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Proteinaceous sperm motility inhibitory factor from the female Indian garden lizard *Calotes versicolor*

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Abstract. Female sperm storage is an intriguing adaptation exhibited by a wide array of both vertebrates and invertebrates. The mechanisms underlying female sperm storage have remained elusive. Using the Indian garden lizard *Calotes versicolor* as a model organism, we investigated the role of low and high molecular weight factors in this phenomenon. Previously, we demonstrated three distinct phases of the reproductive cycle in this animal with live, motile spermatozoa recovered from the uterovaginal region during the reproductive phase. In the present study, we analysed the uterovaginal contents using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and identified an abundant protein band corresponding to ~55 kDa regardless of the phase of the reproductive cycle. Analysis of the purified protein by liquid chromatography–tandem mass spectrometry suggested a unique protein without any homology to the National Center for Biotechnology Information database. Exogenous addition of this protein to washed spermatozoa derived from the epididymis reversibly inhibited sperm motility in a concentration- and time-dependent manner, suggesting it plays a key role in sperm storage. These studies are likely to offer new avenues to unravel the secrets of female sperm storage seen across the animal taxa and may have novel applications not only in reproductive biology, but also in general cell storage and preserving endangered animal species.

Additional keywords: lactic acid, sperm storage tubule, uterovaginal flushing.

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Introduction

Animals of various taxa, both vertebrates (Birkhead and Moller 1993; Holt and Lloyd 2010) and invertebrates (Bernasconi *et al.* 2002; Baer *et al.* 2006; King *et al.* 2011; Wolfner 2011; Ribou and Reinhardt 2012; Novo *et al.* 2013), have evolved different strategies for reproductive success. One such strategy is synchronising the release of the spermatozoa and egg, so that their chance of meeting each other is enhanced (Willaert *et al.* 2016). However, in many internally fertilising species, the reproductive cycle of the male and female is not synchronised (Girons 1982; Schuett 1992; Roy and Krishna 2011), resulting in the evolution of new strategies for enhanced reproduction. Sperm storage in the female reproductive tract has been one such fascinating adaptation, with a variety of specialised structural modifications in the oviduct to store sperm. These modifications are referred to as spermathecae and seminal receptacles in insects (Wolfner 2011), spermathecae in amphibians (Kuehnel and Kupfer 2012), sperm storage tubules (SSTs) in birds (Matsuzaki *et al.* 2015) and reptiles (Kumari *et al.* 1990; Birkhead and Moller 1993; Holt and Lloyd 2010) and sperm reservoirs in mammals (Crichton *et al.* 1982; Holt and Fazeli 2010; Suarez 2010). The storage of spermatozoa, in a motile and viable condition, for

variable lengths of time is likely responsible for extending the female reproductive phase beyond that of the male in a species-specific manner (Birkhead and Moller 1993; Holt and Lloyd 2010).

In an earlier study using *Calotes versicolor*, we described the histological and ultrastructural features of SSTs during the reproductive phase of this reptile (Shankar *et al.* 2015). Briefly, the SSTs are formed by the fusion of the distal ends of the mucosal folds lining the oviduct. At the junction between the uterus and vagina, called the uterovaginal junction, spermatozoa are found in abundance in these tubules, along with copious secretory material, apparently secreted by the epithelial cells of the SST (Shankar *et al.* 2015). Various molecular components have been shown to be required for long-term sperm survival in different groups of animals, including Ca^{2+} (Holm *et al.* 2000), Zn^{2+} (Bakst 1985; Holm *et al.* 2000), lactic acid (Matsuzaki *et al.* 2015), neuromodulators such as octopamine and tyramine (Avila *et al.* 2012), heme peroxidase 15 (Shaw *et al.* 2014), androgen (Liu *et al.* 2016), seminal vesicle protein 2 (Kawano *et al.* 2014), oviducal secretions (King *et al.* 1994; Abe *et al.* 1995; Satoh *et al.* 1995), male accessory gland secretions and female spermathecal fluid secretions (den Boer *et al.* 2009). Despite the prevalence of female sperm storage in many animal species, very little is known about the molecular mechanism(s) involved. Some of the extreme examples of prolonged female storage of spermatozoa, in a viable condition at body temperature, call for a deeper understanding of this phenomenon. Such studies can take us a long way to solving many modern problems in the field of reproductive biology. The aim of the present study was to examine the ultrastructural details of SSTs during three phases of the reproductive cycle of *C. versicolor* and to elucidate the effect of abundant uterovaginal protein on epididymal sperm sustenance *in vitro*.

