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An ultrastructural study of spermiogenesis in two species of *Sitophilus* (Coleoptera: Curculionidae)

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ABSTRACT: The spermiogenesis of *Sitophilus zeamais* and *Sitophilus oryzae*, the maize and the rice weevil, respectively, was studied by light microscopy and scanning and transmission electron microscopy. *Sitophilus* spp. is the most widespread and destructive primary pest of stored cereals in the world. The spermiogenesis occurs within cysts. There are approximately 256 germ line cells per cyst. Inside each cysts, all the spermatids are in the same stage of maturation. The ultrastructure of the spermatozoa of *S. zeamais* and *S. oryzae* is similar to that described for other beetles. The head is formed by a three-layered acrosome with the perforatorium, the acrosomal vesicle, the extra-acrosomal layer and the nucleus. The flagellum has the typical axoneme formed by a 9+9+2 microtubules arrangement, two mitochondrial derivatives and two accessory bodies. The typical pattern for Curculionidae spermatozoa described here may provide useful information for future phylogenetic analysis of the superfamily Curculionoidea.

Introduction

The order Coleoptera (beetles) comprises 250,000 known species, many of which are able to exploit human-made and human-modified habitats, and, therefore, these beetles are important pests. Members of some 40 families of beetles have been recorded in stores worldwide (Halstead, 1986). However, almost all of the species of major importance as storage pests belong to one of seven families: Bostrichidae, Bruchidae, Cucujidae, Curculionidae, Dermestidae, Silvanidae, and Tenebrionidae (Rees, 1996).

According to Anderson (1993, 1995), weevils (Coleoptera: Curculionidae) are the largest family of known organisms, with about 48,000 valid species. High-level classification of weevils is difficult and a subject of debate and disagreement among specialists (Morimoto, 1962; Crowson, 1967; Thompson, 1992; Kuschel, 1995). Most of these studies are focused on adults, therefore, the knowledge about immature stages is relatively poor (Marvaldi, 1997).

The rice weevil, commonly referred to as *Sitophilus oryzae*, is divided into two distinct species: *S. zeamais* (Motschulky) and *S. oryzae (Linnaeus)* (Halstead, 1963). These two species are indistinguishable by external characteristics; to differentiate between them, the dissection and examination of genitalia are required (Rees, 1996).

According to Rees (1996), *Sitophilus* spp. are among the most widespread and destructive primary pests of stored cereals in the world. *S. zeamais* and *S. oryzae* are cosmopolitan, but are especially abundant as pests in warm temperate to tropical regions. *S.*

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zeamais is a common pest of stored maize but is frequently found in other cereals including milled and paddy rice; *S. oryzae* is most often found attacking small-grained cereals (rice, wheat, and sorgum) (Haines, 1981).

The development of germinative cells in Coleoptera, as in the majority of insects, takes place within cysts, contained in the testicles (Phillips, 1970).

The classic spermatozoa of Coleoptera are characterized by enormous accessory bodies and large and almost fully crystallized mitochondrial derivatives in the tail region. The head region is formed by an acrosome (three-layered) and nucleus (Baccetti *et al*., 1973; Burrini *et al*., 1988; Baccetti and Daccordi, 1988; Báo, 1996).

According to Burrini *et al.* (1988), the great number of representatives in the superfamily Curculionoidea make it difficult to provide a systematic arrangement based on phylogenetic considerations. In this case, comparative spermatology research can help resolve taxonomic and philogenetic problems.

This study aimed to provide information about the process of spermiogenesis and additional data about the mature spermatozoon in two species of the genera *Sitophilus* (*S. zeamais* and *S. oryzae*).

Material and Methods

The insects utilized were male adults of *S. zeamais* and *S. oryzae* (Coleoptera: Curculionidae) obtained from the colonies maintained at UFV (Universidade Federal de Viçosa – Minas Gerais /Brasil) and CONAB (Companhia Nacional de Abastecimento – Brasília-DF/ Brasil), respectively.

Light microscopy

Testes used in transmission electron microscopy were sectioned (4 μm), stained with 0.25% toluidine blue, pH 11, observed, and photographed with a Zeiss® Axiophot light microscope.

