

Unusual Intestinal Lamellae in the Nematode *Rhabditophanes* sp. KR3021 (Nematoda: Alloinematidae)

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ABSTRACT The free-living nematode *Rhabditophanes* sp. has recently been placed in a clade of animal parasites and may be a unique example of a reversal to a nonparasitic lifestyle. Detailed morphological analysis of the intestine reveals the unusual and unique structure of splitting microlamellae forming a meshwork with cavities along the entire intestinal tract. Secretion vesicles were observed along the whole tract and along the length of the lamellae. It is suggested that these lamellae are adaptations to a different digestive strategy where low food availability and a low absorption surface are compensated for by maximizing the nutrient uptake efficiency along the entire length of the intestine. The likely reversal to a free-living life cycle may have caused drastic changes in diet, providing the necessary driving forces to such morphological changes. *J. Morphol.* 264:223–232, 2005.

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Based on SSU rDNA, the free-living nematode *Rhabditophanes* sp. KR3021 has been placed within a clade of parasitic nematodes with alternating life cycles, comprising *Strongyloides*, *Parastrongyloides*, and *Rhabdias* (Dorris et al., 2002). Furthermore, it has been suggested that *Rhabditophanes* sp. may represent a reversal to a nonparasitic lifestyle (Dorris et al., 2002). Combined with its ease of culturing, *Rhabditophanes* sp. may be an excellent genotypic model for studies of parasitism. The evolution towards parasitism must have entailed the complex coordinated acquisition of many new and different phenotypic traits. The reversal must have been as complex. This raises questions as to the detailed morphology of *Rhabditophanes* sp. and whether some morphological “remnants” may still be present. Detailed morphological analysis may shed light on how evolution towards parasitism from a free-living ancestor has evolved. To this end, we are performing a detailed embryological and ultrastructural analysis of *Rhabditophanes* sp. We are especially focusing on structures, for example, a nematode’s intestinal microvilli, that due to their anatomical location and physiological function could be directly influenced by parasite–host interactions.

The unusual structure of the microvilli in the intestine of *Rhabditophanes* sp. is presented here.

MATERIALS AND METHODS

The *Rhabditophanes* sp. KR3021 sample was obtained from the west coast of Vancouver Island near Bamfield, British Columbia, Canada. The nematodes were grown on *Escherichia coli* OP50 in agar plates (1% agar) at 15°C and generally handled as described by Brenner (1974). *Rhabditophanes* sp. reproduces parthenogenetically with an average lifespan of 2–3 weeks.

The first juvenile stage was obtained by placing eggs overnight in an embryo dish with distilled water. The following morning the juveniles were collected for fixation. All other juvenile stages were obtained by placing the eggs in an embryo dish with phosphate-buffered saline (PBS, Sigma, St. Louis, MO) and collecting the juveniles at ~6-h intervals after hatching. Young adults were obtained directly from the Petri dish. Young adults were taken because the eggshell in gravid females causes poor fixation and tissue damage when sectioning. Life stages were used in all analyses. Live observations were done by directly observing feeding specimens of all stages through a Leica dissecting microscope.

Transmission Electron Microscopy (TEM)

The nematodes were fixed in a Karnovsky solution diluted 50% with distilled water (2% paraformaldehyde, 1% glutaraldehyde in 0.2 M Na-cacodylate buffer pH 7.2) (Van De Velde and Coomans, 1989) at 60°C for 30 min. The nematodes were placed at 4°C overnight. Each step done at 4°C involved a rotary motion of the solution during the incubation period. The following day the specimens were rinsed in 0.2 M Na-cacodylate buffer, pH 7.2, for 8 h at room temperature and postfixed overnight in reduced osmium at 4°C. The nematodes were transferred in 0.2 M Na-cacodylate buffer, pH 7.2, to rinse away the osmium. They were

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subsequently dehydrated in a 50%, 70%, 90%, and 100% ethanol series for 20 min each at room temperature. The nematodes were transferred to 100% alcohol/Spurr's resin (1:1) at 4°C overnight, brought to 100% alcohol/Spurr's resin (1:2) for 8 h (4°C), and transferred to 100% Spurr's resin and left overnight at 4°C. Polymerization was done at 70°C for 12 h. Seventy-nm-thick sections were made using a Reichert Ultracut S Ultramicrotome. Formvar-coated single slot copper grids were used. Longitudinal and cross sections were made. Sections were poststained with a Leica EM stain for 30 min in uranyl acetate at 40°C and 5 min in lead stain at 20°C. Electron microscopy was done using a JEM 1010 Jeol, operating at 80 kV. Micrographs were taken on Kodak electron image film (Esthar Thick Base, SO-163, Laborimpex) and processed using Brovira Speed paper (Agfa), Neutol liquid (Agfa) for development, and Agefix (Agfa) fixative.

3D Serial Reconstruction From TEM Photography

To study the spatial details of the intestinal microvilli, graphical 3D reconstructions were generated based on photographs of ultrathin serial sections using Surfdriver (3.5, Lozarnoff).

