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Reconstruction of the neuromuscular system of the swimming-type larva of *Loxosomella atkinsae* (Entoprocta) as inferred by fluorescence labelling and confocal microscopy

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Abstract

Entoprocta is one of the most enigmatic phyla of the Animal Kingdom. The morphology of their larvae has been little investigated, with details on the larval musculature lacking entirely and immunocytochemical data on the larval nervous system available for only 2 species. Here, we provide the first detailed study of the muscular bauplan and the serotonergic nervous system of an entoproct swimming-type larva. The overall muscular architecture of the larva of *Loxosomella atkinsae* Bobin & Prenant, 1953 is complex and includes several sets of ring, longitudinal, and diagonal muscles. The dorsal region of the larva (episphere) and the apical organ comprise an outer layer of tightly packed ring muscles. Beneath this layer lie sets of prominent longitudinal and diagonal muscles that run in dorso-ventral direction. The prototrochal musculature is composed of compact layers of outer ring and inner longitudinal muscles. The serotonergic nervous system consists of 3–4 flask-shaped serotonergic cells in the apical organ and a paired nerve passing the frontal neuropil and connecting to the serotonergic prototroch nerve ring. We show here that the entoproct larval stage, in addition to the adult stage, provides several morphological characters for evolutionary inferences. Comparative data on entoproct swimming-type larvae suggest ring and longitudinal muscles underlying the prototroch, a paired main longitudinal muscle, and an unpaired abfrontal longitudinal muscle as part of the ancestral muscular groundpattern of loxosomatid swimming-type larvae.

Keywords: Phylogeny; Lophotrochozoa; Spiralia; Serotonin; Muscle

Introduction

The phylogenetic relationships of the phylum Entoprocta (Kamptozoa) are highly controversial. Earlier, a close relationship of entoprocts with Ectoprocta was proposed, based on some similarities in life cycle and larval morphology (Nielsen 1971, 2001). However, this relationship has been challenged by a number of developmental, morphological, and molecular data. While entoprocts are spiral cleavers, ectoprocts show a radial cleavage pattern. Moreover, adult entoprocts are entirely acoelomate, whereas adult ectoprocts differentiate true coelomic cavities. Molecular studies also argue against a sistergroup relationship between Entoprocta and Ectoprocta (Mackey et al. 1996), with some of them

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suggesting close relations of Entoprocta to Micrognathozoa or Cycliophora, respectively (Giribet et al. 2004; Passamaneck and Halanych 2006; Baguñà et al. 2008). The latter assemblage has also been proposed based on shared morphological characters, including the presence of mushroom-shaped extensions from the basal lamina and microvilli that underlie the cuticle in some species (Sørensen et al. 2000). However, the earlier argument to unite Entoprocta, Ectoprocta, and Cycliophora based on their larval 'brains' (apical organs) disappearing during metamorphosis is not a decisive factor, because this developmental feature appears to be common in spiralian neurogenesis (see Nielsen 2004). Some most recent studies aiming at resolving the entire metazoan tree of life have used molecular datasets of considerable size. When Entoprocta are included they usually nest within Lophotrochozoa, but their exact position is controversial and remains unresolved (Baguñà et al. 2008; Dunn et al. 2008). This might at least in part be due to insufficient taxon sampling within Entoprocta (e.g., only a single colonial entoproct being used as a representative of the entire phylum). However, numerous morphological characters such as a chitinous cuticle that is not molted, a sinusial circulatory system, a complex serotonergic larval apical organ, tetraneury, and the ultrastructure of the foot (with anterior cirri, a pedal gland, intraepithelial mucous cells, and a ventrally intercrossing dorso-ventral musculature) are shared between some larval Entoprocta and Mollusca, and thus clearly argue in favor of a monophyletic assemblage of the 2 phyla (Haszprunar and Wanninger 2007; Wanninger et al. 2007).

With respect to the entoproct life cycle, the sexually produced larva constitutes an important stage. Entoproct larvae can be assigned to one of 2 major types, the creeping-type larva or the swimming-type larva. Compared to the creeping-type larva, the swimming-type larva is small, does not carry a well developed and pronounced, ciliated creeping foot, and is found among the solitary Loxosomatidae and presumably in the single species of colonial Loxokalypodidae (Nielsen 1971; Emschermann 1972). However, although larval morphology has been investigated in several entoproct species, applying mainly serial sectioning and light and electron microscopy techniques (Nielsen 1964, 1966, 1967, 1971, 2001, 2002; Nielsen and Rostgaard 1976; Nielsen and Jespersen 1997), immunocytochemical data are available for only one each creeping- and swimming-type larva (Hay-Schmidt 2000; Wanninger et al. 2007), and muscular anatomy is largely unknown in both larval types. Most entoproct larvae have a large apical organ, from which a paired nerve runs via a frontal ganglion to a prototroch nerve ring. The frontal ganglion often makes contact with the frontal organ, a structure that is either paired (as in the solitary Loxosomatidae and colonial Loxokalypodidae) or unpaired (as in the colonial Pedicellinidae and Barentsiidae). This organ is a protruding structure that can be ciliated and is often supported by a ring of gland cells or houses a pair of eyespots (Woollacott and Eakin 1973). In the larvae of Loxosomatidae, this organ is used for attachment to the substrate during settlement (Nielsen 2001). In some loxosomatid larvae the frontal organ innervates a pair of lateral sense organs. The observation of a ventral ganglion in *Barentsia* by Mariscal (1965) has never been confirmed. However, the general description of the entoproct nervous system, consisting of an apical organ and a paired nerve running from the apical organ to the frontal ganglion and further to the prototroch nerve ring, has been supported by an immunocytochemical study visualising the serotonergic nervous system of the swimming-type larva of *Loxosoma pectinaricola* Franzén, 1962 (Hay-Schmidt 2000). This larva exhibits a median and 2 lateral neurons in the apical organ, and a paired nerve which extends from the lateral neurons to a thin neuropil (corresponding to a part of the earlier-described frontal ganglion) and further to the prototroch nerve ring. This condition differs considerably from the one found in the entoproct creeping-type larva of *Loxosomella murmanica* (Nilus, 1909), which comprises 6–8 centrally positioned and 8 peripheral serotonergic cells in the apical organ. In addition, the larva of *L. murmanica* has a very complex overall neural anatomy that includes a prototroch nerve ring, an anterior nerve loop, an oral nerve ring, a paired buccal nerve, and 2 pairs of longitudinal nerve cords that correspond to the tetraneurous condition found in molluscs (Wanninger et al. 2007).

