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The ultrastructure of the spermatheca of *Mordellistena brevicauda* (Coleoptera, Tenebrionoidea) and the associated bacterial cells

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A B S T R A C T

The ultrastructural study on the female reproductive system of the beetle *M. brevicauda* (Mordellidae) confirmed the positive correlation between the length of the sperm and the size of the female seminal receptacle (Spermatheca).

The spermatheca of the species is characterized by an apical bulb-like structure where the spermathecal duct forms numerous folds filled with sperm. At this level many bacterial cells are present intermingled with the duct folds. Some are organized in large structures, such as bacteriomes, while other are single bacteriocytes. The latter are often found near the basal lamina of duct epithelium. In addition, some bacteria are visible in the cytoplasm of the duct epithelial cells. Interestingly, bacterial cells have never been observed in the duct lumen. The possible function of the bacterial cells is discussed.

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1. Introduction

It has been established that natural selection acts so that the female reproductive organs drive the evolution of male genital system; more specifically multiple evidences support a correlated evolution between the dimensions of the sperm storage organ and the sperm length. Studies on different insect groups have confirmed this correlation (Dybas and Dybas, 1981; Presgraves et al., 1999; Morrow and Gage, 2000; Miller and Pitnick, 2002; Minder et al., 2005; García-González and Simmons, 2007; Higginson et al., 2012; Dallai et al., 2012, 2014; Kotrba and Heβ, 2013).

It has been also suggested that females have preference for sperm of a given length and are able to discriminate among competing sperm of different length (Keller and Reeve, 1995).

The adaptive significance of the sperm length is supported by sperm competition, since longer sperm have some advantage over shorter ones at the time of fertilization. In particular, longer sperm would be more effective at both displacing and resisting displacement by shorter competitor sperm (Pattarini et al., 2006; Hunt et al., 2002; Werner and Simmons, 2011; Lüpold et al., 2016).

A recent ultrastructural study on the sperm structure of basal groups of Tenebrionoidea, the Mordellidae and Ripiphoridae (Dias et al., 2022a) has indicated that these groups not only have long sperm but also a sperm flagellar posterior region that exhibits the axoneme with only accessory tubules (Dias et al., 2022b). These findings suggest investigating the female reproductive apparatus of a species of Mordellidae to verify whether this apparatus and the sperm storage organ, the spermatheca in particular, showed morphology and a size that support the occurrence of a positive correlation with the sperm size. The present work aims to answer to this question.

During the study on the spermathecal ultrastructure of *Mordellistena brevicauda*, we found intermingled with the spermathecal ducts, a large number of cell bodies of different size full of bacteria, commonly defined as bacteriocytes, forming the so-called bacteriome. Bacteriocytes have been commonly found in different insect organs (Buchner, 1965; Baumann, 2005; Ferrari and Vavre, 2011; Masson et al., 2016), but only a few examples concerned the spermatheca, where bacteria were always detected within the lumen of this organ (Marchini et al., 2014; Raina et al., 2007).

2. Materials and methods

Three females of *M. brevicauda* were collected near Siena (Campiglia d'Orcia, 42.94582° N, 11.66472° E) during summer 2022. They were dissected in 0.1 M, pH 7.2 phosphate buffer (PB) to which 3 % of sucrose was previously added. The whole female genital apparatus was isolated and photographed with a Leica DMRB light

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phase contrast microscope equipped with a Zeiss MRC5 digital camera. The spermatheca of two females was isolated from the whole apparatus and fixed overnight at 4 °C in 2.5 % glutaraldehyde solution in PB. After rinsing with PB, the material was post-fixed with 1 % Osmium tetroxide in PB for 2h, then carefully rinsed and dehydrated in a series of alcohol (50 %−100 %) and embedded in a mixed Epon-Araldite resin. Semithin sections, obtained with a Reichert Ultracut ultramicrotome, were stained with 0.5 % Toluidine blue. Ultrathin sections were routinely stained with uranyl acetate and lead citrate and observed with a CM10 Philips electron microscope operating at 80 KV.

3. Results

The female reproductive apparatus of *M. brevicauda* consists of two ovaries, each one with 4−5 ovarioles showing almost mature eggs at their proximal region. The lateral oviducts are about 400−500 μm long and flow into a common oviduct about 1.2 mm long (Fig. 1A, B). At the end of this oviduct a sperm storage organ, the spermatheca, is evident. It consists of an elongated structure, 1.2 mm long, with an irregular diameter along its length: with short regions about 60 μm wide alternated with others about 120 μm wide (Fig. 1B). Apically, the spermatheca ends with a bulb-like structure 220 × 130 μm (Fig. 1B, C). The spermatheca has a quite thin spermathecal duct, about 25 μm wide, that opens at its almost distal end in a cavity about 50 × 100 μm wide. From this cavity, the spermathecal duct gives rise to two parallel thin ducts, each one 200 μm long, opening into the bulb-like terminal structure. The ducts form a rolled complex (Fig. 1D).

