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The sperm structure of *Clinidium canaliculatum* (Costa): A contribution to the systematic position of Rhysodidae (Coleoptera: Carabidae)

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ABSTRACT

The systematic position and the phylogenetic relationship of Rhysodidae members is still debated, with some authors considering the group as a separate family of Adephaga, while for others they could be a subfamily of Carabidae. The group have morphological traits quite different from Carabidae and an aberrant behaviour compared to ground beetles being not predaceous. The sperm ultrastructure of *C. canaliculatum* was studied comparatively with other species of beetles, Carabidae in particular. The results indicate that the sperm structure of this species is similar to that of the Carabinae species. As in these species, *C. canaliculatum* has sperm conjugates with an apical conical cap protecting the heads and the initial region of flagella. This sperm appearance is also shared by another species of Rhysodidae, *Omoglymmius hamatus*. The material of the apical cap consists of an electron-dense material with a peculiar outer net configuration. Many species of Carabidae, however, can present a different type of sperm conjugation, the spermatostyle: a long rod-like structure where the individual sperms have only the most apical part inserted in the cortical area and the flagella are completely free. *C. canaliculatum* sperm are endowed with a mono-layered acrosome, a nucleus of variable shape along its length, a flagellum consisting of a typical axoneme 9 + 9+2, provided with 16 protofilaments in the tubular wall of accessory tubules, two asymmetric mitochondrial derivatives with the left one larger than the opposite one, and the right accessory body elongated and larger than the opposite one. These sperm characteristics, which are shared also by another member of the group, suggest the demotion of the family Rhysodidae to the subfamily Rhysodinae within Carabidae, a result also supported by recent molecular data.

1. Introduction

Rhysodidae is the greatest enigma in Adephaga systematics (Beutel et al., 2008). The group has a quite different larval and adult morphology compared to members of Carabidae. Moreover, they exhibit an aberrant behaviour compared to ground beetles, being not predaceous insects but rather using slime moulds (Myxomycetes, now Mycetozoa) as a food source (Bell and Bell, 1962; Beutel, 1993, 2016; Bell, 1998; Giglio et al., 2022). Some authors considered the group as a tribe within the family Carabidae (Crowson, 1955; Lawrence and Newton, 1995). The taxonomic rank was based on a series of autapomorphies connected to the life habits of larval and adult members which live on rotting wood (Bell and Bell, 1991; Beutel, 2016; Beutel et al., 2020).

Molecular data (McKenna et al., 2015, 2019) suggested a position of Rhysodidae close to the root of Carabidae, while Hunt et al. (2007), López-López and Vogler (2017), Baca et al. (2021) and Vasilikopoulos et al. (2021) indicated for the group the rank of subfamily Rhysodinae.

A series of ultrastructural studies on spermatozoa from numerous carabid species have clarified that the sperm can be organized according to different patterns, some carabid beetles showing isolated sperm (singleton sperm) but the majority have conjugated sperm with a variable sperm number (Takami and Sota, 2007; Hodgson et al., 2013; Schubert et al., 2017; Dallai et al., 2019a, 2020; Gomez and Maddison, 2020).

When sperm are conjugated they can be taken together by an apical cap (the simple rod−type described in Gomez and Maddison,

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2020) as in Carabinae (Dallai et al., 2019a, 2020), or inserted into the cortical part of elongated rod-like structure, the spermatostyle, one by one (the sheet-type of Gomez and Maddison, 2020). The spermatostyles are, however, remarkably variable among the species either in length and width and also on the modality of the sperm attachment to the elongated structure (Dallai et al., 2019a, 2020; Gomez and Maddison, 2020). In both types of sperm conjugates these structures are secreted by the epithelial cells of deferent ducts (Hodgson et al., 2013; Dallai et al., 2019a). Sperm conjugation with a spermatostyle appears to have been present very early in the carabid evolution. A similar result was described in the diving beetles (Dytiscidae) and even in other Adephaga in which sperm conjugation was found in the ancestral condition (Higginson and Pitnick, 2011). Several basal lineages of Carabidae have conjugated sperm with apical cap or spermatostyle and this suggests an early origin of this characteristic (Maddison et al., 2009). Nebriinae, Cicindelinae, many Trechinae, Apotominae, and the tribe Paussini are exceptions to this evidence showing single sperm in their male deferent ducts. Singleton sperm, however, are present in several other species of Carabidae together with sperm conjugates and spermatostyles (Gomez and Maddison, 2020).

The aim of our work is to explore the sperm ultrastructure of *Clinidium canaliculatum* (Costa) being aware that sperm morphology has been successfully used to infer the phylogenetic position of taxa (Jamieson et al., 1999; Dallai, 2014; Dallai et al., 2016).

2. Material and methods

2.1. Specimens

Males of *C. canaliculatum* (Costa) were hand-collected under rotten pine barks in the Sila National park (39° 21' 16,79″ N, 16° 37,57' 64″ E), San Giovanni in Fiore, Cosenza, Southern Italy in June 2023. Males and females of *Harpalus affinis* (Schrank) were collected by R.A. Gomez in the Farma Natural Reserve, Siena (43° 04,55' 69″ N, 11° 12' 13,76" E) in October 2022. The sperm of three other species of Carabidae (*Calathus fuscipes latus* Serville, *Brachinus italicus*, Dejean and *Carabus preslii*) were from material already studied by Dallai et al. (2019a, 2020).

2.2. Fluorescence microscopy

Bundles of sperm of *C. canaliculatum* were isolated from deferent ducts of adult males. The material was placed on a microscope slide in a small drop of 0.1 M phosphate buffer (pH 7.2) to which 3 % of sucrose was previously added (PB). DNA was stained for fluorescence observations by incubating the sperm bundles for 3–4 min in Hoechst 33258 1 μg/ml (Sigma). The material was then mounted in a drop of 90 % glycerol. The sperm bundles were observed and photographed using interference contrast and epifluorescent microscopy with a Leica DMRB microscope equipped with AxioCam HR camera (Carl Zeiss).

2.3. Scanning electron microscopy (SEM)

For SEM study, *C. canaliculatum* sperm bundles were spread onto glass coverslips previously treated with 1 % Poly-L-lysine. The coverslips were placed in 2.5 % glutaraldehyde in PB for 30 min at 4 °C and then rinsed several times in PB. Specimens on glass coverslips were dehydrated in a graded series of ethanol and then processed by the critical point drying method in a Balzer's CDP 030 unit. The coverslips were sputtered with about 20 nm gold in a Balzer's MED 010 sputtering device and finally observed in a Quanta 400 SEM (FEI Company) operating at electron accelerating voltage of 20 kV.

2.4. Transmission electron microscopy (TEM)

For TEM observations, adult males of *C. canaliculatum* and *H. affinis* were dissected in PB to isolate the testes and deferent ducts. The material was fixed overnight in 2.5 % glutaraldehyde in PB. After careful rinsing, the material was post-fixed in 1 % osmium tetroxide for 2 h. After rinsing, the material was dehydrated with ethanol series (50%–100 %), then transferred to propylene oxide and finally embedded in a mixture of Epon-Araldite epoxy resins. Part of material was also treated with tannic acid, omitting osmium fixation, according to Dallai and Afzelius (1990). Ultrathin sections were obtained with a Reichert Ultracut IIE ultramicrotome, routinely stained with uranyl acetate and lead citrate and observed in a TEM Philips CM10 operating at electron accelerating voltage of 80 kV.

