Immunocytochemical localization of tubulins in spermatids and spermatozoa of Euptoieta hegesia (Lepidoptera: Nymphalidae)

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Abstract

A comparative analysis of the distribution of tubulin types in apyrene and eupyrene sperm of Euptoieta hegesia butterflies was carried out, also verifying the presence of tubulin in lacinate appendages of the eupyrene sperm. Ultrathin sections of LR White embedded spermatids and spermatozoa were labeled for alpha, beta, gamma, alpha-acetylated and alpha-tyrosinated tubulins. Apyrene and eupyrene spermatids show the same antibody recognition pattern for tubulins. All tubulin types were detected in axonemal microtubules. Alpha and gamma tubulins were also detected on the cytoplasmic microtubules. However, for beta and tyrosinated tubulins only scattered labeling was detected on cytoplasmic microtubules and acetylated tubulin was not detected. In apyrene and eupyrene spermatozoa only the axoneme labeling was analyzed since cytoplasmic microtubules no longer exist in these cells. Alpha, beta and tyrosinated tubulins were easily detected on the apyrene and eupyrene axoneme; gamma tubulin was strongly marked on eupyrene axonemes but was scattered on the apyrene ones. Acetylated tubulin appeared with scattered labeling on the axoneme of both sperm types. Our results demonstrate significant differences in tubulin distribution in apyrene and eupyrene axonemal and cytoplasmic microtubules. Extracellular structures, especially the lacinate appendages, were not labeled by antibodies for any tubulin.

Keywords: Immunocytochemistry; Tubulin; Apyrene; Eupyrene; Lacinate appendages

1. Introduction

The best-known case of sperm polymorphism occurs in butterflies and moths, which produce two distinct sperm types called apyrene and eupyrene spermatozoa (Riemann, 1970, Katsuno, 1977, Lai-Fook, 1982, Kubo-Irie et al., 1998, Jamieson et al., 1999, França and Báo, 2000, Mancini and Dolder, 2001, Mancini and Dolder, 2003, Mancini and Dolder, 2004a and Mancini and Dolder, 2004b). In general, eupyrene spermatozoa contain a nucleus and an acrosome, constituting an elongated head, and a long tail, whereas apyrene spermatozoa have a proximal tip covered by a dense cap and also a long tail.

However, the most evident difference is the elaborate extracellular structures present in eupyrene spermatozoa, which undergo morphological modifications along the male and female reproductive tract (Riemann, 1970, Phillips, 1971, Riemann and Thorson, 1971, Friedländer and Gitay, 1972, Lai-Fook, 1982, Kubo-Irie et al., 1998 and Mancini and Dolder, 2003). In the testis, they possess two exclusive appendages, called the lacinate and reticular

appendages (Phillips, 1970, Phillips, 1971 and Jamieson et al., 1999). In the extra testicular regions, the eupyrene sperm lose their lacinate appendages and acquire a complex coat (Phillips, 1971, Riemann and Thorson, 1971, Lai-Fook, 1982, Kubo-Irie et al., 1998, Mancini and Dolder, 2001 and Mancini and Dolder, 2003). The apyrene spermatozoa, however, present a less complex extracellular coat, acquired only in the extra testicular portions of the reproductive tract (Phillips, 1971, Friedländer and Gitay, 1972, Kubo-Irie et al., 1998, Garvey et al., 2000, Mancini and Dolder, 2001 and Mancini and Dolder, 2003).

The chemical composition of these appendages as well as the coats of both sperm types still remains unclear. Previous ultrastructural studies suggested that the lacinate appendages were tubulin-containing structures; its mean, intracellular formations being derived from transient microtubules of elongating eupyrene spermatids (Friedländer, 1976, Friedländer and Gershon, 1978 and Jamieson et al., 1999).

Microtubules are found in almost all eukaryote cell types associated with many cellular functions, such as cell division, morphogenesis, flagellar and ciliary motility, intracellular transport and cytoskeletal organization. This multiplicity of functions is thought to rely on the differentiation of various intracellular microtubule populations. These populations are generated by tubulin types, products of multigenic families, as well as post-translational modifications of tubulins and differential binding of associated proteins (Schulze et al., 1987, MacRae, 1997 and Ludueña, 1998).