In order to increase the datasets on the anatomy of the still poorly known swimming-type larva of Entoprocta, we describe herein the gross morphology, the muscular bauplan, and the serotonergic nervous system of the swimming-type larva of *Loxosomella atkinsae* Bobin & Prenant, 1953, using scanning electron microscopy (SEM) and fluorescence labelling in combination with confocal laser scanning microscopy (CLSM). We discuss our findings in the light of recent data on other spiralian clades, and aim at contributing to insights into the evolution and phylogenetic relationships of Entoprocta.

Material and methods

Populations of loxosomatid Entoprocta (Kamptozoa) were found associated with individuals of the sipunculan *Phascolion strombus* (Montagu, 1804) which inhabit empty shells of the gastropod *Turritella* and the scaphopod *Antalis*. The molluscan shells were collected with benthic dredges from muddy bottom in the vicinity of the Kristineberg Marine Research Station at Gåsö

Ränna in Gullmarsfjord on the West coast of Sweden in September 2005. Animals were kept in small water containers until after some days adult entoprocts started to release larvae. The latter moved rapidly through the water column and were determined as swimming-type larvae of *Loxosomella atkinsae* (a voucher sample has been deposited at the Zoological Museum, University of Copenhagen; registration no. ZMUC-ENT-26). The specimens were fixed at room temperature for 0.5–1 h in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4), rinsed in PB, and stored at 4 °C in PB containing 0.1% sodium azide (NaN_3). At the time of fixation, the larvae did not show any signs of settlement, metamorphosis or budding.

Scanning electron microscopy

After fixation in PFA and storage as described above, larvae were rinsed in distilled water and postfixed in 1% osmium tetroxide (OsO_4). Subsequently, specimens were rinsed in distilled water, dehydrated in an ascending acetone series, and critical-point dried. The larvae were placed on carbon-mounted stubs and coated with copper. SEM images were recorded with a JEOL JSM-6335F scanning electron microscope (Jeol Ltd., Tokyo, Japan).

Fluorescence labelling

Muscular system

After fixation in PFA, larvae were rinsed in PB and transferred to PB containing 0.1% Triton X-100 to enhance tissue permeability. F-actin was labelled at room temperature with Oregon Green 514 phalloidin (Molecular Probes; Eugene, OR, USA) for 1 h in the dark. Larvae were washed in 0.1 M PB prior to mounting in Vectashield (Vector Laboratories; Burlingame, CA, USA) on glass slides.

Nervous system

Specimens fixed in PFA and stored in 0.1 M PB with 0.1% NaN_3 were transferred to 0.1 M PB containing 0.1% NaN_3 and 0.1% Triton X-100 (PTA) for 1 h. Larvae were then incubated in 6% normal goat serum (DakoCytomation; Glostrup, Denmark) (BlockPTA) over night at 4 °C to block unspecific binding sites. Subsequently, the larvae were incubated in an anti-serotonin (5-HT) primary antibody (Calbiochem; Cambridge, MA, USA) diluted in BlockPTA (1:400) over night at 4 °C. After several washes in BlockPTA over night at 4 °C, the secondary antibody conjugated with TRITC or FITC (Jackson ImmunoResearch; West Grove, PA, USA), respectively, was applied in BlockPTA (anti-rabbit TRITC: 1:100; anti-mouse FITC: 1:300) over night at 4 °C. This was followed by rinsing the larvae in PB. Finally, the specimens were mounted in Vectashield on glass slides.

Visualisation and image processing

Fluorescence-labelled muscular and nervous tissue was visualised using a Leica TCS SP2 AOBS confocal laser scanning microscope. Confocal stacks were merged into projection images with greater focal depth. Observations were also made with a Leica DMRXA light microscope with epifluorescence equipment, and images were taken with an attached digital camera Evolution MP and the program ImageProPlus 5.0 (both Media Cybernetics Inc., Silver Spring, MD, USA). Images were processed and arranged on plates using Adobe ImageReady 7 (Adobe Systems Inc., San Jose, CA, USA) and CorelDRAW 11 (Corel Graphics Suite 11, Corel Corporation, Ottawa, ON, Canada).

Results

Gross morphology and orientation in the larva of *Loxosomella atkinsae*

The apical organ marks the dorsal side of the entoproct swimming-type larva; the oral–anal plane defines the ventral side (Fig. 1A, G, and H). The region dorsal of the prototroch including the apical organ is referred to as episphere, the ventral region below the prototroch as hyposphere (Fig. 1A and B). The U-shaped digestive tract is positioned below the apical organ (Fig. 1H). Mouth and anus open on the ventral side (Fig. 1E).

