2001

Ultrastructure of 'Turbellaria' (Platyhelminthes)

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ULTRASTRUCUTURE OF 'TURBELLARIA'

(PLATYHELMINTHES)

By

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Mag. rer. nat., University of Salzburg, Austria, 1999

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

(in Zoology)

The Graduate School

The University of Maine

August, 2001

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Thesis Advisor: Dr. Seth Tyler

An Abstract of the Thesis Presented
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The systematics of turbellarian platyhelminths (also known as free-living flatworms) has proven difficult since few taxonomically useful characters can be discerned in them. Generally, features of the reproductive tract, observed through conventional light microscopy, provide key taxonomic characters. Through the newer techniques of electron microscopy and fluorescence microscopy, new characters are emerging that provide better clues to the phylogenetic relationships of these animals. We have applied both of these microscopies to representatives of two groups of turbellarians whose phylogenetic positions are uncertain and controversial, the Acoela and the Genostomatidae.

Because fluorescence microscopy of phalloidin-labeled acoel turbellarians has provided new taxonomically relevant characters in the arrangement of muscles, we studied another feature that has emerged in such phalloidin-labeled

preparations, namely sensory receptors. By correlating electron micrographic images and fluorescence images of *Convoluta pulchra*, we determined that these structures are sensory receptors with a central cilium surrounded by a collar of microvilli. The collared sensory receptors were inserted between epidermal cells, and each bore a central cilium surrounded by a collar of 6–18 microvilli and an additional centrally positioned 2–7 microvilli of which 2 or 3 were associated with a modified rootlet called the swallow's nest (Bedini et al. 1973). Confocal scanning laser microscopy resolved the core of actin filaments within the microvilli of the collar and their rootlet-like connections to the base of the sensory cell.

Many other acoels have similar receptors in various patterns of distribution across the epidermis, and so further study of these organs in these other species may provide meaningful characters for deciphering phylogenetic relationships.

Whereas assignment of *Convoluta pulchra* to the Acoela is straightforward, another species, *Genostoma kozloffi* cannot be readily assigned to any higher taxon within the Rhabditophora. Some have proposed that the family in which it is classified, the Genostomatidae, is part of the sister group to the Neodermata, the major group of parasitic flatworms, and so stands as a representative of the link between turbellarians and parasitic flatworms. Characters of spermiogenesis and spermatozoa have been used by others to

distinguish between turbellarians and the Neodermata. By applying electron microscopy to elucidate the process of spermiogenesis in *Genostoma kozloffi*, we found that its sperm are more like those of the turbellarians than those of the Neodermata. Spermiogenesis in *Genostoma kozloffi* occurs in a distal-proximal fashion, just as in those free-living turbellarians in which the axoneme is incorporated into the body of the sperm. Mature spermatozoa are filiform, possess an elongate rod-like nucleus and one short single, fully incorporated, axoneme. A rod of multiple, fused mitochondria accompanies the nucleus and axoneme and an array of cortical microtubules with thickened walls are present. In this study more evidence in sperm and spermiogenesis, linking *Genostoma kozloffi* to Kalyptorhynchia, more specifically to Schizorhynchia, was found.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisor Dr. Seth Tyler for giving me the opportunity to take part in his research and for his prompt and helpful advice. I would also like to thank my committee members Mary Tyler and Irv Kornfield for reviewing my thesis and Kelly Edwards for the help in the EM lab, especially the technical support in handling computers and microscopes. Last but not least, I would like to thank my "co-advisor" Matt Hooge for his valuable comments and extensive talks, as well as his help whenever needed.

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Chapter 1

INTRODUCTION

The phylum Platyhelminthes comprises å variety of acoelomate, bilaterally symmetrical, dorsoventrally flattened animals (Bogitsh and Harrison 1991). It is considered primitive in the system of the Metazoa and is placed between coelenterates and the remaining bilaterians in most phylogenetic trees (Tyler 1999). According to one classification system (Cavalier-Smith 1998) the phylum consists of two Subphyla: Turbellaria and Neodermata (Cavalier-Smith 1998). The members of the Turbellaria are generally free-living predators, dwelling in terrestrial, marine, and freshwater habitats (Bogitsh and Harrison 1991). They possess an epidermis of multiciliated epithelial cells and use their cilia for locomotion (Rieger et al. 1991). Neodermatans are all parasitic and have an epidermis apparently specialized for this lifestyle - non-ciliated, syncytial and insunk (Ehlers 1985, Tyler and Tyler 1997). Platyhelminths are hermaphrodites that transfer sperm directly onto or into a partner and discharge their eggs through the mouth, through the body wall by rupture (Rieger et al. 1991), or through a uterine pore (in Neodermata).

While the Neodermata are a well-defined monophyletic taxon (Rohde et al. 1995), systematically, the group Turbellaria has an uncertain status since it forms a paraphyletic group whose members share only pleisiomorphic features relative to the major parasitic platyhelminth taxon, the Neodermata (Trematoda, Monogenea, Cestoda) (Rieger et al. 1991). Since this violates cladistic principles,

the validity of this taxon has been questioned (Ax 1987, Ax et al. 1988) but Cavalier-Smith (1998) accepts such paraphyletic groups as useful taxonomic entities. In his system, the Turbellaria consist of two Infraphyla, Mucorhabda and Rhabditophora (Cavalier-Smith 1998). Mucorhabda possess mucoid non-lamellate rhabdoids and have either no protonephridia or ones with two cilia; Rhabditophora on the other hand have lamellate rhabdites and protonephridia with many cilia (Cavalier-Smith 1998).

The systematics of Turbellaria has proven difficult since few taxonomically useful characters can be discerned in them. Acoel turbellarians (Mucorhabda) are particularly difficult because most are entirely soft without any sclerotized parts, such as copulatory spicules, that form the basis for classification of many other groups of lower worms (Brüggemann 1986; see also Hooge and Tyler 1999). Generally, features of the reproductive tract provide the key taxonomic characters, especially the male copulatory organ (Dörjes 1968). A fairly new character for phylogenetic placement are patterns of muscles which can be revealed by phalloidin labeling of the filamentous actin within them. These patterns are surprisingly complex, and certain arrangements may be distinctive of certain taxa (see for example Tyler and Hyra 1998, Hooge and Tyler 1999, Hooge and Tyler 1998, Ladurner and Rieger 2000, Hooge in progress).

Convoluta pulchra is a generally well known species. Tyler and Rieger (1999), therefore, used it as a model specimen for muscle patterns in their work. In the same fluorescence preparations used for revealing muscles, various non-muscular structures are seen to fluoresce as well. Our goal was to identify those

structures with the prospect of being able to use them as a new and easy-to-examine character for phylogeny.

Whereas assignment of *Convoluta pulchra* to the Acoela is straight-forward, another species, *Genostoma kozloffi* (Rhabditophora) cannot be readily assigned to any higher taxon within the Rhabditophora. For some species that have been proposed to be related to it, characters of spermiogenesis and spermatozoa provide key clues of relationship (Watson and Schockaert 1996, Joffe and Kornakova 2001). In this case, our hope was that such characters could provide the synapomorphies necessary to establish that relationship.

The objective of this study was to contribute to the knowledge of these two turbellarians and to analyze characters that could be used to better establish their phylogenetic position. For *Convoluta pulchra*, this entailed identifying certain fluorescent non-muscular structures in its epidermis, and for *Geonostoma kozloffi* this entailed reconstructing the three dimensional morphology of the spermatozoa and the developmental process by which those spermatozoa arise. Both of these cases highlight the importance of basic ultrastructural studies, using fluorescence microscopy and, most importantly, transmission electron microscopy, to address questions of phylogenetics.

Chapter 2

IDENTIFICATION OF FLUORESCENT NON-MUSCULAR ELEMENTS IN THE EPIDERMIS OF *CONVOLUTA PULCHRA* (PLATYHELMINTHES: ACOELA)

2.1. Chapter abstract

Phalloidin-stained whole mounts of acoel turbellarians show brightly fluorescing club-shaped structures distributed over the epidermis and concentrated especially at the anterior and posterior tips of the body. By correlating electron micrographic images and fluorescence images of *Convoluta pulchra*, these structures can be seen to be sensory receptors with a central cilium surrounded by a collar of microvilli. The other candidate for the fluorescence in the epidermis, namely gland necks, can be ruled out since their distribution is too dense to resemble the distribution of the fluorescent structures. The collared sensory receptors were inserted between epidermal cells, and each bore a central cilium surrounded by a collar of 6–18 microvilli and an additional centrally positioned 2–7 microvilli of which 2 or 3 were associated with a modified rootlet called the swallow's nest (Bedini et al. 1973). Confocal scanning laser microscopy resolved the core of actin filaments within the microvilli of the collar and their rootlet-like connections to the base of the sensory cell. Such receptors could be identified by fluorescence microscopy in several other species of acoel turbellarians as well.

2.2. Introduction

Fluorescently tagged phalloidin is commonly used to reveal musculature because of its strong affinity for the filamentous actin (F-actin) in the contractile apparatus of muscle fibers (Tyler and Hyra 1998; Hooge and Tyler 1999; Tyler and Rieger 1999; Ladurner and Rieger 2000; Hooge 2001). Muscular structures other than the body wall that contain F-actin and, therefore, stain with this method include muscles of the male copulatory bulb and the prominent actin-rich cells of the bursal mouthpiece. Phalloidin also binds to other structures containing F-actin, however, such as the cell web in epithelial cells. Usually the signal is so strong from muscle that these other structures constitute only a kind of background noise in muscle preparations. Notable exceptions to this typically weak non-muscle staining in acoel flatworms are peg-like projections that stand out prominently in the epidermis as bright points against the background of body-wall musculature. In some publications, the peg-like structures have been identified as gland cells (Ladurner and Rieger 2000), in others as actin-rich sensory cells (Hooge and Tyler 1999), but mostly were not labeled at all. Since phalloidin-linked fluorescent stains specifically bind to F-actin (Faulstich et al. 1988), these bright points have to be rich in that form of actin. Cells in the epidermis containing structures with such a concentration of microfilaments are rhabdoid glands (in their zonulae adherentes) and collared sensory cells (in their microvilli).

The objective of this study was to identify the nature of these labeled non-muscle structures by correlating their distribution and shape in fluorescence preparations with distributions and shape of actin-containing elements of the epidermis in the higher-resolution images of the transmission electron microscope.

2.3. Materials and methods

Specimens were collected at Otter Cove, Acadia National Park, Maine, in September 1998, and extracted from the sediment using magnesium-chloride anesthetization (Sterrer 1971; Higgins and Thiel 1988).

2.3.1. Phalloidin-labeled whole mounts

Whole mounts were stained with fluorescently labeled phalloidin (Alexa 488) to reveal F-actin. Initially the animals were relaxed in magnesium chloride for five minutes and then fixed with 4% formaldehyde in 0.01 M PBS (Phosphate Buffered Saline), rinsed in PBS, and transferred onto poly-L-lysine-coated coverslips. Specimens were then permeabilized in 0.2% Triton X-100 in 0.01 M PBS for one hour and then stained with Alexa 488. After three short rinses in PBS, the specimens were mounted using Gel-Mount. The whole mounts were

examined in a Leitz Ortholux microscope with epifluorescence and images were recorded on a digital video recorder (Sony; Cohu CCD camera). Some were also examined on a Confocal Microscope (Leica TCS SP2). Images were imported into CorelDRAW 8 for Macintosh to map out individual receptors.

After fixation in 4% formaldehyde we attempted to store specimens for later staining in 30% sucrose in PBS, which turned out unsatisfactory in that the animals would tend to stick together, forming clumps, and the staining of epidermal structures was less stable. Altogether, 13 whole mounts were prepared, of which 5 were suitable for investigation of epidermal features. The density of muscle bands in very thick animals made it hard to discern epidermal features.

