BIOCELL
2004, 28(3): 299-310

ISSN 0327 - 9545
PRINTED IN ARGENTINA

Protein detection in spermatids and spermatozoa of the butterfly *Euptoieta hegesia* (Lepidoptera)

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Key words: ultrastructure, protein, apyrene, eupyrene.

ABSTRACT: This study was undertaken to detect protein components in both sperm types of the butterfly *Euptoieta hegesia*. These spermatozoa possess complex extracellular structures for which the composition and functional significance are still unclear. In the apyrene sperm head, the proteic cap presented an external ring and an internal dense content; basic proteins were detected only in external portions. In the tail, the paracrystalline core of mitochondrial derivatives and the axoneme are rich in proteins. The extratesticular spermatozoa are covered by a proteic coat, which presented two distinct layers. In eupyrene spermatozoa, acrosome and nucleus were negatively stained, probably because of their high compaction. In the tail, there is no paracrystalline core and the axoneme presented a very specific reaction for basic proteins. The lacinate and reticular appendages are composed of cylindrical sub-units and presented a light reaction to E-PTA and a strong reaction to tannic acid. A complex proteic coat also covers the extratesticular spermatozoa. We found similarities between both extratesticular coats, indicating a possible common origin. Both spermatozoon types are rich in proteins, especially the eupyrene appendages and the extratesticular coats. We believe that both coats are related to the sperm maturation and capacitation processes.

Introduction

Butterflies and moths produce two sperm types, the eupyrene and apyrene ones, which are formed in different stages of testicular development. However, they can be found simultaneously in the imago (Leviatan and Friedländer, 1979; Friedländer and Benz, 1981; Katsuno, 1989).

The apyrene spermatozoa are devoid of a nucleus and the anterior end is composed only of a dense cap (Phillips, 1971; Friedländer and Gitay, 1972; Medeiros and Silveira, 1996; Mancini and Dolder, 2001a, 2004). Eupyrene spermatozoa, however, present a typical head, consisting in a tubular acrosome and nucleus (Lai-Fook, 1982; Kubo-Irie *et al*., 1998; Mancini and Dolder, 2004). Two mitochondrial derivatives and a 9+9+2 axoneme constitute the organelles of the flagella of both sperm types.

In eupyrene spermatozoa, there are two types of extracellular appendages attached to the membrane, called lacinate and reticular, which extend along the entire sperm length (Phillips, 1970; Leviatan and Friedländer, 1979; Mancini and Dolder, 2001b, 2004). Both sperm types undergo several morphological changes after they leave the testis. The apyrene spermatozoa acquire several concentric layers while eupyrene ones acquire a complex coat and lose their lacinate appendages (Phillips, 1971; Riemann and Thorson, 1971; Lai-Fook, 1982; Kubo-Irie *et al*., 1998; Mancini and Dolder, 2003).

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Received on March 19, 2004. Accepted on August 17, 2004.

Despite the abundant morphological studies of Lepidoptera sperm, there are few analyses concerning their cytochemistry. Wolf and collaborators provided a very expressive contribution in immunolocalization in light microscopy of microtubules in spermatocytes of Lepidoptera (Wolf, 1992, 1996a, 1996b, 1997; Wolf and Bastmeyer, 1991a, b; Wolf and Joshi, 1996). However, cytochemical studies involving apyrene and eupyrene spermatozoa, with their elaborate extracellular structures, are rare (Friedländer and Gershon, 1978; França and Báo, 2000).

The composition and functional significance of the eupyrene appendages and the extracellular coat of both sperm types are still unclear. Here we detected proteins in *Euptoieta hegesia* sperm, in an effort to collaborate towards understanding the chemical composition and the ultrastructural transformations of these spermatozoa as they move along the male reproductive tract.

Materials and methods

Testes, deferent ducts and seminal vesicles of adult *Euptoieta hegesia* butterflies were processed according to two methods for protein detection in Transmission Electron Microscopy.

Tannic acid for General Proteins (Dallai and Afzelius, 1990)

Specimens were fixed in 2.5% glutaraldehyde, 1% tannic acid, 1.5% sucrose and 5 mM calcium chloride in 0.1 M sodium phosphate buffer for three days at 4°C. The materials were rinsed in buffer and contrasted in an aqueous solution of 1% uranyl acetate for 2 hours at room temperature. Finally, they were dehydrated and embedded in Epoxy Resin. The ultra thin sections were contrasted with solutions of uranyl acetate and lead citrate.

Ethanolic-Phosphotungstic acid (E-PTA) for Basic Proteins (Modified from Bloom and Aghajanian, 1968)

Specimens were fixed in 2.5% glutaraldehyde, 1.5% sucrose and 5 mM calcium chloride in 0.1 M sodium phosphate buffer for 4 hours at 4°C. They were then rinsed in buffer, dehydrated in ethanol at 4°C and finally contrasted in 2% PTA (phosphotungstic acid) in absolute ethanol for 2 hours at room temperature or up to 24 hours at 4°C. The specimens were embedded in Epoxy Resin. The ultra thin sections were observed without further contrasting.

Results

Apyrene Spermatozoa

In apyrene spermiogenesis, early spermatids contain several micronuclei scattered in the cytoplasm as a result of an atypical meiotic division. These micronuclei contain amorphous chromatin, which presented an electron-dense reaction to E-PTA (Fig. 2A). In late spermatids, such micronuclei are degenerated and eliminated from the posterior tip.