3. Results

3.1. C. canaliculatum

The male genital apparatus of *C. canaliculatum* is provided with helical unifollicular testes with several germ cysts in their apical region. Each cyst contains about 64 germ cells as results of 2^6 cell divisions (Fig. 1A). Cysts in mid-stage spermiogenesis contain elongated early spermatids measuring 1.3 μm wide in cross section (Fig. 1C and D), with apical flattened mono-layered acrosomes overlying the polygonal nucleus that, in cross section, has a variable shape along its length. The nucleus measures about 0.26–0.34 μm and shows an irregular outline; then it increases its diameter up to 0.82 μm becoming elliptical (Fig. 1C). At this stage, it is surrounded by a layer of microtubules (Fig. 1C and D). In a posterior nuclear infolding is housed the centriole, embedded in a scanty centriole adjunct material. A cross section through this level reveals a centriole, a small nucleus and a mitochondrial derivative (Fig. 1D). Beneath this region, in a cross section, the presence of a conventional 9 + 9+2 flagellar axoneme and two elongated mitochondrial derivatives of different size are visible (Fig. 1E). The one on the left side is clearly larger than the other and it contains an electron-dense matrix, particularly on the side facing the axoneme. In cross section, the two mitochondrial derivatives have a width of 0.16–0.25 μm and 0.10–0.16 μm, respectively. These structures are surrounded by a layer of microtubules (Fig. 1E) and are 0.50 μm in largest diameter. Two accessory bodies are also visible with that one on the right side of the axoneme elongated, banana shaped, made of electron-dense material. On the contrary, on the opposite side of the axoneme, only a small electron-dense dot is visible (Fig. 1E).

Once spermiogenesis is complete, in the proximal region of deferent ducts, at the level of thin diverticula (Fig. 2B), possibly seminal vesicles, the sperm cells are 280–300 μm long and have a nucleus 20 μm long (Fig. 2G). About 45–60 elements are assembled into bundles in which the sperm are apically embedded with their head region and a short tract of the flagella into a moderately electron-dense conical structure about 25–30 μm long and 2.6–4.0 μm wide (Fig. 2A, C-F, 4A).

When the sperm bundles are observed after Hoechst staining, they appear strongly fluorescent in their anterior region corresponding to the conical structure containing the sperm nuclei (Fig. 2C and D). At the scanning electron microscope, the sperm bundles show that the apical conical structure can have a different length and shows a smooth surface. The sperm flagella are extruding from the posterior region of the structure at different levels (Fig. 2E and F).

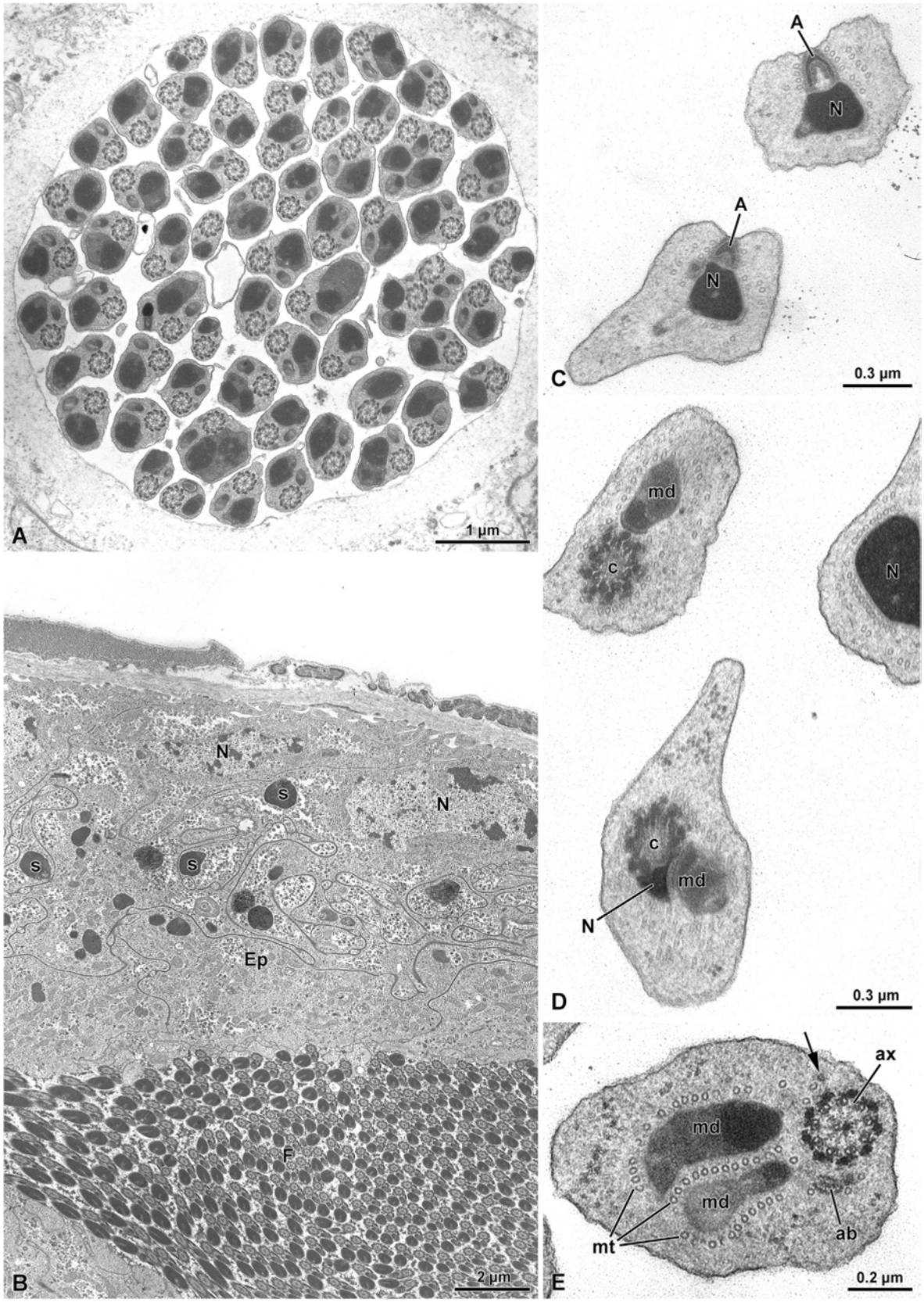


Fig. 1. **A**) Cross section through a cyst with 64 sperm. **B**) Cross section of the deferent duct to show the numerous sperm flagella (F) in the lumen. The cytoplasm of the epithelial cells (Ep) contains several electron-dense secretions (s). N, nuclei. **C**) Cross section of spermatid cells showing the apical acrosome (A) and the polygonal nucleus (N). **D**) Cross section of a spermatid showing the centriolar region with the centriole (c) a mitochondrial derivative (md) and a small portion of the nucleus (N). **E**) Cross section through spermatids showing the axoneme (ax), the two mitochondrial derivatives of different size (md) and the right accessory body (ab). On the left side of the axoneme, only a small electron-dense dot is visible (arrow). The structures are surrounded by microtubules (mt).

