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Cellular and Muscular Growth Patterns During Sipunculan Development

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Abstract

Sipuncula is a lophotrochozoan taxon with annelid affinities, albeit lacking segmentation of the adult body. Here, we present data on cell proliferation and myogenesis during development of three sipunculan species, *Phascolosoma agassizii*, *Thysanocardia nigra*, and *Themiste pyroides*. The first *anlagen* of the circular body wall muscles appear simultaneously and not subsequently as in the annelids. At the same time, the rudiments of four longitudinal retractor muscles appear. This supports the notion that four introvert retractors were part of the ancestral sipunculan bodyplan. The longitudinal muscle fibers form a pattern of densely arranged fibers around the retractor muscles, indicating that the latter evolved from modified longitudinal body wall muscles. For a short time interval, the distribution of S-phase mitotic cells shows a metameric pattern in the developing ventral nerve cord during the pelagosphaera stage. This pattern disappears close to metamorphic competence. Our findings are congruent with data on sipunculan neurogenesis, as well as with recent molecular analyses that place Sipuncula within Annelida, and thus strongly support a segmental ancestry of Sipuncula.

The phylogenetic position and evolutionary origin of the sipunculans, a small and exclusively marine group of coelomate, vermiform animals that show no obvious segmental organization in the adult stage, has been controversial for decades. They have been related to taxa as diverse as holothurians, echiurids, priapulids, phoronids, mollusks, or annelids (e.g., Åkesson, '58; Hyman, '59; Rice '85; Scheltema, '93; Cutler, '94). Recently, a number of independent molecular phylogenetic analyses have suggested a close relationship to Annelida (including Echiura) or even a nested position within this phylum (Boore and Staton, 2002; Staton, 2003; Jennings and Halanych, 2005; Bleidorn et al., 2006; Struck et al., 2007; Dunn et al., 2008; Hejnol et al., 2009; Mwinyi et al., 2009; Shen et al., 2009; Sperling et al., 2009; Zrzavy et al., 2009). The latter scenario has received significant support by a recent study, whereby topology tests significantly reject the sistergroup relationship of Sipuncula and Annelida (Dordel et al., 2010). Moreover, ultrastructural

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AUTHORS' CERTIFICATION

The authors declare that they agreed to be listed as such, and that they have read and approved the final version of the manuscript.

similarities have been found in the foregut of certain sipunculans and polychaetes as well as in their collagenous cuticle (Tzetlin and Purschke, 2006). This notion is further supported by recent developmental studies on sipunculans and echiurans that have revealed segmental traits during neurogenesis (Hessling, 2002, 2003; Hessling and Westheide, 2002; Kristof et al., 2008; Wanninger et al., 2009). Given their proposed inclusion within Annelida is correct, it seems plausible to assume secondary loss of a once segmented body plan rather than an initial evolutionary step toward segmentation in these animals. Interestingly, the lack of certain annelid key features, such as segmentation, coelomic cavities, nuchal organs, and chaetae, is also known for a variety of interstitial, parasitic, and sessile annelid representatives, but to a much lesser extent for large burrowing forms, such as earthworms (reviewed in Bleidorn, 2007).

Segmentation is usually considered a concerted repetition of organs or organ systems that form subsequently from a posterior growth zone along the anterior–posterior axis of an animal (Willmer, '90). Studies on the cell proliferation patterns and the ontogeny of organ systems typically associated with annelid segments (e.g., subsequently emerging sets of paired perikarya associated with the ventral nerve cords, body wall ring muscles, nephridia) have proven to be ideal markers to assess whether or not a taxon has derived from a segmented ancestor (Müller and Westheide, 2000; Hessling, 2002, 2003; Hessling and Westheide, 2002; de Rosa et al., 2005; Seaver et al., 2005; Bergter et al., 2007; Brinkmann and Wanninger, 2008; Kristof et al., 2008; Wanninger, 2009). Furthermore, the growing number of immunocytochemical studies on neuro- and myogenesis of a number of lophotrochozoan taxa allows for a comparison of nervous and muscle system patterning pathways in putatively segmented and nonsegmented clades (e.g., Hay-Schmidt, 2000; Croll and Dickinson, 2004; McDougall et al., 2006; Bergter et al., 2007; Wanninger, 2008; Wanninger et al., 2008, 2009). Although some variation in annelid segment formation has been reported (Seaver et al., 2005; Brinkmann and Wanninger, 2010), the segmentation process driven from a posterior growth zone is considered to be the ancestral condition for Annelida (Anderson, '66; de Rosa et al., 2005; Seaver et al., 2005; Wanninger et al., 2009). Herein, we compare the tempo–spatial distribution of proliferating cells in *Thysanocardia nigra* and *Themiste pyroides* with growth patterns reported for annelids. We supplement this work with data on myogenesis in *Phascolosoma agassizii*, *T. nigra*, and *T. pyroides*, and thus provide insights into the evolution of the myogenic bodyplans within the Lophotrochozoa.

MATERIAL AND METHODS

Animals

Adult *P. agassizii* were collected in the vicinity of the Friday Harbor Laboratories (Washington) and were kept in the laboratory until gametes were released. After fertilization, the developing larvae were maintained in natural seawater at ambient temperature (10°C). Development was followed until 15 days post-fertilization (dpf) when animals had reached the late pelagosphera stage. Adult specimens of *T. nigra* and *T. pyroides* were obtained from *Crenomytilus grayanus* mussel beds. Mussel aggregations were collected by scuba divers at depths of 4–8 m from the Vostok Bay, Sea of Japan (Russia).

