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The dipteran sperm tail: ultrastructural characteristics and phylogenetic considerations

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The sperm tail from representatives of several families of Diptera has been examined by high resolution electron microscopy and a computer analysis that improved the visualization of recorded patterns. A considerable variability in sperm tail structure is found within Diptera, and is actually greater than that of any other insect order. The 'generalized insect sperm axoneme', which is characterized as a 9+9+2 axoneme and by the accessory microtubules having 16 protofilaments, was found only in some dipterans; these are members of Mycetophilidae. From this fact we conclude that Mycetophilidae is likely to be the most primitive extant dipteran group. Another mycetophilid, *Boletina*, was seen to have accessory tubules with 15 protofilaments as have members of families Dixidae, Chironomidae, Culicidae, and Bibionidae. The last two families have spermatozoa of a type designated as 9+9+'1'; there is a central rod rather than two microtubules. We regard this 9+9+'1' pattern with 15 protofilaments to represent a synapomorphic feature. Representatives of the nematoceran families Tipulidae and Trichoceridae have accessory tubules with 13 protofilaments as do examined members of several brachyceran families. Brachycera is hence likely to be derived from the vicinity of the tipulid family. The intertubular material is small in Mycetophilidae and most nematoceran groups, whereas in Tipulidae and Brachycera it is enlarged; here it bridges the space between the accessory tubules and contains various inclusions.

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Introduction

Diptera is one of the largest orders of pterygote insects. It belongs to the Panorpida complex, which also includes the orders Mecoptera and Aphaniptera (Kristensen 1989). The classification of Diptera presents many problems to systematists. Whereas there is no doubt as to the primitive status of suborder Nematocera or about the origin of Brachycera from Nematocera, other major questions remain: (1) which is the most primitive family within the Nematocera? and (2) from which group or groups within the Nematocera are the Brachycera derived?

Edwards (1925) and White (1949) have claimed that crane-flies (Tipulidae) on morphological grounds are to be regarded as the most archaic group of extant Diptera. Spermatological data, on the other hand, have indicated that fungus-gnats (Mycetophilidae) might be the primitive group from which other dipterans have evolved (Dallai and Mazzini 1983*b*).

New data will be presented here which indicate that family Mycetophilidae indeed is likely to be the most primitive dipteran group and that the family Tipulidae is close to the base of the evolutionary line leading to the Brachycera. These data have been obtained from analysis of the sperm tail in several dipteran families, using a new fixation method that includes the use of tannic acid and uranyl acetate but omits osmium tetroxide (Dallai and Afzelius 1990) and by employing computer analysis in order to improve the visualization of the findings (Lanzavecchia *et al.* 1991*a*).

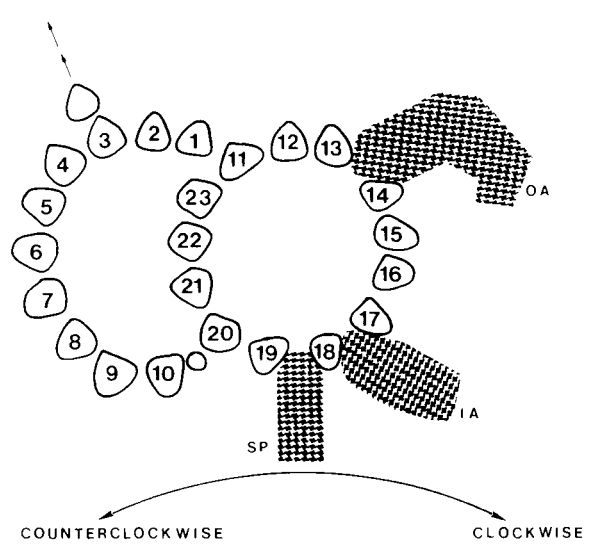
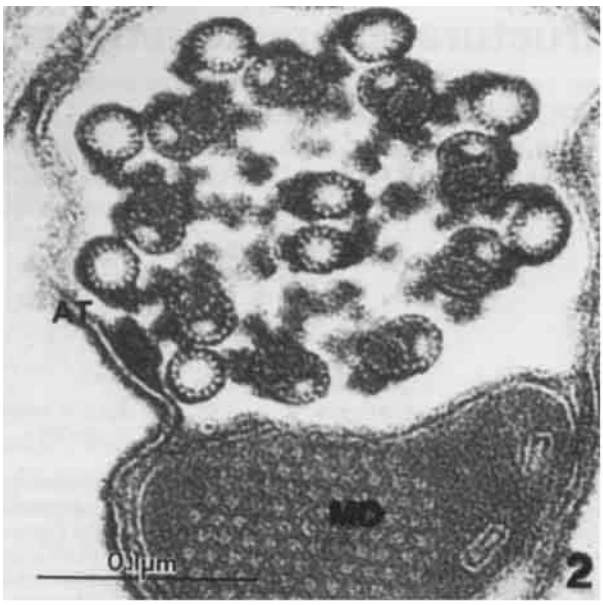


