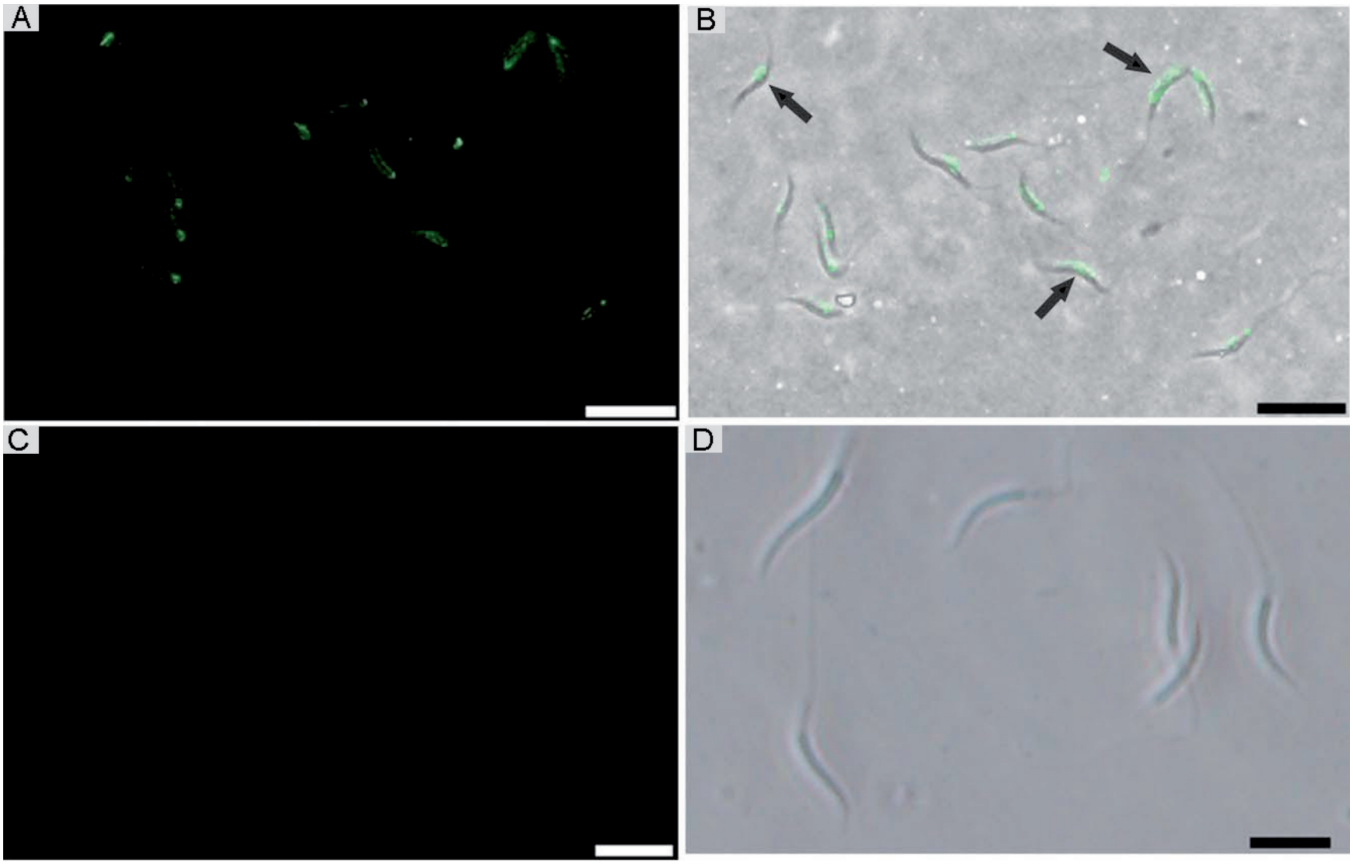

Fig. 8. Immunolocalization of TLR2 in epididymal spermatozoa. Quoted from (Hu et al., 2016). **A, B.** TLR2 is expressed in the midpiece and the posterior segment of the head (arrow) of the spermatozoa during hibernation. **C, D.** The negative controls for TLR2. Scale bars: A, B, 25 μm; C, D, 10 μm.

active immune cells in the female reproductive tract, and the differential regulation of these cells in the distinct compartments of the tract is critical for reproductive success (Dunbar et al., 2012). Seminal fluid not only induces the expression of proinflammatory cytokines and chemokines in the cervix, but also causes a major influx of macrophages, dendritic cells, and memory T cells in mammals (Sharkey et al., 2012). The stored spermatozoa were believed to induce an increase in immune cells of the oviduct, thus removing alloantigens, whereas, in fact, fewer immune cells are found in the mucosa of the female genital tract in *P. sinensis*. It is difficult to detect intraepithelial lymphocyte (IEL), lamina propria lymphocytes (LPLs), plasma cells, dendritic cells, neutrophils, macrophages, or mast cells in the layer of epithelium, except for some lymphocytes inside the blood vessel in oviduct lamina propria (Fig. 7) (Chen et al., 2015). Research on ants has shown that if more males contribute to the quantity of stored spermatozoa, this could reduce female immune response during the stage of sperm storage (Baer et al., 2006). Furthermore, in chickens, immune tolerance is considered be necessary for the storage of antigenic spermatozoa in order to extend the survival period (Bakst, 2011). The sparse immune cells may benefit spermatozoa hidden from the immune attack in the female reproductive tract, which suggests that spermatozoa might be immune-privileged during storage in the oviduct of *P. sinensis*. In other words, sperm storage may induce immune tolerance in female reproductive tract of this species.