Scanning Electron Microscopy (SEM)

Nematodes were picked out with a platinum needle, heat (80°C)-relaxed for 1 min, and fixed in a 4% formaldehyde solution for 24 h. Nematodes were transferred to an embryo dish containing a solution of 4% formaldehyde plus 1% glycerin, and placed in a closed glass vessel containing 95% ethanol at 25–30°C overnight. Next, the glass dish was placed, partially closed, in an oven at 35°C and every 2 h, 4–5 drops of a solution of 95% ethanol and 5% glycerin were added. This allowed the ethanol to evaporate until the nematodes were in pure glycerin. The higher viscosity of glycerin compared to water made cutting the nematodes easier and more precise. Longitudinal and cross sections were made using a scalpel. The glycerin was substituted by distilled water by adding the latter drop by drop. Subsequently, the nematodes were dehydrated using an ethanol series (30%, 50%, 75%, 95%); after this initial dehydration that took about 8 h, the specimens were left overnight in 100% ethanol. To avoid collapsing the nematodes, they were critically point-dried with CO₂ using a Balzers CPD 020. The dried nematodes were then removed individually, placed on a glass rod on a standard specimen stub, sputter-coated with gold using a Balzers SCD 040, and observed using a Jeol JSM-840. Images were taken on Agfa APX120 film and developed commercially in a local photography store.

Actin Labeling

Worms were collected in an embryo dish and put in a small drop of water on a poly-L-lysine (MW > 300,000, Sigma) covered slide. A coverslip was added and excess water removed. The slide was subsequently submerged in liquid nitrogen. The coverslip was dried and the slide immersed in precooled (–20°C) methanol for 5 min followed by 5 min in precooled (–20°C) acetone. The slides were rinsed in PBS (3 × 5 min), transferred to PBS + 0.1% Tween 20 (Sigma), and incubated in phalloidin-TRITC (Sigma, 1:20) for 60 min at room temperature. The slides were washed three times in PBS + 0.1% Tween 20 for 5 min each. A drop of glycerin was added and the slide was covered with a coverslip and sealed with nail polish. Observations were made using a Zeiss Axiovert 135 equipped for fluorescence microscopy.

Nomenclature: Microvilli vs. Microlamellae.

Traditionally, microvilli are considered fingerlike projections anchored in a terminal web and internally supported by a skeleton ending in a darker staining top of yet unknown composition (Borgonie et al., 1995a). The projections observed in *Rhabdito-*

phanes sp. share some of these characteristics as the internal actin containing, skeleton and the darker staining top. They differ from microvilli by the sideways budding, splitting, rejoining, and the lamella-like, rather than finger-like, shape. Because of these differences we propose to use the term microlamellae for these structures.

RESULTS

No differences were observed between the intestinal lamellae of the different life stages analyzed. All data presented are from female adult specimens. In any given cross-section of the intestine, the number and length of visible implanted lamellae is variable, so no representative count can be given. However, the density of the lamellae does increase from anterior to posterior (Fig. 1A–D). Immediately posterior to the pharynx (Fig. 1A) the lamellae are farther apart than in the posterior intestine (Fig. 1D), where they are more closely packed. This difference in lamellae density was observed in all life stages (data not shown).

The lamellae have a clearly discernable darker inner skeleton (Figs. 2B–D, 4A–C,E), and a somewhat knotty darker staining apical end. The lamellae curve and bend extensively along the intestinal tract (Figs. 2A–D, 3, 4E). A terminal web could not be visualized. Many lamellae exhibit branching at several places along their length (Figs. 2B,C, 4A,C). There is no discernable pattern evident for the branching, either along the length of the intestinal tract or between specimens. In at least one occurrence where the lamellae were long enough and the opposing intestinal cells came close enough, lamellae formed a meshwork spanning the entire intestinal lumen (Fig. 3). Some lamellae seem to originate in one cell, run across the lumen, and almost reach the opposite intestinal cell. We did not observe this phenomenon in any other sections made. Phalloidin staining confirms the presence of F-actin in the lamellae, staining a fuzzy wide band at the brush border position (Fig. 3D). Serial sectioning and 3D reconstructions show a complex pattern of stand-alone lamellae fusing with neighboring lamellae to form cavities (Figs. 5A,B, 6A–D).

Secretions emanating from the lamellae can occur at any position along the length of the lamellae (Figs. 2D, 3, 4A–C). Secretion activity is considerable, as observed from the number of vesicles identified in the lumen, and was evident along the entire length of the intestinal tract, albeit to a varying degree. Typically, vesicles seem to burst open once the lumen is reached (Figs. 2B,D, 3, 4B).

The glycocalyx is easily identified and all the cavities are formed within the glycocalyx. No lamellae were seen protruding into the lumen beyond the darker-staining glycocalyx.

