

A New Species of Orthonectida That Parasitizes *Xenoturbella bocki*: Implications for Studies on *Xenoturbella*

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Abstract. Orthonectida is a phylum of marine invertebrates known to parasitize many invertebrate animals. Because of its simple body plan, it was suggested that it belong to Mesozoa, together with Dicyemida, and that it represent the evolutionary step between unicellular organisms and multicellular animals. Recent studies, including analyses of its genomes, have clarified its phylogenetic position as a member of the Protostomia, but details such as the species diversity within the phylum and how it infects the host remain unknown. Here we report orthonectids discovered from the marine worm *Xenoturbella bocki*. Orthonectids were found from sections of four xenoturbellid specimens, collected eight years apart. Live females were also discovered on three separate occasions. These recurring instances of orthonectids found from *Xenoturbella* show that they are parasitic to the animal and not just chance contaminations. Based on morphological characters such as the presence of sexual dimorphism, the arrangement of oocytes within the female body, and the presence of crystalline inclusions in the male epidermal cells, we regard this orthonectid as a new species, *Rhopalura xenoturbellae* sp. nov. Since orthonectids are present within the xenoturbellid adult body, caution is needed when interpreting morphological, molecular, and experimental data from *X. bocki*. Further studies on *R. xenoturbellae* will yield important information on the fundamental biological details of orthonectids that remain unknown.

Introduction

Orthonectids, with about 30 described species, are microscopic animals known as parasites in various marine invertebrates, possessing a peculiar life cycle (Kozloff, 1992, 1993). They have been reported to parasitize acoels, platyhelminthes, annelids, molluscs, nemerteans, echinoderms, and chordates; and it has been suggested that they are present in the host animals as multinucleate plasmodia (Slyusarev and Miller, 1998). However, the plasmodia may also be a mass of host cells that has degenerated due to the parasites, and therefore not a viable stage of the orthonectid life cycle (Kozloff, 1994, 1997). Male and female adults are produced from germinal cells within the plasmodia, and the ciliated adults swim out from the hosts. The adults have a very simple morphology lacking digestive, excretory, or circulatory organs. Muscular and nervous systems have previously been reported to be absent; but the presence of both structures, although very reduced, has been confirmed (Kozloff, 1969, 1971; Slyusarev and Starunov, 2016). Instead of these organs, the orthonectid adult body is full of gametes. Mating results in internal fertilization, and the ciliated larva swim out of the female and search for new hosts (Caullery and Lavalley, 1908, 1912; Atkins, 1933). Once inside the host, the larva disintegrates and forms new plasmodia. However, the complete life cycle of a single species remains to be observed; how they find and enter the hosts, oogenesis, spermatogenesis, and mating all remain poorly studied. Research on other details of orthonectids, such as diversity within the phylum, physiology, and nutrient uptake, have also been scarce.

Owing to their simple body plan, orthonectids were grouped together with Dicyemida, microscopic parasites found in cephalopods, in a clade called Mesozoa, a sister group to Metazoa. However, recent studies suggest that the two groups are not basal animals but bilaterians that have secondarily simplified as a result of their parasitic lifestyle. The spiral cleavage, musculature (Slyusarev and Starunov, 2016), and fine structure of ciliated cells and ciliary rootlets (Slyusarev and Kristensen,

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Abbreviations: SMNH, Swedish Museum of Natural History; SMRC, Shimoda Marine Research Center.

Online enhancements: videos.

2003) of Orthonectida show similarities to those of annelids; therefore, it has been suggested that they be a member of the Protostomia. Molecular phylogenetic analyses have been in accord with this view (Hanelt *et al.*, 1996; Pawlowski *et al.*, 1996; Petrov *et al.*, 2010), with genomic data of *Intoshia linei* strongly supporting this affinity (Mikhailov *et al.*, 2016). Studies using transcriptomic data of a dicyemid, *Dicyema japonicum*, have also suggested that orthonectids are protostomes (Lu *et al.*, 2017); and another analysis on mitochondrial genomes and nuclear genes suggested that orthonectids are members of the phylum Annelida (Schiffer *et al.*, 2018).

