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Morphology of male and female reproductive systems in the ground beetle *Apotomus* and the peculiar sperm ultrastructure of *A. rufus* (P. Rossi, 1790) (Coleoptera, Carabidae)

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ABSTRACT

Relatively few studies have focused on evolutionary losses of sexually selected male traits. We use light and electron microscopy to study the male and female reproductive anatomy of *Apotomus* ground beetles (Coleoptera, Carabidae), a lineage that we reconstruct as likely having lost sperm conjugation, a putative sexually selected trait. We pay particular attention to the structure of the testes and spermatheca. Both of these organs share a strikingly similar shape—consisting of long blind canals arranged into several concentric overlapping rings measuring approximately 18 mm and 19.5 mm in total length, respectively. The similarity of these structures suggests a positive evolutionary correlation between female and male genital organs. Males are characterized by unifollicular testes with numerous germ cysts, which contain 64 sperm cells each, and we record a novel occurrence of sperm cyst "looping", a spermatogenic innovation previously only known from some fruit fly and Tenebrionid beetle sperm. The sperm are very long (about 2.7 mm) and include an extraordinarily long helicoidal acrosome, a short nucleus, and a long flagellum. These findings confirm the structural peculiarity of sperm, testis, and female reproductive tract (FRT) of *Apotomus* species relative to other ground beetles, which could possibly be the result of shifts in sexual selection.

1. Introduction

Insect sperm are notorious for their morphological diversity (Jamieson et al., 1999; Pitnick et al., 2009a; Dallai, 2014). However, patterns in sperm evolution are surprisingly underexplored, and relatively little is known regarding how sperm form evolves with other male and female reproductive traits (Pitnick et al., 2009a, b). Postcopulatory sexual selection is widely assumed to be responsible for the diversification of sperm, but the function and adaptive value of most sperm traits are unknown (Lüpold and Pitnick, 2018). The resolution of sperm trait–function relationships requires studying sperm in their selective environment, which is the female reproductive tract (FRT) in internally fertilizing organisms (Eberhard, 1996; Pitnick et al., 2009b). Despite sperm structure being known from thousands of species, data on FRT anatomy for these same species are critically lacking (Ah-King et al., 2014). Thus, our ablitity to decipher patterns in sperm morphological evolution hinges on both a detailed understanding of sperm and FRT traits as well as their phylogenetic distributions.

Traditional research on sexual selection has generally focused on the gain of elaborate male traits (Darwin, 1871; Fisher, 1930; Lande, 1980; Andersson, 1994), but recent phylogenetic studies have revealed a surprising pattern: widespread loss of sexually selected male traits (Wiens, 2001). These losses imply that other forces can overcome the power of sexual selection or that selection might become weaker or reverse direction over time, or both (Wiens, 2001). Several complex sperm traits show repeated patterns of losses (Pitnick et al., 2009a; Higginson et al., 2012a; Schärer et al., 2011), but few studies have investigated male and female reproductive anatomy and sperm ultrastructure in lineages inferred

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to have lost an elaborate sperm trait. Such studies could shed light on the evolutionary dynamics accompanying these losses.

Sperm conjugation — a biological phenomenon where two or more sperm join together for travel through the female reproductive tract prior to dissociation and fertilization (Higginson and Pitnick, 2011) — is a complex male trait that shows repeated losses in some clades of insects (beetles of the suborder Adephaga (Fig. 1), Higginson et al., 2012a, 2015; Gomez and Maddison, 2020; Sasakawa, 2020). Sperm conjugation is presumed to be an adaptation in sperm competition (Moore et al., 2002; Immler, 2008; Higginson and Pitnick, 2011), but relatively little is known regarding the mechanisms driving sperm conjugation evolution or how sperm conjugation co-diversifies with sperm form and the FRT (but see Higginson et al., 2012a, b; Pitnick et al., 2020). If sperm conjugation is being driven and maintained by sexual selection, under what conditions is it lost and what changes in male and female reproductive traits accompany its loss? Here, we contribute to this topic by studying the sperm and reproductive anatomy of *Apotomus* Illiger ground beetles, a lineage suspected of subtending a loss of sperm conjugation (Gomez and Maddison, 2020; Gomez, R.A., unpubl. data).

Ground beetles (Carabidae) vary in sperm conjugation presence and type (Fig. 1; Dallai et al., 2019, 2020; Gomez and Maddison, 2020; Sasakawa, 2020), and they are emerging as a fruitful system for studying pattern and process in reproductive evolution. With nearly 40,000 described species (Lorenz, 2005, 2021), Carabidae exhibits a great diversity of male and female genitalia (Liebherr and Will, 1998; Will et al., 2005) and complex sperm structure (Dallai et al., 2019, 2020; Gomez and Maddison, 2020), and several of these traits vary widely among species allowing for comparative and experimental studies of sexual traits in a dominant clade of terrestrial organisms (Chen, 1984; Yahiro, 1996, 1998; Carcupino et al., 2002; Will et al., 2005; Sasakawa, 2007, 2020; Takami and Sota, 2007; Hodgson et al., 2013; Schubert et al., 2017; Gomez and Maddison, 2020). Males of most ground beetles conjugate their sperm into groups by embedding them via their heads in a novel structure termed a spermatostyle (Fig. 1; Breland and Simmons, 1970), a hyaline matrix produced in the testes (Hodgson et al., 2013; Schubert et al., 2017). The spermatostyle can show obvious morphological differences among species, varying in shape from a simple apical cap to an elaborate rod (Dallai et al., 2019, 2020; Gomez and Maddison, 2020). Singleton sperm characterize the closely related tiger beetles (family Cicindelidae) and occurs in several higher-order groups of carabids including: Trechitae, the tribe Paussini, and various phylogenetically isolated lineages including the monogeneric Apotomini (Dallai et al., 2019, 2020; Gomez and Maddison, 2020; Sasakawa, 2020). The lack of a robust comprehensive carabid phylogeny has limited the identification of derived losses of conjugation, but ancestral state reconstruction using a low-resolution phylogenetic hypothesis suggests

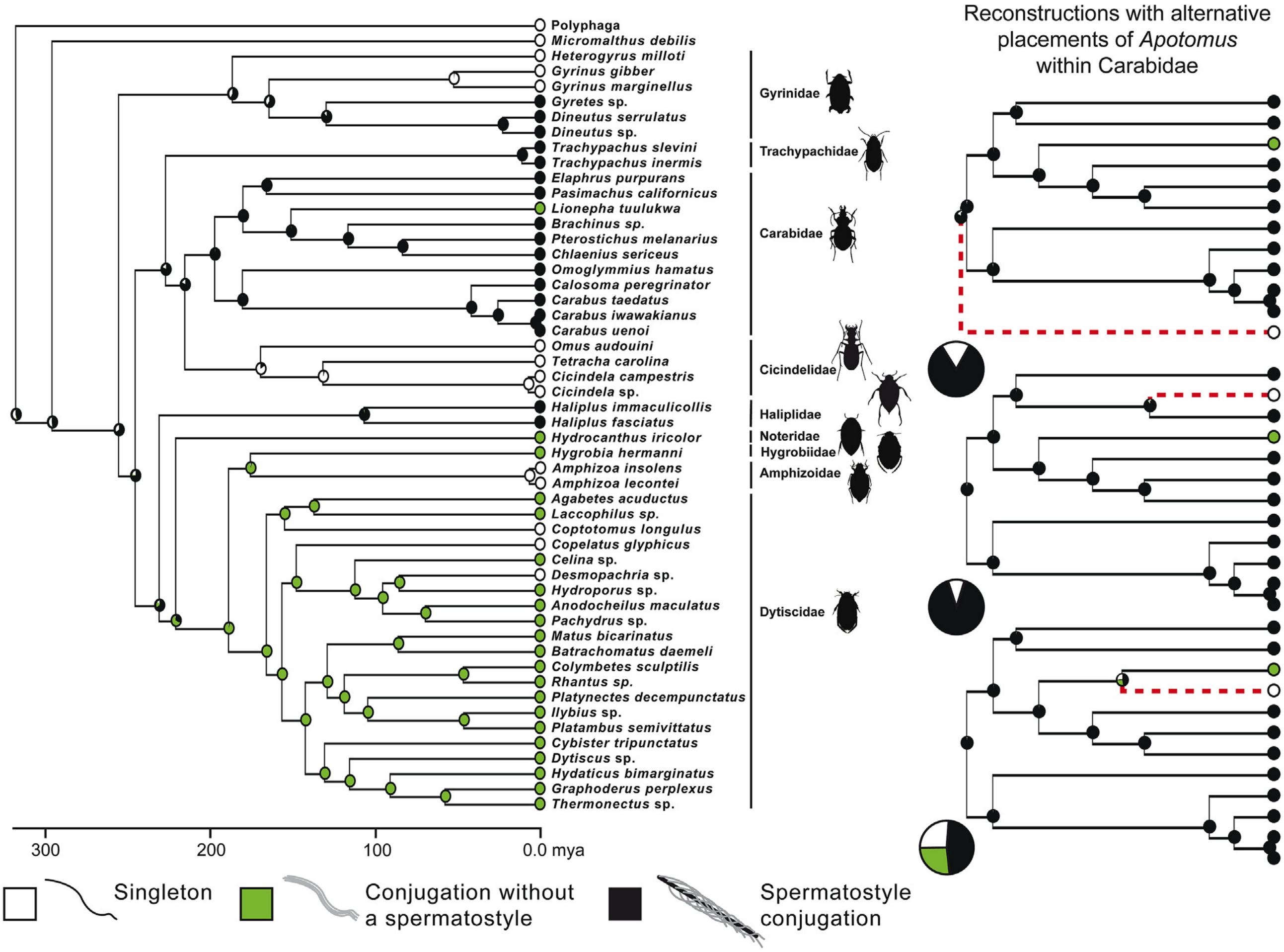

Fig. 1. Time-calibrated phylogeny of Adephaga and outgroup beetles from Baca et al. (2021) with ancestral states for sperm conjugation reconstructed under maximum likelihood (see methods). Three examples of different possible placements for *Apotomus* on the Carabidae phylogeny are shown on the right inset (dashed red lines), and the ancestral state probablities of its immediate parental node are shown next to each reconstruction.

that sperm conjugation with a spermatostyle evolved early in the ground beetle tree and has been lost independently at least three times (Gomez and Maddison, 2020).

Among ground beetles with singleton sperm, the genus *Apotomus* stands out because of its numerous divergent morphological traits (Erwin, 1980; Baehr, 1990; Will et al., 2005) and its putative isolated phylogenetic position within Carabidae (Liebherr and Will, 1998; Maddison et al., 1999). The aim of the present work is to provide high-resolution morphological data on the testis, sperm ultrastructure, and FRT of *Apotomus* species, focusing particularly on *A. rufus* (Rossi), and we evaluate the hypothesis that its possession of singleton sperm is due to an earlier gain and loss of sperm conjugation by reconstructing ancestral states across a broad sampling of adephagan beetles. These data will allow for a better characterization of the tribe Apotomini and may prove useful for uncovering the dynamics and consequences of losing a complex sperm trait presumed to be favored by sexual selection.

