Final report of UGC-major research project, entitled

"Unraveling the biology of sperm storage in the oviduct of female garden lizard, *Calotes versicolor*"

Introduction

Animals of various taxa have evolved different mechanisms for optimal reproductive success. Success in fertilization is best achieved by synchronizing the release of the sperm and egg so that their chance of meeting each other is enhanced. However in many species the reproductive cycle of the male and the female do not synchronize (1-5), forcing them to evolve certain novel mechanisms for enhanced reproduction. Sperm storage in the female reproductive tract has been one such intriguing adaptation, among many reported in a variety of animals including insects (6-8), fish (2), amphibians (9-11), reptiles (12-19), birds (20, 21), and mammals (1, 2, 22). In spite of prevalence of many such reports very little is known about the mechanism involved in the sperm storage. Some of the extreme examples of prolonged female sperm storage, in a viable condition at body temperature call for deeper understanding of its mechanism. Such studies can take us a long way in solving many modern problems in the field of reproductive biology.

The Indian garden lizard, *Calotes versicolor* is widely distributed in India. The females (Fig. 1 A) can be readily identified by the absence of dorsal crest beyond the neck region, the dull green yellow banding pattern on their dorsal body and usually smaller body size compared to male (Fig. 1 B). It is a multi-clutched, seasonal breeder (Fig. 2). The male reproductive phase lasts from April to August with complete testicular regression from September to March (18). However, in female, vitellogenic follicles are observed in ovary from as early as March and eggs are laid in multiple clutches between May to October and in a few females even as late as January, when the males are reproductively inactive and mating cannot occur (Fig. 2). Histological (18) and ultrastructural (19) studies have shown that sperm are stored at the sperm storage tubules (SSTs) found at the junction between uterus and vagina called the uterovaginal junction. This provides circumstantial evidence for the stored sperm possibly being used for fertilization beyond male reproductive phase. The SSTs are formed by fusion of the distal ends of mucosal folds lining the oviduct. Further, stored sperm has been shown to be distributed in a

homogeneous substance, previously referred to as the "carrier matrix" (15), presumably of glycoproteinaceous nature and possibly playing an important role in sperm sustenance for a prolonged period. Various functions have been attributed to the "carrier matrix," ranging from nutritive function (17) up to sustenance of stored sperm, although nothing is certain till date (16). The uterovaginal flushings from the SST viewed under the light microscope showed the presence of motile sperm with intact morphology. The sperm density was found to maximum during the reproductive phase while it reduced towards the phase of regression. The uterovaginal flushings when subjected to SDS-PAGE consistently showed the presence of several protein bands along with an abundant protein band of ~55kDa molecular weight irrespective of the phase of the reproductive cycle of this animal (Fig. 10). Purifying this protein by conventional chromatographic techniques and subjecting the purified protein to LCMS/MS analysis suggested the uniqueness of this protein without any homology to the NCBI database (Fig. 14, Table 1) Exogenous addition of this purified protein to washed sperms derived from epididymis, reversibly inhibited the motility of the latter in a concentration and time-dependent fashion (Fig. 16 & 17). These studies are likely to offer new tool to unravel the secrets of sperm storage among females across the animal taxa and may have unpresidential applications not only in reproductive biology, but may have far reaching applications in general cell storage.

Figure 1: *Calotes versicolor* (A) Female (B) Male

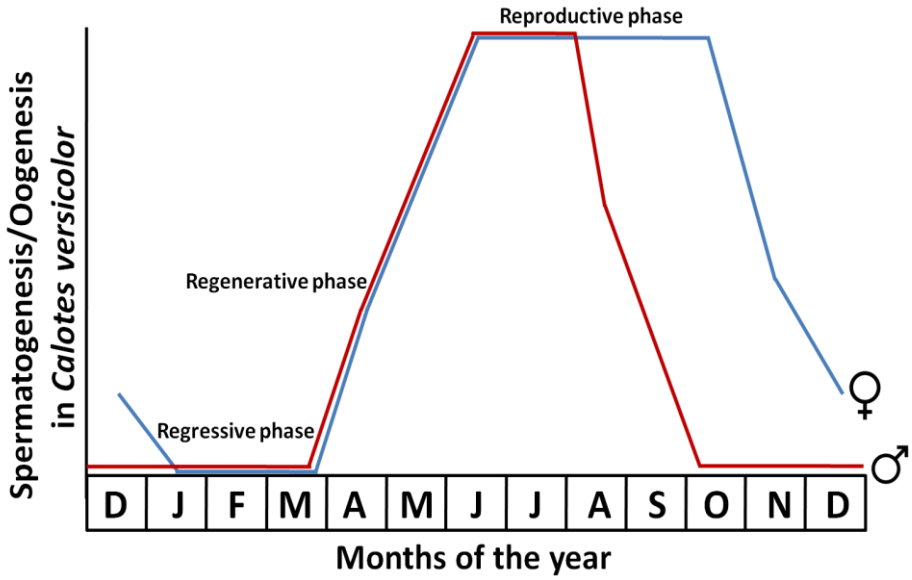

Figure 2: Different phases of reproductive cycle of male and female *Calotes versicolor.*

Objectives

1. Investigation on histochemical and ultrastructural details of sperm storage pockets
2. Analysis of pro-survival factors, in flushings of sperm storage pockets using classical biochemical techniques.
3. Testing the efficacy of the pro-survival factor(s), in *in vivo* and/or *in vitro* condition

Work Completed

Materials and methods

1. Histology and histochemistry of the oviduct of *C. versicolor*

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 Mysuru, South India. Permission to capture and use *C. versicolor* for research was obtained from The Principal Chief Conservator of Forests (Wildlife) Karnataka, Bengaluru, India (No. PS/PCWL/CR-27/2013-2014) and the study plan was approved by the Institutional Animal Ethics Committee, University of Mysore, Manasagangotri, Mysuru-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 and histochemical 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.

2. **Transmission electron microscopy of SST during the three phases of the reproductive cycle**

   For ultrastructural studies, the uterovaginal junction of the oviduct, having the specialized sperm storage tubules (SST), during reproductive, regressive and regenerative phases were 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 60ºC for 48 hr for polymerization.

   Ultramicrotomy of the SST: 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) (24). 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.

3. **Purification of abundant ~55kDa protein of the uterovaginal flushings**

   The uterovaginal region of the oviduct dissected out from 5-6 garden lizards during different phases of the reproductive cycle were flushed with physiological saline and the flushings were centrifuged at 1000*g* for 10 min at 4ºC. The supernatant obtained was

concentrated using centricon tubes (cut off >3kDa). The concentrated protein solution when subjected to SDS-PAGE analysis followed by silver staining showed the presence of a major protein band with an apparent molecular weight of ~55kDa along with minor bands (Fig. 10, 12 & 16C). This ~55kDa protein was subjected to purification by conventional chromatographic techniques using Cibacron blue 3G-A pseudo-affinity and DEAE-cellulose anion exchange column chromatography. Briefly, concentrated uterovaginal flushings (containing 5mg/ml of protein) was loaded on to a Cibacron blue 3G-A column (0.5 x 15cm) equilibrated with 10mM sodium phosphate buffer pH 7.4. ~55kDa protein was eluted in the void volume as determined by SDS-PAGE analysis followed by silver staining. The void volume fraction was pooled and concentrated using Centricon tubes (cut off >3kDa) and loaded onto a DEAE-cellulose column (0.5 x 5cm) equilibrated with 10mM sodium phosphate buffer pH 7.4 and the fractions were eluted with increasing molarity (10mM - 1M) of NaCl. ~55kDa protein eluted when salt concentration was between 200-250mM.

During purification of ~55kDa protein by Cibacron blue 3G-A column chromatography, we also observed that long term incubation of Cibacron blue 3G-A fraction caused sperm agglutination and dislodging of head and tail from the sperm even though it contained an appreciable amount of ~55kDa protein as noted by silver staining. These fractions in addition to ~55kDa protein contained a prominent high molecular weight protein (~100kDa) and few minor bands. Further purification and enrichment of ~55kDa protein by anion exchange chromatography on DEAE-cellulose column eliminated the agglutination and dislodging ability of the other unidentified factors.