The height of the larva from the base of the hyposphere to the apical organ is approximately 50–100 μm (excluding ciliation). The apical organ comprises several cilia with a length of about 12 μm. Some single cilia of the apical organ are surrounded by a circle of microvilli. With the exception of the apical ciliary tuft, the episphere is not ciliated, but covered by a more or less dense layer of particles of unknown composition and different shapes and sizes. However, apical organ, prototroch and hyposphere are without such deposits.

The frontal organ is reduced in larvae of *L. atkinsae*; only a slight, unciliated, elongated evagination on the frontal side of the episphere is seen in strongly retracted larvae (not shown). However, we found 2 pairs of protruding structures with a small invagination (pore) on their tips on the lateral sides of the episphere below the apical organ, and 2 pairs of these structures above the prototroch (Fig. 1C). The paired structures do not seem to be ciliated; eyespots were not observed either (Fig. 1D).

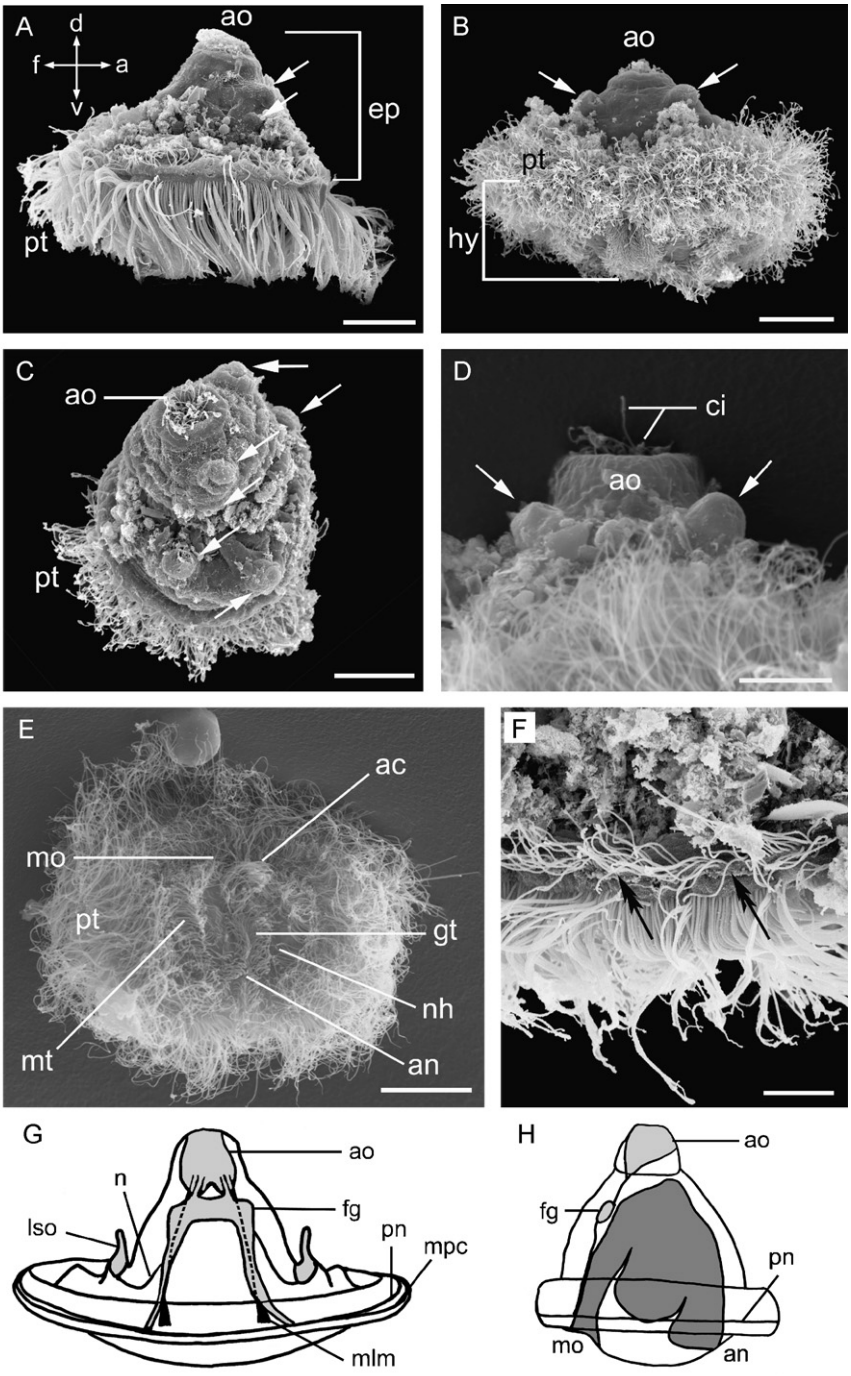

Fig. 1. SEM micrographs of swimming-type larvae of *Loxosomella atkinsae* (A–F), and semi-schematic line drawings of major nervous and muscle structures and digestive tract of *Loxosomella pectinaricola* and *L. elegans*, respectively (G, H; modified after Nielsen 1971): (A) lateral view of larva; arrows point to 2 protruding structures, (B) abfrontal view of larva; arrows point to one of 4 pairs of protruding structures, (C) dorso-lateral view of larva, frontal side facing to left; arrows point to 4 pairs of protruding structures, (D) abfrontal side of slightly retracted apical organ with some cilia; arrows point to most dorsal pair of enlarged structures with a small pore, (E) ventral side of larva showing ciliation of hyposphere, (F) detail of prototroch; arrows point to short cilia of pretrochal ciliary ring, (G) frontal view of swimming-type larva of *L. pectinaricola*; main parts of nervous system in light grey and (H) lateral view of swimming-type larva of *L. elegans*; digestive tract in dark grey. Abbreviations: a = abfrontal, ac = long adoral cilia, an = anus, ao = apical organ, ci = cilia, d = dorsal, ep = episphere, f = frontal, fg = frontal ganglion, gt = gastrotroch, hy = hyposphere, lso = lateral sensory organ, mlm = main longitudinal muscles, mo = mouth, mpc = main prototroch constrictor, mt = metatroch, n = nerve, nh = non-ciliated area of hyposphere, pn = prototroch nerve ring, pt = prototroch, and v = ventral. Scale bars A–E: 20 μm; F: 10 μm.

The prototroch defines the border between episphere and hyposphere and consists of compound cilia (Fig. 1A). It has an oval to roundish shape and is about 60–120 µm in diameter. The prototroch curves slightly inwards at the anal side; the cilia appear to be shorter in this region (Fig. 1E). A pretrochal ciliated ring with a single line of short cilia lies dorsal to the prototroch (Fig. 1F). The hyposphere is heavily ciliated; several separate ciliary bands can be distinguished (Fig. 1E). Inside the prototroch lies a ciliated food groove with short cilia (length about 6 µm) and a metatroch (sensu Jägersten 1964). A separate ciliary band can be observed between the mouth and the anal region. We refer to it as a "gastrotroch" (sensu Nielsen 2002). The gastrotroch can be subdivided into an area with long cilia directly behind the mouth (cilium length about 10 µm) and a band of short cilia that lead to the metatroch (cilium length about 4 µm). The areas between metatroch and gastrotroch are not ciliated.