Microtubules are comprised of heterodimeric complexes of alpha and beta tubulin types (Fosket and Morejohn, 1992 and Ludueña, 1998). The third type, the gamma tubulin, has a 28–35% sequence identical to the classical alpha and beta tubulin (Oakley and Oakley, 1989 and Ludueña, 1998). In addition, alpha and beta tubulin undergo a variety of post-translational modifications that include acetylation, detyrosynation, tyrosynation, phosphorylation, polyglutamylation and polyglycylation (MacRae, 1997 and Ludueña, 1998). The functional significance of these isoforms is being elucidated (Huitorel et al., 1999, Huitorel et al., 2002 and Kierszenbaum, 2002). Tubulin in highly stable microtubules is almost invariably acetylated and tyrosinated, although the relationship between post-translational modifications and stability remains unclear (Bulinski and Gundersen, 1991).

During the spermiogenesis, two tubulin-containing structures are assembled: a transient manchette and a stable axoneme. There are few studies about the tubulin composition of the insect sperm axoneme (Wilson and Forer, 1989, Fernandes and Báo, 1996, Fernandes and Báo, 2001, Mencarelli et al., 2000 and Taddei et al., 2000). Here, we made a comparative analysis of the different tubulin distribution in apyrene and eupyrene sperm of

Euptoieta hegesia butterflies and a verification of the possible presence of tubulin in lacinate appendages.

2. Materials and methods

Adult males of the butterfly E. hegesia were collected on the Campus of the Universidade Estadual de Campinas (SP, Brazil). Testis and seminal vesicle were dissected and used for transmission electron microscopy and immunocytochemistry.

2.1. Transmission electron microscopy

2.1.1. Conventional methods

Specimens were fixed in 2.5% glutaraldehyde, 4% paraformaldehyde, 1.5% sucrose and 5 mM calcium chloride in a 0.1 M sodium phosphate buffer for 12 h at 4 °C. After fixation, they were rinsed in the same buffer, post-fixed in 1% osmium tetroxide in sodium phosphate buffer for 3–5 h at 4 °C, dehydrated in acetone and embedded in Epoxy resin.

Specimens were fixed in 2.5% glutaraldehyde, 1% tannic acid, 1.5% sucrose and 5 mM calcium chloride in a 0.1 M sodium phosphate buffer for 3 days at 4 °C. The materials were rinsed in the same buffer and contrasted in an aqueous solution of 1% uranyl acetate for 2 h at room temperature (Dallai and Afzelius, 1990). They were dehydrated in acetone and embedded in Epoxy resin.

Specimens were fixed in 2.5% glutaraldehyde, 1% ruthenium red in a 0.1 M sodium cacodylate buffer for 2 h, in the dark, at room temperature and then washed in the same buffer. They were post-fixed in 1% osmium tetroxide in sodium cacodylate buffer for 1 h, followed by 1% osmium tetroxide, 1% ruthenium red in sodium cacodylate buffer, in the dark, at room temperature and then washed in the same buffer. They were dehydrated in acetone and embedded in Epoxy resin.

The ultrathin sections obtained were contrasted with uranyl acetate and lead citrate and observed in a transmission electron microscope (LEO 906).

2.2. Immunocytochemistry

Specimens were fixed in 0.5% glutaraldehyde, 4% paraformaldehyde, 0.2% picric acid, 3% sucrose and 5 mM calcium chloride in a 0.1 M sodium phosphate buffer for 3 h at room temperature. After rising in the same buffer, free aldehyde groups were quenched with 50 mM glycin in 0.2 M sodium phosphate buffer overnight at 4 °C and contrasted with 2% uranyl acetate in 15% acetone for 2 h also at 4 °C. The specimens were dehydrated in acetone and embedded in LR White resin.

The 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 in monoclonal antibodies against alpha tubulin (clone DMIA), alpha-acetylated tubulin (clone 6-11B-1), alpha-tyrosinated tubulin (clone TUB-1A2), beta tubulin (clone TUB 2.1) and gamma tubulin (clone GTU-88), diluted in the proportion of 1:100 (British Biocell International, UK). After washing with PBS-BSA, the grids were incubated for 1 h with the respective labeled secondary antibody-Au (mouse or rabbit-IgG-Au-conjugated 10 nm) at a dilution of 1:20. After incubation, the grids were washed with PBS and distilled water, stained with uranyl acetate and lead citrate and observed in a transmission electron microscope.

