

Fine Structure of Sperm of *Ekphymatodera thomasoni* (Heteroderinae, Nemata)¹

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Abstract: Fine structure of developing sperm of the monospecific genus, *Ekphymatodera*, was compared with other Heteroderinae as part of a study to recognize diversity and phylogenetically informative characters within the subfamily. Sperm of *Ekphymatodera* originate from germ cells connected to a central rachis, a character which is shared with *Globodera*, but not with other Heteroderinae. In *Ekphymatodera*, and cyst-forming genera, a layer of cortical microtubules lies just beneath the surface of the plasma membrane. Sperm of *Ekphymatodera* are unique among Heteroderinae examined by the presence of spiral surface elevations on the filopodia, a character that may prove to be a synapomorphy for Sarisoderini. Fibrous bodies are abundant in spermatids; however, they do not persist in sperm of *Ekphymatodera* as they do in *Meloidodera* and *Verutus*. The male gonad of *Ekphymatodera* is lined by epithelial cells, which are greatly enlarged near the ejaculatory canal. These enlarged cells contain vesicles with concentric lamellar inclusions, not observed in other genera of the subfamily. Sperm of Heteroderinae are rich in diversity, and examination of additional representative species may indicate new phylogenetically informative characters.

Key words: *Ekphymatodera thomasoni*, filopodia, fine structure, Heteroderinae, lamellar inclusion, male gonad, nematode, ontogeny, phylogeny, pseudopodia, sperm, systematics, ultrastructure.

Sperm are morphologically and physiologically diverse, even where there are no other phenotypic variations among closely related species. Baccetti and Afzelius (1) suggest that speciation frequently begins as barriers to cross fertilization, and these changes are often first seen in sperm. Sperm diversity, however, has not been explored as a source of characters for nematodes, nor specifically for testing and refining controversial relationships indicated in a hypothetical phylogenetic tree for Heteroderinae Filip'ev and Schuurmans Stekhoven, 1941, sensu Luc et al., 1988 (2,4).

The cyst-forming genera, *Heterodera* and *Globodera*, are considered to have the most derived set of character states among Heteroderinae. Shepherd et al. (19) and Walsh and Shepherd (23) detected differences in sperm fine structure between these genera. Recently, we advanced Shepherd's

work to consider fine structure of sperm in *Verutus* and *Meloidodera*, putatively the Heteroderinae with the most primitive set of character states (7). As fine structure is extended throughout Heteroderinae and outgroups, the range of character states of sperm will provide a new tool for testing relationships within the subfamily.

Ekphymatodera Baldwin et al., 1989, like other Sarisoderini Husain, 1976, sensu Baldwin and Schouest, 1990, has submedial lips fused with the labial disc in second-stage juveniles and males, wide vulva-anus distance, females with a thick cuticle and lack of a cyst stage (4,12,25). We propose to investigate fine structure of sperm of *Ekphymatodera* as an intermediate representative between the "primitive" non-cyst genera, *Verutus* and *Meloidodera* (7), and the "derived" cyst genera of Heteroderinae, including *Heterodera*, *Globodera*, and *Punctodera* (6).

Because sperm morphology changes rapidly with development, comparisons among taxa must consider equivalent stages throughout the range of development (7). Terminology for designation of germ cell developmental stages of spermatogonium, primary and secondary spermatocytes, spermatid and spermatozoon, follows Shepherd (16).

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MATERIALS AND METHODS

Specimens of *Ekphymatodera thomasoni* Baldwin et al., 1989, the only species of the genus, were originally collected from Yosemite National Park, California, and maintained in greenhouse cultures on the type host, *Juncus effusus* L. var. *pacificus*. Collection of fourth-stage juveniles (J4) with developing testis and males from soil, and of females from the roots, was as described in Cares and Baldwin (7).

For scanning electron microscopy (SEM), sperm were collected from males in sperm media on a poly-L-lysine precoated coverglass. Fixation with 4% glutaraldehyde, dehydration with ethanol, infiltration with freon, air drying, and mounting on stubs for SEM were as previously described (7). The specimens were observed and photographed in a Jeol 35C scanning electron microscope at 15 kV.

For transmission electron microscopy (TEM), sperm from juveniles with developing testis and males as well as from inseminated females were studied. Fixation was with the fast technique modified from Hobbs (11), as previously described (7). Intact bodies of juveniles, males, and females were fixed for 1 hour in 39% O_5O_4 in CCl_4 , dehydrated in an ethanol series, and embedded in the "hard" formulation of epoxy (20). A Sorvall MT6000 ultramicrotome was used to obtain nearly serial sections. Thin sections were mounted and stained in 1% aqueous uranyl acetate and in lead citrate (15). A H-600 Hitachi electron microscope at 75 kV was used for observation and photography.

RESULTS

Although *E. thomasoni* shares several characters of the testis and sperm with other Heteroderinae, some striking features, including certain enlarged epithelial cells with concentric lamellar inclusions and sperm filopodia with spiral surface elevations, are unique to *Ekphymatodera*.

