Microtubular Diversity in Insect Spermatozoa: Results Obtained with a New Fixative

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The architecture of the tail axoneme of spermatozoa from 49 insect species representing 20 insect orders has been examined. Whereas the microtubular walls of the nine doublet tubules and the two central singlet tubules were found to consist of a fixed number of protofilaments, the walls of the accessory tubules may consist of 13, 15, 16, 17, or 19 microtubules and have diameters from 24 to 38 nm. Cytoplasmic microtubules in the spermatid cytoplasm, outside the axoneme, may have 13, 14, or 16 protofilaments. The number of protofilaments seems constant for each class of accessory tubule and, except in Diptera, seems fixed in each insect order. Pterygote insects have 16 protofilaments and this was found in all orders except Ephemeroptera and Psocoptera with 13, Phasmida with 17, and Trichoptera with 19 protofilaments. Within Diptera, 16 protofilaments were found within the family Mycetophilidae, which for this reason can be regarded as the most primitive, extant dipteran group; the majority of dipteran flies have 13 protofilaments. Two nematoceran families, Bibionidae and Culicidae, have axonemes of a 9 + 9 + "1" type and accessory tubules with 15 protofilaments. We feel that axonemal ultrastructure is important in studies of insect phylogenv. We conclude that spermatids can effect specification of different protofilament numbers with numerical exactitude for the different types of microtubules. © 1990 Academic Press, Inc.

INTRODUCTION

Fixation with a glutaraldehyde-tannic acid mixture followed by uranyl acetate postfixation has been shown to preserve proteinaceous structures so accurately that studies of the cell at high magnification have become meaningful. By employing this technique, Afzelius (1988) found that all protofilaments in the axoneme of a stick insect could be clearly resolved and their numbers could be counted without ambiguity. It was also possible to recognize the enantiomorphism (i.e., the "handedness") of the individual protofilaments and to recognize certain other features of the protofilament arrangement (Afzelius *et al.*, 1990).

The sperm tail of the stick insect-and in fact of most insect species—is characterized by the presence of 9 accessory tubules in a circle around the doublets; the axoneme is hence described as having a 9 + 9 + 2 structure, which is the shorthand notation for 9 accessory tubules, 9 microtubular doublets, and 2 central microtubular singlets. The accessory tubules have been described as resembling ordinary cytoplasmic microtubules and also the two central singlets of the axoneme. This resemblance was strengthened by the finding by Phillips (1966), of 13 protofilaments in the accessory tubules of a fly sperm, and the study by Stanley et al. (1972) who determined the number of these protofilaments to be about 12 or 13. Perotti (1969) estimated the number of protofilaments to be between 10 and 12 in an examination of negatively stained fruitfly spermatozoa. Cytoplasmic microtubules from all sources have been found to consist of 13 protofilaments with only a few exceptions (Dustin, 1984).

It was therefore somewhat of a surprise to find that the number of protofilaments in the accessory tubule of the stick insect was 17 rather than 13 (Afzelius, 1988). Fujiwara and Tilney (1975) have given some reasons why a prime number of protofilaments is favored in the construction of microtubules used in cells: to ensure instability so that microtubules can polymerize and depolymerize more easily and to build elasticity into a tubule cluster so that relaxation can occur without expenditure of energy.

We therefore decided to undertake an investigation of spermatozoa from a number of insect orders to determine whether there are 13 or 17 protofilaments in an accessory microtubule. Neither was found to be case in most instances. Seventeen protofilaments were thus found to be characteristic of phasmid insects, but of no other insect group; thirteen protofilaments were found to be characteristic of brachyceran flies (these were the insects examined by Stanley, Perotti, and Phillips), but of only a few other insect groups. For most insect orders the number of protofilaments is 16, an amount which has also been given by Kaye (1970) in his study of the spermatozoon of the house cricket.

We found that the pattern of accessory tubules and of the dense material between the tubules and associated with them differed in different insect orders and we feel that the diversity of axonemal pattern may be important in studies of insect phylogeny.

MATERIAL AND METHODS

Spermatids and spermatozoa from the following insect orders have been examined in this study:

Order Diplura:	Campodea sp.
Order Archeognatha:	Machilinus sp.
Order Zygentoma:	Lepismodes inquilinus
Order Odonata:	Calopteryx sp.
Order Ephemeroptera:	Cloëon dipterum
Order Blattodea:	Blattella germanica, Leucophaea ma- derae
Order Mantodea:	Mantis religiosa
Order Phasmida:	Baculum sp., Extatosoma tiaratum, Li- bethra regularis, Bacillus rossius
Order Orthoptera:	Pezotettix giornai, Acrida bicolor, Gryl- lus campestris, Gryllotalpa gryllotalpa
Order Dermaptera:	Forficula auricularia
Order Psocoptera:	Lepinotus inquilinus
Order Hemiptera:	Pyrrhocoris apterus, Raphigaster nebu- losa, Notonecta reuteri, Cercopis sp.
Order Raphidioptera:	Raphidia sp.
Order Strepsiptera:	Stylops sp.
Order Coleoptera:	Lytta vesicatoria, Tenebrio molitor, Chrysomela sp.
Order Mecoptera:	Panorpa germanica
Order Hymenoptera:	Solenopsis sp., Apis mellifera
Order Trichoptera:	Stenophylax permistus, Limnephila sp.
Order Lepidoptera:	Pieris brassicae, Pieris rapae, Syntoma sp., Arctia sp.
Order Diptera:	Ôrfelia sp., Tipula sp., Bibio sp., Sciara sp., Culex pipiens, Anopheles gambiae, Asphondylia ilicola, Empis sp., Scatophaga sp., Bombylius sp., Ceratitis capitata, Calliphora vomitoria, Family Ephydridae species.

The two cockroach species, the blowfly, and one of the stick insects were taken from cultures kept in laboratories; the other insects were collected in the wild in either Italy or Sweden. Testes, deferent ducts, and spermatheca 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 resins. This technique by Afzelius (1988) is modified from that of Mizuhira and Futaesaku (1974) mainly in that the osmium postfixation is omitted. After sectioning and section staining with uranyl acetate and lead citrate (sometimes according to Daddow, 1983) the sections were examined in a JEOL 100S or a Philips 400 electron microscope.

RESULTS

Our observations are presented here following the classification of insects given in Jamieson (1987). In

order to avoid repetitiveness, the number of protofilaments in the accessory tubules is given only in those cases in which it deviates from the "standard value" of 16. The number of protofilaments in the nine doublets and two central microtubules seems invariant: subtubule A and the central microtubules have 13 protofilaments whereas subtubule B is incomplete with 10 full-sized protofilaments and 1 smaller one. The generalized pattern of an insect axonemal doublet and some terms used in this manuscript are shown in Chart 1.

Order Diplura

Campodea sp. The spermatid of this apterygote insect has a 9 + 9 + 2 axoneme like that seen in pterygotes (Baccetti and Dallai, 1973; Bareth, 1974). During spermiogenesis the accessory tubules detach from the doublets and migrate to a position along one side of the axoneme, namely along doublets 6–9 (Baccetti and Dallai, 1973). The accessory tubules become distributed either in a straight row or in two groups divided by the mitochondrion. They have 13 protofilaments (Figs. 1 and 3). Each accessory tubule is removed from the microtubular doublets but attached to the mitochondrion by a short bridge. There is no intertubular material.

Order Archeognatha

Machilinus sp. The Machilinus spermatozoon resembles that of the closely related species Machilis distincta, which has previously been described (Dallai, 1972). The spermatid has an axoneme that at an early stage shows a symmetrical distribution of the



CHART 1. Schematic drawing of the protofilaments of an axonemal doublet with the position of the outer dynein arms (OA), inner dynein arms (IA), spokes (SP), and the outgrowing accessory tubule. The numbering of the protofilaments is according to Witman *et al.* (1972). The doublet is oriented as seen from the basal body toward the ciliary tip.

accessory tubules, and thus has a regular 9 + 9 + 2pattern. During maturation the accessory tubules detach from the doublets (Dallai, 1972) and form two groups, which-unlike in Campodea-lie on both sides of the plane of flagellar beating, five tubules along doublets 2-5 and four tubules along doublets 7-9 (Fig. 2). Another and probably more significant difference from the two dipluran sperm is the number of protofilaments in the accessory tubule, which is 16 rather than 13 (Fig. 5). Accessory tubules that have been properly preserved and are well oriented in the section have an inner substructure that contains what appears as a circle of several microcylinders surrounding a central microcylinder. The accessory tubule may hence have a 16 + 9 + 1 pattern, although there is a constancy only in the number of outer protofilaments, 16. An electron-dense material lies between the microtubules and is called the intertubular material. Sometimes a row of electronlucid units is seen along its outer surface.

Order Zygentoma

Lepismodes inquilinus. The spermatozoon of this apterygote insect is similar to that of the related species, the silver fish Lepisma saccharina (Wingstrand, 1972). The axoneme is of the symmetrical 9 + 9 + 2 type and the accessory tubules have 16 protofilaments and an electron-dense interior (Figs. 4 and 7). Intertubular material is present.

Order Odonata

Calopteryx sp. Only the spermatides of this species have been examined by us and also by Rosati *et al.* (1976). The mature spermatozoon of another dragonfly species has been studied by Kessel (1970). At least in the stages examined by us, there is an electron-dense intertubular material, which, however, does not seem to be in contact with the accessory tubules. The cytoplasmic microtubules have 13 protofilaments as is common whereas the accessory tubules have 16 protofilaments (Figs. 6 and 8).