The anterior end of apyrene cells, devoid of a nucleus, is composed of a long cap, which consists of an external ring and an internal dense material, strongly stained by tannic acid (Figs. 1A and B), when compared with conventional fixation methods (not shown). The presence of basic proteins was only detected in the external portions (Fig. 2B).

In the spermatid flagella, a positive reaction due to general and basic proteins was observed in the paracrystalline core of mitochondrial derivatives (Figs. 1C and 2C). However, it is not possible to clearly distinguish the paracrystalline arrangement of this structure. On the other hand, in spermatozoa, despite the positive reaction of the paracrystalline core using both techniques (Figs. 1E-G and 2D-F), it is only possible to clearly identify the paracrystalline substructure with tannic acid (Figs. 1E-G).

In the axoneme, the walls of the peripheral and accessory microtubules are very evident using E-PTA (Figs. 2C-F), if compared with the central ones. The lumen of one of the peripheral microtubule doublets and, generally, one of the central pair, presented basic proteins (Figs. 2C and E). The proteins that link the axoneme elements do not have a positive reaction with this technique.

However, tannic acid reveals rich details of the axoneme proteins (Figs. 1C-G). The dynein arms and the radial spokes presented an electron-dense reaction and the microtubule walls can be easily identified as being composed of protofilaments. The accessory microtubules present 16 protofilaments and the central ones, 13 (Fig. 1D). The interior of the A microtubules of the peripheral pairs and, generally, one of the central microtubules stand out with an electron-dense reaction. Although the lumen of the accessory microtubules was

not electron-dense, it was possible to identify, in cross section, 7-9 circular electron lucid sub-units (Fig. 1D). These sub-units are organized in a circle of micro cylinders surrounding a central micro cylinder.

The plasma membrane of apyrene spermatids and spermatozoa showed an electron-dense reaction with E-PTA (Figs. 2C-F). The reaction becomes more intense in the extra testicular spermatozoa, where a thick coat, composed of several concentric layers, is acquired (Fig. 2E).

Using tannic acid, this coat, found on apyrene spermatozoa from the deferent duct and seminal vesicle, also