Scanning electron microscopy

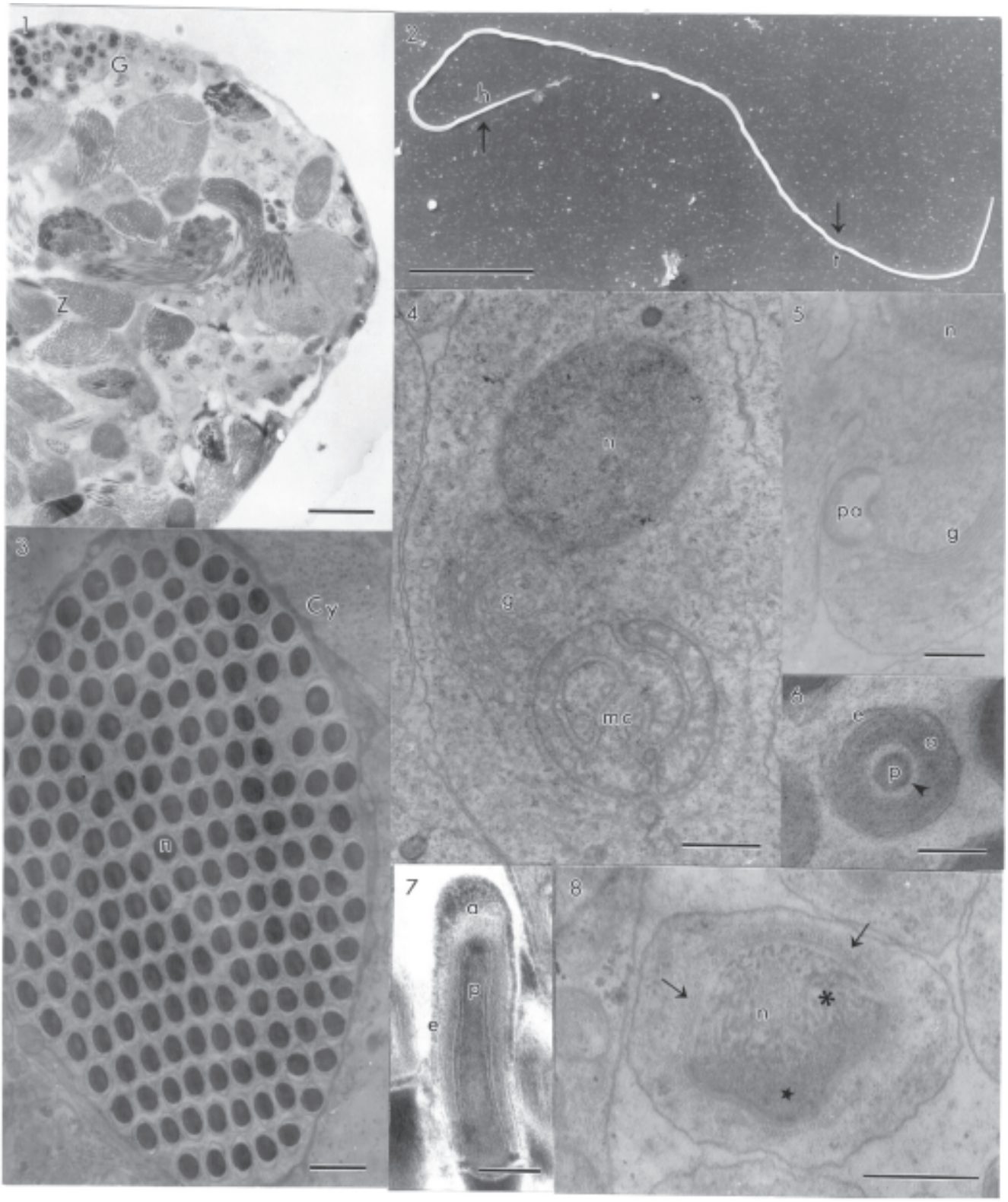
For observation with the scanning electron microscope, *S. orizae* spermatozoa were isolated from the seminal vesicle with phosphate-buffer saline (PBS), pH 7.0. Spermatozoa were attached to poly-L-lysine-coated glass chips, and the chips were immersed in fixative solution. Fixation was carried out at room temperature for 3 h in a solution containing 3% glutaraldehyde, 3% paraformaldehyde, and 0.1% picric acid buffered in 0.1 M sodium cacodylate, pH 7.4 (Erlandsen *et al*., 1989). Subsequently, the samples were risend in the same buffer and post fixed for 30 min in osmium-potassium ferricyanide solution. After fixation, the glass chips with adherent spermatozoa were processed through an ascending ethanol series, dried to a critical point in CO_2, covered with a layer of gold, and observed in a JEOL JSM 840 scanning electron microscope at an accelerating voltage of 5.0 kV.

