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Ultrastructure and Tubulin Immunocytochemistry of the Copulatory Stylet-Like Structure in *Childia* Species (Acoela)

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ABSTRACT One of the main characters used in acoel taxonomy is the male copulatory organ. Despite this, ultrastructural studies of this structure are scarce. We studied the ultrastructure of the copulatory organ in eight species of acoels belonging to the taxon *Childia*. Members of *Childia* possess a well-developed conical or cylindrical stylet-like structure composed of needles. Immunogold cytochemistry of tubulin was used to determine the composition of the needles. Stylet-like structures of *Childia* species at the ultrastructural level are basically similar. Stylet needles show intracellular differentiations. As shown both by ultrastructural and immunocytochemical methods, the stylet needles, in all species studied, are composed of long, parallel microtubules, either tightly packed or polymerized. We report unusual polymerization of microtubules, resulting in formation of a honeycomb-like structure in cross section. Variations of ultrastructure among *Childia* species include numbers and arrangement of stylet needles, shape of needles, needle compactness, microtubule polymerization, direction of stylet growth, and presence/absence of different types of granules. The stylet-like structures are homologous within *Childia*, but are likely to prove nonhomologous with the other needle-like structures found in acoel copulatory organs. Stylets in Platyhelminthes are not homologous with stylet-like structures in acoels. J. Morphol. 268:166–180, 2007.

KEY WORDS: Acoela; stylet; stylet needles; ultrastructure; microtubules; polymerization; tubulin

The Acoela are small benthic, predominantly marine, worms. Acoels are characterized by a statocyst with a single statolith, the absence of an epithelized gut, near absence of extracellular matrix (except for the statocyst capsule and myoepithelial junctions), and epidermal ciliary rootlets interconnected at two levels (e.g., Smith et al., 1986; Ehlers, 1992; Tyler and Rieger, 1999). The foundation of the current classification of acoels is the male copulatory organ (Dörjes, 1968). Emphasis on a single structure has resulted in a misbalance among the different families, where about half of the acoel species fell into one family, the Convolutidae, while most of the other families comprised only few species, or were monotypic (see Dörjes, 1968; Tyler et al., 2005). Molecular studies involving large data sets of acoels as well as spermatological and muscular system data have played a significant role for solving these problems and revealed numerous inconsistencies in Dörjes' system (Hooge, 2001; Raikova et al., 2001; Hooge et al., 2002; Jondelius et al., 2002; Hooge and Tyler, 2005). The male copulatory organs carry a phylogenetic signal, since some taxonomic groups based on this feature are also supported from other sources of data (e.g., molecular data, sperm ultrastructure, and muscular system) (Raikova et al., 2001, 2006; Hooge et al., 2002; Hooge and Tyler, 2005; Tekle et al., 2005, 2006). Dörjes (1968) predominantly used light microscopic techniques to compare the male copulatory organs of acoels from live specimens and from traditional histological preparations. More detailed study of the copulatory organ involving advanced techniques is required in order to determine the primary homology of copulatory organs that appear similar at the light microscopic level. Recent investigations using fluorescence techniques to study the musculature of copulatory organs have necessitated revisions of two families: the Convolutidae (Hooge and Tyler, 2005) and the Childiidae (Tekle et al., 2005; Raikova et al., 2006).

Despite the extensive use of the male copulatory organ in acoel systematics, ultrastructural studies of this structure are scarce. Mainitz (1977) studied copulatory stylet ultrastructure of *Paratomella rubra* along with stylets in Gnathostomulida. Her

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study showed that the stylet of *P. rubra* was structurally not similar to that of Gnathostomulids. Similarly Brüggemann studied the ultrastructure of the bursa mouthpiece (1985) and the stylet (1986) of *Philocelis cellata*. To the best of our knowledge, there are no other published ultrastructural studies of the stylet, with main focus on the Acoela. Generally, the hard structures of the copulatory organs in lower Bilateria are either intracellular differentiations (e.g. Mainitz, 1977; Lanfranchi, 1978; Ehlers and Ehlers, 1980; Doe, 1982, 1986; Brüggemann, 1984, 1986; see also Rieger et al., 1991) or derivatives of basal lamina (e.g., Martens and Schockaert, 1981; Martens, 1984). Rieger and Doe (1975) were the first to point out this dual nature of sclerotized structures in platyhelminths. Westblad (1948) described the hypothetical transformations of copulatory organs among acoels and in relation to rhabditophoran flatworms, which were supposed to be closely related at that time, based on histological comparisons. In the Acoela, most of the taxa with well-developed copulatory organ in the form of conical or cylindrically shaped penis stylet were placed in the family Childiidae (Dörjes, 1968). The new taxon Actinoposthiidae consisting of a subset of former *Childia* species was recognized on the basis of body-wall musculature characters (Hooge, 2001). Recently, Tekle et al. (2005) revised Childiidae and defined the taxon *Childia*, according to the PhyloCode, based on three different molecular markers and 50 morphological characters. Here, we examined the stylet in eight members of *Childia* to provide first hand data on stylet ultrastructure in acoels and to study its evolution within a monophylum. Immunogold cytochemistry of tubulin was used to determine the composition of the stylet needles.

MATERIALS AND METHODS

Animals

Specimens of *Childia brachyposthium* (Westblad, 1942), *C. crassum* (Westblad, 1942), *C. cycloposthium* (Westblad, 1942), *C. groenlandica* (Levinsen, 1879), *C. macroposthium* (Steinböck, 1931), *C. submaculatum* (Westblad, 1942), *C. vivipara* (Tekle et al., 2006), and *C. trianguliferum* (Westblad, 1942) were sampled on muddy bottoms at 30–60 m depth in the Koster area and in the vicinity of the Kristineberg Marine Research Station at the Gullmar Fjord in August and September 1999 and August 2005 on the Swedish West coast.

Transmission Electron Microscope

Mature specimens were fixed at 4°C in 3.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2), 2% $CaCl_2$, and 8% sucrose, and postfixed in 1% osmium tetroxide with the same buffer. After dehydration in a series of ethanol, specimens were embedded in Spurr or Epon-Araldite. Ultrathin sections were cut with a LKB 1 Ultracut equipped with a diamond knife, stained with the aqueous solution of saturated (6%) uranyl acetate followed by Reynolds' (1963) lead citrate, and examined with a Zeiss Supra 35-VP (Carl Zeiss SMT, Oberkochen, Germany) field emission SEM equipped with a STEM detector for transmission electron microscopy (TEM).

One to four specimens of each species were examined. Five to 10 sections of the stylet-like structure were examined for each specimen. Needles were counted on cross sections or on oblique sections.

Postembedding TEM Immunocytochemistry (Immunogold)

Living worms of *C. groenlandica* were fixed for 1 h in 2% glutaraldehyde in a buffer solution of 0.1 M sodium cacodylate at pH 7.2 at 4°C. After rinsing in the same buffer (3 × 10 min), the animals were embedded in LR White resin (medium grade). The resin was polymerized in tightly capped gelatin capsules for 10 h at 60°C. Ultrathin sections were made on nickel grids. Grids were rinsed (PBS, 3 × 5 min), and nonspecific antigens were blocked with goat antiserum (Sigma) diluted at 1/30 in PBS for 1 h. A monoclonal anti-tubulin antibody (anti-α-tubulin, clone DM 1A, Sigma, or anti-β-tubulin, clone TUB 2.1, Sigma, or anti-acetylated-tubulin, clone 6-11B-1, Sigma) diluted at 1/100 in PBS, was applied for 40 min at room temperature. After rinsing (PBS, 3 × 5 min) the gold-conjugated antibody (Goat anti-mouse, 10 nm gold beads, 1/20 in PBS) was applied for 1 h at room temperature. After a final rinse (PBS 3 × 5 min, then distilled water, 3 × 5 min), the grids were stained for 5 min with uranyl acetate followed by lead citrate and examined with a Zeiss Supra 35-VP (Carl Zeiss SMT, Oberkochen, Germany) field emission SEM equipped with a STEM detector for transmission microscopy.

RESULTS

General Observations on the Stylet-Like Structures in *Childia*

All eight species of *Childia* we studied have prominent male copulatory organs with one, two, or several stylet-like structures. The stylet-like structure lies in a male antrum, which opens on the ventral side of the animal, near the posterior end. Stylet-like structures of *Childia* species are composed of several intracellular needles (Figs. 1–10). The needles lie in 1–7 concentric layers leaving an empty central space—the stylet canal, which is often filled with sperms (Figs. 1A, 2A, 3A, 4A, 5A, 10).

