Invertebrate Biology

Invertebrate Biology 132(3): 207–225.

DOI: 10.1111/ivb.12023

The peculiar nemertean larva *pilidium recurvatum* belongs to *Riserius* sp., a basal heteronemertean that eats *Carcinonemertes errans*, a hoplonemertean parasite of Dungeness crab

Terra C. Hiebert, George von Dassow, Laurel S. Hiebert, and Svetlana Maslakova[a]

Oregon Institute of Marine Biology, University of Oregon, Charleston, Oregon, USA 97420

Abstract. A typical nemertean pilidium larva resembles a hat with ear flaps. But one type, called *pilidium recurvatum*, looks more like a sock, swimming heel first. This distinctive larva was discovered in 1883 off the coast of Rhode Island and subsequently found in plankton samples from other parts of the world. Despite the long time since discovery, and its significance in discussions of larval evolution, this larva remained unidentified even to the family level. We collected *pilidium recurvatum* larvae from plankton samples in Coos Bay, OR, and identified them as belonging to the heteronemertean genus *Riserius* based on juvenile morphology and DNA sequence data. Phylogenetic analysis suggests that two distinct types of *pilidium recurvatum* from Oregon represent two new species within this currently monotypic genus. We describe the morphology of *pilidium recurvatum* using confocal microscopy and compare it to that of the typical pilidium, discussing possible implications for larval feeding. We also report our surprising discovery that juveniles of *Riserius* sp. from Oregon prey on another nemertean, *Carcinonemertes errans,* an egg predator of *Cancer magister* (Dungeness crab), a commercially important species. We speculate that the species-level diversity and geographic distribution of *Riserius* may be much greater than currently appreciated.

Additional key words: Pilidiophora, *Riserius pugetensis*, *Carcinonemertes errans*, *Cancer magister*

Introduction

Most nemerteans (Phylum Nemertea, ribbon worms) have a biphasic life cycle with a benthic adult and a planktonic larval stage (Norenburg & Stricker 2002). The pilidium is a charismatic long-lived planktotrophic larval form found in one clade of nemerteans called, for that reason, the Pilidiophora (Thollesson & Norenburg 2003; Maslakova 2010a). The pilidium is remarkable for its mode of development (via imaginal discs) and catastrophic metamorphosis in which the emerging juvenile rapidly devours the larval body (Cantell 1966a; Lacalli 2005; Maslakova 2010b; Maslakova & von Dassow 2012). These larvae are frequently encountered in plankton samples and have many distinct morphotypes (e.g., Dawydoff 1940; Cantell 1969; Lacalli 2005) and yet the species-, genus-, or even family-identity of most of these larvae remains a mystery, because the development of most nemerteans is undescribed (e.g., Johnson 2001).

Originally described in 1847 by the German anatomist and physiologist Johannes Müller, a typical pilidium looks like a transparent deerstalker cap with its ear flaps pulled down (Fig. 1A). Although he suspected that it might be the larva of an animal, Müller did not know that the pilidium is the larva of a nemertean worm, and he originally assigned it the binomen *pilidium gyrans* (in reference to its hat-like shape and rotating swimming motion) (Müller 1847). Since then, many other pilidial morphotypes have been described based on unidentified specimens collected from plankton, and, following Müller's lead, assigned various binomena that reflect their distinct morphology, e.g., *pilidium auriculatum* (Leuckart & Pagenstecher 1858), *pilidium recurvatum* (Fewkes 1883), and *pilidium depressum* (Dawydoff 1940). (These binomial designations, which we do not capitalize to avoid confusion with proper species names, are a convenient way, pending matches to species or species group, to refer to larval morphotypes, as it is

[a]Author for correspondence.
E-mail: svetlana@uoregon.edu

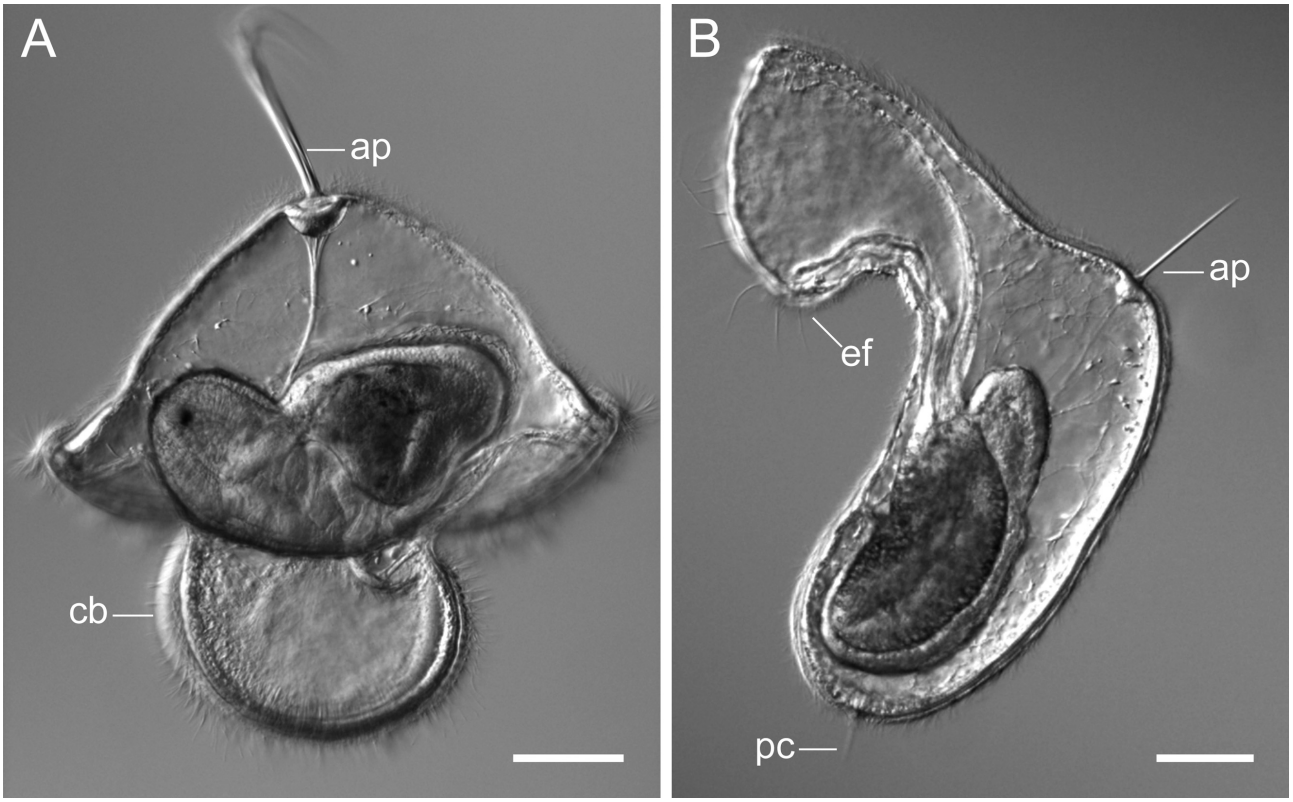


Fig. 1. Overall morphology of the typical hat-like pilidium larva (A) and *pilidium recurvatum* from Oregon (B). **A.** A typical pilidium larva from Coos Bay plankton with a prominent apical tuft (ap) at the larval anterior end and ciliated band (cb) spanning the lobes and lappets at the larval posterior end. The anterior–posterior axis of the developing juvenile is perpendicular to that of the larva (eyes mark the juvenile anterior). **B.** A sock-like *pilidium recurvatum* larva from Coos Bay plankton. The larval anterior is marked with the apical tuft (ap), and the larval posterior with a small cirrus (pc). The anterior–posterior axis of the developing juvenile inside is parallel to that of the larval body. The prominent larval esophageal funnel (ef) is extended perpendicular to the larval/juvenile anterior–posterior axis, and lacks a prominent ciliated band along its margin. Scale bars=100 μm.

not always clear whether a particular pilidial morphotype represents a species, a group of related species, or unrelated species: Bürger 1895; Schmidt 1930; Dawydoff 1940).

One of the most remarkable pilidial morphotypes is *pilidium recurvatum*. If a typical pilidium looks like a hat, *pilidium recurvatum* looks more like an athletic sock, swimming heel first and toe trailing behind (Fig. 1B). It was discovered by Walter Fewkes in 1883 in a plankton sample taken off the coast of Rhode Island (Fewkes 1883; Fig. 2A) and named in reference to the characteristic curvature of its anterior end. Similar morphotypes have since been reported from other parts of the world: the Bay of Nha Trang, Vietnam (Dawydoff 1940; Fig. 2B), Gullmarfjord, Sweden (Cantell 1966a; Fig. 2C), the Sea of Japan, Russia (Chernyshev 2001; Fig. 2D), and the NE Pacific off Washington and Oregon (Schwartz 2009; Maslakova 2010a). Fewkes (1883) believed *pilidium recurvatum* to be the larva of a heteronemertean from the genus *Lineus* (Family Lineidae) and, later, Cantell speculated that this larva may belong to the heteronemertean family Baseodiscidae (1966a) or Valenciniidae (more specifically the genus *Oxypolella*) (1966b), but until now, the identity of *pilidium recurvatum* remained undetermined.

Because of its distinctive morphology and the orientation of the juvenile anterior–posterior axis with respect to the larval axes, Jägersten (1972) suggested that *pilidium recurvatum* may represent an evolutionary intermediate between the juvenile-like planuliform nemertean larva, found in non-pilidiophoran nemerteans and presumed to be ancestral for the phylum (reviewed in Maslakova 2010a), and the typical pilidium. Evaluating this hypothesis depends on being able to determine the phylogenetic position of *pilidium recurvatum*.

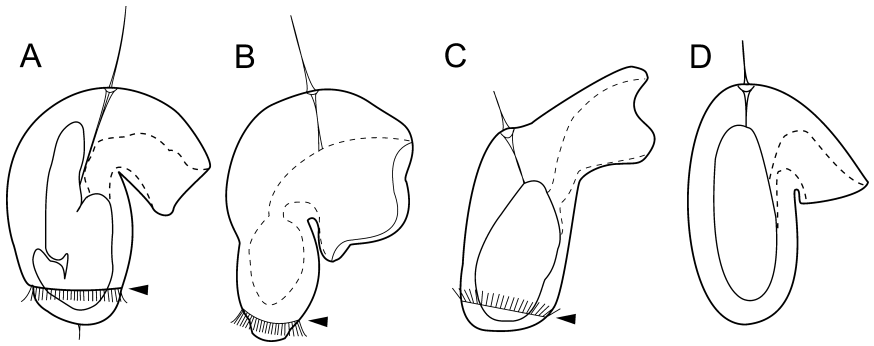


Fig. 2. Diagrams highlighting the morphology of the previously described *pilidium recurvatum*-like larvae. **A**. A larva from the northeast Atlantic (after Fewkes 1883). **B**. A larva from the Bay of Nha Trang, Vietnam (after Dawydoff 1940). **C**. A larva from Gullmarfjord, Sweden (after Cantell 1966a). **D**. A larva from the Sea of Japan (after Chernyshev 2001). Note presence of a posterior transverse ciliated band in *pilidium recurvatum* from the northeast Atlantic, Bay of Nha Trang, and Gullmarfjord (arrowhead in A, B, C).

