Arthropod Structure & Development 73 (2023) 101250

Contents lists available at ScienceDirect

Arthropod Structure & Development

journal homepage: www.elsevier.com/locate/asd

Fine structure of the female genital system of diving beetle *Stictonectes optatus* (Seidlitz, 1887) (Dytiscidae-Hydroporinae) and evidence of mating plug formation

Romano Dallai*, David Mercati, Pietro Paolo Fanciulli, Pietro Lupetti

Department of Life Sciences, University of Siena, Italy

ARTICLE INFO

Article history:
Received 25 November 2022
Received in revised form
13 February 2023
Accepted 20 February 2023
Available online 16 March 2023

Handling Editor: Dr G. Scholtz

Keywords:
Diving beetle reproduction
Female genital system
Mating plug formation

ABSTRACT

The general organization of the female genital system of the diving beetle *Stictonectes optatus* was studied, clarifying the complex structure of the spermatheca and spermathecal gland. The two structures adhere closely to each other, sharing a small area of their cuticular epithelium. A long duct connects the bursa copulatrix to the spermatheca, where the sperm are stored. The sperm reach the common oviduct, where egg fertilization occurs, via a fertilization duct. The spermathecal gland cells have extracellular cisterns where secretions are stored. Thin ducts composed of duct-forming cells transport these secretions to the apical gland region and into the spermathecal lumen. Soon after mating, the bursa copulatrix is almost completely occupied by a plug secreted by the male accessory glands. The secretions of the bursa epithelium seem to contribute to plug formation. Later this plug becomes large and spherical, obstructing the bursa copulatrix.

1. Introduction

Sexual selection is considered to be the main force inducing variability in female genital morphology and in male reproductive traits interacting directly with the female reproductive system (Parker, 1979; Anderson, 1994; Eberhard, 1996; Rowe and Houle, 1996; Arnqvist and Rowe 2005; Pitnick et al., 2009). According to Lüpold and Pitnick (2018) “sexual selection will promote those female preferences that precisely target those condition-dependent male traits that honestly signal male genetic-condition, as females would benefit by producing offspring that inherit those qualities”. In insect populations, male sexual competition arises when two or more males compete to fertilize a female's eggs (Parker, 1970). Sperm competition gives rise to a variety of adaptations in males, increasing their fertilization success and limiting female remating.

The anatomy of the female reproductive tract may have driven evolution of sperm features in the Dytiscidae (Higginson et al., 2012a). Studies on these beetles have also established that the female reproductive tract diversifies: different internal organs often vary in shape and size in relation to changes in sperm size, head shape and loss of sperm conjugation (Higginson and Pitnick, 2011; Higginson et al., 2012b). In a recent study (Mercati et al., 2023), we investigated sperm ultrastructure in two species of hydroporinae (diving beetle): *Stictonectes optatus* (Seidlitz, 1887) and *Scarodytes halensis* (Fabricius, 1787), both with long sperm, and for the first time in Dytiscidae (*S. optatus*), we reported a spermatostyle surrounding sperm-head stacks.

As observed by Miller (2001a), there is disagreement about female genital morphology in Dytiscidae. Burmeister (1990) and De Marzo (1997) differ dramatically in their interpretations of female genital morphology. Limiting observations to the genitalia of Noteridae, Hydroporinae and Dytiscinae, Miller (2001a, 2009) described the following configuration.

a) simple bursa/vagina with spermathecal and fertilization ducts leading to the spermatheca and common oviduct, respectively: noterid-type;
b) separate bursa and vagina with spermathecal and fertilization ducts leading to the spermatheca and common oviduct, respectively: hydroporid-type;

* Corresponding author.
E-mail addresses: romano.dallai@unisi.it (R. Dallai), david.mercati@unisi.it (D. Mercati), paolo.fanciulli@unisi.it (P.P. Fanciulli), pietro.lupetti@unisi.it (P. Lupetti).

https://doi.org/10.1016/j.asd.2023.101250

c) bursa absent, separate spermathecal and fertilization ducts leading from the vagina to the spermatheca and common oviduct, respectively: dytiscid-type.

Other interpretations also concern the shape and size of the spermatheca and spermathecal gland, as well as with the presence/absence of a fertilization duct joining the spermatheca to the common oviduct in Hydroporinae (Angus, 1985; De Marzo, 1997; Miller, 2001a; Miller and Bergsten, 2014).

We therefore decided to study the fine structure of the female genital tract of the diving beetle *S. optatus* in order to clarify whether this species has a fertilization duct. We found quite a large plug in the bursa copulatrix of mated females, suggesting that males of the species actively compete with each other. We describe the morphology of this plug and investigate whether the female contributes to its formation.

2. Material and methods

Six mated and four virgin females of *S. optatus*, captured in June (six specimens) and October 2022 (four specimens) in a stream near Grosseto (Italy), were studied. The specimens, reared in a container of pond water, were monitored for mating. The specimens 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 material was fixed overnight at 4 °C in 2.5% glutaraldehyde in PB. After careful rinsing in PB, the material 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. Semithin sections, obtained with a Reichert ultramicrotome, were stained with 0.5% toluidine blue and observed and photographed with a Leica DMRB light

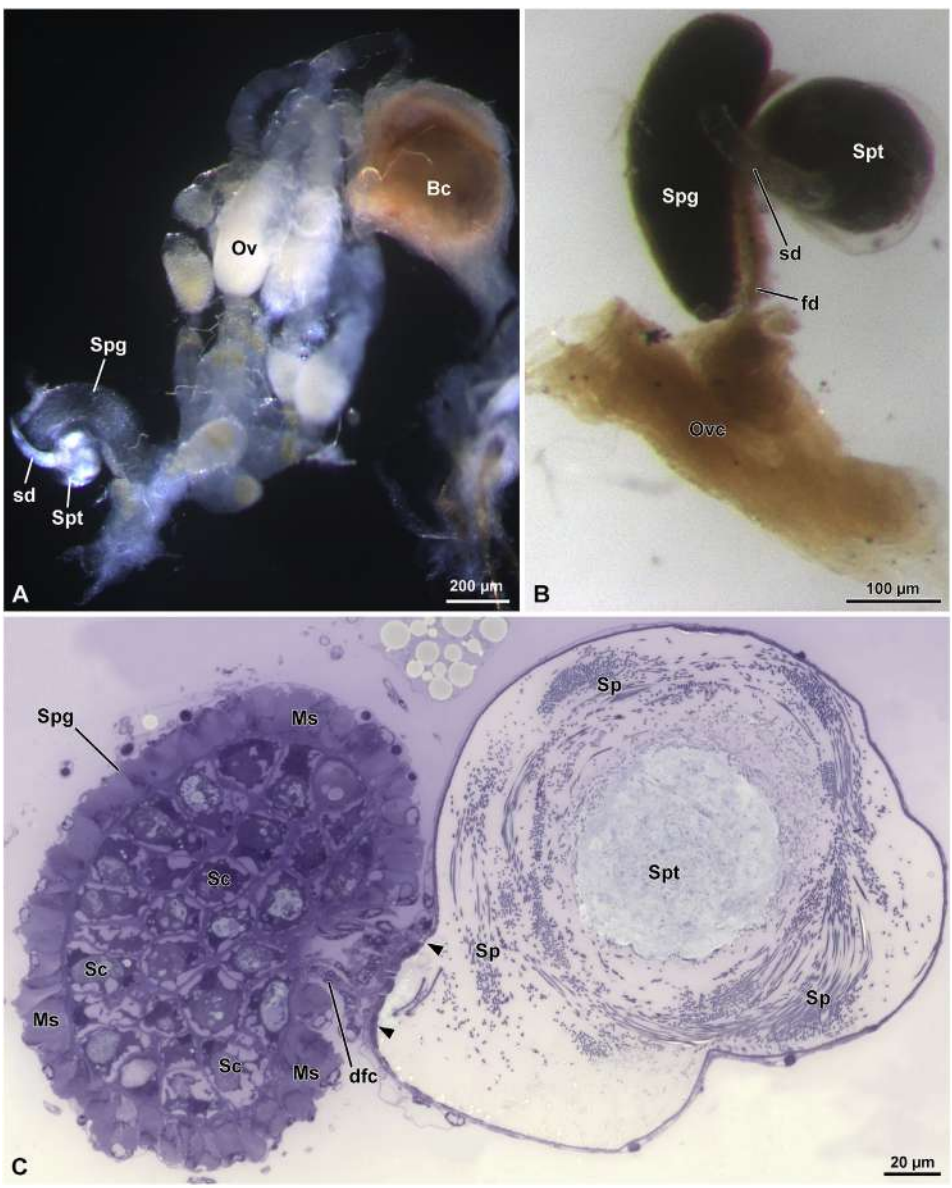

Fig. 1. A - General organization of the female genital apparatus. Ov, ovary; Spg, spermathecal gland; Spt, spermatheca. On the right upper side, a bursa copulatrix (Bc) is visible. Note a short tract of the spermathecal duct (sd). **B** - Detail at light microscopy of the spermatheca (Spt), the spermathecal gland (Spg), the fertilization duct (fd) and the spermathecal duct (Sd). Ovc, common oviduct. **C** - Semithin section of the spermatheca (Spt) and the closely adherent spermathecal gland (Spg). Arrowheads indicate the common cuticular tract between the two organs, through which thin ducts open into the spermathecal lumen. dfc, duct-forming cells; Ms, muscle cells; Sc, secretory cells; Sp, sperm.

microscope. Ultrathin sections, stained with uranyl acetate and lead citrate, were observed with a Philips CM10 transmission electron-microscope at 80 kV.