2. Material and methods

2.1. Phylogeny and ancestral state reconstructions of sperm conjugation

To visualize evolutionary patterns in sperm conjugation across ground beetles, we reconstructed ancestral states for sperm conjugation across 44 beetle species using the phylogenomic tree of Baca et al. (2021) and sperm conjugation data compiled from the literature (Table 1 and references therein). The phylogeny of Baca et al. (2021) is currently the most comprehensive fossil-based dated framework of adephagan beetles as it includes all currently recognized families of Adephaga and several outgroups. *Apotomus* has not been sampled in any modern molecular phylogenetic study aside from older studies based on single gene fragments (Maddison et al., 1999), and although Baca et al. (2021) did not sample *Apotomus* for their study, their phylogeny includes a broad sampling of most major lineages of ground beetles and their near relatives. Because the phylogenetic placement of *Apotomus* within Carabidae is ambiguous, we explored the impact of different placements of *Apotomus* on ancestral state reconstructions by adding it randomly to the Carabidae tree.

We inferred ancestral states of sperm conjugation under maximum likelihood using the Mk1 model as implemented in Mesquite version 3.61 (Maddison and Maddison, 2019). Briefly, this evolutionary model treats evolutionary losses and gains as equally probable and considers branch lengths in its calculations. We added *Apotomus* to the tree randomly in Mesquite by using the ‘Randomly Modify Current Tree’ option while also selecting the option to consider branch lengths. This method generated many biologically realistic permutations, three examples of which are shown in Fig. 1. We coded sperm conjugation into three character states (Table 1): (0) conjugation absent, (1) conjugation without a spermatostyle, (2) conjugation with a spermatostyle. Some of the species sampled by Baca et al. (2021) have not been studied for sperm morphology. In cases where close relatives of these species have been studied, we merged data from the two taxa, which were typically either congeners or sister taxa (Table 1). The presence or absensce of sperm conjugation appears to be typically stable within most carabid and dytiscid genera studied to date (Higginson et al., 2012a; Gomez and Maddison, 2020), but a few groups are known to vary in this trait among their constituent species (*Gyretes* in Gyrinidae, Higginson et al., 2015, Salazar et al., 2022; *Pachydrus* in Dytiscidae, Higginson et al., 2012a). Because the maximum likelihood calculations in Mesquite cannot handle polymorphic character data, taxa with and without sperm conjugation (0 and 1 or 0 and 2) were coded as monomorphically possessing sperm conjugation (1 or 2).

2.2. Specimens

We studied two male specimens of *Apotomus rufus* from central Italy for transmission electron microscopy and eight specimens (two males and six females) of an unidentified species of *Apotomus* from southern Africa prepared for light microscopy and scanning electron microscopy. The specimens of *A. rufus* were collected in the clay hills in the vicinity of Siena, central Italy on March 12, 2001. The southern African specimens were previously preserved in 100% ethanol and stored in a −20 °C freezer. These specimens have the following label data: (one male and one female) Republic of South Africa, Limpopo, Makuya Nature Reserve, Mutale Falls Camp, LED light trap, 301m, 02.ii.2018. Gomez, Kanda, Pflug; (one male and five females) Mozambique, Sofala, Parque Nacional da Gorongosa, Chitengo camp, searching in camp, 21m, 08−15.ii.2018. RA Gomez. We note that the southern African specimens appear to be the same or similar species based upon morphological examination of the male genitalia. However, we were unable to identify these specimens to species as there is no modern revision of global *Apotomus*, and the group is known to have significant undocumented diversity in Africa (Erwin, 1987).

2.3. Light and epifluorescence microscopy

We dissected two females and two males of unindentified species of *Apotomus* from Africa under a light microscope and isolated the male and female genitalia using standard dissection procedures (Liebherr and Will, 1998). We stained some of these samples for DNA in order to visualize the sperm nucleus. Briefly, we added a drop of 1 μg/mL of Hoechst 33258 dye in 0.1 M PB to fragments of the samples and allowed them to incubate for a few minutes before mounting them in a drop of 90% glycerol with a coverslip. We observed and photographed the samples using interference contrast and epifluorescence microscopy with a Leica DMRB microscope equipped with an axioCam HR camera (Carl Zeiss).

2.4. Scanning electron microscopy (SEM)

We isolated and prepared the female sperm-storage organ (spermatheca) from one of the African specimens (*Apotomus* sp.) for observation with SEM. We rinsed the material in PB before cutting the long tubular canal into several parts, allowing for the removal of stored sperm from the lumen of some fragments. We transferred the spermathecal fragments onto 1% poly-ʟ-lysine-pretreated glass cover slips and fixed them with 1% osmium for 1 h. We then rinsed the samples in distilled water and dehydrated them in alcohol prior to critical point drying in a Balzers CDP 010. We mounted the material on aluminium stubs and coated them with gold using a Balzers Med 010. We examined the gold-coated samples using a Philips XL20 scanning electron microscope operating at 15 kV.

2.5. Transmission electron microscopy (TEM)

We dissected two males specimens of *A. rufus* and isolated their testes in 0.1 M phosphate buffer solution (pH 7.2) supplemented with 3% sucrose (PB). We fixed the tissue overnight in 2.5% glutaraldehyde in PB at 3 °C. After a careful wash, we post-fixed the tissue in 1% osmium tetroxide in PB for 2 h. Following another wash step, we dehydrated the material with an alcohol series ranging from 50 to 100% ethanol before embedding the tissue in a mixture of Epon-Araldite resin (50/50). We prepared a portion of the material according to the methods of Dallai and Afzelius (1990) by employing a tannic acid impregnation step without osmium

Table 1
Trait data and taxon sampling for ancestral state reconstructions of sperm conjugation. Sperm conjugation data are organized into three discrete states: sperm conjugation absent (0), sperm conjugation present but without a spermatostyle (1), and spermatostyle conjugation present (2). Dashes '–' refer to duplicated entries for taxa sampled for DNA data and sperm data.

Species sampled by Baca et al. (2021)	Species sampled for sperm morphology	Sperm conjugation (0, 1, 2)	References
Agabetes acuductus	–	1	Higginson et al. (2012a)
Amblycheila cylindriformis	*Omus audouini*	0	Gomez and Maddison (2020)
Amphizoa insolens	–	0	Higginson et al. (2012a)
Amphizoa lecontei	–	0	Higginson et al. (2012a)
Andocheilus exiguus	*Andocheilus maculatus*	1	Higginson et al. (2012a)
Batrachomatus nannup	*Batrachomatus daemeli*	1	Higginson et al. (2012a)
Brachinus cyanipennis	*Brachinus* spp.	2	Gomez and Maddison (2020)
Calosoma frigidum	*Calosoma peregrinator*	2	Gomez and Maddison (2020)
Carabus iwawakianus	–	2	Takami and Sota (2007)
Carabus taedatus	–	2	Gomez and Maddison (2020)
Carabus uenoi	–	2	Takami and Sota (2007)
Celina sp.	*Celina* spp.	1	Higginson et al. (2012a)
Chlaenius sericeus	–	2	Gomez and Maddison (2020)
Cicindela pulchra	*Cicindela* spp.	0	Werner (1965); Gomez and Maddison (2020)
Cicindela sexguttata	*Cicindela campestris*	0	Werner (1965)
Colymbetes sculptilis	–	1	Higginson et al. (2012b)
Copelatus chevrolati	*Copelatus glyphicus*	0	Higginson et al. (2012a)
Coptotomus longulus	–	0	Higginson et al. (2012a)
Cybister fimbriolatus	*Cybister tripuncatus*	1	Higginson et al. (2012a)
Desmopachria granum	*Desmopachria* spp.	0	Higginson et al. (2012a)
Dineutus serrulatus	–	2	Gustafson and Miller (2017)
Dineutus sp.	–	2	Higginson et al. (2015); Gustafson and Miller (2017)
Dytiscus circumcinctus	*Dytiscus* spp.	1	Higginson et al. (2012a)
Elaphrus aureus	*Elaphrus purpurans*	2	Gomez and Maddison (2020)
Graphoderus perplexus	–	1	Higginson et al. (2012a)
Gyretes sp.	–	0, 2	Higginson et al. (2015); Salazar et al. (2022)
Gyrinus marinus	*Gyrinus gibber*	0	Higginson et al. (2015)
Gyrinus minutus	*Gyrinus marginellus*	0	Higginson et al. (2015)
Haliplus fluviatilis	*Haliplus immaculicollis*	2	Higginson and Pitnick (2011); Higginson et al. (2015)
Haliplus tocumensis	*Haliplus fasciatus*	2	Higginson and Pitnick (2011); Higginson et al. (2015)
Heterogyrus milloti	–	0	Gustafson and Miller (2017)
Hydaticus bimarginatus	–	1	Higginson et al. (2012a)
Hydrocanthus debilis	*Hydrocanthus iricolor*	1	Higginson et al. (2015)
Hydroporus ruficeps	*Hydroporus* spp.	1	Higginson et al. (2012a); Higginson et al. (2015)
Hygrobia hermanni	–	1	Higginson et al. (2012a)
Ilybius piciceps	*Ilybius* spp.	1	Higginson et al. (2012a)
Laccophilus gentilis	*Laccophilus* spp.	1	Higginson et al. (2012a)
Lionepha sp.	*Lionepha tuulukwa*	1	Gomez and Maddison (2020)
Matus ovatus	*Matus bicarinatus*	1	Higginson et al. (2012a)
Micromalthus debilis	–	0	Scott (1936)
Omoglymmius hamatus	–	2	Gomez and Maddison (2020)
Pachydrus sp.	–	0, 1	Higginson et al. (2012a)
Pasimachus viridans	*Pasimachus californicus*	2	Gomez and Maddison (2020)
Platambus maculatus	*Platambus semivittatus*	1	Higginson et al. (2012a)
Platynectes sp.	*Platynectes decempunctatus*	1	Higginson et al. (2012a)
Pterostichus melanarius	–	2	Gomez and Maddison (2020)
Rhantus suturellis	*Rhantus* spp.	1	Higginson et al. (2012a)
Scirtidae sp.	Polyphaga	0	Baccetti and Daccordi (1988); Dias et al. (2015); Jamieson et al. (1999); Dallai et al. (2018); Dias et al. (2022a)
Tetracha carolina	–	0	Gomez and Maddison (2020)
Thermonectus intermedius	*Thermonectus* spp.	0, 1	Higginson et al. (2012a)
Trachypachus gibbsii	*Trachypachus slevini*	2	Gomez and Maddison (2020)
Trachypachus inermis	–	2	Gomez and Maddison (2020)

fixation. We obtained semithin sections with an ultramicrotome Reichert Ultracut, which we stained with 0.1% toluidine blue and observed with a Leica DMRB interference light microscope equipped with a Zeiss Axiocam digital camera. Ultrathin sections, routinely stained with uranyl acetate and lead citrate, were observed with a Philips CM 10 transmission electron microscope operating at 80 kV.

