GLENDA SAMARA DIAS SANTOS

MORFOLOGIA DOS ESPERMATOZOIDES DOS COLEÓPTERAS *STICTOLEPTURA CORDIGERA* E *TRIBOLIUM CASTANEUM* E O PRIMEIRO REGISTRO DE GREGARINA EM VESÍCULA SEMINAL DE INSETOS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de *Doctor Scientiae.*

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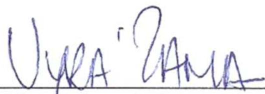

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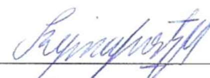

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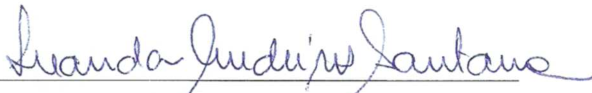

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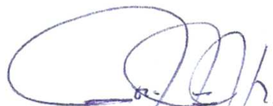

José Lino Neto
(Orientador)

Ensinar é um exercício
de imortalidade. De alguma forma
continuamos a viver naqueles cujos olhos
aprenderam a ver o mundo pela magia
da nossa palavra.
O professor, assim, não morre
jamais...
Rubem Alves

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À minha vó Lourdes (*in memoriam*), o meu mais belo exemplo de simplicidade e amor.

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RESUMO

SANTOS, Glenda Samara Dias, D.Sc., Universidade Federal de Viçosa, abril de 2017. **Morfologia dos espermatozoides dos coleópteras S*tictoleptura cordigera* e *Tribolium castaneum* e o primeiro registro de gregarina em vesícula seminal de insetos.** Orientador: José Lino Neto.

Nesta tese descrevemos a morfologia dos espermatozoides dos coleópteras *Stictoleptura cordigera* (Cerambycidae) e *Tribolium castaneum* (Tenebrionidae) e, pela primeira vez, a ocorrência de parasitos Gregarina (*Ascogregarina* sp.) em vesícula seminal de insetos. Ao longo do processo evolutivo, os espermatozoides acumularam inúmeras variações morfológicas, tornando-se as células mais diversas dos metazoários, sendo especialmente observada nos insetos. Por essa razão, o conhecimento sobre a morfologia dessas células tem auxiliado extensivamente à sistemática de diversos grupos de insetos, podendo ser usado para distinguir diferentes níveis taxonômicos e, até mesmo, espécies filogeneticamente próximas. Dentre estes, os Coleoptera constituem a maior e mais significativa ordem em riqueza de espécies. E, como para a maioria dos grandes grupos, as suas relações filogenéticas permanecem controvérsias nos diversos níveis taxonômicos. Neste contexto, procuramos, através de diferentes métodos de microscopia, descrever a morfologia dos espermatozoides dos besouros *S. cordigera* e *T. castaneum*, buscando fornecer dados que possam contribuir na sistemática das famílias as quais pertencem, bem como dos Coleoptera em geral. Os espermatozoides de *S. cordigera* apresentaram: na região de cabeça, um acrossomo em duas camadas (vesícula acrossomal e perforatorium) e um núcleo alongado fortemente eletrondenso, e na região flagelar, um axonema com 9 + 9 + 2 microtúbulos, dois derivados mitocondriais assimétricos parcialmente cristalizados, e dois corpos acessórios levemente assimétricos, com duas regiões de densidades diferentes. Nessa espécie, a morfologia dos espermatozoides exibe estreita semelhança àquela descrita para *curculionídeos,* indicando uma estreita relação filogenética entre Chrysomeloidea e Curculionoidea. Os espermatozoides de *T. castaneum*, assim como os de *S. cordigera*, apresentaram, na região da cabeça, um acrossomo e um núcleo, e no flagelo, um axonema com a mesma organização microtubular, dois derivados mitocondriais assimétricos e dois corpos acessórios. Contudo, em *T. castaneum*, o acrossomo é do tipo três camadas (além da vesícula acrossomal e o perforatorium, apresenta um material extra-acrossomal), e os corpos acessórios exibem apenas a região de maior densidade. A

presença de dois feixes de espermatozoides por cisto dispostos antiparalelamente, descrita para outros tenebrionídeos, foi aqui também observada. Ainda, nesses espécimes de *T. castaneum*, observamos degeneração em massa das células espermáticas em muitos cistos testiculares e, surpreendentemente, nas vesículas seminais, a presença de parasitos Gregarina. Os quais, segundo estudos morfológicos e moleculares, pertencem ao gênero *Ascogregarina*.

ABSTRACT

SANTOS, Glenda Samara Dias, D.Sc., Universidade Federal de Viçosa, April, 2017. **Sperm morphology of the *Stictoleptura cordigera* and *Tribolium castaneum* coleopteran and the first record of gregarina in seminal vesicle of insects.** Adviser: José Lino-Neto.

In this thesis we describe the sperm morphology of the coleopterans *Stictoleptura cordigera* (Cerambycidae) and *Tribolium castaneum* (Tenebrionidae) and, for the first time, the occurrence of Gregarina parasites in seminal vesicle of insects. Troughout the evolutionary process, the sperm cell accumulated numerous morphological variations, becoming the most diverse cells of the metazoan, being especially observed in the insects. For this reason, the knowledge about the morphology of these cells has extensively assisted the systematics of different insect groups, being used to distinguish different taxonomic levels and, even, close phylogenetically species. The Coleoptera comprise the largest and most significant order in species richness. And, as for most large groups, their phylogenetic relationships remain controversial at the various taxonomic levels. In this context, we describe the sperm morphology of the *S. cordigera* and *T. castaneum* beetles using different microscopy methods, searching to provide data that may contribute to the systematics of the families that belong to them, as well as to Coleoptera in general. The spermatozoa of *S. cordigera* exhibited: in the head region, an acrosome in two layers (acrosomal vesicle and perforatorium) and an elongate nucleus strongly electron-dense; in the flagellar region, an axoneme with 9 + 9 + 2 microtubules, two partially crystallized asymmetric mitochondrial derivatives, and two slightly asymmetric accessory bodies with two regions of different densities. In this species, the sperm morphology exhibits close similarity to that described for Curculionidae, indicating a close relationship between Chrysomeloidea and Curculionoidea. The spermatozoa of *T. castaneum*, as those of *S. cordigera*, showed an acrosome and a nucleus in the head region, and in the flagellum, an axoneme with the same microtubular organization, two asymmetric mitochondrial derivatives and two accessory bodies. However, in *T. castaneum*, the acrosome is of the three-layer type (besides the acrosomal vesicle and the perforatorium, an extra-acrosomal material), and the accessory bodies exhibit only the greater density region. The presence of two bundles of spermatozoa per cyst arranged antiparallelly, described for other tenebrionids, was also observed in this beetle. Furthermore, in these *T. castaneum*

specimens, we observed a mass degeneration of spermatic cells in many testicular cysts, and, surprisingly, in the seminal vesicles, the presence of Gregarina parasites. Molecular and morphological studies indicated that these parasites belong to the genus *Ascogregarina*.

1. Introdução Geral

1.1. Ordem Coleoptera

Com mais de 400 mil espécies descritas, Coleoptera constitui a ordem mais rica e diversa da classe Insecta, representando quase 25% de todas as formas viventes conhecidas (Hunt et al. 2007; Jäch & Balke 2008). Cerca de 170 famílias são encontradas em diferentes regiões do mundo, sendo 104 registradas no Brasil o que corresponde a aproximadamente 30.000 espécies (Costa 2000).

O sucesso desse grupo possivelmente está relacionado à ocorrência de metamorfose completa (holometabolia), evitando desta forma competição intra-específica, bem como a um conjunto de caracteres que o permite a ocupar locais bastante diversificados sem perder a habilidade de voo (Grimaldi & Engel 2005). Por exemplo, são os únicos insetos que possuem as asas anteriores rígidas e altamente esclerotizadas (élitros), protegendo-os da dessecação e de choques mecânicos. Ainda, os élitros protegem as asas membranosas e o corpo frágil, permitindo que os besouros explorem ambientes inacessíveis a outros insetos (Grimaldi & Engel 2005; Triplehorn & Johnson 2011). Os coleópteras apresentam grande diversidade em forma, cor e tamanho. Eles podem variar de menos que 1 mm, como os Ptiliidae, até 200 mm, como o cerambycídio *Titanus giganteus*, considerado o maior inseto do mundo, encontrado na Amazônia (Gullan & Cranston 2012).

Os besouros são considerados um grupo monofilético, cuja principal sinapomorfia é a presença dos elétritos. Os besouros, provavelmente, divergiram a partir de um ancestral comum com Neuropterida e a grande diversificação do grupo ocorreu a partir do Jurássico, há 160 milhões de anos, período em que já existiam os grupos de Coleoptera mais derivados, como Curculionidae, Chrysomelidae e Cerambycidae (Grimaldi & Engel 2005).

A ordem Coleoptera é dividida em quatro subordens: Archostemata, Myxophaga, Adephaga e o grande grupo Polyphaga (Triplehorn & Johnson 2011). Esta última, com cerca de 300 mil espécies descritas, contém mais de 90% dos besouros (Grimaldi & Engel 2005; Vanin & Ide 2002). A classificação interna de Polyphaga compreende as superfamílias: Hidrophiloidea, Staphylinoidea, Scarabaeoidea, Buprestoidea, Byrrhoidea, Elateroidea, Bostrichoidea, Tenebrionoidea, Lymexyloidea, Chrysomeloidea, Curculionoidea, Cleroidea e Cucujoidea, com as seis últimas

constituindo o agrupamento Cucujiformia, que representa mais da metade de todos os besouros (Hunt et al. 2007; Gullan & Cranston 2012)

1.2 Aspectos filogenéticos e morfologia dos espermatozoides

Traçar a história evolutiva e compreender o sucesso adaptativo dos insetos em um período de existência de cerca de 400 milhões de anos tem sido um grande desafio (Grimaldi & Engel 2005). Muitos trabalhos vêm sendo feitos envolvendo a sistemática dos insetos, organismos de indiscutível importância para os ecossistemas em especial por constituírem o grupo mais diverso da fauna atual (Dallai et al. 2016). A morfologia dos espermatozoides tem oferecido um conjunto de dados, o qual tem auxiliado na sistemática de diferentes grupos animais, incluindo os insetos. Por ser um tipo celular muito diverso nos diferentes grupos de insetos, mas bastante conservada na espécie, faz com que ele seja uma importante ferramenta para fins filogenéticos e taxonômicos. As variações estruturais e ultraestruturais dos espermatozoides têm sido comumente utilizadas para resolver dúvidas sobre a filogenia de vários grupos de insetos, incluindo os besouros (Burrini et al. 1987; Baccetti & Daccordi 1988; Jamieson et al. 1999; Dias et al. 2013a, b; 2015a, b). Apesar do número limitado de componentes subcelulares, os espermatozoides são caracterizados por um surpreendente conjunto de caracteres (Dallai et al. 2016). Ake Franzén (1955) foi o primeiro a realizar estudos utilizando a morfologia e ultraestrutura destas células no estabelecimento de relações filogenéticas (ver Birkhead et al. 2009). Em seguida, vários autores deram continuidade a esta linha de pesquisa (Baccetti et al. 1973; Lino-Neto et al. 1999; Zama et al. 2005; Dolder et al. 2008, 2009; Dias et al. 2013a, b; Dallai et al. 2016). Em Coleoptera, um dos primeiros trabalhos comparativos sobre a morfologia dos espermatozoides foi descrito por Dlugosz & Harrold (1952) em *Pitnus tectus* (Ptnidae).

A ultraestrutura dos espermatozoides de Coleoptera segue um padrão do espermatozoide típico de Pterygota. Eles são filiformes, na região da cabeça contem um acrossomo de duas ou três camadas, e um núcleo com cromatina bastante compacta, e na região flagelar, um axonema de 9 + 9 + 2 microtúbulos com material intertubular, dois derivados mitocondriais e dois corpos acessórios ao longo de praticamente todo flagelo. Na região de transição núcleo-flagelo observa-se um adjunto de centríolo, geralmente pouco desenvolvido (Jamieson et al. 1999, Dallai 2014; Dias et al. 2015a, b). Variações deste tipo ocorrem, predominantemente, no tamanho, número e arranjo

dessas estruturas. Por exemplo, uma estrutura adicional associada a cada corpo acessório é observada no flagelo de Chrysomelidae e Curculionidae, a qual foi denominada “puff-like” por ter uma aparência amorfa e difusa (Burrini et al. 1987; Baccetti & Daccordi 1988).

