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ORIGINAL ARTICLE

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Characteristics of the sperm flagellum in fungus gnats (Insecta, Diptera, Mycetophiloidea)

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Abstract. Spermatozoa from several members of the closely related Mycetophilidae and Keroplatidae were examined by electron microscopy using a fixative that contains glutaraldehyde and tannic acid, followed by a post-fixative that consists of uranyl acetate rather than osmium tetroxide. With this fixative, the detailed architecture of the flagellar axoneme and its various microtubules could be resolved. The so-called accessory tubules, which surround the central 9+2 unit of the sperm axoneme, were found to have 16 protofilaments in several examined Mycetophiloidea, but in no other Diptera. As 16 is the common number in holometabolic insects, it is presumably the plesiomorphic condition in Diptera. Other fungus gnats have accessory tubules with 15 or 14 protofilaments. The intertubular material situated between the accessory tubules is smaller in the examined members of the Mycetophilidae than in the Keroplatidae. The acrosome consists of an apical vesicle, which in one species, *Macrorhyncha ancae,* has three microtubular doublets in its anterior part and two large and three small extensions which extend posteriorly along the sperm axoneme.

A. Introduction

Comparative studies of spermatozoa have shown interspecific variations in structure that are without parallel in other animal cell types. Such studies can give valuable indications on the phylogenetic position of an animal. Examples of this are given in two recent monographs by Jamieson (1987, 1991). He coined the term 'spermiocladistics' for this discipline of zoology.

In some previous studies, we present evidence for the fungus gnats Mycetophilidae being similar to the stem group from which other families of the Diptera are derived (Dallai and Mazzini 1983; Dallai and Afzelius 1990; Dallai et al. 1993). This opinion is compatible with data on the external morphology, although the family Tipulidae (crane flies) has also been proposed by White (1949) to be the most basic dipteran group.

Most Pterygota have a flagellar axoneme that contains nine accessory microtubules outside the familiar 9+2 unit. The axoneme is then described by the shorthand notation 9+9+2. In all Pterygota except a few, the accessory tubules consist of 16 protofilaments; thus, this situation seems to be plesiomorphic in Pterygota (excepting Ephemeroptera). Such tubules have been found in Mycetophilidae and Keroplatidae but in no other dipteran family. [In this paper we will adhere to the terminology by Zaitzev (1994) in which Keroplatidae is a separate family of fungus gnats and together with Mycetophilidae forms the Mycetophiloidea. In our previous papers, the Mycetophilidae were thought to include Keroplatinae and Mycetophilinae].

The aim of the present investigation has been to examine, by electron microscopy, spermatozoa from several species of Mycetophilidae and Keroplatidae. The information will then be used with the hope that it will shed some light on the relationship of various genera within these families.

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B. Materials and Methods.

Spermatozoa and spermatids from the following mycetophiloid species have been examined in this study: Keroplatidae: *Keroplatus reaumurii* (Dufour, 1839); *Macrorhyncha ancae* Matile, 1975; *Macrocera* sp.; *Orfelia persimilis* Caspers, 1991; *Neoplatyura nigricauda* (Strobl, 1893); Mycetophilidae: *Allodia alternans* (Zetterstedt, 1938); *Brevicornu crassicorne* (Stannius, 1831); *Exechia fusca* (Meigen, 1804); *E. seriata* (Meigen, 1830); *Phthinia* sp.; *Boletina* sp.

The animals were collected in the neighbourhood of Siena. The testes and deferent ducts were dissected and fixed in a mixture of 2% glutaraldehyde, 1% tannic acid and 1.8% sucrose in a 0.1 M phosphate buffer. The material was block stained in 1% uranyl acetate in distilled water, dehydrated and embedded in epoxy resin. This technique is modified from Mizuhira and Futaesaku (1972) in that the osmium postfixation is omitted. After sectioning and section-staining with uranyl acetate and lead citrate, the sections were examined in a Philips CM 10 electron microscope. The micrographs have been printed in such a way that the dynein arms have a clockwise orientation. This is equivalent to viewing the flagellum from the basal body towards the distal end.

