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Morphology of the male reproductive system and sperm ultrastructure of *Leucoptera coffeella* (Lepidoptera: Lyonetiidae)

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Abstract

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The male reproductive tract of *Leucoptera coffeella* was processed for light and transmission electron microscopy. In the testis, the eupyrene cells are arranged in individual cysts, while the apyrene cysts form aggregates, never observed in other Lepidoptera. Both cysts contain 128 spermatozoa, which differ from the typical pattern. In the seminal vesicle, both types of spermatozoa are dispersed in the lumen, also different from other Lepidoptera. The apyrene spermatozoa are similar to those observed for other Lepidoptera. They present an anterior region covered by a dense cap and the flagellum is composed of a 9 + 9 + 2 axoneme and two mitochondrial derivatives. The eupyrene spermatozoa, however, differ from the typical pattern for Lepidoptera. Their anterior region contains a nucleus, an acrosome and a peculiar arc of eight accessory microtubules connected to the plasma membrane by dense bridges. In the nucleus–flagellum region, the ninth accessory microtubule is assembled between both mitochondrial derivatives, to participate in the axoneme. The flagellum comprises a 9 + 9 + 2 axoneme and two mitochondrial derivatives with paracrystalline cores. External to the plasma membrane and close to the accessory microtubules, there are tufts of an amorphous material, suggesting reduced lacinate appendages, while the reticular ones are absent. The reduction of lacinate appendages and the absence of sperm bundles in the seminal vesicle support the concept that the appendages of other Lepidoptera could be associated with the eupyrene aggregations. The characters 'number of spermatozoa per cyst' and 'absence of bundles' should be considered plesiomorphic, supporting the position of this taxon in the base of the Ditrysia.

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Introduction

The sperm dimorphism that occurs in the order Lepidoptera is one of the most evident, well-studied and complex examples of sperm polymorphism. In this case, the male produces two sperm types, called eupyrene (nucleated) and apyrene (enucleated) spermatozoa (Zylberberg 1969; Phillips 1970; Riemann 1970; Friedländer and Gitay 1972; Lai-Fook 1982; Medeiros 1986, 1997; Corsatto-Alvarenga *et al.* 1987; Jamieson 1987; Kubo-Irie *et al.* 1998; Jamieson *et al.* 1999; França and Báo 2000; Mancini and Dolder 2001, 2003, 2004a; Mancini 2003). The eupyrene spermatozoa possess a nucleus and an acrosome in the anterior region, while the apyrene spermatozoa are devoid of both nuclei and acrosome, and covered only by a dense cap over their anterior region. Both sperm types present a 9 + 9 + 2 axoneme and two mitochondrial derivatives in the tail region. The testicular eupyrene spermatozoa possess, as an exclusive characteristic of this order, two extracellular structures called lacinate and reticular appendages, which extend along the entire sperm length (Phillips 1971; Lai-Fook 1982; Medeiros 1986, 1997; Kubo-Irie *et al.* 1998; França and Báo 2000; Mancini and Dolder 2004a).

In the testis, apyrene and eupyrene spermatozoa are produced in cysts that contain approximately 256 germ cells (Phillips 1970; Jamieson 1987; Jamieson *et al.* 1999). When they leave the testis, both sperm types lose the surrounding cystic cells, and, in the seminal vesicle, the apyrene spermatozoa are found dispersed in the lumen, while the eupyrene ones are organized into bundles (Phillips 1971; Lai-Fook 1982; Medeiros 1986, 1997; Kubo-Irie *et al.* 1998; Mancini and Dolder 2001, 2003).

Both sperm types are transferred to the female (Riemann 1970; Phillips 1971; Riemann and Thorson 1971; Friedländer and Gitay 1972; Lai-Fook 1982; Friedländer *et al.* 2001). Eupyrene spermatozoa fertilize eggs while the apyrene spermatozoa participate in this process indirectly to guarantee successful paternity by diminishing the sperm competition (Silberglied *et al.* 1984; Drummond 1984; Cook and Wedell 1996, 1999; Snook 1997, 1998; Wedell and Cook 1999).

The use of sperm morphology for phylogenetic analyses has proven to be an important tool in entomological systematics (Baccetti 1970; Phillips 1970; Jamieson 1987; Dallai and Afzelius 1993, 1999; Jamieson *et al.* 1999). In Lepidoptera, the sperm ultrastructure is very conserved and there are few publications that contribute to phylogenetic studies (Sonnenschein and Hauser 1990; Hamon and Chauvin 1992; Jamieson *et al.* 1999).

The coffee-leaf-miner butterfly *Leucoptera coffeella* (Guérin-Mèneville, 1842) (Dystrisia suborder, Lyonetiidae family), with the adult measuring 3–4 mm, is considered the principal coffee pest (Parra 1981; Reis and Souza 1984; Souza *et al.* 1998). The larvae penetrate the leaf tissue and eat the cells between the superior and inferior epidermis, leaving tunnels that cause leaf necrosis. Severe attacks destroy the parenchyma, reducing the photosynthetic region, which causes premature leaf fall.