Here, we use a combination of mtDNA sequence data and juvenile morphology to reveal the identity and phylogenetic position of *pilidium recurvatum* from Oregon: it is the larva of a member of *Riserius*, previously a monotypic genus within the order-level taxon Pilidiophora. We document with video-microscopy the catastrophic metamorphosis of *pilidium recurvatum*, and reveal, for the first time, its morphology by use of confocal microscopy. We also report our surprising discovery of the prey choice of juveniles from *pilidium recurvatum*—they preferentially fed on the larvae and juveniles of *Carcinonemertes errans*, a hoplonemertean predator on crab eggs. This may be relevant to the biology of the Dungeness crab, a commercially harvested species.

Methods

Collecting and maintaining live larvae

Nemertean larvae were collected opportunistically from plankton samples taken from the Charleston Marina docks in Charleston, OR using a plankton net with 153 μm mesh. We found one individual of the *pilidium recurvatum* morphotype on each of 9 and 30 October 2008, one or two in Spring of 2009, and several individuals (<10) on 11 October and 1 November 2011. The largest number of individuals (~50) was encountered in samples from 14 to 17 August 2012, and small numbers of these larvae were present in plankton samples taken from August to October 2012. All individuals were photographed, and some were preserved for DNA extraction or confocal microscopy. The most developmentally advanced larvae were maintained live in 150 mL bowls containing filtered seawater (FSW, 0.45 μm) and partly submerged in a sea table with flow-through seawater (9–12°C) to document metamorphosis and juvenile morphology. We were able to prompt and observe metamorphosis in some of these individuals by compressing them gently between a glass slide and a coverslip supported over the slide using small clay feet.

Larvae of *Micrura alaskensis* Coe 1901 used here for comparison to illustrate the morphology of a typical pilidium were reared from eggs fertilized *in vitro* as previously described (Maslakova 2010b). The larva depicted in Fig. 1A was collected from plankton in Coos Bay, OR on 5 December 2011, and has been identified by DNA sequence as probably belonging to one of the local *Lineus* spp. (e.g., *L. rubescens* Coe 1904, *L. pictifrons* Coe 1904, or an undescribed species).

Photo- and video-microscopy

Live larvae were photographed on an Olympus BX51 microscope equipped with DIC using either a Leica DFC400 or a Grasshopper2 (Point Grey Research) camera. Video recordings of metamorphosis were done on the same microscope using a Grasshopper2 camera and Astro IIDC v. 4.07 imaging program.

DNA extraction and PCR amplification

Individual live larvae were photographed and cryopreserved (−80°C) in a small drop of sea water. DNA from larval samples was extracted using a Chelex-based method (InstaGene, BioRad). To obtain reference sequences from the local species *Micrura wilsoni* Coe 1904, we collected adult specimens from

Middle Cove, Cape Arago, OR in September 2009 and preserved tissue samples in ethanol, later extracting DNA using a DNeasy Blood and Tissue Kit (Qiagen). We amplified two mitochondrial gene regions: a ~460 bp fragment of the large subunit ribosomal DNA (16S), and a 658 bp "barcoding" region of the cytochrome *c* oxidase subunit I (COI). In one case, we also amplified a ~300 bp region of the nuclear gene encoding histone H3. We used previously published "universal" primers: 16SArL [5′ CGCCTGTTTATCAAAAACAT 3′] and 16S BrH [5′ CCGGTCTGAACTCAGATCACGT 3′] (Palumbi et al. 1991) for 16S rDNA; LCO 1490 [5′ GGTCAACAAATCATAAAGATATTGG 3′] and HCO 2198 [5′ TAAACTTCAGGGTGACCAAAA AATCA 3′] (Folmer et al. 1994) for COI; and H3NF [5′ ATGGCTCGTACCAAGCAGAC 3′] and H3R [5′ ATATCCTTRGGCATRATRGTGAC 3′] (Colgen et al. 2000) for histone H3. In several instances, nemertean-specific primers designed and kindly provided to us by Dr. Jon Norenburg (Smithsonian Institution) proved to be more successful for COI amplification (especially when used in combination with the Folmer primers): COIDr [5′ GAGAAATAATACCAAAACCAGG 3′] and COILf [5′ TTTCAACAAATCATAAAGATAT 3′]. PCR thermocycling was carried out as follows using 8 μl of undiluted template DNA: 95°C for 2 min; 35 cycles of 95°C for 40 s, 45–55°C (45–52°C for COI, 52°C for 16S and H3) for 40 s, and 72°C for 1 min; and a 2 min final extension at 72°C. PCR products were purified using Wizard SV Gel and PCR Cleanup kit (Promega) and sent to Sequetech (Mountain View, CA) to sequence in both forward and reverse directions using PCR primers.

Sequence analysis

Sequences were proofread and trimmed in Codon Code Aligner v. 3.7.1 (Codon Code Corp, MA). To determine the identity of *pilidium recurvatum,* we conducted phylogenetic analyses including all of the 16S rDNA sequences obtained from the larval samples, as well as adults of local nemertean species *Micrura wilsoni*, and GenBank sequences from a broad range of pilidiophoran taxa (see Table 1 for accession numbers). All sequences were aligned using ClustalX v. 2.1 (Larkin et al. 2007) with default gap penalties. We conducted a neighbor-joining analysis using ClustalX. Uncorrected pairwise distances were calculated for all larval samples using PAUP* v. 4b1.0 (Swofford 2002). Phylogenetic analysis using parsimony was also carried out in PAUP* using heuristic search (random sequence addition with 1000 replicates, 10 best trees held at each step, and TBR branch-swapping algorithm). Clade support was estimated using 1000 bootstrap replicates (Felsenstein 1985). Phylogenetic analysis using Bayesian inference was carried out using MrBayes v. 3.2.1 (Ronquist et al. 2012) with evolutionary model parameters determined with jModelTest v. 2.1 (Posada 2008) using Akaike and Bayesian Information criteria. Both criteria determined the TN93 (Tamura & Nei 1993) evolutionary model (gamma distributed with invariant sites) to be most appropriate for our data. Markov chain Monte Carlo (MCMC) was set for 10^6 generations, sampling every 100 generations with the first 25% of trees discarded as burn-in. Tree topologies were viewed using FigTree v. 1.3.1 (Rambaut 2009).

Antibody labeling and confocal microscopy

Ten *pilidium recurvatum* were relaxed in a 1:1 mixture of 0.34 M $MgCl_2$ and filtered seawater (0.45 μm, FSW) for ~30 min prior to fixation. Larvae were preserved in 4% paraformaldehyde (made up in FSW from 20% ultrapure paraformaldehyde, Electron Microscopy Sciences) for 1 h at room temperature (RT). Fixative was removed by a few quick (<1 min) washes in phosphate buffered saline (PBS, Fisher Scientific). Larval tissues were permeabilized in PBS with 0.1% Triton X-100 and 0.1% Bovine Serum Albumin (PBT+BSA) in three consecutive 10-min washes. To block non-specific labeling, larvae were incubated in 5% Normal Goat Serum in PBT+BSA for 2 h at RT. To reveal the structure of the serotonergic nervous system, larvae were incubated in rabbit anti-serotonin (1:500) primary antibody (Immunostar, Cat# 20080) at 4°C for 48 h, followed by three 10-min washes in PBT+BSA, and labeling with Alexa Fluor 488 goat anti-rabbit secondary antibody (1:200) for 2 h at RT. To help visualize the cell nuclei and the muscles, larvae were additionally labeled with Hoechst 33342 (2 μmol L^{-1}) and Rhodamine Phalloidin (Sigma, 165 nmol L^{-1} in PBT+BSA). Fluorescently labeled larvae were washed in PBS and imaged in PBS or 90% glycerol in glass coverslip-bottomed Petri dishes (MatTek). Glycerol-mounted larvae were noticeably more clear. Confocal stacks of 0.5–0.75 μm sections were obtained using an Olympus FluoView 1000 confocal system on an Olympus IX81 inverted microscope equipped with UPlanSApo 20X 0.85 NA oil lens. Z-projections were reconstructed using ImageJ v. 1.46 (Wayne Rasband, National Institutes of Health, Bethesda, MD) and

Table 1. Classification, accession numbers, and references for sequences used in the 16S phylogenetic analysis. See text (Results: DNA sequence analysis) for accession numbers of *pilidium recurvatum* 16S rDNA and COI sequences.

	Accession number	Reference
Ingroup taxa		
Nemertea, Anopla, Heteronemertea		
Baseodiscus delineatus	EF124860	Schwartz and Norenburg, unpubl. data
Baseodiscus hemprichii	EF124862	Schwartz and Norenburg, unpubl. data
Baseodiscus mexicanus	EF124863	Schwartz and Norenburg, unpubl. data
Baseodiscus quinquelineatus	EF124864	Schwartz and Norenburg, unpubl. data
Baseodiscus unicolor	EF124865	Schwartz and Norenburg, unpubl. data
Carinoma mutabilis	AJ436832	Thollesson & Norenburg 2003
Cerebratulus lacteus	JF277575	Andrade et al. 2011
Cerebratulus marginatus	AJ436821	Thollesson & Norenburg 2003
Cerebratulus montgomeryi	EF124875	Schwartz and Norenburg, unpubl. data
Dushia atra	EF124878	Schwartz and Norenburg, unpubl. data
Evelineus tigrillus	EF124879	Schwartz and Norenburg, unpubl. data
Lineus acutifrons	JF277573	Andrade et al. 2011
Lineus alborostratus	AJ436822	Thollesson & Norenburg 2003
Lineus bicolor	AJ436823	Thollesson & Norenburg 2003
Lineus bilineatus	JF277571	Andrade et al. 2011
Lineus longissimus	AJ436825	Thollesson & Norenburg 2003
Lineus torquatus	JF277572	Andrade et al. 2011
Micrura akkeshiensis	EF124887	Schwartz and Norenburg, unpubl. data
Micrura alaskensis	AJ436827	Thollesson & Norenburg 2003
Micrura callima	EF124889	Schwartz and Norenburg, unpubl. data
Micrura fasciolata	JF277585	Andrade et al. 2011
Micrura purpurea	JF277577	Andrade et al. 2011
Micrura verrilli	EF124899	Schwartz and Norenburg, unpubl. data
Micrura wilsoni	—	Maslakova, unpubl. data
Notospermus geniculatus	AJ436824	Thollesson & Norenburg 2003
Oxypolella alba	AF103767	Sundberg & Saur 1998
Parborlasia corrugatus	AJ436829	Thollesson & Norenburg 2003
Ramphogordius lacteus	JF277584	Andrade et al. 2011
Riserius pugetensis	AJ436831	Thollesson & Norenburg 2003
Zygeupolia rubens	JF277574	Andrade et al. 2011
Nemertea, Anopla, Palaeonemertea		
Hubrechtella dubia	AJ436834	Thollesson & Norenburg 2003
Outgroup taxa		
Nemertea, Anopla, Palaeonemertea		
Cephalothrix filiformis	AJ436835	Thollesson & Norenburg 2003
Tubulanus sexlineatus	JF277574	Andrade et al. 2011

overlaid and false-colored in Adobe Photoshop CS3.