Xenoturbella, with six reported species, is another animal with a simple body plan (Nakano, 2015; Rouse *et al.*, 2016; Nakano *et al.*, 2017). Although not parasitic, sessile, or microscopic, the animal lacks a centralized nervous system, anus, coeloms, and reproductive organs. Because of the simple morphology, its phylogenetic position has long remained undecided. Recent molecular phylogenetic analyses mostly agree that it forms the clade Xenacoelomorpha together with the Acoelomorpha (Hejnlol *et al.*, 2009; Philippe *et al.*, 2011; Cannon *et al.*, 2016; Robertson *et al.*, 2017). Analyses of their mitochondrial genome suggest a deuterostome affinity of the clade (Philippe *et al.*, 2011; Robertson *et al.*, 2017), but large-scale phylogenomic studies suggest that it is a sister group to all other extant bilaterians (Nephrozoa) (Hejnlol *et al.*, 2009; Cannon *et al.*, 2016). Therefore, Xenacoelomorpha and *Xenoturbella* will contribute to the studies of early bilaterian or deuterostome evolution. However, mainly because of the difficulties in collecting the animals, research on *Xenoturbella* has been lagging (Nakano, 2015). For example, except for reports on cleavage stage embryos and swimming hatchlings (Nakano *et al.*, 2013; Nakano, 2015), the complete life cycle of *Xenoturbella* is still a mystery, despite its breeding season being first reported nearly 70 years ago (Westblad, 1949).

Here we report orthonectids discovered from the marine worm *Xenoturbella bocki* Westblad, 1949. They were found from sections of four specimens, three of which were collected and fixed in April 1998 and the other in March 2006. Live female orthonectids were observed in May and November 2006 and in March 2008. These recurring instances of orthonectids suggest that they are parasites of *Xenoturbella*

and are not just chance contaminations. Based on the host organism and morphological features, we regard the newly found orthonectid as a new species, *Rhopalura xenoturbellae* sp. nov. We consider its probable effect on the reproduction of its host and discuss the parasite's importance for studies on *X. bocki*. Many fundamental biological details that remain unknown for orthonectids will be revealed through research on various species, including this species.

Materials and Methods

Xenoturbella specimens were collected in Gullmarsfjord, Sweden (approx. 58°17'N, 11°31'E), at 80–150-m depth, using a Waren's dredge and the research vessel *Oscar von Sydow*, Sven Lovén Centre for Marine Sciences-Kristineberg, University of Gothenburg. The collection dates and the number of collected xenoturbellids are shown in Table 1. The collected xenoturbellids were kept in laboratories at 4–10 °C in either of the following methods: (1) closed system: animals were kept in closed 50-mL tubes (about 115 mm in length and 30 mm in diameter) with natural seawater, without any flow-through water, or (2) flow-through system: animals were placed in containers with a mesh (100- μ m pore size) placed on both the inflow and outflow tubes, with constant flow of natural seawater. Details on the collection and rearing methods are described in Nakano *et al.* (2013) and Nakano (2015). All individuals were identified as *Xenoturbella bocki* based on their morphology. Xenoturbellid specimens were fixed in 4% paraformaldehyde in filtered seawater for 6–16 h at 4 °C, washed, and paraffin sectioned (6 μ m), according to Stach *et al.* (2005). The sections were stained with hematoxylin and eosin.

Photographs and movies of live orthonectid specimens and sections of a xenoturbellid collected on March 6, 2006, were taken with a Canon S40 color camera (Tokyo, Japan) on Leica DMRBE or MZFLIII microscopes (Wetzlar, Germany).

Newly collected live orthonectid specimens were fixed in 4% paraformaldehyde in filtered seawater or 2.5% glutaraldehyde in 0.1 mol L⁻¹ sodium cacodylate buffer (pH 7.2) overnight, washed, and kept in 70% ethanol.

Three *Xenoturbella* specimens deposited in the Swedish Museum of Natural History (SMNH) were observed at the

Table 1

Occurrences of Rhopalura xenoturbellae sp. nov. from *Xenoturbella bocki*

<i>R. xenoturbellae</i>	Date live <i>R. xenoturbellae</i> found	Date host <i>Xenoturbella</i> collected	No. host <i>Xenoturbella</i> collected
12 females	May 23–26, 2006	May 17, 2006	31
1 female	November 21, 2006	November 13, 2006	44
1 female	March 12, 2008	February 18 and March 4, 2008	12, 5
Numerous larvae	February 22, 2006	February 7, 2006	25
Females in sectioned hosts	...	April 14, 1998	SMNH slides
Males in sectioned host	...	March 6, 2006	31

SMNH, Swedish Museum of Natural History.

museum. *Xenoturbella* specimens SMNH 5009, 5010, and 5012 are a series of sectioned and hematoxylin-stained slides of specimens that were collected off the island of Syd-Hällsö, on the west coast of Sweden, at 60–70-m depth on April 14, 1998. They are logged at SMNH as *Xenoturbella westbladi*, but since recent molecular analyses have shown that it is a junior synonym of *X. bocki* (Rouse *et al.*, 2016), these *Xenoturbella* specimens (SMNH 5009, 5010, and 5012) are treated as *X. bocki* in this study.