2.3.2. TEM fixation

TEM fixation was done with 2.5% glutaraldehyde in 0.1 M phosphate buffer and then in 1% osmium tetroxide in 0.1 M phosphate buffer under microwave-enhanced conditions. For aldehyde fixation, specimens on ice were placed in the center of a microwave oven (Samsung II, 650 Watts) and irradiated on a 7/20/7 seconds cycle (seven seconds on high- power setting, 20 seconds off, and again seven seconds on high-power setting - Giberson and Demaree 1995). Two buffer rinses in two 7/20/7 cycles followed. For the osmium fixation, the buffer was replaced by 1% osmium tetroxide and irradiated on a 7/20/7 cycle (see

also Hooge and Tyler 1999). Following dehydration in acetone, specimens were embedded in Epon-Araldite and cured at 60 °C for 24 hours.

Ultrathin sectioning was performed with glass knives as well as a diamond knife on a Sorvall MT2-B Ultramicrotome. The posterior end of one animal was sectioned sagittally as well as frontally. The serial sections were stained with uranyl acetate and lead citrate and examined with a Phillips CM10 electron microscope. Images were captured by a digital CCD camera (Advantage 12HR, AMT corp.) and imported into CorelDRAW 8 for Macintosh where individual receptors were mapped out.

2.4. Results

2.4.1. Fluorescent images

Within the body wall, distinct close-woven layers of muscles were observed in phalloidin-labeled specimens. The epidermis, distinguishable through a fainter labeling of the cell web, covered this layer. At low-magnification, bright points could be observed across the body, but they were especially concentrated at the posterior tip (Figs. 2.1A, 2.1B). Another concentration could be found at the anterior tip; fewer points occurred across the mid-part of the body. In profile at the margins of the whole mounts, their shape was brush-like (Fig. 2.1B). They extended through the epidermis and slightly beyond the epidermal cell web. In views looking down on the surface of the epidermis, mainly points of indistinct

shape appeared, but sometimes they were elongated and teardrop or club-like. Some fluorescent images exhibited detailed structure within the club-shape. Especially in the higher-resolution confocal images (Fig. 2.1C) the shape of these structures could be discerned as resembling a stem with branches. No obvious pattern of distribution could be found. Also, depending on the shape of the posterior tip of the specimen (i.e., on various fixations) more or fewer points could be discerned, making comparisons with TEM images difficult.

2.4.2. TEM images

Rhabdoid gland cells as well as collared sensory receptors were traced through the same series of sections as far as it was possible. The density of the gland cells was approximately five times as high than the density of collared sensory receptors (Fig. 2.2), and both appeared to be randomly distributed.

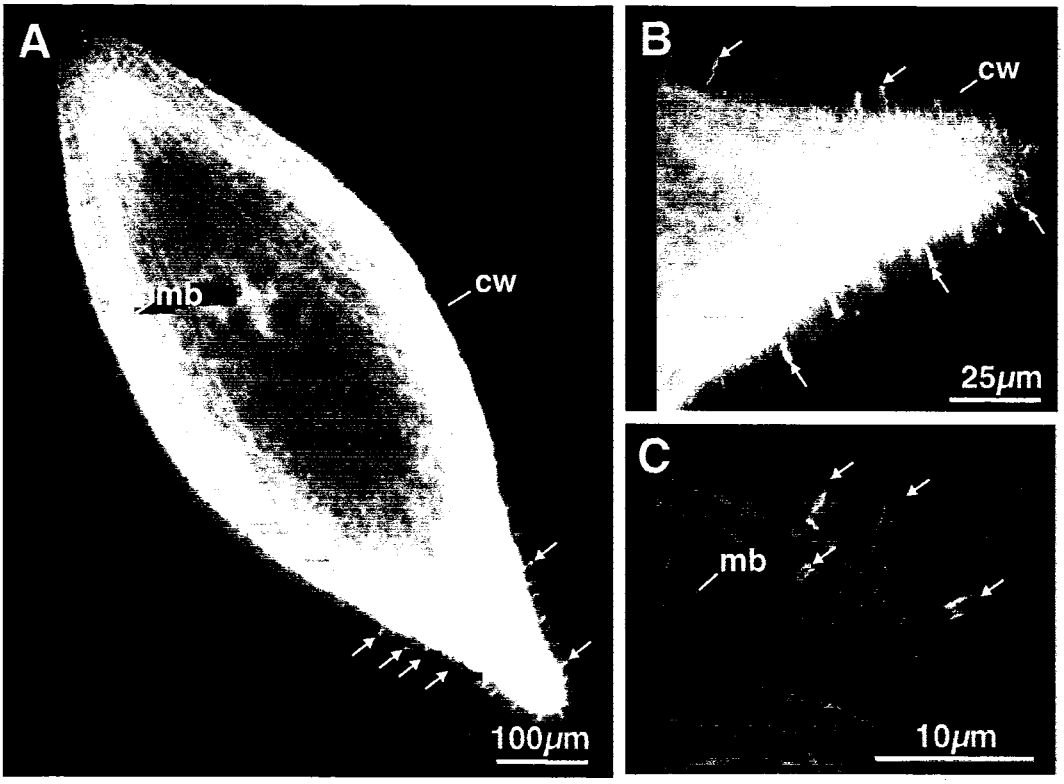

Figure 2.1. -Whole mount of *Convoluta pulchra* stained with phalloidin-Alexa 488. - **A.** Whole mount of immature *Convoluta pulchra.* The epidermal cell web (cw), muscle bands (mb), and other non-muscular, fluorescent peg-like structures (arrow) fluoresce. Scale bar: 100 μm. - **B.** Enlargement of the posterior end of a whole mount showing the peg-like shape of the fluorescent structures (arrow) at the margins, protruding slightly beyond the epidermal cell web (cw). Scale bar: 25 μm. - **C.** Confocal optical section of phalloidin-labeled whole mount showing brush-like projections above the muscles of the body wall, which conform well to the arrangement of microvilli within collared sensory cells. Scale bar: 10 μm

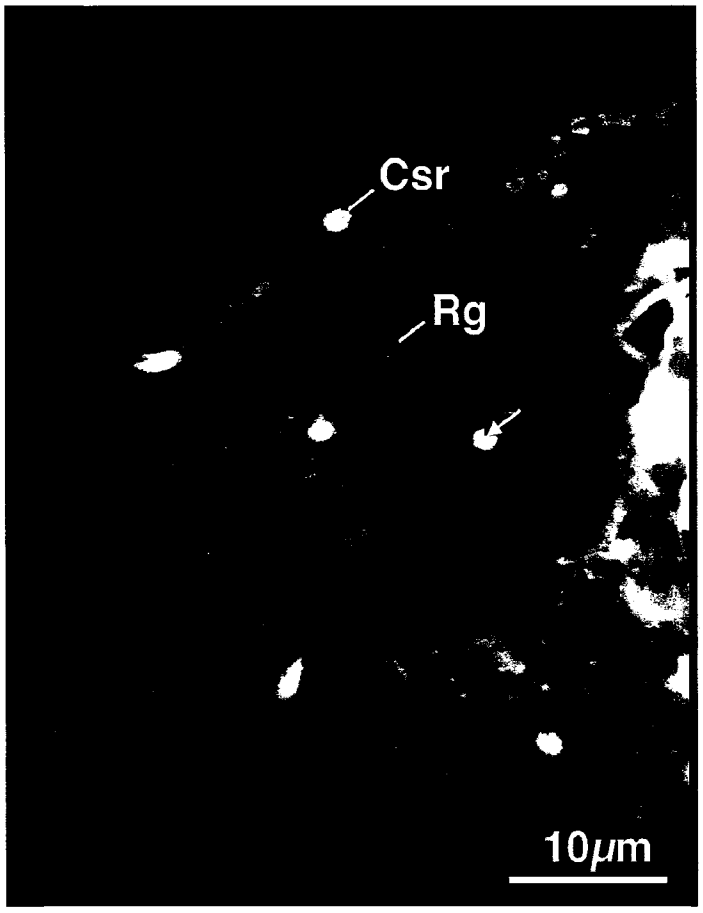

Figure 2.2. - Confocal optical section of *Convoluta pulchra* differentiating the zonulae adherentes of gland necks (Rg) and the more densely stained and more widely scattered sensory receptors (Csr). The receptors comprise a ring-like array of microvilli forming a collar and a few central microvilli within the collar (arrow). Scale bar: 10 μm.

2.4.3. Ultrastructure of monociliary collared sensory receptors

Collared sensory receptors, first described by Bedini et al. (1973) in *Symsagittifera psammophila*, are sensory cells bearing a collar of microvilli surrounding a central cilium. Like collared sensory receptors described by Bedini et al. (1973), those we found in *Convoluta pulchra* were inserted in between epithelial cells (Figs. 2.3, 2.4, 2.5) and appeared pear-shaped in TEM sections, exhibiting a narrowed apex (1.0 μm in diameter), a wide basal cell body containing the nucleus, and a narrowed process leading basally into the muscle layer (Figs. 2.3, 2.5). It was connected to the adjoining epidermal cells through a zonula adherens and a septate junction and exhibited some differences from epithelial cells. The central cilium in the receptor cell appeared stiffly straight and possessed a modified rootlet, the so-called swallow's nest. The receptor cell lacked epitheliosomes, which contain mucus and can also bear pigment (Tyler 1984). The microvilli of epidermal cells were considerably shorter and narrower (0.8 μm long by 0.07 μm wide - Rieger et al. 1991) than the microvilli present in receptor cells (2.6 μm long by 0.1 μm wide). The collar of a receptor cell bore 8–16 microvilli (Figs. 2.4, 2.6). There was no indication that receptors with a specific number of microvilli are confined to certain areas of the body. Receptors with high and low numbers of microvilli could be found in all body regions (posterior, anterior, medial). The central cilium (0.2 μm wide by 5μm long) protruded from a level slightly lower than the microvilli and the epithelial surface (Fig. 2.7).