Adults were placed in small tanks (15–30 specimens each) with ambient seawater (20–22°C) until spawning occurred. In addition, fertilization experiments were performed. Adult specimens were cut open and gametes were transferred into glass jars. The eggs were fertilized with a few drops of a diluted sperm suspension. Embryos, larvae, and juveniles were reared in Petri dishes and glass vessels at 17–19°C. Elongation of the anterior–posterior axis started at 3 dpf in both species. Metamorphosis occurred at 10 dpf in *T. nigra* and at 15 dpf in *T. pyroides*. Development was followed in both species until the first juvenile stages, which already showed the *anlagen* of the primary tentacles (i.e., at 18 dpf in *T. pyroides* and 15 dpf in *T. nigra*).

EdU Labeling, F-Actin Staining, Confocal Laserscanning Microscopy, and 3D Reconstruction

Proliferating cells were visualized by in vivo labeling with the nucleotide analogue 5-ethynyl-2'-deoxyuridine (EdU) that is incorporated during the synthesis phase (S-phase) of the cell cycle. Larvae were incubated in EdU (Invitrogen, Taastrup, Denmark), diluted in filtered seawater in the following concentrations and time intervals at 17–19°C: 250 µM for 1 hr, 8 µM for 6 hr, and 5 µM for 24 hr. After EdU treatment and before F-actin staining, larvae were anesthetized by adding drops of a 3.5 or 7% MgCl₂ solution to the seawater and were subsequently fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.3) for 1.5 hr at room temperature or overnight at 4°C. This procedure was followed by three washes (15 min each) in 0.1 M PBS (pH 7.3) with 0.1% sodium azide (NaN₃). Until further processing, samples were stored in 0.1 M PBS with 0.1% NaN₃ at 4°C.

After storage, larvae were rinsed in 0.1 M PBS (pH 7.3) for more than 6 hr, followed by incubation in a blocking and permeabilization solution (saponin-based permeabilization and wash reagent with 1% BSA) overnight at 4°C. Incorporated EdU was detected with a Click-iT EdU Kit (Cat# C3005, Invitrogen, Taastrup, Denmark). The larvae were incubated with the reaction cocktail provided by the supplier (7.5 µL Alexa Flour 488 azide, 30 µL CuSO₄, 1313 µL EdU reaction buffer, and 150 µL EdU buffer additive) for 24 hr at 4°C. Some specimens were additionally incubated for 24 hr at 4°C in a polyclonal rabbit anti-serotonin primary antibody (Calbiochem, Cambridge; dilution 1:200). The specimens were rinsed three times for 15 min in PBS. This was followed by incubation with a goat anti-rabbit Alexa 594 secondary antibody (dilution 1:300; Invitrogen, Taastrup, Denmark) in 0.1 M PBS for 24 hr at 4°C. Finally, the specimens were washed three times for 15 min each in the saponin-based wash reagent with 1% BSA, incubated with the nucleic acid stain 4', 6-diamidino-2-phenylindole (DAPI; 1:10 dilution; Invitrogen, Taastrup, Denmark), and mounted in Fluoromount G (Southern-Biotech, Birmingham, Alabama) on glass slides.

For F-actin labeling, the stored larvae were washed three times for 15 min in 0.1 M PBS, permeabilized for 6 hr in 0.1 M PBS containing 4% Triton X-100 at 4°C, and incubated in a 1:40 dilution of Alexa Flour 488 phalloidin (Invitrogen, Taastrup, Denmark) in 0.1 M PBS in the dark for 24 hr at room temperature. Thereafter, the samples were washed three times for 15 min and mounted in Fluoromount G on glass slides. Some specimens were double labeled for DAPI and F-actin.

Analysis and digital image acquisition was performed on a Leica DM IRBE microscope equipped with a Leica TCS SP2 confocal unit (Leica Microsystems, Wetzlar, Germany). Optical sections taken at intervals of 0.5–0.2 μm were digitally merged into maximum projection images. Images were further processed with Photoshop 9.0.2 (Adobe Systems, San Jose, CA) to adjust contrast and brightness. 3D reconstructions were created from selected confocal stacks using isosurface algorithms of the imaging software Imaris v. 4.1 (Bitplane, Zürich, Switzerland). Digital line drawings were created with Corel Draw 11.0 (Corel Corporation, Ottawa, Ontario, Canada).

RESULTS

Cell Proliferation During Larval Development of *T. pyroides* and *T. nigra*

Dividing cells were labeled with the thymidine analogue EdU during development of *T. pyroides* and *T. nigra*. Combination of EdU and DAPI labeling enabled identification of proliferating cells and their location in the specimens (Fig. 1). Subsequent clones of S-phase cells that have incorporated EdU show progressive loss of staining intensity in the respective daughter cells, which allows following the direction of proliferation (Fig. 1B, D, E; cf. Chehrehasa et al., 2009).