C. Results

Keroplatus reaumurii

The sperm tail consists of a simple 9+2 axoneme adhering to a mitochondrial derivative with a semicircular cross-section (Fig. 1). The A tubules have 13 protofilaments as in all other examined species. The incomplete B tubule has 10 protofilaments plus a thinner so-called 11th filament at the inner border between the A and B tubules. The lumen of the A tubule contains a characteristic inclusion, which in cross-section is seen as a pentagonal structure with a dark centre. This structure is termed the pentagon (Afzelius et al. 1990). The B tubule has a larger inclusion with an elliptic cross-sectional profile. Dynein arms and spokes are well visible, but the nexin links are only rarely seen in preparations of this kind. The acrosome consists of an elongated vesicle and there is no subacrosomal material.

Neoplatyura nigricauda

The sperm flagellum of this species has been previously described (Dallai and Afzelius 1990), although at that time it was incorrectly classified as an *Orfelia* species. The axonome has nine accessory tubules and is, thus, of 9+9+2 type. Each accessory tubule has 16 protofilaments (Fig. 2).

Macrorhyncha ancae

This fungus gnat has spermatozoa of a kind that seems not to exist in any other insect. The axoneme as well as the acrosome extend for almost the entire length of the cell. The acrosome has a peculiar shape. Anteriorly it projects in front of the centriolar region of the axoneme and envelops three microtubular doublets with regular A and B tubules, but with no dynein arms (Figs. 5, 6). At the centriolar region, the acrosomal vesicle is branched into two units. These flank the centriole which contains a reduced number of microtubular doublets and singlets but no triplets. The slender anterior tip of the mitochondrial derivative is also visible at this level as is a homogeneous substance that will be called the centriolar adjunct (Fig. 7).

Further posteriorly, the cell diameter is gradually increased, mainly because of a larger size of the mitochondrial derivative. The crystal inside the mitochondrial derivative has a simple, approximately square cross-section anteriorly (Fig. 8) and an anchor-like profile posteriorly (Fig. 9). The axoneme at these levels is of the 9+9+2 type and each of the nine accessory tubules has a wall of 16 protofilaments. Five branches of the acrosome are seen at these levels: two larger ones on opposite sides of the axoneme and three smaller ones which alternate with four of the accessory tubules. The acrosome, therefore, has five fingers posteriorly, which all are extensions from a single anterior vesicle. One of the microtubular doublets (with index number 3) is connected to the mitochondrial derivative by a prominent bridge, two of the other ones (numbers 1, 6, terminology according to Afzelius 1959) are connected to the large acrosomal branches. These connections can be regarded as extensions of the so-called intertubular material.

The nuclear region of the sperm is positioned at a rather posterior level. The nucleus partly envelops the mitochondrial derivative, which in its turn partly surrounds the axoneme (Fig. 10). The posterior end of the spermatozoon consists of the 9+9+2 axoneme only (Fig. 11) which distally has a reduced number of microtubules.

Macrocera sp

The spermatozoon of this species has a more conventional appearance. Most of its length consists of a 9+9+2 axoneme in which, however, the accessory tubules have 15 protofilaments (Fig. 3). The axoneme is accompanied by a mitochondrial derivative which along much of the sperm's tail has a cross-sectional diameter of about 1 μm. Its crystal is relatively small and has a hexagonal profile.

Orfelia persimilis

The flagellum consists of a 9+9+2 axoneme that is unique among examined insects species in one respect, in that it has 14 protofilaments in the accessory tubules. The centriole and the anterior portion of the 9+9+2 axoneme are located within a deep invagination in the nucleus. Further posteriorly, the axoneme is only partly surrounded by the nucleus, as is also the mitochondrial derivative (Fig. 4), or is flanked only by the mitochondrial derivative (not illustrated). The lengths of the accessory tubules are somewhat variable, those close to doublets number 7, 8 being longer than the others.