For comparative purposes, we included confocal images from pilidium larvae of *M. alaskensis*. Antibody labeling was carried out as described above, larvae were mounted in Vectashield (Vector Laboratories), and confocal stacks were obtained at the Friday Harbor Laboratories (University of Washington) using a BioRad Radiance 2000 laser scanning confocal system mounted on a Nikon Eclipse E800 microscope with a 40X 1.3 NA oil lens. Images were processed in ImageJ and Adobe Photoshop as described above.

Results

Larval morphology and behavior

Pilidium larvae of the *pilidium recurvatum* morphotype from Oregon have an elongated transparent body and a large recurved "trunk" or esophageal funnel (Figs. 1B, 3A–D). Each larva is equipped with a prominent apical tuft at the anterior end and a small ciliary cirrus at the posterior end (Fig. 3C). The apical plate is connected to the developing juvenile by an apical muscle (Figs. 3C, 4A), which can be more or less conspicuous depending on the

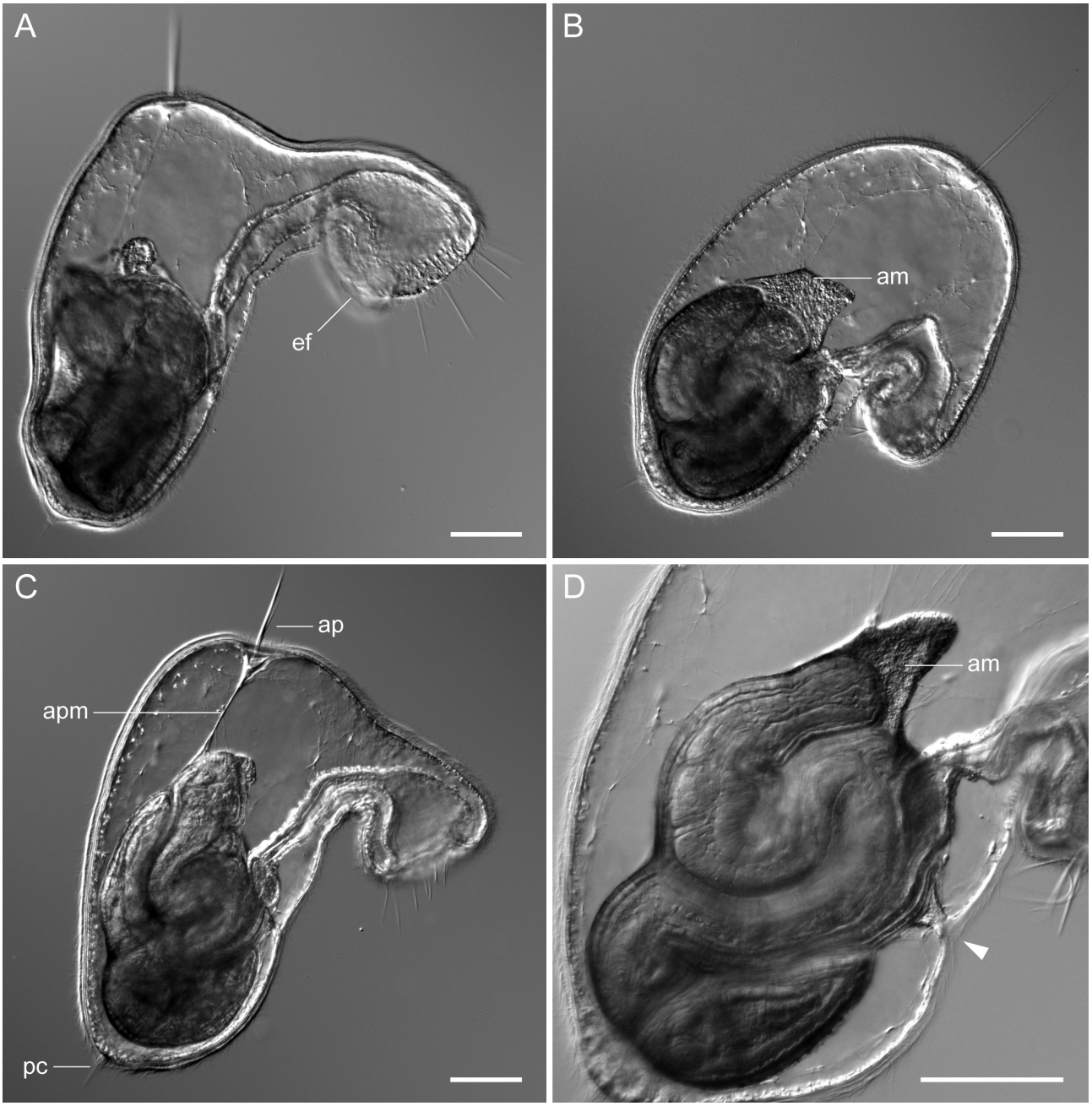


Fig. 3. Morphology and behavioral postures of *pilidium recurvatum* larvae from Coos Bay, OR. **A**. 'Feeding posture' in *pilidium recurvatum*. Note several stiff ciliary cirri along the margin of the esophageal funnel (ef). **B**. The 'swimming posture', in which the esophageal funnel is contracted. Note the coiled juvenile nemertean inside the thin amniotic membrane (am). **C**. An intermediate posture with esophageal funnel partially outstretched. Note the blade-like anterior apical tuft (ap) and posterior ciliary cirrus (pc). Connecting the apical tuft to the juvenile amnion is an apical muscle (apm). **D**. The juvenile amnion (am) opens to the outside by the ventral amniotic pore (arrowhead). Scale bars=100 μm.

individual (its developmental stage and overall condition). The pilidial body is uniformly ciliated except for the distinct apical tuft, the posterior cirrus, and ~20 conspicuous (~100 μm long) ciliary cirri arranged along the margin of the esophageal funnel (Fig. 3A). These larvae lack the posterior transverse ciliary band, referred to as the telotroch (compare Figs. 1B and 2A–C).

Neurons in the serotonergic nervous system of *pilidium recurvatum* are distributed within the larval epidermis, and their processes form a fine sub-epidermal network (Fig. 4B). Unlike in typical pilidia (e.g., see Salensky 1912; Maslakova 2010b; Fig. 1A, here), there is no distinct ciliary band along the margin of the esophageal funnel of *pilidium recurvatum* (Figs. 1B, 3A–C). Accordingly, there is no prominent muscle strand analogous to the one spanning lobes and lappets of a typical pilidium (compare Fig. 4C,D, also see 4E,F), nor a thick serotonergic nerve cord (Fig. 4B,C,F) present in a typical pilidium (Fig. 4D). Instead, there are numerous fine muscle and nerve fibers (not unlike elsewhere in the body) running along the margin of the esophageal funnel (Fig. 4A–C,F).

The marginal ciliary band of a typical pilidium possesses several rows of collar cells, each equipped with a single stationary cilium (data not shown) supported by a cone of microvilli (Fig. 4D, inset). These collar cells, which are spaced out regularly along the margin of lobes and lappets (at a distance of ~8 μm), likely serve to detect food particles (G. von Dassow & S. Maslakova, unpubl. data). The margin of the esophageal funnel in *pilidium recurvatum* lacks these characteristic collar cells (Fig. 4E). Instead, the margin of the esophageal funnel bears the long cirri (Fig. 3A), each composed of multiple cilia (data not shown) that originate from a single cell (judging from the number of associated nuclei on Fig. 4E) and are supported by a 10–12 μm wide field of microvilli. These fields of microvilli are separated from each other by a distance of ~25–35 μm (Fig. 4E). At the same time, we noticed what appear to be collar cells dispersed throughout the inner surface of the esophageal funnel (Fig. 4A) of *pilidium recurvatum*. Accordingly, we noticed many single stationary cilia inside the funnel (data not shown), similar to those originating from the collar cells in a marginal ciliary band of a typical pilidium.

We observed two distinct postures or behaviors in *pilidium recurvatum* from Oregon, which we provisionally refer to as the "swimming posture" and "feeding posture." In the swimming posture, the funnel is contracted and folded back against the larval body (Fig. 3B). Larvae in this posture swim apical organ forward while revolving along the antero-posterior axis. In the feeding posture, the funnel is dramatically expanded and positioned perpendicularly to the larval body, or flexed further toward the apical organ (Fig. 3A). Larvae in this posture do not swim, but slowly drift in the water column. In this posture, the marginal cirri protrude stiffly outward, not unlike the hair-like projections of the Venus flytrap. This uncanny resemblance is further strengthened by the bi-lobed appearance of the *pilidium recurvatum* funnel, as the margin of the funnel bears a distinct ventral notch (Fig. 4F). The inner surface of the funnel is ciliated densely, but not entirely uniformly. There is a distinct tract of denser ciliation lining the ventral esophageal groove that originates deep within the funnel and terminates at the ventral notch. Corresponding to this ciliary tract, there is a concentration of fine serotonergic nerve and muscle fibers running along the ventral esophageal groove (Fig. 4F). Although we introduced individuals of *pilidium recurvatum* to a variety of potential food items, including small phytoflagellates (*Rhodomonas* and *Dunaliella*), and larger prey, such as marine invertebrate larvae (molluscan veligers, echinoderm blastulas, bipinnarias and plutei, a variety of hoplonemertean larvae), euglenids, diatoms, and *Noctiluca,* none of these elicited a response. We also tried offering *pilidium recurvatum* natural plankton (excluding items larger than 100 μm), but did not observe any change in gut contents after an overnight trial. We have not yet observed actual feeding, or direct evidence thereof, in these larvae.

Catastrophic metamorphosis

The antero–posterior (AP) axis of the developing juvenile inside *pilidium recurvatum* coincides with the larval AP axis (Fig. 1B), unlike in a typical pilidium where the axes are approximately perpendicular (Fig. 1A). A juvenile approaching metamorphosis is long compared with the larval body, and is typically coiled inside the amnion (Fig. 3), which opens to the outside via a short ciliated canal and a ventral pore (Fig. 3D). We observed metamorphosis in numerous individuals, and captured two on film (e.g., see Video S1). In all cases we observed, the posterior portion of the larval body dramatically contracted at the beginning of metamorphosis (Fig. 5A–C). The juvenile then emerged posterior end first by rupturing the ventral larval epidermis posterior to the amniotic pore (Fig. 5B,C). As the juvenile emerged, the amnion collapsed and appeared as a dark brown mass inside the larva (Fig. 5D). Once the juvenile head was out, the larval body was drawn into the juvenile mouth, at a considerable distance away from the anterior tip (Fig. 5D,E). Metamorphosis was complete within minutes in all but one of the instances we witnessed, and the entire larval body was swallowed by the juvenile worm (Fig. 5F). Although we did not witness metamorphosis in all collected individu-

als, we frequently noted dark brown gut contents in recently metamorphosed juveniles, which suggest that they ingest the larval body (including the amnion) as a matter of course (Fig. 5F). The juvenile epidermis was rather sticky, and individuals often remained twisted on the bottom of culture dishes in a tight coil for several days after metamorphosis.

Juvenile morphology

Newly metamorphosed juveniles are ~1.5 mm in length and lack a caudal cirrus or lateral cephalic furrows. The other characteristic features of juvenile morphology include an unusually long pre-oral region (about 40–50% of the total juvenile body length immediately after metamorphosis: Fig. 5D), a pair of conspicuous cerebral organ pits (Fig. 6), and a V-shaped transverse cephalic furrow located immediately posterior to the cerebral organ pits, but anterior to the mouth (Fig. 6).