In the trochophore stages, mitotic cells are scattered through-out the ectoderm (not shown). At the beginning of elongation (“teardrop stage”; age: 3 dpf), proliferating cells are densely arranged around the eyes, mouth, metatroch, ventral trunk region, and along the lateral and dorsal epidermis (Figs. 1A, 2A and B). Moreover, the S-phase cells are symmetrically arranged in two lateral stripes in the introvert (Figs. 1A and 2A). Slightly later (3.5 dpf), proliferating cells appear in the area of the developing digestive system (Figs. 1B, 2C and D). The highest number of proliferating cells is found along the developing ventral nerve cord (Figs. 1B, 2C and D), which at this stage has three pairs of serotonergic perikarya associated with the serotonergic nerve cords (Fig. 1C). Moreover, intensity loss toward the posterior pole of the ventral trunk region indicates an anterior to posterior direction of cell proliferation (Figs. 1B, 2C and D). As in the 1 hr incubated pelagosphera larvae aged 8 dpf, few units of proliferating cells appear in the posterior tip of the ventral nerve cord. The signal intensity of these cells decreases from posterior to anterior (Figs. 1D, 2E and F). In addition, few weakly stained mitotic cells appear in the developing digestive system and in the posterior trunk region (Figs. 1D, 2E and F). As expected, 8 dpf old pelagosphera larvae that had been exposed to EdU for 6 hr show a higher number of proliferating cells in the ventral nerve cord, the developing digestive system, around the mouth, and in two bilateral stripes in the introvert (Figs. 1E, 2G and H). Moreover, the number of S-phase cells has increased in the posterior trunk region, and more units of proliferating cells appear in the posterior part of the ventral nerve cord (Figs. 1E, 2G, H). Lateral to the ventral nerve cord, bilateral patches of stained nuclei seem to migrate into the developing digestive system (Figs. 1E, 2G and H). As in the 1 hr incubated specimens, the 6 hr incubated pelagosphera larvae show the same pattern of intensity decrease from posterior to anterior (Figs. 1E, 2G and H). As the larvae grow, most proliferating cells occur in the developing digestive system, around the anus, and in the anterior part of the ventral trunk area (Figs. 1F, 2F, G). We found no units of S-phase cells in the posterior ventral trunk region (Figs. 1F, G, 2I, J).

In metamorphic competent larvae (18 dpf), the majority of proliferating cells is detected in the anterior trunk, the introvert, and the digestive system (Fig. 1H). Post-metamorphic stages show proliferating cells mostly in the introvert and tentacle *anlagen* (not shown). Overall, the distribution pattern of proliferating cells during ontogeny of *T. pyroides* and *T. nigra* does not unambiguously reveal a textbook-like posterior growth zone. However, a distinct tissue formation zone appears temporarily in the posterior ventral trunk region in the pelagosphera stage.

Myogenesis in *P. agassizii*

In the early trochophore larva of *P. agassizii* (i.e., at 6 dpf), numerous circular body wall muscles appear simultaneously, together with the *anlagen* of the longitudinal retractor muscles (Figs. 3A inset and 4A). Slightly later, the longitudinal retractors can be distinguished as one pair of ventral, one pair of dorsal, and one single unpaired terminal organ retractor muscle (Fig. 3B). In addition, the mouth opening is visible and situated anterior to the *anlage* of the buccal musculature (Fig. 3B). The ventral and dorsal retractor muscles and the terminal organ retractor muscle are well developed in the late trochophore larva before the onset of longitudinal growth at 7 dpf (Figs. 3C and 4B). At the same time, numerous circular muscles of the future buccal musculature emerge posteriorly to the mouth opening (Figs. 3C and 4B). In addition, the entire length of the larval trunk is covered by circular body wall muscles (Figs. 3C and 4B). Their number remains constant during the early phases of elongation (7–8 dpf) (Fig. 3D and E). By contrast, additional longitudinal body wall and introvert retractor muscle fibers start to form (Fig. 3D and E). At the same time, the esophageal ring, intestinal ring, and longitudinal muscle fibers, together with the anal ring muscles of the digestive system, are established (Figs. 3D, E and 4C). As the pelagosphera larva grows (9–2 dpf), new circular body wall muscles are synchronously added along the entire anterior–posterior axis by fission from existing myocytes (Figs. 3F and 4D). Accordingly, the formation of new circular body wall muscle fibers does not occur in a directional anterior–posterior process. The ventral longitudinal retractor muscles contribute to the muscles that encircle the mouth opening and surround the musculature of the buccal organ from either side (Figs. 3F, 4C and D). Similarly, the dorsal pair of longitudinal retractor muscle bands passes the prominent esophageal ring muscle fibers, which are situated dorsally to the buccal organ (Figs. 3F, 4C and D). In addition, the two branches of the terminal organ retractor muscle, which insert at the body wall dorsal to the anal ring muscles, can be distinguished (Figs. 3F, 4C and D). Close to metamorphosis (15 dpf), the myoanatomy of *P. agassizii* is composed of four solid longitudinal introvert retractors, which persist into adult-hood, a prominent longitudinal terminal organ retractor muscle with two distinct roots, and a strongly developed buccal and esophageal musculature (Figs. 4E and 5). In addition, the dorsal retractors intersect in the anterior introvert region (Figs. 4E and 5B). The body wall consists of numerous longitudinal muscle fibers which are loosely arranged in the mid-body region and in bands near the retractors, together with homogeneously arranged circular body wall muscles along the entire anterior–posterior axis (Figs. 4F, 5A, C and D). Moreover, new helicoid muscles, which probably connect the gut to the body wall, appear close to the insertion areas of the longitudinal retractor muscles in the late pelagosphera larva (Figs. 4E, 5A and D). No oblique muscles were found in the body wall during myogenesis of *P. agassizii*.

