THE MOLECULAR AND DEVELOPMENTAL BASIS OF BODYPLAN PATTERNING IN SIPUNCULA AND THE EVOLUTION OF SEGMENTATION
PhD thesis
Alen Kristof

The molecular and developmental basis of bodyplan patterning in Sipuncula and the evolution of segmentation

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April, 2011
Cover illustration:

Front: Frontal view of an adult specimen of the sipunculan *Themiste pyroides* with a total length of 13 cm.

Back: Confocal laserscanning micrograph of a *Phascolosoma agassizii* pelagosphera larva showing its musculature. Lateral view. Age of the specimen is 15 days and its total size approximately 300 µm in length.
“In a world that keeps on pushin’ me around,
but I’ll stand my ground,
and I won’t back down.”

Thomas Earl Petty, 1989
Preface

The content of this dissertation comprises three years of research at the University of Copenhagen from May 1, 2008 to April 30, 2011. The PhD project on the development of Sipuncula was mainly carried out in the Research Group for Comparative Zoology, Department of Biology, University of Copenhagen under the supervision of Assoc. Prof. Dr. Andreas Wanninger. I spent nine months working on body patterning genes in the lab of Prof. Dr. Pedro Martinez, Department of Genetics, University of Barcelona, Spain. Within these three years, research trips of altogether 16 weeks were made to collect, rear, and fix different sipunculan species at the Sven Lovén Center for Marine Sciences in Kristineberg, Sweden (6 weeks), at the Espeland Marine Biological Station in Bergen, Norway (4 weeks), and at the Vostok Station of the A. V. Zhirmunsky Institute of Marine Biology in Vladivostok, Russia (6 weeks). Furthermore, PhD courses had a great impact on my scientific knowledge and skills. During my studies I attended the EMBO course Marine Animal Models in Evolution and Development at the University of Gothenburg, Sweden, Comparative Embryology of Marine Invertebrates at the Friday Harbor Laboratories, University of Washington, USA, and Scientific Writing at the University of Copenhagen, Denmark. This PhD project was funded by the European Research Council (MEST-CT-2005-020542) and the Faculty of Science of the University of Copenhagen.

This thesis is composed of four chapters. The first chapter gives a general introduction to the topics of the present study, its objectives, and the discussion of the results in a broader perspective, leading to conclusions and perspectives for future research. Chapters II to IV contain the major findings of this PhD project in three published papers.

Copenhagen, April 2011

Alen Kristof
Acknowledgements

I am heavily indebted to my principal supervisor Andreas Wanninger for more than three years of support, guidance, education, motivation, and invaluable help. His overall excitement for evolution, invertebrates, science, and finally life had the greatest impact on me. Thank you Andi!

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I thank Prof. Dr. Danny Eibye-Jacobsen that he agreed to act as the head of my defence committee at the University of Copenhagen, as well as Prof. Dr. Stefan Richter and Prof. Dr. Anja Schulze for kindly reviewing this thesis.

Thanks to the working group for Comparative Zoology for a wonderful time in Copenhagen. All these years of the PhD project would not be the same without these members and associates. Therefore, special thanks are going to my friends Tim Wollesen, Uwe Spremberg, Judith Fuchs, and my crazy office mate Jan Bielecki. Special thanks are also going to Anders Garm who helped me with the Danish abstract. Thanks man! In addition, I would like to thank Dany “Sahne” Zemeitat, Ricardo Neves, Nora Brinkmann, Lennie Rotvit, Andreas Altenburger, Birgit Meyer, Henrike Semmler, Christopher Grubb, Tommy Kristensen, Jens Høgh, Lisbeth Haukrogh, and Åse Jaspersen.

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For comments, important discussions and collaboration, I highly acknowledge Mary Rice (Smithsonian Research Center, Florida, USA) and Katrine Worsaae (University of Copenhagen, Denmark).
I am deeply grateful for the never ending support from my family, in particular my parents. Za sve što ste napravili da krenem ovim uspješnim putem, jednostavno se zahvaljulem vama tisuću puta. Puno hvala mama! Puno hvala tata i puno hvala mom burazu Jožefu!