The cylindrical telogonic testis of *E. tho-*

masoni males extends slightly posterior to midbody and opens into the cloaca near the posterior end of the nematode. The wall of the gonad consists of a single layer of epithelial cells enclosed within a basal lamina (Figs. 1B–D; 2A,C; 3C). Bundles of intestinal fibers are adjacent to the gonad (Figs. 1B–D; 2A; 3C). The anterior tip of the testis is characterized by a thin epithelium (Figs. 1B; 2A; 3A,B), but posteriorly the epithelium thickens gradually with maximum thickness occurring in a few cells surrounding the ejaculatory canal (Fig. 1D).

As the gonad epithelium thickens, there is an increase in numbers of large electron-lucent membrane-bound structures (vesicles) within the epithelial cells (Figs. 1C,D; 2B,C; 3C). These structures vary in size and shape and typically contain one or two spherical electron-dense granules (Figs. 1C,D; 2B,C). Nearly serial cross sections from slightly anterior to the ejaculatory canal toward the cloaca show that these spherical electron-dense granules posteriorly become enlarged and surrounded by concentric electron-dense lamellae (Figs. 1D; 2C–F). Further posteriorly, additional whorls encircle the electron-dense granules, and in some cases the complex lamellar inclusions occupy most of the volume of the vesicles (Fig. 2C). Cases of apparent deterioration of the lamellar bodies were observed.

Spermatocytes were present only in the anterior end of the testis of J4 and young males; in older males only spermatids and spermatozoa were observed. Spermatocyte organization in males suggests connection of germ cells to a central rachis (Figs. 1B; 2A; 3A). The spermatocytes are round cells about 4 μm in diameter (Figs. 1B; 2A; 3A), with rare pseudopodia (robust cytoplasmic projections with a blunt end), and filopodia (long slender straw-like cytoplasmic projections). Ribosomes are abundant in the granular cytoplasm. Contrary to primary spermatocytes, in secondary spermatocytes, a nuclear envelope was no longer present, and a reduced nucleolus was observed in a few cases. One to four clumps