Materials and methods

Animals

Permission to capture and use *C. versicolor* from the wild for research purposes 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 (No. UOM/IAEC/20/2013), as described previously (Shankar *et al.* 2015).

Transmission electron microscopy of SSTs

Transmission electron microscopy (TEM) studies of SSTs were performed during the reproductive (June), regressive (January) and regenerative (April) phases of *C. versicolor*, as described previously (Shankar *et al.* 2015). Briefly, the trimmed uterovaginal tissue (~1 mm^3 blocks) of the oviduct, containing SSTs, was fixed in modified Karnovsky's fixative (4% paraformaldehyde + 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) for 24 h and later post-fixed in 1% osmium tetroxide for 1 h. Subsequently, tissues were dehydrated and stained en bloc with 2% uranyl acetate in 95% ethanol for 1 h at room temperature (25–30°C), and then cleared in propylene oxide. Then, the tissues were impregnated overnight at room temperature (25–30°C) with a 1 : 1 mixture of propylene oxide : araldite resin, which was later increased after overnight incubation to a 1 : 3 ratio mixture of propylene oxide : araldite resin for 3 h, followed by pure araldite resin for 2–3 h. Finally, tissues were embedded in a flat embedding mould and kept at 60°C for 48 h for polymerisation. The uterovaginal tissue blocks were cut using an EM UC6 ultramicrotome (Leica). Ultrathin sections (50–60 nm) 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) operated at 50 kV, and representative areas were photographed using an inbuilt Olympus Megaview III charge-coupled device camera. The reagents used for TEM were obtained from TAAB Laboratories.

Purification of abundant ~55-kDa protein of uterovaginal flushings

The uterovaginal region of the oviduct, dissected out from five to six garden lizards during different phases of the reproductive cycle, was flushed with physiological saline and the flushings were centrifuged at 1000*g* for 10 min at 4°C. The supernatant obtained, free of spermatozoa, was concentrated using Centricon tubes (Merck Millipore) (cut-off >3 kDa). The concentrated protein solution, when subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis followed by silver staining, showed the presence of a major protein band with an apparent molecular weight of ~55 kDa, along with minor bands. This ~55-kDa protein was subjected to purification by conventional chromatographic techniques using Cibacron blue 3G-A (Sigma), pseudo-affinity and DEAE-cellulose anion exchange column chromatography. Briefly, pooled and concentrated uterovaginal flushing (containing 5 mg mL^{-1} protein) was loaded on to a Cibacron blue 3G-A column (0.5 cm × 15 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.4. The ~55-kDa 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 >3 kDa) and loaded onto a DEAE-cellulose column (0.5 cm × 5 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.4, and the fractions were eluted with increasing molarity of NaCl (10 mM–1 M). The ~55-kDa protein eluted when the salt concentration was between 200 and 250 mM.

Sperm motility assay

Spermatozoa were isolated by gently squeezing the epididymis down the ampulla of the vas deferens (Akbarsha and Meeran 1995) in 0.5 mL Earle's balanced salt solution (EBSS; Sigma) supplemented with 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 0.1% bovine serum albumin. The epididymal sperm suspension was centrifuged at 200*g* for 8 min at 37°C. Subsequently, the pellet was washed twice in EBSS and centrifuged at 200*g* for 5 min at 37°C. Following this, the final pellet was carefully overlaid with 200 µL EBSS. The tubes were then inclined at an angle of 45° and samples were incubated for 1 h at room temperature for direct swim-up. Viable