Finally, thousand thanks to my lovely girlfriend Maria Eracleous for being the one. Maria mou, ise i zoi mou! Also, I want to thank all members of her family, especially Despina, Iraclis, and Georgia for treating me as one of them.

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### Chapter II  

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Chapter I

Danish abstract

Abstract
Peanut worms (Sipuncula) have been variously related to a number of animals in the past, but, recently, and despite their non-segmented adult body, molecular phylogenetic analyses place them within Annelida. In order to contribute to the question whether or not sipunculan worms show traces of a segmental pattern in their ontogeny, neuro- and myogenesis as well as the distribution of proliferating cells were analysed in three different sipunculan species. The data show that the rudiments of numerous circular muscles of the body wall musculature and the longitudinal retractor muscles appear at the same time in the early trochophore larva. In addition, throughout development newly formed ring muscles emerge along the entire anterior-posterior axis by fission from already existing myocytes. In contrast to that, neurogenesis does follow a segmental pattern by subsequently emerging pairs of serotonergic perikarya along the anterior-posterior axis, which are associated with a paired ventral nerve cord and are arranged in four distinct repetitive units. This metameric pattern, however, disappears prior to metamorphosis and the ventral nerve cords fuse to form the single ventral nerve of the adult. Congruently, the distribution pattern of mitotic cells show similarities to an annelid-like posterior growth zone that also disappears in metamorphic competent pelagosphera larvae. Accordingly, these developmental and morphological data corroborate recent molecular analyses and show that sipunculans stem from a segmented ancestor. Furthermore, the loss of segmentation in these relatively large, free living animals indicates that body segmentation may easier be lost during evolution than previously assumed. The ontogenetic establishment and (secondary) loss of segmentation renders Sipuncula ideal for developmental and evolutionary studies on the segmentation process in Metazoa.
Short abstract

In this PhD thesis, three sipunculan species were investigated by immunocytochemistry in conjunction with confocal laserscanning microscopy and 3D reconstruction software, in order to clarify whether or not cryptic segmentation can be found during sipunculan ontogeny. The results show that sipunculan myogenesis does not follow a segmental manner, but for a short period of time neurogenesis and the distribution of mitotic cells show transitional stages of segmentation during sipunculan development, thus supporting a sipunculan/annelid affiliation. Moreover, the establishment of an *in situ* hybridisation protocol for the model sipunculan *Themiste pyroides* for gene expression analyses pave the way for future studies on the molecular processes underlying sipunculan segmentation that might give important insights into the evolution of segmentation.
General introduction

Annelids and arthropods are highly successful animals that have a “segmented” body plan. Therefore, understanding the origin of segmentation as well as the mechanisms of segment formation and diversification has been of scientific interest for decades. Segmented animals exhibit repeated units at the cellular, tissue or organ system level along the anterior-posterior axis (Wilmer 1990, Scholtz 2002). While many animal clades show serial repetition of organs along the anterior-posterior axis (i.e., serially arranged shell plates in polyplacophoran mollusks, ring muscles in platyhelminths, or commissures in numerous worm-shaped invertebrates), only the above mentioned animal clades display the definition of “true” segmentation, i.e., multiple organ systems that are generated successively along the anterior-posterior axis (Couso 2009, Chipman 2010). Recently, our view on animal interrelationships has changed dramatically, splitting them roughly into three major lineages (Deuterostomia, Ecdysozoa, and Lophotrochozoa) and placing the segmented animals all in separate clades (e.g., Aguinaldo et al. 1997, Halanych 2004, Dunn et al. 2008, Hejnol et al. 2009). Consequently, the “new” animal tree of life has led to two equally possible and hotly debated scenarios: either, the common ancestor of the so-called Bilateria (bilaterally symmetrical animals) was very simple and segmentation evolved independently in each superclade; or, their last common ancestor was more complex and segmented, with segmentation having been lost in the vast majority of animal clades (Arendt 2005, De Robertis 2008, Couso 2009, Chipman 2010). Accordingly, the monophyly of segmentation is in conflict with the long-standing argument that segmentation is an evolutionary key invention in metazoan (multicellular animals) evolution that is unlikely to get lost (Seaver 2003). Interestingly, recent interpretations of gene expression data on representatives of the segmented phyla have revealed remarkable similarities, proposing a segmented ancestor of all Bilateria (e.g., Arendt 2005, De Robertis 2008, Couso 2009). Since these data largely come from a very limited number of model system animals such as Drosophila (fly), Tribolium (beetle), Capitella (polychaete), Hirudo (leech), Cupiennius (spider), and so forth, developmental studies of non-model system taxa are needed in order to either verify or falsify this hypothesis. Moreover, comparative molecular, developmental, and morphogenetic approaches, namely gene expression, cell proliferation, and organ system formation, have proven useful in elucidating the evolution of body patterning.

