Ultrastructural Features of Sperm Storage Tubules in the Oviduct of the Indian Garden Lizard, *Calotes Versicolor*

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ABSTRACT

This study provides the first description of the ultrastructural features of sperm storage tubules (SSTs) in the uterovaginal region of the oviduct of the Indian garden lizard, *Calotes versicolor*. Abundant spermatozoa along with copious secretory material were found in the lumen of the SSTs. These secretory granules appeared similar in electron density to those found in the epithelial cells lining the SSTs indicating their similar origin. The close physical proximity of sperm with these granules suggests an intimate association between the two. The present study is also the first report of recovery of motile sperm from the flushings of SSTs in *C. versicolor*. The density of sperm found in the flushings varied, being most abundant during the reproductive phase and minimum/absent during the regressive phase. Understanding the microenvironment of the SSTs, the nature of the secretory granules and their interaction with sperm can guide us in unraveling the biology of oviductal sperm storage. Anat Rec, 298:1932–1937, 2015. © 2015 Wiley Periodicals, Inc.

Key words: uterovaginal junction; sperm storage tubules; secretory material

Abbreviations used: PAS = periodic acid-Schiff; SST = sperm storage tubule; TEM = transmission electron microscopy

This article includes an AR WOW Video, which can be viewed at http://bcove.me/5kcvjc1b.

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INTRODUCTION

During the course of evolution reptiles had to develop various novel reproductive strategies in the dry, terrestrial environment (Birkhead and Moller, 1993; Sever and Hamlett, 2002; Shanbhag, 2002; Holt and Lloyd, 2010). In addition to synchronization of male and female reproductive cycles, the reptilian oviduct performs a wide array of functions based on the parity pattern of the species. Besides being the site of fertilization, secreting albumin, egg shell and nurturing the embryos during gestation, the oviduct in many species also stores sperm for a prolonged period (Girling, 2002; Sever and Hamlett, 2002; Siegel and Sever 2008a; Holt and Lloyd, 2010). Sperm storage in these reptiles, for variable lengths of time, in a motile and viable condition is likely responsible to extend the female reproductive phase beyond that of the male in a species specific manner (Girling, 2002; Holt and Lloyd, 2010). Laying of fertilized eggs by females, isolated from males for a long time, provides circumstantial evidence for the stored sperm being used for fertilization without subsequent mating (Cuellar, 1966; Adams and Cooper, 1988). Female sperm storage is not unique to reptiles and has also been reported in quite a few species of fishes, amphibians, birds and mammals in various regions of the oviduct (King et al., 1999; Sever et al., 2001; Wang et al., 2008; Ito et al., 2011; Liu and Avise, 2011; Moura et al., 2011; Roy and Krishna, 2011; Kuehnel and Kupfer, 2012). However, in comparison to higher vertebrates nothing much is known about the biology of oviductal sperm storage in reptiles.

The histological and histochemical features of SSTs found in the uterovaginal junction of the oviduct in *C. versicolor* were described in a previous study (Kumari et al., 1990). These tubules are formed by fusion of the distal ends of mucosal folds lining the oviduct. Further, stored sperm was shown to be distributed in a homogeneous substance, previously referred to as the "carrier matrix" (Halpert et al., 1982), presumably of glycoproteinaceous nature and possibly playing an important role in sperm sustenance for a prolonged period. Knowledge of the ultrastructural details of SSTs in *C. versicolor* is likely to shed some light on the origin of the "carrier matrix," the nature of the relationship of sperm with the lining epithelium of the SSTs and other cellular details. The present study was carried out to examine the ultrastructure of SSTs of the Indian garden lizard, *C. versicolor* during its breeding season, using transmission electron microscopy (TEM).

MATERIALS AND METHODS

Adult healthy female *Calotes versicolor* (snout-vent length > 8.5 cm), six per month, during reproductive phase (May to July) were captured from wild in the surrounding areas of Mysore, South India. Permission to capture and use *C. versicolor* for research was obtained from The Principal Chief Conservator of Forests (Wildlife) Karnataka, Bangalore, India (No. PS/PCWL/CR-27/2013-2014) and the study plan was approved by the Institutional Animal Ethics Committee, University of Mysore, Manasagangotri, Mysore-570006, Karnataka, India (No. UOM/IAEC/20/2013). The lizards were anaesthetized using chloroform within 24 hr of their capture, autopsied and the ovarian condition was recorded. The left oviduct was fixed in aqueous Bouin's fixative for routine histological studies. The uterovaginal region of the right oviduct was processed for TEM studies. Care was taken to ensure that all the lizards were at the same stage of their reproductive cycle having vitellogenic follicles prior to ovulation.