spermatozoa recovered from direct swim-up were used for all other studies. Briefly, the washed epididymal spermatozoa (2×10^5) were resuspended in a total volume of 100 μL of EBSS and treated for 0–180 min with various concentrations of purified ~55-kDa protein (0–75 μg) or crude uterovaginal junction proteins (100 μg) at 37°C. Finally, at 180 min all the above treated spermatozoa were washed twice with EBSS and resuspended in EBSS. The final washing process was carried out to remove the uterovaginal proteins from the treated samples. A total of 200 spermatozoa in 10 random fields (×400 magnification) at different time intervals (0–180 min) was visually assessed under a conventional microscope and classified as either motile (progressive and non-progressive motility) or immotile, with percentage motility subsequently calculated. Vitality assessment for the treated spermatozoa was based on the dye exclusion method as described previously (Björndahl *et al.* 2003) with minor modifications. Approximately 10 μL sperm sample was gently mixed with 20 μL of 1% eosin and 30 μL of 10% nigrosin. The smear was prepared immediately using a cover glass and allowed to air dry. The stained slides were observed under an oil immersion objective (×1000 magnification). Viable spermatozoa appeared white, whereas dead spermatozoa took up the dye and appeared pink (Fig. 1*h*). A minimum of 100 spermatozoa was evaluated, with viability expressed as a percentage.

Lactic acid estimation

The uterovaginal flushings were subjected to ultrafiltration using Centricon tubes (cut-off >3 kDa). Lactic acid concentrations were determined in the flow-through fractions using a spectrophotometric method (Figenschou and Marais 1991). In a parallel experiment, various concentrations (0–20 mM) of lactic acid were exogenously added to washed epididymal spermatozoa (2×10^5) and the sperm motility was assessed as described earlier for 0–180 min.

Results

TEM of SSTs during different phases of the reproductive cycle

Female *C. versicolor* store live, motile spermatozoa in the SSTs located at the uterovaginal junction of the oviduct over a prolonged period for future use, as described previously (Kumari *et al.* 1990; Shanbhag and Prasad 1993; Shanbhag 2002; Shankar *et al.* 2015). Briefly, the reproductive cycle of *C. versicolor* generally comprises reproductive (May–October), regressive (November–February) and regenerative (March–April) phases. During these phases of the reproductive cycle, several drastic changes occur in oviducal structure and mass (Fig. 1*a–c*) that are also associated with changes in the secretory activity of the SSTs, as reflected by TEM observations (Fig. 1*d–f*). During the reproductive phase, abundant electron-lucent secretory granules are found in the lumen and epithelial cells lining the SSTs. The granules in the lumen are found in close association with the stored spermatozoa (Fig. 1*d*). During the regressive phase, these cells contain electron-dense secretory granules, with degenerating spermatozoa and minimal secretory material in the lumen (Fig. 1*e*). Sever (1992) also reported similar degenerating spermatozoa in the salamander *Eurycea cirrigera*. During the regenerative phase there is a reappearance of secretory activity similar to that seen during the reproductive phase but without any spermatozoa (Fig. 1*f*). The nuclei of epithelial cells of the SSTs appear clearer during this phase compared with the other two phases. Motile spermatozoa could be recovered from the uterovaginal flushings over a period much longer (beyond October and as far as up to January) than the reproductive phase of the cycle (May–October; Shankar *et al.* 2015), indicating their prolonged storage and the possibility of being used to fertilise subsequent clutches of eggs (Shanbhag and Prasad 1993).

Analysis and purification of an abundant ~55-kDa protein from uterovaginal flushings

Sperm-free uterovaginal flushings subjected to 12.5% SDS-PAGE analysis showed the presence of protein bands with molecular weights ranging from 17 to 198 kDa. One major protein band of ~55 kDa appeared regardless of the phase of the reproductive cycle (Fig. 1*i*). To investigate the role of this major secretory protein from uterovaginal flushings, it was purified using conventional chromatographic techniques on a Cibacron blue 3G-A column followed by DEAE-cellulose anion exchange chromatography (Fig. 2*e*). In general, uterovaginal flushings from an individual garden lizard yielded approximately 300 μg total protein, of which approximately 25–75 μg accounted for the ~55-kDa protein, with the highest amount present during the reproductive phase and the lowest amount present during the regressive and moderate during the regenerative phase. Mass spectrometry analysis of the purified ~55-kDa protein suggested a unique protein without any homology to the National Center for Biotechnology Information (NCBI) database (data not shown).

