

THE SPERMATOOZON OF ARTHROPODA

XXX. The Multiflagellate Spermatozoon in the Termite

Mastotermes darwiniensis

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ABSTRACT

In this paper the spermatozoon of the termite *Mastotermes darwiniensis* is described. It is the first example of a multiflagellate sperm cell in animals. The sperm consists of a conical head and ~100 flagella. Other remarkable features of this sperm cell are the absence of an acrosome, the presence of centrioles containing doublet microtubules instead of triplets, and the presence of axonemes devoid of central tubules and with doublets bearing only one arm. The flagella are feebly motile.

KEY WORDS spermatozoon multiflagellate · axoneme doublets · termites · microtubules · centrioles

Studies in comparative spermatology carried out for many years by light and electron microscopy have demonstrated that in plants (22) the spermatozoid, when present, is provided with one or many pairs of flagella (only a few exceptional fungi have only a single axoneme), whereas in animals (3) the spermatozoon generally has a single flagellum arising from the distal centriole, and only in a few instances do two flagella arise from both centrioles. Two axonemes have been found in most platyhelminths (17), one gastrotrichian (33), some Rhynchotoid insects (7), a cecydomyid dipteran (4), several teleost and diploid families (25), and the Japanese frog *Rhacophalus arborea* (3). Only the microgamete of the protozoan *Eimeria perforans* has three axonemes (28). During our investigations on Arthropoda spermatozoa, we found that spermatozoa of the termite *Mastotermes darwiniensis* invariably have ~100 flagella. We will describe here for the first time this multiflagellate spermatozoon in animals.

MATERIALS AND METHODS

Adult males of *Mastotermes darwiniensis* termites were supplied to us alive by Prof. D. Waterhouse (Division of Entomology, Canberra, Australia). Living spermatozoa in the male body fluid were observed under the interference Nomarski microscope.

The termites were dissected and the testes and ducts were fixed in 4% paraformaldehyde and 5% glutaraldehyde in cacodylate buffer, pH 7.2. The dehydrated tissue were embedded in Epon, sectioned on an LKB III Ultratome (LKB Produkter, Bromma, Sweden), stained with uranyl acetate and lead citrate, and observed in the Philips 301 electron microscope. DNA was detected with the EDTA regressive method of Bernhard (9).

For scanning electron microscopy, a few sperm were fixed in glutaraldehyde, washed, dehydrated in ethanol and dried between silver disks at the critical point. Photographs were taken in the Jeol scanning microscope JSM 2.

RESULTS

Examined under the Nomarski interference microscope, the living, mature spermatozoon of *Mastotermes darwiniensis* appears to consist of a conical body from the base of which ~100 undu-

lating flagella arise (Fig. 1*a*). The flagella are feebly motile. Under the scanning electron microscope (Fig. 1*b*), the picture is the same but some membranous material seems to surround the base of the cell body. The cone is 9 μm long and reaches 1.5 μm in diam at the base, and the flagella are 100 μm long. In thin sections, several regions can be identified in the sperm body. The most anterior region (Fig. 2*a*) is occupied by the nucleus, which is conical in form, 7 μm long, and reaches a maximal basal diam of 1.5 μm . It can be resolved as a homogeneous osmiophilic central cone surrounded by a marginal palisade of longitudinal fibers 40 nm thick (Figs. 2*a* and 5*a*). The base of the nucleus is concave and accommodates clumps of osmiophilic granular material in which many centrioles are embedded (Fig. 2*b*). In a cross section of a sperm (Fig. 2*c*) there appear to be ~ 100 centrioles, and the wall of the centriole is made up of doublet instead of the usual triplet microtubules. The diameters of the centrioles and microtubules are similar to those of these structures in other cells, and the center of each is occupied by an osmiophilic core. The centrioles are unusually long, 2.5 μm , and are perfectly parallel and hexagonally arranged (Fig. 2*b*). They occupy the posterior segment of the body (Fig. 2*b*). In this region, some vesicles project outward from the cell surface (Fig. 2*a* and *b*). A long axoneme arises from the distal end of each cen-