The stylet needles of all *Childia* species are composed of long microtubules, aligned parallel to the needle axis (Figs. 1D, 4D, 5B, 6B,C, 7B, 8E, 9A, 10). Sometimes, the microtubules are tightly packed and look polymerized to form a compact structure, honeycombed in cross section (Figs. 1F, 2C, 3B,C, 4D). In this case, each microtubule is tightly surrounded by six others (Fig. 3B). Individual microtubules, recognizable by their shape and size (25 μm), could be detected at higher magnifications around the edges of a needle cross section (Figs. 1C, 5D). In longitudinal sections, parallel lines of the walls of the individual microtubules were easily detectable (Figs. 1D, 4D, 5B, 6B,C, 7B, 8E, 9A). In all species studied, the needle occupied most of the cytoplasm of the needle cell. Occasionally, endoplasmic reticulum (Fig. 1F), mitochondria (Fig. 7C,D), myelin bodies (Figs. 1E,F and 5C,D), vacuoles (Fig. 3C),

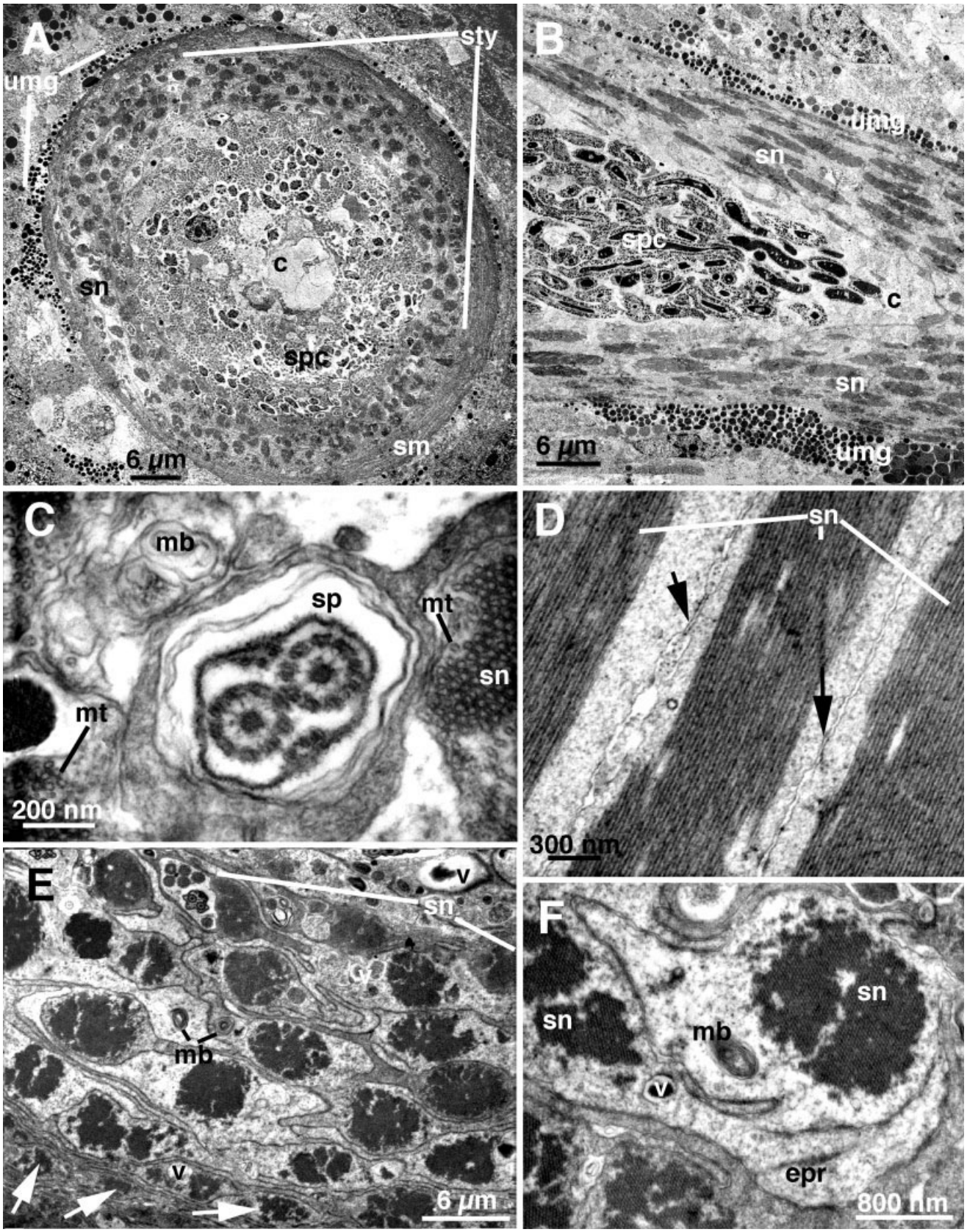
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Figure 1

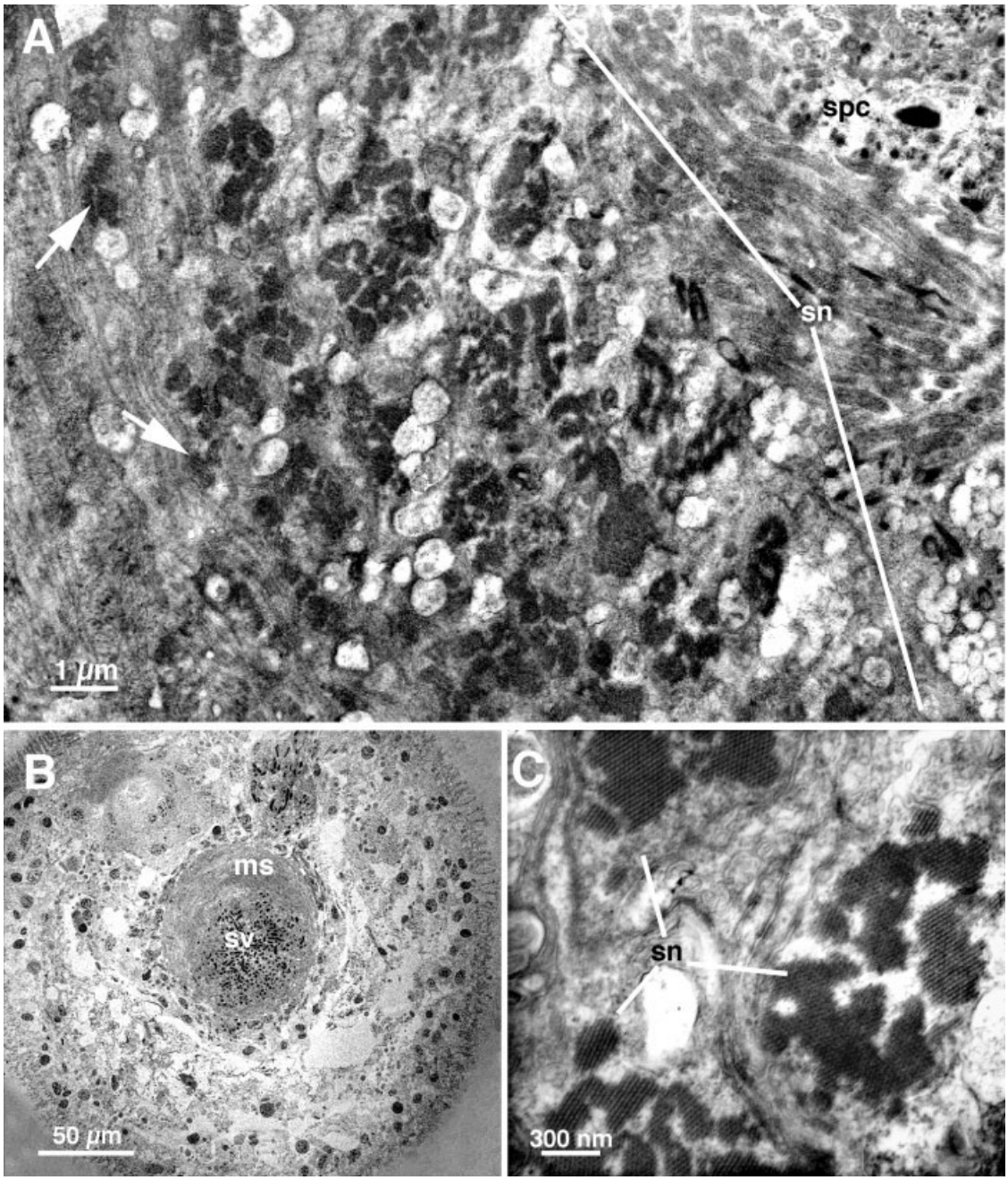

Fig. 2. *Childia brachyposthium*; cross sections (TEM). **A:** Stylet needles (sn) in concentric layers around the stylet canal (c) with sperm cells (spc). Outer needles are less compact and smaller in size (arrows). **B:** Distal region of the seminal vesicle (sv) with thick layer of muscles (ms) that is also associated with the stylet. **C:** Close-up view of needles (sn) showing polymerized microtubules.