Effect of total uterovaginal flushings and purified ~55-kDa protein on sperm motility and viability

In an attempt to understand the role of this major (~55-kDa) protein, washed epididymal spermatozoa (2×10^5), in a total volume of 100 μL, were treated with either 100 μg crude uterovaginal protein or different concentrations (0–75 μg) of purified protein. Untreated spermatozoa showed no significant change in motility over a 3-h incubation period. In contrast, treatment with crude uterovaginal protein progressively decreased sperm motility from 91 ± 3% at Time 0 to 63 ± 5% after 3 h incubation ($P < 0.001$; Fig. 2*a*; see also Video S1, available as Supplementary Material to this paper). The purified ~55-kDa protein (0–75μg) also inhibited sperm motility in a concentration- and time-dependent manner (Fig. 2*a*, *c*; Video S2). In the presence of 25 μg purified ~55-kDa protein, sperm motility decreased from 91 ± 3% at Time 0 to 69 ± 4% after 3 h incubation. Increasing the concentration of ~55-kDa protein to 50 μg decreased sperm motility within 30 min from 90 ± 5% to 16 ± 4% ($P < 0.001$), whereas at 75 μg, ~55-kDa protein caused a marked decline in sperm motility within 30 min (from 87 ± 4% to 5 ± 2%; $P < 0.0001$; Fig. 2*c*; Video S2). This inhibitory effect of the purified ~55-kDa protein was not the result of reduced sperm viability (Fig. 2*b*, *d*), as assessed by eosin–nigrosin staining (Fig. 1*g*, *h*). In addition, sperm motility