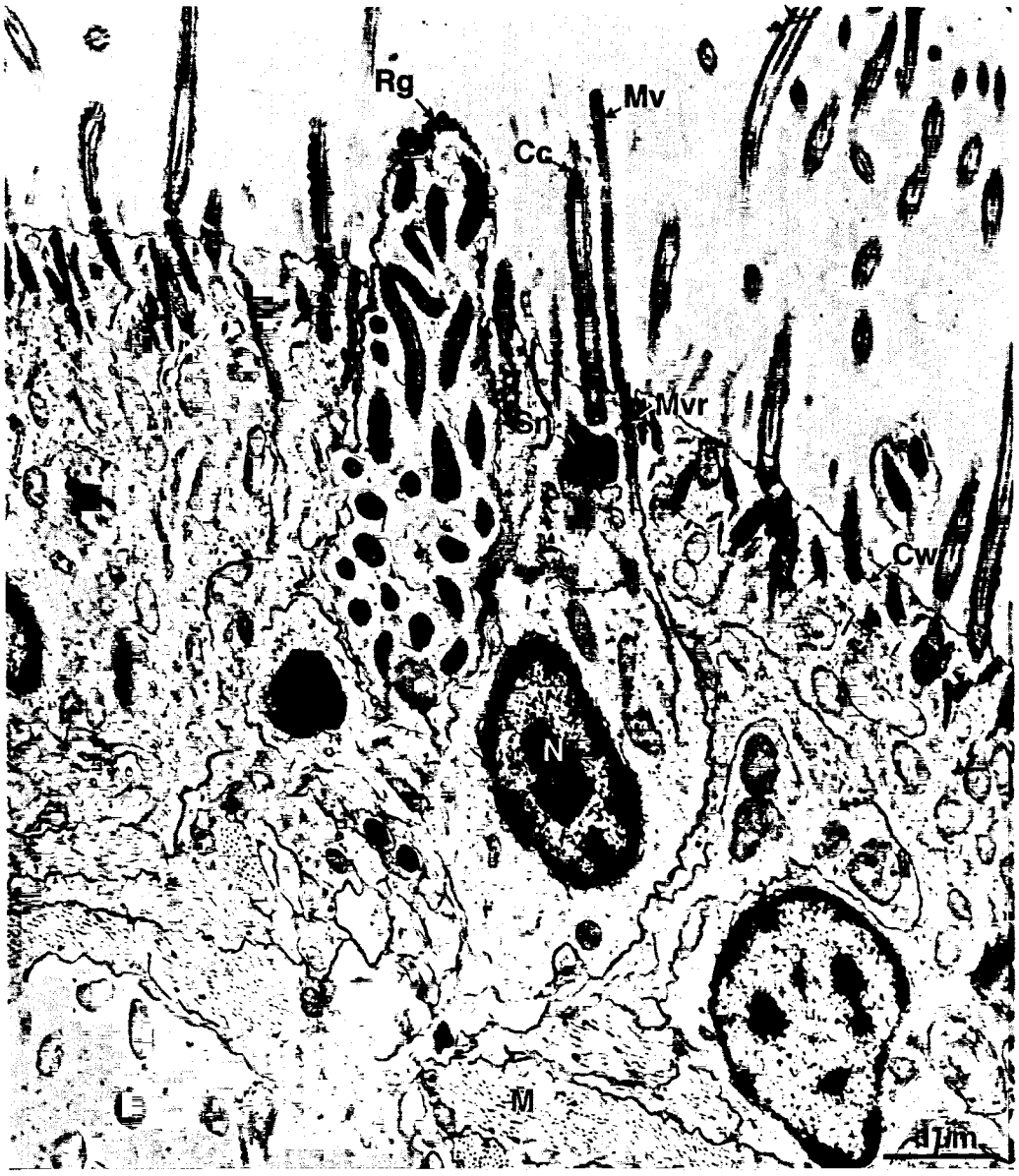

Figure 2.3. - Transmission electron micrograph of the epidermis of *Convoluta pulchra* through the neck of a rhabdoid gland (Rg) and a collared sensory receptor cell with its central cilium (Cc), swallow's-nest ciliary rootlet (Sn), microvilli (Mv), and microvillar-rootlets (Mvr). (Cw) cell web; (M) muscle; (Mt) mitochondria; (Za) zonula adherens. Scale bar: 1 µm.

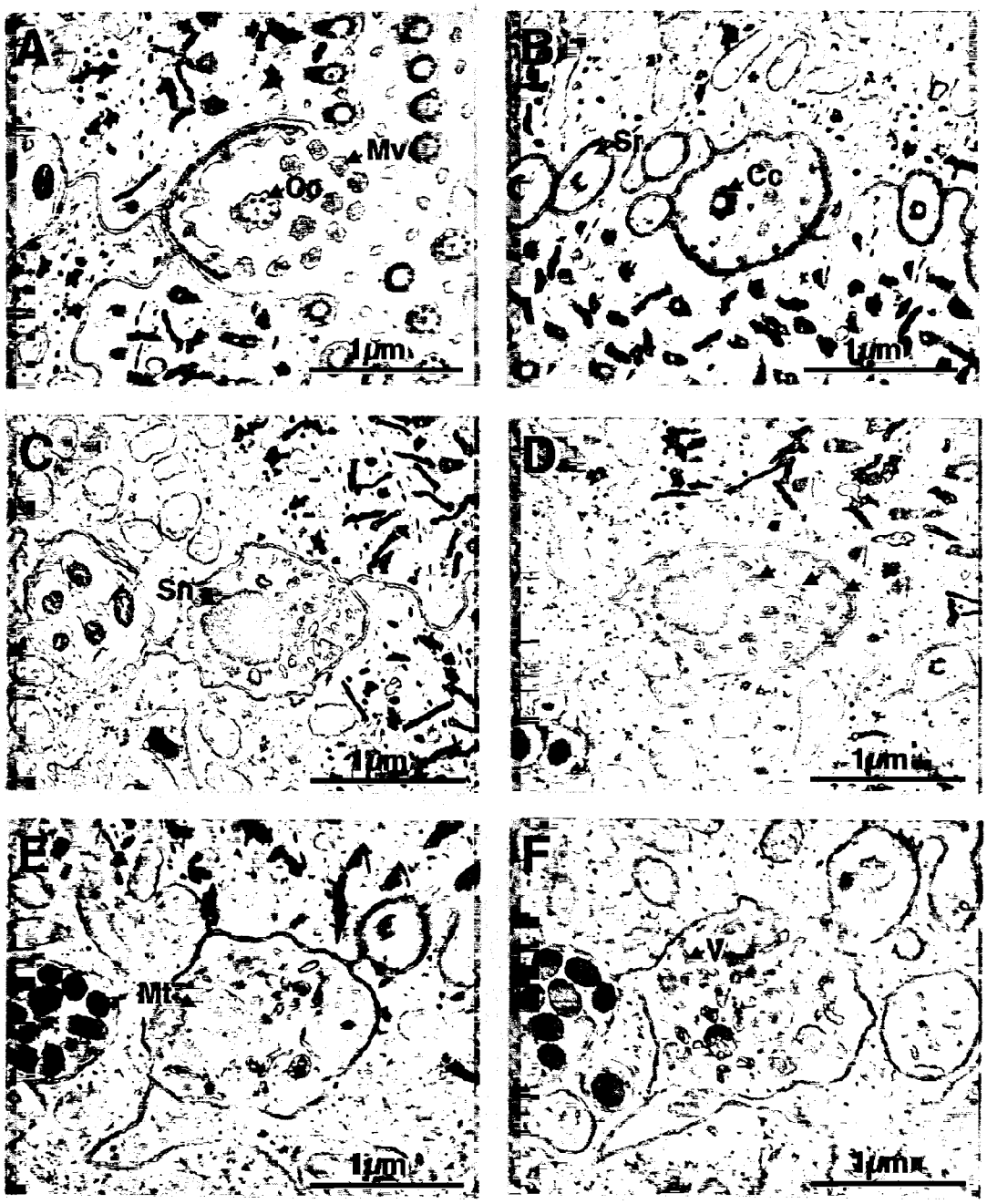

Figure 2.4.

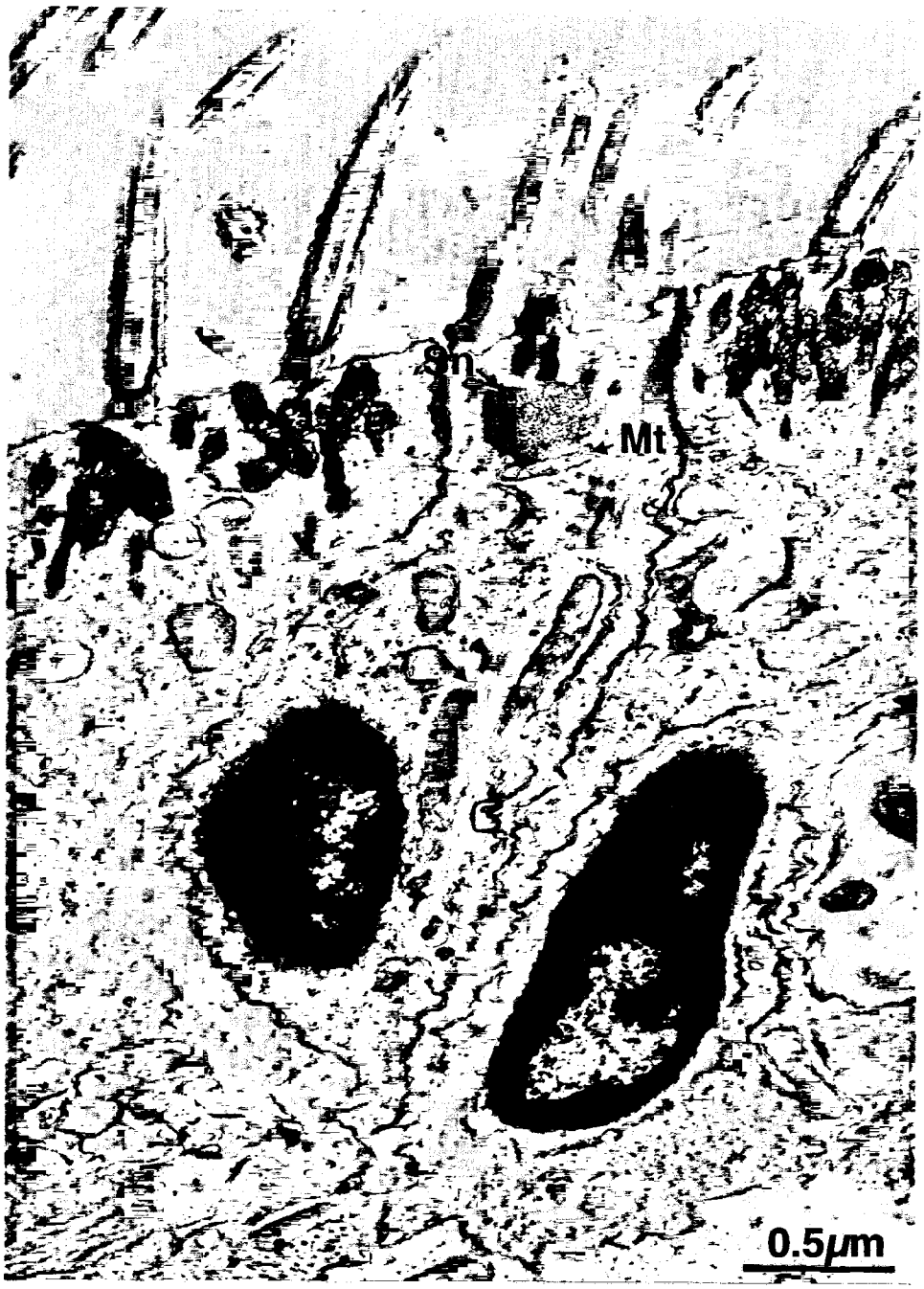

Figure 2.5. - Slightly oblique longitudinal TEM section through a collared sensory cell of *Convoluta pulchra*. The rootlets of the microvilli unite at the level of the nucleus (arrow). (Sn) swallow's nest; (Mt) mitochondria. Scale bar: 0.5 μm.

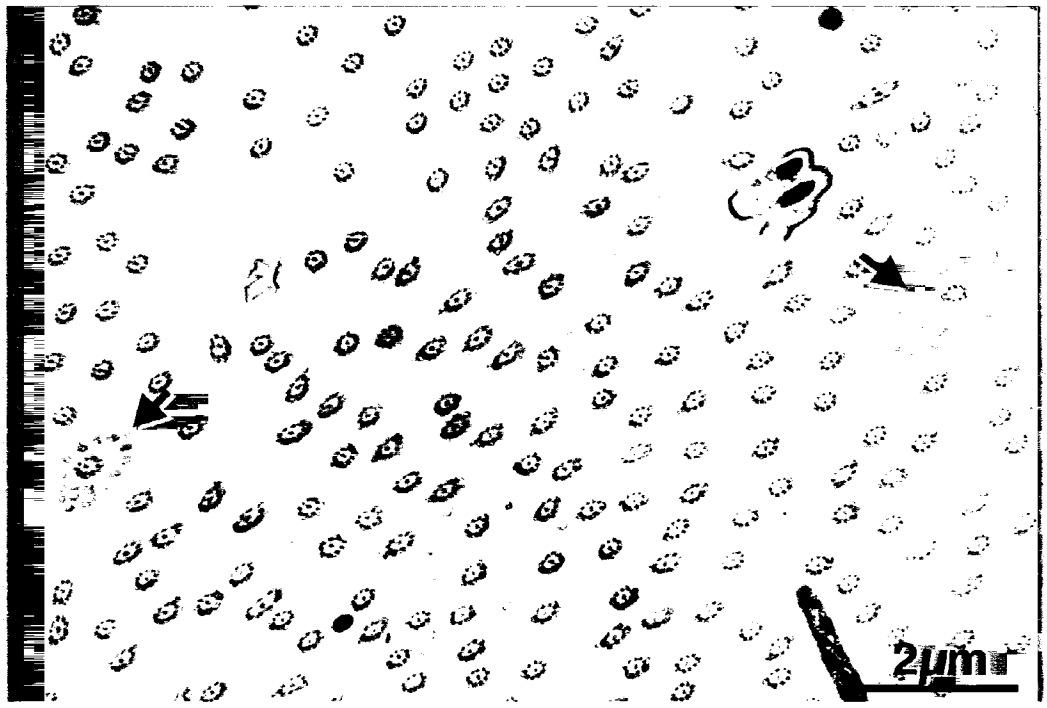

Figure 2.6. - Section tangential to epidermal ciliary fields and showing cross sections of *Convoluta pulchra* through collared sensory cells (arrow) epidermal cilia and microvilli from the posterior end of the animal. Scale bar: 2 µm.