Lamellae and secreting vesicles were observed within the cavities, and in a majority of TEM images the glycocalyx enclosed in the cavity is somewhat less contrasted than the glycocalyx outside the cav-

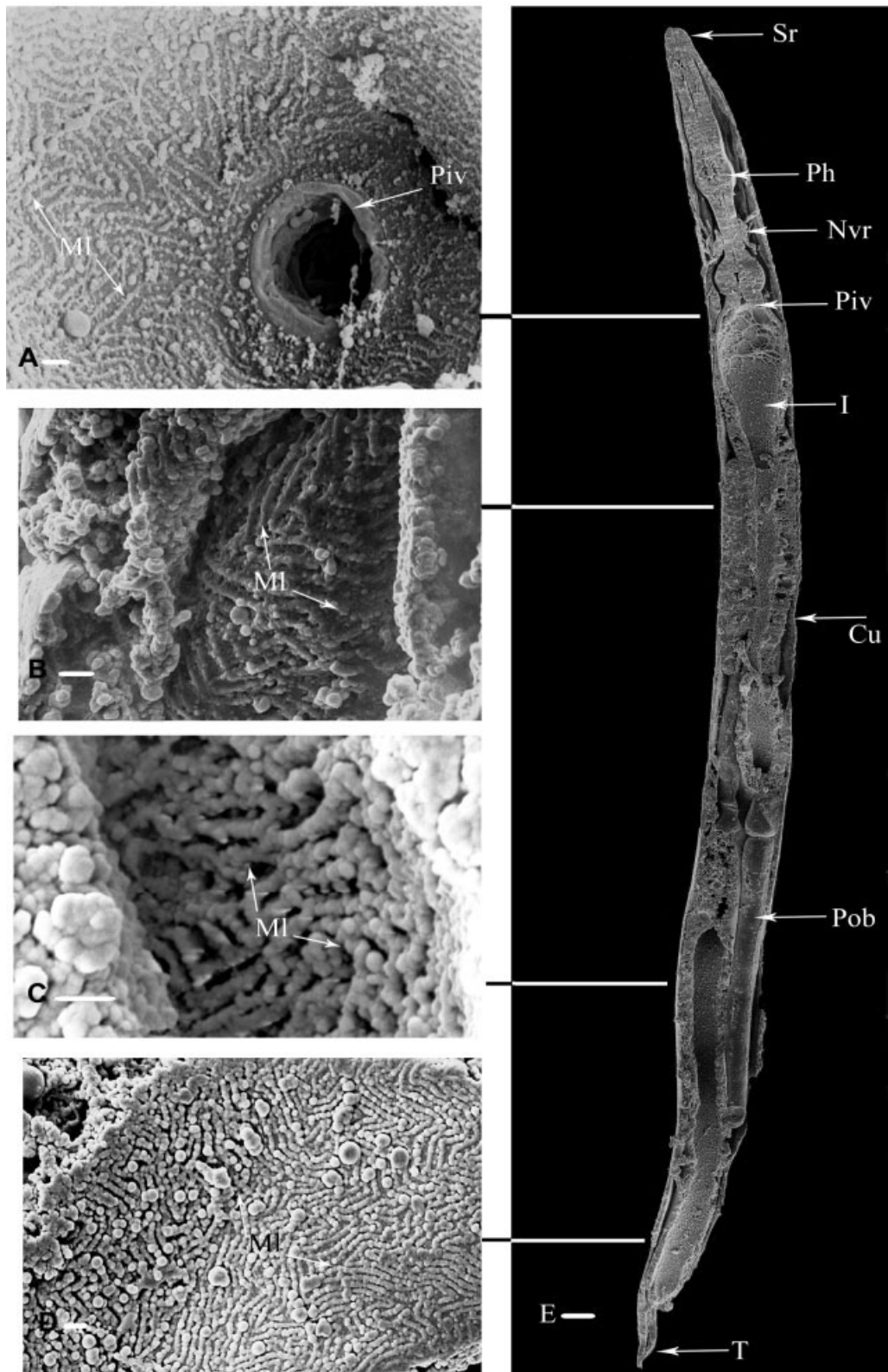


Fig. 1. *Rhabditophanes* sp. Longitudinal section of whole-mount (E) with details of spatial distribution of intestinal lamellae (A–D) along the intestinal tract. SEM. Micrographs (A) through (D) show an increasing density of lamellae: posterior to the pharynx (A) the lamellae are farther apart than in the posterior intestine (D). Cu, cuticle; I, intestine; MI, microlamellae; Nvr, nerve ring; Piv, pharyngo-intestinal valve; Ph, pharynx; phb, pharynx bulb; Pob, posterior ovary branch; Sr, stoma region; T, tail. Scale bars = 35 μ m.