Fig. 1. Schematic drawing of the axonemal doublet with the protofilaments indexed according to Witman *et al.* (1972). The positions of the outer dynein arm (OA), the inner dynein arm (IA), and the spoke (SP) is indicated. The doublet is oriented as seen from the basal body toward the axonemal tip. (Reproduced with permission from Dallai and Afzelius 1990.)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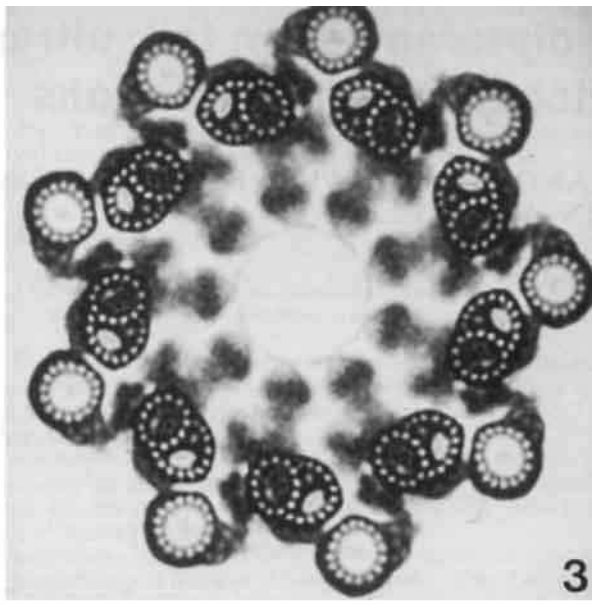
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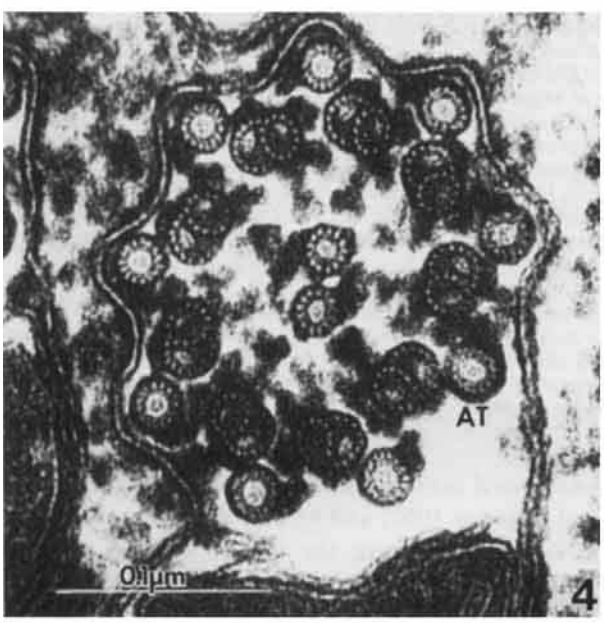
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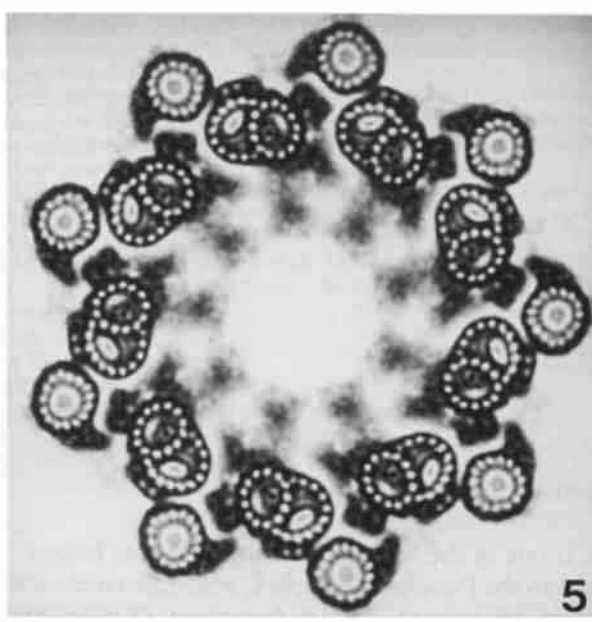
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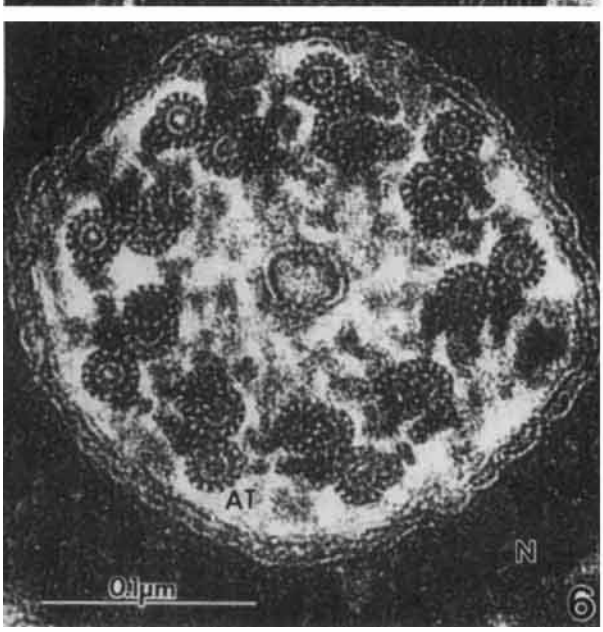
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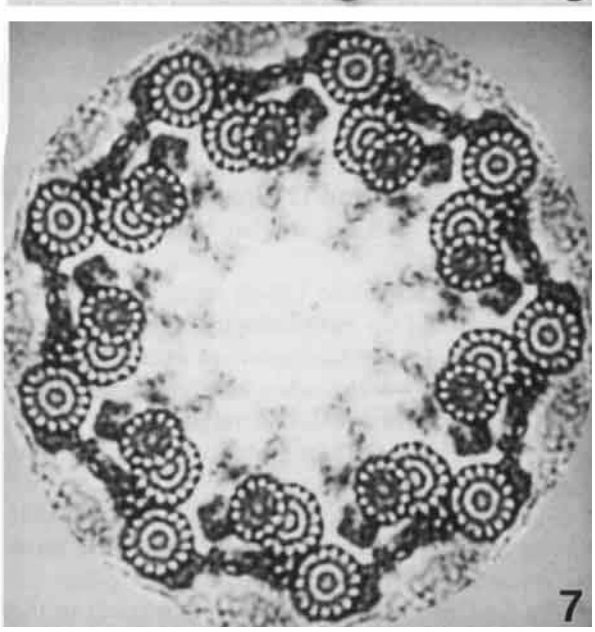
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Table 1. *Comparison of the sperm axoneme in different families of Diptera. A question mark in the third or fourth column signifies that no information is available*

	Axoneme formula	Protofilament number in the accessory tubule	Relative size of intertubular material	Reference
Nematocera				
Mycetophilidae:				
Orfelia	9+9+2	16	+	Dallai & Afzelius 1990
Exechia	7+9+2	16	+	This paper
Tarnania	2+9+2	?	?	Dallai & Mazzini 1983*b*
Boletina	9+9+2	15	+	,,
Keroplatus	9+2	0	0	,,
Psychodidae	9+9+0	?	?	Dallai *et al.* 1984
Chironomidae	9+9+2	15	++	This paper
Dixidae	9+9+2	15	0	,,
Culicidae	9+9+1	15	++	,,
Bibionidae	9+9+1	15	++	,,
	9+1	0	0	Trimble & Thompson 1974
Simulidae	9+9+3	?	?	Baccetti *et al.* 1974
Sciaridae	N+N+0	13	0	Dallai & Afzelius 1990
Cecidomyiidae	9+3	0	0	Dallai & Mazzini 1983
	0+9+0	0	0	Lanzavecchia *et al.* 1991*b*
Tipulidae	9+9+2	13	+++	This paper
Trichoceridae	9+9+2	13	+++	,,
Brachycera				
Bombyliidae	9+9+2	13	++++	This paper
Empididae	9+9+2	13	++++	,,
Drosophilidae	9+9+2	13	++++	Dallai & Afzelius 1991
Tephritidae	9+9+2	13	++++	,,
Sepsidae	9+9+2	13	?	Phillips 1966
Scatophagidae	9+9+2	13	++++	This paper
Calliphoridae	9+9+2	13	++++	Dallai & Afzelius 1990
Gasterophilidae	9+9+2	13	++++	This paper