4. **LC-MS/MS analysis of ~55kDa uterovaginal protein** (collaboration with Belinda Willard, The Cleveland Clinic Foundation, USA):

   The LC-MS/MS analysis was carried out to determine the sequence of a single abundant protein of the uterovaginal flushings. For the protein digestion, the bands were cut to minimize excess polyacrylamide, divided into a number of smaller pieces. The gel pieces were washed with water and dehydrated in acetonitrile. The bands were then reduced with DTT and alkylated with iodoacetamide prior to the in-gel digestion. All bands were digested in-gel using trypsin, by adding 5 μL 10 ng/μL trypsin in 50 mM ammonium

bicarbonate and incubating overnight digestion at room temperature to achieve complete digestion. The peptides that were formed were extracted from the polyacrylamide in two aliquots of 30µL 50% acetonitrile with 5% formic acid. These extracts were combined and evaporated to <10 µL in Speedvac and then resuspended in 1% acetic acid to make up a final volume of ~30 µL for LC-MS analysis.

The LC-MS system was a Finnigan LTQ-Obitrap Elite hybrid mass spectrometer system. The HPLC column was a Dionex 15 cm x 75 µm id Acclaim Pepmap C18, 2µm, 100 Å reversed phase capillary chromatography column. 5 µL volumes of the extract were injected and the peptides eluted from the column by an acetonitrile/0.1% formic acid gradient at a flow rate of 0.25µL/min were introduced into the source of the mass spectrometer on-line. The microelectrospray ion source was operated at 2.5 kV. The digest was analyzed using the data dependent multitask capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in successive instrument scans. The data were analyzed by using all CID spectra collected in the experiment to search the full NCBI database with the MS-Batch program available at Protein Prospector web site. The data was also manually interpreted and the resulting amino acid sequences were subjected to Blast searches.

5. Lactic acid estimation

Matsuzaki *et al*., (2015) (23) reported a massive production and release of lactic acid from SSTs of Japanese quail, *Coturnix japonica* under a hypoxic condition leading to the inactivation of resident sperm. This is also favourable for sperm survival because it leads to a reduced production of reactive oxygen species by sperm respiration. Thus it was tempting to check whether a similar mechanism of sperm quiescence also occur in case of *C. versicolor*.

Principle: Lactic acid was estimated based on the method of Figenschou and Marais (1991) (26). Lactic acid was quantitatively oxidized to acetaldehyde by cerium (IV). Oxidation was later terminated by reducing cerium (IV) to cerium (III) with nitrite. The acetaldehyde was then reacted with copper ions and oxalyldihydrazide under alkaline conditions to form an intense blue complex which was measured spectrophotometrically at 610nm (Fig. 3).

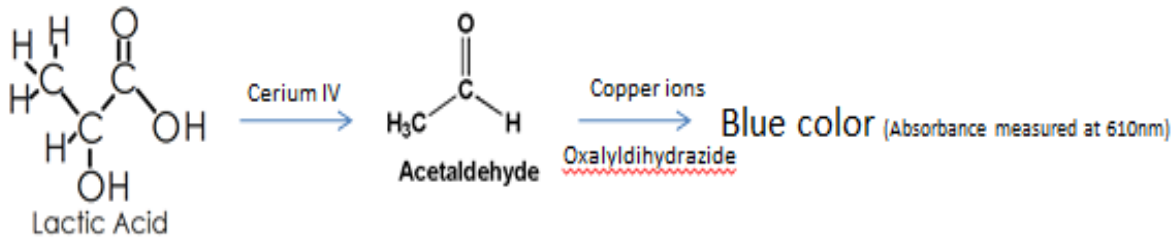

Figure 3: Principle of colorimetric estimation of lactic acid.

Reagents:

a) Oxidizing reagent: Ceric Ammonium nitrate (0.033 M), Ammonium sulphate (0.17 M) Copper sulphate (0.533 mM), Nitric acid (0.33 M), Perchloric Acid (0.8 M)

b) Alkalizing reagent: tri-Sodium citrate (0.2 M), Disodium hydrogen phosphate (0.232 M), Sodium hydroxide (0.174 M), Disodium tetraborate (0.115 M)

c) Sodium nitrite (0.6 M)

d) Oxalyldihydrazide (5 mM)

Procedure:

Lactate standard solution was taken in the range of 0-30µl (conc. 0-150µg) and the volume was made up to 1 ml with distilled water. Lactate standard and blank (distilled water) were treated similarly. 1.5ml of oxidizing reagent was added to all the tubes and was incubated at room temperature. After the oxidation period of 15 minutes, 0.6 M nitrite solution (0.2 ml) was added, followed by alkalizing reagent (5 ml) and 5 mM oxalyldihydrazide (ODH) solution (1 ml). The samples were mixed thoroughly and centrifuged (1000g, 1 min). After color development (30 min) at room temperature, the absorbance of the supernatant solution was measured (610 nm) against the blank in a Biomate 3S UV-Visible Spectrophotometer.

6. **Epididymal sperm extraction by direct swim up**

   The spermatozoa were extracted by squeezing the epididymis in 0.5 ml of pre-warmed Earle's Balanced Salt Solution (EBSS) (Sigma, USA) supplemented with 25mM of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 0.1% BSA (bovine serum albumin). The epididymal sperm suspension was centrifuged at 200×g for 8 min. Subsequently, the supernatant was discarded and washed with pre-warmed EBSS medium, centrifuged at 200×g for 5 min and once again the supernatant was discarded. Following this, 200µl of the sperm preparation medium (EBSS) was carefully added to the final pellet. The tube was inclined at an angle of 45° and incubated for 1 hr. at room temperature for swim up. After incubation period the supernatant was carefully pipetted out into a new tube and was assessed for sperm concentration, motility and normal morphology.

7. **Effect of partially purified single abundant protein of uterovaginal flushings on washed epididymal sperms**

   The washed epididymal sperm were resuspended in a total volume of 100µl containing 2 x 10^5 sperm and were treated with various concentrations (25 - 75µg) of ~55kDa uterovagional proteins and/or major purified protein and its effect on sperm motility and viability was assessed by eosin-nigrosin stained smears (Fig. 17C & D).

Results and discussion:

I. General morphology, histology and histochemistry of sperm storage tubules

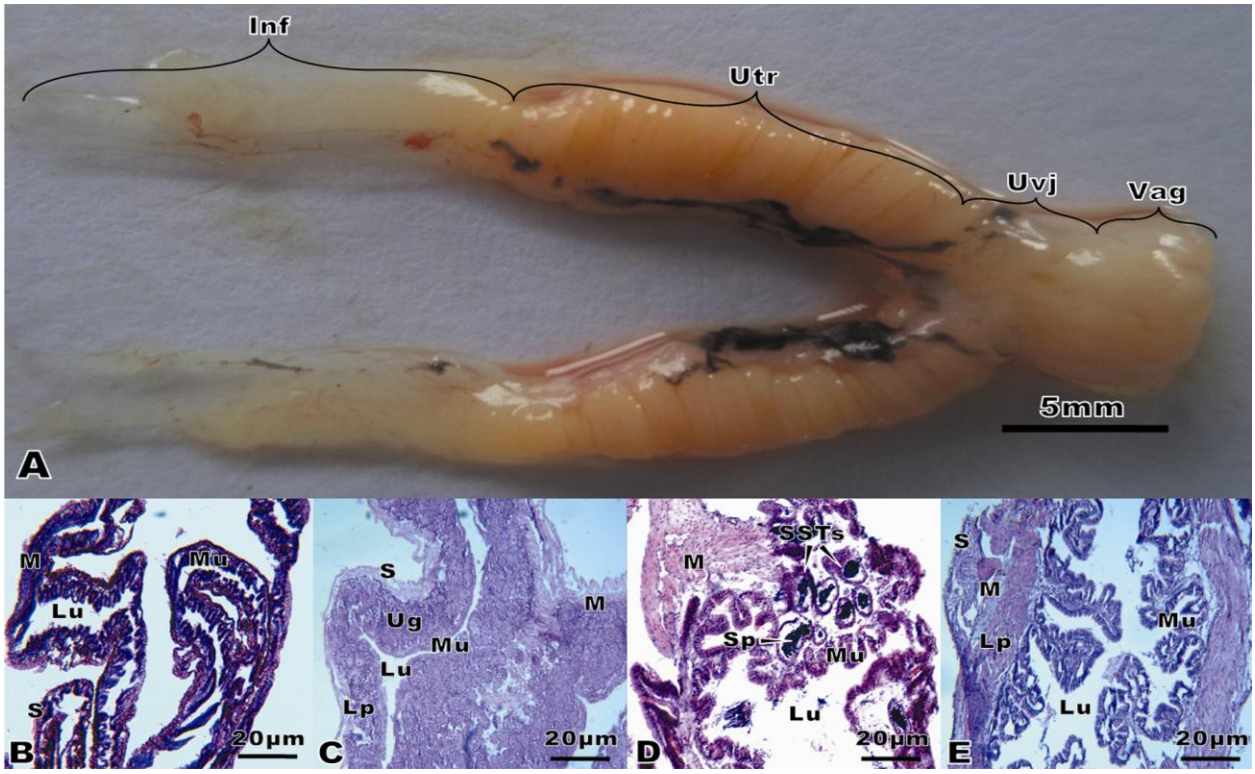

Figure 4: Different regions of the oviduct of *Calotes versicolor* (A) with corresponding histological sections [infundibulum (B), uterus (C), uterovaginal junction (D) and vagina (E)]. Inf, Infundibulum; Lp, lamina propria; Lu, Lumen; M, Muscularis; Mu, Mucosa; S, Serosa; Ug, Uterine glands; Utr, Uterus; Uvj, Uterovaginal junction Vag, Vagina.