The present research furnished a description of the male reproductive system of *L. coffeella*, a morphological and organizational characterization of the apyrene and eupyrene spermatozoa from the testis and the seminal vesicle. The singular structures of this species have been recognized, contributing to a better understanding of dichotomic spermatozoa in Lepidoptera.

Materials and Methods

Adults of *L. coffeella* were collected in the Federal University of Viçosa (MG, Brazil) and in the 'Alcides Carvalho' Center for Analysis and Technological Research of Coffee at the Agronomical Institute of Campinas (SP, Brazil). The male reproductive system was dissected and processed for light and transmission electron microscopy.

Light microscopy

Male reproductive system morphology. The reproductive systems were fixed in 4% paraformaldehyde in a 0.1 M sodium phosphate buffer, pH 7.2 and were photographed under a stereomicroscope.

Histology. The reproductive systems were fixed in 2.5% glutaraldehyde, 4% paraformaldehyde, in a 0.1 M sodium phosphate buffer for 12 h at 4 °C. After fixation, they were rinsed in buffer, dehydrated in ethanol and embedded in historesin. The material was sectioned (1–3 μm), collected on histological slides, stained with 1% toluidine blue and 0.5% sodium carbonate, and finally photographed under the light microscope (Olympus BX60).

Sperm suspension. For morphometric analysis, drops of sperm suspension, obtained from the seminal vesicle, were spread on histological slides and fixed in 4% paraformaldehyde, in a 0.1 M sodium phosphate buffer at room temperature. The materials were stained with Giemsa for 15 min, rinsed with distilled water and observed with a light microscope (Olympus BX60).

Transmission electron microscopy

Conventional. The reproductive systems were fixed in 2.5% glutaraldehyde, 4% paraformaldehyde, 1.5% sucrose, 5 mM $CaCl_2$ in a 0.1 M sodium phosphate buffer for 12 h at 4 °C. After fixation, they were rinsed in buffer, post-fixed in 1% osmium tetroxide for 3–5 h at 4 °C, dehydrated in acetone and finally embedded in Epoxy resin.

Tannic acid fixation. In some cases, the reproductive tract was fixed in 2.5% glutaraldehyde, 1% tannic acid, 1.5% sucrose and 5 mM $CaCl_2$, buffered with 0.1 M sodium phosphate at pH 7.2, for 3 days at 4 °C. After fixation, they were rinsed in buffer and block-stained in 1% uranyl acetate for 2 h at room temperature (Dallai and Afzelius 1990). They were dehydrated in acetone and embedded in Epoxy resin.

Ultrathin sections were collected on copper grids, stained with uranyl acetate and lead citrate and then photographed in the transmission electron microscope (Zeiss, LEO 906).

Results

Male reproductive system morphology

The male reproductive system of *L. coffeella* (Fig. 1A) comprises a pair of fused testes (Fig. 1B) connected to a pair of thin efferent ducts that are transformed into two seminal vesicles (Fig. 1C). Two long deferent ducts extend from the seminal vesicles and converge, together with the accessory glands, to form a single, thin ejaculatory duct (Fig. 1D). The spermatozoa develop in the testis and are stored in the seminal vesicles until mating occurs.