Fig. 1. *Childia macroposthium* (TEM). **A:** Cross sections of the proximal stylet region. Note several tightly packed concentric layers of stylet needles (sn), stylet canal (c) filled with sperm cells (spc), and muscles (sm) binding the stylet. Uniform membrane granules (umg) are located outside the stylet in the cells lining the walls of the male antrum. **B:** Oblique section of the proximal part of the stylet. Note sperm cells (spc) inside the stylet canal (c). Granules (umg) are abundant in the male antrum walls on both sides of the stylet needles (sn). **C:** Portions of developing stylet needles (sn) with free nonpolymerized microtubules (mt) corresponding in size to the microtubules in the sperm (sp). Note also myelin bodies (mb). **D:** Longitudinal section of intracellular stylet needles (sn). Note that each needle cells are closely packed, together with small spaces between them (arrows). **E:** Cross sections of a portion of the stylet with stingray-shaped needle cells bearing vacuoles (v) and myelin bodies (mb) in their cytoplasm. Note that smaller and less compact needles are located within the outer concentric layer (arrows) **F:** Needle cells with needles of polymerized microtubules (sn), endoplasmic reticulum (epr), myelin bodies (mb) and vacuoles (v).

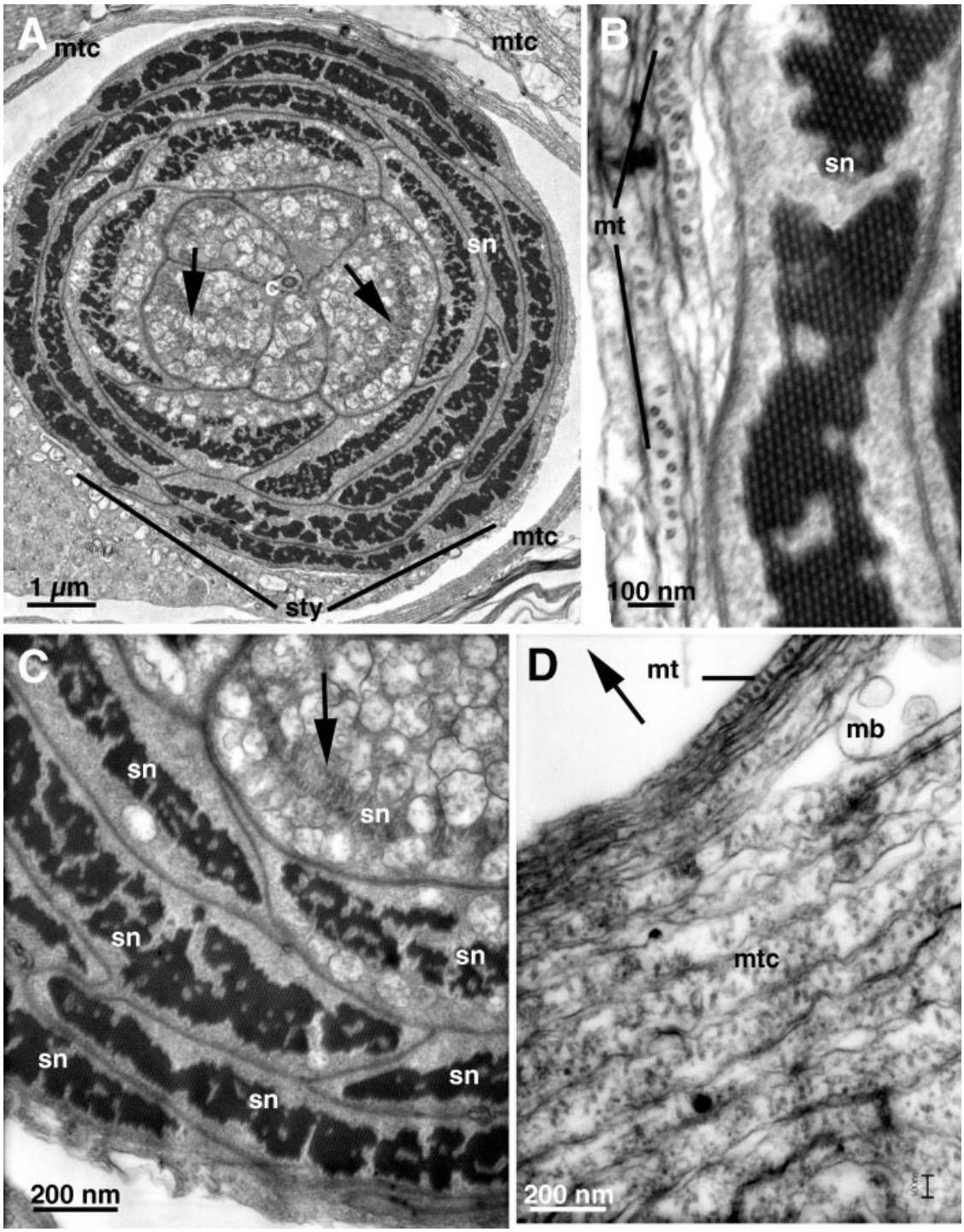

Fig. 3. *Childia vivipara*; cross sections (TEM). **A:** Distal region of stylet (sty) with 15 needle cells (sn) and 5 central cells arranged in closely packed concentric layers around the stylet canal (c). Less-developed needles (arrows) are located within central cells. Note flat microtubule cells (mtc) around the stylet. **B:** A close-up of mature needle cell (sn) with highly polymerized microtubules and microtubule cells with free microtubules (mt) aligned parallel to the needles. **C:** A close-up view of stylet needles (sn). Note the less-developed needle cell (arrow). **D:** Microtubule cells in the vicinity of the stylet. Note that the cells closer to the stylet have more mature microtubules than the cells located far way from the stylet. The arrow indicates the direction of the stylet location.

and granules (Figs. 6C, 7C,D, 8A–E) were also found. Though it was difficult to determine the location of the needle cell nuclei in most of the species examined, in *C. cycloposthium*, they were found in the proximal ends of the long needle cells (Fig. 8F). Presumably, in other species the needle cell nuclei are located proximally within the seminal vesicle (Fig. 10).