Allodia alternans

The proximal portion of the axoneme is contained in a tubular nucleus and has the centriole near its anterior end

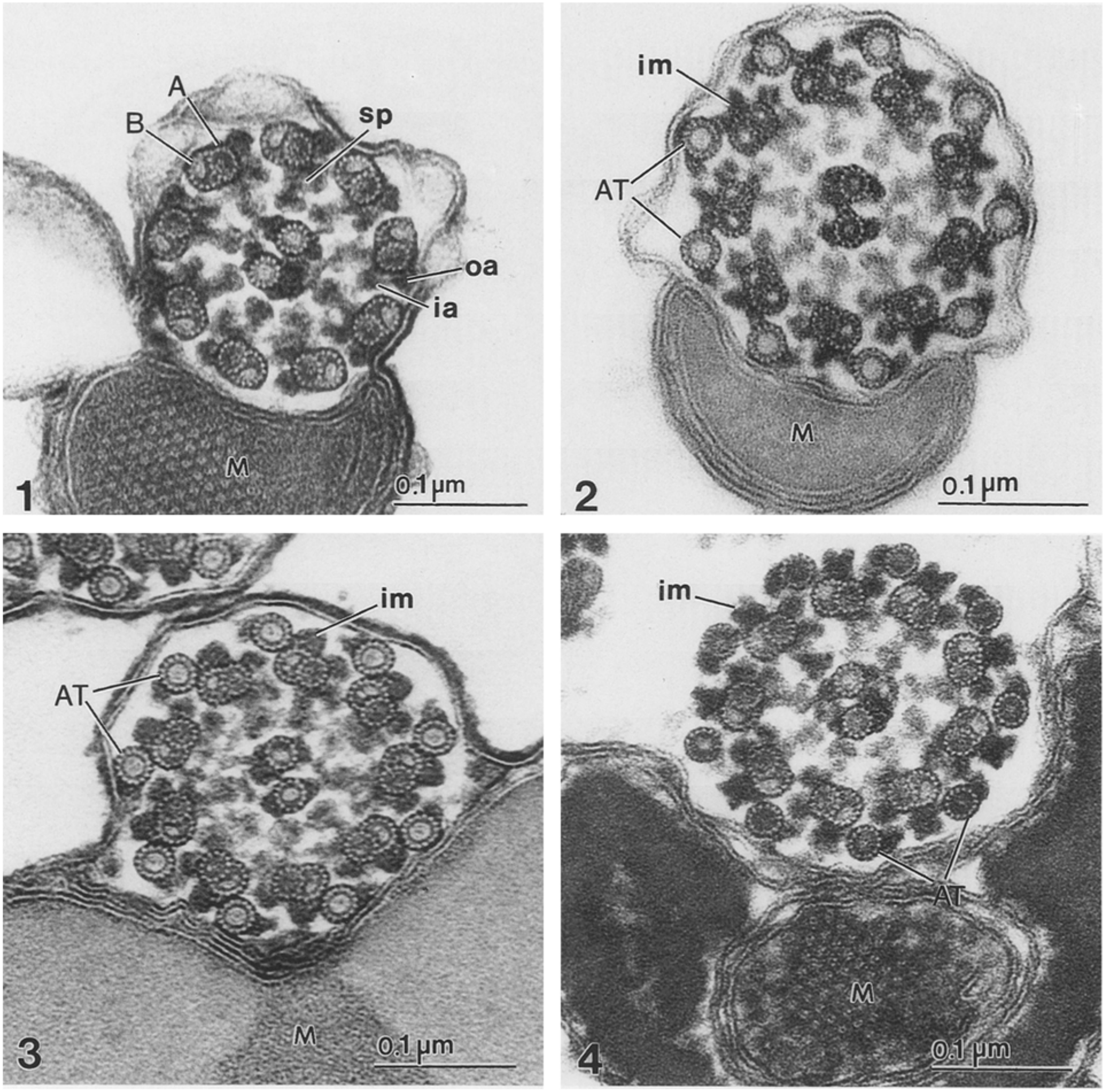


Fig. 1–4 Transversely sectioned sperm tails from four species of the fungus gnat family Keroplatidae.

