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Male reproductive tract and spermatozoa ultrastructure in the grasshopper *Orphulella punctata* (De Geer, 1773) (Insecta, Orthoptera, Caelifera)

Daniela Santos Martins Silva[1] | **Jamile Fernanda Silva Cossolin**[2] | **Marcelo Ribeiro Pereira**[3] | **José Lino-Neto**[4] | **Carlos Frankl Sperber**[4] | **José Eduardo Serrão**[4]

[1]Programa de Pós-Graduação em Entomologia, Departamento de Entomologia, Universidade Federal de Viçosa, Viçosa, Minas Gerais 36570-000, Brazil

[2]Programa de Pós-Graduação em Biologia Celular e Estrutural, Departamento de Biologia Geral, Universidade Federal de Viçosa, Viçosa, Minas Gerais 36570-900, Brazil

[3]Instituto de Ciências Biológicas e da Saúde, Universidade Federal de Viçosa, campus de Rio Paranaíba, Rio Paranaíba, Minas gerais 38810-000, Brazil

[4]Departamento de Biologia Geral, Universidade Federal de Viçosa, Viçosa, Minas Gerais 36570-900, Brazil

Correspondence
Daniela Santos Martins Silva, Programa de Pós-Graduação em Entomologia, Departamento de Entomologia, Universidade Federal de Viçosa, UFV, Zip Code 36570-000, Viçosa, Minas Gerais, Brazil.
Email: danielasantos.biology@gmail.com

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Abstract

Identification Orphulellini grasshoppers (Acrididae: Gomphocerinae) species has been difficult due to high polymorphism rate. *Orphulella* Giglio-Tos, 1894 is a genus with widespread geographical distribution and poor descriptions. *Orphulella punctata* (De Geer, 1773) has an extensive record of occurrence and available information about the phallic complex, however, there is poor data describing other parts of the male reproductive tract. The objective of this study was characterizes the internal organs of the male reproductive system and spermatozoa of *O. punctata*. *Orphulella punctata* testes are of *Fountain* type, each having only four follicles. Spermatozoa into the seminal vesicle are arranged in bundles with c.a. 2320 μm length, with a nucleus 110 μm long. The spermatozoa are covered by a glycocalyx, the nucleus is cylindrical with condensed chromatin and connected to the flagellum by a dense and lamellar centriole adjunct. The axoneme have 9 + 9 + 2 pattern and present two symmetrical mitochondrial derivatives. A fibrous net and two flat membranous cisternae fill the space between the axoneme and mitochondrial derivatives. This is the first description of the reproductive system of a Gomphocerinae representative.

KEYWORDS
grasshoppers, reproduction, spermatogenesis, spermiogenesis

1 | INTRODUCTION

Orphulella Giglio-Tos, 1894 is endemic to the New World and has 24 species (Cigliano, Braun, Eades, & Otte, 2017). Their identification is hard due to polymorphism, insufficient descriptions, wide geographic distribution, and the difficulty for type species access (Otte, 1979). *Orphulella punctata* (De Geer, 1773) has an extensive record of occurrence and available information about the phallic complex (see Rowell, 2013), but, like in other Caelifera, data regarding other components of the reproductive system are scarce.

The comparative investigations on the anatomy of the Orthoptera species suggest that studies of male genitalia combined with molecular data and the development of the "*Orthoptera Species File*" online taxonomic database have contributed to advances in the taxonomy and systematics of this group (Song, 2010). However, structural features in other parts of the reproductive system (i.e., in addition to the genitalia) may also aid in the determination of Orthopteran groups (Laird, 1943). Among these structures, the testes in Orthoptera are paired organs enveloped together by connective tissues (Uvarov, 1966). Grasshoppers and other orthopteroids have testes with finger-shaped follicles

(White, 1954) which, when mature, can vary in position in relation to the *vas deferens* and spermatozoa production (Snodgrass, 1937).

