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Sperm structure of the diving beetle *Deronectes moestus incospectus* (Leprieur, 1876) (Hydroporinae, Dytiscidae) and considerations on extracellular material surrounding sperm bundles

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ABSTRACT

The sperm cells of the diving beetle *Deronectes moestus incospectus* are characterized by sperm conjugation leading to the formation of sperm bundles of 64 units each. These bundles are formed at the end of spermatocyte cell divisions occurring in the testes and can be detected in the anterior region of the deferent ducts (first type of sperm conjugation). Fusions of some sperm bundles can occur at the end of the deferent ducts. The sperm bundles show sperm-head stacks (sperm *rouleaux*) and are surrounded by a cup of extracellular material secreted by the epithelial cells of the deferent ducts. This material extends posteriorly around the sperm bundle to cover the nuclei and the initial region of the sperm flagella. The cup extracellular material consists of fine tubules, and is no longer visible in sperm bundles at the posterior end of the deferent ducts. The sperm cells of *D. moestus incospectus* have an axoneme with a 9 + 9 + 2 pattern and unusual mitochondrial derivatives having a matrix showing dense dots and a small crystallized domain. Two thin elongated accessory bodies are located between the mitochondrial derivatives and the axoneme. The extracellular material can have different morphologies in the various families of Adephaga, but all are produced by the epithelium of the deferent ducts. Thus it is reasonable to assume that it has the same function in the different groups.

1. Introduction

Sperm play a central role in sexual reproduction and their morphology may often change in response to pressure, such as sperm competition. An unusual variant of sperm appearance is aggregation (or conjugation), where two or more sperm join to form a sperm bundle. Sperm conjugation is widespread among Invertebrate and in some insect orders (Higginson and Pitnick, 2011) and also occurs in a few mammals. It is considered an efficient male reproductive strategy since transfer of a mass of sperm to the female seminal receptacle during mating can prevent females from remating with a second male. Fertilization success can also be achieved by other strategies, such as male guarding and/or production of a mating plug that obstructs the female genital opening (Immler, 2008; Higginson et al., 2012; Aiken, 1992; Simmons, 2001; Pitnick et al., 2009). Sperm conjugation can occur in different ways and may lead to bundles grouping different numbers of sperm cells, depending on how many sperm cysts are involved.

Higginson and Pitnick (2011) described how conjugation develops and distinguished two types of conjugation, depending whether the sperm of the bundle came from a single sperm cyst, and the sperm components were therefore sister cells (first type of conjugation), or whether the sperm of the bundle came from fusion of multiple sperm cysts (second type of conjugation), as often happens after sperm reach the deferent ducts or even the seminal vesicle.

Sperm ultrastructure and the eventual presence of sperm bundles have only been reported in a few species of the monophyletic and diversified group of diving beetles Dytiscidae (Mackie and Walker, 1974; Werner, 1976; Dallai and Afzelius, 1985, 1987; Higginson and Pitnick, 2011; Higginson et al., 2012). With more than 4300 species, Hydroporinae is the most specious subfamily of Dytiscidae: they have reduced body size and modifications of mandibular morphology and movement (Beutel et al., 2020). The group also shows great variation in the organization of the male and female genital systems and particularly spermathecal ducts length and the size and structure of the spermatheca and the spermathecal gland (Miller, 2001; Miller and Bergsten, 2014; Dallai et al., 2023a, 2023b).

A recent study of sperm structure in two members of the group indicated new structural differences in the large family of Dytiscidae

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(Mercati et al., 2023). The two species of Hydroporinae, *Stictonectes optatus* and *Scarodytes halensis,* both showed long sperm bundles with nuclei arranged in orderly sperm-head stacks with free flagella, as in the particular sperm assembly called sperm *rouleaux* by Higginson and Pitnick (2011). According to these authors, sperm conjugation in Dytiscidae can be classified as: 1) sperm pairing, when two sperm are assembled in antiparallel via the ventral sides of heads with scanty material between them (Mackie and Walker, 1974; Werner, 1976; Dallai and Afzelius, 1985, 1987); 2) sperm *rouleaux*, when the cone-shaped side of one sperm head adapts to the concave surface of the neighboring sperm-head to form a long stack (Higginson and Pitnick, 2011).

We detected a third type of sperm bundle in *S. optatus:* it shows a thick layer of extracellular material around the sperm-head stacks; the layer is secreted by the epithelium of the apical deferent ducts region (Mercati et al., 2023). In this study we observe sperm bundles in the species *Deronectes moestus,* in which the sperm-head stacks and the anterior region of the flagella are surrounded by extracellular secretory material. Here we describe this new finding, with new and additional considerations on the comparative analysis of the extracellular material surrounding the sperm bundles of Adephaga.

2. Materials and methods

Males and females of *Deronectes moestus incospectus*, captured in a stream near Grosseto (Italy), were studied. The specimens, kept in a container of pond water, were identified by Dr. Saverio Rocchi of the Museum "La Specola" in Florence (Italy).

After dissection under a light microscope in 0.1 M phosphate buffer with 3 % sucrose (PB), the unifollicular testes, deferent ducts, seminal vesicles and female spermatheca were isolated. From a small tract of the anterior region of deferent ducts, sperm bundles were isolated and spread over histological slides and photographed with a Leica DMRB light phase-contrast microscope.