3. Results

3.1. Female genital tract

The female genital tract of *S. optatus* consists of two ovaries each with 6–8 ovarioles. The ovaries are continuous with short lateral oviducts that unite to form the common oviduct, into which a short fertilization duct opens. The latter is about 200 μm long and arises from a complex spheroidal spermatheca, about 160 μm in diameter (Fig. 1A and B). The spermatheca is closely linked to a long, elliptical, kidney-shaped spermathecal gland, measuring 350–400 μm by 120–200 μm (Fig. 1A and B). A duct about 1.3–1.6 mm long arises from a large bursa copulatrix, about 450–500 μm in diameter (Figs. 1A, 2C-E), and extends to the spermatheca (Figs. 1A and 2D and video in supplementary material). The bursa copulatrix is connected by a short duct to the vagina and the gonocoxal apparatus.

3.2. The spermathecal duct

A long twisted canal (about 1.3–1.6 mm long) with a large elliptical lumen (64 μm × 38 μm) extends from the bursa copulatrix to the spermatheca (Figs. 1A, 2D and 3A). Its lumen is lined with thin epithelium (3.5–6 μm) and a thin cuticle (0.3–0.5 μm) (Fig. 3A). The epithelial cells contain few organelles and inclusions and are adapted to the insertion of muscle fibres surrounding the duct. Bundles of microtubules, anchored to the basal cuticle by hemidesmosomes, and directed towards the muscle fibres, are visible all along the epithelium (Fig. 3B and C). Beneath the epithelium, 20–25 longitudinal muscles fibres and a few circular muscle fibres form a layer 2.5–4 μm thick (Fig. 3A–C). Muscle fibres are large cylindrical structures, up to 6 μm in diameter, with an axial nucleus and typical contractile material organized in fibrils separated by mitochondria (Fig. 3B and C). Isolated sperm embedded in secretory material consisting of apparently empty vesicles are visible in the duct lumen (Fig. 3A and B).

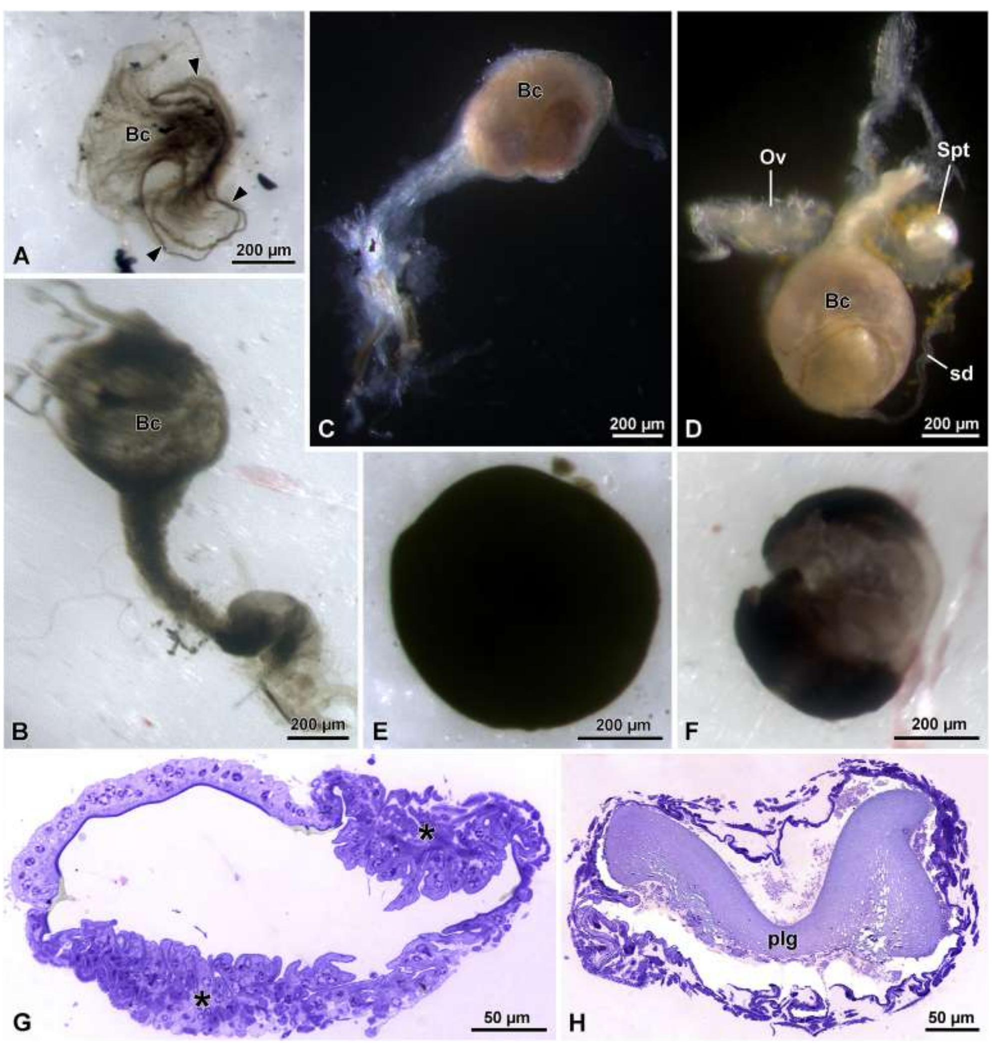

Fig. 2. A - Light microscopy of the bursa copulatrix (Bc) in a virgin female. Note the dense lines corresponding to thicker epithelium (arrowheads). **B** - Light microscopy of a bursa copulatrix (Bc) from which the plug was removed. **C** - Light microscopy of a bursa copulatrix (Bc) in a mated female. Note the dense pink-coloured plug in the lumen. **D** - Light microscopy of a bursa copulatrix (Bc) in a mated female with evident plug. Note also the spermatheca (Spt) filled with lucent sperm and an ovary (Ov). **E, F** - Light microscopy of two plugs extracted from the bursa copulatrix. In E the structure was not damaged, while in F it was opened to show that the inner part is less dark than the peripheral layer. **G** - Semithin section of a bursa copulatrix in a virgin female. Asterisks indicate the regions with thicker epithelium corresponding to dark lines in A. **H** - Semithin section of a plug (plg) in a mated female.

3.3. Spermatheca

The spermatheca of *S. optatus* is large and globular or slightly elliptical (Fig. 1A–C, 2D), apparently integral with the spermathecal gland. It is actually distinct but shares a portion of the cuticular layer lining the epithelium with the spermathecal gland (Fig. 1C).

Spermathecae of mated females are about 170–200 μm in diameter and appear translucent, possibly due to sperm actively swimming in the lumen (Fig. 2D). The epithelium lining their large cavity is thin, 1.2–1.4 μm, and in the apical part bears a cuticle 0.5–0.6 μm thick (Figs. 1C and 4A, C). Scattered elliptical nuclei (1.4 μm × 1.2 μm) are visible in the epithelium. The cytoplasm contains a few small dense bodies and ovoidal granules. A few scattered muscle fibres are observed beneath the epithelium (Fig. 3A–C).