Laird (1943) evaluated the testes follicle in 97 grasshopper species and characterized three main types of testes based on follicle insertion in the *vas deferens*: (1) *Fountain*, in which numerous follicles connect to the proximal end of the blind end of the *vas deferens*; (2) *Radiating*, in which follicles are arranged parallel to the *vas deferens* for the entire length of the gonads; and (3) *Intermediate*, in which follicles are arranged in the *vas deferens* to half the length of the gonads.

In insects, spermatazoa are produced in the testes and stored in the seminal vesicle. The male reproductive tracts of orthopteroids have tubular accessory glands that opens at the ejaculatory duct (Gregory, 1965), producing secretions that are essential for transfer spermatozoa to the females (Gillott, 2003). Sperm acceptance and transfer occurs through genital structures in orthopteroids, which principally includes phallic organs during copulation (Snodgrass, 1937).

Caeliferan males place the final abdominal region under the female abdomen during mating, where the aedeagus is introduced between the ovipositor valves. The aedeagus is filled with hemolymph, and sperm is transferred to the female due to pressure exerted by the visceral muscles (Uvarov, 1966). Within grasshopper, the structures related to reproduction, only phallic sclerites are widely studied because they provide information for distinguishing different species (Dirsh, 1957, 1961; Eades, 1961, 2000; Roberts, 1941; Slifer 1940a,b; Snodgrass, 1935, 1936, 1937; Song & Mariño-Perez, 2013).

In this work, the morphological aspects of the male reproductive system and spermatozoa ultrastructure of *O. punctata* has been examined with the aim to assess whether new characters have potential use for taxonomy and phylogeny.

2 | MATERIALS AND METHODS

2.1 | Source of animals

Orphullela punctata were collected in an Atlantic Forest fragment at Federal University of Viçosa (20°45′S, 42°52′W). Entomological nets were used to capture specimens, which were then transferred to laboratory. Grasshoppers were kept at 25°C with 70% RH and 12 h photoperiod until dissection. Reproductive systems from eight males *O. punctata* were dissected in sodium phosphate buffer solution (PBS) 0.1M, pH 7.2, transferred to (3:1) ethanol: acetic acid solution, and photographed using a Zeiss Stereo Discovery V.20 stereomicroscope.

2.2 | Light microscopy

Males of *O. punctata* were dissected in PBS and the reproductive systems transferred to 2.5% glutaraldehyde in PBS for 24 h. The testes and seminal vesicles were separated and postfixed in 1% osmium tetroxide in sodium cacodylate buffer (0.1M) for 2 h, dehydrated in a graded ethanol series and embedded in historesin (Leica). Sections 1 μm thickness were stained with hematoxylin and eosin or toluidine blue-borax. Sections were examined and photographed under a light microscope (Olympus BX-60).

2.3 | Transmission electron microscopy

Seminal vesicles were dissected out rapidly and fixed in a solution containing 2.5% glutaraldehyde, in 0.2M sodium cacodylate buffer for 24 h. After rinsing buffer, they were postfixed with 1% osmium tetroxide in the same buffer for 2 h. Dehydration was carried out in acetone and ethanol, followed by embedding in Epon resin. Ultrathin sections were stained with the 1% uranyl acetate and lead citrate and observed using an electron microscope Zeiss EM 109.

2.4 | Spermatozoa

After dissection, drops of spermatozoa suspension extracted from the seminal vesicles were spread onto histological slides in PBS and mixed with 4% paraformaldehyde for 15–20 min at room temperature. Slides were rinsed in distilled water and air dried. Spermatozoa samples were stained for 15 min with Giemsa solution and examined under a light microscope. To obtain nuclei sizes, some samples were stained with 4,6-diamino-2-phenylindole (DAPI) 0.2 μg/mL in PBS for 20 min, rinsed with water, air-dried, and mounted with 50% sucrose for analysis using an epifluorescence microscope (Olympus BX-60). Fifty spermatozoa and 50 nuclei in total were measured using Image Pro Plus software. The average was calculated.

3 | RESULTS

3.1 | Morphology of the reproductive apparatus

The reproductive tract of *O. punctata* had two testes joined by an orange-colored conjunctive sheath. Each testes opens in a narrowed *vas deferens* that leads to the ejaculatory duct. In the end of the *vasa deferentia* were found a pair of enlarged opaque diverticles that correspond to the seminal vesicles among tubular accessory glands of different sizes (Figure 1).