Os curculionídeos, crisomelídeos e tenebrionídeos estão entre os maiores grupos que compõem a secção Cucujiformia. Há quase 30 anos foi descrita a morfologia dos espermatozoides de várias espécies de Curculionoidea (Burrini et al. 1987) e Chrysomelidae (Baccetti & Daccordi 1988). Quanto aos tenebrionídeos, a primeira descrição dos espermatozoides foi realizada em *Tenebrio molitor* por Baccetti et al. em 1973.

Entretanto, considerando o grande número de espécies em Coleoptera apenas uma pequena fração delas tem sido examinada com respeito à ultraestrutura dos espermatozoides. Portanto, estudar a morfologia dessas células em um número maior de espécies dessa ordem possibilitará a identificação de caracteres que poderão ser usados na sistemática do grupo.

1.3 Gregarina (Apicomplexa)

As gregarinas são um dos mais antigos parasitos conhecidos, formando um grupo heterogêneo dentro de Apicomplexa. Aproximadamente 1.650 espécies distribuídas em 250 gêneros têm sido registradas (Clopton 2000). Eles são caracterizados por indivíduos grandes e extracelulares, infectando uma variedade de invertebrados, principalmente artrópodes (Valigurová & Koudela 2005; Lantova & Volf 2014). Entre os insetos hospedeiros, Orthoptera, Odonata, Blattodea, Diptera e Coleoptera são os mais infectados (Desportes & Schrével 2013). Nestes grupos, as gregarinas são principalmente observadas em corpos gordurosos, túbulos de Malpighi e trato digestivo de larvas e adultos (Lantova & Volf 2014). Já no sistema reprodutor esses parasitos têm sido descritos apenas em glândulas acessórias e espermateca de fêmeas (Warburg & Ostrovska 1989; Lantova et al. 2010; Lantova & Volf 2014). Portanto, este é o primeiro relato de uma espécie de gregarina em vesícula seminal de insetos. Os ciclos de vida da gregarina incluem os seguintes estádios: esporozoítos (forma celular que infecta novos hospedeiros); Trofozoítos ou gamontes (grandes estádios vegetativos extracelulares); Gametocistos (pares de gamontes nos quais gametas são produzidos); E oocistos (esporo resistente, com parede espessa, que contém

os estádios infectantes). Em geral, a contaminação por gregarina ocorre através da transmissão oro-fecal, quando os parasitos entram no corpo por ingestão de oocistos contendo vários esporozoítos. Em seguida, os esporozoítos atingem a cavidade intestinal, ligam-se às células hospedeiras, e desenvolvem extracelularmente em estágios vegetativos maiores (Desportes & Schrével 2013. Estes diferentes estádios de desenvolvimento, foram descritos, nesta tese, usando as microscopias de luz e eletrônica de transmissão. Análises de uma sequência de rDNA indicaram que este protozoário pertence ao gênero *Ascogregarina*.

2. Objetivos

Esta tese tem como objetivo geral fornecer informações sobre a morfologia dos espermatozoides de Coleoptera, buscando descrever elementos que contribuam para o entendimento da sistemática do grupo. Como objetivo específico, descrever a estrutura e ultraestrutura dos espermatozoides de *Stictoleptura cordigera* (Cerambycidade) e *Tribolium castaneum* (Tenebrionidae). Durante a investigação dos espermatozoides de *T. castaneum* observamos a presença de gregarinas nas vesículas seminais e, a partir dessa descoberta, realizamos a descrição morfológica e molecular desse parasito.

Durante a realização desta tese foram produzidos três trabalhos, os quais encontram-se publicados. São eles:

1) The sperm ultrastructure of Stictoleptura cordigera (Füssli, 1775) (Insecta, Coleoptera, Cerambycidae);

2) The sperm ultrastructure and spermiogenesis of Tribolium castaneum (Coleoptera: Tenebrionidae) with evidence of cyst degeneration;

3) First record of gregarines (Apicomplexa) in seminal vesicle of insect.

Capítulo I

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The sperm ultrastructure of *Stictoleptura cordigera* (Füssli, 1775) (Insecta, Coleoptera, Cerambycidae)

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ABSTRACT

The spermatozoa of the longhorn beetles *Stictoleptura cordigera* were ultrastructurally described in this paper. They have an apical bilayered acrosome, an elongated nucleus, a centriole with star-shape links, two asymmetric mitochondrial derivatives partially crystallized and a 9+9+2 flagellar axoneme with accessory tubules provided with 16 protofilaments in their wall. A centriole adjunct is present and gives rise to two thick laminae as accessory bodies, also asymmetrical, to which two relatively small puff-like structures of different size are connected. These features were previously found in the sperm of the cerambycid *Morimus asper*. The strict similarity of the cerambycid sperm characters with those of curculionoids indicates a clear phylogenetic relationship between Chrysomeloidea and Curculionoidea.

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1. Introduction

The morphology of the spermatozoa of various groups of Coleoptera has been described, providing useful information for phylogenetic studies. Burrini et al. (1988), for instance, realized an extensive work with spermatozoa of different curculionoid families; similarly, Baccetti and Daccordi (1988) investigated sperm structure in the family Chrysomelidae and Dallai et al. (1998) described the sperm structure of Bruchidae, Chrysomelidae and several curculionoids. However the morphological description of the spermatozoa of Cerambycidae remains practically unknown, limited to only a short description by Dallai et al. (1998) who examined the species *Morimus asper*.

With an estimated 4000 genera and 35,000 described species, the Cerambycidae is one of the largest beetle families (Lawrence and Newton, 1982). Cerambycids, longhorn beetles, are a diverse and economically important group of insects (Hanks, 1999; Canettieri and Garcia, 2000). They are among the most serious wood boring pest species around the world, affecting many agricultural crops, lumber products and ornamental trees (Paine et al., 1995; Meshram, 2009). Despite their economic importance and biological diversity, the phylogenetic relationships within the group remain unclear (Santos-Silva et al., 2010).

The aim of the present study is to describe the sperm structure of the cerambycid beetle *Stictoleptura cordigera* (Füssli, 1775), that represents a useful complement to previous works.

2. Materials and methods

Adult males of *S. cordigera*, collected in Sardinia, Italy, were dissected under a light stereo microscope and their testes and seminal vesicles were transferred in 0.1 M phosphate buffer (PB) pH 7.2 to which 3% of sucrose was added. The material was fixed overnight in 2.5% glutaraldehyde in PB, rinsed in PB and postfixed in 1% osmium tetroxide in the same buffer for 1 h. After washing in PB the material was dehydrated with a graded series of alcohol and embedded in Epon–Araldite mixture. Ultrathin sections, obtained with a Reichert Ultracut IIE ultramicrotome, were stained with uranyl acetate and lead citrate and observed at a CM10 Philips transmission electron microscope (TEM) operating at 80 kV. Part of the material was fixed according to Dallai and Afzelius (1990) using tannic acid impregnation.

3. Results

Spermatozoa of *S. cordigera* are slender and long, and exhibits an apical bi-layered acrosome. The acrosome is 2.5 μm long and consists of a flattened acrosomal vesicle with a compact

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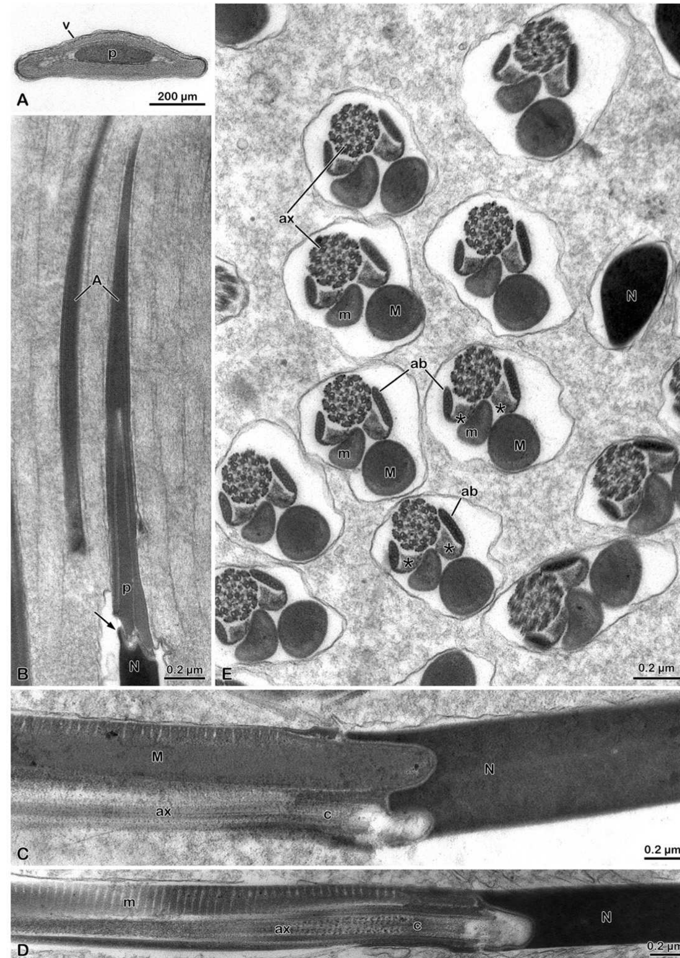

Fig. 1. Transmission electron micrographs of *S. cordigera* sperm. (A and B) Cross (A) and longitudinal (B) sections showing the acrosome formed by a flattened acrosome vesicle (v) containing an compact subacrosomal perforatorium (p). Note the perforatorium and the acrosomal vesicle inserted into two small depressions in the nuclear apical tip (arrow). (C–E) Longitudinal (C and D) and transversal (E) sections through the nucleus-flagellum transition region. Note: in (C) the larger mitochondrial derivative (M) and centriole (c) inserted in different nuclear cavities (N); in (D) the smaller mitochondrial derivative (m) begins below nuclear base; (E) cross-sectioned through one nucleus and several sperm tails. Note the elliptical appearance of the nucleus (N) and that all components of the flagellum are asymmetrical. Accessory bodies (ab); axoneme (ax); "puff"(*).

subacrosomal perforatorium that extends from the small depression in nuclear tip to the middle region of the acrosome vesicle (Fig. 1A and B). The nucleus has an elliptical appearance in cross-section; it is filled with dense chromatin and gradually tapers towards its posterior end (Fig. 1C–E). At this level the nucleus shows two deep invaginations, one containing the centriole and the other the major mitochondrial derivative (Fig. 1C); three microtubule doublets of the centriole and the larger mitochondrial derivative are surrounded by a thin extension of the nucleus and by the expanded centriole adjunct material. The doublets are devoid of dynein arms (Fig. 2A). Furthermost the complete centriole is present showing nine microtubule doublets; the two central tubules are missing at this level, but accessory tubules are already visible. Each peripheral doublets is connected to the central region, which consists of an axial dense cylinder, by radial links giving to the complex the appearance a star-like structure with 9 rays (Fig. 2B). The flagellum consists of one axoneme, two mitochondrial derivatives and two accessory bodies. The axoneme has the 9+9+2 microtubule pattern (9 accessory tubules with an axial dense filament, 9 peripheral doublets provided with both outer and inner dynein arms and 2 central microtubules). Intertubular material is evident and formed by a short dense ribbon adherent to the subtubule B of doublets and one dense beak on the accessory tubules (Fig. 2C and D). The wall of the accessory tubules is formed by 16 protofilaments (Fig. 2D). From the peripheral doublets extend radial spokes along which evident spok-heads are visible. The mitochondrial derivatives, in cross-section, are asymmetric in size and shape, with that having a greater size almost circular, located distant from the axoneme and filled with paracrystalline material; the smaller has a kidney-shaped appearance and the inner paracrystalline material is restricted to a small region (Fig. 2C and D). In longitudinal section peripheral cristae are evident with