Fig. 1 *Keroplatus reaumurii*

Fig. 2 *Neoplatyura nigricauda*

Fig. 3 *Macrocera* sp.

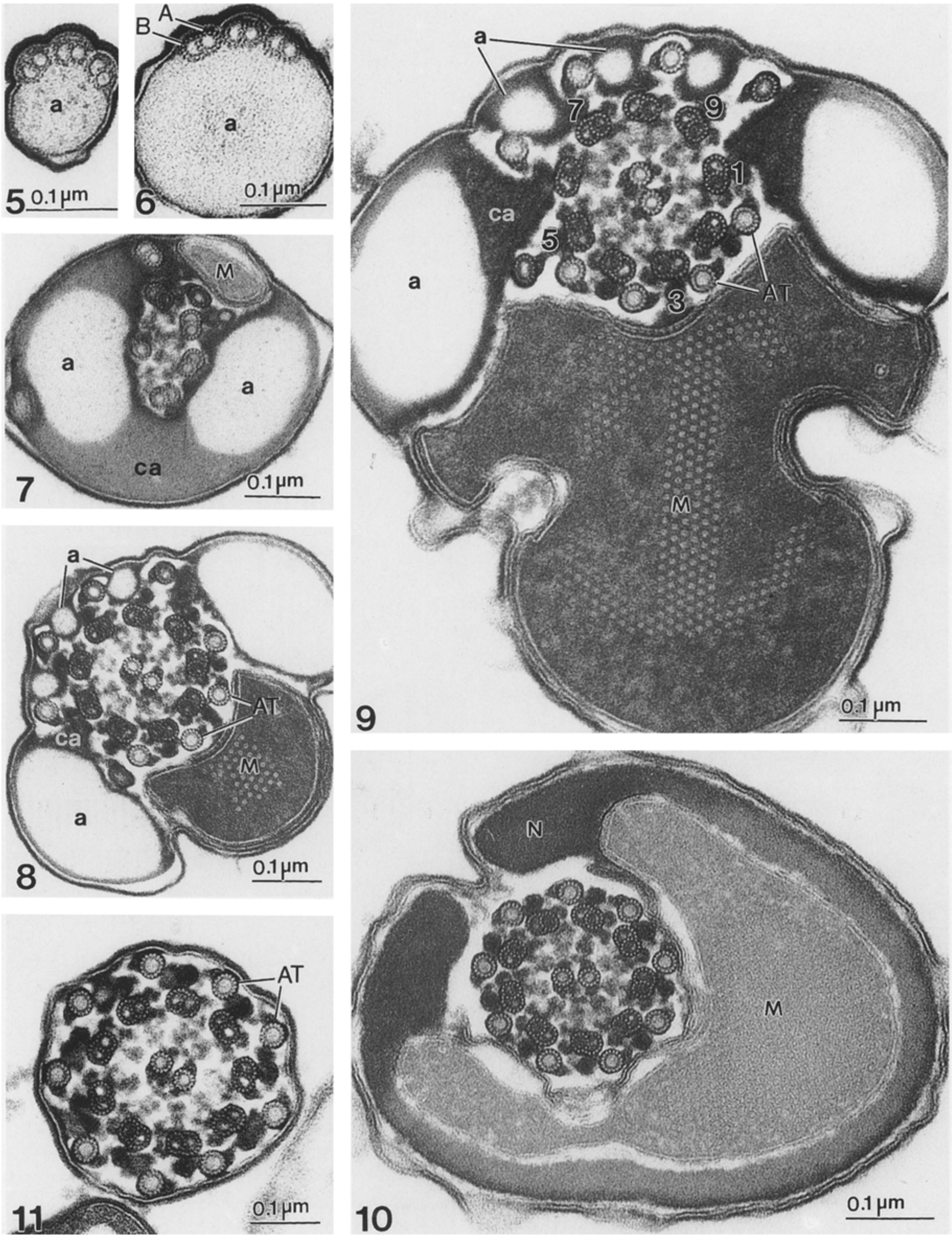
Fig. 4 *Orfelia persimilis*

There are no accessory tubules in the *Keroplatus* axoneme, those in *Neoplatyura* and *Macrocera* have a wall consisting of 16 protofilaments and those in *Orfelia* have 14 protofilaments only. *A* subtubule A of the microtubular doublet, *AT* accessory tubule, *B* subtubule B, *ia* inner dynein arm, *im* intertubular material, *M* mitochondrial derivative, *oa* outer dynein arm, *sp* spoke