Toll-like receptors expression in spermatozoa and oviduct, which plays critical roles in detecting and responding to invading pathogens and then protects the storing spermatozoa from microbial infections

Immune regulation is a crucial factor affecting sperm storage *in vivo*. In human studies, infection with foreign microorganisms can reduce the spermatozoa quantity and quality (Fujita et al., 2011), thus influencing sperm storage and possibly ultimately reducing fertilization ability. Toll-like receptors (TLRs) have evolved to recognize pathogen-derived molecules or pathogen associated molecular patterns (Girling and Hedger, 2007). The mRNA and protein expression of TLR2/4 are observed in spermatozoa of *P. sinensis* via RT-PCR and Western blot analyses, suggesting that spermatozoa have a powerful defense system to protect themselves from pathogens (Hu et al., 2016). Spermatozoa exhibit immune cell-like functions in human, as they possess TLRs in response to the bacterial endotoxins LPS and peptidoglycans (Fujita et al., 2011). In *P. sinensis*, immunofluorescence against TLR2/4 is localized to the midpiece and the posterior segment of the sperm head, where the huge CDs are located (Fig. 8). In human spermatozoa, however, TLR2/4 is localized to the acrosomal and tail regions (Fujita et al., 2011). The observed expression of TLR2/4 in CDs shows that sperm CDs play important immune roles during sperm storage in *P. sinensis*.

The initiation of innate immunology system may play an important role in the aspect of protection for long-term sperm storage when the spermatozoa enter the oviduct and come contact with the epithelium. TLR2/4 protein and mRNA are expressed in the oviduct of *P. sinensis*, and the immunohistochemistry results show that TLR2 protein is mainly localized to the membranes of ciliated cells, the cilium, and the membranes of secretory gland vesicles, while in other parts of the oviduct, less expression is observed. The expression of TLR4 protein, which is more widely distributed than TLR2 protein in the oviduct, is observed in nearly every part of the oviduct, particularly in the epithelial cell membrane and secretory gland vesicle membrane. Both the protein and mRNA levels of TLR2/4 are lower in the magnum than in the isthmus, uterus, and vagina during hibernation season. The expressions of TLR2 protein and mRNA in the magnum, isthmus and uterus decrease in April, compared with that in November. The expressions of TLR4 protein and mRNA in the magnum, uterus and vagina increase in April, compared with that in November (Li et al., 2015). Ozoe et al. suggested that, in hens, the lower part of the tract expresses more TLR4 protein than the upper part in the oviduct (Ozoe et al., 2009), which is not consistent with observations in the turtle, where the expressions of TLR2/4 mRNA and protein in the oviduct are higher in the lower part than that in the upper part of the reproductive tract both in November and April. When specific ligands bind to TLR2/4 receptors, the TLR2/4 signal pathway cascade is activated, leading to the release of a series of inflammatory factors, including IL-1, IL-6, IL-10, IFNb/a, and TNF-β, which protects the organism from damage (Takeuchi and Akira, 2010; Vaure and Liu, 2014). In addition, when spermatozoa are inseminated into the oviduct, these cells pass through the vagina and reach the storage site, uterus and isthmus. It is proposed that TLR2/4 protein expression would increase at the site where the spermatozoa are stored. However, the TLR2/4 expression profile during the initiation of storage remains elusive (Peng et al., 2005). Based on the results obtained in *P. sinensis*, the expressions of TLR2 protein and mRNA are decreased in the magnum, isthmus, uterus, and vagina in April compared with that in November. Perhaps TLR2 protein plays a role in the early stages of hibernation during sperm storage. However, in the oviduct, TLR4 protein and mRNA showed greater expression in April than that in November, suggesting that TLR4 protein plays a role in last stages of hibernation.

Studies have shown that TLR2/4 expression is widely distributed throughout the reproductive tract (Young et al., 2004; Fazeli et al., 2005). In hens, TLR4 immunoreactivity is observed on the surface of the epithelium, and in spindle-shaped subepithelial cells and leukocytes in the lamina propria of the isthmus, uterus, and vagina of the oviduct (Ozoe et al., 2009), which is

consistent with the results obtained in *P. sinensis*. However, in contrast to the findings reported herein, the expression of TLR2/4 protein is also detected in the epithelial cell membrane, cilia, muscles, vascular endothelium, and the membranes of secretory gland vesicles in the turtle oviduct.

There are structural barriers in the epididymis and oviduct, by which the nourishment exchange and the microenvironment maintenance are ensured

Epithelia cells are connected by cell junctions including tight junctions, intermediate junctions and desmosomes in the epididymis of *P. sinensis*. Moreover, cell junctions are concentrated in the apical half of the epithelial cells in the epididymis. In addition to contributing to the structural integrity of the epithelium, stability of the microenvironment in the cavity of the epididymis is also assured by the junctions. It has been confirmed that the epididymis provides an appropriate microenvironment for the spermatozoa to maintain their fertility, even across an entire year (Han et al., 2008). On the other hand, a prominent feature of the SSTs in *P. sinensis* is the presence of a barrier, which is called blood-epithelium barrier here. Another principal feature of the SSTs is the presence of cell junction complexes between cells, including tight junction, intermediate junction, desmosome, and lateral interdigitations in the oviduct of *P. sinensis*. These junctions serve not only as sites of adhesion but also as seals to prevent the flow of materials through the intercellular space and to provide a mechanism for communication between adjacent cells (Han et al., 2008). As a special structure of sperm storage, in addition to contributing to the structural integrity of the SSTs, which are stretched considerably as ova descend through the albumen region of the oviduct, these junctions in the turtle may constitute an immunological barrier between stored spermatozoa and host, thus facilitating the long-term storage. Similar with blood-brain barrier and blood-testis barrier, or blood-air barrier, which have been reviewed extensively, the blood-epithelium barrier might play an important role in the microenvironment maintenance and the nourishment exchange in the oviduct.

Sperm heads are always embedded among the cilia and even intercalate into the apical hollowness of the ciliated cells in the oviduct during sperm storage, suggesting that the ciliated cells can support the stored spermatozoa.