Material and methods

The following species have been examined:

Suborder Nematocera	
Family Mycetophilidae	*Orfelia* sp.
	Exechia seriata
	Boletina sp.
Family Cecidomyiidae	*Asphondylia ruebsaameni* (Lanzavecchia *et al.* 1991*b*)
Family Sciaridae	*Sciara* sp. (Dallai and Afzelius 1990)
Family Chironomidae	*Chironomus* spp. (three unidentified species)
Family Culicidae	*Culex pipiens*
	Anopheles maculipennis
Family Dixidae	*Dixa* sp.
Family Bibionidae	*Bibio* sp.
Family Trichoceridae	*Trichocera hiemalis*
Family Tipulidae	*Tipula* sp.
Suborder Brachycera	
Family Bombyliidae	*Bombylius* sp.
Family Empididae	*Ramphomyia* sp.
Family Drosophilidae	*Drosophila melanogaster*
Family Tephritidae	*Dacus oleae*
	Ceratitis capitata
Family Scatophagidae	*Scatophaga* sp.
Family Calliphoridae	*Calliphora vomitoria*
Family Gasterophilidae	*Gasterophilus intestinalis*

Testes and vasa deferentia were removed and processed according to methods described in an earlier paper (Dallai and Afzelius 1990). Selected micrographs of cross-sectioned sperm tail axonemes were used for computer-aided image reconstruction.

Images of entire axonemal sections were recorded in digital form from the electron microscopical negatives by a solid state camera (Sony XC77e), mounted on a zoom microscope, and connected to a frame grabber (Matrox VIP-640). The grabber is part of a small workstation based on M68030 and M68882 microprocessors. All computation was performed on this machine.

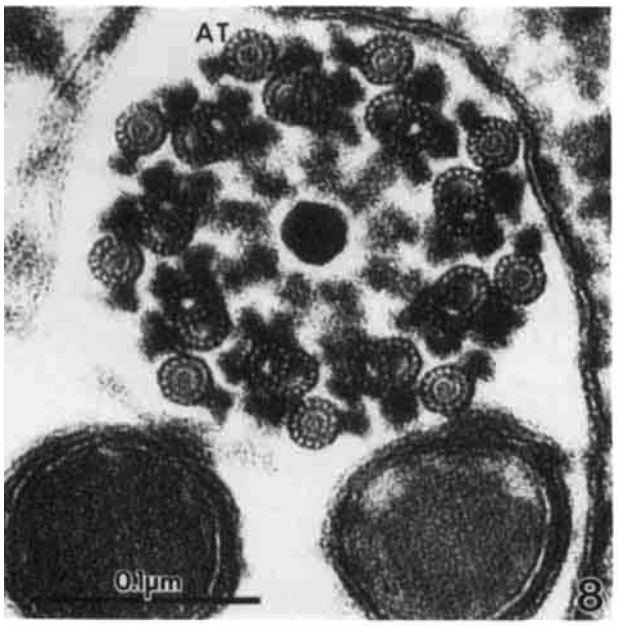
The peripheral region of the sections was reconstructed by averaging the equivalent portions according to the overall nine-fold symmetry. This process is performed on the polar representations of the images with a technique of distortion removal that allows corresponding elements of the nine slices to be accurately superimposed (Lanzavecchia *et al.* 1991*a*). Each reconstruction has been obtained from a number of sections as previously described (Afzelius *et al.* 1991).

Image analysis was performed by using software packages developed by PLB and SL in Milan. Extensive use was made of packages CFFT (Bellon and Lanzavecchia 1989) and POLCA (Bellon and Lanzavecchia 1990) and of a set of programs written in order to remove the relative distortions in structures possessing axial symmetry.

Results

The sperm tails of several dipteran species have been examined in this study. The overall structure of the