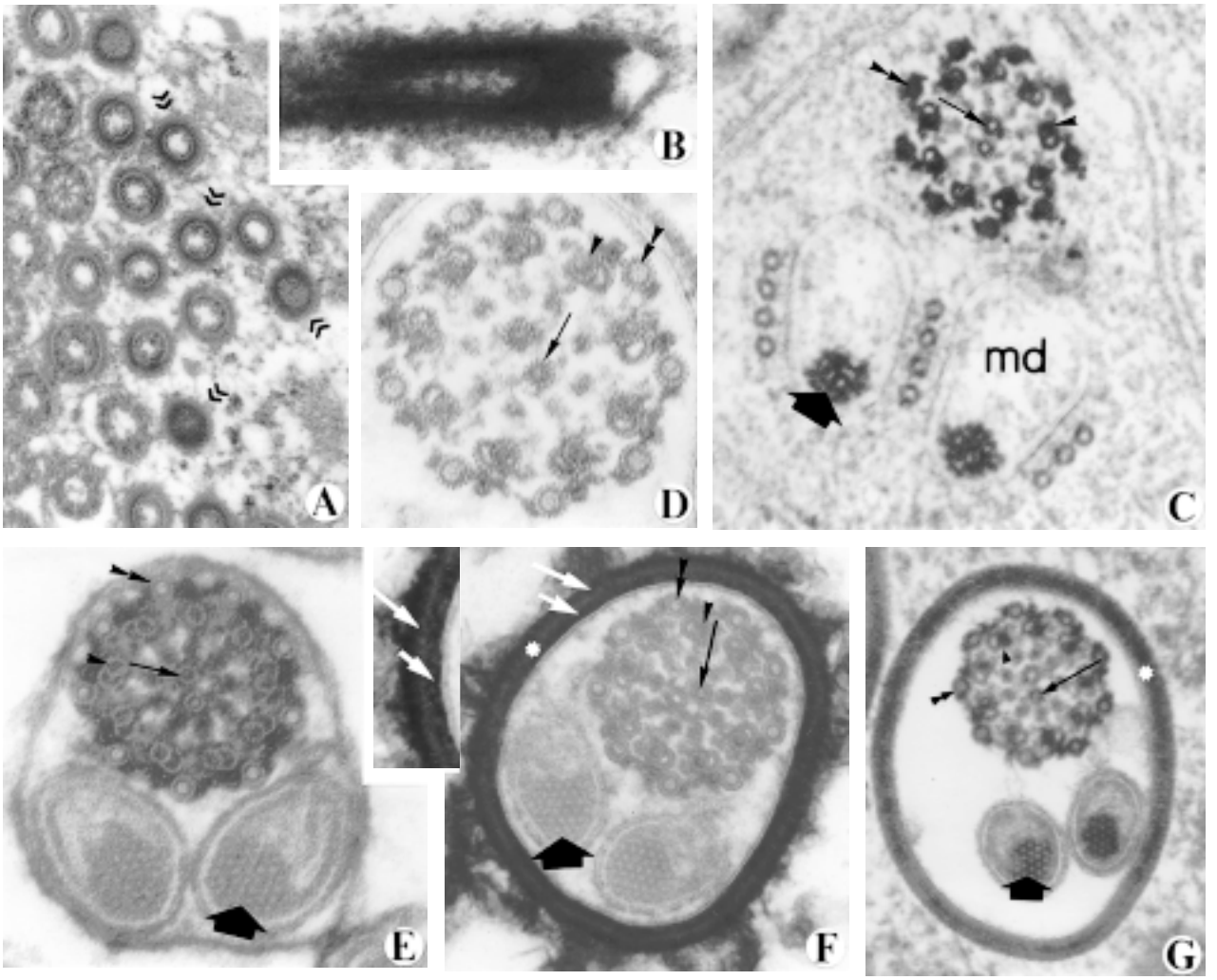


FIGURE 1. Apyrene spermatozoa with the tannic acid method
A and B: Cross and longitudinal sections, respectively, of the spermatid anterior cap (double arrowheads). X 25,100; X 64,800
C: Spermatid flagellum with positive reaction in the paracrystalline core (arrow) of the mitochondrial derivatives (md). On the axoneme, the electron density lumen of the accessory microtubules (double arrowhead) and one of the peripheral ones (arrowhead). Notice the electron dense reaction in one of the central pair of microtubules (arrow). X 90,000
D: Axoneme detail, showing the electron density of the lumen of the central pair (arrow), and the peripheral microtubules (arrowhead). Notice the electron lucid circular sub units within the accessory microtubules (double arrowhead). X 180,000
E: Spermatozoon from testis. On the axoneme: accessory microtubules with electron lucid cores (double arrowhead), peripheral ones (arrowhead) and one of the central pair (arrow) with electron dense reactions. Notice the heavy staining of axoneme and membrane proteins. Paracrystalline core (large arrow). X 138,000
F and G: Spermatozoa from deferent duct and seminal vesicle, respectively. Notice the thick external coat (white asterisk). In the deferent duct, this coat presents two regions: an external amorphous layer (longer arrow) and an internal one divided into small dense, aligned sub-units (small arrow). On the axoneme: accessory microtubules have electron lucid cores (double arrowhead); peripheral ones (arrowhead) and one of the central pair (arrow) are electron dense. Paracrystalline core (large arrow). X 124,000; X 85,000

