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Ultrastructure of the female reproductive organs of the diving beetle *Deronectes moestus incospectus* (Leprieur, 1876) (Dytiscidae, Hydroporinae)

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ABSTRACT

We describe the ultrastructure of the female reproductive organs of *Deronectes moestus* (Dytiscidae Hydroporinae). The long spermathecal duct has a simple epithelium lined internally by a thin cuticle and externally by a thick layer of muscle cells. The wide duct lumen contains electron-dense material, among which remnants of extracellular material are visible. This material consists of tubular structures assembled around sperm bundles previously described in the male deferent ducts. The so-called gland, disposed along the spermathecal duct, is a structure with epithelial cells lined by an irregular cuticle bearing a rich system of microvilli. Many mitochondria are visible in the apical cytoplasm of the epithelial cells, and a few spheroidal bodies are close to the basal nuclei. Since the epithelial ultrastructure of the gland suggests it is involved in fluid uptake from the lumen rather than secretory activity, the term *gland*, coined by other authors to describe this organ, is inappropriate. The spermatheca is a large structure with a complex epithelium showing secretory and duct-forming cells. The lumen of this organ contains sperm with the distinctive ultrastructural features of those described in the male deferent ducts, namely having a mitochondrial matrix with a small crystallized area and electron-dense dots. Because to its overall organization, the spermatheca of *D. moestus* can be considered a more integrated organ than those in previously studied hydroporine species.

1. Introduction

Dytiscidae is the most successful group of Adephaga by their efficient adaptations to aquatic life. More than 4300 species belonging to this group have been described up to now (Beutel et al., 2020). The large subfamily Hydroporinae comprises many taxa that show complex differences in the shape and size of their bursa copulatrix, spermatheca, and spermathecal gland, as well as variations in the length of their spermathecal and fertilization ducts (Miller, 2001). There is disagreement in the literature about the names used for defining the spermatheca. The two-chambered structure was called "diverticulum" by Angus (1985) and, more inclusively, "receptacle" by Miller (2001). De Marzo (1997) used the term "receptacle" to indicate a capsule opening into the common oviduct, and also described a "gland" into which the spermathecal duct flows. The general variable configuration of the female genital tract was confirmed by recent ultrastructural studies of two species: *Stictonectes optatus* (Seidlits, 1887) and *Scarodytes halensis* (Fabricius, 1787). The former has a relatively short spermathecal duct and a complex consisting of the spermatheca closely juxtaposed to the spermathecal gland (Dallai et al., 2023a). In *Sc. halensis*, the two districts are fused to form a long structure with a common lumen (Dallai et al., 2023b). However, an important distinction, from an ultrastructural point of view, is that the epithelia of the two districts maintain their own ultrastructural characteristics: the spermatheca has a simple non-secretory epithelium, while the spermathecal gland shows a glandular epithelium with secretory cells. The secretions pour into the spermathecal lumen via fine ducts generated by duct-forming cells, as commonly seen in ectodermal glands (Quennedey, 1998).

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Here we investigated a third species of Hydroporinae, *Deronectes moestus,* to establish the ultrastructural organization of the above two districts. Light microscopy observations indicated that this species is unusual because the spermatheca (receptacle) and the gland are distant from each other, as originally described by De Marzo (1997) (Fig. 1A). The aim of the present study was to verify whether the ultrastructure of the two districts in *Deronectes moestus* is similar to those described in *Stictonectes optatus* and *Scarodytes halensis*.

2. Material and methods

Five specimens of *Deronectes moestus incospectus* (Leprieur, 1876) were collected in a small river near Grosseto (Italy) and classified by Dr. Saverio Rocchi, Museum "La Specola", Florence. Three females were dissected under light microscopy in 0,1 M, pH 7,2 phosphate buffer, to which 3% of sucrose was previously added (PB). The genital organs were fixed at 4 °C overnight in 2,5% glutaraldehyde in PB, observed and photographed with an Olympus stereomicroscope equipped with a Zeiss MRC5 digital camera. After rinsing in PB, the material was post-fixed in 1% OsO_4 for 2 h, rinsed again in PB, and after alcohol dehydration (50%–100%) it was