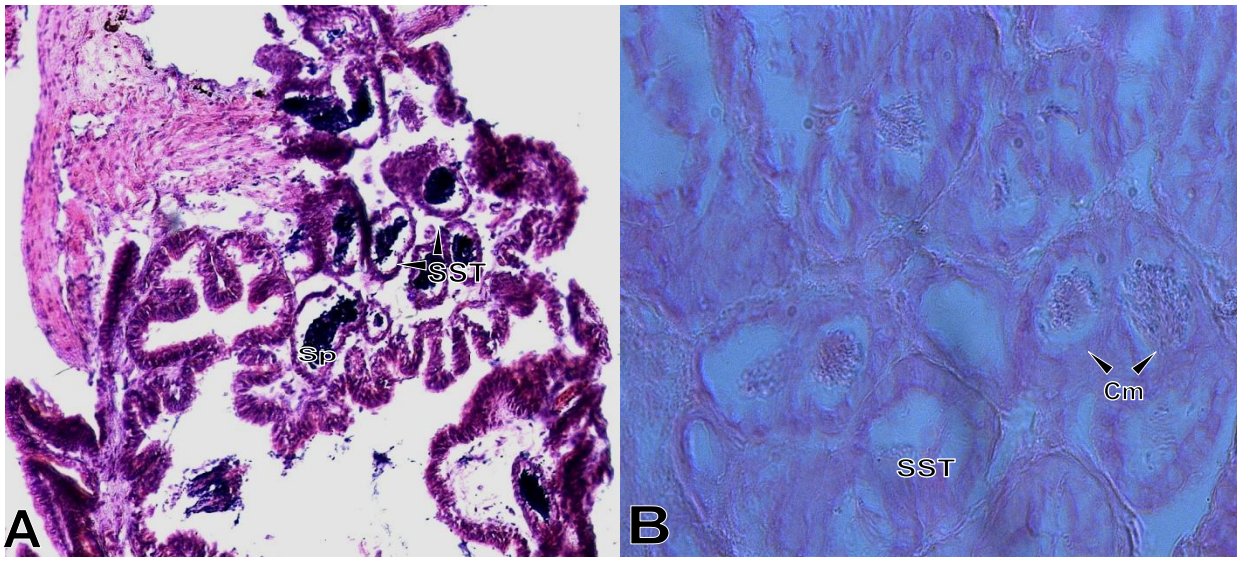

Figure 5: Transverse sections of the uterovaginal junction of the oviduct of *C. versicolor*.A:Sperms inside the sperm storage tubules.B: Carrier matrix inside the sperm storage tubules showing PAS positive reaction(Fig. 3A x100, 3B x400). Cm, Carrier matrix; Lu, Lumen; Sp, Sperms; SST, Sperm storage pocket.

The oviductal cycle of *C. versicolor* has three distinct phases; reproductive, regressive and regenerative phase showing drastic changes in its weight and structure in close correlation with that of the ovary. The oviduct is elongated, thin and fragile which is both morphologically and functionally divisible into anterior extremely thin walled infundibulum, where fertilization occurs, middle uterus lodging the oviductal eggs and posterior vaginal region receiving the sperm during mating (Fig. 4A). Histologically however, the wall of the oviduct possesses the same layers; viz. outer serosa, followed by muscularis, lamina propria and inner most mucosa, all along its length. The relative proportion of the thickness of these layers and the extent of folding of mucosal layer as well as the height of these folds are the variable parameters in the different regions of the oviduct (Fig. 4B, 4C, 4D, 4E).

The muscularis is the thinnest in the infundibulum, while it is very thick in uterine and vaginal regions. Similarly, the mucosal lining is highly folded in infundibular and vaginal regions while smooth and unfolded in the uterine region. The short region between uterus and vagina; the uterovaginal junction has the highest and most complex mucosal folds. The free ends of these folds fuse and form at their base, pocket like structures; storing the sperms and hence called sperm storage tubules (SST) (Fig. 5A). 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 are very similar to that of the latter. The lining of SST consists of ciliated cells interspersed with non-ciliated cells. The stored sperm bundles are found with a homogenous substance, which shows positive reaction with “Periodic acid Schiff’s (PAS) reagent (Fig. 5B). However, the stored sperms do not show any intimate, physical contact with the epithelium of the storage tubules (Fig. 5A).

II. Electron microscopy of sperm storage tubules.

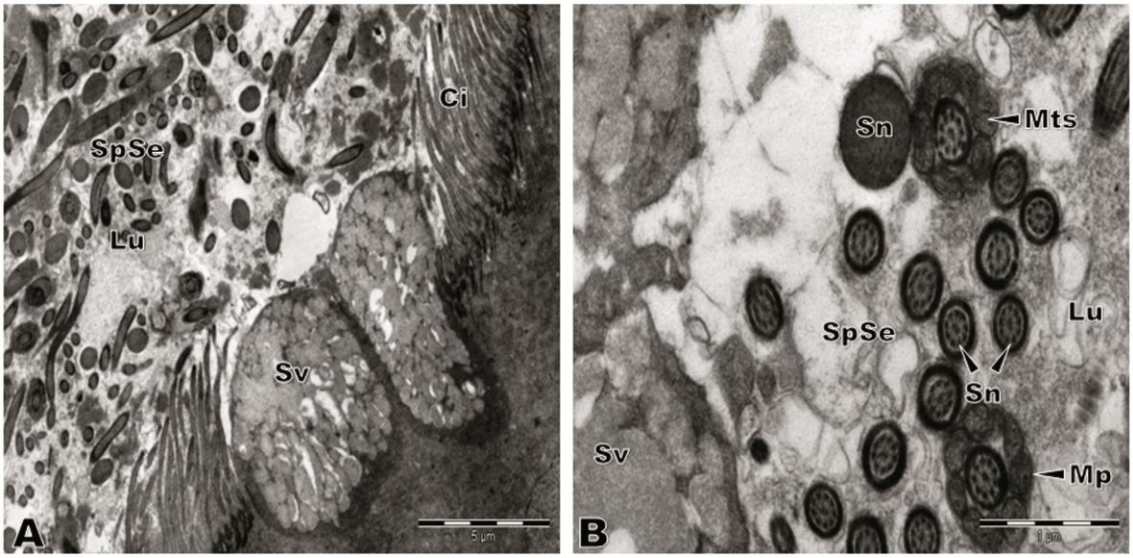

Figure 6: Electron micrograph of apical portion of the cells lining SST and lumen of the SST of *C. versicolor* during reproductive phase. A: Epithelial lining of the SST with ciliated and secretory cell. B: Lumen of the SST with various parts of the sperms along with secretory material. Ci, Cilia; Lu, Lumen; Mp, Middle piece of sperm; Mts, Mitochondrial sheath; Mv, Microvilli; SpSe, Sperm with Secretory material; Sv, Secretory vesicles.