(Fig. 16). The acrosome is an elongated vesicle, although one without subacrosomal and periacrosomal material (Fig. 16). The centriole consists of nine doublets joined to the nuclear membrane (Fig. 17). From it emerges the axoneme, which anteriorly is a simple 9+2 unit (Fig. 18), but further posteriorly also contains some accessory tubules (Fig. 19). Where the flagellum emerges from the nucleus it contains a 9+9+2 axoneme in which the nine accessory tubules are enforced by an electron-dense material (Fig. 12) .Throughout most of its length, the axoneme has a reduced number of accessory tubules (Figs. 19–21). The accessory tubules have 16 protofilaments.

Brevicornu crassicorne

As in the previous species, much of the axoneme is enclosed within the tubular nucleus (Fig. 13). All nine ac-



Figs. 5–11 Transversely sectioned spermatozoa from the keroplatid fungus gnat *Macrorhynchus ancae.*

Figs. 5, 6 Acrosome with its three microtubular doublets

Fig. 7 Section at the level of the centriole

Figs. 8, 9 sections from levels posterior to the centriole

Fig. 10 Section at the level of the nucleus

Fig. 11 Section of the posterior tip. The wall of the accessory tubule has 16 protofilaments. The acrosome extends along most of the spermatozoon and is branched into five fingers posteriorly. The elongated mitochondrion contains a crystalloid of a complex cross-sectional shape. *A* subtubule A of the doublet, *a* acrosome, *AT* accessory tubule, *B* subtubule B, *ca* centriolar adjunct, *M* mitochondrial derivative, *N* nucleus