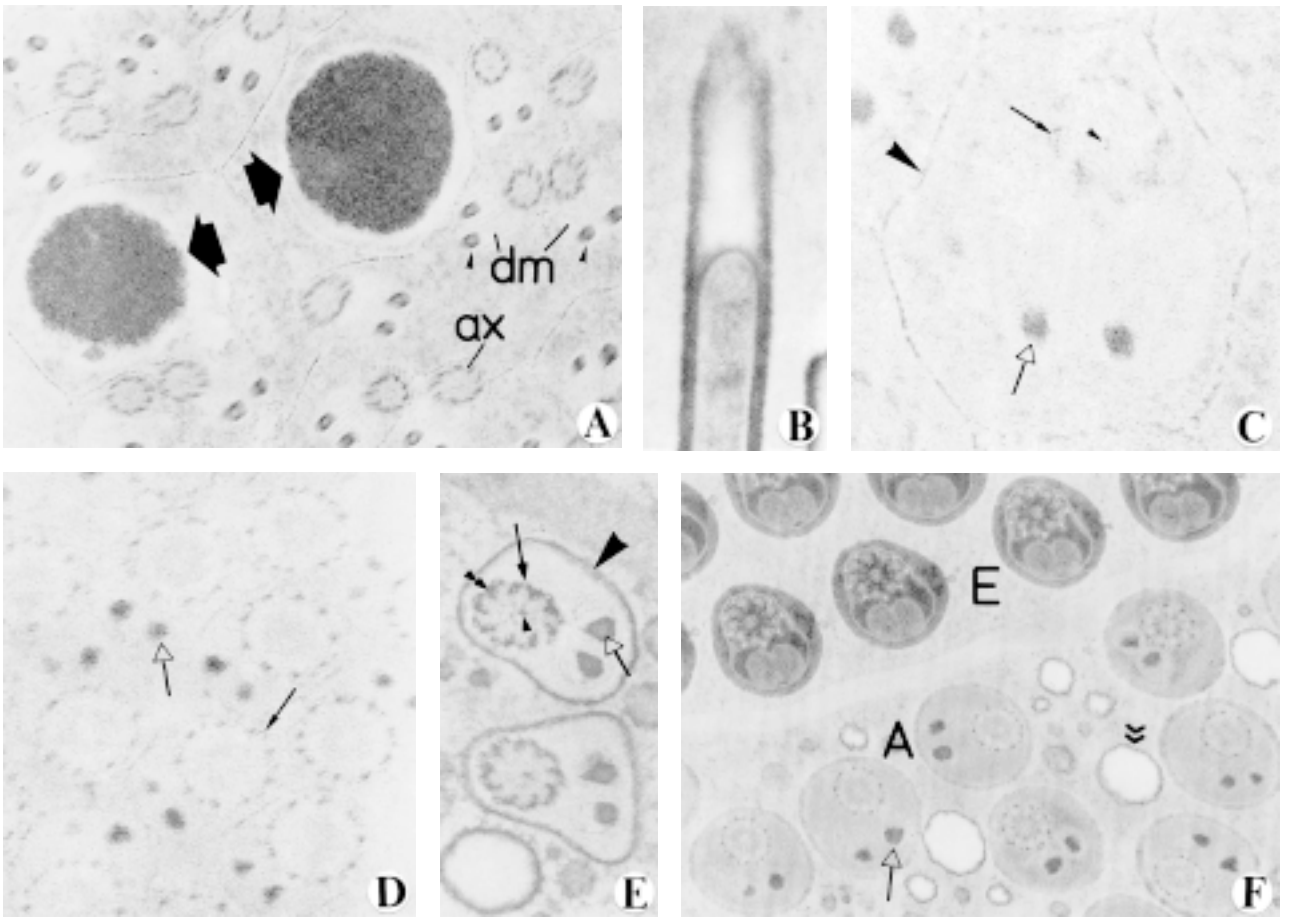
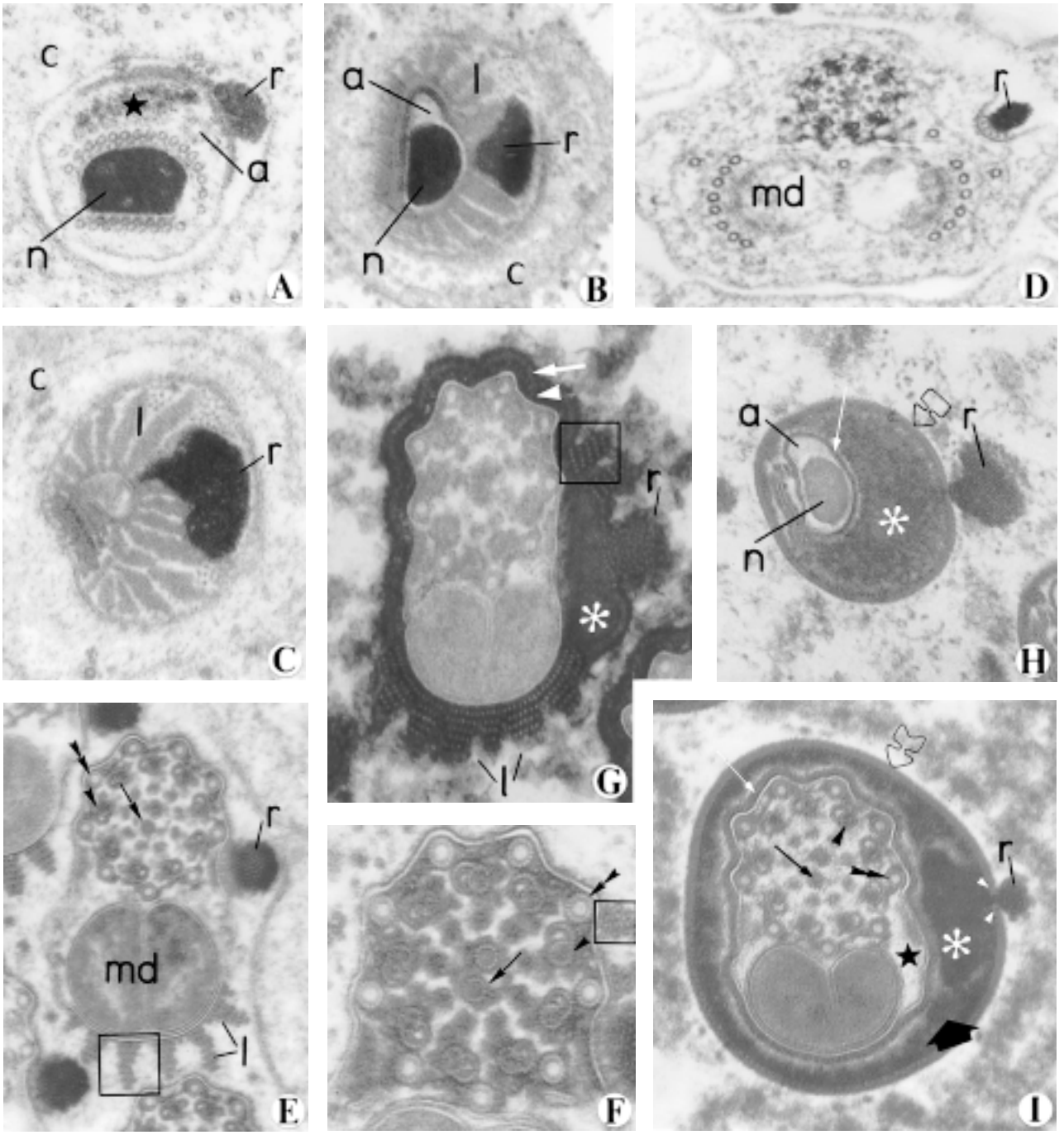


FIGURE 2. Apyrene Spermatozoa with E-PTA method
A: Spermatids with E-PTA positive micronuclei (arrows). Light staining occurs on axoneme (ax) and the paracrystalline core (arrowhead) of mitochondrial derivatives (dm). X 25,000
B: Basic proteins on the surface of the anterior cap. X 90,000
C: Spermatid flagellum with an E-PTA positive paracrystalline core (open arrow) and plasma membrane (large arrowhead). On the axoneme: accessory microtubules (arrow) and one of the central pair (arrowhead) are electron dense. X 55,600
D: Spermatozoa flagellum from testis. Paracrystalline core (open arrow) and accessory microtubules (arrow) of the axoneme are E-PTA positive. X 55,600
E and F: Apyrene spermatozoa (A) and Eupyrene sperm bundles (E) from the seminal vesicle. Apyrene coat (arrowhead) and paracrystalline core (open arrow) show an electron-dense reaction. Accessory microtubules, peripheral (arrow) and one of the central pair (arrowhead) are E-PTA positive. Secretion globule (double arrowhead) with basic proteins marked on its surface. X 45,200; X 25,000.