Order Ephemeroptera

Cloëon dipterum. The various families of Ephemeroptera are characterized, among other ways, by their spermatozoa having a 9 + 9 + 0 axoneme, a 9 + 9 + 1 axoneme, or no axoneme (Fink and Yasui, 1988). *Cloëon* spermatozoa belong to the first category (Baccetti *et al.*, 1969) and are further characterized by lacking both outer dynein arms and intertubular material. During spermiogenesis the accessory tubules are formed as outgrowths from the B-subtubule of the doublet (unpublished observations). The accessory tubules have 13 protofilaments only; inside these there is a circle of usually 7 other units, thus forming a 13 + 7 + 1 pattern (Fig. 9). Each axonemal doublet has an inner dynein arm with a prominent end knob. The spokes are connected with a central sheath.

Order Blattodea

Blattella germanica. The developing spermatozoa of the German cockroach have been described by Breland *et al.* (1968). All 9 + 9 + 2 microtubules appear to be filled with an electron-dense substance rather than being hollow (Fig. 10). The intertubular material extends from the accessory tubules in a clockwise direction and contains a single electron-lucid spot that is located near doublet protofilament 3 (Witman's nomenclature). The cell membrane is covered by a thick (about 100 Å) homogeneous and electron-dense layer.

Leucophaea maderae. The description given for Blattella germanica is also valid for the Madeiran cockroach.

Order Mantodea

Mantis religiosa. Baccetti (1987) has shown that the spermatozoon of the praying mantis has a structure similar to that of many other orthopteroid insects. We can now add that it has a characteristic so far not seen in any other insect examined by the glutaraldehyde-tannic acid fixation method: there is a row of nine electron-lucid spots, similar to the tubulin protofilaments, extending in a curved fashion from protofilament 3 (Figs. 11 and 12). This row of particles projects in a clockwise direction and encircles part of the intertubular material. The accessory tubules, by contrast, have detached from the doublets (and have the usual 16-protofilament pat-

FIGS. 1-38. Electron micrographs of sperm tails from the indicated insect species (insect order given within parentheses). The specimens have been prepared to reveal the subunit pattern of the various microtubules.

FIG. 1. Campodea sp. (Diplura). The nine accessory tubules have a wall of 13 protofilaments; they are aligned along one side of the axoneme. \times 220 000.

FIG. 2. Machilinus sp. (Archeognatha). The accessory tubules have a 16-unit wall and form two groups. \times 250 000.

FIG. 3. Detail of Fig. 1. × 440 000.

FIG. 4. Detail of Fig. 7. \times 400 000.

FIG. 5. Detail of Machilinus spermatozoon. × 450 000.

FIG. 6. Detail of Fig. 8. × 400 000.

FIG. 7. Lepismodes inquilinus (Zygentome). Accessory tubules have 16 protofilaments and an electron-dense interior. \times 270 000.

FIG. 8. Calopteryx sp. (Odonata). Accessory tubules with 16 protofilaments, cytoplasmic microtubules with $13. \times 220000$.



tern). The spermatid cytoplasm outside the axoneme and mitochondria contain some microtubules, some of which have 13 protofilaments, and others that have 16.

Order Phasmida

Baculum sp. The axoneme of the stick insect Baculum sp. is characterized by having accessory tubules with 17 protofilaments (Afzelius, 1988; Afzelius *et al.*, 1990) (Figs. 13 and 16). The intertubular material is divided into two portions, a larger one in contact with the doublet microtubule only and a smaller one that extends counterclockwise from the accessory tubules. As in many other insects there are one or two protofilament-like spots within the intertubular material and close to protofilament 3.

Extatosoma tiaratum, Libethra regularis, and Bacillus rossius. In most respects the fine structure of the axoneme is indistinguishable from that of Baculum. As in that species the accessory tubules have 17 protofilaments and an interior that often is seen as a circle of about 7 or 8 roundish units around a central unit, thus forming a 17 + 7 + 1 or 17 + 8 + 1 pattern.

Order Orthoptera

Pezotettix giornai. A characteristic of many orthopteran species is the presence of a thick, "hairy" glycocalyx that is prominent also after traditional fixation methods (Baccetti *et al.*, 1971a; Yasuzumi and Asai, 1978). It has been termed the "locust model" and differs from that of, e.g., cockroach spermatozoa, by being much thicker (about 300 Å) and by consisting of densely packed rodlets. The axoneme itself is rather unremarkable; the intertubular material is abundant and projects clockwise from the accessory tubules (Figs. 14 and 17). An unexpected finding was that the cytoplasmic microtubules of spermatids consist of 14 rather than 13 protofilaments (Fig. 15).