Numerous cross-sectioned sperm flagella, often embedded in a reticular secretion, are visible in the spermathecal lumen (Fig. 4B and C). Sperm flagella can be observed together with a few sperm nuclear stacks; some seem broken into fragments similar to those observed in the distal region of the male deferent ducts. These sperm nuclear stacks do not show their spermatostyles, unlike those in the deferent duct (Fig. 4C). In cross-section, sperm flagella

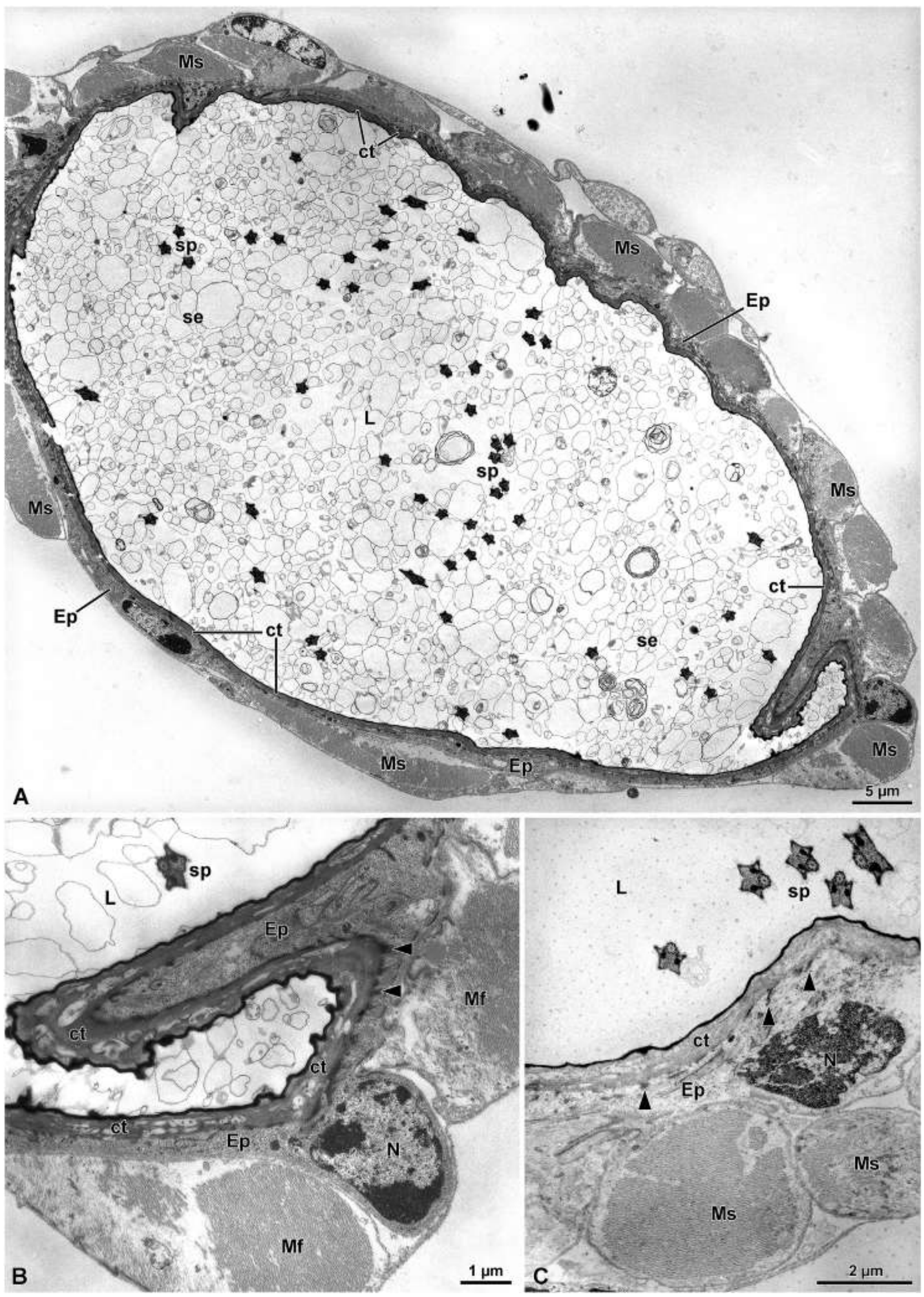

Fig. 3. A - Cross section through a spermathecal duct. Note the thin epithelium (Ep) lined by a cuticle (ct) beneath which a series of longitudinal muscle fibers (Ms) are orderly arranged. In the lumen (L) several sperm (sp) embedded in a secretion (se) are visible. **B, C -** Cross sections of the epithelial cells (Ep). Beneath the deeper layer of cuticle (ct) some densities are visible (arrowheads) representing points of the microtubule insertion to the cuticle. Ms, muscle fibers; N, nucleus of the muscle cells. In the lumen (L) some sperm (sp) are visible.

have the shape typical of those in the deferent duct, but the texture of their matrices seems less homogeneous.

3.4. Spermathecal gland

The spermathecal gland is elongated and closely associated with the globular spermatheca (Fig. 1A–C). It consists of secretory and duct-forming cells, as commonly found in the ectodermal glands of insects. The most remarkable structure is the cuticular epithelium of the apical gland cells, which for a short stretch is continuous with that of the spermatheca (Figs. 1C, 6C-E). The base of the spermathecal gland is surrounded by a muscle fibre layer 18 μm thick (about 50–60 fibres) (Figs. 1C and 5A, D). Fibre thickness varies from 4.5 to 6.5 μm and the nucleus is spheroidal (4.5 μm in diameter) (Fig. 5A). The region in front of the spermatheca has an epithelial layer about 1.8–2.5 μm thick, lined by a cuticle 2.5–3.0 μm thick (Fig. 6C–E).

The main central region of the spermathecal gland consists of various secretory cells separated from each other by a thick layer of connective tissue consisting of fine fibres (Fig. 5D). The secretory cells have an elliptical nucleus (12.5 μm × 5 μm), the shape of which depends on the size of the extracellular cistern containing each cell (Fig. 5D). The diameter of this cistern varies from 14 to 20 μm, depending on the amount of secretory material it contains. The cisterns are bordered by long microvilli (Fig. 5B, D, 6A) and their lumen contain different types of secretory material: fine granules, often quite electron-dense, giant spheroidal multilamellated bodies up to 6.5 μm wide and vesicles with variable density (Fig. 5A, C, D,

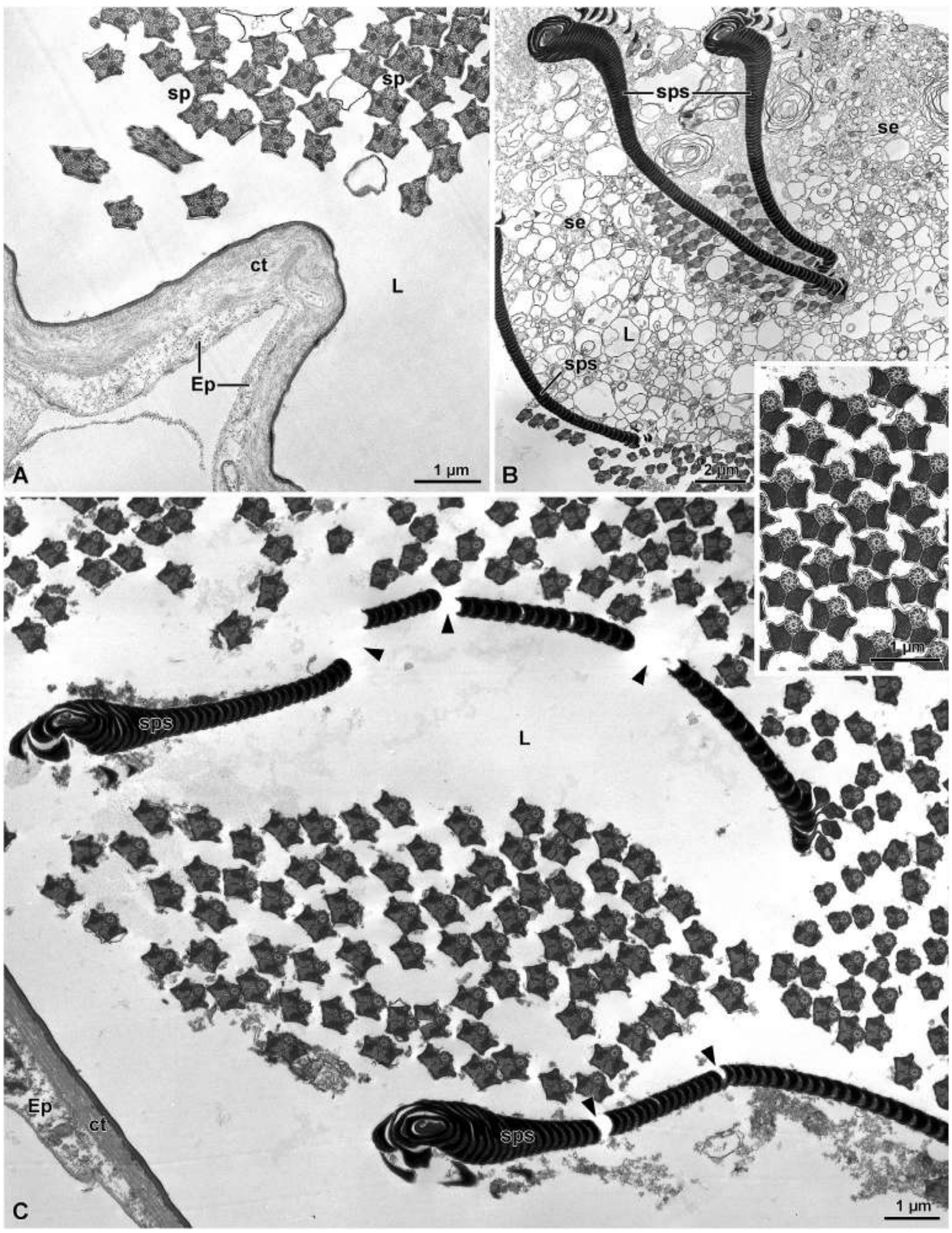

Fig. 4. **A** – Cross section of the spermatheca showing the thin epithelium (Ep) lined by a cuticle (ct). In the lumen (L) many sperm flagella (sp) are visible. **B** – Cross section through the spermathecal lumen (L) showing three sperm-nuclear stacks (sps) embedded in a secretion (se). **C** – Cross section of the spermathecal lumen (L) showing two sperm-nuclear stacks (sps) fragmented at different levels (arrowheads). Ep, epithelium; ct, cuticle. In the inset, high magnification of the sperm flagella.