Myogenesis in *T. pyroides* and *T. nigra*

Because myogenesis in *T. pyroides* and *T. nigra* is strikingly similar, a detailed description is provided for *T. pyroides* only. Development from fertilization to the crawling juvenile is 11 dpf at 17–9°C in *T. nigra* and 18 dpf in *T. pyroides*. In contrast to *P. agassizii*, *T. pyroides* and *T. nigra* have a lecithotrophic development. Interestingly, an earlier report mentions direct development in *T. pyroides* populations from the Pacific North-east (Rice, '67), whereas indirect development is known for this species from the Sea of Japan, i.e., the West Pacific (Adrianov et al., 2008; Adrianov and Maiorova, 2010).

In *T. pyroides*, the first muscles appear simultaneously in 2 dpf old larvae as numerous circular body wall muscles, together with the *anlagen* of two pairs of longitudinal retractor muscles (Fig. 6A and F). In the tear-drop stage, where anterior–posterior axis elongation begins (2.5 dpf), the two pairs of longitudinal retractor muscles can be distinguished (Fig. 6B). In addition, the ventral retractor muscles encircle the mouth opening and more circular body wall muscles appear (Fig. 6B). Slightly later (at approximately 3 dpf), new circular body wall muscles form by fission from existing myocytes along the entire anterior–posterior axis (Fig. 6C and G). In addition, the longitudinal retractor muscles are well developed and numerous longitudinal body wall muscles can be distinguished as bands near the retractor muscles (Fig. 6C and G). During elongation of the anterior–posterior axis in the pelagosphera larva (3.5 dpf), the retractor muscles become denser and new circular and longitudinal body wall muscles form (Fig. 6D). In late pelagosphera larvae (8 dpf), the retractor muscles become prominent and the body wall musculature consists of homogeneously arranged circular muscles along the entire anterior–posterior axis, as well as numerous longitudinal muscles around the retractors and few in the mid-body region (Fig. 6E and H). The body wall musculature of early juveniles (18 dpf) comprises ring muscles and underlying longitudinal muscles, which are densely arranged around the strongly developed paired ventral and dorsal retractor muscles (not shown). As in *P. agassizii*, no anterior to posterior development of newly formed circular body wall muscles and no oblique body wall muscle fibers were found during myogenesis of either *T. pyroides* or *T. nigra*.

DISCUSSION

Proliferation Zones and Segmentation

Traditional descriptions of annelid segment formation have mainly dealt with clitellates (leeches and oligochaetes). In these groups, segments are formed in anterior–posterior progression from a posterior growth zone that contains both mesodermal teloblasts and ectoblasts (e.g., Weisblat et al., '88; Shankland, '91; de Rosa et al., 2005; Seaver et al., 2005; Brinkmann and Wanninger, 2010). Divisions of the mesoteloblasts result in mesodermal bands that extend anteriorly and then form segmentally iterated muscle units. In the ectoderm, a ring of ectoteloblasts localized in the growth zone generates the segmental ectoderm (Anderson, '66, '73). In polychaetes, segments are added sequentially from proliferating cells located in one posterior or two lateral growth zone(s), and these cells might be homologous to the clitellate teloblasts (Shankland and Seaver, 2000; Seaver et al., 2005). Interestingly, a distinct posterior region of increased cell proliferation comparable to

an annelid-like growth zone seems to be present in certain echiurans, a derived polychaete taxon (Hessling, 2003). In *T. pyroides* and *T. nigra*, proliferating cells were constantly present in tissues of developing organ systems, such as the digestive system, the ventral nerve cord, and the post-metamorphic tentacles. First, S-phase cells were scattered throughout the ectoderm, then they appeared in the endodermal tissue of the developing gut before they formed metameric clusters (units) along the posterior ventral nerve cord during the pelagosphera stage. The intensity of these EdU-labeled cells decreased in their respective daughter cells in anterior direction, which also indicates the direction of proliferation (Chehrehasa et al., 2009). However, toward metamorphosis and in post-metamorphic stages, these posterior clusters of mitotic cells disappear.

Interestingly, in regenerating segments of *Platynereis dumerilii* juveniles, BrdU experiments showed many stained cells on the ventral side and around the ventral nerve cord. Subsequently, these cells migrate laterally as the segments become older (intensity loss in respective daughter cells; de Rosa et al., 2005). However, BrdU pulse (15 min incubation with BrdU) and chase experiments (fixation after 24 hr and 48 hr, respectively) in regenerating segments of *P. dumerilii* juveniles revealed a distinct band of proliferating cells between the pygidium and the newly formed segment (de Rosa et al., 2005). A recent study on the polychaetes *Capitella* and *Hydroides* showed that larval segments in these distantly related polychaetes are formed from a field of dividing cells from the ventrolateral region of the body, and that only post-metamorphic segments arise from the posterior growth zone (Seaver et al., 2005). Likewise, neither an obvious posterior band of proliferating cells were detected in the larval segments of metatrochophores nor in later larval stages of *Sabellaria alveolata* (Brinkmann and Wanninger, 2010). These data illustrate the ontogenetic plasticity of segment formation in annelids on a cellular level, a fact that is also reflected in the highly variable mode of establishment of the various parts of the segmental nervous and muscle system in some polychaete annelids (Brinkmann and Wanninger, 2008, 2010).