Figs 2–7.—2. Cross-sectioned axoneme of an *Exechia seriata* (Mycetophilidae) spermatozoon. There are only seven accessory tubules (AT), each with 16 protofilaments. A portion of the mitochondrial derivative (MD) is also shown.—*3.* Computer average of several *Exechia* axonemes. A microtubular doublet is shown at higher magnification in Fig. 24. Note that the presence of nine accessory tubules is an artefact from the averaging procedure.—*4.* Cross-sectioned axoneme of a *Boletina* (Mycetophilidae) spermatozoon. The wall of the accessory tubules (AT) consists of 15 protofilaments.—*5.* Computer average of several *Boletina* axonemes.—*6.* Cross-sectioned axoneme of a *Chironomus* (Chironomidae) axoneme sectioned at a proximal level within the nucleus (N), where central tubules are lacking. The accessory tubules (AT) have 15 protofilaments. No pentagon can be seen in the lumen of the A-tubule.—*7.* Computer average of several *Chironomus* axonemes.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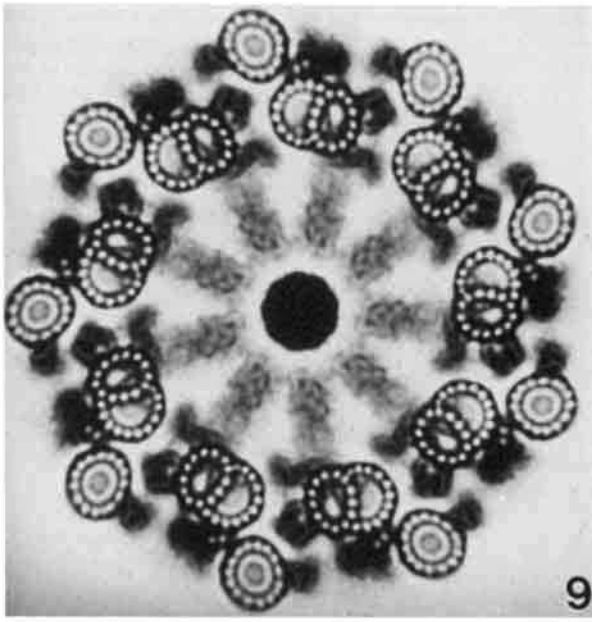
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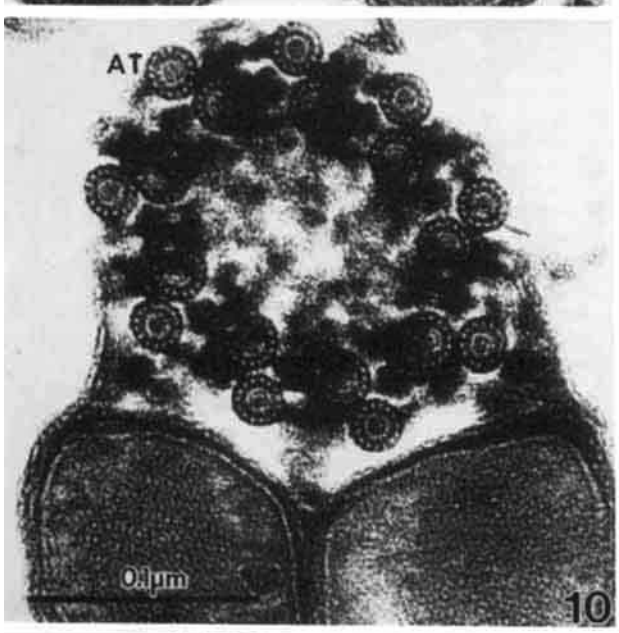
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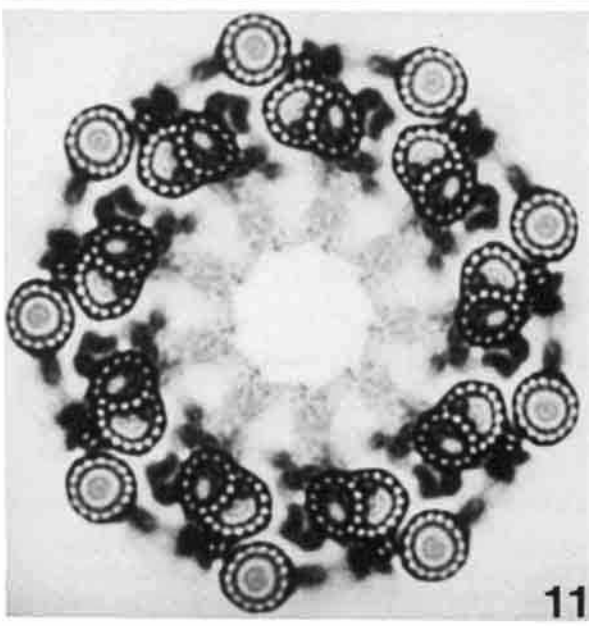
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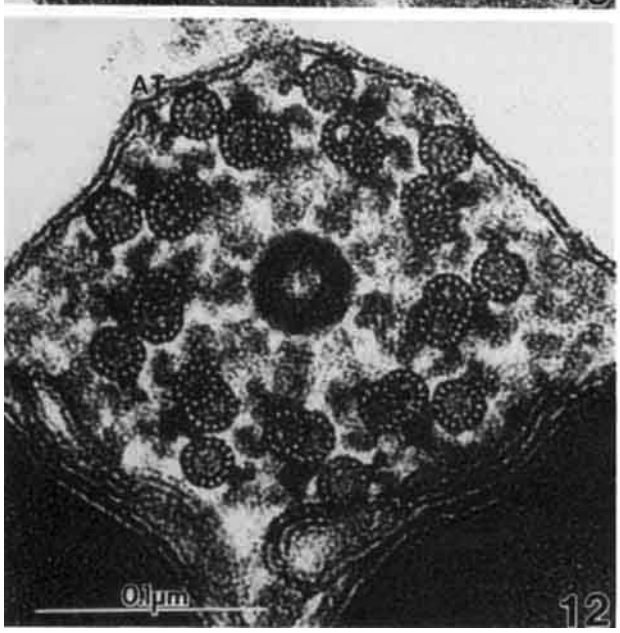
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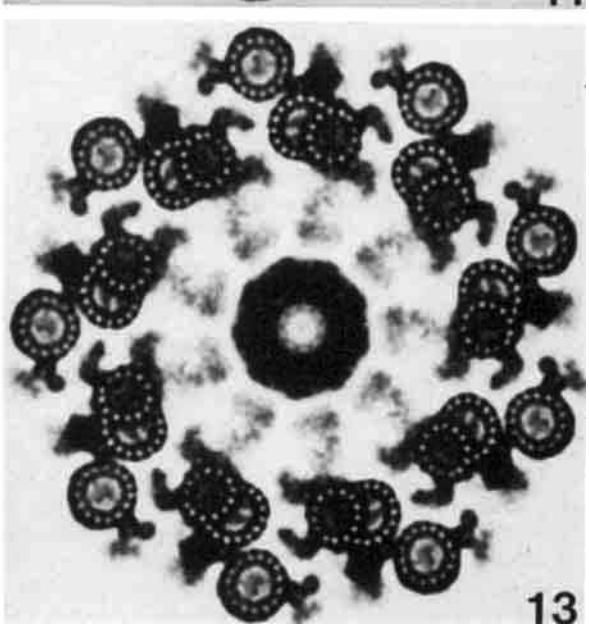
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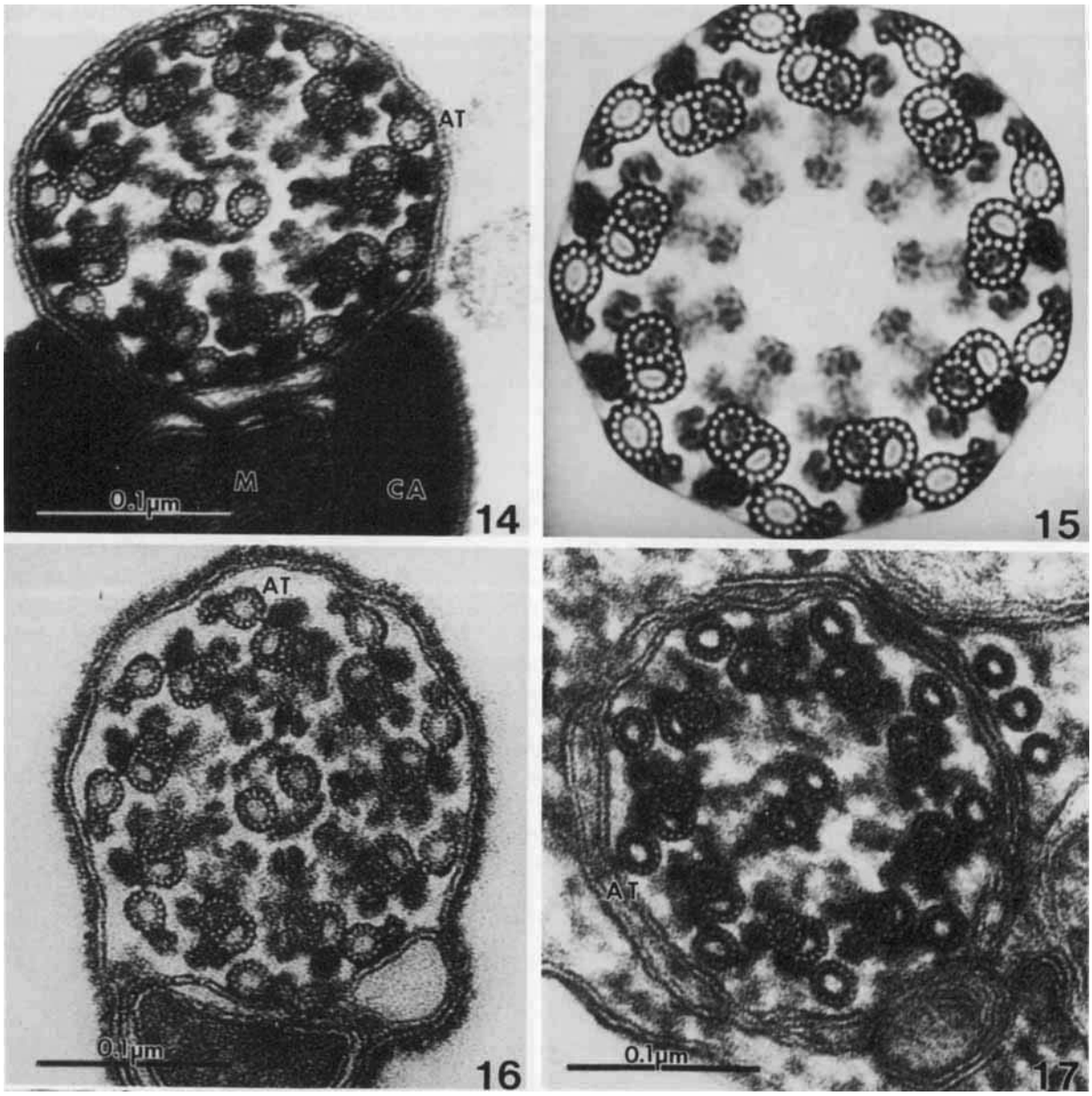
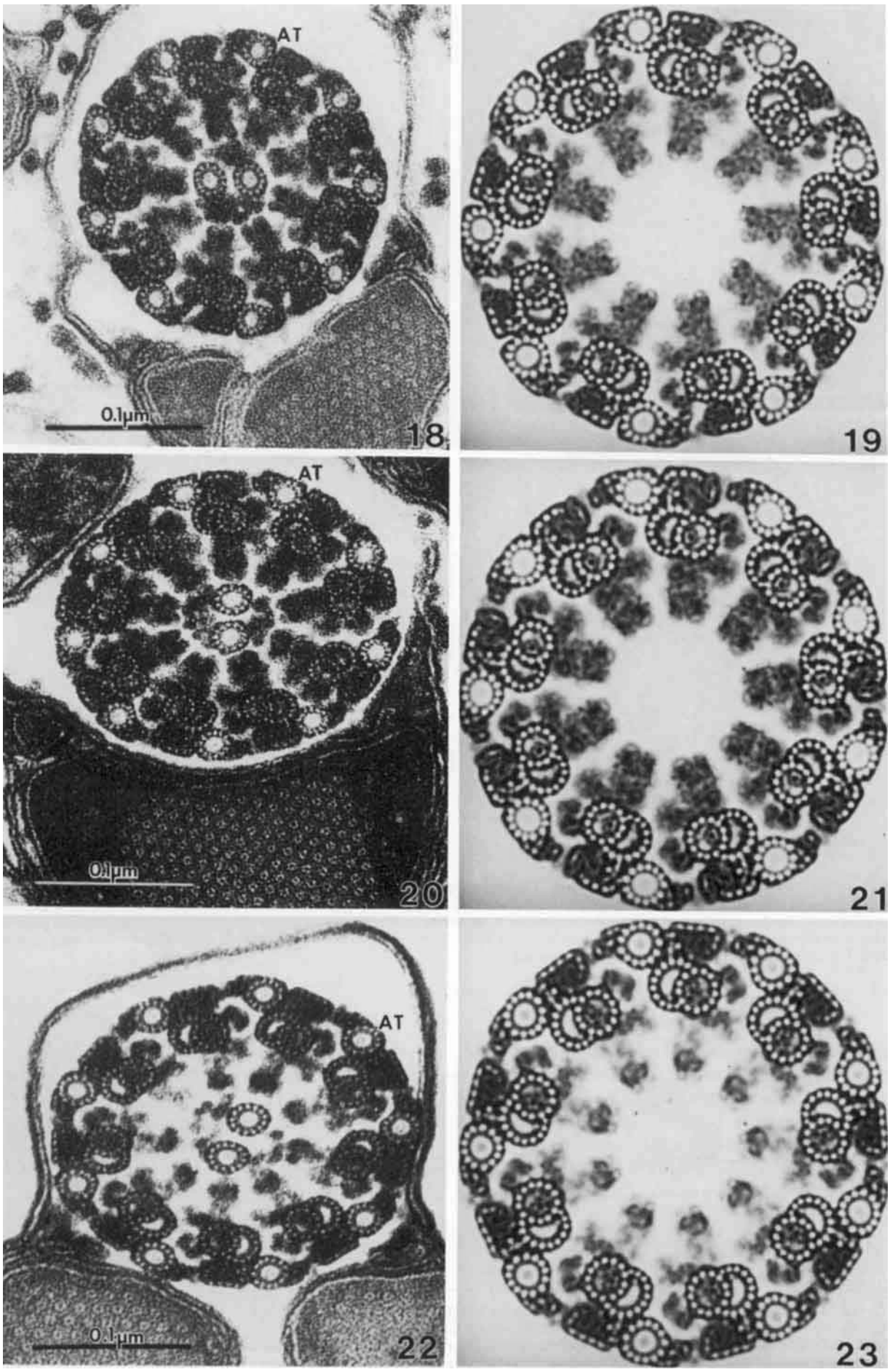