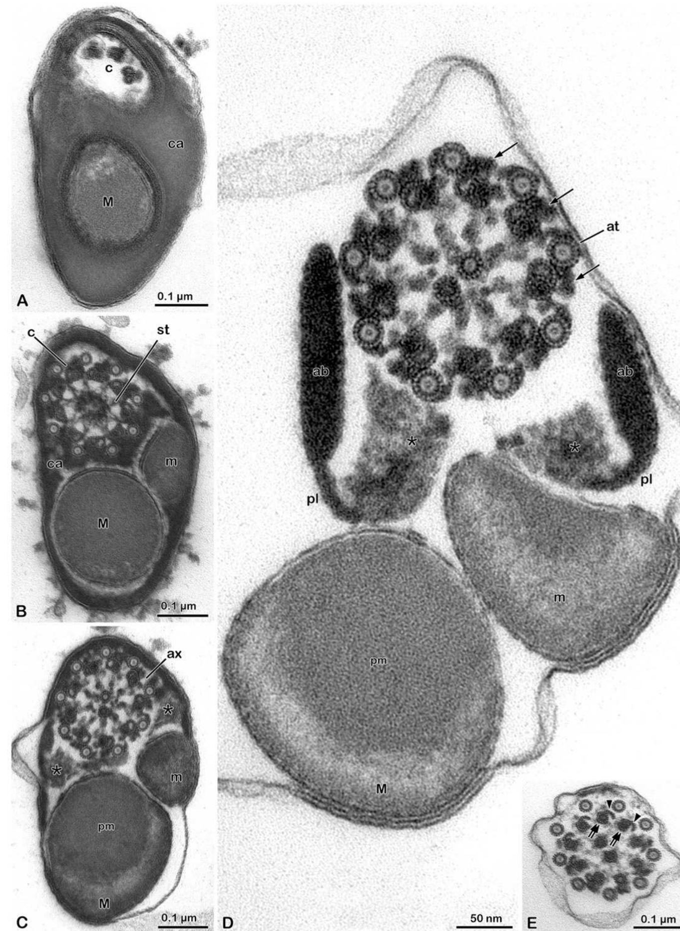

Fig. 2. Cross-sections of *S. cordigera* sperm though the centriolar (A and B) and flagellar (C–E) regions. (A) Note the larger mitochondrial derivative (M) and a few doublets of the centriole (c) inserted in different cavities of the nuclear base (N), surrounded by the centriole adjunct material (ca). (B) The centriole (c) and the two mitochondrial derivatives are surrounded by the centriole adjunct material. Note the peculiar star-like pattern (st) of the links connecting the doublets to the axial dense cylinder. (C) Cross section through the initial flagellar region. Note that the two accessory bodies are small and consist of amorphous material. The two mitochondrial derivatives are of different size. (D) Cross-section through the sperm flagellum showing the 9 + 9 + 2 axoneme (ax). Note the accessory tubules (at) with 16 protofilaments in their tubular wall. These tubules are separated by intertubular material (arrows). Two asymmetric mitochondrial derivatives (M,m) and two thick laminae of the accessory bodies (ab) are visible. Note that the larger mitochondrial derivative (M) is almost completely filled with paracrystalline material (pm) and each accessory body is connected to a puff-like structure (*) by a dense prolongation (pl). (E) Flagellar tip, showing the disorganization of axoneme with the subtubules B (arrowhead) detached from the subtubules A (double arrow).

a repeat of about 45 nm (Fig. 1C and D). The two accessory bodies, which are in continuity with the centriole adjunct material, extend on both sides along the flagellum between the axoneme and the two mitochondrial derivatives. At the anterior sperm tail they are irregular rods of amorphous material (Fig. 2C). Further they have in cross-section the shape of two thick laminae (0.36 nm large) with different length (0.18 and 0.14 μm, respectively); both have a stried cortex with a repeat of 18 nm (Fig. 2D). Each of them exhibits two irregular triangular expansions (puff-like) of amorphous material connected to the laminae by thin curved prolongations (Fig. 2D).

At the tail posterior end, the subtubules B of the axonemal doublets detach from the subtubules A, displaying a hook-shaped structure; and at this level the regular array of the radial spokes is no longer evident (Fig. 2E).

4. Discussion

Sperm ultrastructure in the vast insect order Coleoptera has been investigated in many different families and was found to match basically the classical pterygote type. *S. cordigera* has a bi-layered acrosome, an elongated nucleus, a centriolar region with asymmetric position, two mitochondrial derivatives, a centriole adjunct material giving rise to two characteristic accessory bodies and a 9 + 9 + 2 flagellar axoneme with accessory tubules provided with 16 protofilaments in their tubular wall. The most peculiar trait of *S. cordigera*, however, is the presence in cross-section of two thick laminae as accessory bodies to which two relatively small puff-like structures are connected, giving to each complex a "J"-like appearance. Under this aspect, the species is similar to the other cerambycid *M. asper* (Dallai et al., 1998) so far known,

which shows, as in many other coleopterans, two accessory bodies and puff-like structures of different shape and size. The accessory bodies with elongated "J"-shape described in *M. asper* (Dallai et al., 1998) and *S. cordigera* are very similar to those observed in some species of Chrysomelidae; it is one of several characters supporting a close affinity between cerambycids and chrysomelids (Baccetti and Daccordi, 1988). In Bruchidae (Bawa and Kanwar, 1975), Chrysomelidae (Baccetti and Daccordi, 1988), Curculionidae (Burrini et al., 1988; Dallai et al., 1998; Name et al., 2007) and Dryophthoridae (Paoli et al., 2014) the puff-like structures, may be evident in one or both accessory bodies. When present in both, one of the puff-like structure is greater than the other. However, Tenebrionidae (Baccetti et al., 1973; Dias et al., 2013), Ripiphoridae, Meloidae (Nardi et al., 2013; Dallai, 2014), Staphylinidae (Werner et al., 1999, 2002) and Scarabaeidae (Werner and Simmons, 2011) lack these structures altogether. According to Baccetti and Daccordi (1988) these different extensions of the accessory bodies seem to compensate for the high degree of asymmetry due to the different sizes of the mitochondrial derivatives. Interestingly, the size and shape of accessory bodies has a large variation among Coleoptera families and makes these structures one of the best diagnostic characters in this group. In the examined chrysomelids, accessory bodies have a pyriform shape (Baccetti and Daccordi, 1988), while in curculionoids they have a hook-like appearance surrounding the axoneme (Burrini et al., 1988; Dallai et al., 1998; Name et al., 2007). Similar diversification of accessory bodies were found in Phasmatodea (Baccetti, 1987; Gottardo et al., 2012) and within Zoraptera (see Dallai et al., 2011, 2012, 2014a,b,c).

S. *cordigera* also exhibits an interesting feature at the centriolar level. The peripheral doublet microtubules are connected by long radial links to an axial dense cylinder, giving rise to a star-shape pattern. A similar organization has been observed in other insects: Dermaptera, Odonata and Phasmatodea (Baccetti et al., 1984 and personal observations) even though the links have in these groups a clockwise direction. A very similar organization to that here shown was also described by Perotti (1970) in *Drosophila melanogaster*. *S. cordigera* centriole is houses in a cavity at the posterior end of the nucleus where doublets are embedded in the dense material of the centriole adjunct. A similar position was also observed in Curculionoidea and Chrysomelidae (Baccetti and Daccordi, 1988; Burrini et al., 1988).

The centriolar adjunct in the initial flagellar region is reduced and surrounds the axoneme and one of the mitochondrial derivatives; further it extends along the flagellum in the evident accessory bodies. Such appearance is common to other Coleoptera, but in the staphylinid *Aleochara bilineata* sperm the centriole adjunct material is well developed for most of the flagellum length (Werner et al., 1999).

The flagellar axoneme of *S. cordigera*, with 9 + 9 + 2 microtubules, has the same microtubular pattern observed in most pterygote insects. Only in Attelabidae and Rhynchitidae the organization of the axonemal microtubules is 9 + 9 + 0, indicating a close relationship between these two curculionoid families (Burrini et al., 1988). In *S. cordigera* and *M. asper*, the accessory tubules have 16 protofilaments in their tubular wall, as reported for most insects (Dallai and Afzelius, 1990; Jamieson et al., 1999).

Another interesting character of the cerambycid is the presence of two asymmetric mitochondrial derivatives. In the two species of Cerambycidae studied so far, *M. asper* and *S. cordigera*, the small mitochondrial derivatives have a different shapes and size, while the larger mitochondrial derivative retains a similar slightly oval shape and is partially filled with paracrystalline material. These features of mitochondrial derivatives are shared with chrysomelids (Baccetti and Daccordi, 1988) and curculionoids (Burrini et al., 1988) and under this aspect, they might be regarded as a synapomorphy between the Curculionoidea and Chrysomeloidea to which cerambycids belong. On the contrary, rhipiphorid and meloid families (Tenebrionoidea) exhibit mitochondrial derivatives with a marked symmetry (Nardi et al., 2013; Dallai, 2014).

In conclusion, *S. cordigera* and *M. asper* share a similar sperm general organization and in particular the shape of their accessory bodies, which seem to be the most important tract of Cerambycidae. Finally, the spermatological characters described in this paper, supports the close phylogenetic relationship between the two superfamilies, Chrysomeloidea and Curculionoidea (Lawrence et al., 1999; Hunt et al., 2007; Marvaldi et al., 2009).

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Capítulo II

Dias, G., Lino-Neto, J., Mercati, D. & Dallai, R. (2015b). The sperm ultrastructure and spermiogenesis of Tribolium castaneum (Coleoptera: Tenebrionidae) with evidence of cyst degeneration. Micron, 73, 21-27.

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The sperm ultrastructure and spermiogenesis of *Tribolium castaneum* (Coleoptera: Tenebrionidae) with evidence of cyst degeneration

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ABSTRACT

Previous studies on the spermatogenesis of tenebrionid beetles showed the unusual formation of two antiparallel sperm bundles per cyst. In this work we reported this feature also in *Tribolium castaneum* using light and transmission electron microscopy. The sperm structure of *T. castaneum*, similar to other tenebrionids, consists of a three-layered acrosome, an elongated nucleus and a flagellum with a 9 + 9 + 2 axoneme, two accessory bodies and two asymmetric mitochondrial derivatives. The presence of two antiparallel sperm bundles per cyst also in Meloidae and Rhipiphoridae suggests that it is a strong trait synapomorphic for Tenebrionoidea. The huge degeneration of whole sperm cells in several cysts of testes during spermiogenesis is also described.

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1. Introduction

Spermatogenesis is a biological process of gradual transformation of germline stem cells into spermatozoa. In insects this process occurs within groups of cells, the germ cysts, and involves spermatogonial proliferation by repeated mitotic divisions resulting in a group of primary spermatocytes. These spermatocytes undergo two successive meiotic divisions and give rise to spermatids, which, after deep morphological change (spermiogenesis), form sperm bundles (Dumser, 1980; Chapman, 1998; Dallai, 2014). A functional spermatogenesis requires an intrinsic regulation, which is managed by a conserved genetic program that underlies the development of germ cells ensuring the sperm viability (Fuller, 1998; Jiang and White-Cooper, 2003; White-Cooper, 2010; Ewen-Campen et al., 2013). The lack of an efficient control mechanism may lead to spermatogenetic alterations, which in turn can result in the production of damaged spermatozoa.

In different animal groups there are variations in the spermatogenesis process and especially in the morphological organization of spermatozoa. This diversity has been considered as a useful character system to understand phylogenetic and taxonomic relationships in insects (Burrini et al., 1988; Jamieson et al., 1999; Dallai et al., 1996, 2005, 2011; Lino-Neto et al., 2000; Dias et al., 2013a,b; Santos et al., 2013; Dallai, 2014).

Recent publications have shown an uncommon spermiogenesis in tenebrionid beetles (Dias et al., 2012, 2013a). In this group, during spermatid elongation, the nuclei migrate to opposite regions of the cyst resulting in unusual formation of two antiparallel sperm bundles (see Dias et al., 2012, 2013a).

The present work deals with the ultrastructural aspects of the spermatogenesis of *Tribolium castaneum*, a common pest and an important model organism, with a special focus on cyst degeneration. It is adding new information to ongoing investigations of sperm in the family Tenebrionidae (Dias et al., 2012, 2013a,b).

2. Materials and methods

Male specimens of *T. castaneum* were obtained from colonies maintained at the Federal University of Viçosa (UFV), in Viçosa, Minas Gerais State, Brazil.