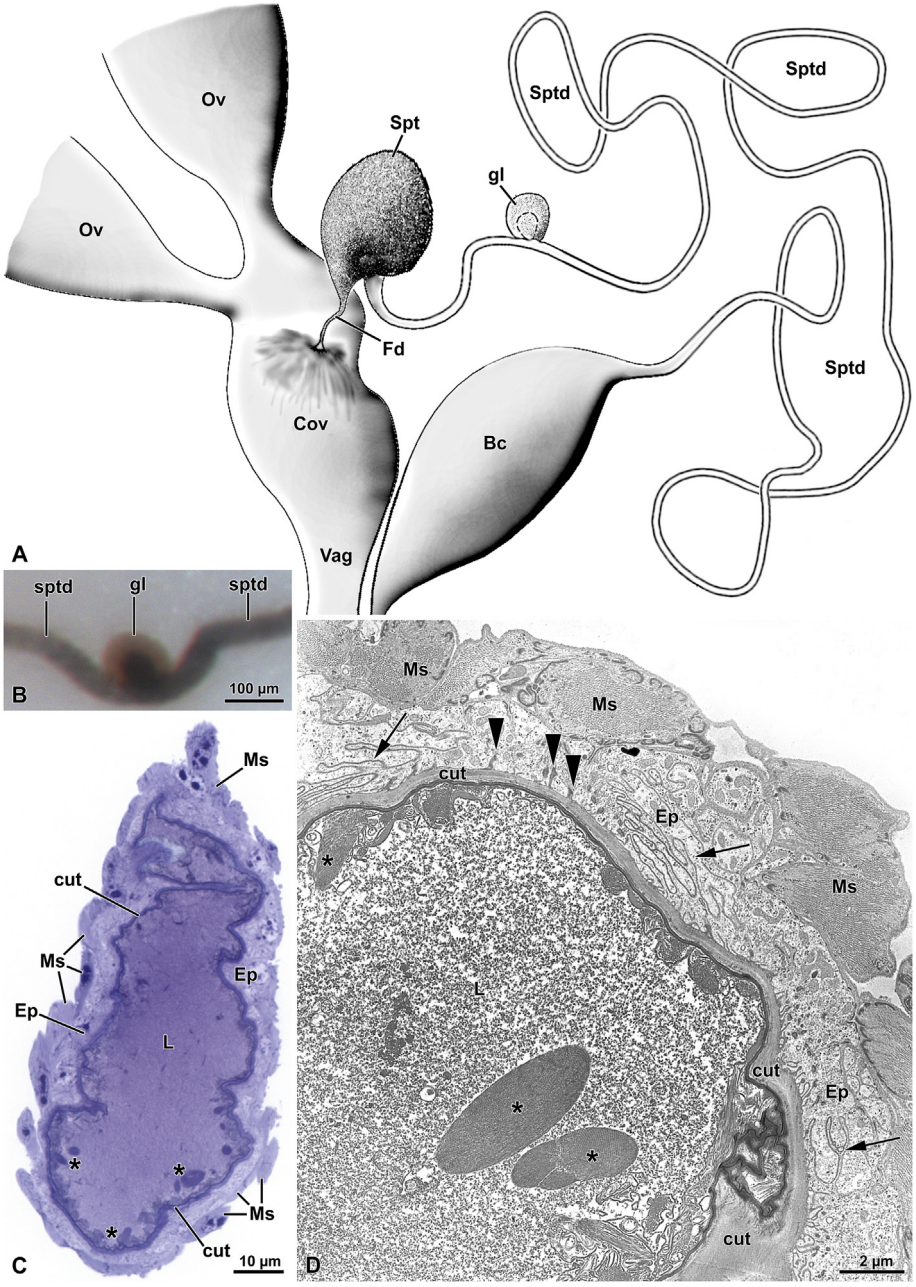

Fig. 1. A- Schematic drawing of the female reproductive apparatus of *D. moestus*. Bc, bursa copulatrix; Cov, common oviduct; Fd, fertilization duct; gl, gland; Ov, ovary; Spt, spermatheca**;** Sptd, spermathecal duct; Vag, vagina (from De Marzo, 1997, modified). **B**- Light microscope view of the spermathecal duct (Sptd) flowing into the gland (gl). **C**- Semi-thin section of the spermathecal duct; cut, cuticle; Ep, epithelium; Ms, muscle fibres; electron-dense bodies (asterisks) in the lumen (L). **D**- Cross section through the spermathecal duct showing the thin epithelium (Ep) with interconnected cells by membrane infoldings (arrows). A thin layer of cuticle (cut) surrounds the wide lumen (L). This contains an electron-dense material and large amounts of filamentous structures (asterisks). Ms, muscle fibres. Arrowheads indicate the hemiadherens junctions beneath the cuticle.

embedded in a mixture of Epon-Araldite. Semi- and ultrathin sections were obtained with a Reichert Ultracut IIE ultramicrotome. Semithin sections were stained with 0.5% Toluidine blue, observed, and photographed with a light microscope Leica DMRB equipped with a Zeiss MRC5 digital camera.

Ultrathin sections were stained with uranyl acetate and lead citrate, observed and photographed with a transmission electron microscope Philips CM10 operating at an electron accelerating voltage of 80 kV.