3. Results

Ultrathin sections of LR White embedded spermatids and spermatozoa were labeled against alpha, beta, gamma, alpha-acetylated and alpha-tyrosinated tubulins. The results are summarized in Table 1.

Table 1.
Summary approach of tubulins types detection

Antibodies (anti-tubulins)	Spermatid		Spermatozoa		
	Axoneme	Cytoplasm	Axoneme		Extracellular structures
			Apy	Eup	
Alpha	++++	+++	++++	++++	–
Beta	++++	+	++++	+++	–
Gamma	+++	++	+	++	–
Alpha-acetylated	+	–	+	+	–
Alpha-tyrosinated	++	+	++	++	–

The signals represent the approximated number of particles per region (axoneme and cytoplasm): (+): 1–5; (++): 6–10; (+++): 11–15; (++++): 15–20; (–) particles not found.

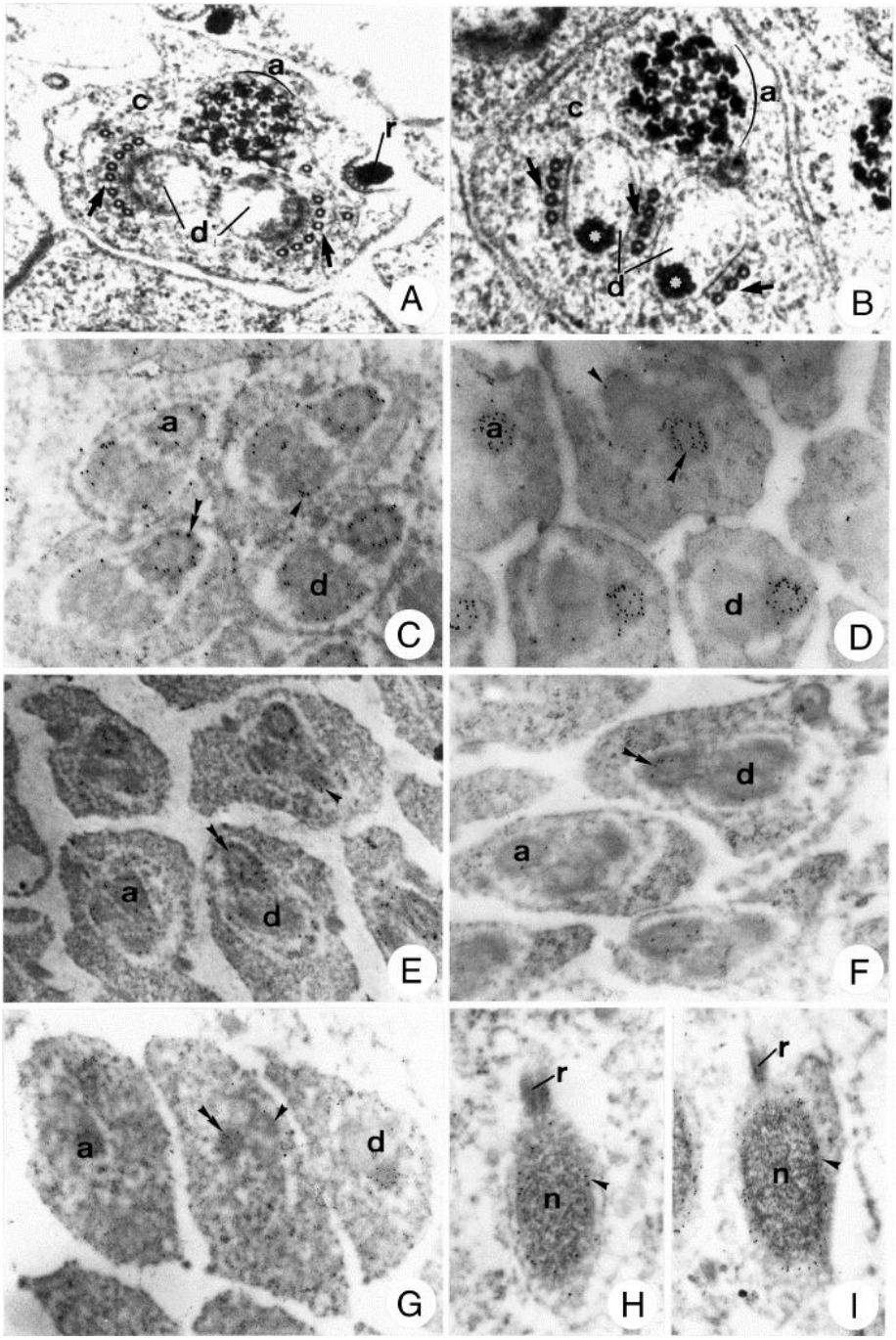

Fig. 1. Apyrene and eupyrene spermatids. Conventional method (tannic acid). Transverse sections on the tail of eupyrene (A) and apyrene (B) spermatids. Axoneme (a), mitochondrial derivatives (d), with paracrystalline core (white asterisk) in the apyrene spermatid, and the reticular appendage (r) on the eupyrene one. In the cytoplasm (c) notice the cytoplasmic microtubules (arrows). (A) 55,500×; (B) 72,000×. Immunocytochemical method. Transverse sections of the tails of spermatids labeled for alpha (C), beta (D), gamma (E), alpha-acetylated (F) and alpha-tyrosinated (G) tubulins. Axoneme (a) with a different labeling pattern (double arrowhead); cytoplasmic microtubules (arrowhead) surrounding mitochondrial derivatives (d). (C) 42,000×; (D) 40,000×; (E) 25,000×; (F) 32,000×; (G) 24,000×. Immunocytochemical method. Transverse sections on the head of eupyrene spermatids labeled for gamma (H) and alpha-tyrosinated (I) tubulins. Cytoplasmic microtubules (arrowhead) surrounding the nucleus (n). Reticular appendage (r). 11,500×.