A semithin section of the apical bulb structure shows numerous cross-sectioned thin ducts with their lumen filled with numerous sperm (Fig. 2A). Among the duct sections more or less large bodies are also visible, sometimes in contact with the duct epithelium. The duct cross sections show a different width, some of them reaching about 20 μm while others are only 10−12 μm wide (Fig. 2A). A cross section through the region where the large cavity is visible shows a conspicuous muscle layer surrounding the epithelium (Fig. 2A). A cross section through the apical region of the spermatheca, where the bulb structure exhibits the numerous rollings of the spermathecal duct, shows that each duct section is lined by epithelial cells, 7.5−9.5 μm high and about 8 μm width, hosting a globular nucleus about 4.4 μm in diameter. The epithelial cells are laterally delimited by a sinuous plasma membrane along which apical *zonulae adhaerentes* and long gap junctions are visible. Often, due to the close position of two duct folds, the epithelia are in contact with their basal laminae (Fig. 2D). Epithelial cells are apically lined by a cuticular layer, only 1 μm high with a thin epicuticle, a middle layer, 0.3 μm thick, of compact electron-dense material and a deep layer 0.6 μm high of procuticle apparently consisting of fibrous moderately electron-dense material (Fig. 2B-C).
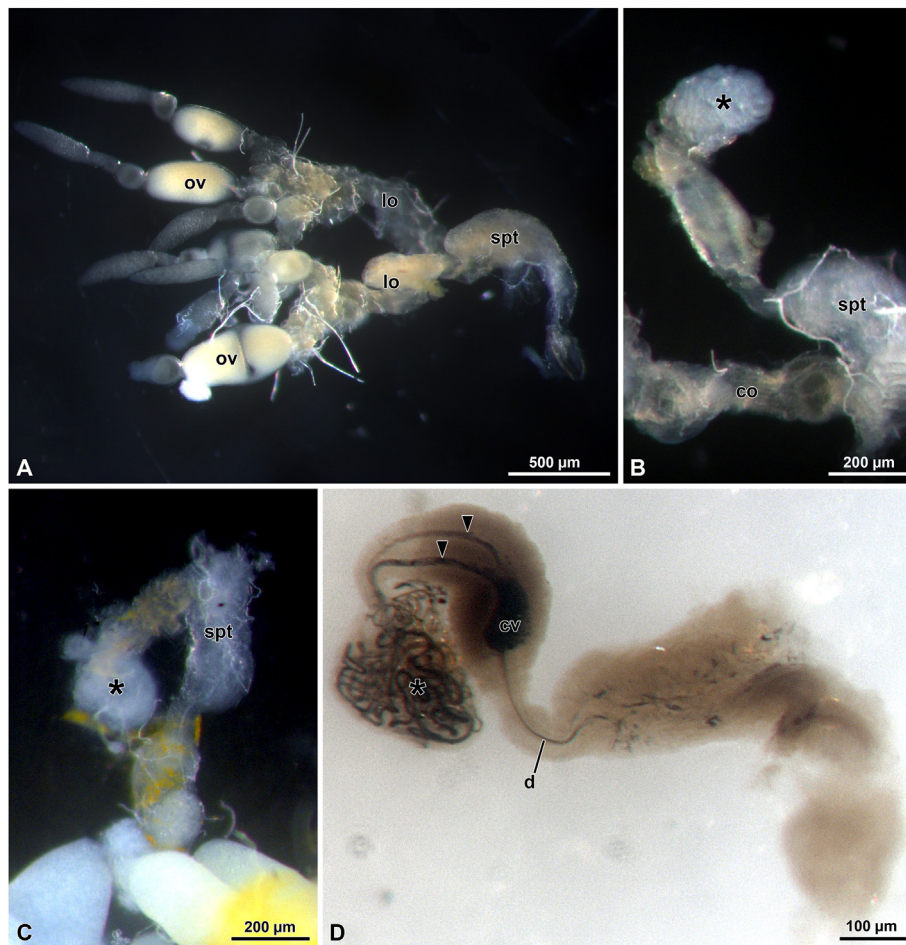