The material fixed overnight at 4 °C in 2.5 % glutaraldehyde in PB, after careful rinsing in PB, was post-fixed in 1 % osmium tetroxide for 2 h, then rinsed again and dehydrated in an ethanol series (50–100 %). The material was embedded in Epon-Araldite resin. Ultrathin sections of the male genital tracts and of spermatheca, stained with uranyl acetate and lead citrate, were inspected and photographed with a Philips CM10 transmission electron-microscope operating at an electron accelerating voltage of 80 kV.

3. Results

3.1. The sperm bundle

The sperm are formed at the apical region of the unifollicular testes from sperm cysts each consisting of 64 sperm cell as consequence of 2^6 germ cell divisions (Fig. 1A, B). The sperm bundles are regularly aligned along the anterior region of the deferent ducts and proceed towards the end of these ducts.

A cross section through the apical region of the deferent ducts shows numerous sperm bundles sectioned at different levels and orderly arranged into a hexagonal array (Fig. 1B, C). Each of the observed sperm bundles consisted of 64 units and no fusion events among the neighboring bundles was detected. In the anterior region of the deferent ducts the apical region of each sperm bundle is embedded in a moderately electron-dense extracellular material (Fig. 2A–C). A cross section

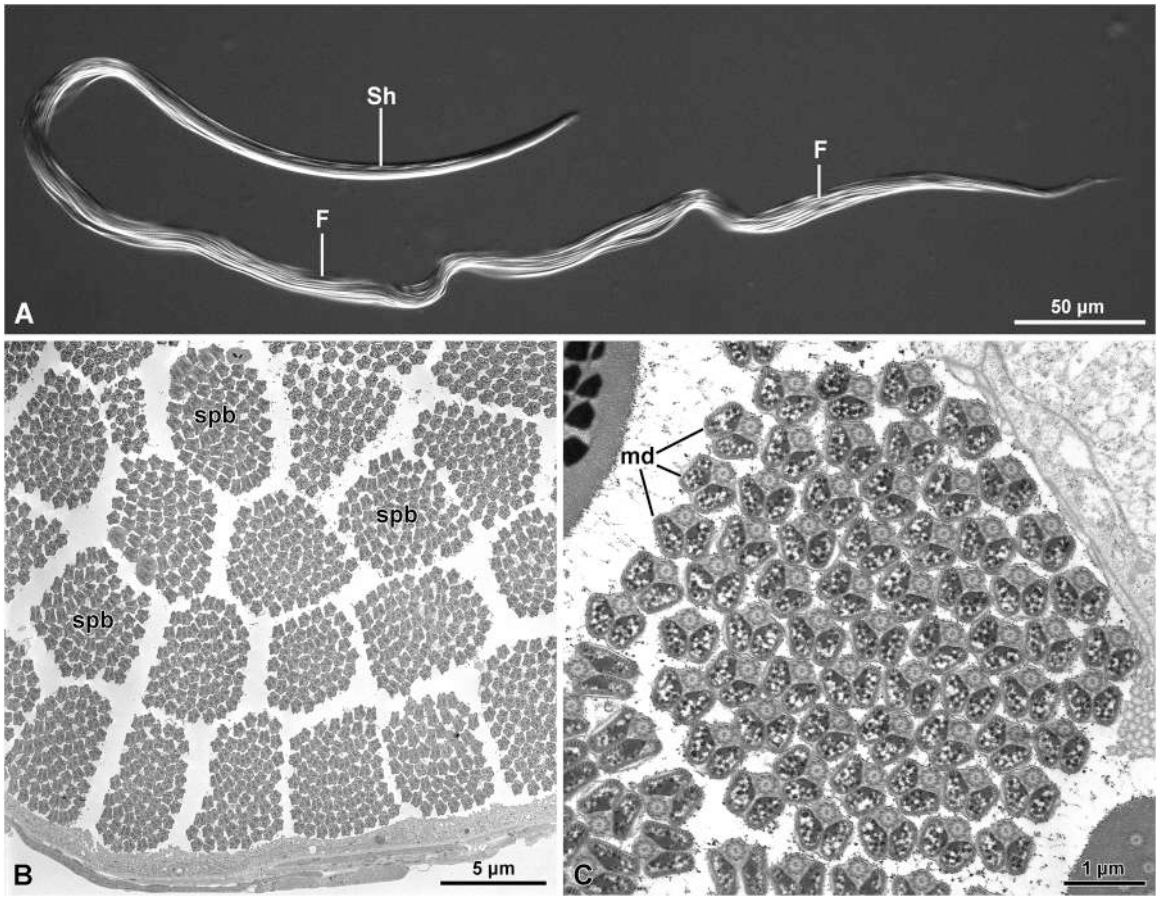

Fig. 1. A. A sperm bundle taken from the deferent ducts. Sh, sperm heads; F, Flagella. **B.** Cross section through the deferent ducts showing many sperm bundles (spb), crossing at the flagella level, each consisting of 64 units. C. Detail of a sperm bundle with 64 sperm cells. Note the peculiar appearance of the matrix of the mitochondrial derivatives (md).

