UNIVERSIDADE ESTADUAL DE CAMPINAS

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"ESTUDO COMPARATIVO DA ESPERMIOGÊNESE NORMAL E DIAPÁUSICA EM INSETOS PERTENCENTES AO COMPLEXO PERCEVEJO DA SOJA (HEMIPTERA: PENTATOMIDAE)"

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural na área de Biologia Celular.

Orientadora: Profa. Dra. Sônia Nair Báo

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Dedico

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Índice

1. Resumo

Percevejos fitófagos (Hemiptera, Pentatomidae) são as principais pragas de colheitas economicamente importantes ao redor do mundo, como por exemplo soja, arroz, coco, café, entre outras. Apesar da grande quantidade de informação sobre as espécies-praga, seu dano potencial nas plantas de importância econômica e as formas de controle usadas, fazem com que o impacto dessas pragas na produção de colheitas permaneça em níveis indesejáveis. A caracterização ultra-estrutural da espermiogênese desses insetos poderá, possivelmente, ser importante para direcionar e programar um controle bioquímico e a manipulação genética através de machos estéreis ou semiestéreis.

Estudos ultra-estruturais e citoquímicos foram desenvolvidos elucidando as mudanças nucleares e a formação do acrosoma durante a espermiogênese de percevejos fitófagos. Além do mais, foi realizada a detecção ultra-estrutural de cálcio e a imunolocalização de calmodulina e de diferentes formas de tubulinas.

A espermiogênese envolve elongação nuclear, condensação cromatínica, formação do acrosoma e desenvolvimento flagelar, com a formação do axonema e dos derivados mitocondriais. O desenvolvimento do núcleo envolve mudanças na forma e no grau de condensação da cromatina que apresenta padrão fibrilar em alguns estágios de diferenciação. O acrosoma é formado pelo complexo de Golgi e pode apresentar características morfológicas pouco comuns aos outros Heteroptera durante o seu desenvolvimento, que conta com a participação de resíduos específicos de carbohidratos e de enzimas como fosfatase ácida, glicose-6-fosfatase e tiaminopirofosfatase.

A detecção / localização de cálcio e a imunolocalização de calmodulina indicam uma possível ação conjunta desses dois elementos como reguladores de vários eventos celulares durante a espermiogênese dos percevejos. Ainda, utilizando a imunocitoquímica foram detectados vários isotipos de tubulina durante o período de diferenciação celular. Esses dados indicam que deve existir alguma diferença estrutural e funcional entre os microtúbulos citoplasmáticos e axonemais.

Além das modificações morfofuncionais que ocorrem durante a espermiogênese foram descritas as modificações enzimáticas sofridas pelo espermatozóide 20 h e 40 h após a cópula.

O processo de espermiogênese também foi analisado durante a diapausa adulta de duas espécies de percevejos fitófagos. Os resultados indicam que durante esse período a espermiogênese é descontinuada e para isso conta com a participação de eventos com padrão apoptótico e fagocítico.

2. Abstract

Phytophagous stink bugs (Hemiptera, Pentatomidae) are the main pests of economically important crops throughout the world, such as soybean, rice, cocoa and coffee among others. Despite the amount of information regarding pest species, their damage potential to economically important plants, and control measures used, the pests impact on the production of crops remains at undersirable levels. The ultrastructural characterization of these insects spermiogenesis would be important to programs directed towards the biochemical control of these insect pests and genetic manipulation through sterile and semisterile males.

Ultrastructurals and cytochemical studies were carried out on nuclear changes and acrosome formation during spermiogenesis of the phytophagous bug. Indeed, was done the ultrastructural detection of calcium and the immunolocated of calmodulin as well as several tubulins isoforms.

The spermiogenesis involves nuclear elongation, chromatin condensation, acrosomal formation and flagellar development along with formation of the axoneme as well as mitochondrial derivatives. The development of the nucleus involves changes in the shape and in degree of chromatin condensation which presents fibrillar pattern during some stages of differentiation. The acrosome is formed by the Golgi complex and can be present unusual morphological features with the other Heteroptera during its development, whose take the participation of specific carbohidrate residues and enzymes as acid phosphatase, glucose-6-phosphatase and thiaminepyrophosphatase.

The calcium detection and the immunolocated of calmodulin suggest that they act toghether as regulators of several cellular events during the bugs spermiogenesis. Moreover, using immunocytochemistry were detected several tubulins isotypes during this cellular differentiation period. These data suggest that could be exist some structural and funcional diferences between cytoplasmic and axonemal microtubules.

Besides the morphofunctional modifications undergone during spermiogenesis, we described the enzimatic modifications undergone to the spermatozoon 20 h and 40 h after copulation.

The spermiogenesis process was analised during adult diapause of two phytophagous bugs species. The results indicate that during this period the spermiogenesis is discontinued and for this, takes off cellular events with apoptotic and phagocytic pattern.

3. Introdução

A família Pentatomidae é uma das maiores famílias dentro dos Heteroptera, com mais de 4000 espécies descritas (Panizzi, 1997). A maioria dos pentatomídeos fitófagos é polífaga, alimentando-se em plantas cultivadas ou nativas. Eles são a maior peste de colheitas economicamente importantes ao redor do mundo, incluindo leguminosas como a soja (Turnipseed & Kogan, 1976; Panizzi & Slansky, 1985; Kogan & Turnipseed, 1987); cereais como o arroz e o trigo (Oliver et al., 1971; Nilakhe, 1976; Jones & Cherry, 1986; Foster et al., 1989) e colheitas de árvores como citrus (James, 1992), coco (Dolling, 1984) e café (Greathead, 1966). Pentatomídeos fitófagos se alimentam em várias estruturas da planta hospedeira e, como conseqüência, a natureza da injúria que causam é variável, sendo que sementes e frutas imaturas são os principais sítios de alimentação.

O controle desses percevejos, assim como da grande maioria dos insetos vetores e pragas, tem sido feito com o uso indiscriminado de inseticidas, em detrimento ao equilíbrio biológico. Este método de controle, além de causar problemas ao homem e ao meio ambiente de um modo geral, pode eventualmente perder sua efetividade com a inevitável seleção de resistência pelo animal. Com estas preocupações, nestas últimas décadas, esforços foram concentrados com o intuito de desenvolver sofisticados métodos de controle, sendo que os mais usados são técnicas utilizando feromônios (Boake et al., 1996) e esterilidade, bem como outros métodos de controle genético (Rossler, 1982). Uma nova possibilidade de controle biológico pode ser estudada a partir da diapausa, que tem lugar durante a vida adulta de alguns desses percevejos fitófagos.

Durante determinadas fases da vida de algumas espécies de insetos, pode ocorrer um retardo no desenvolvimento. Esse retardo é conhecido como quiescência - quando é uma conseqüência direta de condições ambientais - ou como diapausa - quando é um fenômeno adaptativo que habilita o inseto a sobreviver em condições adversas que ocorrem regularmente.

Em regiões temperadas, a diapausa está, geralmente, associada à sobrevivência do inseto no inverno gelado, quando a taxa de crescimento normal não é possível. Já nos trópicos, ela pode facilitar a sobrevivência durante a estação seca que é caracterizada pela baixa umidade e pela diminuição da oferta de alimento. Em insetos que sofrem uma diapausa obrigatória em cada geração, o estágio particular de entrada na diapausa é determinado geneticamente. Mas quando a diapausa ocorre somente em certas gerações, as mudanças no metabolismo são iniciadas por algum sinal vindo do ambiente, o qual, embora não desfavorável por ele mesmo, indica o advento de uma condição adversa (Chapman, 1998). Assim sendo, os insetos são capazes de sobreviver na ausência de alimento e sob condições climáticas adversas pela entrada em períodos de dormência e inatividade reprodutiva.

Durante a diapausa larval e pupal a espermiogênese sofre profundas alterações devido ao novo balanço endócrino estabelecido. Essas alterações na espermiogênese são causadas pela lise das células germinativas em desenvolvimento que tenham alcançado um certo grau de maturação e este é característico para uma dada espécie (Chippendale & Alexander, 1973). No final da diapausa, o balanço endócrino é restabelecido e as células germinativas param de ser lisadas, fazendo com que a espermatogênese prossiga ininterruptamente como antes da diapausa (Herman, 1981; Friedländer, 1982; 1997; Friedländer & Reynolds, 1992).

A maioria dos trabalhos sobre gametogênese, durante a diapausa adulta, tem sido feita durante a oogênese (Denlinger, 1985). Os dados publicados sobre a diapausa adulta de machos referem-se, principalmente, sobre o comportamento sexual, as glândulas acessórias e a competição de espermatozóides (Ferenz, 1975; Glitho & Huignard, 1990; Pener, 1992). Assim, pouco se conhece sobre a espermatogênese durante esse período (de Wilde, 1954; Tran & Huignard, 1992); Friedländer & Scholtz, 1993). Também não há informações sobre as mudanças estruturais e ultra-estruturais ocorridas na espermiogênese de insetos adultos no período da diapausa.

3.1. Morfologia Geral dos Espermatozóides de Heteroptera

Os espermatozóides típicos de insetos apresentam forma alongada e diâmetro reduzido, escasso citoplasma e duas regiões distintas: a cabeça e a cauda, geralmente conectadas pelo adjunto do centríolo (Philips, 1970; Baccetti, 1972). A cabeça é constituída pelo acrosoma e o núcleo. A cauda é formada pelo axonema, derivados mitocondriais e estruturas acessórias. O acrosoma é formado a partir do complexo de Golgi e o seu conteúdo é rico em enzimas hidrolíticas (Baccetti, 1972; Báo et al., 1989; Báo & de Souza, 1992). O axonema, na grande maioria dos insetos, apresenta o padrão de 9+9+2 microtúbulos, sendo 2 microtúbulos centrais, 9 duplas periféricas e 9 microtúbulos acessórios (Phillips, 1970; Baccetti, 1972). Geralmente apresentam dois derivados mitocondriais flanqueando o axonema, que podem ser do mesmo tamanho e conter matriz paracristalina (Phillips, 1970; Baccetti, 1972; Baccetti et al., 1977).

Além das características comuns aos espermatozóides dos demais insetos, os de Heteroptera apresentam algumas que não são encontradas nos espermatozóides de outros insetos: dois ou três corpúsculos paracristalinos nos derivados mitocondriais e pontes entre os derivados mitocondriais e dois dos microtúbulos axonemais (Afzelius et al., 1976; 1985; Dallai & Afzelius, 1980; Dolder, 1988; Afzelius & Dallai, 1989; Báo & de Souza, 1994; Fernandes & Báo, 1998).

3.2. Objetivos

Este trabalho teve como objetivo geral disponibilizar maiores informações sobre a morfologia dos espermatozóides dos Heteroptera e como objetivos específicos:

⇒ Descrever a estrutura e ultra-estrutura da espermiogênese normal de *Edessa meditabunda, Piezodorus guildini* e *Thyanta perditor.*

⇒ Descrever as alterações ultra-estruturais e citoquímicas ocorridas nos espermatozóides de *Acrosternum aseadum* após a cópula.

⇒ Caracterizar o processo de formação do acrosoma e as mudanças nucleares ocorridas durante a espermiogênese de *Euchistus heros.*

⇒ Detectar a participação de cálcio e calmodulina na espermiogênese de *Acrosternum aseadum, Euchistus heros, Nezara viridula* e *Piezodorus guildini.*

⇒ Detectar a presença de diferentes isotipos de tubulinas na espermiogênese de *Acrosternum aseadum* e *Euchistus heros.*

⇒ Descrever e caracterizar a espermiogênese diapáusica em espécimens adultos de *Edessa meditabunda* e *Nezara viridula.*

4. Artigos Publicados ou Submetidos á Publicação

Durante a realização desta tese foram produzidos seis trabalhos, dos quais quatro já foram publicados, um aceito para publicação (*in press*) e um submetido a apreciação. São eles:

4.1. **Fernandes, A.P.** & Báo, S.N. (2001). Ultrastructural studies on spermiogenesis and spermatozoa of insect pests (Hemiptera, Pentatomidae. In: Sarma, R. *Current Res. Adv. in Agricul. & Entomol.* Kerala-India, World Wide Research, 2001. p. 13-26.

4.2. **Fernandes, A.P.** & Báo, S;N. (2000). Ultrastructural and cytochemical studies on the spermatozoa of *Acrosternum aseadum* (Hemiptera: Pentatomidae) after copulation. *J. Submicrosc. Cytol. Pathol.* 32(4): 547-553.

4.3. **Fernandes, A.P.**; Curi, G.; França, F.G.R. & Báo, S.N. (2001). Nuclear changes and acrosome formation during spermiogenesis in *Euchistus heros* (Hemiptera: Pentatomidae). *Tissue & Cell* 33(3): 286-293.

4.4. **Fernandes, A.P.** & Báo, S.N. (2001). Detection of calcium and calmodulin during spermiogenesis of phytophagous bugs (Hemiptera: Pentatomidae). *BioCell* 25(2): 173-177.

4.5. **Fernandes, A.P.** & Báo, S.N. (2002). Immunoelectron microscopy detection of tubulins during the spermiogenesis of phytophagous bugs (Hemiptera: Pentatomidae). *Invertebrate Reproduction. Development* "in press"

4.6. **Fernandes, A.P.**; Peixoto, F.L. & Báo, S.N. Ultrastructural and cytochemical studies on diapause spermiogenesis in phytophagous bugs (Hemiptera: Pentatomidae). Submetido à publicação.

4.1. **Fernandes, A.P.** & Báo, S.N. (2001). Ultrastructural studies on spermiogenesis and spermatozoa of insect pests (Hemiptera: Pentatomidae. In: Sarma, R. *Current Res. Adv. in Agricul. & Entomol.* Kerala India, World Wide Research, 2001. p. 13-26.

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Ultrastructural studies on spermiogenesis and spermatozoa of insect pests (Hemiptera: Pentatomidae)

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ABSTRACT

Phytophagous stink bugs (Hemiptera, Pentatomidae) are the main pests of economically important crops throughout the world. Despite the amount of information regarding pest species, their damage potential to economically important plants, and control measures used, the pests impact on the production of crops remains at undesirable levels. The ultrastructural characterization of these insect spermiogenesis would be very important to programs directed towards the biochemical control of these insect pests and genetic manipulation through sterile and semisterile males. The spermiogenesis involves nuclear elongation, chromatin condensation, acrosomal formation and flagellar development along with formation of the axoneme as well as mitochondrial derivatives. The nucleus development involves changes in the shape and in the degree of chromatin condensation, with specific aggregation patterns of DNA-histone complex occurring during this process. The acrosome showed a three-layered and the acrosomal content a tubular arrangement. The axoneme presents a 9+9+2 microtubule pattern and bridges occurs between axonemal microtubules doublets 1 and 5 and mitochondrial derivatives. Two paracrystalline structures embedded in

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amorphous regions were observed in the mitochondrial derivatives. An electron dense rod was observed near the centriolar adjunct, which may be involved in the microtubule organization during spermiogenesis of these insects.

INTRODUCTION

The Heteroptera or true bugs is a suborder of the insect order Hemiptera. The heteropterans cause various physiological and biochemical changes in the tissues of host plants, destroying or reducing the fecundity and fertility of reproductive structures desired also by humans, or forcing the plant into undertaking energetically cost repairs (for reviews see 1).

The Pentatomidae is one of the largest families of the Heteroptera. Within this family there are eight subfamilies: Asopinae, Cyrtocorinae, Discocephalinae, Edessinae, Pentatominae, Phyllocephalinae, Podopinae and Serbaninae, where the subfamiiy Pentatominae is the largest and consists entirely of plant feeders (2).

Phytophagous stink bugs, in general, are characterized by being round or ovoid, with five-segmented antennae, three-segmented tarsi and a scutellum that is short, usually narrowed posteriorly and more or less triangular. They are called stink bugs because they produce a disagreeable odor by means of scent glands that open in the region of metacoxae (3).

Most phytophagous pentatomids are polyphagous, feeding on cultivated and uncultivated plants. Since that several plants are species of economic importance they are regarded as major pests of economically important crops throughout the world, including legumes such as soybean (4, 5), cereals such as rice (6, 7), and tree crops such as citrus (8), oil palms and coconut (9), cocoa (10), and coffee (11).

Phytophagous pentatomids feed on various structures of the host plant, and as consequence, the nature of the injury is also variable, resulting in plant wilt and, in many cases, abortion of fruits and seeds. During the feeding process, they also may transmit plant pathogens, which increase their damage potential (for reviews see references in 12).

The phytophagous pentatomids of economic importance, belong to the subfamilies Edessinae, with some pest species in the genus *Edessa*, and Pentatominae, which contains the majority of species that are pests of crops (2). In this study we work with three species of these subfamilies: *Edessa meditabunda* (Edessinae), *Piezodorus guildini* and *Thyanta perditor* (Pentatominae).

Edessa meditabunda is a neotropical pentatomid which is pest of many species of Solanaceae, including tomato and potato, and of Leguminosae, including peas, soybean and alfafa. It also feeds on cotton, eggplant, tobacco, sunflower, papaya and grapes (13 - 16).

Piezodorus guildini, the small green stink bug, is a Neotropical pentatomid found from the southern United States to Argentina. It is the major pest of soybean in South America. The list of food plants includes some economically important plants in addition to soybean, mostly legumes such as common bean, pea, and alfafa. Indeed, it is associated with several microorganisms present on damage soybean seeds (17).

Thyanta perditor was not considered a pest species prior to its first appearance in significant numbers during the late 1970s in Brazil; then, no published information was found in the literature related to its economic importance. Today, it is considered a pest of some Gramineae, although it only is the minor pest of soybean, at least in Brazil (18, 19).

The general control of these insect pests is made throughout the use of chlorinated hydrocarbon, organophosphate, and organochlorophosphate insecticides (20). However, if insecticidae controls are not timed properly, then this management strategy is useless and in fact, actually intensifies the stink bug problem in the main crop besides causing serious damage to environment and human health. Some progress has been made in the development of host plant resistance (see references in 5, 20, 21) and in inoculative releases of the egg parasitoid (Scelionidae) (22). Indeed, studies about plant/ herbivore/ parasitoid semiochemical relationship in conventional and genetically engineered soybean as well as study means to conserve and augment

native biological control agents through semiochemical manipulation had began (Borges and Aldrich - personal communication).

Despite the vast amount of information regarding pest species and control measures used, their damage potential to crop production remains high and appears obviously that more knowlegment about the basic biology and ecology of most heteropteran pests as well as new efficiently alternatives of biological control must be necessary.

Among several aspects of the basic biology the reproductive biology may be one of the most important. Within this aspect the gametogenesis in the male insects had received some attention in the last years. The complete sequence of events in the male gametogenesis encompasses production of primary spermatogonia by stem-cell division, sequential differentiation of secondary spermatogonia during the course of a species-specific number of incompleteiy cytokinetic cell divisions culminating in the primary spermatocyte. After prophase maturation and the ensuing meiotic divisions, occur extensives morphological alterations of the spermatid termed spermiogenesis, by which the mature sperm is formed. The formation of spermatids and spermiogenesis is general delayed until the last larval or pupal stadium in a manner analogous to puberty. During this and earlier stages, the testis maintains a level of differentiation consistent with the insect's somatic development. The ability of the male insect to maintain a level of differentiation of the germ line in phase with its somatic development argues strongly that there must exist rate-regulating mechanisms or regulatory points within the developmental sequence. Certain events within the spermatogenic sequence can be recognized as potential regulation points or physiological mechanisms by which the insect balances its own sperm production. The value of such information to programs directed toward chemical or biological control of insect pests or genetic manipulation of populations through sterile and semisterile males is obvious (23). Then, the structural and ultrastructural characterization of spermatozoa may be used with one of the aspects on the manipulation of point controls of insect

development.

The spermatozoa are very specialized and highly differentiated cells. They have lost various organelles essential to cell metabolism while the remaining organelles are modified in a manner unparalleled in other process of cell differentiation. The main compartments of a typical insect spermatozoon are the head, containing nucleus and acrosome, and the tail which contains axoneme and mitochondrial derivatives (for reviews see 24, 25).

Heteropteran spermatozoa have certain characteristic that are not found in other insects: two or three crystalline bodies within the mitochondrial derivatives and bridges between the mitochondrial derivatives and two double axonemal microtubules (26 - 32).

In the present study we used electron microscopy to trace the morphofunctional events which take place during spermiogenesis of *Edessa meditabunda*, *Piezodorus guildini* and *Thyanta perditor*.

MATERIALS AND METHODS

The insects utilized were male adults of the phytophagous bugs *Edessa meditabunda*, *Piezodorus guildini* and *Thyanta perditor* (Hemiptera, Pentatomidae), obtained from a colony maintained in the National Center of Genetic Resource and Biotecnology (CENARGEN), Brasilia – Brazil.

Transmission electron microscopy

The testes were dissected and fixed for 4h in 2,5% glutaraldehyde, 4% paraformaldehyde, 5mM $CaCl_2$ and 3% sucrose, buffered with 0,1M sodium cacodylate, at pH 7.3. After fixation, the specimens were rinsed in buffer, and postfixed in 1% osmium tetroxide, 0,8% potassium ferricyanide, and 5mM $CaCl_2$ in 0,1M sodium cacodylate buffer. In some cases the specimens were fixed in a mixture of 2,5% glutaraldehyde, 1% tannic acid in 0,1M sodium phosphate buffer, at pH 7.3, followed by block-staining in 1% uranyl acetate in distilled water (33). The material was dehydrated in a series of ascending acetones (30-100%) and embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined in a JEOL 100C transmission electron microscope.

Enzyme cytochemistry

The testes of *Thyanta perditor* were dissected and briefly fixed for 15 min at 4°C in 1% glutaraldehyde buffered with 0,1M sodium cacodylate at pH 7.2. After fixation, the specimens were washed with buffer and used for the cytochemical experiments.

Acid phosphatase activity:

The fixed-washed testes were incubated for 1h at 37°C in the following medium: 0,1M Tris-maleate buffer, pH 5.0, 7mM cytidine-5'-monophosphate, 2mM cerium chloride and 5% sucrose (34). For controls, the substrate was omitted.