Acrida bicolor. A cross section of the tail from the Acrida grasshopper spermatid is practically indistinguishable from that of *Pezotettix giornai* spermatid. The resemblance also encompasses the cytoplasmic microtubules with their 14 protofilaments.

Gryllotalpa gryllotalpa. This mole-cricket has a long sperm tail that is immotile along its distal half (Baccetti *et al.*, 1971b). The proximal and motile half is provided with an abundant intertubular material that bridges the entire distance between adjacent accessory tubules (Fig. 18).

Gryllus campestris. The spermatozoon of the field cricket is motile and its sperm tail shows a strong resemblance to that of Gryllotalpa, although its intertubular material does not span the entire distance between the accessory tubules. As in that species and other primitive orthopteran families the glycocalyx is homogeneous rather than fibrillar.

Order Dermaptera

Forficula auricularia. The spermatozoon of the earwig is a typical filiform insect sperm (Baccetti et al., 1974). Its axonemal cross section also is rather unremarkable except for a W-shaped structure between the axoneme and the two mitochondria (Fig. 19). There are indications of two connecting bridges between the axoneme and the mitochondria; probably these extend from doublets 2 and 5 (compare with the study by Afzelius and Dallai, 1989). The two central singlets have a dense central filling whereas the accessory tubules (with 16 protofilaments) have not (Fig. 21). The intertubular material is abundant and fills most of the space between pairs of accessory tubules; it contains a single electronlucid spot near doublet protofilament 1 or 2. Each spoke ends with a double spoke head somewhat similar to that in the brachyceran flies.

Order Psocoptera

The spermatozoon of a psocopteran, probably *Lepinotus inquilinus*, will be described separately. Here it will suffice to mention that its accessory tubules have 13 protofilaments.

Order Hemiptera

Pyrrhocoris apterus. The insect order Hemiptera is divided into two large groups, Heteroptera and Homoptera, which are spermatologically distinct. The heteropteran spermatozoa have certain synapomorphic features that have not been found in spermatozoa from other animals: the presence of three crystals, rather than one, within the mitochondrial derivatives and the presence of connecting bridges between mitochondria and doublets 1 and 5 (Dallai and Afzelius, 1980). The bulk of the intertubular material is connected to the doublets but not to the accessory tubules; it contains one or two electronlucid dots near doublet 3 (Figs. 20 and 22). There is also a small portion of the intertubular material at the counterclockwise side of the accessory tubule, again with a small light spot. In the center of many accessory tubules there is a small circle of electronlucid material (Fig. 20).

Raphigaster nebulosa. A micrograph of the axoneme of this stink bug has been published by Afzelius and Dallai (1989) and shows that the description given above for *Pyrrhocoris* is also valid for this species.

Notonecta reuteri. Spermatozoa of the backswimmer have an enormous length as described by Afzelius *et al.* (1976). When examined by the glutaral-



FIG. 9. Cloëon sp. (Ephemeroptera). Accessory tubules with 13 protofilaments surrounding 7 smaller units. \times 480 000. FIG. 10. Blattella germanica (Blattodea). Accessory tubules with 16 protofilaments. All 9 + 9 + 2 microtubules have an electron-dense interior. \times 420 000.



FIG. 11. Mantis religiosa (Mantodea). Accessory tubules with 16 protofilaments. There is also a row of 9 other units along the outer circumference of the intertubular material. \times 340 000.

- FIG. 12. Detail of Fig. $11. \times 770\ 000.$ FIG. 13. Detail of Fig. $16. \times 640\ 000.$ FIG. 14. Detail of Fig. $17. \times 440\ 000.$

- FIG. 15. Detail of the same sperm tail as in Fig. 17. × 440 000.
 FIG. 16. Baculum sp. (Phasmida). Accessory tubules have 17 protofilaments. × 280 000.
- FIG. 17. Pezotettix giornai (Orthoptera). Accessory tubules have 16 protofilaments. \times 220 000.



FIG. 18. Gryllotalpa gryllotalpa (Orthoptera). Accessory tubules have 16 protofilaments and are joined by a prominent intertubular material. \times 200 000.

FIG. 19. Forficula auricularia (Dermaptera). Accessory tubules have 16 protofilaments. \times 150 000.

FIG. 20. Detail of Fig. 22. × 500 000.

FIG. 21. Detail of Fig. 19. × 400 000.

FIG. 22. Pyrrhocoris apterus (Hemiptera). Accessory tubules with 16 protofilaments. Note also the electron-lucid spots within the intertubular material. \times 200 000.

FIG. 23. Cercopis sp. (Hemiptera). Accessory tubules with 16 protofilaments. \times 200 000.

dehyde-tannic acid method the axoneme is shown to conform to the pattern of *Pyrrhocoris* and *Raphigaster*.