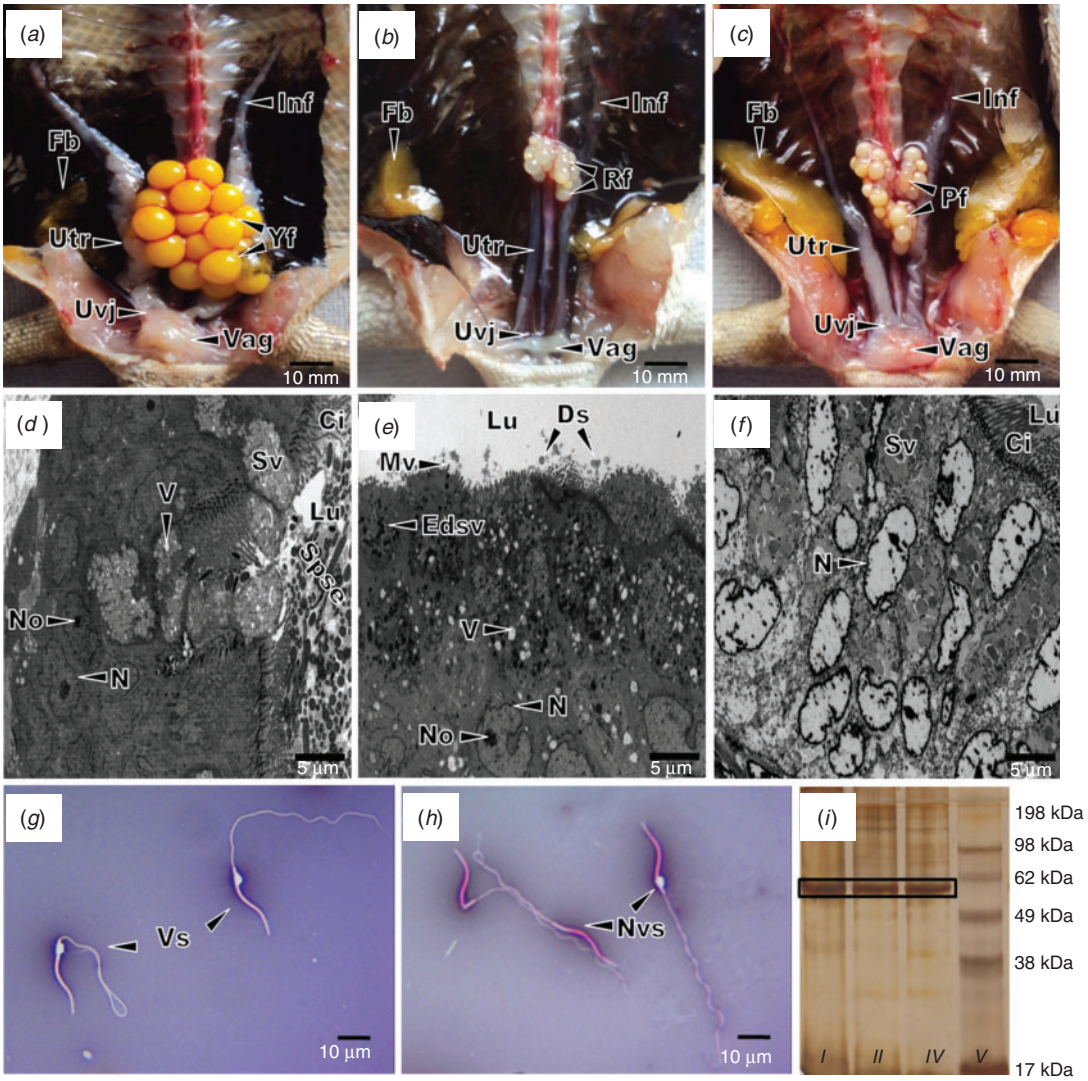

Fig. 1. Representative images of (*a–c*) dissection and (*d–f*) transmission electron microscopic (TEM) views of the reproductive organs of female *Calotes versicolor* with a sodium dodecyl sulfate–polyacrylamide gel (12.5%) electrophoresis banding pattern of crude uterovaginal junction proteins and visualisation of both viable and non-viable spermatozoa. There are three distinct phases in *C. versicolor* reproduction: (*a*, *d*) reproductive, (*b*, *e*) regressive and (*c*, *f*) regenerative phases. Cellular and secretory content varies among these phases, as shown by both dissection and TEM images (see text for details). Ci, cilia; Ds, degenerating spermatozoa; Edsv, electron-dense secretory vesicle; Fb, fat body; Inf, infundibulum; Lu, lumen; N, nucleus; No, nucleolus; Ov, ovary; Pf, previtellogenic follicles; Rf, regressed follicles; Spse, spermatozoa along with secretory material; Sv, secretory vesicle; Utr, uterus; Uvj, uterovaginal junction; Vag, vagina; Yf, yolky follicles. (*g*, *h*) Photographs of viable (*g*) and non-viable (*h*) spermatozoa stained with eosin–nigrosin. Nvs, non-viable spermatozoa; Vs, viable spermatozoa. (*i*) Sodium dodecyl sulfate–polyacrylamide gel (12.5%) electrophoresis analysis of uterovaginal flushings during the reproductive (Lane I), regressive (Lane II) and regenerative (Lane III) phases. Lane IV, molecular weight marker. Representative images of three reproductive seasons.

was regained after different concentration of ~55-kDa protein (0–75 µg) or crude uterovaginal protein (100 µg) was washed off with EBSS at 180 min (Fig. 2*a*, *c*; Video S3). Thus, the effect of this protein on sperm motility is reversible. All the above data represents mean ± s.d. of three independent experiments.