FIGURE 3. Eupyrene Spermatozoa with Tannic Acid method
A and B: Anterior regions of spermatid and spermatozoon from testis, respectively. Electron lucid acrosome (a), nucleus (n), lacinate (l) and reticular (r) appendages. In fig. (A) the dense amorphous mass (star). Cystic cell (C). X 54,300; X 60,400
C: Region anterior to the nucleus, showing the elaborate lacinate (l) and reticular (r) appendages, this last one with an electron-dense reaction. Cystic cell (C). X 100,700
D: Spermatid flagellum from the testis with reticular appendage (r) and mitochondrial derivatives (md) surrounded by microtubules. X 55,600
E: Spermatozoon flagellum from the testis. Lacinate appendages (l) with aligned tubular sub-units (square) and paracrystalline reticular appendage (r). Electron density of microtubules of central pair (arrow) and of A-sub-units of the peripheral (arrowhead); accessory microtubules have electron lucid cores (double arrowhead). Mitochondrial derivatives (md). X 96,000



F: Axoneme detail. Electron density of the central pair (arrow), peripheral (arrowhead) and accessory (double arrowhead) microtubules. Notice the tubular sub-units of lacinate appendages (square). X 153,300
G: Spermatozoa from the proximal deferens duct. Electron-dense reaction of the extracellular portions: first coat with an external thin amorphous layer (white arrow) and an internal one (white arrowhead), dense material (asterisk), paracrystalline reticular (r) and lacinate (l) appendages with tubular sub-units (square). X 126,000
H: Spermatozoon head region from the seminal vesicle. The coat can be divided into: external layer (open arrow), dense material (asterisk) and internal layer (white arrow). Electron lucid acrosome (a), nucleus (n), paracrystalline reticular appendage (r). X 83,400
I: Spermatozoon flagellum from the seminal vesicle. In the coat: external layer (open arrow) with a break (white arrowheads), amorphous material (large arrow), dense material (white asterisk) and internal layer (white arrow). In the axoneme: central pair (arrow), peripheral (arrowhead) and accessory (double arrowhead) microtubules. Reticular appendage (r). Notice that cytoplasmic material is not marked (star). X 100,000

presented a positive reaction for proteins (Figs. 1F and G). Especially in the deferent duct, the tannic acid technique indicated two regions in the coat: an external amorphous layer and an internal one, next to the cell membrane, that is divided into small dense, aligned cylindrical sub-units (Fig. 1F).

Eupyrene Spermatozoa

The anterior region of eupyrene spermatozoa is made up of an acrosome and a nucleus. The acrosome is formed initially by a spherical vesicle that elongates and becomes a tubular structure in the spermatozoon. In early spermatids, the spherical acrosomal vesicle (Fig. 4A), as well as the following elongating phases (Figs. 4B and C) are E-PTA positive. However, in late spermatids and spermatozoa, where this structure has acquired its definitive tubular form, no positive reaction for E-PTA was observed (Figs. 4E and F). For general proteins in spermatids and spermatozoa, there was no reaction and the acrosome appears electron lucid (Figs. 3A, B and H).

Simultaneously to the acrosomal changes there are nuclear modifications. The spermatid nucleus, in different chromatin condensation phases presented a positive reaction to E-PTA (Figs. 4A-C and E). In spermatozoa, however, the compact nucleus is E-PTA negative (Figs. 4F and J). With tannic acid there was no evident reaction in spermatid and spermatozoon nuclei (Figs. 3A, C and H) that differ from that observed with conventional methods (not shown).

The eupyrene flagella are composed of an axoneme and two mitochondrial derivatives. In contrast to apyrene flagella, the eupyrene mitochondrial derivatives do not present paracrystalline cores and no reaction was observed in this region, using both techniques. The centriolar region, in the anterior flagellar extremity, presented a strongly electron-dense reaction to E-PTA (Fig. 4D). The proteins that link the axoneme elements were strongly marked with both techniques (Figs. 3D-F, I and 4G, H, K). The central microtubule wall is E-PTA negative, and the lumen of the A microtubules of the peripheral pairs and the central ones are E-PTA positive (Figs. 4G, H and K).

With the tannic acid method, the walls were very well outlined, with 16 protofilaments in the accessory microtubules and 13 in the central ones (Fig. 3F). The lumen of the A microtubules of the peripheral pairs and the central ones were electron dense (Figs. 3D-F and I). No micro cylinder sub-units were observed in their lumen.

FIGURE 4. Eupyrene Spermatozoa with E-PTA method

A: Early spermatid with spherical nucleus (n) and an E-PTA positive acrosomal vesicle (v). Notice the positive reaction on the nucleus periphery (arrow). X 20,000

B and C: Longitudinal section of spermatids with an E-PTA positive nucleus (n). The tubular acrosome (a), reticular appendage (arrow), centriolar region (c) are E-PTA positive. A very light reaction occurs on the membrane (double arrowhead). X 20,000

D: Longitudinal section of spermatid with an E-PTA negative nucleus (n) and centriolar region (c) with abundant basic protein. X 20,000

E: Transverse section of spermatid with electron dense nucleus (n) and electron lucid acrosome (a). Reticular appendage (r) and amorphous material (star) with positive electron dense reaction. Cellular membrane (double arrowhead). X 54,000

F: Spermatozoa from testis. Nucleus (n) and acrosome (a) are electron lucid. Reticular appendage (r) with basic protein evident at the surface. Lacinate appendages (l) are E-PTA positive. X 116,000