Sipuncula – a neglected taxon with annelid affinities

Sipuncula is a small, worm-shaped, exclusively marine lophotrochozoan taxon that uniformly exhibits an unsegmented adult body, which is subdivided into a posterior trunk and a retractable anterior introvert. Sipunculans are filter or deposit feeders that live in soft sediments, rock crevices, dead corals, or vacant mollusc shells, and are widespread throughout the oceans (Rice 1975a). Their fossil record is generally sparse but a description of six well preserved sipunculan specimens found in southwest China dates their origin to more than 520 million years ago (Huang et al. 2004). Furthermore, the fossilised outer and inner morphology resembles extant sipunculan species, suggesting only limited changes since the Early Cambrian (Huang et al. 2004). Cladograms based on morphological characters divide sipunculans into two classes, Sipunculidae and Phascolosomatidea, comprising four orders and six families (Aspidosiphonidae, Phascolosomatidae, Golfingiidae, Phascolionidae, Themistidae, and Sipunculidae) (Cutler and Gibbs 1985, Cutler 1994). Today, the use of DNA sequence data has changed this morphology-based view of the relationships among the currently recognised 147 species of Sipuncula (Maxmen et al. 2003, Schulze et al. 2005, 2007). The two classes have not been recognised by Maxmen et al. (2003) and Schulze et al. (2005), whereas the class Phascolosomatidae was recovered in the by far most comprehensive analysis on sipunculan phylogeny of Schulze et al. (2005). Congruently, all analyses strongly support *Sipunculus nudus* as the sister group to the remaining Sipuncula, while most of the previously recognised families and orders were not recovered as monophyletic (Maxmen et al. 2003, Schulze et al. 2005, 2007). However, based on morphological characters and DNA sequence data, the monophyly of Sipuncula is strongly supported (Maxmen et al. 2003, Schulze et al. 2005, 2007).

In general, sipunculans are dioecious (separate sexes), but there are a few known hermaphroditic and parthenogenetic species (asexual reproduction) (Åkesson 1958, Rice 1970, Rajalu and Krishnan 1969, Pilger 1978, Cutler 1994). Four different, one direct, and three indirect developmental pathways have been described for Sipuncula (Fig. 1; Rice 1975b, c). Since sipunculans share certain developmental features such as spiral cleavage, that gives rise to a trochophore larva with an apical
tuft and a ring of cilia used for locomotion, they have commonly been placed within the clade Spiralia, which, among others, also comprises annelids and mollusks (Jägersten 1972, Rice 1985, Cutler 1994, Rouse 1999).

![Figure 1. The four distinct developmental modes in Sipuncula. Development may be direct, whereby the embryo emerges from the egg as a crawling worm, which eventually transforms into the juvenile stage without an intermediate larval form (black line and arrows). In some species the lecithotrophic trochophore, after a brief swimming period, transforms into a vermiform stage succeeded by the juvenile (red line and arrows). In the lecithotrophic indirect developmental pathway the trochophore larva starts to elongate and metamorphoses into a second larval stage, the pelagosphera, which swims in the plankton for a short time, settles, and metamorphoses into a juvenile (blue line and arrows). In the planktotrophic indirect developmental mode the lecithotrophic trochophore transforms into a pelagosphera, which remains in the plankton for up to several months before it settles and metamorphoses into a juvenile (green line and arrows). Drawing modified from Rice 1975b, c.]