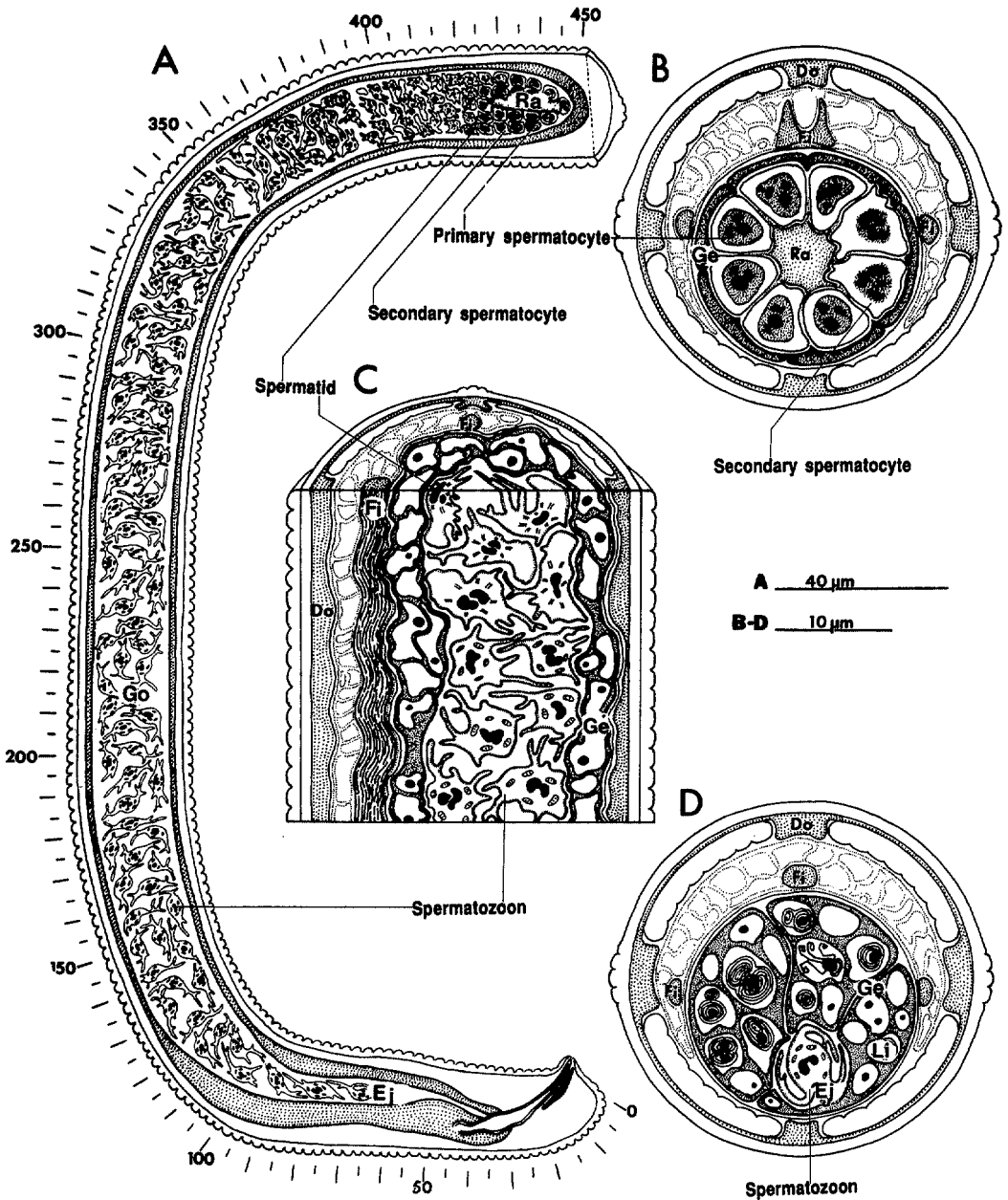


FIG. 1. Diagrammatic representation of male reproductive system of *Ekphymatodera thomasoni*. A) Gonad (Go) of an adult. Scale units indicate the distance in micrometers (μm) from the posterior extremity of the nematode. B) Cross section of the gonad of an adult including the rachis (level 425, Fig. 1A). C) Three-dimensional view from a combination of cross and tangential sections of the gonad of an adult (level 370–410, Fig. 1A). D) Cross section of the gonad of an adult including the ejaculatory canal (Ej) (level 55, Fig. 1A). Do = dorsal chord, Ej = ejaculatory canal, Fi = bundle of intestinal fibers, Ge = gonad epithelium, Li = lamellar inclusion, and Ra = rachis.

of chromatin were observed in the central region of the cell (Figs. 1B; 2A; 3A).

Young spermatids are round to ovoid, about 3–4 μm in diameter. The spermatid

plasma membrane, including that of the filopodia, is internally lined by a single layer of cortical microtubules. The cytoplasm is full of conspicuous fibrous bodies