Fig. 1. A) The female genital apparatus with the ovaries (ov), the lateral oviducts (lo) and the spermatheca (spt). **B, C)** The spermatheca (spt) consists of a long structure showing a different wideness along its length. Note its distal globular end (asterisks). co, common oviduct. **D)** Spermatheca after fixation and inclusion. Note the thin duct (d) that in the distal region flows into a large cavity (cv) from which two ducts (arrowheads) open into the apical globular structure (asterisk) forming a complex of tightly folded ducts.
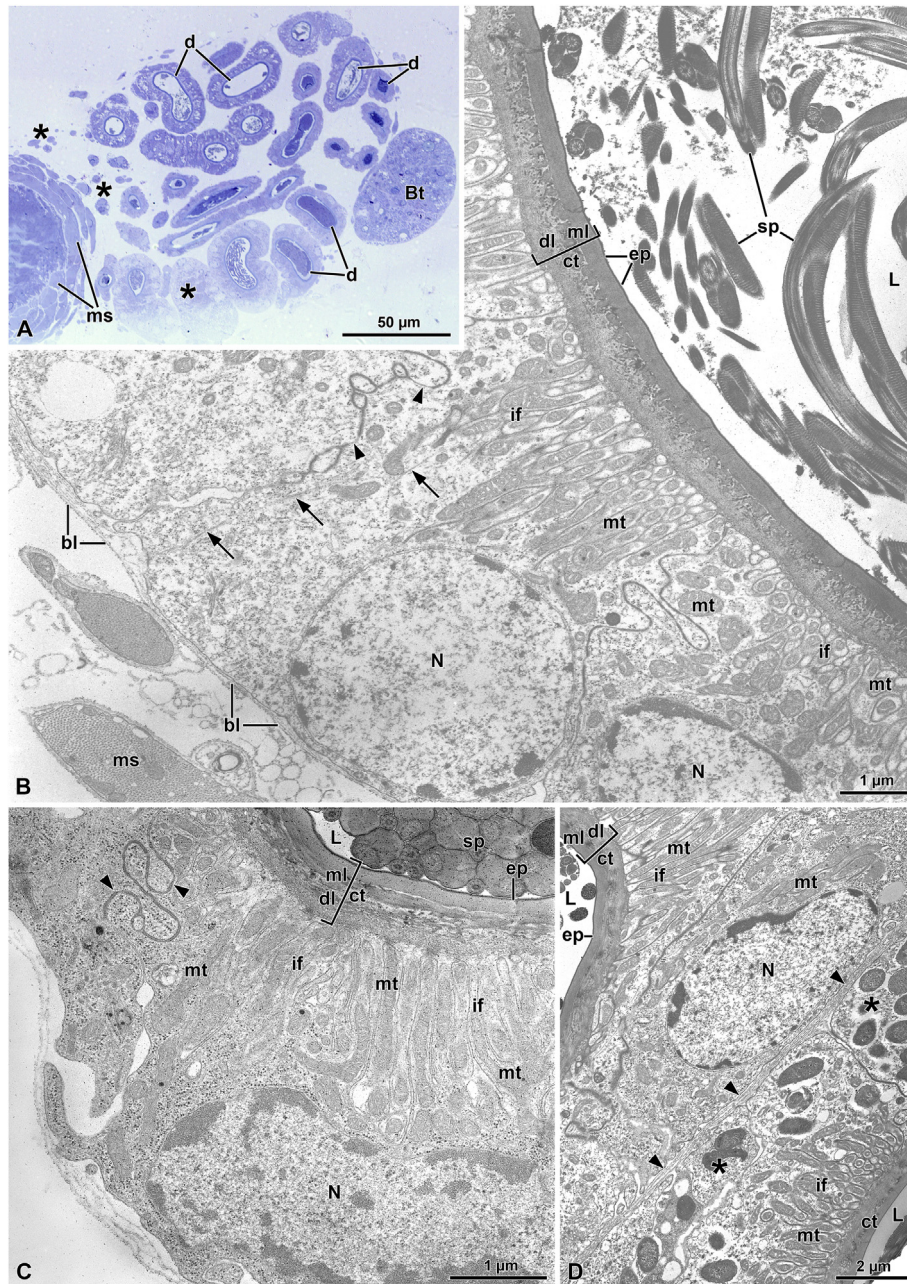
Fig. 2. A) Semithin section of the apical spermathecal bulb showing several cross-sectioned ducts (d) with their lumen filled with sperm. Note the more or less large bodies corresponding to the bacteriome (Bt) and bacteriocytes (asterisks). ms, muscle fibers. **B**, **C)** Cross sections through the duct wall with the apical cuticle (ct) beneath which a series of deep membrane infoldings (if) are visible. Arrowheads, gap junctions; arrows, cistern of endoplasmic reticulum; bl; basal lamina; dl, deep cuticle layer; ep, epicuticle; ml, middle cuticle layer; ms, muscle fibers; mt, mitochondria; N, nucleus; sp, sperm in the lumen (L). **D)** Cross section through the contact between two ducts epithelia (arrowheads). Asterisks, bacterial cells; ct, cuticle; dl, deep cuticle layer; ep, epicuticle; if, membrane infoldings; L, duct lumen; ml, middle cuticle layer; mt, mitochondria; N, nucleus.