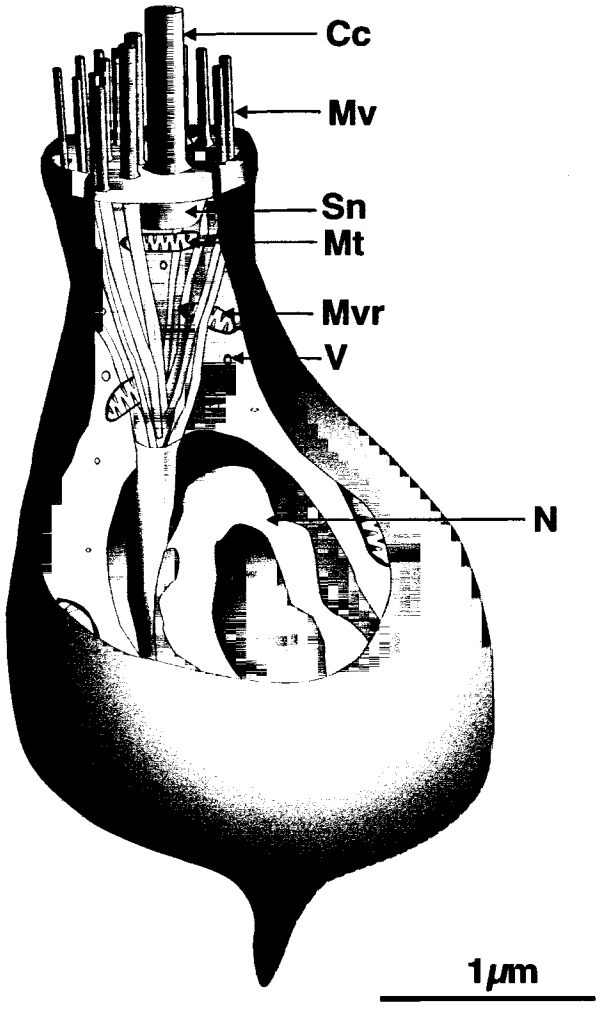

Figure 2.7. - Three-dimensional reconstruction of a collared sensory receptor cell of *Convoluta pulchra*. (Mt) mitochondria, always found underneath the swallow's nest; (Mv) microvilli; (Mvr) microvilli rootlet; (N) nucleus; (Sn) swallow's nest; (V) vesicles. Scale bar: 0.5 μm.

Between the central cilium and the collar of microvilli a number (between 2 and 7) of other large microvilli were inserted (Figs. 2.4, 2.6). Two or three of those (approximately 0.14 μm in diameter) were associated with a structure underlying the central cilium, the swallow's nest (Fig. 2.4C; terminology of Bedini et al. 1973). The swallow's nest was a bowl-shaped mass (0.46 μm in diameter) and consisted of densely packed granules (Fig. 2.3, 2.4C, 2.4D, 2.5).

Whereas the two or three microvilli associated with the swallow's nest showed only a slight enlargement (27% thicker) over the other microvilli (collar microvilli and other central microvilli) their rootlets were considerably larger (Fig. 2.4B, 2.4C, 2.4D), with a diameter of approximately 0.12 μm compared to 0.07 μm for the rootlets of the collar microvilli (i.e., 71% thicker).

All rootlets of the microvilli, collar microvilli as well as central microvilli, united at the level of the nucleus, forming a single bundle there (Fig. 2.5).

At least one mitochondrion was always positioned underneath the swallow's nest; and a large number of vesicles were distributed throughout the cell (Figs. 2.3, 2.4B, 2.4C, 2.4D, 2.4E, 2.4F, 2.5).

Though several receptors could be found in proximity to nerves (Figs. 2.3, 2.5), synapses on the receptor could not be observed.

2.5. Discussion

2.5.1. Correlation of fluorescence and TEM images

By comparison of shape and distribution patterns, it seems highly likely that the bright points in the epidermis of phalloidin-stained *Convoluta pulchra* correspond to monociliary collared sensory receptors. Collared sensory receptors contain a high number of large microvilli whose rootlets reach through the thickness of the epidermis, forming an elongate brush-like structure (Fig. 2.5). These microvilli bear a core of microfilaments, F-actin as indicated by the phalloidin staining.

In several regions, the shape of the fluorescing structure can be discerned, being clearly elongated and forming a brush-like shape at the tip (Fig. 2.1B). Especially in the confocal images (Fig. 2.1C) the brush-like form matches the arrangement of the microvilli and their rootlets in collared sensory receptors (Fig. 2.8). The F-actin-rich zonula adherens in gland cells is located apically in the cell and would not exhibit an elongate shape but rather a ring shape, which could also be detected in fluorescence images (Fig. 2.2; see also Ladurner and Rieger 2000); also the distribution of the gland cells, is far too dense to possibly match the bright spots in phalloidin-labeled whole mounts. Neither other receptors nor rhabdoid gland cells possess such a concentration of F-actin throughout the entire cell. They only exhibit a concentration in the zonulae adherentes, very shallow at the surface of the epidermis.

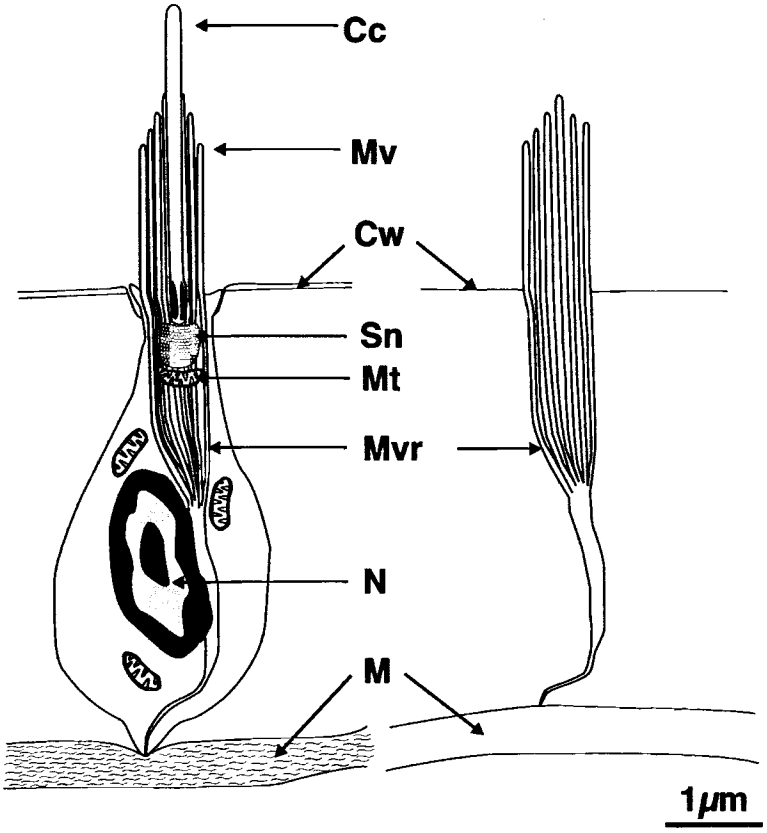

Figure 2.8. - Correlation of the appearance of the collared sensory receptors in fluorescence images (right) with that in electron microscopic images (left) of *Convoluta pulchra*. - **Right**. The microvillar-rootlets (Mvr) unite at the level of the nucleus (N), forming a single bundle extending to the base of the cell. (M) muscle layer; (Mt) mitochondria; (Mv) microvilli; (Sn) swallow's nest. - **Left**. The shape of the projections is brush-like, extending beyond the cell web (Cw) and narrowing down at approximately the same level where the microvillar-rootlets unite. The cores of the microvilli, as well as their rootlets, fluoresce, because they consist of bundles of filamentous actin. Scale bar: 1 μm.

While the number of receptors in TEM reconstruction and fluorescent structures in fluorescent images do not match completely, such a difference could be just a matter of the sizes of the investigated animals. More important is that the gland cells occur in far too high numbers to correlate with the bright spots in fluorescence images. Therefore, the most likely structures causing the fluorescence are the collared sensory receptors.

2.5.2. Comparison of monociliary collared sensory receptors in *Convoluta pulchra* with those in *Symsagittifera psammophila*

Bedini et al. (1973) described monociliary sensory cells in turbellarian acoels, one of which was *Symsagittifera psammophila*. Generally their description of this species' receptors conforms well to the collared receptors found in *Convoluta pulchra* (Fig. 2.7). In both species, the sensory cells are inserted in between epithelial cells, rather than being enwrapped by them as occurs with receptors of higher turbellarians, the central cilium of the collared receptors possesses a modified rootlet, the swallow's nest (Bedini et al. 1973), and both have a collar of microvilli (Figs. 2.3, 2.4, 2.5).

However, *Symsagittifera psammophila* possesses an "insunk" epithelium, which means that most nucleated portions of the cells are located underneath the muscle layer (Tyler 1984). *C. pulchra* does not possess such an epithelium but simple cuboidal epidermal cells with the cell bodies above the muscle layer (Fig.

2.3). This fact could be due to a greater degree of development of the muscle layer in *C. pulchra*, so packed that it confines the cell bodies to the epidermal layer. *S. psammophila* does not possess such a dense muscle layer, and this provides a potential for the cell bodies to "slip" through it, since, as in all acoels, no basal lamina is present.

The number of microvilli forming the collar also seems to be of greater variability in *C. pulchra*, ranging from 8 to 16 (Fig. 2.6), compared to 16 – 18 in *S. psammophila*. It might be possible that those variations in the number of microvilli are related to either the developmental stage or size of the animal as well as the size of the receptor itself. On the other hand it might be a true variability occurring in collared sensory cells of *C. pulchra*. Generally, stable numbers can be observed in higher turbellarians, such as members of the Proseriata and Rhabdocoela, which possess collared sensory receptors that always have eight microvilli forming the collar (Proseriata: see Bedini et al. 1975; Ehlers 1977; Ehlers and Ehlers 1977; Rhabdocoela: see Reuter 1975; Schockaert and Bedini 1977).

Also, a greater variability, not described in Bedini et al. (1973), within the central microvilli was apparent in *Convoluta pulchra*. In addition to the two or three microvilli associated with the swallow's nest, up to five clearly centered microvilli, which did not seem to belong to the collar, were observed. They seemed intermediate between the collar microvilli and the swallow's-nest-associated microvilli in diameter (0.12 μm) as well as in diameter of their rootlets (0.10 μm). We could not determine whether all microvilli are of the same length

or if those central microvilli intermediate in width are also intermediate in length, but since the diameter of their rootlets was intermediate this is a possibility.

The entire construction of the receptor cell clearly showed a polarity of the microvilli with respect to the central cilium, with the central microvilli concentrated on one side of the swallow's nest. This structure suggests the abitity to detect the direction of stimuli (Bedini et al. 1973).

Believing the morphology relates to the functional activity of these receptors, Bedini et al. (1973) suggest a hypothesis concerning reception and transduction of stimuli: The stimulus can come from deformation or displacement of the basal body relative to the modified rootlet. This hypothesis is similar to the one proposed by Horridge (1969) for the receptors of Ctenophora. It implies that the microvilli may act only as a frame for the mechanical anchorage of the modified rootlet.