3. Results

3.1. The spermathecal duct

The female genital apparatus of *Deronectes moestus* is characterized by a long spermathecal duct which, when stretched out, is about 10 mm long (Fig. 1A). In the duct, not far from the spermatheca, an ovoid structure is present, defined as a "gland" by De Marzo (1997) (Fig. 1A and B). The duct has a diameter of 25–27 μm and a thin epithelium, only 2.5–3.0 μm high, lined internally by an 0.5 μm thick cuticular layer, with an electron-dense epicuticle, about 25–30 nm thick (Fig. 1C and D). A very thin basal lamina is present. Surrounding the epithelium, a series of longitudinal muscle fibres are visible, about 3.2 μm in diameter (Fig. 1C and D), each connected with the neighboring fibres by thin connective bridges. Epithelial cells are elongated, reaching 7.0–8.2 μm in height and are interconnected with adjacent cells by deep plasma membrane infoldings (Fig. 1D), with typical *zonulae adhaerentes* in the most apical region. From the basal cuticular region, electron-dense thin and short projections directed to the cytoplasm are visible. Bundles of microtubules anchored on these structures prolong into the cytoplasm up to the basal lamina. At this level, analogous electron-densities are visible on which microtubules adhere. These structures are typical hemiadherens junctions (according to the definition used by Bitsch and Bitsch, 2002) or MicroTubule Associated junctions (MTAj as mentioned previously by Noirot and Noirot-Timothée, 1998) homologous to hemidesmosomes connecting the muscle fibres to the epithelium (Fig. 2A). The muscle fibres show, along their peripheral sarcolemma in contact with the basal lamina, electron-densities apposed to those present along the epithelium (Fig. 2A).

Only a few organelles and inclusions, represented by elongated mitochondria and short cisterns of endoplasmic reticulum, are visible in the cytoplasm of the spermathecal duct epithelial cells, also scattered small electron-dense inclusions are sometimes visible. A small nucleus (3.7 μm × 2.5 μm) is present in the basal region (Fig. 2A). The large lumen of the spermathecal duct, 17–19 μm wide, is filled with an electron-dense homogeneous and finely granular material in which some inclusions of different size are present (Fig. 1C and D; 2 B-E). They consist of oval or elongated structures, 1.6–3.0 μm long but sometimes reach up to 6.5 μm. Often these inclusions are aligned along the epicuticle to form an irregular layer (Fig. 1D), or they are embedded in the electron-dense material filling the lumen and forming elongated bundles long about 3.0–3.5 μm (Fig. 2B–D). These inclusions consist of a complex of thin tubules about 17–18 nm in diameter in cross-section (Fig. 2E). Such tubules can also join to form larger structures of variable dimensions.

3.2. The "gland"

This gland is a small structure where the spermathecal duct pours its secretions (Fig. 3A). It consists of an elliptic body, not easy to detect at the dissection of the female genital apparatus, as the involutions of the long spermathecal duct often hide it (Fig. 1B). The gland is about 125 μm long and about 100–110 μm wide (Fig. 3A and B). The epithelium is about 25–30 μm high and is lined by a 1.6 μm thick cuticle that, in some regions, thickens up to 4.0 μm (Fig. 3B and C). The gland lumen is 50–60 μm wide and contains a homogeneous electron-dense material. The epithelial cells, about 25–32 μm height, have lateral sinuous membranes and basal, roundish nuclei 4.0–6.0 μm in diameter with a prominent nucleolus (Fig. 3E). Close to the nuclei, few spheroidal inclusions, 1.2 μm in diameter and with a narrowed and less electron-dense peripheral region than their core, are visible (Fig. 3E). Other smaller inclusions are visible at different levels. A rich system of mitochondria is present in the apical cell region beneath the complex of cytoplasmic membrane invaginations beneath the cuticle forming a 2.4 μm high labyrinth (Fig. 3C, D, F). Epithelial cells lay on a thin basal lamina, 0.25 μm thick. Unlike the spermathecal duct, muscle fibres are not visible surrounding the basal lamina of the gland (Fig. 3E).

3.3. The spermatheca

After pouring its content into the gland, the spermathecal duct continues for a short tract maintaining the same above described structure up to the spermatheca, where the duct end opens. The spermatheca is a large piriform structure, 360 μm long and 240 μm wide (Fig. 4A), with an epithelium about 1.5–10.0 μm thick, lined by a cuticular layer of 3.0 μm (Fig. 4B and C). Surrounding the epithelium, a thick layer of muscle fibres, about 6.0–8.5 μm, is present. A large lumen, about 250 μm wide, is filled with a fine homogeneous material in which are embedded numerous free sperm (Fig. 4B). These sperm have the same structure previously described in the male deferent ducts, but in the spermatheca, they are present as independent cells, not associated in bundles. Their mitochondrial derivatives still have the peculiar structure with the electron-dense dots and the small crystallized area in the mitochondrial matrix they showed in the male deferent ducts (Fig. 5E). The spermathecal epithelium consists of secretory and duct-forming cells (Figs. 4C, 5A, C, D). Secretory cells are large and have an irregular globular shape (Figs. 4C, 5A, C). Their diameter can vary from 10 μm to 25 μm. Their cytoplasm is rich in rough endoplasmic reticulum, Golgi complexes, and scattered mitochondria (Figs. 4C, 5A, C). They have an elliptical nucleus 6.5–8.6 μm long and 5.0 μm wide (Fig. 4C). The secretory cells do not reach the apical cuticle lining the epithelium, and their basal region adheres to the basal lamina, 0.25 μm thick. As expected, secretory cells have a variably expanded extracellular cistern, 3.5–4.0 μm long, lined by long microvilli (Fig. 5A, C). The cistern contains an electron-dense secretion that is transferred to the apical cell region, and further poured into the spermatheca lumen by thin ducts formed by duct-forming cells intermingled with the secretory cells (Figs. 4C, 5C, D). The duct-forming cells are numerous and take contact with the cisterns of the secretory cells by the so called "end apparatus", consisting of fine tubular structures embedded in an electron-dense material (Fig. 5B and C). The duct-forming cells are elongated with elliptical nuclei, 5.5–7.5 μm long, often located beneath the apical cuticle lining the epithelium, but can also adapt to fill the spaces between adjacent secretory cells (Figs. 4C, 5A, C, D). Duct-forming cells are distinguishable by their electron-transparent cytoplasm, the scarcity of organelles and inclusions, and thin efferent ducts, 0.37 μm wide, crossing their cytoplasm. These ducts can be visible for long tracts crossing the secretory cells (Fig. 4A) and then the apical cuticle, where they pour the secretion produced by the secretory cells.