Beneath the cuticular layer, a complex of membrane infoldings are present which extend for 1.2–1.5 μm deep into the cytoplasm. Several mitochondria are hosted in the infoldings. In the deep cytoplasm only a few mitochondria and rare cisterns of endoplasmic reticulum are visible. The cells are lined basally by a thin basal lamina beneath which scattered muscle fibres are present. The duct lumen has a quite variable diameter (6.5–8.5 μm wide) and contains many cross sectioned sperm (Fig. 3A–C). These have the peculiar ultrastructure described recently (Dias et al., 2022a, 2022b), consisting of a thin and short acrosome, a cylindrical nucleus and a long flagellum, the posterior region of which appears

dense, straight and immotile, containing only a crown of 9 accessory tubules (Fig. 3C).

The epithelial cells are often associated with more or less large bodies consisting of cells containing numerous bacterial cells. These bodies can be very large, up to 37 μm wide, contain 3-4 nuclei 5.3 μm in diameter, filled with numerous bacterial cells, to constitute a bacteriome; smaller bodies commonly defined as bacteriocytes, only 6.5 μm wide, are also visible lining the duct. Degenerating bacterial cells are also visible within some of such bodies (Figs. 2A; 4A-B; 5A-C).
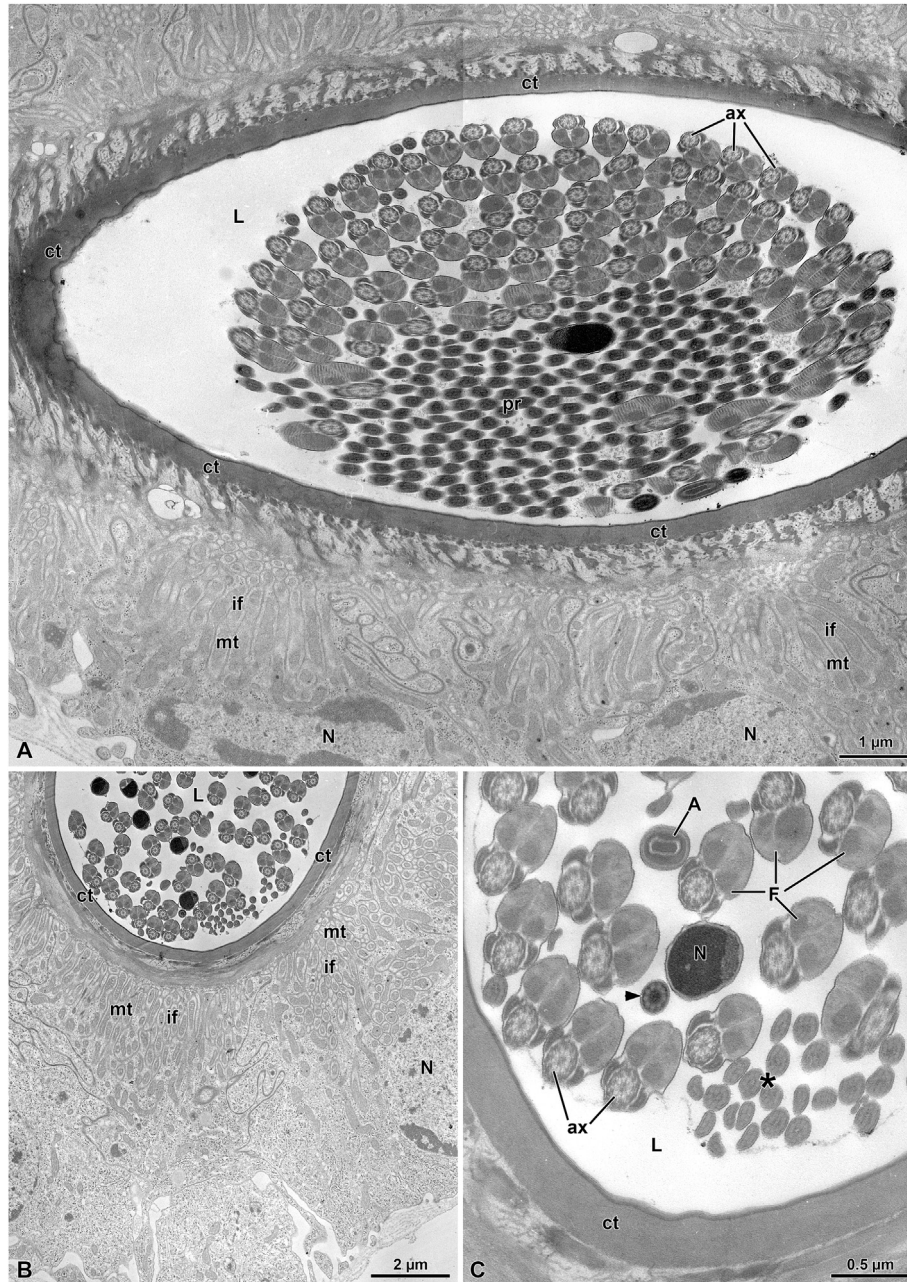
Fig. 3. A) Cross section of a duct filled with a sperm bundle in the lumen (L). Note that such a bundle consists of sperm bending showing two different flagellar levels: the sub-nuclear region with the complete axoneme organization (ax) and the posterior region (pr) with only accessory tubules. ct; cuticle; if, membrane infoldings; mt, mitochondria; N, nucleus. **B)** Cross section of a duct showing several sperm in the lumen (L). Beneath the cuticle (ct) many membrane infoldings (if) associated with mitochondria (mt) are visible. N, nucleus. **C)** Detail of sperm in the duct lumen (L). A, acrosome; arrowhead, posterior flagellar region with only accessory tubules; asterisk, end part of flagellum; F, anterior flagellar region; N, nucleus.

4. Discussion

Mordellidae, to which *M. brevicauda* belongs, is a basal family of Tenebrionoidea. This family, together with Ripiphoridae, is characterized by having long sperm. Although *M. brevicauda* sperm are about 300 μm long, much longer sperm (1200 μm or more) were observed in other species of the two families (Nardi et al., 2013; Dias et al., 2022a, 2022b). These two families, in addition to the peculiar antiparallel array of sperm within the germ cysts during the spermiogenesis (Dias et al., 2012), show an atypical sperm flagellar structure with the tail end of peculiar incomplete axonemal organization (Dias et al., 2022b). These evidences are consistent with the indication that both the mentioned families are closely related and form a sister group of all Tenebrionoidea (Zhang et al., 2018; McKenna et al., 2019; Dias et al., 2022b).