Glucose-6-phosphatase activity:

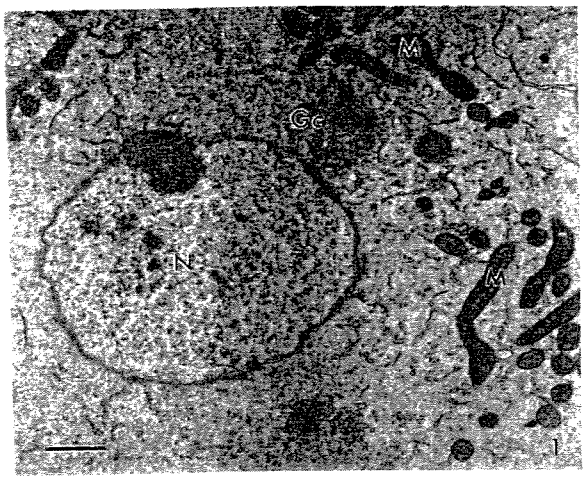
The fixed-washed testes were incubated for 1h at 37°C in the following medium: 5mM glucose-6-phosphatase, 5mM manganese chloride, 4mM cerium chloride, 5% sucrose and 0,1M Tris-maleate buffer, pH 6.5 (35). The controls were incubated in the same medium from which the substrate was omitted.

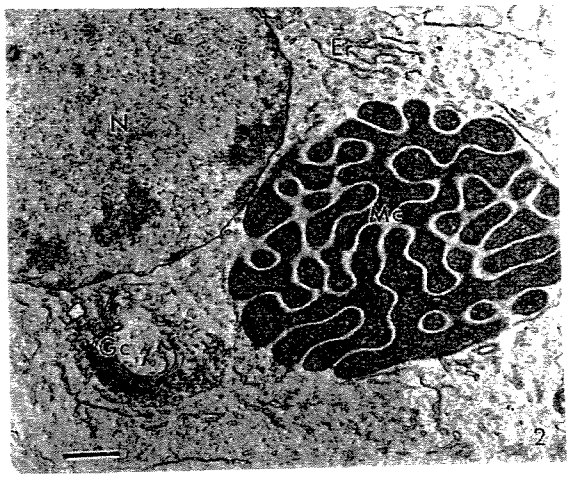
After incubation of the testes in one of the media as described above, the specimens were washed with sodium cacodylate buffer and fixed again for 3h at 4°C in a solution containing 4% paraformaldehyde, 2% glutaraldehyde in 0,1M sodium cacodylate buffer, pH 7.2. The specimens were then washed in clean buffer, and postfixed in a solution containing 1% osmium tetroxide, 0,8% potassium ferricyanide and 5mM $CaCl_2$ in 0,1M sodium cacodylate buffer. Subsequently, they were dehydrated in acetone and embedded in Spurr resin. Thin sections were stained with uranyl acetate and lead citrate, and examined in a Jeol 100C transmission electron microscope.

RESULTS

The spermiogenesis of *Edessa meditabunda*, *Piezodorus guildini* and *Thyanta perditor* are very similar and will be described together.

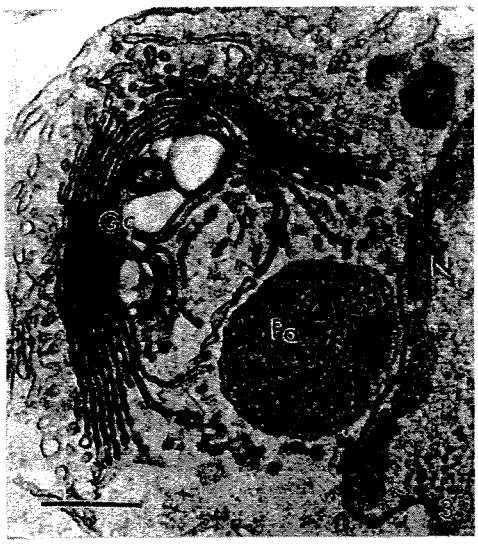
During the early spermatid phase, the nucleus resembles that of somatic cells, with electron dense areas of chromatin in some regions (Fig. 1). The scarce surrounding cytoplasm contains a well-developed Golgi complex and several mitochondria. In the subsequently stage, a large

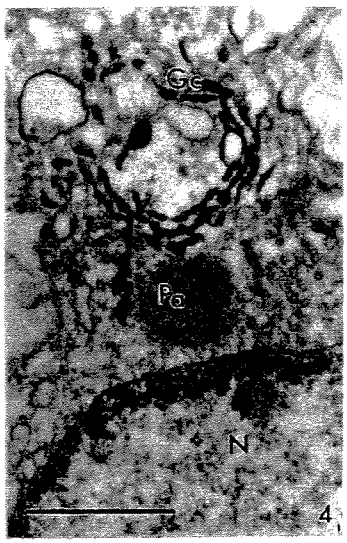


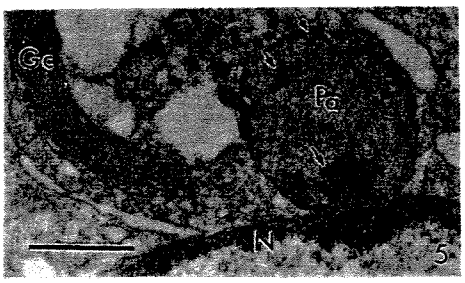


Figures 1 and 2: Spermatids of *E. meditabunda* in the initial stage of differentiation, showing the nucleus (N) and a scarce surrounding cytoplasm with a well developed Golgi complex (Gc), abundant mitochondria (M), which fuse together forming the mitochondrial complex (Mc). Endoplasmic reticulum (Er). Bars : 1µm

number of mitochondria of early spermatids fuse together forming a labyrinth-like structure, known as the mitochondrial complex or nebenkern (Fig.2). Indeed, the Golgi complex and the endoplasmic reticulum are well-developed. In a immediately subsequent stage, a large vesicle, called the proacrosomal granule and derived from vesicles of the Golgi complex is formed and attaches to the nuclear envelope. The nucleus shows two distinct regions: one near the nuclear envelope showing homogeneously condensed chromatin attached to it and the other in the central region of the nucleus showing descompacted chromatin (Fig. 3-5). In this phase the spermatid which were incubated on a medium containing cytidine-5'-monophosphate as substrate and cerium as capture agent, led to the appearance of an electron dense reaction product, indicating the presence of acid phosphatase activity in association with the Golgi complex (Fig.4) and scattered on the proacrosomal granule (Fig. 5). As differentiation progresses is observed the reorganization of the proacrosomal granule into an

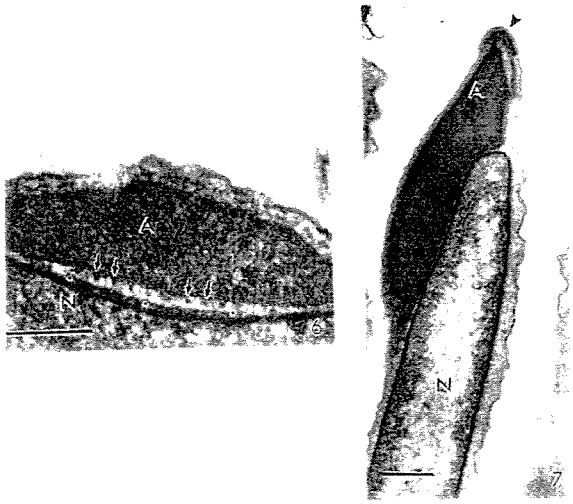






Figures 3-5: Spermatids of *T. perditor* showing the first steps of acrosomal formation from vesicles of the Golgi complex (Gc). Note the presence of acid phosphatase activity in the Golgi cisternae (Fig. 4), and scattered (arrows) in the proacrosomal granule (Pa). Nucleus (N). Bars: 0,5 μm

acrosomal complex, localized anteriorly at the nucleus. During the early spermatid phase, it appears as an amorphous and electron dense mass (Figs. 6 and 7), connected to the nucleus by small electron dense pits attached in a light electron dense lamina-like structure (Fig.6). Indeed, in this phase can be observed the beginning formation of the extra-acrosomal thin layer (Fig. 7). This amorphous mass is observed in other stages of differentiation (Fig. 8) and became in a cap-like



Figures 6 and 7: Spermatids of *T. perditor* showing the acrosome (A) as an electron dense mass attached to the nucleus (N) by the small electron dense pits (arrows) connected with a light electron dense lamina-like (asterisks). Note the beginning formation of the extracrosomal thin layer (arrowhead). Bars: 0,1 µm and 0,5 µm, respectively.

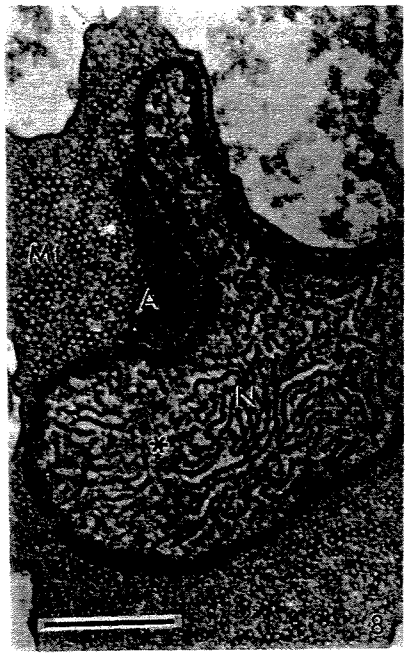
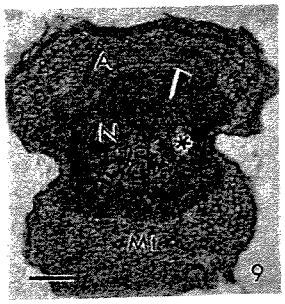
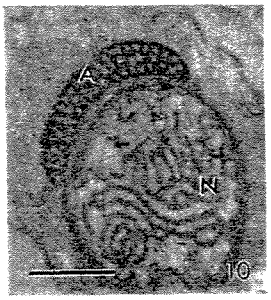


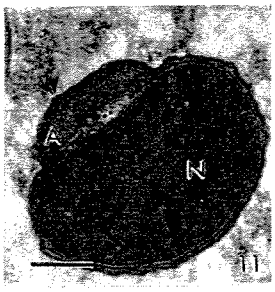
Figure 8: Spermatid of *E. meditabunda* in hte intermediate stage of differentiation. The contents of the nucleus (N) shows a fibrillar arrangement (asterisk); the acrosome (A) appears as an amorphous mass. Microtubules (Mt). Bar: 0,5 µm

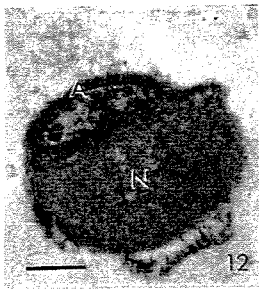
acrosome in the late spermatids, with its contents showing a tubular arrangement (Fig. 9). In this phase, a light reaction product of glucose-6-phosphatase activity can be seen surrounding the tubular structure of the acrosome (Fig. 10). The final form of acrosome consists of an electron lucent inner cone, the acrosomal content – that shows a tubular arrangement – and an outer most extra acrosomal thin layer (Fig. 11). In this final stage we can observed the reaction product of acid phosphatase activity surrounding the acrosomal membrane and scattered on the acrosomal content

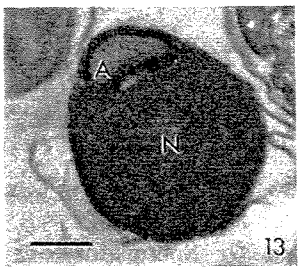




Figures 9 and 10: Spermatids of *P. guildini* and *T. perditor*, respectively, showing the nucleus (N) with the compacted chromatin (arrowhead) near to the nuclear envelope, and fibrillar arrangement (asterisk) in the central region. The acrosome (A) shows a tubular arrangement with glucose-6-phosphatase activity (Fig. 10). Microtubules (Mt). Bars: 0,2 μm







Figures 11-13: Spermatids of *P. guildini* (Fig. 11) and *T. perditor* (Figs. 12 and 13) in the late stage of differentiation. The nucleus (N) shows the chromatin full compacted. The acrosome consists of an electron lucent inner cone (double asterisk), the acrosomal content (A) and the outer most extracrosomal thin layer (double arrowhead). Note the presence of acid phosphatase activity surrounding the acrosome and scattered in the acrosomal content (Fig. 12), and the glucose-6-phosphatase activity (Fig. 13) on the acrosomal membrane. Bars: 0,1 μm

(Fig. 12) and the reaction product of glucose-6-phosphatase activity on the acrosomal membrane (Fig. 13).

The nuclear material presents a peculiar organization during spermatid differentiation. After the stage where two regions can be distinguished in the nucleus (Figs. 3 and 7), the chromatin continues with condensation process, and the

nuclear material shows a fibrillar arrangement (Figs. 8 and 14). The condensation process occurs in proximal and distal portion in relationship to the acrosomal region (Fig. 9) leading the appearance

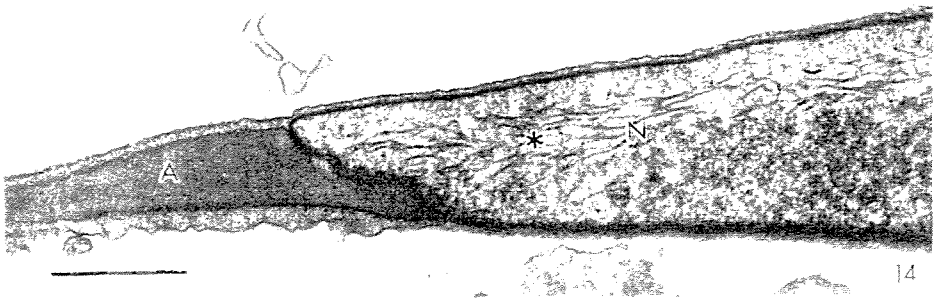
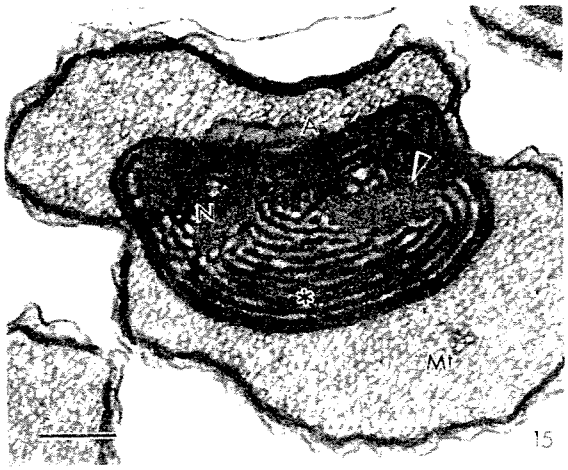
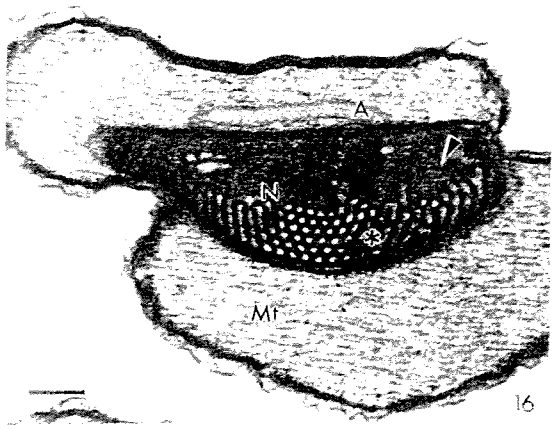


Figure 14: Spermatid of *P. guildini* showing the chromatine-fibrillar arrangement (asterisk). Acrosome (A), nucleus (N). Bar: 0,5 μm





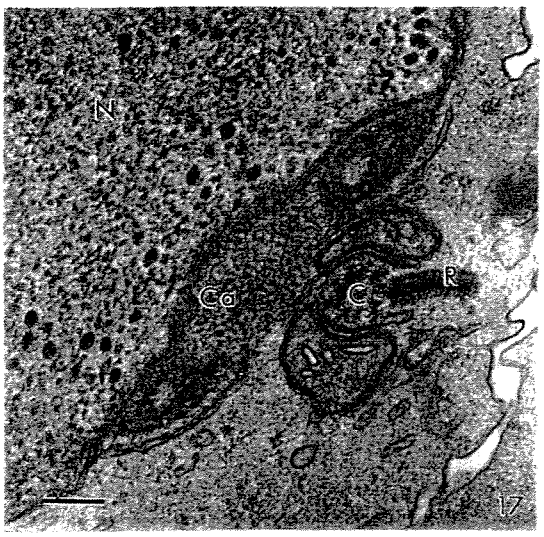
Figures 15 and 16: Spermatid of *P. guildini* on intermediate stage of differentiation showing two distinct regions in the nucleus (N): one with well compacted chromatin (arrowhead) and another with fibrillar arrangement (asterisk). Acrosome (A); microtubules (Mt). Bars: 0,1 μm and 0,2 μm, respectively.

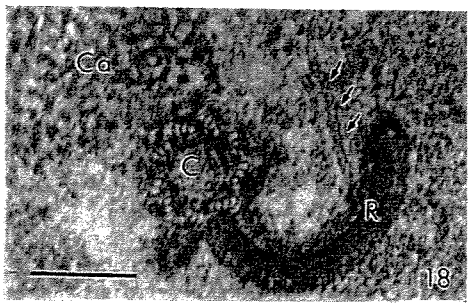
once more of two distinct regions in the nuclear content: one near the acrosomal region totally compacted and electron dense and the other on the posterior region of the nucleus showing a fibrillar arrangement (Fig. 15 and 16). After complete chromatin coalescence, a very compact and electron dense nucleus can be observed, without fibrilar arrangement (Fig. 11).

In early spermatids the centriolar adjunct is located posterior to the nucleus, in the region of flagellum implantation. This structure appears electron dense with some electron lucid areas (Figs. 17 and 18).

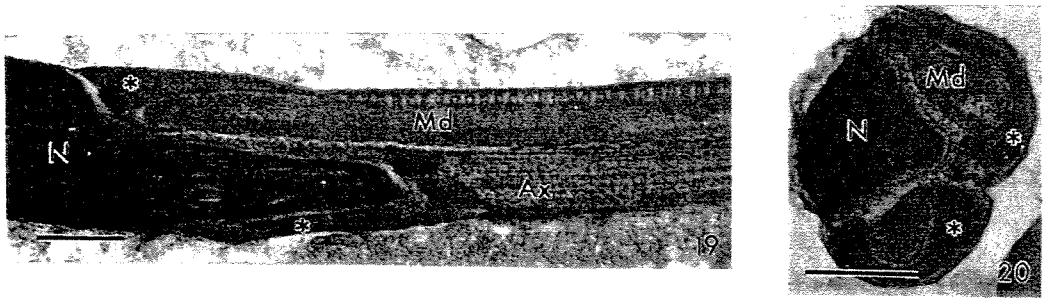
The nucleus is attached to the flagellum by a centriolar adjunct-like structure which penetrates into the basolateral nuclear groove (fig. 19) and involves the mitochondrial derivatives (Figs. 19 and 20).

Near the centriolar adjunct we observed an electron dense rod (Figs. 17 and 18). A microtubule network was observed anchored to this electron





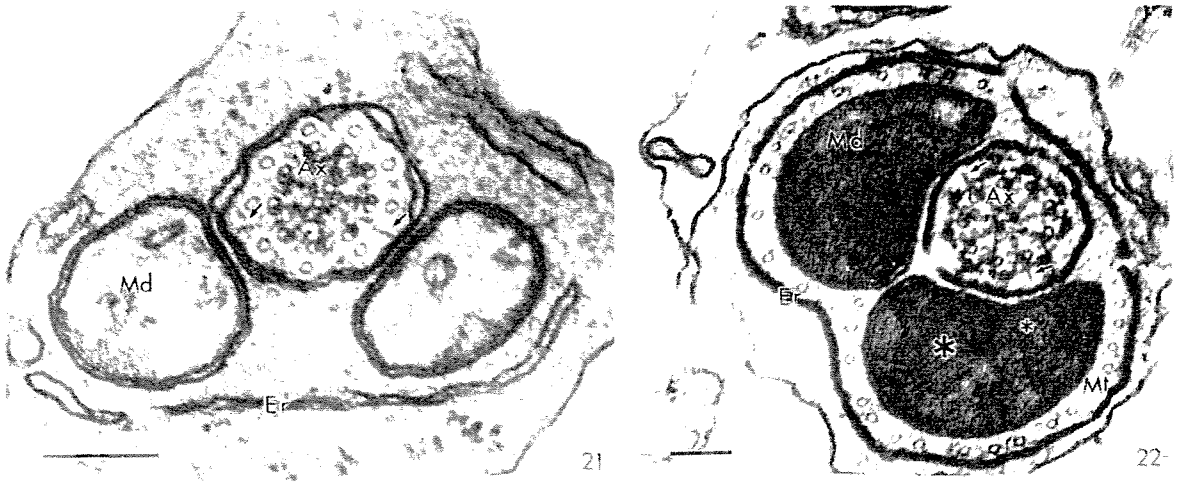
Figures 17 and 18: Sections through region of flagellum implantation of *E. meditabunda* and *T. perditor* early spermatids, respectively. Note the presence of the centriolar adjunct (Ca), the centriole (C) and of an electron dense rod (R) where microtubules (arrows) are anchored. Nucleus (N). Bars: 0,2 μm



Figures 19 and 20: Longitudinal and cross section through connection region of the nucleus (N) and the flagellum of *P. guildini* and *E. meditabunda* spermatids, respectively. Note the presence of centriole adjunct-like structure (asterisk) involving the initial part of the mitochondrial derivatives (Md). Axoneme (Ax). Bars: 0,2 μm

dense rod (Fig. 18).