Spermatozoa stored in SSTs are likely to be in a quiescent state in order to retain their viability (Bakst 1985; Bakst and Richards 1985; Jones and Bavister 2000). However, when suspended in EBSS media, the spermatozoa were motile, suggesting that many endogenous factors, including the ~55-kDa protein, probably inhibit sperm motility *in vivo*. In this way, spermatozoa are likely kept quiescent until they become motile and functional (Jones and Bavister 2000). Although, how quiescent spermatozoa become motile in *C. versicolor* is not clear. Using Japanese quail (*Coturnix japonica*), Hiyama *et al.* (2014) demonstrated that heat shock protein 70 can activate and support the migration of

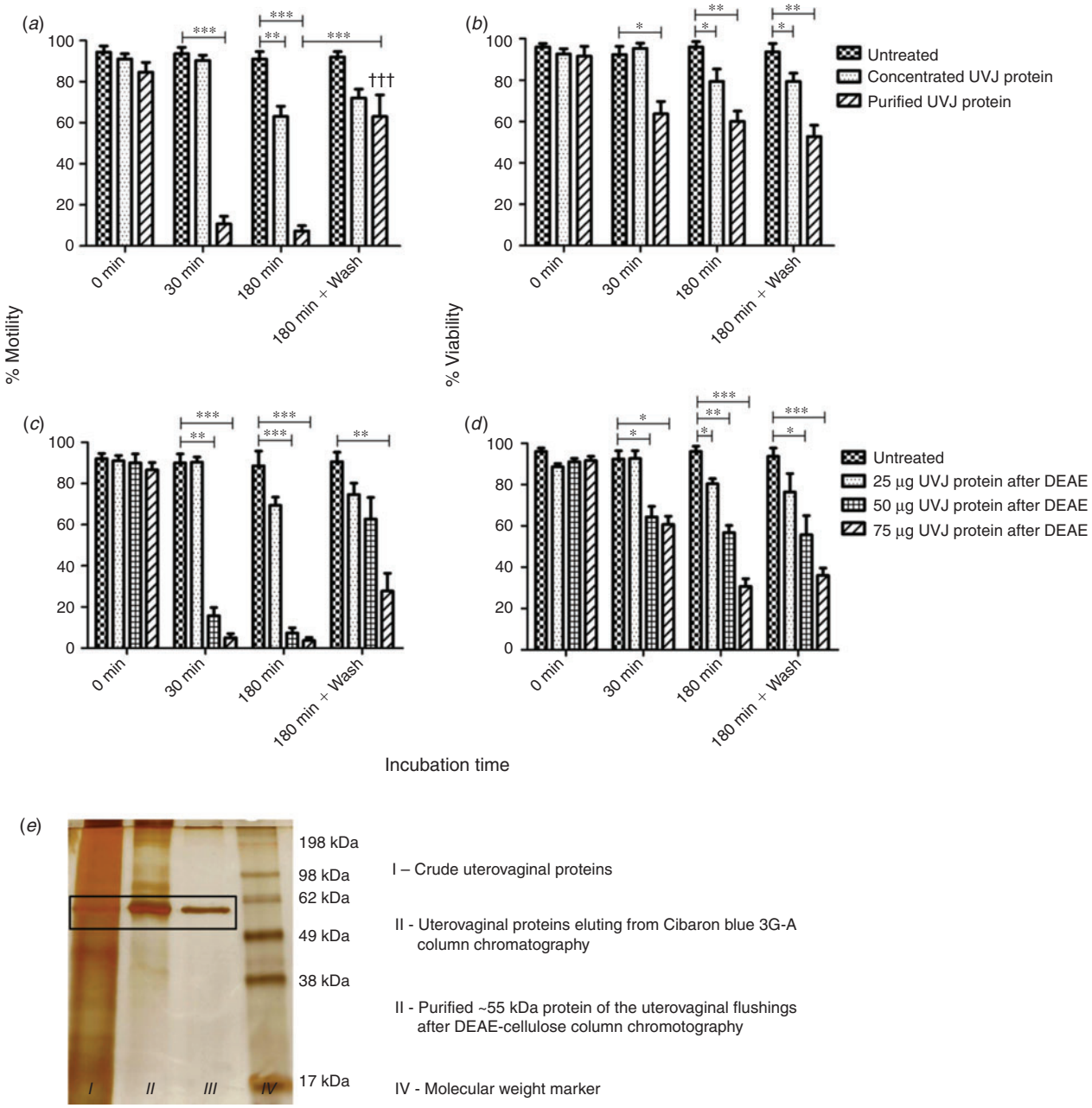

Fig. 2. Effects on washed epididymal sperm (*a*, *c*) motility and (*b*, *d*) viability over a 3-h incubation period and after washing out. (*a*, *b*) Reversible and time-dependent effects of 50 μg purified ~55-kDa protein and 100 μg crude uterovaginal junction (UVJ) protein. (*c*, *d*) Reversible and concentration-dependent effects 25, 50 and 75 μg purified (~55-kDa) UVJ protein. Data are the mean ± s.d. of three independent experiments. $*P < 0.01$, $**P < 0.001$, $***P < 0.0001$. (*e*) Sodium dodecyl sulfate–polyacrylamide gel (12.5%) electrophoresis banding pattern for various stages of purification of ~55-kDa uterovaginal junction protein.

spermatozoa stored in SSTs via voltage-dependent anion channel protein 2.

Estimation of lactic acid in uterovaginal flushings

Matsuzaki *et al.* (2015) showed increased production and release of lactic acid (i.e. 14 ± 3 mM, mean ± s.e.m. of increased level of lactic acid) from SSTs of Japanese quail under hypoxic conditions that resulted in the inhibition of sperm motility. To test whether lactic acid is present in uterovaginal flushings of *C. versicolor* and its possible involvement in inhibition of sperm motility, we measured lactate in uterovaginal flushings using a spectrophotometric method (Figenschou and Marais 1991). Lactate concentrations in the uterovaginal

flushings were approximately 2 ± 1 mM, which is lower than that reported by Matsuzaki *et al.* (2015). In addition, we added exogenous lactate to washed epididymal spermatozoa and found lactate to be inhibitory with regard to motility only at concentrations >15 mM (data not shown).

Discussion

Maintenance of stored spermatozoa in a viable condition is critical for reptilian survival. However, the mechanism(s) underlying this phenomenon is not known. Although a few studies in honey bees (Collins *et al.* 2004; den Boer *et al.* 2009; King *et al.* 2011) and *Drosophila* (Wolfner 2011; Lee *et al.* 2015; Avila *et al.