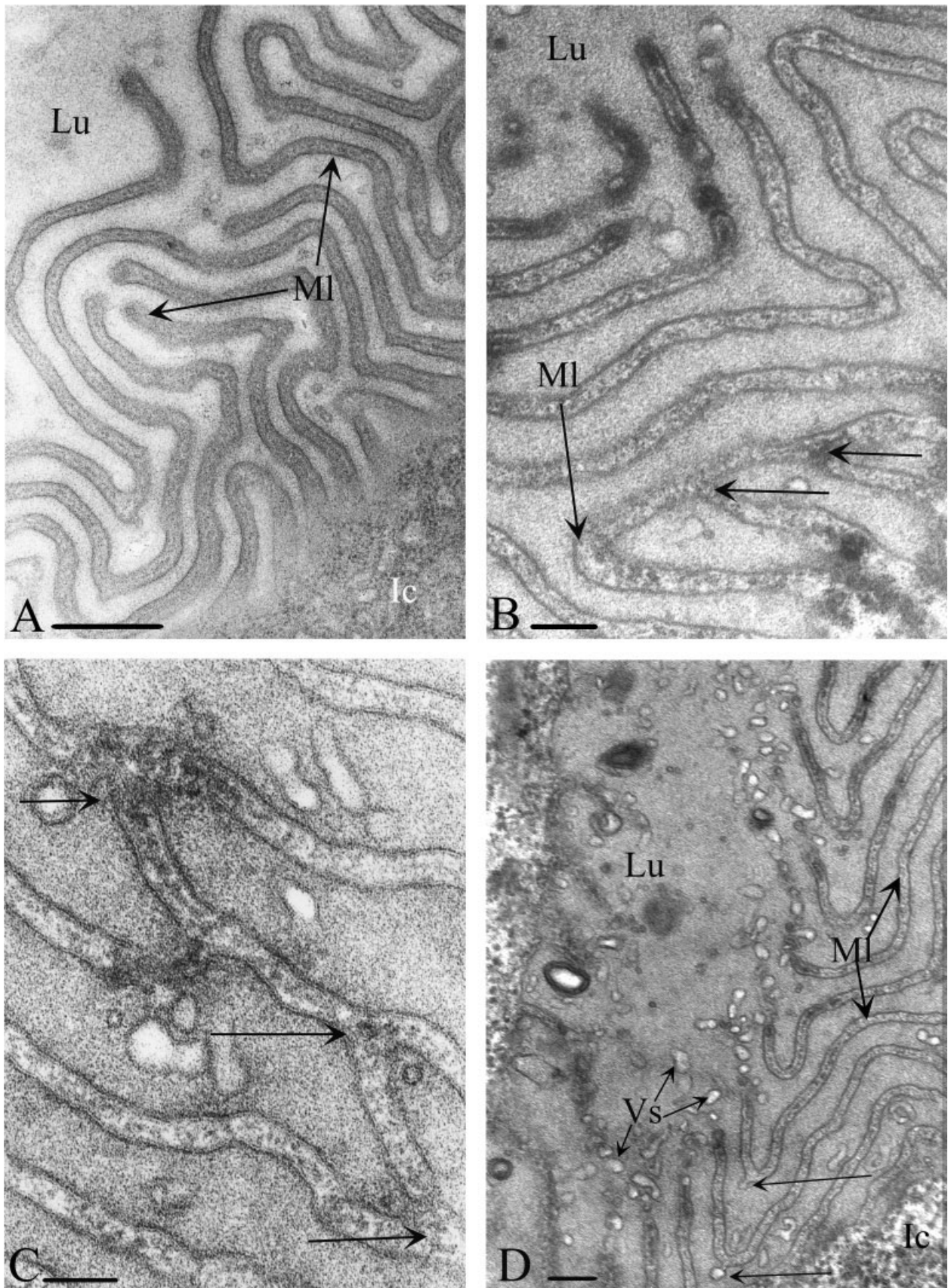


Fig. 2. *Rhabdtiophanes* sp., ultrastructure of the intestinal lamellae. TEM. All micrographs represent transverse sections through the lumen of the intestinal tract. **A:** Meshwork of microlamellae. Several microlamellae lie between each other; others have protrusions into the intestinal lumen. **B,C:** Branching microlamellae (arrows). Note the branches in **C** forming connections between neighboring microlamellae. **D:** Sideways budding microlamellae. Secretions not only occur at the apical end of the microlamellae but also on the side (arrows). Note the darker staining top and inner skeleton of the lamellae. Glx, glycocalyx; Ic, intestinal cell; Lu, lumen; MI, microlamellae; Vs, vesicles. Scale bars = 500 nm in **A**; 200 nm in **B**; 100 nm in **C**; 200 nm in **D**.