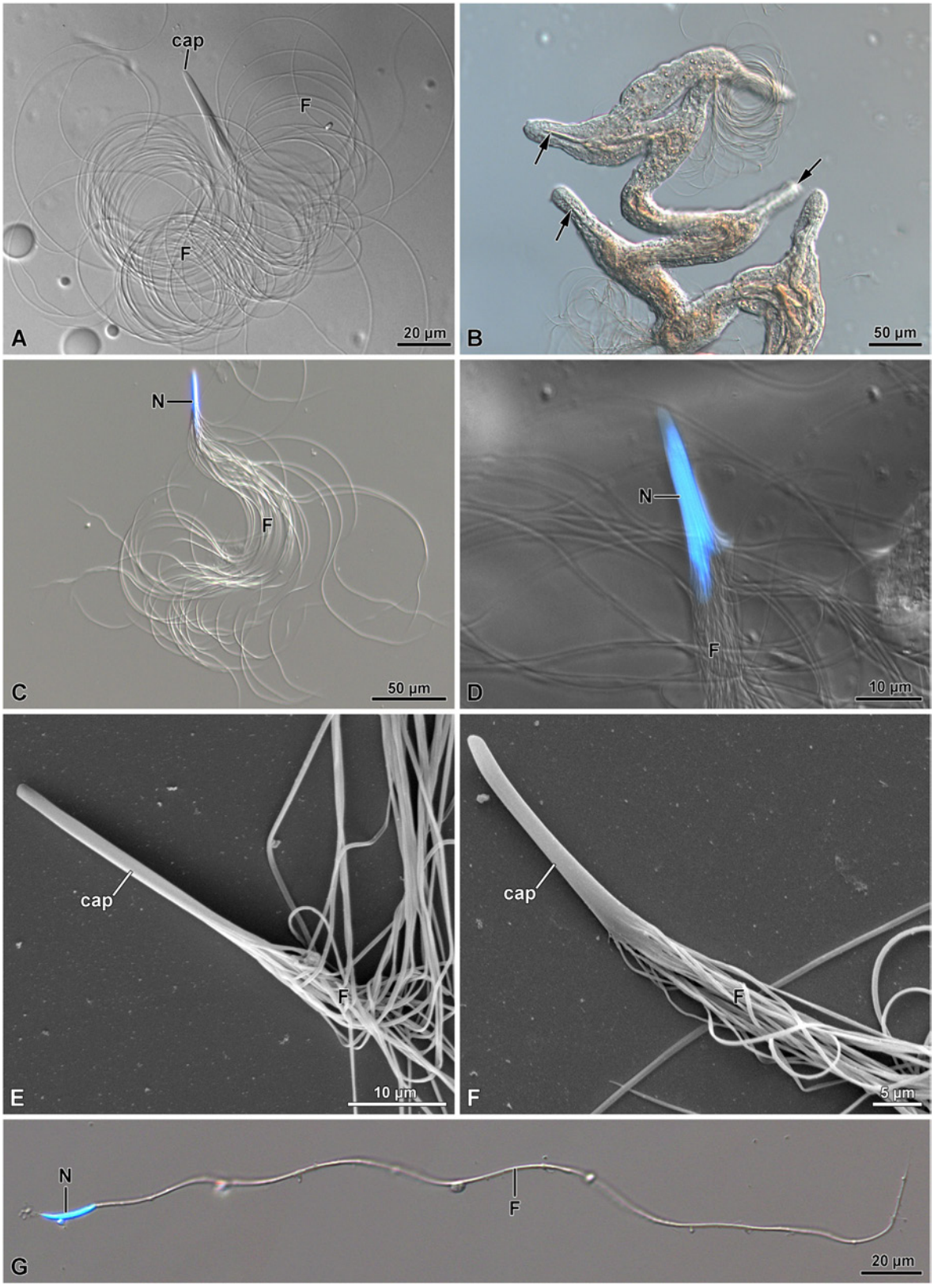


Fig. 2. **A**) Sperm bundle at interference contrast microscope showing the long conical cap (cap) and the flagella (F). **B**) The proximal region of deferent duct with the sperm bundles (arrows) in the duct lumen. **C**-**D**) Hoechst staining of the sperm bundle to show the position of nuclei (N). F, flagella. **E**-**F**) Scanning electron micrographs of the anterior region of the sperm bundles protected by the smooth cap (cap). From the posterior regions of the structures many flagella (F) are visible. **G**) An isolated sperm after Hoechst staining. N, Nucleus; F, flagellum.

The conical structure at the apex of each sperm bundle has an elliptical shape in cross section and its width varies along its length from 2.6 μm in the distal end to 4.0 μm towards the posterior region (Fig. 2A–F, 3, 4A-D, 5E). The structure that holds sperm together consists of a very elaborated material, particularly at its periphery. In cross section it appears as a fine lace, 0.16 μm thick at half length of the structure, reaching up to 0.23 μm in the apical region (Fig. 4B–D, 5E). Further posteriorly, the region, 4.0 μm wide, is interested by the progressive disappearance of the material embedding the sperm; it is also characterized by the ends of nuclei and the beginning of the flagella (Fig. 4A–E). In the most anterior region of the conical structure, the acrosome can be observed in longitudinal section, consisting of 1.1 μm long acrosomal vesicle containing a uniform electron-dense material, which extends posteriorly to surround the nuclear apex (Fig. 5A and B). In a cross section the acrosome is a flat structure 0.30 μm x 50–70 nm that shows in the centre the nuclear apex, only 23 nm in diameter (Fig. 4B).

At a more posterior level the nucleus increases its diameter up to 0.14 μm wide becoming elliptical in cross section (Figs. 3 and 4C). Further posteriorly, the nucleus takes a triangular shape in cross section and is 0.31 μm wide. This size is maintained up to its end (Figs. 3 and 4D, E). At this level, the nucleus shows, in longitudinal section, two infoldings. A mitochondrial derivative is hosted in the larger cavity, while the smaller one contains the centriole from which the flagellum will originate (Fig. 5C and D). When observed in cross section, these two infoldings are positioned at the opposite side (Fig. 5E).

The flagellar axoneme has the typical 9 + 9+2 complex of microtubules (Fig. 1E, 4E and 6). After the tannic acid preparation of the material, it was observed that the accessory tubules have 16 protofilaments, as is common among insects (Fig. 7). The axoneme apparently maintains its regular pattern up to the tail end. The flagellum, in cross section, shows two mitochondrial derivatives; the one on the left side of the axoneme is larger than the opposite one, it has an ovoidal shape (0.33 × 0.27 μm) and is filled with a homogeneous electron-dense material exhibiting a crystallization well visible only after tannic acid preparation (Figs. 6 and 7). The second mitochondrial derivative, which is at the right side of the axoneme, has a significantly reduced size compared to the opposite one and is only 0.18 × 0.1 μm wide (Figs. 6 and 7).

Two accessory bodies are located between the axoneme and the mitochondrial derivatives. They also have a different size. That one on the right side is elongated and it looks like an electron-dense rod (0.1 μm long and 33 nm wide) adhering to the plasma membrane, while, at the left side of the axoneme only a small electron dense spot surrounded by a cloud of less electron-dense material is visible (Figs. 6 and 7).