Until they were considered a distinct taxon among the spiralians, the phylogenetic position of Sipuncula has long been an enigma. Accordingly, they were placed close to sea cucumbers (holothurian echinoderms), as derived annelids, or as an in-group of Gephrea (sipunculans, echiurans, and priapulids), then as relatives of phoronids, bryozoans, and brachiopods (Prosoygii), until developmental studies proposed a close relationship to spiralians (reviewed in Rice 1985). From there on,
they have been variously seen as either a sister taxon to Annelida or to Mollusca (e.g., Åkesson 1958, Rice 1975b, c, Scheltema 1993, 1996, Cutler 1994, Zrzavy et al. 1998, Giribet et al. 2000). Nowadays, a growing number of morphological and molecular analyses strongly suggest a close relationship to annelids, leaving the only question whether sipunculans cluster within Annelida or constitute their sister taxon (e.g., Wanninger et al. 2005a, Tzetlin and Purschke 2006, Struck et al. 2007, Dunn et al. 2008, Mwinyi et al. 2009, Shen et al. 2009, Sperling et al. 2009, Zrzavy et al. 2009, Dordel et al. 2010, Hausdorf et al. 2010, Struck et al. 2011). Accordingly, the inclusion of Sipuncula within Annelida suggests a secondary loss of a previously segmented body plan in Sipuncula rather than an initial evolutionary step towards segmentation in these animals (Dordel et al. 2010, Struck et al. 2011).

**Thesis objectives**

In the light of the proposed sipunculan-annelid assemblage, it was the aim of the present PhD thesis to elucidate sipunculan body plan patterning and to deal with the following scientific issues:

1. Neuro- and myogenesis were described in three different sipunculan species, _Phascolosoma agassizii_, _Themiste pyroides_ and _Thysanocardia nigra_ (Fig. 2). Since nervous and muscle systems follow a segmental development in annelids, these experiments should clarify whether or not sipunculans show cryptic segmentation during their ontogeny.

2. The distribution of proliferating cells in _T. pyoides_ and _T. nigra_ were described throughout development to assess whether sipunculans possess a posterior growth zone similar to the segmented annelids.

3. Comparative analysis of the acquired developmental data with those available for annelids and other lophotrochozoans (e.g., Mollusca) were carried out in order to infer shared and possible ancestral neuromuscular features.

4. The establishment and application of an _in situ_ hybridisation protocol for analyses of tempo-spatial expression patterns of selected candidate genes known to be involved in body patterning (e.g., neural, muscular and so-called “segmentation” genes (_hox1-9_, _even-skipped_, _hairy_)).
In the following, the main results are summerised and a general discussion is presented, dealing with these key issues of the present thesis. More detailed discussions are found in the chapters II to IV, which comprise three published manuscripts. Finally, future perspectives of this project are proposed.

**Figure 2.** Adults of investigated sipunculan species in the present PhD study. All in lateral view. Tentacles, which are at the top, mark the anterior end of animals. A. *Phascolosoma agassizii*, total length approximately 15 cm. B. *Themiste pyroides*, total length approx. 10 cm. C. *Thysanocardia nigra*, total length approx. 12 cm.

**Material and methods**

In the present PhD project, several sipunculan species were investigated by developmental-morphological markers such as immunolabelling, confocal microscopy, and 3D reconstruction software (Fig. 2). In addition, developmental stages of *Themiste pyroides* were used for cloning genes known to be involved in the formation of segmentation in arthropods and annelids, and for establishment of an *in situ* protocol for gene expression analyses (see below). An overview of the species investigated by morphological methods is given in Table 1. Further details of the respective techniques are provided in the chapters II to IV.
Table 1. List of species investigated in the course of the PhD project by fluorescence markers; Serotonin and FMRFamide – neurotransmitters/peptides; Phalloidin – F-actin of the musculature; Dapi (4’, 6-diamindino-2-phenylindole) – cell nuclei marker, EdU (5-ethynyl-2’-deoxyuridine) – proliferating cells.