Interaction between spermatozoa and oviductal cells can lengthen the life span of spermatozoa, regulates spermatozoa maturation, and affect the fertilizing ability of spermatozoa in mammals (Miller, 2011). *In vitro*, the binding of spermatozoa to the oviductal epithelium has been shown to prolong the motile life span of spermatozoa in several species (Raychoudhury and Suarez, 1991; Pacey et al., 1995; Apichela et al., 2009). Strategies underlying sperm binding have been considered, especially in terms of preovulatory sperm storage and suppression of full membranous maturation (Hunter, 2011). On the other hand, the protective effects of spermatozoon-oviductal epithelial cell interaction against oxidative stress in human spermatozoa have been demonstrated (Huang et al., 2013). Direct interactions between spermatozoa and oviduct have been reported in diverse mammal species (Yeste et al., 2009). However, there have been few reports in reptiles and avians (Long et al., 2003). As far as we are aware, spermatozoa are described as residing free in the lumen of sperm storage sites, without direct connection with the oviductal tissue in some reptiles (Kumari et al., 1990; Gist and Fischer, 1993; Gist et al., 2008). In the keeled earless lizard, *Holbrookia propinqua*, the spermatozoa are rarely considered to be partially embedded in oviductal tissue (Adams and Cooper, 1988). The difference of the sperm-epithelium relationships in reptilian species implies diverse mechanisms of the long-term sperm storage. In *P. sinensis*, direct interactions between spermatozoa and oviduct are found. The sperm heads are always embedded among cilia and even intercalate into the apical hollowness of the ciliated cell in epithelium during storage in the oviduct. There is no lysosome around the apical hollowness, indicating that the ciliated cell can support the spermatozoon instead of phagocytosing in the oviduct (Fig. 9) (Chen et al., 2015). Therefore, the ciliated cells probably have a role in maintaining sperm storage within the oviduct for a long time in the turtle. The stored spermatozoa often go into the gland conduit of the oviduct (Chen et al., 2015). The protective effects of the spermatozoon-oviductal epithelial cell interaction may enhance the ability of resistance to oxidative stress in turtle spermatozoa, to extend spermatozoon life span during long-term sperm storage. Furthermore, the turtle spermatozoa are often present in the gland conduit of oviduct, where they can easily get the gland secretion for nourishment during sperm storage.

The survival factor Bcl-2 expression in the oviduct is associated with sperm storage occurrence

Holt (2011) suggested that the spermatozoa often make intimate and chemically specific contact with the surface of oviductal epithelial cells (Holt, 2011); and the epithelial cells respond by de novo gene transcription (Ellington et al., 1993) and the synthesis of new proteins (Fazeli et al., 2004; Georgiou et al., 2007). Taking into consideration the known Bcl-2 function of anti-apoptosis, it is hypothesized that Bcl-2 is involved in the sperm storage. Studies on sperm storage in a bat revealed that Bcl-2 is required for prolonged survival of stored sperm (Roy and Krishna, 2011). Another study reported that Bcl-2 expression increases over time in the stroma of ovarian epithelial cancer cells, which supports the idea that Bcl-2 could be secreted (Anderson et al., 2009) and potentially provide a suitable microenvironment (McLachlan et al., 2002). Western

blot analysis confirms the presence of Bcl-2 in *P. sinensis* oviduct. IHC and H&E results show that the time of occurrence of intense positive Bcl-2 reaction in glands, gland ducts, and cilia is almost synchronous with the period when sperm storage occurs. IHC results reveal that Bcl-2 is evenly distributed throughout the oviduct epithelium and the host glands. Bcl-2 immunostaining in gland cells and gland ducts is intense