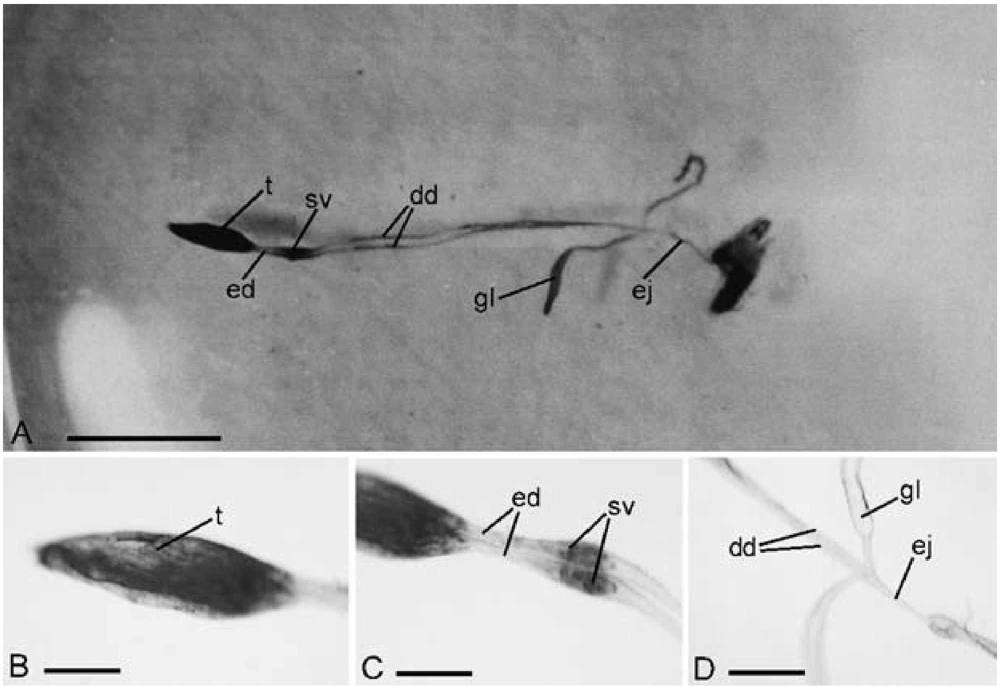


Fig. 1—Male reproductive system of *Leucoptera coffeella*. —**A**–**D**. It is composed of a pair of fused testes (t), a pair of efferent ducts (ed), two seminal vesicles (sv), two deferent ducts (dd), two accessory glands (ag) and a single ejaculatory duct (ej). Scale bars: (A) 200 µm; (B–D) 50 µm.

The testis is made up of a large number of elongated follicles composed of many cysts, that contain either apyrene or eupyrene cells in the same developmental stage (Fig. 2A,B). Spermatogenesis is centripetal; the early cysts (spermatogonia and spermatocytes) are located at the testis periphery, while the more mature cysts (spermatids and spermatozoa) are found in the central testis region (Fig. 2A,B). When leaving the testis, both apyrene and eupyrene spermatozoa lose their cystic organization and are found dispersed in the lumen (Figs 2C and 3E).

The eupyrene cysts occur individually (Fig. 3B), while the apyrene ones form aggregates of cysts with their content isolated by the cystic cells (Fig. 3A). Apyrene (Fig. 3C) and eupyrene (Fig. 3D) cysts contain, approximately 128 germ cells per cyst.

Apyrene spermatozoa

The apyrene spermatozoa are filiform, measure about 1,400 µm in length and are devoid of nucleus and acrosome. They possess only a dense cap over the anterior end that is embedded in the cystic cells (Fig. 4A). Below the dense cap is the axoneme. The flagellum is composed of an axoneme and two mitochondrial derivatives (Fig. 4B). The axoneme presents the 9 + 9 + 2 microtubular pattern, with nine accessory microtubules, nine peripheral microtubule doublets and two central microtubules. The lumens of the A-microtubule of the doublets and one of the central pair are electron dense. The mitochondrial derivatives have similar size and shape in cross-section. They are located parallel to the axoneme reaching the posterior flagellar region and are devoid of a paracrystalline core. In the extratesticular regions, these spermatozoa acquire an extracellular coat along the entire sperm length (Fig. 3E).

Eupyrene spermatozoa

The eupyrene spermatozoa are filiform, measuring about 2,500 µm. Differing from the apyrene ones, the eupyrene spermatozoa comprise a nucleus, an acrosome and accessory microtubules in the anterior region (Fig. 5A–C). The flagellar region, as in the apyrene spermatozoa, is composed of an axoneme and two mitochondrial derivatives (Fig. 5F).

The nucleus is elongated, with compact, homogeneous chromatin, and extends until the beginning of the flagellum (Fig. 5A–D). For most of the nucleus' length it is almost completely surrounded by the acrosome (Fig. 5A–C), and at its base, it is located between the mitochondrial derivatives (Fig. 5D).

The acrosome extends bilaterally to the nucleus and contains two large portions that surround the nucleus (Fig. 5A–C). Beyond these structures, this anterior region is composed of an arc of eight accessory microtubules individually connected to the membrane by dense bridges (Fig. 5A–C). On the tip, the next to last microtubules at each extremity of this arched row have longer dense bridges, making them stand