3. Results

3.1. Ancestral state reconstructions of sperm conjugation

Sperm conjugation is present in many diverse lineages across the phylogeny of Adephaga, and it is commonly recovered as the most probable ancestral state across numerous deep nodes (Fig. 1). The presence or absence of sperm conjugation is ambiguous towards the base of the tree of all beetles, but most families sampled thus far for sperm data are recovered as likely having some form of sperm conjugation near their base, excluding Amphizoidae, Cicindelidae, and, possibly, Gyrinidae (Fig. 1). Conjugation with a spermatostyle is reconstructed as ancestral to most Carabidae, and it likely predates the origin of the family. It is not clear if spermatostyle conjugation has evolved repeatedly in Adephaga, but evolutionary losses of conjugation are estimated to outnumber their gains. Ancestral states across the tree do not change dramatically with the random addition of *Apotomus* to the

Carabidae tree (Fig. 1), and most reconstructions of its parental node show a high probability of having spermatostyle conjugation. The reconstructions with *Apotomus* placed as sister to *Lionepha tuluukwa* are notable in that the probabilitiy of spermatostyle conjugation in the parental node of *Apotomus* drops relative to other placements within Carabidae that we observed (Fig. 1).

3.2. Female reproductive tract

The upper FRT of *Apotomus* sp. consists of two ovaries measuring between 1,2–1,3 mm in length, and each ovary includes 3–4 ovarioles (Fig. 2A). The two short oviducts join a common oviduct posteriorly, which attaches to the bursa copulatrix. An elongate, slender, and tubular spermatheca joins the bursa in this area (Fig. 2A). The spermatheca consists of a blind looped canal that forms a series of 13 overlapping concentric rings (Fig. 2A, C). Individual loops vary in diameter from 490 to 540 µm. The spermatheca measures about 19,500 µm in total length when uncoiled, and its various sections measure about 28–30 µm in width. The spermathecal epithelium is lined by a thin layer of cuticle that bears scattered rear-facing spines, which are visible under SEM (Fig. 6A). The spermathecal lumen is filled with tightly compacted sperm (Fig. 6B); their apical regions show a tight and variable helicoidal array of acrosomes (Fig. 6C–E). Two small ellipitical pygidial defense gland reservoirs are visible lateral and posterior to the genital opening (Fig. 2A).

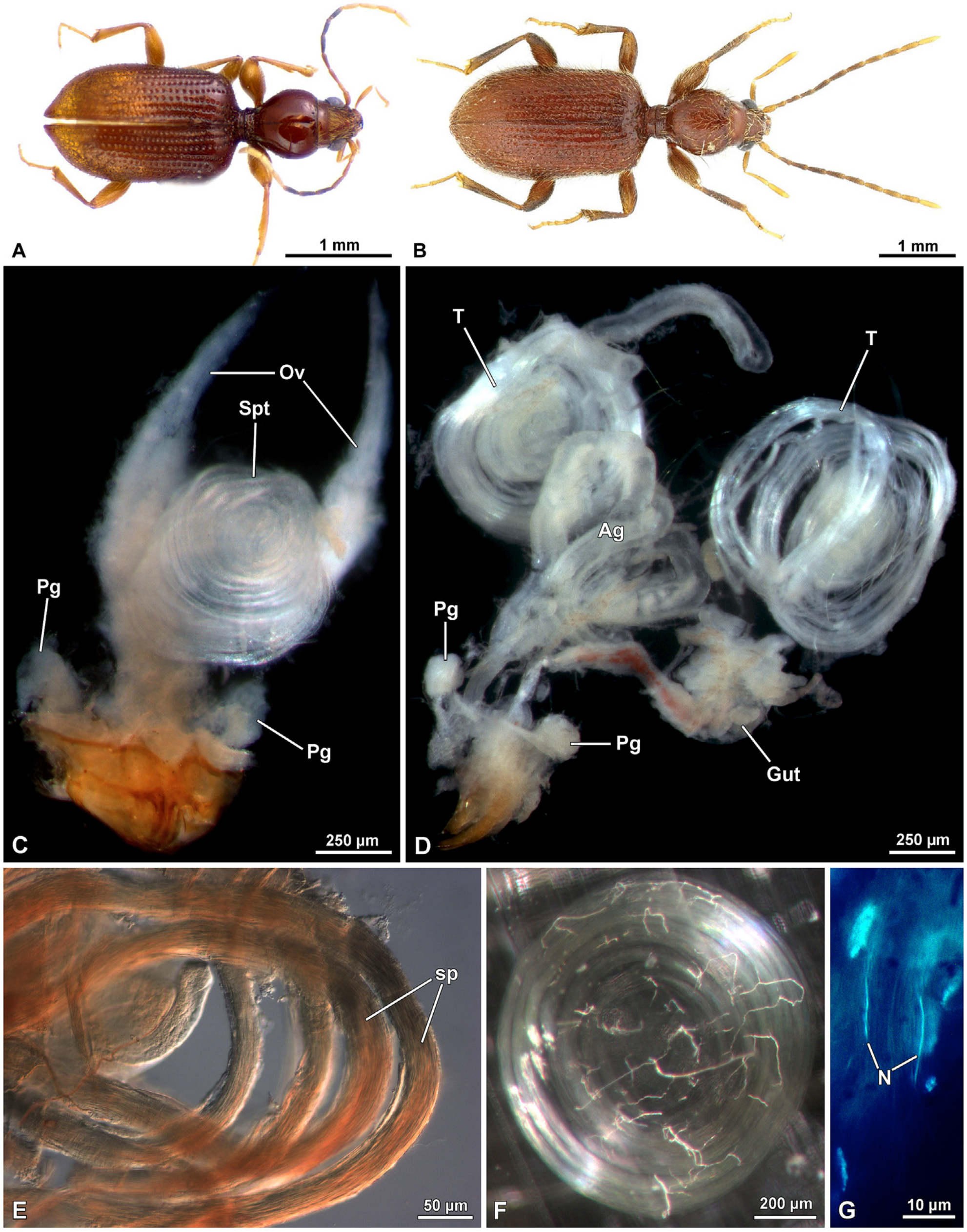

Fig. 2. Dorsal habitus photographs of *Apotomus* sp. from Mozambique (**A**) and *Apotomous rufus* (**B**). Photo credits: (**A**) RA Gomez; (**B**) Cédric Alonso. **C -** Female genitalia of *Apotomus* sp. Note the long ovaries (Ov)**,** the several rings of the spermatheca canal (Spt), and the pygidial defense gland reservoirs (Pg). **D -** Male genitalia of *Apotomus rufus* with monofollicular testes (T), male accessory glands (Ag), and the pygidial defense gland reservoirs (Pg). **E -** A close-up of the female tubular spermatheca of *Apotomus* sp. with stored sperm (sp). **F -** An intact disc-like testis of *Apotomus rufus*. **G -** Hoechst staining showing the fluorescent nuclei (N) in sperm taken from spermathecal storage of *Apotomus* sp.

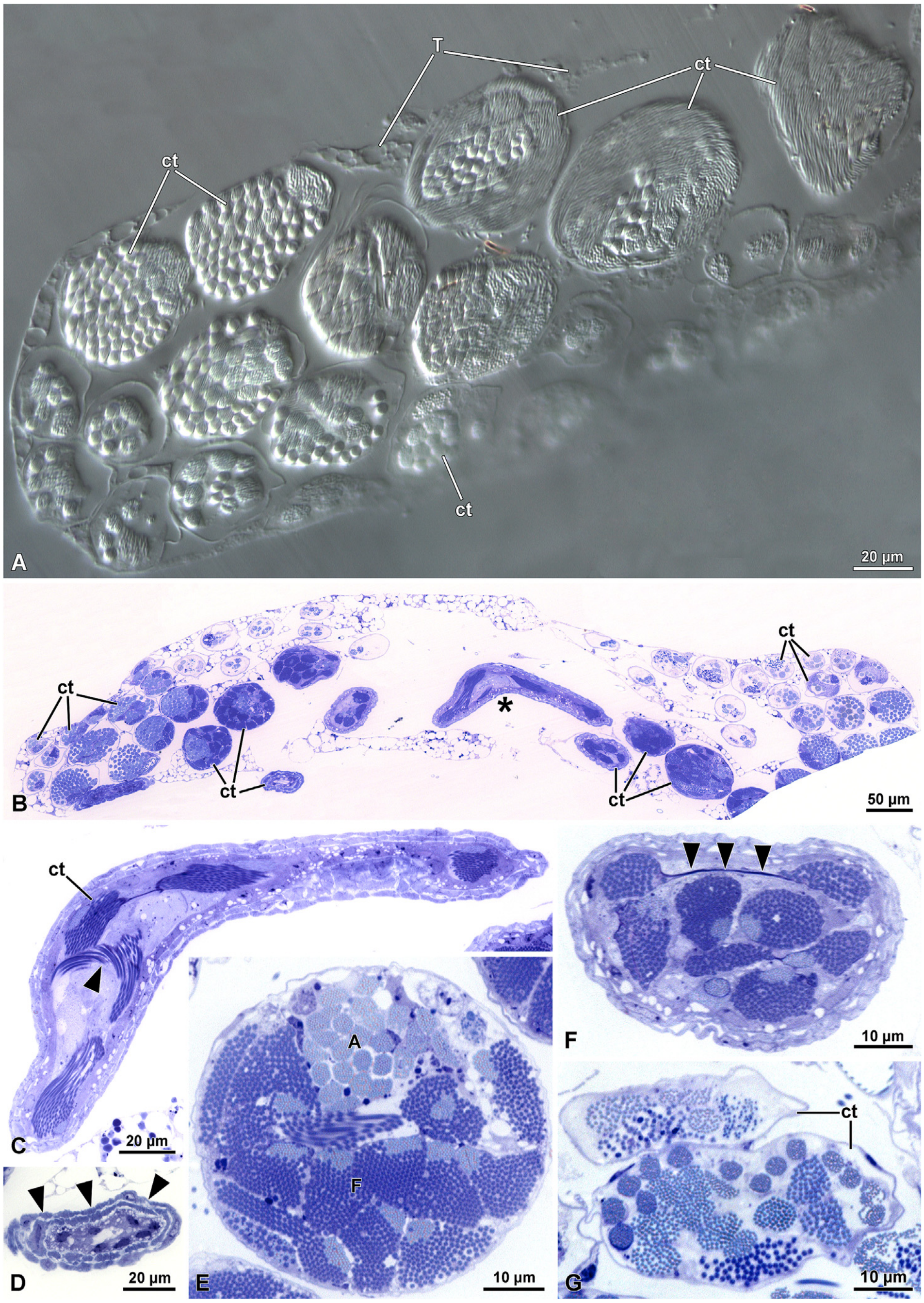

Fig. 3. A - Thick section of testis of *Apotomus rufus* showing the different cylindrical turns (ct) each consisting of a different number of sperm cysts. T, testis. **B -** Semi-thin cross section of testis showing several cylindrical turns (ct) each consisting of a number of sperm cysts. Note the central elongated structure (asterisk). **C -** Detail of the elongated turn (ct) showing a sperm cyst with sperm cyst looping (arrowheads). **D -** Semi-thin cross section of a cylindrical turn without sperm cysts and surrounded by a thick layer of muscle cells (arrowheads). **E -** Semi-thin cross section of a cylindrical turn with numerous sperm cysts sectioned at different levels allowing for the visualization of acrosomes (A) and both acrosome and flagella (A + F). **F -** Semi-thin cross section through different levels of the previous figure showing fewer sperm cysts. Note in the upper region two sections of the same sperm cyst connected by a longitudinal thin bundle of flagella (arrowheads). **G -** Semi-thin cross section of two cylindrical turns sectioned at two different levels. There are few sperm cysts in the upper section whereas the bottom section includes numerous sperm cysts that are cut at different levels. Toluidine blue staining (**B**–**E**). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. *Male genitalia and sperm*

