SPERMATOZOON OF MAYFLIES (EPHEMEROPTERA): AN ULTRASTRUCTURAL APPROACH

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ABSTRACT

The spermatozoa of some species of Ephemeroptera belonging to the Heptageniidae (*Electrogena grandiae, Ecdyonurus* gr. *venosus*) and Leptophlebiidae (*Habroleptoides umbratilis, Habrophlebia eldae, Choroterpes picteti*) are described.

In the flagellate spermatozoa of the Heptageniidae, the sperm head consists of a short monolaminar acrosome and an elongated nucleus. The tail contains a partially crystallized mitochondrial derivative, two paired crystalline accessory bodies and an axoneme characterized by a 9+9+0 microtubular pattern. Even though such an array represents the most representative mayfly axoneme model, in *Electrogena grandiae* sperm of a same specimen may also show a centrally located dark spot. The designation 9+9+"1" for this peculiar arrangement is proposed.

The spermatozoon of the Leptophlebiidae is an aflagellate spherical-shaped cell provided with acrosome. Sperm features differ according to the systematic position of the above mentioned species.

The importance of sperm architecture for both taxonomic purposes and fertilization biology is discussed.

INTRODUCTION

Spermatozoon ultrastructure represents an important tool for understanding the systematic position of many groups of animals (see reviews Baccetti and Afzelius, 1976 and Baccetti, 1979a) and in particular for tracing the phylogeny of different insect orders (see reviews Baccetti, 1979b and Dallai, 1979).

In the Ephemeroptera, histological studies on spermatogenesis and light microscope observations on spermatozoa have been published by Soldàn

(1979a,b) but our knowledge of sperm ultrastructure is very scarce if we consider that mayflies are the most primitive extant pterygote insects. During the past 20 years, ultrastructural characteristics have been pointed out in mayflies and these concern only seven species encompassed by different families. To date three main sperm models have been described in the following chronological sequence: a) the 9+9+0 axoneme pattern, characterized by 9 outer singlets, 9 doublets and no central elements, in *Cloeon dipterum* (Baetidae) (Baccetti *et al.*, 1969), in *Pentagenia vittigera* (Palingeniidae), in *Tricorythodes* sp. (Tricorythidae), *Hexagenia* sp. (Hexageniidae) (Phillips, 1969) and in *Siphlonurus croaticus* (Grimm, 1985); b) the aflagellate sperm in *Habrophlebia lauta* (Grimm, 1985); c) the 9+9+1 axoneme pattern, characterized by a central element in *Dolania americana* (Behningiidae) (Fink and Yasui, 1988). Even though these ultrastructural studies demonstrated that the 9+9+0 array of tubules is the most representative sperm model, mayfly spermatozoa differ not only in flagellum structure but also in their cytological organization. Considering that mayfly sperm morphology is variable, our aim is to add new data to this field to improve the knowledge of this primitive insect group from a spermatological point of view.

MATERIAL AND METHODS

The following species have been examined:
Heptageniidae:

Electrogena grandiae (Belfiore), (E. Gaino leg. et det.);

Ecdyonurus gr. *venosus* (Fabricius), (E. Gaino leg. et det.).
Leptophlebiidae:

Habrophlebia eldae Jacob & Sartori, (E. Gaino leg. et det.);

Habroleptoides umbratilis (Eaton), (E. Gaino leg. et det.);

Choroterpes picteti (Eaton), (E. Gaino leg. et det.).

Live and aldehyde-fixed spermatozoa were observed with a phase contrast light microscope.