Processing of the Oviduct for Transmission Electron Microscopy

For ultrastructural studies, the uterovaginal junction of the oviduct, having the specialized sperm storage tubules (SSTs), was trimmed into ~1mm blocks and fixed in modified Karnovsky's fixative (4% paraformaldehyde + 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) for 24 hr. Tissues were subsequently washed in phosphate buffer and post-fixed in 1% osmium tetroxide for 1 hr. Later the tissues were dehydrated in a graded series of ethanol. During dehydration tissues were en-block stained by treating with 2% uranyl acetate in 95% ethanol for 1 hr and proceeded to 100% ethanol and were cleared in propylene oxide. Then the tissues were impregnated overnight at a 1:1 ratio of propylene oxide: araldite resin, which was increased to a 1:3 ratio followed by pure araldite resin for 2-3 hr. Later the tissues were embedded in flat embedding mould and kept at 60°C for 48 hr for polymerization.

Ultramicrotomy of the SSTs

Tissue blocks containing the uterovaginal junction were cut under a EM UC6 ultramicrotome (Leica, Austria). Initially 1 μm thick sections collected on plane glass slides were stained with 1% toluidine blue and viewed using a light microscope to find the specific area of interest and to study light microscopic features. Later 50–60 nm ultrathin sections collected on copper grids were stained with uranyl acetate (saturated with 50% methanol) and 0.1% lead citrate as described by Frasca and Parks (1965). Following staining, the ultrathin sections were examined under a transmission electron microscope (Tecnai G2 Spirit Bio-twin, FEI, Netherlands) and representative areas were photographed using an inbuilt *Megaview III* CCD camera. All the reagents used for TEM were procured from TAAB Laboratories, England.

Analysis of Uterovaginal Flushings

The uterovaginal region of the oviduct was dissected out during the reproductive phase. The uterovaginal tube was flushed with physiological saline. An aliquot of this saline flush was taken on a glass slide and viewed under the light microscope to check for the presence/absence of sperm.

RESULTS

General Morphological and Histological Features of the Oviduct

The oviductal cycle of *C. versicolor* showed three distinct phases: reproductive (May–October), regressive (November–February) and regenerative (March–April). Each phase exhibited drastic changes in oviductal mass

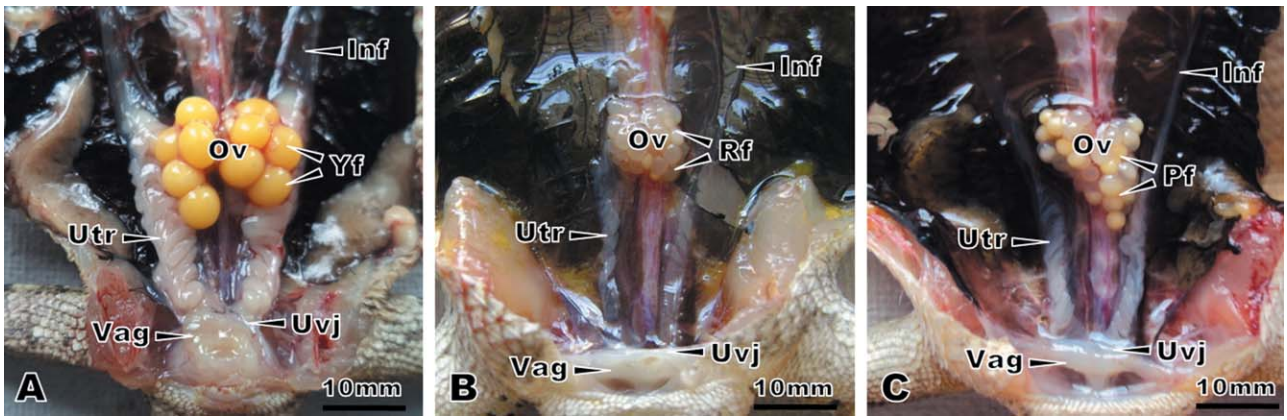

Fig. 1. Dissectional view of reproductive organs of female *Calotes versicolor* during the reproductive (**A**), regressive (**B**) and regenerative (**C**) phase. Inf, Infundibulum; Ov, Ovary; Pf, Previtellogenic follicles; Rf, Regressed follicles Utr, Uterus; Uvj, Uterovaginal junction; Vag, Vagina; Yf, Yolky follicles.