In some larvae roundish vesicles with diameters of about 10–20 µm are present on the episphere and make contact with the cilia of the prototroch ("stalked vesicles"; see Jägersten 1964; Sensenbaugh 1987; Nielsen 2002). The number of vesicles varies; a direct connection between vesicles and larval epidermis was not observed.

Muscular system

The musculature of the larvae consists of a broad band of outer ring muscles that follows the extension of the prototroch (Fig. 2A, B, H, and I). The most ventral of these muscles, the main prototroch constrictor, exceeds the other fibres in thickness (Fig. 2C). The prototroch ring muscles have fewer fibres in the anal region, where the muscles curve towards the anus. Inside the prototroch ring muscles there are about 40 short longitudinal muscles ('prototroch longitudinal muscles'). They insert with small branching fibres on the ventral side near the main prototroch constrictor and end just above the most dorsal prototroch ring muscles (Fig. 2A–C). The episphere of the larva is surrounded by fine muscle fibres, the episphere ring muscles, which form broad, loosely arranged circular bands around the digestive tract of the larva. They extend from the ventral plane of the larva to below the apical organ (Fig. 2A and F). The apical organ is surrounded by apical ring muscles (Fig. 2A and B).

A pair of fine muscles runs on the ventral side from the outer ring muscles on both sides of the anus and terminates in the vicinity of the inner ring muscles (abfronto-ventral muscles in Fig. 2C). Several longitudinal and diagonal fibres are present inside this layer of episphere ring muscles. A pair of frontal diagonal muscles inserts lateral to the mouth. Ventrally, these muscles come in close contact with the main prototroch constrictor. The muscles cross each other at about the level of the frontal ganglion and end just below the apical ring muscles (Fig. 2A).

A pair of prominent longitudinal muscles inserts at a region lateral to the digestive tract and reaches into the inner part of the apical organ (Fig. 2A, C and D). Here, the main longitudinal retractor muscles insert with broad, triangular-shaped fibres in the apical organ (Fig. 2G). The digestive tract is connected to the apical organ by a pair of median muscles. They insert on the dorsal side of the U-shaped gut of the larva and terminate inside the apical ring muscles (Fig. 2A and E).

The paired lateral longitudinal muscles insert with several fibres on the ventral side of the larva; some fibres reach the prototroch ring muscles (Fig. 2A, C and D). The muscles continue in dorsal direction and split on each side at about the level of the frontal ganglion. The fibres insert below the apical ring muscles (Fig. 2G). Some fibres run to the most apical pair of protruding structures. On the abfrontal side of the larva, a pair of diagonal muscles inserts close to the prototroch ring muscles, passes the layer of episphere ring muscles, and intercrosses close to the anus (Fig. 2C). The 2 muscles run in dorsal direction and terminate below the apical ring muscles at the uppermost pair of the epispheral protruding structures (Fig. 2B).

A short abfrontal muscle inserts in close proximity to the abfrontal diagonal muscles and inserts deep inside the apical organ. The terminal endings of the short abfrontal muscle and of the main longitudinal retractor muscles come in contact inside the apical ring muscles of the apical organ (Fig. 2G).