triole and extends caudally from the plasma membrane at the base of the spermatozoon (Fig. 2*a* and *b*). Each axoneme is completely enclosed in an extension of the cell membrane and forms an individual flagellum. The flagella number ~ 100 (from 95 to 110 by our count); the peripheral flagella are entirely independent of one another from their beginnings (Fig. 2*d* and *e*), whereas those more centrally situated are enclosed in a common membrane for a short distance (Fig. 2*e* and *f*). In cross section, each axoneme shows a 9 + 0 pattern and is made up of only peripheral doublet microtubules (Fig. 2*d-f*). The central, single microtubules are consistently lacking, but, not infrequently, in the caudal region of the axonemes one of the doublets is displaced inward and occupies the axis of the axoneme (Fig. 2*g*). Only the outer arm is present on the doublets in cross sections of the axoneme (Fig. 2*d-g*). No other structures, such as accessory tubules, spokes, nexin links, or projections, have been detected. This spermatozoon is therefore multiflagellate and devoid of an acrosome (Fig. 3).

Study of spermatogenesis provides additional details as to the origin of the flagella. The early spermatids (Fig. 4*a*) are conventional cells with many small mitochondria, a large Golgi complex, and a pair of typical centrioles. Numerous large vesicles with an osmiophilic content arise in the Golgi complex (Fig. 4*b*) and accumulate around the nucleus. Later in spermiogenesis, the vesicles become concentrated near the apex of the cell body and finally are eliminated in the cytoplasmic droplets without giving rise to an acrosome. The centrioles have the conventional orientation perpendicular to one another, but their wall is made up of doublet microtubules instead of triplets (Fig. 4*e*). The spheroidal nucleus is $\sim 5.6 \mu\text{m}$ in diam, and is relatively poor in chromatin content as revealed by Bernhard's method (9). It is certainly not polyploid, and DNA does not seem to have been eliminated from it. As spermiogenesis proceeds, the number of centrioles increases many times (Fig. 4*d*). This process of proliferation takes place in the region of the cell opposite that occupied by the Golgi vesicles, and involves the loss of the binary arrangement of the centrioles (Fig. 4*c* and *d*), the appearance of new centrioles, and elongation (Fig. 4*c*) of the old centrioles which ultimately reach a length of 2.5 μm . Both procentrioles and stages of centriolar assembly (Fig. 4*g*) can be found (Fig. 4*f*). In the advanced spermatid the anterior part of the cell is filled

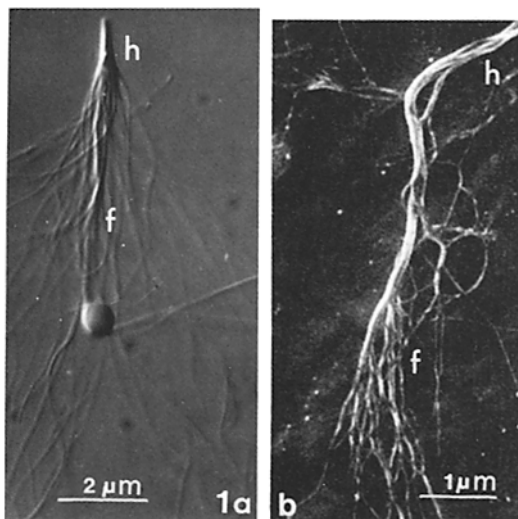


FIGURE 1 The mature spermatozoon of *Mastotermes darwiniensis* as seen in the Nomarski microscope and in the scanning microscope. Many flagella (*f*) arise from the head (*h*). (*a*) $\times 600$; (*b*) $\times 1,000$.