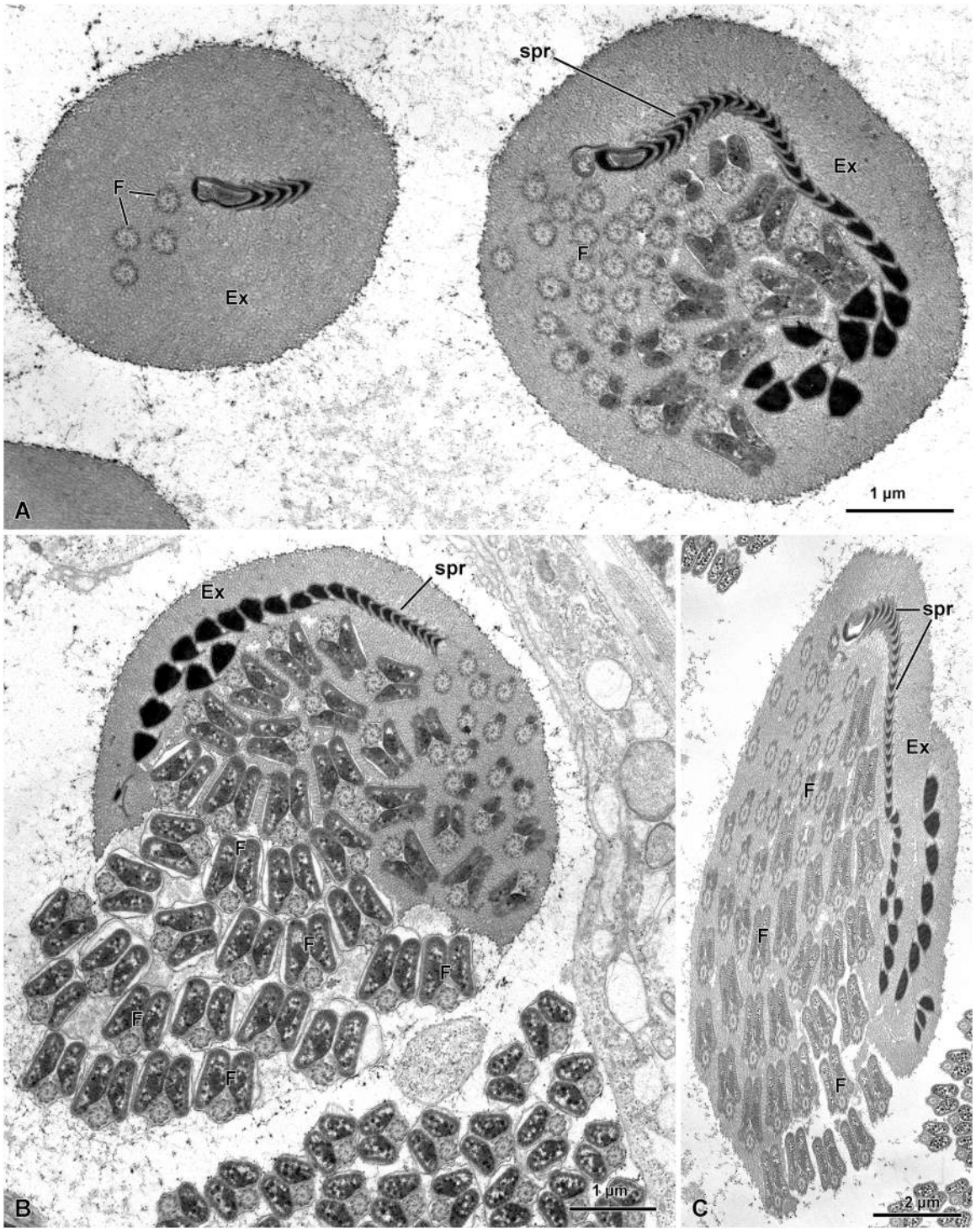

Fig. 2. A. Cross sections through two levels of the anterior region of the sperm bundles. It is a cup of electron-dense extracellular material (Ex) embedding the sperm bundles. In the sperm bundle on the left side the cross section was conducted through the most anterior region and a few initial flagella axonemes were visible. In the sperm bundle on the right side, 64 sperm sectioned at different levels were visible. Note the sperm-head rouleaux (spr). F, flagella. B. Cross section of a sperm bundle similar to the previous one but sectioned slightly posteriorly where the extracellular material is reduced (Ex). spr, sperm-head rouleaux; F, flagella. C. Longitudinal section through a sperm bundle to show the extension of the extracellular material (Ex) forming the cup embedding the sperm-head rouleaux (spr) and the flagella (F).

through this region of the sperm bundle is circular or slightly elliptical in shape and contains the sperm-nuclear stack and the initial flagellar region (Fig. 2A–C); the extracellular material extends further along one side of the bundle accompanying a tract of the sperm flagella (Fig. 2C). At its most anterior region the structure is only 2.2 µm wide and it shows only a short nuclear chain with one or two flagellar sections (Fig. 2A). Further posteriorly, the cross section through the sperm bundle becomes wider, measuring 4.4 µm, and it contains the whole series of 64 cross sectioned sperm. The complex has a bell-like configuration. The extracellular material has an unusual appearance; in cross section it consists of a uniform array of numerous thin tubules with a diameter of about 16–17 nm, spaced about 53 nm from each other, embedded in an

amorphous material (Fig. 3A, B). In the posterior region of the sperm bundle, the embedding material is no longer present and only free flagella are visible (Fig. 1B, C). The sperm grouping in bundles of 64 sperm each, is maintained for most of the length of the deferent ducts and only in the posterior end of these ducts the sperm-nuclear stacks can lose their orderly array with some sperm nuclei detaching from the stacks (Figs. 4–5, 6A, B).