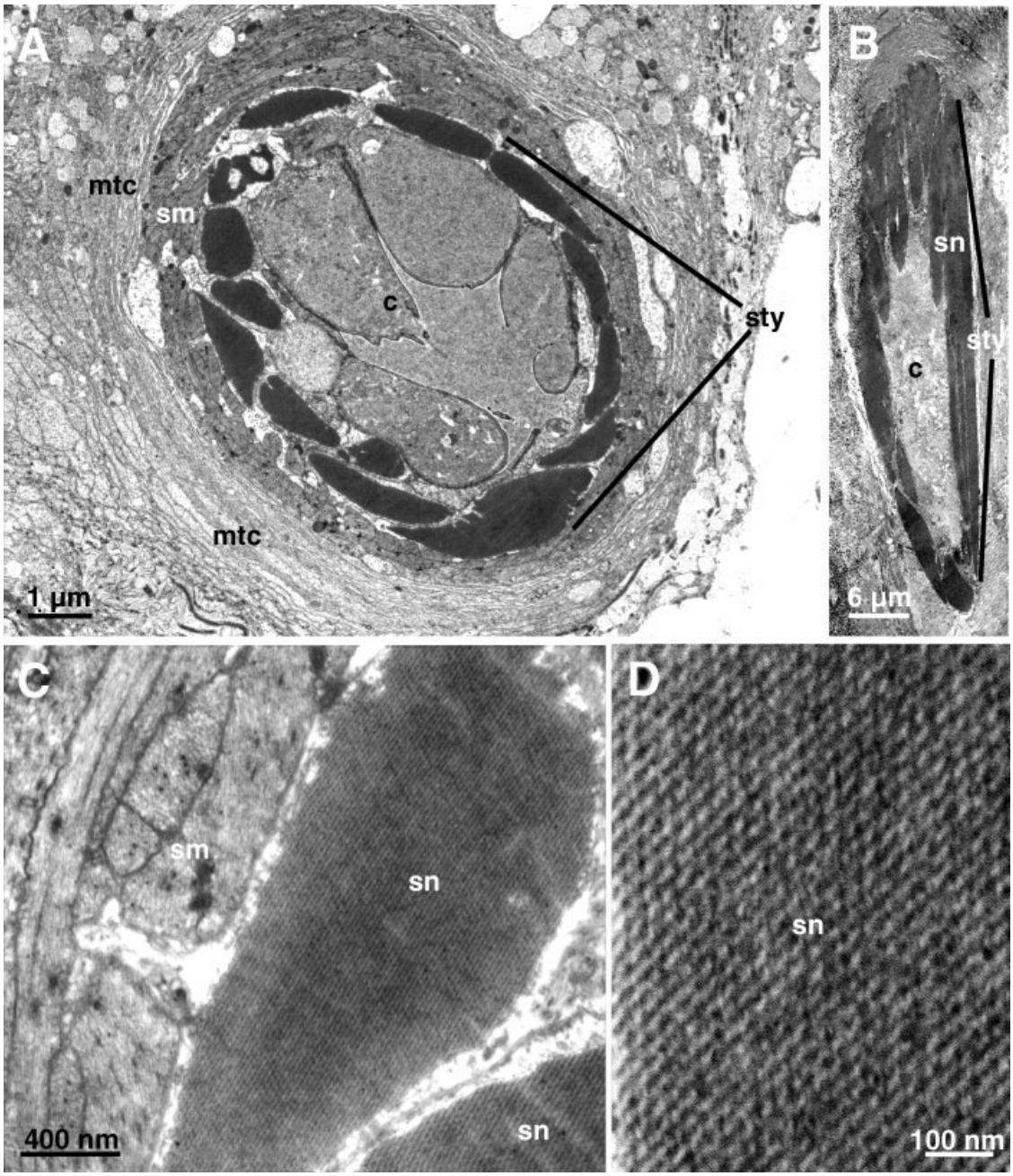

Fig. 4. *Childia groenlandica* (TEM). **A:** Stylet (sty) cross section with stylet needles (sn) in one circle; stylet canal (sc) contains six large cells. Note muscles (sm) around stylet and layers of flat microtubule cells (mtc) forming the antrum walls. **B:** Longitudinal section of the stylet (sty) showing needles (sn) and the canal (c). **C:** Close-up view of the stylet needle cells (sn) and adjacent muscles. **D:** Highly polymerized needle (sn) microtubules in oblique section.

Variations of ultrastructure observed among *Childia* species include numbers and arrangement of stylet needles, shape of needles, needle compactness, microtubule polymerization, direction of needle growth, and presence/absence of different types of granules.

The number of needle cells making up the stylet-like structure of *Childia* species ranges from 14 to over 200. *C. macroposthium* and *C. brachyposthium* have the largest number of stylet needles, while *C. groenlandica* (Fig. 3) has the lowest number of needles (around 14). The species with lowest and highest numbers of concentric layers of needles are *C. groenlandica* and *C. macroposthium*, respectively. The shapes of needle cells and needles in cross section are mostly round (oval) in *C. crassum* and *C. trianguliferum*, and more irregular in *C. brachyposthium*. However, in *C. macroposthium* the nee-

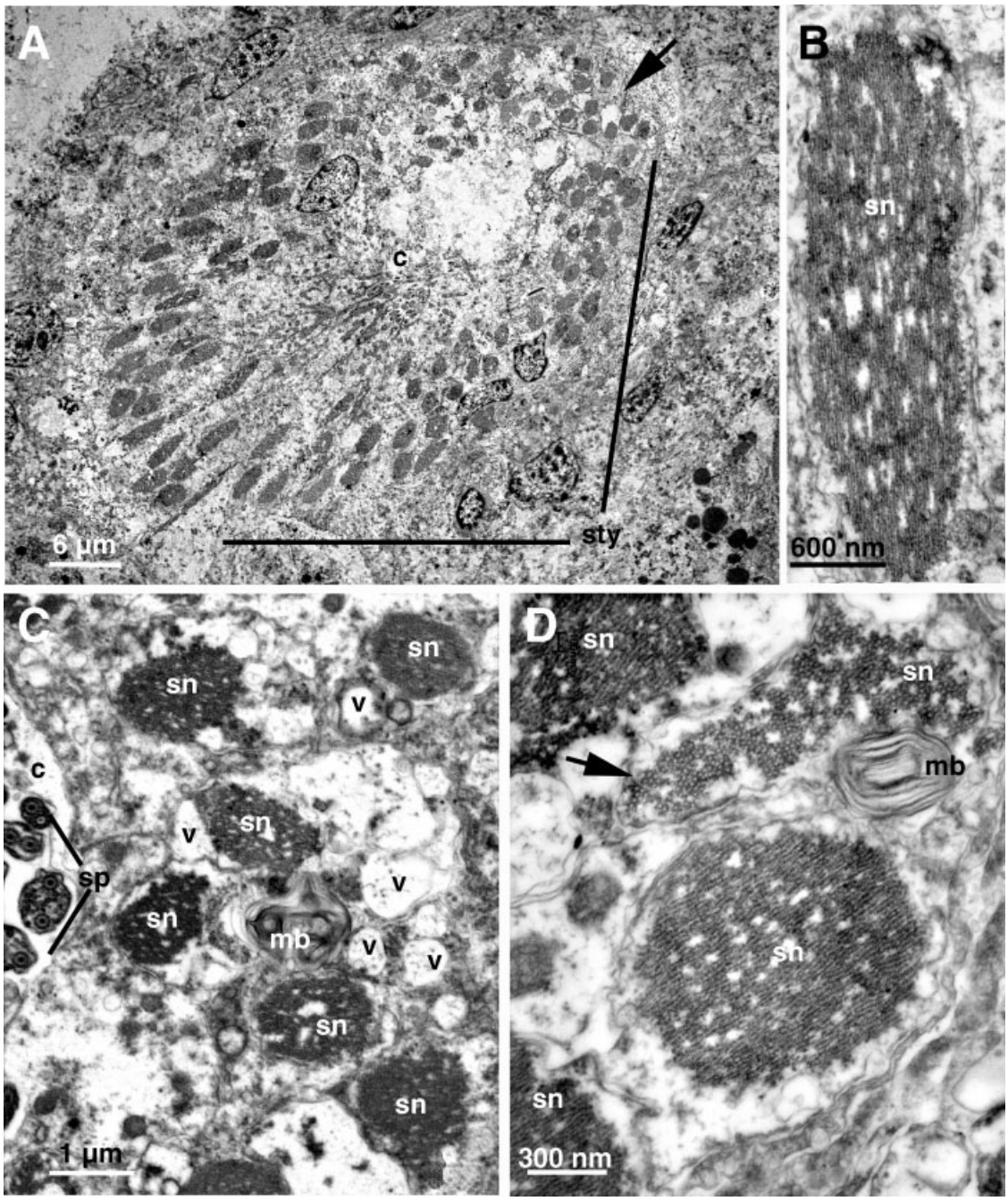

Fig. 5. *Childia crassum* (TEM). **A:** Stylet (sty), its canal (c) and stylet needles, some cut transversally (arrow). **B:** Longitudinal section of a stylet needle (sn). Note nonpolymerized intracellular microtubules. **C:** Cross section of stylet needles (sn). Note vacuoles (v) and myelin bodies (mb) within the needle cell cytoplasm, and sperm cells (spc) in the stylet canal (c). **D:** Cross section of stylet needles (sn). Note near-absence of microtubule polymerization and some clearly nonpolymerized microtubules (arrow) in the needle cells with myelin bodies (mb).

dle cells are stingray-shaped in cross section (Fig. 1A,E,F). *C. vivipara* and, to a much lesser degree, *C. groenlandica* have slender and crescent-shaped needle cells in cross section (Figs. 3 and 4). Data on the shape of the needle cell are not available for *C. cycloposthium* and *C. submaculatum*, because of shortage of material.