Results

Observations of living specimens

Living orthonectid females were observed on three separate occasions (Table 1). Thirty-one specimens of *Xenoturbella bocki* were collected on May 17, 2006, and maintained in closed 50-mL tubes. Between May 23 and 26, 12 swim-

ming orthonectids were found when the contents of the tubes were examined under a stereomicroscope. On November 21, 2006, a single swimming female was found in a closed tube containing 8 of the 44 xenoturbellids collected 8 days earlier. Another swimming animal was found on March 12, 2008, on the mesh placed on the outflow tube of a flow-through system container with 17 xenoturbellids inside. The animals were collected on February 18 and March 4, 2008.

We were able to follow the development of a single female, observed to exit the xenoturbellid body on May 23, 2006 (Fig. 1). The orthonectid came out through a rupture at the posterior end of the host (Fig. 1A). It showed an elongated ovoid shape, with a tapering anterior tip (Fig. 1B). This specimen gradually elongated (Fig. 1C); and after one day, a constriction was formed at one-third of its body length from the anterior (Fig. 1D). The front of the constriction narrowed toward the anterior (Fig. 1E, F), whereas a pointed tip also formed at the posterior end (Fig. 1G). Its body was filled

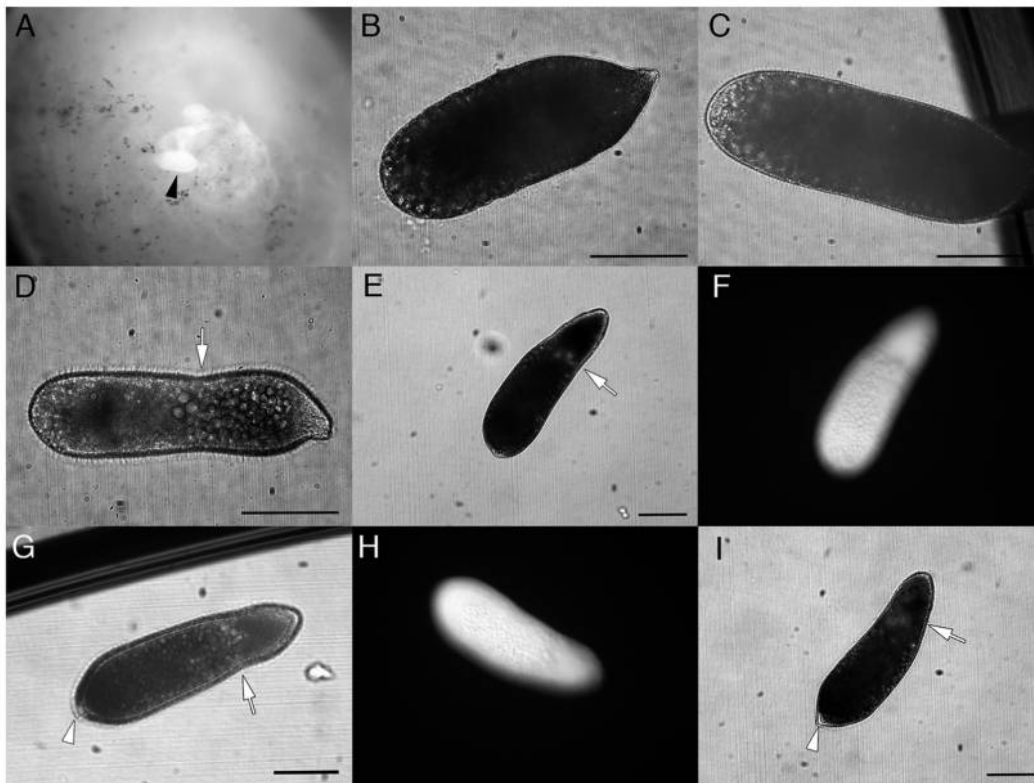


Figure 1. *Rhopalura xenoturbellae* sp. nov. female specimen, found on May 23, 2006 (anterior to the right in B–D, G, H and to the top in E, F, I). (A) The specimen (black arrowhead) exited the *Xenoturbella bocki* body from a rupture in the body wall, situated at the posterior end of the host. (B) Specimen just after leaving the host. (C) Twenty-four hours after exiting the host. (D) Thirty-six hours after exiting the host. A constriction (white arrow) has formed at one-third the body length from the anterior. (E) Two days after exiting the host. White arrow indicates constriction. (F) Same stage as in (E), showing oocytes present in the body. (G) Three days after exiting the host. White arrow indicates constriction; white arrowhead indicates pointed tip at the posterior end. (H) Same stage as in (G), showing oocytes present in the body. (I) Four days after exiting the host. White arrow indicates constriction; white arrowhead indicates pointed posterior tip. Scale bars = 100 μ m.

with numerous round oocytes (Fig. 1F, H). No further external morphological changes were observed beyond this stage (Fig. 1I). It continued swimming for nine days after exiting its host but was lost after the ninth day.