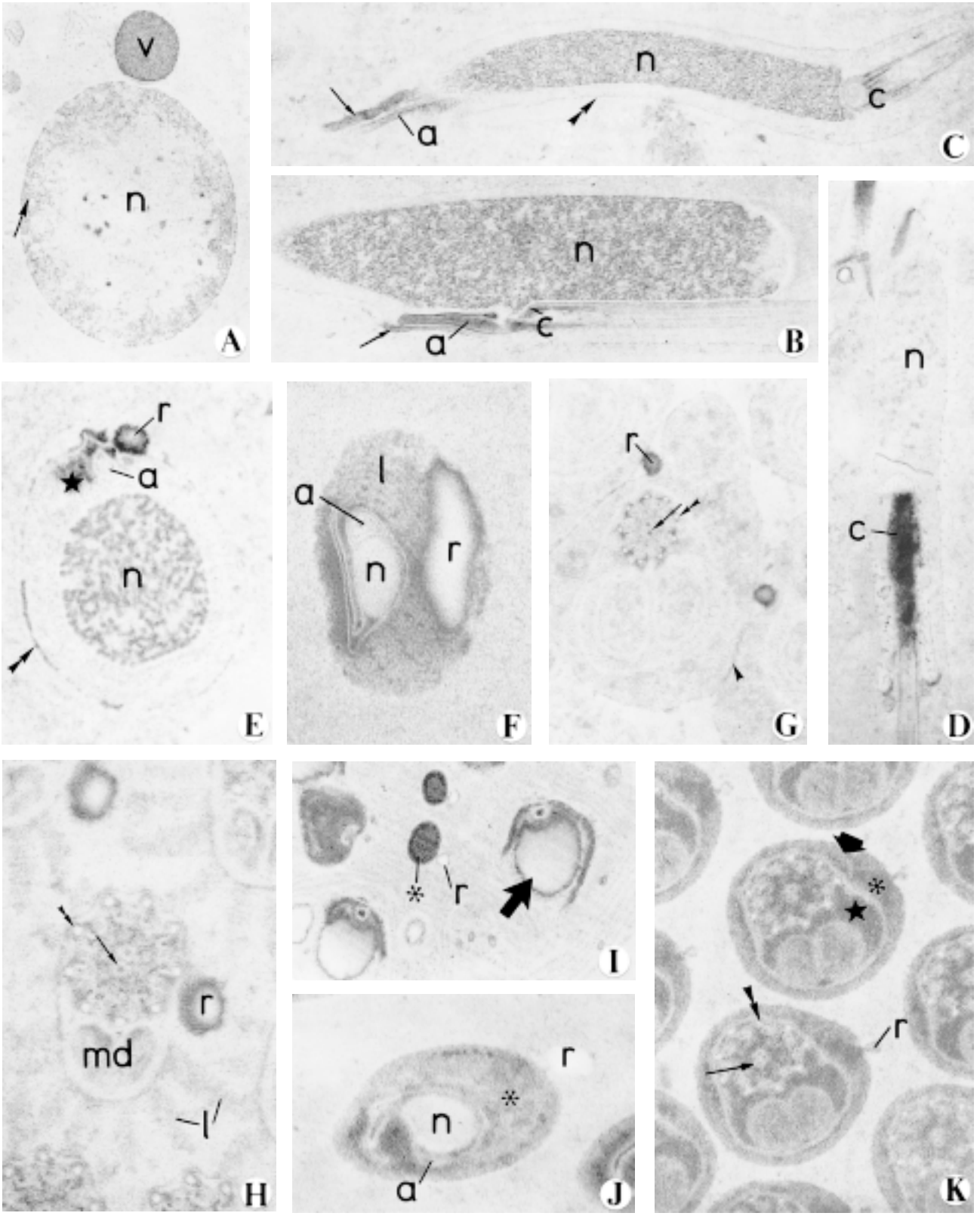
G: Spermatid flagellum. Positive reaction in reticular appendage (r). In the axoneme: the lumen of the central pair (arrow) and walls of the accessory microtubules (double arrowhead) are electron dense. Cellular membrane (arrowhead). X 55,600

H: Spermatozoon flagellum from the testis. Axoneme with basic protein; electron dense accessory microtubule wall (double arrowhead); central pair (arrow) with dense lumen and electron lucid wall. Reticular appendage (r) is E-PTA positive on the surface. Mitochondrial derivatives (md) and lacinate appendages (l) show a light reaction. X 116,000

I: Anterior ends of seminal vesicle spermatozoa. The globular structure (arrow) has a heterogeneous distribution of basic proteins. Spermatozoon coat (asterisk) and electron lucid reticular appendage (r). X 25,000

J: Anterior region of a seminal vesicle spermatozoon. Acrosome (a), reticular appendage (r) and nucleus (n) are E-PTA negative. Dense coat (asterisk). X 54,000

K: Spermatozoan flagella from the seminal vesicle with electron-dense reaction product in the coat (large arrow), principally in the dense material (asterisk) and the cytoplasmic material (star). In the axoneme: observe the dense core of the central pair of microtubules (arrow) and clear accessory and peripheral microtubules (double arrowhead). X 59,000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Exclusively external to the plasma membrane, the eupyrene sperm present appendages, called lacinate and reticular. The lacinate ones presented a positive reaction to E-PTA in the anterior regions, where they are well developed (Fig. 4F). However in the flagellar regions they presented a very light reaction (Fig. 4H). With tannic acid, these structures clearly showed a regular structure (Figs. 3C, E and F), which is much more defined than when observed using conventional fixation methods (not shown). In the square of figure 3F, it is possible to note that this regular structure consists of aligned tubular sub-units.