Towards the end of the flagellum the larger mitochondrial derivative reduces its size, thus becoming similar to the other one. At

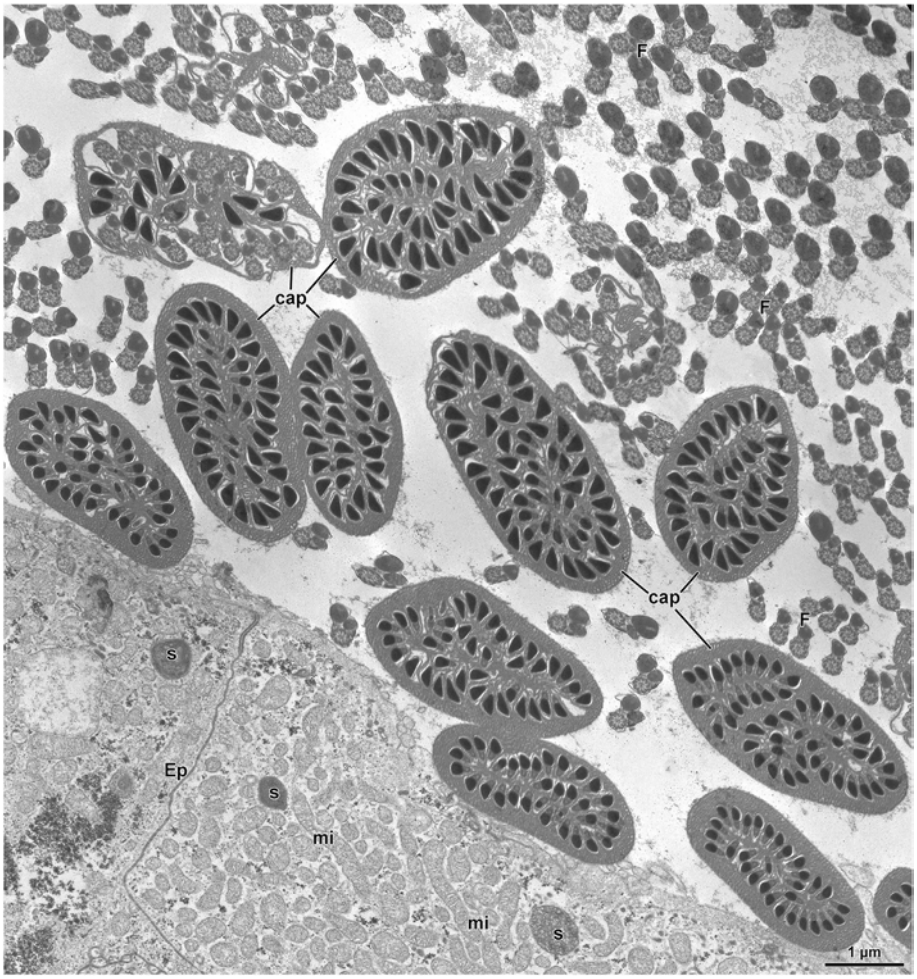
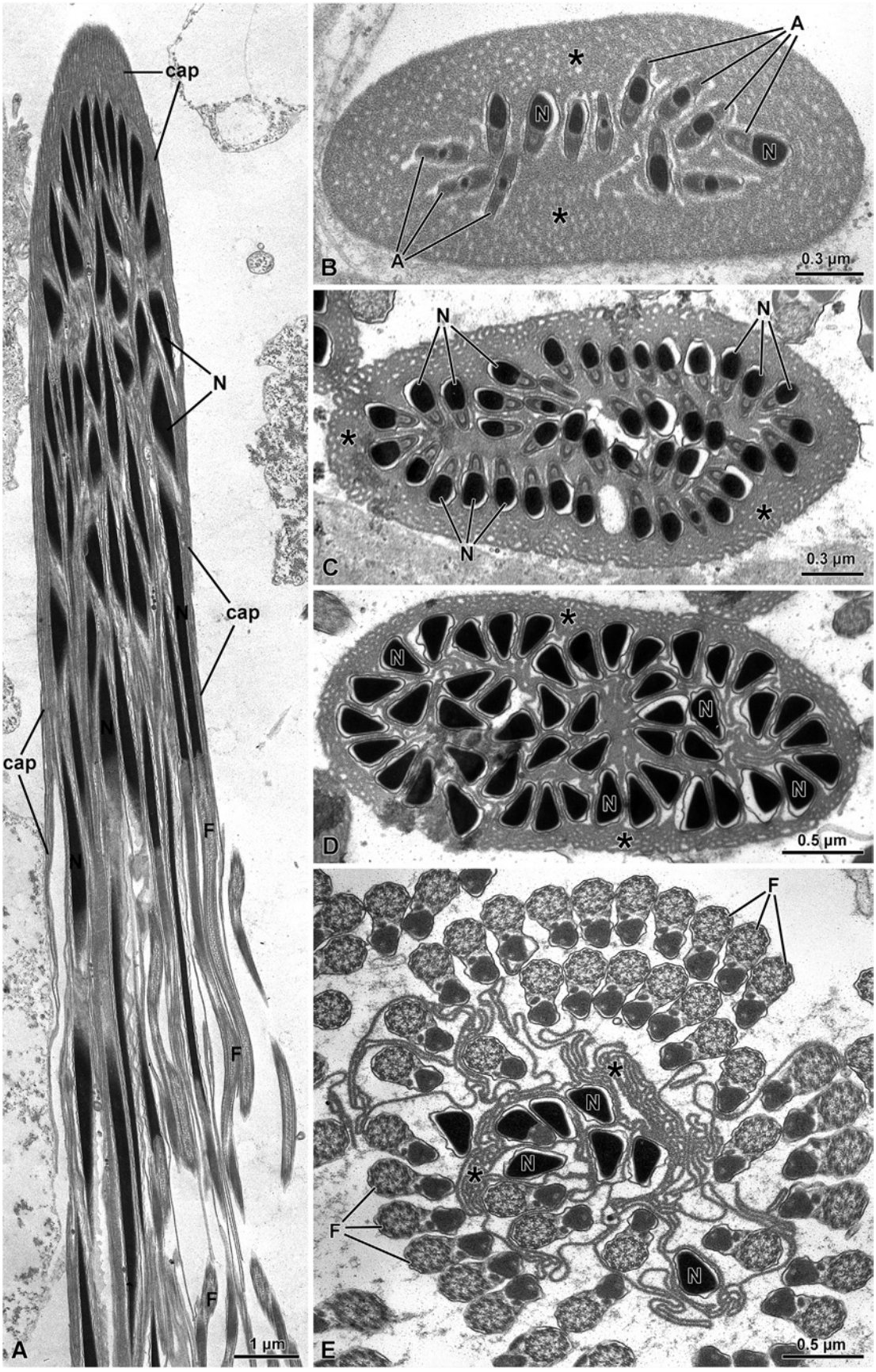


Fig. 3. Cross section through a deferent duct lumen to show several elliptical cap structures (cap) and the numerous flagella (F) close to the epithelial cells (Ep). mi, mitochondria; s, cell secretions.