Generally, *C. macroposthium*, *C. brachyposthium*, *C. vivipara*, and *C. groenlandica* have a compact stylet-like structure, though there are variations in needle compactness (the absence of free spaces within the needles). *C. groenlandica* has the most compact needles, followed by *C. vivipara.* Among the other species, *C. cycloposthium* and, especially, *C. trianguliferum*

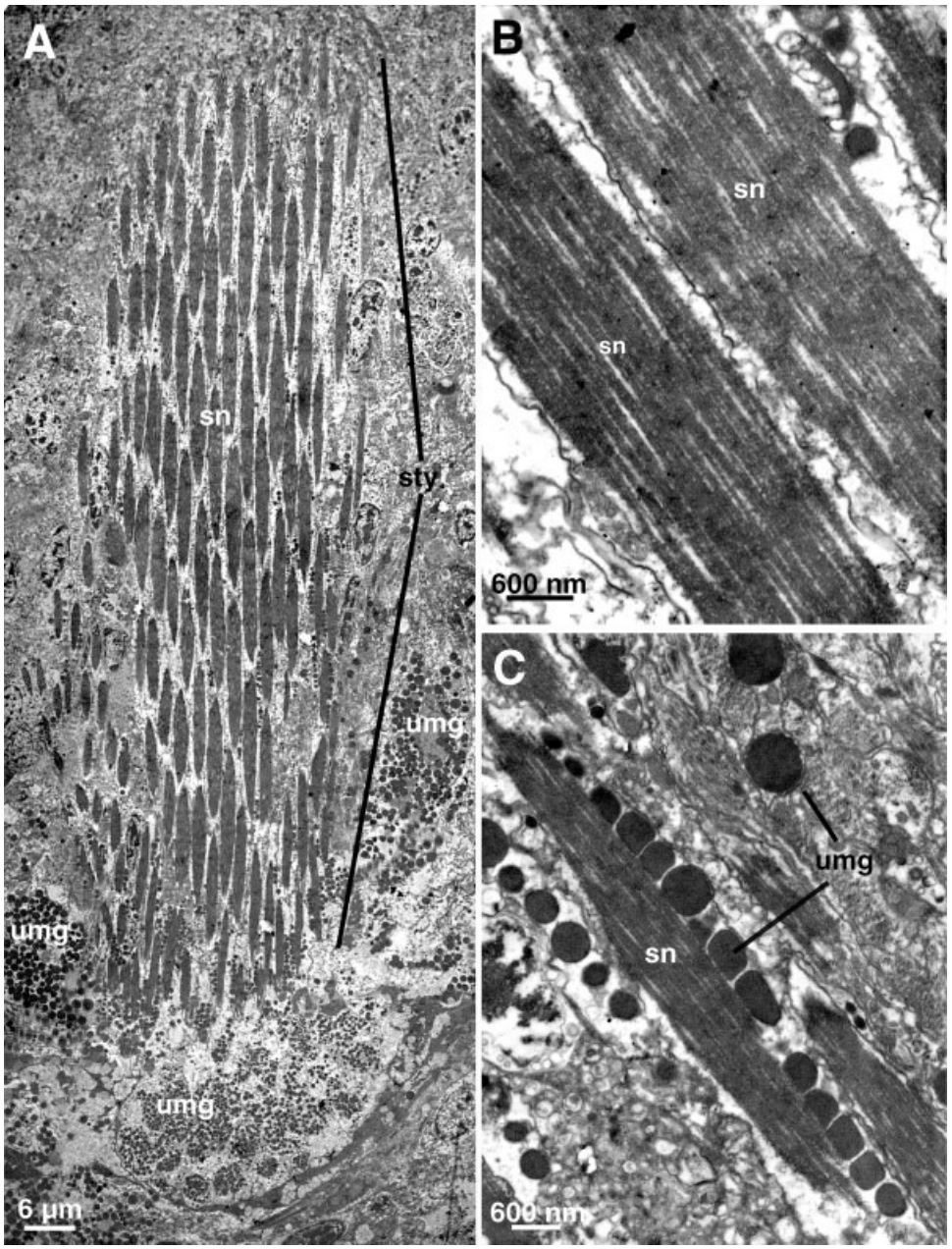

Fig. 6. *Childia submaculatum* (TEM). **A:** Cylindrical stylet (sty) of numerous needles (sn). Note abundance of granules (umg) in antrum walls at posterior region of stylet. **B, C:** Longitudinal sections of intracellular stylet needles (sn) showing nonpolymerized microtubules and granules (umg) (C).

have the least compact needles. The microtubule polymerization in the stylet needles is quite pronounced in *C. vivipara*, *C. groenlandica*, *C. macroposthium*, and *C. brachyposthium*, occasional in *C. crassum* and *C. submaculatum*, while in *C. cycloposthium* and *C. trianguliferum*, polymerization is not observed. Furthermore, the stylet needles of the four latter species were observed to be easily detached upon application of pressure on squeezed preparations, compared to the stylet-like structures of *C. macroposthium*, *C. brachyposthium*, *C. vivipara*, and *C. groenlandica*, which are also tightly bound by muscles (Figs. 1A, 2A, 4A,C).

The direction of growth of the stylet-like structure (addition of new layers of needles) might vary from species to species. In *C. macroposthium* (Fig. 1E) and *C. brachyposthium* (Fig. 2A), the smallest and the least compact needles are located within the outer layer of the stylet-like structure, while in *C. vivipara*, the developing needles are observed in the inner concentric layer and in the central cells

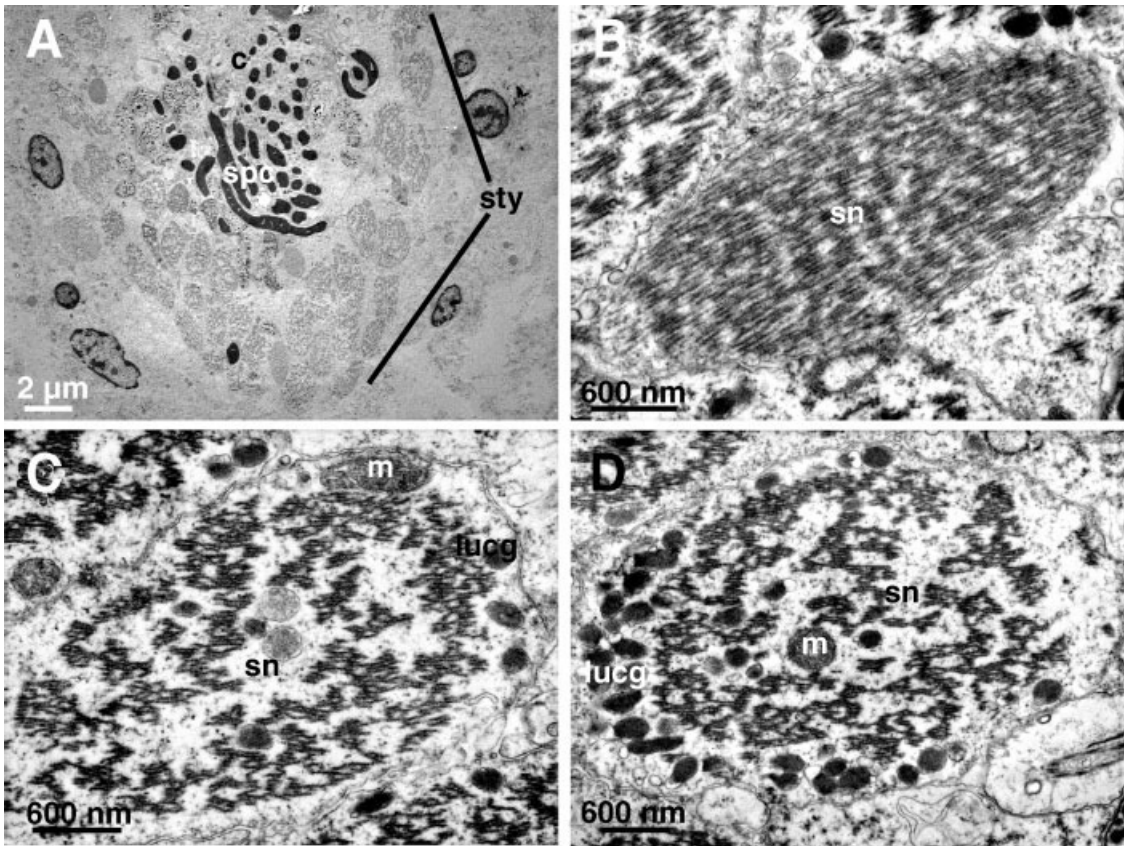

Fig. 7. *Childia trianguliferum* (TEM). **A:** Conical stylet (sty) with sperm cells (spc) in the proximal part of the stylet canal (c). **B:** Oblique section of needles (sn). **C, D:** Cross sections of stylet needles (sn). Note nonpolymerized and fuzzy-looking needle microtubules, loose membrane granules with uniform core (lucg), and mitochondria (m).

