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Removal of extracellular coat from giant sperm in female receptacle induces sperm motility in *Mytilocypris mytiloides* (Cyprididae, Ostracoda, Crustacea)

Renate Matzke-Karasz[1] · **Robin J. Smith**[2] · **Martin Heß**[3]

Abstract Previous studies of cypridoidean ostracods have noted that (1) their giant spermatozoa are immotile inside the male, (2) these spermatozoa are motile in the female seminal receptacle and (3) these receptacles are often filled with empty sperm coats. Such findings have led previous authors to hypothesize that sperm must shed their coats in the female receptacle to become motile. We present light and electron microscopy results and video recordings of mating experiments with virgin specimens of *Mytilocypris mytiloides*. We show that the empty sperm coats frequently found in the female receptacles are not the result of sperm molting but are the resistant inner coats of exhausted sperm not used for egg fertilization. In contrast, we show that an outer granular coating material is successively removed from the sperm while resident inside the female receptacles before first oviposition occurs. During this period, previously immotile sperm gain motility, showing strong movement shortly before first oviposition takes place. By correlation of these phenomena, we suggest that dissolution of the outer coat material is required for motility to develop.

Keywords Sperm coat · Sperm degradation · Sperm movement · Spiral canal · Zenker organ

Introduction

Reproduction in cypridoidean ostracods is either fully parthenogenetic or fully sexual; neither cyclic parthenogenesis, nor hermaphroditism occurs. Both genders in cypridoidean ostracods possess a paired reproductive system, with one set on each side of the body and without a connection between the two sets. In males, four testes on either side of the body produce the spermatids, which, on their journey through the long seminal vesicle, develop into so-called giant sperm.

The term giant sperm includes all animal male gametes that are characterized by extraordinary length, although their morphology can vary considerably, depending on the taxon in which they are found. Ostracods of the superfamily Cypridoidea possess filiform aflagellate sperm, typically subdivided into a thin anterior region (without mitochondria) and a thicker posterior region (containing two long mitochondria and contractile organelles). The longest spermatozoa known in ostracods are those of *Australocypris robusta* De Deckker (11.8 mm) and although these are not the longest sperm on record in the animal kingdom, they qualify as the ones with the largest volume (Smith et al. 2016b).

In cypridoidean ostracods, these giant sperm are transferred directly into the female during copulation, without the formation of spermatophores. A sperm pump (Zenker organ) present in the distal part of each of the two vasa deferentia pump the sperm through the penis (consisting in two hemipenes joined at their bases) into the two vaginas, while the penis is firmly

✉ Renate Matzke-Karasz
r.matzke@lrz.uni-muenchen.de

1 Department of Earth and Environmental Sciences, Palaeontology, Ludwig-Maximilians-Universität München & GeoBio-Center LMU, 80333 Munich, Germany

2 Lake Biwa Museum, 1091 Oroshimo, Kusatsu, Shiga 525-0001, Japan

3 Department of Biology, Ludwig-Maximilians-Universität München & GeoBio-Center LMU, 82152 Munich, Germany

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attached to the genital lobes of the female. Once in the female, the sperm travel through the paired, long, partly wound up sperm ducts and are stored in the two seminal receptacles. These are closed off, sac-like organs, which are not connected to the paired ovaries. The ovaries have distinct openings on the female genital lobes, posterior to the vaginas. Thus, egg fertilization most probably takes place when the oviporus opens up to let an egg pass; however, egg fertilization has not yet been observed in this group and thus insights into the way that it occurs rely purely on morphological deductions. In contrast, the entire giant sperm is known to enter the egg upon fertilization (Matzke-Karasz 2005).

In spite of a long history of research into ostracod sperm biology (Fig. 1), one important aspect has remained obscured up to now; the process between insemination of the female and egg fertilization. Zenker (1854) found that sperm were motile only inside the female receptacles but not in the male and he frequently saw a large number of empty sperm coats in the female seminal receptacles. Zenker concluded that the secretion of a "slime gland" covers the sperm and keeps them immotile by hardening. Later, in the receptacle, these hardened coats swell, break apart and finally, the sperm molt and become motile, leaving their empty coats behind in the seminal receptacle. Although Zenker's "slime gland" was later found to be a sperm pump (Stuhlmann 1886) and was named after Zenker (Vávra 1891), the hypothesis that the sperm shed a coat to become motile was followed by a few authors in the subsequent 160 years, although it has neither been fully verified nor falsified. The presence and number of sperm coats varies between studies, as does the interpretation of empty sperm coats in the seminal receptacles (for a detailed chronology of this dispute

First illustration and description as enigmatic `hair vessels´ (1)

1808

1836 Short note on long, filiform, and spiraled spermatozoa (2)

Ostracod sperm and the male reproductive system; spermatogenesis in ostracods; first overviews on different ostracod sperm morphologies (3)

1854-1912

1935 Motility in ostracod giant sperm; first correct interpretation of the sperm's orientation and direction of movement (4)

Electron microscopic studies on relation between external appearance and internal organization of sperm (5)

1967-1988

2001, 2012 Mummified sperm preserved in Holocene ostracod valves (6)

Giant sperm coiling in the egg after fertilization (7)

2005

2009, 2014 Evidence of fossil giant ostracod sperm with preserved cell organelles and the evolutionary longevity of reproduction with giant sperm in the group (8)

Micromorphology, function, and formation of the cypridoidean sperm pump (Zenker organ) (9)

2012, 2014

2016 Distribution of sperm lengths and their variability within cypridoidean ostracods (10)

Fig. 1 Chronology of investigations of giant sperm in Ostracoda. References: (1) Ramdohr (1808); (2) Wagner (1836); (3) Zenker (1854), Plateau (1868), Stuhlmann (1886), Müller (1889), Retzius (1909), Schmalz (1912); (4) Lowndes (1935); (5) Tétart (1967), Gupta (1968), Reger and Florendo (1969a,1969b), Reger (1970), Zissler (1969a,1969b, 1970), López-Camps et al. (1979), Wingstrand (1988); (6) Matzke-Karasz et al. (2001), Iepure et al. (2012); (7) Matzke-Karasz (2005); (8) Matzke-Karasz et al. (2009, 2014); (9) Yamada and Matzke-Karasz (2012), Yamada et al. (2014); (10) Smith et al. (2016a,2016b)

see Supplementary Online Material). The most recent study covering these issues, namely Wingstrand's (1988) monograph on ostracod sperm, generally followed Gupta's (1968) conclusions that initially two distinct coats are present around the sperm. However, whereas Gupta dismissed the idea of sperm molting in the seminal receptacle, Wingstrand considered that both molting and disintegration of the outer coat were possible. Therefore, the source of the empty sperm coats in the seminal receptacles has not, so far, been satisfactorily explained and reasons for the emergence of sperm motility in the female and the disappearance of the outer sperm coat remain speculative.

The present study was designed to test whether the presence or absence of a sperm coat can be correlated with sperm motility or the lack thereof in non-marine ostracods. A further aim was to verify the presence of empty sperm coats in the female seminal receptacle, their origin and their possible relationship to sperm motility. We present here the results of an investigation of sperm morphology and motility, after controlled inseminations of ovigerous virgin females by mature virgin males of the ostracod species *Mytilocypris mytiloides* (Brady, 1886) (Mytilocypridinae, Cyprididae).

Materials and methods

For experiments, the athalassic saline ostracod species *Mytilocypris mytiloides* was chosen because of its relatively quick development from egg to sexual maturity (30–40 days), large size (carapace length: ca. 3 mm) and easy visibility of its eggs (bright orange). This species inhabits saline lakes in Australia, with several generations per year. As with other cypridoidean species, *M. mytiloides* is promiscuous; copulations take place at a high frequency and females can lay eggs over several weeks. Sperm length in this species is ca. 4 mm.

Stock cultures of *Mytilocypris mytiloides* were kept in a climate chamber at 22 °C and under a 12h light/dark cycle (plant growth light), in glass jars, at 30–40 ‰ salinity (Tropic Marine Bio Active sea salt). From these stock cultures, juveniles of the pre-adult stage A-1 were isolated into slots of multiwell culture plates (six wells Cellstar, Greiner, Germany), containing water of the same salinity and a mixture of quartz sand (Roth, Germany) and Caribbean Life Sand (CaribSea Inc, Florida, USA). These plates were examined three times per week and food was added if required (food flakes for fish; Vitakraft, Germany). After the final molt, the date of the molt and the gender of the animal were noted. Since sexual maturity was expected to be attained about two weeks after the last molt (Yamada et al. 2014), copulation experiments were carried out after this lapse of time.