cap
N
cap
cap
N
F
F
F
1 μm
A
A
N
N
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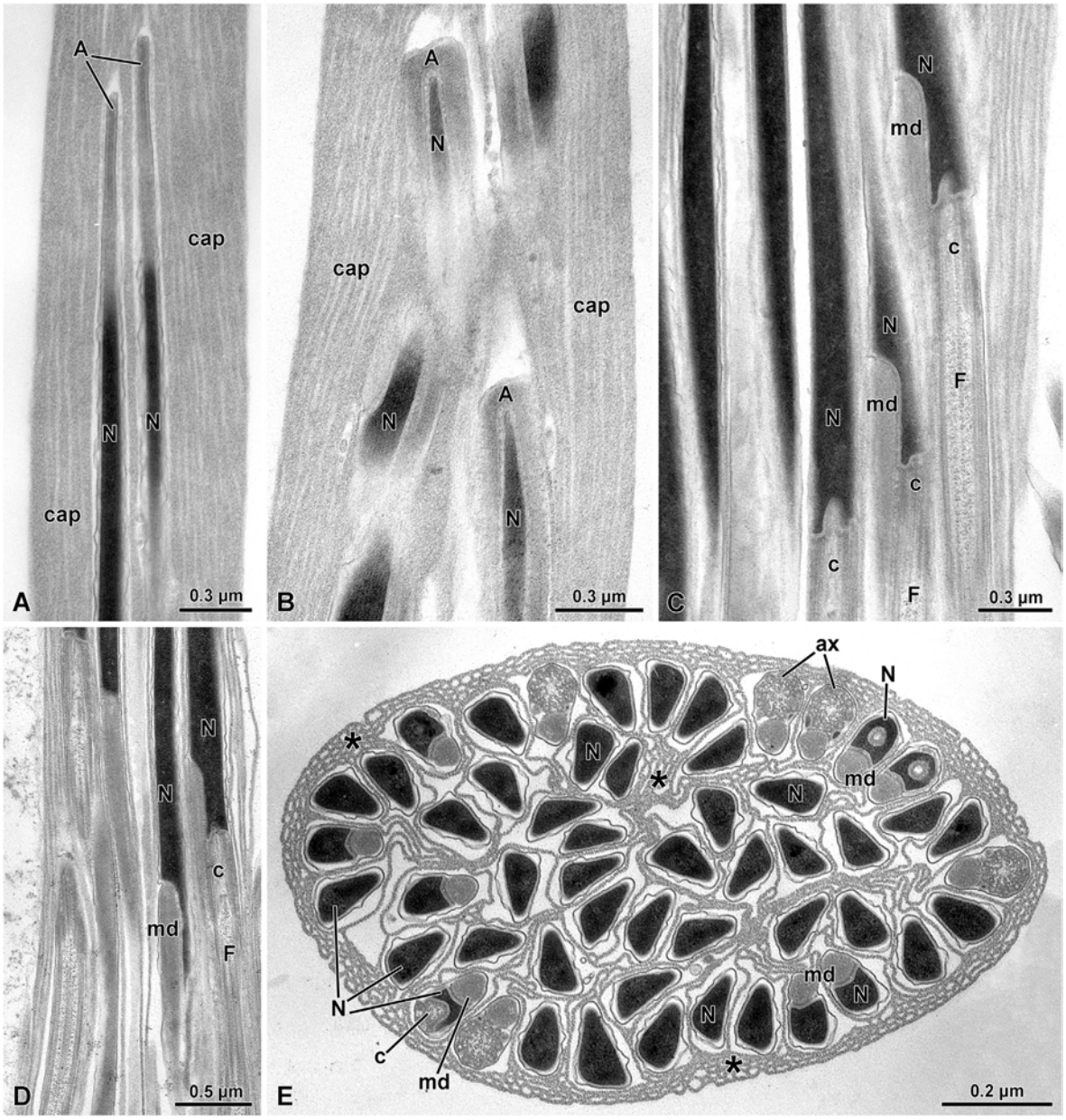

Fig. 5. **A**-**B**) Longitudinal section of the apical conical cap (cap). Note the mono-layered acrosome (A), devoid of inner perforatorium, covering the apical nucleus (N). **C**-**D**) Longitudinal sections of the transition region between nucleus (N) and flagellum (F). The basal region of nucleus shows two infoldings in the deeper of which is hosted the larger mitochondrial derivative (md), while, in the other small infolding is placed the centriole (c) from which start the flagellum (F). **E**) Cross section through the basal region of the conical cap protecting the sperm bundle. Note the triangular nuclei (N), the centrioles (c) and the beginning of the larger mitochondrial derivative (md) hosted in the nuclear infoldings. The elaborated material of the cortical region of the cap is well visible (asterisks).

the flagellum end, the two mitochondrial derivatives are no longer visible.

3.2. *H. affinis*

The sperm structure of *H. affinis* is quite similar to that of previously studied Carabinae provided with spermatostyle. The heads of the spermatozoa are inserted inside cylindrical structures of different sizes (the spermatostyles) while the flagella remain strictly adherent to the peripheral region of these structures for long traits before being free (Fig. 8D).

4. Discussion

The systematic position of Rhysodidae, the wrinkled bark beetles, is one of the highest debated point of Geadephaga taxonomists (Jeannel, 1941; Crowson, 1955, 1967, 1981; Nagel, 1979; Beutel, 1992, 2016; Makarov, 2008; Bousquet, 2012; Beutel et al., 2019;

Fig. 4. **A**) Longitudinal section through a whole cap conical structure (cap). The nuclei (N) are visible within the cap, while the flagella (F) exit from the basal region and are free. **B**-**E**) Cross sections through the different levels of the conical cap starting from the apical (**B**) to the posterior region (**E**). The apical acrosomes (A), the different shapes of nuclei (N) and flagella (F) coming out from the conical structure are visible. Note the electron-dense material forming the cap (asterisk).

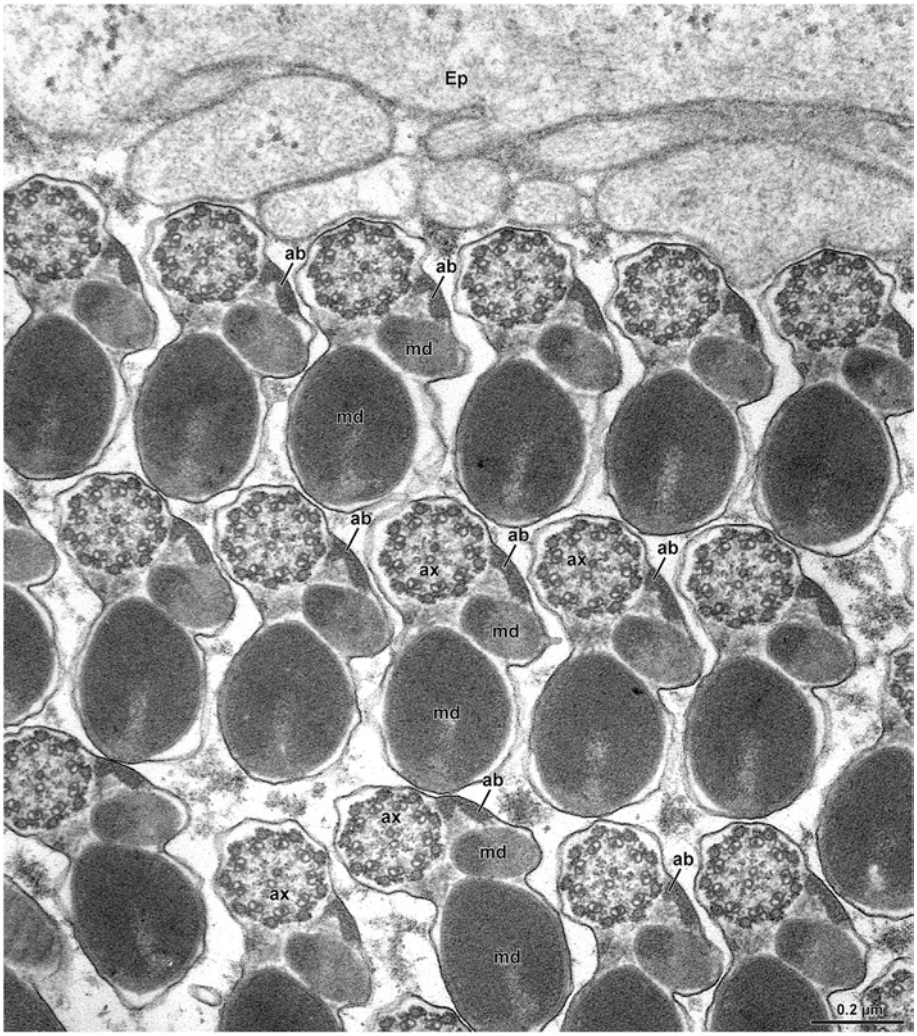

Fig. 6. Cross section through flagellar sperm bundle. Note the axoneme (ax), the large right accessory body (ab), the two mitochondrial derivatives (md) the left of which is larger than the other, Ep, epithelial cell.