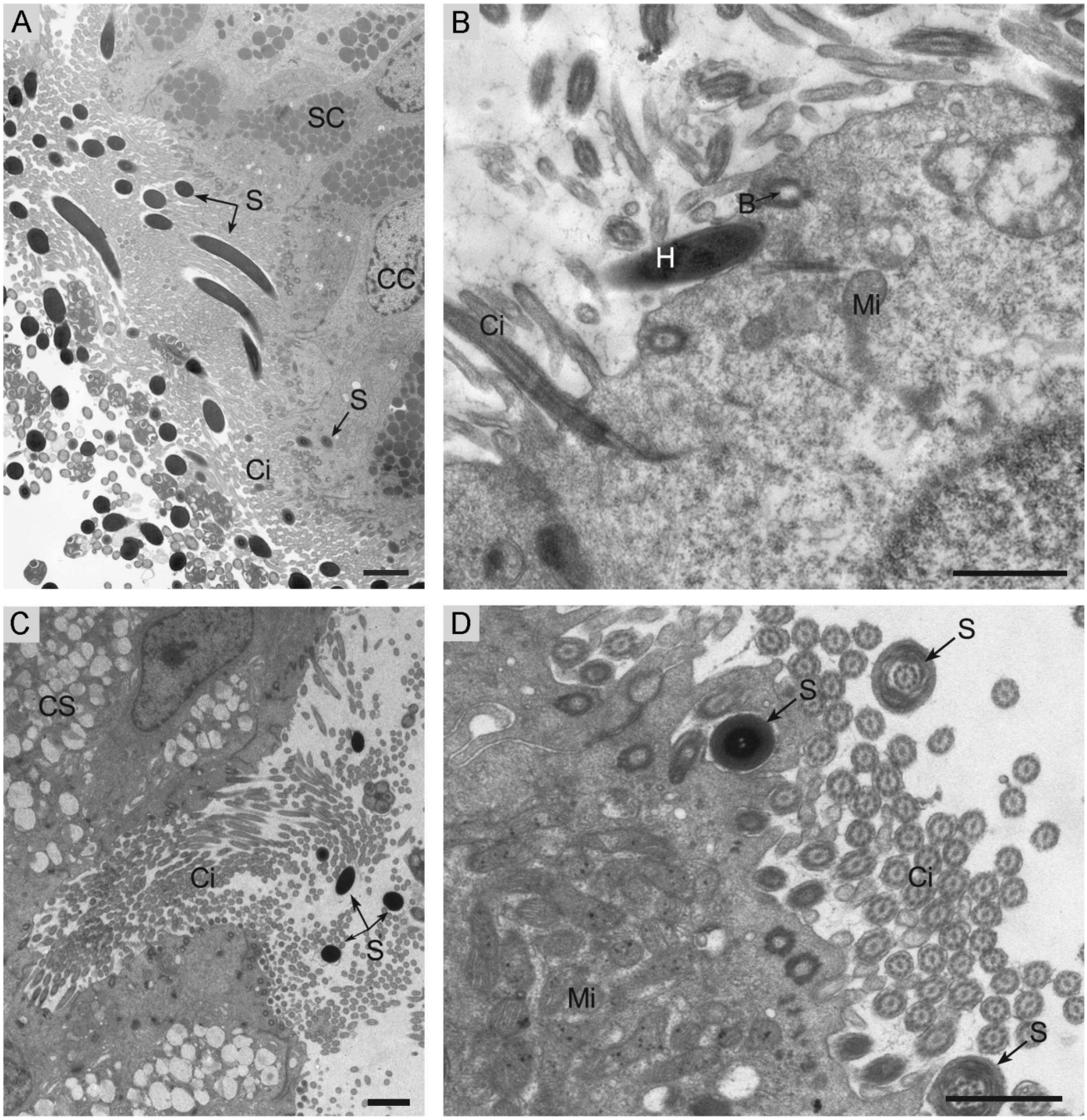

Fig. 9. Transmission electron microscopy showing spermatozoa attachment in the oviduct of *P. sinensis*. Quoted from (Chen et al., 2015). **A.** Many spermatozoa (S) are embedded among cilia (Ci) and inserted into the apical hollowness of the ciliated cell (CC). SC: secretory cells. **B.** A spermatozoon inserted its head (H) into the hollownes which is surrounded by no lysosome in ciliated cell. Ci: cilia; Mi: mitochondia; B: basal body. **C.** Spermatozoa are present at the opening of gland conduit. Ci: cilia; S: spermatozoa. **D.** Cross section of the spermatozoa head in the hollowness of ciliated cell and the spermatozoa midpiece among cilia. S: spermatozoa; Ci: cilia; Mi: mitochondia; Scale bars: A, C, 2 μm; B, D, 1 μm.

in November, and increase in intensity until April; the intensity progressively decreases from July to October. On the surface of the ciliated epithelial cells, the Bcl-2 immunostaining become intense from January to April, weakened in July and is almost nonexistent in October (Le et al., 2015).

Very intense positive immunostaining in glands and gland ducts was first observed in oviducts collected in November, whereas more intense immunostaining on the surface of the ciliated epithelial cells appeared in late January oviducts. This increase in immunostaining might be related to the amount of time it takes for Bcl-2 to transfer and accumulate in cilia in *P. sinensis*. Study on *P. sinensis* demonstrates the presence of many mitochondria in the ciliated epithelial cells, as Bcl-2 is a mitochondrial membrane protein. It is strongly suggested that Bcl-2 produced by gland cells to form a microenvironment with cilia, might support sperm storage in *P. sinensis* oviduct, in which the spermatozoa are protected against apoptosis (Le et al., 2015).

The localization and the variation of androgen receptor in the oviduct demonstrate the crucial regulating roles of androgens in sperm storage.

Androgens are essential for sexual development and health throughout the life span of male vertebrates, including the outward development of secondary sex characters and the initiation and maintenance of spermatogenesis (McLachlan et al., 2002). The relationship between androgens and the prolonged sperm storage has primarily been studied in males. Jones (2004) reported that the epithelial cells of the cauda epididymidis could be stimulated through androgens, thereby ensuring sperm survival for a prolonged period, while the withdrawal of androgens from epithelial cells ultimately results in the dissolution and degradation of spermatozoa (Jones, 2004). In the male lizard (*Sceloporus undulates*), androgens are highly correlated with size and weight of the epididymis, and the stored spermatozoa are lost from the regressing epididymis (McKinney and Marion, 1985). Furthermore, high circulating levels of androgen are also present in the blood of female reptiles and amphibians during certain periods of their seasonal reproductive cycles (Lind et al., 2010). In female *P. sinensis*, the concentrations of circulating testosterone (T) and dihydrotestosterone (DHT) show relatively higher levels during the hibernation season than that during the non-hibernation season. These observations are consistent with the studies in female bats: a high concentration of T is necessary to maintain the integrity and viability of stored spermatozoa in the bat oviduct (Roy and Krishna, 2010). These results imply the probable relationship between androgens and prolonged sperm storage in female *P. sinensis* (Liu et al., 2016).

Androgens modulate multiple developmental and physiological processes through binding to androgen receptor (AR), a member of the nuclear receptor superfamily (Staub and Beer, 1997). The localization of AR in sperm storage tubules would provide evidence for the roles of androgens. IHC and Western blot analyses

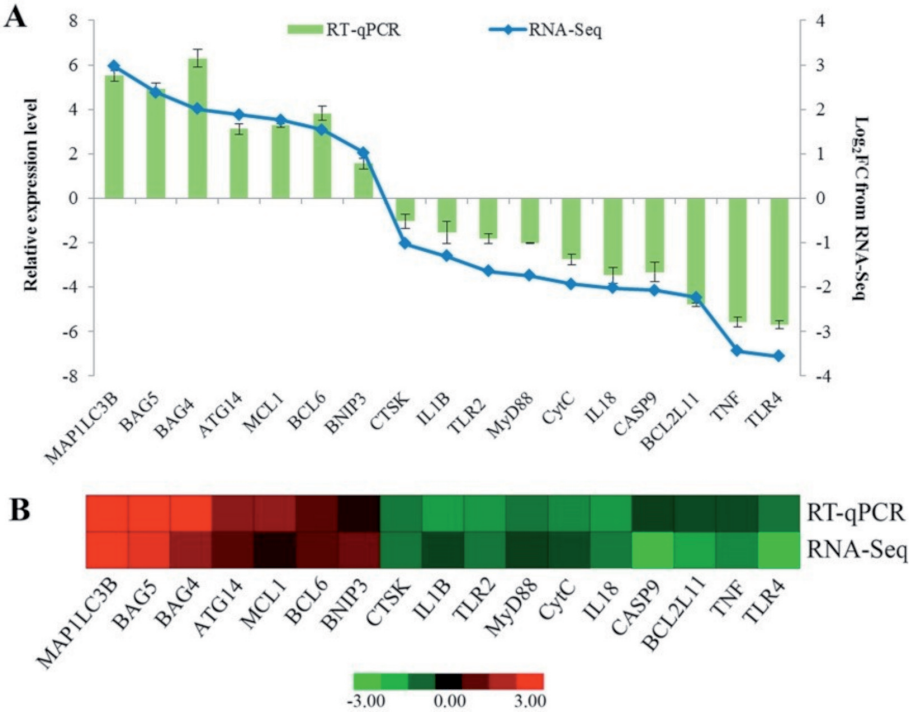

Fig. 10. Expression validation of selected DEGs related to sperm storage. Quoted from (Liu et al., 2016b). **A.** The relative expression levels of DEGs by RT-qPCR were compared with the transcript abundances from RNA-Seq. **B.** Heat map diagram of the expression patterns of DEGs in *P. sinensis*. The red and green colours indicate up- and down-regulated genes in the FU_2 library compared with those in the FU_1 library, respectively.