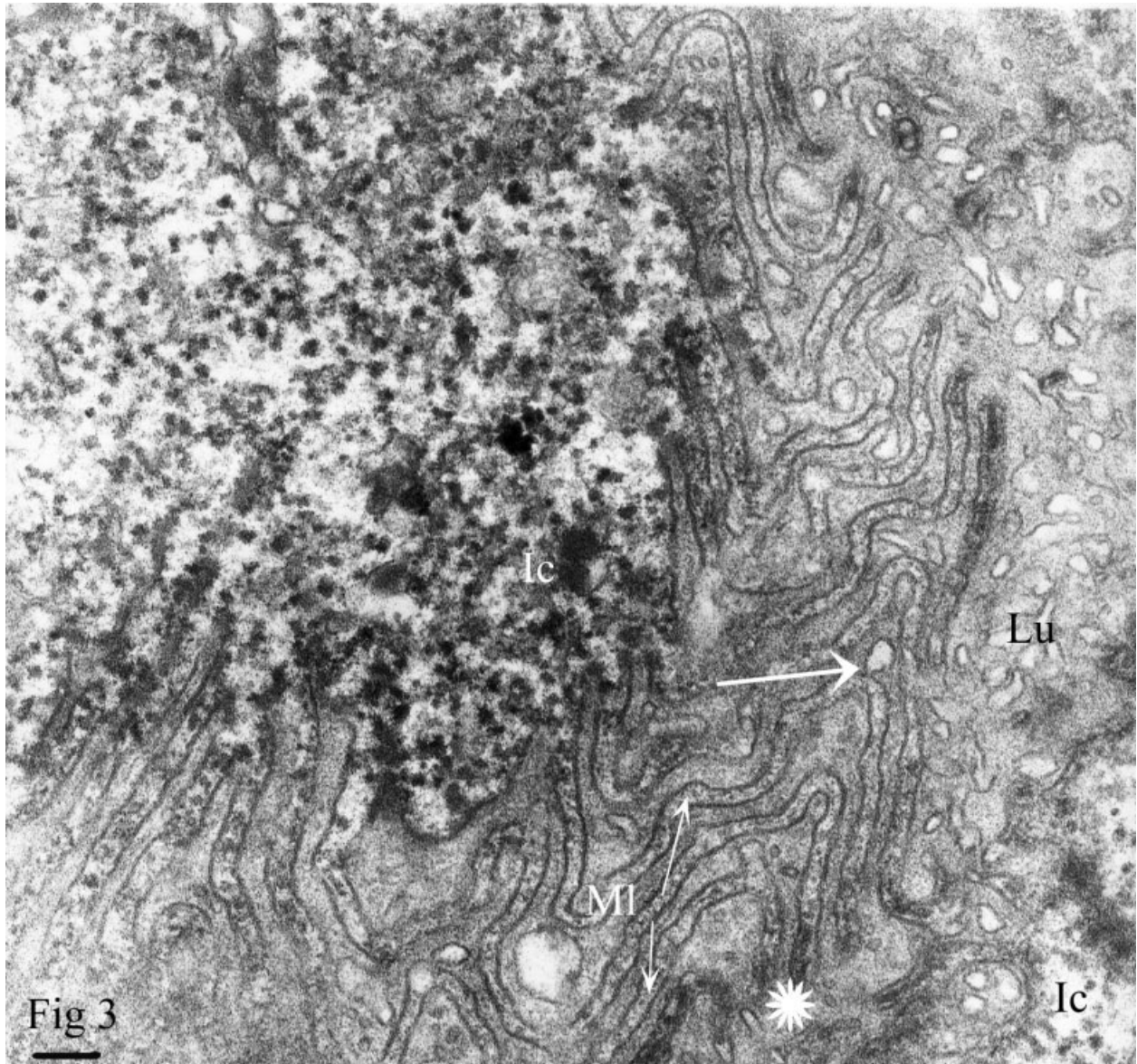


Fig. 3. *Rhabditophanes* sp. The intestinal lamellae. TEM. Micrographs represent transverse sections through the lumen of the intestinal tract. Elongated microlamellae span the entire lumen. Some lamellae reach to the opposite intestinal cell, almost obstructing the lumen (asterisk). Note the sideways budding of the microlamellae (arrows). Ic, intestinal cell; Lu, lumen; ML, microlamellae; Vs, vesicles. Scale bar = 200 nm

ities. No other inclusions (e.g., bacteria) were identified within any of the studied cavities.

DISCUSSION

General Morphology

The general morphology of the intestine of *Rhabditophanes* sp. is identical to that of other free-living and some marine nematodes (Deutsch, 1978; Boronie et al., 1995b), including the increase of the number of microlamellae with the highest density at the posterior end of the digestive tract.

The most remarkable difference from any other free-living nematode studied so far is the presence of lamellae instead of finger-like microvilli in *Rhabditophanes* sp. Not only do these lamellae form a complex 3D maze network but they also fuse, forming cavities of varying length. Branching and backfolding of lamellae are frequent, so sometimes the beginning of the lamellae cannot be distinguished from the ending. On some occasions this results in the formation of cavities within cavities. Although the images only give a static representation of a dynamic process, we propose three distinct possibilities of how cavities may be formed: 1) lamellae