Mating experiments were carried out by transferring a virgin male into a well in which a virgin female had been raised to adulthood. Once the male attached to the female, the successful introduction of the hemipenes into the female genital field was monitored microscopically and either the time of insemination was measured (oviposition experiments and video recordings of live sperm) or the couples were allowed to mate several times, until, after 1 h, the male was replaced into its original well (oviposition experiments, TEM and SEM studies). A small piece of wood (broken barbecue stick) for egg attachment was added to the well slots. Females treated in this way were either used (1) to monitor the behavior and success of oviposition, (2) for light microscopy investigation of sperm, (3) for video recording of living sperm, (4) for transmission electron microscopy (TEM) of sperm, or (5) for scanning electron microscopy (SEM) of sperm.

1. *Oviposition:* After controlled matings of 1 h duration and the subsequent removal of the males, the culture wells with females were observed regularly starting approximately 12 h after the first insemination to determine the timing of the first oviposition. In ten females, the total numbers of deposited eggs were monitored. Occasionally, oviposition behavior could be directly observed.

   Three females were allowed to be inseminated only once (time was monitored) and subsequently to lay their eggs. The numbers of eggs were noted and eggs were removed into separate well slots to be monitored for hatching success. One female was killed 15 days after having deposited the last egg and checked for motile sperm and empty sperm coats. Two females were cultured until they died and were then observed for empty sperm coats in their receptacles.

2. *Video recordings of living sperm:* After anesthetization with H_2CO_3, the female seminal receptacles or the seminal vesicles in the males were dissected from the body under a Leica M165FC microscope and observed on a glass slide, after being mounted in a Carcinus saline solution under a standard cover slip. The Carcinus saline solution was prepared after Pantin (1934).

   Videos were recorded by using a Canon EOS 650D Digital Camera attached to a Leica DMLB2 transmission light microscope. Videos were processed with Adobe Photoshop CS6 and iSkysoft iMedia Converter.

3. *Light microscopy:* Preserved spermatozoa (70 % ethanol) were examined by using distilled water or Hydromatrix medium (MicroTechLab, Graz), standard glass slides and coverslips and a Leica DMLB2 microscope under brightfield illumination and images were captured with a Canon EOS 650D camera.

4. *Transmission electron microscopy:* Females and males were anesthetized (H_2CO_3) and dissected. The valves were removed and the soft bodies were initially fixed in 2.5 % glutaraldehyde in 0.05 M phosphate buffer at 4 °C and then post-fixed in 2 % osmium tetroxide in the same buffer for 2 h at 4 °C. After being washed in buffer, the samples were dehydrated by a graded series of acetone/water solutions. Dehydrated samples were kept in a diluted Epon epoxy resin mixture (1:1 with 100 % acetone) for 2 h and kept

in 100 % Epon overnight. They were subsequently embedded in Epon with a DPM-30 (Sigma) accelerator at 60 °C according the manufacturer's instructions. Samples were initially serially sectioned with glass knives at a thickness of 1.5 μm and stained by using Richardson's solution (Richardson et al. 1960). After digital alignment of light micrographs and definition of appropriate regions (e.g., tip of sperm head), ultrathin sections were taken from selected remounted semithin slices with a diamond knife (thickness 70 nm, Leica EM UC6 Ultramicrotome). Stained ultrathin sections (1 % potassium permanganate solution in distilled water and lead citrate after Reynolds [1963]) were studied by using a FEI Morgagni 268 TEM at 80 kV.

In female specimens, sections were taken from filled seminal receptacles. In male specimens, the vasa deferentia each form an expansion (called the sperm vesicle) just in front of the entry of the Zenker organs. In sexually mature males, a sperm vesicle is filled with sperm of a similar age, coiled tightly in a thick ring. These rings were sectioned for investigation by transmission electron microscopy.

5. *Scanning electron microscopy:* Fixed samples were dehydrated, critical-point-dried (BalTec CPD 030), coated with gold (Polaron Sputter Coater) and studied by using a Leo 1430VP (Zeiss) scanning electron microscope at 15 kV.

Results

Anatomy of female sperm storage tract in *M. mytiloides*

The reproductive organs in male and female cypridoidean ostracods are paired and in both genders, the two parts are not interconnected. Hence, the female genital area possesses two vaginas and two openings of the oviduct (Fig. 2a, b). In *M. mytiloides*, no internal connecting duct between oviduct and sperm storage tract has been found; the sperm most likely have to exit through the vagina shortly before oviposition to come into contact with the egg.

The male pumps sperm of approximately 4 mm length into the vaginas and through an approximately 12 mm long, wound up, so-called spiral canal (Fig. 2c, d), which terminates in a cuticular sac-like extension, the seminal receptacle (Fig. 2c, e). Here, sperm are stored before they travel back through the spiral canal to come into contact with a mature egg. The receptacles in Fig. 2a, c, e are all partially filled with sperm forming a relatively regular circle.

Oviposition

During experiments, females of *M. mytiloides* preferentially laid their eggs into small cavities between wood fibres formed at the tips of broken barbecue sticks. Eggs were laid either singly or in small clutches per day (average around 3 eggs/day). The shortest time span measured between insemination and oviposition in the model species was 22 h. However, most females did not start oviposition until about 24 h after insemination. Oviposition after a single insemination event, with females kept in isolation (*n* = 3), occurred up to 47 days after insemination. At death, all females had >10 eggs of the "final" size in their oviducts.

Empty sperm coats in female receptacles

In seminal receptacles of females of *M. mytiloides* of undefined age, taken from a stock culture, empty sperm coats were frequently present. Scanning electron microscopy clearly showed solid helicoidal strands representing the former inner sperm coats (Fig. 3). Coats had split along their long axis into curled strands, consisting in four coaxial bands.

Empty coats were also visible by transmitted light microscopy (Fig. 4). A female specimen, dissected 57 days after a single insemination and 15 days after having laid the last egg (out of 149 eggs), carried ca. 50 sperm in each receptacle. All sperm were immotile and ca. 50 % were visibly decayed, as represented by empty coats. Two more females treated in the same way were kept until natural death and were subsequently dissected, with one carrying a total of around 40 sperm and the other around 80 sperm, many of them as empty coats.