We found two distinct *pilidium recurvatum* morphotypes in Coos Bay, OR, easily differentiable by the presence or absence of juvenile eyes, which become apparent in advanced larvae (Fig. 7).

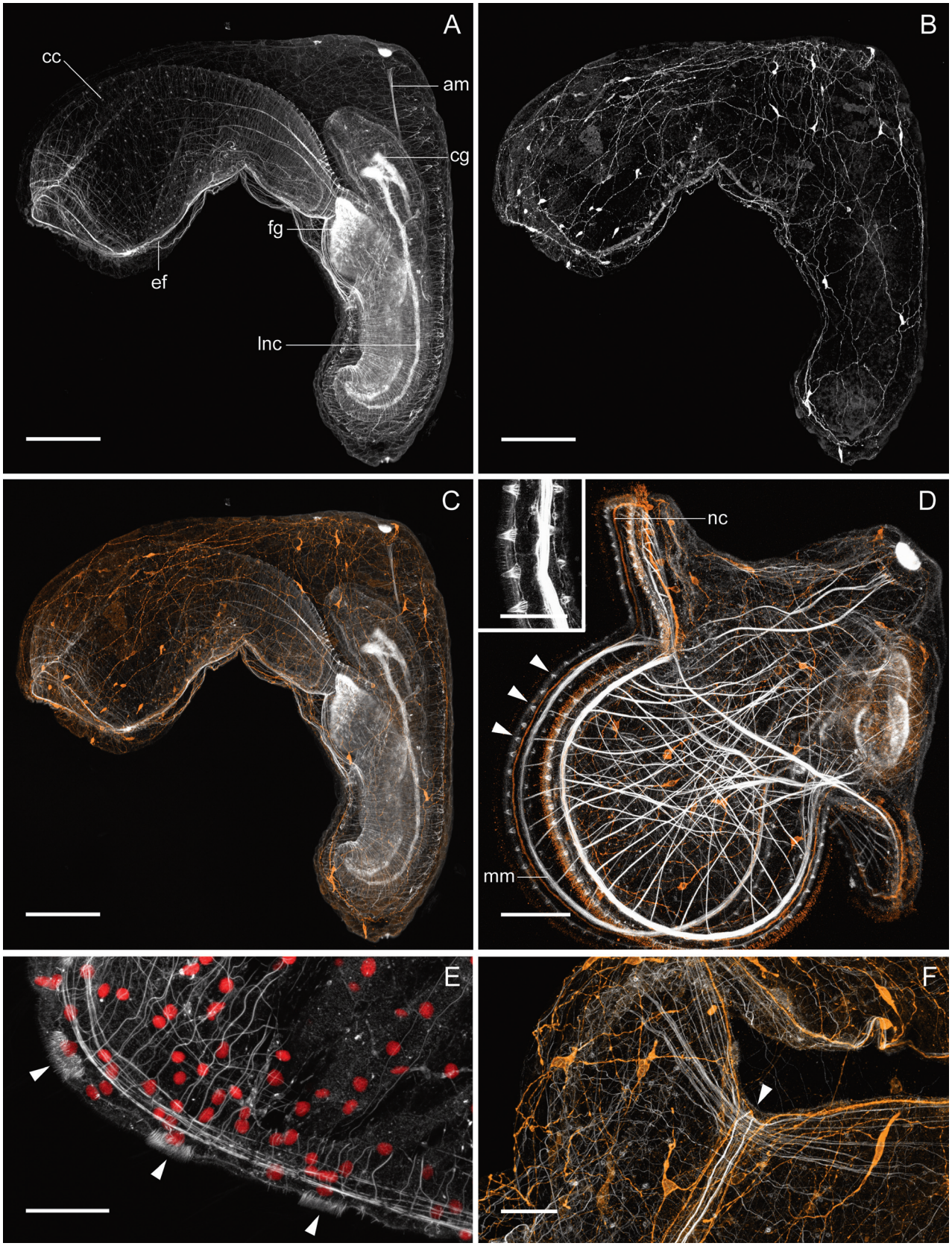
DNA sequence analysis

We obtained sequences of 16S and COI gene regions from ten and eight individuals of *pilidium recurvatum*, respectively (GenBank accession numbers KC777021–KC777038). For the phylogenetic analyses, we used all acquired *pilidium recurvatum* sequences, a sequence of *Micrura wilsoni* from Oregon (obtained by us), and a diverse selection of heteronemertean sequences from GenBank (Table 1).

The 16S alignment comprised 43 individuals (representing 35 species) and was 571 bp long. Of 571 characters, 312 were parsimony informative. Maximum parsimony analysis yielded a strict consensus of three most parsimonious trees (tree length=2139, consistency index=0.3406, homoplasy index=0.6594, Fig. 8), which was nearly identical in topology to both the Bayesian and the neighbor-joining trees (data not shown). Both types of *pilidium recurvatum* formed a monophyletic clade with *Riserius pugetensis* Norenburg 1993 with high bootstrap support (BST=100) and posterior probabilities (PP=100, Fig. 8). The two larval types formed two distinct and well-supported clades (*pilidium recurvatum* with eyes [BST=100, PP=100] and *pilidium recurvatum* without eyes [BST=100, PP=100]). *Riserius pugetensis* appeared more closely related to *pilidium recurvatum* without eyes (BST=99, PP=89). Our 16S data are reported here because this gene region is known to resolve species level relationships well among nemerteans (e.g., Schwartz & Norenburg 2005; Strand et al. 2005), which is not the case for COI.

Average sequence divergences are shown as uncorrected p-distances for 16S and COI gene

Fig. 4. Muscular and neural anatomy in *pilidium recurvatum* from Oregon (A–C, E–F), and a typical pilidium (**D**, inset), as revealed by confocal microscopy. Apical plate is up. **A.** Confocal z-projection of a *pilidium recurvatum* stained with phalloidin. Note the lack of a prominent muscle band, or the collar cells along the margin of the esophageal funnel (ef). On the other hand, what we think are numerous collar cells (cc), visible here as small dots, are scattered over the inner surface of the funnel. The juvenile foregut (fg) is brightly labeled probably due to the dense lining of microvilli. The juvenile cerebral ganglia (cg), lateral nerve cords (lnc), and apical muscle (am) are also highlighted with phalloidin. Scale bar=100 μm. **B.** The same larva as on (A) labeled with anti-5HT antibody showing sub-epidermal serotonergic nerve network. Note the absence of a prominent serotonergic nerve cord along the margin of the esophageal funnel. Scale bar=100 μm. **C.** Same larva as on (A) and (B). An overlay of phalloidin (gray) and 5-HT (orange) channels, shows the relative position of muscles and serotonergic neurons and fibers. Scale bar=100 μm. **D.** A confocal projection of a conventional pilidium larva *(Micrura alaskensis)*, stained with phalloidin (gray) and anti-5HT antibody (orange). Note the prominent marginal muscle (mm) and nerve cord (nc), which correspond to the marginal ciliated band along the pilidial lobes and lappets. Also note regularly spaced prominent collar cells (arrowheads and inset) arranged along the marginal ciliated band. Scale bar=100 μm; inset scalebar=20 μm. **E.** A close-up view of the margin of the esophageal funnel in a *pilidium recurvatum* stained with phalloidin (gray) and Hoechst (red). Note the absence of collar cells found in a conventional pilidium (D, inset). Instead, there are somewhat wider spaced dense microvillar fields (arrowheads), each originating from a single cell (as evident from the arrangement of the nuclei). On the basis of number and distribution of these microvillar fields, we think that they probably correspond to the marginal cirri (Fig. 3A). Scale bar=25 μm. **F.** Ventral view of the funnel margin of *pilidium recurvatum* stained with phalloidin (gray) and anti-5HT antibody (orange) showing the ventral notch (arrowhead) and multiple fine muscle fibers and serotonergic nerves along the margin and the ventral groove of the funnel, as opposed to the single prominent muscle and nerve running along the marginal ciliated band in the typical pilidium (D). These morphological differences probably reflect the feeding strategies of the two larval types. Scale bar=25 μm.


A
cc
am
cg
fg
ef
lnc
B
C
D
nc
mm
E
F

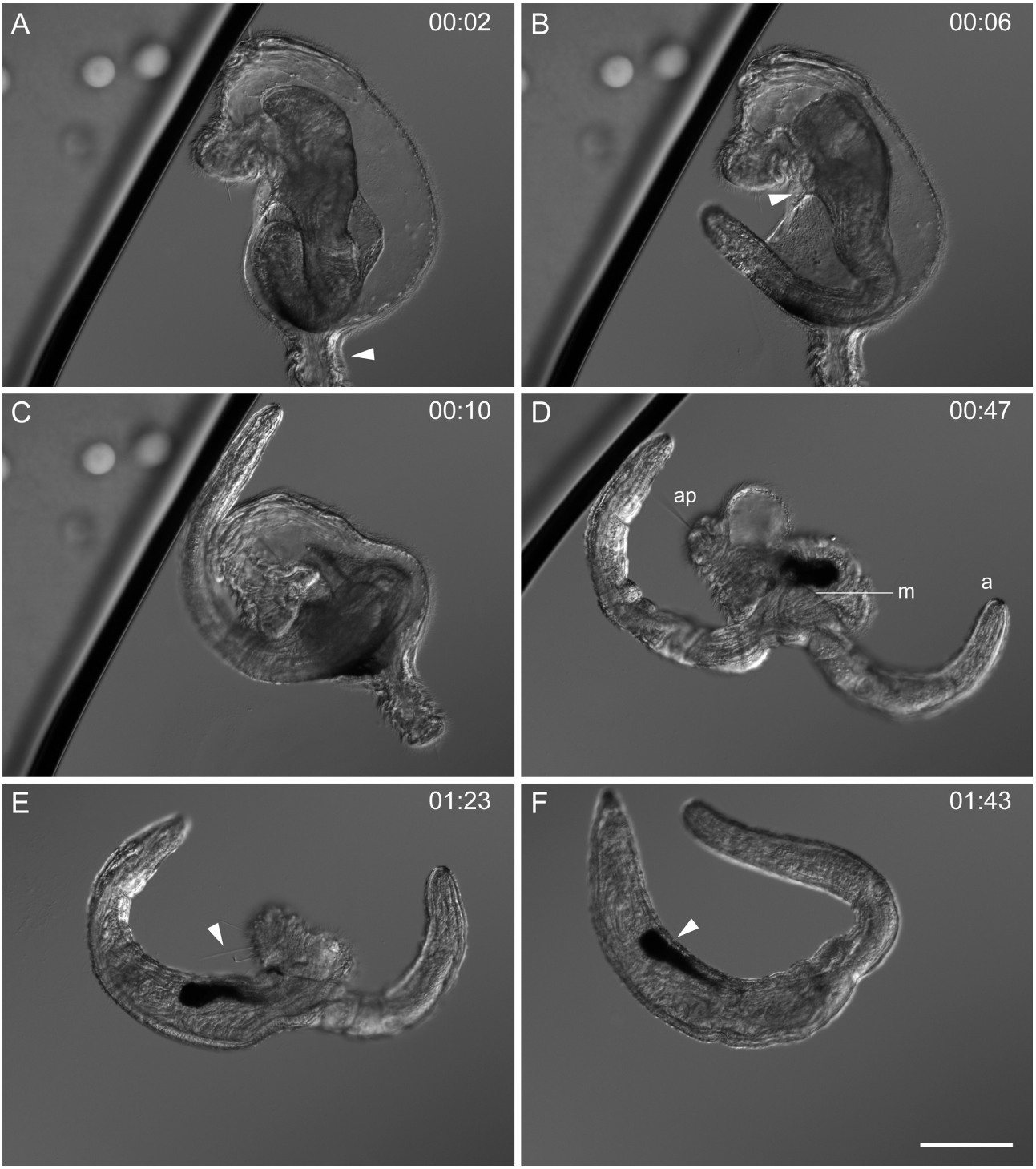


Fig. 5. Metamorphosis of *pilidium recurvatum*. Selected frames from the Video S1. Time stamp (min:sec) is at upper right. **A**. Metamorphosis begins with the contraction of the larval posterior (arrowhead). **B–C**. The juvenile posterior end emerges first by rupturing the ventral larval epidermis posterior to the amniotic pore (arrowhead). **D**. The anterior end of the juvenile (a) emerges, and the juvenile begins to swallow the larval body, including the dark collapsed amnion. The margin of the mouth (m) and the larval apical tuft (ap) are sharply in focus. Note the characteristically long pre-oral end, and the lack of the juvenile caudal cirrus. **E**. The stiff ciliary cirri (arrowhead) arranged along the margin of the larval esophageal funnel are among the last structures to be ingested. **F**. The collapsed amnion (black body, arrowhead) can be seen inside the gut of metamorphosed juveniles. Scale bar=200 μm.