reveal that AR is localized to the vagina, uterus and isthmus of female *P. sinensis*. AR is distributed throughout the oviduct epithelium and gland cells. In the oviduct epithelium of *P. sinensis*, there are two primary cell types: ciliated cells and secretory cells (Han et al., 2008). A variety of functions have been attributed to ciliated cells. Some studies have shown that ciliated cells are involved in the movement of mucus and cellular debris in the oviduct (Palmer and Guillette, 1988). Other studies have suggested that ciliated cells are involved in spermatozoa survival and might be important in sperm transport and ova movement (Girling et al., 1997). In *P. sinensis*, almost all AR immunostaining are observed in the cilia of ciliated cells. Furthermore, H&E staining and TEM analysis demonstrate that spermatozoa are closely attached to epithelial cilia during sperm storage. These results reveal that androgens might play a role in sperm storage via the cilia cells. In addition, AR is localized to the cytoplasm in gland cells (Liu et al., 2016a,b). Similar cytosolic AR localization has been documented in the oviduct of the turtle *Trachemys scripta*, which also exhibits prolonged sperm storage (Selcer et al., 2005). Androgens exhibit direct effects mediated through AR to induce receptor dimerization and recruit co-regulators to promote target gene expression (Heinlein and Chang, 2002; Lee and Chang, 2003). However, increasing

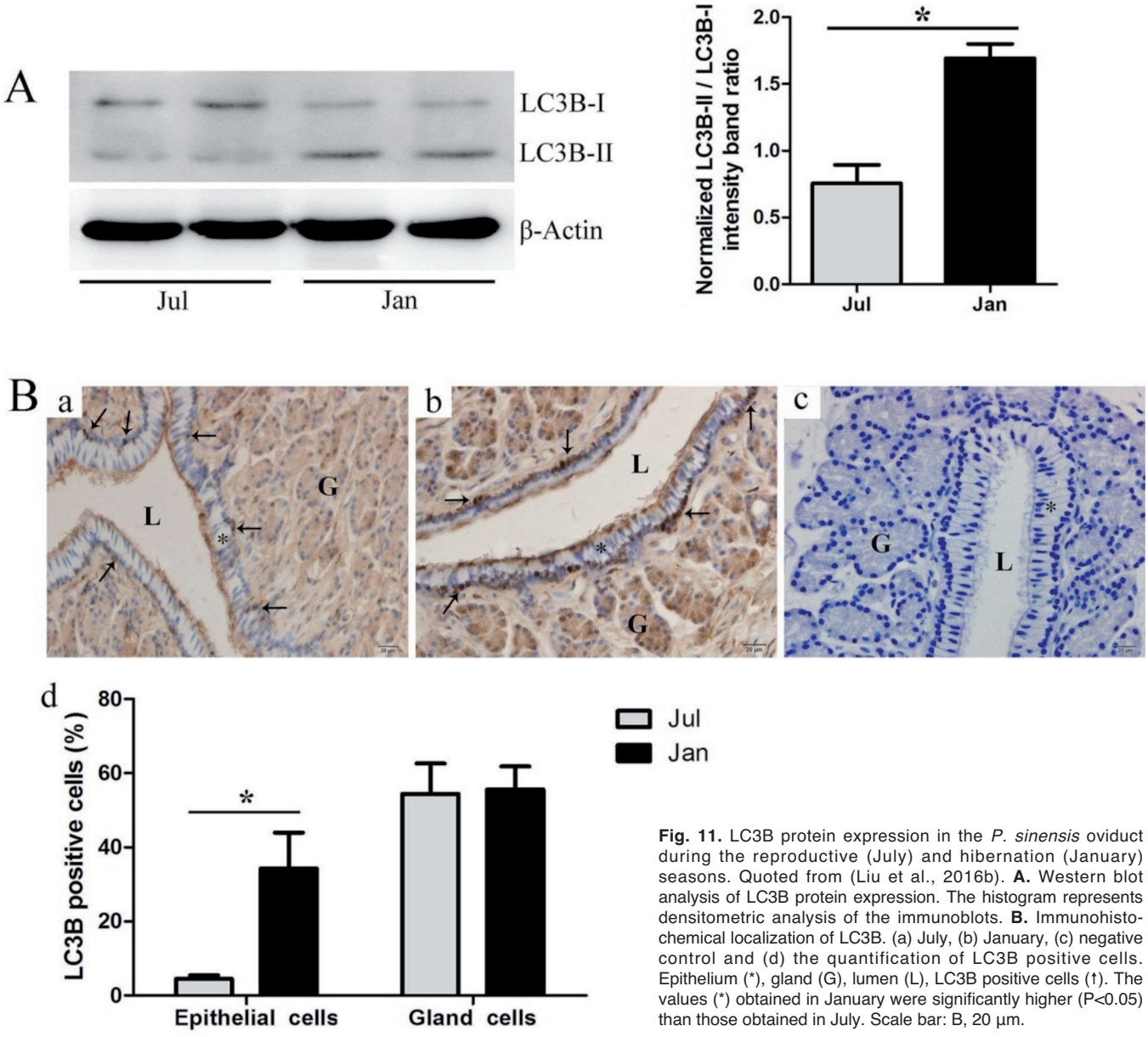

Fig. 11. LC3B protein expression in the *P. sinensis* oviduct during the reproductive (July) and hibernation (January) seasons. Quoted from (Liu et al., 2016b). **A.** Western blot analysis of LC3B protein expression. The histogram represents densitometric analysis of the immunoblots. **B.** Immunohistochemical localization of LC3B. (a) July, (b) January, (c) negative control and (d) the quantification of LC3B positive cells. Epithelium (*), gland (G), lumen (L), LC3B positive cells (↑). The values (*) obtained in January were significantly higher ($P<0.05$) than those obtained in July. Scale bar: B, 20 μm.

evidence has suggested the physiological effect of non-genomic androgen action (Simoncini and Genazzani, 2003; Foradori et al., 2008). In many cases, AR is localized outside the nucleus and might trigger biological responses through non-genomic mechanisms. Baron et al. report that the AR activates the PI3-K/AKT pathway through which androgens could protect epithelial cells against apoptosis (Baron et al., 2004).