For electron microscope studies, testes and ducts were dissected from nymphs, subimagoes and adults as follows:

a) *Transmission electron microscopy* (TEM) - Selected material was fixed in Karnovsky's medium (1965) in cacodylate buffer (pH 7.2, 0.1 m), rinsed in the same buffer, postfixed in 1% osmium tetroxide-cacodylate buffer, dehydrated in ethanol and embedded in epon-araldite mixture. Sections were obtained with Reichert ultratome and mounted on formvar-coated copper grids, stained with uranyl acetate and lead citrate and observed in both Philips 300 and Zeiss EM9 electron microscopes.

b) *Freeze-fracturing analysis* - Inner reproductive systems were briefly fixed in Karnovsky's medium-cacodylate buffer as above and infiltrated in glycerol to a final concentration of 30%. They were then rapidly frozen in freon 22 cooled to the temperature of liquid nitrogen. Fracture and platinum carbon coating were carried out in a Balzers BAF 301 freeze etching apparatus set at -115° C. Replicas were digested with Clorox, picked up with form-

var-coated copper grids, and examined with Philips 300 and 400 electron microscopes.

RESULTS

A. Sperm organization in the Heptageniidae (figs. 1-11)

Sperm of the examined species of Heptageniidae belong to the flagellate model and show active movements when observed in biological fluids. Spermatozoon architecture has been reconstructed for *Electrogena grandiae* by means of ultrastructural serial sections (fig. 1).

In *Electrogena grandiae* living mature spermatozoa observed with contrast phase microscope appear to consist of a narrow straight body of 9 μm, from the base of which the flagellum arises (mean value about 14 μm) (fig. 2). In thin sections, spermatozoa reveal the presence of several constitutive regions. The most anterior region is occupied by the nucleus (8.7μm long with a maximum diameter of 0.4 μm), containing rather electron-opaque chromatin with many clear areas inside it (fig. 3). Apically located and close to the nucleus there is a small, rounded monolaminar acrosome (0.3 μm as long as thick) (figs.3-4). The basal portion of the nucleus shows a deep nuclear fossa in which are lodged both the centriole and the initial portion of the axoneme originating from the classical centriole. A mitochondrial derivative is located along the axoneme and in front of it as clearly evident in cross sections (fig. 5). The partially crystallized mitochondrial derivative presents a series of septa, perpendicular to the major mitochondrial axis (fig. 4), which in freeze-fracture replicas show a regular pattern with about a 200 Å periodicity (fig. 6). In transverse sections the mitochondrial derivative, characterized by a peripheral bilayer, includes homogeneous, unstructured and weakly electron-dense material (fig. 7), with some translucent areas. The mitochondrial derivative is flanked by two crystalline bodies. This complex is wrapped in a membrane formed by two electron-dense layers separated by a thin light space which broadens in some areas (fig. 7). The two crystalline structures arise beneath the nucleus and are located at both sides of the mitochondrial derivative (fig. 7). In longitudinal sections they show parallel rows of short electron-dense lines with a 200 Å periodicity. In transverse section each crystalline body seems to arise from two parallel indented structures linked to each other along their length (fig.7). The axoneme of about 200 nm in diameter, is composed of a 9+9+0 pattern made up of outer singlets and doublets (fig. 7), these latter bearing only the innermost dynein arm. Both central sheath and radial spoke are evident. The central element is generally absent, even though in some sections the axoneme pattern presents a centrally located dark spot (fig. 7).

Living mature spermatozoa of *Ecdyonurus* gr. *venosus* when observed in contrast-phase microscope show clearly an arch-shaped head (about 15.5 μm in length), longer than the flagellum (fig. 8). The ultrastructural sperm organization of *Ecdyonurus* gr. *venosus* is very similar to that of the previous species, with the exception of the acrosome, which is longer (0.6 μm in

length and 0.3 μm in width) as also showed by freeze-fracture replicas (fig.9). This technique, besides confirming the arch-shaped body, gives evidence for plasma membrane particles packed at the top of the sperm, for mitochondrial derivative organization and position. This organelle, located in front of the axoneme and laterally flanked by the two crystalline bodies, is characterized by regularly spaced septa with a periodicity of about 160 Å (fig. 11).