Transmission electron microscopy

The testes and seminal vesicles were dissected from adult males and fixed for 4 h in 2.5% glutaraldehyde, 4% paraformaldehyde, 5 mM $CaCl_2$, and 3% sucrose buffered in 0.1 M sodium cacodylate at pH 7.2. After fixation, the specimens were rinsed in the same buffer, and postfixed in 1% osmium tetroxide, 0.8% potassium ferricyanide, and 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer. In some cases, the specimens were fixed in a mixture of 2.5% glutaraldehyde, 1% tannic acid in 0.1 M phosphate buffer, at pH 7.2, followed by block-staining in 1% uranyl acetate in distilled water (Afzelius and Dallai, 1988). The material was dehydrated in an ascending acetone series (30%-100%) and embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a Jeol® 100 transmission electron microscope at 80 kV.

FIGURES 1-8.

Fig. 1. Light micrograph of follicles with several cysts in different developmental stages. **Scale Bar:** 20μm. **Fig 2.** Scanning electron micrograph of *S. oryzae* spermatozoon. **Scale Bar:** 10μm. **Figs. 3-8**. Transmission electron micrographs of *Sitophilus* spermatozoon. **(3)** Cyst showing cross-section of spermatozoa head region. **(4)** Spermatid at initial stage of differentiation. **(5)** Early spermatid showing a proacrosomal vesicle (pa). **(6)** Transverse section of the acrosome complex. The arrowhead showed an electron transparent layer between the acrosome and perforatorium. **(7)** Longitudinal section of the sperm head region. **(8)** Transverse section shows the immature nucleus with peripheral compacted chromatin (star), and fibrillar chromatin in the central region (asterisk). Microtubules (arrows) are observed surrounding the nucleus. **Abbreviations:** (a) acrosome; (e) extra acrosomal layer; (g) Golgi complex; (h) head; (G) spermatogonia; (Z) spermatozoon; (t) tail; (n) nucleus; (cy) cystic cells; (mc) mitochondrial complex; (p) perforatorium. **Scale Bars: 3, 4:** 1μm; **5, 8:** 0.5μm; **7:** 0.2μm; **6:** 0.1μm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Results

The spermiogenesis in *S. zeamais* and *S. oryzae* are very similar. The males of each of these species have two testes, and these testes are subdivided into follicles containing many cysts in different developmental stages (Fig. 1). The differentiation of the spermatids of *Sitophilus* occurs within the cysts. Inside each cysts, all the spermatids are in the same stage of maturation. There are approximately 256 germ line cells per cyst (Fig. 3).

Spermiogenesis in insects involves processes such as nuclear elongation, chromatin condensation, acrosomal formation, and flagelar development, along with the formation of the axoneme as well as mitochondrial derivatives.

When examined by scanning electron microscopy, the spermatozoon showed a threadlike appearance, were approximately 78.13 μm long, and lacked distinct head and tail regions (Fig. 2). The sperm structure consists of the head, which is formed by an acrosome and nucleus, and the flagellum, which is formed by two mitochondrial derivatives, an axoneme, and two accessory bodies.