Serotonergic nervous system

The larvae of *L. atkinsae* bear either 3 or 4 flask-shaped serotonergic cells in the apical organ. Two of these cells lie in a slightly abfrontal position within the apical organ (cells 1 and 2 in Fig. 3A–I). They have elongated somata and their necks approach each other and appear to lead to the cilia of the apical organ. The remaining serotonergic cells lie in a more frontal position within the apical organ (cells 3 and 4 in Fig. 3). One of these (cell 3) always shows strong immunoreactivity. Cell 4 is only weakly labelled or entirely absent in some larvae. In most larvae, however, cell 4 is well developed and shows clear serotonin immunoactivity. Where developed, cell 4 lies frontally and in close contact with cell 3.

All serotonergic cells in the apical organ are connected to each other by axons. A pair of nerves reaches from the serotonergic cells of the apical organ to

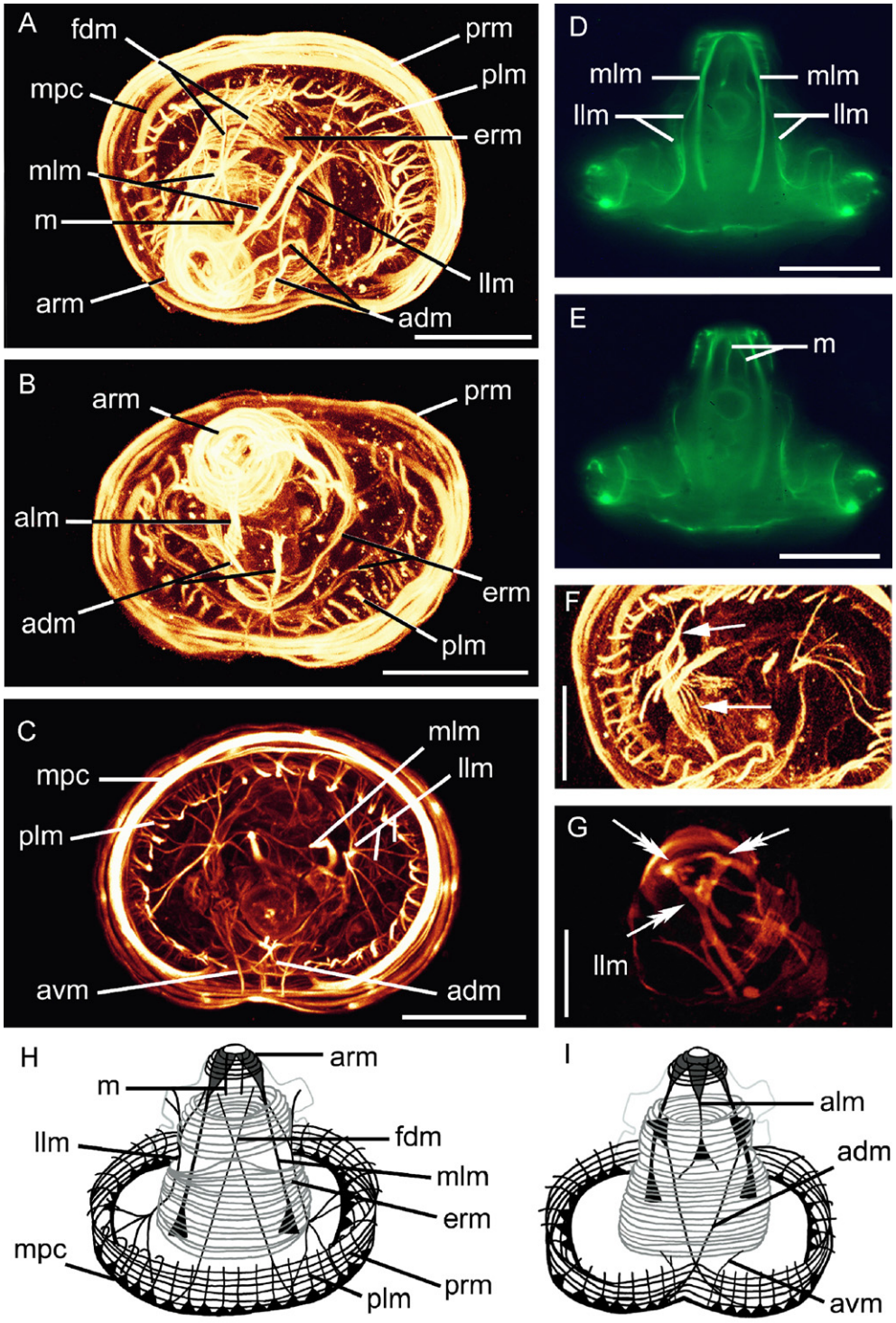

Fig. 2. *Loxosomella atkinsae*; CLSM micrographs of myoanatomy of swimming-type larva (A–C, F, G), epifluorescence micrographs (D, E), and semi-schematic line drawings (H, I): (A) dorso-lateral view of larva, frontal side at top, showing all main muscle systems, (B) abfrontal muscle system, (C) dorsal view of hyposphere visualising insertions of main muscle systems, (D) frontal view of larva, (E) median view of larva, (F) dorsal view of part of episphere ring muscles (arrows), (G) apical organ showing insertion of 3 muscle systems (arrows) inside apical ring muscles and (H, I) main muscular systems in frontal and abfrontal view, respectively; light grey lines indicate larval epidermis. Abbreviations: adm = paired abfrontal diagonal muscles, alm = abfrontal longitudinal muscle, arm = ring muscles of apical organ, avm = paired abfronto-ventral muscles, erm = episphere ring muscles, fdm = pair of frontal diagonal muscles, llm = paired lateral longitudinal muscles, m = pair of median muscles, mlm = pair of main inner longitudinal retractor muscles, mpc = main prototroch constrictor, plm = prototroch longitudinal muscles, and prm = prototroch ring muscles. Scale bars A–E: 30 μm; F, G: 20 μm.