B. Sperm organization in the Leptophlebiidae (figs. 12-15)

The sperm cells of the species of Leptophlebiidae examined belong to the aflagellate model. In biological fluids, the sperm have a spheroidal shape and do not show active movement.

The spermatozoon of *Habroleptoides umbratilis* (about 2 μm, along its major axis), has a clear constriction about 1/3 along its length which is detectable with light microscopy (fig. 12). In ultrastructural sections sperm show two regions differing in size and appearance (fig. 13). The former is wider and almost completely occupied by a nucleus with tightly packed chromatin; the latter contains the acrosome complex consisting of a monolaminar acrosome that includes a well defined perforatorium with a fibrillar content (fig. 13). This complex defines the anterior region of the spermatozoon. A single small mitochondrion is located beneath the nucleus and apparently lacks any crystallized appearance or crystalline-like inclusions (fig. 13).

In *Habrophlebia eldae* the spermatozoon (approx. 1 μm, along its major axis) presents a very simplified architecture consisting in the nucleus and superimposed acrosome only. Neither mitochondrion, mitochondrial derivatives nor perforatorium are visible (fig. 14).

In *Choroterpes picteti* the spermatozoon (about 1.5 μm, along its major axis) shows a small knob-like acrosome with a fibrillar perforatorium and a mitochondrion laterally located in the area between the acrosome and the nucleus (fig. 15). The nucleus is separated from the cell membrane by a sheath of cytoplasm containing some electron-dense bodies with a crystalline organization (fig. 15).

DISCUSSION

Phylogenetic trees based on sperm organization have been traced in insects taking into particular account the architecture of the acrosome complex, the morphological variations of the mitochondria, accessory bodies and axoneme microtubule arrays.

A fairly simple acrosome model is typical of many insect groups whose eggs are provided with a thick chorion and large micropyles. This relationship may also be envisaged for flagellate sperm of Ephemeroptera, where the acrosomal architecture is constructed according to a primitive, cap-shaped, apically located organelle without perforatorium.

Insects have had to adapt to terrestrial life and consequently have tried out different solutions to fulfil internal fertilization. According to Dallai

(1979), the deviation from the most representative sperm model may be related to this fact, thus emphasizing that the shape of the spermatozoon is influenced by reproductive biology (Afzelius, 1979). In this context, the acquisition of a supplementary set of nine tubules, external to the 9+2, makes the flagellum longer and the 9+9+2 array becomes the most common model of pterygote insect spermatozoa, well adapted to terrestrial life. By contrast, in Ephemeroptera evolutionary steps have evolved the 9+9+0 pattern, which represents the most common sperm model in this order, even though in *Dolania* (Behningiidae) a central element, defined as dark (Fink and Yasui, 1988), is present. Regarding this central dark element some thoughts may be put forward. Deviation from a central pair of tubules towards a single central element was observed in mosquitoes by Phillips (1969), who described this element as more solid than tubular in appearance. An axoneme with a rod-like electron-dense element in its central region was found in schistosomes and designated "1" to point out its non-microtubule feature (Justine and Mattei, 1981). This element was subsequently observed in spermatozoa of other Platyhelminthes (Justine and Mattei, 1988 a) and Diptera (Justine and Mattei, 1988 b). When the ultrastructure of the *Dolania* axoneme pattern is compared with that of the above-mentioned groups, the central element does not seem to be a true microtubule. In addition, our observations on the axoneme pattern of *Electrogena grandiae* showed that a central dark spot is present in transverse sections of several sperm of the same specimen. This fact underlines the need for caution in defining the new 9+9+1 model in Ephemeroptera. We suggest that, until more conclusive findings are available, the designation 9+9+ "1" be introduced in order to focus on this peculiarity. Flagellate sperm are motile because neither their peculiar axonemal model nor the preservation of only one dynein arm, the innermost, prevents sperm movement (Baccetti *et al.*, 1969; Fink and Yasui, 1988). As has recently been stressed, the presence of a single dynein arm, whether the outer or the inner one, is sufficient for sperm locomotion (Jazdowska Zagrodzinska and Dallai, 1988).