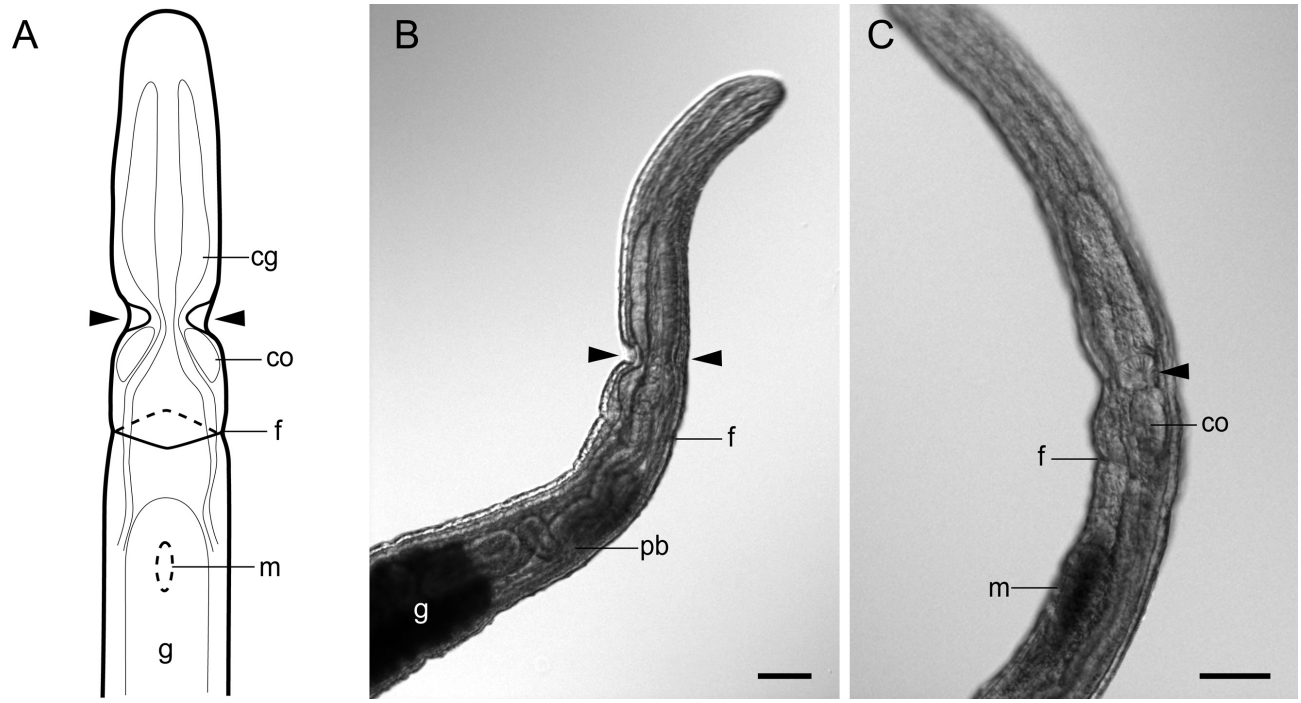


Fig. 6. Morphology of a recently metamorphosed juvenile from *pilidium recurvatum* (i.e., *Riserius* sp.). **A**. A diagram in dorsal view showing the location of two deep cerebral organ openings (arrowheads) located posterior to the cerebral ganglia (cg) and anterior to the cerebral organs (co) themselves, and the transverse cephalic v-furrow (f) located posterior to the cerebral organs and anterior to the mouth (m). Dorsally, the apex of the "v" points toward the posterior and ventrally—toward the anterior. An unusual characteristic of *Riserius* sp. is the long pre-oral end, as indicated by the position of the mouth and gut (g). **B**. Juvenile anterior end in dorsal view, showing deep cerebral organ openings (arrowheads) and the v-furrow (f). One can also see the proboscis (pb) and the gut (g). **C**. Juvenile anterior in lateral view showing one of the cerebral organ openings (arrowhead) which leads to the cerebral organ (co), the v-furrow (f) and the mouth (m). Scale bars=100 μm.

regions (Table 2). Mean divergence distances between the two types of *pilidium recurvatum* were 0.11585 (16S) and 0.18928 (COI). In comparison, mean divergences between *R. pugetensis* and *pilidium recurvatum* with eyes were 0.09274 (16S) and 0.17071 (COI), and between *R. pugetensis* and *pilidium recurvatum* without eyes 0.05974 (16S) and 0.19037 (COI). Sequence divergence observed between individuals of the same morphotype was much less (Table 2). Mean intraspecific divergences among *pilidium recurvatum* without eyes was 0.00049 (16S, n=8) and 0.00516 (COI, n=6), and among *pilidium recurvatum* with eyes p=0.0000 (16S, n=2) and 0.00516 (COI, n=2).

The surprising diet of juveniles of *Riserius*

Recently metamorphosed juveniles derived from *pilidium recurvatum* without eyes readily preyed on the larvae of the hoplonemertean *Carcinonemertes errans* Wickham 1978 collected from the plankton (Fig. 9A). We discovered this by accident, because both kinds of nemertean larvae shared a bowl in our sea table and one (*pilidium recurvatum*) metamorphosed and ingested the other (*C. errans*) (Fig. 9B). We also offered a variety of other potential prey items (e.g., larvae and small adults of other hoplonemerteans, bivalve pediveligers, cyphonautes, echinoid plutei, ophiuroid juveniles, and variety of polychaete nechtochaetes and juveniles), but without success. We have not attempted to feed the juveniles of *pilidium recurvatum* with eyes.

The discovery that metamorphosed juveniles from *pilidium recurvatum* feed on juveniles of *C. errans* was surprising and fortuitous. *Carcinonemertes errans* (Fig. 9C) makes a living as a symbiotic egg predator on the Dungeness crab (*Cancer magister* Dana 1852), a commercially important species. We initially supplied the juveniles from *pilidium recurvatum* with larvae of *C. errans* collected by plankton tow, but as these waned in abundance and the juveniles from *pilidium recurvatum* grew, we offered them juveniles of *C. errans* (Fig. 9D,E), which we scraped off in large numbers from live

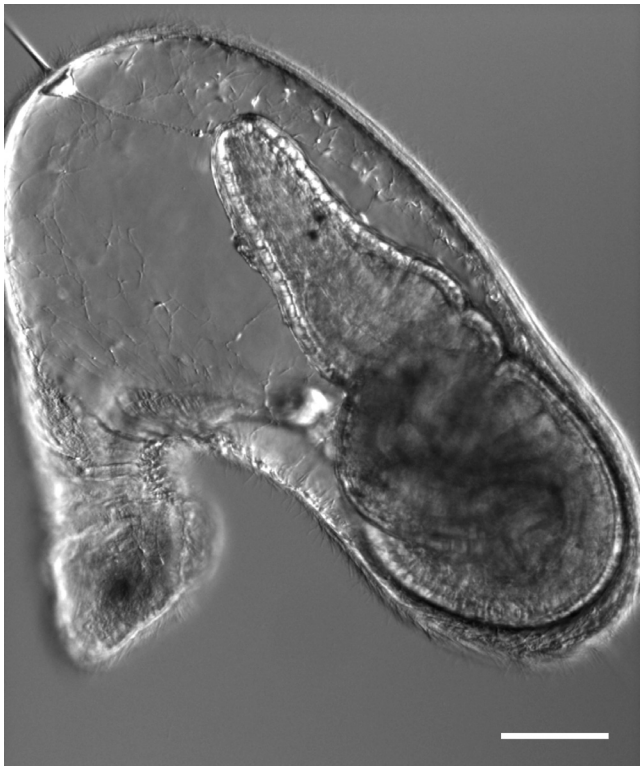


Fig. 7. The "eyed" *pilidium recurvatum* morphotype encountered in a plankton sample from Coos Bay in November 2011. Note the two eyes in the juvenile head inside the larva. Scale bar=100 μm.

male Dungeness crabs purchased at a market in Charleston, OR. To date, we have raised fourteen juveniles from *pilidium recurvatum* on the diet of *C. errans*. Two of these have been maintained for over a year, and developed oocytes. We found that these juveniles ate and grew better with a thin layer of sand on the bottom of their culture dish. Over half of the metamorphosed juveniles from wild-caught *pilidium recurvatum* readily ate *C. errans* (64%); some of those who did not may have metamorphosed at too small a size, as it was difficult to find *C. errans* for them that were smaller in any dimension, and they exhibited no interest in *C. errans* juveniles that had been chopped into pieces with glass needles. We observed an ~20-mm long individual of *Riserius* sp., raised from a wild *pilidium recurvatum*, ingest several dozen juveniles of *C. errans* within a half hour (Fig. 9F), whereafter its appetite abated. Ingestion usually involved a quick eversion of the proboscis, which was wrapped around *C. errans* and sometimes moved the prey toward the mouth (Video S2). In other instances, the proboscis was withdrawn, and the heteronemertean engulfed *C. errans* (sometimes several at a time) by pulling its mouth over the prey. Proboscis eversion was nearly always preceded by direct contact between the tip of the head and the body of the prey. Although predation appeared to be aided by the proboscis, it did not apparently disable the prey, which was often swallowed while wriggling. Occasionally, juveniles of *C. errans* escaped from the heteronemertean predator. Within as little as 3 months, juveniles of *Riserius* sp. raised from wild-caught *pilidium recurvatum* exceeded 20 mm in length, more than ten times their length at metamorphosis, on a diet of *C. errans* (Fig. 9E). While this manuscript was in revision, we found another acceptable prey item—an unidentified species of *Tetrastemma* (Hoplonemertea; Nemertea), collected by us from the blades of surfgrass in Middle Cove, Cape Arago, OR. We observed several attacks and feeding by several of our laboratory-reared *Riserius* sp. on small adults of this species.