Our findings on *T. pyroides* and *T. nigra* show different distribution patterns of proliferating cells during development. Only after the metameric arrangement of the nervous system has been established, proliferation increases in the ventral posterior trunk area and seems to progress from posterior to anterior. Accordingly, the distribution of mitotic cells in the sipunculan pelagosphera larva—but not in the earlier stages—corroborates the data on neurogenesis, which follows a segmental pattern (Wanninger et al., 2005, 2009; Kristof et al., 2008). Interestingly, a similar situation has recently been described for the polychaetes *Hydroides* and *Capitella*, whereby the posterior growth zone was established only in later larval stages after the first segments had already been formed (Seaver et al., 2005).

In *T. pyroides* and *T. nigra*, this metameric pattern of ectodermal S-phase cells is lost toward metamorphosis, as is the case for the segmental arrangement of the nervous system in *P. agassizii* (Kristof et al., 2008) and *T. pyroides* (unpublished data). Future studies including expression patterns of genes known to be involved in the establishment of segmentation in annelids and arthropods, such as *caudal*, *engrailed*, *brachyury*, and *even-skipped*, should shed further light on the evolution of segmentation loss in Sipuncula.

Comparative Aspects of Myogenesis in Sipunculans and Annelids

The *anlagen* of the longitudinal retractor muscles and a considerable number of circular muscles appear simultaneously in the early trochophore larvae of *P. agassizii*, *T. nigra*, and *T. pyroides*. Slightly later, the entire trunk is covered by circular muscle fibers. Their number remains constant and increases only during the later larval stages. Thereby, newly formed circular muscle fibers of the body wall appear to be formed by fission from already existing myocytes along the entire length of the body axis. In *Phascolion strombi*, which has a shortened larval phase and an entirely lecithotrophic mode of development, a different pattern of muscle formation is found. Here, new circular body wall muscle fibers are added between the already existing circular muscles along the entire anterior–posterior axis, whereby splitting of existing myocytes was not observed (Wanninger et al., 2005).

Accordingly, the process of simultaneous ring muscle differentiation along the entire anterior–posterior axis seems to be independent of a planktotrophic or a lecithotrophic life style, and thus probably ancestral to sipunculans (Wanninger, 2009). This is in striking contrast to the situation found in the segmented annelids, where ring muscles, if present, are typically formed in an anterior–posterior progression, probably owing to the existence of the posterior growth zone (e.g., Hill and Boyer, 2001; Seaver et al., 2005; Bergter et al., 2007; Hunnekuhl et al., 2009; Wanninger, 2009).

In all sipunculans investigated so far, the longitudinal body wall musculature develops later than the circular muscles, and because metamorphosis occurs without major dramatic gross morphological changes, these larval muscles form the scaffold of the adult musculature (Jaeckle and Rice, 2002; Wanninger et al., 2005; Schulze and Rice, 2009). A body wall comprising an outer layer of circular and an inner layer of longitudinal muscle fibers as expressed in a number of oligochaetes is often considered as basal for Annelida (Dales, '63; Lanzavecchia et al., '88; Gardiner, '92), although ring muscles are absent in various polychaete clades (Purschke and Müller, 2006). In contrast to the uniform muscle layers found in clitellates, polychaetes exhibit a high diversity of complex muscle patterns of distinct longitudinal muscle bands and sometimes absent, incomplete, or only weakly developed circular muscles (Tzetzlin and Filippova, 2005; Purschke and Müller, 2006). This may be correlated with the lifestyle of many polychaete taxa that use their parapodia for walking and swimming, thus probably positively selecting for a pronounced longitudinal musculature rather than a prominent ring musculature. However, irrespective of their developmental modes, one ventral and one dorsal pair of longitudinal muscles form the first rudiments of the longitudinal musculature in polychaetes and oligochaetes, whereas additional muscles, if present, arise considerably later (McDougall et al., 2006; Bergter et al., 2007, 2008; Brinkmann and Wanninger, 2010). In contrast to circular body wall muscles, which are absent in the majority of polychaetes, it seems likely that these two pairs of primary longitudinal body wall muscles are part of the annelid muscular groundpattern.

Several recent phylogenetic analyses suggest that the last common annelid ancestor had a polychaete-like morphology with parapodia and an aquatic, epibenthic lifestyle (Rousset et al., 2007; Struck et al., 2007; Zrzavy et al., 2009). Because body wall ring muscles are common in most spiralian, it must be assumed that these are part of the spiralian groundplan. Accordingly, two scenarios seem possible: (1) circular body wall muscles were

lost at the base of Annelida (including the echiurans and sipunculans) and independently regained in some polychaetes, oligochaetes, echiurans, and sipunculans or (2) circular body wall muscles were present in the last common ancestor of Annelida and lost several times independently within several subtaxa of the phylum. Interestingly, a study by Bergter et al. (2007) showed that in the hirudinean *Erpobdella octoculata* the circular muscles do not show a segmental appearance from anterior to posterior, but are formed simultaneously along the anterior–posterior body axis, as is the case in the sipunculans. Because Hirudinea (and maybe also Sipuncula; see Struck et al., 2007; Dordel et al., 2010) is considered a derived annelid taxon that does not show coelomic segmentation in the adults, the nonsegmental mode of myogenesis in these two clades is most likely a secondary condition that evolved independently in these taxa (Erseus and Källersjö, 2004; Erseus, 2005; Jamieson and Ferraguti, 2006; Siddall et al., 2006; Bergter et al., 2007).