and structure that are in close correlation with that of the ovary (Fig. 1A–C). The oviduct appeared as an elongated, thin and fragile structure. It can be divided both morphologically and functionally into an anterior extremely thin walled infundibulum, where fertilization occurs, a middle uterus lodging the oviductal eggs and a posterior vaginal region receiving the sperm during mating (Fig.2A). Histologically, however, the wall of the oviduct displayed the same layers; viz. outer serosa, followed by muscularis, lamina propria and inner most mucosa, along its entire length. The relative proportion of the thickness of these layers and the extent of the folding of mucosal layer as well as the height of these folds were the variable parameters in the different regions of the oviduct (Fig. 2B–E). The muscularis was thinnest in the infundibulum, while it was very thick in the uterine and vaginal regions (Fig. 2B,C,E). Similarly, the mucosal lining was highly folded in the infundibular and vaginal regions while smooth and unfolded in the uterine region (Fig. 2B,E). The short region between the uterus and vagina, the uterovaginal junction, had the highest and most complex mucosal folds. The free ends of these folds are fused at their base to form, pocket-like structures storing the sperm and hence called SSTs (Fig. 2D). As the SSTs are formed by the mucosal folding of the uterovaginal junction of the oviduct, the histology and the staining properties of these tubules were very similar to that of the oviduct. The lining of SSTs consisted of ciliated cells interspersed with non-ciliated cells. The stored sperm bundles were found along with a homogenous substance. However, the stored sperm did not show any intimate, physical contact with the epithelium of the SSTs (Fig. 2D).

Recovery of Motile Sperm in the Flushings of SSTs

The flushings from the SSTs viewed under the light microscope showed the presence of motile sperm with intact morphology (Supporting information video). The sperm density was found to be maximum during the reproductive phase while it reduced towards the phase of regression (Unpublished data).

TEM of the Sperm Storage Tubules (SSTs)

TEM studies showed that SSTs were lined by columnar ciliated and non-ciliated cells with a large voluminous nucleus, which was either ovoid or elongated. The nuclei were situated centrally in some cells while basally in other cells. The cilia with the central axoneme connected to the basal body were clearly visible at the apical end of the ciliated cells (Fig 3C,D). The cross section of the cilia showed a typical microtubular arrangement (Fig. 3C-E). The plasma membrane at the apical end of the non-ciliated cells was projected into a large number of finger-shaped microvilli (Fig. 3A,C). These cells had a basally situated large, ovoid nucleus with a prominent nucleolus. The cytoplasm of both cell types had a large number of circular vacuoles of a lower electron density distributed throughout the cell (Fig. 3A,G).

The most striking feature of the cells lining the SSTs was the presence of abundant secretory material in the form of discrete assemblies of granules. These assemblies were found either at the apical end of the secretory cells or at their supranuclear region. The size, shape and number of these granules varied considerably in each assembly (Fig. 3A–E,G). The content of these granules was found to be released into the lumen of either the SSTs or that of the oviduct through exocytosis (Fig. 3D,G).

Abundant sperm were found in the lumen of the SSTs and also in that of the oviduct. The sperm were randomly oriented without any apparent physical contact with the cells lining the SSTs. They were found to be surrounded by a flocculent ground substance, which was similar in appearance and electron density to the content of the secretory granules released by the cells lining the SSTs (Fig. 3B,D–F,H). At higher resolution, the sperm nuclei with intensely stained dense, compact chromatin material were visible. Also various other regions of sperm, the middle portion with its characteristic central axial filament and the peripheral circlet of the mitochondrial sheath and the sperm tail in cross section were observed (Fig. 3D,F). The sperm appeared normal without any signs of degradation (Fig. 3E,H). Below the epithelium lining the SSTs, was the basement membrane with blood capillaries, muscle fibers and connective tissues (Fig. 3A).