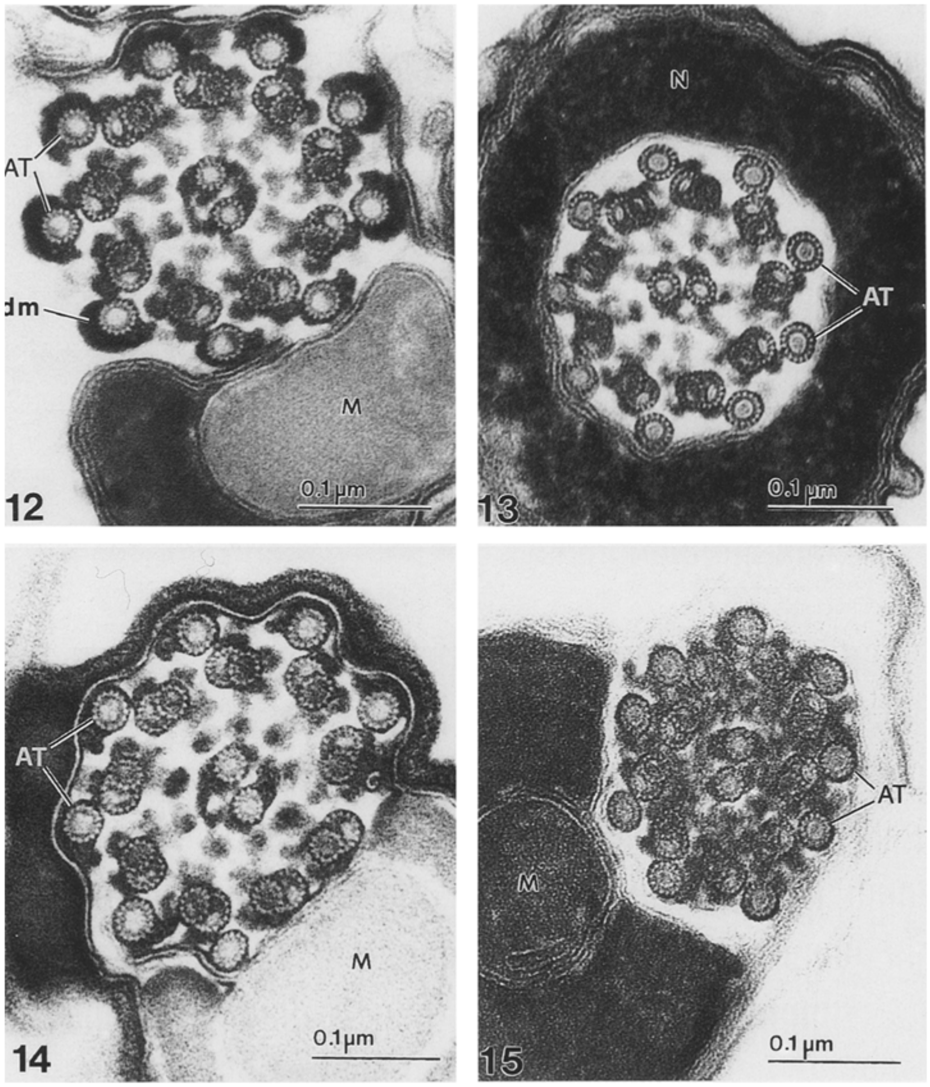


Fig. 12–15 Transversely sectioned sperm tails from four species of the fungus gnat family Mycetophilidae

Fig. 12 *Allodia alternans*

Fig. 13 *Brevicornu crassicorne*

Fig. 14 *Exechia fusca*

Fig. 15 *Phthinia* sp.

The accessory tubules of the first three species have a wall of 16 protofilaments whereas that of *Phthinia* has 15 protofilaments. *AT* accessory tubules, *dm* dense material, *M* mitochondrial derivative, *N* nucleus

cessory tubules are, however, present along most of the axoneme, both within the nucleus and outside it. Distally there are only two accessory tubules which run close to the junction between the axoneme and the mitochondrial derivative. The accessory tubules have 16 protofilaments.

Exechia fusca and *E. seriata*

The latter species has been previously described by Dallai et al. 1993. The free flagellum in both species has a 7+9+2 axoneme; thus, all doublets except numbers 7, 8 (close to the mitochondrial derivative) are connected to accessory tubules (Fig. 14). The seven accessory tubules have 16 protofilaments. The proximal part of the axoneme is located in the tubular nucleus and contains a single accessory tubule only, namely that connected to doublet number 4.

Phthinia sp

The free portion of the flagellum consists of a 9+9+2 axoneme and a small mitochondrial derivative. The accessory tubules have 15 protofilaments (Fig. 15).

Boletina sp

As previously stated (Dallai et al. 1993), the cross-sectional appearance of the axoneme is practically identical to that of *E. seriata* except that the accessory tubules have 15 rather than 16 protofilaments.

D. Discussion

I. General remarks

Spermatozoa display an enormous diversity in their ultrastructure, a diversity that has proven to be valuable in phy-