6A). Cisterns of rough endoplasmic reticulum, Golgi complexes and dense bodies of different sizes are visible in the cytoplasm (Fig. 5C and D, 6A). The end-apparatus of the duct-forming cell is often visible in the centre of the cistern (Fig. 6A); it consists of fine filamentous material surrounding the proximal end of the efferent duct. These ducts transport secretory material in the cistern to the apical cell region. As already mentioned, at this level, a small area of the epithelium lined by a cuticle, is shared by the spermathecal gland and the spermatheca (Figs. 1C and 6C). The cuticular layer is traversed by thin ducts (only 0.7 μm wide) of duct-forming cells that are intermingled with the secretory cells (Figs. 1C, 6B-E). The duct-forming cells are juxtaposed in an orderly manner in long series across the group of secretory cells, up to the apical cuticle. They often form clusters of a few units (Fig. 6C). The ducts cross the

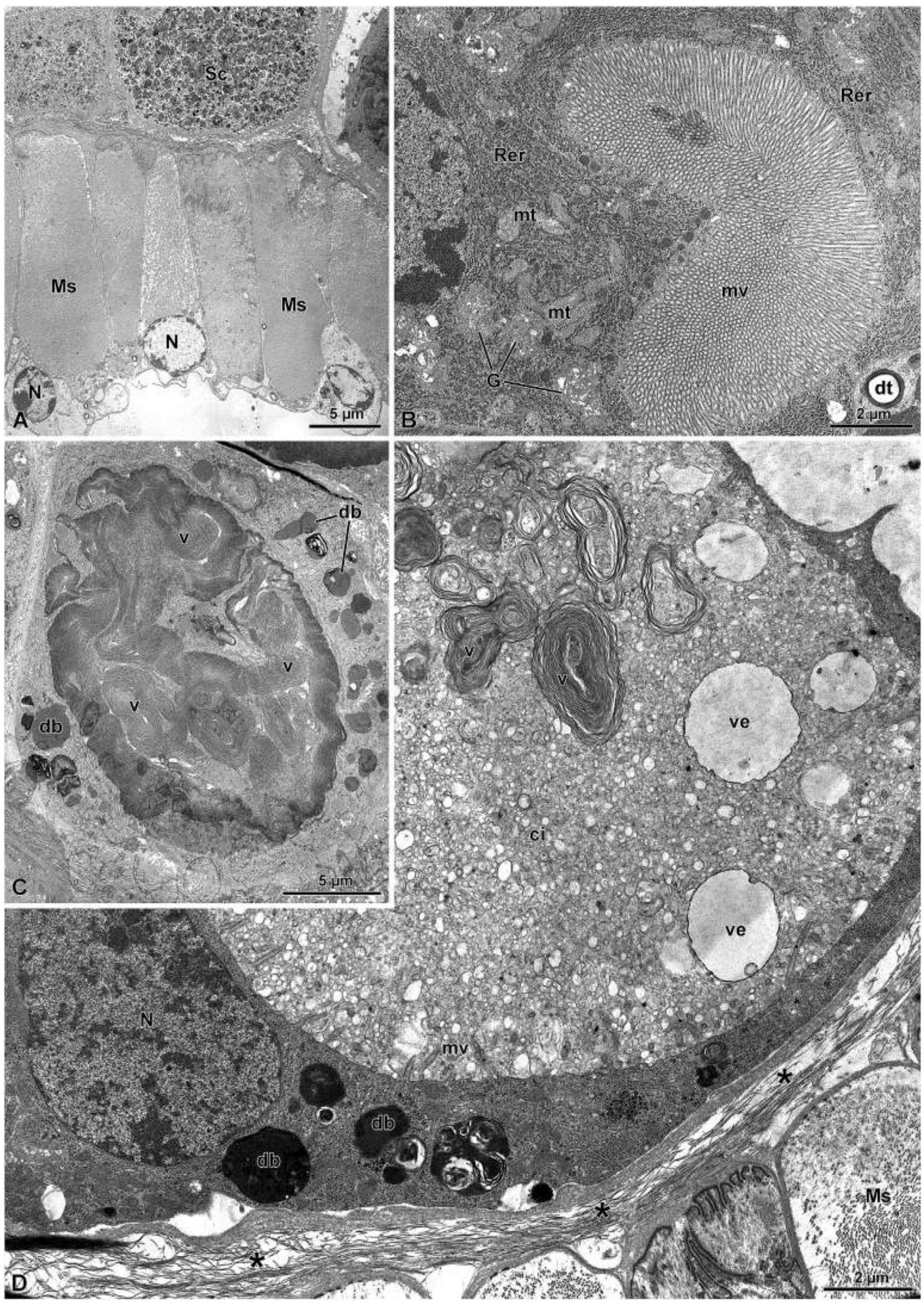

Fig. 5. A - Cross section through the basal region of a spermathecal gland to show the series of longitudinal muscle fibers (Ms). Sc, secretory cells of the gland. N, nuclei of muscle cells. **B -** Cross section of a secretory cell with the large extracellular cistern (ci) filled with long microvilli (mv). In the cytoplasm mitochondria (mt), Golgi apparatuses (G) and rough endoplasmic reticulum (Rer) are visible. dt, a tubular duct of duct-forming cell. **C** - Cross section of a secretory cell of the spermathecal gland with a cistern filled with vortices of membranes (v). In the cytoplasm several dense bodies (db) are visible. **D** - Cross section of a secretory cell with the cistern showing some vortices of membranes (v) and secretory vesicles of various shape (ve). N, nucleus; db, dense body in the cytoplasm. Beneath the cell, a layer of filaments of connective tissue (asterisks) is between the secretory cells and the muscle fibers (Ms).

apical cuticle and convey spermathecal gland secretions into the spermathecal lumen (Fig. 6C–E).

The epithelial cells, which produce the cuticular layer of the spermathecal gland, are only 1.8–2.3 μm thick. They contain very few cytoplasmic organelles and have a roundish nucleus. They form a single layer that also contains duct-forming cells. This epithelium is continuous with that surrounding the main part of the spermatheca.

3.5. Fertilization duct

The spermatheca is joined to the common oviduct by the so-called "fertilization duct" (Fig. 1B). About 200 μm long and 28–30 μm wide, this duct has an epithelium only 1–3 μm thick (Fig. 7A and B). A muscle fibre layer up to about 7 μm thick is visible beneath the epithelium (Fig. 7A and B). The epithelium is lined by a fine cuticle 0.4 μm thick (Fig. 7A and B). Mitochondria and small dense bodies are scattered in the cell cytoplasm. As is common in insects, muscle fibres are anchored by apical hemidesmosomes to the cuticle lining the epithelial cells. Bundles of microtubules anchored to the cuticle lining the epithelial cells by hemidesmosomes, cross the cytoplasm and reach the sarcolemma of the muscle fibres, where they form desmosomes (Fig. 7A and B).