The testes and seminal vesicles of five specimens were dissected in 0.1 M sodium cacodylate buffer, pH 7.2, and fixed in a 2.5% glutaraldehyde solution containing 0.2% picric acid, 3% sucrose and 5 mM $CaCl_2$ in the above buffer, for approximately 24 h. The material was post-fixed in a 1% osmium tetroxide solution for 2 h, dehydrated in an increasing alcohol series, infiltrated and finally embedded in epoxy resin (Epon 812). Ultrathin sections obtained with a Reichert Ultracut II E ultramicrotome, were routinely stained and then observed with a Philips CM 10 electron microscope operating at 80 kV. Semithin sections (1 μm) were cut with a Reichert

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Ultracut E ultramicrotome, mounted on glass slides, stained lightly with 1% toluidine blue, and viewed under a Leica DMRB light microscope. Images were taken with an Axiocam digital photocamera (Carl Zeiss).

To observe cysts in whole mount, testes of three specimens were dissected in a sodium phosphate buffer solution, 0.1 M, pH 7.2; follicles were placed on histological slides, dissociated using needles, fixed in a solution of 4% paraformaldehyde in the sodium phosphate buffer, for 15 min, washed with distilled water and dried at room temperature. To determine the length of the sperm nucleus, seminal vesicles of four specimens were dissected in the same buffer and the spermatozoa were spread onto histological slides and fixed in the same way as the follicles. Follicles and spermatozoa were stained for 15 min with 0.2 mg/ml DAPI (4.6-diamino-2-phenylindole), washed in distilled water and mounted with 50% sucrose. The images were captured with an epifluorescence microscope (Olympus BX-60), equipped with a BP360-370 nm excitation filter. The average nuclear length was obtained from measurements, with the software Image Pro-Plus, version 4.5 (Media Cybernetics Inc., MD), of 50 nuclei photographed.

3. Results

3.1. Spermiogenesis

After spermatogonial cell divisions, primary spermatocytes are formed (Fig. 1A). These cells are characterized by uniformly diffuse chromatin, in which synaptonemal complexes are visible (Fig. 1A). In the nuclei some dense masses, possibly nucleoli are visible (Fig. 1A). Close to the nucleus one pair of centrioles is found, oriented at right angle to each other, consisting of the typical microtubular triplets and an axial hub (Fig. 1B). In the same cyst it is not

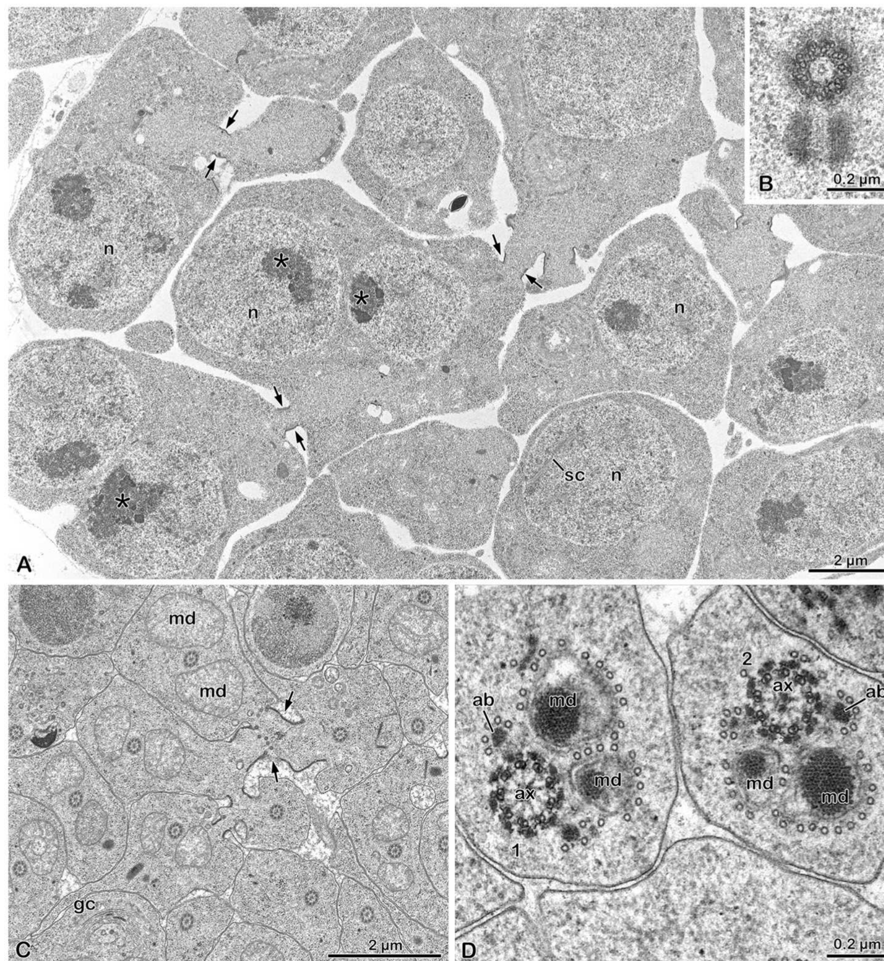

Fig. 1. TEM micrographs of *T. castaneum* spermatogenesis. (A) Primary spermatocyte. Note the synaptonemal complexes (sc) and cytoplasmic bridges (arrows) between germ cells. The nuclei (n) have diffuse chromatin with some small masses (asterisk). Note also the occurrence of two nuclei in same cytoplasm. (B) Detail of one pair of centrioles consisting of the typical microtubular triplets. (C) Early spermatids interconnected by cytoplasmic bridges (arrows) with spherical nuclei and large amount of cytoplasm; the mitochondria are already modify into two mitochondrial derivatives (md). (gc), Golgi complex. (D) Cross-section through the equatorial region of a cyst in more advanced stage of spermiogenesis, showing spermatids with axonemal doublets (ax) clockwise (1) and counterclockwise (2) oriented, indicating an antiparallel arrangement of the sperm cells. (md), mitochondrial derivatives; (ab), accessory bodies.

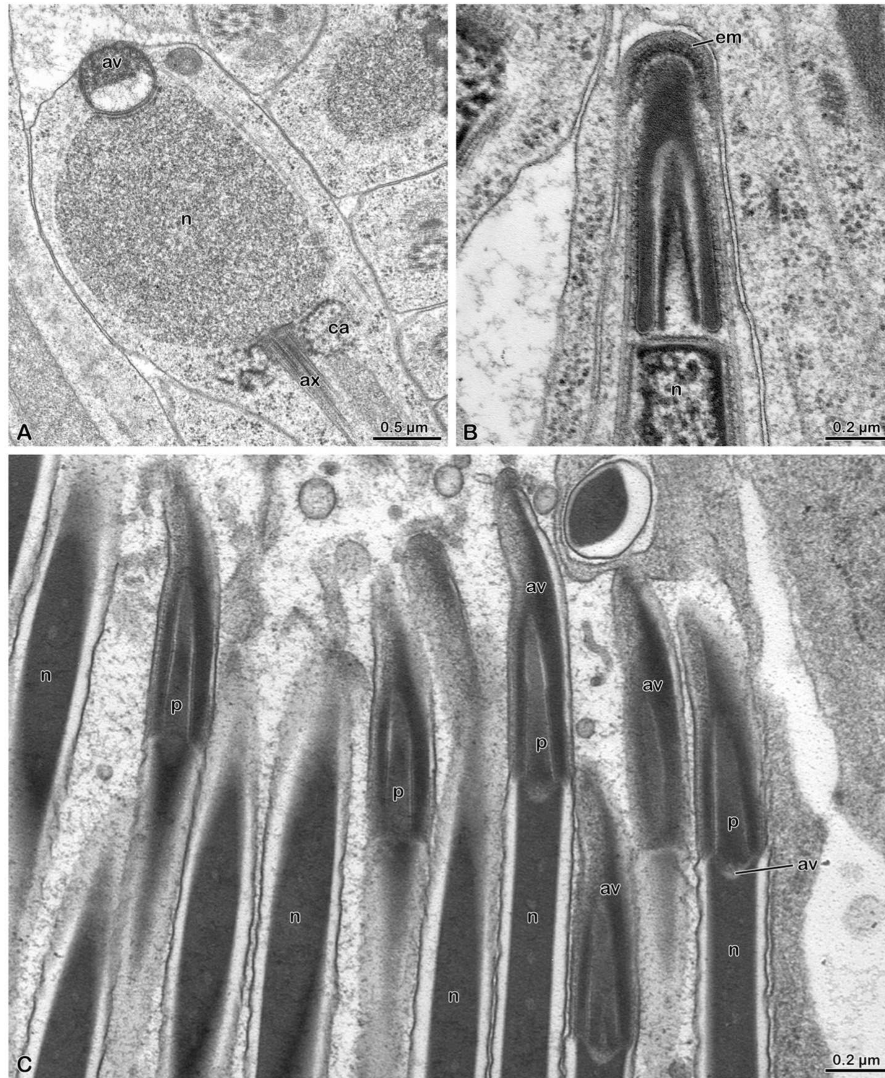

Fig. 2. Longitudinal sections showing the acrosomal development during spermiogenesis. (A) Early spermatid shows only a small acrosomal vesicle (sv) placed at anterior nuclear region. The initial region of the axoneme (ax) is surrounded by the centriole adjunct material (ca). (B) Spermatid at a later stage with elongated nucleus. Observe that the acrosomal vesicle is cone-shape, and has an extra-acrosomal material (em). (C) Mature sperm with the perforatorium (p) housed in a small nuclear cavity. (av), Acrosomal vesicle; (n), nuclei.

rare to observe cells with two nuclei (possibly spermatogonia) in the same cytoplasm, indicating the occurrence of an asynchrony in the cell division (Fig. 1A). At the beginning of spermiogenesis, spermatids are still interconnected by cytoplasmic bridges and exhibit a large amount of cytoplasm (Fig. 1C). The axoneme starts beneath the nucleus and is surrounded by flocculent material of the centriole adjunct. Two large mitochondrial complexes which give rise to mitochondrial derivatives are also observed (Fig. 1C). The chromatin of the nuclei gradually condenses and its electron density increases. During spermiogenesis, the acrosomal complex starts with the formation of an acrosomal vesicle partially filled with electron dense material, which is attached to the nuclear apex (Fig. 2A). As it elongates, the perforatorium is formed, and subsequently an extra-acrosome layer (Fig. 2B). The acrosomal vesicle is conical and coats the perforatorium, which is compact and has its base into a small cavity at the nuclear tip (Fig. 2C). In a transverse section, both the acrosomal vesicle and the perforatorium have an elliptic outline (Fig. 4B).

During elongation, the spermatid undergoes evident modifications, changing from spheroid in shape (Fig. 2A) to an elongated fusiform profile (Figs. 3A–C and 4A). The entire process results in unusual formation of two antiparallel sperm bundles clearly observable in fluorescent preparations (Fig. 3C). This arrangement is also visible in longitudinal sections where the nuclear apex and the sperm posterior end of the flagellum are close to each other, indicating an antiparallel organization (Fig. 4A).

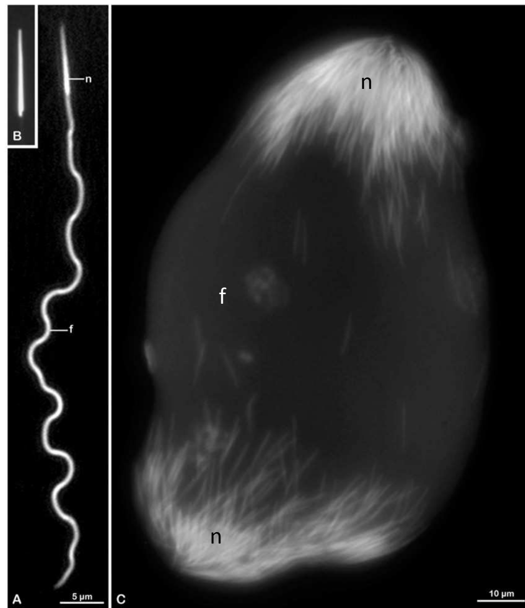

Fig. 3. (A) *T. castaneum* spermatozoon at phase contrast microscope showing the nuclear (n) and flagellar (f) regions. (B) Nucleus stained with DAPI. (C) Cyst in the advanced stages of spermiogenesis stained with DAPI. Note the presence of nuclei at opposite ends of the cyst, indicating the antiparallel arrangement of these sperm cells.