The large number of mitochondria of early spermatids which fused together forming the mitochondrial complex (Fig. 2) turns into two mitochondrial derivatives, in the course of its differentiation, concomitantly with an axoneme outgrowth (Fig. 21). These mitochondrial derivatives had the same size and containing two paracrystalline structures which are embedded in an amorphous material (Figs. 22 and 23). In the axoneme a 9 (outer singles) + 9 (intermediate doublets) + 2 (central singles) pattern of microtubules is observed (Figs. 21 to 23). The accessory microtubules are composed of 16 protofilaments clearly visible when the specimens are fixed in the presence of tannic acid (Fig. 23, inset). Bridges that connect the mitochondrial derivatives to axoneme are observed in the axonemal microtubules doublets 1 and 5. These bridges occur in the region of the small paracrystalline structure (Fig. 22). In the early stages of differentiation during the tail formation there are endoplasmic reticulum cisternae surrounding the mitochondrial derivatives and the axoneme (Figs. 21 and 22). In this phase the acid phosphatase reaction product can be seen on the axoneme mainly surrounding the central pair of microtubules and scattered on the cytoplasm of the tail region (Fig. 24). At the late phases of spermiogenesis a light reaction product of



Figures 21 and 22: Cross section on flagellar region of *T. perditor* and *E. meditabunda* spermatids, respectively. The flagellum consists of the axoneme (Ax) with 9+9+2 microtubule pattern, two mitochondrial derivatives (Md) with two crystalline structures of different sizes (asterisks), and bridges (arrows) that connect the axoneme to the mitochondrial derivatives. Endoplasmic reticulum (Er); microtubules (Mt). Bars: 0,2 μm and 0,1 μm, respectively.

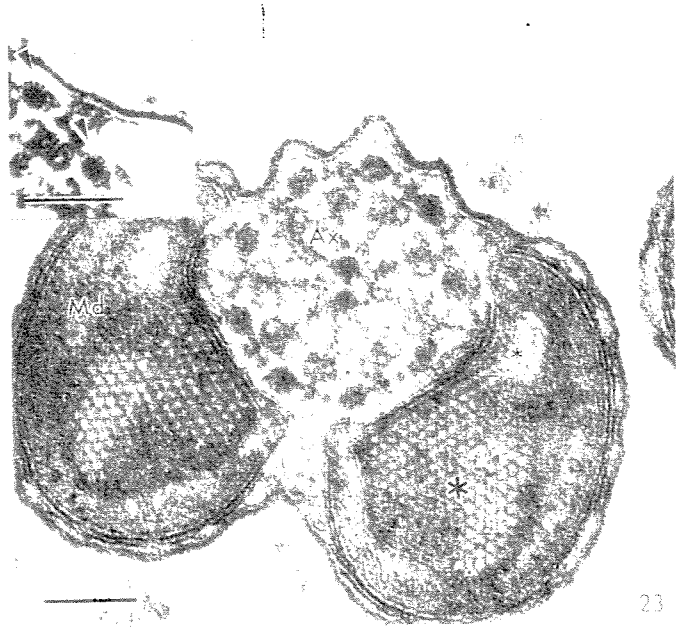
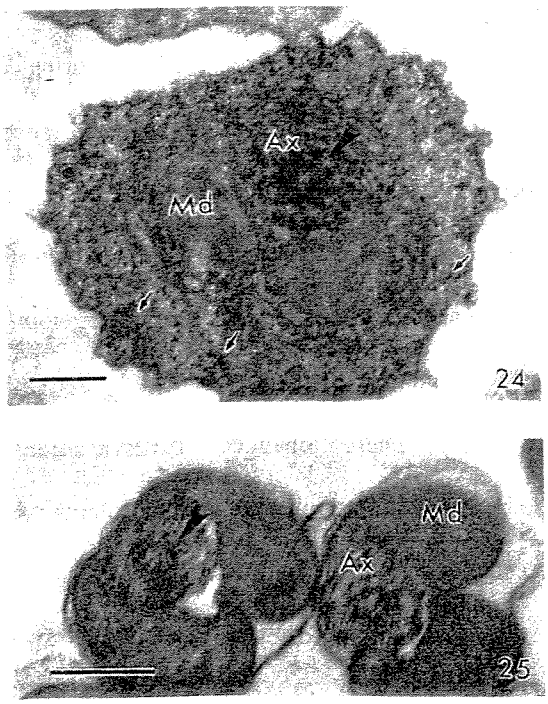


Figure 23: Cross section through flagellar region of *E. meditabunda* late spermatids, showing mitochondrial derivatives (Md) with crystalline structures (asterisks) and the axoneme (Ax). The accessory microtubules (arrowhead) appear with 16 protofilaments (inset). Bars: 0,05 μm



Figures 24 and 25: Sections through intermediate and late stages of *T. perditor* spermatids showing the acid phosphatase and glucose-6-phosphatase activity (arrowhead), respectively, on the axoneme (Ax) and scattered on the cytoplasm of intermediate spermatids (arrow). Mitochondrial derivatives (Md). Bars: 0,1 µm and 0,2 µm, respectively.

glucose-6-phosphatase can be observed on the axoneme (Fig. 25).

During almost all this differentiation process a

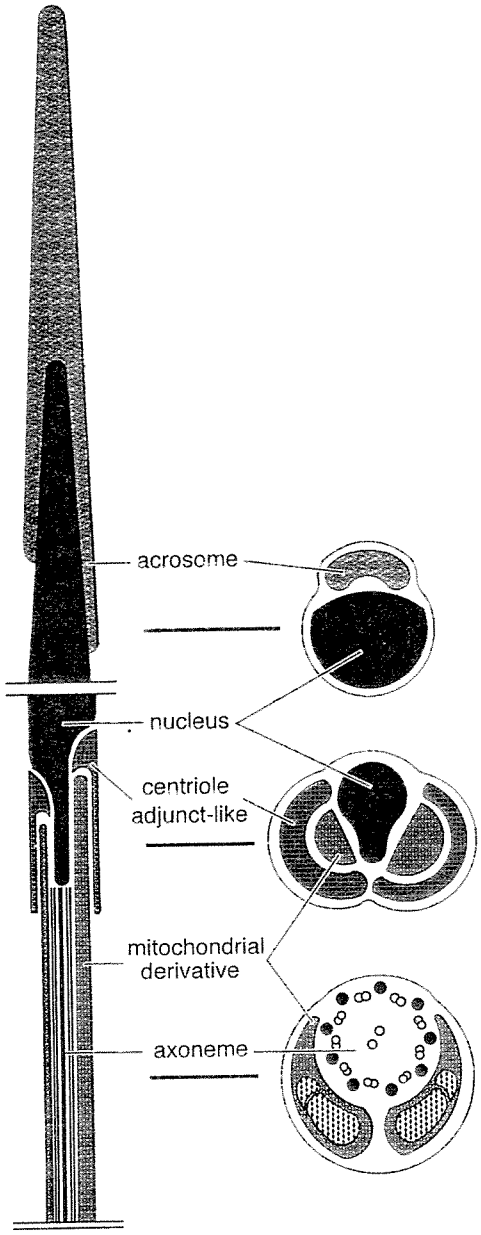


Figure 26: Diagram of the spermatozoon, and corresponding transverse section. Scales of various components are only approximate. Bar = 0,5 µm.

large number of microtubules was observed surrounding the nucleus (Figs. 8, 9,15 and 16), but only a few cytoplasmic microtubules were observed in the flagellar region (Fig. 22). At the end of spermatid elongation the microtubular material is eliminated with the disappearance of the cytoplasmic microtubules in the spermatozoa (Fig. 11and 23).

The spermatozoa of *E. meditabunda*, *P. guildini* and *T. perditor*, after finished the differentiation process are filiform, consisting of a head (acrosomal complex and nucleus), and a tail region (axoneme and mitochondrial derivatives) connected by a centriolar adjunct-like structure (Fig. 26).

DISCUSSION

The insects utilized in this study - *Edessa meditabunda* (Edessinae), *Piezodorus guildini* and *Thyanta perditor* (Pentatominae) (Hemiptera, Heteroptera, Pentatomidae) (for taxonomic reviews see 36) - present the spermiogenesis process very similar to that observed in other Pentatomids (32).

In these species, as well as in the majority of insects, the development of germinative cells takes place within cysts (37). During the spermiogenesis the spermatids undergo specific morphofunctional modifications which involve nuclear elongation, chromatin condensation, acrosomal formation, and flagellar development with axoneme and mitochondrial derivatives formation. These changes involve transient establishment of cytoplasmic compartments related to cell elongation and cytoplasmic elimination.

As described for other insects (32, 38 - 40), the acrosome is derived from Golgi complex, or again, the Golgi complex contributes with material to the developing acrosomic system, like at occur in mammalian spermatids (41, 42). In the great part of the insects, where this process has been studied, the formation of a spherical body, the proacrosomal granule, occurs on the concave side of the Golgi complex. This vesicle, found in early spermatids, between the Golgi complex and the nucleus, is gradually modified, taking on a characteristic shape in the last stages of spermiogenesis (38, 39). The acid phosphatase activity has been associated with the Golgi

complex, because by definition, the trans Golgi network is the site where proteins finally exit from the Golgi to their respective cellular sites (43, 44), i.e. plasma membranes, secretion granules and lysosomes. In contrast to that occurs with *A. aseadum* and *N. viridula* spermatids (32) the proacrosomal vesicle of *T. perditor* presents the acid phosphatase activity product scattered in this structure. The acrosome formation of *E. meditabunda*, *P. guildini* and *T. perditor* pass through distinct phases: first it consists of an amorphous and electron dense mass, which presents small electron dense pits probably connecting this mass to the nucleus by a lamina-like structure; subsequently, it shows a tubular arrangement. Already, in mature spermatozoon the acrosome present a three-layered arrangement, that consists of an electron lucent inner cone, the acrosomal content with tubular arrangement, and an outermost extra-acrosomal thin layer. These characteristics of acrosome formation and mature acrosome differ from what described for some other heteropterans (27, 45 - 47), but are similar to that occur in *A. aseadum* and *N. viridula* (32). As the acrosome of spermatozoa is involved in the penetration of this cell through the membrane of the oocyte, it is natural that acrosomic system contains several hydrolytic enzymes including acid phosphatase and glucose-6-phosphatase (48 - 50), and may therefore be considered a lysosome-like organelle (51), which can redistribute its enzymes considerably different during acrosome maturation, besides this distribution vary among species (38, 50, 52, 53).

Sperm nucleus development is characterized by a change from a spherical to an elongated shape. The nuclear materials pass through a stage of conversion from a loose to a more compact form. These events follow the pattern described for *Eurydema ventralis* (54), *Leptocoris trivittatus* (45), *Triatoma infestans* (55). Similar to that occur with *A. aseadum* and *N. viridula* (32) the nucleus shows the chromatin with fibrilar aspect since intermediate stages until final stages of development. In the immediately anterior stage before total coalescence of the chromatin, the nucleus shows its content with two distinct regions: one near to the acrosome, with compacted

chromatin and another, more posterior, with fibrilar arrangement. These results indicate the existence of a variety of chromatin arrangement during spermiogenesis, suggesting that these organization patterns may be characteristic of the spermatid differentiation stage.

In early spermatids the centriolar adjunct is located posterior to the nucleus, in the region of flagellum implantation. This structure appears electron dense with some electron lucid areas. As suggested by Taffarel and Esponda (56) the centriolar adjunct - which is associated with the centrioles - can functioning as a connection piece between the nucleus and flagellum. At indeed may participate in the developing axial tail filament bundle similar to what described for grasshopper spermatids (57). Although other related articles do not any mention of the presence of centriolar adjunct in late spermatids of mature spermatozoa. The late spermatids analyzed in this study show a centriolar adjunct-like structure similar the centriolar adjunct described in some Hymenoptera (58, 59), which involve the initial part of the mitochondrial derivatives and that probably acts as a mechanical support to the flagellar axis. The electron dense rod, that appear in the young spermatids and disappears in the final stages of spermiogenesis seems to be involved in microtubule organization as suggest anteriorly (32, 60)

In the initial stages of differentiation, the complex process of rearrangement and fusion of the mitochondria takes place giving rise to two mitochondrial derivatives of same shape and size flanking the axoneme along its length. The mitochondrial derivatives are occupied by two crystalline structures which are embedded in amorphous material. These crystalline structures may be an energy storage or it could play mechanical role, functioning as a stabilizing element for the very long sperm of these insects, as suggested for *Notonecta glauca* (61). The bridges that occur between the axoneme and mitochondrial derivatives are always located in the region of the small crystalline structures and microtubules doublets 1 and 5 as described for other heteropterans (30). Bridges with regular distribution between the flagellar organelles have

been shown in spermatozoa of *T. infestans* (29) and *Rhodinius prolixus* (31). Previous studies suggest that mitochondrial derivatives and their association with the other flagellar components play some role in the control of the movement of the axoneme (62), and regulation of the wavelength (37).

The axoneme presents the 9+9+2 microtubule pattern, i.e. 9 outer singlets + 9 intermediate doublets + 2 central singlets. The accessory microtubules are composed of 16 protofilaments and a dense lumen, similar to described for others Heteroptera (63). The presence of acid phosphatase activity on the axoneme of *T. perditor* intermediate stages is coincident with known descriptions of the spermatozoa of other insects (31, 50, 64, 65). This enzyme is apparently involved in the metabolism of phosphate important to flagellar motility. The detection of glucose-6-phosphatase activity surrounding the central pair of microtubules of late spermatid is similar to that occurs in some Coleoptera species (53), and suggest that this enzyme may also participate in the metabolism of phosphates.

During spermatid-spermatozoon differentiation numerous cytoplasmic microtubules are involved in the shaping of the cell, and these microtubules are eliminated simultaneously with the cytoplasmic remainder at the end of spermiogenesis. Microtubule participation in spermiogenesis has been described in other insects (32, 54, 66 - 69).

As described for other heteropteran species (27, 29, 31, 32, 70) the insects utilized in this study present the spermatozoon with two synapomorphic characteristics: 1) bridges between the axoneme and mitochondrial derivatives, and 2) two crystalline structures in the mitochondrial derivatives.

The data obtained with this studies give new information about basic biology of these insect-pests which could be contribute with the knowledge of their reproductive biology.

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4.2. **Fernandes, A.P.** & Báo, S.N. (2000). Ultrastructural and cytochemical studies of the spermatozoa of *Acrosternum aseadum* (Hemiptera: Pentatomidae) after copulation. *J. Submicrosc. Cytol. Pathol.* 32(4): 547-553.

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Ultrastructural and cytochemical studies of the spermatozoa of *Acrosternum aseadum* (Hemiptera: Pentatomidae) after copulation

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SUMMARY - Beside the morphofunctional modifications undergone during spermiogenesis, the spermatozoon could undergo other modifications after copulation. Since no structural modification occurs in the spermatozoon of *Acrosternum aseadum* after copulation, we used cytochemical studies to show the enzymatic activities variations of acid phosphatase, thiamine pyrophosphatase, glucose-6-phosphatase and cytochrome C oxidase, when the spermatozoon passes through the spermatheca. The enzymatic activity, few hours after copulation, is strong and specifically located. However, 40 h after copulation, there is considerable loss of enzymatic activity, with the exception of thiamine pyrophosphatase, which shows the same activity. This result indicates that the spermatozoon of *A. aseadum* undergoes physiological modifications when passing through the spermatheca and that these modifications may be involved with survival in this organ as well as with the fertilization process.

KEY WORDS *Acrosternum aseadum - cytochemistry - enzymes - hemiptera - spermatozoa - ultrastructure*

INTRODUCTION

The spermatozoon is the result of a complex process of cellular differentiation. During this process, morphofunctional modifications occur based on biochemical and cytochemical changes (for reviews see Phillips, 1970; Baccetti, 1972; Fawcett, 1975). The constructive and regressive cellular alterations occurring during spermiogenesis make spermatozoa capable of reaching and fertilizing immotile oocyte. They lose various organelles essential to cell metabolism while the remaining ones are modified as in no other process of cell differentiation (Phillips, 1974; Baccetti and Afzelius, 1976). The main compartments of a typical insect spermatozoon are the head, containing nucleus and acrosome, and the tail, which contains axoneme and mitochondrial derivatives (Phillips, 1970; Baccetti, 1972). After copulation, the spermatozoon can undergo other modifications in the spermatheca, an organ of the female reproductive system of many invertebrates, which is the main responsible for the storage of spermatozoa from copulation up to the fertilization process. The cytochemical study is useful to determine the functional role of the different elements of the spermatozoon, in its movement and in the fertilization process. The present paper analyses the localization of acid phosphatase, cytochrome C oxidase, glucose-6-phosphatase and thiamine pyrophosphatase in spermatozoa of *Acrosternum aseadum*, which is one of the main pests of economically important crops throughout the world (Panizzi, 1997).

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MATERIALS AND METHODS

The insects utilized were female adults of the phytophagous bug *Acrosternum aseadum* (Hemiptera: Pentatomidae), obtained from a colony maintained in the National Center of Genetic Resource (CENARGEN), Brasília, Brazil.

Transmission electron microscopy

Spermatheca from mated females were dissected and fixed for 4 h in 2.5% glutaraldehyde, 4% paraformaldehyde, 5 mM $CaCl_2$ and 3% sucrose, buffered with 0.1 M sodium cacodylate, at pH 7.2. After fixation, the specimens were rinsed in 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, 1988). The material was dehydrated in a series of ascending acetones (30%-100%) and embedded in Spurr. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a JEOL JEM 100C or in a Zeiss 912 transmission electron microscope.

Enzyme cytochemistry

Spermatheca from mated females were dissected 20 h and 40 h after the beginning of copulation, and were briefly fixed for 15 min at 4 °C in 1% glutaraldehyde buffered with 0.1 M sodium cacodylate buffer, pH 7.2. After fixation, the specimens were washed with buffer and used for the cytochemical experiments.

Acid phosphatase activity - The fixed-washed spermatheca were incubated for 1 h at 37 °C in the following medium: 0.1 M Tris-maleate buffer, pH 5.0, 7 mM cytidine-5'-monophosphate, 2 mM cerium chloride and 5% sucrose (Pino *et al.*, 1981). For controls the substrate was omitted.

Glucose-6-phosphatase activity - The fixed-washed spermatheca were incubated for 1 h at 37 °C in a medium containing 5 mM glucose-6-phosphate, 5 mM manganese chloride, 4 mM cerium chloride, 5% sucrose and 0.1 M Tris-maleate buffer, pH 6.5 (Robinson and Karnovsky, 1983). The controls were incubated in the same medium from which the substrate was omitted.

Thiamine pyrophosphatase activity - The fixed-washed spermatheca were incubated for 1 h at 37 °C in a medium containing 2.2 mM thiamine pyrophosphate, 5 mM manganese chloride, 4 mM cerium chloride, 5% sucrose and 0.1 M Tris-maleate buffer, pH 7.2 (Angermuller and Fahimi, 1984). The controls were incubated in the same medium from which the substrate was omitted.

Cytochrome C oxidase activity - The fixed-washed spermatheca were incubated for 1 h at 37 °C in a medium consisting of 3 mM 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma), 10 mM cytochrome C (Sigma), and 5% sucrose in 0.1 M phosphate buffer, pH 7.2 (Seligman *et al.*, 1968). In control preparations, cytochrome C was omitted and 5 mM potassium cyanide was added to the incubation medium.

After incubation of the spermatheca in one of the media as described above, the specimens were washed with cacodylate buffer and fixed again overnight at 4 °C in a solution containing 4% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Then, the specimens were washed in clean buffer, and postfixed in a solution containing 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM calcium chloride in 0.1 M sodium cacodylate buffer. Subsequently, they were dehydrated in acetone and embedded in Spurr. Thin sections were stained with uranyl acetate and lead citrate, and examined in a JEOL JEM 100C or in a Zeiss 912 transmission electron microscopy.

RESULTS

The spermiogenesis of *Acrosternum aseadum* has been described in detail previously (Fernandes and Báo, 1998) as well as the detection of enzymatic activity during this

FIGURES 1 and 2 Transverse section on spermatozoon of *A. aseadum* showing the compact nucleus (N), an acrosome (A), the tail with 9 + 9 + 2 microtubule pattern on the axoneme (Ax) and two mitochondrial derivatives (Md). Bar = 0.2 µm.

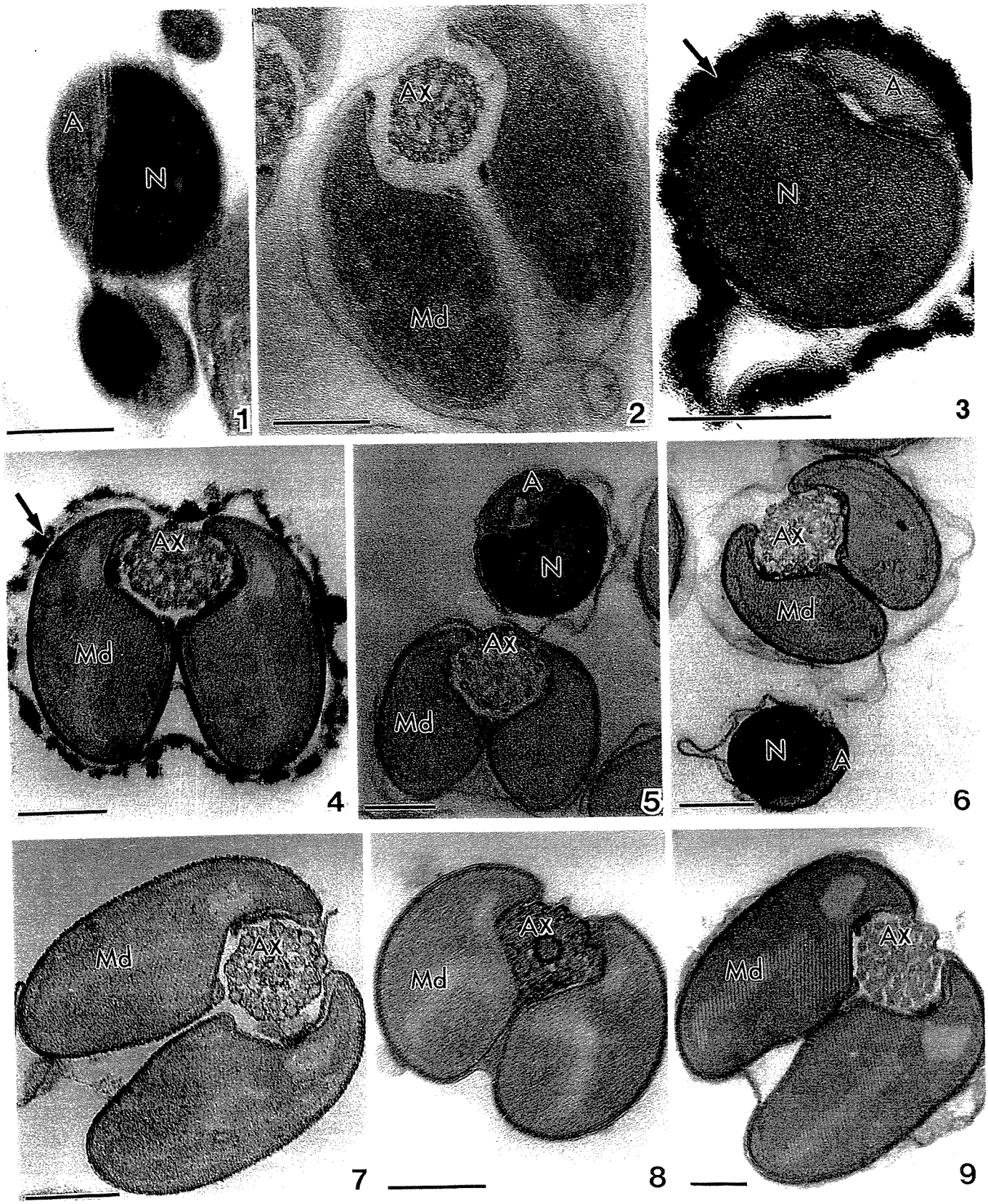
FIGURES 3 and 4 Localization of acid phosphatase activity 20 h a.c. The reaction product is associated with the plasma membrane (arrows). Acrosome (A); axoneme (Ax); mitochondrial derivatives (Md); nucleus (N). Bar = 0.2 µm.