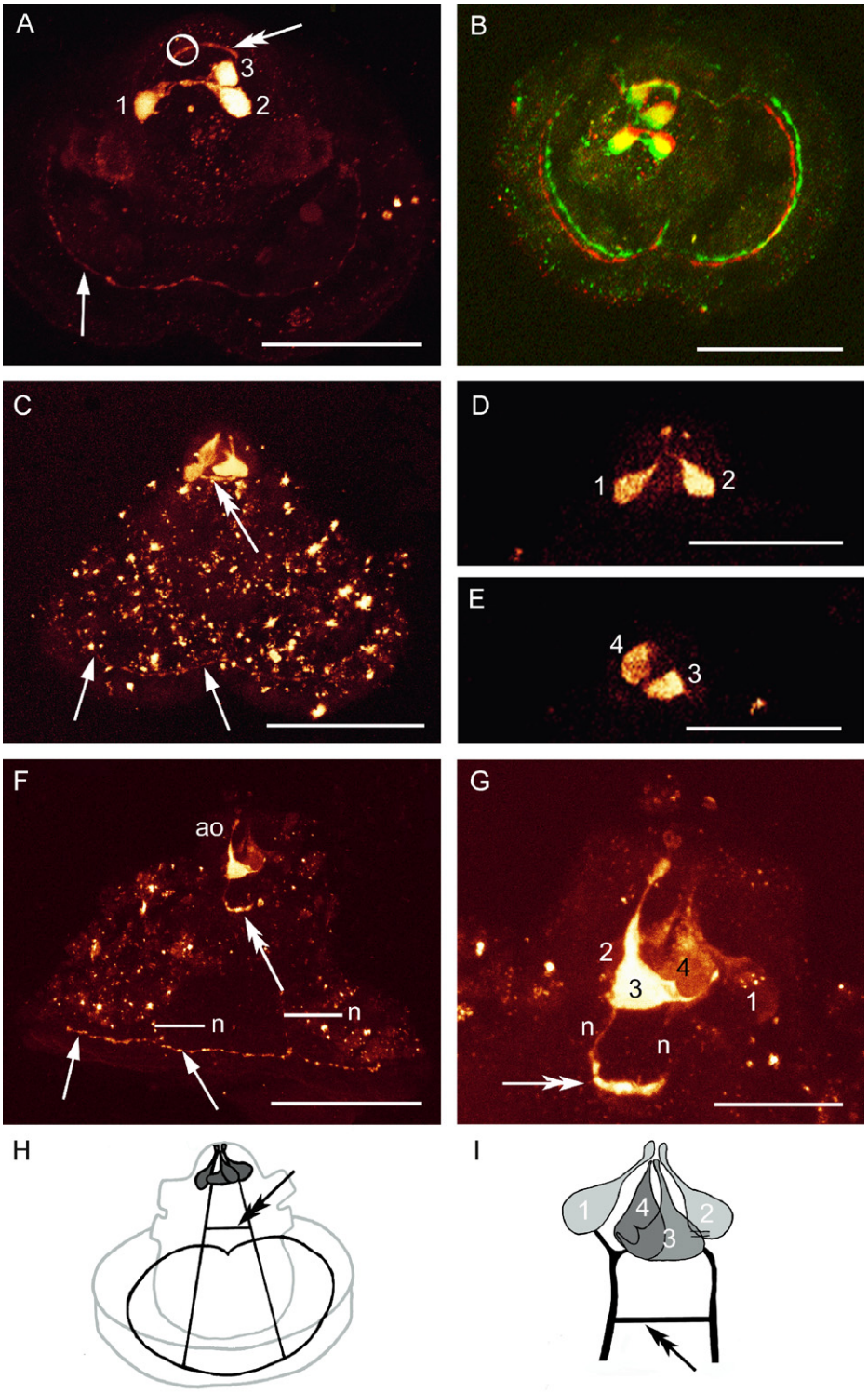

Fig. 3. *Loxosomella atkinsae*; CLSM micrographs of serotonergic nervous system of swimming-type larva (A–G), and semi-schematic line drawings (H, I): (A) serotonergic nervous system of larva, indicating 3 intensely stained flask-shaped cells in apical organ and one faintly stained spot (circle), (B) stereo image of larva, frontal area at top, (C) larva in abfrontal view, (D, E) close-ups of the 4 serotonergic cells, (F) larva in frontal view showing serotonergic nerves, (G) detail of apical organ and paired nerve reaching from cells 3 and 4 to frontal neuropil, and (H, I) frontal views of serotonergic nervous system and detail of apical organ, respectively; epidermis of larva indicated by light grey lines, frontal cells in dark grey. Arrows point to serotonergic prototroch nerve; double arrows indicate frontal neuropil; 1–4 = serotonergic cells of the apical organ. Abbreviations: ao = apical organ, n = paired serotonergic nerve from the "ao" to the neuropil, and from the latter to the prototroch nerve ring. Scale bars A–F: 30 μm; G: 10 μm.

a serotonergic neuropil on the frontal side of the larva (Fig. 3F–I). From this frontal neuropil the pair of nerves runs to the prototroch nerve. The prototroch nerve is circular with a slight invagination at the abfrontal side of the larva and underlies the entire extension of the ciliated prototroch.