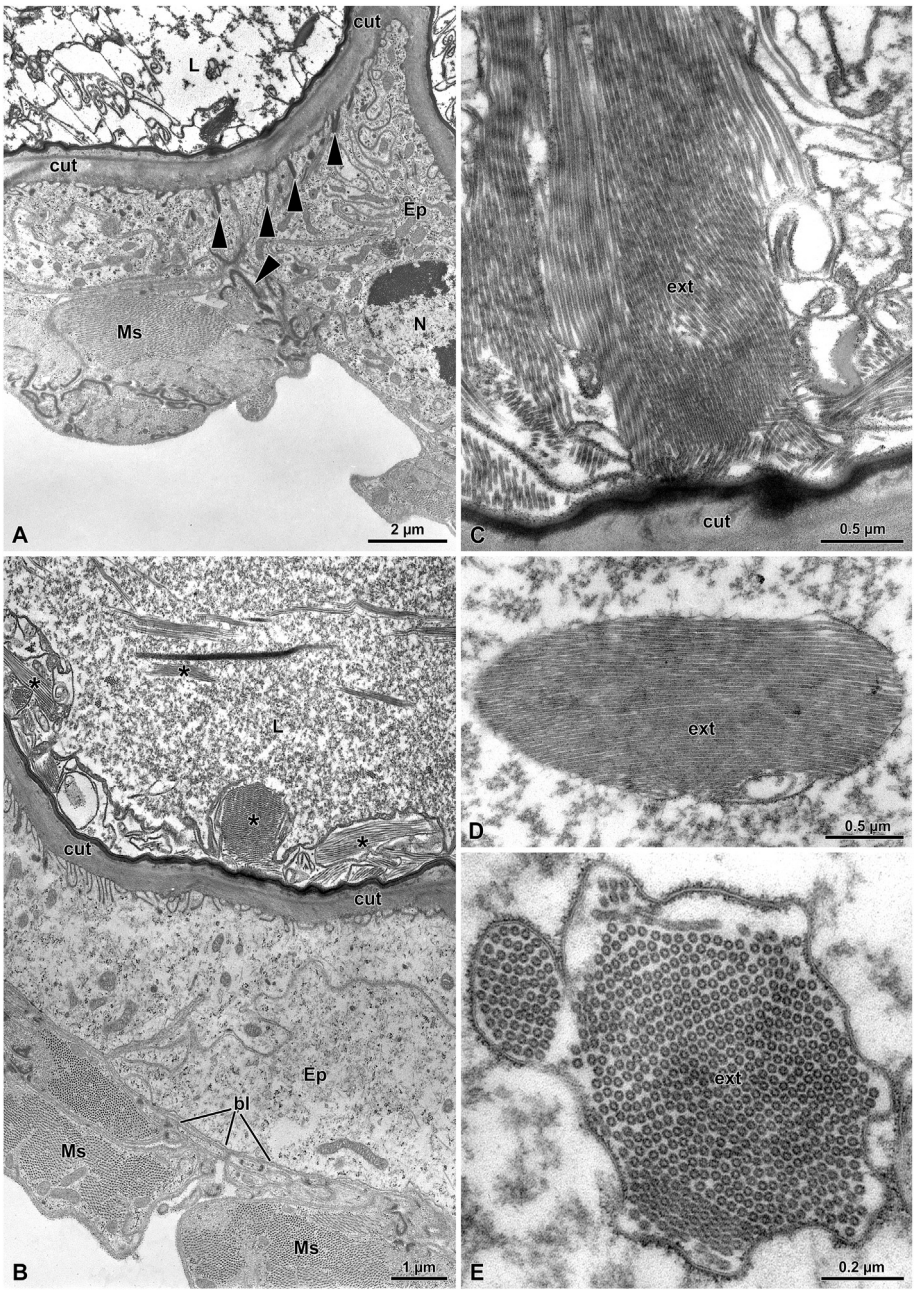

Fig. 2. A- Cross section of the spermathecal duct epithelium (Ep) lined by an apical cuticle (cut) from which dense projections extend into the cytoplasm to give attach to microtubules forming a typical hemiadherens- or microtubule-associated junction (arrowheads). Analogous densities are visible at the muscle levels. L, lumen; Ms, muscle fibres N, nucleus. **B**- Cross section through the epithelium (Ep) lined by a cuticle (cut). In the lumen (L) an electron-dense secretion is present with remnants (asterisks) of the tubular extracellular material present around the sperm bundles in the male deferent ducts. bl, basal lamina; Ms, muscle fibres. **C, D, E**- Cross sections and high magnification of the tubular structures of the extracellular material (ext) present in the spermathecal duct lumen. cut, cuticle.