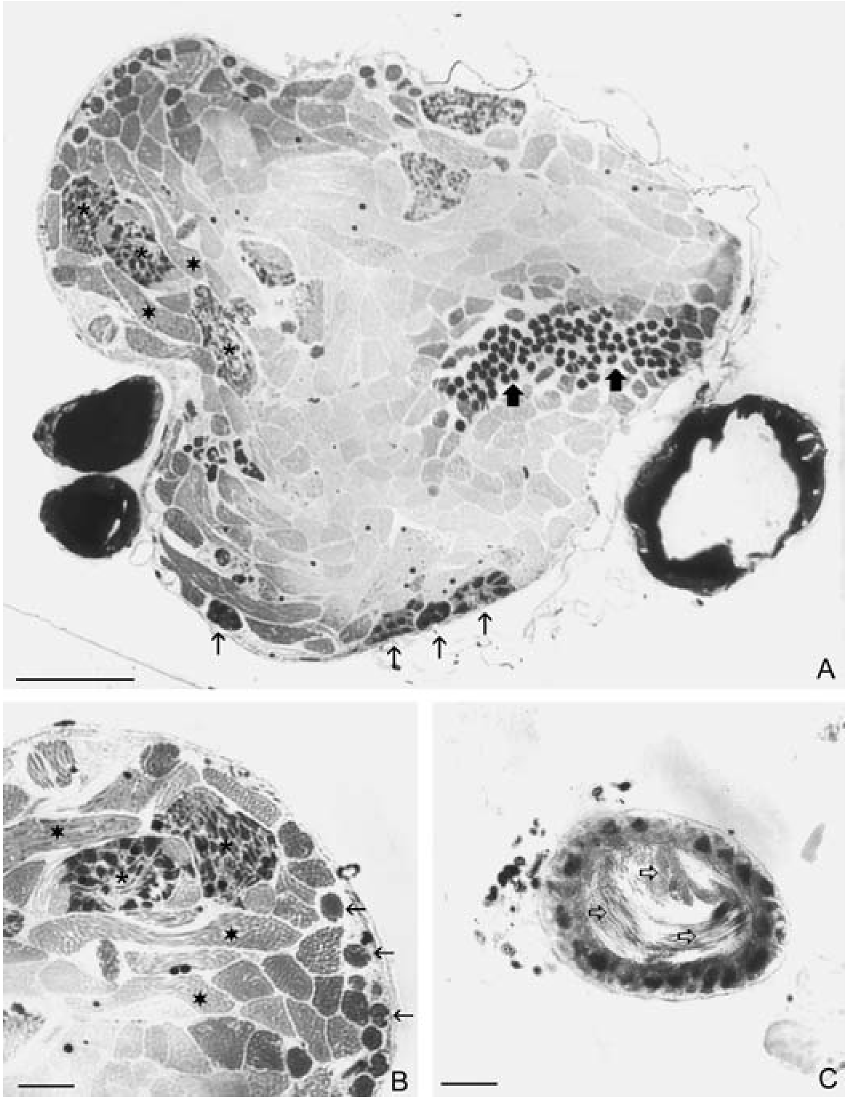


Fig. 2—Light microscopy. —**A**, **B**. Testis stained with toluidine blue. Spermatogonia (small arrows), spermatocyte cysts (asterisks), spermatid cysts (stars) and spermatozoon cysts (large arrows). —**C**. Seminal vesicle stained with toluidine blue. Notice the spermatozoa (arrows) dispersed in the lumen. Scale bars: (A) 220 µm; (B) 60 µm and (C) 75 µm.

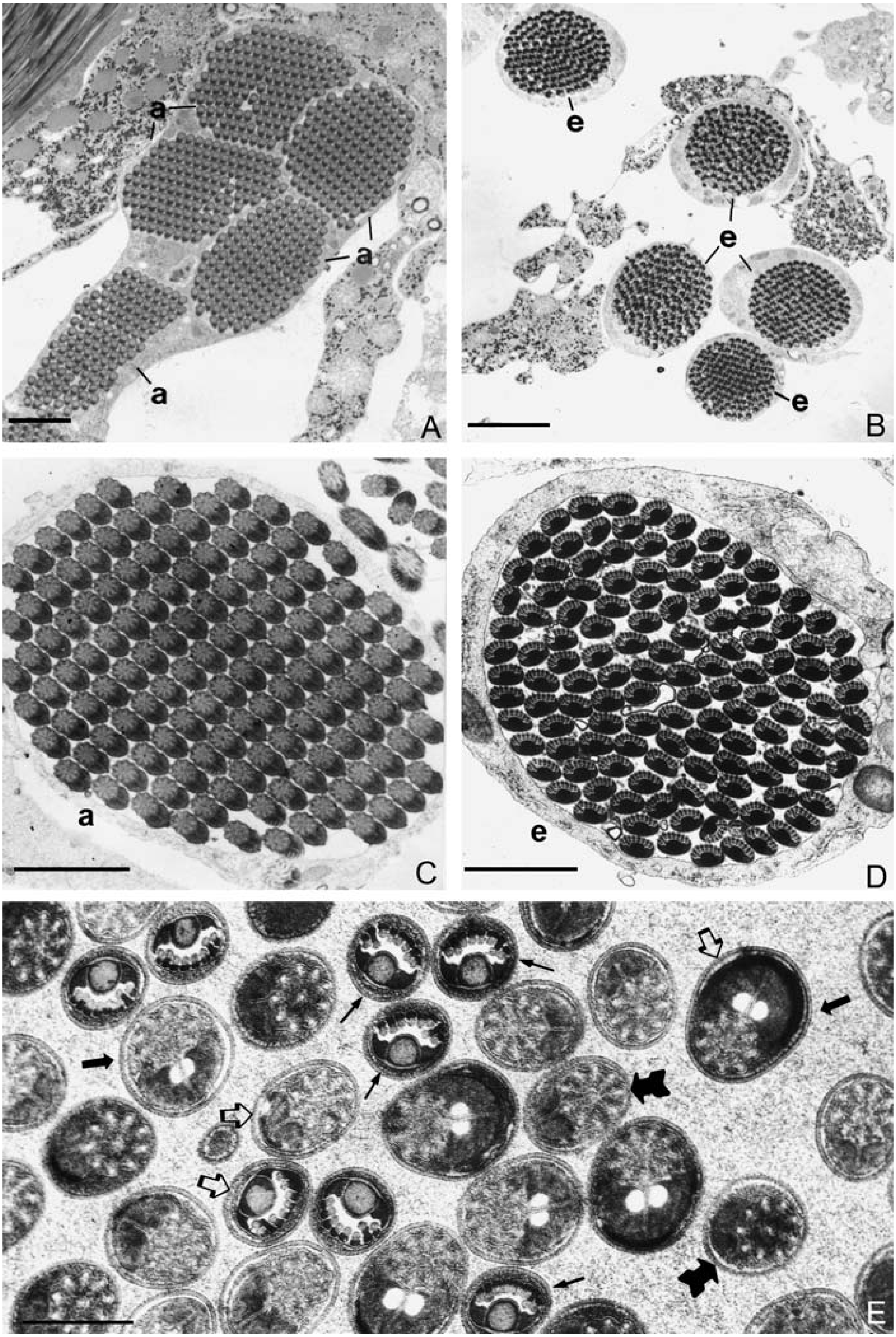
out at a different level in the arc (Fig. 5A). Each accessory microtubule is formed by 16 protofilaments. There are tufts of amorphous materials attached externally to the plasma membrane next to each dense bridge (Fig. 5A–C).