Figs 14–17.—14. Cross-sectioned axoneme of a *Tipula* sp. (Tipulidae) spermatozoon. The accessory tubules (AT) have 13 protofilaments and the intertubular material is rather prominent. A single mitochondrion (M) is surrounded by material of the centriolar adjunct (CA).—*15.* Computer average of several *Tipula* axonemes. A microtubular doublet is shown at higher magnification in Fig. 26.—*16.* Cross-sectioned axoneme of a *Trichocera hiemalis* spermatozoon. The accessory tubules (AT) have 13 protofilaments.—*17.* Cross-sectioned axoneme of a *Dixa* sp. (Dixidae) spermatid. The accessory tubules (AT) have 15 protofilaments.

axoneme is nearly the same in all species treated here and, in fact, is similar to that of the sperm flagellum of most other insects (Dallai and Afzelius 1990). The number of microtubules is given in Table I. Each axoneme thus has nine microtubular doublets each consisting of an A-subtubule carrying dynein arms and a B-subtubule. The wall of the A-subtubule consists of 13 protofilaments; these are indexed according to the system of Witman *et al.* (1976) as pfs 11–23 (Fig. 1). The wall of the B-subtubule is incomplete and contains 10 tubulin protofilaments plus a smaller subunit of unknown composition. The 10 protofilaments are indexed as pfs 1–10 starting with the unit at the outer border to the A-subtubule. The two central microtubules have 13 protofilaments. The protofilaments

Figs 8–13.—8. Cross-sectioned axoneme of a *Culex pipiens* (Culicidae) spermatozoon. The accessory tubules (AT) have 15 protofilaments and the two central mictrotubules are replaced by a prominent central rod.—*9.* Computer average of several *Culex pipiens* axonemes.—*10.* Cross-sectioned axoneme of an *Anopheles maculipennis* (Culicidae) spermatozoon. The accessory tubules (AT) have 15 protofilaments.—*11.* Computer average of several *Anopheles maculipennis* axonemes.—*12.* Cross-sectioned axoneme of a *Bibio* sp. (Bibionidae) spermatozoon. The accessory tubules (AT) have 15 protofilaments and there is a central structure somewhat similar to that in spermatozoa from the culicid family.—*13.* Computer average of several *Bibio* spermatozoa. A microtubular doublet is shown at higher magnification in Fig. 25.


AT
0.1µm
18
19
AT
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21
AT
0.1µm
22
23

appear as electron lucid areas surrounded by an electron dense complex consisting of tannic acid and uranyl acetate. The outer dynein arm makes two bends, whereas the inner dynein arm is shorter and straighter. A radially oriented spoke projects from the inner side of the A-subtubule and—typical for dipteran species—has a prominent spoke head, which usually appears double.

Most microtubular doublets are accompanied by an accessory tubule, which during spermiogenesis emerges as an outgrowth of the B-subtubule, close to pf 3 (Fig. 1). Outside the B-subtubule there is also an electron dense mass named the intertubular material; this material usually is divided into two portions: one adhering to the B-subtubule, one adhering to the accessory microtubule. It has been noticed in a previous study that the electron dense border between the different protofilaments is somewhat widened at those places, where the spoke, dynein arms and intertubular materials are anchored to the microtubular wall (Afzelius *et al.* 1991). The contents of the A- and B-subtubules and of the accessory tubules vary from one dipteran species to another, as does the appearance of the accessory tubules and of the intertubular material. However, a pentagon is visible in the lumen of most A-subtubules carrying two dynein arms (representatives of families Chironomidae and Culicidae seem not to have a pentagon in their A-subtubule).

Such structural features that characterize the axoneme of the various dipteran group will now be described. The computer reconstructions have provided images of each cross-sectioned axoneme that are much more distinct than the original electron micrographs, but inspection of these shows that each feature that we describe is present already before image processing (see, however, figure legend to Fig. 3).

Suborder Nematocera

Orfelia sp. (Mycetophilidae). The structure of the axoneme has recently been published and is of the common 9+9+2 type (Dallai and Afzelius 1990). The wall of the accessory tubules contains 16 protofilaments. The intertubular material is sparse and appears as two separate electron dense masses: one unit with triangular cross-section and attached to the accessory tubule and another rather flat unit that adheres to the B-subtubule.

Exechia seriata (Mycetophilidae) (Figs 2, 3 and 24). The axoneme resembles that of *Orfelia* except that doublets numbers 7 and 8 have no accompaning accessory tubules. There are thus only 7 accessory tubules (each with a wall of 16 protofilaments) and the axoneme hence has a 7+9+2 pattern. The lumen of the A-subtubule is fairly electron dense but it contains a pentagonal structure close to pfs 20–22 and another electron lucid streak close to pfs 12–14. The lumen of the B-subtubule also contains an electron dense substance although with a less dense elliptic area close to pfs 3–5 and another such area halfway between pfs 9 and 21. The portion of the intertubular material that projects from the accessory tubule will meet the outer dynein arm; the other portion is small and flat and adheres to the B-subtubule.

Boletina sp. (Mycetophilidae) (Figs 4 and 5). The cross-sectional appearance of the axoneme is practically identical to that of *Exechia* except that the accessory tubules have 15 rather than 16 protofilaments. In most cross-sections the cell membrane can be seen to adhere rather tightly to the accessory tubules and therefore to have an undulating profile.

Chironomus spp. (Chironomidae) (Figs 6 and 7). Three examined, unidentified species of the *Chironomus* genus have a sperm tail that contains two mitochondrial derivatives and a 9+9+'2' axoneme. The nine accessory tubules have 15 protofilaments and a lumen with a central cylinder. No pentagonal structure is visible in the A-subtubule. The B-subtubule resembles a partial accessory tubule also because it has an, albeit incomplete, internal cylinder. The intertubular material appears as a thin bridge that spans neighbouring tubules and that contains a few electron lucid dots.