Discussion

Gross morphology of entoproct swimming-type larvae

Ciliation in *Loxosomella atkinsae* matches the description of the ciliation in the swimming-type larva of *Loxosoma pectinaricola*, constituting a prototroch, a metatroch, a ciliated food groove, and a gastrotroch (cf. Nielsen 2002). In *L. atkinsae*, a small pretrochal ciliary ring, also mentioned in an earlier description of the larva of this species (Nielsen 1971), is found dorsal to the prototroch. Due to the fixation method the compound cilia often separate, but we believe that the entoproct prototroch and metatroch comprise compound cilia as proposed earlier for entoproct larvae (Nielsen 2002).

Interesting characters of the larvae of *L. atkinsae* are the 4 pairs of enlarged structures, 2 pairs on the higher and 2 on the lower part of the episphere, which are also mentioned in the description by Nielsen (1971). However, we have not found serotonergic nerve innervation of these structures. The pair of structures closest to the apical organ is connected to the lateral longitudinal muscles and probably also to the abfrontal muscles. When the larva is fully retracted, the 4 enlarged structures are also partly retracted by the main muscle systems. The function of these cells or organs is currently unknown.

Functional and comparative aspects of the musculature of entoproct swimming-type larvae

The muscular system of the swimming-type larva of *L. atkinsae* is surprisingly complex with 4 main layers of muscles, including the prototroch ring and longitudinal muscles, the epispheral ring, and the epispheral longitudinal and diagonal muscles. The prototroch ring muscles and the numerous short prototroch longitudinal muscles together form a prominent muscle complex underlying the prototrochal region, thus enabling contraction of the latter. The layer of epispheral ring muscles contains prominent ring muscles surrounding the apical organ and fine fibres enveloping the lower parts of the episphere. This layer together with the sets of inner longitudinal and diagonal muscles enables contraction of the episphere and the hyposphere. The most prominent epispheral inner longitudinal muscles are the main longitudinal muscles which run from the apical organ to a region lateral of the esophagus. These are possibly used for contraction of the ventral side (hyposphere) of the larva. The pair of short median muscles connects the apical organ with the dorsal part of the intestine. The lateral longitudinal muscles send small fibres to the most apical pair of the protruding structures of the episphere, thus probably being at least partly used for contraction and expansion of these structures. The abfrontal muscles all insert close to or directly at the intestine. The paired abfrontal diagonal muscles may also be involved in moving the protruding structures of the episphere.

In earlier descriptions of the swimming-type larvae of *Loxosomella elegans* Nielsen, 1964 and *Loxosoma pectinaricola*, some individual muscles were observed, all of which correspond to muscles described herein for *L. atkinsae* (cf. Nielsen 1971, Figs. 4 and 5; 1997). In all 3 species, the prominent pair of main longitudinal muscles was found running from the apical organ along the paired nerves and inserting on the sides of the esophagus close to the mouth (Figs. 1G and 2A, C, D). In addition, an unpaired abfrontal muscle originating in the apical organ and inserting at the intestine was found in *L. elegans*. This muscle corresponds to the unpaired abfrontal longitudinal muscle in *L. atkinsae* (Fig. 2B). In *L. elegans*, some small muscles are present between the lateral parts of the episphere and the hyposphere. These correspond to the numerous prototroch longitudinal muscles in *L. atkinsae* (Fig. 2A). The main prototroch constrictor is present in all 3 species investigated. Accordingly, we propose the ancestral muscle bauplan of the loxosomatid swimming-type larva to comprise muscles underlying the prototroch (prototroch ring muscles and short longitudinal prototroch muscles), a paired main longitudinal muscle, and an unpaired abfrontal longitudinal muscle.

It appears that the larval entoproct muscular architecture is at least as complex as that of the adults, despite the much smaller size of the larvae. Both life-cycle stages comprise ring, longitudinal, and diagonal muscles. However, a distinct outer layer of ring muscles is lacking in the fully grown adults of the species investigated so far (Wanninger 2004; Fuchs et al. 2006). Development of the entoproct muscle system is currently known only from the asexual budding process; it is unknown which, if any, larval muscle systems contribute to the adult bauplan. Thus, homology assumptions for entoproct larval and adult muscles are still impossible. The complexity of the entoproct larval muscle systems, however, may very well provide important characters for both phylogenetic and taxonomic analyses (see also Wanninger 2004). However, data on the myoanatomy of additional species (especially of creeping-type larvae) are required for further insights into the ancestral larval muscle bauplan of Entoprocta.

Comparison of the entoproct larval nervous system with other Spiralia and Ectoprocta, and evolution of entoproct larval types

The nervous system of entoproct larvae in general seems to be more variable than previously believed. The serotonergic nervous system of *Loxosomella atkinsae* includes 3 or 4 serotonergic flask-shaped cells in the apical organ and a paired nerve on the frontal side of the episphere which passes the frontal neuropil and connects to the prototroch nerve ring. The serotonergic nervous system has been described for only one additional swimming-type larva, namely that of *Loxosoma pectinaricola* (Hay-Schmidt 2000). That account is in accordance with our description, except that only 3 serotonergic cells were found in the apical organ of *L. pectinaricola*.

In contrast to the swimming-type larvae, the entoproct creeping-type larva of *Loxosomella murmanica* exhibits a much more complex serotonergic system with 6–8 central cells and 8 peripheral cells in the apical organ, 2 pairs of longitudinal nerve cords (tetraneury), as well as buccal, oral, and prototrochal nerves, thus expressing striking similarities with the neural architecture of basal Mollusca (Wanninger et al. 2007). Since the creeping type is considered as the basal entoproct larval type (Nielsen 1971; Jägersten 1972), the ancestral larval neural bauplan of entoprocts appears to be more complicated than that of the swimming-type larvae.