A central amorphous dense mass, absent in the tip (Fig. 5A), appears in the lower head portion, and is gradually compacted, separating the nucleus and acrosome from the more accentuated microtubule arc (Fig. 5B–D).

In the upper centriolar region, the two mitochondrial derivatives appear next to the nuclear base (Fig. 5D) and at this level, a ninth accessory microtubule can be found between the two mitochondrial derivatives, parallel to the arc of eight accessory microtubules that accompany the nucleus. The centriole is composed of nine peripheral microtubule doublets devoid of dynein arms and these are associated with the pre-existing accessory microtubules and the central amorphous mass to form a dense hollow cylinder (Fig. 5E).

In the flagellar region, the axoneme has a 9 + 9 + 2 arrangement, originating from the accessory microtubules that begin in the head region. This arrangement is made up of nine external accessory microtubules, nine peripheral doublets and two central single microtubules (Fig. 5F). The lumens of the doublet microtubules and one of the central pair are electron dense (Fig. 5F).

Fig. 3—Spermatozoa arrangement. —**A**. Aggregated apyrene cysts (a) in the testis. —**B**. Isolated eupyrene cysts (e) in the testis. —**C**. Apyrene cyst (a) with 128 spermatozoa. —**D**. Eupyrene cyst (e) with 128 spermatozoa. —**E**. Apyrene and eupyrene spermatozoa dispersed in the lumen of the seminal vesicle. Eupyrene anterior region (thin arrows), eupyrene flagellar region (medium arrows), apyrene flagellar region (large arrows) and extracellular coat (open arrows) on both sperm types. Scale bars: (A) 3.3 µm; (B) 5.2 µm; (C,D) 1 µm; (E) 0.4 µm.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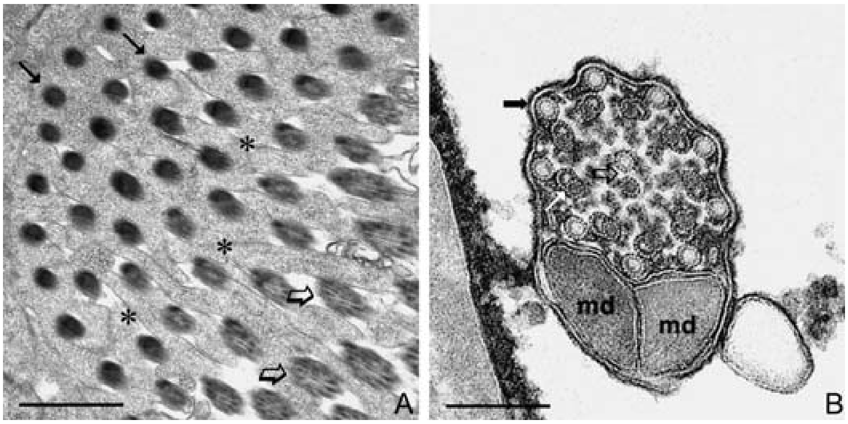