3.2. The spermatozoon

The sperm of *D. moestus* are commonly found in the deferent ducts in bundles with 64 units and about 650 μm long (Fig. 1A). The sperm

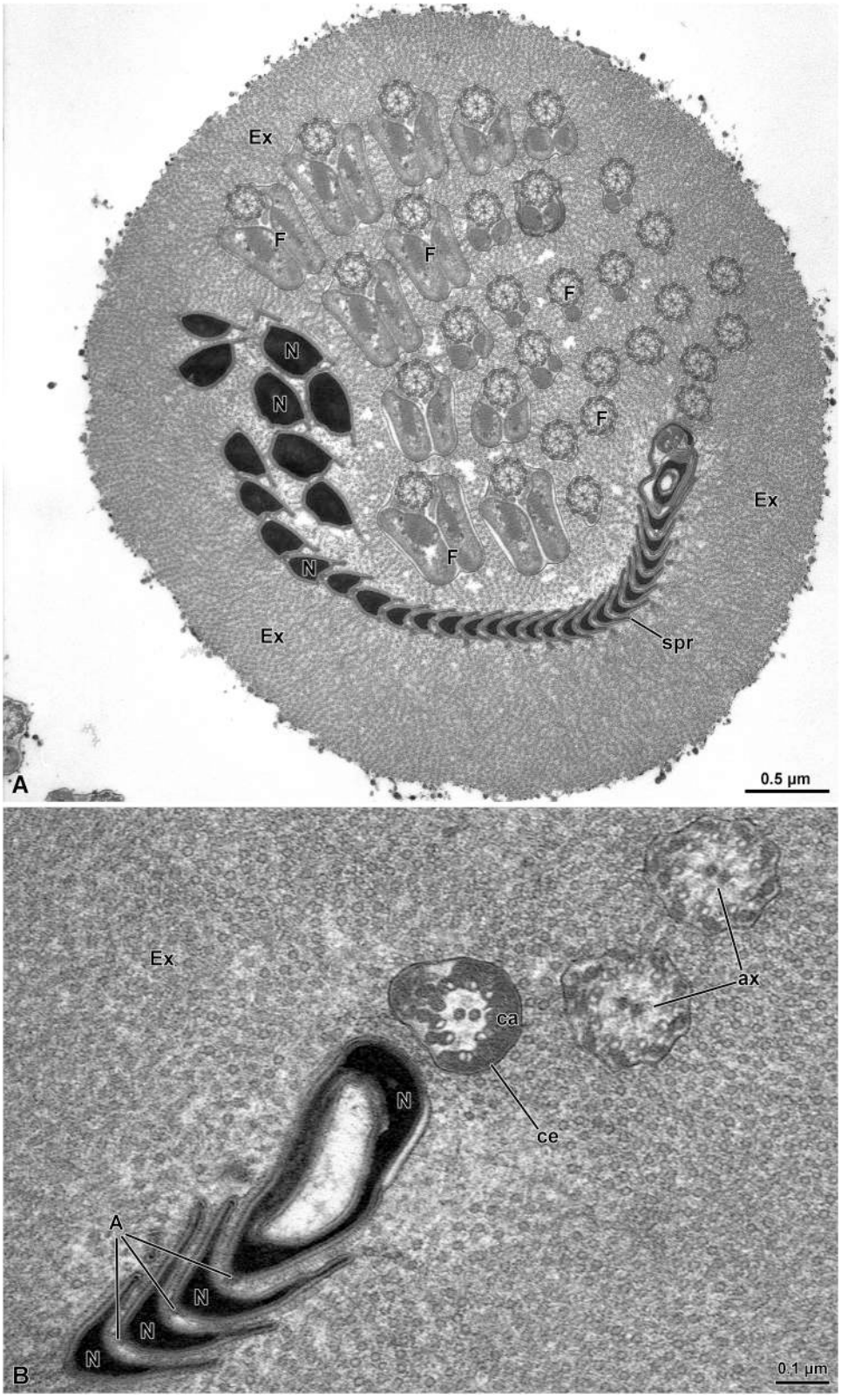

Fig. 3. A. Cross section of a sperm bundle with the sperm-head rouleaux (spr) and the flagella (F). Note the peculiar extracellular material (Ex) surrounding the sperm bundle. B. High magnification of a sperm bundle to show the details of the extracellular material (Ex) consisting of numerous fine tubules in an amorphous material. Note the centriolar region (ce) with the doublet microtubules embedded in the electron-dense material of the centriole adjunct (ca). A, acrosome; ax, flagella axoneme; N, nuclei.

