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The formation of accessory tubules in spermatids of the red firebug, *Pyrrhocoris apterus* (Hemiptera: Pyrrhocoridae)

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Abstract. Sperm of most insects are characterized by the presence of a 9+9+2 pattern of microtubules. In the present fine structure study, the development of the accessory tubules is described in spermatids of *Pyrrhocoris apterus* using transmission electron microscopy of ultrathin sections. The first step in the assembly of the accessory tubules consisted in the formation of hook-shaped protofilament ribbons attached to the B-tubules of the axoneme. Subsequently the protofilament ribbons detached from the B-tubules and formed individual accessory tubules. This mode of formation of accessory tubules has been described previously in Orthoptera, Plecoptera, Diptera, Strepsiptera, Trichoptera and Coleoptera species and, possibly, is common to most insects. The detachment of protofilament sheets from the B-tubulus under natural conditions corroborates experimental evidence that the lateral interaction among protofilaments is very weak. The study revealed also a high incidence of multiflagellate sperm and this phenomenon is documented in the present article.

INTRODUCTION

Sperm structure has been studied in a variety of insect species (for a review, see Jamieson, 1987). With few exceptions (Phillips, 1969), the sperm tail contained a central pair of microtubules (MTs), nine peripheral doublets and a set of nine accessory tubules located lateral to the doublets. This arrangement of MTs is typical of insect sperm and referred to as the 9 + 9 + 2 pattern. In *Tenebrio molitor* (Coleoptera) (Cameron, 1965), *Limnephilus rhombicus* (Trichoptera), *Gryllus campestris* (Orthoptera), *Isoperla* sp. (Plecoptera), *Ceratitis capitata* (Diptera) and *Stylops* sp. (Strepsiptera) (Dallai & Afzelius, 1993) the origin of the accessory tubules has been studied. They were found to originate as C-shaped appendages of flagellar B-tubules. For the terminology of flagellar components, the reader is referred to Witman (1990).

The structure of the sperm tail has been examined in a series of Hemiptera species including the red firebug *Pyrrhocoris apterus* (Pyrrhocoridae: Hemiptera). Consistently the studies revealed the 9 + 9 + 2 pattern of tubules (Tandler & Moriber, 1966; Folliot & Maillet, 1979; Trandaburu, 1973; Rosati et al., 1976; Dallai & Afzelius, 1980; Itaya et al., 1980; Afzelius & Dallai, 1989; Motzko, 1992; Wolf, 1995). The accessory tubules of two Hemiptera species studied in this respect, *P. apterus* and *Cercopis* sp., possessed 16 protofilaments (Dallai & Afzelius, 1990). Protofilaments are longitudinal assemblies of tubulin molecules. The lateral interaction of protofilaments leads to complete MTs (for a review of microtubular structure, see Burns & Surridge, 1994). In the present article, the route of formation of accessory tubules in *P. apterus*, a model organism of the Hemiptera (compare Socha, 1993), is studied. Ultrathin cross sections through spermatid tails in the

bug have been analysed using electron microscopy. The bug has served for several studies revolving around structural aspects of spermatogenesis in the past (Godula, 1979; Wolf & Motzko, 1995; Wolf & Joshi, 1996; Wolf, 1996b).

MATERIAL AND METHODS

Wild-type larvae of *Pyrrhocoris apterus* originating from laboratory cultures at the Institute of Entomology, České Budějovice (Czech Republic) were reared in the laboratory on linden seeds (*Tilia* sp.) at room temperature in July 1994. For more information on the biology of this species, the reader is referred to the review by Socha (1993). Testes of young adults were prepared for electron microscopy according to Wolf (1994). In brief, the gonads were excised and transferred to Ringer's solution containing 2.5% glutaraldehyde. After 5 min, 3 volumes of 8% tannic acid (Merck) in phosphate buffer (0.067M, pH 6.8) were added. The gonads were postfixed in phosphate-buffered OsO_4 (1%, 1 h), dehydrated in ethanol and embedded in Epon 812. Ultrathin sections were analysed with a Philips EM 400 transmission electron microscope operated at 80kV.