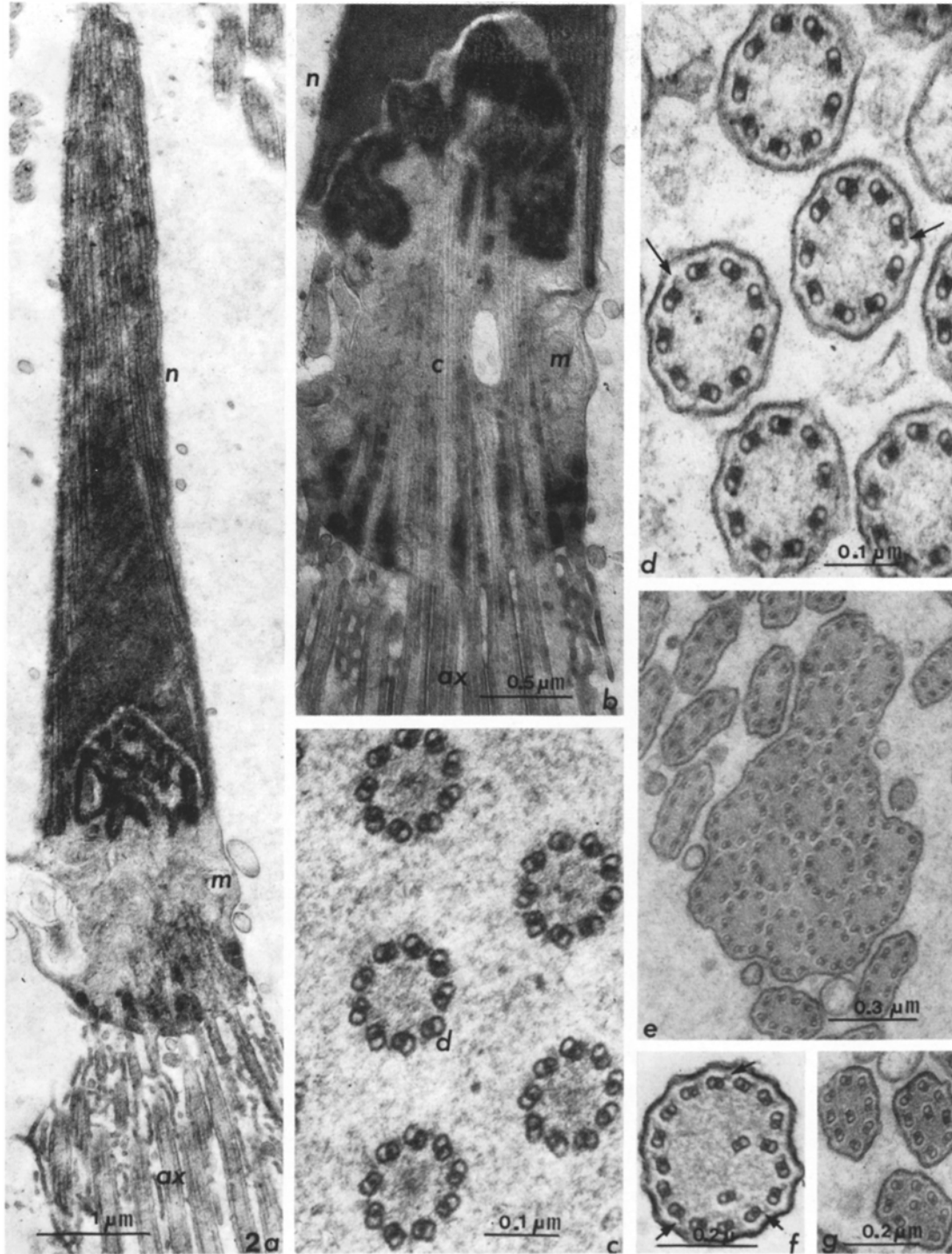
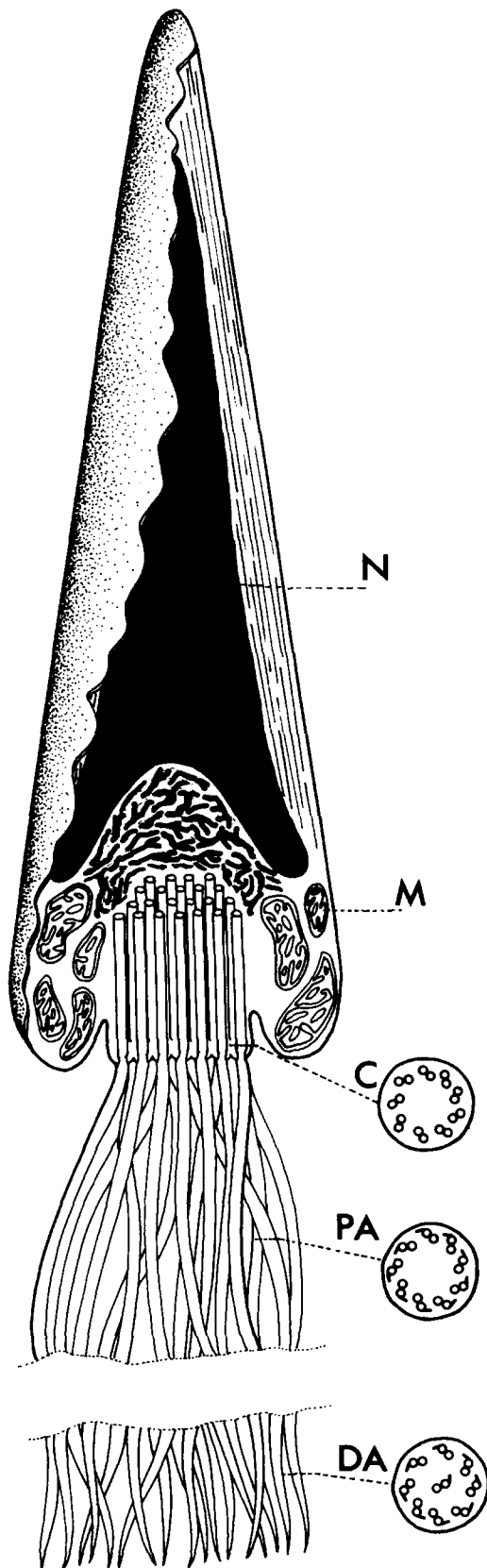