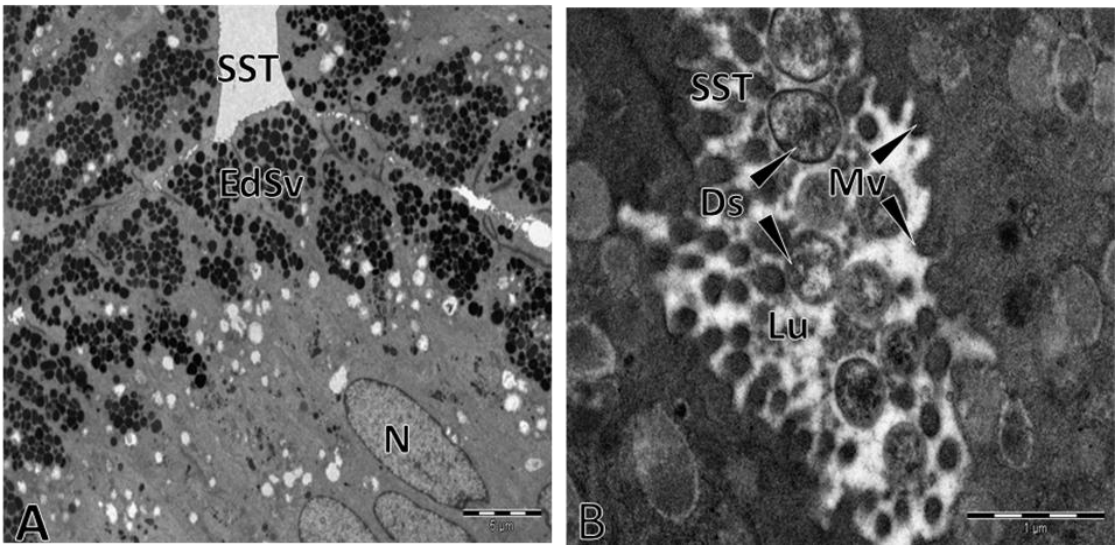

Figure 7: Electron micrograph of apical portion of the cells lining SST and lumen of the SST of *C. versicolor* during regressive phase. A: Epithelial lining of the SST with microvillus. B: Lumen of the SST containing denegerating sperms. Ds, Degenerating sperms; EdSv, Electron dense secretory vesicles; Lu, Lumen; Mv, Microvilli; N, Nucleus.

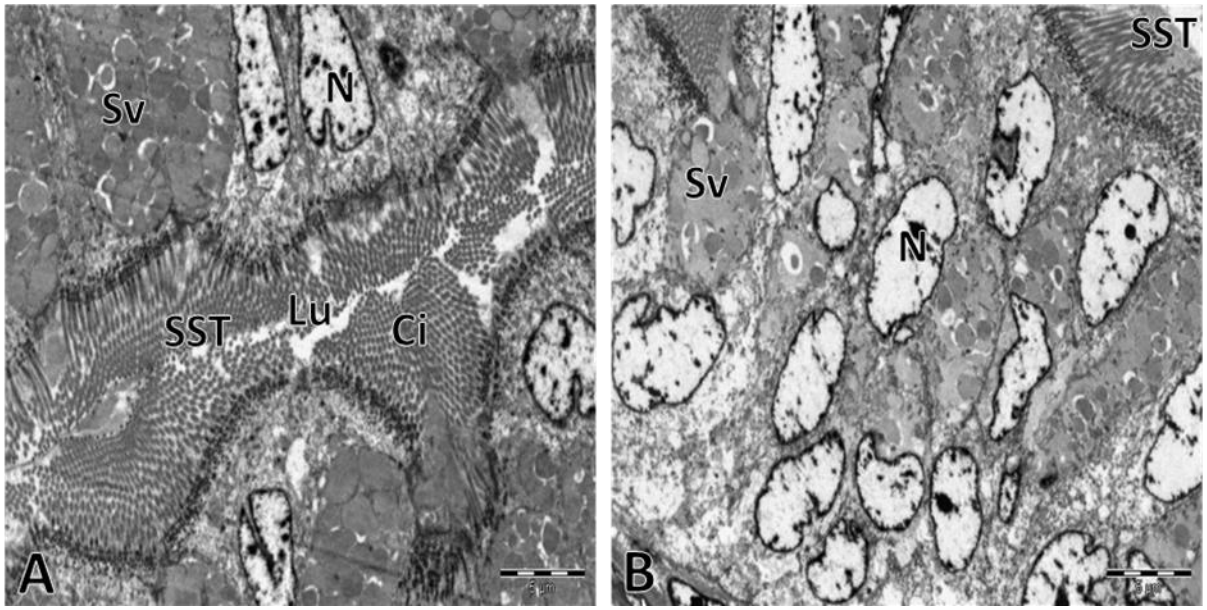

Figure 8: Electron micrograph of apical portion of the cells lining SST and lumen of the SST of *C. versicolor* during regenerative phase. A: Epithelial lining of the SST with ciliated and secretory cell. B: Epithelial lining the of the SST along with nucleus. Ci, Cilia; Lu, Lumen; Sv, Secretory vesicles.

During the reproductive phase, the cells of the columnar epithelial lining of the SST show the presence of large nucleus of varied shape (Fig 6A, 6B). The epithelium has both ciliated and non-ciliated cells interspersed with each other. The apical end of ciliated cells possesses abundant long cilia while the non-ciliated ones have microvilli. In the supranuclear region of these cells, large assemblages of vesicular secretory granules are found. These granules appear to have similar structure and electron density. These masses of secretory granules, throughout the lining of the SST, move towards the apical end of the cells, empty their contents into the lumen of the tubule. Abundant sperm without any apparent physical contact with the epithelium nor a specific pattern of arrangement are found within the lumen of the SST. The sperm mass is found distributed in a homogenous ground substance having close resemblance with the secretions of the lining epithelium in both its texture and staining properties, indicating its probable origin. The spermatozoa are intact with normal structural details (Fig 6A, 6B).

Though the same cell types, both ciliated and non-ciliated are found lining the SST even during regressive phase, there is a marked reduction in the height of cilia while the microvilli remain unchanged (Fig 7A, 7B). The nucleus and nucleolus do not show much change in their structure. However, many of the secretory vesicles have electron dense material in them and there are a large number of empty vacuoles (Fig 7A, 7B). Function of this electron dense material is not yet

clearly understood. Intercellular junction between the adjacent cells looks markedly conspicuous. Membranous organelles such as Golgi complex appear prominent. Sperm density in the lumen of the SST appears almost nil. Even a few spermatozoa found near the epithelium are highly degenerative with loss of their structural integrity. The flocculent ground material found in the lumen of SST along with the sperm during breeding season is not found during this phase (Fig 7A, 7B). The overall structure of the oviduct and that of the lining of SST shows regenerative changes during the recrudescent phase (March-April) of reproductive cycle. The connective and muscle tissues appear more evident. The epithelial cells show abundant contingent of long cilia with non-ciliated cells in between. These cells show large, prominent nuclei with nucleoli. Also assemblages of large secretory vesicles are found in the cells. These vesicles show the same kind of uniform texture and density as found during the reproductive phase. There is complete absence of spermatozoa while often some kind of filamentous debris can be found in the lumen of the SST (8A, 8B).

Research article in relation to the above work has been published in an International Journal "The Anatomical Record" manuscript entitled **"Ultrastructural features of sperm storage tubules in the oviduct of the Indian garden lizard, *Calotes versicolor*" (doi: 10.1002/ar.23257. 2015 Aug 13)**

III Recovery of motile sperm from the flushings of the SST

From histology and ultrastructural studies we know that sperm are stored at the junction between uterus and vagina referred as 'uterovaginal junction'. At first we wanted to flush out these sperms from the SST and verify whether these sperms are still alive?

Initially the uterovaginal region of the oviduct was dissected out from the animal during different phases of the reproductive cycle. The uterovaginal tube was flushed with physiological saline (Fig. 9A). An aliquot of this saline flush was taken on a glass slide and was viewed under the light microscope showed the presence of numerous motile sperms with intact morphology (Fig. 9B). Later the process of uterovaginal flushings was carried out during regressive and regenerative phases. The sperm density was found to be nil during the regenerative phase, maximum during the reproductive phase while it reduced towards the phase of regression.

Motile and morphologically intact sperm were also observed in the uterovaginal flushings of females isolated (10 weeks) from males during the reproductive period.

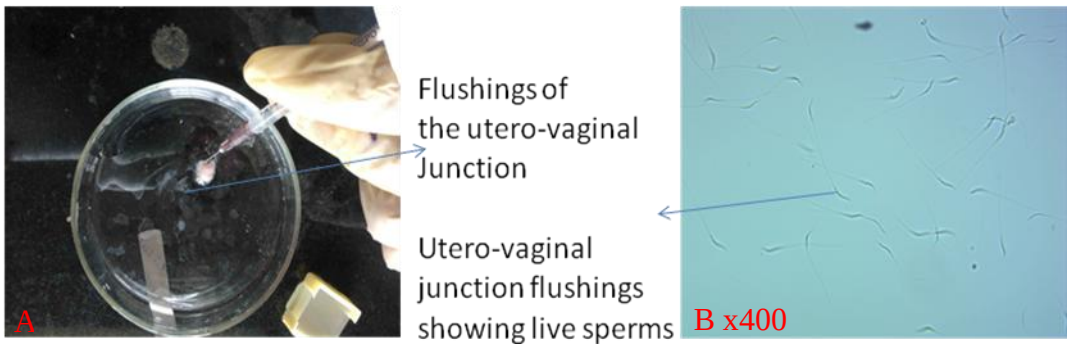

Figure 9: Flushings of the utero-vaginal junction (A). Utero-vaginal junction flushings showing live sperms (B).