The presence of long sperm has a double consequence from an evolutive point of view: I) they are the results of a positive correlation with the female storage organ dimension, and II) they are positively selected by the female as it was well established that larger sperm are more efficient at displacing shorter sperm and resisting to displacement from the organ closest to the site of egg fertilization by shorter sperm (Pattarini et al., 2006; Higginson et al., 2012). The present study on the spermatheca of *M. brevicauda* confirms such a positive correlation. Also, it
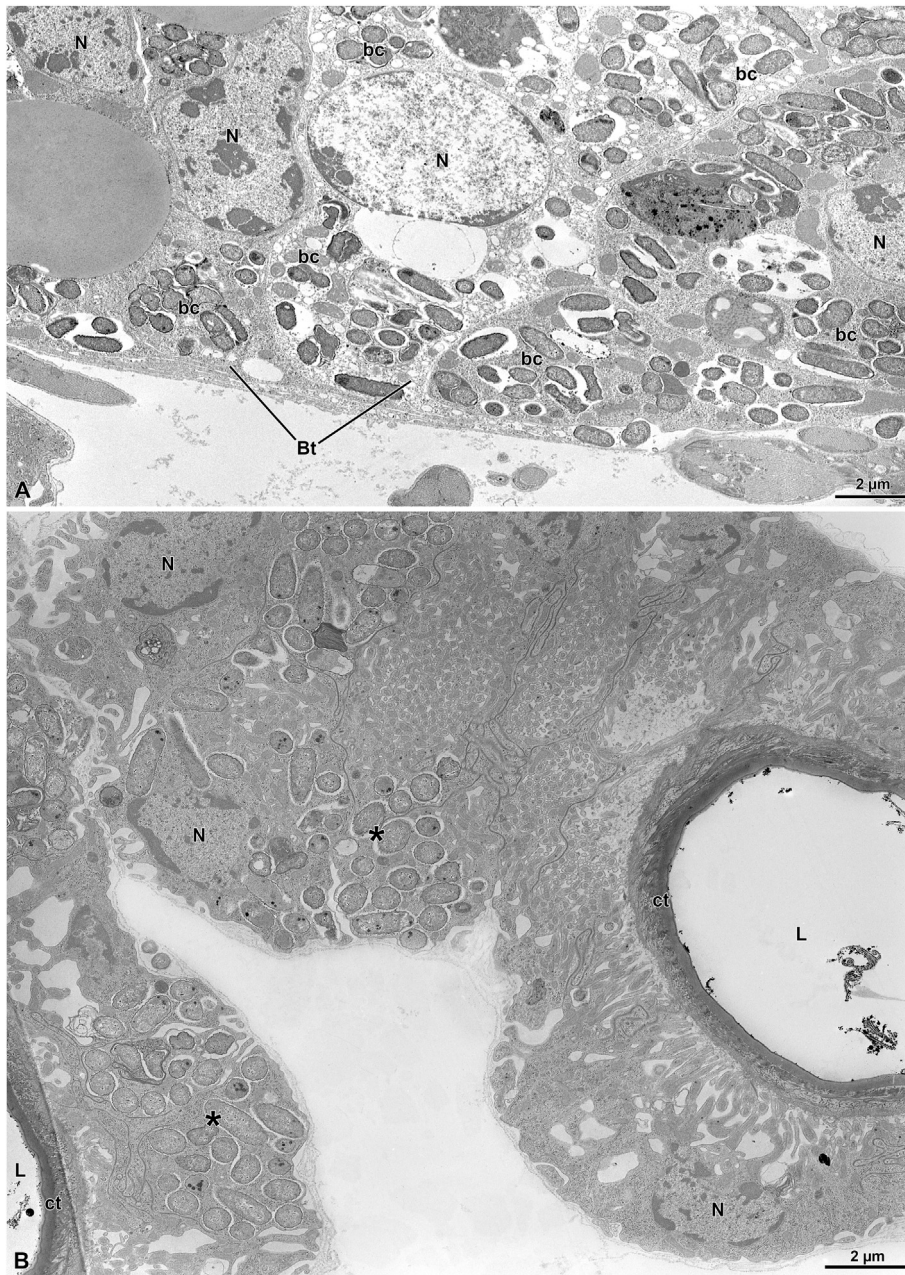
Fig. 4. A) Cross section of a bacteriome (Bt) containing numerous bacterial cells (bc). N, nucleus. **B)** Cross section through bacteriocytes within the duct epithelial cells. Some bacterial cells (asterisks) are intermingled with the epithelial cells. ct, duct cuticle; L, duct lumen; N, nucleus.

underlines that, while the general shape of the spermatheca of the species has a pretty conventional structure, it is apparently not extraordinarily long as it occurs, for instance, in the Heteroptera *Gerris* and *Hydrometra* (Dallai et al., 2021a, Dallai et al., 2021b) or in the Diptera *Drosophila bifurca* (Pitnick et al., 1999), the spermathecal duct is apparently extremely long and it is confined in the apical region of the spermatheca. At this level the thin duct forms a rolling complex, each section level of which shows a duct lumen filled with numerous sperm. The epithelial cells of the duct have the typical organization of cells involved in fluid reabsorption being endowed with a series of apical membrane infoldings and associated mitochondria located beneath the cuticular layer (Berridge and Oschman, 1972; Wall et al., 1975; Bradley, 1985).