4. Discussion

Like all Dytiscidae except Dytiscinae, Hydroporinae diving beetles have two female genital openings: the bursa copulatrix to receive sperm and secretions from the male, and the vaginal opening through which the female deposits her eggs (Heberdey, 1931; Jackson, 1960; Miller, 2001; Miller and Bergsten, 2014). The bursa copulatrix is connected to the spermatheca by a spermathecal duct. Sperm and secretions contained in the spermatheca reach the common oviduct via a fertilization duct (Dallai et al., 2023a, 2023b). In some recently studied Hydroporinae, the spermathecal duct was relatively short in *S. optatus* (Dallai et al., 2023a) and extremely long in *Sc. halensis* (Dallai et al., 2023b). In both it consists of a simple epithelium, and in *Sc. halensis* the epithelium has an external thick layer of muscle fibres whose contractions help convey sperm and secretions towards the spermatheca-spermathecal gland complex (Dallai et al., 2023b). The two species have a differently organized reproductive organ. In *S. optatus* the spermatheca is separate from the spermathecal gland and the two structures are only connected by a short tract of their epithelia. On the contrary, in the *Sc. halensis*, the two organs are fused together and share the same lumen, however the two domains around the common lumen maintain their own structures, that is, the spermatheca has a simple epithelium and the spermathecal