IV Purification of a single abundant protein from the flushings of sperm storage tubules using pseudo-affinity column.

Since the protein of our interest was observed throughout the year irrespective of the reproductive cycle and had a molecular weight ~55kDa (Fig. 10) which is nearer to the molecular weight of bovine serum albumin (~66.5kDa), we first wanted to eliminate the possibility of albumin in the uterovaginal flushings. So we carried out Cibacron blue 3GA pseudo-affinity column chromatography. Cibacron blue 3GA is a sulfonated polyaromatic blue dye covalently attached to dextran, called blue dextran and binds to several protein and enzymes like, dehydrogenases, kinases, restriction endonucleases, albumin etc. This blue dextran complexes with wide range of proteins because it is specific for a super-secondary structure called the dinucleotide fold, which forms the binding sites for substrates and effectors on a wide range of proteins (Thompson et al 1974). 1ml of Cibacron blue 3GA gel has a capacity for albumin binding of greater than 11mg/ml. The protein of our interest did not bind to Cibacron blue 3GA gel and was eluted in the void volume itself.

The graph of absorbance v/s fraction number eluted with 10mM phosphate buffer of pH 7.4(void volume) shows single peak which contain a single abundant protein of the uterovaginal flushings along with other unbound proteins (Fig. 11) as evident from Tricine-SDS-PAGE analysis on a 12.5% gel using silver and comassie brilliant blue staining (Fig. 12).

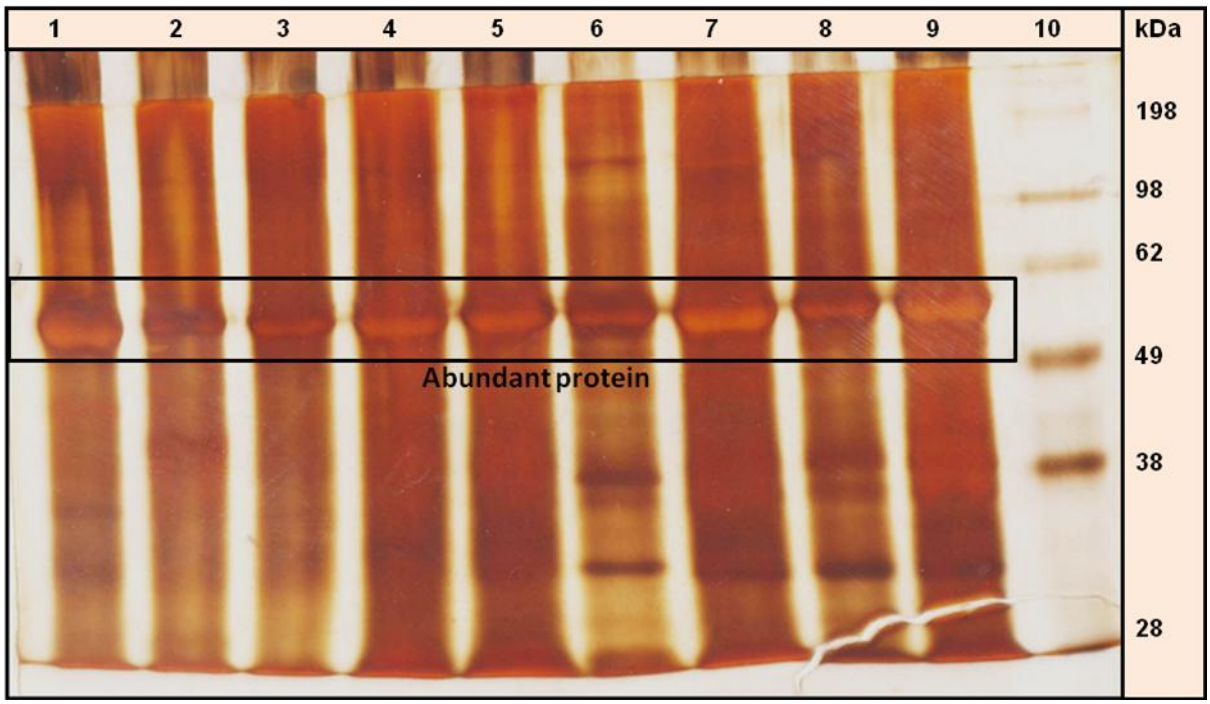

Figure 10: 10% SDS-PAGE banding pattern of concentrated uterovaginal flushings for different months of the year 2014 after silver staining [Lane 1, uterovaginal flushings for the month of April; 2, May; 3, June; 4, July; 5, August; 6, September; 7, October; 8, November; 9, December; 10, Molecular weight marker]. Data represents typical banding pattern obtained in more than 3 experiments.

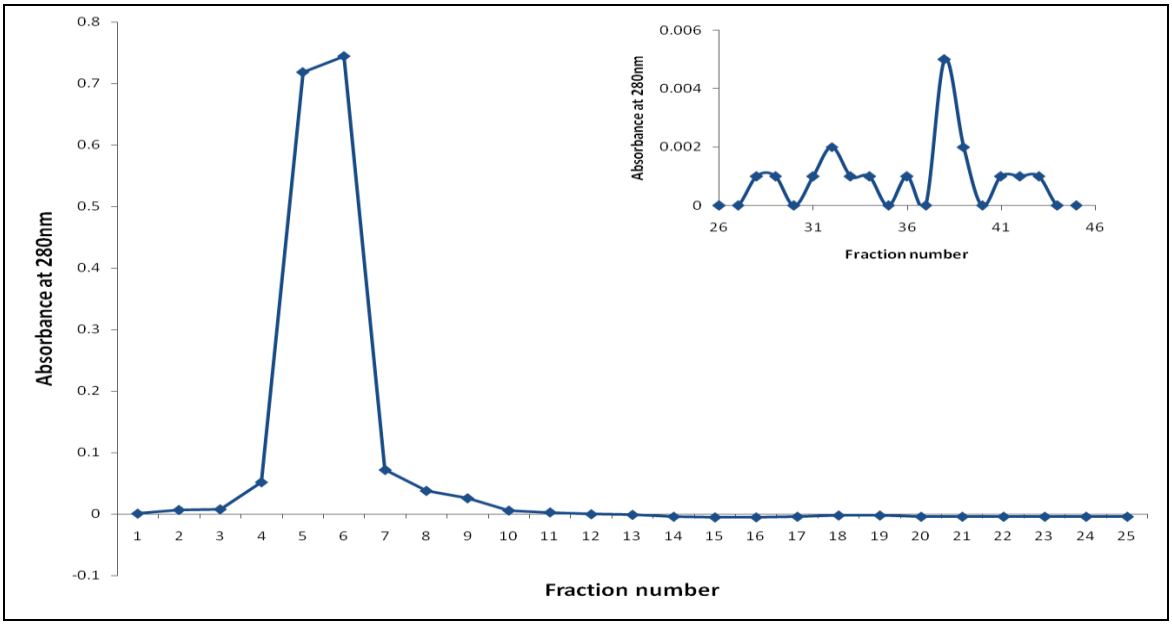

Figure 11: Elution profile of the Void Volume fraction of uterovaginal flushings on Cibacron blue pseudo affinity column. The void volume was eluted using phosphate buffer pH 7.4, whereas the bound albumin was eluted by the same buffer containing 1M NaCl.