During the early spermatid phase, the nucleus resembles that of a somatic cell, with electrondense chromatin in the peripheral region and diffuse chromatin in the central region (Fig. 4). The structural reorganization undergone by the chromatin during nuclear condensation does not follow the same course in all insect species. In *S. zeamais* and *S. oryzae*, chromatin condensation is not uniform. During some stages, chromatin is more condensed around the periphery of the nucleus than in the center. Two regions could be distinguished; one showing the chromatin more homogeneously condensed and another showing the chromatin with a fibrillar aspect (Figs. 8 and 9). Thus, nuclear chromatin gradual condensed and its electron density increased, and the resulting pattern resembles a honeycomb (Fig. 9). After complete chromatin coalescence, we could observe a very compact nucleus (Fig. 10) surrounded by a layer of microtubules (Figs. 8-10) in the mature spermatid.

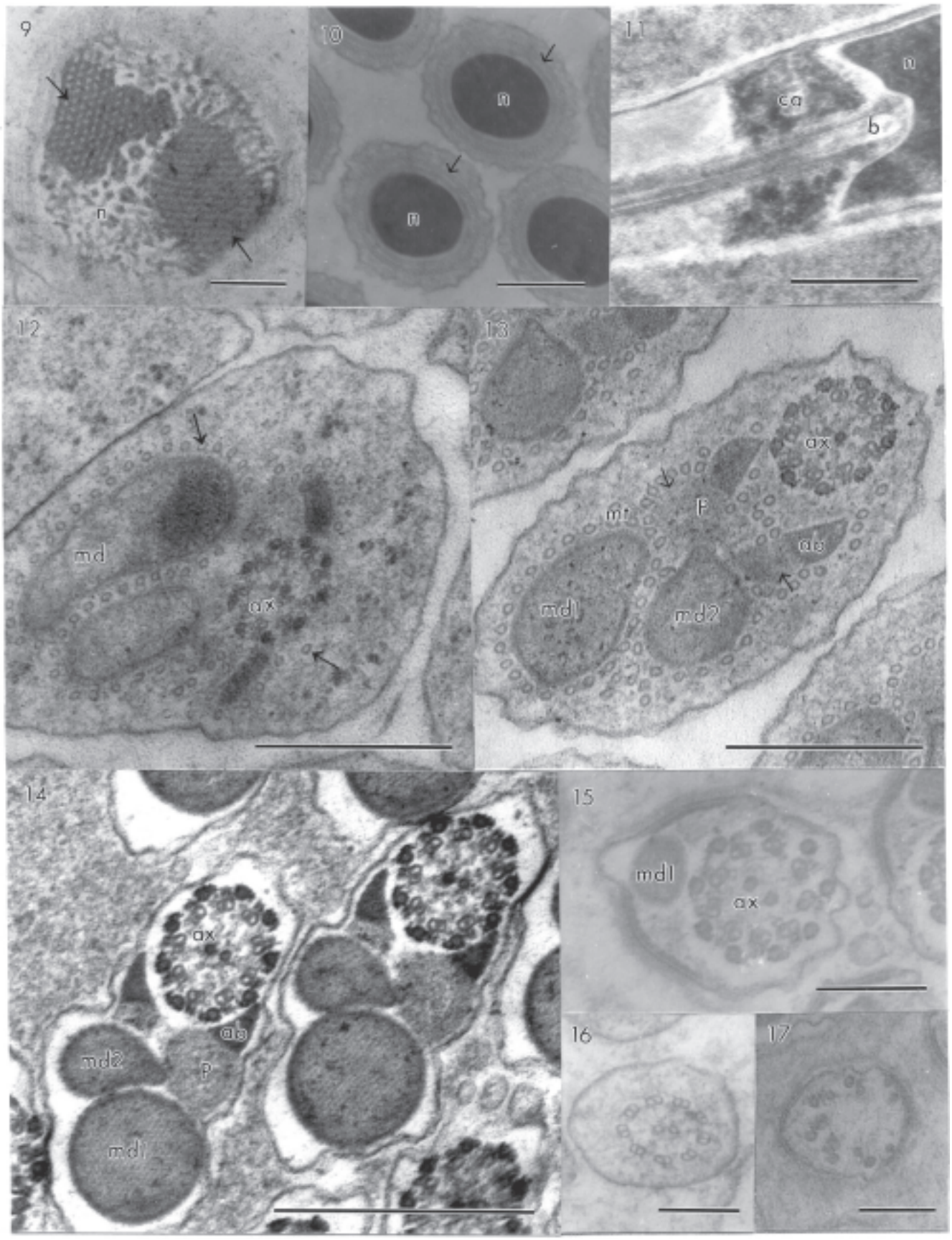
The acrosome formation actively involves the Golgi complex. We observed numerous vesicles of this structure during the first stages of spermiogenesis. We also observed, during the first stage, the formation of a large vesicle called the proacrosomal granule, which is derived from vesicles of the Golgi complex (Figs. 4 and 5). The acrosome is located in an anterior portion of the nucleus and consists of a prominent three-layered acrosome: the perforatorium-like inner cone, which is an electrondense and filamentous structure; the electrondense acrosomal content; and the outermost extra-acrosomal layer. The acrosomal vesicle is cone shaped with a rounded tip and covers the perforatorium up to the beginning of the nucleus (Fig. 7). In transverse sections, the acrosomal vesicle and the perforatorium have profiles that vary from circular to oval. Along the acrosome, an electron transparent layer covers the perforatorium and separates it completely from the acrosomal vesicle (Fig. 6).