FIGURE 2 Sections of the mature spermatozoon of *Mastotermes darwiniensis*. In (a) the anterior region, longitudinally sectioned can be seen ($\times 17,000$). In (b), the same section of the centriole region, at higher magnification ($\times 27,500$). In (c), cross sections of centrioles showing the doublets ($\times 112,000$); in (d), of proximal axonemes ($\times 112,000$); in (e), of many proximal axonemes, some of which are in a common membrane ($\times 45,000$); in (f), of two fused axonemes ($\times 75,000$); in (g), of three distal axonemes, showing a displaced doublet ($\times 57,000$). All doublets bear only one arm (arrows). ax, axoneme; c, centriole; d, doublet; m, mitochondrion; n, nucleus.



with Golgi vesicles, the nucleus is surrounded by a thick layer of longitudinally disposed microtubules, and the chromatin is concentrated at the periphery (Fig. 5a). The centrioles become oriented parallel to one another (Fig. 5b-d) and migrate toward the posterior pole of the nucleus, where they come to occupy a shallow concavity (Fig. 5b). The elongating axonemes start to project caudally surrounded by individual membranes (Fig. 5e), and the connections between them are gradually lost, giving way to vesicular remnants (Fig. 5f). The morphogenesis of the spermatozoon is completed by elimination of excess cytoplasm containing the apical Golgi vesicles, the perinuclear microtubules, the excess periaxonemal cytoplasm, and the vesiculated interaxonemal membranes.

DISCUSSION

The main peculiarities of the *Mastotermes* spermatozoon are the absence of an acrosome, the presence of numerous flagella, "9 + 0" axonemes, the absence of inner arms on the doublets, and the presence of centrioles whose walls are formed by doublet microtubules instead of the usual triplets. Each of these features deserves some comment.

Absence of the acrosome is an involution not uncommon among the higher animals, and it is usually associated with the occurrence of a micropyle traversing the egg chorion (3). It occurs quite frequently in insects (2). The other known termites, *Reticulitermes* and *Calotermes*, also lack an acrosome (8). *Mastotermes* is somewhat more primitive than those two genera, and the acrosome has probably been lost in their ancestral form. In fact, Embioptera, a closely related order, has a monolayered acrosomal complex (8), while the other classical orthopteroid insects even have a trilaminar acrosome (2).

The multiflagellarity of the sperm is by far the most unusual peculiarity of *Mastotermes*, and it is not shared with any other animal species thus far described. We were puzzled as to how it came to occur. An initial speculation was to interpret the *Mastotermes* sperm as a syncytium and to consider the multiple flagella as the tails of individual

FIGURE 3 Scheme of the mature spermatozoon of *Mastotermes darwiniensis*: C, centriole; DA, distal axoneme; M, mitochondria; N, nucleus; PA, proximal axoneme.