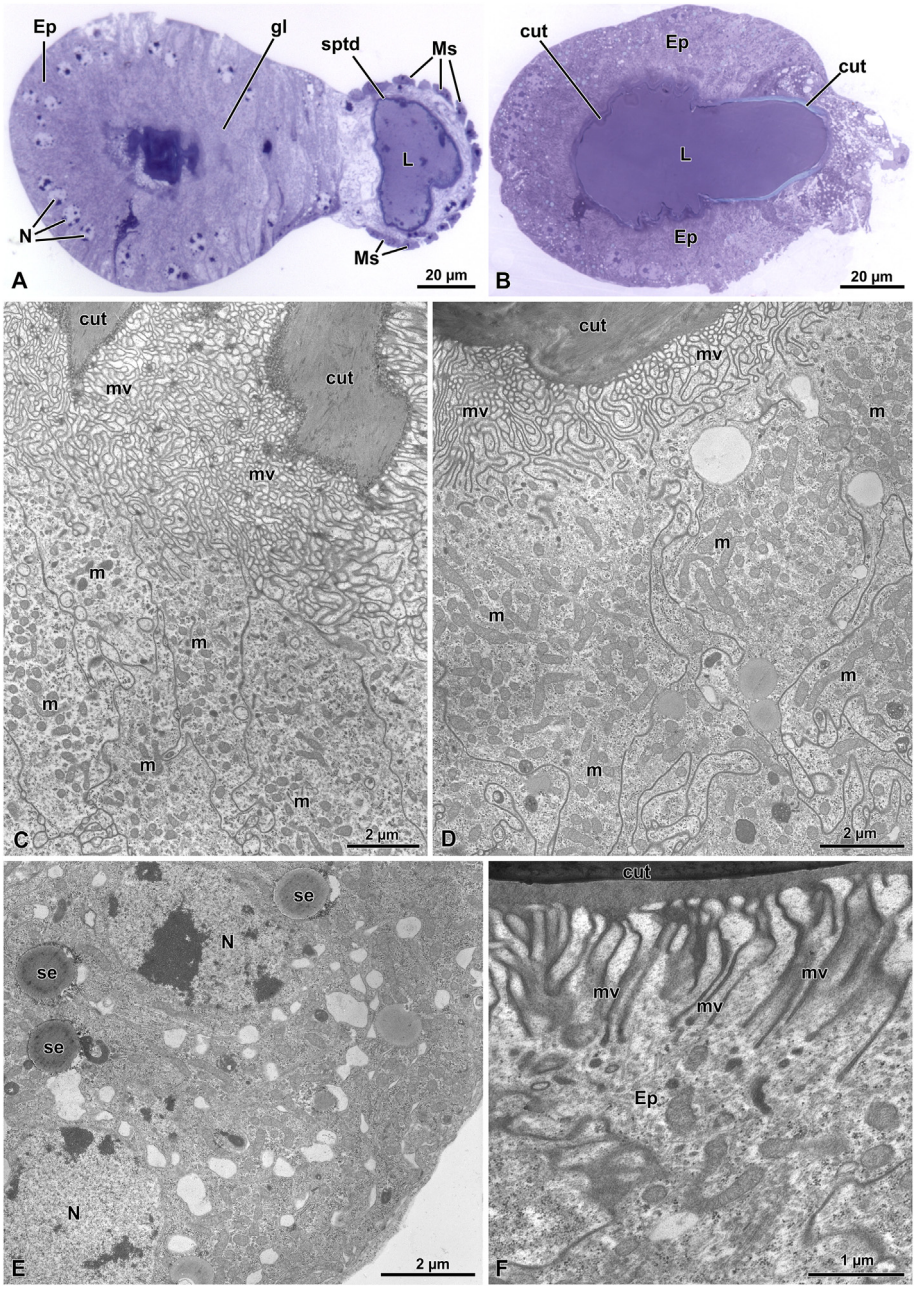

Fig. 3. A-B- Semi-thin sections through the connection between the spermathecal duct (Sptd) and the gland (gl). Note the different structure, with the duct showing a thin epithelium (Ep) lined by a uniform cuticle (cut), while the gland epithelium is higher and with an irregular cuticle. Beneath the epithelium, muscle fibres (Ms) are present only at the spermathecal duct level. L, lumen; N, nucleus. **C-D-** Cross sections of the apical regions of the duct epithelial cells. Beneath the cuticle lining the epithelium (cut), a rich system of membrane invaginations forms a microvillated complex (mv). In the cytoplasm many mitochondria (m) are present. **E**- Cross section through the basal epithelial region. N, nuclei; se, roundish secretory bodies. No muscle fibres are visible beneath the epithelium. **F**- Cross section of the apical epithelial cells (Ep) showing an orderly series of microvilli (mv). cut, cuticle.

gland a more complex secretory epithelium with secretory as well as duct-forming cells.

In this study on the female genital apparatus of *D. moestus*, we demonstrated that the long spermathecal duct has a structure similar to that described in *Sc. halensis* with a thick layer of muscle fibres beneath the epithelium (Dallai et al., 2023b). We also obtained ultrastructural evidence that the epithelium of the structure defined as gland by De Marzo (1997) has a simple organization without the organelles and inclusions typical of secretory cells. Therefore, we concluded that it cannot be a true secretory organ. The numerous mitochondria in the apical region of the epithelial cells and the great extension of membrane infoldings beneath the cuticle lining the epithelium, forming a compact layer of microvilli, suggest that this organ reabsorbs fluid (Phillips, 1970; Flower and Walker, 1979; Martoja and Ballan-Dufrançais, 1984; Dallai et al., 1991; Bradley, 1998; Chapman, 1998; Noble-Nesbitt, 1998). Scattered spheroidal bodies in the basal cytoplasmic region of the epithelium further sustain the possibility of fluid uptake (Dallai et al., 2016). The position of the structure in the spermathecal duct, and the fact that it contains secretions of male accessory glands and extracellular material protecting the sperm bundles, further sustain such activity. Should this interpretation be correct,