Transmission electron microscopy of sperm: investigated in males

Surrounding the compact homogeneous extracellular inner coat, sperm had a second or outer coat composed of a loosely packed material, externally being mostly defined by a darker and denser line. The thickness and appearance of the coat varied in the different regions of the sperm. The material of the outer coat filled the grooves of the inner coat along the posterior parts and enveloped the drills along the anterior part. At the anterior-most tip, the outer coat had no clear outer boundary and the coating material surrounded the cross-section resembling a cloud (Fig. 5a-c). In the thin anterior part of the sperm, the outer coat was circular in cross-section and between 0.5 and 0.7 μm thick (Fig. 5d, e). Toward the posterior, a transition was apparent in which the mitochondria made up only a small part of the cross-section, whereas the thick posterior region was defined by mitochondria at their maximum thickness. In the former, the outer coat measured up to 0.7 μm (Fig. 5e), whereas in the latter, it varied between very narrow regions of 0.1 μm in thickness and very wide areas at which the outer coat adhered to those of the adjacent sperm (Fig. 5f-h). In the latter case, a single darker line marked the boundary of two adjacent outer coats (Fig. 5f-i, black arrows). This adherence of sperm outer coats might explain the increased difficulty of separating sperm taken from male sperm
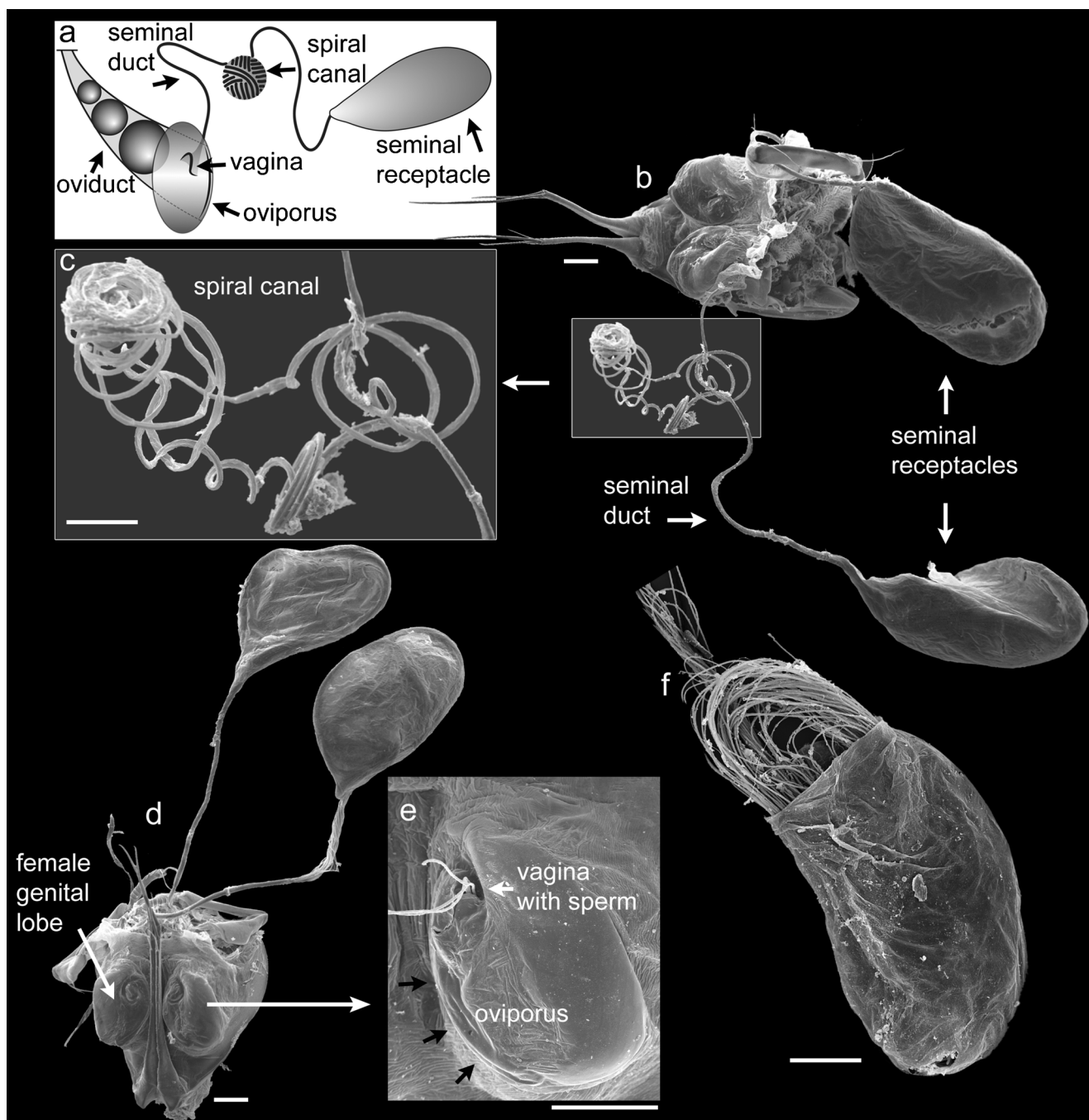
Fig. 2 Paired reproductive system in female *Mytilocypris mytiloides* (**b-f** scanning electron micrographs). **a** Representation of the general anatomy (organs of left body side omitted), ventral view of the right female genital lobe. **b** Posterior body with one entire seminal duct freed. **c** Detail of the highly coiled spiral canal. **d** Ventral view of the posterior body, showing the paired genital lobes, with the caudal rami folded between them. Parts of the two seminal ducts, leading to the seminal receptacles, are dissected free. **e** Higher magnification of the genital lobe with sperm hanging out of the s-shaped vaginal opening (*white arrow*) and a fine long line (*black arrows*) representing the oviporus. **f** Seminal receptacle partly opened, with stored sperm uncovered. *Bars* 20 μm (**c**), 100 μm (**b, d-f**)

vesicles; this is especially noticeable in ethanol-preserved specimens compared with sperm taken from females.

In some transmission electron microscopy sections, sperm and the sperm duct lining were in close contact (Fig. 5j), with small gaps filled with the granular material forming the outer sperm coats (Fig. 5j, white arrowheads).

Transmission electron microscopy of sperm: investigated in females

In sperm stored in the female receptacle for ca. 1 h (*n* = 2), the outer coats were similar in appearance to those seen in the males; they were comparatively thick in the anterior regions

Fig. 3 Scanning electron microscopy images of sperm in a seminal receptacle of a female *M. mytiloides*. **a** In addition to unaltered sperm, the receptacle contains several sperm that have degenerated, with only the empty inner sperm coat remaining, the cell content having gone. **b, c** Higher magnifications of *white frames* in **a**. *Bars* 10 μm (**a**), 3 μm (**b, c**)
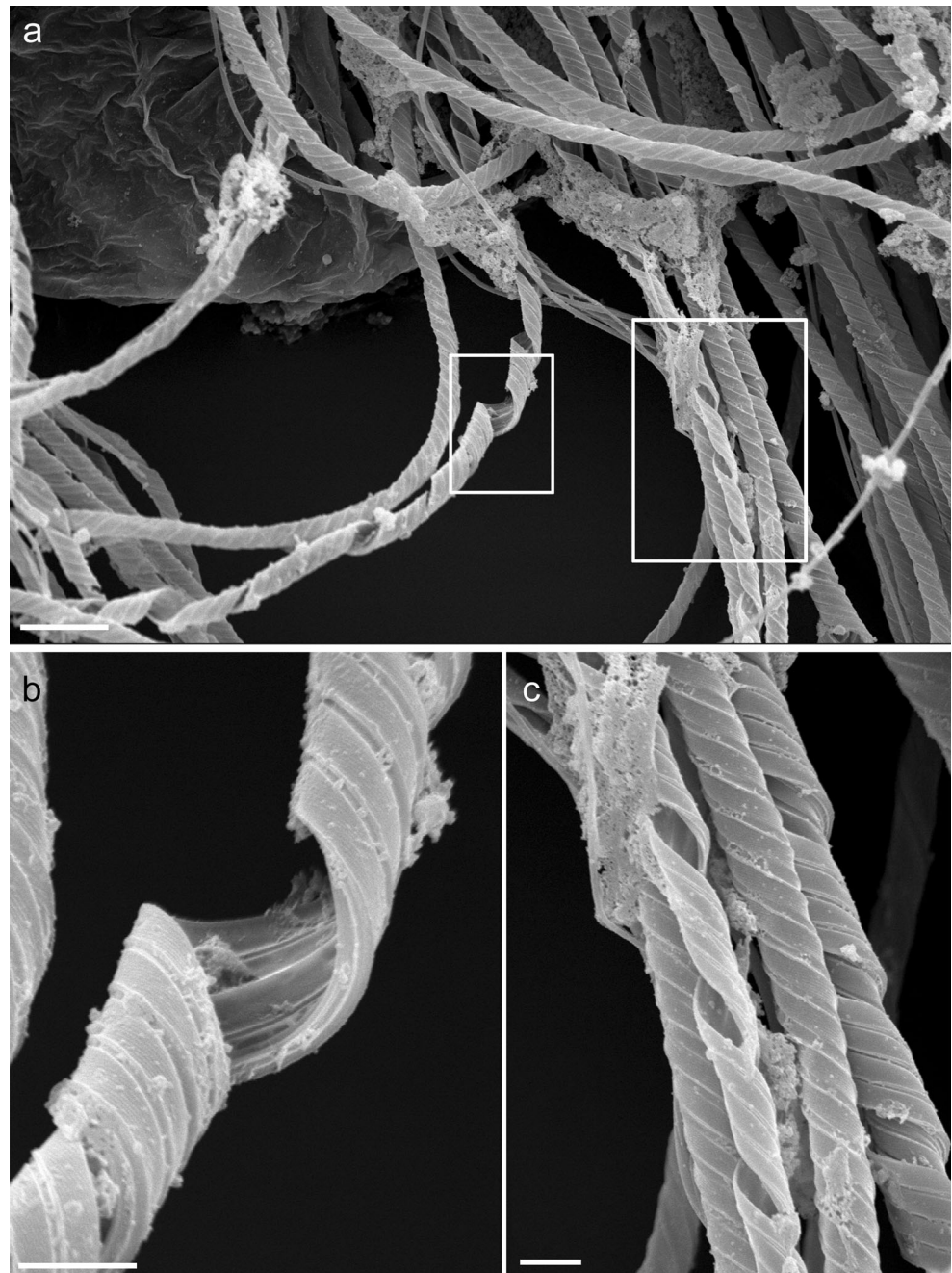

(Fig. 6a) and much thinner in the posterior regions of the cell. The fine line marking the boundary of the outer coat was still present but it appeared slightly weaker and on rare occasions, it seemed perforated. However, a noticeable difference with sperm in the male was that the outer sperm coats no longer adhered to adjacent sperm (Fig. 6a-c).