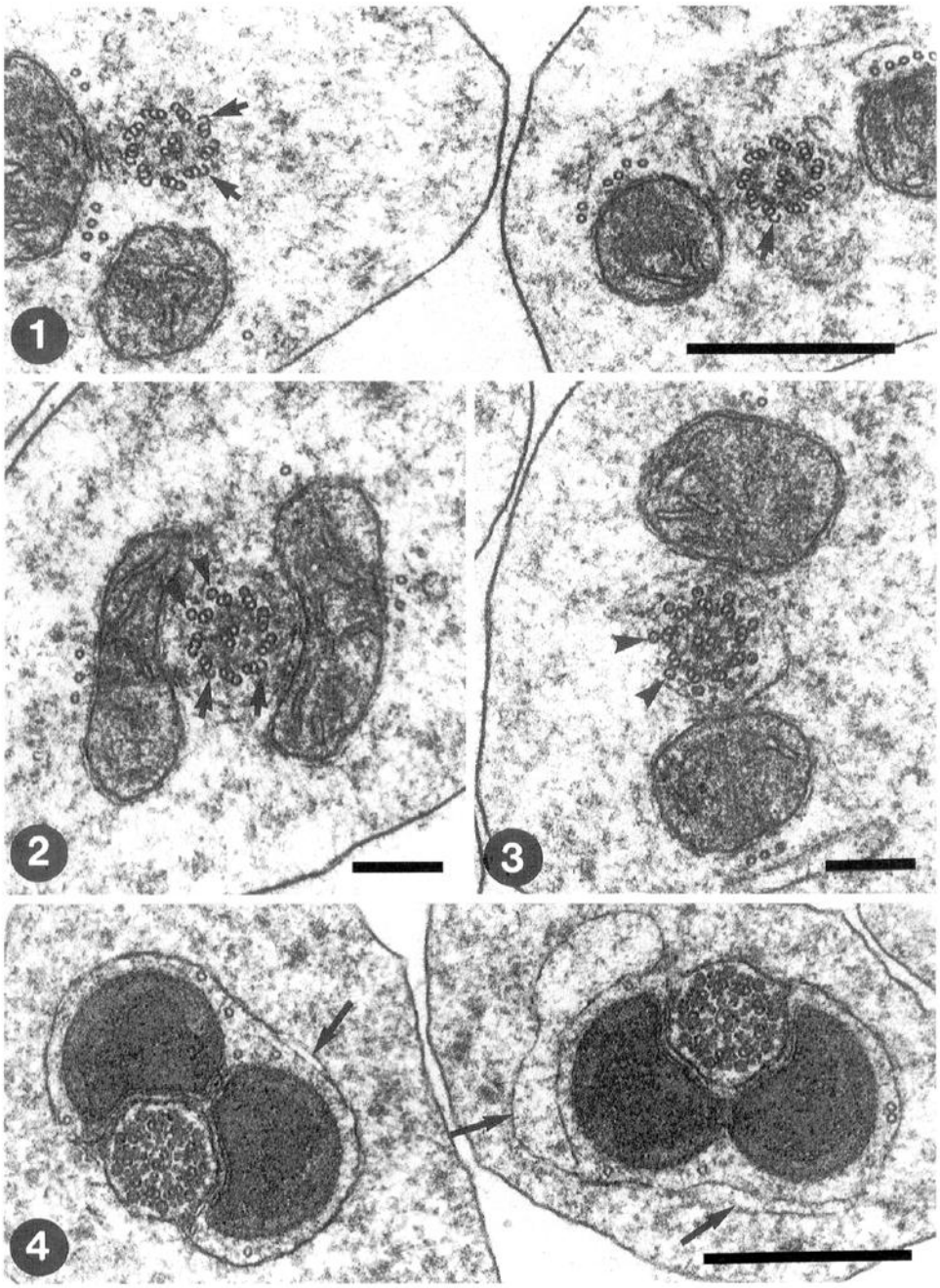
RESULTS

During the progression of spermiogenesis of *P. apterus*, the electron density of the mitochondrial matrix increased and agranular endoplasmic reticulum (ER) developed within the sperm tail. In the present study, young spermatids are those whose mitochondria possess a matrix of only slightly higher electron density than the lateral cytoplasm. Agranular ER is scarce in the spermatid tails and it has a wide lumen. Advanced spermatids, in turn, have mitochondria with an electron-dense matrix and agranular ER is present in higher quantity. Most often its lumen is not detectable.