Brandmayr, 2021). The discussion deals with whether the systematic position of the group must be outside Carabidae or it is a member of the family with a rank of subfamily or another rank (Bell and Bell, 1962, 1978; Reichardt, 1977; Bell, 1998; Maddison et al., 1999, 2009; Ober, 2002; Hunt et al., 2007; Beutel et al., 2008, 2020; López-López and Vogler, 2017; Baca et al., 2021; Vasilikopoulos et al., 2021).

Among ground beetles, as indicated by numerous results obtained by several authors, the sperm can be found as single cells (singleton) or, more often, as sperm bundles with the conjugated sperm 1) showing an apical cap that protects the sperm head region and sometimes also the first flagellar tract or 2) with the sperm cells attached with their anterior region to a more or less long rod, the spermatostyle described by Breland and Simmons (1970) in the whirligig *Dineutus* sp. The ultrastructural appearance of the spermatostyle can be quite different, some species showing a long rod that in cross-section has a peripheral electron-dense layer, as in *B. italicus* (Dallai et al., 2020) (Fig. 8C) or *Pterostichus nigrita* and *P. morio* (Hodgson et al., 2013; Dallai et al., 2019a) or a sort of peripheral electron-dense net where group of sperm are enveloped as in *C. fuscipes latus* (Dallai et al., 2020) and *H. affinis* (Fig. 8A, B, D). Other different arrays of the sperm attachments, however, are also possible (Dallai et al., 2019a, 2020; Gomez and Maddison, 2020). The two structures of sperm bundle protection are secreted by the epithelial cell of the deferent ducts as it occurs in other Adephaga (Breland and Simmons, 1970; Salazar et al., 2022). Sometimes, as in Broscinae, both individual sperm and sperm conjugates with spermatostyle were observed (Gomez and Maddison, 2020; Sasakawa, 2020).

Despite the different ways in which sperm can be conjugated, the chemical composition of the structure that holds sperm together remains uncertain; it is supposed to be constituted by polysaccharides and proteins (Hodgson et al., 2013; Schubert et al., 2017; Salazar et al., 2022).

The sperm of *C. canaliculatum* belong to the first type of conjugation as their anterior head region is protected by a long conical structure, the cap, made by a quite elaborated secretion. A similar organization was also described in *Omoglymmius hamatus*, another member of Rhysodidae (Gomez and Maddison, 2020).

An important aspect characterizing carabid sperm is the relative position of the sperm components within the cells, with many species showing the centriole in an anterior position with the flagellar axoneme running parallel to the nucleus, sometimes up to the tail end. This unusual position of the nucleus does not seem to be due to the anterior shifting of the basal body as described in *Cicindela campestris* (Werner, 1965), nor it depends on the nuclear

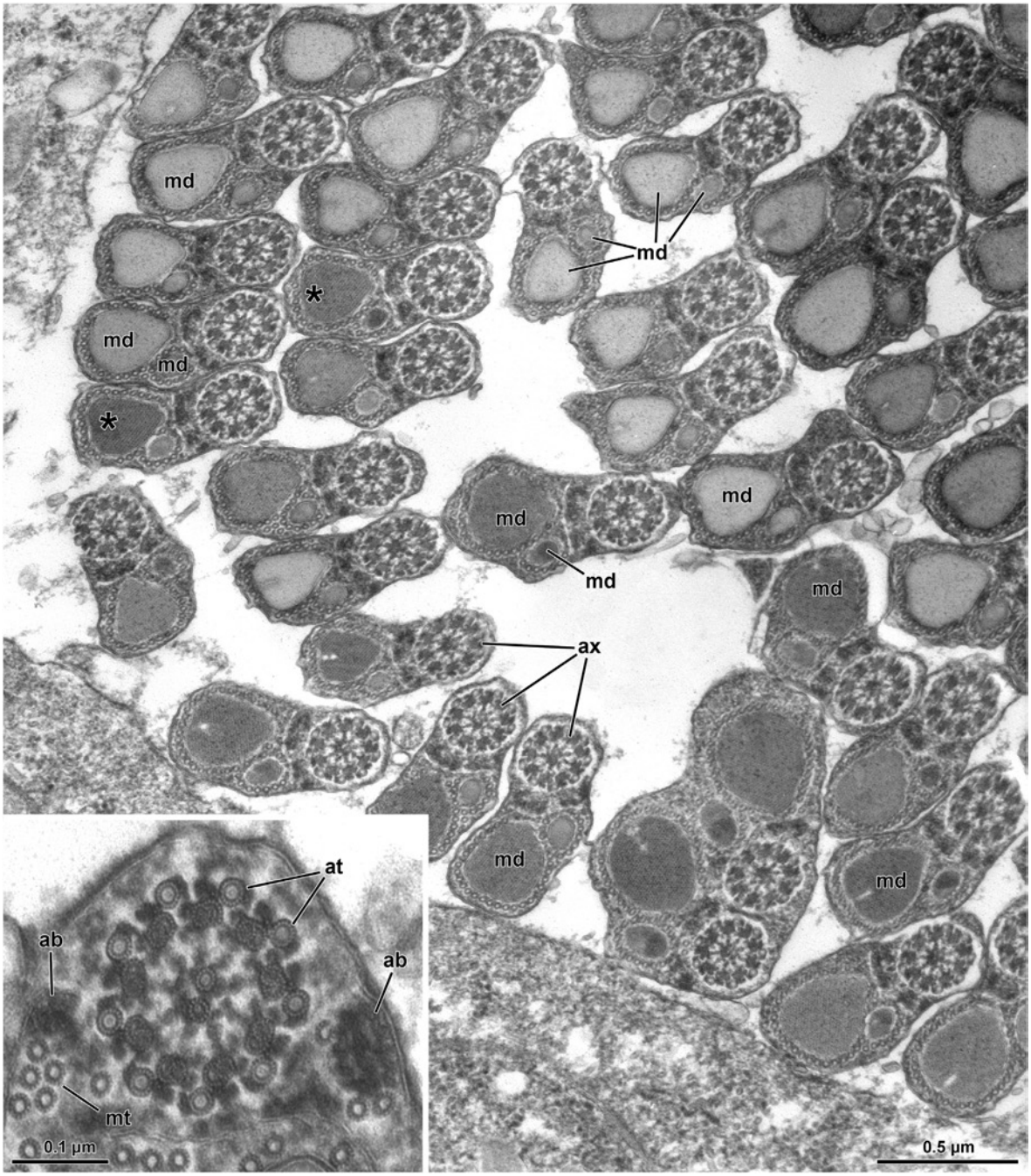

Fig. 7. Cross section of a mature spermatid bundle after tannic acid preparation. ax, axoneme; md, mitochondrial derivatives with inner crystallization. In the inset, note the 16 protofilaments in the tubular wall of the accessory tubules (at) and the layer of microtubules (mt) surrounding the structures. ab, accessory body.

length as result of the large number of chromosomes of Carabidae (White, 1973; Serrano and Galián, 1998). It is evident that such anterior position of the nucleus could have some consequences in the functional sperm motility. The peculiar anterior position of the centriole and of the axoneme in the sperm was recently described in the ladybirds (Coccinellidae) (Dallai et al., 2018, 2019b).