In sperm stored in the female receptacle for ca. 5 h (*n* = 2), the outer coats of the anterior region were less compact, partly deformed and partly reduced. In the posterior regions, the coats were thinner, often being only visible in the grooves and their outer boundary had become less visible in some places.

In sperm stored in the female receptacle for ca. 10 h (*n* = 3), the condition of the outer sperm coats was not homogeneous in the various samples. In most sperm of these samples, the coat was still present, even with outer lines present, whereas in others, the coat was strongly reduced, with some of the typical granular material lying around the sperm but without a clear boundary (Fig. 6d-f).

In sperm stored in the female receptacle for ca. 15 h (*n* = 2), even weak traces of the outer sperm coat were doubtful; in some cross-sections, a denser aggregation of particles could be tentatively interpreted as the remains of the outer coats but no clear boundaries were

Fig. 4 Light microscopy images of sperm in a seminal receptacle of *M. mytiloides*. Areas in *white frames* and in *inset* show typical appearances of empty inner sperm coats remaining from decayed sperm. *Bars* 10 μm
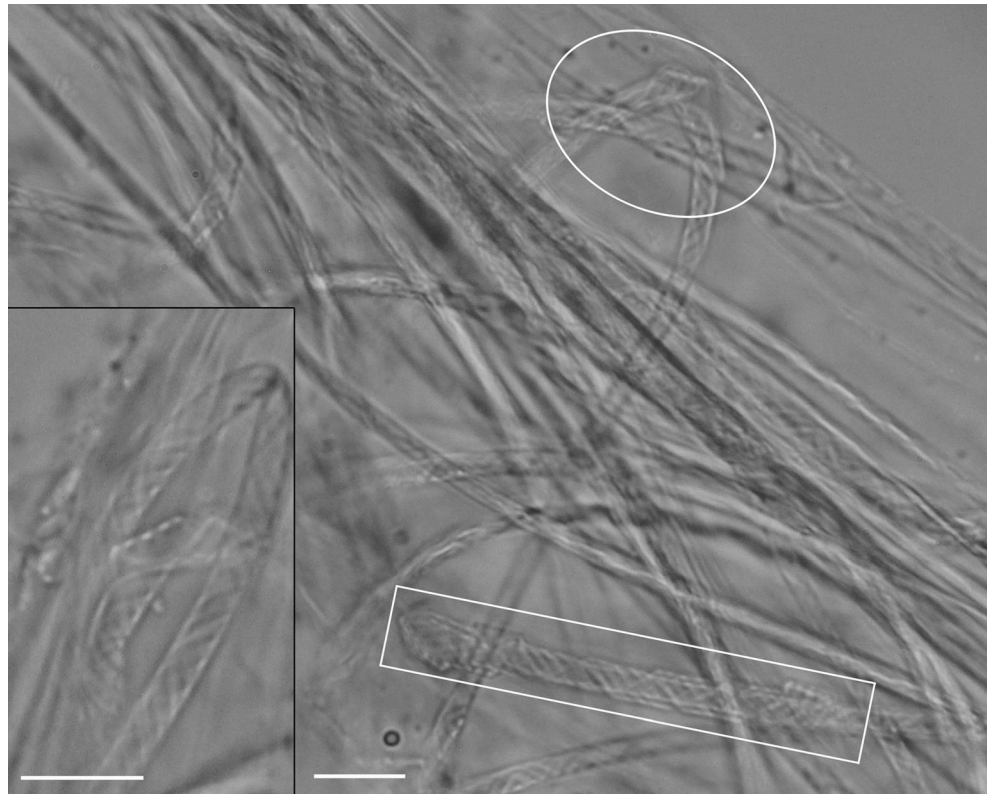
visible. In many cross-sections, however, no outer sperm coat material was visible (Fig. 6g-i).

In sperm stored in the female receptacle for 24–27 h (*n* = 3), no traces of the outer coat were visible on any sperm (Fig. 6j-l).

Light microscopy of sperm

Anterior regions of sperm taken from a male and a female appeared to be different by light microscopy; the outer coat of sperm taken from a male formed a halo along the border of the cell, whereas sperm taken from the female receptacle (later than 24 h after insemination) had no such halo and the anterior drill was clearly visible (Fig. 7). This small but nevertheless distinct, difference in bright field light microscopy images has not been previously reported.

Scanning electron microscopy of spermatozoa

By scanning electron microscopy, the anterior drill was faintly visible in sperm from males (Fig. 8a, a'), although transmission electron microscopy images had suggested that it should be completely covered by the outer coat (Fig. 5d, e). However, in sperm from females, the drill was much more obvious (Fig. 8b, b'). A clearer difference could be observed in the posterior region; the fine surface pattern of the sperm seen in females was not visible in sperm taken from males (Fig. 8c, d).

Observation of live sperm

No motility occurred in sperm taken from the seminal vesicles of males (*n* = 4). A total of 15 dissections were made to observe sperm motility in female *M. mytiloides* (Fig. 9). Sperm stored for less than 17 h in the receptacles showed no motility (*n* = 3, not included in Fig. 9). All observations of motile sperm (*n* = 11) were recorded by video. In one of the two specimens dissected at 17 h after insemination, extremely weak motility of the sperm was observed. In four dissections made between 22 and 23.5 h after insemination, sperm were initially immotile but then gradually gained motility over the observation period. The clearly motile sperm dissected from a female at 24.5 h after insemination developed much stronger motility during the observation period. In two dissected specimens, the movement of the chaotic sperm mass became homogeneous over a period of ca. 30 min by the constant rotation of the sperm. This synchronization of the sperm mass resulted in their posterior ends becoming organized in a parallel fashion (Fig. 9, Videos V1, V2). In dissections in which the female seminal receptacles were completely ruptured and the sperm lay free in the physiological saline solution, no initiation of motility was observed.

In a specimen that naturally died at 22.5 h after insemination, no motility of sperm occurred over more than 5 h of observation.