Apyrene and eupyrene spermatids show the same antibody recognition pattern for tubulins. Alpha tubulin was detected on the axonemal microtubules as well as on the cytoplasmic microtubules that surround the mitochondrial derivatives (Fig. 1C). Beta tubulin was detected on the axonemal microtubules but their occurrence on the cytoplasmic microtubules appears to be scattered (Fig. 1D). Gamma tubulin was detected on the axoneme and on the cytoplasmic microtubules that surround mitochondrial derivatives (Fig. 1E) and nucleus (Fig. 1H). Acetylated tubulin was detected scattered on the axoneme but not in the cytoplasm (Fig. 1F). Tyrosinated tubulin was detected on the axoneme and scattered in the cytoplasmic microtubules of the tail (Fig. 1G), as well as on the head where cytoplasmic microtubules surround the nucleus (Fig. 1I).

3.2. Apyrene and eupyrene spermatozoa

The tail of apyrene and eupyrene spermatozoa is made up of a 9 + 9 + 2 axoneme type and two mitochondrial derivatives (Fig. 2 and Fig. 3, respectively). The eupyrene one also presents two exclusive extracellular structures, denominated reticular and lacinate appendages, which present a paracrystalline organization, with circular subunits observed in transverse sections, when the tannic acid and ruthenium red techniques are applied (Fig. 3A and B, respectively). In the extra testicular regions, the eupyrene sperm lack the lacinate appendages, and both sperm types acquire an extracellular coat (Fig. 2 and Fig. 3).