If the possibility is accepted that the microvilli-central-cilium polarization could be a morphological feature correlated with their function, a comparison with cochlear hair cells in vertebrates (Hudspeth 1989) should be considered.

If the microvilli that are closer to the central cilium of collared sensory cells turn out to be, in fact, longer than the collar-microvilli (which is implied by the different diameters of microvilli in different positions), one could expect a depolarization of the cell membrane if a mechanical deformation of microvilli in the direction of the central cilium occurs, a hyperpolarization at an opposite deformation (see also Flock 1965).

2.6. Conclusion

Considering that the fluorescing brush-like structures in the epidermis of phalloidin-labeled fluorescence preparations of *C. pulchra* match the structure and arrangement of the collar of microvilli in the monociliated collared sensory receptors discerned by TEM, these structures must be the self-same sensory receptors.

Chapter 3

SPERM AND SPERMIOGENESIS IN *GENOSTOMA KOZLOFFI* (PLATYHELMINTHES, RHABDOCOELA)

3.1. Chapter abstract

Genostoma kozloffi is a symbiotic turbellarian living underneath the carapace of *Nebalia* sp., a leptostracan crustacean. Its spermiogenesis occurs in a distal-proximal fashion, just as in free-living turbellarians in which the axoneme is fully incorporated. Mature spermatozoa are filiform, possess an elongate rod-like nucleus and one short single, fully incorporated, axoneme. A rod of multiple, fused mitochondria accompanies the nucleus and axoneme, and an array of cortical microtubules with thickened walls is present. Neither dense bodies nor acrosomal vesicles could be found. The phylogenetic position of *Genostoma kozloffi* has been in dispute because of its similarities to three different groups of flatworms: its epidermis is like that of the Neodermata, its pharynx and genitopharyngeal atrium are arranged like those of opisthoporate prolecithophorans, and the arrangement of reproductive structures, the presence of a tunica surrounding its testes, and the ultrastructure of its spermatozoa are like those of the Kalyptorhynchia. We found evidence in sperm and spermiogenesis that links *Genostoma kozloffi* to Kalyptorhynchia, specifically to the Schizorhynchia.

3.2. Introduction

While the Neodermata constitutes a clearly monophyletic group within the Platyhelminthes and must have arisen from a turbellarian-like (or "free-living plathelmith") ancestor, just which turbellarian taxon would be the outgroup for the Neodermata is far from clear. Several small groups of aberrant turbellarians, with uncertain systematic position themselves, have been proposed to occupy that out-group position. Among them are the Fecampiidae (as suggested by Ehlers 1985, Xylander 1989, and Watson and Rohde 1993a,b), *Urastoma* (as suggested by Watson 1997), and *Notentera* (as suggested by Joffe and Kornakova 1998, Kornakova and Joffe 1999). These turbellarians are all parasitic, and they share not only this lifestyle but also a peculiar feature of spermiogenesis with the Neodermata, namely that the spermatids develop with a polarity that is the reverse of that of the free-living turbellarians. While turbellarian spermatozoa develop in such a way as to position the basal bodies of the axonemes at the distal end of the spermatid and the nucleus proximal, those of the Neodermata, Fecampiidae, *Urastoma*, and *Notentera* develop with the basal bodies at the proximal end, and the nucleus moves to the distal end of the spermatid before the spermatid detaches from the cytophore (from which multiple spermatids develop). The sharing of this character in spermiogenesis of the Neodermata and these particular parasitic turbellarians led Kornakova and Joffe (1999) to group them together in a taxon they called the Revertospermata.

Another candidate for the Revertospermata is the Genostomatidae, a group of turbellarians symbiotic with leptostracan crustaceans and teleosts (Hyra 1993, Syromjatnikova 1949). *Genostoma kozloffi* in this family (and presumably other members as well; Syromjatnikova 1949) has an epidermis that, alone among turbellarians, is syncytial in the same way as is the epidermis of the Neodermata. By virtue of its similarities to *Urastoma*, including a plicate pharynx and a common oro-genitial pore at the posterior end of the body, Joffe and Kornakova (2001) placed the Genostomatidae in the Revertospermata. Whether the sperm actually develop in the revertospermatan fashion, however, was not known.

In general, platyhelminth sperm are biflagellate and lack a distinct head or tail (Watson and Rohde 1995). The basal bodies of the flagella typically lie at one end of the sperm; the flagella can be of a variety of lengths and either be free, superficially attached, incorporated within the sperm body, or secondarily lost. Most platyhelminth sperm show a number of "longitudinal cortical microtubules" just underneath the cell membrane. Mitochondria can be found in most of the taxa in various forms, for example scattered throughout the sperm body, arranged in one or more rows, or fused together into rods. Many free-living taxa also possess "dense bodies," granules of various composition and size (Watson 1999). The flagellar end of mature platyhelminth sperm is commonly referred to as anterior, the end containing the nucleus as posterior; the end that detaches last from the cytoplasmic mass is referred to as proximal, the opposite end as distal (Watson 1999).

Sperm of *Genostoma*, as well as of some kalyptorhynch turbellarians and cestode neodermatans, have a single axoneme, presumably by secondary loss of the second axoneme, and this style axoneme of the 9+'1' structure of microtubules (Hyra 1994) is characteristic of the large group Trepaxonemata that includes higher rhabditophorans and neodermatans. The sperm are filiform, in excess of 100 μm long, and bear cortical microtubules and rod-like mitochondria (Hyra 1994).

We have studied the spermiogenesis of *Genostoma kozloffi* to determine whether it is properly included in the Revertospermata. If it were to have reverse polarity in spermiogenesis, then this character, in combination with that of its neodermis-like epidermis, would make it the best model for the outgroup to the Neodermata. As intriguing as such a position would be, we found, instead, that spermiogenesis follows a course of turbellarian-type genesis.

3.3. Materials and methods

Specimens of *Nebalia pugettensis* were collected by E. N. Kozloff on San Juan Island, WA, near Argyle Lagoon in November of 1992. They were brought to Maine and dissected for symbionts. One small and 7 large specimens of *Genostoma kozloffi* Hyra, 1993, were gathered and fixed according to the procedure of Eisenmann and Alfert (1982). Specimens were prefixed in glutaraldehyde/osmium prefixative for 10 minutes, fixed in 4% glutaraldehyde for

one hour and rinsed in 3 buffer washes for a total of 30 minutes. Afterwards, specimens were postfixed in 1% osmium fixative for one hour, rinsed in distilled water, dehydrated in a series of consecutively stronger solutions of ethanol and embedded in Epon-Araldite. Serial sections were prepared using a Sorvall MT2-B ultramicrotome. They were stained with uranyl acetate and lead citrate and examined with a Phillips CM10 electron microscope. Images were captured with a digital CCD camera (Bioscan, Gatan).

3.4. Results

As already described by Hyra (1993), the paired testes of *Genostoma kozloffi* are proportionally large in size and lie immediately posterior to the brain. They are bound by a tunica and contain several follicles. The follicles do not appear tightly packed but separated by narrow spaces, which contain spermatids and mature spermatozoa. The two testes are separated from each other by the anterior intestine. The sperm ducts emerge posterolaterally from the testes and run along the lateral margins of the body to the seminal vesicles. The seminal vesicle is located slightly to the right of the body midline and is ovoid in shape. From there the ejaculatory duct leads to the prostatic gland and then to the sclerotized penis stylet. The male gonopore can be found in the genitopharyngeal atrium dorsal to the pharynx.

3.4.1. Spermiogenesis

Spermatocytes of *Genostoma kozloffi* appear very large in size and contain nuclei with a loose arrangement of heterochromatin and a homogeneous cytoplasm with only a few mitochondria. Occasionally, Golgi complexes could be observed as well. Spermatocytes located more anteriorly appear to be more differentiated than posterior ones (Fig. 3.1). They also appear larger in size and arranged more loosely than posterior ones (Fig. 3.1).

In the course of differentiation, the spermatocytes undergo mitotic division without cytokinesis and then harbor a number of nuclei within the same cytoplasmic mass, the so-called cytophore (Fig. 3.2). A number of tightly packed concentric arrays of rough endoplasmic reticulum appear as well (Fig. 3.2). The nuclei migrate close to the cell membrane setting off a peripheral zone of differentiation from the central mass of cytoplasm; next to the nucleus lie two perpendicular centrioles (Fig. 3.3.1). The centrioles pose as basal bodies, of which only one will later develop a flagellum.

A number of mitochondria become apparent as the cytoplasm forms an outgrowth into which basal bodies, nucleus, and mitochondria migrate. This process occurs simultaneously at multiple sites along the edge of the cell (Fig. 3.4). Along the margins of these outgrowths, microvillus-like protrusions become apparent (Fig. 3.4A, 3.5B), which might have nutritive function (Lundin and Hendelberg 1998).

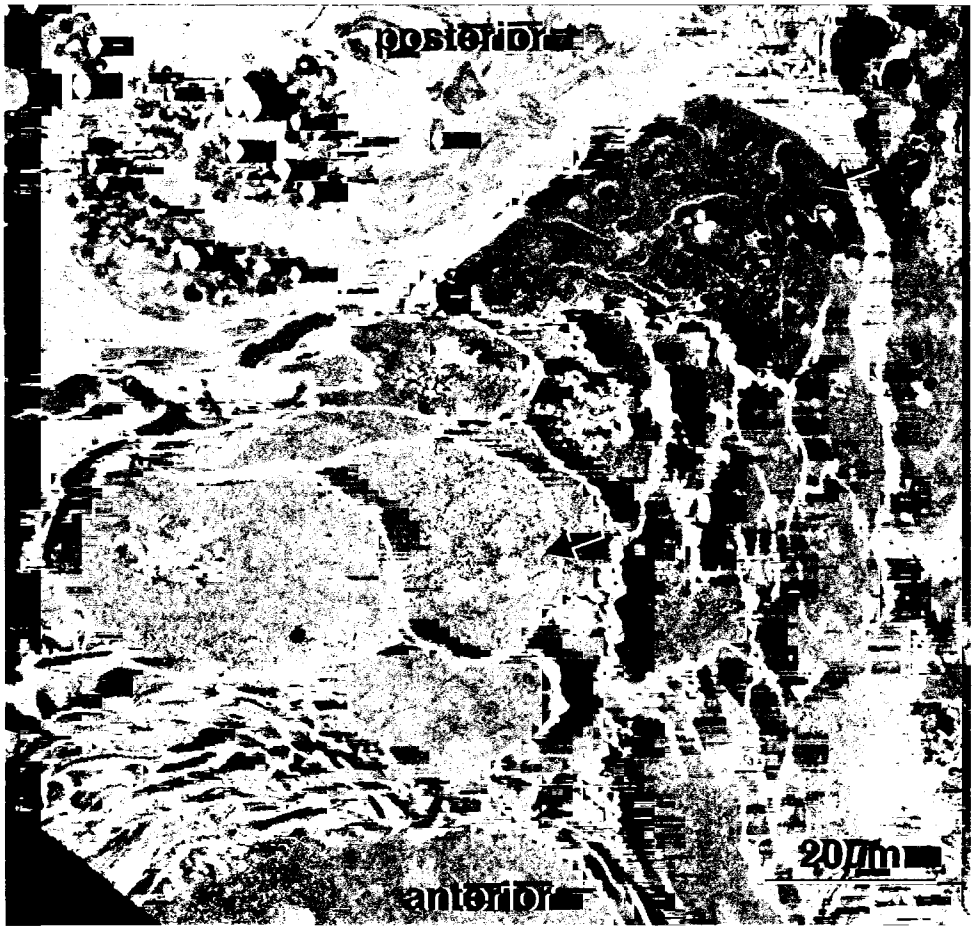

Figure 3.1. - Longitudinal TEM section through the left testis of *Genostoma kozloffi*. More posteriorly located spermatocytes are less differentiated, smaller and more tightly packed than anterior ones (arrows). Scale bar: 2µm.