The reticular appendage is present since early spermatid formation and presented a positive reaction to E-PTA only in the external portions (Figs. 4E-H). With tannic acid, this appendage was highly contrasted in the anterior region (Figs. 3A-C) and presented a paracrystalline organization only in the flagellar portion (Figs. 3D and E).

Eupyrene spermatozoa in the proximal deferent duct region start to acquire a coat, strongly contrasted by tannic acid. It is possible to distinguish two regions: an amorphous external layer and an internal one, next to the cell membrane, that also presents small cylindrical sub-units, as seen for apyrene spermatozoa. Beyond this first coat, there is an accumulation of dense material between the cell membrane and the reticular appendage. The lacinate appendages, still with a regular organization, start to disintegrate (Fig. 3G).

In the seminal vesicle, these spermatozoa are totally encapsulated by the coat, which presents an electron-dense reaction for both techniques (Figs. 3H-I and 4J-K). The lacinate appendages are absent.

Here, the anterior extremity does not have a coat, but is composed of a large globular structure, which is externally E-PTA positive (Fig. 4I). The anterior region, at the level of the nucleus and acrosome, and the flagellar region are coated.

The internal layer next to the cell membrane, described for the deferent duct spermatozoa, is maintained in the seminal vesicle (white arrows in Figs. 3H and I) and the external one develops into a thick amorphous layer (large arrow in Fig. 3I). The dense material, next to the reticular appendage is well developed (white asterisk in Fig. 3I). Covering all the structures described above, except the reticular appendage, is the external layer of the coat (open arrow, Figs. 3H and I) that shows a break (white arrowheads, Fig. 3I) where it contacts the reticular appendage. In the nuclear region, the amorphous material does not exist and the eupyrene coat is uniformly filled with dense material (Figs. 3H and 4J). It is interesting to notice the presence of basic protein in the cytoplasmic material of these spermatozoa (star, Fig. 4K) and the absence of reaction of this material to tannic acid (star, Fig. 3I).

Discussion

There are several cytochemical studies involving spermiogenesis and/or spermatozoa of different insect orders: Coleoptera (Báo, 1991, 1996, 1998; Báo and Hamú, 1993; Craveiro and Báo, 1995; Fernandes and Báo, 1996), Diptera (Perotti, 1971, 1986; Perotti and Riva, 1988; Quagio-Grassiotto and Dolder, 1988; Báo and Dolder, 1990; Báo *et al*., 1992; Báo and Souza, 1992, 1993, 1994; Cattaneo *et al*., 1997; Perotti and Pasini, 1995; Pasini *et al*., 1996, 1999), Hemiptera (Báo, 1997; Fernandes *et al*., 1998; Fernandes and Báo, 1999, 2000), Orthoptera (Kierszenbaum and Tres, 1978) and Hymenoptera (Lino-Neto *et al*., 1999, 2000).

In the Lepidoptera order, however, these cytochemical analyses are very rare (França and Báo, 2000; Friedländer and Gershon, 1978) and most of them are related to very immature cells (Wolf, 1992, 1996a, 1996b, 1997; Wolf and Bastmeyer, 1991a, 1991b; Wolf and Joshi, 1996). So, this discussion is mostly based on comparisons with other insect orders.

Because of the structural complexity of extracellular structures of apyrene and eupyrene spermatozoa and their various morphologic alterations along the male and female reproductive tracts, a cytochemical study contributes expressively towards the understanding of this elaborate sperm dimorphism.

Due to the excellent preservation of the tissue with the tannic acid method, images of conventionally fixed specimens were not included in the present work (see Mancini and Dolder, 2001a). The tannic acid method developed by Dallai and Afzelius (1990), specific for proteins, was revealed to be extremely interesting for the morphologic description, mainly in detailing elements of axoneme and the extracellular coat.

The anterior cap of apyrene spermatozoa of *Euptoieta hegesia* presented basic proteins in the most external region, in contrast with the observation of França and Báo (2000) and Medeiros (1997), which detected a homogeneous basic protein reaction. The present research used different incubation times for E-PTA (2 to 24 hours), however, in none of them was the reaction homogeneous, as observed by the above authors. We believe this could reflect a difference in the compaction or the composition of this structure in dif-

ferent species. It is known that, in the testis, apyrene anterior regions are embedded in the cytoplasm of cystic cells. That could make penetration of the E-PTA stain more difficult. In *E. hegesia* we observed a large quantity of rough endoplasmic reticulum in the cytoplasm of cystic cells and we believe that the cap could be a product of these sites of protein production.

The protein detection of the paracrystalline core of mitochondrial derivatives in apyrene flagella is similar to the observations of França and Báo (2000), Medeiros (1997) and Mancini and Dolder (2001a, 2004). The presence of a paracrystalline core in mitochondrial derivatives is common to many orders of insects (Jamieson *et al.,* 1999) and its function is unclear.

In the eupyrene spermatozoa, the proteins that connect the axonemal elements were more strongly stained than in the apyrene ones. Because of the clear details of the axoneme seen with the tannic acid method, it was possible to count the protofilaments, as also observed by Medeiros (1997) in apyrene spermatozoa, and by Dallai and Afzelius (1990, 1995) and Jamieson *et al*. (1999) in eupyrene spermatozoa.

Observations of sub-units in the lumen of accessory microtubules in apyrene spermatozoa were made only by Medeiros (1997). Dallai and Afzelius (1990, 1995) also detected micro cylinder sub-units in many insect orders.