In early spermatids, the centriolar adjunct is located posterior to the nucleus, in the region of flagellum implantation. This structure appears to be electrondense with some electronlucid areas (Fig. 11).

The mitochondrial complex is formed by the process of fusion and aggregation of a large number of mitochondria and this process occurs in the early spermatid stage (Fig. 4). This structure turns into two mitochondrial derivatives during the course of its differentiation that is concurrent with axoneme outgrowth. In the early spermatid, these two structures are separated from each other and there are microtubules in the surrounding cytoplasm (Fig. 12). These two mitochondrial derivatives are different sizes, and only the large derivative has a dense crystalloid in the mitochondrial matrix (Figs. 12 and 14).

The flagellum of *S. zeamais* and *S. oryzae* spermatozoa are similar and consist of two mitochondrial derivatives, an axoneme, and two accessory bodies (both

FIGURES 9-17. Transmission electron micrographs of *Sitophilus* spermatozoon. **(9)** Transverse section showing an evident honey-comb structure in the chromatin texture of the nucleus (arrows). **(10)** Compact nucleus in mature spermatids. Microtubules (arrows) are observed surrounding the nucleus. **(11)** Longitudinal section of posterior region of the nucleus **(12-17)** Transverse sections of the sperm tail. **(12)** Microtubules (arrows) surrounding the axoneme and mitochondrial derivatives in formation. **(13)** Flagellum in development. The accessory body and the "puff"-like corpuscle with electrondense areas (arrows), are observed parallel to the axoneme. **(14)** The flagellum shows axoneme with a 9+9+2 microtubule pattern. A paracrystaline organization is observed in the large mitochondrial derivative. **(15-17)** Terminal region of the flagellum showing the disappearance of some structures and disorganization of the axonemal subunits. **Abbreviations:** (n) nucleus**;** (ca) centriolar adjunct; (b) basal body; (md) mitochondrial derivatives; (ax) axoneme; (ab) accessory body; (P) "puff"-like corpuscle; (md1) large mitochondrial derivative; (md2) minor mitochondrial derivative; (mt) microtubules. **Scale Bars**: **11-14:** 0.5μm; **9, 10, 15-17:** 0.2 μm.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expanded in a "puff"-like portion). The two mitochondrial derivatives are different sizes and are laterally located in relation to the nucleus. In the axoneme, we observed a 9 (outer singlets) + 9 (intermediate doublets) + 2 (central singlets) pattern of microtubules (Fig. 13). The axoneme originates from a basal body of a differentiated centriole (Fig. 11). We observed, in cross section, two accessory bodies (of unequal sizes) between the axoneme and the mitochondrial derivatives. The accessory bodies appear to be electron dense in their "puff"-like portion (Figs. 13 and 14).