Members of the genus *Apotomus*, either from Italy (*A. rufus*) or Africa (*A.* sp.), have morphologically divergent testes compared to all other carabid beetles surveyed to date (Will et al., 2005). Each testis appears to consist of a single coiled follicle in the general shape of a thick disc formed by up to 15 tight concentric overlapping loops, which individually measure about 400 μm in diameter (the testis has been uncoiled slightly in Fig. 2B). The testis measures 30–50 μm in width and reaches a maximum width of 70 μm in its most proximal region where it attaches to the accessory glands (Fig. 2B). Seminal vesicles were not observed. We estimate that the total length of the follicle can reach approximately 18 mm. Each portion of the disc-like testis of *A. rufus* contains several cylindrical sperm cyst complexes of variable length and size based on careful observation of thick and semithin cross sections

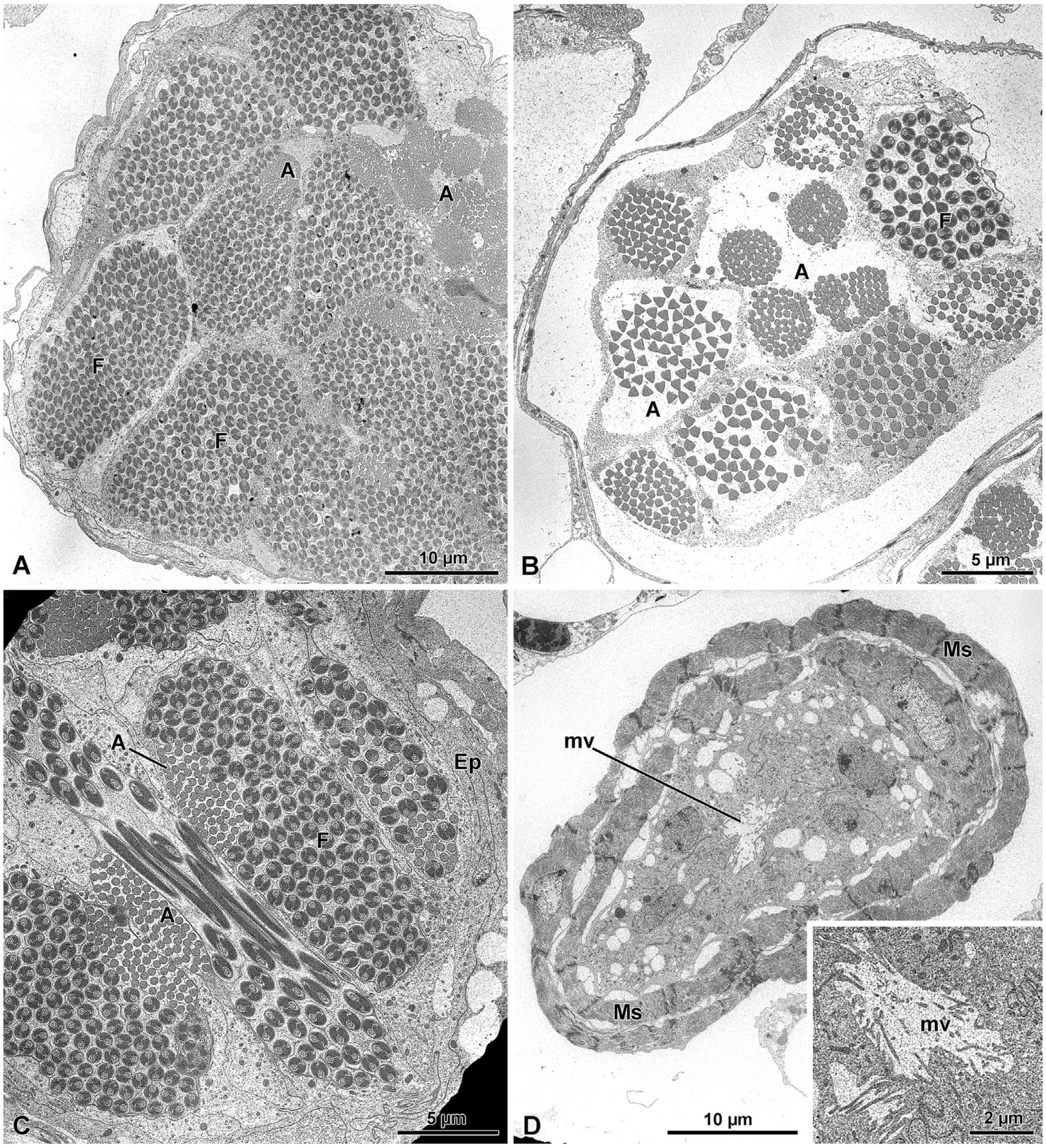

Fig. 4. **A -** Cross section of a portion of the cylindrical testis of *Apotomus rufus* with many sperm cysts sectioned at the levels of the acrosome (A) and flagella (F). **B -** Cross section of a cylindrical turn with several sperm cysts cut at the level of the acrosome (A) and the flagella (F). **C -** Cross section through a cylindrical turn showing *in situ* sperm cyst looping as the number of flagella (F) correspond to twice the number of acrosomes (A). Note the thick epithelial layer of (Ep) and the muscle cell layer. **D -** Cross section through a cylindrical turn without sperm cysts in the lumen, which is possibly one of the ends of the testis. Note the lumen is filled with microvillated cells (mv) surrounded by muscle cells (Ms). The inset shows in detail the lumen lined by cells with microvilli.

taken through several loops of the testis (Fig. 3A–G). The outer cylindrical turns show sections of testis with 30–50 sperm cysts, which entirely fill the canal lumen. Each sperm cyst consists of 64 sperm cells, as a result of 6 cell divisions (Fig. 5A). The number of cysts drastically reduces to only 6–10 in the narrower sections of the testis (Fig. 3F; 4B, C; 5B) and in places where adjacent sperm cysts fuse (Fig. 3E; 4A). The large sections of testis that are filled with sperm cysts exhibit a thin epithelial wall surrounded by few stretched muscle fibres (Fig. 5B). The thinner sections of testis do not exhibit any sperm cysts within the lumen. Their inner contents consist of a few cells provided with microvilli (Fig. 3D; 4D), and their outer wall shows a thick layer of muscle cells. We observed only a single testis, the right testis, in a male of *A.* sp. collected in Limpopo, RSA, but the male we studied from Mozambique of *A.* sp. possessed both testes. With the exception of the male from Limpopo, all other *Apotomus* males studied possessed the typical diorchid condition.

A. rufus sperm are long cells measuring approximately 2.7 mm. It is characterized by an extraordinarily long acrosome that measures at least 800 µm (Fig. 6B). The length of the acrosome is such that some cross sections of testis loops exhibit sperm cysts with only acrosomes (Fig. 5A). The acrosome features an elongate anterior region that is longitudinally arranged into a helix with a repeating pattern that varies widely in length from 0.13 µm to 0.8 µm (Fig. 6C–E; 7B). When viewed in cross section, this repeating region measures about 0.33 µm in width, and its outline varies according to the section level: from being triangular or almost quadrangular in the anterior region (Fig. 7A, C, D) to circular in the posterior end (Fig. 5A; 7A). The contents of the acrosome have a homogeneous, moderately electron-dense appearance. A perforatorium is missing.

The sperm nucleus is relatively short, about 20–22 µm long, and includes electron-dense chromatin material, easily observed with

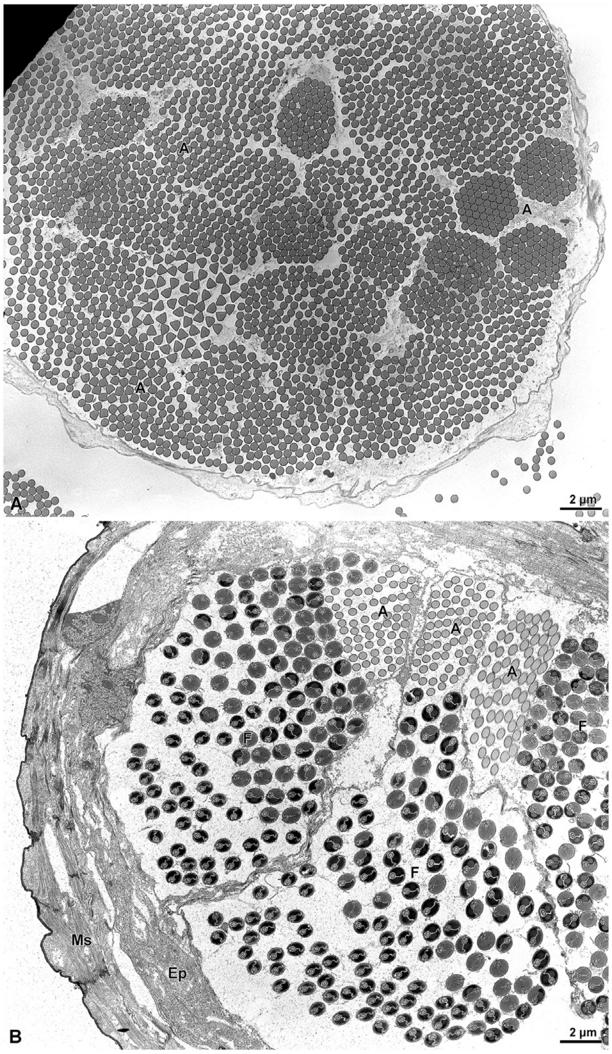

Fig. 5. *Apotomus rufus* testes. **A -** Cross section of a cylindrical turn with numerous sperm cysts, all cut at the acrosomal level (A). **B -** Cross section of a cylindrical turn with few sperm cysts each showing acrosomes (A) and flagella (F). Note the thick outer wall with epithelial cells (Ep) and muscle cells (Ms).