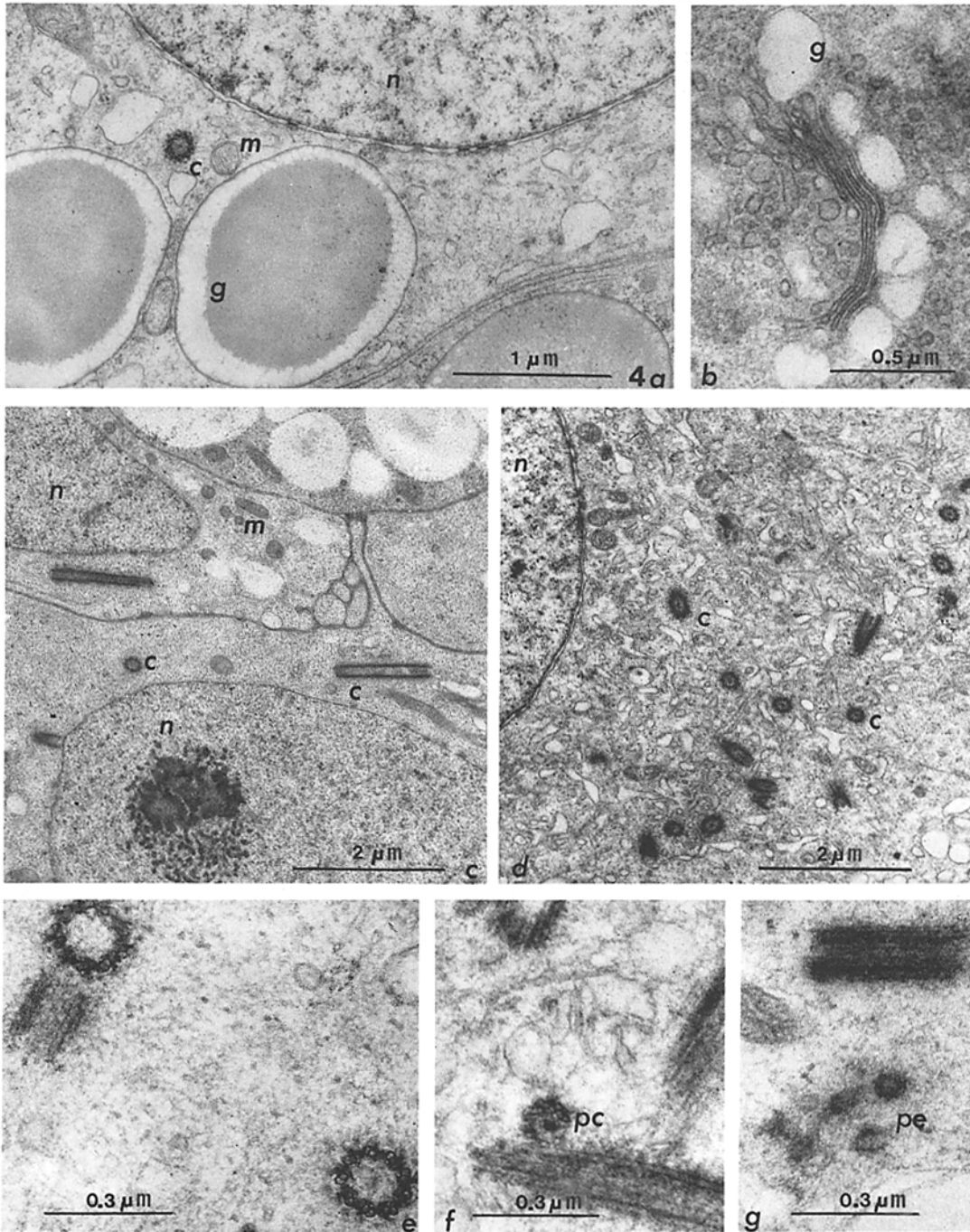


FIGURE 4 The early spermatid of *Mastotermes darwiniensis*. In (a) a single centriole, a few mitochondria, and many Golgi-derived vesicles can be seen ($\times 23,000$); in (b), the activity of the Golgi complex ($\times 37,500$); in (c), elongating centrioles ($\times 11,000$); in (d), multiplied centrioles, without binary structure ($\times 11,000$); in (e), binary centrioles, made up of doublets and not yet elongated ($\times 62,500$); in (f), a pro-centriole ($\times 62,500$); in (g), many proliferative elements ($\times 62,500$). c, centriole; g, Golgi vesicles; m, mitochondrion; n, nucleus; pc, pro-centrioles; pe, proliferative elements.