Mayfly sperm are also variable in number, shape and the localization of accessory bodies. Two crystalline-like accessory bodies were found by Baccetti *et al.*, (1969) in *Cloeon* and by us in *Electrogena grandiae*, while in other species they did not have a crystalline appearance (Phillips, 1969). In *Dolania* a single bi-lobed accessory body has been described (Fink and Yasui, 1988). In addition, accessory bodies differ in position: they are located either between the mitochondrial derivative and axoneme or else lie well separated and laterally on both sides of the mitochondrial derivative, as in *Dolania*, *Electrogena grandiae* and *Siphlonurus croaticus*.

Aberrant sperm models have been examined in order to point out their cytological organization and to evaluate their influence on motility (Baccetti, 1972; Phillips, 1974). Motionless sperm are not unusual in insects, and have been described in Protura (Baccetti *et al.*, 1973), termites (Baccetti *et al.*, 1974; Baccetti *et al.*, 1981), and Psychodidae (Baccetti *et al.*, 1973 b). In cecidomyiids, sperm evolution has followed two different lines, one of which

gives sperm with a more variable shape (Dallai and Mazzini, 1989), but whose immotility is due to the absence of dynein arms (Dallai, 1979). A peculiar progressive axoneme degeneration takes place during spermatogenesis in Homoptera, giving rise to an aflagellate and non-motile sperm in the Coccoidea (Baccetti and Dallai, 1977).

The non-motile condition in leptophlebiids is related to the loss of the flagellum. Since aflagellate sperm are found in the more specialized groups (Dallai, 1979), aflagellarity may be regarded as the advanced stage of evolution. From a spermatological point of view and accordingly to previous statments the Leptophlebiidae may be regarded as a specialized group within the Ephemeroptera. However, the presence in this family of a well differentiated fibrous perforatorium hitherto unobserved in all other mayfly sperm models and of the uncrystallized mitochondrial derivative, indicate more conservative organization. The aflagellate condition is related to the necessity of placing sperm cells in contact with eggs. In leptophlebiids deferentes are surrounded by a muscle ring that accomplishes this function through peristaltic muscle movements (Grimm, 1977; 1985). In the Psychodidae sperm are deposited in the female genital ducts, whose contractions allow them to reach the ovary (Baccetti *et al.*, 1973). In *Habrophlebia eldae*, a muscle cell sheath has been observed enveloping the female genital duct (research in progress) and presumably this performs the same function.

As for other mayfly families, a parallel adaptation of the female genital apparatus might be suggested by comparing its different structure with sperm morphology, at least at the light microscope level. Considering the anatomy of several female genital systems described by Grandi (1955) and the sperm organization studied by Soldán (1979b), a relationship between sperm structure and fertilization biology may be envisaged. Indeed the outlet of the oviducts into a common vestibulum gives rise to a specialization of this latter towards the acquisition of a spermatheca. This process takes place through the differentiation of a sperm diverticulum (as in *Electrogena grandiae*, *Ecdyonurus* gr. *venosus*) which becomes more and more complex as vestibulum size decreases (as in *Siphlonurus lacustris*). In *Ephemerella ignita*, in keeping with the presence of a well differentiated spermatheca for storing sperm, spermatozoa show a very short flagellum. It seems acceptable that flagellum size reduces according to the acquisition of a pocket for sperm preservation. Sperm storage devices in mayflies are usually consistent with the greater adult longevity (Grandi, 1955). After mating, eggs may be fertilized during their passage towards oviposition without sperm having to move intensively to reach them.

In conclusion, data on the ultrastructure of sperm, and of male and female reproductive systems may allow a better understanding of the reproductive biology of this primitive insect group and to outline family relationships according to the demands posed by structural and behavioural traits.