Histological cross sections showed that each testes had four follicles with a short *vas efferens* (Figure 2A). The testes follicles were filiform and contain spaces along their entire length (Figure 2B). The terminal portion of the follicles had a germarium region with polygonal spermatogonia with well-developed nucleus containing decondensed chromatin. The growth zone of the testes follicle was characterized by the presence of spherical spermatocytes with well-developed nuclei rich in decondensed chromatin (Figure 3A). The maturation and differentiation zone had spermatids with spherical nuclei and a strongly stained structure named Nebenkern that corresponds to compact, dense, and fused mitochondria (Figure 3B).

In the onset of spermiogenesis, the Nebenkern in spermatids arisen two elongated mitochondrial derivatives (Figure 3C,D). During spermatid maturation, the cytoplasmic volume gradually reduces, and a small, dense body named centriolar adjunct appeared adjacent to the nucleus and the base of the flagellum (Figure 3D–F). As spermiogenesis proceed, the acrosome was positioned opposite the centriolar adjunct (Figure 3G), and cytoplasm reduction and elongation processes increase until spermatid differentiation in spermatozoa was completed (Figure 3H).

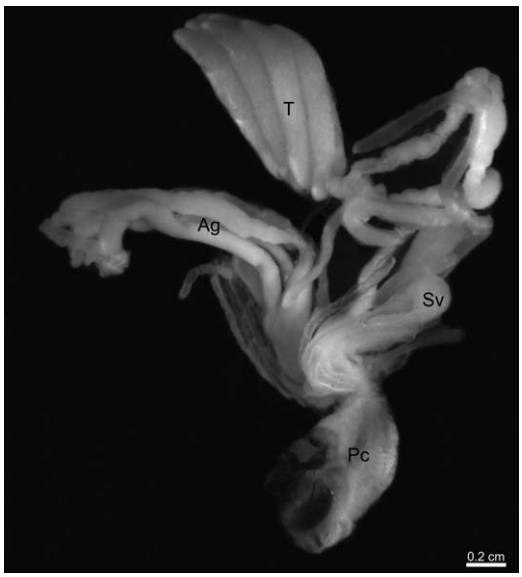

FIGURE 1 Reproductive system of *Orphulella punctata* (T) testes; (Ag) accessory glands, (Sv) seminal vesicle; (Pc) phallic complex

Approximately 450 spermatozoa occurred in each spermatic cyst, resulted from nine cell division cycles (i.e., seven mitotic and two meiotic divisions). The seminal vesicle (Figure 4A) had a wall with a single layer of cubic epithelial cells containing extensive brush border and well-developed striated border and a nucleus containing condensed chromatin cloths (Figure 4B,C). Spermatozoa remained in bundles in the seminal vesicle (Figure 4B), which was easily loosen when the seminal vesicle wall was disrupted. *Orphulella punctata* spermatozoa is extremely long and thin with 2320 ± 568.82 μm total length and head length 110.03 ± 10.79 μm (Figure 5).

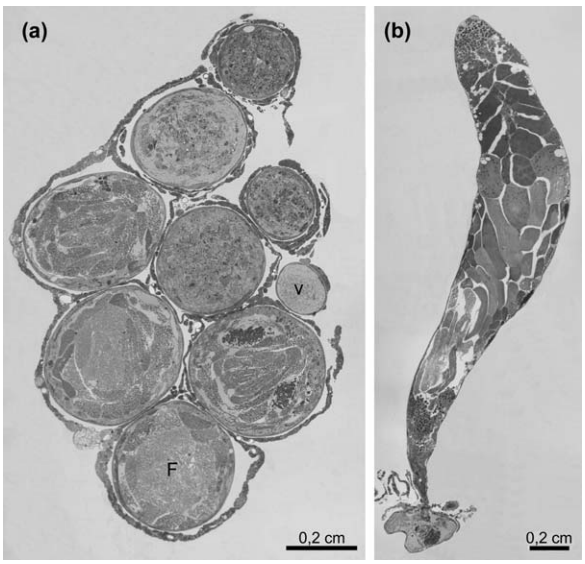

FIGURE 2 Histological sections of *Orphulella punctata* testis and follicles. A, Testes are joined by a connective sheath (cross-section). Each has four testicular follicles, wherein (f) represents each follicle and (v) a vessel. B, Follicle showing the cysts in various stages of maturation (longitudinal section)