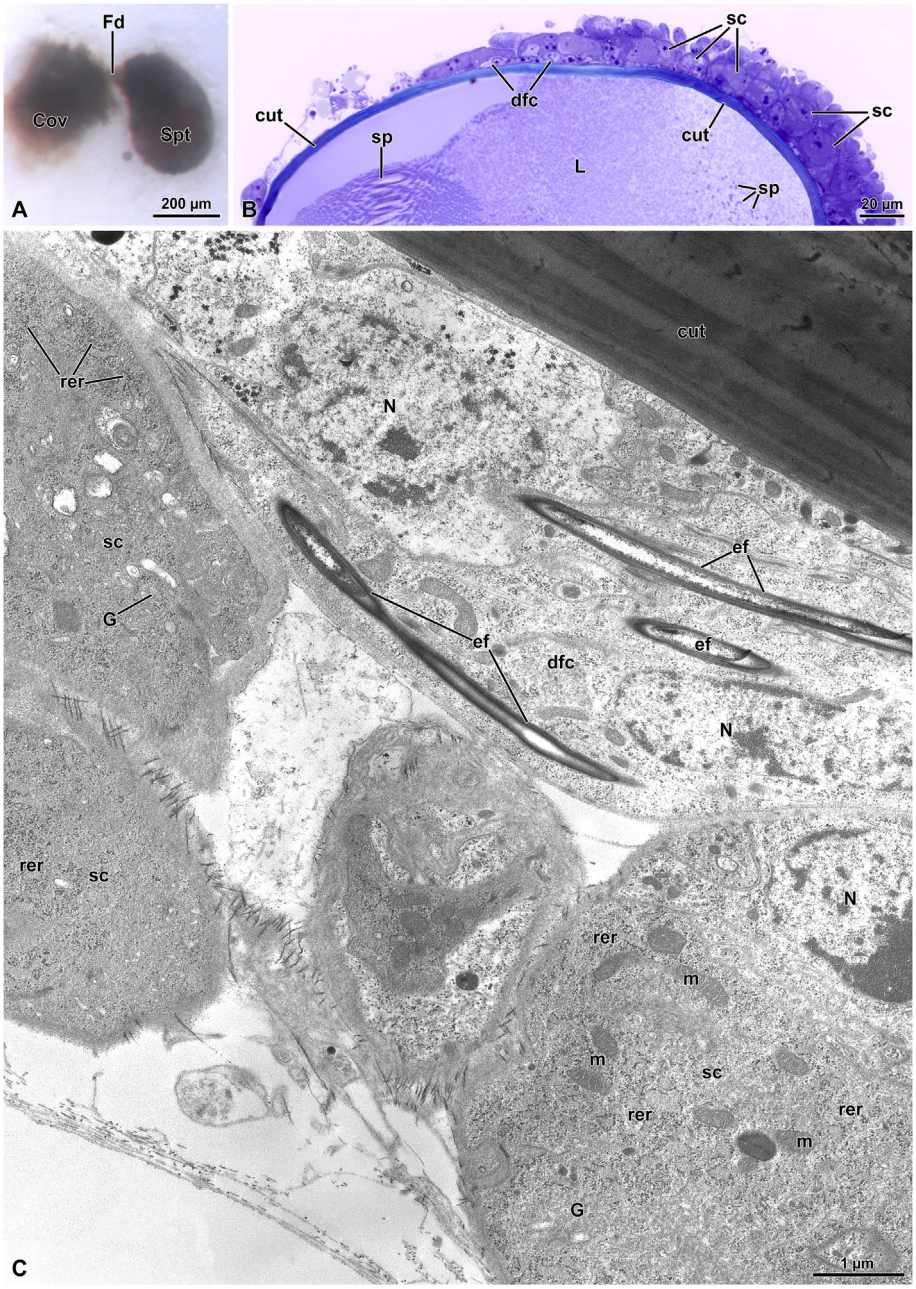

Fig. 4. A- Light microscope view of the spermatheca (Spt) from which a thin fertilization duct (Fd) starts directed to the common oviduct (Cov). **B**- Semi-thin section of the spermatheca wall with the epithelium lined by a cuticular layer (cut). The epithelium has secretory (sc) and duct-forming cells (dfc). In the lumen (L) many sperm (sp) are visible. **C**- Cross section through the spermatheca showing the large irregular shape of the secretory cells (sc). The cytoplasm is rich of rough endoplasmic reticulum (rer), Golgi complexes (G) and mitochondria (m). Duct-forming cells (dfc) are intermingled with secretory cells. Long efferent ducts (ef) of the former cells cross the epithelium. cut, cuticle; N, nuclei.

the term "gland", literally intended to define a secretory organ, is inappropriate and the organ should be considered a structure with fluid uptake functions, identified for the first time in the female genital tract of Hydroporinae.

Comparative analysis of the spermatheca of *D. moestus* and those of *S. optatus* and *Sc. halensis* suggests that the latter two species have a spermatheca with a simple epithelium devoid of the organelle specialization involved in secretory activity, which is instead only performed by the spermathecal gland, whose epithelia are rich in secretory cells. On the contrary, the spermatheca of *D. moestus* has an epithelium with secretory and duct-forming cells, suggesting a sperm storage function and the secretory activity of a typical spermathecal gland. Among the species studied, *D. moestus* seems to have the most functionally efficient structure that resembles the spermatheca of many other insects (Happ and Happ, 1975; Huebner, 1980; Dallai et al., 1993, 2012; Fritz and Turner, 2002).

A further question is the presence of remnants of the extracellular material from the male in the spermathecal duct. This material protects the anterior domain of sperm bundles flowing in the male deferent ducts. In a previous study on Hydroporinae (Mercati et al., 2023), we described extracellular material surrounding the sperm bundles. However, we were unable to detect this material at the end of the deferent duct or in the female genital tract of the species