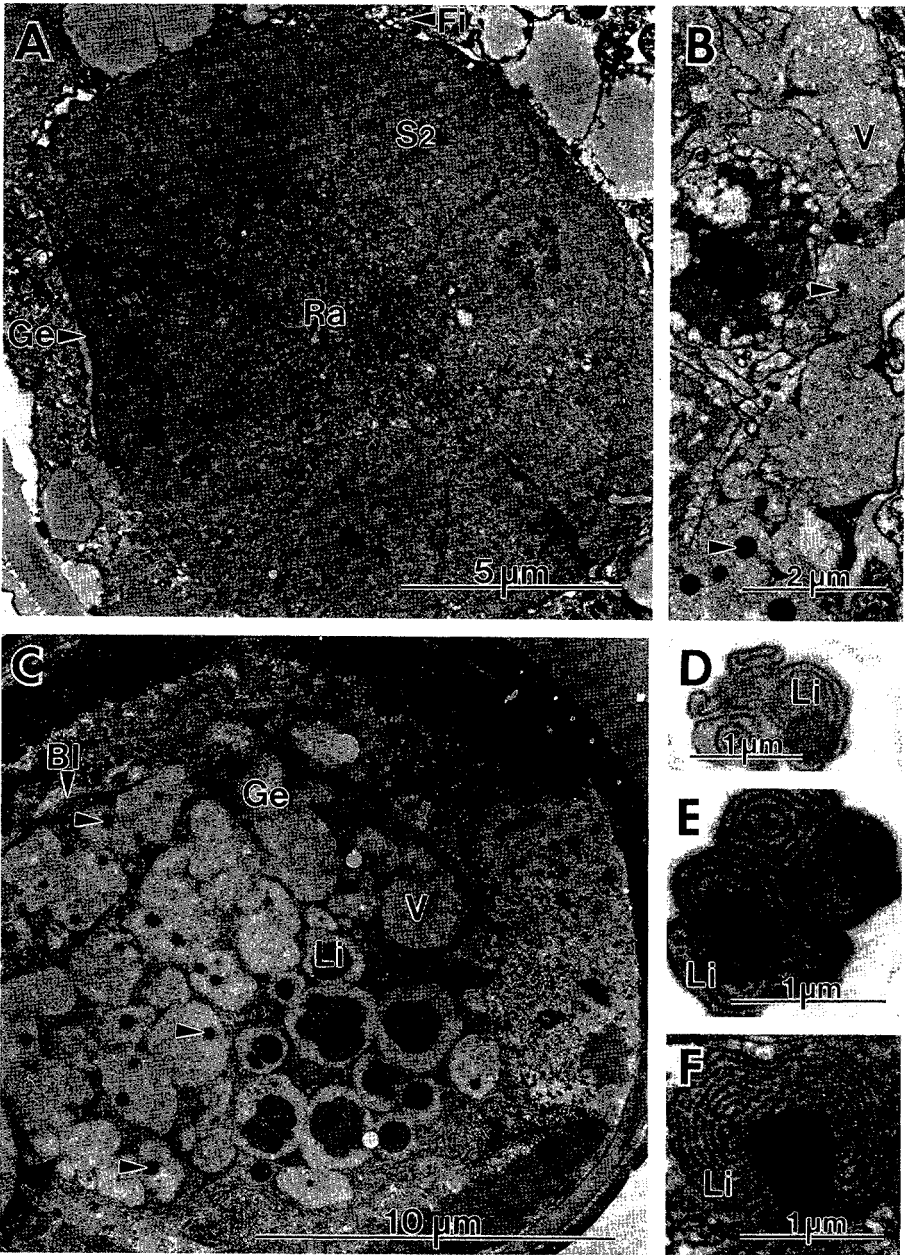


FIG. 2. Cross sections of the gonad of males of *Ekphymatodera thomasoni* viewed with TEM. A) Secondary spermatocytes surrounding the remains of a rachis (Ra), all enclosed by a thin gonad epithelium (level 425, Fig. 1A). B) Portion of the gonad epithelium including electron-lucent vesicles, some of them enclosing electron dense granules (arrows) (level 300, Fig. 1A). C) Enlarged cells of the *vas deferens* epithelium completely occluding the ejaculatory canal; dense granules (arrows) or lamellar inclusions are enclosed by electron-lucent vesicles in the epithelial cells (level 50, Fig. 1A). D) Lamellar inclusion apparently in the initial steps of lamellae arrangement around the central electron-dense core. E) Lamellar inclusion with a complex organization of the concentric lamellae. F) Lamellar inclusion with beaded concentric lamellae sectioned from a different plane from Fig. 2D,E. Bl = basal lamina, Fi = bundle of intestinal fibers, Ge = gonad epithelium, Li = lamellar inclusion, S2 = secondary spermatocyte, and V = electron-lucent vesicle.