FIGURE 5 Localization of acid phosphatase activity 40 h a.c. No reaction product was observed. Acrosome (A); axoneme (Ax); mitochondrial derivatives (Md); nucleus (N). Bar = 0.2 µm.

FIGURE 6 The spermatozoon prepared as a control, showing no enzymatic reaction product. Acrosome (A); axoneme (Ax); mitochondrial derivatives (Md); nucleus (N). Bar = 0.2 µm.

FIGURES 7 and 8 Localization of thiamine pyrophosphatase activity 20 h and 40 a.c. respectively. Reaction product was observed on the axoneme (Ax) of the spermatozoa. Mitochondrial derivatives (Md). Bar = 0.2 µm.

FIGURE 9 Spermatozoa prepared as a control of thiamine pyrophosphatase activity. No reaction product was found. Axoneme (Ax); mitochondrial derivatives (Md). Bar = 0.2 µm.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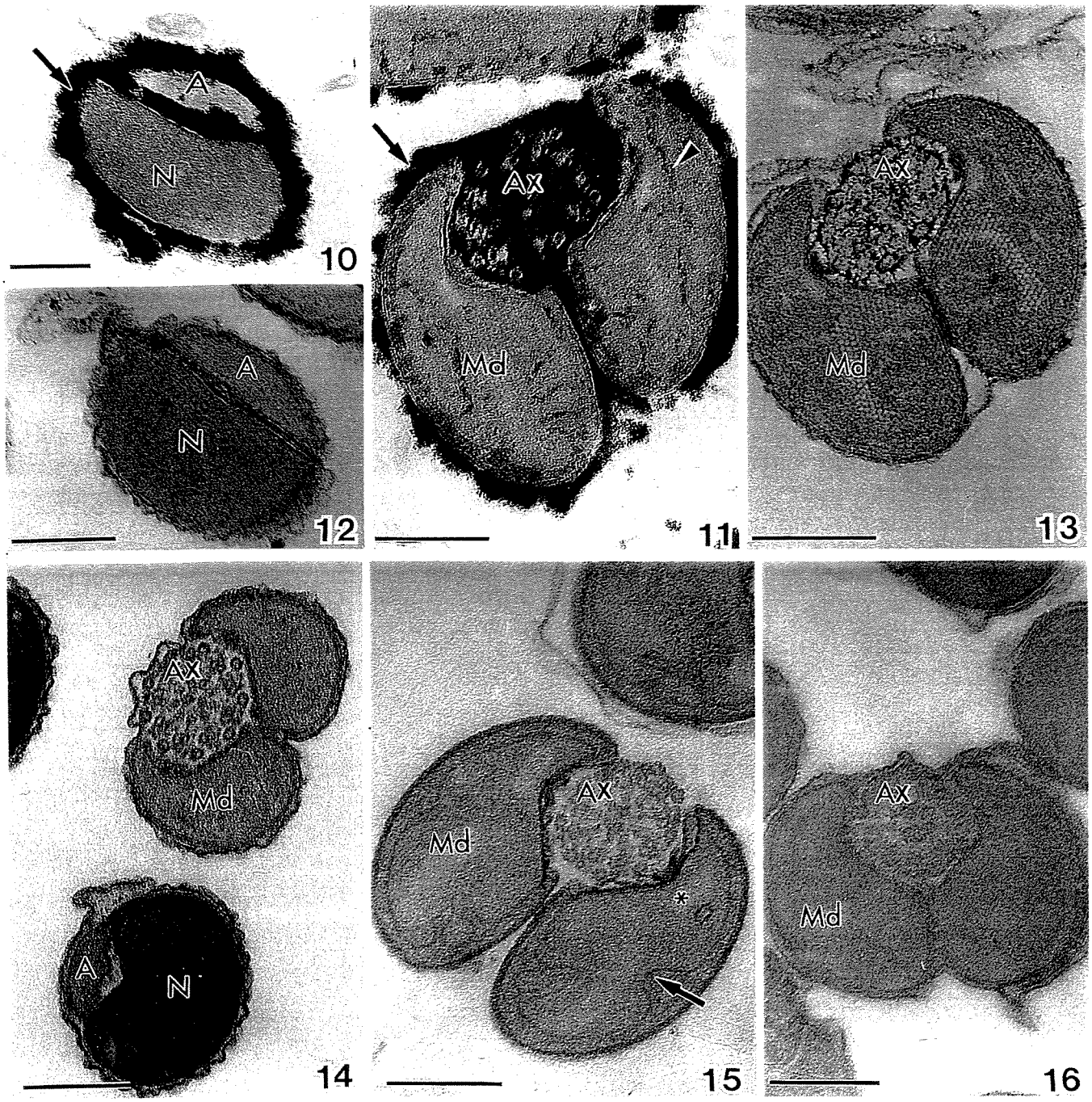

FIGURES 10 and 11 Localization of glucose-6-phosphatase activity 20 h a.c. Transverse sections through *A. aseadum* spermatozoa showing the reaction product on plasma membrane (arrows) on the basal portion of acrosome (A) and on the axoneme (Ax). The reaction product is also observed in the membrane complexes (arrowhead) that surround the paracrystalline structures in the mitochondrial derivatives (Md); nucleus (N). Bar = 0.2 μm.

FIGURES 12 and 13 Localization of glucose-6-phosphatase activity 40 h a.c. No reaction product was observed on plasma membrane. A light deposition was found in the axoneme (Ax). Acrosome (A); mitochondrial derivatives (Md); nucleus (N). Bar = 0.2 μm.

FIGURE 14 Spermatozoa prepared as control of glucose-6-phosphatase activity. No reaction product was observed. Acrosome (A); axoneme (Ax); mitochondrial derivatives (Md); nucleus (N). Bar = 0.2 μm.

FIGURE 15 Localization of cytochrome C oxidase activity 20 h a.c. A light reaction product was found on the remaining cristae (arrow) surrounding the paracrystalline structures (asterisk). Axoneme (Ax); mitochondrial derivatives (Md).

FIGURE 16 Localization of cytochrome C oxidase activity 40 h a.c. No reaction product was found. Axoneme (Ax); mitochondrial derivatives (Md).

process (Fernandes and Báo, 1999). The spermatozoa of these insects are highly differentiated cells, which consist of two morphologically and functional distinct regions: an elongate rod-like head, which contains the nucleus and the acrosome (Fig. 1), and the tail, which is formed by an axoneme and two mitochondrial derivatives (Fig. 2).

Acid phosphatase activity

Incubation of spermatozoa in a medium containing cytidine-5'-monophosphate as substrate, and cerium as capture agent, led to the appearance of an electron dense reaction product, indicating the presence of acid phosphatase activity. Twenty hours after copulation (a.c.) the reaction product was seen on the plasma membrane surrounding the head (Fig. 3) and the tail (Fig. 4). However, no reaction product was observed in the acrosome, neither in the axoneme. Fourty hours a.c. no reaction product was observed in the spermatozoa (Fig. 5). The plasma membrane showed no reaction product, as well as the other structures of spermatozoa. No reaction product was found in the control spermatozoa (Fig. 6).

Thiamine pyrophosphatase activity

Reaction product, indicative of thiamine pyrophosphatase, was seen on the axoneme of spermatozoon, mainly around the central pair of microtubules and surrounding the accessory ones, 20 h a.c. (Fig. 7) and 40 h a.c. (Fig. 8). No reaction product was observed on the mitochondrial derivatives. In the spermatozoa prepared as control no reaction product was observed (Fig. 9).

Glucose-6-phosphatase

Following incubation of spermatozoa in a medium containing glucose-6-phosphate as substrate and cerium chloride as capture agent, reaction product was seen, 20 h a.c., in association with plasma membrane (Figs. 10 and 11) as well as with the basal portion of the acrosome (Fig. 10). A considerable deposit was observed on the axoneme (Fig. 11), in the radial spokes that extend from the central pair of microtubules towards the 9 microtubule doublets, as well as surrounding the accessory microtubules. The reaction product was also observed on the internal membrane of mitochondrial derivatives and surrounding the paracrystalline structures. Fourty hours a.c. no reaction product was found on the plasma membrane, neither on the acrosome (Fig. 12). A light deposit of reaction product was observed on the axoneme (Fig. 13) surrounding the central pair of microtubules as well as the accessory ones. No reaction product was found in the control spermatozoa (Fig. 14).

Cytochrome C oxidase activity

After incubation in a medium with cytochrome C as substrate and diaminobenzidine tetrahydrochloride (DAB) as capture agent, the cytochrome C oxidase activity was evidenced 20 h a.c. in the mitochondrial derivatives on the remaining cristae that surround the paracrystalline structures (Fig. 15). Fourty hours a.c. no reaction product was found on the spermatozoa (Fig. 16). In the spermatozoa prepared as control no reaction product was observed (not shown).

DISCUSSION

The spermiogenesis process involves the structural and physiological transformation of organelles to more adapted forms, leading to the fertilization process. These events, and the participation of some enzymes involved in this remodeling on *Acrosternum aseadum* spermiogenesis were previously described (Fernandes and Báo, 1998, 1999).

In most invertebrates species, the spermatozoon, after copulation, passes through the spermatheca – an organ of the female reproductive system associated with sperm storage, digestion and absorption, according to the ultrastructural and cytochemical studies (Reeder and Rogers, 1979; Rogers *et al.*, 1980; Kitajima and Paraense, 1983). In spermatheca several morphological modifications occur, as in some gastropods (Selmi *et al.*, 1989), and in some insects (Hughes and Davey, 1969; Rieman, 1970). In other invertebrate species, as occurs in *Aiolopul strepens* (Renieri and Talluri, 1974) and in *Acrosternum aseadum*, the spermatozoon in female spermatheca maintains all the ultrastructural characteristics it had in the testes, whereas its physiological features undergo modifications.

As observed previously, during acrosome maturation in the testes, the response to the acid phosphatase reaction is considerably modified (Báo *et al.*, 1989; Souza and Azevedo, 1986; Furtado and Báo, 1996; Fernandes and Báo, 1999), also in the female spermatheca. Twenty hours after copulation no reaction product of acid phosphatase activity was found in the acrosome, as observed in the testes. However, a strong reaction product was observed on the plasma membrane of spermatozoa on the head and on the tail. This result suggests that acid phosphatase in the plasma membrane could function as a hydrolytic enzyme to help the spermatozoa absorb the energy source of spermatheca secretion. Spermatheca secretion as an energy source was reported for various species (Davey and Webster, 1967; Gupta and Smith, 1969; Happ and Happ, 1970; Bhatnagar and Musgrave, 1971; Hueber, 1980). This mass could be digested by acid phosphatase found on the spermatozoon membrane to supply energy for other modifications occurring in the spermatozoa and for the survival of these cells in the spermatheca for long periods. The loss of acid phosphatase activity on the membrane of

spermatozoa 40 h after copulation could indicate that there are no energy sources to digest after some hours, or that the digestion capability decreases with time.

The presence of acid phosphatase activity on the axoneme, 20 h after copulation, could be involved in the metabolism of phosphate, important to flagellar motility. Indeed, this enzyme could regulate the content of hydrolyzable substrate and the activities of other enzymes – as glucose-6-phosphatase and tiamine pyrophosphatase – in the axial filament. It could also control the free phosphates pool necessary for the resynthesis of ATP and other high-energy phosphate compounds used in the kinetic and metabolic functions of the spermatozoon, as suggested by Allison and Hartree (1970). The loss of this enzymatic activity 40 h after copulation could indicate that the spermatozoon movement capability has ceased.

Although the glucose-6-phosphatase normally catalyses glucose production, it could be functioning as an hydrolytic enzyme. The cytochemical localization of this enzyme in the spermatids of *Acrosternum aseadum* (Fernandes and Báo, 1999) and its detection on the basal portion of the acrosome support this hypothesis. Since glucose can not be stored in significant amounts in the spermatozoon it must be ejected from another source, that in this case could be the spermatheca secretion. The presence of glucose-6-phosphatase activity on the plasma membrane of spermatozoon 20 h after copulation could indicate that this enzyme, together with acid phosphatase, takes part in the digestion of spermatheca secretion and also in the incorporation of energy from another source. The presence of this enzyme on the internal membrane of mitochondrial derivatives and around the paracrystalline structures confirms this hypothesis, since mitochondrial derivatives function is related to the production of energy to flagellar movement (Perotti, 1973; Tokuyasu, 1974, 1975). The presence of glucose-6-phosphatase activity on the axoneme could be related with phosphate metabolism for flagellar movement, supporting the action of acid phosphatase and thiamine pyrophosphatase in this region. The loss of glucose-6-phosphatase activity on the plasma membrane of spermatozoon 40 h after copulation corroborates the statement that after some time there is no spermatheca secretion to digest or that the spermatozoon metabolic capability decreases with time. The light reaction product deposits on the axoneme of spermatozoon 40 h after copulation could be the rest of metabolic activities in this area related with flagellar movement.

Thiamine pyrophosphatase is an important indicator of cellular metabolic activity (Thomopoulos *et al.*, 1992). Indeed, as observed by Nelson (1959, 1962) pyrophosphate inhibits flagellar ATPase activity and a Mg^{++} dependent inorganic pyrophosphatase localized in the vicinity or the axonemal elements in spermatozoa. Under this scheme suggested by Nelson (1962, 1967) the interaction of complexes formed between pyrophosphate and ATP with Mg^{++} ion under mediation of a pyrophosphatase, could provide the mechanism of contraction and relaxation during sperm motility. By this way, the presence of thiamine pyrophosphatase activity on the axoneme of the spermatozoon 20 h and 40 h after copulation indicates that this enzyme has a metabolic activity related with flagellar movement, and that this activity is not lost with time. The loss of thiamine pyrophosphatase activity on the paracrystalline structures of mitochondrial derivatives indicates that there could be a change of metabolic activity in the spermatozoon after copulation.

The cytochrome C oxidase activity observed on the membrane complexes that surround the paracrystalline structures 20 h after copulation was also observed in other insect species (Bigliardi *et al.*, 1970; Baccetti *et al.*, 1973*a*; Perotti, 1973; Báo *et al.*, 1992). This result confirms the idea that after copulation the spermatozoon changes its metabolism, since in the spermatozoon in the testes this enzyme activity was not observed. As cytochrome C oxidase activity could be related with respiration (Andre, 1962; Baccetti *et al.*, 1973*b*), its presence in this area indicates that spermatozoon metabolism is active, and that this activity is lost with time.

Although morphological modifications cannot be observed in the spermatozoon found in spermatheca, there are no doubts that several physiological changes occur in these cells before fertilization. These changes could be related with the preparation of spermatozoa for fertilization or again could be related with the survival of these cells in the spermatheca for long periods.

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Nuclear changes and acrosome formation during spermiogenesis in *Euchistus heros* (Hemiptera: Pentatomidae)

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Abstract. Ultrastructural and cytochemical studies were carried out on nuclear changes and acrosome formation during the spermiogenesis of the phytophagous bug *Euchistus heros*. The development of the nucleus involves changes in the shape and in degree of chromatin condensation: initially it is dispersed and with a low-electron density, then assumes a fibrillar arrangement and finally compacts in an electron-dense material. The acrosome is formed by the Golgi complex and presents unusual morphological features during its development. The reaction product of acid phosphatase, glucose-6-phosphatase and thiamine pyrophosphatase activities were detected during various stages of acrosome development. In contrast, residues of α-N-acetylgalactosamine and basic proteins were only reported in the intermediate and late stages of the differentiation process, respectively. © 2001 Harcourt Publishers Ltd

Keywords: bugs, insect, lectins, phosphatases, phytophagous, spermatozoon

Introduction

The spermatozoon is a highly specialized cell which has many unusual features. The main compartments of a typical insect spermatozoon consist of the head (nucleus and acrosome) and the tail (axoneme and mitochondrial derivatives) (for reviews see Phillips, 1970; Baccetti, 1972). Sperm nucleus development is characterized by the transition from a spherical into a highly asymmetric configuration and by the chromatin conversion from a dispersed to a very condensed state (Tokuyasu, 1974). The process of sperm chromatin condensation occurs in a specific fashion which can be characteristic of both the differentiation stage and species (Werner & Bawa, 1988).

The acrosome is essential for the recognition and penetration of the sperm within the egg, leading to fertilization. This organelle is formed by the Golgi complex (Phillips, 1970; Baccetti, 1972). The acrosome development begins with a spherical body, the proacrosomal granule. This structure results from the fusion of vesicles produced by the Golgi complex, and is gradually modified until it reaches its final shape. The size, shape and internal structure of the mature acrosome are variable for the different animal species (Anderson & Personne, 1975).

The morphogenetic changes that occur during spermiogenesis involve the participation of several enzymes (including phosphatases) and carbohydrate-rich molecules (Yanagimachi, 1994). In recent published accounts on spermiogenesis, the detection of several enzymes and/or carbohydrate rich molecules in insects has been done throughout cytochemical and biochemical studies (Perotti & Riva, 1988; Báo et al., 1989; Báo & de Souza, 1992, 1994; Craveiro & Báo, 1995; Furtado & Báo, 1996;

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Cattaneo et al., 1997; Báo, 1997; Fernandes & Báo, 1999; Pasini et al. 1999).

A cytochemical approach is useful to determine the functional role of the different sperm elements in the movement and in the fertilization process, and particularly to detect the role of enzymes and carbohydrate-rich molecules during the differentiation process of these cells. We use ultrastructural and cytochemical techniques to analyze the morphological changes of the nucleus and the acrosomal complex formation during the *Euchistus heros* spermiogenesis. This insect is polyphagous, feeding on soybean, legumes, and on some species of Solanaceae, Brassicaceae and Compositae (Panizzi, 1997), and is therefore considered a pest of economically important crops throughout the world.

Materials and methods

The insects studied were adult male of the phytophagous bug *E. heros* (Hemiptera, Pentatomidae), obtained from a laboratory colony reared at the National Center of Genetic Resource (CENARGEN), Brasília, Brazil.

Transmission electron microscopy

Part of the material was fixed for 4 h at 4 °C in 2.5% glutaraldehyde, 4% paraformaldehyde, 5 mM $CaCl_2$ and 3% sucrose, in 0.1 M sodium cacodylate buffer, pH 7.3. 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 cacodylate buffer. The material was dehydrated in a graded series of acetone (30–100%) and embedded in Spurr. Ultrathin sections were stained with uranyl acetate and lead citrate.

For the alcoholic phosphotungstic acid method (E-PTA), the procedure used was that reported by Bloom and Aghajanian (1968). Specimens were block stained with a solution of 3% PTA in absolute ethanol for 16 h at 4 C. The material was then embedded and sectioned as above described; their sections were observed without staining.

Enzyme cytochemistry

The testes were dissected and briefly fixed for 15 min at 4 C in 1% glutaraldehyde buffered with 0.1 M sodium cacodylate pH 7.2. After fixation, the specimens were washed with buffer and incubated for 1 h at 37 C in the following media:

1. *Acid phosphatase activity:* 0.1 M Tris-maleate buffer, pH 5.0, 7 mM cytidine-5-monophosphate, 2 mM cerium chloride and 5% sucrose (Pino et al., 1981);
2. *Glucose-6-phosphatase activity:* 5 mM glucose-6-phosphate, 5 mM manganese chloride, 4 mM ceriumchloride, 5%sucrose and 0.1 M Tris-maleate buffer, pH 6.5 (Robinson & Karnovsky, 1983).
3. *Thiamine pyrophosphatase activity:* 2.2 mM thiamine pyrophosphate, 5 mM manganese chloride, 4 mM cerium chloride, 5% sucrose and 0.1 M Tris-maleate buffer, pH 7.2 (Angermuller & Fahimi, 1984). The controls for acid phosphatase, glucose-6-phosphatase and thiamine pyrophosphatase activities were incubated in the same medium from which the specific substrates were omitted.

After incubation of the testes in one of the media as described above, the specimens were washed with sodium cacodylate buffer and fixed again for 3 h at 4 °C in a solution containing 4% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Then, the specimens were washed in clean buffer, and postfixed in a solution containing 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM calcium chloride in 0.1 M sodium cacodylate buffer. Subsequently, they were dehydrated in acetone and embedded in Spurr. Thin sections were stained with uranyl acetate and lead citrate.