Hoechst staining (Fig. 2D). It has a cylindrical shape with a diameter of nearly 0.31 μm (Fig. 8A). The nucleus prolongs into a saddle-like shape dorsal to the flagellar axoneme, which becomes apparent when viewed in cross section (Fig. 8D and E). The posterior nuclear region hosts the centriole (Fig. 8B and C) from which a long flagellar axoneme extends. The centriole consists of central tubules and doublets devoid of dynein arms (Fig. 8B and C); sparse centriole adjunct material is present in this region (Fig. 8B and C).

The flagellum measures 0.8 μm in diameter and is surrounded by a plasma membrane with a very thin glycocalyx showing a fine structural organization (Fig. 9B). The flagellum consists of a typical 9 + 9+2 axoneme with microtubule doublets with dynein arms, radial spokes, and two central tubules. A crown of 9 accessory tubules with 16 protofilaments in their tubular wall is located external to the central microtubular array (Fig. 9B). Intertubular material can be found associated with the accessory tubules and between two adjacent tubules (Fig. 9B). The elongate spermatid flagella fold in on themselves or "loop" within cysts based on examination of the position of the dynein arms on the axonemal doublets, which shows that flagellar axonemes within the same cyst can have opposite orientations. Due to cyst looping, some axonemes have dynein arms oriented clock-wise whereas others are oriented counter clock-wise (Fig. 8E and F; 9A). The looping can be easily observed when the flagella of the sperm cyst are sectioned at different levels. For example, in Fig. 8D, it is obvious that the 64 acrosomes visible in this section of a single sperm cyst are accompanied by 128 flagella. This pattern results from sperm cyst looping, which doubles the number of flagellar sections.

Two expanded mitochondrial derivatives embrace the axoneme dorsally in cross sections. They are bilaterally asymmetrical with the left mitochondrial derivative possessing a protrusion that fits within a cleft within the opposite side (Fig. 8E and F; 9A, B). In longitudinal sections, the mitochondrial derivatives are helicoidally arranged around the axoneme (Fig. 9D); their peripheral region includes orderly series of cristae (Fig. 9C). The majority of the mitochondrial matrix is crystallized (Fig. 9B). Two small accessory bodies are visible adhering to the apical narrow region of each mitochondrial derivative (Fig. 9A and B). They are also asymmetrical both in form and in position with respect to the mitochondrial derivatives; that on the left side being a little larger than the right one and with a more apical position (Fig. 9A and B). The posterior flagellar region is characterized by the following features: the central tubules disappear, the microtubule doublets lose their dynein arms and intertubular material (Fig. 10C), and the mitochondrial derivatives progressively reduce in diameter until ultimately dissappearing (Fig. 10A and B). The accessory bodies begin to separate from the mitochondria at the posterior end of the flagellum (Fig. 10B). The posterior end of the cell is cylindrical and measures 0.4 μm in diameter, and its contents appear homogenous featuring both a central region of moderately electron-dense material and a peripheral electron-dense crescent-shaped region that appears more (Fig. 10, C).

4. Discussion

4.1. Evolutionary loss of a complex sperm trait

Research on sexual selection has traditionally focused on the gain of male traits and female preferences, but losses of these traits can be widespread and can even outnumber gains (Wiens, 2001). The study of these losses is relevant to the field because they imply that other forces are able to overcome sexual selection or that sexual selection can weaken and/or change direction (Morris et al., 2005; Weigel et al., 2015; Heinen-Kay and Zuk, 2019). However, these patterns have largely been neglected perhaps because they require identification using a phylogeny, and current models are generally inadequate for explaining why sexually selected traits are so frequently lost (Wiens, 2001). Most research on sexual trait evolution has focused on traits involved in precopulatory dynamics such as horns (Emlen et al., 2007) or plumage (Dunn et al., 2015), but much less is known about how traits involved in *postcopulatory* dynamics, such as sperm, seminal fluid, and FRTs, evolve (Pitnick et al., 2009b). For example, Wiens (2001) identified several phylogenetic studies documenting examples of evolutionary losses of male traits, and all but one of the 14 examples included traits involved in precopulatory events. In the case of many sperm traits, theory predicts that FRT trait changes drive changes in sperm — divergence in FRT design is thought to precede and induce concomitant changes to sperm (Pitnick et al., 2009b; Higginson et al., 2012b). We contribute to this topic by studying male genitalia, sperm, and FRT design in *Apotomus* ground beetles and by inferring ancestral states of sperm conjugation across Adephaga, and we interpret our detailed morphological results in the context of current phylogenetic thinking for the placement of *Apotomus* (Fig. 1).

The occurrence of singleton sperm in *Apotomus* is likely due to a secondary (i.e., derived) loss of conjugation based on maximum

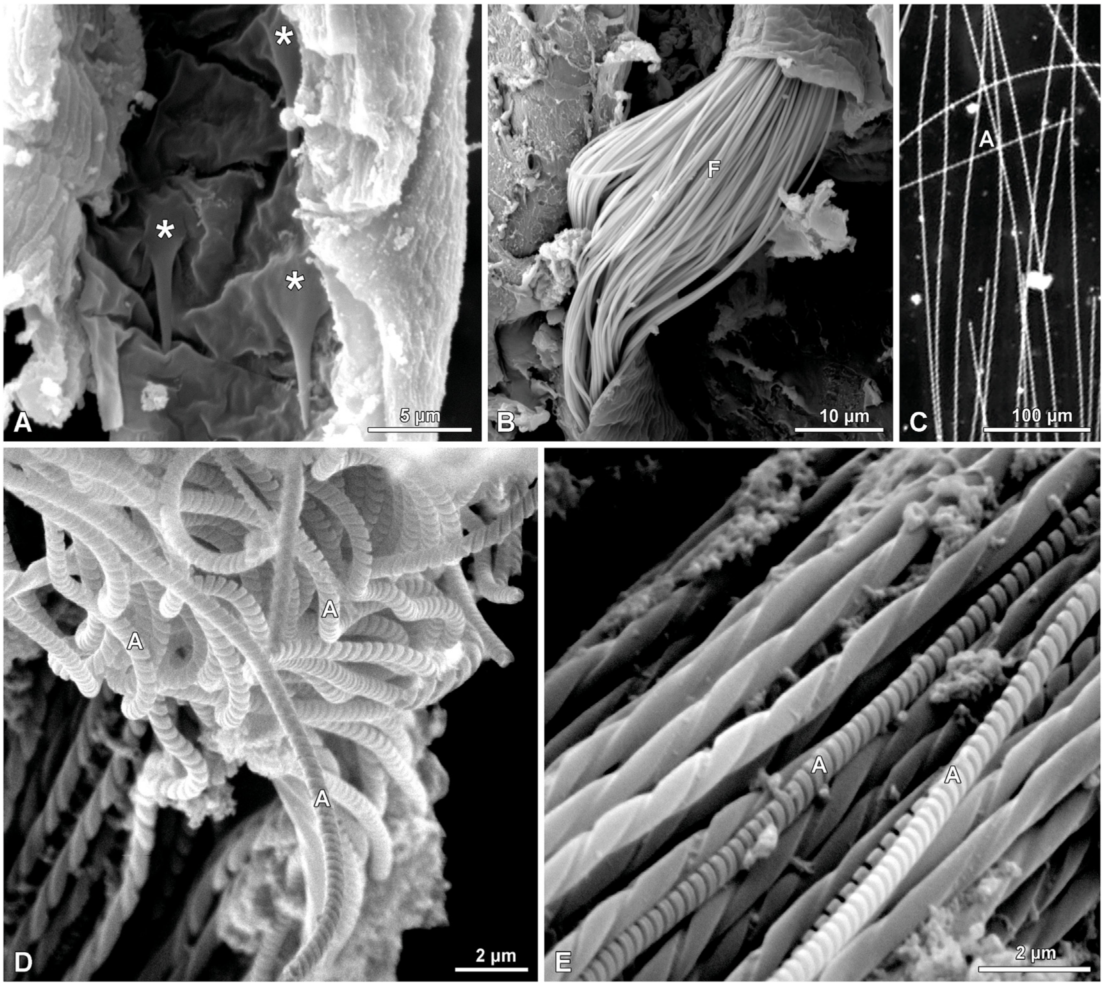

Fig. 6. SEM preparations of the spermatheca of *Apotomus* sp. showing the lumen and its surrounding cuticle (**A**, **B**, **D**, **E**) and a darkfield microscopy image of the external form of acrosomes (**C**). **A -** backwards pointing spines are evident in the spermathecal epithelium (asterisks). **B -** Numerous flagella (F) are visible within the spermathecal lumen. **C -** Group of acrosomes (A) showing their longitudinal helicoidal pattern. **D**, **E -** SEM preparations of the anterior region of acrosomes (A) showing the variable repeat of the helicoidal array.

likelihood reconstruction of ancestral states (Fig. 1). Sperm conjugation is typically rare across animal phyla (Higginson and Pitnick, 2011), but it is widespread in beetles of the suborder Adephaga, a dominant clade of insects with nearly 10,000 species that includes ground and diving beetles (Fig. 1; Higginson and Pitnick, 2011; Higginson et al., 2012b, 2015; Gomez and Maddison, 2020; Sasakawa, 2020). Sperm conjugation is assumed to be an adaptation under postcopulatory sexual selection (Moore et al., 2002; Immler, 2008; Higginson and Pitnick, 2011), and its pervasiveness throughout Adephaga's nearly 250 million year old phylogeny (Baca et al., 2021) suggests that it is frequently maintained (Fig. 1; Gomez and Maddison, 2020; Sasakawa, 2020). Ancestral character state reconstruction supports an early origin of spermatostyle conjugation in the phylogeny of Carabidae, and it is estimated to be present throughout its backbone regardless of uncertainties in the phylogeny (Gomez and Maddison, 2020). The exact phylogenetic position of *Apotomus* within Carabidae has long been ambiguous (Maddison et al., 1999), and although it has not been sampled in recent phylogenomic studies, its placement within the ground beetles has never been seriously questioned. Ancestral state reconstuctions suggest that conjugation was lost at some point along the branch leading to *Apotomus*, and we show that different possible placements of *Apotomus* within Carabidae have a minimal impact on ancestral states across the tree, including estimates of its parental node (Fig. 1). Recent phylogenetic evidence from 6 nuclear gene fragments for about 550 adephagan beetles places it sister to *Gehringia* and in a clade with the enigmatic tribe Melaenini (Maddison, D.R, unpubl. data). Comparative sperm-FRT data for these near relatives of *Apotomus* are scant, but they suggest that sperm-FRT traits have diverged substantially in this clade. It is not clear how long the lineage subtending *Apotomus* has had singleton sperm, but we surmise that it has been for most of its evolutionary history, as *Gehringia* also makes singleton sperm (Gomez and Maddison, 2020). In addition to making singleton sperm, *Gehringia* is unusual among carabids in their possession of two sperm-storage organs (Liebherr and Will, 1998). The sperm of Melaenini have not been studied in any species. However, the FRT of several Melaenini have been studied, and they possess elongate and narrow sperm-storage organs with tortuous diverticula that are reminiscent of the elongate tubules of *Apotomus* spermathecae though they lack their tightly coiled organization (Ball and Shpeley, 2005).