The observed females showed differences in morphology (Fig. 2). The body length ranged between 270 and 450 μm and the body width between 90 and 160 μm . Some retained their ovoid shapes, with no constrictions or pointed posterior tip, even after a few days (Fig. 2A). Some individuals possessed a rounded lump at the anterior tip (Fig. 2B). In one of the specimens, a ring of narrow epidermal cells was visible (Fig. 2C). Regardless of their shape, the interior was packed with round oocytes, not arranged in any rows (Figs. 1F, H, 2E). The females swam while rotating in a straight line or in spirals, using their cilia (Video 1, available online).

On February 22, 2006, numerous orthonectid ciliated larvae were found in a closed tube containing *X. bocki* collected 15 days earlier (Fig. 3A, B). Most were found trapped in or near mucus excreted from xenoturbellids (Fig. 3C). The larval body was composed of a cluster of cells, with long cilia present all around the body. They moved around by using the long cilia (Video 2, available online).

Observations of sectioned specimens

During observations of *X. bocki* sections deposited at SMNH, orthonectids were found inside three different animals: SMNH 5009, 5010, and 5012. All three of the animals contained multiple female orthonectids, but males were not found (Fig. 4A). Females were present in either the gastric cavity or the space between the muscle layer and the intestine (Fig. 4A, B). The largest section measured about 440 μm in

length and 135 μm in width (Fig. 4B). The females were filled with round oocytes (Fig. 4C). Sections near the surface of the orthonectid showed patterns of the epidermal cells and cilia (Fig. 4D).

Observations of sections of a xenoturbellid collected (in Gullmarsfjord, Sweden, on March 6, 2006) and sectioned by the authors revealed about five male specimens inside (Fig. 4E). The body length of the orthonectids was about 170 μm and the body width about 40 μm , smaller than the female specimens. Crystalline inclusions were observed in the males (Fig. 4E).

Taxonomic Description

Genus *Rhopalura* Giard, 1877

Rhopalura xenoturbellae sp. nov.

Figures 1–4, with illustrations of males and females presented in Figure 5.

Etymology: The species name refers to the generic name of the host.

Materials examined: Holotype, deposited in Shimoda Marine Research Center (SMRC), University of Tsukuba, as SMRC-Ortho1, female specimen, found on May 23, 2006, fixed and stored in 70% ethanol. Paratypes: SMRC-Ortho2, swimming larvae found on February 22, 2006, fixed and stored in 70% ethanol; SMRC-Ortho3, serially sectioned male specimen from *Xenoturbella*, collected at type-locality on March 6, 2006. All examined specimens are summarized in Table 1.

Type-host: *Xenoturbella bocki* Westblad, 1949.