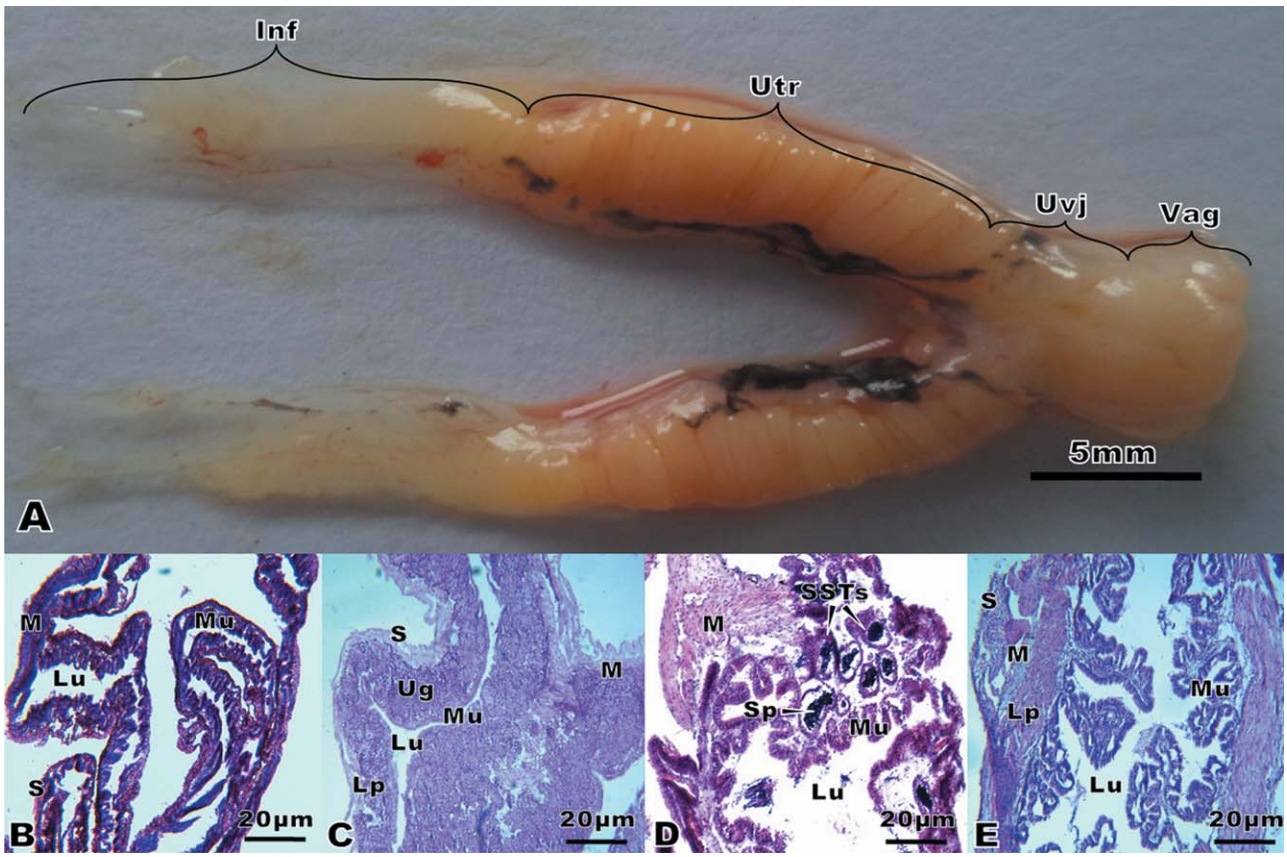

Fig. 2. Different regions of the oviduct of *Calotes versicolor* (**A**) with corresponding histological sections [infundibulum (**B**), uterus (**C**), uterovaginal junction (**D**) and vagina (**E**)]. Inf, Infundibulum; Lp, lamina propria; Lu, Lumen; M, Muscularis; Mu, Mucosa; S, Serosa; Ug, Uterine glands; Utr, Uterus; Uvj, Uterovaginal junction Vag, Vagina.