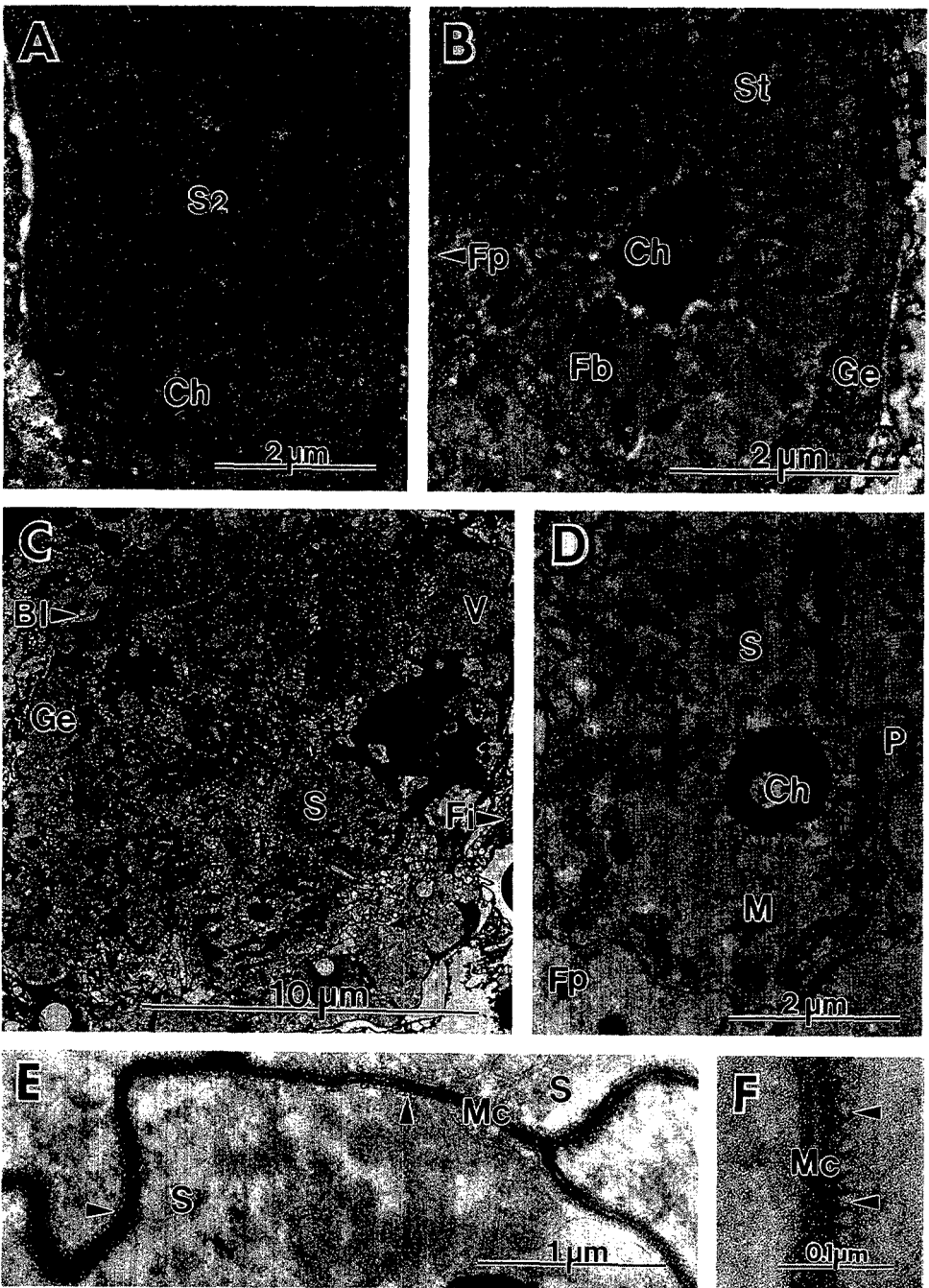


FIG. 3. Cross sections of the gonad of males (A–D) and females (E, F) of *Ekphymatodera thomasoni* viewed with TEM. A) Secondary spermatocyte with condensed chromatin enlarged from Fig. 2A. B) Young spermatid enclosing many fibrous bodies (level 420, Fig. 1A). C) Several spermatozoa enclosed by the gonad epithelium (level 300, Fig. 1A). D) Spermatozoon in the testis enclosing several mitochondria (level 250, Fig. 1A). E) Portions of inseminated sperm in the female spermatheca with cortical microtubules (arrows) lining the inner surfaces of the plasma membrane complex between adjacent sperm. F) Membrane complex, including cortical microtubules (arrows), enlarged from Fig. 3E. Bl = basal lamina, Ch = chromatin, Fb = fibrous body, Fi = bundle of intestinal fibers, Fp = filopodium, Ge = gonad epithelium, M = mitochondrion, Mc = membrane complex, P = pseudopodium, S = spermatozoon, St = spermatid, and S2 = secondary spermatocyte.

radially distributed around the compact chromatin (Fig. 3B). Numbers of filopodia increase as spermatids mature.

Sperm remain loosely packed in the testis (Fig. 3C). Mature testicular sperm are diverse in shape, varying from round to elongate, about $4 \times 6\text{-}\mu\text{m-d}$. About seven filopodia occur, and they are unevenly dispersed on one side of the body (Fig. 5A). The filopodia may be branched and have a unique ringed or spiral-like surface elevation (Fig. 5B). Pseudopodia are also present (Fig. 3D). Microtubules not only underlie the plasma membrane, but are also dispersed throughout the sperm cytoplasm. However, the fibrous bodies, abundant in the spermatids, are absent in stored sperm of the testis. Organelles, including mitochondria, are evenly distributed throughout the sperm cell, excluding the filopodia (Fig. 3D). The morphology of the compact chromatin does not change from the spermatid to the spermatozoa (Fig. 3B,D).

Inseminated spermatozoa are compactly stored within the female spermatheca, where pseudopodial extensions of one sperm interdigitate with those of neighboring sperm (Figs. 3E; 4A,B). The close sperm-to-sperm attachment results in the formation of a thick electron-dense membrane complex (Figs. 3E,F; 4C). Apparently, inseminated sperm also establish attachment with the spermatheca epithelium, sometimes doing so with a long robust pseudopodium that interdigitates with the surface of the epithelium (Fig. 4B). Although only a few filopodia remain after insemination, cortical and cytoplasmic microtubules persist (Figs. 3E,F; 4B,C). Cytoplasmic microtubules are sometimes closely associated with the external surface of mitochondria. Mitochondria remain abundant, and the chromatin appears to be unchanged in inseminated spermatozoa (Fig. 4B).

DISCUSSION

Sperm of *Ekphymatodera* are morphologically distinct from those of other recently investigated Heteroderinae, *Verutus*, and

Meloidodera (7). While fewer in number, the filopodia in sperm of *Ekphymatodera* have spiral surface elevations, and cortical microtubules are present under the surface of the plasma membrane. In addition, the male gonad of *Ekphymatodera* has a testicular rachis, and the posterior portion of the testis has distinctive, enlarged epithelial cells enclosing vesicles with unusual lamellar inclusions.

The epithelium of the male reproductive tract of nematodes has long been recognized as secretory, and it also has been shown to actively engulf residual bodies and defective sperm (8–10,17,19,22). In males of *Ekphymatodera*, just posterior to the cap cell, the epithelium is thin and uniform with no apparent secretory function. We have shown, however, that at the posterior end of the testis, the epithelium thickens and becomes irregular in shape. These enlarged epithelial cells are filled with robust vesicles that could be interpreted as reservoirs for secretory products, including spherical, lipid-like electron-dense granules. Similar vesicles were observed in the male gonad of *Verutus* and *Meloidodera* (7).