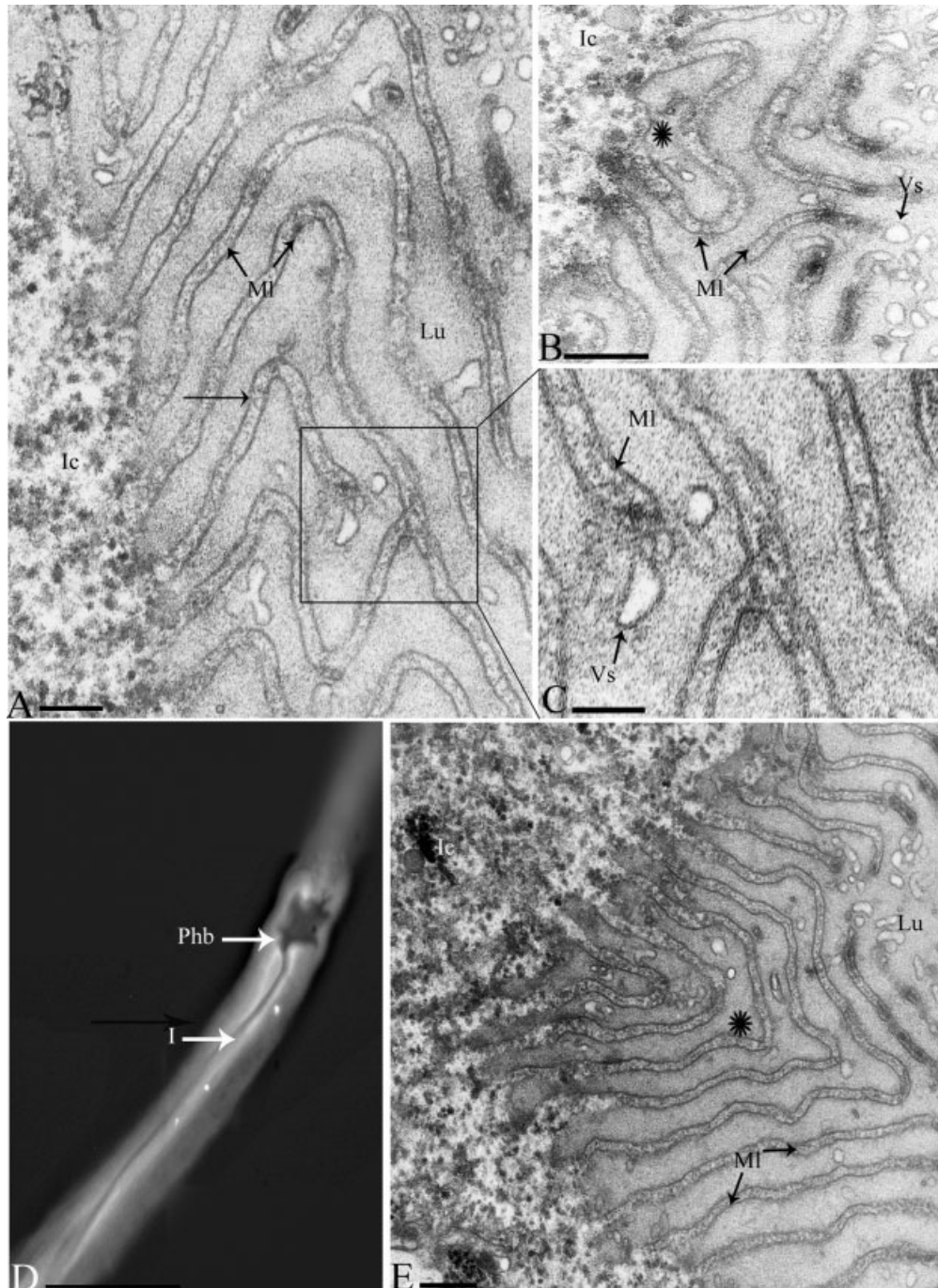


Fig. 4. *Rhabdtiophanes* sp. Ultrastructure of microlamellae (A–C, E; TEM) and whole-mount F-actin labeling (D). A–C, E: Formation of cavities by microlamellae. A: Microlamellae enclosed within a cavity (arrow) formed by two other microlamellae. B: Cavity (asterisk). Note the presence of vesicles within the cavity. C: Detail of Figure 3A. Note the secretion of vesicles at the top of the lamellae. E: Formation of cavities within cavities. D: Whole-mount F-actin labeling of intestinal tract (anterior is to the top). The bright delineation of the lumen confirms the presence of polymerized actin in the brush border. I, intestine; Ic, intestinal cell; Lu, lumen; MI, microlamellae; Phb, pharynx bulbus; Vs, vesicles. Scale bars = 200 nm in A; 200 nm in B; 200 nm in C; 100 μ m in D; 500 nm in E.

project into the lumen, recoil, and fuse with the apical intestinal membrane; 2) branched lamellae where one end projects towards the intestinal lumen and the other branch fuses with the apical intestinal

membrane; and 3) two neighboring lamellae fuse at the apical end to form a cavity.

To our knowledge, in none of the nematodes studied so far has a phenomenon of branching lamellae-