3.2 | Sperm ultrastructure

The entire surface of the spermatozoa plasma membrane is characterized by a thick glycocalyx (Figure 6A–F). The nucleus is cylindrical and had condensed chromatin (Figure 6A). The centriole adjunct hold the base of nucleus and it is electron-dense and semicircular in cross section. Besides it is partially embracing the beginning of the axoneme, structured in several thick longitudinal elements that are laminated (Figure 6B).

The centriole adjunct becomes smaller in size with axoneme formation and with diameter increase of mitochondrial derivatives that appear in the posterior part of centriole adjunct (Figure 6C–E). At the flagellum level, the axoneme cross section shows 9 + 9 + 2 microtubules pattern, which the central and outer single microtubules filled by dense material (Figure 6D–F).

The two mitochondrial derivatives had almost triangular shape, symmetrical, extend to end of flagellum and have few longitudinal cristae deeply penetrating in a crystalline matrix (Figure 6E–F). Two flat membranous cisternae are found between the mitochondrial derivatives and axoneme (Figure 6D). A fibrous material filled the inner space between mitochondrial derivatives and axoneme along the flagellum (Figure 6F).

4 | DISCUSSION

The phallic organs in Orthoptera have an abundant morphological variety in different families (Snodgrass, 1959) and the testes generally have size and shape according to the number, form, and arrangement of the sperm tubes (Snodgrass, 1937). However, despite this variety, Laird′s hypothesis (1943) proposed that uses of structural features of testis are important for phylogenetic analyses within Acrididae subfamilies. Laird (1943) examined a variety of subfamilies and the *Fountain* type testis were found only in Acridinae, Oedipodinae, and Cyrtacanthacridinae.

Orphulella punctata has eight testis follicles, the lowest number among studied Caeliferan species. *Chortophaga viridifasciata* (De Geer, 1773) has 26–28 follicles (Carlson and Handel, 1988), Akicerinae (Caelifera: Pamphagidae), c.a. 30 follicles (Laird, 1943), *Melanoplus differentialis* (Thomas, 1865), 188 follicles (Nelsen, 1931) and *Romalea microptera* (Palisot de Beauvois, 1817), 250 follicles (Laird, 1943). The spaces found among the testis follicles in *O. punctata*, suggest that the studied specimens may had already ceased sperm production. This absence in sperm production is probably related to the cessation of this function in adult stage, because similar situations were observed in insects by Dumser (1980), mainly in some species of short duration.

The sperm components and their arrangement in the *O. punctata* are similar to the majority of the known grasshoppers. Primary spermatogonia undergo cytoplasmic division in the germarium region of the testis follicles, and change in spermatocytes in the differentiation zone (Dumser, 1980). Spermatocytes of *O. punctata* then differentiate into spermatids, with two mitochondrial derivatives from a single Nebenkern. These mitochondrial derivatives have been used as relevant taxonomic character in many insects and in grasshopper, being partially