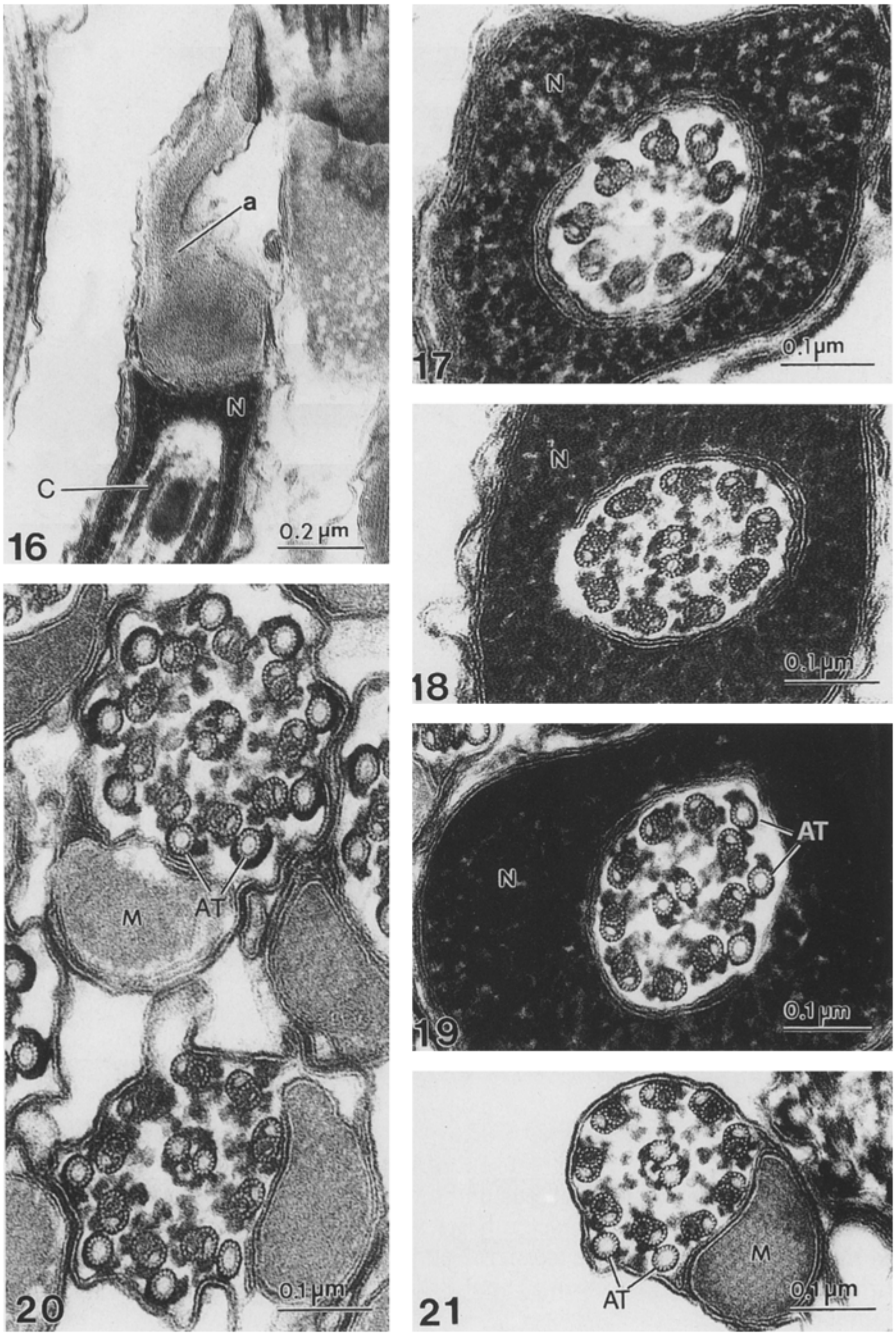


Fig. 16 Longitudinal section through the anterior portion of the spermatozoon of *Allodia alternans*

Figs. 17–21 Transverse views of this spermatozoon at different levels from the centriole anteriorly (**Fig. 16**) to the distal tail end posteriorly (**Fig. 21**)

The acrosome consists of an acrosomal vesicle but has no subacrosomal material. The centriole is located near the anterior end of the nucleus and the axoneme lies both inside a nuclear canal (**Figs. 17–19**) and distal to the nucleus (**Figs. 20, 21**). The accessory tubules have 16 protofilaments and extend along a part only of the microtubular doublets. *a* acrosome, *AT* accessory tubule, *C* centriole, *M* mitochondrial derivative, *N* nucleus

logenetic studies. The monograph on insect spermatozoa and insect phylogeny by Jamieson (1987) gives many examples of this. Most ultrastructural studies of spermatozoa have been performed after fixation with what can be called 'standard fixatives'. This gives a good view of the overall morphology of the sperm cell, but practically no information on the flagellar axoneme other than whether its pattern is of the 9+9+2 or the 9+2 type, or something else.