(Fig. 3C). The cells producing needles are characterized by an extensive development of vacuoles with flocculent or vesicular content, while the cells with fully developed needles have more electron-dense, homogeneous cytoplasm (Fig. 3A,C).

Two types of intracellular granules associated with the stylet-like structure were observed in some of the species examined. Uniform membrane granules were found in *C. macroposthium*, *C. submaculatum*, and *C. cycloposthium* (Figs. 1A,B, 6A–C, 8A–E). The granules in *C. macroposthium* and *C. submaculatum* are more abundant outside the stylet-like structure than inside the needle cells, while in *C. cycloposthium* they are mostly contained within the latter. Second type of granule, with a loose membrane and a uniform core, was found in *C. trianguliferum* and *C. cycloposthium* (Figs. 7B,C and 8A,B). In the latter taxon, the granules are characteristically arranged in groups of round clusters (Fig. 8B,C).

Childia macroposthium. Among all *Childia* species examined, *C. macroposthium* has the biggest stylet-like structure, cylindrical in shape, with the highest number of stylet needles, more than 200 (Fig. 1A). The needles are tightly packed around a prominent stylet canal, forming up to seven concentric layers (Fig. 1A,E). The stylet canal is continuous with the seminal vesicle; both are filled with spermatozoa (Fig. 1B). Rarely a few sperm cells could be observed between the needle cells and seemingly inside the needle cell vacuoles (Fig. 1C), probably because of the intrusion of the distal ends of the spermatozoa into the needle cell cytoplasm. The proximal region of the stylet-like structure is tightly bound by muscles (Fig. 1A). The distal part of the stylet lies in a tubular male antrum, which opens in a subterminal position at the posterior end of the body. The walls of the male antrum are lined by cells, filled with uniform membrane granules (Fig. 1A,B).

Smaller and less compact needles are located more within the outer concentric layer of the stylet-like structure (Fig. 1A,E). Each needle, as far as we can tell, is contained within its own needle cell (Fig. 1D,E). The needle cells, in cross section, have a characteristic stingray shape, i.e., an oval body with an extended tail-like ridge (Fig. 1E,F). The needles are round to oval in cross section, the microtubules are tightly packed in the central region of the needle, and lying loose at the periphery, where individual microtubules can be easily detected (Fig. 1C–F). The cytoplasm of the needle

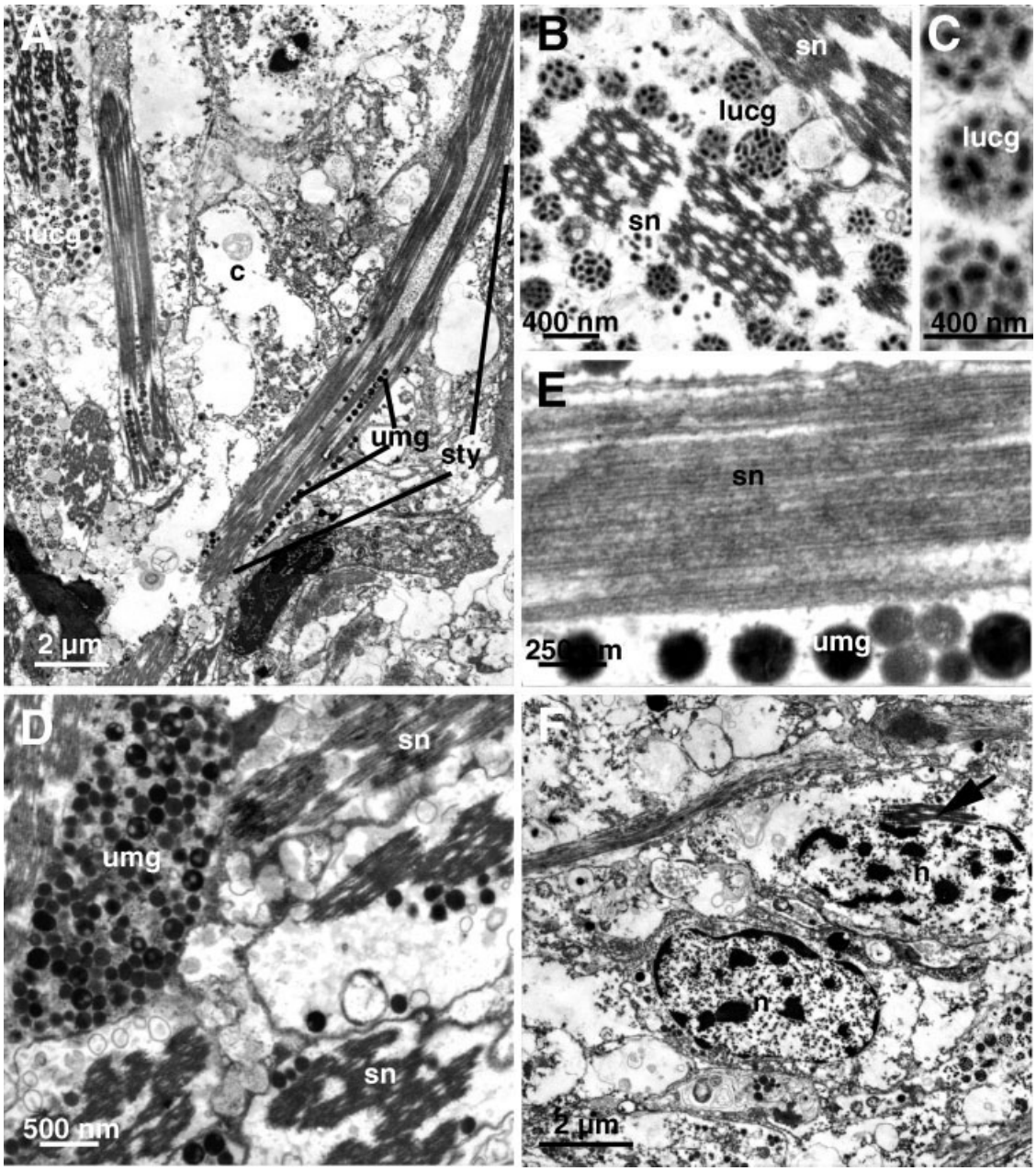

Fig. 8. *Childia cycloposthium* (TEM). **A:** Several stylets (sty) with stylet canals (c), cut at different angles. Note numerous granules (umg, lucg). **B:** Needle cell with needle (sn) and round clusters of loose membrane granules with uniform core (lucg). **C:** Granule clusters. **D:** Needle cells with needles (sn) and uniform membrane granules (umg). **E:** Longitudinal section of a stylet needle cell (sn) showing nonpolymerized and fuzzy looking microtubules and uniform membrane granules (umg). **F:** Needle cells nuclei (n). Note needles close to one nucleus (arrow).

cells contains few inclusions such as endoplasmic reticulum, myelin bodies, and very few granules (Fig. 1F). Numerous uniform membrane granules are mostly located in specialized gland cells, located around the stylet-like structure and are more numerous around its posterior end (Fig. 1A,B).

Childia brachyposthium. The large conical stylet-like structure of this species comprises more than 160 needles, closely packed around the stylet canal, filled with spermatozoa (Fig. 2A,B). The proximal region of the stylet-like structure is tightly bound by muscles (Fig. 2A). The stylet needles are arranged in four to six irregular concentric layers. Smaller and less compact needles are located within the outer concentric layer (Fig. 2A). In cross sections, the needles are of very irregular shape, and the regions of tightly packed polymerized microtubules intermingled with regions of electron-lucent cytoplasm (Fig. 2A,C). The needle cells are of irregular shape and contain numerous

vacuoles filled with flocculent material (Fig. 2A,C). No granules associated with the proximal portion of the stylet were observed.

Childia vivipara. The stylet-like structure is conical, long and thin, progressively tapering distally. The stylet needles are relatively few (15–40) (Fig. 3A). The stylet is compact; the microtubules in the needles are polymerized (Fig. 3A–C). Needle cells are tightly packed, forming four to five concentric layers (Fig. 3A). The needle cells as well as the needles within are crescent-shaped in cross section. Cross sections of the innermost needles are shorter and more curved than those of the outer needles, which are long and slender, and appear more compact. Some of the outer needle cells extend around one-third of the stylet circumference (Fig. 3A,C). Along the axis of the stylet-like structure, no canal was observed; instead, the center of the cross section was occupied by 5–6 vacuolated cells (Fig. 3A). Some of the central cells contain half-formed needles at different stages of maturation, where single microtubules forming the needles could still be discerned (Fig. 3C). Within the more compact needles, regions of tightly packed polymerized microtubules are intermingled with regions of electron-lucent cytoplasm (Fig. 3C). The vacuoles filling the central cells are electron-lucent and contain flocculent material. The cytoplasm of the needle cells of the inner circle also contains similar vacuoles. Unlike other *Childia* species (except *C. groenlandica*, see later), the two specimens of *C. vivipara* we have sectioned had no sperm within the penis canal. The stylet-like structure lies within the male antrum lined by layers of flattened cell projections that are filled with cytoplasmic microtubules (Figs. 3A,B,D and 10). The microtubules in the cells adjacent to the stylet-like structure are aligned parallel to the stylet axis and parallel to the polymerized microtubules in the stylet needles, while the outer projections have randomly oriented microtubules (Figs. 3B,D and 10). Granules associated with the stylet-like structure were not observed in this species.