Carbohydrate detection

For lectin labeling, testes were fixed for 3 h at 4 °C in a solution of 4% paraformaldehyde, 0.5% glutaraldehyde, 0.2% picric acid, 3.5% sucrose and 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer, pH 7.2. After several rinses in the same buffer, free aldehyde groups were quenched with 50 mM ammonium chloride in 0.1 M sodium cacodylate buffer for 1 h, followed by block-staining in 2% uranyl acetate in 15% acetone for 2 h at 4°C (Berryman & Rodewald, 1990). Specimens were dehydrated in 30–90% acetone. Embedding was performed in LRGold resin. Ultrathin sections were collected on nickel grids, pre-incubated in phosphate buffered saline (PBS) containing 1.5% bovine serum albumin (PBS-BSA) and 0.01% Tween 20, and subsequently incubated for 1 h at room temperature in the presence of *Helix pomatia* agglutinin (HPA) gold-labeled in PBS-BSA pH 8.0 at a dilution of 1:10. After incubations the grids were washed first with PBS, then in distilled water and finally stained with uranyl acetate and lead citrate. Controls consisted in the addition of 200–300 mM of the corresponding monosaccharide to the incubation medium. The lectin used was obtained from Sigma Chemical Company. The glycoprotein was labelled with colloidal gold particles (8–10 nm), according to Roth (1983).

All observations were performed in a Jeol 100C transmission electron microscope.

Results

The spermatids of *Euchistus heros* undergo specific morphofunctional modifications during spermiogenesis. The acrosome and flagellum formation occurs simultaneously with the nuclear transformations involving the shape and the degree of chromatin condensation.

During the early spermatid stage, the nucleus resembles that of somatic cells and presents electron dense areas of chromatin near the nuclear envelope (Fig. 1). Subsequently, there is a gradual condensation of the nuclear chromatin with an increase in its electron density, showing a granular aspect (Fig. 2). In the next stage, the nuclear chromatin shows a fibrillar arrangement (Fig. 3), and then continues with its condensation process, that occurs from the margin to the central portion of the nucleus. The chromatin may have a paracrystalline aspect (Fig. 4) before becoming completely compact, homogeneous and electron dense (Figs 5 & 6).

During the differentiation process, numerous microtubules can be observed surrounding the nucleus (Figs 2–5). At the end of the spermatid elongation, the cytoplasmic microtubules are eliminated (Fig. 6). Reaction products of the enzymatic activity as well as basic proteins have not observed during the nuclear differentiation process. Nevertheless, few α-N-acetyl-galactosamine residues, showed by the gold-labeled HPA, appear in the nucleus during late differentiation (Fig. 7).

The acrosome formation actively involves the Golgi complex. During the first stages of spermiogenesis, numerous vesicles of the Golgi complex are observed (Fig. 1). These vesicles join into a large proacrosomal vesicle (Fig. 1) which adheres to the nuclear envelope. At this stage, the acid phosphatase activity is located at the level of the cisternae of the Golgi complex, mainly on the cis and trans Golgi network; a diffuse weak reaction is also visible in the proacrosomal granule (Fig. 8). The reaction product of glucose-6-phosphatase activity is also observed in the Golgi complex, but only in the trans Golgi and trans Golgi network (Fig. 9).

Simultaneously with the chromatin condensation, and the nuclear and cellular elongation, a reorganization of the proacrosomal vesicle into an acrosomal complex is seen at the anterior end of the nucleus. In early spermatids, the proacrosomal vesicle presents an electron lucent cap appearance, surrounded by microtubules, and its content appears with a tubular arrangement (Fig. 2). Posteriorly, it becomes a large three-layered structure, consisting of: an electron dense inner cone which adheres to the nucleus; the acrosomal content, that shows a tubular organization: and an outermost extra acrosomal vesicle, with a granular aspect (Figs 3–5). The morphology of the acrosomal complex is maintained until the end of chromatin condensation. During this intermediate stage of development, acid phosphatase activity is scattered in the acrosomal vesicle (Fig. 10), in the plasma membrane and in the remnants of cytoplasm (Fig. 11). At the same stage, glucose-6-phosphatase activity is detected scattered in the acrosomal vesicle (Fig. 12) and thiamine pyrophosphatase activity is present on the membrane surrounding the acrosomal vesicle (Fig. 13). In sections from LRGold embedded material, the presence of α-N-acetyl-galactosamine shown by the gold-labeled HPA is initially evident in the acrosomal content, then it decreases progressively (Figs 14 & 15) and disappears at the end of spermiogenesis (Fig. 7). The posterior domain of the acrosomal vesicle regresses and the acrosomal complex appears more compact, assuming its final shape. At a later stage of spermiogenesis, acid phosphatase activity is detectable on the plasma membrane in the acrosomal vesicle (Figs 16 & 19); a glucose-6-phosphatase activity that appears only on the acrosomal membrane (Fig. 20). At this stage, a positive reaction of the acrosomal membrane to the ethanolic phosphotungstic acid treatment is observed (Fig. 18). Mature spermatozoa show a compact nucleus and two acrosomal regions: the inner one, which appears electron lucent and without sugar residues or phosphatase activities, and the outer one, whose membranes are positive to the glucose-6-phosphatase activity (Fig. 17).

The controls for enzymatic activities and detection of carbohydrate residues are negative (not shown), demonstrating the specificity of the reactions.

Discussion

During spermiogenesis, the spermatids undergo specific morphofunctional modifications which involve nuclear elongation, chromatin condensation, acrosomal formation and flagellar development with axoneme and mitochondrial derivatives formation. Several enzymes and glycoproteins may be involved in this remodeling, as well as in the chemical changes that occur during this process.

Sperm nucleus development of *E. heros* is characterized by a transition from a spherical to an elongated shape. This event follows the pattern described for other Heteropterans (Trandaburu, 1973; Itaya et al., 1980; Dolder, 1995; Fernandes & Báo, 1998). The organization of the nuclear material during spermatid differentiation resembles that of *Acrosternum aseadum* and *Nezara viridula*, previously reported by Fernandes and Báo (1998). Although the E-PTA method has shown positive results in spermatids of beetles (Báo & Hamú, 1993) and fruit-flies (Quagio-Grassiotto & Dolder, 1988). The presence of basic proteins has not been reported during nucleus development of *E. heros*. Thus, other molecules other those basic proteins participate in the chromatin condensation process in *E. heros*. Residues of α-N-acetyl-galactosamine have been detected using gold-labeled HPA lectin on the late spermatid nucleus of *E. heros*. Biochemical and cytochemical studies have demonstrated the presence of sugar residues in intracellular compartments, mainly in the nucleus, associated with the dense chromatin (Vannier-Santos et al., 1991; Báo & de Souza, 1992; Craveiro & Báo, 1995, Báo, 1997). Despite the fact that the role of nuclear glycoproteins is unclear, they seem to modulate the physicochemical environment of the nucleoplasm and

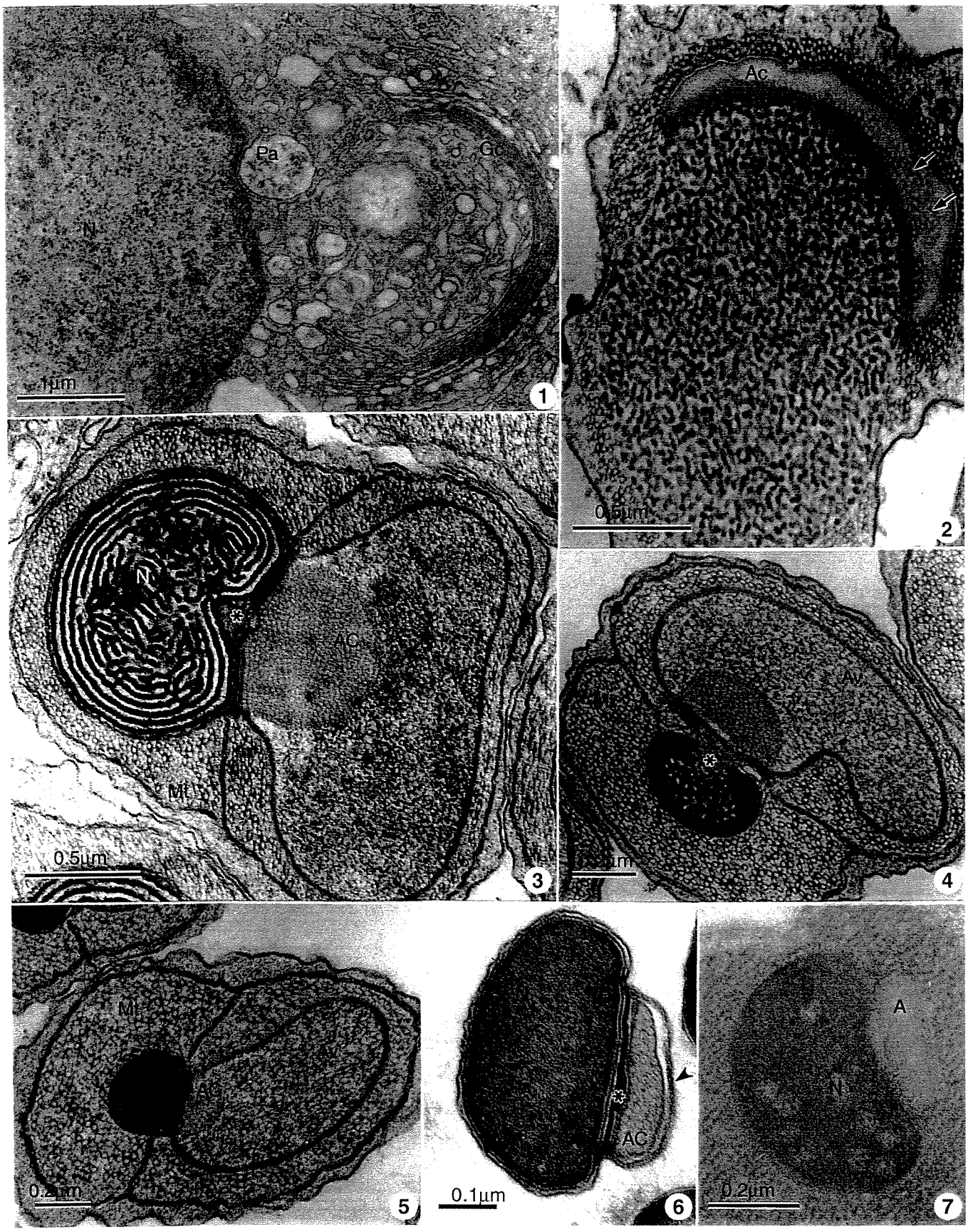
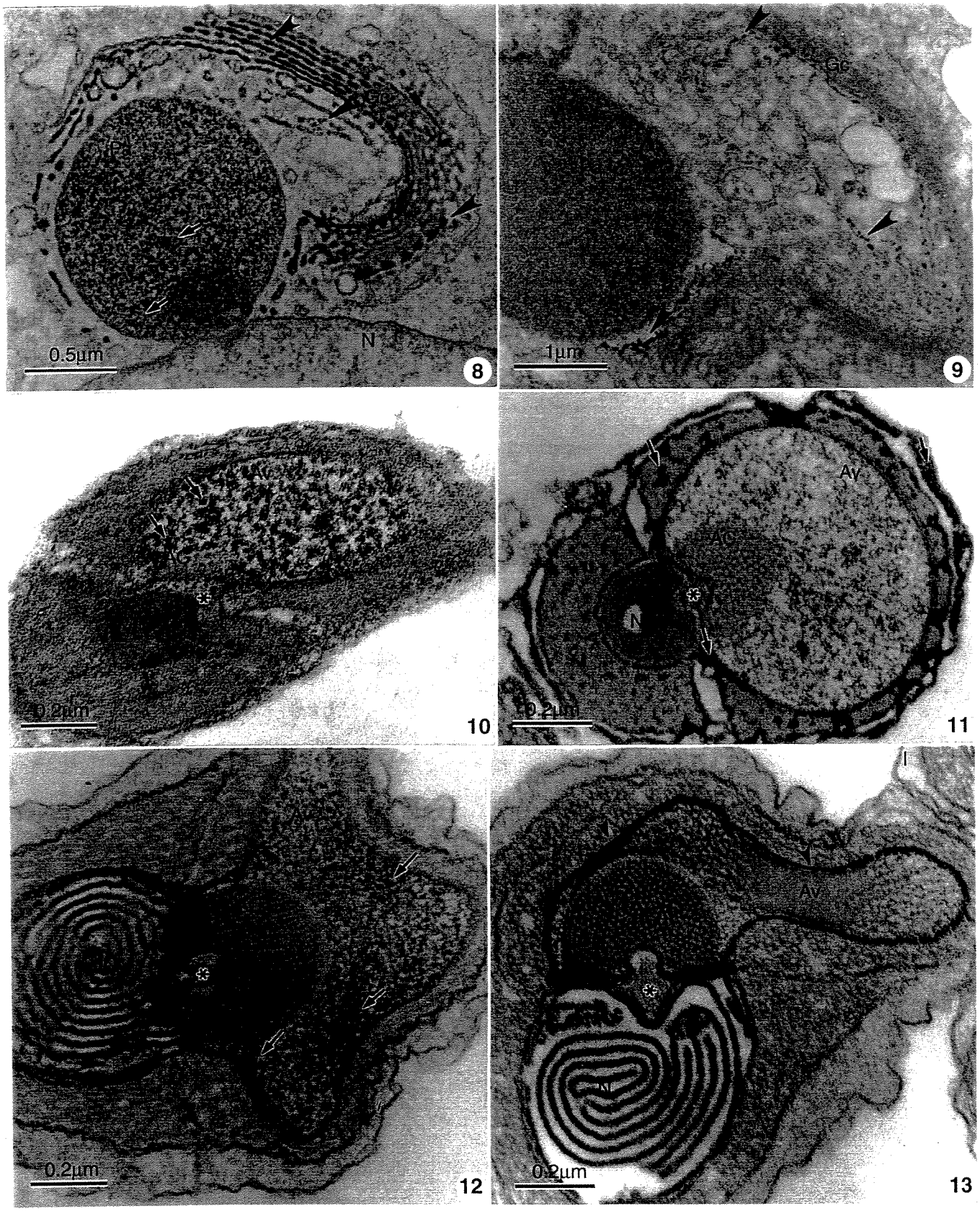
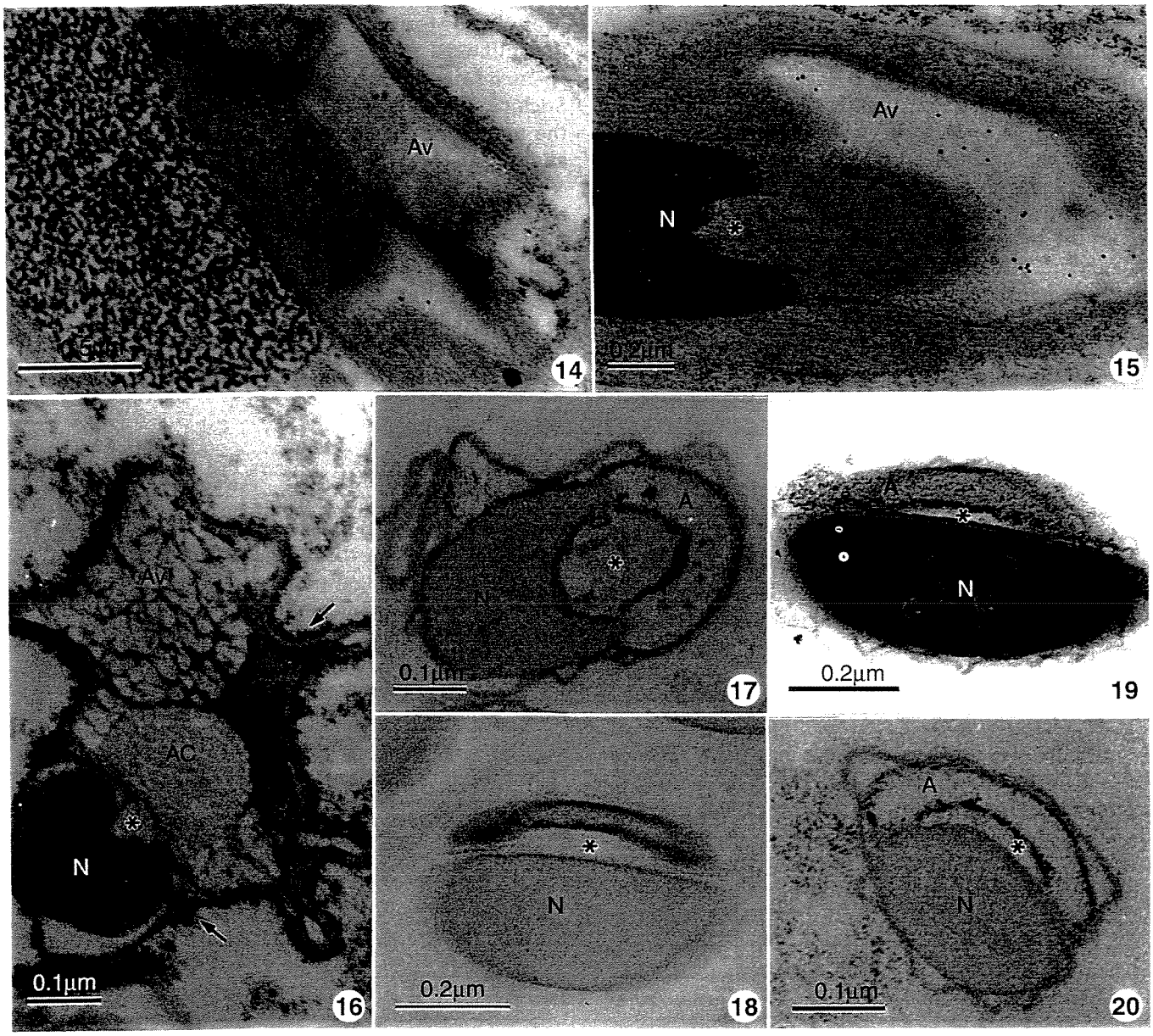


Fig. 1 Early spermatids showing the proacrosomal vesicle (Pa) formation from the Golgi complex (Gc). Nucleus (N). X 19 000 **Figs 2–6** Spermatids from early to late stages of differentiation showing the gradual condensation of chromatin and the acrosome formation. Acrosomal complex (Ac); acrosomal content (AC); acrosomal vesicle (Av); inner cone (*asterisk*); microtubules (Mt); tubular arrangement of acrosomal complex (*arrows*); outer most acrosomal layer (*arrowhead*); nucleus (N). X 42 000; X 42 000; X 56 000; X 52 000 and X 100 000, respectively. **Fig. 7** Late spermatids showing a light labelling (*arrow*) for *Helix pomatia* agglutinin (HPA) in some regions of the nucleus (N). Acrosome (A). X 83 000.



Figs 8 & 9 The reaction products of acid phosphatase and glucose-6-phosphatase activities, respectively, are associated with are Golgi, trans Golgi and trans Golgi network (*arrowheads*). Light reaction product of acid phosphatase activity (*arrows*) was observed on the proacrosomal granule (Pa). Nucleus (N); Golgi complex (Gc). X 32 000 and X 16 000, respectively. **Figs 10 & 11** Localization of acid phosphatase. Note that the electron dense reaction product (*arrows*) in the intermediate stage of differentiation is present mainly on the acrosomal vesicle (Av), and scattered on the cytoplasm and plasma membrane. Acrosomal content (AC); inner cone (*asterisks*); nucleus (N). X 67 600; X 69 300, respectively.

Figs 12 & 13 Localization of glucose-6-phosphatase and thiamine pyrophosphatase activities. The reduced reaction product of the first enzyme (*arrows*) is scattered on the acrosomal vesicle (Av), while an intense thiamine pyrophosphatase activity is located only on the acrosomal vesicle membrane (*arrowheads*). Acrosomal content (AC); inner cone (*asterisks*); nucleus (N). X 70 200 and X 70 200, respectively.



Figs 14 & 15 Spermatids in intermediate stage of development showing an intense labelling by *Helix pomatia* agglutinin (HPA), mainly on the acrosomal content (AC) and on the acrosomal vesicle (Av). Inner cone (*asterisk*); nucleus (N). X 39 000 and X 52 000, respectively. **Fig. 16** Localization of the acid phosphatase activity in the final stage of spermatid differentiation. The reaction product can be observed on the plasma membrane (*arrows*) and on the acrosomal vesicle (Av). Acrosomal content (AC); inner cone (*asterisk*); nucleus (N). X 112 000. **Fig. 17** Localization of glucose-6-phosphatase activity. Note the presence of the reaction product on the acrosomal membrane (A). Inner cone (*asterisk*); nucleus (N). X 112 000. **Figs 18–20** Detection of basic proteins by E-PTA, acid phosphatase and glucose-6-phosphatase activity, respectively, in the late spermatids. Note the presence of basic proteins and the enzymes on the acrosomal membrane (A). Nucleus (N); inner cone (*asterisk*). X 85 000; X 85 800 and X 145 200, respectively.

or participate directly in localized molecular interactions at specific sites of the genome (Kan & Pinto da Silva, 1986).

During the formation of the proacrosomal vesicles, the acid phosphatase and glucose-6-phosphatase activities have been detected cytochemically in the Golgi complex, resembling those in *A. aseadum* and *N. viridula* spermatids (Fernandes & Báo, 1999). On the contrary, in *E. heros*, the acid phosphatase activity appears scattered in the proacrosomal vesicle. Since the trans Golgi network is the site where proteins finally exit from the Golgi to reach their final cellular sites (Griffiths & Simons, 1986; Grab et al., 1997), such as plasma

membranes, secretion granules and lysosomes, these enzymatic activities have been associated with the Golgi complex. Indeed, these outcomes indicate an important role of these enzymes during acrosome formation.

Despite the fact that the thiamine pyrophosphatase has been generally considered a cytochemical marker for the trans side of the Golgi complex in many cell types (Cheetham et al., 1971; Angermuller & Fahimi, 1984; Roth et al., 1985), this is true for *E. heros*, *A. aseadum* and *N. viridula* spermatids (Fernandes & Báo, 1999), while it was not possible to detect this enzyme in the Golgi complex.