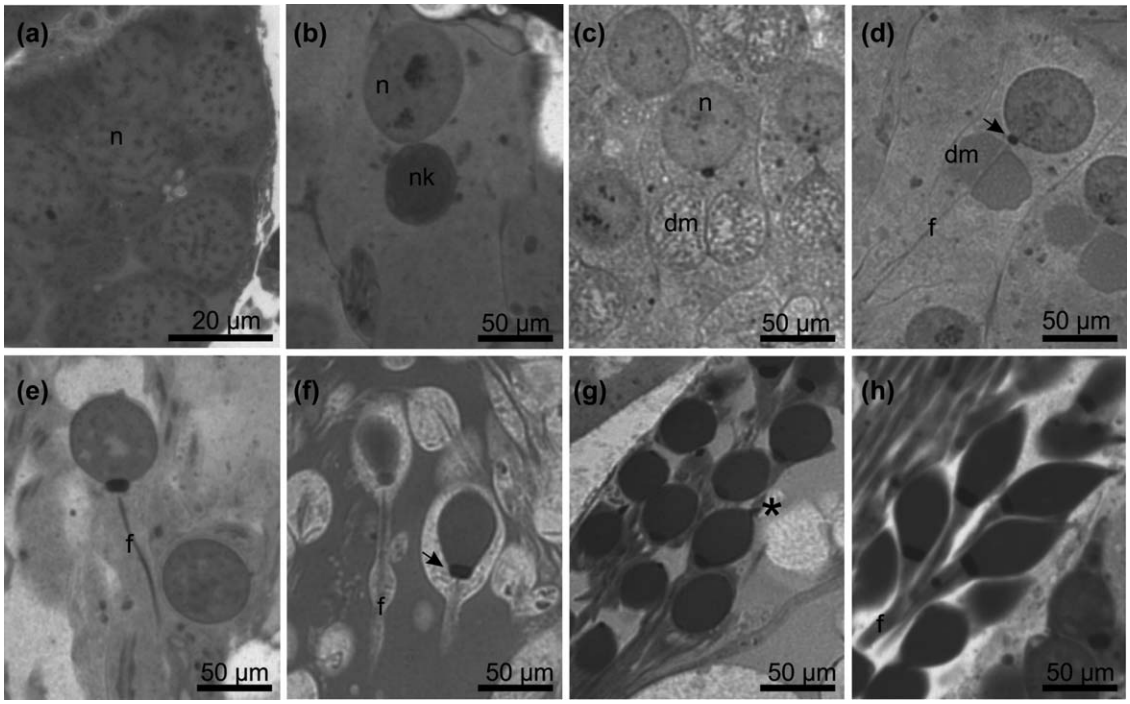

FIGURE 3 Light micrographs of spermiogenesis in *Orphulella punctata*. A, large spermatocytes, rounded and with loose chromatin, with nuclei indicated by (n); B, spermatid with Nebenkern mitochondria (Nk); C and D indicate separating and elongation of mitochondrial derivative (dm), where (f) indicates the formation of flagellum and (arrow) indicates the centriolar adjunct; E, flagellar elongation; F, cytoplasmic reduction and development of centriolar adjunct; G, position of the acrosome during elongation indicated by (*) and (H) spermatids at an advanced developmental phase

crystalline in appearance, with transverse cristae and a few longitudinal cristae whose features varies among families and subfamilies (Jamieson, Dallai, & Afzelius, 1999).

According Virkki (1969), sperm bundles are found in Odonata, Neuroptera, Hemiptera, Diptera, Coleoptera, Lepidoptera, Tricoptera, Orthoptera and all basal insects that have more sperm per bundles