Cross sections through the tails of young spermatids revealed a central pair of MTs and nine peripheral microtubular doublets. These had C-shaped appendages attached to their B-tubules (Fig. 1). The C-shaped appendages were clearly visible in most cases. However, the impression of microtubular triplets arose, when the appendages had a very flat curvature. Also electron-dense material, lying inadvertently at the open end of the C-shaped protofilament ribbons, obscured the nature of the C-shaped appendages.

Consistently, the C-shaped protofilament appendages were attached to the B-tubule at a site close to their junction with the A-tubule. The spermatid tail possessed two nebenkern derivatives, which appeared round or elongated in cross sections. The mitochondria had few flat cristae. Usually, a small number of MTs was usually situated close to the

Figs 1–4. Transmission electron micrographs of cross-sections through spermatids of *Pyrrhocris apterus*. 1 – two monoflagellate young spermatids. In addition to the nebenkern derivatives, the spermatid tails contains some individual microtubules and the axonemes. Note the C-shaped appendages (arrows) of the B-tubules close to their junction with the A-tubules. Elements of the agranular endoplasmic reticulum are visible close to the axoneme in the spermatid at the right hand side. Scale bar represents 0.5 μm. 2 – monoflagellate young spermatid. C-shaped protofilament appendages project from the B-tubules (arrows). At the same level of the spermatid tail complete accessory tubules are visible (arrowheads). Scale bar represents 200 nm. 3 – monoflagellate young spermatid. Nine complete accessory tubules (arrowheads) are visible in addition to the peripheral doublets and the central microtubular pair of the axoneme. Note the sheet of agranular endoplasmic reticulum with a wide lumen around the axoneme and close to the nebenkern derivative at the bottom. Scale bar represents 200 nm. 4 – two monoflagellate advanced spermatids. The axonemes and the nebenkern derivatives are surrounded by agranular endoplasmic reticulum. With the exception of a few sites (arrows), a lumen is not detectable. Note that the accessory tubules possess a content of medium electron density. Scale bar represents 0.5 μm.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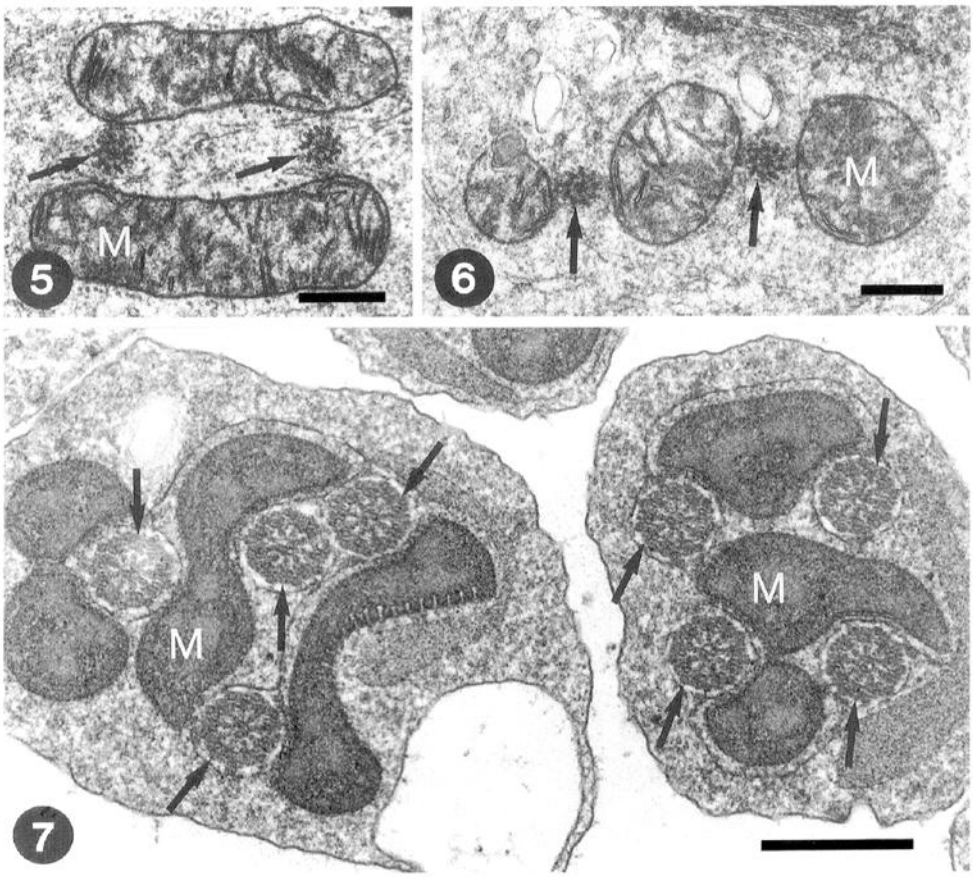