Fig. 4—Intratesticular apyrene spermatozoa. —**A**. Tangential section of the anterior end. Dense cap (small arrows), beginning of the axoneme (large arrows) and cystic cells (asterisk). —**B**. Cross-section of the flagellar region composed of an axoneme and two mitochondrial derivatives (md). The axoneme consists of two central microtubules (large arrow), nine peripheral doublets of microtubules (white arrow) and nine accessory microtubules (black arrow). (B) Tannic acid method. Scale bars (A) 0.8 µm; (B) 0.1 µm

Mitochondrial derivatives are located parallel to the axoneme up to its terminal portion. They present the same shape and area, in cross-section, and paracrystalline cores (Fig. 5F). The eupyrene spermatozoa, as well as apyrene ones, present an extracelullar coat along the entire sperm length in extra-testicular portions of the reproductive tract (Fig. 3E).

Discussion

The male reproductive system and spermatozoon morphology, as well as their organization in the testis and seminal vesicle of *L. coffeella*, differ very much from those observed in most of the known butterflies and moths.

The reproductive system of *L. coffeella* presents the same elements as have been described for Lepidoptera, with a fused testis, two efferent ducts, two deferent ducts, two seminal vesicles, one ejaculatory duct and two accessory glands (Ehrlich 1961). Some Lepidoptera present the efferent ducts converging with the accessory glands to the seminal vesicles, which join in a single ejaculatory duct (Ehrlich 1961; Kubo-Irie *et al.* 1998; Justus and Mitchell 1999; Mancini and Dolder 2003).

In *L. coffeella*, as well as for other Lepidoptera (Riemann and Thorson 1976; Lai-Fook 1982; Medeiros 1986; Giebultowicz *et al.* 1988, 1996, 1997; Riemann and Giebultowicz 1991; Giebultowicz and Brooks 1998), the seminal vesicles are followed by the deferent ducts and converge together with the accessory glands to form a single ejaculatory duct. The testicular organization of *L. coffeella*, including follicles composed of cysts and a centripetal spermatogenesis, is similar to that observed in insects and for the majority of the butterflies and moths (Medeiros 1986; Corsatto-Alvarenga *et al.* 1987; Mancini and Dolder 2004a).

As described for the majority of Lepidoptera species (Phillips 1971; Jamieson 1987; Jamieson *et al.* 1999; Swallow and Wilkinson 2002), *L. coffeella* presents sperm polymorphism, with the production of apyrene and eupyrene spermatozoa. The exception for this dimorphism was found in the basal genus *Micropterix* (Micropterigidae) (Sonnenschein and Häuser 1990; Hamon and Chauvin 1992), in which only the eupyrene spermatozoa were observed. The number of germ cells per cyst in Lepidoptera for both sperm types is, in general, about 256 (2^8) (Phillips 1971; Lai-Fook 1982; Medeiros 1986, 1997; Kubo-Irie *et al.* 1998; Jamieson *et al.* 1999; Mancini and Dolder 2003, 2004a). However, in *L. coffeella* it is about 128 (2^7) germ cells per cyst. This number was registered only in the leaf miner butterfly *Tischeria angusticolella* (Tischeriidae) (Virkki 1969) and in the basal genus *Micropterix* (Sonnenschein and Häuser 1990). Even with the few data obtained for basal groups of Lepidoptera, the numbers contradict the general hypothesis that derived insects tend to have fewer spermatozoa per cyst than the basal species (resulting in reduction of sperm production and limited genetic variability, that aid populations to adapt to specific niches) (Virkki 1969).

Aggregated apyrene cysts have not yet been described for other Lepidoptera species. In the Noctuidae *Alabama argillacea* (cotton pest) (Medeiros 1986) and in the Nymphalidae *Euptoieta hegesia* (Mancini and Dolder 2003), voluminous apyrene cysts were described; however, they were formed by the fusion of two or three cysts. In *L. coffeella*, cystic cells continue to limit each cyst in the aggregate.

In *L. coffeella*, apyrene and eupyrene spermatozoa are found dispersed in the lumen of the seminal vesicle. This organization was noticed only in *Korscheltellus lupulinus* (Hepialidae) (Hamon and Chauvin 1992). In all the species studied, the eupyrene spermatozoa are organized in bundles maintained by a matrix, while the apyrene ones are dispersed in the lumen, between the eupyrene bundles (Phillips 1971; Lai-Fook 1982; Medeiros 1986, 1997; Kubo-Irie *et al.* 1998; Mancini and Dolder 2001, 2003).