<table>
<thead>
<tr>
<th>Species (Family)</th>
<th>Neurogenesis</th>
<th>Myogenesis</th>
<th>Cell nuclei, cell proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Themiste pyroides</em> (Themistidae)</td>
<td>Serotonin, FMRFamide</td>
<td>F-actin (chapter IV)</td>
<td>Dapi, EdU (chapter IV)</td>
</tr>
<tr>
<td><em>Thysanocardia nigra</em> (Golfingidae)</td>
<td>Serotonin, FMRFamide (chapter I)</td>
<td>F-actin (chapter IV)</td>
<td>Dapi, EdU (chapter IV)</td>
</tr>
<tr>
<td><em>Phascolosoma agassizii</em> (Phascolosomatidae)</td>
<td>Serotonin, FMRFamide (chapters I-III)</td>
<td>F-actin (chapter IV)</td>
<td>-</td>
</tr>
</tbody>
</table>

RNA isolation and cDNA synthesis

Total RNA was purified from *Themiste pyroides* embryos at 15, 28, 40, 48, 62, 72, 84, and 111 hours post fertilization (hpf) (miRCURY RNA isolation kit, Exiqon, Vedbaek, Denmark). cDNA samples were synthesised by reverse transcription (RETROSript, Ambion, Woodward St. Austin, TX, USA), and stored at -20 °C until use.

Degenerate PCR

To clone homeobox genes, a range of degenerate primers were designed referring to Martinez et al. (1997), Nederbragt et al. (2002), Seaver et al. (2006), and Paps et al. (2009). The sequences of the oligonucleotides used are given in Table 2.

A touchdown PCR was performed with the degenerate primers given in Table 2. The amplification parameters were: 3 min at 94 °C, 10 cycles of 45 sec at 94 °C, 45 sec at 52 °C (every cycle -1 °C), 30 sec at 72 °C, and 30 cycles of 45 sec at 94 °C, 45 sec at 42 °C, and final 30 sec extension at 72 °C. The PCR products were purified by a gel extraction kit (QIAquick, QIAGEN, Copenhagen, Denmark), and on 1 µl of this reaction another PCR with the same parameters was performed. The samples were displayed by electrophoresis on a 1% agarose gel, purified, concentrated by speedvacuum (GENEVAC EZ-2plus, Ipswich, UK), resuspended in 10 µl distilled water, and directly ligated into pGEM-T Easy vector using a ligation kit (Promega,
Nacka, Sweden). The inserted DNA fragments were sequenced using BigDye Terminator 3.1 Cycle Sequencing kit with an ABI Prism 377 DNA sequencer (Applied Biosystems, Carlsbad, CA, USA).

Degenerate primers were also designed to clone a number of muscular and neural genes for establishment of a whole mount \textit{in situ} hybridisation protocol (Table 2). Subsequently, all recovered gene fragments were identified by the similarity of their nucleotide sequences to already known genes that are placed at the public online source of the National Centre for Biotechnology Information (NCBI) using basic local alignment searches (BLASTs).