The peculiar antiparallel sperm arrangement can also be established in a cross-section through the equatorial region of the sperm cyst; some sperm flagella show the dynein arms of their flagellar axoneme clockwise orientated and others have a counterclockwise orientation (Fig. 1D).

3.2. Sperm ultrastructure

Each cyst contains up to 512 spermatozoa as a result of nine division cycles (2^9); these sperm are distributed equally into two bundles arranged in antiparallel fashion (Fig. 3C). The spermatozoa of *T. castaneum* are long and slender, measuring 92.45 ± 1.90 μm (n = 50) in length and consist of the head and flagellar regions (Fig. 3A and B). The head contains an acrosome and a nucleus (Figs. 3A,B and 4A,B), while the flagellum contains an axoneme, two elongate mitochondrial derivatives of different size and two symmetric accessory bodies (Fig. 4C). The acrosome consists of three-layers: a cone shape acrosomal vesicle, a long and dense perforatorium, and a less dense extra-acrosomal layer that extends anterior-laterally to the acrosome (Fig. 2C). The nucleus is very thin and long, measuring 10.20 ± 1.20 μm (n = 50) in length (Fig. 3A), and consists of a homogenously compact chromatin and appears fusiform in cross section (Fig. 4B). The nucleus tapers gradually anteriorly (Figs. 3A and 4A). Its posterior extremity shows an oblique profile and in an indentation it houses the larger mitochondrial derivative (Fig. 4A). In the flagellum, the axoneme begins at the base of the nucleus surrounded by a small amount of the centriole adjunct material and has a 9 + 9 + 2 microtubule pattern (Fig. 4A): nine outer single accessory tubules with extended intertubular material a portion of which is adherent to the doublets and the other, as a beak-like, to the accessory tubules, nine microtubule doublets and two central single microtubules. The mitochondrial derivatives differ in length and diameter. They run parallel along the axonemal length (Fig. 4A and C). The larger one appears first, adjacent to the nuclear base and is oval in cross section, and its diameter is at least twice that of the smaller one (Fig. 4C). The smaller mitochondrial derivative is pear shaped in cross section. Two small, dense accessory bodies are present between the axoneme and the mitochondrial derivatives (Fig. 4C). Each accessory body seems to be connected, to the close mitochondrial derivative by a thin amorphous material (Fig. 4C).

3.3. Spermatid and sperm degeneration

During spermiogenesis several cysts with multiple degenerating sperm were observed intermingled with cysts with normal development (Fig. 5A). The sperm degeneration concerns the flagellum but apparently the acrosome and the nucleus are not affected (Fig. 5D). Cross-sections of flagella show remarkable alterations of the axoneme with a complete dissociation of microtubule doublets and the typical axoneme pattern replaced by bundle of dense microtubules (Fig. 5A–D). The mitochondrial derivatives and the accessory bodies have also modified their shape and position becoming fragmented (Fig. 5A–C).

4. Discussion

In *T. castaneum* the spermatogenesis occurs within cystic structures as in most insects (Jamieson et al., 1999; Klowden, 2013; Dallai, 2014). In tenebrionids this process is characterized by successive cell divisions, producing spermatids that through a complex and still unclear cellular mechanism give rise to two sperm bundles arranged diametrically opposed with the nuclei at the two extremities of the cyst (Dias et al., 2012, 2013a). Cross-sections through the sperm bundles of a cyst in the rhipiphorid *Macrosiagon tricuspidata* (Fig. 1C in Nardi et al., 2013) exhibited some flagella with axonemes clockwise oriented together with others showing axonemes with a counterclockwise orientation. Similarly, the meloid *Hycleus scutellatus* and *Mylabris variabilis* (Fig. 2A and B in Nardi et al., 2013) exhibited the nuclei arranged at the two ends of the sperm bundle. These observations show that the antiparallel arrangement of sperm in the cysts, observed in Tenebrionidae, may also occur in the Rhipiphoridae and Meloidae tenebrionoids. According Levkaničová (2009), the tenebrionoids families can be arranged in four clades, being the tenebrionid clade more basal and ripiphorid-mordellid-meloid the more derived clade. Thus, it is conceivable to assume that the antiparallel arrangement of sperm is a trait shared by all tenebrionoid families. Therefore this character gives support to previous works that considered the Tenebrionoidea as a monophyletic group (Lawrence and Newton, 1995; Levkaničová, 2009; Beutel and Friedrich, 2005; Bocák et al., 2014; Kergoat et al., 2014).

In beetles the number of spermatids or of sperm cells per cyst is usually 256 as a result of eight cycles (2^8) of cell divisions (Phillips, 1974; Name et al., 2007). However, previous works in *T. castaneum* and other tenebrionid pests have shown two sperm bundles per cyst, each one with 256 spermatozoa, for a total of 512 sperm cells, generated by nine cycles (2^9) of cell divisions (Dias et al., 2012). This high number of sperm per cyst may be related to reproductive behavior of these insects, since adults are highly promiscuous and the females can adjust the amount of sperm accepted depending on male phenotypic quality (Fedina and Lewis, 2008).

Primary spermatocytes of *T. castaneum* are characterized by the presence of synaptonemal complexes; the meiotic process, however, seems to develop asynchronically in the cyst as some cells with two nuclei in the same cytoplasm can be found due to a still incomplete cytokinesis, together with others in meiotic prophase. Spermatocytes are provided with two centrioles consisting of a central hub surrounded by nine microtubular triplets. Similar

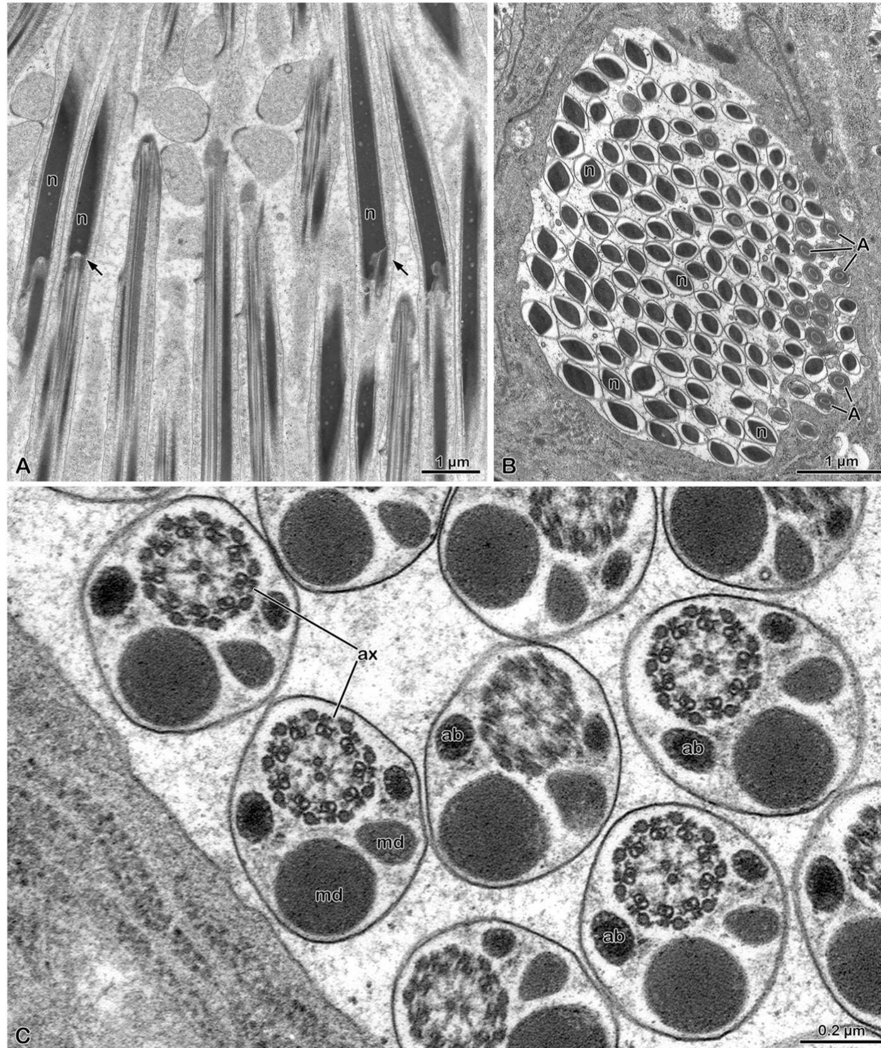

Fig. 4. Transmission electron micrograph of *T. castaneum* sperm. (A) Longitudinal section of the nucleus (n) and transition region (arrows) between head and flagellum. (B) Cross-section through the sperm at the acrosomal (A) and nuclear level (n). (C) Cross section through the flagellar region showing a 9 + 9 + 2 axoneme (ax), two symmetric accessory bodies (ab) and two asymmetric mitochondrial derivatives (md).

organization of the centriole was described by Wolf and Joshi (1995) in *Tenebrio molitor* spermatogenesis. Spermatids of *T. castaneum*, as commonly found in insects, however, have only one centriole, due to the lack of a centriole duplication at the second meiotic division (Gonzáles et al., 1998; Callaini et al., 1999).

The early spermatids of *T. castaneum* exhibit a plesiomorphic three-layered acrosomal complex as it occurs in many pterygote insects and is also found in species of Tenebrionidae such as *T. molitor* (Baccetti et al., 1973) and *Lagria villosa* (Dias et al., 2013b). This type of acrosome does also occur in some Curculionoidea (Burrini et al., 1988) and Chrysomelidae (Baccetti and Daccordi, 1988). *T. castaneum* exhibits a typical insect sperm consisting of an apical acrosome, elongated nucleus, two crystallized and asymmetric mitochondrial derivatives, two accessory bodies and a 9 + 9 + 2 flagellar axoneme. These characters are shared by other tenebrionids and in particular with *T. molitor* (Baccetti et al., 1973) and *Zophobas confusa* (personal observations) whereas *L. villosa* has symmetric mitochondrial derivatives. The nuclear outline is fusiform in cross section but it is circular in *L. villosa*, which has also a flattened acrosome (Dias et al., 2013b).

Undoubtedly the accessory bodies represent one of the best diagnostic characters within Tenebrionidae; they exhibit always an oval shape when observed in cross section. In contrast, in several families of beetles including Curculionidae (Burrini et al., 1988), Chrysomelidae (Baccetti and Daccordi, 1988; Dallai et al., 1998) and Cerambycidae (Dallai et al., 1998) these accessory bodies have a different organization with an asymmetric shape and the presence of an additional puff-like extension. Baccetti et al. (1973) have demonstrated that in *T. molitor* the accessory bodies have intense ATPase

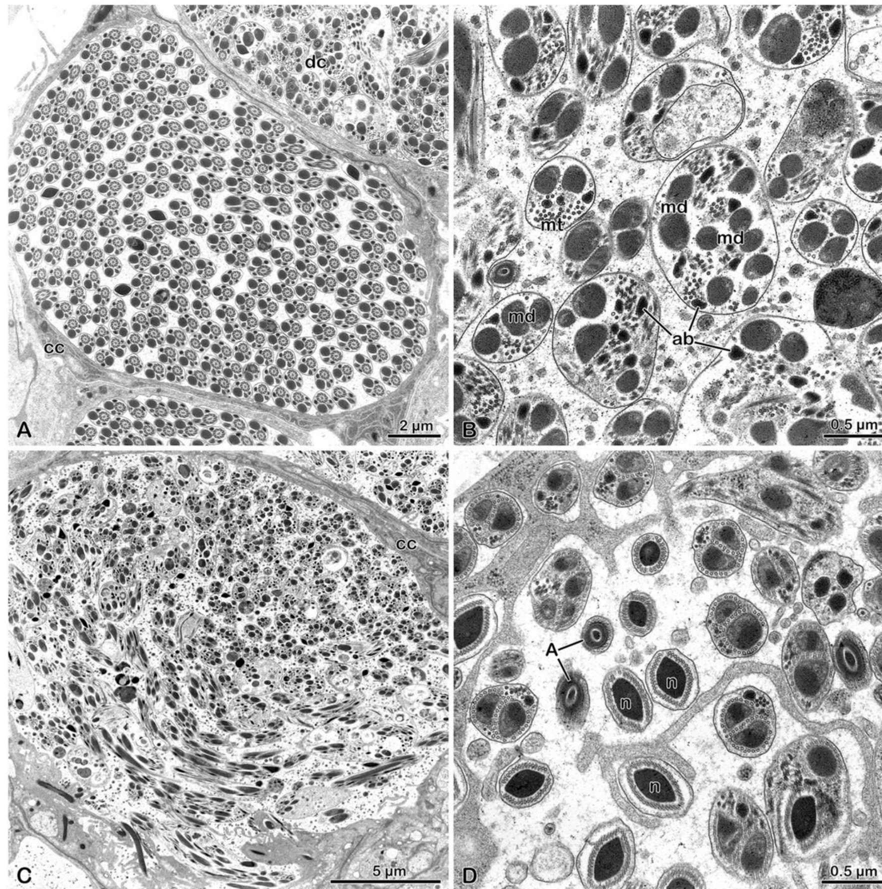

Fig. 5. Cross-sections of *T. castaneum* testes, showing a cyst with normal spermatozoa (A) intermingled with cysts filled with abnormal spermatids and spermatozoa (B–D). (B) Detail of sperm degeneration. Note a complete disorganization of axonemal microtubules (mt) together with supernumerary number of mitochondrial derivatives (md) and accessory bodies (ab). (C) Cross section of a cyst showing all the sperm in degeneration. (D) Cross section of a cyst with degenerating spermatids. Note that the acrosome (a) and nuclei (n) seem not affected by degeneration. cc, cyst cells.

and UTPase activities. However the function of these structures is not well understood (Dallai, 2014).