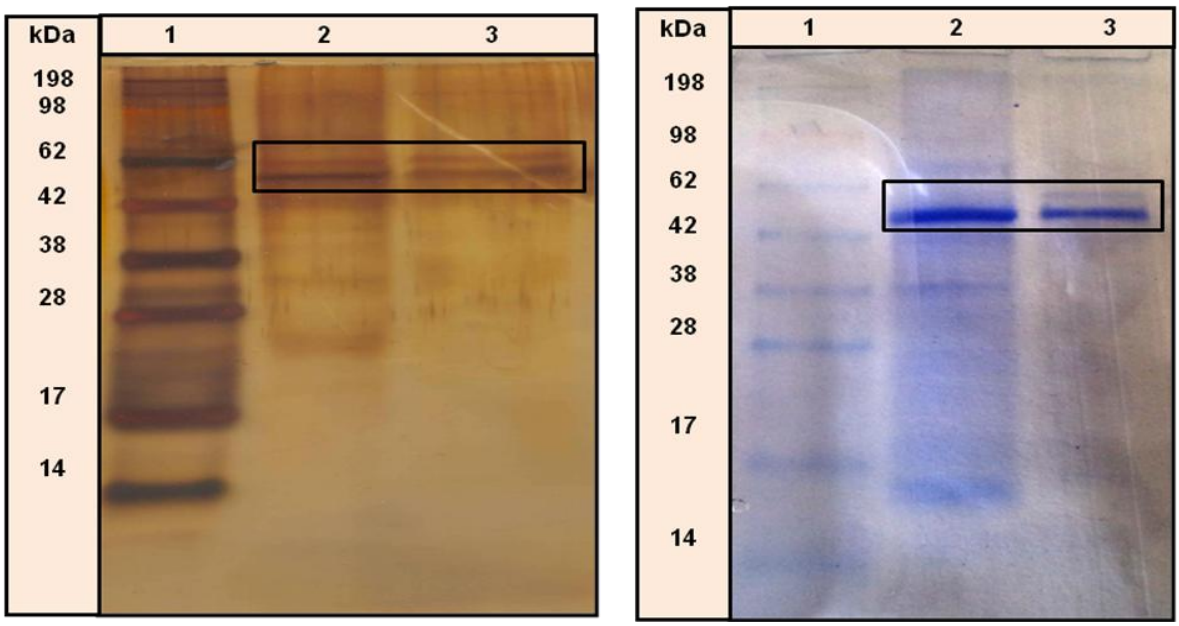

Figure 12: 10% Tricine SDS-PAGE banding pattern of concentrated and void volume fraction of uterovaginal flushings after cibacron blue column [Lane 1, Molecular weight marker; 2, Concentrated uterovaginal flushings; 3, Concentrated cibacron blue void volume fraction of uterovaginal flushings]. Data represents typical banding pattern obtained in more than 3 experiments.

Purification of single abundant protein from the uterovaginal flushings using DEAE cellulose anion exchange column following cibacron blue 3GA column chromatography

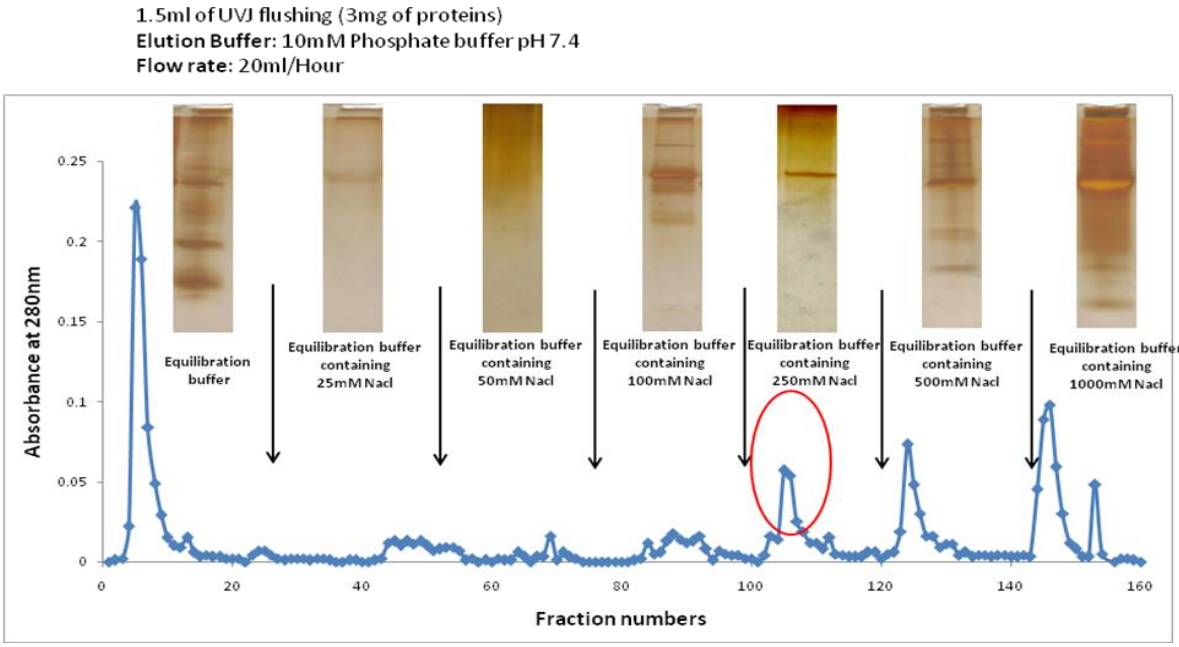

Figure 13: Elution profile of concentrated uterovaginal flushings void volume fraction of cibacron blue column on DEAE-Cellulose column. Single abundant protein of the uterovaginal flushings was purified from the concentrated void volume fraction of cibacron blue column by anion exchange chromatography and was eluted with 50mM phosphate buffer containing 250mM NaCl.

The pooled peak from void volume of Cibacron blue column was desalted and concentrated using Centricon tubes (3kDa cut off) and loaded on to a pre-packed DEAE-cellulose column and eluted with 10mM phosphate buffer (pH 7.4) containing increasing molarity of NaCl (0mM, 25mM, 100mM, 250mM, 500mM and 1000mM) (Fig. 13).The single abundant protein of uterovaginal flushings was eluted in the phosphate buffer containing 250mM NaCl fraction. This was confirmed by SDS-PAGE analysis on a 10% gel using silver staining (Fig. 13).

LC-MS/MS analysis of a major purified protein of the uterovaginal flushings

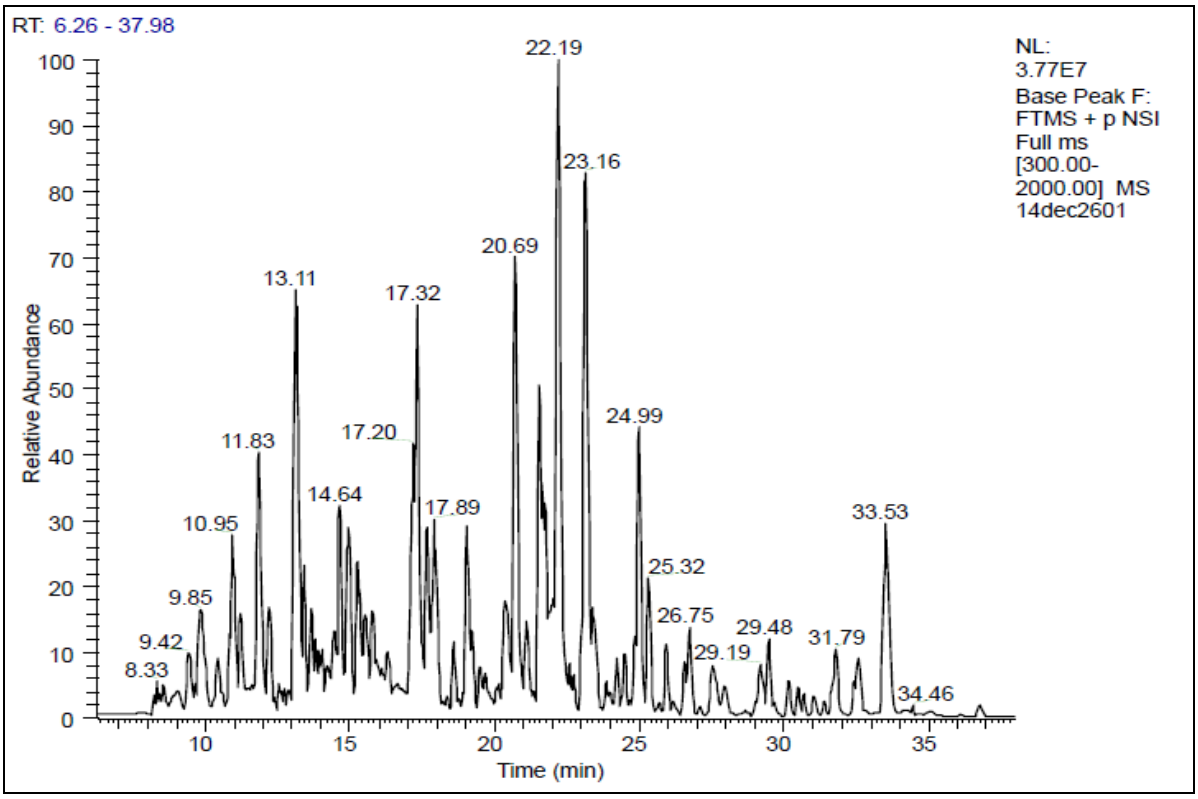

Figure 14: Mass spectral data of the trypsin digested protein purified from uterovagianl flushings.