If conjugation is maintained by sexual selection, the placement of lineages with singleton sperm within clades with sperm conjugation implies that the selective environment for sperm (i.e., the FRT) has changed. We hypothesize that the absence of sperm conjugation in *Apotomus* beetles is due to the evolution of FRT sperm preference traits that no longer favored social cooperation

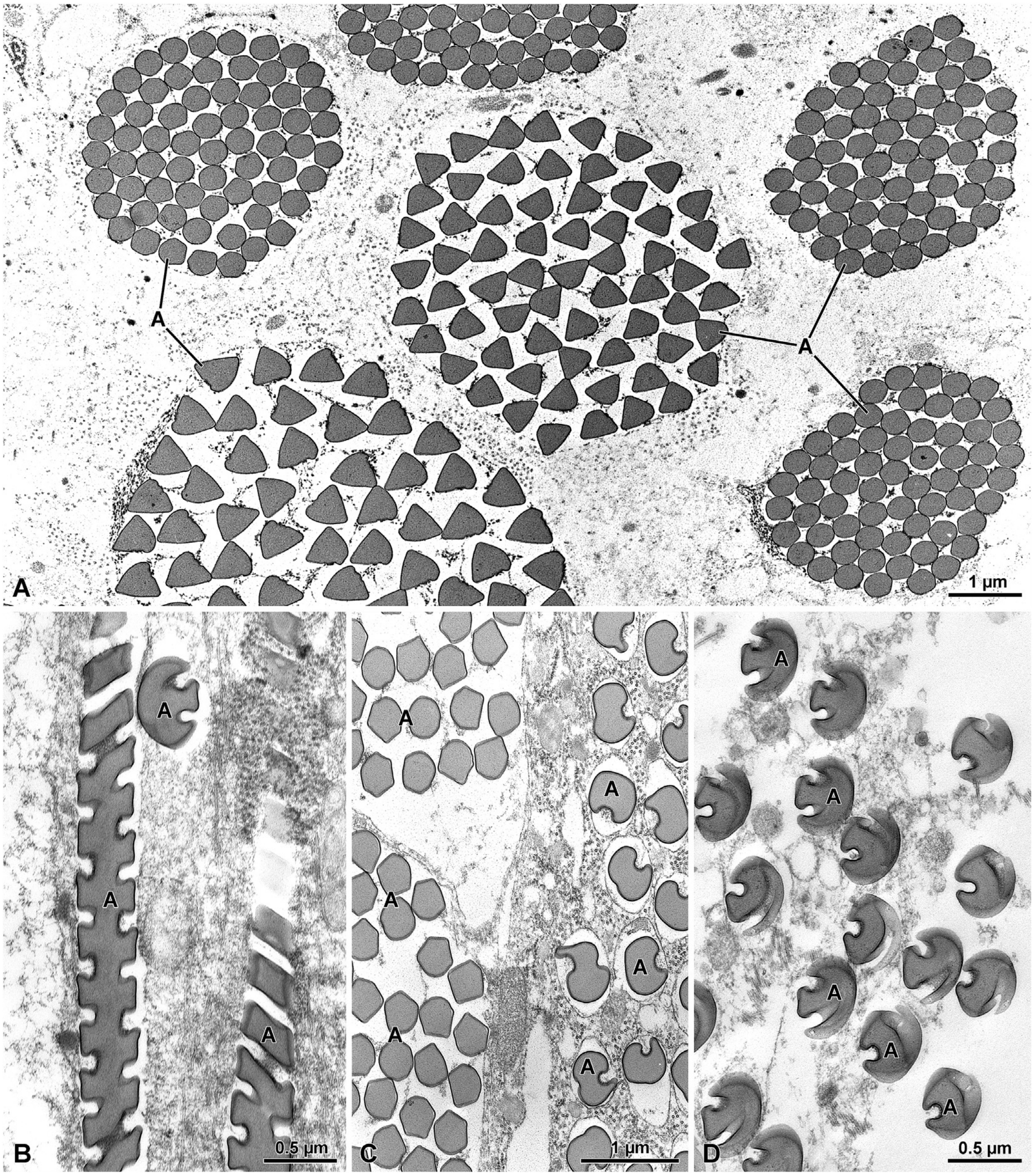

Fig. 7. A - Cross section of the single testis follicle of *Apotomus rufus* showing numerous cysts with 64 sperm cells sectioned at the acrosomal level (A). **B -** Longitudinal sections of acrosome (A) showing helicoidal pattern. **C**, **D -** Cross sections of the acrosomes (A) at different levels showing variation in its shape along its length.

among sperm via conjugation. A more rigorous evaluation of this hypothesis and alternative explanations for the absence of sperm conjugation in *Apotomus* await a richer understanding of the carabid phylogeny, and, for now, we can only speculate on the prcocesses underlying sperm-FRT co-diverisfication in this group. The FRT is thought to be a morphological proxy for female sperm preference traits (Eberhard, 1996), and there is substantial evidence for correlated evolution between sperm length and female sperm-storage organ length (or its associated duct) from a wide variety of animal taxa including birds (Briskie et al., 1997), mammals (Anderson et al., 2006), beetles (Dybas and Dybas, 1981; Rugman-Jones and Eady, 2008; Higginson et al., 2012b), flies (Pitnick et al., 1999; Presgraves et al., 1999; Minder et al., 2005; Holman et al., 2008), moths (Morrow and Gage, 2000), and snails (Beese et al., 2009). Sperm and FRTs, however, vary along many morphological axes, not just length (Pitnick et al., 2009a, b; Puniamoorthy et al., 2010), but little is known regarding the evolution of complex multivariate sperm traits such as conjugation (Higginson and Pitnick, 2011). Comparative data on multivariate sperm-FRT traits across 42 species of diving beetles indicate that elongation of the FRT drives the loss of sperm conjugation in this group (Higginson et al., 2012b). Sasakawa (2007) also found a correlation between conjugate length and spermatheca length in various species of Pterostichini ground beetles suggesting that sperm conjugation is evolving with the FRT, but this analysis did not correct for possible phylogenetic effects.

We speculate that the unusual singleton sperm of *Apotomus* evolved due to shifts in selection mediated by the FRT that favored individualization and later elaboration of individual sperm. Under this scenario, the loss of sperm conjugation along this branch occurred prior to the evolution of its divergent male traits including sperm cyst looping, long helicoidal acrosomes, and long sperm. Perhaps some sperm traits are unlikely to evolve when sperm are conjugated? There is evidence that sperm conjugation covaries

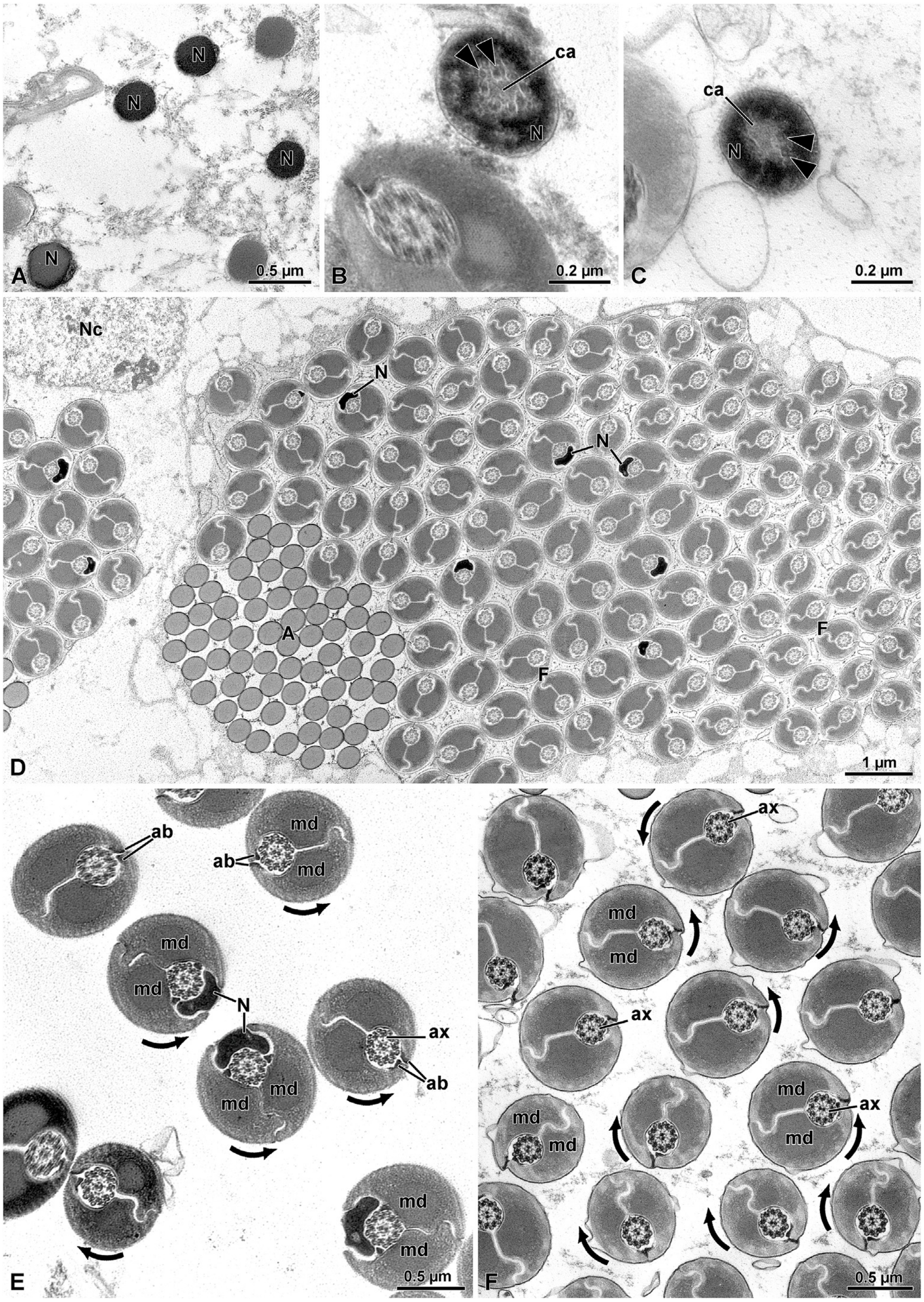

Fig. 8. *Apotomus rufus*. **A -** Cross sections of the cylindrical nucleus (N). **B**, **C -** Cross sections through two levels of the nuclear (N) posterior region hosting the centriole. Some centriolar doublets are visible (arrowheads), and there is minimal centriole adjunct material (ca). **D -** Cross section of a sperm cyst showing the acrosome (A), the nucleus (N), and the flagella (F). The presence of 64 acrosome and 128 flagella sections is indicative of sperm cyst looping. **E**, **F -** Closeup of the previous figure showing variation in orientation of sperm axonemes as a result of sperm cyst looping (see arrows). ax, axoneme; md, mitochondrial derivatives; ab, accessory bodies; N, nucleus. Note the position of the acccessory bodies at the nuclear extension above the axoneme.

with sperm head size (Higginson et al., 2012a; Gomez and Maddison, 2020), and it seems intuitive that following sperm conjugation loss, sperm would be permitted to evolve into new morphological spaces.