The most interesting and unusual occurrence in the spermathecal structure is the presence, at the level of the apical bulb, of several bodies housing bacterial cells intermingled with the spermathecal ducts. Some of these structures are large, pluri-nucleate and contain numerous bacterial cells as are the bacter-iomes. More often, however, smaller formations compatible with the so-called bacteriocytes are visible close to the basal duct epithelium, adhering to the basal lamina or even housed into the epithelial cells. Apparently the epithelial cells seem not to be altered in their structure and presumably in their functional activity as their apical region with the membrane infoldings with associated mitochondria do not show ultrastructural modifications. Some isolated bacteria are also visible in the cytoplasm of the epithelial cells intermingled with cytoplasmic components.

The presence of bacteriocytes in insects is not a rare event, and
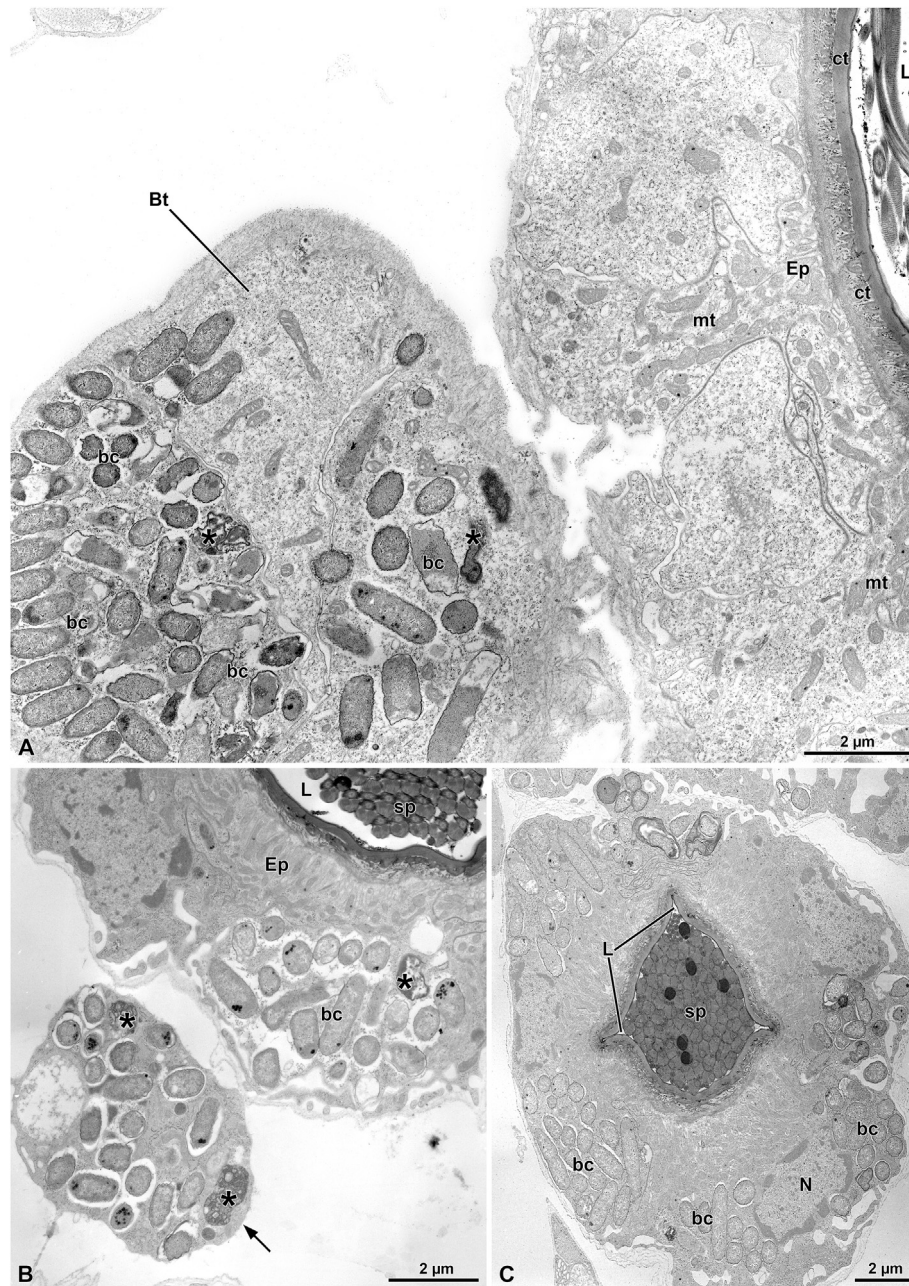
Fig. 5. A) Cross section through a bacteriome (Bt) rich of bacterial cells (bc) approaching the duct epithelium (Ep). Asterisks, degenerating bacterial cells; ct, cuticle lining the duct lumen (L); mt, mitochondria. Asterisks, degenerating bacterial cells. **B)** A small bacteriocytes (arrow) is close to the duct epithelium (Ep) and a cluster of bacterial cells (bc) is intermingled with the epithelial cell components. L, duct lumen filled with sperm (sp). **C)** Cross section of a duct with a narrow lumen (L) filled with sperm (sp). Bacterial cells (bc) are housed in the epithelial cells. Asterisks, degenerating bacterial cells; N, nucleus.