Cercopis sp. Folliot and Maillet (1970) have described the spermatozoon of *Cercopis sanguinea* and those of a number of other homopteran bugs. The distribution of the intertubular material is roughly the same as that in the heteropteran species examined (Fig. 23). The central microtubules, the Asubtubules, and the accessory tubules appear filled with a dense material. No other distinguishing features of the axoneme have been found.

Order Raphidioptera

Raphidia sp. The sperm tail of the snake fly contains the axoneme, two slender mitochondria, two accessory bodies, and some material between these (Afzelius and Dallai, 1988). The intertubular material of the axoneme is divided into two portions: a larger one extending clockwise from the accessory tubules and a smaller beaklike one at the counterclockwise side. Within the central singlets and the accessory tubule lumen a substructure of small units can be seen (Figs. 24 and 26). Accessory tubules may show a 16 + 10 + 4 pattern.

Order Coleoptera

Lytta vesicatoria. Sperm ultrastructure in the Spanish fly has not previously been reported. As in most other beetles the sperm tail contains two mitochondria, two accessory bodies, and the axoneme. (The accessory bodies are inconspicuous at the spermatid stage shown in Fig. 25.) The appearances of accessory tubules and central singlets resemble those of *Raphidia* and so does the distribution of the intertubular material.

Tenebrio molitor. The appearance of the sperm axoneme in this species is similar to that of Lytta vesicatoria.

Chrysomela sp. In contrast to the situation in *Lytta* and *Tenebrio* the axoneme of *Chrysomela* was seen to have inner singlets with an electron-dense interior and accessory tubules with an inner circle of electron-lucid units. The distribution of the intertubular material seems to resemble that of the other beetles and snake flies examined.

Order Strepsiptera

Stylops sp. The ultrastructure of Stylops sperm cell will be described in a separate paper.

Order Mecoptera

Panorpa germanica. The sperm axoneme in mecopterans are of a simple 9 + 2 variety and hence lack accessory tubules and intertubular material. The nine doublets and two singlets, when seen after

fixation with the glutaraldehyde-tannic acid mixture, are unremarkable.

Order Hymenoptera

Solenopsis sp. The spermatozoa of the fire ant, Solenopsis saevissima, has been examined by Thompson and Blum (1967). Our data agree with theirs and we can add only a few details: The intertubular material is fairly abundant and spans most of the distance between the accessory tubules (Fig. 28). In many cases one or two small light dots are seen in this dark material near doublet protofilament 3.

Apis mellifera. In its general traits the sperm axoneme of the honeybee is similar to that of the ant (see also Cruz-Höfling *et al.*, 1970); we find the intertubular material to be even more abundant than it is in the ant.

Order Lepidoptera

Pieris brassicae. Spermatozoa from *Pieris* and other butterflies have complex outgrowths from the cell membrane, termed the lacinate and reticular appendages (André, 1962). As shown by our fixation technique, the reticular appendages consist of straight rows of particles that have a stained outer rim and an unstained interior (Fig. 29). The intertubular material consists of two portions: a thicker one at the clockwise side of the accessory tubules and a thinner one extending from it toward the neighboring accessory tubule. The thick portion contains two or three small lucid spots that are close to protofilament 3 (Figs. 27 and 29).

Pieris rapae, Syntoma sp., and Arctia sp. The axoneme is identical to that of Pieris brassicae.

Order Trichoptera

Stenophylax permistus. The ultrastructure of the spermatozoon of this caddiesfly species has not previously been examined. In its main features it resembles that of the related species Mystacides azurea (Baccetti et al., 1970). A particularly striking feature is the large diameter of the accessory tubules. These were seen, in the present study, to consist of 19 protofilaments and to have a diameter of 38 nm (Fig. 30). The cross-sectioned sperm tail hence contains 404 cross-sectioned protofilaments (9 \times 19 + 9 \times 23 + 2 \times 13). The accessory tubules have a set of inner globules that form a pattern that often is 19 + 10 + 4 (but where only 19 seems constant). The intertubular material consists of two portions, a small portion at the centripetal side of the accessory tubule and a longer one extending from doublet protofilament 3 and containing a row of about 5 or 6 electron-lucid spots. The cell membrane has a glycocalyx of uniform electron density.



Raphidia sp. (Raphidioptera). Accessory tubules with 16 protofilaments. \times 240 000. Lytta vesicatoria (Coleoptera). Accessory tubules with 16 protofilaments. \times 200 000. F1G. 24. F1G. 25.

F1G. 26. Detail of Fig. 24. \times 450 000.

FIG. 27. Detail of Fig. 29. \times 350 000.

F1G. 28. Solenopsis sp. (Hymenoptera). Accessory tubules with 16 protofilaments. \times 220 000.