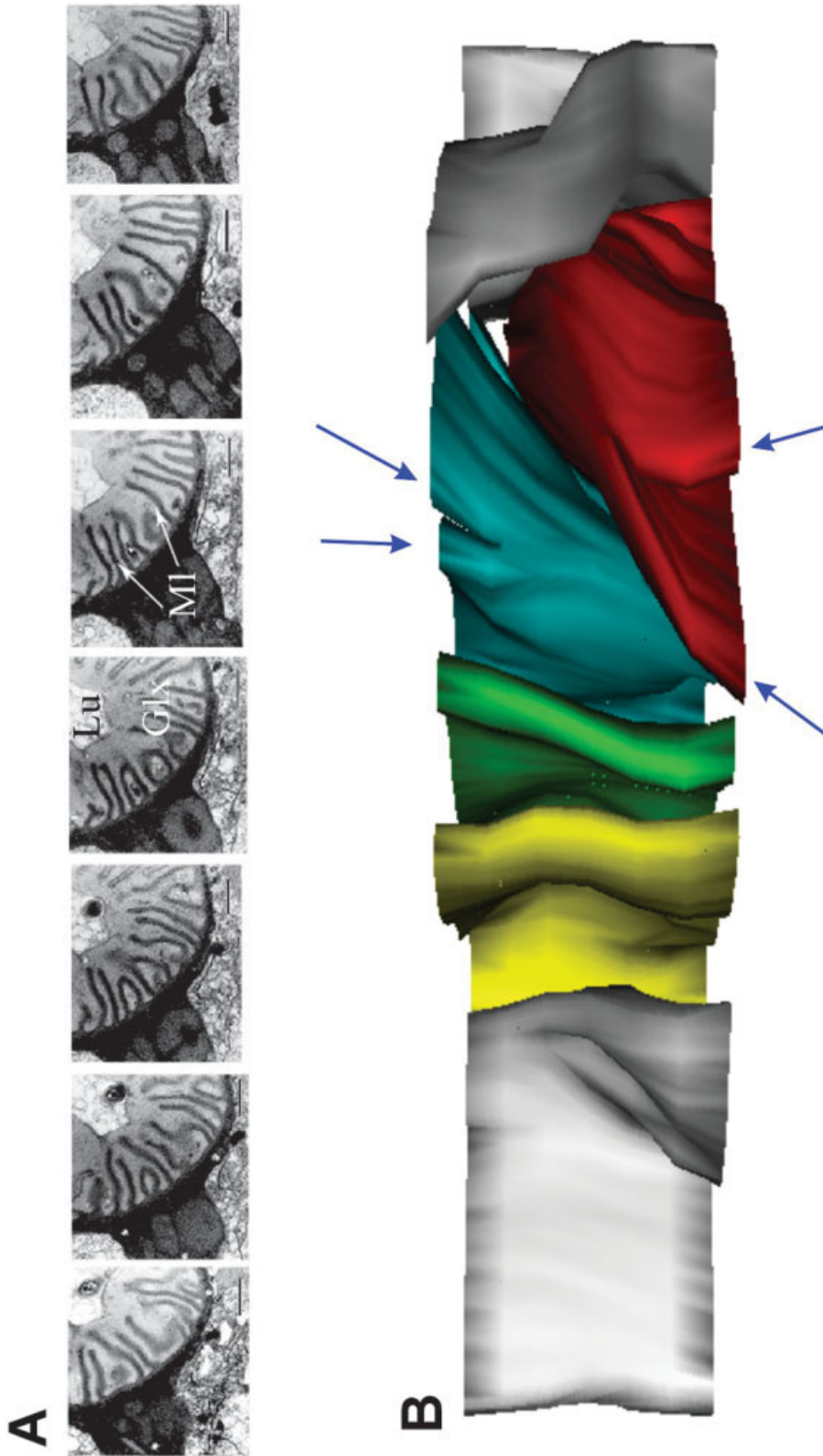


Fig. 5. *Rhabditophanes* sp.; 3D-reconstruction (B) of lamellae based on TEM micrographs of serial transverse sections (A) of the intestinal tract. Arrows indicate fusion of single microlamellae to form cavities. B: Top view. Ca, cavity; Gly, glycocalyx; Lu, lumen; MI, microcavity. Scale bars = 500 nm.



Fig. 6. *Rhabditophanes* sp.; 3D-reconstructions (A–D) of lamellae based on TEM micrographs of serial transverse sections (Fig. 4A) of the intestinal tract. Arrows indicate fusion of single microlamellae to form cavities. **A:** Left side view. **B:** Right side view. **C:** Right oblique view. **D:** Left oblique view. Ca, cavity; Glx, glycocalyx; Lu, lumen; MI, microlamellae. Scale bars = 500 nm.

like villi and cavity formation ever been recorded. Furthermore, the budding of vesicles from villi along their length has also not been recorded before in nematodes.

Are the Microlamellae an Artifact Due to Infection or Suboptimal EM Preparation?

Only actively moving, well-fed specimens were processed for EM purposes. Infection (due to ingestion of a toxic bacteria strain) can be excluded since *Rhabditophanes* sp. was cultured monoxenically on *Escherichia coli* OP50. Stress/anoxia due to slow fixation causes shortening of microvilli, widening of the intestinal lumen, or rolling up of rough endoplasmic reticulum (Hayat, 1981). None of the TEM images taken revealed any indication of infection or unusual morphology indicative of stress or infection.

Moreover the techniques employed in the preparation of *Rhabditophanes* sp. sections were identical to those applied to other free-living bacterivorous nematodes, which possess simple, uniform finger-like microvilli (Borgonie et al., 1995c).

Different Digestive Strategy?

The absence of data other than ultrastructural images precludes a detailed explanation of the function of the cavities. However, we believe that the differences in the intestinal morphology of *Rhabditophanes* sp. may be related to a different digestive strategy. Low food availability and low absorption surface seems to be compensated for by maximizing nutrient uptake efficiency.

Rhabditophanes sp. is a nematode that does not survive temperatures higher than 17°C in culture.

Furthermore, *Rhabditophanes* sp. cannot be cultured easily on nutrient agar since bacteria easily overgrow the nematode culture. As 17°C is the maximum tolerable temperature, *Rhabditophanes* sp. seems better suited for colder environments where bacteria are less abundant. In contrast, *Caenorhabditis elegans*, which has a maximum temperature of 25°C, is prevalent in places of massive decomposition of organic material where food is plentiful.

If *Rhabditophanes* does live in a habitat with fewer bacteria, and maximum extraction of nutrients is a prerequisite for survival, then a strategy of maximizing the intestinal absorption surface would be expected. However, lamellae offer less increase in surface per area unit than would finger-like villi. In that view, the lamellae and the cavities they form must yield additional advantages that outweigh surface area loss.

The first possible advantage might be that the close meshwork of lamellae may act as a barrier and block the smooth passage of bacteria in the intestine, thereby allowing more time for digestion. Other nematodes, like *Caenorhabditis elegans*, are known to ingest huge amounts of bacteria but also defecate the entire content of the intestine every 40–45 sec (Thomas, 1990; Borgonie et al., 1995b). However, in observing live animals we did not see an accumulation of bacteria in the intestine at this close-knit meshwork.