Discussion

Larval morphology and behavior

Pilidium larvae with the *recurvatum* morphotype from Oregon most resemble *pilidium recurvatum* from the Sea of Japan (Chernyshev 2001) in that they lack the posterior transverse ciliary band, also referred to as the telotroch, which characterizes the original *pilidium recurvatum* from Rhode Island (Fewkes 1883), as well as the Swedish (Cantell 1966a) and the Vietnamese forms (Dawydoff 1940).

We noted with interest an important difference in larval morphology between the typical pilidium larva (e.g., as described in Maslakova 2010a,b) and *pilidium recurvatum* (aside from the obvious difference in body shape). In a typical pilidium, the larval mouth is surrounded by a set of lobes (anterior and posterior) and lappets (typically two lateral) whose margins are spanned by the larval ciliary band. Internally, the ciliary band is supported by a prominent muscle band (Fig. 4D; Maslakova 2010a,b) and serotonergic nerve (Fig. 4D; Maslakova 2010a). Absence of the marginal ciliary band in the *pilidium recurvatum* is correlated with the absence of the marginal muscle and serotonergic nerve cord, and the monociliated collar cells. The marginal ciliary band in the typical pilidium larva is involved in capturing unicellular algae (G. von Dassow, R. Emlet, and S. Maslakova, unpubl. data). Absence of this structure in *pilidium recurvatum* correlates with our observation that *pilidium recurvatum* did not feed on unicellular algae (von Dassow, unpubl. data), such as the cryptomonad *Rhodomonas lens* Pascher & Ruttner (CCMP739), which is an excellent food for typical pilidia (Maslak-

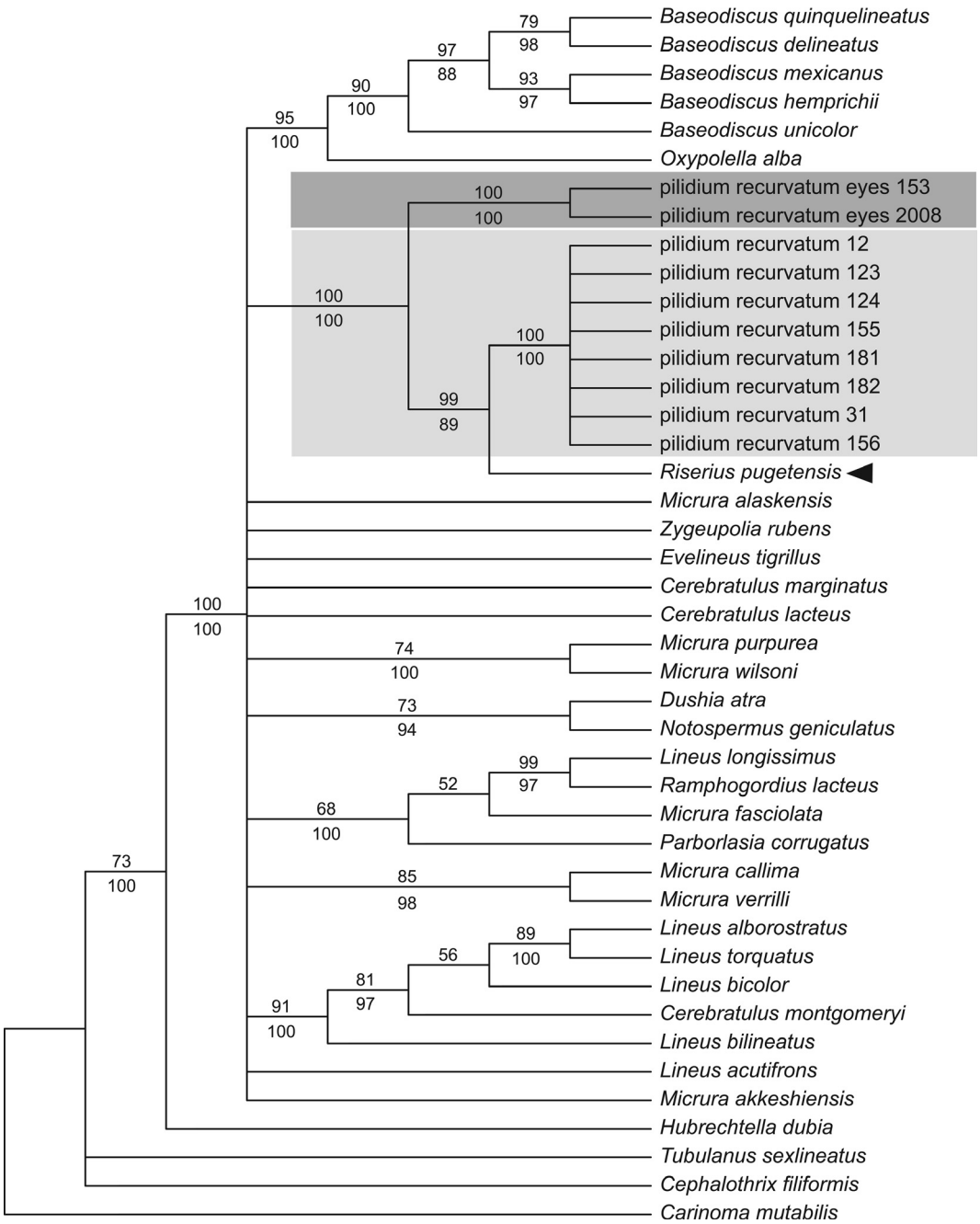


Fig. 8. A strict consensus of three most parsimonious trees resulting from the analysis of 16S rDNA sequence data, including all available *pilidium recurvatum* sequences and a selection of pilidiophoran sequences from GenBank (accession numbers listed in Table 1). The topology resulting from the Bayesian analysis was nearly identical. Numbers above branches are bootstrap values (1000 replicates) and numbers below are Bayesian posterior probabilities, which are only shown for clades that were represented in both types of analyses. The two *pilidium recurvatum* morphotypes each form a strongly supported monophyletic clade (gray and light gray boxes), and together they form a well-supported monophyletic clade with the only described species within the genus *Riserius* – *R. pugetensis* (arrowhead).

ova 2010a; T. Hiebert, S. Maslakova, and G. von Dassow, unpubl. data).

At the same time, we strongly suspect that *pilidium recurvatum* is a planktotroph. Young *pilidium recurvatum* maintained in the lab for several weeks shrank in size over time, presumably from lack of suitable food, and did not proceed to develop imaginal discs. The long ciliary cirri arranged along the

Table 2. Average uncorrected divergences (p-distances) for 16S rDNA (top number) and COI, with divergence ranges included in parentheses.

	pilidium recurvatum	*pilidium recurvatum* with eyes	*Riserius pugetensis*
pilidium recurvatum	0.0005 (0.0000–0.0020)	0.1159 (0.0989–0.1220)	0.0597 (0.0595–0.0615)
	0.0052 (0.0046–0.0106)	0.1893 (0.2027–0.2070)	0.1904 (0.1885–0.1949)
pilidium recurvatum with eyes	—	0.00000	0.0927 (0.0866, 0.0989)
	—	0.00300	0.1707 (0.1690, 0.1724)

margin of *pilidium recurvatum* esophageal funnel (Fig. 3A) are unique to this pilidial type, and, we speculate, have something to do with how these larvae detect or capture food. We also suspect that the larval posture in which the esophageal funnel is outstretched (Fig. 3A) may be involved in feeding, although we did not succeed in identifying acceptable prey for these larvae, and never observed larval feeding.

No previous authors (Fewkes 1883; Dawydoff 1940; Cantell 1966a; Chernyshev 2001) reported the prominent ciliary cirri along the margin of the esophageal funnel, although Cantell (1966a) describes that the "rim of the funnel consists of thickened epithelium with longer cilia." It is interesting to note that Fewkes (1883) also observed the "feeding posture" in one of his *pilidium recurvatum* larvae, but thought that it might be an "individual peculiarity," and Cantell (1966a) depicted a larva in a "feeding posture," but did not comment on it.

Metamorphosis

Similar to *pilidium recurvatum* larvae observed by Fewkes (1883) and Cantell (1966a), larvae from Oregon undergo catastrophic metamorphosis in which the juvenile escapes and devours the larval body. This type of catastrophic metamorphosis has been observed in most other types of pilidium larvae (Cantell 1966b, 1969; Maslakova 2010a,b), with the exception of *pilidium auriculatum*, which belongs to the non-heteronemertean pilidiophoran family Hubrechtidae (Cantell 1966b, 1972).

Identification of *pilidium recurvatum*

Absence of the lateral cephalic furrows and caudal cirrus in the metamorphosed juveniles of *pilidium recurvatum* suggests that they do not belong to the Lineidae, as originally suggested by Fewkes (1883). Absence of the secondary cephalic furrows suggests that they do not belong to Baseodiscidae, as speculated by Cantell in 1966 (a). Cantell later (1969) suggested the possibility that *pilidium recurvatum* may be the larva of *Oxypolella alba* Bergendal 1903, but several features in metamorphosed juveniles do not conform to the diagnosis for this genus (e.g., large cerebral organs and long pre-oral end) (Cantell 2005).

At the same time, juveniles from *pilidium recurvatum* possess a highly unusual combination of morphological features (conspicuous cerebral organ pits, V-shaped transverse cephalic furrow, and long pre-oral end) that is only known in one pilidiophoran—a mesopsammic species from Puget Sound, Washington, USA, *Riserius pugetensis* (Norenburg 1993). The familial affiliation of *Riserius* is uncertain, but molecular phylogenetic analysis suggests that the species is basal within the Heteronemertea (Thollesson & Norenburg 2003).

Analysis of DNA sequence data (16S rDNA and Cytochrome Oxidase I) confirms our morphological identification: the two types of *pilidium recurvatum* larvae from Coos Bay form a clade with *R. pugetensis* (Fig. 8). On the basis of available GenBank 16S sequence data, Mahon et al. (2010) calculated the average interspecific divergence between congeneric nemertean species as ~4.8%. However, Meyer & Paulay (2005) argued that minimum interspecific divergences are better suited for species delimitation than the average. Minimum interspecific divergences for pilidiophoran 16S are on the order of 3% (S. Maslakova, unpubl. data). Either way, the p-distances between the two types of *pilidium recurvatum* from Coos Bay and *R. pugetensis* are too large (e.g., 6–11% for 16S) to consider them conspecific (Table 2). As *R. pugetensis* is the only described species of *Riserius*, we speculate that the two types of *pilidium recurvatum* larvae from Oregon represent two new (undescribed) species of *Riserius*.

The *pilidium recurvatum* larvae we describe and identify here are the first instance of an indirect-developing nemertean larva of apparent mesopsammic origin. *Riserius pugetensis* is a mesopsammic species (Norenburg 1993) and, as such, was not expected to have a long-lived planktotrophic larva (Swedmark 1964; Norenburg 1988; Geire 2009).