The most active movement was found in sperm taken from females 24–26 h after insemination (see Video V3). Sperm
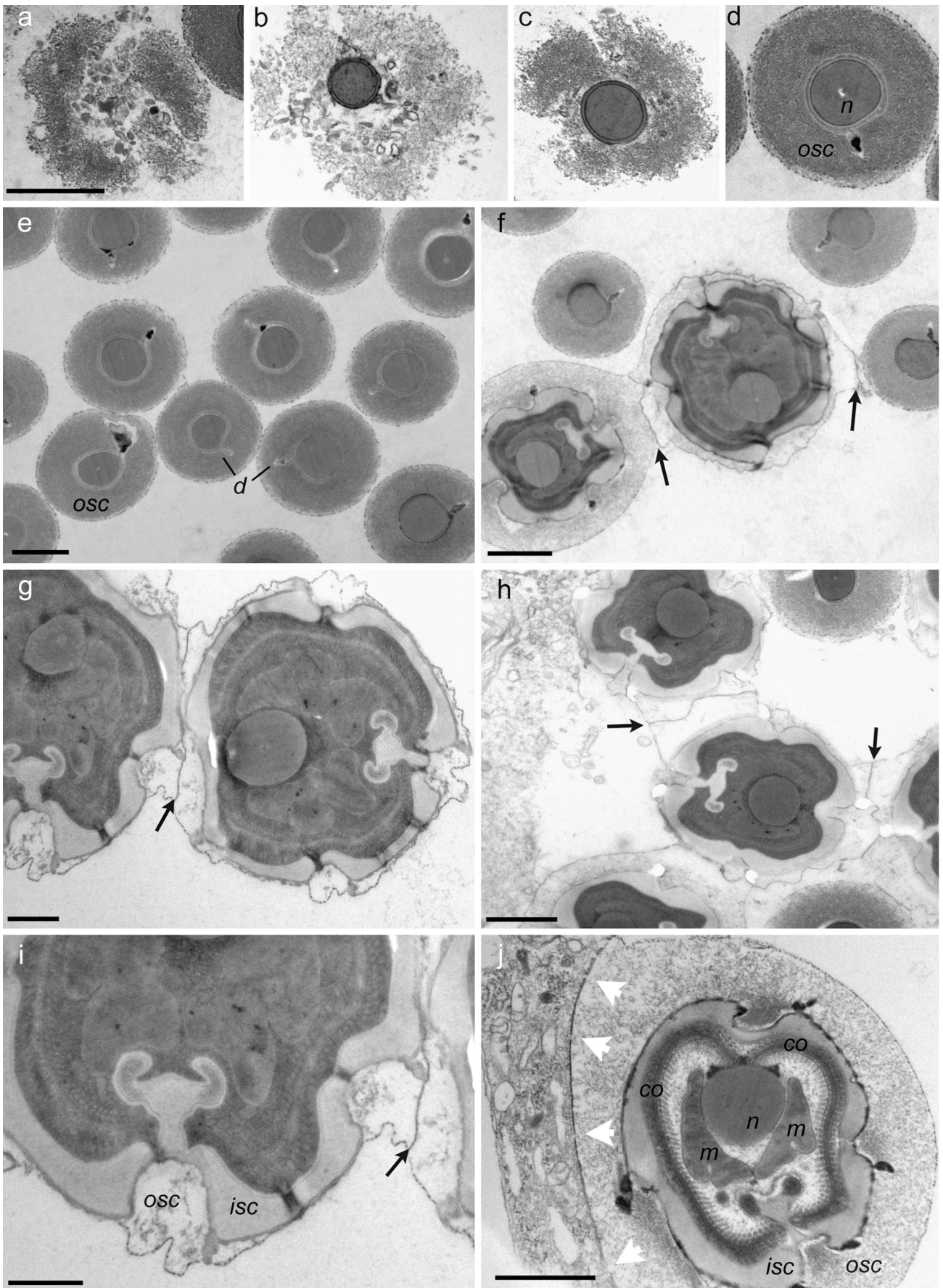
◄ Fig. 5 Transmission electron microscopy images of sperm in *M. mytiloides* males. **a–d** Sections of various points of the anterior region, from the anterior-most tip (**a**) downward (**b–d**; same magnification). Material of the outer sperm coat (*osc*) is less compact at the anterior tip of the sperm than in the rest of the sperm. **e** Section through a number of anterior regions with their inner sperm coats forming drills (*d*), which are embedded in the thick outer sperm coats. **f–i** Sections showing interconnections of sperm via their outer sperm coats (*black arrows*). Interconnections exist between sperm along their posterior regions and between the posterior and anterior regions (**f**) but not among anterior regions alone (*isc* inner sperm coat). **j** Tight connection between a cell of the sperm duct (*left*) and the outer coat of a sperm. Pores in the cell membrane (*white arrowhead*) suggest that the material forming the outer sperm coat is produced by the wall of the sperm duct (*co* contractile organelle, *m* mitochondrium, *n* nucleus). *Bars* 1 μm (**a–f**, **j**), 0.5 μm (**g**, **i**)

rotated around their long axis, frequently switching the direction of rotation. Where punctures in the receptacle allowed exits from the confined space, rotation often resulted in a supercoiling of the sperm, which, as the rotation direction reversed, uncoiled again (Video V4, first and third appearing arrow). Vigorously moving sperm quickly exited the limited space of the stretched receptacle cuticle. Once the sperm could expand horizontally under the cover slip, they showed fast and straight forward movement, obviously driven by the rotation of the cells (Video V5). Sperm moved with the thin anterior region ahead (= forward in the sense of Lowndes 1935), only occasionally changing rotation direction to move "backward", i.e., with the thick region ahead (Video V4, second appearing arrow).

Of five dissections in which sperm mobilization could be observed for more than 3 h, three showed a gradual slowing down of movements toward the end of the observation period. This loss of motility is presumably attributable to the non-physiological conditions on the dissection slide (microscope lamp heat and changes in oxygen and ion content of the saline solution), rather than to a natural process. Two of these observations were stopped after 6 h and 6.5 h, respectively and sperm were still highly motile.

Discussion

Outer (non-permanent) sperm coats

Experiments with *M. mytiloides* showed that eggs were laid after a delay of at least 22 h after insemination. If sperm undergo a morphological change to become motile and thus fertile, this change must happen before the females release the first eggs, in our model species, at 22–26 h after insemination.

Our transmission electron microscopy investigations of sperm taken from male and female *M. mytiloides* support previous studies (Tétart 1967; Gupta 1968) in that sperm taken

from males have an outer coat, whereas this coat is absent from sperm taken from females. In the present study, sperm taken from males had an outer sperm coat composed of a loosely aggregated granular material. Although the thickness of this coat is variable in the different sperm regions, the material generally fills and smooths out the surface contours formed by the underlying inner sperm coat. In the anterior-most region, the coat is cloud-like, lacking a defined outer boundary. The continuous solid extracellular inner coat most likely prevents the sperm from extruding such material around itself and ultrathin sections showing sperm in contact with the vasa deferentia (Fig. 4j) suggest that the outer sperm coat material originates from the vasa deferentia. Our results thus concur with studies of *Notodromas monacha* by Gupta (1968) and *Cypridopsis* sp. by Reger and Florendo (1969a, 1969b). The frequent adherence of sperm to other sperm by their outer coat material (Fig. 5f) suggests a role in keeping the long sperm attached to each other, at least along their posterior lengths. This bond between individual sperm may aid (passive) transport into the female, when sperm are maneuvered in small parallel batches through the Zenker organs and hemipenes. The immobile sperm in the male implies passive transport within the vasa deferentia, which agrees with observations of the Zenker organ acting as an effective sperm pump (Yamada and Matzke-Karasz 2012). We postulate that the Zenker organs pull off clumps of mature sperm from the main clump in the male seminal vesicle during insemination.

A continuous reduction in the thickness and an increasing lack of a defined outer boundary of the sperm outer coats were observed in samples taken from the female receptacles over a period of 1 to 24 h after insemination (see Results for details). These findings confirm previous hypotheses that the outer sperm coats (fibrous coat of Gupta 1968; deciduous coat of Wingstrand 1988) seen in sperm taken from males are lost in the female receptacles. In addition to previous studies, we provide evidence for a successive dissolution of this coat (no "shedding" of a solid coat) and correlate this process with the time lapse between insemination and oviposition.

Sperm motility

After at least 17 h since insemination, sperm had the potential to move actively, although in most observations, motility arose only around 23–24 h after insemination. The most vigorous movements were seen in sperm that were in female receptacles for more than 24 h, which coincided with the average delay encountered between insemination and first oviposition. We can thus assume that motility is a prerequisite for the giant sperm of ostracods to fertilize the eggs, even if details of the latter process are unknown.