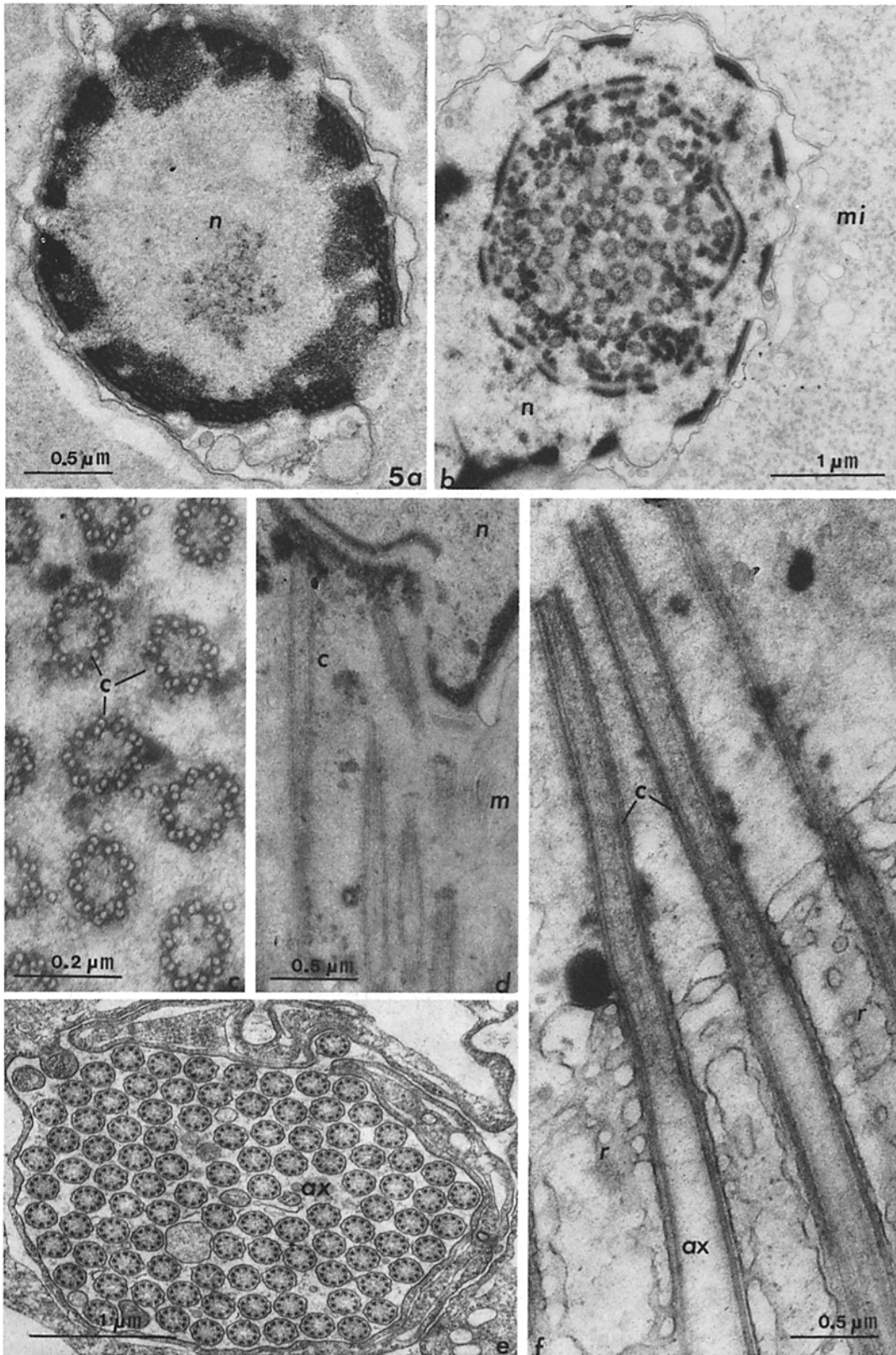


FIGURE 5 The aged spermatid of *Mastotermes darwiniensis*. (a) The nucleus with peripherally condensed chromatin and fibrous material ($\times 27,500$). (b) Cross-sectioned centrioles in the nuclear concavity ($\times 95,000$). (c) Centrioles at higher magnification ($\times 18,000$). (d) Longitudinally sectioned centrioles and mitochondria among them ($\times 27,500$). (e) Cross-sectioned flagella ($\times 23,000$). (f) The origin of the axonemes ($\times 27,500$). ax, axoneme; c, centriole; m, mitochondria; mi, microtubules; n, nucleus; r, remnants of plasma membrane.