3.6. The bursa copulatrix

The bursa copulatrix of *S. optatus* is a conspicuous spherical structure near the end of the female genital tract. Before mating, it

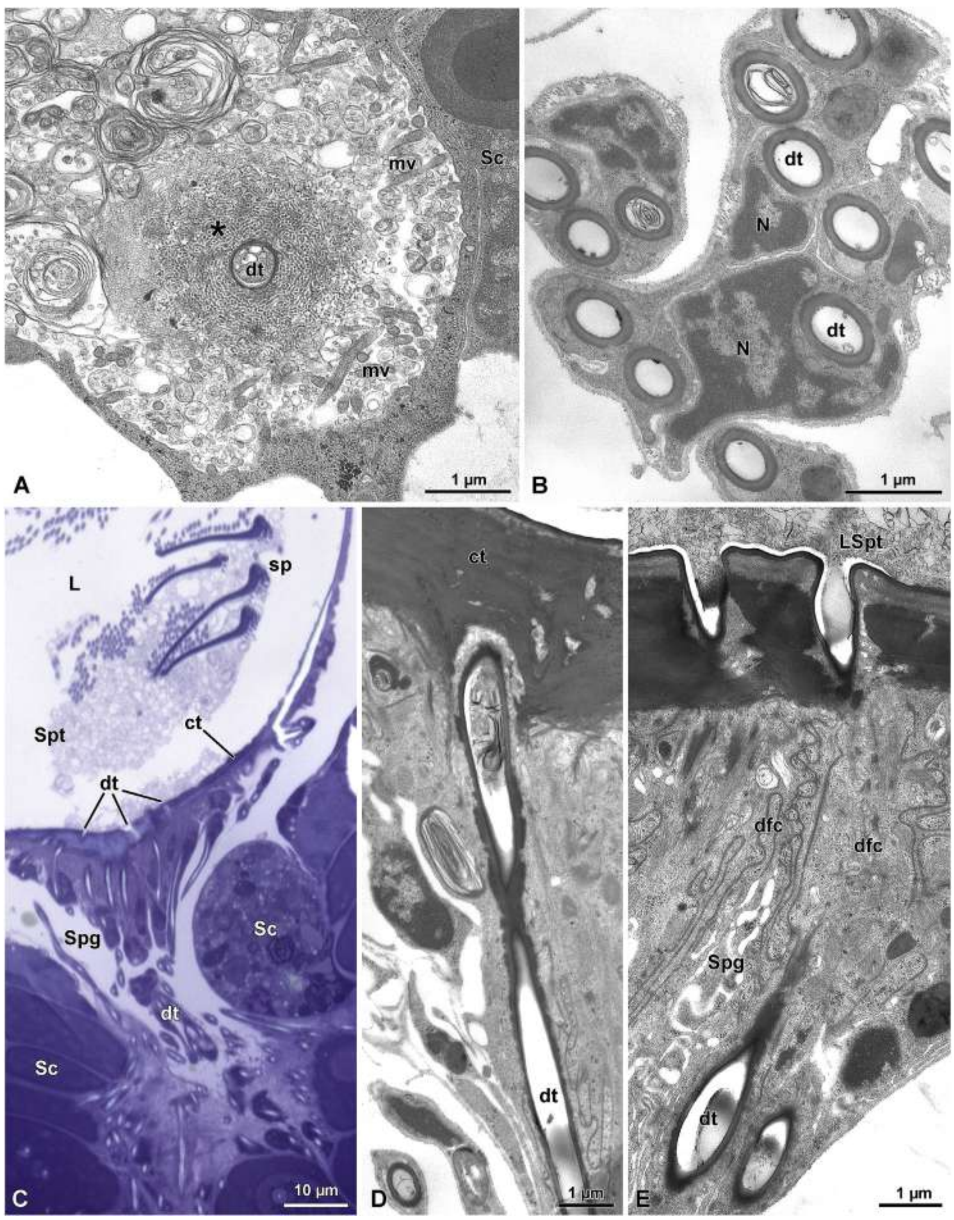

Fig. 6. A - Cross section of the spermathecal gland showing the end-apparatus of a duct-forming cell connecting the cistern of a secretory cell (Sc) to the beginning of a thin duct. mv, microvilli. Note the filaments (asterisk) surrounding the central duct (dt). **B** - Cross section of the small duct forming cells with their ducts (dt). N, nucleus. **C -** Semithin section of the region between the spermathecal gland (Spg) and the spermatheca (Spt). Note the numerous ducts (dt) crossing the apical cuticle and flowing their secretion into the spermathecal gland lumen (L). sp, sperm; Sc, secretory cells. **D, E** - Two longitudinal sections showing the ducts (dt) of duct-forming cells (dfc) crossing the thick apical cuticle (ct) of the spermathecal gland (Spg). LSpt, lumen of spermatheca.

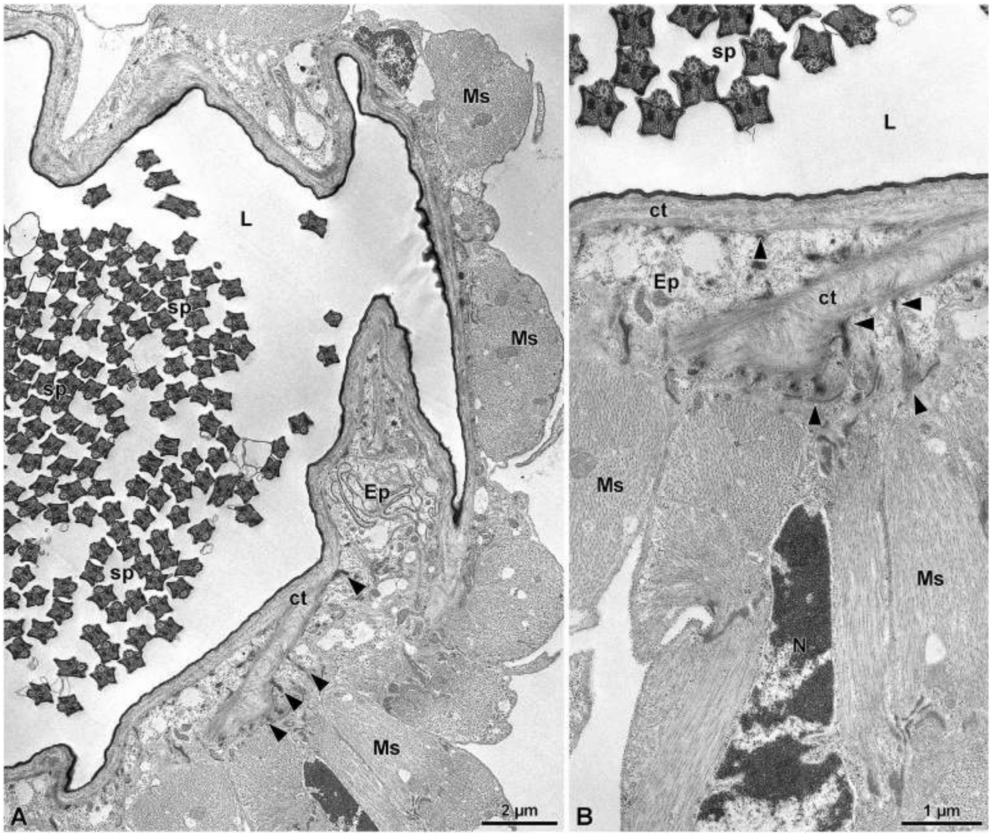

Fig. 7. A, B -Cross sections through the epithelium (Ep) of the fertilization duct. Thin densities beneath the cuticle (arrowheads) are visible. They are sites of microtubule insertion. Analogous densities are visible in the basal region where muscle fibers are present (Ms). N, nucleus of muscle cells. In the lumen (L) of the duct, cross sectioned sperm are present (sp). ct, cuticle.

is a deflated sac showing cuticular reinforcements in its epithelial wall (Fig. 2A). Semithin sections of the bursa show that the epithelium is thin in most areas and very thick in some regions (Fig. 2G). Ultrastructural analysis showed an epithelium, 3.5–4 μm thick, an apical cuticle about 0.7–1 μm thick, and an epicuticle only 0.1 μm thick (Fig. 8A and B). In the regions where the epithelium is thicker, it reaches 10–12 μm and the cuticle is 7 μm thick. Isolated muscle fibres lie beneath the epithelium. Where the cuticle lining the epithelium is thicker, it shows scattered groups of dense droplets, some of which reach the apical epicuticle, in which some very small pores are visible (Fig. 8A).

In thicker convoluted epithelial regions, the cells show finger-like expansions that contain the nucleus (Fig. 8B). Here the cuticle contains large masses of dense material. The cell cytoplasm contains large mitochondria, isolated Golgi complexes and dense bodies (Fig. 8B).

The bursa copulatrix of mated females has a quite different appearance. It shows a more uniformly thick epithelium (Fig. 2H) surrounding a large spheroidal plug, up to 500 μm in diameter (Fig. 2B–D). This plug is of hard consistency (Fig. 2E and F) and is retained by the female for up to 7–10 days after mating.