The acrosome is a large secretory vesicle and carries a variety of hydrolytic enzymes, stored in the form of proenzymes, as well as several proteases and glycosidases which are essential for successful fertilization (for reviews see Yanagimachi, 1994). During the intermediate stages of development, a significant concentration of phosphatases in the large acrosomal vesicle of *E. heros* spermatids has been observed. The presence of thiamine pyrophosphatase activity on the acrosomal vesicle membrane differs from the results obtained from *A. aseadum* and *N. viridula*, where this enzyme was not detected in the acrosomal components during initial and intermediate stages of differentiation (Fernandes & Báo, 1999). These enzymes, at least in *E. heros*, seem to be involved in the remodelling and the enzymatic condensation of the acrosome. This indicates that the acrosome has different responses to the same enzyme during the differentiation process.

During early spermiogenesis, an intense presence of α-N-acetyl-galactosamine residues on the acrosomal complex has been observed. Later on, these residues were not detected, indicating that this carbohydrate is involved in the acrosome maturation but is not essential in the final stages of spermiogenesis. Recent studies using gold-labeled lectins have shown that glycoproteins of mosquito, blood-sucking bug, fruit-fly and beetle acrosomes contain different specific sugars (Báo & de Souza, 1992; Perotti & Riva, 1988; Perotti & Pasini, 1995; Craveiro & Báo, 1995) and that sugar residues are not uniformly distributed within the acrosome. Furthermore, this distribution may be variable during the process of development. The glycoconjugates are essential to provide specificity for the recognition and fusion of gametes (Yanagimachi, 1994). Some previous cytochemical and biochemical investigations on the spermatozoa of Drosophila suggest that the sperm plasma membrane can be characterized by α-Man/ α-Glc residues concentrated in the acrosomal region (Perotti & Riva, 1988; Perotti & Pasini, 1995; Cattaneo et al., 1997; Pasini et al., 1999). As these sugar residues could not be found in the spermiogenetic process of *E. heros*, this indicates that other sugar residues may be involved in acrosomal maturation and in the oocyte-recognition process.

In the last stages of differentiation, we observed both enzymatic and basic proteins on the acrosomal membrane, but not in the acrosomal content. The internal tubular arrangement of the acrosomal content of *E. heros* was observed both in the first stages of development and in the mature sperm. This in analogy to what was observed in mature acrosome of the water-strider Gerris (Tandler & Moriber, 1966; Werner & Werner, 1993), and the milkweed bug Oncopeltus (Barker & Riess, 1966), *A. aseadum* and *N. viridula* (Fernandes & Báo, 1999). Such paracrystalline material, however, is not present in the mature acrosome of Leptocoris (Itaya et al., 1980) and *Notonecta* (Werner et al., 1988).

The elucidation of the enzymatic activities and the localization of carbohydrate residues contributes to the enlightening of some particular aspects of spermiogenesis in *E. heros*. Our results show that different species use different carbohydrate residues and enzymes to control their own development, indicating that the presence and functional role of carbohydrates and enzymes during the spermiogenetic process seems to be species-specific.

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Brief Note:

Detection of Calcium and Calmodulin During Spermiogenesis of Phytophagous Bugs (Hemiptera: Pentatomidae)

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Key words: calcium, calmodulin, immunocytochemistry, spermiogenesis, ultrastructure

ABSTRACT: The ultrastructural detection of calcium using pyroantimonate, and the immunocytochemical localization of calmodulin using monoclonal antibody were carried out during the spermiogenesis of phytophagous bugs. The presence of calcium was observed on the Golgi apparatus during the initial phases of spermiogenesis. In the other stages the calcium was observed in association with the nucleus and in some regions of acrosome. Indeed, it was detected surrounding the mitochondrial derivatives and specific axonemal microtubules on the tail region. The immunocytochemical detection of calmodulin showed the presence of this protein approximately in the same regions where the calcium was detected, indicating that calcium and calmodulin could work together during spermiogenesis of this phytophagous bugs, suggesting their involvement on the regulation of flagellar beating, nuclear compactation and acrosome formation.

Introduction

Considerable information about regulatory mechanisms of the main biological processes characteristic of spermatozoa is now available. It is well established that calcium ions and calmodulin - the ubiquitous and multifunctional calcium dependent regulator - play a significant role in metabolic processes, epididymal sperm maturation, motility, capacitation and acrosome reaction (for reviews see Hisanaga and Pratt, 1984; Camatini *et al.*, 1986; Aitken *et al.*, 1988; Tash, 1989). Despite the existence of these studies, there are little information about the roles played by calcium and/or calmodulin during insects spermatogenesis (Motzko and Ruthmann, 1990; Motzko, 1992).

Phytophagous stink bugs (Hemiptera, Pentatomidae) are important pests of high economical value crops throughout the world (for reviews see Panizzi, 1997). Structural and cytochemical studies of spermiogenesis and spermatozoa of these insects can be a valuable source of information to improve programmes directed towards their biochemical control and genetic manipulation (Dumser, 1980).

In the present paper we localized calcium – using the pyroantimonate technique - and calmodulin – using immunocytochemistry – during the different stages of spermiogenesis in four phytophagous bugs.

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Materials and Methods

The insects utilized were male adults of the phytophagous bugs *Acrosternum aseadum*, *Euchistus heros*, *Nezara viridula* and *Piezodorus guildini* (Hemiptera: Pentatomidae), obtained form a colony maintained in the EMBRAPA - Recursos Genéticos e Biotecnologia, Brasília-Brazil.

Localization of Calcium

The testes were dissected and fixed for 4h at 4°C in a solution containing 2.5% glutaraldehyde and 5% sucrose in 0.1M sodium cacodylate buffer, pH 7.2. After fixation, the specimens were rinsed in 0.1M potassium phosphate buffer pH 7.2. Thereafter, the specimens were postfixed in 1% OsO_4, 5% pyroantimonate buffered in 0.1M potassium phosphate pH 7.2 for 1h at 4°C. Then, the specimens were dehydrated in acetone and embedded in Spurr resin. After staining with uranyl acetate and lead citrate, the ultrathin sections were observed in a Jeol 100C transmission electron microscope.

For the controls the specimens were postfixed in a solution without pyroantimonate.

Detection of Calmodulin

The testes were fixed in a mixture containing 4% paraformaldehyde, 0.5% glutaraldehyde and 0.2% picric acid in 0.1M sodium cacodylate buffer pH 7.2, for 5h. After washing the specimens with several changes of the same buffer, free aldehyde groups were quenched with 50mM ammonium chloride in buffer for 1h, followed by block-staining in 2% uranyl acetate in 15% acetone for 2h at 4°C (Berryman and Rodewald, 1990). Specimens were dehydrated in 30-90% acetone and embedding was performed in LRGold resin. Ultrathin sections were collected on nickel grids, pre-incubated in phosphate buffered saline (PBS) containing 1.5% bovine albumin (PBS-BSA), and 0.01% Tween 20, and subsequently incubated for 1h in mouse monoclonal antibody against calmodulin (1:10) (Sigma). After washing with PBS-BSA, the grids were incubated for 1h with the labelled secondary antibody goat anti-mouse-IgG-Au (10nm) at a dilution of 1:20. After incubation, the grids were washed with PBS and distilled water, and floated, before staining, with uranyl acetate and lead citrate. The sections were examined with a Jeol 100C transmission electron microscope.

FIGURE 1: Spermatid of *P. guildini* showing the presence of calcium (electron dense deposits) in the cis Golgi cisternae (arrowhead). Golgi complex (Gc); nucleus (N); proacrosomal granule (Pa). X 19,000

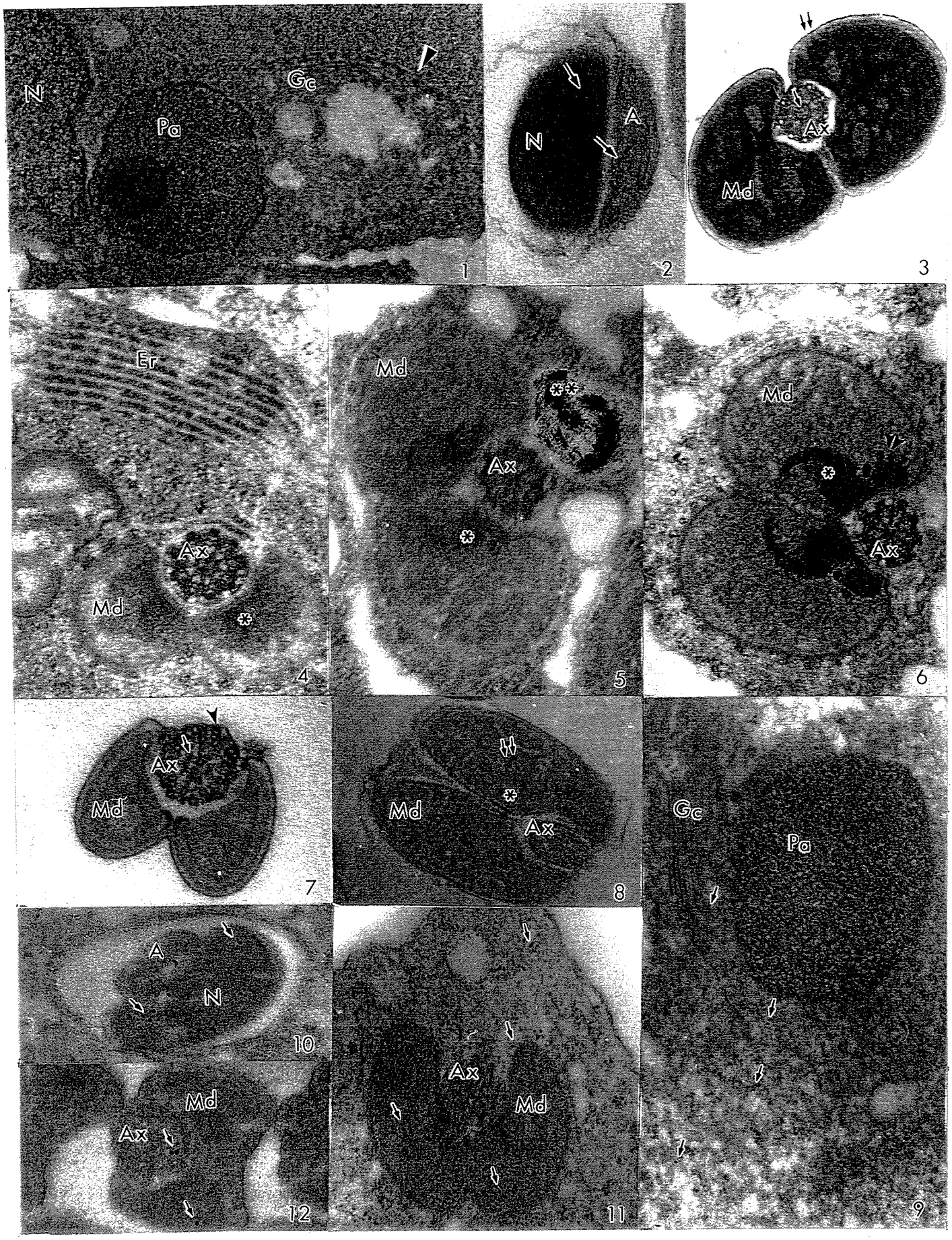
FIGURE 2: Detection of calcium on late spermatid of *A. aseadum*, showing calcium deposits (arrows) scattered in the acrosome (A) and in the nucleus (N). X 104,000

FIGURES 3-6: Detection of calcium on the tail region of *E. heros* (Fig. 3), *P. guildini* (Fig. 4) and *A. aseadum* (Figs. 5 and 6) on intermediate stages of differentiation. Deposits of calcium can be seen in the endoplasmic reticulum cisternae (Er), plasma membrane (double arrow), as well as surrounding the central pair of microtubules (arrow), the accessory microtubules (arrowheads) and the minor paracrystalline structures (double arrowhead). Indeed, the calcium deposits can be observed in a great endoplasmic reticulum cisternae (double asterisk). Axoneme (Ax); mitochondrial derivatives (Md); paracrystalline structures (asterisk). X 43,000; X 68,000; X 55,000 and X 41,000, respectively.

FIGURES 7 and 8: Detection of calcium on the tail region of *E. heros* and *A. aseadum*, respectively, on the late stages of differentiation. Deposits of calcium can be seen surrounding the central pair of microtubules (arrow), the accessory microtubules (arrowhead) and the mitochondrial derivatives (double arrow). Axoneme (Ax); mitochondrial derivatives (Md); paracrystalline structures (asterisk). X 88,000 and X 34,000, respectively.

FIGURES 9 and 10: Localization of calmodulin on head region of *N. viridula* and *E. heros* spermatids, respectively, showing the presence of this protein (arrows) on the Golgi cisternae (Gc), scattered throughout the cytoplasm during the proacrosomal granule formation (Pa) and in the nucleus (N). Acrosome (A). 34,000X and 66,000X, respectively.

FIGURES 11 and 12: Localization of calmodulin on tail region of *A. aseadum* and *N. viridula* spermatids, respectively, showing the presence of this protein (arrows) on the axoneme (Ax) and in the mitochondrial derivatives (Md), as well as scattered on the cytoplasm of intermediate spermatids. X 36,000 and X 70,000, respectively.


N
Gc
Pa
1
N
A
2
Ax
Md
3
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Ax
Md
4
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6
Ax
Md
7
Md
Ax
8
Gc
Pa
9
A
N
10
Ax
Md
11
Md
Ax
12

The control grids were incubated only in the PBS-BSA solution and on the labelled secondary antibody (goat anti-mouse-IgG-Au) in the same conditions.

Results and Discussion

During spermiogenesis, the spermatids undergo a series of modifications that results in the formation of a highly differentiated cell, the spermatozoon. This cell consists of two anatomically and functionally distinct regions: a head, containing the nucleus and the acrosome, and the tail, formed by an axoneme and two mitochondrial derivatives, which contains two paracrystalline structures (Fernandes and Báo, 1998).

Calcium ions are know to play a key role in many cell events, and they are an important second messenger in eukaryotic cells, modulating a wide range of cell processes including cell motility, adhesion, regulation of the cytoskeleton, division, secretion and proteins synthesis. Many of these calcium regulated processes are mediated by calmodulin, a ubiquitous calcium binding protein that translates the rise in intracellular calcium into a physiological response by regulating the activity of a large number of proteins (for reviews see Means and Dedman, 1980).

The presence of calcium, detected by pyroantimonate, was observed as a reaction product which shows an electron dense precipitated in specific regions. On the head, this electron dense precipitated was observed in the cis portion of the Golgi apparatus during proacrosomal granule formation, which happens during the early phases of spermiogenesis (Fig. 1). No calcium was detected on the head region during intermediate stages of differentiation. However, on the late stage of spermiogenesis, the reaction product of calcium detection was seen scattered in the acrosome and in the nucleus (Fig. 2).

On the tail region, the reaction product of calcium detection began to be seen just after mitochondrial derivatives formation, before the cristae rearrangement in paracrystalline structures. In early stages, calcium was detected surrounding the central pair of axonemal microtubules as well as the accessory microtubules. Indeed, deposits of reaction product was seen on the plasma membrane (Fig. 3). After paracrystalline structures formation, calcium was detected in the endoplasmic reticulum cisternae (Figs. 4 and 5) and in small concentrates surrounding the minor paracrystalline structures on the mitochondrial derivatives (Fig. 6). On intermediate stages, no calcium was detected on the axoneme. However, on the late stage of spermiogenesis, the reaction product of calcium detection was seen again on the axoneme, surrounding the central pair of microtubules and the accessory microtubules (Fig. 7), as well as scattered on the mitochondrial derivatives surrounding the two paracrystalline structures (Fig. 8).

Ultrathin section of LRGold embedded spermatids were used for the localization of calmodulin, a calcium binding protein involved in many intracellular events stimulated by calcium. In these species, calmodulin was detected in the similar regions where calcium was located, i.e. on the Golgi apparatus during the proacrosomal granule formation (initial stages of spermiogenesis) (Fig. 9) and scattered in the nucleus (late stages) (Fig. 10). This results confirm the idea that these elements could be involved in some nuclear functions including DNA replication, DNA repair, expression of some genes and the phosphorylation of nuclear proteins (for reviews see Bachs *et al.*, 1992).

The presence of calcium and calmodulin in the Golgi complex on the early spermatid during the proacrosomal granule formation could suggest the participation of these elements in the acrosome formation. Although the presence of calmodulin in the head region on the spermatids of some mammalian species appear evident (Kann *et al.*, 1991), and related with capacitation (Leclerc *et al.*, 1990), acrosome reaction and sperm-egg fusion (Jones *et al.*, 1980; Moore and Dedman, 1984; Weinman *et al.*, 1986 a,b; Camatini *et al.*, 1986; Aitken *et al.*, 1988). We did not observe the presence of calmodulin on the acrosome as well as on the nucleus during intermediate stages of differentiation (not shown) suggesting that in insects these events could be done by other mechanisms without the calmodulin and calcium participation.

The presence of calcium and the role of calmodulin in the regulation of sperm flagellar motility has been extensively studied (Gibbons, 1982; Tash, 1989); as well as the participation/association of calmodulin with motor proteins as dynein, and cytoskeleton proteins as microtubules and actin (Scholey *et al.*, 1980; Hisanaga and Pratt, 1984; Piazza and Wallace, 1985).

The calmodulin was detected scattered in the mitochondrial derivatives and on the cytoplasm that surrounds the tail region, during the early stages of differentiation (Fig. 11). In the late stages, little amount of this protein was observed on the axoneme as well as on the mitochondrial derivatives (Fig. 12).

Although calmodulin is not abundant on the flagellar region of these phytophagous bugs, its presence on the axoneme and on the mitochondrial derivatives of late

spermatids (similar regions where calcium was detected) suggests that calmodulin could be involved in the control of flagellar beating by calcium (Tash and Means 1982; Gordon *et al.* 1982, 1983). Indeed, the notably presence of calcium in the endoplasmic reticulum cisternae as well as on the Golgi complex and its posterior scattering in vesicles on the cytoplasm can suggest its participation in other cellular events that could not be related with flagellar beating regulation.

The omission of the pyroantimonate in the postfixation abolished the appearance of reaction product (not shown). Again, incubation in the presence of the labelled secondary antibody (goat anti-mouse-IgG-Au) does not label the cells (not shown).

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Immunoelectron microscopical detection of tubulins during spermiogenesis in phytophagous bugs (Hemiptera: Pentatomidae)

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Summary

Ultrastructural and immunocytochemical studies were carried out in the tail region of spermatids and spermatozoa of the phytophagous bugs, *Acrosternum aseadum* and *Euchistus heros*. The axoneme presented a 9+9+2 microtubule pattern and bridges occurred between axonemal microtubules 1, 5, and mitochondrial derivatives. Two paracrystalline structures, embedded in an amorphous matrix, were observed in the mitochondrial derivatives. The axonemal microtubules contained alpha, acetylated and tyrosinated tubulin. Cytoplasmic microtubules contained alpha, beta and gamma tubulin. Moreover, the gamma tubulin was detected near the electron dense rod, an element associated with the centriole, suggesting that this structure may be a microtubule organizing center.

Key words: Hemiptera, immunocytochemistry, spermiogenesis, tubulins, ultrastructure

Introduction

Spermatozoa are very specialized and highly differentiated cells. They lose various organelles essential to cell metabolism while the remaining organelles are modified in a manner unparalleled in other processes of cell differentiation (Phillips, 1974). The main compartments of a typical insect spermatozoon are the head, containing nucleus and acrosome, and the tail, containing the axoneme and mitochondrial derivatives (for review see Phillips, 1970; Baccetti, 1972).

The axoneme of insects has a typical 9+9+2 arrangement with the central pair of microtubules, nine peripheral doublets and nine accessory microtubules. This structure is among the most stable microtubular assemblies known and contains more than 150 different proteins; tubulins are the most abundant (Redeker et al., 1994).

The tubulin diversity observed in eukaryotic cells is generated by the differential expression of several alpha and beta tubulin isogenes and is increased by post-translational modifications (for a review, see MacRae, 1997). These modifications have been described for alpha-tubulin, with the acetylation of lys40 (L'Hernault and Rosebaum, 1985), and tyrosination at the same position (Kreis, 1987). Acetylation is often, but not always, an indicator of stable microtubules (Schulze et al., 1987). It was originally found in cilia and flagella (Piperno and Fuller, 1985)

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and was later recorded in several cells and organisms (e.g., Wolf, 1994; Delgado-Viscogliosi et al., 1996). Tyrosination has been recorded in various organisms (Warn et al. 1990; Delgado-Viscogliosi et al. 1996), including germ cells (Wilson et al. 1994; Wolf, 1994).

Another member of the tubulin gene family, the gamma-tubulin, has an approximate 35% sequence identity with the classical alpha and beta tubulins (Oakley and Oakley, 1989). Unlike alpha and beta tubulins, gamma-tubulin is excluded from the bulk of the microtubule lattice. Gamma-tubulin is detected in centrosomes and in other microtubule organizing centers of distinct morphologies found in many divergent species and cell types (e.g., Oakley, 1992; Joshi, 1993; 1994).

In the present paper we used electron microscopy and immunocytochemical analysis to trace the localization of microtubules and of alpha, beta and gamma tubulins, as well as the distribution of modified tubulins during spermiogenesis of phytophagous bugs, *Acrosternum aseaudum* and *Euchistus heros*. In Brazil, these phytophagous bugs are the major component of the pentatomid pest complex on soybean. They feed on pods of soybeans, and the extent of damage is related to the stage of seed development, resulting in pod-abscisiion or abortion of young seeds, deformed seeds and foliar retention (Schaefer and Panizzi, 2000).

Materials and Methods

Male adults of the phytophagous bugs *A. aseadum* and *E. heros* (Hemiptera: Pentatomidae) were obtained from a colony maintained in the National Center of Genetic Resource (CENARGEN), Brasilia, Brazil.