Near the ejaculatory canal, the enlarged epithelial cells of *Ekphymatodera* possess vesicles with complex lamellar inclusions, which may be analogous to the crystalline granules enclosed in vesicles of enlarged epithelial cells in the *vas deferens* of *Aphelenchoides blastophthorus* (17). There have been no attempts to determine the nature and function of these inclusions. However, based on their position and comparison with other nematodes (8,10,22), these inclusions could influence sperm transfer and (or) maturation in the female genital tract.

The intestinal bundles of fibers frequently observed adjacent to the male gonad of *Ekphymatodera* are probably intestinal fasciculi, although there is no explanation as to why they are so consistently aligned adjacent to the testis. Similar fasciculi have been reported in other Tylenchida and are not limited to males (3,5).

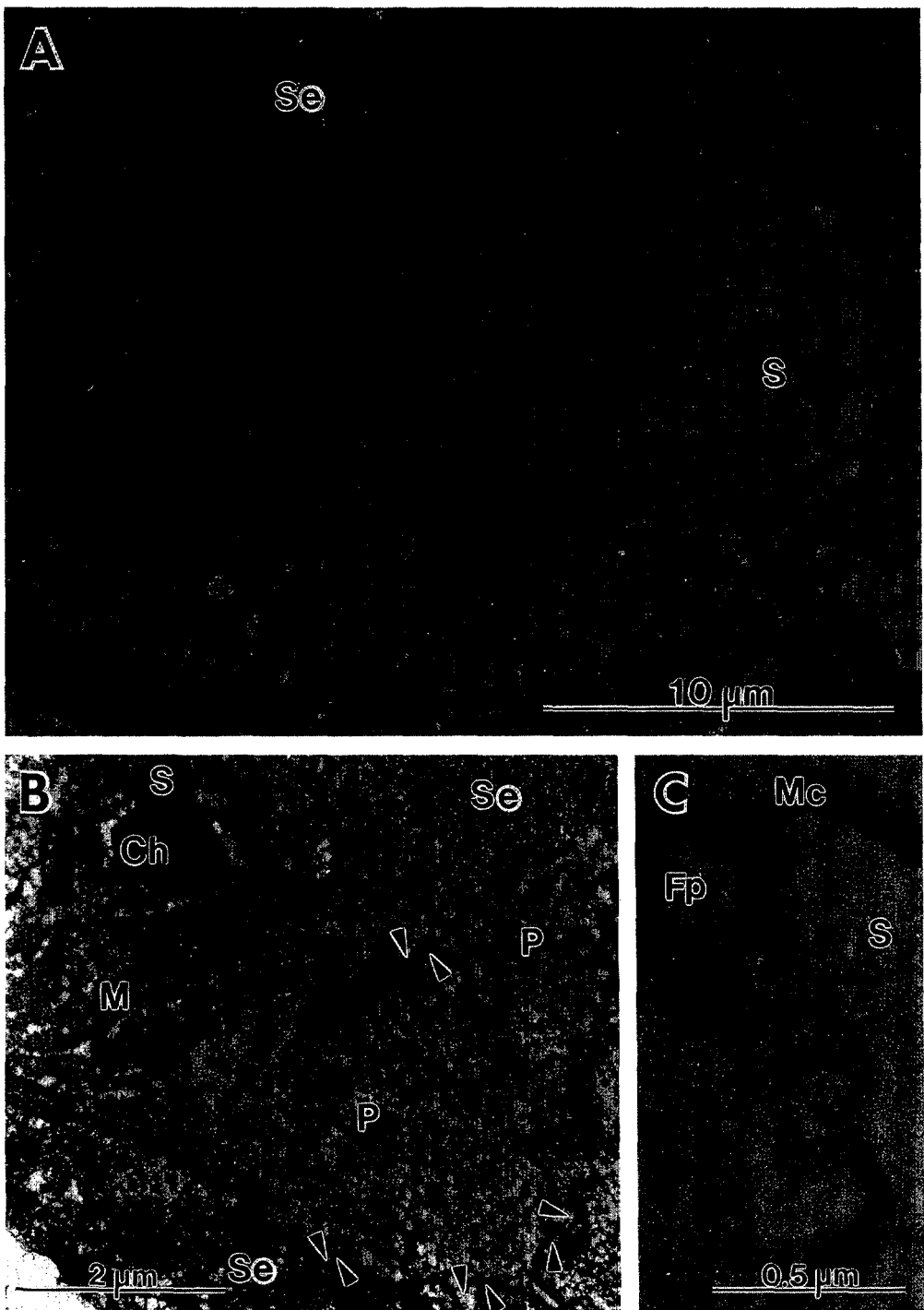


FIG. 4. Sections of the spermatheca of inseminated females of *Ekphymatodera thomasoni* viewed with TEM. A) Inseminated sperm compactly stored in the spermatheca. B) Inseminated sperm with a robust pseudopodium interdigitating (arrows) with the pseudopodium of an adjacent sperm and with the spermatheca epithelium. C) Section of filopodia and portion of the membrane complex between adjacent inseminated sperm. Ch = chromatin, Fp = filopodium, M = mitochondrion, Mc = membrane complex, P = pseudopodium, S = sperm, and Se = spermatheca epithelium.