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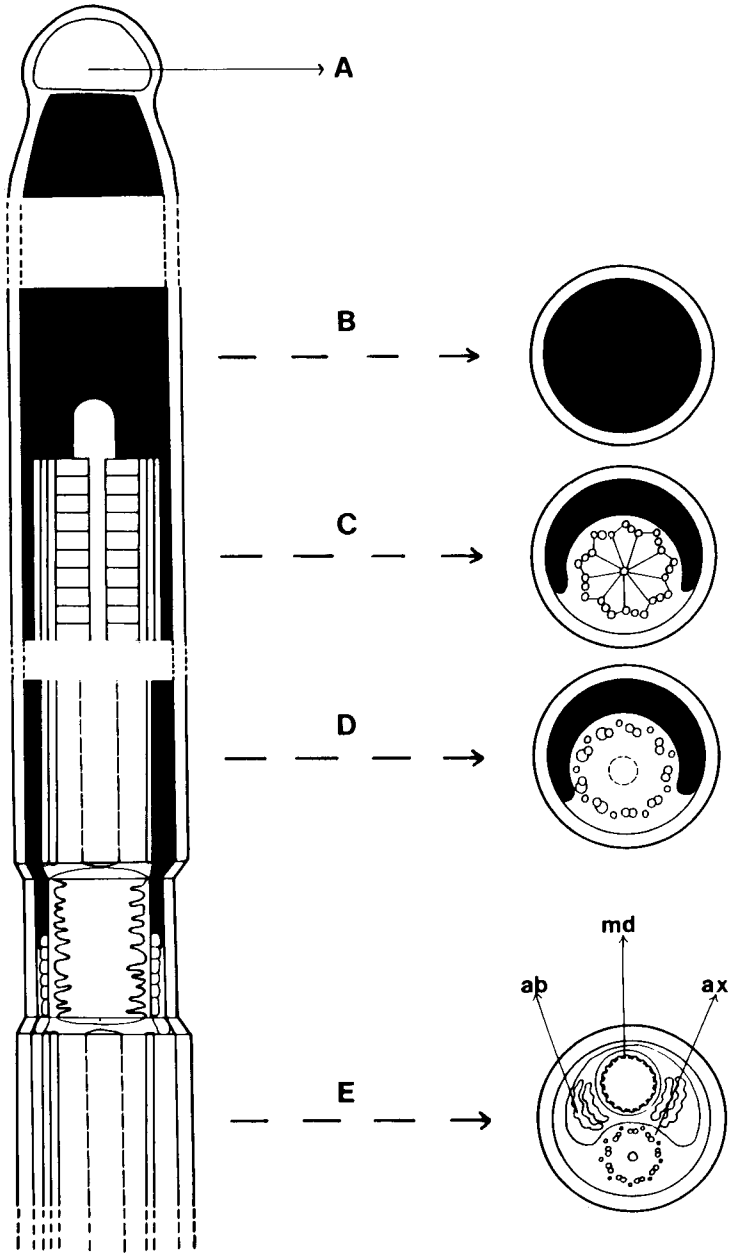