Among the species with sperm conjugated, only Carabini have a relatively short nucleus in a regular anterior position and the flagellum running posteriorly (Dallai et al., 2019a). Also *C. canaliculatum* and *O. hamatus*, have spermatozoa with anterior nucleus and posterior flagella similar to *Carabus* species (Dallai et al., 2019a; Gomez and Maddison, 2020) (Fig. 9A–D). Apparently other members of Carabidae could have a similar pattern in their spermatozoa (see Gomez and Maddison, 2020) and it would be important to further verify this aspect at ultrastructural level.

At the most anterior region, *C. canaliculatum* sperm have a relatively long mono-layered acrosome, quite similar to that of all the Carabidae studied so far. The presence of a simple type of acrosome is retained an evolutionary simplification (Baccetti, 1979). The disappearance of the inner perforatorium in the acrosome of all Carabidae, and also in *C. canaliculatum*, could be considered an apomorphy of the family, as Dytiscidae and Gyrinidae have a bi-layered acrosome with a perforatorium (Afzelius

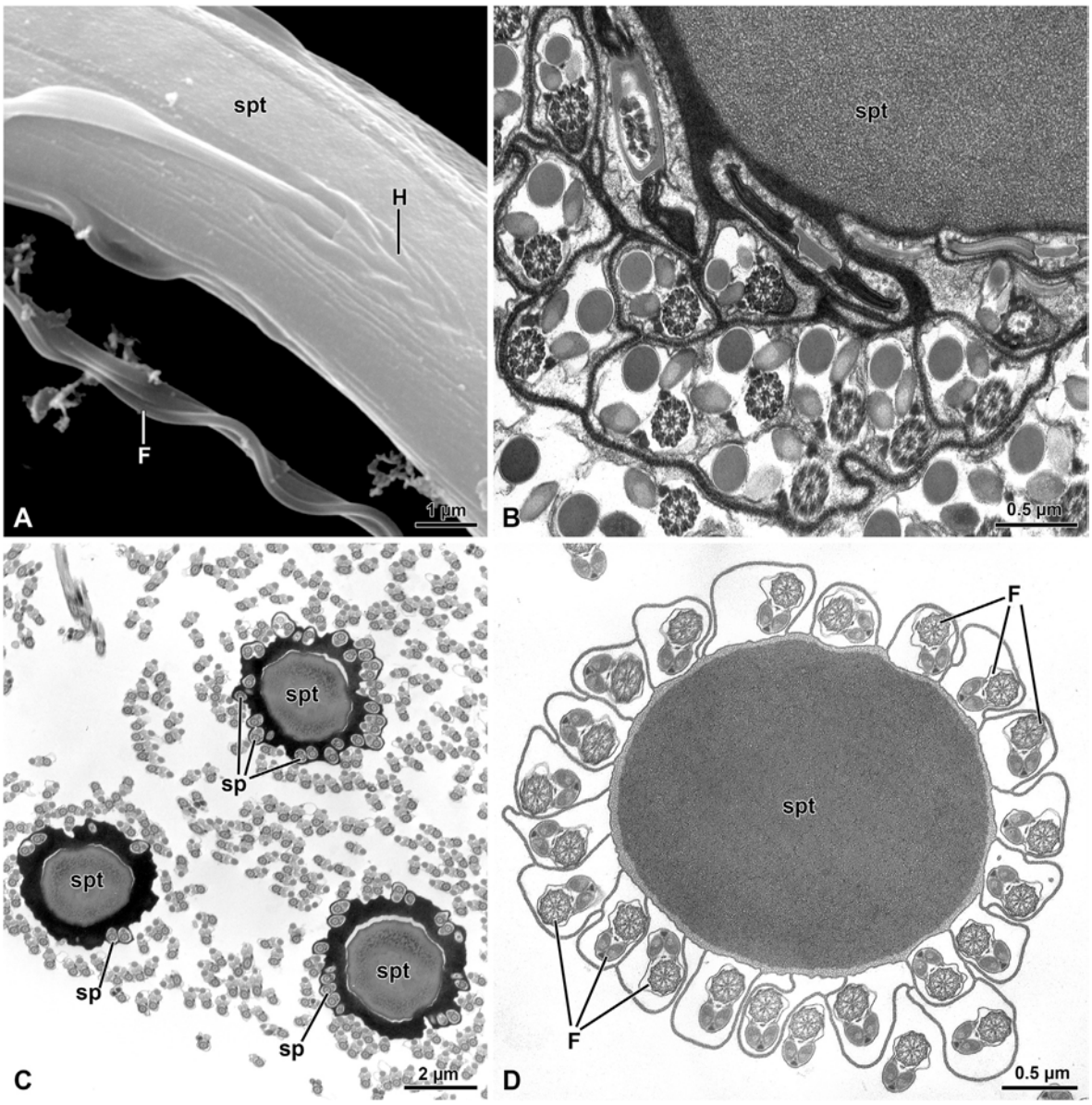

Fig. 8. **A**) SEM micrograph showing the sperm head (H) of *Calathus fuscipes* inserted within the spermatostyle (spt) and the helical array of the flagellum (F). **B**) Cross section through the spermatostyle (spt) of *C. fuscipes*. Note the electron-dense cortical layer giving rise to a net enveloping the sperm groups. **C**) Cross section through the spermatostyle (spt) of *Brachinus italicus* showing the electron-dense cortical layer where the sperm heads (sp) are inserted. **D**) Cross section of a spermatostyle (spt) of *Harpalus affinis*, showing several sperm flagella (F) adhering to the periphery of the structure.

and Dallai, 1987; Salazar et al., 2022).

The *C. canaliculatum* nucleus has a triangular outline in cross section as also *Carabus* species exhibit. The basal nuclear region shows two infoldings to host the centriole and the beginning of the large mitochondrial derivative, respectively. Somewhat similar array is present in *Carabus*, even though in this species, the mitochondrial derivatives are smaller, and the region shows a reduced amount of centriole adjunct material (Dallai et al., 2019a) in *C. canaliculatum*.

What is remarkable in *C. canaliculatum* sperm is the shape and diameter of the right accessory body. This sperm component is quite similar to that observed in *Carabus* (Dallai et al., 2019a) having an elongated shape and being adherent to the plasma membrane. The accessory body present at the opposite side of the axoneme, thus on the left side, has, instead, in both the above mentioned species, a quite reduced size. Generally, Carabidae exhibit two small accessory bodies of similar size. Only in *Demetrias atricapillus* the two accessory bodies are well developed and with an unusual shape; with the one on the right side divided into two parts (Dallai et al., 2019a).

The main characteristic of *C. canaliculatum* sperm, compared to other Carabidae, is the presence of two mitochondrial derivatives of different diameters. The one on the left side is distinctly larger than the opposite one, which is only half of the other mitochondrial derivative. The unequal diameter of the two structures makes sperm flagellar quite irregular and with an asymmetrical outline that could affect sperm motility. The difference in diameter between the two mitochondrial derivatives is determined since early spermiogenesis, soon after their appearance in the early spermatids. This difference has been described in other beetles such as Chrysomeloidea and Curculionoidea (Gassner et al., 1975; Baccetti e Daccordi, 1988; Báo, 1996, 1998; Burrini et al., 1988; Dallai et al.,