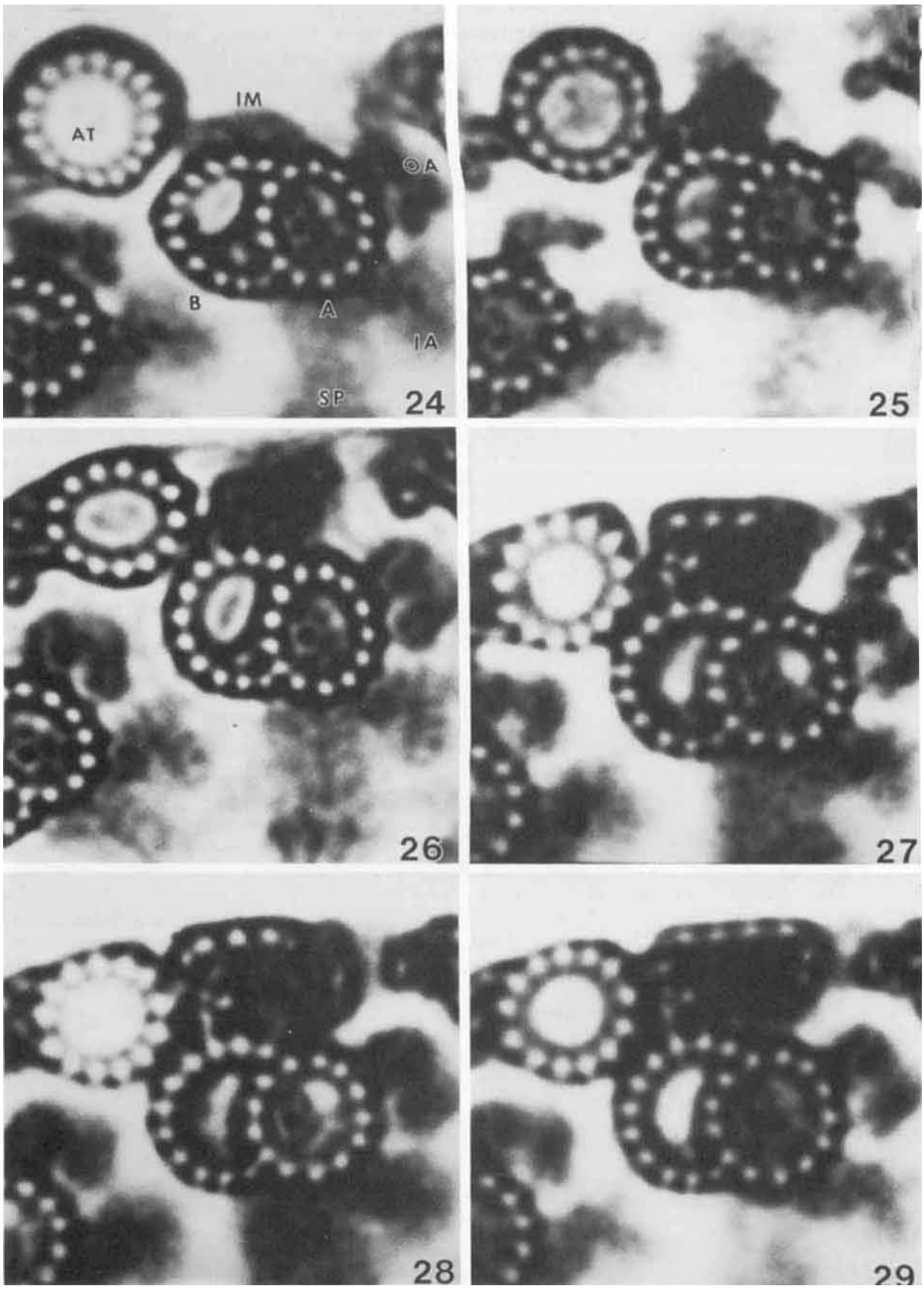
Dixa sp. (Dixidae) (Fig. 17). The characteristics of this species are accessory tubules with 15 protofilaments and an intertubular material that is restricted to a flat rim adhering to the B-subtubule. The examined material is from a late spermatid and it is possible that its axoneme differs slightly from that of the mature spermatozoon.

Culex pipiens (Culicidae) (Figs 8 and 9). In this sperm tail a central rod replaces the two central microtubules. The flagellum has two mitochondrial derivatives and a 9+9+'1' axoneme. The central rod has a diameter of 20 nm. No pentagon is seen in the A-subtubule. The accessory tubules have a wall of 15 protofilaments and a central cylinder. The portion of the intertubular material that adheres to the B-subtubule is more prominent than that of the species described above and contains two or three distinct electron lucid areas. The portion adhering to the accessory tubule, on the other hand, is rather insignificant.

Anopheles maculipennis (Culicidae) (Figs 10 and 11). The flagellum differs from that of the examined *Culex* species only in the central rod being thinner (13 nm in diameter) and at some levels even absent.

Bibio sp. (Bibionidae) (Figs 12, 13 and 25). The structure of the axoneme can be characterized as 9+9+'1' assembly where the central unit consists of an electron

Figs 18–23.—18. Cross-sectioned axoneme of a *Ramphomyia* sp. (Empididae) spermatozoon. The accessory tubules (AT) have 13 protofilaments, the intertubular material contains a row of three electron lucid dots.—*19.* Computer average of several *Ramphomyia* axonemes. A microtubular doublet is seen at higher magnification in Fig. 27.—*20.* Cross-sectioned axoneme of a *Drosophila melanogaster* spermatozoon. The accessory tubules (AT) have 13 protofilaments and the intertubular material is similar to that in *Ramphomyia*.—*21.* Computer average of several *Drosophila* spermatozoa. A microtubular doublet is shown at higher magnification in Fig. 28.—*22.* Cross-sectioned axoneme of a *Scatophaga* sp. (Scatophagidae) spermatozoon. The accessory tubules (AT) have 13 protofilaments and the intertubular material contains a straight row of four electron lucid spots.—*23.* Computer average of several *Scatophaga* axonemes. A microtubular doublet is shown in higher magnification in Fig. 29.



Figs 24–29. Computer averages of axonemal doublets and accessory tubules from *Exechia seriata* (Mycetophilidae) (*Fig. 24*), *Bibio* sp. (Bibionidae) (*Fig. 25*), *Tipula* sp. (Tipulidae) (*Fig. 26*), *Ramphomyia* sp. (Empididae) (*Fig. 27*), *Drosophila melanogaster* (Drosophilidae) (*Fig. 28*), and *Scatophaga* sp. (Scatophagidae) (*Fig. 29*). Note the different numbers of protofilaments in the accessory tubules of *Exechia* and *Bibio* versus the other shown species and the gradual increase in intertubular material in the three brachyceran species (*Figs 27–29*). A = A-subtubule; AT = accessory tubule; B = B-subtubule; IA = inner dynein arm; OA = outer dynein arm; SP = spoke.

dense cylinder with a diameter of 40 nm. The lumen of the A-subtubule contains a pentagon and that of the B-subtubule appears hollow except for some electron dense material along pfs 20–23. The intertubular material resembles that in the three previously described species.

Tipula sp. (Tipulidae) (Figs 14, 15 and 26). The sperm tail consists of a 9+9+2 axoneme, a mitochondrion and, in the proximal part, a horseshoe-shaped (in cross-sections) centriolar adjunct surrounding the mitochondrion. The electron dense centriolar adjunct extends along about half the tail length. The two lumina of the A- and B-subtubules appear much the same as in the mycetophilid species. There are, however, only 13 protofilaments in the accessory tubules of the axoneme. The portion of the intertubular material that adheres to the accessory tubule contains some electron lucid areas, whereas the other portion, although fairly prominent, appears rather homogeneous.

Trichocera hiemalis (Trichoceridae) (Fig. 16). The cross-sectioned sperm tail axoneme is nearly identical to that of *Tipula*.