\textbf{Table 2.} Nucleotide sequences of degenerate primers and their melting temperatures (Tm) used to clone homeobox, muscular and neural genes in \textit{Themiste pyroides}.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Homeobox genes</strong> (Martinez et al. 1997)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT 77</td>
<td>5’-CGGATCCYTIGARYTIGARAARGART</td>
<td>42°C</td>
</tr>
<tr>
<td>CT 78</td>
<td>5’-GGAATTCACTICKRTTYTGRAACCAIATTY</td>
<td></td>
</tr>
<tr>
<td><strong>Engrailed</strong> (Seaver et al. 2006, Nederbragt et al. 2002)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EnEH2</td>
<td>5’-TGGCCTGCITGGGTNTAYTGYAC</td>
<td>42°C</td>
</tr>
<tr>
<td>en2-2</td>
<td>5’-TGRRTTRTANARNCCYTGNGCCAT</td>
<td></td>
</tr>
<tr>
<td>Eng1</td>
<td>5’-ATGGAATTCCNGCNTGGGTNTWYAC</td>
<td>42°C</td>
</tr>
<tr>
<td>Eng4</td>
<td>5’-TGGAAAGCTTRTANARNCCYTSGSCAT</td>
<td></td>
</tr>
<tr>
<td><strong>Myosin heavy chain type II</strong> (Ruiz-Trillo et al. 2002)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mio7 (F)</td>
<td>5’-TGYATCAAYTWYACYAAYGAG</td>
<td>53 °C</td>
</tr>
<tr>
<td>Mi6 (R)</td>
<td>5’-CCYTCMARYACACRTTRCA</td>
<td></td>
</tr>
<tr>
<td><strong>Tropomyosin</strong> (Paps et al. 2009)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TropoF</td>
<td>5’-ATYRAGAGAARATGNBKVGCATG</td>
<td>53 °C</td>
</tr>
<tr>
<td>TropoR</td>
<td>5’-GTHYGRCTCCARTTGNYCACT</td>
<td></td>
</tr>
<tr>
<td><strong>Intermediate filaments</strong> (Paps et al. 2009)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FilF1</td>
<td>5’-TACATCGAGAAGGTGCCTTCTTCTGG</td>
<td>48 °C</td>
</tr>
<tr>
<td>FilR1</td>
<td>5’-CCTCAACCTCCAGCAGTTTCTGTA</td>
<td></td>
</tr>
<tr>
<td>FilF3</td>
<td>5’-TACATCGAGAAGGTGCCTTCTTG</td>
<td>50 °C</td>
</tr>
<tr>
<td>FilR3</td>
<td>5’-CYTCNCCYTCCAGCAGYTTYCTGTA</td>
<td></td>
</tr>
<tr>
<td><strong>β-Actin</strong> (Matsuo and Shimizu 2006)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
β-Actin F1 5'-TGGGAYGAYATGGARAARAT 50 °C
β-Actin R1 5'-GCCATYTCYTGYTCRAA

α-Tubulin (Zheng et al. 1998)
AT16-F 5'-ATHGGHAAYGCNTGYTGGG 50 °C
AT412-R 5'-RAAYTCDCCYTCYTCCATDCC

β-Tubulin (Garant and MacRea 2009)
BT-F 5'-GGNCARTCNGNGCNGGNAAYAAYTGGGCN 55 °C
BT-R 5'-NGGRAANGGNACCATRTTNACNGC

Rapid amplification of cDNA ends (RACE)
Fragments of *Themiste pyroides* homebox containing genes were isolated by degenerate PCR using cDNA of mixed larval stages as template. In addition, 5’ and 3’ cDNA were generated using the same template for rapid amplification of cDNA ends (RACE) with the SMARTer RACE cDNA amplification kit (Clonetech, Mountainview, CA, USA).

For each of the recovered gene fragments, which ranged between 135 and 271 base pairs (bp) (Table 3), gene specific primers (GSP) were designed and used for RACE (Advantage 2 PCR Kit; Clonetech). Touchdown PCRs were performed with following amplification parameters: 5 min at 94 °C, 10 cycles of 45 sec at 94 °C, 45 sec at 57 °C (every cycle -1 °C), 3 min at 72 °C, and 25 cycles of 45 sec at 94 °C, 45 sec at 47 °C, and final 30 sec extension at 72 °C. Reactions were agarose-gel electrophoresed, stained with SYBRsafe (Invitrogen, Taastrup, Denmark), and photographed under UV light. Subsequently, 1 µl of RACE PCR products were used as a template for another RACE PCR with nested GSPs (see Table 3). The parameters were: 5 min at 94 °C, 35 cycles of 45 sec at 94 °C, 45 sec at 60 °C, 30 sec at 72 °C, and a final 10 min extension at 72 °C. Positive fragments were purified from the agarose gel, cloned into the vector pGEM-T easy, and sequenced as described above. However, I was able to amplify into the 3’ direction of two genes only, *hoxl* (47 bp) and *hox8* (73 bp).
Material and methods