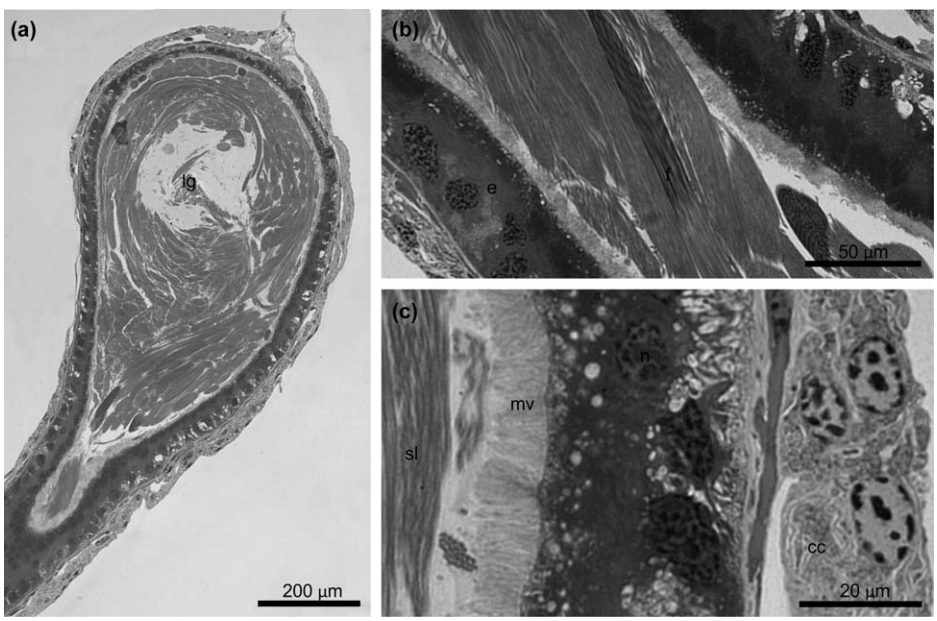

FIGURE 4 Histological sections of seminal vesicle of *Orphulella punctata*. A, seminal vesicle, where (lg) indicates the light, the space where spermatozoa are stored; B, spermatozoa organized in bundles with (f) and (e) arrangement of the vesicle epithelium; C, detail showing the epithelial cells with extended microvilli (mv) in the vesicle, (n) nucleus of seminal vesicle epithelium in longitudinal section (sl). The entire vesicle is covered by a connective capsule (cc) formed by another type of epithelial cell

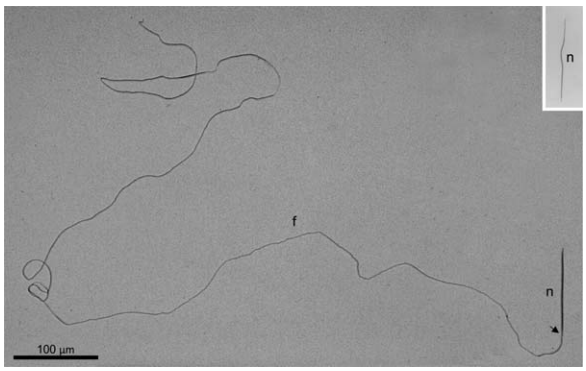

FIGURE 5 Light micrograph of *Orphulella punctata* spermatozoa showing nucleus (n), flagellum (f), and nucleoflagellar transition region (arrow). Insert—nucleus (n). DAPI staining

compared to derived ones. *Orphulella punctata* showed 450 spermatozoa per bundle and for Orthoptera, the presence of sperm in bundles occurs in Eumastacidae and Acrididae with number of sperm per bundles range from 256 to 2048 (White, 1954).

Sperm length also differs among Caeliferan groups. *Cylindraustralia kochii* (Saussure, 1877) (Cylindrachetidae) has narrow and long sperm c. a. 700 μm mm length with a 60 μm head; *Xya varie*gata (Latreille, 1809) (Tridactylidae) has a 300 μm flagellum with a 20 μm head (Jamienson et al., 1999) and *Orthochtha dimorpha* Miller, 1929 (Acridinae) has a 180 μm head with a 3.00 mm flagellum (Baccetti, 1987), *Euchorthippus declivus* (Brisout of Barneville, 1848) 1.60 mm flagellum, and both *Gomphocerippus rufus* (Linnaeus, 1758) and *Arcyptera (Arcyptera) fusca* (Pallas, 1773) measuring 0.3 mm (Baccetti, 1987). However, this information may not be useful for characterizing higher taxa since sperm length varies significantly within each genus (Baccetti, 1987).

Data on the shape of Orthopteran spermatozoa were lacking until 1966 due to the prevailing idea that spermatozoa morphology did not differ in other insects, consisting basically of a single, long spindle cell (Uvarov, 1966). However, Baccetti (1987) in his pioneering study on Caeliferan spermatozoa have pointed out that these reproductive cells are elongated and thin due to cytoskeletal axoneme and two mitochondrial derivatives almost completely filled with crystallomitin as found in *O. punctata*.

In *O. punctata*, the plasma membrane of mature spermatozoa is surrounded by glycocalyx along the entire length. This system is common in derived Orthoptera families and this unique feature probably acts as a desmosome-like, anchoring the spermatozoa each other, facilitating the sperm bundles (Baccetti, 1987).