Moreover, there is a marked variation in AR protein expression in the vagina, uterus and isthmus of female *P. sinensis* during different stages of sperm storage. IHC and Western blot analyses reveal intense AR expression during the hibernation season, consistent with the period of peak circulating T and DHT levels. Furthermore, a similar trend of circulating AR mRNA levels is confirmed through qPCR analysis (Liu et al., 2016a,b). The presence and variation of AR indicate the vital roles of androgen in sperm storage in the oviduct of *P. sinensis*. For example, it has been suggested that androgens control many signalling pathways mediated through the AR. Stereological studies have demonstrated that androgen is responsible for apoptosis in the seminiferous epithelium (Zhang et al., 2008). A study demonstrated that AR protects osteoblasts and osteocytes from apoptosis through the Src/Shc/ERK signalling pathway (Kousteni et al., 2001). Moreover, the involvement of apoptotic pathways in sperm storage has also been reported. Urhausen et al. describe the expression of apoptosis-related proteins in the dog oviduct, suggesting that the control of apoptosis could be a functional component of sperm storage mechanisms prior to fertilization (Urhausen et al., 2011). Furthermore, the oviduct of *P. sinensis* might support sperm storage through anti-apoptosis (Le et al., 2015). Hence, the high AR expression likely indicates that androgens play vital roles in prolonging the spermatozoa storage periods in the oviduct, and androgens are major players in the pathway of sperm storage of *P. sinensis* (Liu et al., 2016).

RNA-seq analysis shows that autophagy, apoptosis, and immune take part in long-term sperm storage in this species

The genome sequences of soft-shelled turtle have been published (Wang et al., 2013), which pvovide a useful database for genomic and functional investigations on some important biological traits. Next-generation sequencing (NGS) technologies have been proven to be an efficient and accurate choice for measuring gene expression under diverse biological conditions (Wang et al., 2009). NGS-based RNA sequencing (RNA-Seq) has been widely employed for global gene expression profiling in some reptile species (Eckalbar et al., 2013; Shaffer et al., 2013). *P. sinensis* oviduct cDNA libraries in reproductive and hibernation seasons have been sequenced using RNA-Seq technology to identify the differentially expressed genes (DEGs) related to sperm storage. A list of DEGs related to the immune response, apoptosis and autophagy signalling pathways are identified and comprehensively profiled (Liu et al., 2016a,b).

Immune-response genes related to prolonged sperm storage

Many genes implicated in the TLR signalling pathway are significantly differentially expressed during the hibernation season compared with the reproductive season in the oviduct of *P. sinensis*. The TLR signalling pathway is responsible for modulating the innate immune responses in vertebrates against parasites, bacteria and viruses (Khan et al., 2013). The expression of several transcripts encoding TLR2, TLR4, CTSK and MyD88 significantly decrease during the hibernation season, suggesting that TLR cascades contribute to the tolerance of the stored spermatozoa in response to immune reaction in female *P. sinensis*.

Moreover, the other immune response, including TNF, IL1β and IL18 are found to be down-regulated during the hibernation season in *P. sinensis* (Fig. 10), which is validated by RT-qPCR and ELISA analyses (Liu et al., 2016a,b). These results indicate that these immune-related genes may modulate the immune response to tolerate the presence of allogeneic spermatozoa in the oviduct for lengthy periods in *P. sinensis*. TNF, as an inflammatory cytokine, is mainly produced by macrophages and monocytes during acute inflammation (Idriss and Naismith, 2000). A previous study revealed that TNF has cytotoxic properties that cause germ cell apoptosis in mammalian cells (Grataroli et al., 2004), inferring that the down-regulation of TNF may be responsible for the stored spermatozoa in the oviduct of *P. sinensis*. Another inflammatory mediator, IL1β, could enhance the immune response induced by various stimuli, including mitogens, cytokines and microbial products (Tabona et al., 1998). Das et al. reported that the changes in mRNA expression of IL1β and TNF-related molecules were significant for spermatozoa survivability in hens, and the decrease in IL1β may permit spermatozoa to survive in SSTs (Das et al., 2009). IL18, also known as IFN-inducing factor, is involved in inflammation, ischemic tissue injury and T-cell-mediated immunity (Akira, 2000; Nakanishi et al., 2001).

Down-regulation of apoptosis genes may favour long-term sperm storage

Apoptosis is a physiological programmed cell death process that plays an essential role in the process of gamete maturation, germ cell death, and sperm storage in the mammalian and bird oviducts. In the oviduct of *P. sinensis*, using TUNEL, which is a routine method for detecting apoptosis, more apoptotic cells are detected in July than in January. Moreover, some up-regulated anti-apoptosis genes and down-regulated pro-apoptosis genes are identified during sperm storage by expression

analysis (Table 2), indicating that these gene expression alterations might contribute to the long-term sperm storage in *P. sinensis* (Liu et al., 2016a,b). Among these up-regulated anti-apoptosis genes, Bcl-6 encodes a Kruppel-type zinc finger transcriptional repressor, and its deficiency in mice causes the obviously sperm apoptosis in response to various stressors (Kojima et al., 2001). MCL1, another member of the anti-apoptotic Bcl-2 family, is an early-response gene in the apoptotic signalling cascade and exerts its function in delaying apoptosis under apoptosis-inducing conditions (Townsend et al., 1998). The functions of anti-apoptotic activities are also shared by BAG4 and BAG5, which belong to the Bcl-2-associated athanogene (BAG)-family. Thus, the up-regulation of these anti-apoptosis genes, including BCL6, MCL1, BAG4 and BAG5, may repress the occurrence of apoptotic effect in the oviduct, thereby protecting the resident spermatozoa in female *P. sinensis*. Moreover, the participation of down-regulated pro-apoptosis genes, such as BCL2L11, is also found

Table 2. DEGs involved in the apoptosis pathway during sperm storage. Quoted from (Liu et al., 2016b).