In *T. castaneum*, the axoneme, which originates from the single centriole present in the spermatid, shows a 9 + 9 + 2 microtubular pattern typical of most pterygote orders (Jamieson et al., 1999; Birkhead et al., 2009; Dallai, 2014). The antiparallel sperm bundles in each cyst can be also recognized in a cross section through the equatorial region of the cysts by the direction of dynein arms of microtubule doublets: they will be clockwise or counterclockwise orientated according to the position of a sperm in the cyst.

Interestingly, in some cysts of *T. castaneum* a degeneration of flagellar components, particularly the axoneme, was observed. The loss of scattered aberrant germ cells is a normal event in insect spermatogenesis (Holmgren, 1901; Friele, 1930; Sara, 1950) and the process is associated with the age of males or with a genetic deficiency (Meyer, 1970; Kiefer, 1970; Fuller, 1998) or even with natural or transformed intersex (unpublished observations). Moreover, it was shown that many environmental factors such as elevated temperatures, food deprivation and high humidity can also affect sperm viability (Szollosi, 1976a,b; Stürup et al., 2013). However, it is not common to find cysts with entirely degenerated sperm. According to Fuller (1998), there are three critical moments during the spermatogenic process: the first occurs at the beginning of the spermatogenesis when the spermatogonial cells start the mitotic cycle; the second occurs between mitotic and meiotic divisions; and the third at the end of meiotic divisions and early of the spermatid differentiation. The sperm degeneration here described can be the result of an irregular mechanism at the third critical moment affecting the early spermatid differentiation. However, at present we do not know yet the cause of this degenerative process in *T. castaneum*. The high extent of the degeneration observed in this species is not comparable to that found in other insects; not only the axonemal components, doublet microtubules and accessory tubules are disorganized and have lost their original appearance, but also the mitochondrial derivatives and the accessory bodies have a wrong position and an irregular shape. Moreover, no dynein arms are observed in any microtubules and this lack together with the irregular position of the microtubule elements, does not allow sperm motility. The absence of degenerated sperm within the seminal vesicle (data not illustrated) strongly suggests that they are eliminated by a process of incorporation in the epithelial wall of the testes (Viscuso et al., 2012).

Despite of the loss of many sperm at the end of the spermiogenesis as result of the cyst degeneration, the reproductive capability of the species is not affected due to the larger number of spermatogonial mitotic divisions and the consequent high number of sperm cell produced after spermiogenesis. Tenebrionidae, among coleopterans, are the group with a higher number of sperm in the cysts. Virkki (1969) and Lachaise and Joly (1991) suggested that a high number of germ cells per cyst is typical for basal insect lineages and probably ancestral for Insecta or Hexapoda. Our results show that this condition also can occur in an advanced group of holometabolous beetles, e.g. cucujiform Coleoptera.

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Capítulo III

Dias, G,. Dallai, R., Carapelli, A., Almeida, J.P.P,. Faroni, L.R.A., Campos, L.A.O. & Lino-Neto, J. (2017). First record of gregarines (Apicomplexa) in seminal vesicle of insect. Scientific Reports, 7 (1), 175.

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First record of gregarines (Apicomplexa) in seminal vesicle of insect

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Gregarines (Apicomplexa) are a diverse group of protozoan parasites, which infects gut and other body cavities of invertebrate hosts. In reproductive system of insects, gregarine has been reported only in the accessory glands and spermathecae of females; therefore, this is the first report of a gregarine species in seminal vesicles of insects. Different developmental stages, including sporozoytes, oocysts and trophozoites were described from morphological descriptions using light and electron transmission microscopy. The parasites were described in seminal vesicles of the beetle *Tribolium castaneum* a model organism and an important insect pest. DNA sequence analysis suggests that the protozoan parasite was an *Ascogregarina* sp.

Gregarines are a heterogeneous group of Apicomplexan protozoan parasites comprising about 1,600 species. Mature forms consist of large and extracellular parasites, typically infecting a variety of invertebrates, especially annelids, arthropods and molluscs[1–6]. These parasites have received great attention from many researchers, including those interested in the parasite-host coevolution as well as those interested in biological control of insects[8–10].

The gregarine life cycles include the following stages: sporozoites (cell form that infects new hosts); trophozoites or gamonts (large extracellular vegetative stages); gametocyst (gamont pairs in which gametes are produced); and oocyst (it is a hardy, thick-walled spore, which contains the infective stages)[11]. In general, the contamination by gregarine occurs via faecal-oral transmission, when the parasites enter the body by oocyst ingestion containing several sporozoites. Then the sporozoites reach the intestinal cavity, attach to the host cells, and develop extra-cellular into larger vegetative stages[11].

Among insect, Orthoptera, Odonata, Blattodea, Diptera, and Coleoptera have been reported to be infected by gregarine[1, 4, 5, 10]. The presence of these parasites is recorded especially in digestive tracts of larvae and adults, Malpighian tubules, fat bodies and eggs[10]. In the reproductive system of insects, gregarines were reported only in accessory glands and spermathecae of females[9, 10, 12]. As part of an ongoing study of spermiogenesis in the stored grain pest, *Tribolium castaneum* (Coleoptera: Tenebrionidae), we report the novel occurrence of a gregarine species in seminal vesicles of insects.

Results

The seminal vesicles of sexually mature *Tribolium castaneum* are characterized by a dilatation of the deferent duct, filled with spermatozoa (Fig. 1A,C). Seminal vesicles of all sampled individuals of the contaminated colony showed parasites (Fig. 1B,D). Under the light microscope, the uncontaminated seminal vesicles in whole mount exhibited a smooth surface (Fig. 1A), while those infected reflects the presence of high contamination showing an uneven surface (Fig. 1B). When the contaminated seminal vesicles were disrupted a large amount of parasites were released together with spermatozoa (Fig. 1B).

Histological sections of the contaminated seminal vesicles showed parasites preferably located close to the vesicular epithelium (Fig. 1D), which was formed by a simple layer of cubic cells. In the uncontaminated seminal vesicles lumen had only spermatozoa (Fig. 1C) and the epithelium was formed by flattened cells. The parasites occurred as single cells with different morphologies, which may represent distinct stages of the life cycle (Fig. 1B,D). The trophozoites were observed closely associated to the epithelial cells of the seminal vesicles

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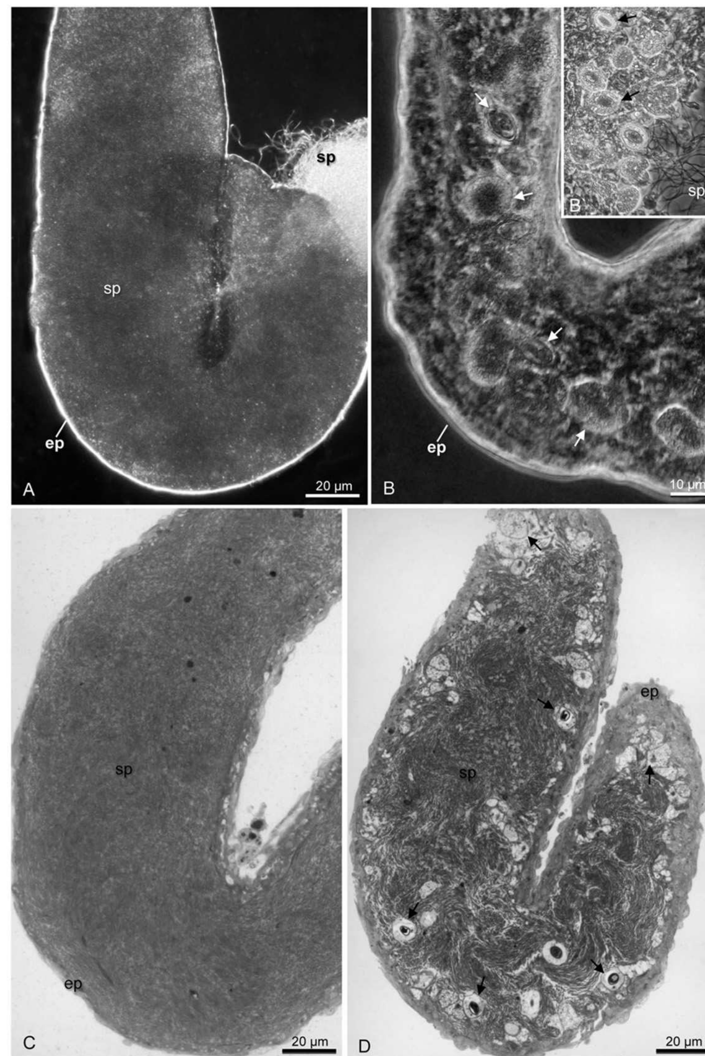

Figure 1. Photomicrographs of seminal vesicles of *Tribolium castaneum* in phase contrast (**A,B,B'**) and histological sections (**C,D**). (**A**) Uncontaminated seminal vesicle in total mount. Spermatozoa (sp); epithelium (ep). (**B,B'**) Contaminated seminal vesicle in total mount. The arrows indicate the parasites. Note in (**B'**) the large amount of parasites from seminal vesicle broken. (**C**) Histological section of uncontaminated seminal vesicle. Observe the lumen completely filled with only sperm (sp). (**D**) Histological section of contaminated seminal vesicle. Note the parasites in different stages (arrows) and located preferentially in the peripheral region.