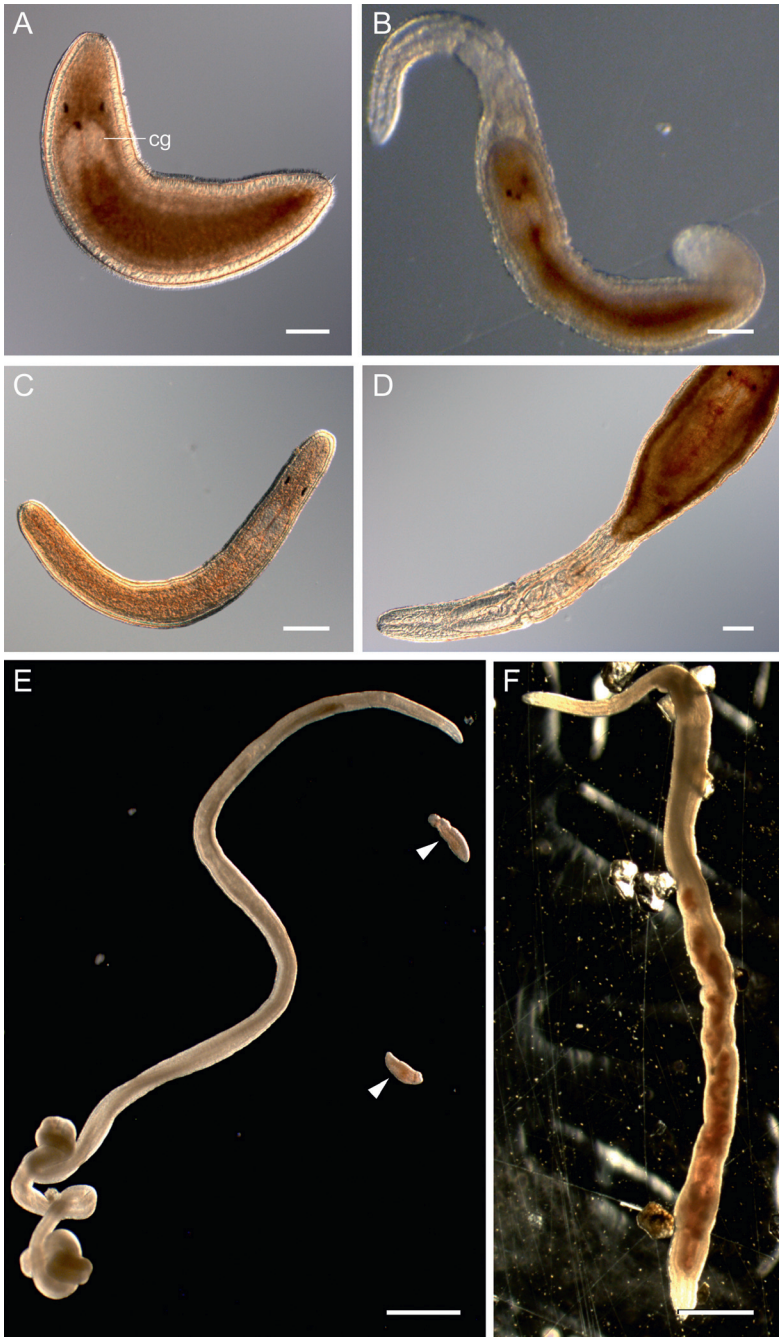


Fig. 9. Juveniles of *Riserius* sp. feeding on larvae and juveniles of *Carcinonemertes errans*. **A**. Larva of *C. errans* collected from Coos Bay plankton, and identified using DNA sequence data. Larvae of *C. errans* are easily differentiable from other hoplonemertean larvae because of their unusual arrangement of eyes: two widely separated anterior, and two closely positioned posterior immediately in front of the cerebral ganglia (cg). The two posterior eyes are so close together that they often appear as one. Scale bar=100 μm. **B**. Larvae like the one depicted on (A) were ingested by newly metamorphosed juveniles of *Riserius*; note orange color and the characteristic eye arrangement in the larva of *C. errans* within the gut of one-month-old specimen of *Riserius* sp. Scale bar=~100 μm. **C**. Juvenile of *C. errans,* with two eyes rather than four (posterior larval eyes are lost after settlement), which was removed from adult male Dungeness crab. Scale bar=100 μm. **D**. Juvenile of *C. errans* ingested by a growing juvenile of *Riserius* sp. Scale bar=100 μm. **E**. A much grown 6-month-old specimen of *Riserius* sp. raised on a diet of *C. errans*. Two juveniles of *C. errans* (arrowheads) are shown for scale. Scale bar=1 mm. **F.** A large individual of *Riserius* raised on a diet of *C. errans* with several dozen juveniles of *C. errans* packed in its gut after a recent meal. Scale bar=1 mm.

However, numerous small eggs (about 300 eggs 50–60 μm in diameter in one 10-mm long female) reported by Norenburg (1993) suggest planktotrophic development. The largest individuals of *Riserius* sp. described here reached at least 25 mm (in gliding) and we observed oocytes of about 80 μm in diameter, which were released from a small body fragment. We estimate that a single female of *Riserius* sp. might be reasonably expected to produce at least on the order of 10^3 eggs. Therefore, our observation of a long-lived likely planktotrophic larva within this genus is not surprising.

Larval evolution

Jägersten (1972) suggested that *pilidium recurvatum* represents an intermediate evolutionary form between planuliform larvae of Palaeonemertea and the conventional hat-like pilidium larva. Assuming that the hat-shaped pilidial forms evolved only once, if the *pilidium recurvatum* is shown to belong to a highly derived pilidiophoran taxon, it would be unlikely to represent an ancestral pilidial form. On the other hand, Jägersten's hypothesis would be consistent with taxa characterized by the *pilidium recurvatum* larva being the sister group of pilidiophorans with a hat-shaped pilidium. Our identification of *pilidium recurvatum* as the larva of *Riserius,* which is the sister clade to the rest of the heteronemerteans (Thollesson & Norenburg 2003), would be consistent with Jägersten's hypothesis if *pilidium auriculatum* (Leuckart & Pagenstecher 1858) of the family Hubrechtidae (sister to Heteronemertea+*Riserius* according to Thollesson & Norenburg 2003; but see Andrade et al. 2011) has evolved separately from hat-like pilidia of heteronemerteans. Jägersten's hypothesis would be supported if *pilidium recurvatum* evolved once, and pilidiophorans with this larval form are paraphyletic with respect to the clade of pilidiophorans with a hat-shaped pilidium.

Distribution and diversity of *Riserius*

Our findings suggest that *Riserius* is a much more diverse and geographically widespread genus than previously appreciated. The two larval types from Oregon clearly represent two new species of *Riserius*. *Riserius* has not been previously reported from Oregon. Based on the similarity of larval morphology, it is possible that the *pilidium recurvatum* from the Sea of Japan (Chernyshev 2001) represents the same species as one of our forms. Trans-Pacific movement of marine species is well documented (e.g., Carlton & Cohen 2007), and we have previously found the larvae of *Hubrechtella juliae* Chernyshev 2003 from the Sea of Japan in Coos Bay, OR (Maslakova 2010b; T. Hiebert and G. von Dassow, unpubl. data). Thus, a conservative estimate of *Riserius* diversity in Pacific waters is three species, two of which are so far known only in their larval form as *pilidium recurvatum* (one with eyes and without telotroch, and one with neither eyes nor telotroch), and one, *R. pugetensis*, so far known only in its adult form.

The Atlantic and the Vietnamese *pilidium recurvatum* are clearly different from the Pacific forms, because they possess a telotroch. It is possible that the original *pilidium recurvatum* (Fewkes 1883) from Rhode Island is different from the Swedish form (Cantell 1966a) because, unlike Fewkes (1883), Cantell (1966a) did not observe ocelli in the recently metamorphosed juveniles. It seems unlikely that the Vietnamese form (Dawydoff 1940) could be the same species as the Atlantic form(s), based on geography alone. Assuming these larvae are congeneric with those described here, *Riserius* has a worldwide distribution, and contains at least five (but probably many more) species. Indeed, J. Norenburg (Smithsonian Institution, unpubl. data) has found adults of *Riserius* in sub-littoral mesopsammon off Pacific Panama, southeast Florida, and southeast Brazil.

Riserius as potential biological control

Our observation that juveniles of *Riserius* sp. fed on *Carcinonemertes errans,* a symbiotic egg predator of *Cancer magister* (Dungeness crab), suggests that species diversity and geographic distribution of *Riserius* might be of importance to zoologists and commercial fisherman alike. Carcinonemertidae is a large hoplonemertean family with worldwide distribution (Gibson 1995). It contains three genera and 18 described species (Sadeghian & Santos 2010), all of which specialize as symbiotic egg predators of different decapod crustaceans, many of which are commercially important, e.g., Dungeness crab *C. magister*, Red King crab *Paralithodes camtschatica* Tilesius 1815, Blue crab *Callinectes sapidus* Rathbun 1896, and American lobster *Homarus americanus* Edwards 1837 (Kuris 1993). *Carcinonemertes* infestations can reach an astonishing thousands of individuals per crustacean host (e.g., Wickham 1980) and make a significant impact on the host population. For example, localized high levels of *Carcinonemertes regicides* Shields, Wickham, & Kuris 1989 infestations and egg predation reduced clutches of Red King crab in the 1980s, which may have led to decreased crab landings and the subsequent closure of commercial fishing

in certain Alaskan regions in 1983 (Kuris & Wickham 1987; Shields et al. 1989; Kuris et al. 1991). Also, predation by *C. errans* may have contributed to the collapse and subsequent non-recovery of Dungeness crab fisheries in central California (Wickham 1979, 1986; Kuris & Wickham 1987). However, recent data of Shanks & Roegner (2007) suggest that annual variation in Dungeness crab abundance correlates with abiotic factors, particularly the timing of the spring transition, which may have a more significant affect on the crab abundance than *Carcinonemertes*.

Larvae of *C. errans* are found in the plankton (Dunn 2011; S. Maslakova, T. Hiebert, L. Hiebert, and G. von Dassow, unpubl. data); however, the adult nemerteans are only found on juveniles and adults of *C. magister* (Wickham 1980). This suggests that if individuals of *Riserius* sp. feed on *C. errans* in nature, they too must be found on, or near *C. magister*. Juveniles and adults of C. *magister*, like many other crustaceans, exhibit a burying behavior in sand (McGaw 2005). Adults of the nemertean *R. pugetensis,* the only described species in the genus, live interstitially between sand grains (Norenburg 1993), and it is possible that other species of *Riserius* also are mesopsammic. Therefore, our working hypothesis is that *Riserius* may encounter *C. errans* on crabs that are buried in sand.

At this point, we only have observed feeding on *Carcinonemertes* in one species of *Riserius*. Because we also observed feeding by *Riserius* sp. on another hoplonemertean species, *Riserius* sp. may not be a specialist predator of *Carcinonemertes* in nature. It is not known whether other species also feed on carcinonemertids or whether and how they encounter *Carcinonemertes* in nature. Nevertheless, this observation leads one to wonder about the potential of *Riserius* to control populations of carcinonemertids on commercially and ecologically important crustaceans.

Concluding remarks

"It's a shirt. It's a sock. It's a glove. It's a hat. But it has other uses. Yes, far beyond that." (*Theodor Seuss Geisel 1971, The Lorax*)

Identifying planktonic larvae of marine invertebrates is important because larvae connect and ensure long-term stability of populations of benthic adults through dispersal and recruitment. Morphological identification of larvae to species is often difficult or impossible because early developmental stages of related species look alike, and the development of very few described species is known. DNA-based identification provides an important alternative. This study highlights additional benefits of DNA barcoding as a tool for identifying marine invertebrate larvae. First, it helps to reveal cryptic and undescribed biodiversity, because some of the species are more likely to be encountered as planktonic larvae than as benthic adults (as is the case with *pilidium recurvatum*). Second, it helps to address questions about larval evolution, as distinct larval morphotypes are placed in phylogenetic context. And sometimes, the benefits may be entirely unexpected and with far-reaching consequences, such as our serendipitous discovery of the unusual predator-prey interaction between *Riserius* and *Carcinonemertes* (whose planktonic larvae we also originally identified based on DNA sequence data), and its possible relevance to the biology of commercially important crustaceans.