Another feature useful in distinguish spermatozoa from different species is by the shape of centriole adjunct. In *O. punctata* the centriole adjunct are electron-dense and have several thick longitudinal laminae, as reported for other Acridomorha (Baccetti, 1987). This structure guides the axoneme development (Jamienson et al., 1999).

Our findings show that *O. punctata* has the lower number of testes follicles and one of the longest spermatozoa among Caelifera. This

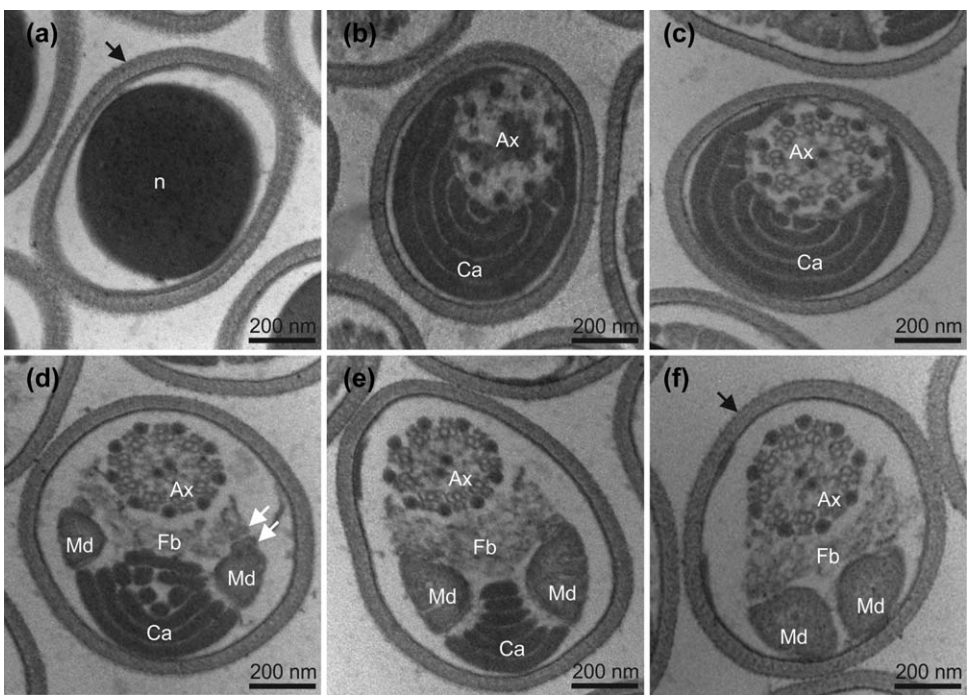

FIGURE 6 Transmission electron micrographs of *Orphulella punctata* spermatozoa in cross section. A, Nucleus (*n*). B, Below the nucleus, the axoneme (Ax) begins the assembling and association with the semi rounded centriole adjunct (Ca). C, Complete axoneme (Ax) enveloping by dense and laminated centriole adjunct (Ca). D, Beginning of mitochondrial derivative (Md) formation on distal part of centriole adjunct (Ca). Note the two flattened membranous cisternae (white arrows) near to mitochondrial derivatives. E, Mitochondrial derivatives (Md) enlargement with concomitant reduction of centriole adjunct (Ca). F, Flagellum with two symmetrical mitochondrial derivatives (Md) almost completely filled by paracristallyne material, axoneme and fibrous net (Fb) between the two structures. Note the gliycocalyx in plasmic membrane along the spermatozoa (black arrows)

species also has shared characteristics with other Gomphocerinae, supporting the use of testes characteristics to distinguish Acrididae species. Currently the grasshopper spermatozoa ultrastructure has not been described and overall knowledge of the fine structure of sperm in Orthoptera remains poorly knowledge. Comparative studies on reproductive system morphology and spermatozoa ultrastructure in Caelifera are needed to find new characters that may help elucidate the relationship among the families, contributing to phylogenetic analyses of the group.

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ORCID

Daniela Santos Martins Silva http://orcid.org/0000-0002-5125-8197
José Eduardo Serrão http://orcid.org/0000-0002-0477-4252

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