P. agassizii, *T. nigra*, and *T. pyroides* exhibit two pairs of introvert retractor muscles, thus corroborating earlier and recent data on sipunculan pelagosphera larvae and early juveniles (Åkesson, '58; Hall and Scheltema, '75; Wanninger et al., 2005; Schulze and Rice, 2009). These findings support the view of a hypothetical ancestral sipunculan with four separate introvert retractor muscles (Cutler and Gibbs, '85; Cutler, '94; Schulze and Rice, 2009). In adult sipunculans, however, the number and arrangement of the introvert retractors varies between four (e.g., *Phascolosoma*) and one (e.g., *Phascolion*), and is therefore considered of taxonomic relevance (Gibbs, '77; Cutler, '94). The number of longitudinal body wall fibers, which underlie the circular body wall muscles, increases markedly during the pelagosphera stages of *P. agassizii*, *T. nigra*, and *T. pyroides*, and these muscles form a pattern of densely arranged fibers in the area of the retractor muscles while they are looser toward the mid-body region. Because all sipunculans investigated so far exhibit this pattern of longitudinal body wall muscles, it seems likely that the introvert retractor muscles evolved from fused longitudinal body wall muscles. The only sipunculans with intermediate oblique body wall muscle fibers are considerably large representatives of the Sipunculidae (Cutler, '94). However, such muscles have neither been found in larvae or juveniles of *P. strombi* (Wanninger et al., 2005) nor in late-stage larvae of *P. agassizii* or juveniles of *T. nigra* and *T. pyroides* (this study). This may be owing to secondary loss of such muscles, because a body wall musculature containing multilayered grids, including oblique muscle fibers, is present in numerous vermiform soft-bodied lophotrochozoans, such as annelids, platyhelminthes, nemertines, or basal mollusks (Solenogastres, Caudofoveata, larval Polyplacophora), and was thus likely a feature of the spiralian stem species (Westheide and Rieger, '96; Haszprunar and Wanninger, 2000; Ladurner and Rieger, 2000; Wanninger and Haszprunar, 2002; Filippova et al., 2005; Sørensen, 2005). A similar arrangement in the muscular bodyplan of Acoela, supposedly the most basal bilaterian offshoot, increases the probability that such a multilayered body wall musculature was also present in the last common bilaterian ancestor (Hooge and Tyler, 2006; Semmler et al., 2008).

CONCLUSIONS

Development of *Phascolosoma* expresses a mosaic of segmental and nonsegmental features. Although the ontogeny of the serially repeated circular body wall muscles does not follow an anterior–posterior pattern, neurogenesis and the distribution of proliferating cells exhibit

transitional stages that resemble a segmental (i.e., annelid-like) mode of formation. This strongly supports a sipunculan–annelid clade, as suggested by recent molecular phylogenetic analyses, and argues in favour of a segmented sipunculan stem species. The obvious loss of segmentation in adult sipunculans indicates that segmented bodyplans may be more prone to evolutionary loss than previously thought and raises the question as to what extent this may have also occurred in other “nonsegmented” metazoan taxa.

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Figure 1.

Confocal micrographs of cell nuclei (blue), proliferating cells (red), and the serotonergic nervous system (green) during ontogeny of *Themiste pyroides*. Incubation with EdU was 1 hr in A and D and 6 hr in B, E, F, G, and H. All aspects are ventral views except B, C and G, which are lateral right views. Anterior faces upwards and scale bars equal 50 μ m in all aspects except for C, in which it equals 10 μ m. The stippled arrow in D and E shows the posterior to anterior decrease of the staining intensity of proliferating cells. Age of larvae is given in days post-fertilization (dpf). (A) Larva at the beginning of elongation (3 dpf)

showing densely arranged proliferating cells throughout the ectoderm, around the eyes (e), the mouth (asterisk), the metatroch (mt), the ventral trunk region, and along the lateral and dorsal epidermis. **(B)** Early pelagosphera larva (3.5 dpf) showing the majority of proliferating cells along the ventral trunk area, the mouth, and the developing digestive system (ds). Along the ventral nerve cord, the intensity of proliferating cells decreases from anterior to posterior; svnc marks the serotonergic portion of the ventral nerve cord (svnc). **(C)** Same specimen as in B showing metamerically arranged perikarya (double arrowheads) associated with the serotonergic ventral nerve cords. **(D)** EdU-stained (1 hr) pelagosphera larva (8 dpf) with numerous proliferating cells in the area of the ventral nerve cord. The highest density of S-phase cells is in the posterior tip of the ventral nerve cord (arrows). Two weakly stained S-phase cells appear in the posterior end of the trunk (arrowheads). **(E)** Same stage as in D, incubated with EdU for 6 hr, showing the highest density and strongest signal of proliferating cells (arrows) at the posterior end of the ventral nerve cord (vnc). Note the stained cells at the posterior end of the trunk (arrowheads), in the developing digestive system, and around the mouth. **(F)** Late pelagosphera larva (15 dpf) with numerous proliferating cells in the developing gut and the ventral nerve cord. Note that the posterior part of the ventral nerve cord shows only few S-phase cells, whereas no proliferation appears in the posterior trunk cells (arrowheads). **(G)** Late stage larva (17 dpf) showing a decrease in mitotic activity in the ventral nerve cord, including the posterior trunk area (arrowhead). The majority of the proliferating cells are found in the digestive system. **(H)** Metamorphic competent larva (18 dpf) showing proliferation in the digestive system, around the mouth, and in the anterior part of the ventral trunk area. Note that the cells in the posterior trunk area (arrowheads) are not mitotic.