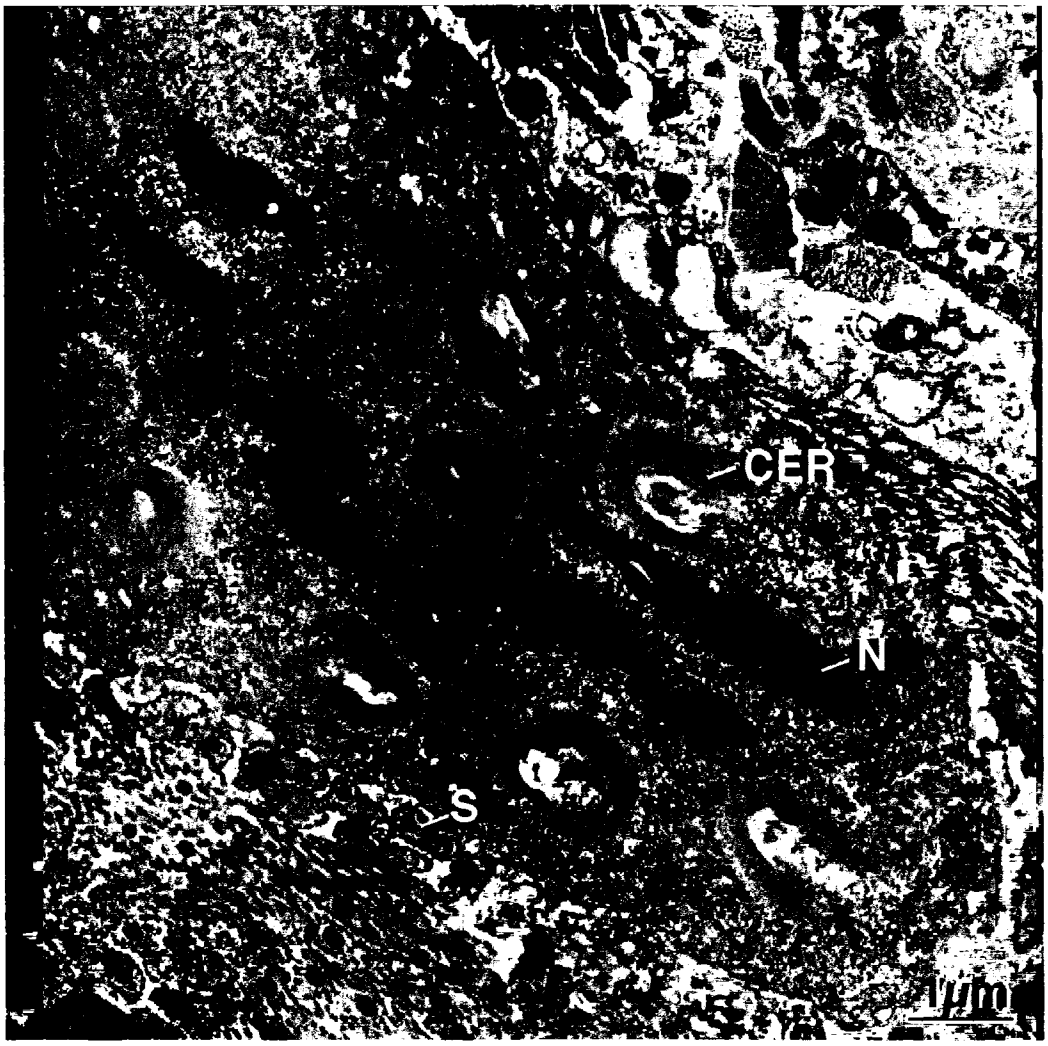

Figure 3.2. – TEM section through a cytophore of *Genostoma kozloffi*. A number of condensed nuclei (N) are visible as well as several concentric rings of rough endoplasmic reticulum (CER). At all margins of the spermatocyte cross sections of immature spermatozoa (S) can be observed. Scale bar: 1µm.

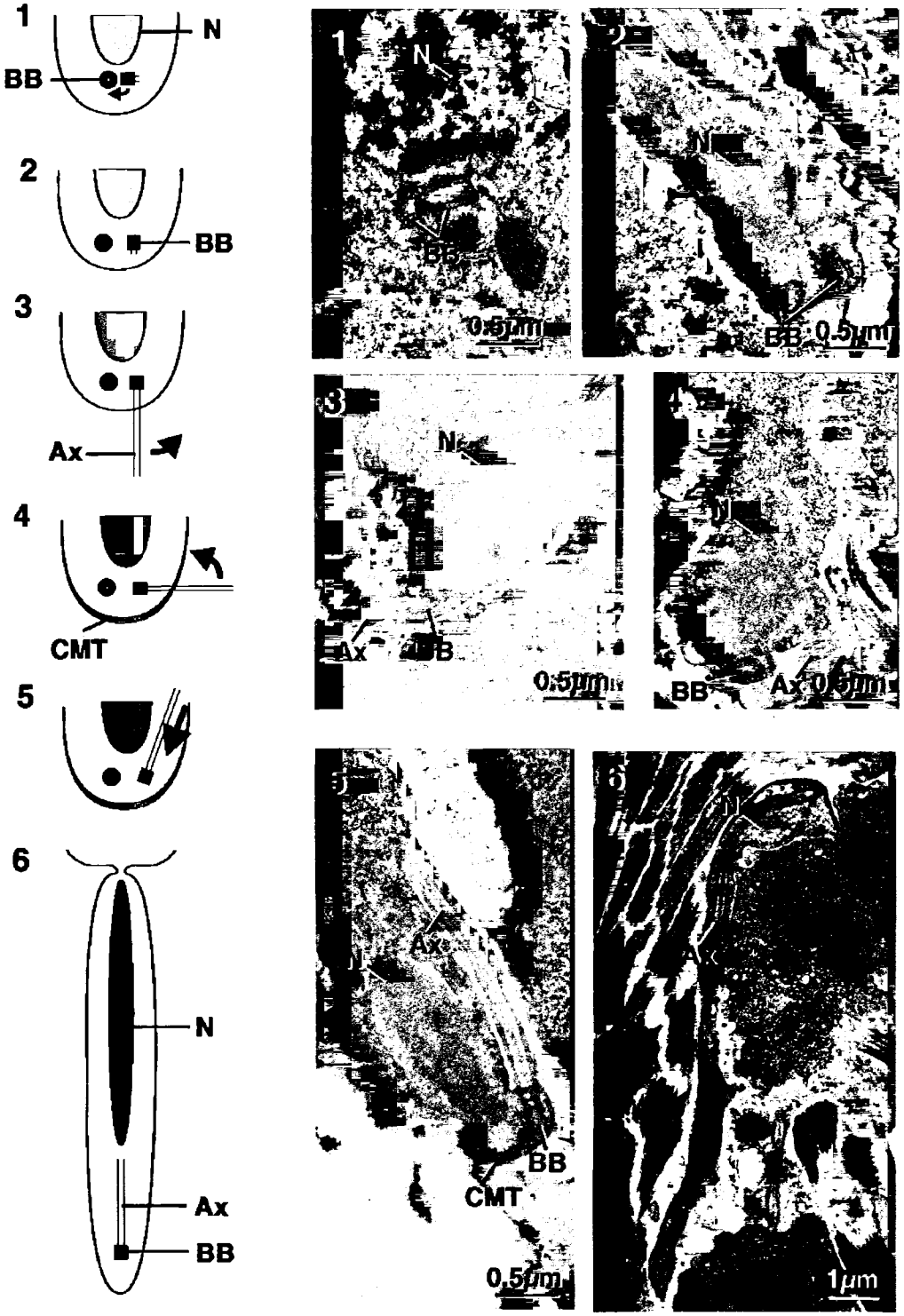
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Figure 3.3.

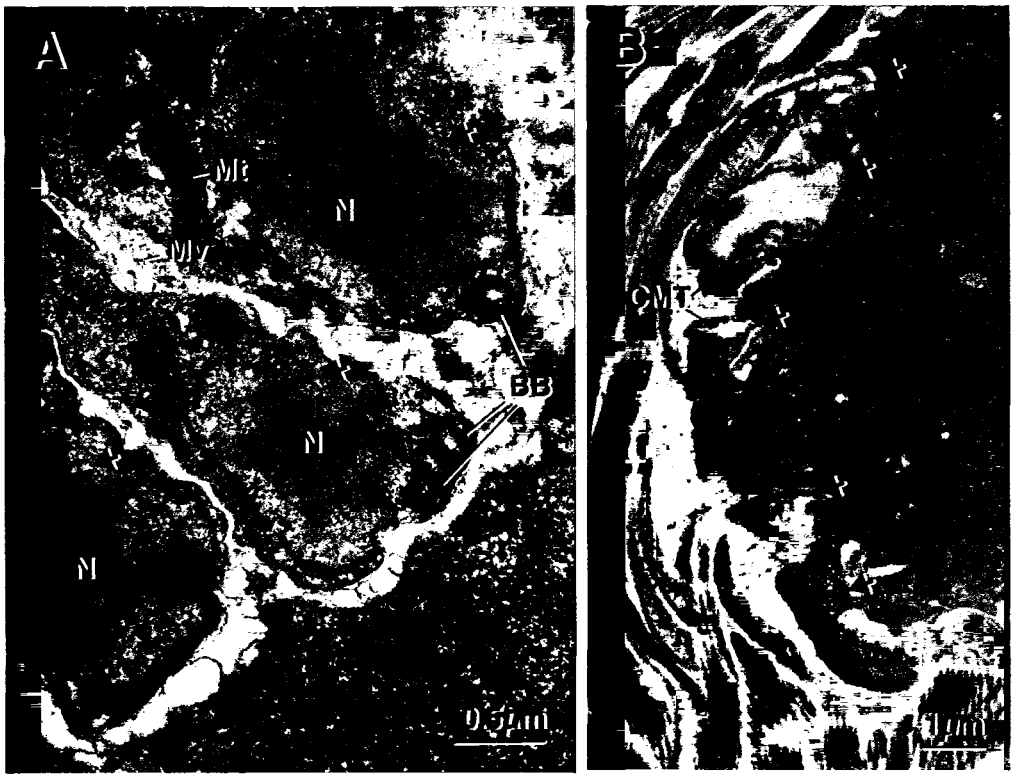

Figure 3.4. – TEM sections through a cytophore of *Genostoma kozloffi* exhibiting a number of cytoplasmic outgrowth. – **A**. The arrows indicate a number of cytoplasmic outgrowths which will develop into spermatids. (BB) basal bodies; (Mt) mitochondria; (Mv) microvillus-like protrusions; (N) nucleus. Scale bar: 0.5µm. – **B**. The arrows indicate spermatids which are still connected to the residual cell. Microtubules (CMT) forming an electron-dense ridge can be seen. (Ax) axoneme; (N) nucleus. Scale bar: 1µm.

At this stage, one basal body rotates at a 90 degree angle but still stays perpendicular to the other basal body. The rotated basal body is also the one that develops a free flagellum (Fig. 3.2).

Once the flagellum has elongated, it starts rotating proximally (Fig. 3.3) and eventually becomes incorporated into the spermatid-outgrowth and then lies alongside the condensed nucleus (Fig. 3.4). At the distal tip of the outgrowth, an electron-dense ridge forms that appears to be a series of microtubules, all in close proximity to the basal body that does not develop a flagellum (Fig. 3.5). These microtubules give rise to the array of large parallel longitudinal microtubules, the cortical microtubules, found just beneath the cell membrane of the mature spermatozoon.

As is characteristic for turbellarians, the basal body of the flagellum moves distally while the nucleus remains in its proximal position (Fig. 3.6). Distal-proximal fusion occurs, and the nucleus sits proximal to the axoneme (Fig. 3.5). In cross sections of these elongating spermatids (Fig. 3.5A), the spermatid closest to the cytophore exhibits a cross section of the nucleus alongside the fully rotated, but not fully incorporated axoneme; consecutively more distal cross sections through spermatids at this same stage show axonemes that are fully incorporated. Since these axonemes are fully incorporated, they appear to represent a stage after the axoneme rotation was completed.