4.2. Insights into sperm-FRT dynamics

Our results provide suggestive evidence of correlated evolution among male and female reproductive systems, and in particular, we focus on the following morphological observations: (1) the male testis and female spermatheca are remarkably similar in structure, consisting of elongate and tightly coiled blind ducts (Fig. 2A–D), (2) the sperm are long, currently the 2nd longest known in Carabidae at 2.7 mm with an elongate and peculiar helicoidal acrosome (Fig. 6B–E; 7; Gomez and Maddison, 2020), and (3) the sperm cysts loop within their testis, which is a novel spermatogenic mechanism

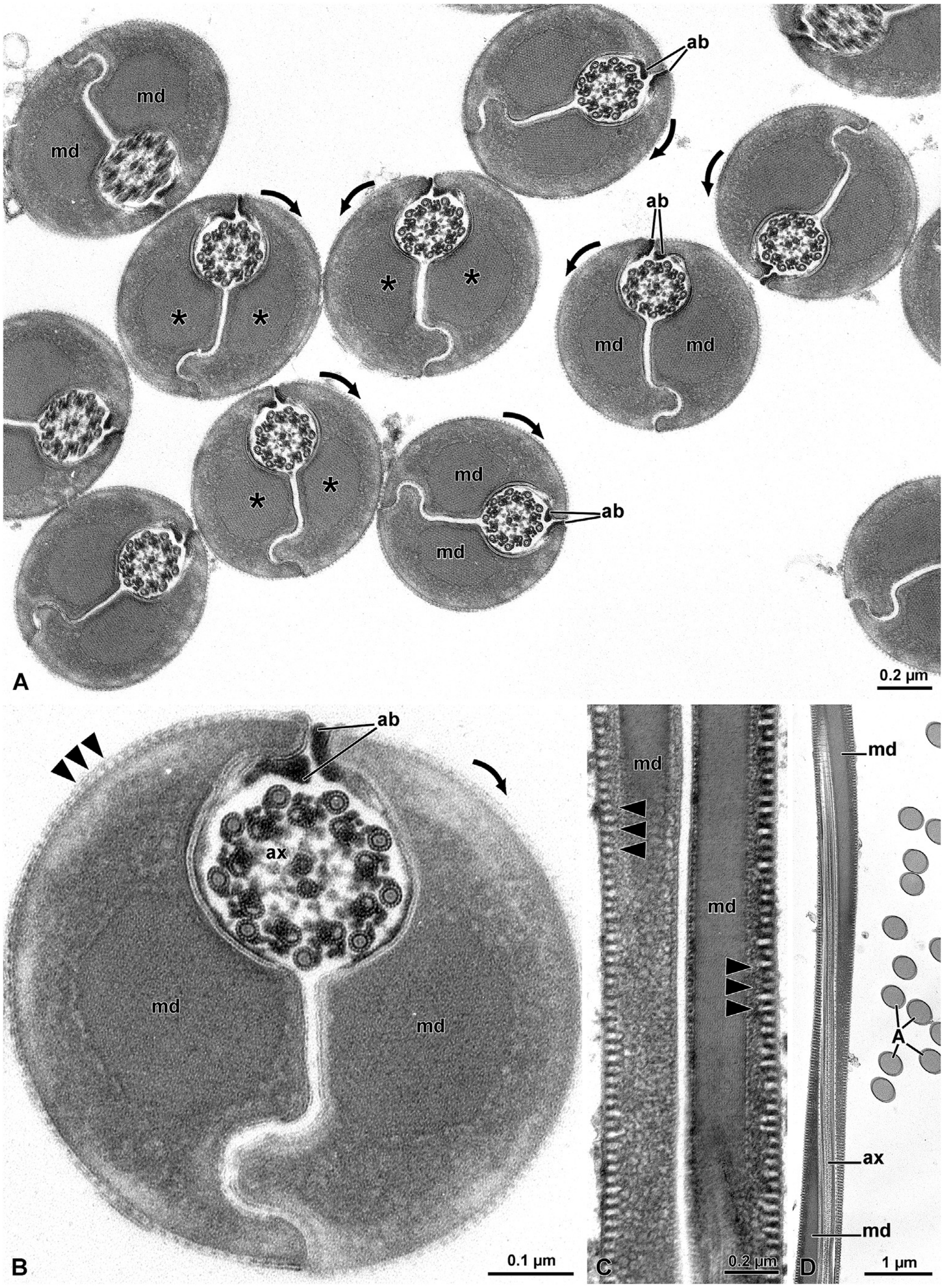

Fig. 9. A - Cross section of sperm flagella of *Apotomus rufus* showing variation in flagellar axoneme orientation based on the positioning of the dynein arms (see arrows). Md, mitochondrial derivatives with a large area of their matrix crystallized (asterisks); ab, accessory bodies. **B -** High magnification of the sperm flagellum showing the elaborate structure of the plasma membrane (arrowheads). The axoneme (ax) and the accessory bodies (ab) adhere to the mitochondrial derivatives (md). **C -** Longitudinal section of mitochondrial derivatives (md) showing the regular repeat of the peripheral cristae (arrowheads). **D -** Longitudinal section of a sperm flagellum with the helicoidal array of the mitochondrial derivatives (md) surrounding the axoneme (ax). A, acrosome.

known to covary with FRT design in some *Drosophila* (Fig. 5B; 8D–F; 9A; Syed et al., 2021). These traits are suspected of being derived based on current phylogenetic reconstructions and comprehensive morphological surveys of sperm (Gomez and Maddison, 2020), FRT (Liebherr and Will, 1998), and testis (Will et al., 2005) traits across the family. Near relatives of *Apotomus*, *Gehringia* and Melaenini have uncoiled testes and FRTs (Liebherr and Will, 1998; Ball and Shpeley, 2005), and *Gehringia* have short singleton sperm without obvious acrosomal modifications (Gomez and Maddison, 2020). In light of recent empirical and theoretical studies of sperm-FRT co-diversification, we argue that these observations collectively provide suggestive evidence for correlated evolution among male and female reproductive systems in *Apotomus*. We discuss each in more detail below.

(1) Testis and FRT form

The extreme similarity, distinctiveness, and likely divergent

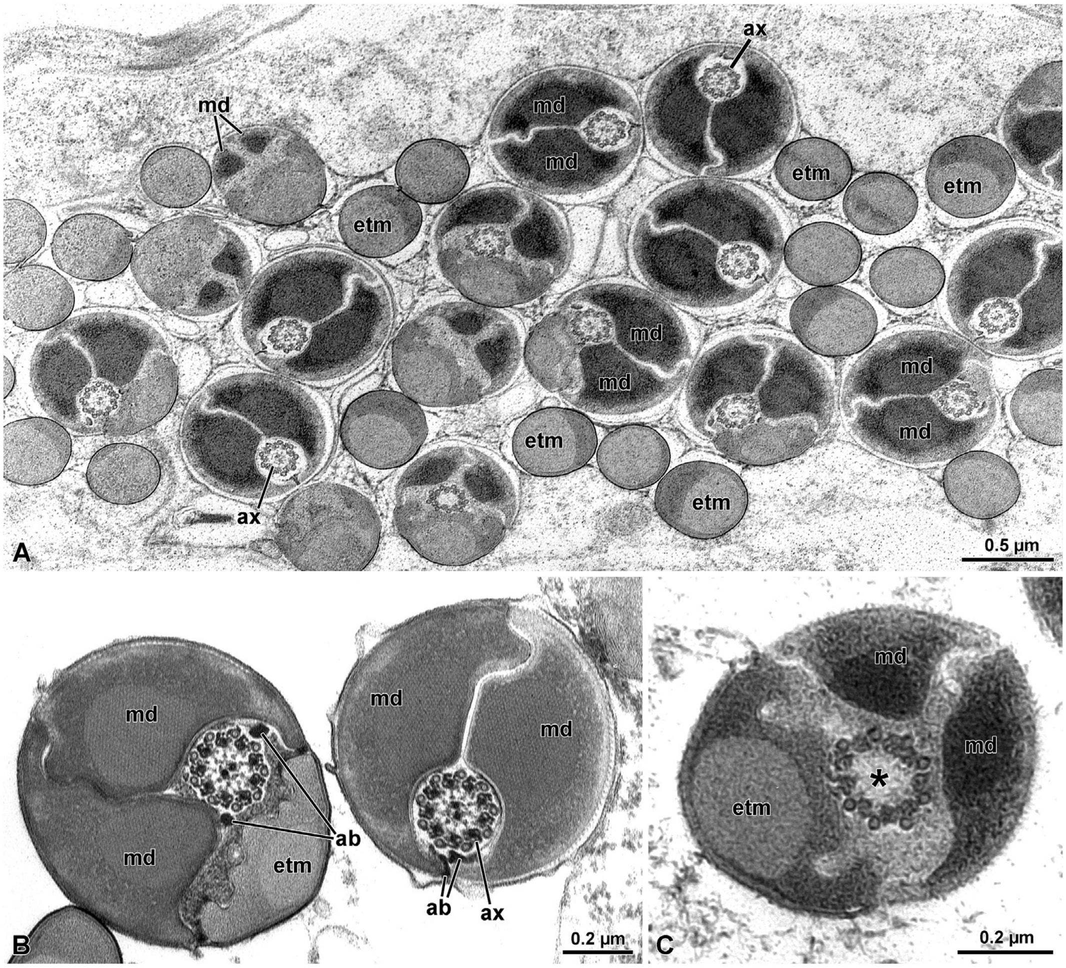

Fig. 10. **A -** Cross section of the posterior region of sperm flagella of *Apotomus rufus* displaying the axonemal end (ax) and the size reduction of the mitochondrial derivatives (md), which can also be missing. The cylindrical end of the flagellum lacks sperm components and contains an electron-transparent material (etm). **B -** Cross section of the posterior flagellum with small mitochondrial derivatives (md) and isolated accessory bodies (ab). ax, axoneme, etm, flagellar end with electron-transparent material. **C -** Detail of the axonemal end with microtubule doublets devoid of arms and central tubules (asterisk). The mitochondrial derivatives (md) have a very reduced size in this area and electron-transparent material (etm) is evident.

history of the coiled and disc-like testis and spermatheca of *Apotomus* suggests that these traits are evolutionarily correlated. In their survey of testis morphology across 820 species of Carabidae, Will et al. (2005) reported that the male reproductive system of *Apotomus* is unique among ground beetles – consisting of a single follicle coiled into a disc. Our study confirms this divergent testis shape by describing in detail that the testis consists of numerous concentric turns or loops of the canal, each one containing a great number of sperm cysts. From our observations it appears that the loops form a continuous series and that the thicker loops include numerous sperm cysts (Fig. 5A), whereas the thinner loops contain few to no cysts (Fig. 4D). Aside from its being tightly coiled, the shape of *Apotomus* testes conforms to the general structure of Carabidae testes, which show variation among species but are universally unifollicular (Lawrence and Britton, 1994; Carcupino et al., 2002; Sasakawa, 2007; Schubert et al., 2017). What is remarkable about *Apotomus* is the extreme similarity of the testis and the spermatheca, which had not been previously studied (Fig. 2A–D). In both cases, these structures are made of a series of overlapping rings of almost the same diameter and length. These traits seem to be particular to the branch leading to *Apotomus* suggesting that they have evolved together. Considering the combination of elaborate male and female traits in these species, our observation of monorchidy in one of our studied southern African specimens is curious, and this finding implies a 4th indepedent derivation of monorchidy in Carabidae (Will et al., 2005). However, we refrain from further discusssion here given our uncertainty in the taxonomic identity of the species we studied, the lack of a modern species revision of African *Apotomus*, and the variation observed in monorchidy among our very limited sampling.