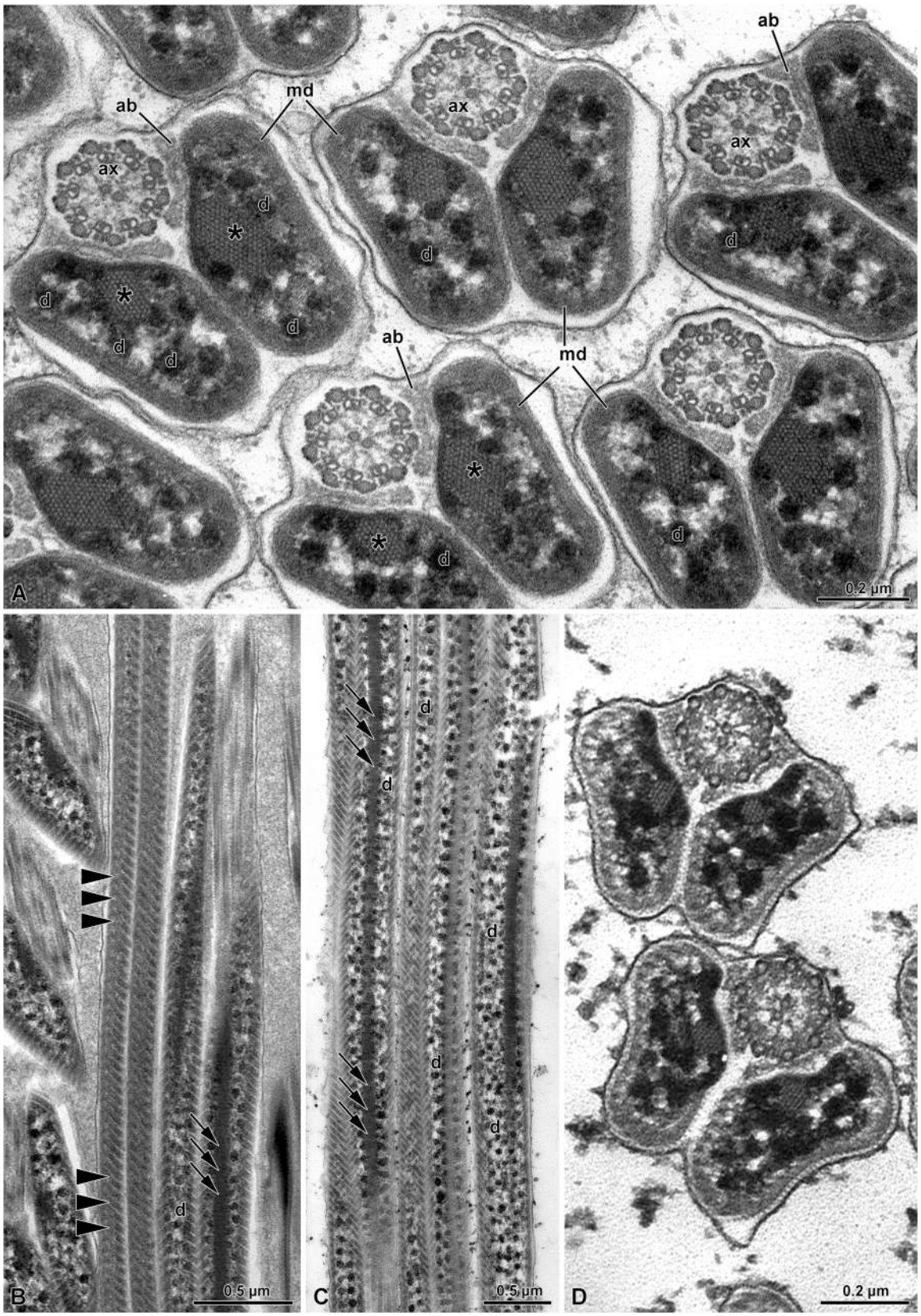

Fig. 4. A. Cross section through the sperm flagella with the 9 + 9 + 2 axoneme (ax), the peculiar mitochondrial derivatives (md) provided with a matrix filled with electron-dense dots (d) and the small crystallized area (asterisk). Note the thin accessory bodies (ab). B–C. Longitudinal sections of the mitochondrial derivatives to show the mitochondrial cristae (arrowheads), the crystallized area (arrows) and many electron-dense dots (d) in the matrix. D. Cross sections of two sperm flagella taken from the female spermatheca. They show an identical structure to that observed in the deferent ducts.