* 2015) have shed some light on this phenomenon, the biology of oviducal sperm storage among reptiles has not been explored. To our knowledge, the present study provides the first description of the molecular features of SSTs in *C. versicolor*, which exhibits cyclic changes in the secretory activity of the epithelial cells lining the SST. In addition, we observed that in a large majority of female lizards isolated from males after mating for up to 70 days, the uterovaginal flushings still contained spermatozoa and these spermatozoa were motile when suspended in EBSS (data not shown); furthermore, the fertilising ability of these stored spermatozoa has been demonstrated previously (Shanbhag and Prasad 1993), whereby females with vitellogenic eggs, reared in the laboratory in the absence of males, were induced to ovulate by injection of pregnant mare's serum gonadotropin (PMSG). The eggs were not only fertilised using stored oviducal spermatozoa, but they also exhibited successful continued embryonic development (Shanbhag and Prasad 1993), thus providing possible circumstantial evidence that the stored spermatozoa are presumably functional.

In internally fertilising organisms, female sperm storage is one of the essential processes required for efficient gamete use and the subsequent maintenance of fertility (Avila *et al.* 2012). Previous studies have shown that oviducal epithelial cells produce secretory proteins (Maillo *et al.* 2016) involved in sperm activation (McNutt *et al.* 1992), fertilisation (King *et al.* 1994), early embryonic development (King *et al.* 1994; Tse *et al.* 2008) and the maintenance of sperm motility (Abe *et al.* 1995; Coy *et al.* 2012) and viability (Abe *et al.* 1995; Satoh *et al.* 1995; Coy *et al.* 2012). However, information regarding the biochemical characteristics and/or biological functions of the oviducal proteins is limited. In our previous study, we had recovered live, motile spermatozoa from uterovaginal flushings of *C. versicolor*, and the density of spermatozoa was found to vary, being highest during the reproductive phase and lowest (or almost zero) during the regressive phase, with no spermatozoa present during the regenerative phase (Shankar *et al.* 2015). Once the ejaculated spermatozoa have entered the female reproductive tract, they can survive for a prolonged time and it is likely that in the lumen of SSTs spermatozoa are naturally immotile and thus appear to be metabolically dormant (Bakst 1985; Bakst and Richards 1985). The occurrence of a sperm motility inhibitory factor has also been reported in the epididymal plasma of chicken (Mohan *et al.* 1995), boar (Iwamoto *et al.* 1992) and caprine (Das *et al.* 2010) species. However, to the best of our knowledge, no reports are available on the purification and characterisation of these factors from the oviducal fluid. In the present study, we purified a unique ~55-kDa protein from the uterovaginal flushings that reversibly inhibited the motility of the washed epididymal spermatozoa. A homology search for this protein using the NCBI database did not yield any fruitful results (data not shown).