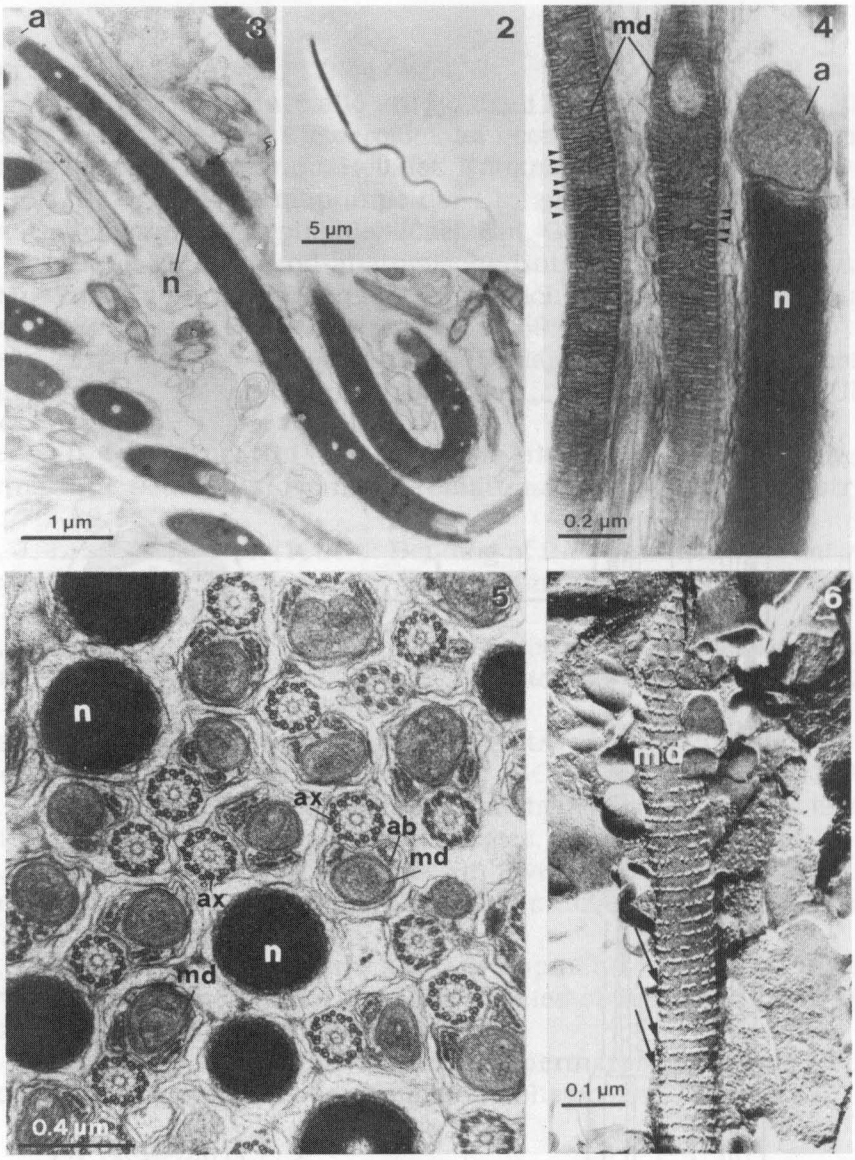
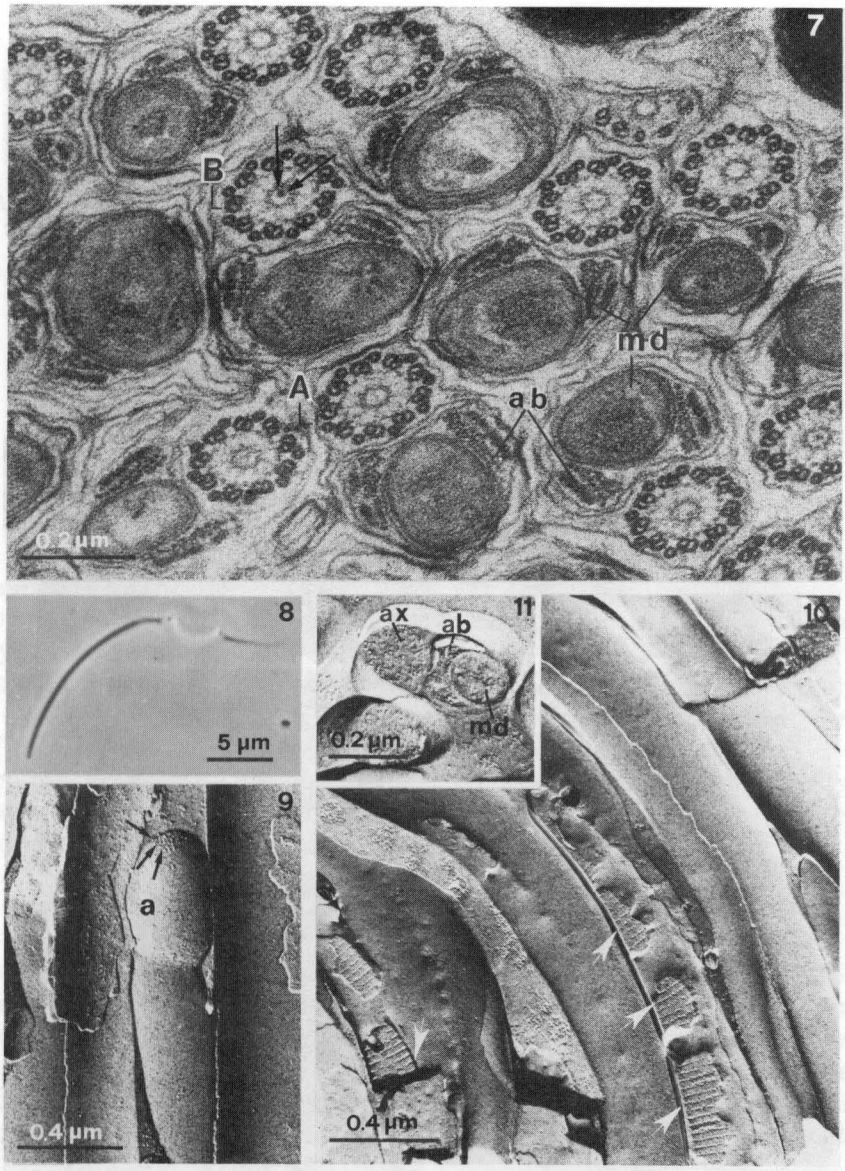