Figs 5–7. Transmission electron micrographs of cross-sections through spermatids of *Pyrrhocoris apterus*. 5, 6 – two biflagellate young spermatids. In one cell (5), there are two elongated nebenkern derivatives (M), whilst in another (6) three round mitochondria (M) are observed. Arrows indicate the axonemes. Scale bars represent 0.5 μm. 7 – two quadriflagellate spermatids in an advanced state. In both cells, three nebenkern derivatives (M) are visible. Arrows indicate the axonemes. Scale bar represents 0.5 μm.

mitochondrial surface. Small cisternae of agranular ER were found within the spermatid tail (Fig. 1).

In young spermatids, the detachment of the C-shaped appendages from the B-tubules and the formation of complete accessory tubules could be observed. There were cells with C-shaped appendages of the B-tubules and complete accessory tubules at the same level within one sperm bundle (Figs 2, 3). C-shaped protofilament ribbons freed from the axonemal tubules were not observed. Thus, detachment is followed immediately by the formation of a complete accessory tubule. With a diameter of approximately 30 nm, the complete accessory tubules were wider than the neigbouring axonemal and cytoplasmic MTs (diameter approximately 25 nm). Other features of the sperm tail such as the

nebenkern derivatives, associated MTs and ER cisternae did not change during this period of accessory tubule formation (compare Figs 1 and 3).

The lumen of accessory tubules in late spermatids contained material of medium electron density, while other axonemal tubules as well as MTs associated with the nenbenkern derivatives appeared empty (Fig. 4). The nebenkern derivatives flanked closely the axoneme and possessed an electron-dense matrix, which was subdivided in an electron-dense central portion and a slightly less electron-dense peripheral area. Cristae were not detectable. The axoneme was surrounded by a membranous sheath which was continuous with the sheath enveloping the nebenkern derivatives, including the associated MTs. It represented agranular ER with a very narrow lumen. This property of the ER was also observed in spermatid tails of another Hemiptera species, *Graphosoma lineatum* (Wolf, 1995). Filamentous bridges between axonemal tubules and the nebenkern derivatives have been described in Hemiptera species (Dallai & Afzelius, 1980, 1990), but were not observed clearly in the present study. It is probable that the bridges form in later stages of spermiogenesis only.

The study revealed numerous multiflagellate spermatids. Almost all cross-sectioned cysts inspected in the present study contained several spermatids with two to four axonemes. In the biflagellate variety, two nebenkern derivatives were found (Fig. 5). Other cells possessed three individually detectable mitochondria (Fig. 6). The quadriflagellate spermatids possessed three extended or lobulate mitochondria (Fig. 7).

DISCUSSION

Microtubules are nucleated in various ways. There are electron-dense masses of various shapes and sizes in the cytoplasm. MTs are attached to the material which is believed to play a role in their organization. The electron-dense material is enriched in a newly discovered tubulin isoform, gamma-tubulin. It is probable that this molecule lies at the surface of the electron-dense material and may be involved directly in the nucleation of microtubular outgrowth (Joshi, 1994; Oakley, 1994). The peripheral doublets of axonemes are organized differently. They extend from the A- and B-tubules of the basal bodies (Witman, 1990). The origin of the central microtubular pair of the axoneme is less clear and it has been speculated that these MTs are nucleated by elements situated within the basal body lumen (Wolf & Kyburg, 1989). These modes of MT formation have in common that the (–) or slowly growing end of MTs is in contact with a distinct organizing structure and that elongation occurs by addition of tubulin subunits throughout the entire microtubular perimeter at the (+) end of the MTs.