The appearance of the bursa copulatrix epithelium varies in relation to the time of mating: soon after this event, it is about 15–25 μm thick and lined with an apical cuticle 0.6–2.5 μm thick (Figs. 8C and 9A, B). The nuclei are at the base of the elongated epithelial cells which show some mitochondria and very few electron-transparent vesicles (Fig. 8C). The most interesting cell structure is a series of microvilli that extend up to 15 μm into the cytoplasm and along the subcuticular space (Fig. 9A and B). Careful observation revealed that the series of microvilli is continuous with a microvillated cistern in the cytoplasm. The microvilli are about 1.3 μm long and surround a lumen which contains scattered dense material (Fig. 9A and B), possibly remains of previous more abundant secretory material. Large masses of electron-dense material can be seen in the cuticle layer (Fig. 8C) and there are isolated muscle fibres beneath the epithelial cells. In the bursa copulatrix lumen, remnants of the plug are visible as electron-dense masses of material intermingled with degenerating sperm (Fig. 8C).

Shortly after mating, the epithelial cells of the bursa, formerly bearing cisterns containing secretory material, become thinner and show apparently empty cisterns with remnants of cytoplasmic membranes (Fig. 9C and D).

4. Discussion

In his important studies, Miller (2001a; Miller and Bergsten, 2014) mentioned the difficulty of acquiring good female genitalia preparations, useful for the identification of species of Dytiscidae (Ordish, 1966, 1985; Biström, 1979, 1980 Franciscolo, 1979; Mazzoldi 1996; Miller 2001b, 2001c). This difficulty was presumably the reason for the different interpretations of the female genital configuration in previous papers. Here we summarize some of the points debated for the species of Hydroporinae analysed in our study.

1) the presence of a simple bursa/vagina with spermathecal duct leading to the spermatheca (dytiscid-type), or separate bursa and vagina with spermathecal duct arising in the anterior bursa and leading to the spermatheca (hydroporid-type);

2) the presence of a fertilization duct connecting the spermatheca to the common oviduct;
3) the structure of the spermatheca and spermathecal gland.

While there is general agreement on the existence of two genital openings, one to the vagina and the other to the bursa copulatrix, in all Dytiscidae, except members of Dytiscinae (Burmeister, 1976; Miller, 2001a; Miller and Bergsten, 2014), the main doubt concerns the "fertilization duct", erroneously interpreted as a "tendon" between the spermatheca and the common oviduct by De Marzo (1997). According to this author, sperm were not conveyed from the spermatheca to the common oviduct, but returned to the bursa copulatrix, the sole opening for sperm at mating and for oviposition. There was also confusion of terminology regarding spermathecal morphology: it was called "diverticulum" by Angus (1985), while Miller (2001a) indicated the spermatheca and any associated structures as "receptacle". The structure can have different shapes in Hydroporinae. Our study on *S. optatus* showed that at least in this species, the spermatheca is a distinct organ, though it adheres closely to the spermathecal gland. A similar appearance of this structure in *Hydroporus lapponum* was also mentioned by Angus (1985). As described here, the spermatheca and the spermathecal gland are united by a small area of the epithelium (lined with cuticle) that surrounds the whole spherical spermatheca and the apical region of the spermathecal gland. The spermathecal gland has various secretory cells with large extracellular cisterns, where the secretory material is stored; a series of thin ducts of duct-forming cells cross the cuticle lining the two structures and conveys the material to the spermathecal lumen. Thus our study on *S. optatus* clarifies the doubts arising from previous studies and

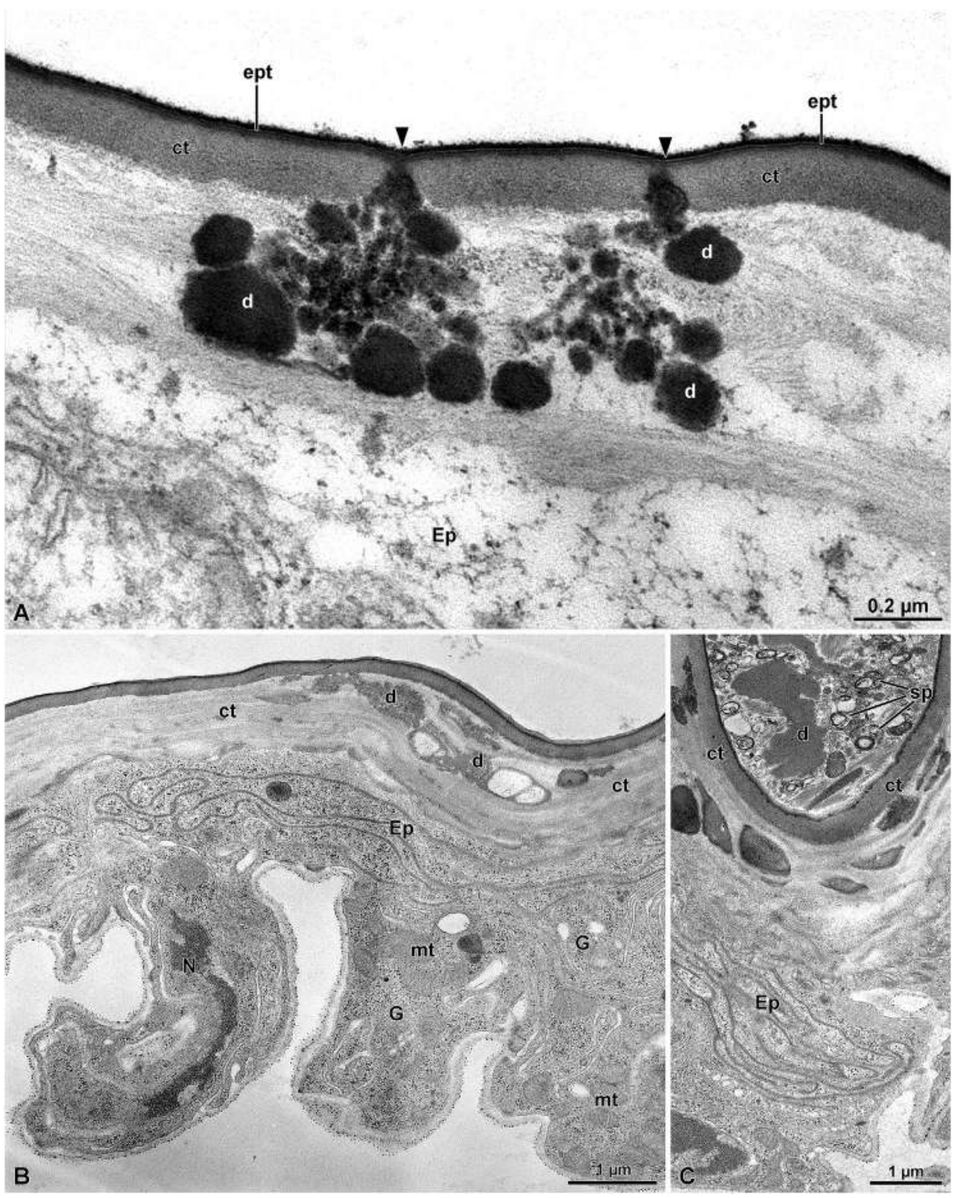

Fig. 8. A - Cross section of the apical cuticle of the bursa copulatrix in a virgin female. Dense droplets are visible (d) some of which apparently directed towards pores at the epicuticular (ept) level (arrowheads). ct, cuticle; Ep, epithelium. **B -** Cross section through the epithelial cells of the bursa copulatrix before mating. The epithelium shows some thick regions with cells having deeply expansions. In the cytoplasm mitochondria (mt) and some Golgi complexes (G) are visible. ct, cuticle; d, dense material stored in the cuticle. N, nucleus. **C -** Cross section of the bursa copulatrix after mating with the plug well formed. In the lumen of the organ dense material (d) of the plug is intermingled with degenerated sperm (sp). ct, cuticle; Ep, epithelium.

establishes the correct significance of these female structures. As regards the structure of the spermathecal gland, our study shows that it is organized according to type 3 insect ectodermal glands (Quennedey, 1998).

The spermatheca is a globular structure where sperm are stored until required for egg fertilization. In *S. optatus,* it is a large structure with a very thin epithelium and very few muscle fibres. This obliges sperm in the spermathecal lumen to swim through the fertilization duct under their own motility, or with the help of contractions of muscles in the duct itself. One interesting observation deals with the presence of sperm-head stacks in the spermathecal lumen. Some of these stacks are fragmented into several parts. This is presumably a preliminary stage of sperm-stack disassembly, in which sperm are released and enabled to fertilize eggs. The epithelium of the fertilization duct has a structure similar to that of the spermathecal duct, which conveys sperm from the bursa copulatrix to the spermatheca.

In our study, we were able to find some mated females with large mating plugs obstructing their bursa copulatrix. These plugs, also described in different species of Dytiscidae (Aiken, 1992; Miller, 2001a; Karlsson Green et al., 2013), had not hitherto been observed in Hydroporinae. The exceptional size of the plugs and their hard consistency make them an interesting subject for research. The plugs are the result of the secretory activity of large accessory glands present in the male reproductive system of *S. optatus* (Mercati et al., 2023).