The band cut from the gel was dark and produced several abundant peptides. The initial analysis of the band involved a search of the full NCBI database with the program MS Batch and no proteins were identified. The next stage of the analysis involved manual interpretation of the most abundant peptides identified in the LC-MS/MS analysis. A total of 35 peptides (Fig. 14) were manually interpreted and the resulting amino acid sequences are given in Table 1. These sequences were subjected to Blast searches against the full NCBI database and no homologous proteins could be identified. This result indicates that the protein present in the gel band is not represented in the NCBI database.

[M+H]	m/z	z	RT	Sequence
799.5	400.26	2	13.1	LVQLAQK
813.4	407.22	2	16.4	__YAYAR (b_2 = 171 Da)
897.4	449.25	2	23.2	FLYDLAR
927.4	464.23	2	17.3	FFCAVQR
932.5	466.74	2	11.8	VACLQEGR
941.4	471.24	2	17.6	FFC^{NEM}AVQR
946.5	473.74	2	12.2	VAC^{174}LQEGR
1018.6	509.79	2	24.9	DLLLGSLCK
1023.5	512.26	2	21.6	DYQDLLEK
1032.6	516.79	2	25.3	DLLLGSLC^{NEM}K
1053.5	527.3	2	21.2	---FLYDPSR
1092.6	546.8	2	22.2	LVDFGSVVEK
1098.6	366.88,549.81	3,2	19.2	LNVDQ---K
1100.2	550.24	2	15.4	CCSGPYSLR
1113.5	557.25	2	15.5	C^{174}CSGPYSLR
1127.5	564.25	2	15.8	C^{174}C^{174}SGPYSLR
1146.6	573.82	2	24.5	LNVDSLMLNK
1162.6	581.81	2	21.8	LNVDSLMoLNK
1177.7	589.33	2	24.2	---LAEVVHAK
1185.6	395.87,593.30	3,2	10.9	LCHEEAVVTK
1194.5	398.86,597.78	3,2	9.9	HFDDPHAVEK
1195.5	398.90,597.85	3,2	18.6	QTVLAEVVHAK
1199.6	400.54,600.31	3,2	11.2	LC^{174}HEEAVVTK
1233.56	411.86,617.29	3,2	9.4	LCHEEAmVTK
1242.6	621.82	2	17.3	PALTDQELAER
1255.7	628.36	2	19	LAPQLSTEQLR
1355.7	678.37	2	20.7	VVTLYQNVEYK
1360.7	454.23,80.85	3,2	10.8	APLEQNEVFADK
1431.7	477.91,716.36	3,2	14.7	LGACCEQPVLER
1445.7	482.58,723.37	3,2	14.9	LGAC^{174}CEQPVLER
1460.7	487.25,730.38	3,2	15.3	LGAC^{174}C^{174}EQPVLER
1490.8	745.9	2	29.5	__VDPEYVPGXFK (b_2 = 228 Da)
1518.7	506.92,759.87	3,2	20.4	KHDDYAEVLCLR
1666	556.00,833.50	3,2	29.2	---PVXPTP---
1910.9	637.64,955.96	3,2	13.2	---XSQHVCSHQAEX---

--- an unknown number of amino acids
_ A single unknown amino acid

Table 1: Peptides of abundant protein of uterovaginal flushings identified by LC-MS/MS analysis

Estimation of lactic acid from the flushings of infundibulum, uterus and uterovaginal region of *Calotes versicolor*

The amount of lactic acid was found to be minimum in the uterus and uterovaginal flushings and almost nil in the infundibular region during the early vitellogenesis in *C. versicolor* and a gradual increase in the amount of lactic acid concentration was observed in all the region of the oviduct along with the gradual development of vitellogenic follicles. Higher concentration of lactic acid was observed in the uterovaginal flushings compared to the uterine and infundibular flushings (Fig. 15).

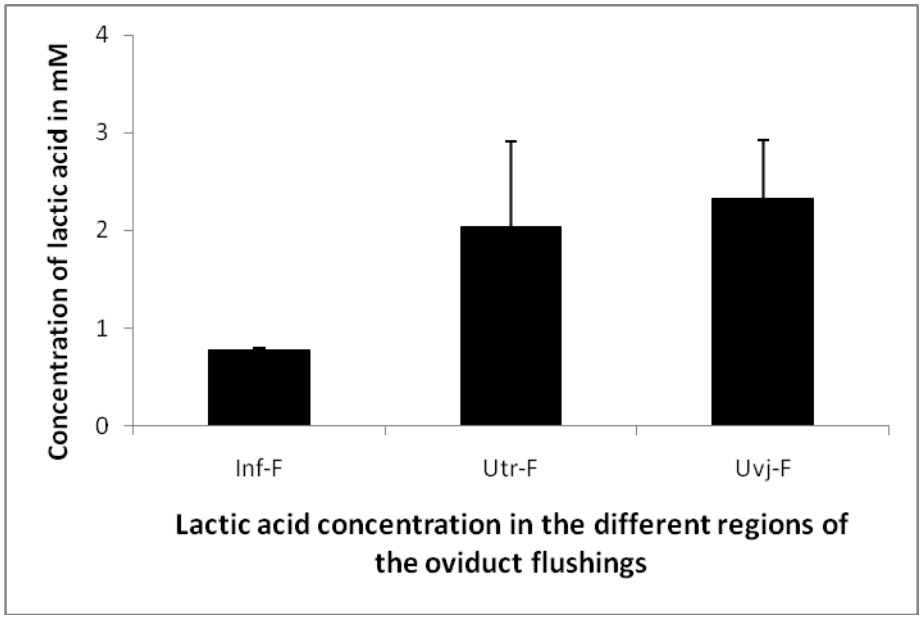

Figure 15: Amount of lactic acid in the flushings of different tissues of the oviduct during various stages of the follicular development. The data are expressed as mean±S.D. of three independent experiments.

Effect of purified single abundant protein of uterovaginal flushings on washed epididymal sperm parameters of *C. versicolor*

Initially washed epididymal sperm in a total volume of 100µl containing 2 x 10^5 sperm treated with 100µg of crude uterovaginal protein showed a progressive decrease in sperm motility from 91±2.64% (0 time) to 63±5% during 3 hours of incubation (Figure 16A). The purified ~55kDa protein of the uterovaginal flushings (25 - 75µg) when incubated with washed epididymal sperm, retarded the sperm motility in a concentration and time-dependent manner (Figure 16A and 17A) with minimum effect on their viability (Figure 16B and 17B) as assessed by eosin-nigrosin staining (Figure 17C and 17D). With 25µg purified protein the sperm motility decreased from 91±2.64% to 69±4.04% during 3 hours of incubation. Increasing the concentration to 50µg decreased the sperm motility within 30 minutes from 90±4.65% to 16±4.04%, while 75µg of this protein decreased the sperm motility from 87±3.51% to 5±2% within 30 minutes (Figure 17A). In a separate experiment, we found sperm regaining the motility and viability when ~55kDa protein is washed off (Figure 17A and 17B). Thus the effect of this protein is reversible.