At the end of the tail, the flagellar components terminate in, first, the accessory bodies, then, the minor mitochondrial derivative, and last, the major mitochondrial derivative (Fig. 15). The several microtubules of the axonemal complex are the last to disappear at different levels (Figs. 16 and 17).

Discussion

Sitophilus oryzae (Linnaeus) and *S. zeamais* (Motschulky), belong to the family Curculionidae, subfamily Rhyncophorinae. The spermiogenesis in these two species of beetles is characterized by specific morphofunctional modifications. The formation of acrosome and of flagellum are followed by nuclear transformations, with changes in shape and condensation of chromatin. These events follow the general pattern described for other Coleoptera (Shay *et al*., 1969; Gassner *et al*., 1975; Hodges, 1982; Báo, 1996).

In *S. zeamais* and *S. oryzae*, as in the majority of insects, the development of germinative cells takes place within cysts (Phillips, 1974). According to Virkki (1969), archaic orders of insects have more sperm per bundle than modern orders. The most modern, or specialized groups, tend to have the least number of sperm per bundle. Examples of this trend are found among the alticid beetles (16 - 256 sperm/bundle) and scarabaeid (128 - 512 sperm/bundle). Less sperm per bundle may indicate reduced sperm production and may have adaptative value in limiting genetic variability. In the Curculionidae family, some variations occur. The species of bool weevil, *Anthonomus grandis*, have a large number of sperm per bundle (512 – 797), which is considered a new record for holometabolous insects (Gassner *et al*., 1975). In contrast, the species used in this study, *S. zeamais* and *S. oryzae*, have a low number of sperm per bundle (approximately 256 sperm/bundle).

The gradual condensation of the nuclear material is accompanied by a change in the shape of nucleus from spherical to elongate. During elongation of the nucleus, the chromatin is reorganized, resulting in a highly compact, long and thin nucleus. In some instances, the nucleus has a honey-comb, instead of homogeneous, texture (Yasuzumi & Ishida, 1957). The nuclear material does not undergo uniform morphological changes during spermiogenesis. Sometimes the central zone, and other times the periphery, is the first to condense (Chevailler, 1970).

Our observations showed a peculiar organization of the nuclear material during the differentiation of spermatozoa in *S. zeamais* and *S. oryzae*. First, we could distinguish two regions of chromatin one was more homogeneously condensed and other had a fibrillar aspect. Then, we observed a gradual condensation of the nuclear material with an increase of electron density. The resulting nucleus had a honey-comb texture. After complete chromatin coalescence, we observed a very compact nucleus in the mature spermatid. These structural changes of nuclear development have been described for other beetles (Báo and Hamú, 1993; Báo, 1996).

During the beetles' nuclear transformations, structures such as microtubules and cytoplasmic membranes were found surrounding the nucleus. These structures have also been reported for several other insects (Kessel, 1966; Tokuyasu, 1974). The presence of these structures in spermatids with this precise orientation with respect to the nucleus suggests that they may be involved in nuclear elongation, given that these structures disappear after this event is completed.

The Golgi complex is important in the acrosome formation in *S. zeamais* and *S. oryzae*. Initially, small vesicles, which originate from the Golgi, fuse to form the proacrosomal vesicle, which is gradually modified during the later stages of the spermiogenesis to take on its characteristic shape. This process appears to be typical for insects (Phillips, 1970; Baccetti, 1972; Báo *et al*., 1989; Báo, 1996) and other animal species (Yasuzumi, 1974).

In *S. zeamais* and *S. oryzae*, the acrosome has three layers: an extra-acrosomal amorphous layer, an acrosome, and a inner cone or perforatorium. This structure is typical in many coleopteras (Baccetti *et al.,* 1973; Baccetti and Daccordi, 1988; Burrini *et al.*, 1988). The inner cone or perforatorium has a filamentous substructure, which may play a cytoskeletal role during the process of sperm-oocyte interaction and fertilization. A similar structure has been reported for the featherwing beetle acrosome (Dybas and Dybas, 1987).