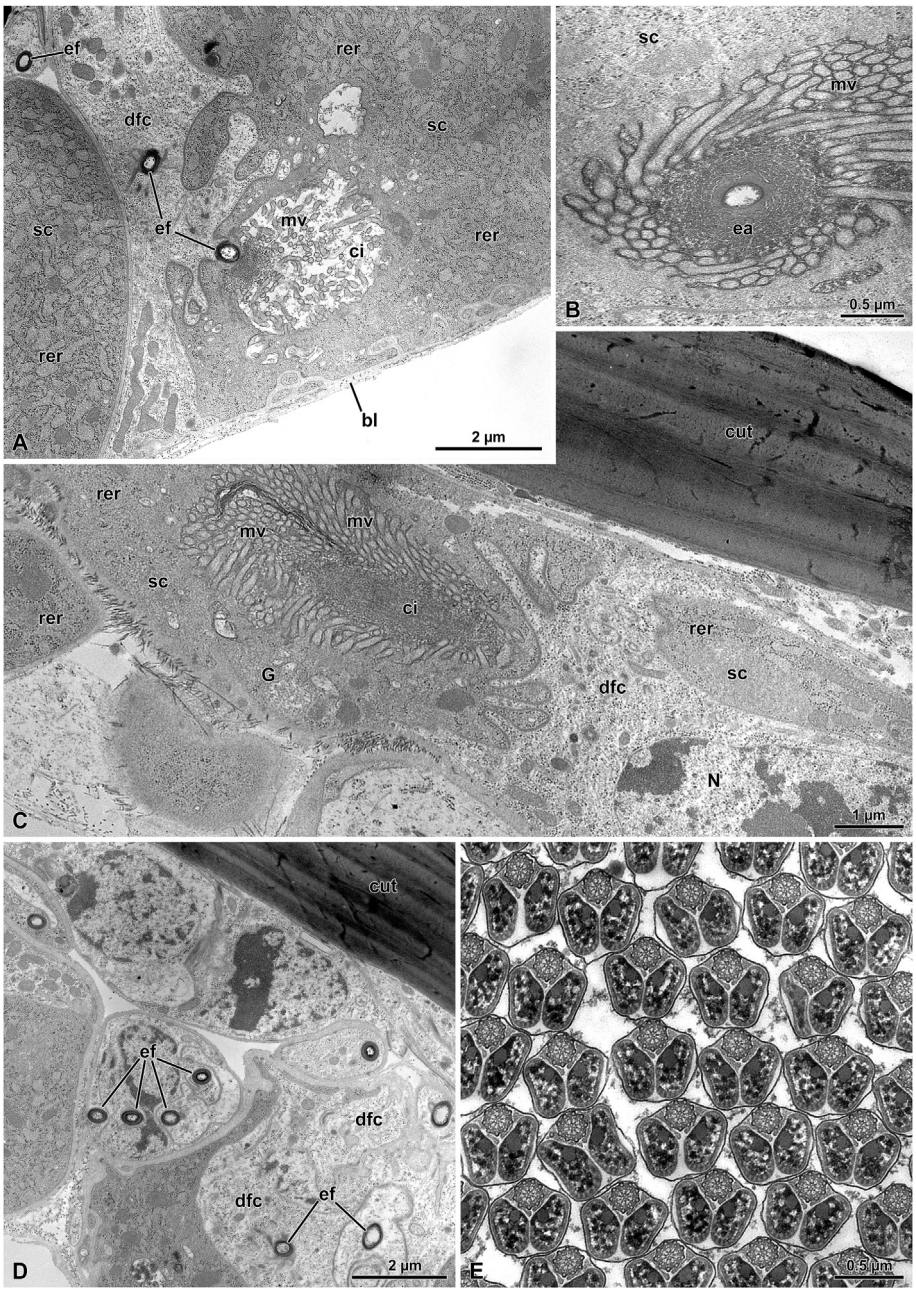

Fig. 5. A- Cross section through secretory cells (sc) rich of rough endoplasmic reticulum (rer). A little portion of an extracellular cistern (ci) rich of microvilli (mv) is visible; duct-forming cells (dfc) and efferent ducts (ef) are placed between two secretory cells. bl, basal lamina. **B**- Cross section of the "end apparatus" (ea) through which the duct-forming cell takes contact with the cistern of a secretory cell (sc). mv, microvilli. **C**- Cross section through the spermatheca epithelium. Beneath the thick cuticle (cut) the secretory cells (sc) and duct-forming cells (dfc) are present. An extracellular cistern (ci) of the secretory cell is lined by microvilli (mv). In the cytoplasm cisterns of rough endoplasmic reticulum (rer) and Golgi complex (G) are visible. N, nucleus. **D**- Cross section of the spermatheca epithelium showing several duct-forming cells (dfc) with their ducts (ef). cut, cuticle. **E**- Cross section through a group of sperm cells from the spermatheca lumen.

analysed (Dallai et al., 2023a). This was presumably due to the structure of the extracellular material, which was homogeneous, moderately electron-dense and difficult to identify ultrastructurally because of its similarity to other material found in the lumen of these organs. In *D. moestus*, on the other hand, the extracellular material has distinctive tubular structure (Dallai et al., 2023c) making it easy to detect in the spermathecal duct lumen as fragments of tubular material. This finding suggests that also in Dytiscidae, the extracellular material surrounding the sperm bundles is transferred at mating to the female, together with the sperm and other secretions produced by the male accessory glands. It may be transferred in the form of simple electron-dense substance cementing the few sperm as in some Colymbetinae and Dytiscinae (Mackie and Walker, 1974; Werner, 1976; Dallai and Afzelius, 1985, 1987; Afzelius and Dallai, 1987) or a cup as in the Hydroporinae *S. optatus* and *D. moestus* (Mercati et al., 2023; Dallai et al., 2023c) and some Carabidae (Dallai et al., 2019), or even spermatostyles, as in other Adephaga, Gyrinidae and Carabidae (Breland and Simmons, 1970; Hodgson et al., 2013; Schubert et al., 2017; Dallai et al., 2019, 2020; Salazar et al., 2022). It is difficult to imagine the functional significance of this material for the female, though a series of studies on different taxa indicate that so-called seminal

fluid proteins, which are produced by the male and transferred to the female at mating, affect the reproductive success of both sexes, including female behaviour and physiology (Swanson et al., 2001; Swanson and Vacquier, 2002; Poiani, 2006; Findlay et al., 2008; Pitnick et al., 2009). Biochemical studies on the extracellular material detected in the few species of Dytiscidae studied so far could help establish the role it plays in the female genital tract.

CRediT authorship contribution statement

Romano Dallai: Conceptualization, Validation, Investigation, Writing – original draft, Writing – review & editing. **David Mercati:** Investigation, Writing – review & editing. **Paulo Henrique Rezende:** Investigation, Writing – review & editing. **Paolo Pietro Fanciulli:** Writing – original draft, Writing – review & editing. **Pietro Lupetti:** Writing – original draft, Writing – review & editing.

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.

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