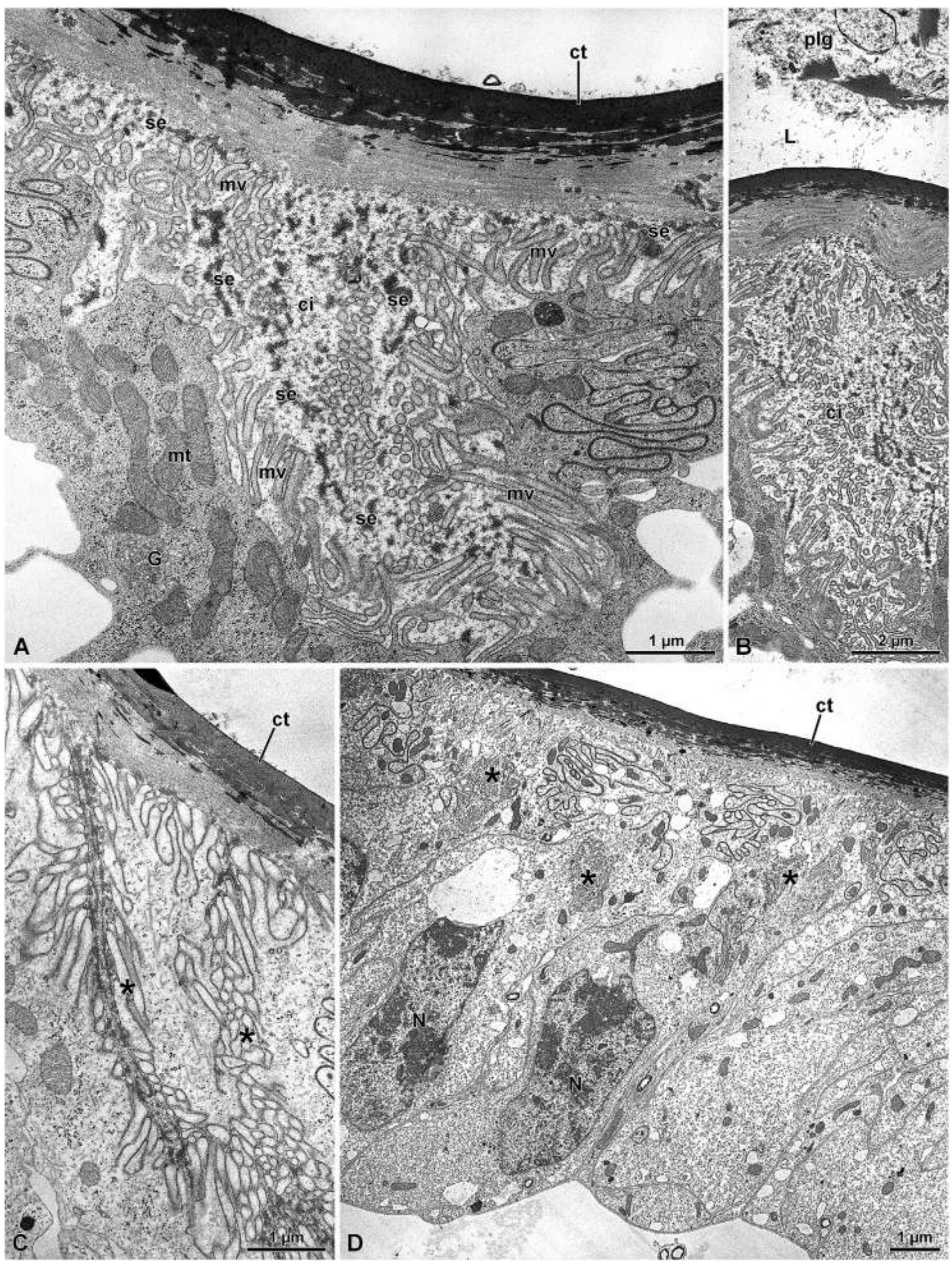

Fig. 9. A - Cross section through a bursa copulatrix just after the mating. Beneath the apical cuticle (ct) lining the epithelium, secretory cells provided with a microvillated apical membrane (mv) delimiting an extracellular cistern (ci). This cistern contains a dense secretion (se). In the cytoplasm, mitochondria (mt) and a Golgi apparatus (G) are visible. A dense secretion is stored between the cuticle and the epithelial cell membrane. **B -** The same type of epithelial cells as in **A**, showing a dense material stored in the lumen (L) of the bursa copulatrix, constituting the plug (plg). ci, cistern. **C, D -** Cross sections of the epithelium in a mated female after a short time from the plug formation. Note that the microvillated cistern are disappeared and remnants of their structure are still visible (asterisks). N, nuclei; ct, cuticle.

It is generally assumed that the role of the mating plug is to reduce sperm competition by erecting a physical barrier to the sperm of rival males, thus inhibiting second male paternity (Parker and Smith, 1975; Parker, 1970; Blum et al., 1962; Giglioli and Mason, 1966; Devine, 1975, 1977; Dickinson and Rutowski, 1989; Polak et al., 1998, 2001; McDonough-Goldstein et al., 2022). Although there have been various studies into the functional significance of mating plugs in different species, it is not yet known whether in a given species plugs are always the system by which males limit female remating or rather whether there are other strategies to obtain the same result.

There is evidence that male guarding (Alcock, 1993), a penile structure that removes sperm from a female's previous mating (Waage, 1979), or even application of anti-aphrodisiacs to the female's terminalia (Gilbert, 1976) can be other effective ways of preventing female remating.

The presence of a mating plug in the female of *S. optatus* was ascertained in late spring (June) and in autumn (October), suggesting that plugs are the rule in these periods. Indeed, plugs were found in seven out of ten females examined. By contrast, in *Dytiscus alaskanus,* mating in autumn resulted in an external mating plug, whereas in spring no external plugs were evident (Aiken (1992).

The unanswered question is whether the mating plugs contain molecules that suppress female sexual receptivity, as previously postulated by Baer et al. (2001). This is a new line of research. Newly formed mating plugs in the butterfly *Pierys rapae* have been found to contain large quantities of female-derived proteases (Meslin et al., 2017; Schneider et al., 2016), and similar findings have been obtained by proteomic analysis of the female reproductive tract of *Drosophila melanogaster* (McDonough-Goldstein et al., 2021).

The contribution of the bursa copulatrix of *S. optatus* to mating plug formation is a new finding of the present study. Before mating, the bursa copulatrix epithelium does not seem engaged in secretory activity but only produces some electron-dense droplets, which are then incorporated in the epicuticle lining the epithelium. On the contrary, in very recently mated females, still having a large fluffy mating plug, the epithelial cells of the bursa copulatrix are clearly involved in secretory activity. They show an apical cell membrane beneath the cuticle lining the epithelium. The membrane is rich in microvilli, which then form microvillated cavities extending deep into the cell body. These cavities contain electron-dense material which is later stored in the narrow space between the cuticular layer and the plasma membrane. Their general appearance strongly recalls the ultrastructural organization of the sternal gland of Termites (the type 1 gland according to Quennedey, 1972, 1998).

Moreover, when the mating plug is formed inside the bursa copulatrix, the epithelial cells of the structure show empty discarded microvillated cisterns with no or very reduced lumen, as well as a residual mass of microvilli and membranes under the apical cuticle. In older bursae, the cisterns of epithelial cells have no microvilli and electron-dense material is stored directly in the cuticular layer.

The mating plug of *S. optatus* is a striking spherical structure, up to 500 μm in diameter, in a beetle only 2.5 mm long, and shows a clear electron-dense cortical layer with some remnants of degenerating sperm and a whitish compact inner part. After mating, the plugs are maintained in the bursa copulatrix without any apparent change for several days. The chemical composition of the plug is still unknown. Biochemical studies are underway to determine whether the plug contains compounds compatible with the chemical composition of the jelly-like secretory material of the male accessory glands, possibly modified by enzyme activity of the bursa copulatrix epithelium.

Authors contributions

Romano Dallai: Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing - Review & Editing, Supervision. **David Mercati:** Investigation, Writing - Review & Editing, Visualization; **Pietro Paolo Fanciulli:** Writing - Review & Editing. **Pietro Lupetti:** Writing - Review & Editing.

Acknowledgements

We thank Dr Saverio Rocchi of the Museum “La Specola”, Florence (Italy) for identifying the species studied. We also thank Helen Ampt for revising the English.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.asd.2023.101250.