The Lepidoptera sperm morphology is evolutionarily highly conserved. For all species already studied, only *L. coffeella* (Lyonetiidae), *Korscheltellus lupulius* (Hepialidae) (Hamon and Chauvin 1992), *Micropterix calthella* and *Micropterix aruncella* (Micropterigidae) (Sonnenschein and Häuser 1990; Hamon and Chauvin 1992) presented a sperm morphology different from that considered the typical

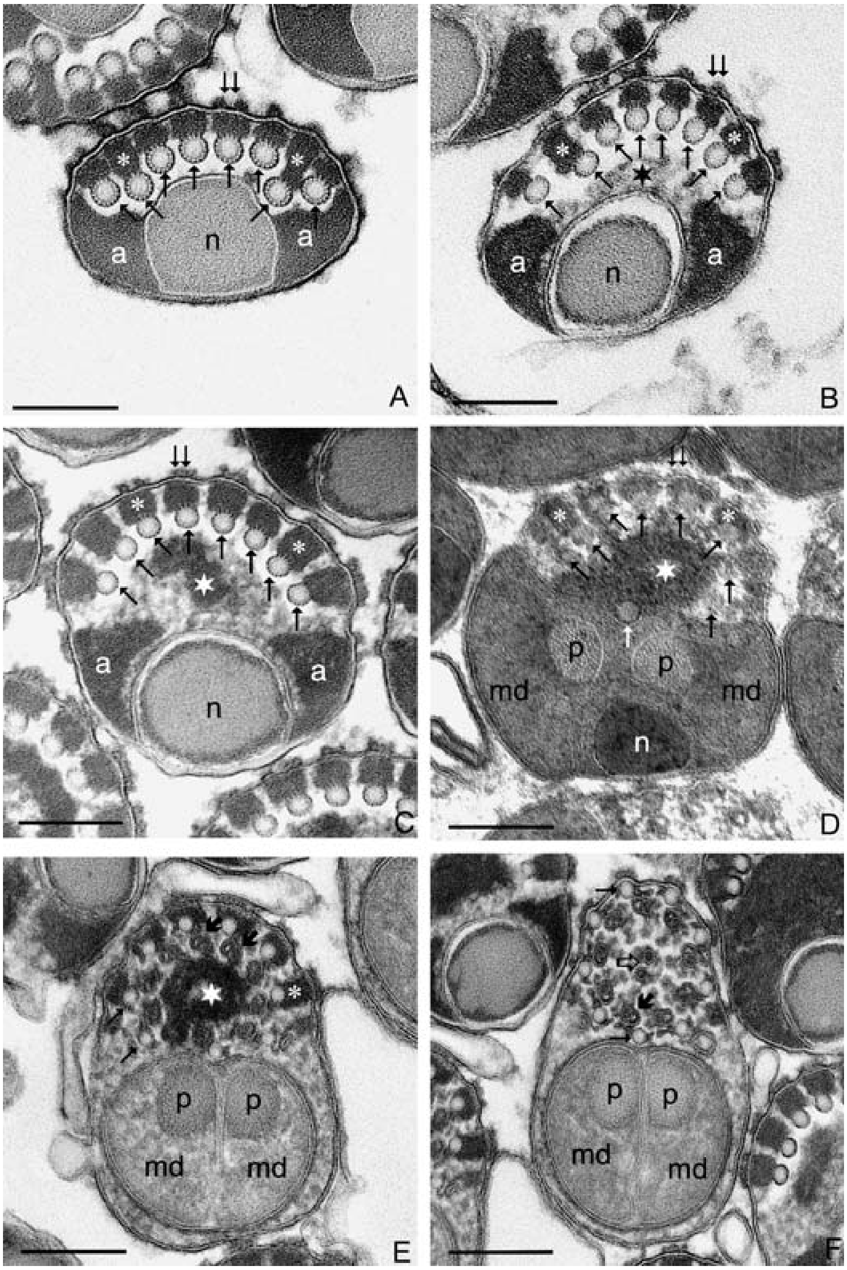

Fig. 5—Intratesticular eupyrene spermatozoa. —**A**. Cross-section of the anterior tip with acrosome (a), nucleus (n), arc of eight accessory microtubules (arrows), extracellular amorphous material (double arrows) and dense bridges (asterisk). —**B**, **C**. Cross-section of the medium anterior region with acrosome (a), nucleus (n), arc of eight accessory microtubules (arrows), extracellular amorphous material (double arrows), dense bridges (asterisk) and a central amorphous mass (star). —**D**. Cross-section of the nucleus–flagellum transition region with nucleus (n), arc of eight accessory microtubules (arrows), dense bridges (asterisk) and central amorphous mass (star). Notice the ninth accessory microtubule (white arrow) and two mitochondrial derivatives (md) with paracrystalline cores (p). —**E**. Cross-section of the centriolar region with nine accessory microtubules (small arrows), nine peripheral microtubules (large arrows), a central amorphous mass (star), dense bridges (asterisk) and mitochondrial derivatives (md) with paracristalline cores (p). —**F**. Cross-section of the flagellar region with complete axoneme, that is nine accessory microtubules (large black arrow), nine peripheral microtubules (small arrow) and two central microtubules (large arrow), and mitochondrial derivatives (m) with paracrystalline cores (p). (A) to (C) and (E) and (F): tannic acid method. Scale bars: (A) 0.09 μm; (B) 0.1 μm; (C) 0.09 μm; (D–F) 0.1 μm.