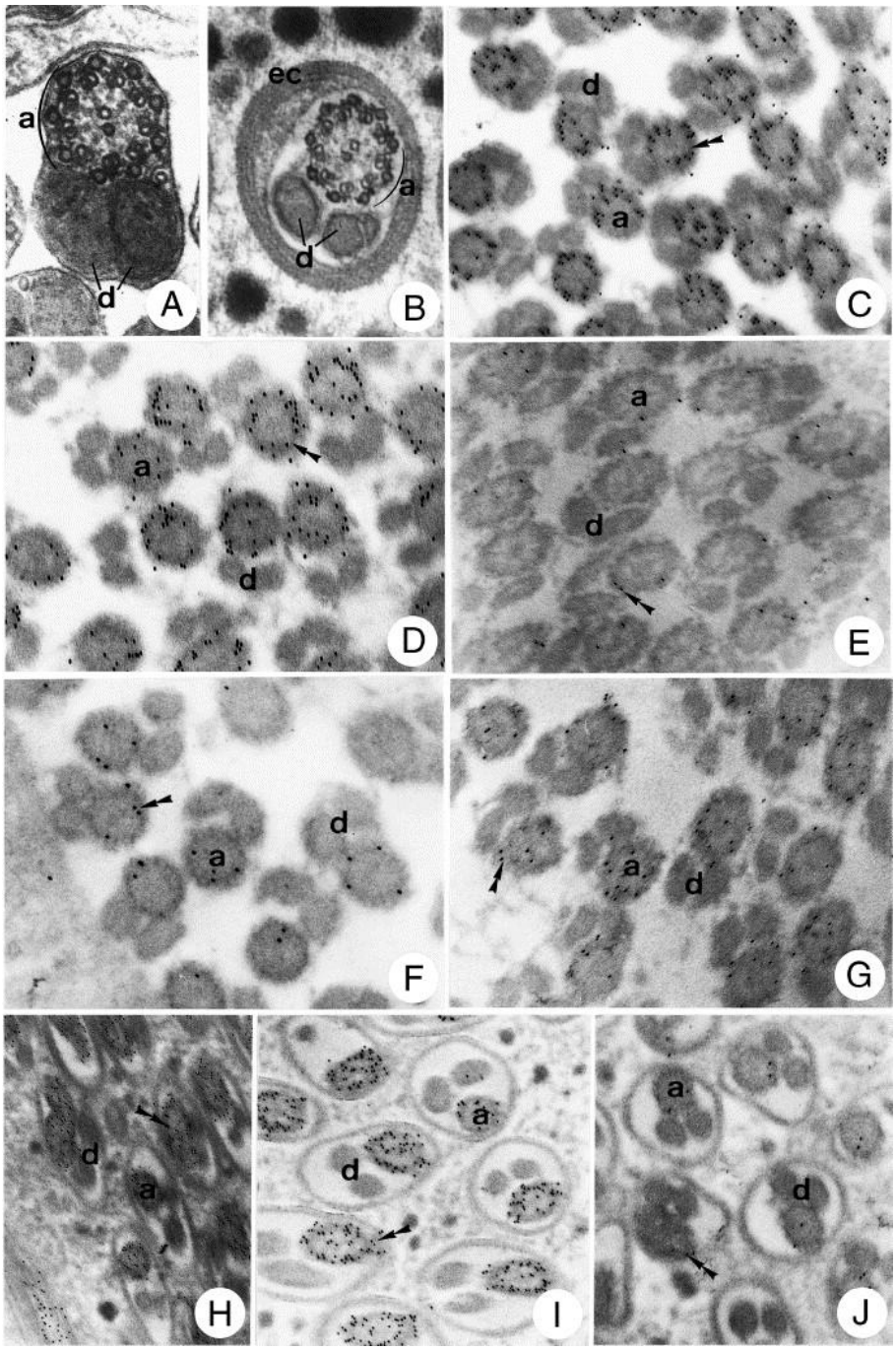

Fig. 2. Apyrene spermatozoa. Conventional method (glutaraldehyde + paraformaldehyde). Transverse sections on the tail of apyrene spermatozoon. (A) Apyrene spermatozoa from testis. (B) Apyrene spermatozoa from the seminal vesicle with an extracellular coat (ec). Axoneme (a), mitochondrial derivatives (d). (A) 90,000×; (B) 70,000×. Immunocytochemical method. Transverse sections of the tails of apyrene spermatozoa labeling for alpha (C), beta (D), gamma (E), alpha-acetylated (F) and alpha-tyrosinated (G) tubulins. Axoneme (a) with different labeling (double arrowhead); no cytoplasmic microtubules surround mitochondrial derivatives (d). (C) 42,000×; (D) 45,000×; (E) 45,000×; (F) 50,000×; (G) 45,000×. Immunocytochemical method. Transverse sections of the tail of apyrene spermatozoa from the seminal vesicle labeled for alpha (H), beta (I) and alpha-acetylated (J) tubulins. Axoneme (a) with different labeling intensity; no labeling on the coat. (H) 26,000×; (I) 32,000×; (J) 32,000×.