Acknowledgments. We thank undergraduate and graduate students who participated in aspects of this study by virtue of taking the Marine Molecular Biology course taught by SAM at the Oregon Institute of Marine Biology in 2008–2012. We acknowledge support of the OIMB staff and faculty. We thank J. Norenburg, B. Pernet, and two anonymous reviewers for thoughtful comments on this manuscript. This study was partially supported by the NSF grants 1120537 and 1030453 to SAM.

References

Andrade SCS, Strand M, Schwartz M, Chen H, Kajihara H, von Döhren J, Sun S, Junoy J, Thiel M, Norenburg JL, Turbeville JM, Giribet G, & Sundberg P 2011. Disentangling ribbon worm relationships: multi-locus analysis supports traditional classification of the phylum Nemertea. Cladistics 28: 141–159.

Bürger O 1895. Die Nemertinen des Golfes von Neapel und der angrenzenden Meeres-Abschnitte. Fauna Flora Golf. Neapel 22: 1–743.

Cantell CE 1966a. Some developmental stages of the peculiar nemertean larva *pilidium recurvatum* Fewkes from the Gullmarfjord (Sweden). Ark. Zool. 19: 143–147.

——— 1966b. The devouring of the larval tissues during the metamorphosis of pilidium larvae (Nemertini). Ark. Zool. 18: 489–492.

——— 1969. Morphology, development and biology of the pilidium larvae (Nemertini) from the Swedish West Coat. Zool. Bidr. Upps. 38: 61–111.

——— 1972. Studies on the morphology, taxonomy, and larval development of heteronemertines (Nemertina). Acta Univ. Upsal. 218: 1–7.

——— 2005. On the anatomy of a new species of *Oxypolella* (Nemertini) and further notes on *Oxypolella bergendali* Cantell 1975. J. Nat. Hist. 39(2): 123–132.

Carlton JT & Cohen AN 2007. Introduced marine and estuarine invertebrates. In: The Light and Smith Manual: Intertidal Invertebrates from Central California to

Oregon, 4th ed. Carlton JT, ed., pp. 28–31. University of California Press, Berkeley, California.
Chernyshev AV 2001. The larvae of unarmed nemerteans in Peter the Great Bay (Sea of Japan). Russ. J. Mar. Biol. 27(1): 58–61.
Clogen DJ, Ponder WF, & Eggler PE 2000. Gastropod evolutionary rates and phylogenetic relationships assessed using partial 28S rDNA and histone H3 sequences. Zool. Scripta 29: 29–63.
Dawydoff C 1940. Les formes larvaires de polyclades et de némertes du plankton Indochinois. Bull. Biol. Fr. Belg. 74: 443–496.
Dunn PH 2011. Larval Biology and Estuarine Ecology of the Nemertean Egg Predator *Carcinonemertes errans* on the Dungeness Crab, *Cancer magister*. Department of Biology, PhD dissertation, University of Oregon, Eugene, Oregon.
Felsenstein J 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.
Fewkes JW 1883. On the developmental stages of certain worm larvae. Bull. Mus. Comp. Zool. 11(9): 167–208.
Folmer O, Black M, Hoeh W, Lutz R, & Vrijenhoek R 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotech. 3: 294–299.
Geire O 2009. Meiobenthology: The Microscopic Motile Fauna of Aquatic Sediments. Springer-Verlag, Berlin. pp. 87–102.
Geisel TS 1971. The Lorax, by Dr. Seuss. Random House, Inc, New York.
Gibson R 1995. Nemertean genera and species of the world: an annotated checklist of original names and description citations, synonyms, current taxonomic status, habitats and recorded zoogeographic distribution. J. Nat. Hist. 29: 271–562.
Jägersten G 1972. Evolution of the Metazoan Life Cycle. Academic Press, New York. pp. 88–102.
Johnson K 2001. Nemertea. In: An Identification Guide to the Larval Marine Invertebrates of the Pacific Northwest. Shanks AL, ed., pp. 26–36. OSU Press, Corvallis, Oregon.
Kuris AM 1993. Life cycles of nemerteans that are symbiotic egg predators of decapod Crustacea: adaptations to host life histories. Hydrobiologia 266: 1–14.
Kuris AM & Wickham DE 1987. Effect of nemertean egg predators on crustaceans. Bull. Mar. Sci. 41: 151–164.
Kuris AM, Blau SF, Paul AJ, Shields JD, & Wickham DE 1991. Infestation by brood symbionts and their impact on egg mortality in the red king crab, *Paralithodes camtschatica*, in Alaska; geographic and temporal variation. Can. J. Fish. Aquat. Sci. 48: 559–568.
Lacalli T 2005. Diversity of form and behaviour among nemertean pilidium larvae. Acta Zool. 88: 267–276.
Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan RA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, & Higgins DG 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23(21): 2947–2948.
Leuckart R & Pagenstecher A 1858. Untersuchungen über niedere Seethiere. Arch. Anat. Phys. Jahrg. 1858: 569–588.
Mahon AR, Thornhill DJ, Norenburg JL, & Halanych KM 2010. DNA uncovers Antarctic nemertean biodiversity and exposes a decades-old cold case of asymmetric inventory. Polar Biol. 33: 193–202.
Maslakova SA 2010a. The invention of the pilidium larva in an otherwise perfectly good spiralian phylum Nemertea. Integr. Comp. Biol. 50: 734–743.
——— 2010b. Development to metamorphosis of the nemertean pilidium larva. Frontiers in Zool. 7:30. doi: 10.1186/1742-9994-7-30
Maslakova SA & von Dassow G 2012. A lecithotrophic pilidium with apparent prototroch and telotroch. J. Exp. Zool. B Mol. Dev. Evol. 9999B: 1–5.
McGaw IJ 2005. Burying behaviour of two sympatric crab species: *Cancer magister* and *Cancer productus*. Sci. Mar. 69(3): 375–381.
Meyer CP & Paulay G 2005. DNA barcoding: error rates based on comprehensive sampling. PLoS Biol. 3(12): e422.
Müller J 1847. Fortsetzung des Berichts über einige neue Thierformen der Nordsee. Arch. Anat. Physiol. 6:159–160.
Norenburg JL 1993. *Riserius pugetensis* gen. n., sp. n. (Nemertina: Anopla), a new mesopsammic species, and comments on the phylogenetics of some anoplan characters. Hydrobiologia 266: 203–218.
Norenburg JL & Stricker SA 2002. Phylum Nemertea. In: Atlas of Marine Invertebrate Larvae. Young CM, Sewall MA, Rice ME, eds., pp. 163–177. Academic Press, San Diego.
Norenburg JL 1988. Nemertina. In: Introduction to the Study of Meiofauna. Higgins RP & Thiel H, eds., pp. 287–292. Smithsonian Institution Press, Washington, District of Columbia.
Palumbi S, Martin A, Romano S, McMillan WO, Stice L, & Grabowski G 1991. The Simple Fools Guide to PCR Version 2.0. Department of Zoology Kewalo Marine Laboratory, University of Hawaii, Honolulu, Hawaii.
Posada D 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25: 1253–1256.
Rambaut A 2009. FigTree version 1.3.1. Available at http://tree.bio.ed.ac.uk/software/figtree/ (accessed on 23 August 2012).
Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, & Huelsenbeck JP 2012. MrBayes 3.2: efficient bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61: 2010–2013.
Sadeghian PS & Santos C 2010. Two new species of *Carcinonemertes* (Hoplonemertea: Carcinonemertidae) living in association with leucosiid crabs from California and Tasmania. J. Nat. Hist. 44: 2395–2409.

Salensky W 1912. Morphogenetische Studien an Würmern. II. Über die Morphogenese der Nemertinen. Entwicklungsgeschichte der Nemertine im Inneren des Pilidiums. Mem. Acad. Sci. St. Petersb. 30: 1–74.

Schmidt GA 1930. Die Pilidien von *Cerebratulus pantherinus* und *marginatus* und die Frage der morphologischen Merkmale der Hauptformen der Pilidien. Zool. Jahrb. Anat. Ontog. 62: 423–448.

Schwartz ML 2009. Untying a Gordian Knot of Worms: Systematics and Taxonomy of the Pilidiophora (phylum Nemertea) from Multiple Data Sets. Columbian College of Arts and Sciences. The George Washington University, Washington, District of Columbia.

Schwartz ML & Norenburg JL 2005. Three new species of *Micrura* (Nemertea: Heteronemertea) and a new type of heteronemertean larva from the Caribbean Sea. Caribb. J. Sci. 41: 528–543.

Shanks AL & Roegner GC 2007. Recruitment limitation in Dungeness crab populations driven by variation in atmospheric forcing. Ecology 88(7): 1726–1737.

Shields JD, Wickham DE, & Kuris AM 1989. *Carcinonemertes regicides* n. sp. (Nemertea), a symbiotic egg predator from the red king crab, *Paralithodes camtschatica* (Decapoda: Anomura), in Alaska. Can. J. Zool. 67: 923–930.

Strand M, Hjelmgren A, & Sundberg P 2005. Genus *Baseodiscus* (Nemertea: Heteronemertea): molecular identification of a new species in a phylogenetic context. J. Nat. Hist. 39(44): 3785–3793.

Sundberg P & Saur M 1998. Molecular phylogeny of some european heteronemertean (Nemertea) species and the monophyletic status of *Riseriellus*, *Lineus* and *Micrura*. Mol. Phyl. Evol. 10(3): 271–280.

Swedmark B 1964. The interstitial fauna of marine sand. Biol. Rev. 39(1): 1–42.

Swofford DL 2002. PAUP*, Phylogenetic Analysis Using Parsimony (*and Other Methods), Vol. 4. Sinauer Associates, Sutherland, Massachusetts.

Tamura K & Nei M 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10(3): 512–526.

Thollesson M & Norenburg JL 2003. Ribbon worm relationships – a phylogeny of the phylum Nemertea. Proc. R. Soc. Lond., Ser. B: Biol. Sci. 270: 407–415.

Wickham DE 1979. Predation by the nemertean *Carcinonemertes errans* on eggs of the Dungeness crab *Cancer magister*. Mar. Biol. 55: 45–53.

——— 1980. Aspects of the live history of *Carcinonemertes errans* (Nemertea: Carcinonemertidae), an egg predator of the crab, *Cancer magister*. Bio. Bull. 159: 247–257.

——— 1986. Epizootic infestations of nemertean brood parasites on commercially important crustaceans. Can. J. Fish. Aquat. Sci. 43: 2295–2302.

Supporting information

Additional Supporting information may be found in the online version of this article:

Video S1. Catastrophic metamorphosis of *pilidium recurvatum* is accomplished within a few minutes, during which the emerging juvenile devours the larval body (also see Fig. 5).

Video S2. A laboratory reared specimen of *Riserius* sp. feeding on juveniles of *Carcinonemertes errans*.