Gene ID	Log_2 Fold change	Associated Gene Name	Interpro Description
up-regulated genes			
ENSPSIG00000014827	2.661	RPAP3	Tetratricopeptide repeat
ENSPSIG00000014929	2.3893	BCOR	Ankyrin repeat-containing domain
ENSPSIG00000001478	2.3731	BAG5	BAG domain
ENSPSIG00000012400	2.0129	BAG4	BAG domain
ENSPSIG00000004968	1.8734	NEK6	Protein kinase-like domain
ENSPSIG00000009078	1.7553	MCL1	Bcl2-like
ENSPSIG00000011287	1.7351	API5	Armadillo-type fold
ENSPSIG00000013461	1.7348	PDCD2	Zinc finger, MYND-type
ENSPSIG00000015104	1.645	IGF1R	Tyrosine-protein kinase, insulin-like receptor
ENSPSIG00000007435	1.5841	SIVA1	Siva
ENSPSIG00000014529	1.5404	BCL6	BTB/POZ fold
ENSPSIG00000012576	1.4298	PRKCI	Protein kinase-like domain
ENSPSIG00000004057	1.4283	GAB1	Pleckstrin homology domain
ENSPSIG00000004826	1.408	HTRA2	PDZ domain
ENSPSIG00000013535	1.381	FAF2	UAS
ENSPSIG00000004383	1.3647	TAOK1	Homeodomain-like
ENSPSIG00000006786	1.3528	ING3	Staphylcoagulase, N-terminal
ENSPSIG00000008015	1.32	PDCD6IP	BRO1 domain
ENSPSIG00000017351	1.2659	ADCK3	Protein kinase-like domain
ENSPSIG00000003952	1.2614	CCNL2	Cyclin-like
ENSPSIG00000015707	1.2495	PSMC3IP	BlaI transcriptional regulatory family
ENSPSIG00000005369	1.1682	NCOA4	Nuclear coactivator
ENSPSIG00000005191	1.1451	DIDO1	Zinc finger, FYVE/PHD-type
ENSPSIG00000015775	1.1177	OPTN	NF-kappa-B essential modulator NEMO, N-terminal
ENSPSIG00000006978	1.0971	MED1	Mediator complex, subunit Med1, metazoa/fungi
ENSPSIG00000016631	1.0249	BNIP3L	BNIP3
ENSPSIG00000017945	1.0182	CCAR2	Nucleic acid-binding, OB-fold
ENSPSIG00000004635	1.0112	NDRG1	Alpha/Beta hydrolase fold
down-regulated genes			
ENSPSIG00000016035	-0.057855	SCOTIN	P53 apoptosis protein
ENSPSIG00000008774	-1.023	PIDD	p53-induced death domain protein
ENSPSIG00000002355	-1.0367	CLDND1	PMP-22/EMP/MP20/Claudin superfamily
ENSPSIG00000004908	-1.1619	MECOM	Zinc finger, C2H2-like
ENSPSIG00000010399	-1.2458	IRF1	Interferon regulatory factor-1/2
ENSPSIG00000010376	-1.5406	MSX2	Homeodomain-like
ENSPSIG00000004029	-1.5816	CASP9	apoptosis-related cysteine peptidase
ENSPSIG00000012821	-1.6969	ELMO2	Armadillo-type fold
ENSPSIG00000017598	-1.7528	FOS	Transcription factor, Skn-1-like, DNA-binding domain
ENSPSIG00000005216	-1.7926	AHSA1	Activator of Hsp90 ATPase, N-terminal
ENSPSIG00000017957	-1.8668	CIAPIN1	S-adenosyl-L-methionine-dependent methyltransferase
ENSPSIG00000002892	-1.9317	CytC	cytochrome c-like
ENSPSIG00000003046	-2.0734	CASP6	Peptidase C14A, caspase precursor p45, core
ENSPSIG00000008544	-2.0876	PARM1	prostate androgen-regulated mucin-like protein
ENSPSIG00000015307	-2.23	BCL2L11	Apoptosis, Bim N-terminal
ENSPSIG00000008949	-3.3761	CARD10	Death-like domain
ENSPSIG00000009528	-3.3981	DTHD1	Death-like domain
ENSPSIG00000016980	-5.0398	SFRP4	Tissue inhibitor of metalloproteinases-like, OB-fold
ENSPSIG00000015419	-6.2964	PERP	TP53 apoptosis effector

during sperm storage. BCL2L11 (also known as BIM) could promote the release of apoptogenic proteins and inactivate the anti-apoptosis BCL-2 proteins to trigger apoptosis (Luo and Rubinsztein, 2013). These findings indicate that the down-regulated pro-apoptosis genes negatively control the initiation of apoptosis in *P. sinensis*.

It has been extensively shown that the p53 signalling pathway could induce DNA damage-triggered apoptosis in various cell types. The effects of oxidative stress are particularly important during sperm storage, whether by cooling or *in vivo*, and reactive oxygen species (ROS) have been suggested as a main factor in the inhibition of sperm longevity (Collins et al., 2004; Ball, 2008). The decreased levels of ROS could suppress the release of CytC from mitochondria to the cytosol, promoting the increase in spermatozoa longevity in the female reproductive system for diverse organisms. The CytC protein could bind to Apaf-1 and, in turn, activate CASP9, triggering apoptosis (Jiang and Wang, 2004). Moreover, the ROS-CytC-Caspase axis in the p53 pathway has been suggested to play key roles in apoptosis (Yang et al., 2015). In *P. sinensis*, the two components of the p53-dependent apoptosis pathway, CytC and CASP9, are down-regulated during the hibernation season. Taken together, these findings imply that the oviduct contributing to prolong sperm storage might suppress oxidative stress-induced apoptosis through the ROS-CytC-Caspase model in the p53 pathway.