pattern for the order. In *M. calthella* and *M. aruncella*, basal Lepidoptera species, only eupyrene spermatozoa were found, with a 9 + 2 axoneme type, two mitochondrial derivatives without paracrystalline cores and a nucleus that extends parallel to the axoneme. In *K. lupulius* (Hepialidae), both apyrene and eupyrene sperm types were found; the eupyrene type had a 9 + 9 + 2 axoneme, two mitochondrial derivatives with paracrystalline cores and a nucleus that also extended parallel to the axoneme. No extracellular appendages were found in these two genera.

Leucoptera coffeella belongs to the Dystrisia suborder, which is considered a very derived group in the Lepidoptera order (http://tolweb.org/tree/phylogeny.html), and therefore an unusual structure was unexpected.

The apyrene spermatozoa from testis and seminal vesicle of *L. coffeella* exhibit similar morphology to that found in the majority of the Lepidoptera species. However, the mitochondrial derivatives of *L. coffeella* do not possess paracrystalline cores, as occur in all apyrene sperm (Phillips 1971; Jamieson *et al.* 1999; Mancini and Dolder 2004a).

Testicular eupyrene spermatozoa, in contrast, present various differences in morphology from that described for most Lepidoptera (Phillips 1971; Medeiros 1986; Kubo-Irie *et al.* 1998; Jamieson *et al.* 1999; Mancini and Dolder 2003, 2004a). For the majority of the Lepidoptera species, the anterior region is composed of a nucleus and a discrete tubular acrosome, parallel to the nucleus, and the flagellar region comprises an axoneme and two mitochondrial derivatives, without paracrystalline cores. In *L. coffeella*, the acrosome is a large structure that involves the nucleus bilaterally. In this species, the significant difference observed in the eupyrene spermatozoa is the arc of eight microtubules in the anterior end that originate the axoneme. This arc has never been described in Lepidoptera, or other insects. Another peculiarity is that, in the nucleus–flagellum contact region, the nucleus extends to the initial portions of the mitochondrial derivatives. In the majority of the Lepidoptera species, the nucleus extends only until the centriolar region and terminates before both mitochondrial derivatives are assembled. This species also presents a paracrystalline core in the eupyrene mitochondrial derivatives, which differs from the other known species, which have a paracrystalline structure only in the apyrene spermatozoa.

Lepidopteran spermatozoa are characterized by exclusive extracellular appendages, called lacinate and reticular, that are present in the intratesticular eupyrene spermatozoa (Phillips 1971; Jamieson *et al.* 1999; Mancini and Dolder 2003, 2004a). Some research has been carried out on the chemical composition of these appendages (França and Báo 2000; Mancini 2003; Mancini and Dolder 2004b; Mancini *et al.* 2005), where protein residues (principally basic proteins) and carbohydrates have been found. Some authors believe that the lacinate appendages, formed in the testis, and lost in the extratesticular portions, reorganize to form the matrix bundles in the seminal vesicle, as well as the eupyrene coat found in the seminal vesicle (Phillips 1971; Riemann and Thorson 1971; Lai-Fook 1982; Riemann and Giebultowicz 1992; Kubo-Irie *et al.* 1998). In *L. coffeella*, the reticular appendage is absent while the tufts of amorphous material, similar to a glycocalix, could represent primitive lacinate appendages. We believe that these reduced testicular appendages are related to the absence of post-testicular eupyrene bundles in *L. coffeella*.

The order Lepidoptera has usually been divided into Monotrysia (a paraphyletic group including many basal families) and Ditrysia (a monophyletic well-supported clade). In this division, *L. coffeella* belongs to a basal group of Ditrysia. Considering *Micropterix* and *Korscheltellus* as potential outgroups of Ditrysia, the states found in *L. coffeella* for the characters 'number of spermatozoa per cyst' and 'absence of eupyrene bundles' should be considered plesiomorphic, supporting the position of this taxon at the base of the Ditrysia clade.

However, the sperm morphology of most Lepidoptera is still not known, and additional speculation cannot be made at this stage of knowledge. Future work in this field should focus on many genera of Monotrysia, and especially in species belonging to the basal Ditrysia. Even if in the literature the sperm morphology of Lepidoptera is considered homogeneous and conserved, the present paper shows that some characters could be very important to the understanding of the early evolution of this group.

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