The origin of accessory tubules in sperm flagella of insects deviates from the modes of MT nucleation described above. It was first observed in a beetle that there is an interdependence between flagellar doublet MTs and accessory tubules. Accessory tubules formed as lateral C-shaped appendages of B-tubules (Cameron, 1965). This route of formation has also been found in a series of insect species (see Introduction) including *P. apterus* (this study) and may be ubiquitous in insects. The longitudinal alignment of tubulin molecules leads to the formation of protofilaments. These, in turn, possess the ability to interact with one another and form regular MTs. The lateral interaction of protofilaments is considered to be very plastic (Burns & Surridge, 1994). In insect spermatids this plasticity is expressed under natural conditions: axonemal doublet MTs have the capacity to laterally

nucleate protofilament ribbons which project into the cytoplasm. Several observations indicate that the process is under strict control. Irrespective of the species, the C-shaped protofilament appendages extend consistently from the B-tubules close to their junction with the the A-tubules (Cameron, 1965; this study). It has been determined, in a previous study involving a series of insect species, that the C-shaped appendages originate near the fourth protofilament of the B-tubules when counting from its attachment site to the A-tubule. This site also gives rise to the C-tubules of basal bodies (Dallai & Afzelius, 1993) and, therefore, appears to have an inherent potential to serve as a template for the assembly of protofilament sheets. Although C-shaped protofilament appendages have also been observed on cytoplasmic MTs (e.g., Turner, 1972; Lee, 1985), only the B-tubules of the axonemal doublets are able to give rise to protofilament appendages, which detach and form individual tubules. The involvement of tectins in the lateral nucleation of microtubules may be excluded. Tectins are a set of MT-associated proteins present in the axonemal A-tubules as stable filaments (Nojima et al., 1995). However, it cannot be ruled out that insect spermatids possess another type of MT-associated protein incorporated at a specific sites along the B-tubules and able to inititate the formation of lateral protofilament ribbons.

The dissociation of protofilament ribbons from the B-tubules of insect spermatids under natural conditions is informative. The lateral interaction of protofilaments is considered to be weak (for a review, see Burns & Surridge, 1994). The connection between the protofilament ribbons and the B-tubules in insect spermatids appears to be even weaker, because it is severed easily, while the axonemal doublets do not disintegrate and the C-shaped protofilament sheets remain intact.

Multiflagellate sperm and spermatids have been observed often in insects (e.g., Kessel, 1967; Woyke, 1984; Führer & Krehan, 1985; Quickie et al., 1992; Wolf, 1996a). In the present study, their occurrence has been demonstrated in a hemipteran species. Multiflagellate sperm which, most probably, contain multiples of the regular haploid chromosome number, are believed to arise when meiotic divisions are flawed or through secondary fusion of cells (Wolf, 1996a). The latter process is facilitated in insects, because cytokinesis is incomplete during gonial mitoses. A complex system of cytoplasmic bridges connects the germ cells throughout spermatogenesis (see Marec et al., 1993 and references therein). The connection between the germ cells may be severed only in spermiogenesis, during cytoplasmic sloughing (Wolf, 1995). It is conceivable that the cytoplasmic bridges widen occasionally and cells fuse with one another. In some Orthoptera, evidence is accumulating that the presence of supernumerary chromosomes, the so-called B chromosomes, raises the incidence of cell fusion in spermatogenesis and the formation of multiflagellate structurally aberrant spermatids (Suja et al., 1987, 1989). A cytogenetic analysis of the *P. apterus* strain used in the present study did not reveal B chromosomes (Wolf, 1996b). For information on B chromosomes, the reader is referred to Jones & Rees (1982). Thus, the development of multiflagellate spermatids is a pathological phenomenon that occurs regularly at a low rate in *P. apterus* possessing an euploid chromosome complement.

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