FIG. 29. Pieris brassicae (Lepidoptera). Accessory tubules with 16 microtubules. The cell membrane is covered by lacinate appendages and other appendages. \times 190 000.



FIG. 30. Stenophylax permistus (Trichoptera). Accessory tubules have 19 protofilaments and a larger diameter than that of the accessory tubules in the other insect species. \times 230 000.

FIG. 31. Orfelia sp. (Diptera, Nematocera, Family Mycetophilidae). Accessory tubules with 16 microtubules. \times 200 000.

Detail from Fig. 31. \times 320 000. FIG. 32.

FIG. 33.

Tipula sp. (Diptera, Nematocera, Family Tipulidae). Sciara sp. (Diptera, Nematocera, Family Sciaridae). Part of the giant axoneme; the accessory tubules have 13 protofilaments. FIG. 34. × 300 000.

Limnephila sp. The cross-sectioned axoneme has an appearance similar to that of Stenophylax with characteristically prominent accessory tubules that consist of 19 protofilaments. See note at end of paper.

Order Diptera

Orfelia sp. There is a remarkable variability in the appearance of the axoneme and of its accessory tubules within the dipteran order. This is in contrast to other insect orders. Orfelia belong to the family Mycetophilidae, which for various reasons has been regarded as the most primitive dipteran family (Dallai and Mazzini, 1980). The cross-sectioned sperm tail has a small crescentic mitochondrion and an axoneme of the 9 + 9 + 2 type. The nine accessory tubules have 16 protofilaments and the intertubular material is divided into two portions (Figs. 31 and 32). Another, as yet undetermined species of the mycetophilid family, has been examined and found to have 16 protofilaments in its accessory tubules.

Tipula sp. The axoneme is of the 9 + 9 + 2 type and has accessory tubules with only 13 protofilaments and intertubular material that is divided into two portions (Fig. 33). As in other dipteran species the spokes terminate in end knobs, spoke heads, that appear double.

Sciara sp. The sperm tail within the Sciaridae family is aberrant and contains a giant axoneme that consists of a great number of microtubular doublets each connected with an accessory tubule (Dallai *et al.*, 1973). The axoneme is permeated by an electron-dense substance in our preparations. Each accessory tubule consists of 13 protofilaments (Fig. 34).

Bibio sp. The sperm tail of bibionid flies has been classified as having a 9 + 9 + "1" axoneme. There are thus 9 accessory tubules, 9 doublets, and a central rod that is not a microtubule (Dallai and Mazzini, 1983). The intertubular material in this species as in most other dipterans has a larger portion attached to the doublet and a smaller one at the counterclockwise side of the accessory tubule. The accessory tubules have 15 protofilaments (Fig. 35).

Culex pipiens. The 9 + 9 + "1" axoneme of the mosquito sperm is strikingly similar to that of the bibionid as has previously been stressed (Dallai and Mazzini, 1983). The similarity can now be extended to the appearance of the accessory tubules—with 15 protofilaments—thus of a type that has been found in these two dipteran groups but in no other animal sperm (Fig. 36).

Anopheles gambiae. The same ultrastructural characteristics as in *Culex* were seen in this species.

Empis sp. The empidid flies belong to the brachyceran suborder of Diptera. Their spermatozoa have not previously been examined by electron microscopy. With the present fixation technique the sperm tail can be seen to contain one mitochondrion (or rather mitochondrial derivative) and one axoneme (Fig. 37). The accessory tubules have 13 protofilaments.

Scatophaga sp. The cross-sectioned sperm tail of Scatophaga is similar to that of the Empis fly, except for the presence of two mitochondrial derivatives and for a somewhat more prominent intertubular material (Fig. 38). Along the outer rim of the intertubular material there is a straight line of electronlucid particles. The accessory tubules have 13 protofilaments.

Calliphora vomitoria. Spermatozoa of the blowfly have accessory tubules containing 13 protofilaments and have intertubular material that has a straight row of four or five electron-lucid particles along its outer margin.

Ephydridae sp. The axoneme of the late spermatids of this fly resembles that of other brachyceran spermatozoa in having accessory tubules with 13 protofilaments and prominent spoke heads. Microtubules in the cytoplasm outside the axoneme also have 13 protofilaments.

Ceratitis capitata. Spermatids of the medfly have been examined and found to have accessory tubules with 13 protofilaments.

DISCUSSION

Cytological Aspects

The fine structures of the glutaraldehyde-tannic acid-fixed spermatozoa from representatives of 20 insect orders have been examined. A distinct substructure could be seen in all microtubules, both those found free in the cytoplasm and those that are components of the flagellum. The unit element in all cases seems structurally similar, if not identical: a protofilament with a somewhat triangular outline such as has been described in Afzelius *et al.* (1990). It is interpreted as the tubulin molecule.