Transmission electronmicroscopy

The testes were dissected and fixed in a mixture of 2.5% glutaraldehyde, 1% tannic acid in 0.1 M phosphate buffer at pH 7.3, followed by block-staining in 1% uranyl acetate in distilled water (Afzelius, 1988). The material was dehydrated in a series of ascending acetones (30–100%) and embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate and lead citrate.

Immunocytochemistry

For ultrastructural immunocytochemistry the specimens were fixed in a mixture containing 4% paraformaldehyde, 0.5% glutaraldehyde and 0.2% picric acid in 0.1 M sodium cacodylate buffer at pH 7.2 for 5 h. The specimens were rinsed several times using the same buffer, and free aldehyde groups were quenched with 50 mM ammonium chloride in this buffer for 1 h, followed by block-staining in 2% uranyl acetate in 15% acetone for 2 h at 4°C (Berryman and Rodewald, 1990). Specimens were dehydrated in 30–90% acetone. Embedding was performed in LRGold resin. Ultrathin sections were collected on nickel grids, pre-incubated in phosphate buffered saline (PBS) containing 1.5% bovine albumin (PBS-BSA) and 0.01% Tween 20, and subsequently incubated for 1 h with antibodies against alpha-tubulin (clone DMIA), α-acetylated-tubulin (clone 6-11B-1), α-tyrosinated-tubulin (clone TUB-1A2) and a polyclonal antibody anti-tubulin (delipidized, whole antiserum) diluted 1:200. Again, beta-tubulin (clone TUB 2.1) and gamma-tubulin (clone GTU-88) were diluted 1:2 (British Biocell International, England). After washing with PBS-BSA, the grids were incubated for 1 h with the respective labelled secondary antibody (mouse or rabbit-IgG-Au-conjugated 10 nm) at a dilution of 1:20. After incubation, the grids were washed with PBS and distilled. The preparations were stained with uranyl acetate and lead citrate and examined in a Jeol 100C transmission electron microscopy operated at 80 Kv.

Results

The detailed spermiogenesis of *A. aseadum* and the interesting aspects of spermiogenesis in *E. heros* were previously described by Fernandes and Báo (1998) and Fernandes et al. (2001) using conventionally embedded tissue. In brief, the spermatozoa of those insects follow the general pattern in that they consist of the head and tail. During spermiogenesis, the mitochondria of the cell fuse, give rise to the so-called nebenkern and finally form two mitochondrial derivatives that flank the axoneme (Fig. 1). These mitochondrial derivatives are the same size and contain two paracrystalline structures which are embedded in an amorphous matrix (Fig. 2). As is typical of insect sperm, in the axoneme a 9 (outer singlets) + 9 (intermediate doublets) + 2 (central singlets) pattern of microtubules is observed (Figs. 1 and 2). The accessory microtubules are composed of 16 protofilaments (Fig. 2, inset). Bridges that connect the mitochondrial derivatives to the axoneme are observed close to the axonemal microtubules 1 and 5 (Figs. 1 and 2). In early spermatids an electron-dense rod is observed nearby the centriole (Fig. 3).

Ultrathin sections of LR Gold embedded spermatids and spermatozoa were used for the localization of

Table 1. Quantitative approach of tubulins detection

Antibodies (anti-tubulins)	*A. aseadum*			*E. heros*		
	YS	AS	MS	YS	AS	MS
Alpha	+++	+++	++++	–	++	+++
Beta	–	+++	–	–	–	–
Gamma	++	++++	+++	++	–	–
Total	–	+++	–	–	–	–
Acetylated	–	++	+	–	–	–
Tyrosinated	++	+	++	–	–	+++

The sinals represent the approximate number of particles per mm². (++++): 31–40 particles; (+++): 21–30 particles; (++) 11–20 particles; (+) 1–10 particles; (–) particles not found. YS, young spermatid; AS, advanced spermatid; MS, mature spermatid.

tubulins, the most abundant protein in the microtubules. The results are summarized in Table 1. The presence of alpha tubulin was detected on the cytoplasmic microtubules (manchette) that surround the nucleus (Fig. 4) as well as on the axonemal microtubules in the intermediate (Fig. 5) and late (Fig. 6) stages of spermiogenesis. Beta tubulin labelling can be detected in cytoplasmic microtubules (Fig. 7). In contrast, beta tubulin could not be detected in axonemal microtubules, but could be seen scattered in the tail (Fig. 8) as well as nearby in the paracrystalline structures. Gamma tubulin, which is known as a microtubule organizing protein, was detected on the cytoplasmic microtubules that surround the nucleus (Fig. 9) and near the electron-dense rod close to the centriole (Fig. 10). Gamma tubulin was also detected scattered throughout the mitochondrial derivatives (Fig. 11) but not on the axonemal microtubules.

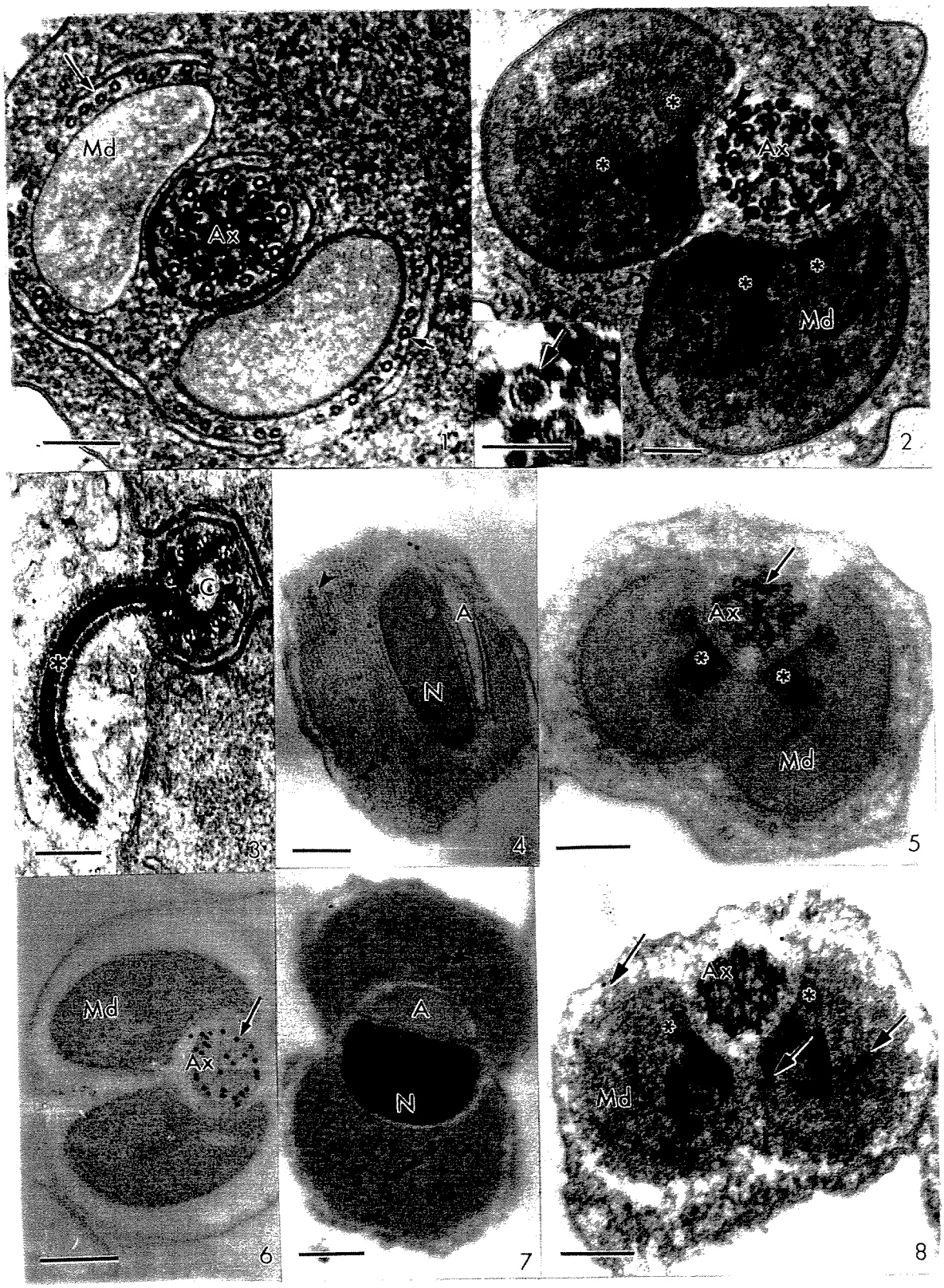
The modified forms of tubulin such as acetylated and tyrosinated tubulins are detected only on the axonemal microtubules in the intermediated stages of spermiogenesis (Figs. 12 and 14) as well as in late stages (Figs. 13 and 15). Moreover, we used an antibody against total tubulin, and this protein was detected in the axonemal microtubules as well as scattered on the mitochondrial derivatives (Fig. 16).

Discussion

During spermiogenesis of *A. aseadum* and *E. heros*, the spermatids undergo specific morphofunctional modifications which involve nuclear elongation, chromatin condensation, acrosomal formation, and flagellar development with axoneme and mitochondrial derivatives assembled as described previously (Fernandes and Báo, 1998; Fernandes et al. 2001).

Tubulin is one of the major proteins found in flagellated spermatozoa of animals. There are several primary tubulin gene products, and this diversity is increased by post-translational modifications. In order to characterize the microtubules in the sperm cell, as well as the kind and the localization of modified tubulins in insects, antibodies against alpha, beta, gamma, acetylated, tyrosinated and total tubulins were used in *A. aseadum* and *E. heros*. Our results demonstrate the presence of significant differences in the tubulin and post-translational modifications that tubulin undergoes in the stages of spermatid differentiation. These differences are indicative of a higher molecular diversity in axonemal and cytoplasmic tubulin and of a partition of tubulin isoforms between the microtubules of the spermatic cells. A specific significant difference in the post-translational modifications that tubulin undergoes in the axonemal microtubules and accessory tubules has been recently demonstrated by biochemical and immunoelectron microscopy characterization in *Apis mellifera* sperm (Mencarelli et al., 2000).

Although both alpha and beta tubulin are known to form microtubules, only alpha tubulin was found in a well defined position at the axonemal microtubules in intermediate and late stages of spermiogenesis in these bugs. Surprisingly, beta tubulin was not detected in the axonemal microtubules, but was found in the microtubules that surround the flagellar region. This result may suggest that, although alpha and beta tubulin form the heterodimers of the microtubules protofilaments, the antigenic epitope of beta tubulin may not be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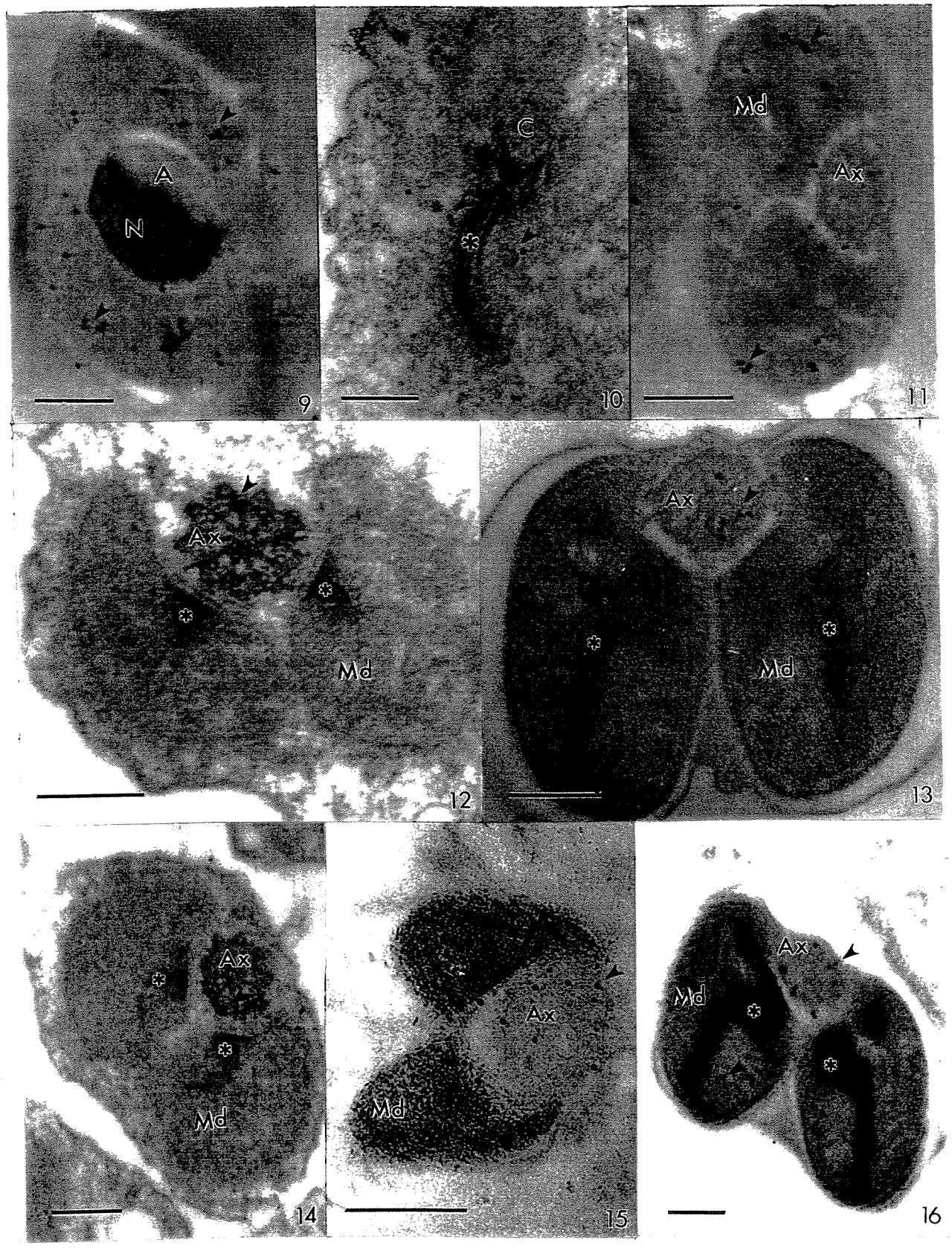
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Figs. 1–3: Conventional embedding. Fig. 1. Transverse section on the tail of early spermatid of *E. heros*. The mitochondrial derivatives (Md) aresurrounded by the microtubules (arrows). The axoneme (Ax) with its 9+9+2 microtubule pattern shows bridges (arrowheads) between axonemal microtubules 1 and 5 and mitochondrial derivatives.×69,300. Bar = 0.2 μm. Fig. 2. Transverse section of tail in a spermatid of *A. aseadum*, showing axoneme (Ax), bridges (arrowheads) between axonemal microtubules and mitochondrial derivatives (Md) which show two paracrystalline structures (asterisks). Inset: accessory microtubules (arrow) with 16 protofilaments. ×100 000. Bar = 0.1 μm. Inset: ×312 000. Bar = 0.05 μm. Fig. 3. Section through the electron dense rod (asterisk) in a spermatid of *E. heros*. Centriole (C). ×60,000. Bar = 0.2 μm. **Figs. 4–16:** LRGold embedding. Figs. 4–6: Thin section of LRGold embedded cells of *E. heros* (4) and *A. aseadum* (5 and 6) labelled with alpha tubulin antibody. Labelling is evident in the area of cytoplasmic (arrowheads) and axonemal microtubules (arrows). Axoneme(Ax); acrosome (A); nucleus (N); mitochondrial derivatives (Md); paracrystalline structures (asterisk). ×52,000, ×65,000 and ×67,600, respectively. Bars = 0.2 μm. Figs. 7 and 8. Cells of *A. aseadum* labelled with an antibody against beta tubulin. Label is observed on the cytoplasmic microtubules (arrowheads) and scattered throughout the tail region (arrows), including the mitochondrial derivatives (Md). Axoneme (Ax); acrosome (A); nucleus (N); paracrystalline structures (asterisks). ×59,800 and ×65,000, respectively. Bars = 0.2 μm. Figs. 9–11. Spermatids of *A. aseadum* (9 and 11) and *E. heros* (10) labelled with an antibody against gamma tubulin. The labelling (arrowheads) is scattered throughout the cytoplasmic region near to the nucleus (N) as well as near to the electron-dense rod (asterisk). Moreover, gamma tubulin is detected within the mitochondrial derivatives (Md) at the tail region. Acrosome (A); axoneme (Ax); centriole (C). ×67,600, ×67,600 and ×78,000, respectively. Bars = 0.2 μm. Figs. 12 and 13. Transverse section through the tail region of *A. aseadum* spermatid and spermatozoa, respectively, showing the binding sites of an antibody against acetylated tubulin. This modified tubulin is detected (arrowheads) only on the axoneme (Ax). Mitochondrial derivatives (Md), paracrystalline structures (asterisks). ×99,000 and ×82,500, respectively. Bars = 0.2 μm. Figs. 14 and 15. Cells of *A. aseadum* and *E. heros*, respectivelly, labelled with an antibody against tyrosinated tubulin. The labelling (arrowheads) is evident on the axoneme (Ax). Mitochondrial derivatives (Md), paracrystalline structures (asterisks). ×62,400 and ×109,200, respectively. Bars = 0.2 μm. Fig. 16. Transverse section through the *A. aseadum* spermatid labelled with total tubulin antibody. Note the label(arrowhead)on the axoneme (Ax) and the mitochondrial derivatives (Md). Paracrystalline structures (asterisks). ×54,600. Bar = 0.2 μm.

exposed in spermatids in the axonemal region. However, the cytoplasmic microtubules present a similar labelling for alpha and beta tubulin, indicating that some difference between axonemal and cytoplasmatic microtubules may exist.

In addition to alpha and beta tubulins, a third type, gamma tubulin, is known, which was originally described in the fungus *Aspergillus nidulans* (Oakley and Oakley, 1989). Unlike alpha and beta tubulins, which assemble to form microtubules polymers, gamma tubulin is excluded from the bulk of the microtubule lattice. The gamma tubulin may be found in the pericentriolar material and in the core of the centriole, as shown by Fuller et al. (1995); it is also part of the basal body in ciliates (Liang et al., 1996) as well as serving as a nucleating site for the minus end of microtubules (e.g., Li and Joshi, 1995; Marschall et al., 1996; Spang et al., 1996). In *A. aseadum* and *E. heros* the gamma tubulin was detected in the cytoplasm near the nucleus and in mitochondrial derivatives, and may be acting as a secondary microtubule organizing center, although the pericentriolar material may be recognized as the main microtubule organizing center. The electron-dense rod that appears in young spermatids seems to be involved in microtubule organization (Godula, 1979). This structure and the cytoplasm nearby present alpha and beta tubulins as described previously (Fernandes and Báo, 1998). Indeed, the cytoplasm region around the electron-dense rod shows the presence of gamma tubulin, confirming that this structure could be a microtubule organizing center.

The tubulin molecule is subjected to a large number of varied modifications (Sullivan, 1988). A wide variety of alpha tubulin has an acetyl group. The acetylation is particularly notable in axonemal microtubules, which are generally quite stable structures. Acetylation has been observed in cilia and flagella from a variety of organisms (see Piperno and Fuller, 1985) including *Chlamydomonas* (LeDizet and Piperno, 1986) and *Trypanosoma cruzi* (Souto-Padron et al., 1993). Acetylation seems to occur in tubulin after it is incorporated into microtubules (Wilson and Forer, 1989). Other than causing an increase in stabilization of microtubules (for reviews see Ludeña, 1998), the precise function of this post-translational modification is not clear.

Tyrosination has been seen in a variety of vertebrates and trypanosomes (Gallo and Precigout, 1988; Xiang and MacRae, 1995; Rutberg et al., 1996). The functional significance of this isotype has not been elucidated. It is likely that some isotopes have no functional significance at all or simply increase an organism's adaptability without perfoming any specific function. It is possible that the function of tubulin isotypes is not always formation of a particular

type of microtubule. Perhaps the isotype composition can determine the overall chemical and physical properties of the microtubules including its nucleation, dynamic behavior, and susceptibility to post-translational modification.

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4.6. **Fernandes, A.P.;** Peixoto, F.L. & Báo, S.N. Ultrastructural and cytochemical studies on diapause spermiogenesis in phytophagous bugs (Hemiptera:Pentatomidae). Submetido à publicação.

ULTRASTRUCTURAL AND CYTOCHEMICAL STUDIES ON DIAPAUSE SPERMIOGENESIS IN PHYTOPHAGOUS BUGS (HEMIPTERA:PENTATOMIDAE)*

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Running title: Diapausic spermiogenesis in bugs

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Adrienne de Paiva Fernandes, Fernanda Lopes Peixoto and Sônia Nair Báo; Ultrastructural and cytochemical studies on diapause spermiogenesis in phytophagous bugs (Hemiptera: Pentatomidae); Braz. J. Morphol. Sci. _____, _____-_____, 2002

ABSTRACT

Diapause is a genetically controlled life phase for which biochemical and behavioral adjustments occur in advance, followed by refractory period of suppressed development. The present study was carried out to elucidate whether spermiogenesis is discontinued in adult diapause and how is the morphology of spermiogenesis during bugs adult diapause. The testes of these insects during diapause present vesicles similar to residual bodies that show acid phosphatase activity suggesting that they are active lysosomes. Moreover, the nucleus of spermatid shows an apoptotic pattern with fragmented chromatin. These results suggest that during adult diapause of these bugs, spermiogenesis is discontinued with the participation of apoptotic and phagocytic events.

Key words: acid phosphatase, diapause, *Edessa meditabunda*, *Nezara viridula*, spermiogenesis

INTRODUCTION

Phytophagous stink bugs (Hemiptera, Pentatomidae) are the main pests of economically important crops throughout the world [16]. Despite the vast amount of information regarding pest species and control mechanisms used, their potential damage to crop production remains high; it appears obvious that more knowledge about the basic biology and ecology of heteropteran pests is needed. It is also necessary to provide new, efficient alternatives of biological control. Among these, a study of their reproductive biology seems very important.

There are numerous investigations on the structure and ultrastructure of Hemiptera spermatozoa and spermiogenesis [1, 2, 5, 7 - 11]. However, little information is available about the reproductive biology of these insects during adult diapause [6, 15 and 18].