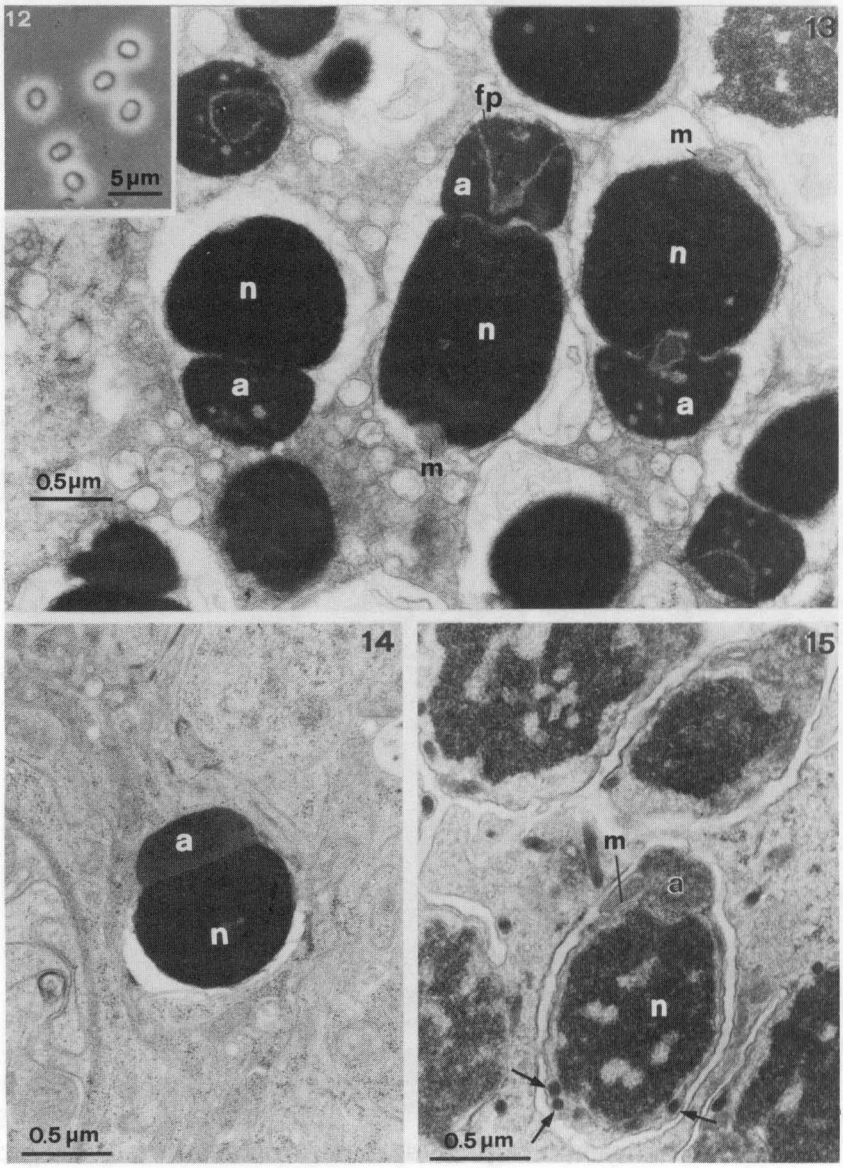
Fig. 1. Schematic drawing of the spermatozoon of *Electrogena grandiae* in longitudinal section (on the left) and in transverse sections at different levels of the body length (on the right). A, acrosome; B, nucleus; C, nucleus and centriole; D, nucleus and axoneme of the 9+9+0 pattern; E, mitochondrial derivative (md), paracrystalline accessory bodies (ab), axonemal pattern (ax).