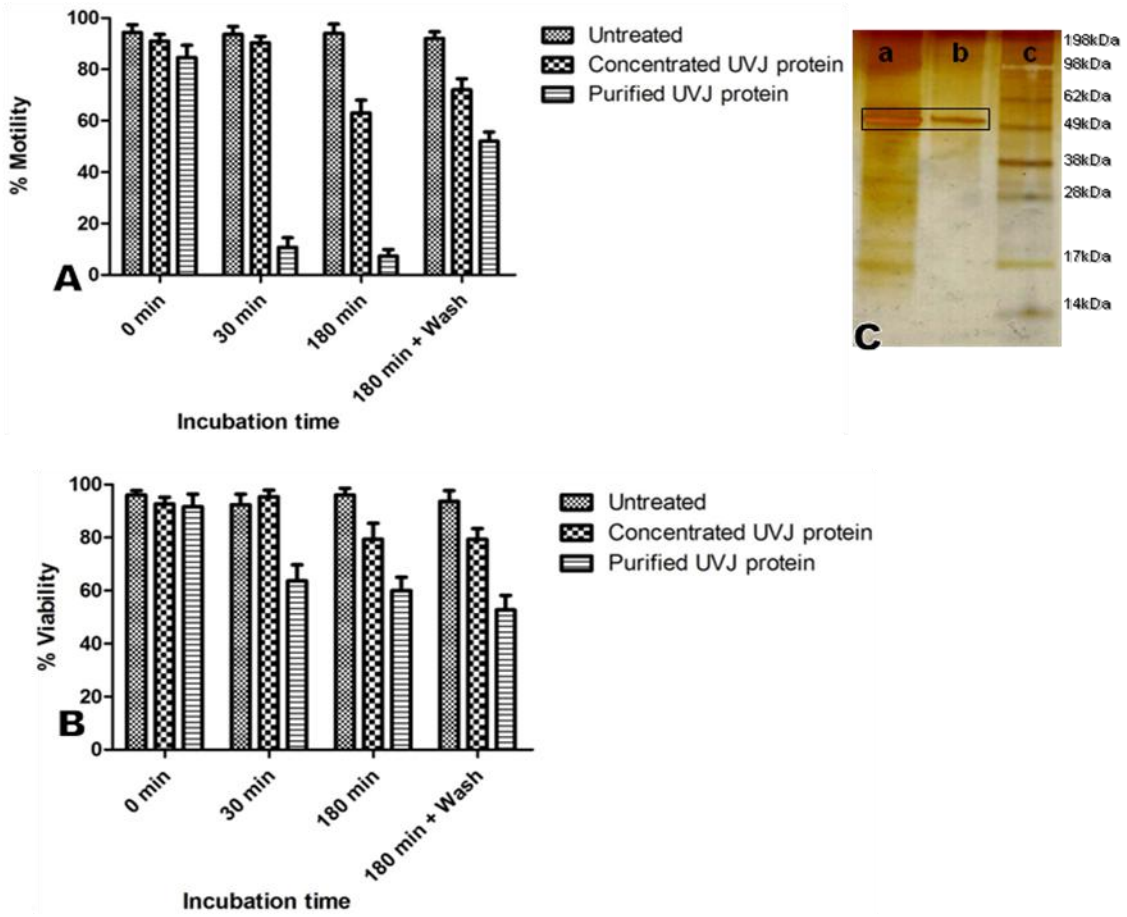

Figure 16: Effect of major uterovaginal protein on sperm motility (A) and viability (B) in a reversible fashion. Effect of crude (100µg) and purified uterovaginal protein (50µg) on washed epididymal sperm motility (A) and viability (B), and SDS-PAGE (12.5%) banding pattern (C) of crude uterovaginal protein (a) purified ~55kDa protein of the uterovaginal flushings (b) and molecular weight marker (c). The last three bars in both A and B represents the sperm motility and viability after 3 hours of incubation with the concentrated and purified uterovaginal protein followed by washing the exogenous protein. The data are expressed as mean±S.D. of three independent experiments.

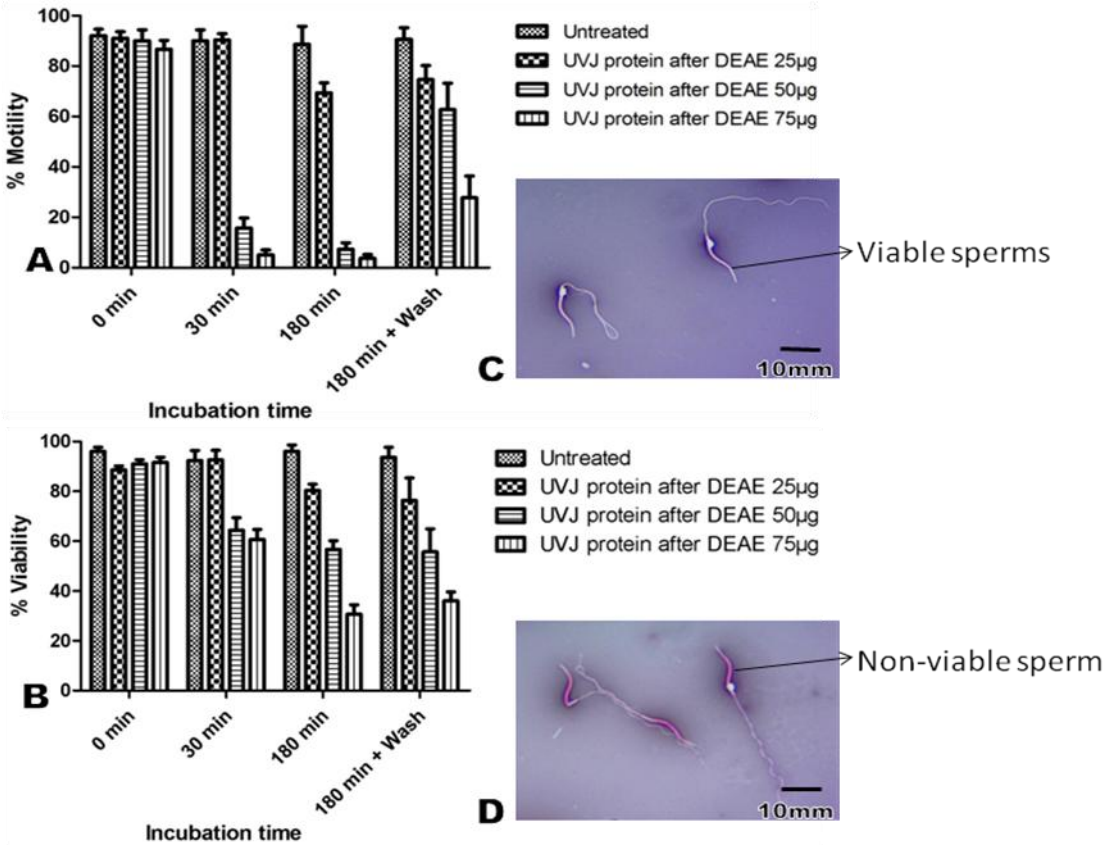

Figure 17: Concentration-dependent effect of ~55kDa uterovaginal protein on washed epididymal sperm motility (A) and viability (B) and recovery of sperm motility and viability after ~55kDa uterovaginal protein is washed off. Photographs of viable (C) and non-viable (D) sperms stained with eosin-nigrosin. The data are expressed as mean±S.D. of three independent experiments.

Conclusion

Sustenance of sperm in a viable and fertilizable condition at body temperature is quite an intriguing phenomenon and dissecting this has potential for far reaching applications. However, the mechanism(s) underlying this phenomenon is hardly understood. Although few studies in honey bee (7) and *Drosophila* (6) throw light on this phenomenon, the biology of oviductal sperm storage among reptiles is not explored. To our knowledge, the current study provides the first depiction of the ultrastructural features of the SST during three phase of the reproductive cycle in *C. versicolor*, which exhibits cyclical changes in the secretory activity of the epithelial cells lining the SST. We also observed that in a large majority of the female lizards isolated from males after mating far up to 70 days, the uterovaginal flushings still possess motile sperm (Data not shown) and the fertilizing ability of these stored sperm has been demonstrated previously (19), where vitellogenic females reared in the laboratory in the absence of males were induced to

ovulate by injecting pregnant mare's serum gonadotropin (PMSG). The eggs were not only fertilized using stored oviductal sperm, but they also successfully continued to embryonic development, thus providing possible circumstantial evidence that the stored sperm are presumably functional. In the current study, a ~55kDa abundant protein was also purified from the uterovaginal flushings that lacked homology to any protein on the NCBI database, and this protein reversibly inhibited the motility of washed epididymal sperm of *C. versicolor* in a concentration and time-dependent fashion (Fig. 16, 17). In our next project we want to look at the effect of this protein on sperm of other animals and also on the effect of peptide fragments resulting from protease digestion from this protein on sperm motility.

In spite of the fact that oviductal sperm storage is reported in large number of reptilian species, the exact mechanism of sperm sustenance for a long time at body temperature is not yet understood. Identification and characterization of factors responsible for prolonged sperm survival at body temperature can help us to devise better mechanisms for storing animal and human sperm and possibly other tissues/organs/cell types for various purposes. In the long run, many of the modern problems related to reproduction can be dealt with better understanding using the knowledge derived from studies like this. It may also open up new avenues in preserving endangered animals in general and reptiles in particular.

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ANNEXURE-IV

Overall assessment of the Guide

It is to certify that the information provided above with this report by the fellow is correct to the best of my knowledge and belief and further it is certified that the progress achieved by **Mr. Goutham S** is in the expected lines.

Date: 04.04.2017
Place: Mysore.

Signature of the guide