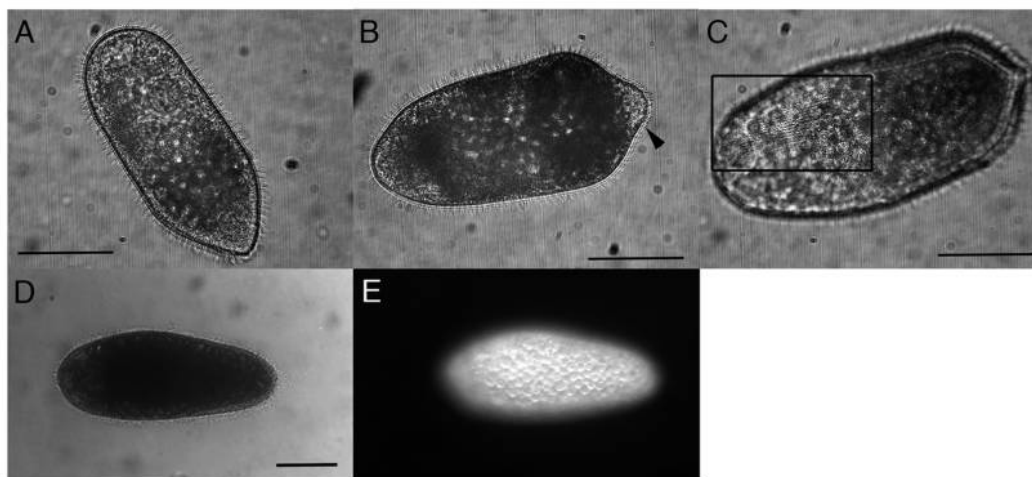


Figure 2. Female specimens of *Rhopalura xenoturbellae* sp. nov. (anterior to the bottom in A and to the right in B–E). (A) Specimen collected in May 2006, possessing a tapering anterior tip. (B) Specimen found in November 2006. A rounded lump (black arrowhead) can be seen at the anterior. (C) Another specimen collected in May 2006. Aligned narrow epidermal cells are visible (inside square). (D, E) An orthonectid discovered in March 2008. The anterior is narrower than the posterior, but it lacks external features seen in other females, such as a tapering tip, rounded lump, or constriction. It is filled with round oocytes. Scale bars = 100 μm .

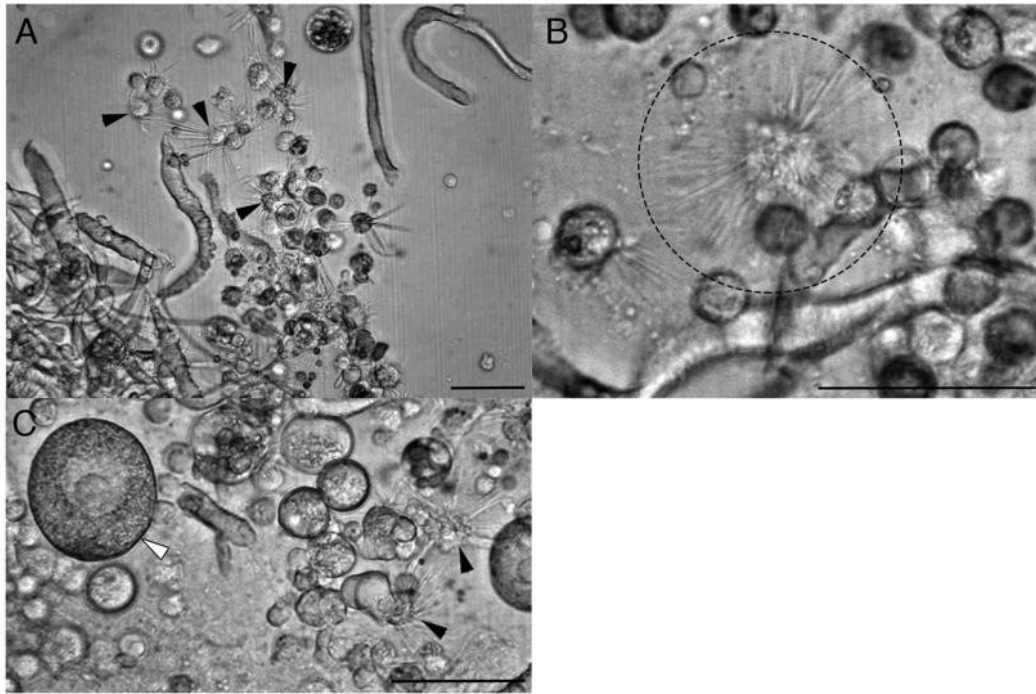


Figure 3. Ciliated larvae of *Rhopalura xenoturbellae* sp. nov. (A) Numerous larvae (black arrowheads) were found. (B) The larvae (one specimen shown inside circle) were a cluster of cells with many long cilia. (C) Larvae (black arrowheads) caught in the mucus of *Xenoturbella*. White arrowhead indicates immature oocyte of *Xenoturbella*. Scale bars = 100 μ m.

Site in host: Males and females present in the gastric cavity and the space between the muscle layer and the intestine of the host xenoturbellid.

Type-locality: Gullmarsfjord, Sweden.

Additional locality: Off the island of Syd-Hällsö, Sweden.