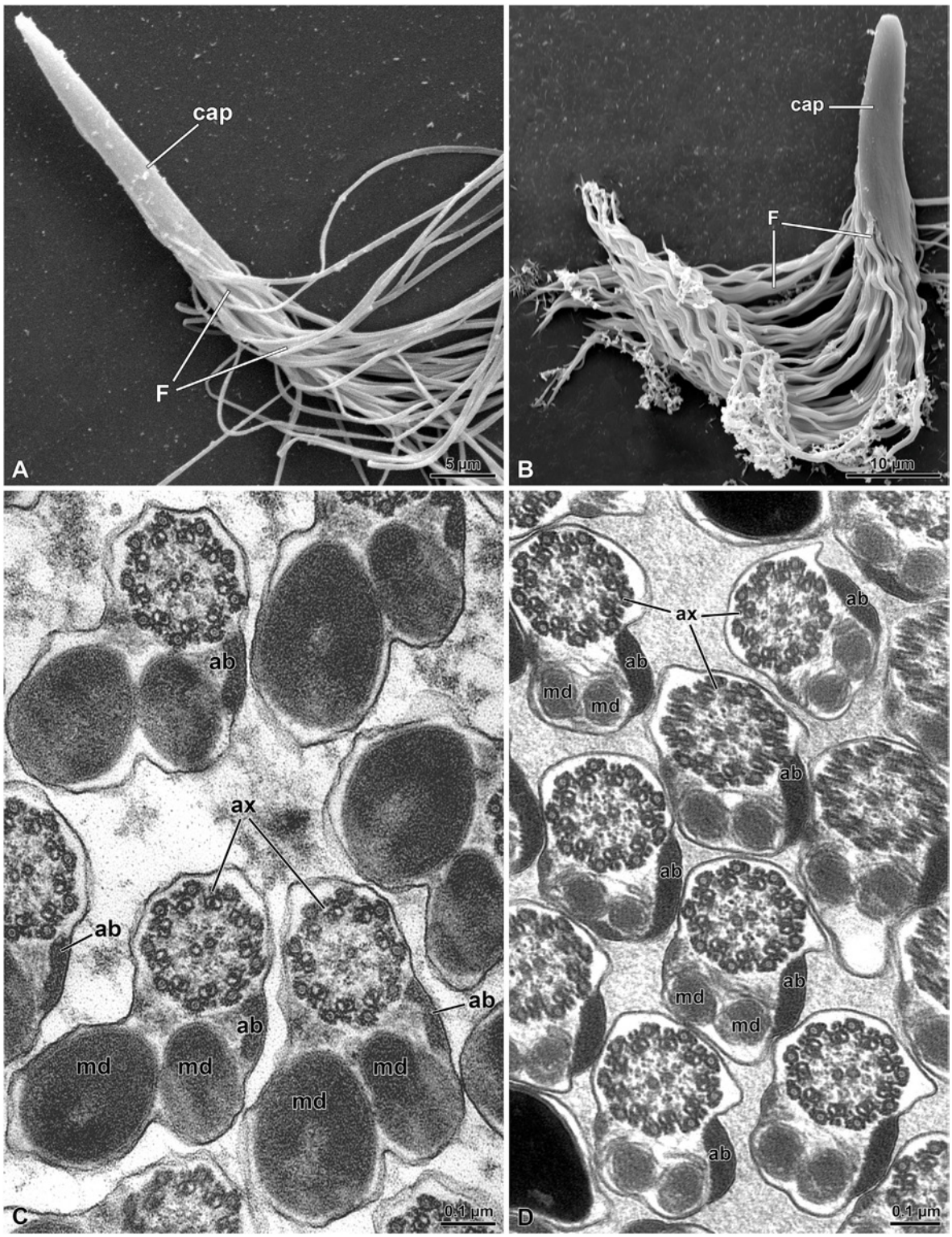

Fig. 9. **A**) SEM micrograph of a *Clinidium canaliculatum* sperm bundle protected by a conical cap (cap) from which many flagella (F) are coming out. **B**) SEM micrograph of the *Carabus preslii sperm* bundle protected by a cap (cap) from which flagella (F) are coming out. **C**) Cross section of *C. canaliculatum* sperm flagella showing the axoneme (ax) the two mitochondrial derivatives of different size (md) and the right large accessory body (ab). **D**) Cross section of *C. preslii* showing the axoneme (ax) the two similar small mitochondrial derivatives (md) and the large right accessory body.

1998; Name et al., 2007), even though in these groups, differently from *C. canaliculatum*, the larger mitochondrial derivative is placed on the right position in a cross section of the sperm flagellum. In these groups, however, the general organization of the sperm flagellum is more complex for the presence of large accessory bodies and the so called "puff-like body", an additional material extending from one of these structures (Burrini et al., 1988; Dallai et al., 1998).

The flagellar axoneme of *C. canaliculatum* is that typical of many insects (Dallai, 2014) it has 9 + 9+2 axonemal pattern and accessory tubules with 16 protofilaments in their tubular wall. As in other beetles, the intertubular material is well evident between the accessory tubules.

The ultrastructural data described in the present work on sperm of *C. canaliculatum* clearly indicate that the species has a sperm bundle similar to those present in Carabinae (Dallai et al., 2019a) with an apical cap of material in the shape of a conical structure (Fig. 9A–D). A similar shape of sperm bundle is also present in another species of Rhysodidae and possibly in other Carabidae (i.e. Trechinae, Paussinae and others) (Gomez and Maddison, 2020). These members do not have a spermatostyle, the long rod on which the sperm are attached (Breland and Simmons, 1970; Dallai et al., 2019a; Gomez and Maddison, 2020; Salazar et al., 2022).

It would be important to extend the study of the sperm structure to other groups of Carabidae to know which Carabidae are provided with spermatostyle and which, instead, have an apical cap. The two patterns of sperm protection, the cap and the spermatostyle, are produced according to two different sequences of realization. In the case of the cap the sperm bundle, as soon as it is formed, becomes covered by a secretion. In the case of the spermatostyle, instead, each single sperm has to stick to the structure after it was secreted. It is not rare to observe, in the deferent lumen, some spermatostyles of different length devoid of attached sperm, an evidence already described in a whirligig species by Breland and Simmons (1970) and also observed in different species of ground beetles (Dallai et al., 2019a, 2020). At mating, after copulation, the two types of sperm bundle protection could have a different fate. The two structures, attached to the innermost part of the bursa copulatrix of the female, will be gradually dissolved within the spermatheca. Those conjugates protected by a cap could allow the sperm to become free simultaneously; differently, those joined in a spermatostyle, could be released from the rod structure individually (Takami and Sota, 2007).

At this phase of our knowledge we are inclined to speak for the inclusion of Rhysodidae within the Carabidae as the subfamily Rhysodinae, as suggested by some authors (Hunt et al., 2007; López-López and Vogler, 2017; Baca et al., 2021; Vasilikopoulos et al., 2021). A possible affinity with Scaritinae, as supposed by some authors (e.g. Bell, 1998), seems unlike, due to the quite diverse sperm morphologies described in Scaritini, Clivinini, and Dyschiriini (Gomez and Maddison, 2020; Dallai et al., 2020). The position of nucleus in these species, opposite to the axoneme, strongly suggests the presence of a spermatostyle. On the other hand, looking at the present hypotheses on the Adephagan phylogeny (Beutel et al., 2020), also the affinities with basic Geoadephagan stocks remain uncertain, as Trachypachids and many other Carabid subfamilies share the spermatostyle (Gomez and Maddison, 2020), but finer details of ultrastructure are missing for the majority of Carabidae and the sperm morphology of the suborder Archostemata is still unknown.

CRediT authorship contribution statement

Anita Giglio: Conceptualization, Resources, Validation, Writing – original draft, Writing – review & editing. **David Mercati:** Investigation, Visualization, Writing – review & editing. **Pietro Lupetti:** Writing – original draft, Writing – review & editing. **Pietro Brandmayr:** Resources, Validation, Writing – original draft, Writing – review & editing. **Romano Dallai:** Conceptualization, Validation, 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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