The phasing or synchronization of motile sperm in the female receptacles results in all posterior ends being localized in the same area in the receptacle (Fig. 10). The bundle of sperm
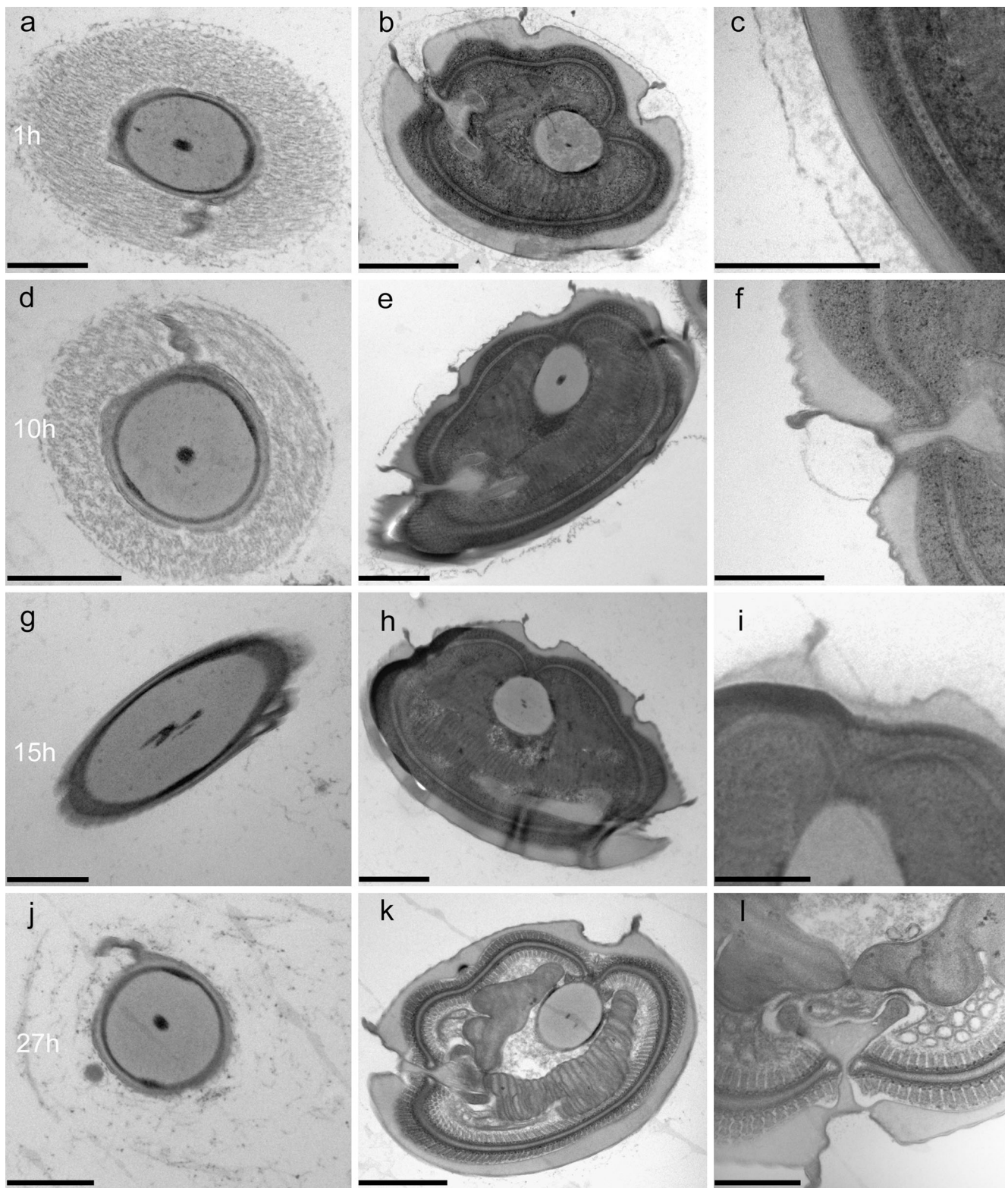
Fig. 6 Transmission electron microscopy images of sperm in *M. mytiloides* females (*left column* anterior region, *middle column* posterior region, *right column* details of sections in posterior region). **a–c** Sperm at 1 h after insemination; outer sperm coats are unmodified. **d–f** At 10 h after insemination, the outer sperm coat is reduced in both sperm regions and, in some sperm, just fills the contours in the inner sperm coat (**f**). **g–i** By 15 h after insemination, the outer sperm coat is either completely dissolved or is reduced to a granular veil (**i**). **j–l** At 27 h after insemination (and after first oviposition), no outer sperm coats can be observed. *Bars* 0.5 μm (**a, c, d, f, g, i, j**), 1 μm (**b, e, h, k**)
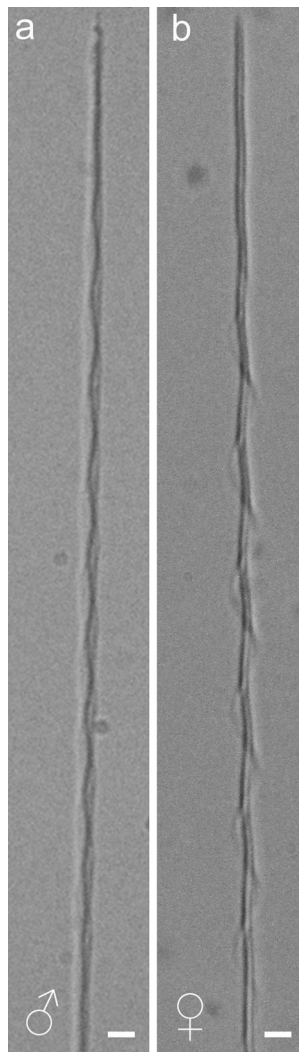
Fig. 7 Light microscopy images of sperm taken from male and female *M. mytiloides*. The drill of the anterior tip (formed by the inner sperm coat) is blurred by the outer sperm coat in a sperm from a male (**a**) and is freed in a sperm from a female (**b**). *Bars* 1 μm

is orientated down toward the spiral duct, suggesting that all anterior tips are positioned closer to the vagina, i.e., the exit of this tract and are assembled at a starting position for movement toward the egg.

Correlating these new data on (1) the progressive removal of the outer sperm coat material and (2) the increasing sperm motility in the female, we assume that the outer sperm coat suppresses motility within the male, whereas its removal in the female seminal receptacle activates motility. This phenomenon may mitigate the limitation of available energy for active sperm movement. If the sperm needs to be motile to reach the egg, the generation of enough power is a decisive factor regarding fertilization success; resources should not be "wasted" while the sperm still reside in the male, where the Zenker organ moves the sperm. After arrival in the female, the sperm must propel themselves as soon as possible to outcompete concurring sperm in the race for egg fertilization and thus

a delay in motility seems disadvantageous for the male. A clear advantage of delayed sperm motility for the female is that incoming sperm will not fertilize on a first-come-first-serve basis. The accumulation of a certain amount of sperm before they gain motility creates a competitive environment and prevents accidental fertilization. Currently, the way that the outer coat is removed remains unknown but as sperm motility only occurs within the receptacle (and not in those sperm removed from the receptacle), a substance, such as a lytic enzyme, in the female is probably responsible. In other taxa, accessory gland proteins (ACPs) and proteins produced by other tissues in the male reproductive tract, when transferred to the female via seminal fluids, can, for example, promote or reduce oviposition (Herndon and Wolfner 1995; Koene et al. 2010), change the behavior of the female (Carvalho et al. 2006), or influence sperm competition (Arienti et al. 1999; for reviews of the effects of factors in the seminal fluids, see Poiani 2006; Avila et al. 2011). Similarly, the dissolution of the outer coat of ostracod sperm could release bioactive molecules (present in the outer coat material) that could play a role in the subsequent processes of fertilization.

Inner (permanent) sperm coats and sperm senescence

Seminal receptacles from females frequently contain empty sperm coats beside functional sperm. Early authors (including Zenker 1854; Stuhlmann 1886; Müller 1889) misinterpreted these empty sperm coats as leftovers of sperm that had undergone an obligatory molt to achieve motility. This view persisted (e.g., Hartmann 1968) until early transmission electron microscopy demonstrated a two-layered sperm coat, of which the outer one is missing in sperm taken from the female receptacles (Gupta 1968; Reger 1970; Zissler 1970; Wingstrand 1988). Our study provides results that concur with the view that these empty sperm coats are not leftovers of sperm that have successfully united with an egg but on the contrary, are aged and deteriorated sperm that have left their empty (inner) coats behind.