Female: (Figs. 1, 2, 4A–D, 5A) Body length between 270 and 450 μ m and body width between 90 and 160 μ m. Ovoid to elongated shape. Tapering anterior tip, anterior round lump, constriction at one-third the body length from the anterior and pointed posterior tip present in some specimens. A ring of narrow ciliated epidermal cells at the posterior part of body. Interior packed with round oocytes, not arranged in any rows. Capable of swimming in a straight line or in spirals while rotating.

Male: (Figs. 4E, 5B) Body length about 170 μ m and body width about 40 μ m. Crystalline inclusions present in the epidermis.

Larvae: Clusters of cells with long cilia present all around the body (Fig. 3). Capable of swimming by using the long cilia.

Discussion

There are four reported genera for the phylum Orthonectida: *Rhopalura*, *Intoshia*, *Ciliocincta*, and *Stoecharthrum*.

The genera are distinguished by morphological features such as the pattern of epidermal cells and ciliation, presence of sexual dimorphism, arrangement of oocytes within the female body, and presence of crystalline inclusions in some of the epidermal cells (Kozloff, 1992). The female body is extensively elongated in *Stoecharthrum* and some species of *Ciliocincta*, but that of specimens in this study was rather ovoid in shape (Figs. 1, 2, 5A). Oocytes are aligned in rows within the female axial mass in *Intoshia*, *Ciliocincta*, and *Stoecharthrum*, but this was not observed in the specimens in this study (Figs. 1F, H, 2E, 4B, C, 5A). Observations on sectioned male specimens within sectioned xenoturbellids suggested a conspicuous sexual dimorphism (Figs. 4, 5), known from *Rhopalura* and some species of *Intoshia*. Crystalline inclusions were observed in the male epidermal cells (Figs. 4E, 5B), which have been previously reported from *Rhopalura*. Swimming larvae, clusters of cells with long cilia (Fig. 3), were similar to those previously reported from *Rhopalura* (Caullery and Lavallee, 1908, 1912; Atkins, 1933). Taken together, all of these morphological features support an affinity to the genus *Rhopalura*. Within the genus, the females of all *Rhopalura* species whose morphology has been reported in detail possess small ovoid epidermal cells in the posterior half of the body (Atkins, 1933; Lang, 1954; Kozloff, 1969, 1992). On the other hand, the females in this study possessed narrow elongated epidermal cells in the posterior half of the body (Figs. 2C, 4D, 5A). Furthermore, orthonectid species mainly