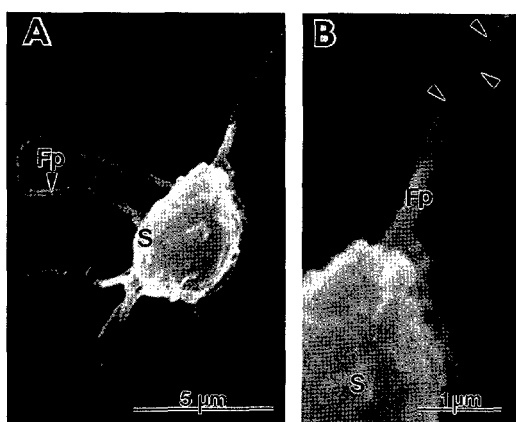


FIG. 5. SEM micrographs of mature testicular sperm (S) of *Ekphymatodera thomasoni*. A) Sperm with few filopodia (Fp) unevenly dispersed on only one side of the cell body. B) Portion of a sperm enlarged from Fig. 5A with part of one filopodium with surface spiral elevation (arrows).

Unlike the spermatocytes of other Heteroderinae (7,19), spermatocytes of *Ekphymatodera* do not persist in mature males; however, a few spermatocytes may briefly remain in the anterior end of the testis of very young males of *Ekphymatodera*. Germ cells in *Ekphymatodera* develop in connection to a central mass of anucleate cytoplasm, the rachis. This character is shared with males of *Globodera* (18) but differs from some other Heteroderinae (7,18). Because a male rachis is widespread throughout the Nemata (10,13,14,18,21, 24), its phylogenetic significance in Heteroderinae may be limited as either plesiomorphic or highly convergent.

Ekphymatodera is placed in Sarisoderini, a noncyst-forming tribe of Heteroderinae that has a number of characters intermediate between the putatively more ancestral Verutini + Meloidoderini and the more derived character sets shared by the cyst-forming tribe Heteroderini (4). Sperm of *Ekphymatodera* are morphologically divergent from sperm of both *Verutus* and *Meloidodera* (7). The number of filopodia extending from testicular sperm of *Ekphymatodera* average about 7, in contrast with more than 20 in *Verutus* and *Meloidodera*. The few filopodia and their uneven distribution are characters shared only with the

cyst-forming *Punctodera* (6). The filopodia of *Ekphymatodera* seem to be unique among nematodes examined, by the presence of a ringed or spiral surface elevation.

Sperm cortical microtubules are present in *Ekphymatodera*, and this is the only morphological trait shared between sperm of *Ekphymatodera* and all members of the cyst-forming group observed so far, including *Heterodera*, *Globodera*, and *Punctodera* (6,19). Conversely, this trait is not shared with noncyst-forming Heteroderinae (7). Although cortical microtubules are apparently absent in *Verutus* and *Meloidodera*, caution must be taken in using the distribution of this character for interpreting phylogeny of Heteroderinae, since cortical microtubules have been reported in members of unrelated outgroups throughout Nemata (7).

Fibrous bodies are abundant in spermatids of *Ekphymatodera*; unlike in *Verutus* and *Meloidodera*, they do not persist after the spermatid matures into a sperm. The ephemeral persistence of the fibrous bodies during spermatogenesis of *Ekphymatodera* is a character shared with the cyst-forming *Globodera* (19), but not with *Heterodera* (19) or with the other two noncyst-forming genera (7). Conversely, sperm polarization after insemination is shared with *Verutus* (7) and *Heterodera*, but not with *Globodera* (19). The unchanged state of chromatin condensation during spermatogenesis in *Ekphymatodera* is shared with *Verutus* (7) and *Heterodera* (19), but not with *Meloidodera* (7) or *Globodera* (19). At present, it is not possible to draw clear conclusions on the phylogenetic relationships of *Ekphymatodera* with other Heteroderinae, considering the pattern of shared morphological characters of sperm. Preliminary observations suggest, however, that sperm of *Ekphymatodera* resemble more nearly those of cyst-forming than other noncyst-forming Heteroderinae with respect to numbers of filopodia, presence of cortical microtubules, and short duration of fibrous bodies.

This investigation, combined with complementary studies on sperm of other gen-

era of Heteroderinae (6,7), testifies to the richness of intergeneric variability of sperm throughout the group. With further understanding, these sperm characters will help to clarify phylogenetic relationships, even in cases where there are few other characters. It will be particularly fruitful to explore morphological characters of sperm in members of the cyst-forming as well as other noncyst-forming Heteroderinae, including Ataloderini, Cryphoderini, and additional Sarisoderini.