Studies of other insect groups also detected different reactions for the axoneme microtubules (Báo, 1991, 1996; Craveiro and Báo, 1995). Besides the possible presence of different types of tubulin in the axoneme (alfa, beta, gamma, acetylated, tyrosinated), there are different proteins that link the axoneme elements and occur in the microtubular lumen. These differences may explain the different staining patterns.

The plasma membrane of intratesticular apyrene spermatozoa is revealed by both techniques. The two methods permit the conclusion that cell membranes of apyrene and eupyrene spermatozoa are rich in proteins and glycoproteins. Glycoproteins are molecules important in cellular recognition and specificity, two essential characteristics of germ cells.

Concentric layers, not always clearly distinguishable, cover the extratesticular apyrene spermatozoa. These layers, in the deferent duct and seminal vesicle, presented a positive reaction to the techniques employed. In the present work, the tannic acid technique showed a similarity between the extracellular coat of the apyrene and eupyrene spermatozoa in the deferent duct. Other authors (Phillips, 1971; Friedländer and Gitay, 1972; Riemann and Gassner, 1973; Riemann and Giebultowicz, 1992) had already found some similarity between the coat of apyrene and eupyrene extra testicular sperm. The formation of apyrene and eupyrene coats occurs simultaneously in the deferent duct, which could also indicate a common origin.

The lacinate and reticular appendages, exclusive of the eupyrene spermatozoa, contain protein. The reticular appendage presented a reaction for basic proteins only in the external regions, possibly due to the difficulty of stain penetration in this highly compact structure. Using the tannic acid technique, it was possible to notice a compact paracrystalline formation in these appendages, as observed by Dallai and Afzelius (1990) and Jamieson *et al*. (1999).

The lacinate appendages reacted positively to E-PTA, mainly in the anterior regions of the spermatozoa. França and Báo (2000) detected intense and homogeneous basic protein in both appendages of *Anticarsia gemmatalis*. Medeiros (1997), however, only found an evident reaction for reticular appendages, also intense and homogeneous.

The globular structure at the anterior end of eupyrene spermatozoa in the seminal vesicle was previously observed by Mancini and Dolder (2001a) and has a positive reaction to E-PTA only on its surface and on the surrounding layer.

The tubular acrosome did not react to either technique. França and Báo (2000), however, had detected a light positive reaction in *A. gemmatalis*. This tubular structure seems to be very compact, which makes penetration difficult for large molecules, such as the stains used.

The nucleus did not have a positive reaction to the E-PTA in mature cells. However, in late spermatids, some areas were marked because of different degrees of chromatin condensation, which expose different sites for linking to basic nuclear proteins. Differences in nuclear reactions for basic proteins between spermatozoa and spermatids have been observed in Lepidoptera (França and Báo, 2000), Coleoptera (Báo and Hamú, 1993; Báo, 1996, 1998), Orthoptera (Kierszenbaum and Tres, 1978) and Diptera (Quagio-Grassiotto and Dolder, 1988). The nucleus of *E. hegesia* spermatozoa is homogeneous and does not present compartments. Zama *et al*. (2004), studying *Meliponini* spermatozoa (Hymenoptera), detected a dense positive reaction to E-PTA in a specific crescent moon nuclear region.

When leaving the testis, in the proximal deferent duct, the eupyrene spermatozoa begin to lose their lacinate appendages (Riemann, 1970; Phillips, 1971; Lai-Fook, 1982; Riemann and Giebultowicz, 1992; Kubo-Irie *et al*.,

1998; Mancini and Dolder, 1999, 2003). Concomitantly, these spermatozoa acquire a surrounding layer with the reticular appendage remaining external; both structures are strongly marked by tannic acid. Many authors suggest that the external layer could be the result of rearrangement of the lacinate appendages (Phillips, 1971; Friedländer and Gitay, 1972; Lai-Fook, 1982; Kubo-Irie *et al*., 1998). We believe that the lacinate appendages may contribute partially to coat formation, but mainly to the matrix formation, which surrounds the spermatozoa organized in bundles in the seminal vesicle.

In the seminal vesicle, the coat, with its three regions, is well developed and involves totally and homogenously the eupyrene spermatozoa. The break, detected in the external layer of the eupyrene coat, which permits extrusion of the reticular appendage, was identified in the seminal vesicle. This structure will later be occupied by the dense plate, which was observed in the spermathecae of *E. hegesia*, as well as in other species (Friedländer and Gitay, 1972; Riemann and Gassner, 1973).

The coat is rich in proteic components, mainly basic protein, as is the cytoplasmic material next to the mitochondrial derivatives. The matrix is also partially composed of protein, but the presence of basic protein is slight.

These results indicate that the extracellular structures of apyrene and eupyrene sperm are rich in proteins. Research on *E. hegesia* sperm indicated also the presence of carbohydrate in these structures (Mancini and Dolder, in preparation). Lacinate appendages of eupyrene sperm and the coats of both sperm types presented positive reactions for the protein detection techniques applied. This could be an indication of lacinate rearrangement to contribute to the coats in the deferent duct. Besides their proteic composition, the similarity of apyrene and eupyrene extra testicular coats indicates that they could have a common origin.

The present study is part of our investigation to understand the various modifications in apyrene and eupyrene sperm types along the male and female tract of *Euptoieta hegesia*, which involves techniques for carbohydrate detection, and methods for lectin and immunocytochemical detection (Mancini and Dolder, in preparation).

Acknowledgments

We would like to thank A. V. L. Freitas for supplying the butterflies and the Brazilian Agency FAPESP for financial support (01/01049-6).

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