Suborder Brachycera

Ramphomyia sp. (Empididae) (Figs 18, 19 and 27). The sperm tail has two mitochondrial derivatives, of unequal diameters, and an axoneme. Each accessory tubule has 13 protofilaments and an electron lucid lumen. The intertubular material is rather prominent and both its portions contain some electron lucid areas. The portion adhering to the B-subtubule contains along its outer surface three electron lucid spots of about the same diameter as the tubulin molecule of the microtubular walls. Close to pf 3 there are three further electron lucid areas.

Bombylius sp. The description given above for *Ramphomyia* is equally valid for *Bombylius* sp. except that the two mitochondrial derivatives are of roughly the same size (not illustrated).

Drosophila melanogaster (Drosophilidae) (Figs 20, 21 and 28). The general structure of the sperm flagellum has been previously described (Dallai and Afzelius 1991). As in *Ramphomyia* the sperm tail has two mitochondrial derivatives of unequal size and a 9+9+2 axoneme, in which the accessory tubules have 13 protofilaments. The only significant difference in the cross-sectional appearance, noted by us, is the more structured appearance of the intertubular material adhering to the B-tubule. Its three equally sized electron lucid dots are flanked by two smaller such dots.

Dacus oleae (Tephritidae). The axoneme of this species is described in Dallai and Afzelius (1991) and shown to resemble that of *Drosophila*.

Ceratitis capitata (Tephritidae). The axoneme of *Ceratitis capitata* also resembles that of *Drosophila* or of *Dacus* (not illustrated).

Scatophaga sp. (Scatophagidae) (Figs 22, 23 and 29). The two mitochondrial derivatives are equal in size and are separated by electron dense material. The 9+9+2 axoneme resembles that of the two previously described species, although the portion of the intertubular material adhering to the B-subtubules contains a row of four, rather than three, units along its outer straight border. There are also some small electron lucid spots along the lateral sides of the intertubular material.

Calliphora vomitoria (Calliphoridae) and *Gasterophilus intestinalis* (Gasterophilidae). The description given above for *Scatophaga* sp. is equally valid for *Calliphora vomitoria* and *Gasterophilus intestinalis*.

Discussion

The main results of this investigation and of some previous studies on dipteran sperm axonemes are summarized in Table I. It is obvious that the axonemal patterns in spermatozoa from Brachycera are relatively uniform, whereas in suborder Nematocera there is a considerable diversity—a diversity which in fact is greater than that in any other insect order. The number of axonemal doublets thus may exceed 2000 in the cecidomyiid *Asphondylia ruebensaameni* (Lanzavecchia *et al.* 1991*b*), the central microtubules may be missing, may be two, or as in some simulid gnats three (Baccetti *et al.* 1974), and the number of accessory tubules may be none, nine or, as in some species within families Mycetophilidae, two or seven. The finding of a greater constancy of axoneme pattern within Brachycera is in good agreement with some previously published data, where the entire sperm structure is considered (Dallai and Mazzini 1983; Dallai *et al.* 1984) and, furthermore, is in good accord with the opinion that brachycerans are derived from nematocerans.

It may be asked which axonemal pattern is the basic one within Diptera. A simple 9+2 axoneme, such as seen in some members of Mycetophilidae, could be the plesiomorphic one in Diptera; it is a character shared with the related orders Mecoptera and Aphaniptera (Dallai and Afzelius 1990). Alternatively a 9+9+2 axoneme in which the accessory tubules have 16 protofilaments is the basic one. This is the axoneme that is found in a majority of the pterygote orders from Odonata to the highest insect orders (Dallai and Afzelius 1990) and is present also in some members of family Mycetophilidae but has been found in no other dipteran group. A loss of some or all accessory tubules would then have occurred in other members of the mycetophilid group. There is also a published (although unillustrated) statement that the hanging fly *Bittacus apicalis* (order Mecoptera) might have a 9+9+2 axoneme (Gassner *et al.* 1972) and this feature has been taken as another argument that 9+9+2 is the basic pattern in Diptera (Jamieson 1988). In either of these two alternatives members of the family Mycetophilidae will be likely to represent the most primitive dipteran group.

Some evolutionary lines can be followed from family Mycetophilidae. One goes to the Chironomidae–Dixidae complex and is characterized by 15 protofilaments in the accessory tubules. Related to this complex is the Culicidae–Bibionidae complex, which is further characterized by an exchange of the two central singlets for a central rod. A central rod of a similar kind has been described from *Psocus* sp. (order Psocoptera) (Phillips 1969) but is otherwise unknown in spermatozoa from insects.

Another evolutionary line goes to the Cecidomyiidae and Sciaridae families where the central microtubules have been lost and where the sperm often is enlarged and contains a great number of microtubular doublets. No accessory tubules are seen in the family Cecidomyiidae, whereas sciarid spermatozoa have accessory tubules with a wall consisting of 13 protofilaments only. This is also the protofilament number in the third evolutionary line, which goes to Trichoceridae and Tipulidae and from this complex further to the suborder Brachycera.

A trend that is seen along all three evolutionary lines is the enlargement of the intertubular material. Whereas this material is relatively sparse in most examined nematoceran families it is prominent in Tipulidae and Trichoceridae and even more so in examined brachyceran species, where it spans almost the entire distance between the accessory tubules. The examined brachyceran species contains three or four linearly arranged spots that appear light against a darker background and hence are interpreted as representing some protein that is not permeated by the electron dense mixture of tannic acid and uranyl acetate. Whether this protein is tubulin or some other protein is presently unknown. It is striking, however, that this row of electron lucid dots seems to originate close to pf 3, that is to say at the same level that the accessory tubules emerge (see Dallai and Afzelius 1990). The role of the intertubular material is unknown. It is reasonable to assume that the accessory tubules together with the intertubular material increase axonemal stiffness, as is the case with the coarse fibres in mammalian spermatozoa (Baltz *et al.* 1990). Several other roles have been hypothesized (compare Curtis *et al.* 1989).

The techniques used here have permitted us to see the sperm axoneme at a higher resolution than is otherwise possible. Minute details such as those that appear in the intertubular material or within the lumen of the A-subtubule could be resolved. In a previous paper we have concluded that a pentagon within the A-subtubule is typical of axonemes containing both sets of dynein arms (Afzelius *et al.* 1991), but may not exist in other axonemes. The present findings are in agreement with that conclusion, although we note that the chironomids and culicids seem not to have a pentagon. We believe that the details which are resolved in this new technique are useful for a better understanding of flagellar movement as well as for studies of insect phylogeny.

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