spermatozoa whose cell bodies and nuclei had fused. Syncytial, but not synkaryal, spermatids are, in fact, of widespread occurrence in insect spermiogenesis (27). But, in fact, this interpretation is not tenable because the nucleus is not polyploid, and there is no evidence for elimination of DNA during spermiogenesis. Moreover, the initial number of spermatid centrioles is only two. The second hypothesis to be considered was that centriole multiplication occurs in the early spermatid, as is reported for plants (14). This hypothesis appears to be correct, because we found in the young spermatids all the classical stages of *de novo* centriole formation, the proliferative elements, and procentrioles, as demonstrated in the chick trachea by Kalnins and Porter (21) and Kalnins et al. (20), and in the mouse oviduct by Dirksen (13), and summarized by Fawcett (15). All the newly formed centrioles elongate and give rise to an axoneme. Elongation at the distal end of the centriole in spermatids before axoneme formation has recently been demonstrated by Maretta (24). All *Mastotermes* centrioles form axonemes; therefore, their elongation is a general feature, and no "proximal" centrioles are retained. Morphogenetic activation of the proximal centriole is the only way by which an animal sperm gains a second flagellum, and it is present in all of the few instances of sperm biflagellarity mentioned in the introduction. But the proximal centriole is rarely entirely inactive during spermiogenesis, as evidenced by the formation of a centriolar adjunct in insects (10), pognophorans (16), and mammals (15). Therefore, no major evolutionary transformations were needed to acquire multiflagellarity in termite sperm; the centrioles simply replicate and all of them form axonemes.

The 9 + 0 pattern has been found many times in animal spermatozoa: in the turbellarian *Childia* (11), the annelid *Myzostomum* (1), several Acanthocephala (23), Pycnogonida (31, 32), and vejevid scorpions (18, 19) among lower Arthropoda; *Plecia* (30) and several cecydomyids (4) among Diptera; and Elopomorpha (25) among fishes. Moreover, among insects, Ephemeroptera have a 9 + 9 + 0 pattern (6, 26). In most of these cases the doublets bear only a single arm, as we have observed in cecydomyids (4), but this is also true in the published pictures of Acanthocephala, Pycnogonida, Elopomorpha, and in the presently described termite. In addition, the 9 + 0 pattern of some Cecydomyids and the 12 + 0

or 14 + 0 pattern of Protura (5) are entirely devoid of arms, and therefore the sperm is immotile. The concomitant occurrence of unusual axoneme patterns and of the lack of dynein arms is not surprising. In these animals at this evolutionary stage, sperm motility may have lost its importance in reproductive biology owing to the development of other devices such as elongate copulatory organs, spermatophores, etc. In fact, some groups closely related to those mentioned above have completely lost the axoneme. The best known examples are Protura (5) and Pycnogonida (32). The termites also exemplify this pathway of evolution.

The final characteristic worthy of emphasis is that the centriole in both spermatids and spermatozoa is made up of doublet microtubules instead of triplets. This has previously been observed only in the sperm of Acanthocephala, whose doublets also have only one arm (23), and in the sperm of Isopoda in which a flagellum is entirely lacking (12). It is tempting to relate this mutation to the incomplete development of the dynein arm. Observation of the centriolar structure in other arm-deficient spermatozoa would be instructive in this regard.

In conclusion, the finding of multiflagellate sperm in animals is certainly novel and unexpected, but to have found it in termites and associated with an axoneme of unusual pattern makes it more credible. The most primitive termites, such as *Termopsis*, apparently have conventional, motile uniflagellate sperm (29); the most evolved termites, *Calotermes* and *Reticulitermes*, have peculiar nonmotile aflagellate sperm (8). *Mastotermes*, which is phylogenetically intermediate, has the unusual form of sperm described here. Thus, we observe, once more, that unusual axonemal mutations can survive when the evolutionary pathway is towards sperm immotility.

This work was supported by the Consiglio Nazionale delle Ricerche Project "Biology of Reproduction".

Received for publication 6 July 1977, and in revised form 24 October 1977.

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