The microtubules described here are of six different classes with 13, 14, 15, 16, 17, or 19 protofilaments and with correspondingly different diameters. The number of protofilaments seems constant for each class of microtubule of a certain insect species. It is also of interest that the protofilaments are visible in all cross-sectioned microtubules; in other words, no class of microtubule with a diffuse or only partially resolved wall was seen. This is remarkable because the tubulin monomers are much smaller than the section thickness, 5.5 nm versus 60–90 nm. Evidently the protofilaments in all six classes run parallel to the microtubular axis or have only a slight helical twist. As half the classes of microtubules have a protofilament number that is a prime



FIG. 35. *Bibio* sp. (Diptera, Nematocera, Family Bibionidae). Axoneme with a 9 + 9 + "1" pattern and accessory tubules with 15 protofilaments. \times 340 000.

FIG. 36. Culex sp. (Diptera, Nematocera, Family Culicidae). Axoneme with a 9 + 9 + "1" pattern; accessory tubules with 15 protofilaments. \times 320 000.

FIG. 37. Empis sp. (Diptera, Brachycera, Family Empididae). Accessory tubules with 13 protofilaments. \times 280 000.

FIG. 38. Scatophaga sp. (Diptera, Brachycera, Family Scatophagidae). Accessory tubules with 13 protofilaments. × 240 000.

number and the other half have not, it would be possible to test the hypotheses by Fujiwara and Tilney (1975) mentioned in the Introduction.

The interpretation of the micrographs is not straightforward. We assume that tannic acid is a mordant that adheres to the proteins and that it chelates uranyl ions which are then seen to outline the protein as in negative staining. An electronlucid spot may thus be either the site of a protein (e.g., the tubulin in the microtubular wall) or an empty spot. In some cases, such as the Sciara axoneme (Fig. 34), the interpretation is simple, in other cases it is not. The central hole in the accessory tubules of Cloëon (Fig. 9) might for instance be interpreted as an empty space surrounded by 13 tubulin molecules and 7 other proteins, or as a single protein surrounded by these 13 + 7 proteins. The success of the technique is dependent on the penetration of tannic acid into the cells. In nearly all of our preparations some spermatozoa were penetrated by tannic acid and uranyl acetate and appeared with well-outlined protofilaments and others were not.

The core of the accessory tubules can be denoted as 17 + 7 + 1 or 17 + 8 + 1 for phasmid spermatozoa and, e.g., 16 + 10 + 4 for *Panorpa* sp. or 19 + 10 + 4 for *Stenophylax*. There is, however, some variability in the number of the inner subunits, and the figures are hence not necessarily valid for all accessory tubules in an axoneme. Whether the pattern variations depend on the degree of tannic acid penetration, on the degree of sperm maturation, or on some other factor has not been determined.

The cores of the nine doublets and two singlets also have certain patterns that differ in different insect groups. In some species they appear solid and in others they appear hollow. The core regions of the doublets in a phasmid have been described in some detail (Afzelius *et al.*, 1990). In spermatozoa from other insect orders the cores may have the same appearance as that of the phasmids or a different one.

A feature that has not been noticed earlier is the presence of electron-lucid spots in the intertubular material. When just a few such spots are seen, they are usually present close to protofilament 3 (Witman's terminology) or at that site and at the outer, counterclockwise side of the accessory tubule. This is of interest since the accessory tubules are formed close to protofilaments 3 or 4 during spermiogenesis (unpublished observations). It thus appears that this specific region of the B-subtubule is involved somehow in the organization of an accessory tubule as well as of another row of molecules, which may or may not be tubulin. The longest row of particles, that in the flagellum of Mantis, actually is strikingly similar to a forming accessory tubule, except that the curvature of the particle row is different in the two structures.

We conclude that spermatids can effect specification of different protofilament numbers from 13 to 19 with numerical exactitude for particular by very closely adjacent microtubules.

Phylogenetic Aspects

The usefulness of comparative spermatology in phylogenetic studies has been stressed by Baccetti and Afzelius (1976) and by Jamieson (1987). Jamieson's monograph gives many striking examples of the value of this new tool in entomology. We believe that the minute details that have been found here and those that will be found in the future with improved fixation techniques might give us a better understanding of the relationship between different animal groups. Grimstone (1959) has warned against relying on ultrastructural evidence in phylogenetic work. Yet we think that there is nothing wrong in basing phylogenetic conclusions on the ultrastructure or biochemistry of the flagellar axoneme, as it is a very complex organelle that requires the participation of as many as 400 polypeptides and thus of a substantial proportion of all genes (Dutcher and Lux, 1989).