Childia groenlandica. *C. groenlandica* possesses a pair of long curved conical stylet-like structures (Fig. 4A). Each structure is composed of 14–15 needles arranged as one concentric layer (Fig. 4A). The needles vary from oval to thick crescent-shaped in cross section. They appear very electron dense, and are composed of very tightly packed microtubules with very few insertions of cytoplasmic spaces within the needle (Fig. 4A–D). The inner part of the stylet along the axis is occupied by several (5–7) cells, with homogenous cytoplasm in the proximal end of the stylet (Fig. 4A), and with vacuolated cytoplasm in the distal end (Fig. 4B). The proximal end of the stylet-like structure is surrounded by muscles (Fig. 4C), while the distal end is surrounded by layers of flattened cell projections (Fig. 4A) containing microtubules (Fig. 9A). Granules associated with the stylet-like structure were not observed in this species.

Childia crassum. The needles composing the stylet-like structure of *C. crassum* are relatively numerous, about 120–130 (Fig. 5A). However, the structure is not very compact. Specifically, the microtubules forming the stylet needles are not polymerized. Individual microtubules were easily detectable. The needle cell cytoplasm is more vacuolated (Fig. 5C,D). Needles are arranged around the stylet canal, forming up to four concentric layers (Fig. 5A). The needles and needle-containing cells are round to oval in cross section (Fig. 5A,C,D). Some stylet needles are less compact than the others, appearing only half-formed (Fig. 5D). The wide stylet canal contains numerous spermatozoa. Granules were not observed neither within the needle cells nor around the stylet-like structure.

Childia submaculatum. Our observations on the copulatory organ ultrastructure of *C. submaculatum* were based only on longitudinal or oblique sections. The long cylindrical stylet-like structure is composed of numerous (at least 150) needles (Fig. 6A). Every needle is contained within its own cell (Fig. 6B,C). Numerous spherical uniform membrane-bound granules 50–800 μm in diameter were observed within the needle cells, lining the needles. The needle cell granules are more numerous along the distal parts of the needles. Even more granules of the same type were found outside the stylet-like structure in specialized secretory cells lying close to its distal end (Fig. 6A).

Childia trianguliferum. The stylet-like structure is conical in shape and slightly curved toward the distal end. The needles are relatively few (40–60), lying in 2–3 irregular concentric layers around the stylet canal (Fig. 7A). Needles and the needle cells are round to oval in cross section (Fig. 7C,D). The microtubules within the needle are packed quite loosely. Individual groups of parallel, nonpolymerized microtubules lie separately in the cytoplasm of the needle cell (Fig. 7C–D). A few granules and mitochondria were found just within the needle or on the periphery of the needle (Fig. 7C,D). The granules have a loose membrane and a uniform core. Some needles are more compact than the others (Fig. 7B). Numerous spermatozoa are present within the proximal region of the stylet canal (Fig. 7A).

Childia cycloposthium. Our observations on the copulatory organ of *C. cycloposthium* were limited to longitudinal or oblique sections. This species possesses 7–10 conical stylet-like structures arranged like petals of a flower around the male opening (Fig. 8A). The number of needles composing each stylet-like structure, as estimated from live specimens and oblique sections, is about 30–40. The needles are composed of groups of individual, not polymerized, microtubules, lying separately in the needle cell cytoplasm (Fig. 8B,E,D). Sometimes

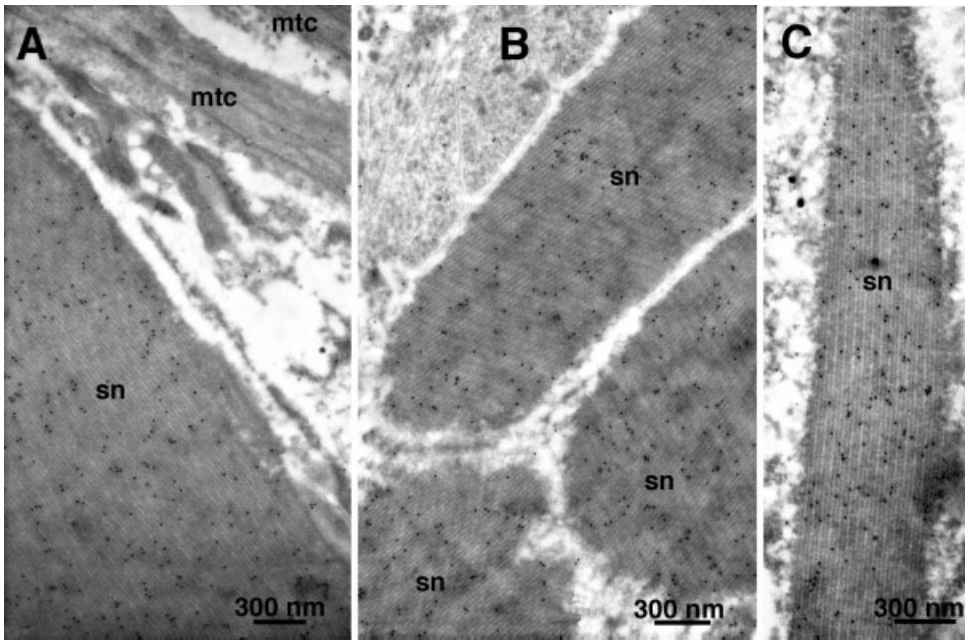

Fig. 9. *Childia groenlandica*. Anti-tubulin immunogold staining of stylet needles. The black dots of immunogold deposits (10 nm) label microtubules within the needle and within the flat microtubule-bearing cells (mtc) lining the male antrum. **A, B:** Anti-α-tubulin labeling. **C:** Anti-β-tubulin labeling.

a needle in cross section has a honeycomb structure (Fig. 8B). Numerous uniform core granules, with either loose or uniform membrane, were found both within the needle cells (Fig. 8B,C,E) and in specialized gland cells around the stylet (Fig. 8A,D). The loose membrane granules are characteristically arranged in spherical clusters (Fig. 8B,C). The needle cell nuclei lie in groups at the proximal ends of the needles. In the proximal region, the needle cells contain numerous vacuoles and inclusions. The nuclei are oval, about 5 × 2.5 μm in size, with patches of condensed chromatin.

Postembedding TEM Immunocytochemistry Results

Immunocytochemical labeling of the stylet of *C. groenlandica* by anti-tubulin antibodies that specifically bind to microtubules showed immunoreactivity to both anti-α-tubulin (Fig. 9A,B) and anti-β-tubulin (Fig. 9C). The reactivity is shown by numerous gold deposits (10 nm) on the section of the stylet-like structure as compared to only slight background labeling of other structures (Fig. 9A–C). The microtubules within the cell projections, lining the stylet canal, were also labeled (Fig. 9A).

DISCUSSION

Composition of the Stylet-Like Structure in *Childia* Species

There have been different definitions of the copulatory hard structures in acoels. At a light microscope level, the stylet-like structure appears composed of several needles that are closely packed in a conical or cylindrical shape. The needles have been described in the older literature as "cuticularized" elements (e.g. Westblad, 1942, 1948; Dörjes, 1968). Later the term "cuticle" was abandoned, as it obviously referred to extracellular material, and so following Karling's (1985, 1986) suggestion, the intracellular hard structures were referred to as "sclerotized" elements (Cannon, 1986; Rieger et al., 1991). Detailed ultrastructural study of the stylet-like structure in *Childia* at higher magnifications enabled us to visualize the finer substructures, microtubules, composing the intracellular stylet needles, and describe microtubule polymerization within the needles. The composition of the needles was confirmed by immunogold tubulin cytochemistry (Fig. 9A,B). It is well established that microtubule formation involves polymerization of smaller tubulin subunits (α-tubulin and β-tubulin monomers). However, polymerization of large groups of microtubules, in the form of closely packed honeycomb structure, as in the stylet-like structures of *C. groenlandica*, *C. vivipara*, *C. brachyposthium and C. macroposthium* is very unusual. Until now, microtubules were known to form doublets or triplets, as is common in all the axonemes of cilia or flagella, but to our knowledge, a more complex tubulin polymerization has not been reported. A similar pattern of polymerization is also known in spines of *Schistosoma* (Cohen et al., 1982). However, the hexagonally packed structures of the spines in *Schistosoma* are composed of actin filaments.