Figure 2.

Schematic representation of cell proliferation patterns during *Themiste pyroides* development. Anterior faces upwards and scale bars represent 50 μm in all aspects. Relative staining intensity is indicated from high (h) to low (l). Ventral views in A, C, E, G, and I and lateral right views in B, D, F, H, and J. Incubation with EdU was 1 hr in A, B, E, and F and 6 hr in C, D, G, H, I, and J. Age of larvae is given in days post-fertilization (dpf). (A) Tear-drop stage larva (3 dpf) with proliferating cells around the mouth (asterisk), along the metatroch (mt), the ventral nerve cord (vnc), the trunk epidermis, and the introvert. Note the

bilateral band of proliferating cells in the introvert, laterally to the eyes (e). at marks the apical tuft, pt the prototroch, and ds the *anlage* of the digestive system. **(B)** Lateral right view of the same specimen as in A, illustrating that the majority of the proliferating cells is found in the area of the ventral nerve cord, the mouth, and the metatroch. **(C)** Early pelagosphera larva (3.5 dpf), showing numerous S-phase cells in the developing digestive system, around the mouth, the introvert, and the ventral nerve cord. Note that the staining intensity in the ventral nerve cord decreases in anterior to posterior direction, and that the proliferating cells are clustered in units. **(D)** Lateral right view of the same specimen as in C. **(E)** Pelagosphera larva (8 dpf) showing the majority of the proliferating cells in the posterior portion of the ventral nerve cord arranged in several units (arrows). The intensity of the proliferating cells along the ventral nerve cord decreases from posterior to anterior (stippled arrow). Note the few weakly stained cells in the introvert and the posterior trunk area (arrowheads). **(F)** Lateral right view of the same specimen as in E. **(G)** Same stage as in D, with an increase of cell proliferation after 6 hr of incubation with EdU compared with the 1 hr incubated specimens. The number of units as well as proliferating cells per unit has increased (arrows) and their intensity decreases from posterior to anterior (stippled arrow). The number of proliferating cells has also increased in the posterior trunk area (arrowheads), the introvert, and the developing digestive system. Note the cluster of S-phase cells in the anterior tip of the ventral nerve cord. **(H)** Lateral right view of the same specimen as in G. **(I)** Late stage larva (17 dpf) showing most mitotic cells in the digestive system and some proliferation in the ventral nerve cord, around the mouth, and the anus (a). Note that the proliferating cells in the ventral nerve cord are not arranged in units and that no mitotic cells are found in the posterior trunk area. **(J)** Lateral right view of the same specimen as in I.

Figure 3.

Confocal micrographs of myogenesis from the trochophore (A–C) to the pelagosphera stage (D–F) of *Phascolosoma agassizii*. Anterior faces upwards and scale bars represent 30 μm in all aspects except for the inset in F, in which it equals 10 μm . A and C are in ventral view, whereas B, D, E, and F are in lateral left view. Age of larvae is given in days post-fertilization (dpf). (A) Early trochophore larva (6 dpf) showing the synchronous appearance of the first circular body wall muscles (cm: see inset) and the *anlage* of the ventral (vr) and dorsal (dm) longitudinal retractor muscles as well as the terminal organ retractor muscle

(trm). (B) Trochophore (6 dpf) with well established retractor muscles and the *anlage* of the buccal musculature (bm); asterisk marks the mouth opening. (C) Late trochophore (7 dpf) with well developed buccal musculature. Note that the entire trunk region is covered by circular body wall muscles. (D) Early pelagosphera larva (7 dpf) at the beginning of anterior–posterior elongation, showing the same number of circular muscles as in C, as well as the anal ring muscles (am). Note the establishment of additional longitudinal retractor muscle fibers (arrowheads). lm marks the longitudinal body wall muscles. (E) Larva (8 dpf), slightly more elongated than in D, but still retaining the number of circular muscles. The musculature of the esophagus (esm) and the intestine (inm) has been established. (F) Elongated pelagosphera larva (12 dpf) with new ring muscles being added. Strongly developed ventral and dorsal longitudinal retractor muscles, a terminal organ retractor muscle with two roots close to the anal ring muscles, and numerous well developed longitudinal body wall muscles (arrow) are present. The inset is a magnification of the boxed area in F. Note that new circular muscles are formed by fission (open triangles) from already existing myocytes (double arrow).

Figure 4.