The first question to be dealt with is the relationship between the various apterygote groups. Three apterygote orders have spermatozoa with accessory tubules: Diplura, Archeognatha, and Zygentoma. On the basis of the number of protofilaments and the presence or absence of intertubular material, there is a dichotomy between Diplura, on one hand, and Archeognatha and Zygentoma, on the other—13 without intertubular material and 16 with, respectively. Accessory tubules may have been acquired independently in Diplura and in Archeognatha and Zygentoma.

Which apterygote order shares a more recent ancestry with the Pterygota? From what apterygote group are the pterygotes derived? What is the relationship between Ephemeroptera, Odonata, and the higher pterygote insects, also called the Neoptera? These questions are more problematic. If sperm data are to be trusted, the situation would be complex. Ephemeroptera and Diplura have sperm tails that are quite similar in that there are 13 protofilaments and no intertubular material. Zygentoma and Odonata plus a majority of pterygote orders have another type of sperm tail with 16 protofilaments and with intertubular material. This probably means that the dipluran sperm tail and the ephemeropteran one appeared independently during evolution.

Boudreaux (1979) sees a sister group relationship between Odonata, on one hand, and Ephemeroptera and Neoptera, on the other. Kristensen (1981) on the contrary sees a sister group relationship between Ephemeroptera, on one hand, and Odonata and Neoptera on the other. Jamieson (1987) too considers Odonata and Ephemeroptera to belong to widely different lineages which must have developed independently from a very early stage in pterygote evolution. We agree with this interpretation.

Within the large group of Neoptera, the original number of protofilaments seems to be 16. Intratubular material is also present and usually is divided into two portions. Sixteen is thus the number seen in Blattodea, Mantodea, Orthoptera, Dermaptera, Hemiptera, Raphidioptera, Coleoptera, Hymenoptera, Lepidoptera, and within one primitive group of Diptera. It might also be the number of protofilaments in those neopteran orders that have not yet been examined in this respect: Plecoptera, Embioptera, Megaloptera, Neuroptera, Zoraptera, and Grylloblattodea. In some of the remaining insect orders the accessory tubules have been lost: Isoptera, Thysanoptera, Mecoptera, and Siphonaptera.

Four orders of neopteran insects fall outside the norm: Phasmida with 17 protofilaments, Trichoptera with 19, Psocoptera with 13, and Diptera in which the situation is complex. The phasmid spermatozoon is unique in several respects, characterized by a lack of mitochondria, its two "lamellated bodies" alongside the sperm axoneme and, we may add, by having accessory tubules with 17 protofilaments. The trichopteran spermatozoon seems to have fewer unique features, although it does contain accessory tubules with 19 protofilaments and thin intertubular bridges and a centripetal portion. It is not known whether all trichopteran spermatozoa with a 9 + 9 + 2 axoneme have such tubules. This is likely, however, since it has been remarked that these tubules are unusually large (Baccetti et al., 1970). Sperm morphology does not give any indications as to the position of the phasmids within the orthopteroid superorder, nor of the trichopterans in relation to other holometabolic insects.

The dipteran order is known to have a great diversity in sperm patterns. Nevertheless we were surprised to find three types of accessory tubules, those with 13, 15, and 16 protofilaments. We interpret these data as follows: Mycetophilidae is a primitive family, which has retained the "standard" pterygote axoneme with its 16-unit accessory tubules. Tipulimorpha is derived from the Mycetophilidae and its sperm pattern is different mainly in that the accessory tubules have reverted to a 13-unit pattern. (An alternative interpretation is that Tipulimorpha are the most archaic dipterans; this would imply that spermatozoa in this group would have mutated back to a 13-unit pattern and that the Mycetophilidae then mutated to the 16-unit pattern, a hypothesis that appears less probable.) The 13unit pattern has been retained in the giant axonemes of the Sciaridae and also in the brachyceran subdivision of Diptera. Two groups of nematocerans, Bibionidae and Culicidae, have been suspected to be rather closely related on spermatological grounds (Dallai and Mazzini, 1983) and their unique possession of a 15-unit accessory tubule can only strengthen this opinion.

The findings presented above must be regarded as provisional. So far only a limited number of the several million insect species have had their sperm ultrastructure examined and they have furthermore been analyzed only with respect to a few characteristics. Other details, for instance the appearance of cytoplasmic microtubules in the spermatids with 13, 14, or 16 protofilaments, have yet to be taken into consideration. It is thus not known whether the 14unit microtubule pattern in spermatids of (at least some) orthopteran species is characteristic of that group.

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Note added in proof. Since the manuscript has been submitted we have examined the sperm tail of a number of additional insect species. The results hereof will be published elsewhere; here it suffices to mention that the accessory tubules of one trichopteran species was found to have 17 rather than 19 protofilaments. Conditions within the trichopteran order thus are more complicated than we first believed.

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