recording their presence in the spermatheca of the three examined females of *M. brevicauda* indicates that these endosymbionts are probably well adapted to the species, and that such a finding is not a random event. Bacteriocytes have already been detected in species of different orders such as Blattodea, Coleoptera, Diptera, Hemiptera, Hymenoptera and Psocodea (Cornwallis et al., 2023; Nozaki and Shigenobu, 2022). It has been estimated that approximately 15 % of all insect species harbor endosymbionts (Douglas, 1989; Baumann, 2005). What is, however, peculiar and remarkable in our finding, is the localization of endosymbionts in bacteriomes outside the spermatheca or within the epithelial cells of the spermathecal duct and never in the duct lumen as is instead the situation

reported in previous records. So far, extracellular bacteria associated with the spermatheca have been reported in few examples, such as the formosan subterranean termite *Coptotermes formosanus* (Raina et al., 2007), the spermatheca of the larvae psyllid *Trioza alacris* (Marchini et al., 2014), and in the gynandrial gland and fecundation canal of the heteropteran *Gerris lacustris* (Dallai et al., 2021a). In these examples, the bacteria were found within the spermathecal lumen intermingled with the sperm. It would be interesting to establish whether the bacteria we found are obligate or primary symbionts or facultative or secondary symbionts sensu Baumann (2005) and Ferrari and Vavre (2011). If they were obligate symbionts, then they could provide some essential components to

the host cells that could possibly be transferred to the sperm present in the lumen. It would be important to verify whether these endosymbionts are present in other tissues of the species and which route they used to reach the spermatheca. According to Masson et al. (2015), bacteriocytes can permanently infect the host germ cells from which they are transmitted to internal organs. It would be interesting to verify whether also male tissues of the species are infected by the same bacterial cells and, if so, whether and how they are transferred to the female.

CRediT authorship contribution statement

Romano Dallai: Writing — review & editing, Writing — original draft, Validation, Methodology, Investigation, Conceptualization. **David Mercati:** Writing — review & editing, Writing — original draft, Visualization, Validation, Investigation. **Pietro Lupetti:** Writing — review & editing, Writing — original draft, Validation.

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.

References

Baumann, P., 2005. Biology bacteriocyte-associated endosymbionts of plant sap-sucking insects. Annu. Rev. Microbiol. 59, 155—189.

Berridge, M.J., Oschman, J.L., 1972. Transporting Epithelia. Academic Press, Inc., New York.

Bradley, T.J., 1985. The Excretory System: Structure and Physiology. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 4. Pergamon Press, Oxford, New York, pp. 421—465.

Buchner, P., 1965. Endosymbiosis of Animals with Plant Microorganisms. Interscience Publisher, New York.

Cornwallis, C.K., van 't Padje, A., Ellers, J., Klein, M., Jackson, R., Kiers, E.T., West, S.A., Lee, M., Henry, L.M., 2023. Symbioses shape feeding niches and diversification across insects. Nat. Ecol. Evol. 7, 1022—1044.

Dallai, R., Mercati, D., Gottardo, M., Machida, R., Mashimo, Y., Beutel, R.G., 2012. The fine structure of the female reproductive system of *Zorotypus caudelli* Karny (Zoraptera). Arthropod Struct. Dev. 41, 51—63.

Dallai, R., Gottardo, M., Mercati, D., Machida, R., Mashimo, Y., Matsumura, Y., Beutel, R.G., 2014. Giant spermatozoa and a huge spermatheca: A case of coevolution of male and female reproductive organs in the ground louse *Zorotypus impolitus* (Insecta, Zoraptera). Arthropod Struct. Dev. 43, 135—151.

Dallai, R., Fanciulli, P.P., Lupetti, P., Mercati, D., 2021a. The ultrastructure of sperm and female sperm storage organs in the water strider *Gerris lacustris* L. (Heteroptera) and a possible example of genital coevolution. Arthropod Struct. Dev. 61, 101043.

Dallai, R., Fanciulli, P.P., Mercati, D., Lupetti, P., 2021b. Coevolution between female seminal receptacle and sperm morphology in the semiaquatic measurer bug *Hydrometra stagnorum* L. (Heteroptera, Hydrometridae). Arthropod Struct. Dev. 60, 101001.

Dias, G., Yotoko, K.S.C., Gomes, L.F., Lino-Neto, J., 2012. Uncommon formation of two antiparallel sperm bundles per cyst in tenebrionid beetles (Coleoptera). Naturwissenschaften 99, 773—777.

Dias, G., Lino-Neto, J., Mercati, D., Fanciulli, P.P., Lupetti, P., Dallai, R., 2022a. The sperm ultrastructure of members of basal Tenebrionoidea (Coleoptera). Arthropod Struct. Dev. 66, 101129.

Dias, G., Mercati, D., Rezende, P.H., Lino-Neto, J., Fanciulli, P.P., Lupetti, P., Dallai, R., 2022b. New findings on the sperm structure of Tenebrionoidea (Insecta,

Coleoptera). Insects 13, 485.

Douglas, A.E., 1989. Mycetocyte symbiosis in insects. Biol. Rev. Camb. Phil. Soc. 64, 409—434.

Dybas, L.K., Dybas, H.S., 1981. Coadaptation and taxonomic differentiation of sperm and spermathecae in feather wing beetles. Evolution 35, 168—174.