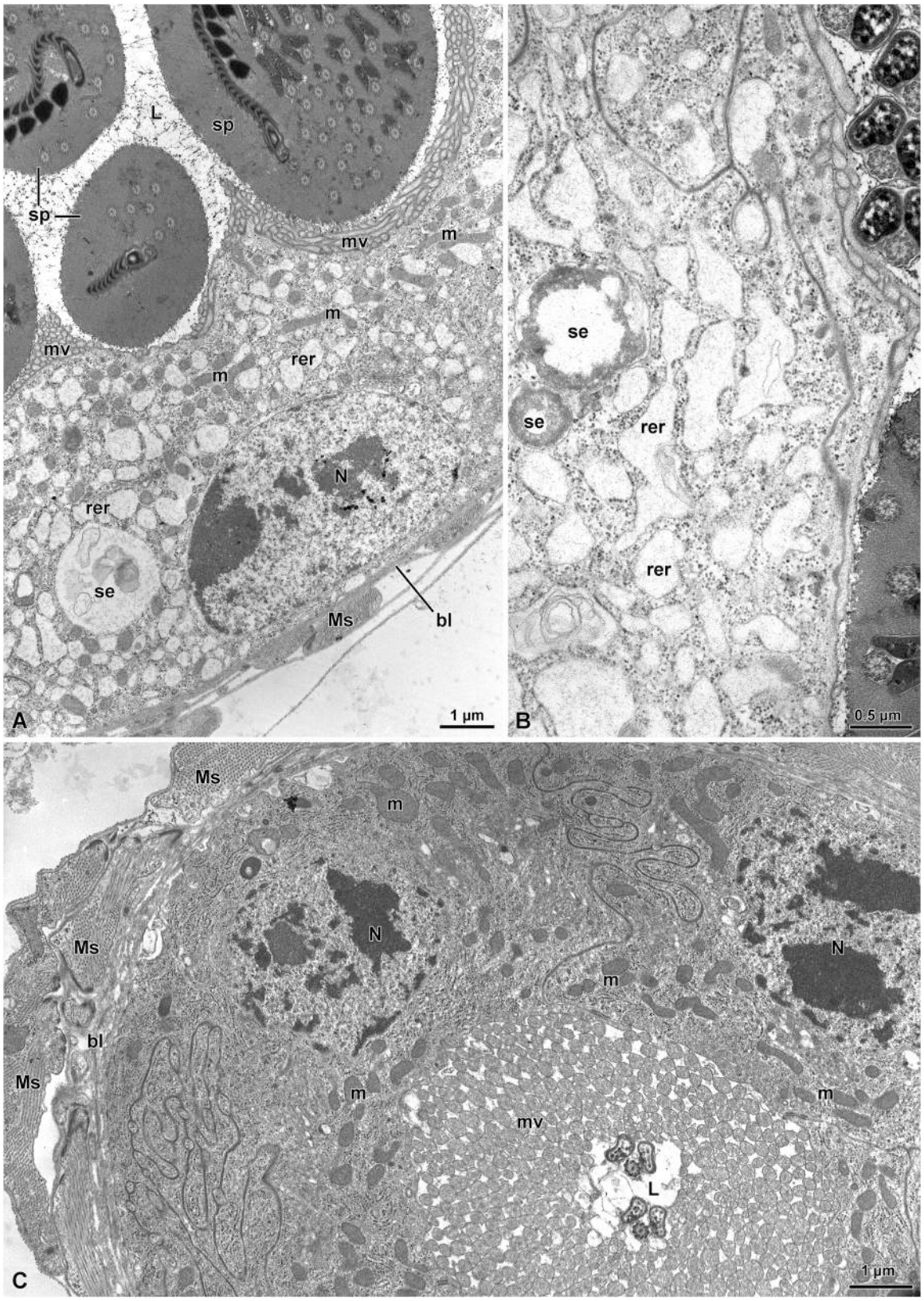

Fig. 5. A. Cross section through the anterior region of the deferent ducts to show the ultrastructure of the epithelial cells. Note the rich system of the rough endoplasmic reticulum (rer) and the few mitochondria (m). Note the short apical microvilli (mv), the nucleus (N), the muscle fibers (Ms) and the thin basal lamina (bl). Sperm bundles (sb) are present in the lumen (L). se, secretion body. B. Detail of the previous micrograph to show the rough endoplasmic reticulum (rer) with the large cisterns filled with electron-transparent content. Note the secretory bodies (se). C. Cross section of the posterior region of the deferent ducts epithelium. The endoplasmic reticulum is very reduced while mitochondria (m) are numerous and mainly located in the apical region of the epithelial cells, beneath the long microvilli (mv). N, nuclei; Ms, muscle fibers; bl, basal lamina.