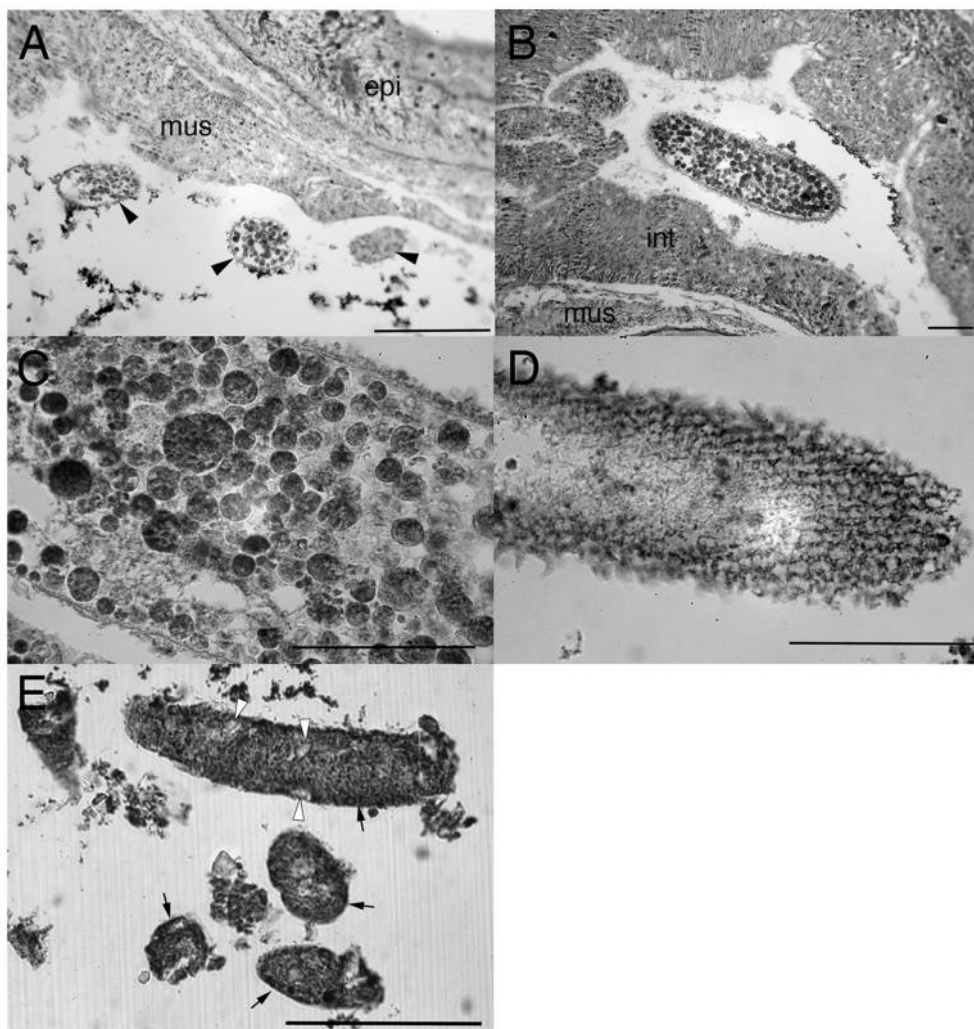


Figure 4. *Rhopalura xenoturbellae* sp. nov. found from sectioned *Xenoturbella bocki* specimens. (A) Three female orthonectids (black arrowheads) were seen in this section. epi, epidermis of the host *X. bocki*; mus, muscle layer of the host *X. bocki*. (B) Female specimen found in the gastric cavity of *X. bocki*. mus, muscle layer of the host *X. bocki*; int, intestinal cells of the host *X. bocki*. (C) Same specimen as in (B). It is filled with round oocytes. Round lipids are seen in the oocytes. (D) Same specimen as in (B), sectioned near the surface. Pattern of epidermal cells and ciliation is visible. (E) Four male orthonectids (black arrows) were seen in this section. Crystalline inclusions (white arrowheads) were present in the epidermis. Scale bars = 100 μ m.

parasitize only a single host species or, in some uncommon cases, multiple related species (e.g., *Rhopalura ophiocomae*, being found from *Amphipholis squamata*, *Ophiothrix fragilis*, and *Ophiura albida*, which are all ophiuroids) (Giard, 1877; Fontaine, 1968; Kozloff, 1969, 1992; Bender, 1972). There have been no previous reports of orthonectids infecting *Xenoturbella*. Based on the above differences in morphology and host species, we regard the animal in this study as an undescribed species and name the observed orthonectid as *Rhopalura xenoturbellae* sp. nov., after its host at the plasmodia stage. Various attempts to extract DNA from the specimens were all unsuccessful, probably because of the fixation and long-term storage. Molecular analysis of new materials will

clarify the phylogenetic position of this animal within the phylum.

The observed female specimens possessed different sizes and shapes (Figs. 1, 2), but it was impossible to determine whether these were artifacts resulting from different collection methods or whether they were natural phenomena. If the latter, the differences could have resulted from different developmental stages of females or from different types of females in a single orthonectid species. In *R. ophiocomae*, two types of females were reported: an elongated type and an ovoid type, differing in overall shape, body size, and epidermal cell arrangement (Kozloff, 1969, 1992). The different types were regarded as the same species, based mainly on

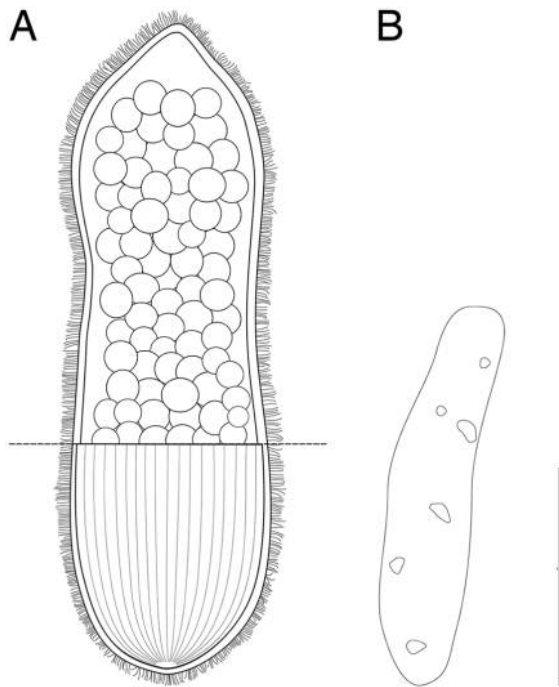


Figure 5. Drawings of *Rhopalura xenoturbellae* sp. nov. (A) Female based on specimen SMRC-Ortho1. The anterior two-thirds show the optical section of a live specimen. The posterior part shows boundaries of epidermal cells. (B) Male based on specimen SMRC-Ortho3. The overall body shape with crystalline inclusions is shown. Scale bar (applicable to both A and B) = 100 μm .