Older females contain a high proportion of empty sperm coats in their receptacle (e.g., Zenker 1854; own observations), suggesting that they accumulate over their lifetime and do not interfere with incoming fresh sperm. Possibly, they are digested at a slower rate than non-chitinous material and will disappear only with a certain delay. An active ejection of such empty sperm coats from the receptacles has not been observed; however, such an ejection might have occurred and been overlooked, e.g., if the female immediately consumed the sperm material. These inner sperm coats are exceptionally resistant and are probably responsible for the presence of (dead) sperm in strongly decayed ostracod bodies (own observation) and for the records of mummified (Matzke-Karasz et al. 2001; Iepure et al. 2012) and petrified (Matzke-Karasz et al. 2014) ostracod sperm. Their chemical nature is

Fig. 8 Scanning electron microscopy images of sperm from *M. mytiloides* males and females. The surfaces differ because of the presence and absence of the outer sperm coat. **a, a'** Anterior region of a sperm taken from a male. The drill along the sperm is covered by the outer sperm coat and is only weakly visible. **b, b'** The same region in a sperm taken from a female. The outer sperm coat is gone and the drill is clearly seen. **c, d** Posterior regions of sperm from male (**c**) with and from female (**d**) without outer sperm coat material. *Bars* 2 μm
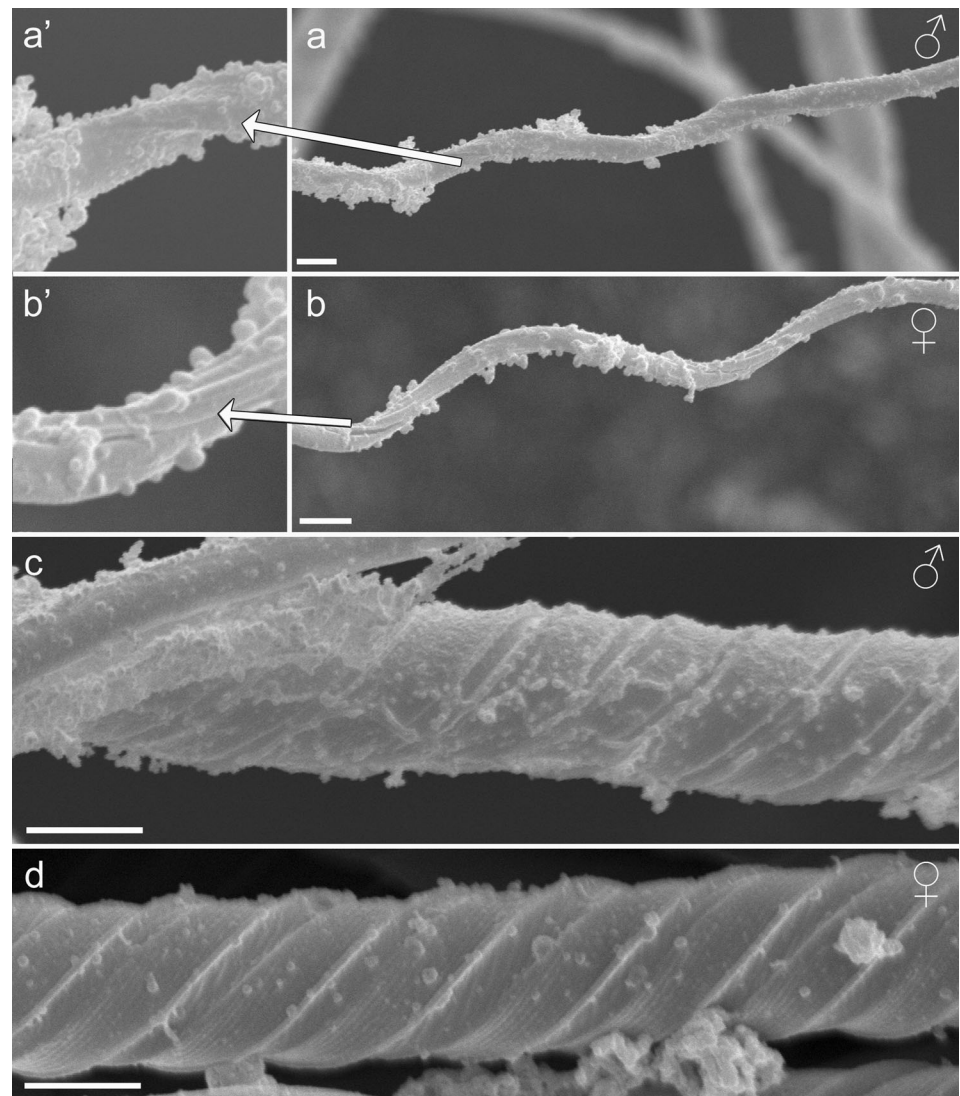
presumed to be chitinous (e.g., Gupta 1968) but no evidence has so far been provided.

In one female specimen, empty inner sperm coats were encountered only 72 h after insemination, suggesting that senescence is unlikely to be the reason for decay in this case.

Such early degeneration can perhaps be attributed to an abnormality, malformation, or the process of activation being unsuccessful. Empty coats appearing later in the reproductive phase of the female might be a result of exceeding the maximum natural lifespan of the sperm. In a second female

Fig. 9 Graph representing experiments carried out to observe live sperm. Each *horizontal bar* represents one dissected female specimen. Sperm motility (over time of observation) is expressed as a *gray scale* as indicated in the *uppermost bar*
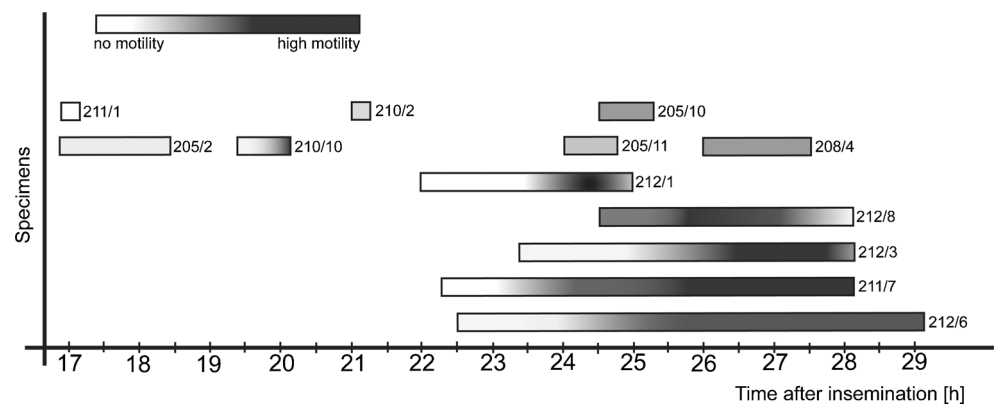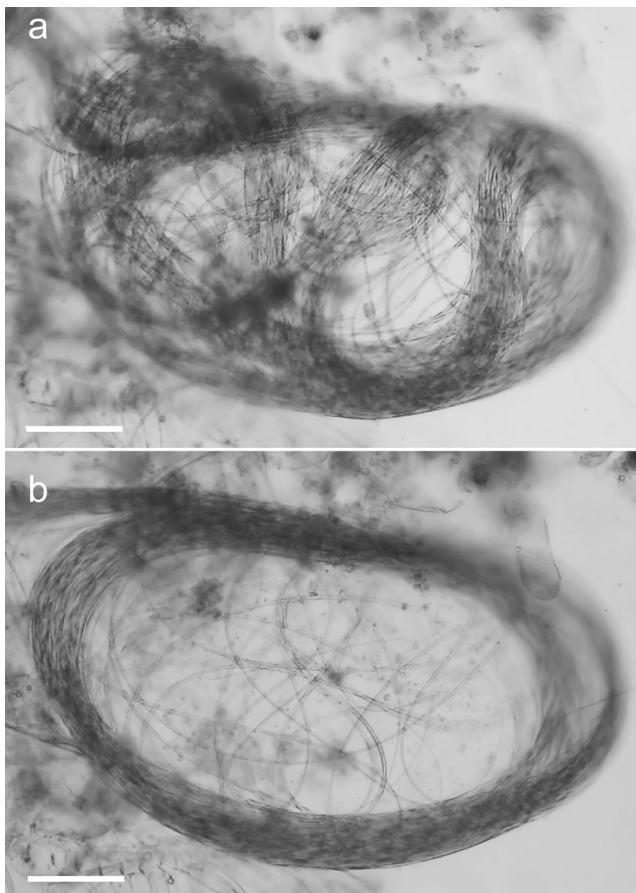
Fig. 10 Still images of Video V3. **a** Motile sperm in the receptacle of a female *M. mytiloides* at 19:15 h after insemination. **b** Same dissection but 40 min later. Movement and rotation of single sperm results in a synchronization of the sperm mass. Previously chaotically stored sperm thus later form a neat bundle of parallel sperm. *Bars* 100 μm

specimen, which had been inseminated only once and had subsequently laid 149 eggs over 42 days, dissection at 15 days after oviposition had ceased revealed large number of sperm in the receptacles. No sperm were motile and ca. 50 % of them had decayed into empty sperm coats. However, the oviducts of this female contained more than 15 eggs close to maturity, showing that egg depletion could not have been the reason for the ceasing of egg deposition. A much more likely reason for the state of this female specimen seems to be the lack of viable and motile sperm.