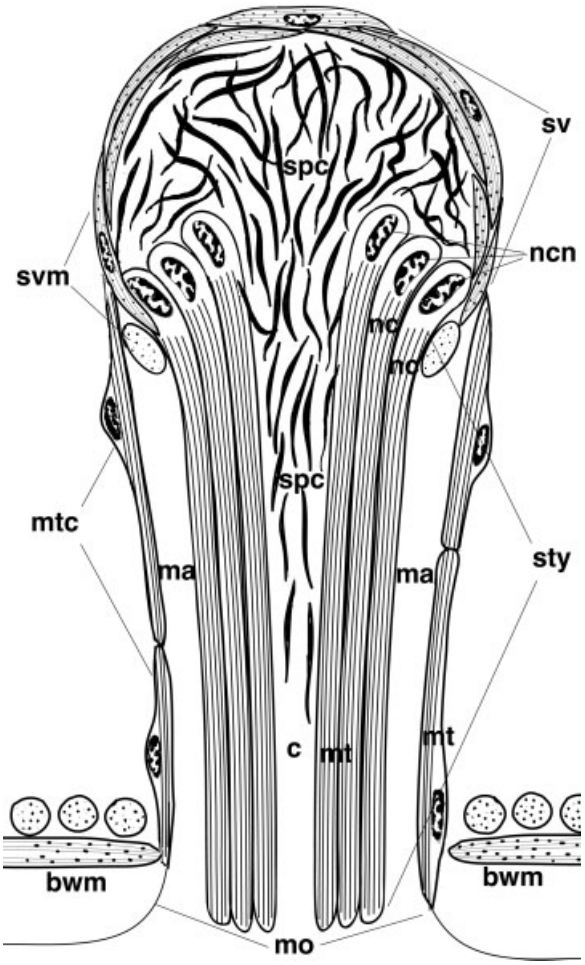

Fig. 10. Schematic interpretation of the male copulatory organ organization in species with microtubule cells lining the walls of the male antrum (*C. vivipara* and *C. groenlandica*). Longitudinal section. Note seminal vesicle (sv) composed of seminal vesicle muscles (svm) continuous with stylet canal (c). Both contain sperm cells (spc). Stylet-like structure (sty) is composed of needle-forming cells (nc) containing needles of polymerized microtubules (mt), with nuclei (ncn) presumably in proximal position inside the seminal vesicle. Walls of male antrum are lined with microtubule cells (mtc) with microtubules aligned parallel to those in stylet needles. Male antrum pierces the layers of body-wall muscles (bwm) and opens to the body surface through male opening (mo).

Evolution of Stylet-Like Structure in *Childia* With Phylogenetic Consideration

Copulatory organ ultrastructure of all *Childia* species examined shows little variety. The following ultrastructural characters of the stylet-like structure are common to all *Childia* species studied: intracellular needles, needles composed of polymerized microtubules, formation of a tubular stylet canal (cylindrical or conical in shape), and closely packed concentric layers of stylet needles (more than 14 needle cells). However, at this stage it is difficult to characterize these characters as apomorphies of the taxon, since we know little about other groups of acoels with stylets. The high degree of the stylet-like structure compactness and microtubule polymerization, and the presence of muscles binding stylet needles and flat cells, mostly with microtubules, lining the male antrum can serve as synapomorphies for the following species: *C. vivipara*, *C. groenlandica*, *C. macroposthium*, and *C. brachyposthium*. Our observation of the latter character is in agreement with Westblad's (1948) observations. Westblad noted that in *C. macroposthium* and *C. brachyposthium* (and we may add, in *C. groenlandica* and *C. vivipara* as well) the epithelium of the male antrum containing the stylet is composed of flat cells. The granules filling some of the cells were stained by iron hemotoxylin, but did not give a similar reaction to mucus. On the basis of these observations, Westblad commented that the function of the mucus secretion is a likely attachment of the animals to each other during copulation. The granular secretions likely only oil the stylet-like structure, because the long stylet itself provides attachment during copulation as well as sperm impregnation (Westblad, 1948). The flat antrum cells closer to the stylet-like structure contained long microtubules, perfectly parallel to the stylet axis, while those further from the stylet contained more randomly arranged or not fully formed microtubules (Fig. 3D). We believe that the microtubules lining the male antrum straighten its walls and allow the stylet-like structure, while it protrudes during copulation, to glide more smoothly without damage to the antrum walls. It is also possible that the numerous flat cells with microtubules might serve as sources of microtubules to the growing needle cells.

The granules may serve as lubricants during copulation (Westblad, 1948). Therefore, they may be absent in specimens not ready for copulation. Furthermore, granules restricted to the extreme distal end of the stylet-like structure may be difficult to observe.

The evolution of the stylet-like structure within *Childia* species led toward increased needle compactness. The stylet-like structures in the basal *Childia* species (Tekle et al., 2005) (*C. trianguliferum*, *C. cycloposthium*, *C. submaculatum*, and *C. crassum*) are less compact, both in terms of space within and between the needle cells and polymerization of the microtubules inside the needle cells, as compared to the stylet-like structures in derived *Childia* species (*C. vivipara*, *C. groenlandica*, *C. macroposthium*, and *C. brachyposthium*).

Copulatory Stylet-Like Structures and Needles in Acoels

Several acoel taxa besides *Childia* species have penial hard structures (Westblad, 1942, 1945, 1948; Dörjes, 1968; Tyler et al., 2005). These types of stylet-like structures are generally less complex at the light microscope level. Westblad (1948) suggested that many of the highly organized copulatory

organs observed in advanced acoels such as *C. groenlandica* might have been derived from the simple copulatory organs with few needles that are found in acoels such as *Paranaperus* and *Haploposthia rubra*. Unfortunately, data on the ultrastructure of the needles in the aforementioned species are not available. According to Mainitz (1977) observations, the stylet of an acoel *Paratomella rubra* is composed of 11 intracellular "cuticular" rod-like structures. The cuticular nature of the rods would exclude any possibility of homology with *Childia* stylets; however, we think the magnification Mainitz (1977, Figs. 7–9) used was too low to reveal the smaller substructures of the stylet needles, and so the possibility of *Paratomella*'s rods being composed of polymerized microtubules cannot be excluded. Similarly, Brüggemann (1986) reported the presence of microtubules in the stylet of *Philocelis cellata*, which were associated with the "cross-striated microfibirils" of the stylet. The magnification of the electron micrograph published by Brüggemann (1986, Fig. 1) does not allow to see the substructure of the needle, but the picture is very similar to the way the needles in *C. groenlandica* look at lower magnifications. Perhaps here again the presence of polymerized microtubules was overlooked. Raikova et al. (2001) mentioned preliminary results on the stylet-like structure of *C. cycloposthium* and mistakenly reported it to be formed by secretory cells, obviously misled by the presence of numerous granules around the needles. Our present study clearly shows that *C. cycloposthium* possess a stylet-like structure composed predominantly of microtubules just like the other *Childia* species. The stylet-like structure of *Actinoposthia beklemischevi* is composed of nonpolymerized microtubules (Raikova et al., 2001). It thus appears that the main component of the needle-like structures in acoels might be microtubules. Nonpolymerized microtubules are also present in gland necks of acoels (Gschwentner et al., 1999), platyhelminths, and other animals (Rieger et al., 1991). It is likely, as Westblad (1948) suggested that structures with a needle-like appearance, including adenodactyles and prostatoid organs, might have some similarity to the copulatory stylet-like structures, which were found to contain microtubules. However, their similarities (homology) in organization, degree of polymerization of individual microtubules in the honeycomb structure, and their abundance within the needle cell need to be investigated. Raikova et al. (2001) reported another type of copulatory spicules, without tubulin, that are formed inside the spermatozoa in *Philactinoposthia* sp. Future studies should aim at studying the ultrastructure of copulatory organs with needles and stylet-like structures, including the needle-like organs (e.g., adenodactyles) in different groups of the Acoela in order to thoroughly evaluate their homologies and to study their evolution within the whole group.

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