Regulation of autophagy involved in sperm storage

Autophagy is a highly conserved catabolic process that is primarily responsible for the nonspecific degradation of redundant and recyclable cellular components (Yu et al., 2008). In the process, portions of the cytoplasm, damaged proteins and organelles are sequestered in double- or multi-membrane structures called autophagosomes, which deliver material to lysosomes for digestion. Recently, increasing reports have focused on the involvement of autophagy in spermatozoa. In *Caenorhabditis elegans*, fertilizing spermatozoa trigger the recruitment of autophagosomes and subsequent paternal mitochondria degradation to prevent paternal mitochondrial DNA transmission (Al Rawi et al., 2011). The expression of autophagy-related proteins in stallion spermatozoa suggested that autophagy plays a role in the survival of cooled stored spermatozoa (Bolaños et al., 2012). Moreover, a series of autophagy-related genes (ATGs) is implicated in the regulation of autophagy. For instance, ATG14 is a critical element of autophagic initiation and can interact with phosphatidylinositol 3-phosphate in the bilayer membrane during autophagosome formation (Zhong et al., 2009). The overexpression of ATG14 has been demonstrated to enhance autophagic activity in yeast and mammals (Xiong et al., 2012). In addition, BNIP3, as a pro-cell death member of the Bcl-2 family, is found to inhibit the mTOR (mechanistic target of rapamycin) pathway and to promote autophagy (Gallo et al., 2014). The knockdown of BNIP3 is reported to inhibit autophagy and promote the necrotic cell death of tumour cells (Tracy et al., 2007). The up-expression of ATG14 and BNIP3 is detected in the process of sperm storage in *P. sinensis* (Table 3), implying that these genes might promote autophagy during the hibernation season and thereby contribute to prolonging the lifespan of stored spermatozoa.

Furthermore, NGS-based expression analysis and RT-qPCR detect the increased transcript abundance of the LC3B gene, and western blot analysis further reveals the high ratio of LC3B-II/LC3B-I in *P. sinensis* oviduct during the hibernation season (Fig. 11). The microtubule-associated protein 1 light chain-3β (LC3B) exists in two forms: LC3B-I and LC3B-II. The

Table 3. DEGs involved in the regulation of autophagy during sperm storage. Quoted from (Liu et al., 2016b).

Gene ID	Log_2 Fold change	Associated Gene Name	Interpro Description
up-regulated genes			
ENSPSIG00000012704	2.9702	MAP1LC3B	Ubiquitin-related domain
ENSPSIG00000002664	2.771	ATG5	Autophagy-related protein 5
ENSPSIG00000006041	2.1118	CALCOCO2	Coiled-coil transcriptional coactivator-like
ENSPSIG00000015566	2.0203	ULK2	Protein kinase-like domain
ENSPSIG00000010882	1.8742	ATG14	UV radiation resistance protein/autophagy-related protein 14
ENSPSIG00000006840	1.6022	SH3GLB1	SH3 domain
ENSPSIG00000009647	1.5666	SQSTM1	UBA-like
ENSPSIG00000002171	1.5491	ATG16L1	STAT transcription factor, coiled coil
ENSPSIG00000017272	1.5223	ATG12	Ubiquitin-related domain
ENSPSIG00000015127	1.2743	RB1CC1	Ubiquitin-related domain
ENSPSIG00000016631	1.0249	BNIP3	BNIP3
down-regulated genes			
ENSPSIG00000005809	-1.0766	STX5	t-SNARE
ENSPSIG00000016695	-1.6898	TECPR2	Regulator of chromosome condensation 1/beta-lactamase-inhibitor protein II
ENSPSIG00000008902	-2.7236	VMP1	vacuole membrane protein 1

unprocessed form of LC3B is cleaved by ATG4 into a cytosolic form, known as LC3B-I. Upon induction of autophagy, LC3B-I is processed to LC3B-II, which is inserted into both the inner and outer membranes of the growing autophagic vesicle (Glick et al., 2010). The conversion from LC3B-I to LC3B-II is a cellular readout of autophagy levels (Vernon and Tang, 2013). Therefore, autophagy is increased during the sperm storage period in *P. sinensis*. Moreover, IHC showed that LC3B-positive cells are mainly located along the oviduct epithelium, in which ciliated cells and secretory cells are distributed. Furthermore, under TEM, autophagosomes containing undigested cytoplasmic material are observed and distributed within the ciliated cells and secretory cells, indicating that autophagy can occur in the oviduct epithelium when spermatozoa are stored during the hibernation season in *P. sinensis* (Liu et al., 2016a,b). It is well known that autophagy serves as an alternative energy source to sustain cellular function under stress (Swampillai et al., 2012). A sufficient energy source at the site of sperm storage is essential for the prolonged survival of spermatozoa (Roy and Krishna, 2013). H&E staining and TEM analysis demonstrate that the spermatozoa are closely attached to the oviduct epithelium during sperm storage. Hence, it is reasonably inferred that autophagy could provide available energy for the oviduct epithelial cells to support the stored spermatozoa in the female *P. sinensis*.

Conclusions

(1) Spermatozoa are stored in the epididymis and oviduct for more than half a year and one year respectively in the Chinese soft-shelled turtle, *Pelodiscus sinensis*, indicating the longer storage time than that of most other reptiles and other class animals.

(2) Huge cytoplasmic droplet (CD) with its content of several large lipid droplets (LDs) may be the transient organelle serving as the energy source essential for long-lived stored spermatozoa in the epidiymis. The consumption of lipoprotein membrane in the onion-like mitochondria can maintain the long-life of spermatozoa. The situations of LDs within CDs and onion-like mitochondrion are turtle natures, instead of other animal ones.

(3) TLR2/4 expression in spermatozoa and oviduct, which plays critical roles in detecting and responding to invading pathogens and then protects the storing spermatozoa from microbial infections.

(4) Androgen and its receptor can regulate sperm storage in the oviduct of the turtle, this function is similar to that found in bats.

(5) Sperm heads are always embedded among the cilia or even intercalate into the apical hollowness of the ciliated cells in the oviduct during sperm storage, suggesting the the ciliated cells can support the stored spermatozoa.

(6) The structural barriers can ensure the microenvironment maintenance and the nourishment exchange in the epididymis and oviduct of the turtle. The survival factor Bcl-2 expression in the oviduct is associated with sperm storage occurrence.

(7) RNA-Seq is firstly used to detect the molecular mechanism of sperm storage, which shows that autophagy, apoptosis and immune take part in long-term sperm storage in this species.

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