With the fixation used in the present study, a greater resolution of the axoneme can be achieved and new information can be obtained of interest for phylogeny and systematics. It has become clear that the axonemal 9+9+2 ultrastructure is far from invariant. In a previous study, we examined spermatozoa from a number of insects representing 20 of the traditional "orders" (Dallai and Afzelius 1990) and found that different taxa have axonemes of different structural appearances. Since then we have extended the study to include most remaining insect "orders" (Dallai et al. 1991; Dallai and Afzelius 1991; Afzelius and Dallai 1994).

The axonemal diversity refers mainly to the aspect of the accessory tubules and the so-called intertubular material that is located between the accessory tubules, whereas the central 9+2 unit is relatively invariant. In most Pterygota, the accessory tubules have 16 protofilaments, whereas some taxa are characterized either by a higher number of protofilaments (Phasmida and Trichoptera) or a lower one (Ephemeroptera, Psocoptera, Mallophaga and Anoplura all with 13 protofilaments). Rhyacophilid trichopterans, which have the most basic characters and life histories, have axonemes with 17 protofilaments along most of the sperm tail, although the plesiomorphic number 16 is seen in the distal part (Dallai et al.1995). Trichopterans with a more specialized morphology have 18–20 protofilaments depending on the family and genus (Dallai and Afzelius 1994).

II. Discussion of the present findings

From the above it can be concluded that the number of protofilaments in the accessory tubules is a useful character in phylogeny and systematics, and that the plesiomorphic number is 16. This number has been found only in the Mycetophiloidea and in this superfamily only in a few genera: *Macrorhyncha* and *Neoplatyura* in Keroplatidae and *Allodia, Brevicornu* and *Exechia* in Mycetophilidae. In both families, a reduction in the number was also found: to 15 in *Macrocera* and 14 in *Orfelia* in Keroplatidae and 15 in *Phthinia* and *Boletina* in Mycetophilidae. Representatives of other dipteran families have either 15 protofilaments (Chironomidae, Dixidae, Culicidae and Bibionidae) or 13 (Sciaridae, Tipulidae, Trichoceridae and all examined groups of Brachycera) protofilaments (Dallai and Afzelius 1990).

We found within Diptera a gradual increase in the size of the intertubular material from families regarded as more 'primitive' to the 'specialized' ones (Dallai et al. 1993). Spermatozoa from Mycetophiloidea have an intertubular material that is rather inconspicuous in *Brevicornu, Allodia* and *Exechia* and more prominent in *Macrorhyncha* and *Orfelia.* Hence spermatozoa from Mycetophilidae, have retained a more plesiomorphic trait. Tipulidae, with 13 protofilaments in the accessory tubules and a conspicuous intertubular material, can be considered a more specialized group.

We have not been able to study the acrosome nor determine the shape of the nucleus in all species here treated. Those acrosomes that we have seen were simple ones and, thus, without subacrosomal material (also termed perforatorium). This is true also of *Macrorhyncha* spermatozoa, although the association of microtubular doublets with the acrosome and the elaborate branching of the acrosome is of a type unknown in other animals. The sperm nucleus in most fungus gnats was found to be elongated with a rather long intranuclear canal containing the anterior part of the axoneme. Similar tubular sperm nuclei have been described in members of other taxa.

The close look at the sperm tail that can be had with the new fixation technique has revealed that there is an unsuspected diversity in the structural organization of the sperm tail axoneme. In all likelihood, this diversity is functionally neutral, in that a spermatozoon will work equally well with thick or thin accessory tubules and with thick or thin intertubular material. Such differences in the axoneme, hence, will survive and can inform the investigator of the phylogenetic position of the animal group.

In conclusion, a plesimorphic axonemal pattern, such as is seen in most Pterygota, has been found in one dipteran taxon only, the Mycetophiloidea and mainly in Mycetophilidae; the axoneme of Tipulidae is more specialized.

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