The present study was carried out to elucidate whether spermiogenesis is discontinued during adult diapause and what are the characteristics of spermiogenesis in *Edessa meditabunda* and *Nezara viridula* during this phase.

MATERIALS AND METHODS

The insects studied were diapausic adult male of the phytophagous bugs *Edessa meditabunda* and *Nezara viridula* (Hemiptera, Pentatomidae), obtained from a laboratory colony reared at the National Center of Genetic Resource (CENARGEN), Brasília, Brazil.

Transmission electron microscopy:

The testes were fixed for 4 h at 4°C in a mixture of 2.5% glutaraldehyde, 4% paraformaldehyde, 5 mM $CaCl_2$ and 3% sucrose, in 0.1 M sodium cacodylate buffer, pH 7.3. After fixation, the specimens were rinsed in the same buffer, and postfixed in 1% osmium tetroxide, containing 0.8% potassium ferricyanide, and 5 mM $CaCl_2$ in sodium

cacodylate buffer. The material was dehydrated in a graded series of acetone (30-100%) and embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate.

Enzyme cytochemistry:

The testes were dissected and briefly fixed for 15 min at 4°C in 1% glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.2. After fixation, the specimens were washed with buffer and incubated for 1 h at 37°C in the following medium: 0.1 M Tris-maleate buffer, pH 5.0, 7 mM cytidine-5′-monophosphate, 2 mM cerium chloride and 5% sucrose [17]. For the controls the substrate was omitted.

After incubation, the specimens were washed with sodium cacodylate buffer and fixed again for 3 h at 4°C in a solution containing 4% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Then, the specimens were washed in plain buffer, and postfixed in a solution containing 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM calcium chloride in 0.1 M sodium cacodylate buffer. Subsequently, they were dehydrated in acetone and embedded in Spurr´s resin. Thin sections were stained with uranyl acetate and lead citrate.

All observations were perfomed in a Jeol 100C transmission electron microscope.

RESULTS

The general structure of the spermiogenesis of *Edessa meditabunda* and *Nezara viridula* by transmission electron microscopy has been described in detail previously [7 and 9]. During normal spermiogenesis the spermatids undergo a series of modifications, resulting in the formation of a highly differentiated spermatozoon, which after capacitation is able to fertilize a oocyte.

During adult diapause, the spermiogenesis begins normally, with the spermatids undergoing the typical modifications: nuclear elongation and tail formation (Fig. 1), that should culminate with formation of a complete spermatozoon, containing a head-piece (with the acrosome and the nucleus) and the tail (with the axoneme and two mitochondrial derivatives) (Fig. 2).

In adult diapause the cytoplasm of the cystic cell presents conspicuous vesicles similar to residual bodies (Figs 3 and 4). Another kind of vesicles was found, containing fragments of spermatozoa (Fig. 5). Another characteristic of spermiogenesis in adult diapause of these insects is the presence of cysts bearing a few or no spermatids at all, but showing several residual bodies (Fig. 6). Although some spermatids became spermatozoa, several of them did not complete development and their nuclei apparently undergo apoptosis, with the chromatin being fragmented in an apoptotic pattern (Fig. 7).

The cytochemical tests for acid phosphatase demonstrate the presence of an electron dense precipitate on the residual bodies, scattered in the cystic cell cytoplasm (Fig. 8), as well as in the active lysosomes that surround early spermatids (Fig.9).

DISCUSSION

The spermiogenesis process involves the structural and physiological transformation of organelles to more adapted forms, at the fertilization process. These changes have been previously described for *Edessa meditabunda* [9] and *Nezara viridula* [7] during normal development.

The diapause is a genetically controlled life phase for which biochemical and behavioural adjustments occur in advance, followed by a refractory period of suppressed

development. In temperate regions, the diapause is associated with the survival during winter, when the normal growth is not possible; in the tropics, diapause could facilitate the survival during drought periods, whose characteristic are low humidity and paucity of food [3]. In this way, insects are able to overcome these adverse conditions by entering periods of dormancy and reproductive inactivity, either undergoing diapause or quiescence.

During larval and pupal diapause the spermiogenesis stops due to the new endocrine balance in a specific developmental phase [4]. By the end of the diapause, the endocrine balance is reestablished and the spermiogenesis proceeds again [12 - 14].

The abnormal development of spermatids during adult diapause of *E. meditabunda* and *N. viridula* and the presence of several conspicuous residual bodies in the cysts cells suggest that the germ cells are eliminated during development. The reaction product for acid phosphatase within these residual bodies indicates that they could be active lysosomes. Moreover, the presence of vesicles containing fragments of spermatozoa could indicate that the germ cells are phagocytosed and later on digested by the active lysosomes.

Another characteristic described here is the apoptotic pattern observed in the nuclei of spermatids, which show fragmented chromatin. This result suggests that apoptosis could be an event present during adult diapause involving lysosomal activity so as to interrupt spermiogenesis during this phase.

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Legends

Figure 1: Section through *E. meditabunda* diapausic testes showing the initial spermatids development. Centriole (C); mitochondrial derivative (Md); nucleus (N). X 18 200. Bar: 1 μm.

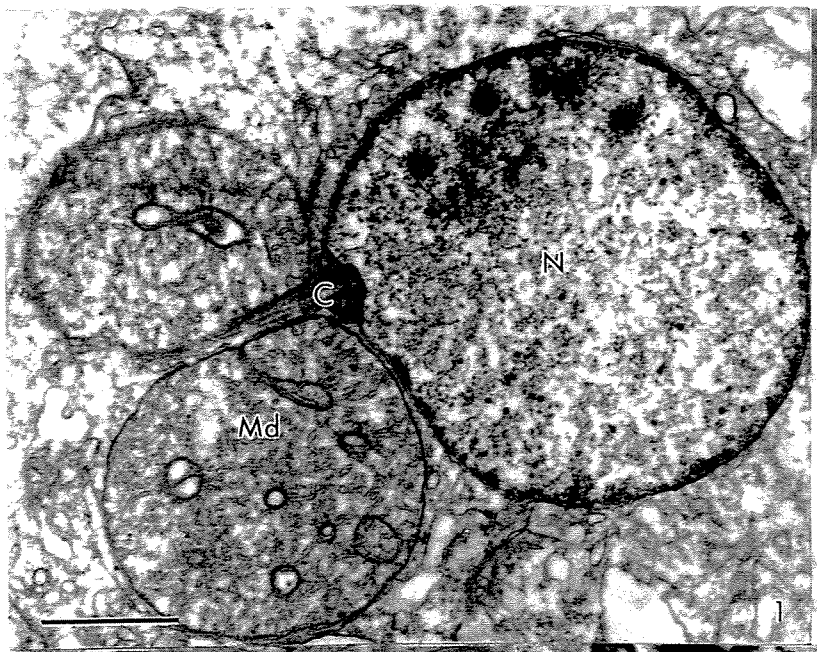
Figure 2: Transverse section through *E. meditabunda* diapausic testes. Observe the completely formed spermatozoa. Acrosome (A); axoneme (Ax); mitochondrial derivatives (Md); nucleus (N). X 99 000. Bar: 1 μm.

Figures 3-5: Sections through diapausic *E. meditabunda* (3 and 5) and *E. meditabunda* (4) cystic cell cytoplasm. The diapausic cystic cell cytoplasm presents residual bodies (Rb). Some vesicles contain fragments of spermatozoa (arrow). Acrosome (A); axoneme (Ax); mitochondrial derivatives (Md); nucleus (N); residual bodies (Rb). X 26 000; X 20 800; X 28 600, respectively. Bars: 0.5 μm.

Figure 6: Section through *E. meditabunda* cystic cell, showing an empty cyst (Ec) and conspicuous residual bodies (Rb). Nucleus (N). X 5 980. Bar: 2 μm.

Figure 7: Spermatids of *E. meditabunda* showing fragmented chromatin (asterisks) similar to an apoptotic pattern. Acrosome (A); nucleus (N). X 13 000. Bar: 1 μm.

Figures 8 and 9: Acid phosphatase reaction. Section through cystic cell of *E. meditabunda* showing the reaction product located on the residual bodies (Rb) and active lysosomes (L). Axoneme (Ax); mitochondrial derivatives (Md); nucleus (N). X 32 000. Bars: 0.5 μm



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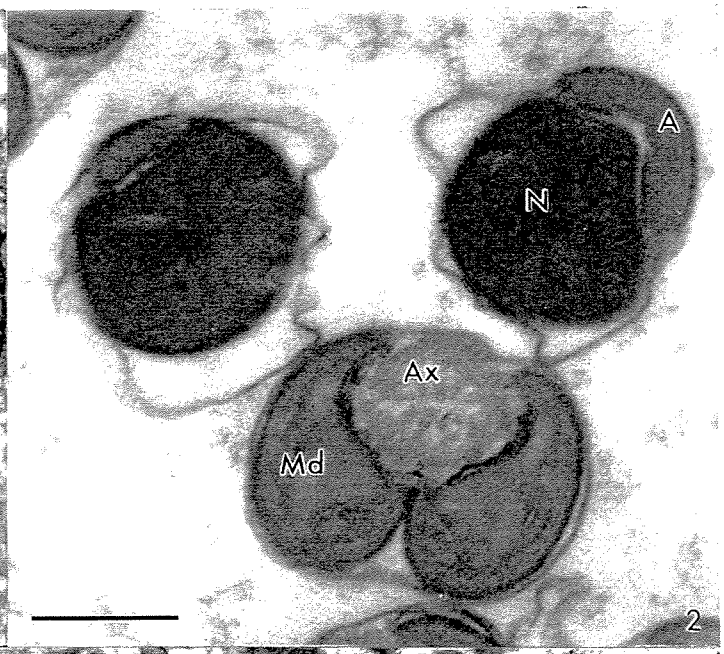

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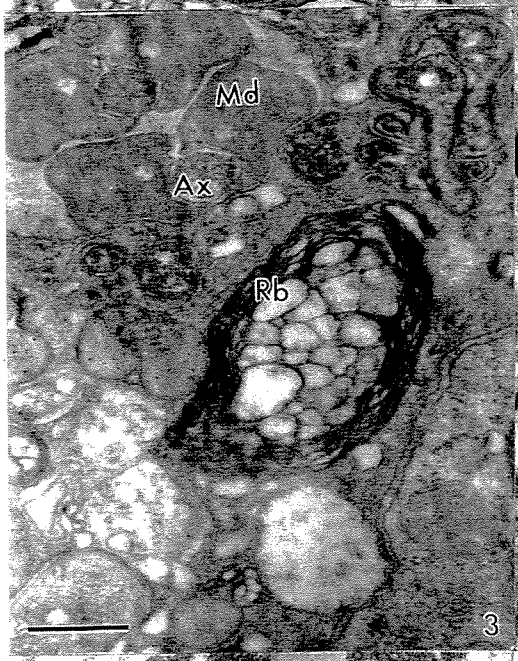

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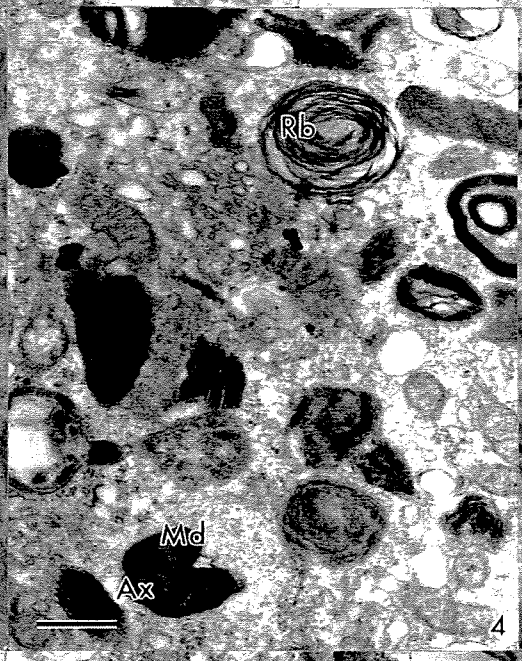

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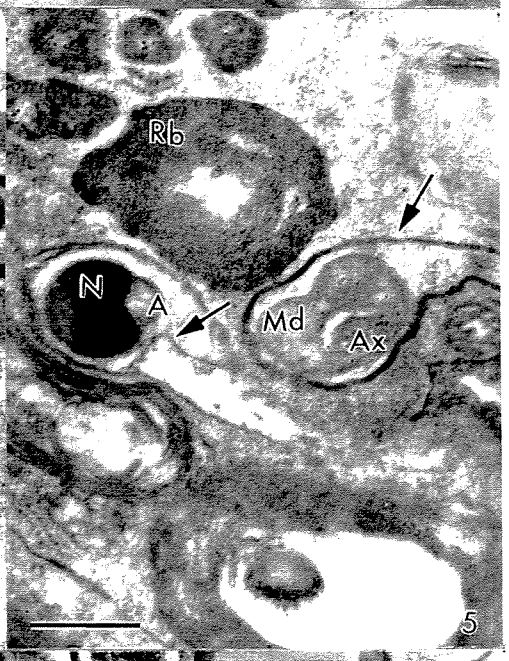

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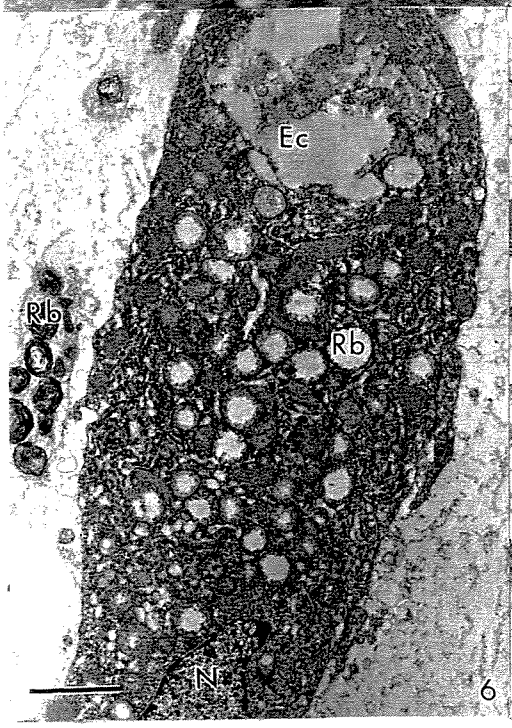

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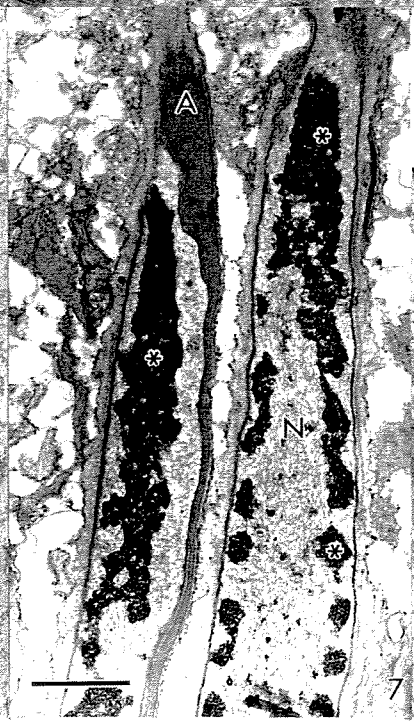

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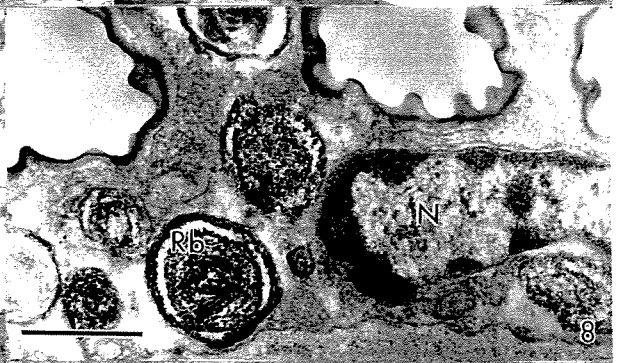

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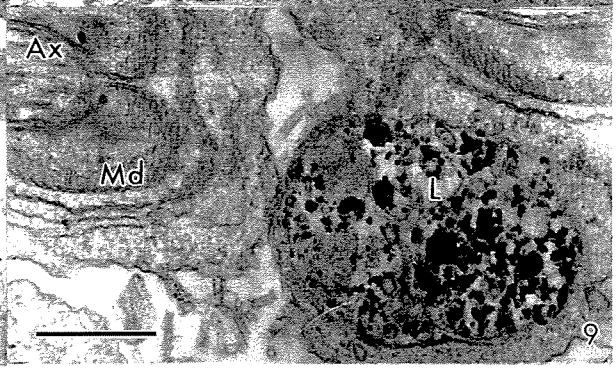

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5. Discussão e Conclusões

Os insetos utilizados nesse estudo têm o processo de espermiogênese muito semelhante ao observado em outros pentatomideos (Fernandes & Báo, 1998). Porém, algumas características como por exemplo, a formação do acrosoma e o desenvolvimento/maturação do núcleo parecem ser bastante específicos (Fernandes & Báo, 2001a; Fernandes et al., 2001a). A formação do acrosoma conta com a participação de várias enzimas como fosfatase ácida, glicose-6-fosfatase e tiaminopirofosfatase, além da presença de resíduos de carbohidratos. Durante os estágios finais, essa estrutura mostra um arranjo tubular que permanece no acrosoma maduro, que por sua vez é constituído de 3 partes distintas: um cone interno eletron lucente, o conteúdo acrosomal (com arranjo tubular) e a membrana extra-acrosomal. Durante a maturação do núcleo a cromatina passa de granulada ou descompactada à totalmente compactada, sendo que nos estágios intermediários apresenta um arranjo fibrilar. A elucidação das atividades enzimáticas e a localização de resíduos de carbohidratos ajudam a esclarecer alguns aspectos particulares da espermiogênese desses insetos. Esses resultados (Fernandes et al., 2001a) mostram que espécies diferentes usam diferentes resíduos de carbohidratos e enzimas para controlar seu próprio desenvolvimento, indicando que a presença e o significado funcional dos carbohidratos e das enzimas durante o processo espermiogênico parece ser espécie-específico.

Outro aspecto importante é a detecção/localização de cálcio e calmodulina durante a espermiogênese dos pentatomideos (Fernandes & Báo, 2001b). Embora

tenha sido descrita a participação desses elementos na formação do acrosoma (Kann et al., 1991), na capacitação (Leclerc et al., 1990) e na reação de fusão do ovo com o espermatozóide (Aitken et al., 1988) em espécies animais diferentes, nos Heteroptera analisados neste trabalho esses eventos parecem ser feitos ou controlados por outros mecanismos sem a participação de cálcio e calmodulina (Fernandes & Báo, 2001b). Entretanto, conforme sugerido por Gibbons (1982) e Tash (1989), esses elementos podem estar envolvidos no controle do batimento flagelar e em outros eventos celulares não relacionados com essa função.

As tubulinas são as principais proteínas encontradas nos espermatozóides de animais. A caracterização dos microtúbulos das células espermáticas e a localização, através de imunocitoquímica, de tubulinas modificadas pós-translacionalmente mostrou que existem diferenças significativas entre os microtúbulos axonemais e citoplasmáticos (Fernandes & Báo, 2002). O significado funcional dessas diferenças ainda não foi elucidado. Porém, parece que a sua composição pode determinar propriedades químicas e físicas dos microtúbulos - componentes do citoesqueleto importantes para o desenvolvimento dos espermatozóides.

Além das transformações ocorridas durante a espermiogênese desses insetos, os espermatozóides maduros já ejaculados sofrem uma série de transformações citoquímicas durante o período que permanecem na espermateca das fêmeas (Fernandes & Báo, 2000). Essas transformações parecem estar relacionadas com a preparação do espermatozóide para a fertilização ou com a sobrevivência dessas células por longos períodos dentro da espermateca. Porém,

são necessários maiores estudos para esclarecer o verdadeiro papel das enzimas nos espermatozóides durante esse período de sobrevivência na fêmea.

A diapausa é uma fase da vida controlada geneticamente onde ocorrem ajustes bioquímicos e comportamentais que garantem a sobrevivência dos insetos em períodos com pouco ou nenhum oferta de alimento e baixa umidade. A espermiogênese de *Edessa meditabunda* e *Nezara viridula* durante a diapausa adulta apresenta algumas características interessantes, como por exemplo a presença de inúmeros corpúsculos residuais que sugerem a eliminação de células germinativas durante o seu desenvolvimento e a interrupção da espermiogênese normal (Fernandes et al., 2001b).

Com base nas características observadas e descritas durante a realização desse trabalho pudemos concluir que:

⇒ A espermiogênese desses percevejos fitófagos é complexa e envolve a elongação nuclear, formação do acrosoma, desenvolvimento flagelar com surgimento do axonema e dos derivados mitocondriais.

⇒ Os espermatozóides desses percevejos apresentam as mesmas características ultra-estruturais descritas anteriormente para outros Heteroptera: núcleo, acrosoma, axonema com o padrão de 9+9+2 microtúbulos, dois derivados mitocondriais contendo duas estruturas paracristalinas cada um e pontes conectando os microtúbulos axonemais 1 e 5 e os derivados mitocondriais.

⇒ As mudanças nucleares e a formação do acrosoma contam com a participação de diferentes enzimas e resíduos de carbohidratos.

⇒ Várias isoformas de tubulinas foram imunocitoquimicamente detectadas durante a espermiogênese desses percevejos.

⇒ Durante a espermiogênese, pôde ser detectada a participação de cálcio e calmodulina que além de estarem envolvidos no controle do batimento flagelar participam de outros eventos regulatórios no desenvolvimento das espermátides.

⇒ Embora não haja mudanças morfológicas nos espermatozóides desses insetos após a cópula, não há dúvidas que ocorrem várias mudanças fisiológicas nessas células antes da fertilização.

⇒ Durante a diapausa adulta sofrida por algumas espécies de percevejos fitófagos, a espermiogênese é descontinuada com a participação de eventos celulares com padrão apoptótico e fagocítico.

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