Ferrari, J., Vavre, F., 2011. Bacterial symbionts in insects or the story of communities affecting communities. Philos. Trans. R. Soc. Lond. B Biol. Sci. 366, 1389—1400.

García-González, F., Simmons, L.W., 2007. Paternal indirect genetic effects on offspring viability and the benefits of polyandry. Curr. Biol. 17, 32—36.

Higginson, D.M., Miller, K.B., Segraves, K.A., Pitnick, S., 2012. Female reproductive tract form drives the evolution of complex sperm morphology. Proc. Natl. Acad. Sci. USA 109, 4338—4543.

Hunt, J., Simmons, L.W., Kotiaho, J.S., 2002. A cost of maternal care in the dung beetle *Onthophagus taurus*? J. Evol. Biol. 15, 57—64.

Keller, L., Reeve, H.K., 1995. Why do females mate with multiple males? The sexually selected sperm hypothesis. Adv. Stud. Behav. 24, 291—315.

Kotrba, M., Heß, M., 2013. Giant spiral shaped spermatozoa of *Diasemopsis comoroensis* (Diptera, Diopsidae) with a unique ultrastructural component. Tissue Cell 45, 443—445.

Lüpold, S., Manier, M.K., Puniamoorthy, N., Schoff, C., Starmer, W.T., Buckley Luepold, S.H., Belote, J.M., Pitnick, S., 2016. How sexual selection can drive the evolution of costly sperm ornamentation. Nature 533, 535—538.

Marchini, D., Ciolfi, S., Gottardo, M., Marri, L., 2014. Bacteria of the genus *Erwinia* found in the spermatheca of the laurel psyllid *Trioza alacris*. Arch. Microbiol. 196, 901—905.

Masson, F., Vallier, A., Vigneron, A., Balmand, S., Vincent-Monégat, C., Zaidman-Rémy, A., Heddi, A., 2015. Systemic infection generates a local-like immune response of the bacteriome organ in insect symbiosis. J. Innate Immun. 7, 290—301.

Masson, F., Zaidman-Rémy, A., Heddi, A., 2016. Antimicrobial peptides and cell processes tracking endosymbiont dynamics. Philos. Trans. R. Soc. Lond. B Biol. Sci. 371, 20150298.

McKenna, D.D., Shin, S., Ahrens, D., Balke, M., Beza-Beza, C., Clarke, D.J., Donath, A., Escalona, H.E., Friedrich, F., Letsch, H., Liu, S., Maddison, D., Mayer, C., Misof, B., Murin, P.J., Niehuis, O., Peters, R.S., Podsiadlowski, L., Pohl, H., Scully, E.D., Yan, E.V., Zhou, X., Ślipiński, A., Beutel, R.G., 2019. The evolution and genomic basis of beetle diversity. Proc. Natl. Acad. Sci. USA 116, 24729—24737.

Miller, G.T., Pitnick, S., 2002. Sperm-female coevolution in *Drosophila*. Science 298, 1230—1233.

Minder, A.M., Hosken, D.J., Ward, P.I., 2005. Co-evolution of male and female reproductive characters across the Scathophagidae (Diptera). J. Evol. Biol. 18, 60—69.

Morrow, E.H., Gage, J.G., 2000. The evolution of sperm length in months. Proc. Roy. Soc. Lond. B 267, 307—313.

Nardi, J.B., Delgado, J.A., Collantes, F., Miller, L.A., Bee, C.M., Kathirithamby, J., 2013. Sperm cells of a primitive Strepsipteran. Insects 4, 463—475.

Nozaki, T., Shigenobu, S., 2022. Ploidy dynamics in aphid host cells harboring bacterial symbionts. Sci. Rep. 12, 9111.

Pattarini, J.M., Starmer, W.T., Bjork, A., Pitnick, S., 2006. Mechanisms underlying the sperm quality advantage in *Drosophila melanogaster*. Evolution 60, 2064—2080.

Pitnick, S., Markow, T.A., Spicer, G.S., 1999. Evolution of multiple kinds of female sperm-storage organs in *Drosophila*. Evolution 53, 1804—1822.

Presgraves, D.C., Baker, H.R., Gerald, S., Wilkinson, G.S., 1999. Coevolution of sperm and female reproductive tract morphology in stalk-eyed flies. Proc. Roy. Soc. Lond. B 266, 1041—1047.

Raina, A., Murphy, C., Florane, C., Williams, K., Park, Y.I., Ingber, B., 2007. Structure of spermatheca, sperm dynamics, and associated bacteria in Formosan subterranean termite (Isoptera: Rhinotermitidae). Ann. Entomol. Soc. Am. 100, 418—424.

Wall, B.J., Oschman, J.L., Schmidt, B.A., 1975. Morphology and function of Malpighian tubules and associated structures in the cockroach, *Periplaneta americana*. J. Morphol. 146, 265—306.

Werner, M., Simmons, L.W., 2011. Ultrastructure of spermatozoa of *Onthophagus taurus* (Coleoptera, Scarabaeidae) exhibits heritable variation. Naturwissenschaften 98, 213—223.

Zhang, S.Q., Che, L.-H., Li, Y., Liang, D., Pang, H., Ślipiński, A., Zhang, P., 2018. Evolutionary history of Coleoptera revealed by extensive sampling of genes and species. Nat. Commun. 9, 205.