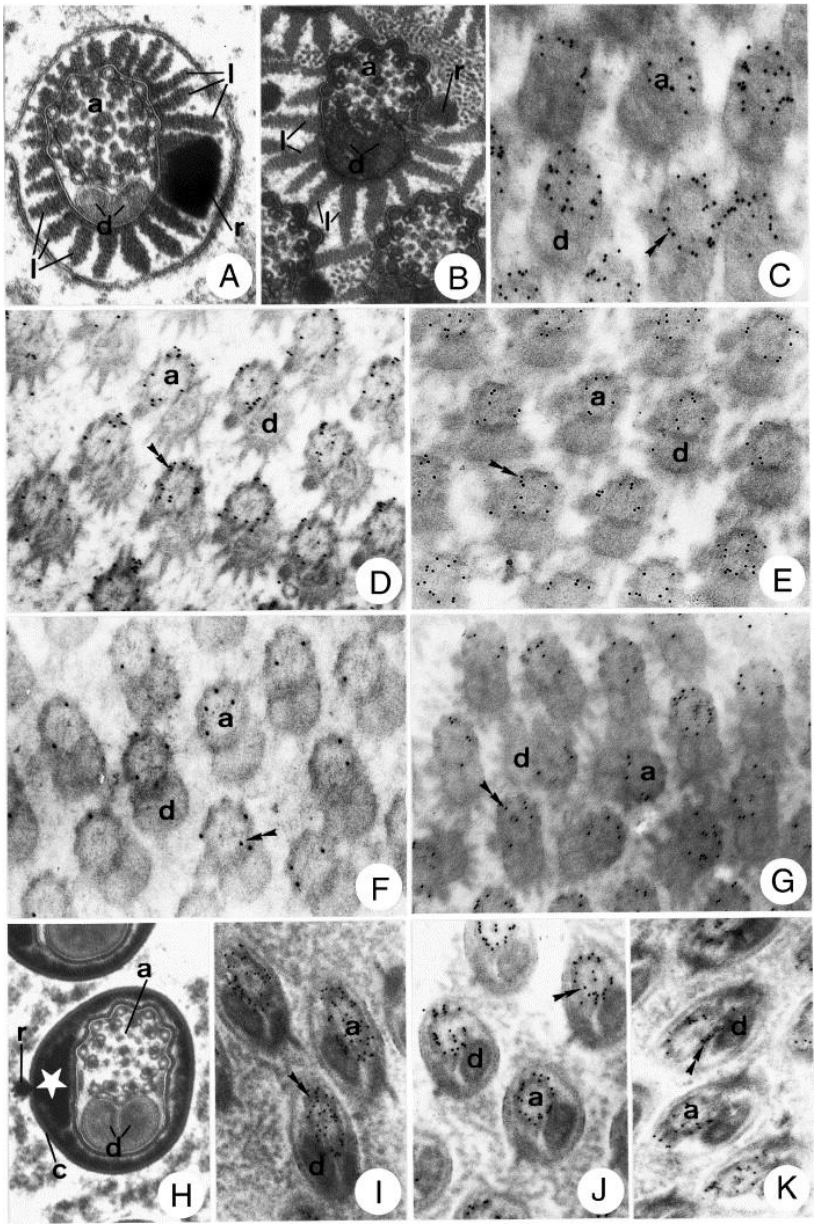

Fig. 3. Eupyrene spermatozoa. Conventional method (tannic acid and ruthenium red, respectively). Transverse sections of the tail of eupyrene spermatozoa from the testis. Axoneme (a), mitochondrial derivatives (d), reticular (r) and lacinate (l) appendages with paracrystalline organization. (A) 97,000×; (B) 70,000×. Immunocytochemical method. Transverse sections of the tail of eupyrene spermatozoa labeled for alpha (C), beta (D), gamma (E), alpha-acetylated (F) and alpha-tyrosinated (G) tubulins. Axoneme (a) with various labeling patterns; no cytoplasmic microtubules surround mitochondrial derivatives (d). No labeling on reticular (r) and lacinate (l) appendages. (C) 42,000×; (D) to (G) 30,000×. Conventional method (tannic acid). Transverse section of the tail of eupyrene spermatozoa from the seminal vesicle. Axoneme (a), mitochondrial derivatives (d), reticular appendage (r) and extracellular coat (c) with dense material (star). 70,000×. Immunocytochemical method. Transverse sections on the tail of eupyrene spermatozoa from seminal vesicle labeling against alpha (I), beta (J) and alpha-tyrosinated (K) tubulins. Axoneme (a) with different labeling, mitochondrial derivatives (d), no labeling on the coat. 30,000×.