DISCUSSION

Despite the fact that female sperm storage is a common feature, enhancing reproductive fitness in many animals, the physiology underlying this phenomenon is hardly understood. Long term sperm sustenance in the reptilian oviduct is still poorly understood, when compared to birds and mammals. Exploring the ultrastructural details of the SSTs specialized for this purpose might be instrumental in unraveling the secret of sperm storage. Most of the available studies are restricted to histological and histochemical aspects and very little is known about the ultrastructural features of SSTs (Sever and Hopkins 2004). A previous study on *C. versicolor* (Kumari et al., 1990) showed the seasonal variation in the abundance of sperm in the SSTs during the reproductive cycle, with a peak during the postovulatory phase (September - October) and almost nil during the regressive phase (January–February). The epithelium of these SSTs shows a positive reaction for periodic acid-Schiff (PAS) indicating the presence of glycoproteins (Kumari et al., 1990). Histochemical studies reported the presence of a similar PAS-positive substance, the "carrier matrix", along with the stored sperm (Hoffman and Wimsatt, 1972; Kumari et al., 1990; Sever and Ryan, 1999). However, the origin and role of this substance have not been elucidated yet. Some authors argued that the carrier matrix originates from the renal sex segment of the male (Shivanandappa et al., 1999), while others believe that it is secreted by the lining of the SSTs (Han et al., 2008). Sever and Ryan, (1999) proposed a dual origin for the carrier matrix. The present ultrastructural study of SSTs in *C. versicolor* shows the presence of copious amounts of secretory material released from the secretory cells lining the SSTs, as such supporting the theory of Han et al., (2008). The nature of the abundant secretory material and its possible role in sperm sustenance are currently under investigation.

Studies by Sever and Hopkins (2004) and Siegel and Sever (2008b) showed large assemblages of secretory granules of various sizes and shapes in the supranuclear region of the secretory cells lining the SSTs. Similar observation were made in *C. versicolor* in the present study. The granules in *C. versicolor* are electron lucid in nature similar to those seen in the ground skink, *Scincella lateralis* (Sever and Hopkins, 2004). Siegel and Sever (2008a) reported a seasonal variation in the electron density of the secretory granules in the viviparous snake, *Agkistrodon piscivorus*. However, whether such a variation also exists in *C. versicolor* is not known, since the present study was carried out only in reproductively