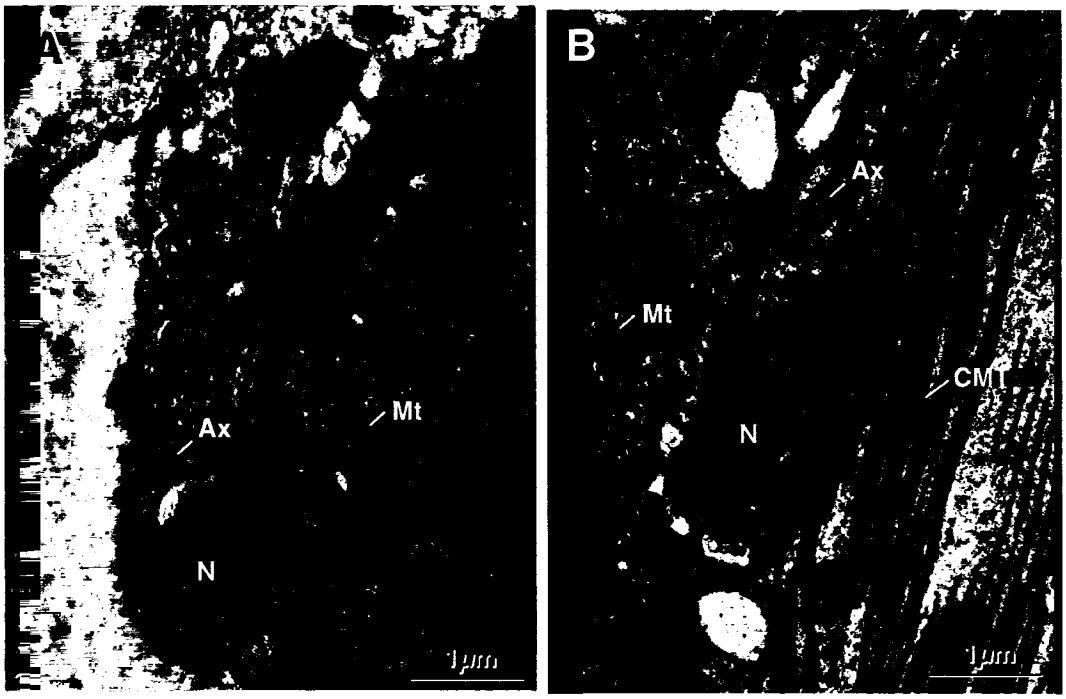

Figure 3.5. – TEM sections though spermatids of *Genostoma kozloffi*. – **A**. Cross sections through a spermatid at the stage described in Fig.3.5. with the axoneme (Ax) incorporated alongside the nucleus (N) and additional, more distal sections of fully incorporated axonemes (arrows). Scale bar: 1µm. – **B**. Longitudinal section through a spermatid. The nucleus remains proximally whereas the basal body and the axoneme have moved distally. Scale bar: 1µm.

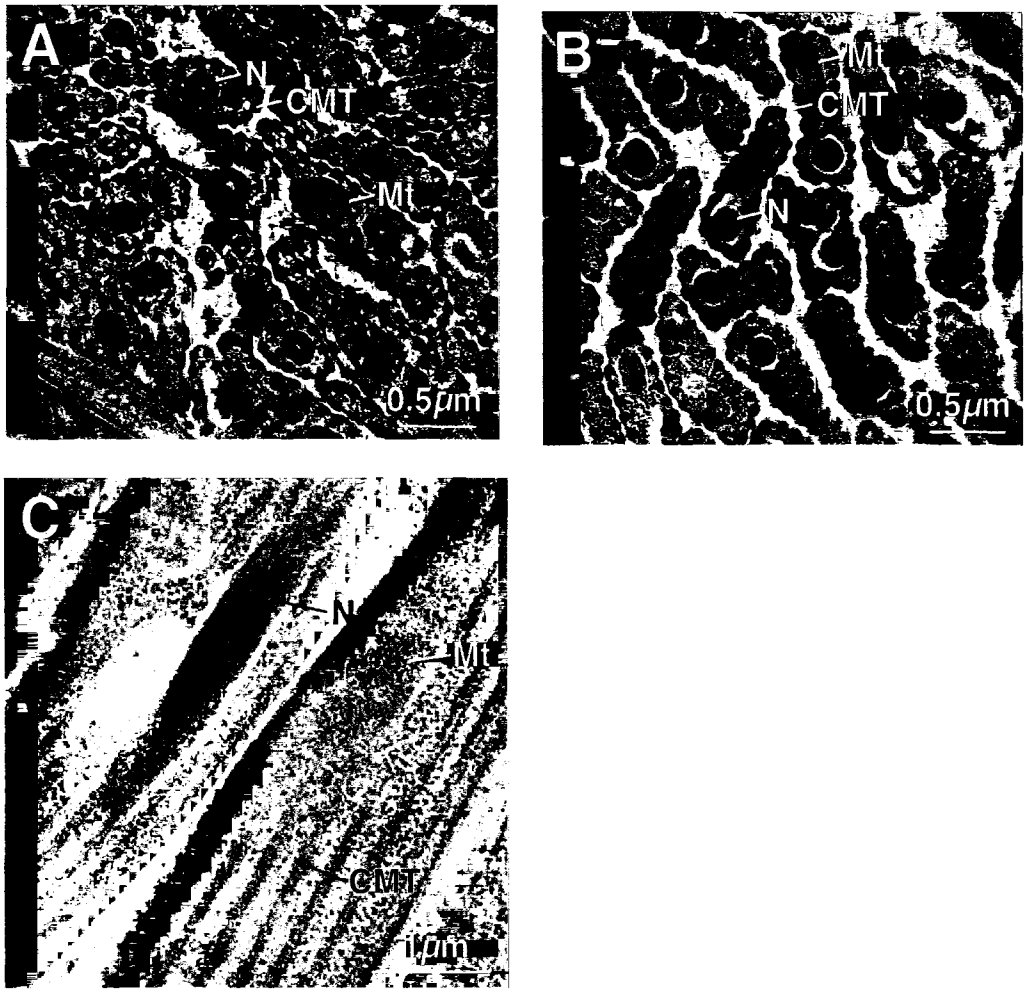

Figure 3.6. – TEM sections through immature and mature spermatozoa of *Genostoma kozloffi*. – **A**. Immature spermatozoa contain a hollow, tube-like nucleus. (CMT) microtubules; (Mt) mitochondria; (N) nucleus. Scale bar: 0.5μm. – **B**. Mature spermatozoa containing a solid, rod-like nucleus. (CMT) microtubules; (Mt) mitochondria; (N) nucleus. Scale bar: 0.5μm. – **C**. Longitudinal section through mature spermatozoa. The cortical microtubules (CMT) run parallel to each other. (Mt) mitochondria; (N) nucleus. Scale bar: 1μm.

Before the spermatozoon detaches from the residual cytophore, the nucleus condenses even more and elongates to its full length of approximately 90 μm. As long as the spermatozoon is still immature the nucleus appears tube-like with a hollow center (Fig. 3.6A); once the spermatozoon is mature the nucleus has become a solid rod (Figs. 3.6B, 3.6C).

3.4.2. Mature spermatozoa

Mature spermatozoa appear ribbon-like and are approximately 100 μm long and between 0.2 and 1 μm wide, depending on the measuring point (Fig. 3.7). A single axoneme with a 9+'1' arrangement of microtubules (Fig. 3.7) occupies approximately 10μm of one end of the spermatozoon. Judging from the way spermiogenesis proceeds, we assume the basal body lies at the distal tip of the mature spermatozoon (Figs. 3.3, 3.5, 3.7); finding this tip among the spaghetti-like profiles of sperm in sections proved impossible, however. With such a position of the basal body, the distal, motile portion of the spermatozoon would be the anterior tip; the proximal portion containing the nucleus would be the trailing posterior tip.

The rod-like nucleus extends throughout the remainder of the length of the spermatozoon (Fig. 3.7). The diameter of the nucleus is marginally smaller (0.18 μm) than that of the axoneme (0.23 μm) (Figs. 3.7D, 3.7E).

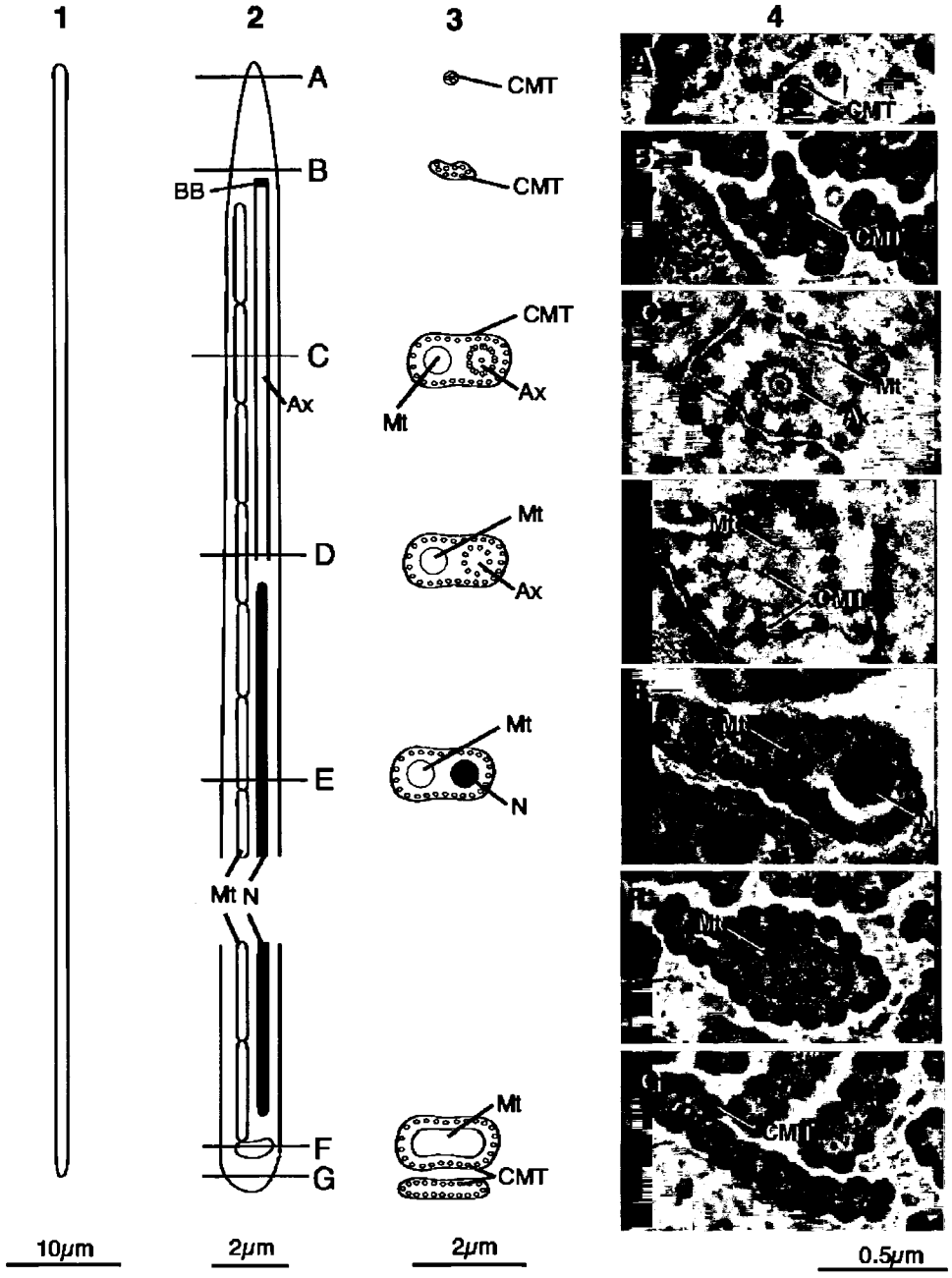
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Figure 3.7.