Schematic representation of myogenesis in *Phascolosoma agassizii*. Anterior faces upwards and the total size of the specimen is approximately 150 μm in A and B, 190 μm in C, 270 μm in D, and 480 μm in E and F. a-p indicates the anterior–posterior axis of the animals. Location of the apical tuft and the prototroch is indicated (purple). Eyes are omitted for clarity. Age of larvae is given in days post-fertilization (dpf). (A) Ventral view of a trochophore larva (6 dpf) with the *anlagen* of the paired ventral (yellow) and dorsal (turquoise) longitudinal retractor muscles, terminal organ retractor muscle (dark blue),

buccal musculature (bm), and synchronously developing early *anlagen* of the circular body wall musculature (red). **(B)** Ventral view of a slightly later stage (6 dpf) with well developed buccal musculature and retractor muscles as well as circular body wall muscles along the entire trunk. Note the *anlage* of the digestive tract with a straight-through hindgut (hg). **(C)** Lateral left view of a longitudinal section through an early pelagosphera larva (8 dpf), showing the well developed U-shaped digestive tract with the dorsal anus (light green) and the retracted terminal organ (to). Note that the buccal musculature and the mouth opening (green) are enclosed by the ventral retractor muscles. The esophagus with its strong musculature (esm) lies between the dorsal retractor muscles, whereas the upper part of the intestine (in) lies between the two arms of the terminal organ retractor muscle. **(D)** Lateral left view of a pelagosphera larva (12 dpf) with newly formed circular muscle fibers. Note that new circular muscle fibers are formed along the entire anterior–posterior axis by fission from existing myocytes (double arrows). **(E)** Lateral left view of a metamorphic competent larva (15 dpf), showing strong and solid longitudinal retractor muscles. During anterior–posterior elongation of the body, the intestine establishes its typical U-shape with newly formed gut-fixing muscles (double arrowheads). Circular muscles omitted for clarity. **(F)** Same specimen as in E highlighting the two-layered body wall musculature with outer circular and inner longitudinal muscle fibers (black). Note that the longitudinal body wall muscle fibers are densely arranged close to the laterally positioned retractor muscles and loosely toward the midbody region. [Color figures can be viewed in the online issue, which is available at wileyonlinelibrary.com]

Figure 5.

Confocal micrographs of the myoanatomy of a metamorphic competent *Phascolosoma agassizii* larva aged 15 days post-fertilization (dpf). All aspects are in lateral left view except for B which is a dorsal view. Anterior faces upwards and scale bars represent 30 μ m in A and 10 μ m in B, C, and D. (A) Larva with the prominent paired ventral (vrn), dorsal (drm), and the unpaired terminal organ retractor muscle (trm), longitudinal (lm) and circular body wall (cm) as well as buccal musculature (bm), helicoid gut-fixing muscles (double arrowheads), and the attachment sites of the longitudinal body wall muscles (asterisks). Left

and right boxed areas are enlarged in C and D, respectively. **(B)** Dorsal view of the partly retracted introvert of the larva showing the intersection of the dorsal retractor muscles (open arrowheads) above the buccal musculature. **(C)** Same specimen as in A, with focal depth being restricted to the trunk region, showing the attachment site of a ventral retractor muscle and the longitudinal body wall muscle fibers (arrows), as well as the attachment sites of the retractor muscle fibers at the body wall (asterisks). **(D)** Same specimen as in A, with focal depth being restricted to the dorsal trunk region, showing the helicoid gut-fixing muscles (double arrowheads), which underlie the dorsal retractor muscle fibers, numerous longitudinal body wall muscle fibers (arrows), and the upended anal opening. am marks the anal ring muscles.

Figure 6.

Myogenesis from the early trochophore (A) to the pelagosphera stage (D-E) of *Themiste pyroides*. Anterior faces upwards and scale bars represent 50µm in all aspects. A-E are confocal micrographs whereas F-H are schematic reconstructions. Color code in F, G, and H is as follows: circular body wall muscles in red, paired ventral and dorsal retractor muscles in yellow and turquoise, respectively, and longitudinal body wall muscles in black. Ventral views in all aspects except E, which is a lateral right view. Age of larvae is given in days post-fertilization (dpf). (A) Early trochophore larva (2dpf) showing the *anlage* of the paired

ventral (vrn) and dorsal (drm) longitudinal retractor muscles as well as numerous circular body wall muscles (cm); asterisk marks the mouth opening. **(B)** Slightly later stage (2.5dpf) at the onset of elongation of the anterior–posterior axis. Elaborated retractor muscles and synchronously developing circular body wall muscles are present. Note that the ventral retractor muscles encircle the mouth opening. **(C)** Early pelagosphera larva (approximately 3dpf), showing body wall musculature with outer circular and inner longitudinal muscles (lm). Note that the entire trunk is covered by circular body wall muscles and that the retractor muscles become more prominent. **(D)** Pelagosphera larva (3.5dpf) with strong retractor muscles as well as circular and longitudinal body wall muscles. Note that longitudinal body wall muscles are more numerous near the retractors than in the mid-body region. **(E)** Late pelagosphera larva (8dpf) with trunk covered by homogeneously arranged outer circular and inner longitudinal body wall muscles that are arranged in bands near the well established retractor muscles. **(F)** Schematic reconstruction of A. Note the early *anlage* of the circular body wall muscles and the paired ventral and dorsal retractor muscles. at marks the apical tuft, cb the ciliary bands, e the eye, eg the egg envelope, and m the mouth opening. **(G)** Schematic reconstruction of C. Note that new circular muscle fibers are formed along the entire anterior–posterior axis by fission from existing myocytes (double arrows), and that longitudinal body wall muscles are numerous close to the retractor muscles. pt marks the prototroch. **(H)** Schematic reconstruction of (E). Note the strong and solid retractor muscles of the pelagosphera larva. [Color figures can be viewed in the online issue, which is available at wileyonlinelibrary.com]