Figs. 2-6. *Electrogena grandiae.* (2) Phase-contrast view of a live spermatozoon. (3) Electron micrograph showing the longitudinal section of the entire sperm head consisting of nucleus (n) and superimposed acrosome (a). (4) Longitudinal section of both sperm head showing monolaminar acrosome (a) and nucleus (n), and two tails whose mitochondrial derivatives (md) present evident regularly spaced cristae (arrowheads). (5) Transverse sections of spermatozoa at different levels. Note the axoneme (ax) in front of the mitochondrial derivative (md) flanked by two paracrystalline accessory bodies (ab); n, nucleus. (6) Freeze-fracture replica showing the periodicity of the septa (arrows) along the major axis of the mitochondrial derivative (md).



Figs. 7-11. (7) Transverse sections of spermatozoa of *Electrogena grandiae* showing in detail both 9+9+0 (A) and 9+9+"1" (B) axoneme pattern, the latter characterized by a dark central spot (arrows); md, mitochondrial derivative; ab, accessory bodies. Figs. 8-11. *Ecdyonurus* gr. *venosus*. (8) Phase-contrast view of a live spermatozoon. (9, 10, 11) Freeze-fracture replicas showing: (9) the anterior region of a sperm whose acrosome (a) presents apically located membrane particles (arrows); (10) the periodicity of mitochondrial derivative septa (arrows); (11) sperm tail with axoneme (ax), mitochondrial derivative (md) and paracrystalline accessory bodies (ab).



Figs. 12-15. Aflagellate sperm in Leptophlebiidae. (12) Phase-contrast view of live spermatozoa of *Habroleptoides umbratilis*. (13) Longitudinal section of sperm of *H. umbratilis*. Note the nucleus (n), the acrosome (a), the fibrillar perforatorium (fp) and the mitochondrion (m). (14) Section of *Habrophlebia eldae* spermatozoon showing the nucleus (n) and superimposed acrosome (a); (15) Section of *Choroterpes picteti* spermatozoon. Note the nucleus (n), the apically located acrosome (a), the mitochondrion (m) and some peripheral electron-dense bodies (arrows).