In apyrene and eupyrene spermatozoa, only the axoneme was labeled (Fig. 2 and Fig. 3). The cytoplasmic layers of microtubules, which surrounded the nucleus and the mitochondrial derivatives in intermediate spermatids, had been eliminated during spermiogenesis. The labeling pattern for tubulins on axonemal microtubules of apyrene and eupyrene spermatozoa was similar. In general, alpha tubulin was most intensely labeled (Fig. 2 and Fig. 3) and beta tubulin was also clearly detected (Fig. 2 and Fig. 3). Gamma tubulin was strongly marked on eupyrene axonemes (Fig. 3E), but it was scattered on apyrene ones (Fig. 2E). Acetylated tubulin label was sparsely scattered on both apyrene (Fig. 2F) and eupyrene (Fig. 3F) axonemal microtubules. Tyrosinated tubulin, however, was clearly detected on eupyrene axonemes (Fig. 3G), as well as on apyrene ones (Fig. 2G).

Extracellular structures were not labeled by antibodies against any tubulin. Reticular and lacinate appendages of intratesticular eupyrene spermatozoa did not show any labeling (Fig. 3C–G) as also happens with the apyrene and eupyrene coats acquired in post-testicular regions (Fig. 2 and Fig. 3). Axonemal microtubules of apyrene (Fig. 2H–J) and eupyrene (Fig. 3I–K) spermatozoa from the seminal vesicle show similar tubulin distribution as seen in these cell types from the testis.

4. Discussion

The sperm polymorphism that occurs in the Lepidoptera order results in two sperm types, which differ in morphology and function. The eupyrene sperm are responsible for egg fertilization, while the apyrene ones, which are devoid of a nucleus, are involved in sperm competition (Drummond, 1984, Silberglied et al., 1984, Gage, 1994, Cook and Wedell, 1996, Cook and Wedell, 1999, Snook, 1997 and Snook, 1998). Their ultrastructure is complicated by the presence of exclusive eupyrene appendages, for which the chemical composition and functions are still not elucidated. Besides this, both sperm types, especially the eupyrene one, undergo several extracellular modifications along the reproductive tracts and the importance of these structures remains unclear.

Only few ultrastructural studies investigated some cytochemical aspects on apyrene and eupyrene sperm (Friedländer, 1976, Friedländer and Gershon, 1978 and França and Báo, 2000). Wolf, 1992, Wolf, 1996a, Wolf, 1996b and Wolf, 1997 and Wolf et al., 1988, Wolf and Bastmeyer, 1991a, Wolf and Bastmeyer, 1991b and Wolf and Joshi, 1996 made important studies using immunofluorescence for tubulin distribution on Lepidoptera spermatocytes and early spermatids.

Here, we carried out a comparative analysis of tubulins and their post-translational modifications in late spermatids and spermatozoa from the testis and seminal vesicle of E. hegesia butterflies. Our results demonstrate distribution differences in tubulins and their post-translational modifications in apyrene and eupyrene axonemal and cytoplasmic microtubules.

All tubulins studied are present in the axonemal microtubules of E. hegesia. In fact, other cytochemical studies (Mancini and Dolder, 2004b) reported differential labeling for protein in axonemal microtubules of apyrene and eupyrene spermatozoa.

Alpha and beta tubulins were most strongly labeled on apyrene and eupyrene axonemal microtubules of spermatids and spermatozoa. Cytoplasmic microtubules presented alpha tubulin but no beta tubulin staining in our conditions. The differential labeling obtained with beta tubulin might be due to a different accessibility of the epitope in the axoneme and to the presence of different microtubule-associated proteins. In contrast, in spermatids of phytophagous bugs, alpha tubulin was detected only in the axoneme and beta tubulin was detected in both axonemal and cytoplasmic microtubules (Fernandes and Báo, 2001). In the beetle Diabrotica speciosa spermatids, alpha tubulin was clearly detected in both axonemal and cytoplasmic microtubules (Fernandes and Báo, 1996).