(2) Long sperm with peculiar acrosomes

We presume that the elongate 2.7 mm sperm of *Apotomus* and its unusual 800 μm helicoidal acrosome have evolved because of FRT sperm preferences. Several studies across a wide variety of taxa have confirmed a correlation between sperm length and FRT length (Pitnick et al., 1999; Briskie et al., 1997; Minder et al., 2005; Anderson et al., 2006; Higginson et al., 2012b), and variation in the acrosome has long been presumed to be indicative of poscopulatory sexual selection due to its role in egg penetration (Afzelius, 1970; Fawcett and Phillips, 1970; Tilney, 1985). We can only speculate as to the function of a long acrosome in *Apotomus* reproduction, and we hypothesize that it evolved due to its contribution in making sperm longer. In *Drosophila*, longer sperm are superior at displacing and resisting displacement by shorter competitor sperm within the seminal receptacle, and the evolution of longer seminal receptacles drives the evolution of longer sperm by enhancing their competitive advantage (Pitnick et al., 1995b; Miller and Pitnick, 2002, 2003; Pattarini et al., 2006; Lüpold et al., 2012, 2016, 2020). The co-occurrence of long sperm with elongate acrosomes and an extremely long tubular spermatheca suggests there has been a correlated response between these traits in *Apotomus* beetles.

The acrosome of *Apotomus* is extraordinarily long and reminiscent of the acrosomes of a few unrelated groups of early-diverging hexapods. It has a variable helicoidal array for most of the anterior region, and it becomes a straight cylinder towards its posterior end. Long acrosomes are not commonly observed in insect sperm, and they are known to occur in the following groups: various Archaeognatha (Dallai, 1972; Wingstrand, 1973; Fanciulli et al., 2015), in some aquatic Heteroptera (Tandler and Moriber, 1966; Afzelius et al., 1976; Lee, 1985; Jamieson et al., 1999; Miyata et al., 2011; Novais et al., 2017; Dallai et al., 2021a, b), in the ground-louse *Zorotypus impolitus* (Dallai et al., 2014), and in several ladybird beetles (Dallai et al., 2018). Nearly all carabids studied to date possess a small acrosome as is typical among insects (Jamieson et al., 1999; Dallai et al., 2019, 2020). In some members of the family, however, the structure becomes very elaborate, increasing in width in *Calathus* and *Pogonus* (Dallai et al., 2020; Dallai, R., unpubl. data).

The function of a long acrosome is unknown, and most research on the acrosome has focused on its role in the phsiological events of fertilization. It is unlikely that the acrosome serves for sperm motility in any way, as this is carried out by activity of the flagellar axoneme (Werner and Simmons, 2008), but the presence of a long acrosomes located anteriorly seems to be a likely obstacle to sperm movement as it has to generate forward motion from its posterior flagellar motor. It is uncertain if the unusual shape of the acrosome contributes to the physiological events in fertilization, as other organisms with short acrosomes complete these events succesfully (Flesch and Gadella, 2000; Evans, 2002; Talbot et al., 2003; Neill and Vacquier, 2004; Rubinstein et al., 2006). Furthermore, the acrosome is not involved in exocytosis in some *Drosophila*; instead, it remains intact after its entry through the micropyle (Wilson et al., 2006). In the water strider *Aquarius,* the long acrosome does not contribute to any physiological process within the fertilized egg as it remains structurally intact even after early gastrulation events (Miyata et al., 2011); something similar also occurs in *Drosophila* (Karr, 1996; Snook and Karr, 1998; Pitnick and Karr, 1998; Karr et al., 2009). Some eggs are not activated by acrosomes whatsoever. It is well known that in many wasps, as well as in *Drosophila*, eggs can be activated by mechanical stimulation, when they are squeezed through the oviduct or the ovipositor before egg laying (Doane, 1960; Callaini et al., 1999; Horner and Wolfner, 2008), and in some stick insects eggs are activated by exposure to air (Went, 1982). Finally, in several insects, the acrosome is missing altogether, as for instance in the dipteran Cecydomyidae (Dallai, 2014). In conclusion, the presence of a long acrosome, like that observed in *A. rufus*, could possibly be involved in a different role in reproduction not directly connected with gamete fusion. For now, we can speculate on the possibility that a long acrosome could enhance the success at fertilization by contributing to the realization of a long sperm.

(3) Sperm cyst looping

The sperm cysts of *Apotomus* loop within their testis allowing for a possible decoupling of sperm and testis length as has been suggested recently for some *Drosophila* fruit flies (Syed et al., 2021). This novel spermatogenic trait was herefore known only from few other species, including a small clade of *Drosophila*, where it is thought to be an adaptation to sexual selection permitting males to produce longer, more competitive sperm in relatively short testes (Syed et al., 2021), and the basal families of tenebrionids Ripiphoridae and Mordellidae (Dias et al., 2022a, b). Cyst looping is thought to occur via a heterochronic mechanism, whereby spermatid tails develop at a faster pace than spermatid and somatic cyst cell membranes, and phylogenetic comparative analyses indicate that this developmental innovation enabled the evolution of long sperm in males while evading delays in reproductive maturation time (Syed et al., 2021). The cost of producing long sperm via longer testes is thought to be a constraint to sperm evolution (Pitnick et al., 1995a), and we hypothesize that like these fruit flies (Syed et al., 2021) as well as the basal Tenebrionids (Dias et al., 2022a, b), male *Apotomus* have partially avoided the evolutionary allometry of making long sperm in their compact testis by sperm cyst looping. This evolutionary novelty covaries with seminal receptacle length (Syed et al., 2021), which is the most rigorously documented mechanism of cryptic female choice in *Drosophila* (Miller and Pitnick 2002, 2003; Pattarini et al., 2006; Lüpold et al., 2012, 2016, 2020). Therefore, the occurrence of cyst looping in *Apotomus* might also be evidence for an evolutionary correlation between sperm and FRT design in this group of beetles.

4.3. Further comments on sperm ultrastructure

The general structure of *Apotomus* sperm components has a great similarity with that exhibited by some aquatic heteropterans such as the water measurer bug, *Hydrometra stagnorum* (Linnaeus), the water strider. *Gerris lacustris* (Linnaeus), and other bug species belonging to the infraorders Nepomorpha and Gerromorpha (Tandler and Moriber, 1966; Dallai and Afzelius, 1980; Miyata et al., 2011; Dallai et al., 2021a, b). The long sperm of *Apotomus* are characterized by a short nucleus (Gomez and Maddison, 2020), which is difficult to find in cross sections but is detectable after Hoechst staining (Fig. 2E). When visible, it appears as a short cylinder that prolongs into a saddle on the dorsal side of the flagellar axoneme. Apart from its small size, its general shape and pattern is not drastically different from other members of the Carabidae (Dallai et al., 2019, 2020). A short nucleus is also found in tiger beetles and various unrelated carabid lineages with and without conjugation, including various Harpalinae (Dallai et al., 2020; Gomez and Maddison, 2020; Schubert et al., 2017). The posterior nuclear region hosts the centriole, which is surrounded by a very poor centriole adjunct material.

The axoneme is typical of many insect orders (Jamieson et al., 1999). It has a 9 + 9+2 microtubule pattern with evident radial links and accessory tubules with 16 protofilaments in their tubular wall (Dallai, 2014). The intertubular material is associated with the accessory tubules, and it is also present between the microtubular doublets.

The mitochondrial derivatives of *Apotomus* are quite peculiar as they strongly diverge from those described in other Carabidae (Dallai et al., 2019, 2020). Curiously, as with the acrosome, the general shape of the mitochondrial derivatives strongly resemble those described in some aquatic heteropteran sperm (Tandler and Moriber, 1966; Dallai and Afzelius, 1980; Lee and Lee, 1992; Miyata et al., 2011; Dallai et al., 2021a, b). They are expanded to embrace the axoneme, appearing as two half-moons (Fig. 9). The left mitochondrion has a protrusion that interlocks with the invagination of the right mitochondrion (Fig. 9A). Its matrix is compact with a large central crystallized region. A cross section through the sperm flagellum shows a perfect circular appearance with the outer plasma membrane possessing a fine structural repeat (Fig. 9B). When observed in longitudinal section (Fig. 9C), the two mitochondrial derivatives surround the axoneme in helicoidal fashion, and their peripheral region shows an orderly series of cristae as is common among insects (Jamieson et al., 1999).

The shape and size of the accessory bodies is quite unusual in *Apotomus* compared to other carabid species. Contrary to the general pattern of large accessory bodies in beetles (Jamieson et al., 1999; Dallai, 2014), Carabidae tend to have small accessory bodies located on top of the mitochondrial derivatives, beneath the axoneme. Their general shape is almost triangular, and frequently one of the accessory bodies, typically the right one, is larger than the other (*Brachinus italicus*, *Calathus fuscipes* and *Pterostichus melanarius*; Dallai et al., 2019, 2020). Typically, each accessory body is separated from the adjacent mitochondrial derivative by a flattened cistern, a residual of the long cistern surrounding the mitochondria during spermiogenesis (Dallai et al., 2020). Unlike most Carabidae, the accessory bodies of *Apotomus* are two small dense elliptical structures that adhere to the extremities of the mitochondrial derivatives located above the axoneme (Fig. 9A and B) without any obvious cistern-like material separating them from the mitochondrial derivatives. They are asymmetrical in their association with mitochondrial derivatives. In a cross section, the one on the right is positioned between the tip of these structures and the axoneme, while the one on the left is placed between the two dorsal extremities of the mitochomdrial derivatives. The small size of the accessory bodies might be linked to the formation of the centriole adjunct as these structures have a common origin (Jamieson et al., 1999; Dallai, 2014).

Authors contributions

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

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