(Fig. 2A,B). Under high resolution micrograph observed an intimate association of the trophozoite membrane to the membrane of the epithelial cells (Fig. 2B). In this stage they were large, about 30 µm, segmented and with irregular shape. They have cytoplasm filled with large amounts of amylopectin granules (Fig. 2A,B), which showed a strong purple color in the PAS tests (Fig. 3A). The gametocyst had two juxtaposed cells (trophozoites or gamontes) surrounded by a thin wall, with a septum between them (Fig. 3E). In this stage there was also a great amount of amylopectin granules (Fig. 3A). The nucleus of the each gamont was evident with DAPI staining preparation (Fig. 3E) as well as those of the sporozoites within the oocysts (Fig. 3F,G). The oocysts were individualized and exhibit shape very uniform (Figs 3B,C,D and 4A,B). They were barrel shape, about 10 µm in length, and exhibited plugs on the both poles, which were also PAS positive (Fig. 3C,D). The cells showed a thick wall, about 2,5 µm, with three layers: the inner, was formed for numerous longitudinal lamellae extending from one pole to another, with a smooth surface associated with the cell membrane, and the opposite side had indentations;

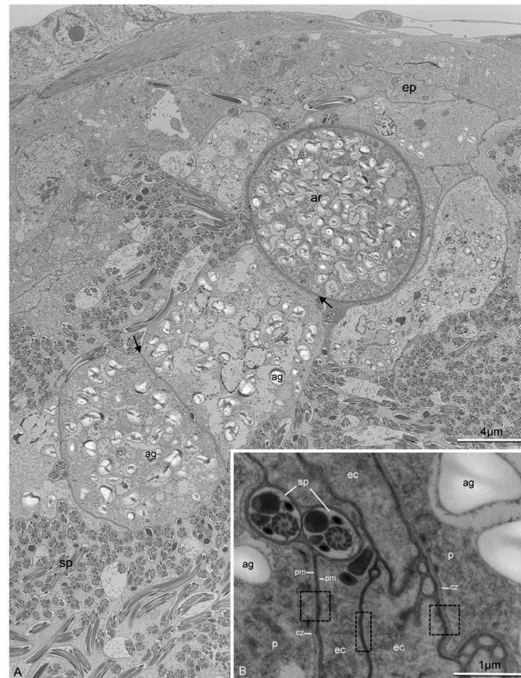

Figure 2. Electron micrographs of contaminated seminal vesicle of *T. castaneum*. (**A**) Segmented trophozoite with the anterior region (ar) inserted into the seminal vesicle epithelium (ep). Note the many amylopectin granules (ag) in all segments (arrows). Spermatozoa (sp). (**B**) Intimate association between the plasma membrane (pm) of the parasite (p) and plasma membrane of epithelial cells (ec). Note the junction between two epithelial cells (rectangle), and junctions between epithelial and parasitic membranes (squares). Cortical zone of the parasite (cz).

the median layer was thicker, with less electrondensity and amorphous with its larger area filled by amylopectin granules and; the outer layer thiner, flat and electrondense (Fig. 4A–D). The oocyst wall enclosed one or more sporozoites (Figs 3F,G and 4C,D), linked by cytoplasmic bridges (Fig. 4D). The sporozoites had nucleus with decondensed chromatin and evident nucleolus, and also amylopectin granules in the cytoplasm (Fig. 4B).

The gregarine DNA fragment was amplified only from the two extractions obtained from infected seminal vesicles, whereas no amplification of the parasite DNA resulted from either non-infected vesicles and whole beetles deprived of the reproductive system. Similarly to the results of Dabert and Dabert[13], the PCR reaction revealed two bands for the extractions from the seminal vesicles, of approximately 1 and 3 kb, respectively. Following the reference paper, we hypothesized that the primer couple, used for the PCR reaction, was unable to exclusively match the protozoan DNA, and that host/parasite selection was only possible by observing the size differences of co-amplified products, as revealed by electrophoresis. In this respect, in agreement with Dabert and Dabert[13] results, the larger band was amplified from the host rDNA, whereas the smaller was obtained from the parasite extraction. Therefore, the two PCR products were separated, excising the targeted band, after a short run on a standard electrophoresis gel. DNA recover from the gel was performed using the kit Wizard SV Gel and PCR Clean-up (Promega, Madison, WI, USA). Purified 1 kb was initially sequenced with both PCR primers, obtaining a clear reading of electropherograms only for f1300. In order to obtain the required double-strand reading of the PCR product, two species-specific primers (Api-18Sf and Api-18Sr) were designed on the sequence obtained with f1300, and than used for additional sequencing reactions. Final consensus rDNA sequences (662 bp in length), encompassing the 18 S and ITS2 rDNA of the parasites DNA present on the seminal vesicles of two *T. castaneum* specimens, were obtained and deposited in GenBank, under accession numbers KY471625 and KY471626. Among the first ten sequences producing significant alignments with the query retrieved in Blastn search, all matches identified three species (barretti, culicis and taiwanensis) of the *Ascogregarina* genus, with a query cover range between 72–73%, E-value = 0 and 92–93% of nucleotide identity. Phylogenetic analysis (Fig. 5) revealed that our sequence forms a well supported cluster (PP = 1) with all *Ascogregarina* sequences present in GenBank. Phylogenetic position inside the genus is uncertain due to low support obtained in this analysis (PP = 0.86).

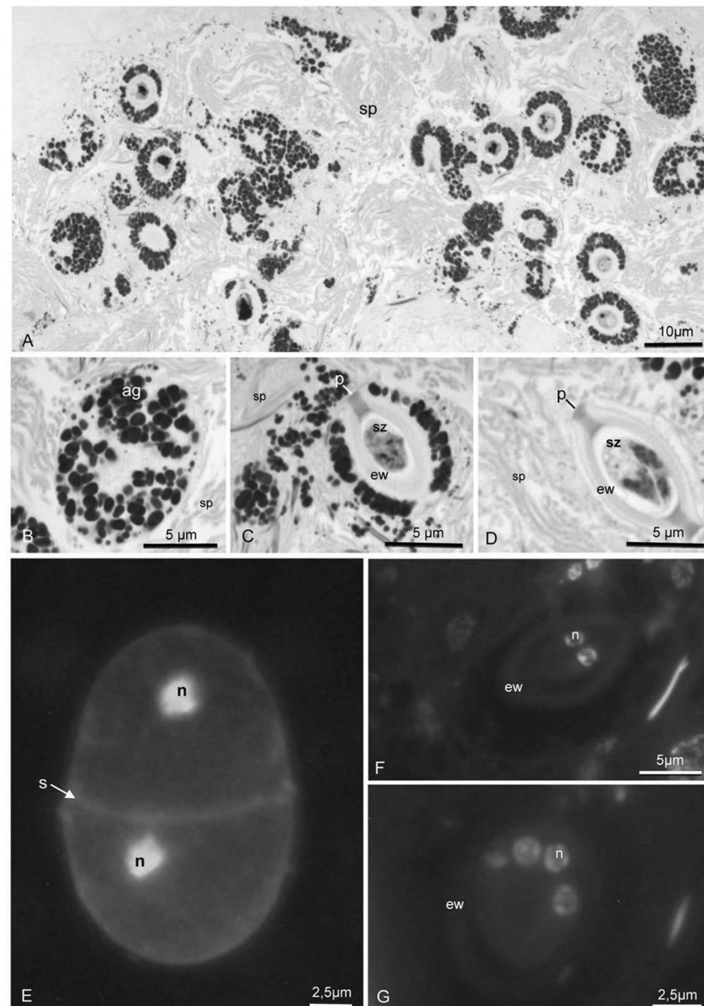

Figure 3. Histological section of contaminated seminal vesicle submitted to periodic acid-Schiff (PAS) reaction (**A–D**), and parasites stained with DAPI (**E–G**). (**A–D**) Gregarines in different stages exhibiting amylopectin granules (ag) in strong purple color. Note in oocytes (**C,D**) the presence of plugs (p) on the both poles and internally the sporozoites (sz). Spermatozoa (sp); extracellular wall (ew). (**E**) Gamont stage; Note the presence of two nuclei (n) and a septum (s). (**F,G**) Oocytes showing two (**F**) and four (**G**) sporozoites surrounded by a thick wall (ew).

Discussion

The seminal vesicles of *Tribolium castaneum*, as in most insects, are dilations of the vasa deferentia, in which sperm are stored before they are transferred to the female[14, 15]. The vesicular epithelium, in several animals including the insects, has glandular that produce nutrients for nourishment and maintenance of the sperm[16, 17]. This makes the seminal vesicles a suitable environment for parasites, as has been observed in studies with earthworms[7, 18]. However, the presence of parasites in the seminal vesicles of insects has not been observed so far, therefore this is the first study that provides the record of gregarine apicomplexan parasites in this reproductive organ. The infection process by gregarine in seminal vesicles of *T. castaneum* probably occurs with a similar mechanism as described by Lantova and Volf[10] for the accessory glands of the female of sand flies. In this study it was reported that the gregarines enter into the intestinal cavity through ingestion, go through the intestinal wall and reach the accessory gland, where they adhere to the wall and finally are released into the gland lumen. The occurrence of intestinal gregarine has already been recorded for several insects, including the tenebrionid beetles[4, 19], and the contamination process is known, which occurs through ingestion of oocysts during feeding[10, 20]. In the

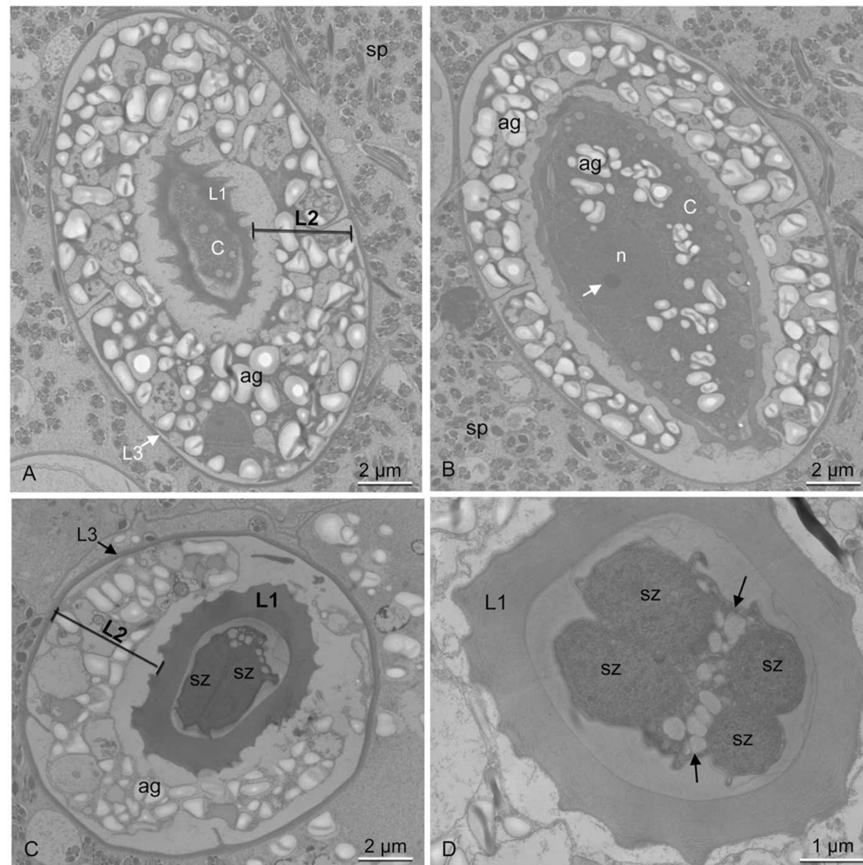

Figure 4. Electron micrographs of oocytes. (**A,B**) Sagittal section of oocytes showing the thick extracellular wall with three layers (L1–L3). In (**B**) note a single sporozoite (sp) showing nucleus (n) and small nucleolus (arrow). Cytoplasm (c); amylopectin granules (ag); spermatozoa (sp). (**C,D**) Transverse section of oocytes showing two (**C**) and four (**D**) sporozoites (sz) linked by cytoplasmic bridges (arrows).

earthworm seminal vesicles the life cycle and infection process were well established and showed that the contamination occurred by an oro-fecal route, with the parasite crossing the intestinal wall to reach the dorsal vessel and the heart from where it was transported to the seminal vesicles[21].

We obtained high-resolution electron transmission micrographs showing also an intimate association between the membrane of parasites and the epithelial cells of the seminal vesicle, suggesting an adhesion mechanism similar to that described by Cox[22]. As known, the apicomplexan members are characterized by the presence of an organelle called apical complex[23]. This structure is a complex assemblage of structural and secretory elements at the apical point of the cell required to in the host cell invasion process and nutrition[24]. However in eugregarine species, for example, this structure is lost during trophozoite development stage suggesting a different mode of nutrition to the group. According Cox[22] the cortex folds of eugregarine trophozoites are likely structural adaptations that create the surface area necessary to effectively absorb nutrients passing through the host intestinal tract.

Trophozoites are the most structurally diverse stages in gregarine life cycles and their ultrastructural traits have been used as a taxonomic too[13,20]. The mature stage is characterized by shape unsegmented or subdivided into distinct regions and the cytoplasm usually appears filled with large amounts of amylopectin granules, which were confirmed from the PAS tests. In this study the trophozoites are segmented and a great number of these granules was also observed in all the other developmental stages, except for sporozoites. Some studies suggest that the role of amylopectines is essential for gametogenesis due to gametocyst wall formation and supplying of energy to the parasite. The oocysts observed in the seminal vesicles of *T. castaneum* are characterized by oval-shaped and contained a thick resistant wall. They exhibit plugs on the poles, which possibly are open for in order to release the sporozoites[25].