In insect sperm, the centriolar adjunct is commonly

a sheath of compact fibrillar material around the basal body (centriole derivative) of the axoneme (Breland *et al.*, 1966; Phillips, 1970). The presence of centriolar adjunct in the spermatid stage, and lack of it in the mature sperm, is similar to that described in *Tenebrio molitor* (Baccetti *et al.*, 1973) and *Anthonomus grandis* (Gassner *et al*., 1975). It has been suggested that the centriole adjunct is a support organelle that stabilizest the insertion of the axoneme at the nuclear base (Phillips, 1970; Baccetti and Afzelius, 1976).

In *S. zeamais* and *S. oryzae*, the flagellum morphogenesis has the same pattern as that described for other Coleoptera, which involves the formation of the axoneme, two mitochondrial derivatives, and accessory structures (Shay *et al*., 1969; Gassner *et al*., 1975).

At the initial stages of differentiation, the complex process of rearrangement and fusion of the mitochondria takes place and gives rise to two mitochondrial derivatives of different shape and size. In *S. zeamais* and *S. Oryzae*, as in most Curculionidae, the spermatozoa possess two mitochondrial derivatives. The bigger mitochondrial derivative is almost as long as the axoneme. It is a dense cylinder full of opaque material, and the cristae, which are orthogonally arranged and differently disposed. The smaller mitochondrial derivative flanks the bigger one, beginning in the nucleus and ending at a more anterior level than before the other. The number and size of the mitochondrial derivatives present in sperm depends on the insect species. In some species, the sperm have two mitochondrial derivatives that are of equal size but are enantiomorphic (Harold and Munz, 1967; Báo and de Souza, 1993). In other species, spermatozoa possess two mitochondrial derivatives that are of unequal size, with one usually extending further posteriorly or anteriorly than the other (Yasuzumi and Oura, 1965; Thompson and Blum, 1967).

The 9+9+2 pattern of the axoneme of *S. zeamais* and *S. oryzae* are similar to that found in other beetles. There are 9 accessory microtubules, 9 microtubules doublets, and 2 central microtubules. This general feature of the Coleoptera spermatozoon is not observed in the family Rhynchitidae (Coleoptera), which appear drastically isolated, because its have a peculiar "9+9+0" axoneme and show, moreover, a limited degree of asymmetry in the tail organelles (Burrini *et al.*, 1988).

In *S. zeamais* and *S. Oryzae*, the flagellum has two accessory bodies of equal size that are adjacent to the axoneme and lateral to the mitochondrial derivatives (pattern similar to most Coleoptera). However, the flagellum of *Coelomera lanio* and *Cerotoma arcuata* (Coleoptera: Chrysomelidae) have a single accessory body (Báo, 1996; 1998), differing from the majority of chrysomelids, which have a pair of accessory bodies (Baccetti and Daccordi, 1988). Thus, the number of accessory bodies may be an important characteristic to be considered in phylogenetic studies.

The "puff"-like corpuscles with a flocculent aspect have different shapes and sizes. Similar structures were observed in other Chrysomelidae (Baccetti and Daccordi, 1988) and Curculionoidea (Burrini *et al.*, 1988). Such "puff"-like corpuscles seem to be characteristic components of the coleopteran flagellum. Probably, these corpuscles help to maintain the equilibrium of the flagellar structure during motility.

The study of the cross-sections of the sperm tail at different levels shows that all the elements progressively disappear. In *S. zeamais* and *S. oryzae*, the accessory bodies disappear first, followed by the smaller of the two mitochondrial derivatives, and finally by the larger one. The several tubules of the axonemal complex are the last to disappear, resulting in the tail's disorganization. This pattern was also described in *Tenebrio molitor* (Coleoptera: Tenebrionidae) (Baccetti *et al.*, 1973), in *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) (França and Báo, 2000), and in Halictidae (Hymenoptera: Apoidea) (Fiorillo *et al.*, 2005).

The patterns of chromatin condensation and the type of acrosomal and flagellar structures are important characters for further taxonomic, phylogenetic, and reproduction biology studies in curculionids.

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