the fact that only a single type of male was observed from the host ophiuroid (Kozloff, 1969, 1992). Similarly, in *Rhopalura xenoturbellae*, only a single type of male was found (Figs. 4E, 5B); but observed males are still few, and more observations of male specimens are needed. Another possibility for different types of females is that they may represent multiple species of orthonectids infecting *Xenoturbella*. Two species of orthonectids, *Intoshia metchnikovi* and *Rhopalura pelse-neri*, have been reported to parasitize the nemertean *Tetras-temma flavidum* (Caullery, 1961); but further morphological studies have suggested that both *I. metchnikovi* and *R. pelse-neri* may be conspecific with *Intoshia linei* (Kozloff, 1992), and no new cases of multiple orthonectid species from a single host have been reported since. Therefore, the possibility of different types of females representing multiple species is low. However, orthonectid species have been previously reported from Swedish waters, the habitat of *Xenoturbella bocki*, such as *Rhopalura philinae* from the gastropod *Philina scabra* (Lang, 1954) and *Intoshia paraphanostomae* from acoels (Westblad, 1942; Dorjes, 1979; Kozloff, 1992). Further morphological and molecular studies of these species and *R. xenoturbellae* are needed to resolve the reasons for multiple types of orthonectid females being present within *X. bocki*.

Males and females of *R. xenoturbellae* were found from both the gastric cavity and the space between the muscle layer and the intestine of the host animal (Fig. 4A, B). Because ma-

ture *X. bocki* eggs have also been reported from both spaces, it is presumed that passage from one to the other is common in this animal. Entrance and exit from the host animal are through ruptures in the body wall (Fig. 1A) and possibly from the mouth as well. These internal parasitizing sites within the host, and the body color of the host, make it impossible to determine just from external observations whether a *Xenoturbella* specimen is infected. Therefore, caution is needed when interpreting morphological, molecular, and experimental data from xenoturbellids. Moreover, reevaluation of past observations—such as degenerating cells (Lundin, 2001; Israelsson, 2006), small brooded embryos less than 100 μm in size (Israelsson, 1999; Israelsson and Budd, 2005), and 5-bromothymidine incorporating bodies (Israelsson and Budd, 2005) inside adult *Xenoturbella*—will be interesting and in some cases may reveal hidden associations with orthonectids.

The orthonectid *Rhopalura ophiocomae* has been reported to have a negative effect on the reproduction of its host, the echinoderm *Amphipholis squamata*, causing total castration of the ophiuroid (Atkins, 1933; Deheyn *et al.*, 1998). A similar effect has also been reported for *Rhopalura granosa* on its host, the bivalve *Heteranomia squamula* (Giard, 1880), and for *Ciliocincta akkeshiensis* on an unidentified turbellarian (Tajika, 1979). In this study, many of the observed specimens were found from *Xenoturbella* collected during February–May (Table 1). This roughly corresponds to the breeding season of *X. bocki* in the examined area (December–March, Westblad, 1949; Nakano, 2015); but no mature gametes were found in parasitized *Xenoturbella*, and the parasite was not found in mature xenoturbellids. *Xenoturbella* has a low rate of animals with mature gametes, even in the breeding season (Westblad, 1949), which is one of the major reasons for its larva not being observed for more than 60 years (Nakano *et al.*, 2013). Further studies are essential, but it is possible that the orthonectids have a negative effect on the reproduction of their host, *X. bocki*.

We have reported here a new species of orthonectid, *Rhopalura xenoturbellae* sp. nov., that parasitizes xenoturbellids, thus revealing that there is still hidden diversity within the phylum Orthonectida. Biological surveys of previously uninvestigated environments, such as the deep sea and the polar regions, and research on various non-model organisms are now becoming prominent. Extensive observations of these samples may uncover more new species of orthonectids. Many fundamental biological details that remain unknown for orthonectids shall be revealed through research on various species, including *R. xenoturbellae* reported here.

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