At present, we cannot exclude that females actively induce sperm degradation, allowing them to control sperm competition inside the receptacles. This would require the release of, for example, lytic enzymes to destroy sperm from the previous mating partner but since this lysis would most probably also affect sperm transferred by the subsequent mating partner, this scenario seems unlikely. Moreover, the simultaneous presence of motile and partly degraded sperm in the female receptacles (e.g., Müller 1889; Gupta 1968; own observations) strongly suggests that sperm decay is part of the sperm's own

physiology, rather than being induced by external factors. Finally, we cannot rule out that ejaculates of subsequent mating partners directly influence the viability of the sperm of previous partners.

Qualitative data collected on the fate of sperm in female *M. mytiloides* after a single insemination suggest the following scenario; after becoming motile in the female receptacle, the sperm are used to fertilize the eggs and the eggs are laid in small batches over a long period. Whereas some sperm do not achieve motility at all because of malformation and will decay after a few days, fully viable sperm will stay motile and be usable for egg fertilization for several weeks. Eventually, their vital functions can no longer be maintained in the receptacle and their contents decay, leaving empty inner coats behind. The female, however, requires a new insemination to fertilize maturing eggs and empty sperm coats accumulate in the receptacles until the animal's death.

In this scenario, the female may control sperm usage by controlling egg release; the longer oviposition is protracted, the weaker stored sperm become in competing with sperm of subsequent mating partners of the female. Thus, regardless of whether sperm decay is a result of natural ageing or is female-induced, both processes can theoretically help the female control sperm usage, representing (at least an indirect) cryptic female choice (Eberhard 1996) in an environment of sperm abundance.

Sperm gigantism, sperm numbers and sperm depletion

In syngamic organisms with tiny sperm, the excess production of sperm is a common strategy to overcome sperm loss related to the small sperm size. Such sperm loss is induced by the female to minimize the risk of polyspermy; mechanisms include, for example, getting lost, becoming immotile and being phagocytosed in the females storage system (First et al. 1968 and references therein) or suffering from abortive interactions with the wall of the female tract (overview in Pitnick et al. 2009). We might therefore expect that, in organisms that have developed sperm gigantism, where the risk of sperm loss is considerably reduced, the transfer of surplus sperm (i.e., a higher number of sperm than can, or will, be used by the female for egg fertilization) is less likely, unless this costly surplus brings other advantages for reproductive success. Additionally, by trading sperm size against sperm numbers, organisms with giant sperm are likely to produce much fewer sperm. Thus, in *Drosophila pachea*, sperm gigantism and the related reduction of sperm numbers and the males partitioning their sperm among mating partners have been found to create potential sperm depletion. Female *D. pachea* therefore show a high remating rate, which in turn leads to an increase of sperm competition (Pitnick 1993). In this light, we were surprised to encounter decayed sperm so frequently in the receptacles of freshwater ostracods, indicating that a certain amount of

sperm material is not being used for egg fertilization. Our first qualitative experiments showed that inseminated females carrying plenty of mature eggs stopped oviposition after 5–7 weeks, most likely because stored sperm had become too old and unusable. We posit here that, in contrast to *D. pachea*, it is not a reduced sperm number (induced by sperm gigantism) but the limited sperm viability in the female that is the dominant factor leading to the female's need for remating and thus to an environment favoring sperm competition. *M. mytiloides* lays eggs in small batches over weeks, so multiple inseminations each with a few sperm would be more favorable than a single insemination with a high number of sperm, since the latter may result in a larger number of decayed sperm in the receptacles.

Removal of sperm coats in other organisms

The here-described removal of a secretion around quiescent sperm in the female, resulting in the mobilization of sperm, is superficially similar to processes described for a variety of other taxa, although these reports most often refer to secretions around more than a single sperm, typically forming a spermatophore. For example, the sperm of the squid *Loligo pealii* are packed into a spermatophore and only gain full motility after its opening (Austin et al. 1964) and in many spiders, several spermatozoa are transferred encapsulated in a sheath (coenospermia) with the sperm being activated after release from the sheath (e.g., Brown 1985; Lipke et al. 2014). Additionally, sperm capacitation (including the acrosome reaction) resulting in a substantial improvement of swimming ability ("hyperactivation"), has been reported in a variety of mammals (Pitnick et al. 2009; Suarez 2016).

Digestion of excess foreign (and own) sperm is known from the stylommatophoran snail *Helix pomatia*. Sperm freed from the spermatophores in the hermaphrodite duct move toward the seminal receptacle (spermatheca) for storage, whereas those remaining in the spermatophore and the spermatophore itself are broken down and digested in the bursa (Lind 1973).

Examples of single sperm being encased prior to transfer to the female are less numerous. One is found in spiders, i.e., in Araneomorphae, where so-called cleistospermia represents single sperm that is encapsulated by a secreted sheath (Michalik and Lipke 2013). In the genital tract of the female in, for example, the spider *Argiope bruennichi*, the extracellular secretion is removed before the sperm becomes activated for egg fertilization (Vöcking et al. 2013). Unlike in ostracods, insemination in spiders is indirect; sperm material is ejaculated and then ingested into the bulbs of transformed pedipalps by the male. During mating, these bulbs empty the sperm into the female. This additional storage step might require a distinct protection of sperm (e.g., against oxidative damage and other putative impacts as discussed in Michalik and Ramirez

2014), which is not the case in the direct internal insemination of ostracods.

A second example is the silk moth *Bombyx mori*, where typical (eupyrene) and atypical (apyrene) sperm are packed into extracellular electron-dense material (typical spermatozoa only) and enclosed in a sleeve when passing through the male's ejaculatory duct. Whereas eupyrene spermatozoa later hatch in the female seminal receptacle, leaving both extracellular coatings behind, the apyrene spermatozoa do not hatch. Stripped naked, only the eupyrene spermatozoa are able to travel further in the genital duct toward the region of egg fertilization (Friedländer and Gitay 1972). However, this system only superficially resembles the here-described mechanisms in *M. mytiloides* sperm. First, the process of "hatching" from their sleeves in *B. mori* eupyrene sperm does not seem to induce motility (instead, an endopeptidase has been shown to activate both kinds of sperm; Nagaoka et al. 2012). Second, *B. mori* apyrene sperm keep their sleeves but are nevertheless described as moving especially energetically (Iriki 1941). Third, *B. mori* eupyrene sperm strip off both the external sleeve and the internal electron-dense material, whereas *M. mytiloides* sperm (just like all other described Cypridoidean sperm) keep the inner of the two extracellular coats until entry into the egg (Matzke-Karasz 2005).

Another example of sperm being gradually stripped of their coats has been reported for the grasshopper *Eyprepocnemis plorans*, where the glycocalix covering the sperm from late spermiogenesis is removed in some of the sperm stored in the female. In this system, the coat removal seems to be related to an inactivation process of surplus sperm (Longo et al. 1993). Single eupyrene sperm of the moth *Ephestia (Anagasta) kuehniella* have been shown to be packed into a compact extracellular sheath prior to insemination. Here, a function related to emerging sperm motility has been tentatively suggested but empty sheaths could not unambiguously be attributed to either programmed sperm molting or to sperm decay (Riemann and Thorson 1971).

Concluding remarks

The alterations occurring to the outer and inner *M. mytiloides* sperm coats, their role in the fate of the sperm and their effects on sperm competition and female cryptic choice are not similar to processes reported in other organisms. The morphology and activation of non-marine ostracod giant sperm are aspects of a unique reproduction mode that still presents numerous enigmas (see also Smith et al. 2016b). Hypotheses explaining the evolution of reproduction with giant sperm in other systems (see references in Pizzari and Parker 2009) cannot be grafted onto what we know so far about ostracods and the more we learn about their reproductive strategies, the more differences appear compared with other systems. Since giant sperm have been used for reproduction in freshwater ostracods

for more than 100 million years (Matzke-Karasz et al. 2009), this successful system undoubtedly deserves and requires, further investigation.

Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

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