LITERATURE CITED

1. Baccetti, B., and B. A. Afzelius. 1976. Sperm structure in relation to phylogeny. Pp. 5-11 in B. Baccetti and B. A. Afzelius, eds. The biology of the sperm cell. Monographs in developmental biology. Basel and New York: Karger.
2. Baldwin, J. G. 1992. Evolution of cyst and noncyst-forming Heteroderinae. Annual Review of Phytopathology 30:271-290.
3. Baldwin, J. G., E. C. Bernard, and M. Mundo-Ocampo. 1989. Four new species of Heteroderidae including *Ekphymatodera* n. gen. from California. Journal of Nematology 21:48-68.
4. Baldwin, J. G., and L. P. Schouest, Jr. 1990. Comparative detailed morphology of the Heteroderinae Filip'ev and Schuurmans Stekhoven, 1941, sensu Luc et al. (1988): Phylogenetic systematics and revised classification. Systematic Parasitology 15:81-106.
5. Byers, J. R., and R. V. Anderson. 1973. Morphology and ultrastructure of the intestine in a plant-parasitic nematode, *Tylenchorhynchus dubius*. Journal of Nematology 5:28-37.
6. Cares, J. E. 1994. Comparative fine structure of sperm of Heteroderinae (Nematoda) and their potential as phylogenetic characters. Ph.D. dissertation, University of California, Riverside.
7. Cares, J. E., and J. G. Baldwin. 1994. Comparative fine structure of sperm in *Verutus volvingensis* and *Meloidodera floridensis* (Heteroderinae, Nematoda). Canadian Journal of Zoology (in press).
8. Foor, W. E., and J. T. McMahon. 1973. Role of the glandular vas deferens in the development of *Ascaris* spermatozoa. Journal of Parasitology 59:753-758.
9. Hess, R., and G. O. Poinar, Jr. 1986. Ultrastructure of the genital ducts and sperm behavior in the insect parasitic nematode, *Heterorhabditis bacteriophora* Poinar (Heterorhabditidae: Rhabditida). Revue de Nématologie 9:141-152.
10. Hess, R., and G. O. Poinar, Jr. 1989. Sperm development in the nematode *Neoaplectana intermedia* (Steinernematidae: Rhabditida). Journal of Submicroscopic Cytology and Pathology 21:543-555.
11. Hobbs, M. J. 1969. Fixation of microscopic fresh-water green algae by 31% O_3O_4 in CCl_4 in an unbuffered, two-phase fixative system. Stain Technology 44:217-221.
12. Luc, M., B. Weischer, A. R. Stone, and J. G. Baldwin. 1986. On the definition of heteroderid cysts. Revue de Nématologie 9:418-421.
13. McLaren, D. J. 1973. The structure and development of the spermatozoon of *Dipetalonema viteae* (Nematoda: Filarioidea). Parasitology 66:447-463.
14. Poinar, G. O., Jr., and R. Hess. 1985. Spermatogenesis in the insect-parasitic nematode, *Heterorhabditis bacteriophora* Poinar (Heterorhabditidae: Rhabditida). Revue de Nématologie 8:357-367.
15. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. Journal of Cell Biology 17:208-222.
16. Shepherd, A. M. 1981. Interpretation of sperm development in nematodes. Nematologica 27:122-125.
17. Shepherd, A. M., and S. A. Clark. 1976. Spermatogenesis and the ultrastructure of sperm and of the male reproductive tract of *Aphelenchoides blastophthorus* (Nematoda: Tylenchida, Aphelenchina). Nematologica 22:1-9.
18. Shepherd, A. M., and S. A. Clark. 1983. Spermatogenesis and sperm structure in some *Meloidogyne* species (Heteroderoidea, Meloidogynidae) and comparison with those in some cyst nematodes (Heteroderoidea, Heteroderidae). Revue de Nématologie 6:17-32.
19. Shepherd, A. M., S. A. Clark, and A. Kempton. 1974. Spermatogenesis and sperm ultrastructure in some cyst nematodes, *Heterodera* spp. Nematologica 19:551-560.
20. Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructural Research 26:31-43.
21. Ugwuuna, S. C., and W. E. Foor. 1982. The function of microtubules during spermatogenesis of *Ancylostoma caninum*. Journal of Parasitology 68:817-823.
22. Wagner, G., and K. A. Seitz. 1983. Structure and function of the vas deferens and the ejaculatory glands of *Pelodera strongyloides* (Rhabditidae, Nematoda) Schneider, 1860. Zoologische Jahrbücher, Abteilung für Anatomie und Ontogenie der Tiere 110:321-341.
23. Walsh, J. A., and A. M. Shepherd. 1983. A further observation on sperm structure in a *Heterodera* sp. Revue de Nématologie 6:148-150.
24. Wolf, N., D. Hirsh, and J. R. McIntosh. 1978. Spermatogenesis in males of the free-living nematode, *Caenorhabditis elegans*. Journal of Ultrastructure Research 63:155-169.
25. Wouts, W. M. 1985. Phylogenetic classification of the family Heteroderidae (Nematoda: Tylenchida). Systematic Parasitology 7:295-328.