References

Aiken, R.B., 1992. The mating behaviour of a boreal water beetle, *Dytiscus alaskanus* (Coleoptera Dytiscidae). Ethol. Ecol. Evol. 4, 245–254.
Alcock, J., 1993. Animal Behavior: an Evolutionary Approach, fifth ed. Sinauer Associates, Sunderland, Massachusetts, p. 620.
Andersson, M., 1994. Sexual Selection. Princeton University Press, Princeton, New Jersey, p. 624.
Angus, R.B., 1985. *Suphrodytes* des gozis a valid genus, not a subgenus of *Hydroporus* clairville (Coleoptera: Dytiscidae). Entomol. Scand. 16, 269–275.
Arnqvist, G., Rowe, L., 2005. Sexual Conflict. Princeton University Press, Princeton, NJ, p. 352.
Baer, B., Morgan, E.D., Schmid-Hempel, P., 2001. A nonspecific fatty acid within the bumblebee mating plug prevents females from remating. Proc. Natl. Acad. Sci. U.S.A. 98 (7), 3926–3928.
Biström, O., 1979. A revision of the genus *derovatellus* sharp (Coleoptera, Dytiscidae) in africa. Acta Entomol. Fenn. 35, 1–28.
Biström, O., 1980. Notes on non-african *derovatellus* species (Coleoptera: Dytiscidae). Entomol. Scand. 11, 78–80.
Blum, M.S., Glowska, S.Z., Taber III., S., 1962. Chemistry of the drone system. II. Carbohydrates in the reproductive organs and semen. Ann. Entomol. Soc. Am. 55, 135–139.
Burmeister, E.G., 1976. Der Ovipositor der Hydradephaga (Coleoptera) und seine phylogenetische Bedeutung unter besonderer Berücksichtigung der Dytiscidae. Zoomorphologie 85, 165–257.
Burmeister, E.G., 1990. On the systematic position of Amphizoidae, emphasizing features of the female genital organs (Insecta: Coleoptera: adephaga). Quaest. Entomol. 26, 245–272.
De Marzo, L., 1997. Revisione anatomiva della spermateca nei Ditiscidi (Coleoptera). Entomol.-Bari 31, 207–219.
Devine, M.C., 1975. Copulatory plugs in snakes: enforced chastity. Science 187, 844–845.
Devine, M.C., 1977. Copulatory plugs, restricted mating opportunities and reproductive competition among male garter snakes. Nature 267, 345–346.
Dickinson, J.L., Rutowski, R.L., 1989. The function of the mating plug in the chalcedon checkerspot butterfly. Anim. Behav. 38, 154–162.
Eberhard, W.G., 1996. Female Control: Sexual Selection by Cryptic Female Choice. Princeton University Press, Princeton, New Jersey, p. 472.
Franciscolo, M.E., 1979. Coleoptera. Haliplidae, Hygrobiidae, Gyrinidae. Dytiscidae. E. Calderini, Bologna, p. 804.
Giglioli, M.E.C., Mason, G.F., 1966. The mating plug in anopheline mosquitoes. Proc. R. Ent. Soc. Lond. 41, 123–129.
Gilbert, L.E., 1976. Postmating female odor in Heliconius butterflies: a male-contributed antiaphrodisiac? Science 193, 419–420.
Higginson, D.M., Pitnick, S., 2011. Evolution of intra-ejaculate sperm interactions: do sperm cooperate? Biol. Rev. 87, 249–270.
Higginson, D.M., Miller, K.B., Segraves, K.A., Pitnick, S., 2012a. Female reproductive tract form drives the evolution of complex sperm morphology. Proc. Natl. Acad. Sci. U.S.A. 109 (12), 4538–4543.
Higginson, D.M., Miller, K.B., Segraves, K.A., Pitnick, S., 2012b. Convergence, recurrence and diversification of complex sperm traits in diving beetles (Dytiscidae). Evolution 66 (5), 1650–1661.
Karlsson Green, K., Kovalev, A., Svensson, E.I., Gorb, S.N., 2013. Male clasping ability, female polymorphism and sexual conflict: fine-scale elytral morphology as a sexually antagonistic adaptation in female diving beetles. J. R. Soc. Interface 10, 20130409.
Lüpold, S., Pitnick, S., 2018. Sperm form and function: what do we know about the role of sexual selection? Reproduction 155 (5), R229–R243.
Mazzoldi, P., 1996. Spermathecal structure in *canthyporus* zimmermann

(Coleoptera, Dytiscidae). Entomol. Basiliensia 19, 593–619.
McDonough-Goldstein, C.E., Pitnick, S., Dorus, S., 2022. Drosophila female reproductive glands contribute to mating plug composition and the timing of sperm ejection. Proc. R. Soc. B 289, 20212213.
Meslin, C., Cherwin, T.S., Plakke, M.S., Hill, J., Small, B.S., Goetz, B.J., Wheat, C.W., Morehouse, N.I., Clark, N.L., 2017. Structural complexity and molecular heterogeneity of a butterfly ejaculate reflect a complex history of selection. Proc. Natl. Acad. Sci. USA 114, E5406–E5413.
Miller, K.B., 2001a. On the phylogeny of the Dytiscidae (Insecta: Coleoptera) with emphasis on the morphology of the female reproductive system. Insect Systemat. Evol. 32 (1), 45–89.
Miller, K.B., 2001b. Descriptions of new species of *Desmopachria* Babington, 1841 (Coleoptera: Dytiscidae: Hydroporinae: hyphydrini) with a reassessment of the subgenera and species groups and a synopsis of the species. Coleopt. Bull. 55, 219–240.
Miller, K.B., 2001c. Revision of the neotropical genus *hemibidessus* zimmermann, 1921 (Coleoptera: Dytiscidae: hydroporinae: bidessini). Aquat. Insects 23 (4), 253–275.
Miller, K.B., 2009. On the systematic of Noteridae (Coleoptera: adephaga: Hydradephaga): phylogeny, description of a new tribe, genus and species, and survey of female genital morphology. Syst. Biodivers. 7, 191–214.
Miller, K.B., Bergsten, J., 2014. Predaceous diving beetle sexual system. In: Yee, D.A. (Ed.), Ecology, Systematics, and Natural History of Predaceous Diving Beetles (Coleoptera: Dytiscidae). Springer, New York, pp. 199–234.
Mercati, D., Fanciulli, P.P., Lupetti, P., Dallai, R., 2023. The sperm structure of the diving beetles *Stictonectes optatus* (Seidlitz, 1887) and *Scarodytes halensis* (Fabricius, 1787) (Dytiscidae, Hydroporinae) with evidence of a spermatostyle in the sperm conjugation. Micron 166, 103412.
Ordish, R.C., 1985. A review of spermathecal structure in New Zealand dytiscids. Proc. Acad. Nat. Sci. Phila. 137, 128–131.
Ordish, R.G., 1966. A systematic revision of the New Zealand water beetles (Coleoptera: Dytiscidae). N. Z. J. Zool 2, 1–10.
Parker, G.A., 1970. Sperm competition and its evolutionary consequences in the insects. Biol. Rev. 45, 526–567.
Parker, G.A., 1979. Sexual selection and sexual conflict. In: Blum, M.S., Blum, N.A. (Eds.), Sexual Selection and Reproductive Competition in Insects. Academic Press, New York, pp. 123–166.
Parker, G.A., Smith, J.L., 1975. Sperm competition and the evolution of the precopulatory passive phase behaviour in *Locusta migratoria migratorioides*. J. Entomol. Ser. A Gen. Entomol. 49, 155–171.
Pitnick, S., Hosken, D.J., Birkhead, T.R., 2009. Sperm morphological diversity. In: Birkhead, T.R., Hosken, D.J., Pitnick, S. (Eds.), Sperm Biology, an Evolutionary Perspective. Academic Press, San Diego, CA, pp. 69–149.
Polak, M., Starmer, W.T., Barker, J.S.F., 1998. A mating plug and male mate choice in Drosophila hibisci Bock. Anim. Behav. 56, 919–926.
Polak, M., Wolf, L.L., Starmer, W.T., Barker, J.S.F., 2001. Function of the mating plug in Drosophila hibisci Bock. Behav. Ecol. Sociobiol. 49 (2/3), 196–205.
Quennedey, A., 1972. Les glandes exocrines des termites. III. Structure fine de la glande sternale de *Trinervitermes geminatus* Wasman (Termitidae, Nasutitermitinae). Z. Zellforsch 130, 205–218.
Quennedey, A., 1998. Insect epidermal gland cells: ultrastructure and morphogenesis. In: Microscopic Anatomy of Invertebrates, 11A. Insecta, Wiley-Liss, Inc., pp. 177–207
Rowe, L., Houle, D., 1996. The lek paradox and the capture of genetic variance by condition dependent traits. Proc. Roy. Soc. Lond. B. 2631415–2631421.
Schneider, M.R., Mangels, R., Dean, M.D., 2016. The molecular basis and reproductive function(s) of copulatory plugs. Mol. Reprod. Dev. 83, 755–767.
Waage, J.K., 1979. Dual function of the damselfly penis: sperm removal and transfer. Science 203, 916–918.