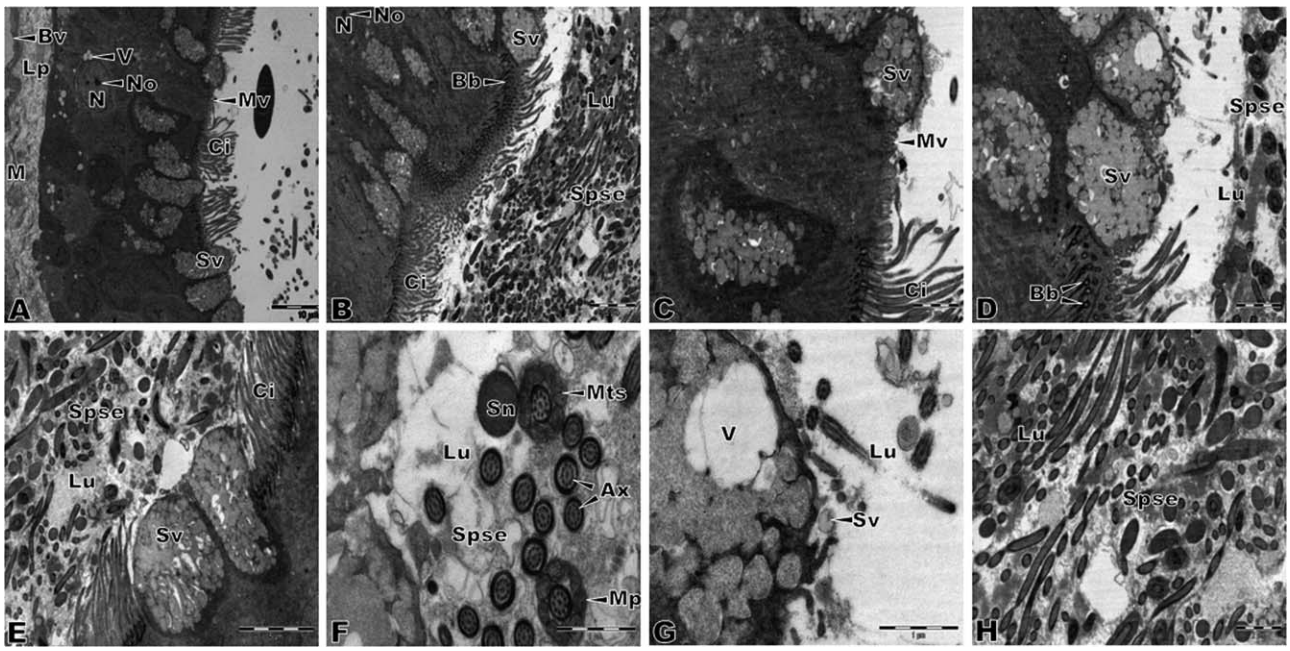

Fig. 3. Electron micrograph of the epithelial lining and lumen of the sperm storage tubules in *Calotes versicolor*. **A**: Portion of lining of SST showing ciliated cells interspersed with non-ciliated cells with microvilli, the oval nucleus with a prominent nucleolus and abundant assemblies of secretory vesicles, muscularis and lamina propria with a blood vessel below. **B**: Lining of SST with ciliated cells, cilia at the base have basal bodies and randomly distributed sperm in the lumen. **C**: Apical portion of the SST with a ciliated cell and a non-ciliated cell with microvilli. **D**: Epithelial lining of the SST with secretory vesicles of various size and shape. **E**: Epithelial lining of the SST with a ciliated and a secretory cell. **F**: Lumen of the SST with various parts of the sperm along with secretory material. **G**: Exocytosis of secretory vesicle into the lumen of SST. **H**: Sperm distributed randomly along with the "carrier matrix" in the lumen of SST. Ax, Axoneme; Bb, Basal bodies; Bv, Blood vessel; Ci, Cilia; Lp, Lamina propria; Lu, Lumen; M, Muscularis; Mp, Middle piece of sperm; Mts, Mitochondrial sheath; Mv, Microvilli; N, Nucleus; No, Nucleolus; Sn, Sperm nucleus; Spse, Sperm with secretory material; Sv, Secretory vesicles; V, Vacuole.

active females. Similar studies in non-breeding and post ovulatory females (with oviductal eggs) may help us understand the role of these secretory granules in sperm survival and the mechanism/s involved therein. The secretory vesicles were found to be protruding into the lumen of the SSTs and the content was released by exocytosis according to a merocrine pattern.

Various functions have been attributed to the "carrier matrix," ranging from nutritive function (Hoffman and Wimsatt 1972, Van Krey et al., 1967) upto sustenance of stored sperm, although nothing is certain to date (Han et al., 2008). Studies in poultry birds have suggested that the stored sperm are immotile and metabolically quiescent with low ATP consumption (Bakst 1985; Bakst and Richards 1985). Also enzymes such as carbonic anhydrase (Holm and Ridderstråle, 1998), alkaline phosphatase (Bakst and Akuffo, 2007) and aquaporins (Zaniboni and Bakst, 2004) are reported to play vital roles in long term sperm storage in birds. Further studies are required to confirm that the PAS-positive "carrier matrix" observed along with the stored sperm under the light microscope (Kumari et al., 1990) and the flocculent material reported in the present study are the same substance. Moreover, the exact nature of the "carrier matrix," its origin and possible roles are yet to be understood. The presence of immature and degenerating spermatozoa in the storage tubules has been reported in reptiles and amphibians (Sever 1992; Han et al., 2008). However, in the present study the stored sperm was normal with a structural integrity. The fertilizing ability of stored sperm has been demonstrated in *C. versicolor* (Shanbhag and Prasad, 1993) and similarly, motile sperm have been also recovered from the oviduct of another sympatric species, the rock lizard, *Psammophilus dorsalis* (Srinivas et al., 1995). Our previous studies (Kumari et al., 1990) have shown that a large proportion of females in the natural population of *C. versicolor* are found with oviductal eggs over an extended period (October to next January), when complete testicular regression is observed, providing strong circumstantial evidence for the hypothesis that the stored sperm is used for fertilizing the eggs laid in multiple clutches without repeated mating (Kumari et al., 1990).

Further characterization of the copious secretory material found in the lumen of the SSTs may help us understand the biology of oviductal sperm storage, which may have long term implications in addressing the modern problems related to reproduction.

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