Gamma tubulin is involved in microtubule nucleation. It appears in the microtubule organizing centers (Joshi, 1994), as also in the spindle pole body (Oakley et al., 1990), the pericentriolar material (Fuller et al., 1995), and the basal body (Liang et al., 1996). It binds to their minus ends (Li and Joshi, 1995) and can self-assemble into a novel tubular structure (Shu and Joshi, 1995). Here, this tubulin type is well distributed on both axonemal microtubules and on cytoplasmic microtubules of eupyrene and apyrene spermatozoa. In the phytophagous bug spermatids, this tubulin is not present on the axoneme microtubules (Fernandes and Báo, 2001).

Acetylation seems to occur in tubulin after it has been incorporated into microtubules (Sasse and Gull, 1988 and Wilson and Forer, 1989). It has been correlated with flagellar assembly (L'Hernault and Rosenbaum, 1985 and Huitorel et al., 2002). It is, generally, an indicator of stable microtubules and is particularly notable in axonemal microtubules (Schulze et al., 1987, Webster and Borisy, 1989, Takemura et al., 1992 and Ludueña, 1998). Nevertheless, in unicellular organisms such as Trichomonas vaginalis, T. foetus and Trypanosoma brunei acetylated tubulin has been demonstrated in unstable microtubules during the elongating phase and mitosis (Sasse and Gull, 1988, Delgado-Viscogliosi et al., 1996 and Lopes et al., 2001). Here, we detected scattered acetylated tubulin on the stable axonemal microtubules. No labeling was observed on cytoplasmic microtubules.

Tyrosination has been seen reported in a variety of cytoplasmic microtubules in vertebrates (Gundersen et al., 1984 and Arregui and Barra, 1995) and is common in the interphase network and in the spindle (Ludueña, 1998). It was detected in the A-tubules of the peripheral doublets of Chamydomonas (Johnson, 1998) and sea urchin axonemes (Multigner et al., 1996). In the Apis mellifera sperm axoneme, the accessory microtubules presented less tyrosinated alpha tubulin than the other axonemal microtubules (Mencarelli et al., 2000). The functional significance of this isoform has not been elucidated. According to Huitorel et al., 1999 and Huitorel et al., 1999 tyrosinated and acetylatilated alpha tubulins do not seem to play a critical role in flagellar motility. On the other hand, polyglutamylation plays a dynamic role in the dynein-based motility process (Huitorel et al., 1999). In E. hegesia, these post-translational modifications were detected on both axonemal and cytoplasmic microtubules; on the latter it was very scattered.

We did not analyze the distribution of these tubulin types along the whole sperm tail as Taddei et al. (2000), where tyrosinated, beta-III tubulin was not homogeneously distributed along the sperm tail of the insect Bacillus rossius.

There was no labeling for tubulins on any extracellular structure of apyrene and eupyrene spermatozoa. Other observations in E. hegesia sperm indicate that proteins compose the extracellular structures: reticular and lacinate appendages and extra testicular coat of both sperm types (Mancini and Dolder, 2004b). According to Friedländer (1976) the lacinate appendages are transitory forms of tubulin, which are destined to generate these appendages or other non-microtubular structures. They may generate these structures after having contributed to the process of nuclear elongation. Additional support for this theory was supplied by treatment in vivo with the antimitotic agent vinblastine sulphate by Friedländer and Gershon (1978). The lacinate appendages are no longer found after vinblastine treatment, as do also the tubulin-containing structures. According to Friedländer and Gershon, the lacinate appendages would be made of microtubule proteins, although they lacked the structure of any of the known polymorphic tubulin forms. Here, we did not find any evidence for this theory.

The cytoplasmic microtubules are located surrounding the nucleus of eupyrene sperm and mitochondrial derivatives of both sperm types. In fact, for the eupyrene spermatozoa the microtubular lining corresponds to the extracellular location of lacinate appendages. We disagree with Friedländer (1976) and Friedländer and Gershon (1978) that these appendages are formed of tubulin. However, these cytoplasmic microtubules could contribute to the formation and orientation of the lacinate appendages. The differential distribution among microtubules of different cytoplasmic regions suggests that the alpha, beta, gamma and their

post-translational modifications play a role in determining the biochemical and functional specificity of microtubules.

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