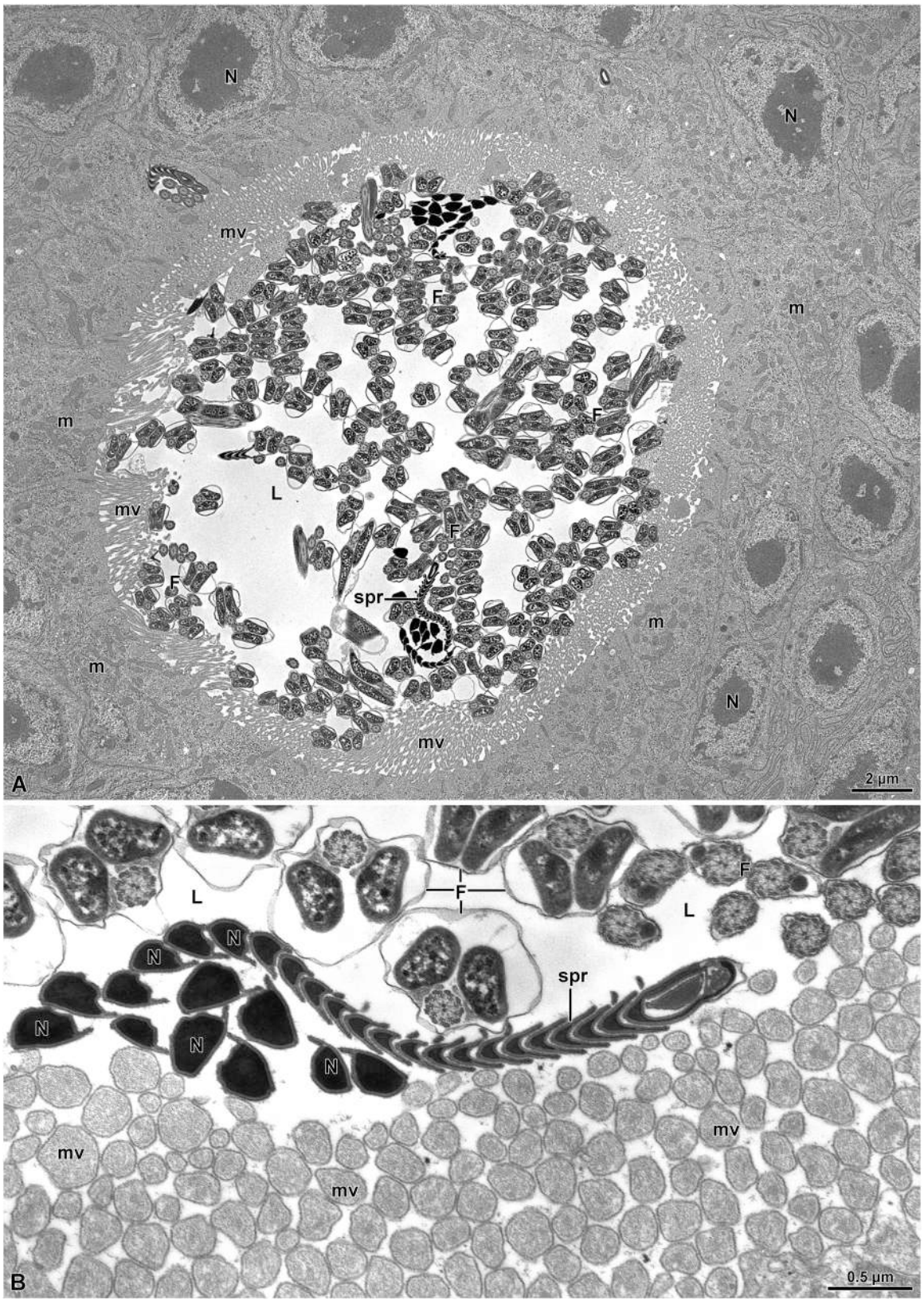

Fig. 6. A. Cross section through the epithelium towards the posterior region of the deferent ducts. Note that the cells are rich of mitochondria (m). In the lumen (L), many cross sectioned sperm flagella (F) and a sperm rouleaux (spr) are visible. N, nuclei; mv, microvilli. B. Detail of the sperm-head rouleaux (spr). mv, microvilli; L, lumen with some cross sectioned sperm flagella (F); N, nuclei.

acrosome is a short-flattened electron-dense structure present on the apical region of the nucleus (Fig. 3B) The nucleus is a long electron-dense structure that in cross section looks like a bell or a cone-shaped structure with lateral extensions (Fig. 3A). This shape allows the interaction with the next sperm to give origin to a long sperm chain (the sperm rouleaux by Higginson et al., 2012). The posterior end of the nucleus shows a thin prolongment where the centriole is hosted (Fig. 3A, B). This latter consists of a complex of peripheral doublet microtubules embedded in the electron-dense material of the centriole adjunct (Fig. 3B).

On a side of the initial region of the axoneme a small dense mitochondrial derivative is visible. Further, this single mitochondrial derivative increases its dimension and is accompanied by a second mitochondrial derivative. Further, these two flagellar components increase in volume, reaching the same diameter and an oval shape (0.6 × 0.26 μm) (Fig. 4A). The most remarkable feature of their inner structure is the partial crystallization of their matrix that show an axis of 0.13 μm in the left mitochondrial derivative and of 0.1 μm in the right one (Fig. 4A). These crystallized regions are positioned near to the mitochondrial membrane facing the axoneme. The remaining of the mitochondrial derivatives matrix is filled with electron dense dots alternated with homogenously dense material that also extends beneath the mitochondrial membrane (Fig. 4A). In longitudinal sections the two mitochondrial derivatives show the crystallized areas as a thick line crossing the dense spots areas filling the matrix (Fig. 4B, C). Close to the mitochondrial membrane an orderly sequence of oblique parallel lines 0,28 μm thick and 0.10 μm distant from one another, correspond to the mitochondrial cristae (Fig. 4B, C). The mitochondrial derivatives reduce their size towards the flagellar end. They maintain their peculiar inner structure even after mating, when sperm reached the lumen of the spermatheca (Fig. 4D). At the tail end, the axoneme loses its orderly array. In a cross section, between the apical region of the mitochondrial derivatives and the axoneme, two peculiar accessory bodies, about 0.21 μm long and 23 nm thick are present, which flank the mitochondrial derivatives up to the tail end (Fig. 4A).

3.3. The deferent ducts

The anterior region of the deferent ducts epithelium has a variable height (from 3.5 to 9.0 μm) with cells provided with short microvilli and a thin basal lamina (Fig. 5A, C). Surrounding the epithelium, a thin layer of muscle fibers is present (Fig. 5A, C). The cytoplasm of epithelial cells contains some elongated mitochondria and is rich of connected cisterns with electron-transparent content (Fig. 5A–C). The presence of ribosomes adherent to the cistern membrane might indicate that each cistern is part of rough endoplasmic reticulum (Fig. 5A, B); in addition to these cell components, dense bodies of variable size are sometimes visible (Fig. 5A, B). Flattened nuclei are scattered along the epithelium (Fig. 5A). Proceeding towards the end of the deferent ducts, the epithelium becomes uniform, about 18.6 μm high, with a uniform apical layer of long microvilli (2.2 μm high) and slightly elliptic muscles (6.7 × 3.0 μm) regularly arranged along the epithelium (Fig. 5C; 6A).

Lateral membranes of the epithelial cells show greatly interrelated infoldings with the cytoplasm rich of mitochondria, mainly located in the apical cell region (Fig. 5C; 6A). The lumen of the ducts is reduced to a diameter of 10 μm, and in cross section it shows both free sperm and nuclear chains (Fig. 6A, B). The orderly array of sperm bundles evidenced in the anterior region of the deferent ducts is no longer visible, even though we cannot exclude that some residues of extracellular material could be still present at the end of deferent ducts.

4. Discussion

With reference to the two types of sperm conjugation defined by Higginson and Pitnick (2011) and Higginson et al. (2012), we can say that unlike *S. optatus* and *Sc. halensis* (Mercati et al., 2023), *Deronectes moestus* shows the first type because the 64 sperm of each bundle are the result of divisions of a single spermatocyte cyst, making the sperm sister cells. The sperm bundles can fuse towards the posterior end of the deferent ducts. In *S. optatus* and *Sc. halensis*, the two other species of Hydroporinae previously studied, fusion of the sperm bundle is already evident in the testes and during sperm transit in the deferent ducts, indicating the second type of sperm conjugation.