Although we only labelled the serotonergic portion of the larval nervous system of *L. atkinsae*, comparison with earlier discriptions of the entire neural anatomy of other swimming-type larvae based on semithin sectioning and light microscopy reveals striking similarities with the data presented herein (cf. Nielsen 1971). The larva of *L. pectinaricola* exhibits a rather compact apical organ, from which a paired nerve connects to the frontal ganglion and to the prototroch nerve ring (Nielsen 1971). An additional pair of fine nerves connecting to the protruding structures is found in *L. pectinaricola* (Nielsen 1971; see also Fig. 1G). This description is congruent with our observations, except that we found a frontal serotonergic neuropil in *L. atkinsae* instead of the frontal ganglion present in *L. pectinaricola*. Due to their similar position in the larval body we consider these structures homologous. The fine nerves connecting to the protruding structures in *L. pectinaricola* were not observed in *L. atkinsae*, but whether they are indeed absent or merely lack serotonin immunoreactivity could not be determined in our study.

In Entoprocta, the development of the serotonergic nervous system from the larval to the adult stage is still unknown. However, immunocytochemical data on the adult nervous system of 2 loxosomatid species show a quite simple serotonergic system comprising a neuropil (part of the bilobed cerebral ganglion), paired nerves in the calyx that are in connection with few serotonergic cells, and nerves innervating the tentacles (Fuchs et al. 2006). The simple adult entoproct nervous system in general is most probably closely linked to the sessile mode of life of the adult stage.

When comparing the serotonergic nervous systems of the cycliophoran chordoid larva to that of entoprocts, it seems that the structures of the chordoid larva resemble more the adult entoproct condition than that of entoproct larvae. A detailed study of the chordoid larva of *Symbion pandora* Funch & Kristensen, 1995 showed that a true apical organ with serotonergic neurons is missing, the only serotonergic and/or FMRFamidergic features being a large frontal bipolar ganglion and 2 pairs of ventral nerve cords (Wanninger 2005).

The serotonergic nervous system of ectoproct larvae appears to be quite different compared to the neural architecture found in spiralian larvae. The coronate larvae of ectoprocts exhibit an apical commissure, a nerve plexus, paired axons that innervate the pyriform organ, paired lateral axons, and a coronate nerve net or equatorial ring (Pires and Woollacott 1997; Shimizu et al. 2000; Wanninger et al. 2005). In contrast, the cyphonautes larva of *Membranipora* sp. has 2 apical nerve cells and a paired nerve that is connected to nerve cells of the pyriform organ and the coronal nerve (Hay-Schmidt 2000). At present, there is no indication for homology of individual nervous structures of ectoproct and entoproct larvae.

Recent comparative investigations of spiralian larval nervous systems show that the actual number of serotonergic cells in the apical organ is more variable within most phyla than previously believed. For example, molluscan apical organs bear 3 (most bivalves, gastropods), 4 (scaphopods), 5 (several gastropods), or initially 3 and later 8–12 serotonergic cells (polyplacophorans) (Marois and Carew 1997; Friedrich et al. 2002; Voronezhskaya et al. 2002; Wanninger and Haszprunar 2003; Croll and Dickinson 2004). However, many larvae share the presence of an apical organ with few serotonergic cells and often a pair of nerves connecting it to the serotonergic prototroch nerve ring (Lacalli 1981, 1983, 1986; Kempf et al. 1997; Marois and Croll 1992; Hay-Schmidt 2000; Page 2002; Wanninger and Haszprunar 2003; Wanninger 2008). Accordingly, the condition found in the swimming-type larva of *L. atkinsae*, with 3–4 serotonergic flask-shaped cells in the apical organ and a paired nerve connecting to the prototroch, is congruent with the situation found in most basal spiralian larvae investigated so far. However, the frontal neuropil in the swimming-type larva does not find a true counterpart in other trochophores and may be a synapomorphy for the entoproct swimming-type larva. As mentioned above, the creeping-type larva of *L. murmanica*, which probably constitutes the basal

entoproct larval type, exhibits a highly complex overall neuroarchitecture which closely resembles that found in basal adult and larval polyplacophoran molluscs (Wanninger et al. 2007). Accordingly, we regard the simple serotonergic neural architecture of the swimming-type larva of *L. atkinsae* as a derived state that has resulted from reduction events during entoproct (larval) evolution. Interestingly, it has been argued earlier that the swimming-type larva has undergone a number of secondary simplifications such as, e.g., loss of the foot due to the transition from a benthic-creeping to a planktonic-swimming lifestyle of the larva (Jägersten 1972). This hypothesis is based on the assumption that the foot of recent entoproct creeping-type larvae constitutes a remnant of an ancestral creeping, free-living entoproct adult. In this scenario, certain adult features, such as the foot, were transferred to the larval stage when the adult adopted a sessile lifestyle. As a consequence, recent entoproct creeping-type larvae would be expected to exhibit both larval and ancestral adult characters. With respect to the anatomy of the serotonergic nervous system, such a situation is indeed exemplified in the creeping-type larva of *L. murmanica*, which expresses a number of neural features also found in adult basal molluscs.

At this stage, our observations make us feel certain that as more data on comparative larval anatomy will become available, further details of the evolutionary pathways of entoproct larval forms will be revealed, thus allowing deeper understanding of entoproct evolution.

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