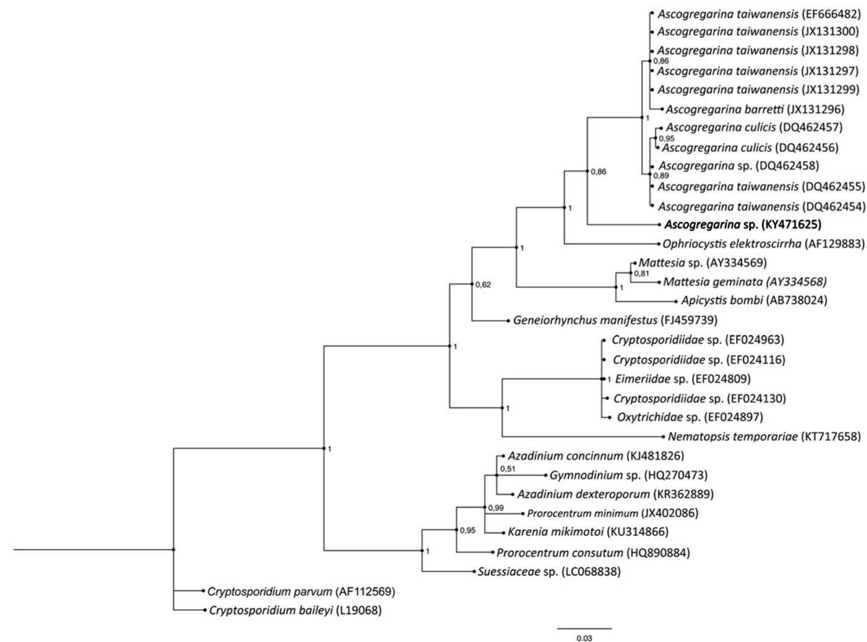

Figure 5. Bayesian tree inferred from partial SSU (small subunit) rDNA sequences showing phylogenetic position of the gregarine from *T. castaneum*. *Cryptosporidium* genus sequences were used as outgroup. Codes in parentheses represent GenBank accession numbers of partial SSU rDNA sequences. Posterior Probabilities (PP) values are shown with the nodes.

The impact of gregarine infection on host fitness and viability is widely discussed. According Valigurová[23], although gregarines are not lethal to the insect hosts, they reduce longevity, fecundity and body size of the host. High levels of infection by gregarines in the digestive epithelium of insects can cause some defects to development or damage the host tissue[26]. This pathogenicity is mainly attributed to trophozoites, which may destroy individual cells through their embedded epimerites or cause significant impact on the host nutritional state by obstruction of the gut[27]. On the other hand, gregarines are relatively harmless to their hosts and some of them even consider that they are essential to the well being of the host[23, 26, 28]. According to Sumner[28] is possible that gregarine secrete essential substances such enzymes or vitamins necessary for larval growth. Others studies have shown that some gregarine species found in mosquito intestines are pathogenic[29, 30], suggesting them as possible agents for biological control[31].

Based on morphological characteristics, we supposed that the species belongs to the eugregarine group. DNA sequence analysis in GenBank showed a clear nucleotide similarity between the parasite hosted in the seminal vesicles of *T. castaneum* and three species of the genus *Ascogregarina*, such as *A. barretti, A. culicis* and *A. taiwanensis*, parasites of the midgut of mosquitoes *Ochlerotatus triseriatus*, *Aedes aegypti*, and *Ae. albopictus*, respectively[32]. Despite the uncertainty of the phylogenetic position inside the genus, our bayesian inference showed the close relation of the parasite sequence obtained in this study with the *Ascogregarina* genus. We therefore conclude that the are strong evidences to suggest that the parasite of the seminal vesicle belongs to this genus *Ascogregarina* (Lecudinidae). The fact of the colony has been reared in the laboratory for more than 10 years and with practically 100% of infected males indicates that this infection is widespread among males. Still considering that no amplification of the parasite DNA resulted from whole beetles deprived of the infected seminal vesicles, it is possible that this parasite is specific of seminal vesicle of these beetles. Thus, given the capacity of transmission and supposed specificity, these parasites most likely belong to a new species of *Ascogregarina*. This finding opens a new avenue for further studies regarding the effect of this parasite on the reproductive fitness of the insect with the potential to use it in pest insect control.

Materials and Methods

Males of *Tribolium castaneum* infected and uninfected by gregarines were obtained from contaminated and uncontaminated colonies maintained at the Laboratório de Manejo Integrado de Pragas de Grãos Armazenados, Departamento de Engenharia Agrícola, Universidade Federal de Viçosa (UFV), in Viçosa, Minas Gerais State, Brazil. To observe the degree of infection 50 males from the contaminated colony and 20 males from the uncontaminated colonies were used.

Light Microscopy. To observe contaminated and uncontaminated seminal vesicles in whole mount, adult males were dissected and seminal vesicles removed in 0.1 M sodium phosphate buffer solution, pH 7.2, fixed for 1 h in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2 and transferred to histological slides covered with coverslips and photographed using an Olympus BX-60 microscope equipped with phase contrast (Olympus Corporation, Tokyo, Japan).

Some isolated seminal vesicles were dissected out and the parasites and spermatozoa were spread on slides, fixed with solution of 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2 for 30 minutes, washed in running water and dried at room temperature. The slides were examined and the parasites were photographed in Olympus BX-60 photomicroscope equipped with phase contrast. To observe the nuclei some slides were stained with 0.2 mg/ml4,6-diamino-2-phenylindole (DAPI).

Histological Sections. To obtain the histological sections, the contaminated and uncontaminated seminal vesicles were dissected and fixed for 2 h in 2.5% glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.2 at 4 °C. The material was washed for 2 h in the same buffer, post fixed in 1% osmium tetroxide for 2 h and dehydrated in alcohol solutions of increasing concentrations: 30, 50, 70, 90 and 100%. The material was immersed in two 4-h baths each at room temperature, the first with a mixture of historesine (Leica Historesin, Heidelberg, Germany) and alcohol (1:1), and the second with pure historesin. For inclusion, the seminal vesicles were immersed in historesin with a catalyst in silicone moulds, which were placed in Petri dishes and transferred to an oven at 58 °C for 24 h. Semithin sections (2 μm) were obtained with a microtome Leica RM 2155 (Leica Corporation, Wetzlar, Germany) with glass knives. These were transferred to histological slides stained with Harris haematoxylin for 15 min, washed in running water for 10 min, stained with eosin for 1 min and rapidly rinsed in tap water. For the detection of neutral polysaccharides some slides were submitted to the periodic acid-Schiff (PAS) reaction. All observations and photographs were made using an Olympus BX-60 microscope.

Transmission Electron Microscopy. The contaminated seminal vesicles were dissected in 0.1 M sodium cacodylate buffer, pH 7.2, and fixed in a 2.5% glutaraldehyde solution containing 0.2% picric acid, 3% sucrose and 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer, pH 7.2, for approximately 24 h at 4 °C. The material was post-fixed in a 1% osmium tetroxide solution for 2 h, dehydrated in an increasing alcohol series, infiltrated and finally embedded in epoxy resin (Epon 812). Ultrathin sections obtained with a Reichert Ultracut II E ultramicrotome, were contrasted with solutions of 3% uranyl acetate of and 0.2% lead citrate and then observed with a Philips CM 10 electron microscope operating at 80 kV (Universitá degli Studio di Siena, Siena–Italy) and a Zeiss EM 109 (Núcleo de Microscopia e Microanálise, Universidade Federal de Viçosa, MG–Brasil).

DNA extraction and PCR. Total genomic DNA was extracted from: two males of either infested or non-infested *T. castaneum* whole specimens, with the reproductive system removed; and from the seminal vesicles isolated from other two specimens for each infested and non-infested coleopteran hosts. Total DNA of the eight samples was extracted with the Wizard SV genomic DNA purification system kit (Promega, Madison, WI, USA) and used for PCR amplifications. A gregarine-specific DNA fragment, encompassing the 18S rDNA and ITS2 regions, was amplified using the primer pair: f1300 (5′-TGCATGGCCGTTCTTAGTTG-3′) and ITS2-28S (5′-ATATGCTTAAATTCAGGGGG-3′)[13]. The PCR reaction, run in a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA) thermal cycler, was performed in a volume of 25 μl containing: 2.5 μl of genomic DNA, 0.5 mM of each primer, 0.2 mM of each dinucleotide, 2.5 mM of $MgCl_2$, 5 μl of Green GoTaq Flexi buffer and 0.625 u of GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA). PCR conditions were: 35 cycles at 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 90 sec, followed by a final extension step at 72 °C for 10 min. PCR products were then purified using the kit Wizard SV Gel and PCR Clean-up (Promega, Madison, WI, USA), and sequenced with the above PCR primers and with other two internal primers, Api-18Sf (5′-GTAATTATTCATCTTGAACGAGGAA-3′) and Api-18Sr (5′-TTCCTCGTTCAAGATGAATAATTAC-3′), specifically designed on the targeted sequence. Sequencing reactions were run on a DNA Analyzer ABI 3730, at the core facility of the Biofab Research Lab (Rome, Italy). The sequence data set was assembled using Sequencher 4.4.2 (Gene Codes Corporation, Ann Arbor, MI, USA).

Sequence analysis. The sequence obtained was searched for highly similar DNA sequences in the database of nucleotide collection of NCBI using the Blastn tool. For Bayesian phylogenetic inference a dataset containing our sequence and 31 reference sequences from Genbank were aligned using ClustalW[33] provided in MEGA 6.0[34] (GenBank accession codes are reported in Fig. 5). To infer the best nucleotide substitution model for the alignment, we used the program MrModelTest 2.3[35] under the Akaike Information Criterion (AIC). The trees were searched using the software MrBayes 3.2[36] provided in the webserver CIPRES[37] with two independent runs, with four Markov chains each (one cold and three heated). Each chain ran for 50,000,000 generations and was sampled every 5,000 generations. A burn-in on the first 25% of the trees was performed before using the remaining topologies to build a consensus topology with its respective branch lengths, which was viewed using FigTree v.1.4.2[38]. Tree was rooted using *Cryptosporidium parvum* (AF112569) and *C. baileyi* (L19068) as outgroup. Statistical support at each node was evaluated by calculating the Posterior Probability (PP).

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Author Contributions

G.D., R.D. and J.L.-N.: Study design, acquisition of microscope images, data analysis and interpretation, and writing of manuscript. A.C.: Acquisition and interpretation of the molecular data. J.P.P.A.: Phylogenetic analysis. L.A.O.C. and L.R.A.F.: Study design.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

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3. Conclusão geral e perspectivas

Os resultados desta tese indicam que ultraestrutura do espermatozoide de *S. cordigera* apresenta uma organização similar a de outros cerambycídeos, principalmente considerando a morfologia dos corpos acessórios, que parecem ser a característica mais marcante de Cerambycidae. Além disso, os caracteres espermatológicos descritos para essa família suportam a estreita relação filogenética entre as duas superfamílias, Chrysomeloidea e Curculionoidea. A ultraestrutura dos espermatozoides de *T. castaneum* apresenta caracteres comuns aos demais tenebrionídeos. Como, por exemplo, corpos acessórios ovais, pequenos, com apenas a região eletrondensa; acrossomo em três camadas e base nuclear chanfrada, onde se associa a extremidade anterior do derivado mitocondrial maior; Além dessas, a disposição antiparalela dos espermatozoides por cisto, aqui descrita, foi também observada em outras famílias de Tenebrionoidea (Meloidae e Rhipiphoridae), sugerindo que esta é uma característica sinapomórfica para Tenebrionoidea. Essas informações reforçam a ideia de que caracteres espermáticos quando adequadamente descritos podem contribuir para resolver dúvidas sobre as relações filogenéticas entre grupos de insetos em diferentes níveis taxonômicos

Em adição, durante a análise morfológica dos espermatozoides de *T. castaneum*, observamos degeneração em massa das células espermáticas em vários cistos testiculares. É interessante observar que, nesses mesmos espécimes que observamos a degeneração, apresentaram as vesículas seminais parasitadas por gregarinas. As quais, a partir de análises moleculares e morfológicas, pertencem ao gênero *Ascogregarina*. Contudo trabalhos futuros deverão ser feitos para analisar se existe uma relação direta entre essas duas observações. Além de buscar entender o ciclo de vida desse parasito e como, de fato, a contaminação ocorre.

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