During purification of the ~55-kDa protein and while studying the exogenous effect of uterovaginal flushings on epididymal spermatozoa, we noticed that long-term incubation with a high concentration (>100 μg) of the Cibacron blue 3G-A uterovaginal junction protein fraction caused sperm agglutination and dislodged the head and tail from the spermatozoa (data not shown), even though this fraction contained an appreciable amount of ~55-kDa protein, as noted by silver staining (Fig. 2*e*). In addition to the ~55-kDa protein, these fractions contained a prominent high molecular weight protein (~100 kDa) and many minor proteins. Further purification and enrichment of the ~55-kDa protein by anion exchange chromatography on a DEAE-cellulose column eliminated the agglutination and dislodging activity of the other unidentified factors, suggesting that there are factors in uterovaginal flushings that counteract the actions of the ~55-kDa protein.

It is possible that the abundant ~55-kDa uterovaginal protein, in combination with other unidentified low molecular weight components, may be involved in the inhibition of sperm motility, thereby keeping the spermatozoa quiescent. However, one such low molecular weight substance identified previously (Matsuzaki *et al.* 2015) was lactate (14 ± 3 mM in the SSTs of Japanese quail). In the present study, lactate was found in the uterovaginal flushings ($\sim 2 \pm 1$ mM). This low concentration of lactate was unable to inhibit sperm motility *in vitro* (data not shown). Thus, lactate alone is unlikely to be the major factor inhibiting sperm motility in our model. The fact that crude uterovaginal flushings have detectable but not pronounced inhibitory effects on sperm motility (Fig. 2*a*; Video S1) suggests that uterovaginal flushings may have other unidentified factor(s) counteracting the actions of the ~55-kDa protein (Satoh *et al.* 1994; Abe *et al.* 1995; Mohan *et al.* 1995; Bernasconi *et al.* 2002; Kareskoski and Katila 2008; den Boer *et al.* 2009; King *et al.* 2011; Kawano *et al.* 2014; Matsuzaki *et al.* 2015). The progressive enrichment of this protein during chromatographic separation (Fig. 2*e*) probably removes the other inhibitory factor(s), making the effect more detectable (Fig. 2*a*, *c*; Video S2).

In conclusion, an abundant ~55-kDa protein was purified from the uterovaginal flushings of *C. versicolor* that lacked homology with any known protein in the NCBI database. This protein reversibly inhibited the motility of washed epididymal spermatozoa of *C. versicolor* in a concentration- and time-dependent manner (Fig. 2*a*, *c*; Videos S2, S3). Further studies are needed to determine whether this protein also blocks sperm motility in other animals and efforts are currently underway to identify specific regions of the peptide responsible for the inhibition of sperm motility.

Despite the fact that oviducal sperm storage has been reported in a large number of reptilian species, the exact mechanism by which spermatozoa are sustained for a long time at body temperature is not yet understood. Identification and

characterisation of factors responsible for prolonged sperm survival at body temperature could help us devise better mechanisms for storing animal and human spermatozoa and possibly other tissues, organs and cell types for various purposes. In the long run, a better understanding of the molecular mechanism(s) gained from knowledge derived from studies like the present one, could help us resolve many of the modern problems related to reproduction. It may also open up new avenues in preserving endangered animals in general and reptiles in particular.

Conflicts of interest

The authors declare no conflicts of interest.

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