Like those of *S. optatus* and *Sc. halensis*, the sperm bundles of *D. moestus* have the aggregation type with the particular mode of nuclear assembly producing sperm *rouleaux;* the nuclear-head is cone-shaped with a concave, hooded base hosting the sperm head of the neighboring sperm. Besides nuclear interlocking, the cup of extracellular material surrounding the apical region of the sperm bundle establishes a new type of sperm organization. However, compared to *S. optatus*, *D. moestus* shows more extensive extracellular material embedding the sperm heads bundle and the initial region of the flagella. This feature was not seen in *S optatus*. Furthermore, the extracellular material of *D. moestus* has a particular structure, consisting of tubules.

Similar tubule secretory material has been described in other systems, such as the salivary glands of the gastropod *Lymnaea stagnalis* (Fain-Maurel, 1969), the male paragonia of *Drosophila paulistorum* (Tandler et al., 1968) and the male accessory glands of *Drosophila* (Bairati, 1966). It has been suggested that these tubules might have some roles in sperm storage (Perotti, 1971).

Biochemical studies on the secretions embedding the sperm bundle of other Adephaga, with particular reference to Carabidae, suggest that they are composed of a mixture of proteins and polysaccharides (Mackie and Walker, 1974; Hodgson et al., 2013; Schubert et al., 2017). Such material is secreted by the epithelium of the apical region of the deferent ducts. Dilated cisterns of endoplasmic reticulum, which often form long chains across the cytoplasm of the epithelial cells, are filled with electron-transparent material involved in secretory activity. On the contrary, the epithelium of the more posterior deferent ducts does not show secretory activity and the many mitochondria in the apical cell region could possibly indicate a reabsorption activity.

Bearing in mind the considerations of Ballowitz (1895) on the nature of the attachment mass, the *"klebemasse"* found in some Dytiscidae, the secretory material embedding the sperm bundle seems to have the same origin in all Adephaga beetles, being produced and secreted by the epithelium of the anterior region of deferent ducts (Carcupino et al., 2002; Takami and Sota, 2007; Hodgson et al., 2013; Schubert et al., 2017; Salazar et al., 2022). This secretion can however take different shapes and size being defined for example as a "cup" in some Carabinae and Dytiscidae Hydroporinae and as a "spermatostyle" in various tribes of Carabinae and some species of Gyrinidae (Breland and Simmons, 1970; Löser and Lampe, 1973; Ferenz, 1986; Sasakawa, 2007; Takami and Sota, 2007; Hodgson et al., 2013; Schubert et al., 2017; Dallai et al., 2019, 2020; Salazar et al., 2022; Mercati et al., 2023).

A further difference between these structures can be established if we consider how sperm are related to the secretory material. In the cup model, the sperm heads, often together with the proximal region of the flagellum, are embedded in the secretion, while in the spermatostyle model, the sperm heads and sometimes even a very short part of the anterior flagella are anchored to a long rod structure.

A relevant aspect for the efficiency of sperm transfer and its use by the female is whether all this material reaches the female spermatheca after mating. In the Carabidae, where sperm conjugation by spermatostyle has been ascertained (personal observation in *Harpalus* sp.), the long rod structure persists in the spermathecal lumen after the sperm has lost connection with it. According to Breland and Simmons (1970) and Schneider and Ferenz (2012), the same seems to occur in *Dineutus* species (Gyrinidae). We presume that this could also be the case in Carabinae species, such *Carabus* sp., whose sperm bundles bear caps. This appearance was not found in *S. optatus* (Mercati et al., 2023), unlike in *D. moestus* here studied. Sperm bundles lose their extracellular secretion as they reach the end of the deferent ducts. These findings are readily

explained by the fact that in Hydroporinae, the sperm are arranged in sperm-head stacks that are quite stable during sperm transfer to the female and persist for some time, almost without modification, in the spermathecal lumen (Dallai et al., 2023a). It therefore seems reasonable to suppose that the extracellular secretion in this group could be a further device to strengthen the sperm bundle, preventing it from breaking up during its journey along the deferent ducts.

It is reasonable to infer that, after its detachment from the sperm bundle, the extracellular material is transferred to the female during mating.

A final consideration concerns comparative analysis of the sperm of the Hydroporinae diving beetles studied so far. Apart from their peculiarly shaped nuclei, *S. optatus* and *Sc. halensis* sperm show a typical $9 + 9 + 2$ flagellar axoneme flanked by two elongated mitochondrial derivatives, whereas *D. moestus* has a mitochondrial derivative matrix containing electron-dense dots and a small area of crystallization. A final remarkable finding is the shape of the accessory bodies of the three species studied so far. In *S. optatus* and *Sc. halensis* they are very small and confined to the apical side of the mitochondrial derivatives. In *D. moestus*, the accessory bodies are two long strips between the axoneme and the mitochondrial derivatives. The different appearance of these structures could be a taxonomic marker indicating that the group with *D. moestus* belongs to a different subtribe to the other two species.

It will be interesting to extend sperm observations to other Hydroporinae beetles, as this large group shows quite pronounced variability in general anatomy, in the organization of the male and female reproductive systems (Miller, 2001; Miller and Bergsten, 2014) and in the fine structure of the latter (Mercati et al., 2023; Dallai et al., 2023a, 2023b).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

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