Figure 3.8. – Longitudinal TEM section through a spermatozoon of *Genostoma kozloffi*. Multiple mitochondria (Mt) form a rod that runs alongside the nucleus and the axoneme throughout the entire length of the spermatozoon. The cortical microtubules (CMT) run parallel to the longitudinal axis. Scale bar: 0.25µm.

A rod of mitochondria (Fig. 3.8) with approximately the same diameter accompanies the nucleus as well as the axoneme (Figs. 3.7C-F). Additionally, a larger mitochondrion can always be found at the proximal tip of the spermatozoon (Fig. 3.7F). Only the flattened proximal (Fig. 3.7G) and the pointed distal tips (Fig. 3.7A, 3.7B) of the sperm do not show cross sections of mitochondria.

A number (approximately 20) of parallel microtubules (Fig. 3.6C) run along the cell membrane (Figs. 3.4, 3.5, 3.6, 3.7, 3.8). These parallel microtubules are termed 'cortical microtubules' (Watson 1999) and, by the way of dense material coating their outer wall, especially along the side adjacent to the plasma membrane, appear larger in diameter than microtubules forming the axoneme (Figs. 3.6, 3.7C).

Most of the length of the mature spermatozoon is somewhat flattened because the two central elements, the nucleus and mitochondrial rod lie next to each other, and the cell membrane and the cortical microtubules are tightly appressed to them (Figs. 3.6A, 3.6B, 3.7). Neither acrosomal vesicles nor 'dense bodies' could be found.

3.5. Discussion

3.5.1. Spermiogenesis

Generally, the spermiogenesis of *Genostoma kozloffi* is, with some exceptions, similar to that of platyhelminths with turbellarian-type

spermiogenesis. Kornakova and Joffe (1999) describe a variant of spermiogenesis in free-living turbellarians with incorporation of the axonemes similar to the condition in *Genostoma kozloffi* (see also Justine 1991).

However, instead of two axonemes forming from the two basal bodies, only one develops a flagellum. The second basal body seems to have completely disappeared within the mature spermatozoon. This arrangement can also be found in all examined species of Schizorhynchia (Table 3.1) (Watson 2001).

A proximal rotation of the axoneme is characteristic of spermiogenesis in turbellarian platyhelminths in which the free axoneme is incorporated (Kornakova and Joffe 1999), and this stage was also found in *Genostoma kozloffi*.

Another fact, and probably the most important one placing *Genostoma kozloffi* within turbellarian platyhelminths, is that distal-proximal fusion occurs, meaning that the basal body moves distally into the developing spermatid while the nucleus remains proximal. In immature spermatids of Neodermata, the nucleus can be found proximally as well. In that case, however, no proximal rotation of the axoneme occurs, leaving the basal bodies positioned close to the nucleus rather than at the distal tip of the spermatid (Kornakova and Joffe 1999).

Unlike other turbellarian platyhelminths, *Genostoma kozloffi* does not develop an intercentriolar body, which is a stack of parallel disc-shaped electron-dense plates between the two basal bodies (Watson and Schockaert 1996). The two basal bodies of *Genostoma kozloffi* rather than being positioned at 180 degrees to each other, always remain perpendicular. Spermatids of most members of the Neodermata also have the two basal bodies parallel to each other and linked

by an intercentriolar body (Kornakova and Joffe 1999). As with other platyhelminths lacking the intercentriolar body, *Genostoma kozloffi* has no indication of rootlets attached to the proximal ends of the basal bodies; such rootlets are found in Polycladida, Lecithoepitheliata, Proseriata, Tricladida, Typhloplanida, Kalyptorhynchia, Dalyeliida and Temnocephalida (Watson and Rohde 1995).

Overall however, the most important characteristics, such as the proximal rotation of the axoneme and, even more importantly, distal-proximal fusion, do place *Genostoma kozloffi* very clearly within the taxa of Platyhelminthes with turbellarian-type spermiogenesis and more specifically the variant with incorporation of the axoneme as defined by Kornakova and Joffe (1999).

3.5.2. Mature spermatozoa

The single, fused axoneme of *Genostoma kozloffi* exhibits the 9+'1' structure (9 peripheral doublets of microtubules and a single complex central structure) that is synapomorphic for Trepaxonemata. The core of the axoneme is comparable to the single structure found in the center of axonemes of other species in this group. Immunocytochemical studies of Iomini and Justine (1997) and Iomini et al. (1998) have shown that this core structure does not possess tubulin and therefore is not a microtubule.

The nuclei of mature spermatozoa of *Genostoma kozloffi* appear very condensed. However, immature spermatozoa show a tube-like arrangement with a hollow center in the nuclear rod. This arrangement might be comparable to that of the sperm of kalyptorhynchs and some typhloplanids in which, early in development, the heterochromatin can be found condensed in a number of separate strands (Watson 2001). However, in these groups no eventual condensation to one solid nuclear rod seems to occur as is found in the mature sperm of *Genostoma kozloffi* (Table 3.1). In two closely related eukalyptorhynch species, *Nannorhynchides herdlaensis* and *Toia calceformis*, a condensation of heterochromatin along the margins of the nucleus can be seen in early spermatocytes as well (Watson 1998), and these condense to a number of tightly packed solid chromatin-rods.

While in other turbellarian platyhelminths the nucleus is accompanied by the two axonemes alongside each other (Kornakova and Joffe 1999; see also Raikova et al. 2001), this is not the case in *Genostoma kozloffi*. In *Genostoma kozloffi* no cross sections of nuclei and axonemes within the same section could be found. This situation applies also to the Neodermata (Kornakova and Joffe 1999).

Mature spermatozoa of *Genostoma kozloffi* contain several mitochondria arranged in a rod that accompanies the nucleus as well as the axoneme. The mitochondria appear to be fused (Fig. 3.7). According to Watson (2001) the arrangement of mitochondria in mature sperm appears to be similar in related species and, therefore, is phylogenetically significant.

Table 3.1. Characters of spermiogenesis and spermatozoa in various platyhelminths.

ICB – Intercentriolar Bodies
F – flagellar fusion
PR – proximal rotation of the axoneme
R – rootlets on basal bodies
DB – dense bodies

Ax# - number of axonemes
AxN – axonemes run alongside the nucleus
Mt – mitochondria
N – nucleus
CMT – cortical microtubules

Characters	***Genostoma kozloffi***	**Schizorhynchia**	**Eukalyptorhynchia**	**Prolecithophora**	**Neodermata**
Reference	present study	Watson and Rohde (1995)	Watson (1998)	Watson and Rohde (1995)	Kornakova and Joffe (1999)
ICB	no	yes	yes	---	yes
F	distal-proximal	distal-proximal	distal-proximal	---	proximo-distal
PR	yes	yes	yes?	---	no
R	no	yes	yes	---	yes
DB	no	no	yes	in some	no
Ax #	1	1	2/1	---	2/1
Ax state	incorporated	incorporated	incorporated	---	incorporated
AxN	no	yes	yes	---	yes
Mt	fused	fused	fused	altered, membranous derivatives	fused
N	rod	multiple rods	multiple rods	lobed	rod
CMT	present, thickened, widely spaced	present	present, widely spaced	present, additional internal microtubules	Polyopisthiocotylea only, otherwise proximal top and dorsal &ventral clusters

Watson and Rhode (1995) investigated a number of species of the Trepaxonemata and found that all examined Kalyptorhynchia, as well as Tricladida and Cotylea (Polycladida), exhibit mitochondria fused into a rod (Table 3.1).

Cortical microtubules can be found in most platyhelminth sperm (Hendelberg 1983). Cross bridges between those cortical microtubules, as observed in other species (see Hendelberg 1983, Henley 1974), could not be seen in *Genostoma kozloffi*. The large cortical microtubules appear too loosely spaced to possess that kind of cross bridges. Also, no other platyhelminth has been found to have such thickened walls due to the cortical microtubules (Table 3.1). The fact that the thickened wall appears at the outer side, facing the cell membrane, might contribute to an enhanced rigidity of the spermatozoon.

Other features typical of Trepaxonemata, such as dense bodies, split posterior ends or spiral anterior projections, could not be found in *Genostoma kozloffi*.

3.5.3. Phylogenetic position

Genostoma kozloffi undoubtedly belongs to the Trepaxonemata because of its 9+'1' arrangement of microtubules within the axoneme. This characteristic is autapomorphic to this group (Watson 1999). However, a more specific placement seems problematic. Hyra (1994) recognized that similarities of *Genostoma*

kozloffi to opisthoporate Prolecithophora (with which it has been classified) in that the position of the pharynx and the presence of a genitopharyngeal atrium were superficial. None of the specific characteristics of spermiogenesis and spermatozoa of prolecithophorans are similar to those of *Genostoma kozloffi*, however (Table 3.1). The epidermis of *Genostoma kozloffi* exhibits several morphological characteristics otherwise unique to Neodermata (Tyler and Tyler 1997). According to Ehlers (1985) and Rohde (1994) these characteristics are "(1) syncytial, (2) insunk, (3) unciliated, and (4) having multiple branching connections between the epidermal perikarya and the surface layer" (Tyler and Tyler 1997). Numerous features, including the general relationship of reproductive structures, the presence of a tunica surrounding the testes, and the ultrastructure of the spermatozoa found in *Genostoma kozloffi* show a similarity to the Kalyptorhynchia (Rhabdocoela) (Hyra 1994), especially Schizorhynchia (Table 3.1).

First, out of all species examined by Watson and L'Hardy (1995) and Watson and Schockaert (1996) Schizorhynchia and *Nannorhynchus heardlaensis*, as well as *Toia calceformis* (Eukalyptorhynchia) are the only species besides *Genostoma kozloffi* to possess a single, incorporated axoneme (Table 3.1) (Watson 1999). Second, a feature that Kalyptorhynchia and *Genostoma kozloffi* have in common is the arrangement of multiple mitochondria fused into a rod (Table 3.1) (Watson and Rhode 1995). Not much data are available for comparison of the structure of the nucleus, but Watson and Rhode (1995) only list

Schizorhynchia as possessing "a dense chromatin rod," very much like that found in *Genostoma kozloffi*.

Keeping in mind that absent characters are problematic for phylogenetics, it stands out that, like *Genostoma kozloffi,* all species of Kalyptorhynchia lack all 4 distinctive characters found in many other rhabdocoels, namely (1) "small granules in longitudinal rows beneath the cortical microtubules of mature sperm," (2) "axonemal spur – a pointed, spur-shaped structure at the base of the sperm axonemes, originating as a dense globular heel during spermiogenesis," (3) "a group of longitudinal microtubules between the embedded ends of sperm axonemes, derived from part of the cortical row by compression during flagellar rotation," and (4) "a fine connection between the nuclear and plasma membranes along much of the length of the sperm length" (Watson 2001). Also, just like Schizorhynchia (Watson and Rohde 1995), *Genostoma kozloffi* does not contain dense bodies (Table 3.1).

3.6. Conclusion

According to prior investigations (Hyra 1993, 1994) as well as the present one, *Genostoma kozloffi* exhibits many similarities to Kalyptorhynchia, especially Schizorhynchia, in features of the male reproductive system and spermiogenesis. Unlike the three other, previously investigated, symbiotic groups, *Kronborgia isopodicola* (Watson and Rhode 1993b), *Urastoma cyprinae* (Watson 1997), and

Notentera ivanovi (Kornakova and Joffe (1999), *Genostoma kozloffi* does not exhibit proximo-distal fusion; rather spermiogenesis occurs in a distal-proximal fashion and, therefore, is similar to that found in turbellarians, specifically the variant exhibiting a full incorporation of the axoneme, as defined by Kornakova and Joffe (1999). Despite its neodermatan-like epidermis, *Genostoma kozloffi* may not serve well as a model for the link between free-living and neodermatan platyhelminths.

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