A second strategy to increase nutrient uptake is secretory activity and absorption in cavities along the whole length of the intestine.

First, in other free-living nematodes secretory activity is restricted mainly, if not exclusively, to the cells immediately posterior to the pharynx. The remaining posterior intestinal cells have an absorptive function (Borgonie et al., 1995b). In *Rhabditophanes* sp., secretion occurs over the entire length of the intestine. All intestinal cells are identical at the ultrastructural level, with no discernable differences in number of organelles, suggesting all intestinal cells are capable of secretion and absorption.

Second, secretion is not restricted to the lamellar apical area, but can occur over the entire length of the lamellae. Vesicles seemingly emanating from lamellae were clearly identified within some cavities. This would allow enzymes to be introduced into the cavity, useful if these cavities were “specialized” digestive chambers.

However, based on the present knowledge of feeding in free-living bacteriophagous nematodes, this possible function does not explain all the observations. The glycocalyx surrounding villi/lamellae is an impenetrable barrier (Borgonie et al., 1995c), implying that only predigested substances in the lumen can pass through the glycocalyx into the intestinal cells. The *Rhabditophanes* sp. images confirm this scenario: no bacteria were ever observed between the lamellae or within the cavities. The observation that all cavities are formed within the

glycocalyx raises the important question: Then what could be digested in the cavities? If the cavities have a digestive function it would imply a role in further digestion of predigested bacterial compounds. The exceptional morphology of the lamellae would then reflect the need for specialized adaptations towards a specific bacterial diet.

The possession of specialized digestive chambers has several advantages for a free-living nematode that is habituated to lower temperatures and relies on a smaller or an erratic food supply. Nutrient uptake efficiency is enhanced by creating cavities for digestion, where enclosed nutrients would be prevented from being defecated and, as such, more time for digestion would be available. Such an alternative digestive strategy would allow *Rhabditophanes* sp. to thrive in niches with small food sources where other free-living nematodes would have difficulty in sustaining themselves.

Remnants of Parasitism?

The Nematoda has large numbers of parasitic groups, and molecular phylogenetic analyses suggest that there have been multiple independent events of gain of parasitism of both animals and plants (Blaxter et al., 1998; Dorris et al., 1999). The evolution of the parasitic phenotype requires coordinate acquisition of many novel traits and thus reversion to a free-living state is thought to be unlikely (Dorris et al., 2002). On the other hand, members of the Nematoda with alternating life histories provide likely candidates for parasitic reversal within the Metazoa (Poulin, 1998).

Since *Rhabditophanes* sp. is the only species within the superfamily Strongyloidea that is considered to have abandoned a parasitic life cycle, representing the first known example of reversal of parasitism within the Metazoa, one could assume that the unusual intestinal morphology is linked to its previous parasitic lifestyle. Other ultrastructural analyses of species within Strongyloidea do not support this, since all have finger-like intestinal microvilli (Moqbel and McLaren, 1980; Dionisio et al., 2000). However, nearly all parasitic nematodes within Strongyloidea seem to have developed some kind of gut-ultrastructure adaptation as a response to their host, such as elongated, dilated microvilli with mini-apocrine function responsible for predigestion of host cells (Jenkins and Erasmus, 1969; Moqbel and McLaren, 1980; Dionisio et al., 2000). Since the cuticle of nematodes is impenetrable, it seems probable that the intestinal epithelium is of paramount importance in the interchange of material between host and parasite. Nutritional adaptations to parasitism were also observed in flatworms (phylum Platyhelminthes). For example, apical microlamellae similar to those of *Rhabditophanes* sp. were observed in the caecal lumen of the parasites *Gyliench nahaensis* and *Echinostoma*

hortense (Platyhelminthes, Digenea) (Fujino and Ishii, 1979; Jones et al., 2000). It was speculated that the function of the microlamellae was twofold: 1) the arrangements of the lamellae into broad sheets at the surface could promote uptake of nutrients, and 2) the microlamellae could perform some auxiliary function in the caeca, such as protection of the cells from the harsh conditions of the lumen due to the highly acidic stomach of the host (Jones et al., 2000). These morphological adaptations of the gut are not solely confined to parasite–host interactions, as illustrated by the variable shape of microvilli (finger-, leaf-, tongue-shaped) in the small intestinal mucosal surface of broilers in relation to age, diet formulation, small intestinal microflora, and performance (Van Leeuwen et al., 2004). To determine functionality of the microlamellae in *Rhabditophanes* sp. further, experiments will be required such as studies using radiolabeled nutrients together with autoradiography, fluorescence experiments to study F-actin dynamics, and ezrin-radixin-moesin immunostainings.

We propose that, in an evolutionary sense, the intestinal epithelium is very receptive to selective pressure. Highly dynamic processes occur during brush border formation. In particular, the number and the length of microvilli increase during the final step of adult enterocyte differentiation, as well as during the last stage of embryogenesis (Heintzelman et al., 1992; Waharte et al., 2004). Food gathering as a selective pressure could have initiated changes in microvillar dynamics within the lineage leading to *Rhabditophanes*, resulting in unusual intestinal microlamellae. Parasitism combined with the likely reversal to a free-living life cycle is likely to have caused drastic changes in diet, providing the necessary driving forces to such morphological changes.

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