Table 3. Isolated DNA fragments of *Themiste pyroides* mixed larval stages. Gene abbreviations: *lab*, labial; *scr*, sex combs reduced; *ftz*, fushi tarazu; *abd-A*, abdominal-A. Other abbreviations: bp, base pairs; e-value, expected value (the similarity between a given sequence and orthologues from the database; high similarities are indicated by considerably low e-values, while high e-values indicate low similarity). Red and underlined parts within the sequences indicate gene specific and nested primers, respectively.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>bp/e-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hox1 (lab)</td>
<td>CGGATCCCTGGAGAACCTGGAAGAAATCCACTTTAACAATAATCTCAGAACAGCGCCATGAATTCCC</td>
<td>136/10^{-10}</td>
</tr>
<tr>
<td>hox3</td>
<td>GGAATCGGGCACTTGGAAGAAATTCATTTAAAAATATCTCACAAGGGCCAGCGGCAGATAGAGATAAGAGCAGATCGCCATGGAATTCCC</td>
<td>135/10^{-14}</td>
</tr>
<tr>
<td>hox5 (scr)</td>
<td>GGAATCCCCTGGAGAACCTGGAAGAAATTCATTTAACAATATCTCGAGAGAGGAATAGAGATAGAGCAGATCGCCATGGAATTCCC</td>
<td>135/10^{-14}</td>
</tr>
<tr>
<td>hox5 (ftz)</td>
<td>GGAATCCCTGGAGAACCTGGAAGAAATTCATTTAACAATATCTCGAGAGAGGAATAGAGATAGAGCAGATCGCCATGGAATTCCC</td>
<td>135/10^{-12}</td>
</tr>
<tr>
<td>hox8 (abd-A)</td>
<td>CGGATCCCTGGAGAACCTGGAAGAAATTCATTTAACAATATCTCGAGAGAGGAATAGAGATAGAGCAGATCGCCATGGAATTCCC</td>
<td>136/10^{-15}</td>
</tr>
<tr>
<td>even-skipped</td>
<td>CGGATCCCTGGAGAACCTGGAAGAAATTCATTTAACAATATCTCGAGAGAGGAATAGAGATAGAGCAGATCGCCATGGAATTCCC</td>
<td>252/10^{-9}</td>
</tr>
<tr>
<td>lox2</td>
<td>CGGATCCCTGGAGAACCTGGAAGAAATTCATTTAACAATATCTCGAGAGAGGAATAGAGATAGAGCAGATCGCCATGGAATTCCC</td>
<td>136/10^{-14}</td>
</tr>
<tr>
<td>caudal (cdx)</td>
<td>GGAATCCCTGGAGAACCTGGAAGAAATTCATTTAACAATATCTCGAGAGAGGAATAGAGATAGAGCAGATCGCCATGGAATTCCC</td>
<td>136/10^{-12}</td>
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<td>137/10^{-14}</td>
</tr>
<tr>
<td>not</td>
<td>CGGATCCCTGGAGAACCTGGAAGAAATTCATTTAACAATATCTCGAGAGAGGAATAGAGATAGAGCAGATCGCCATGGAATTCCC</td>
<td>271/10^{-11}</td>
</tr>
</tbody>
</table>
Whole mount *in situ* hybridisation

Embryos, larvae, and juveniles of *Themiste pyroides* were anesthetised by adding drops of a 3.5 or 7% MgCl₂ solution to the seawater, and were subsequently fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) (pH 7.3) for 20-30 min at room temperature. Subsequently, the fixative was removed by three washes in 70% ethanol (15 min each), followed by the storage of the specimens at -20°C.

Fixed specimens were rehydrated in PBS, washed four times for 5 min in PTw (phosphate-buffer containing 0.1% Tween), and permeabilised with proteinase K (Sigma; 10 µg/ml in PTw for 15 min at room temperature). Digestion was terminated by two washes (5 min each) with PTw containing 2mg/ml of glycine and treated with triethanolamine (TEA, 0,1M pH 7.6, Fluka; 3 x 5 min), following addition of 4µl/ml and 8µl/ml of acetic anhydride without changing the TEA solution to block positive charges. After two 5 min washes in PTw, specimens were post-fixed in 3.7% PFA for 1 hr at room temperature. Subsequently, specimens were rinsed 5 x 5 min in PTw, incubated for 10 min in 50% pre-hybridisation buffer (50% formamide, 5x SSC, 1mg/ml yeast RNA, 0.1 mg/ml heparin, 0.1% Tween 20, 10mM DTT) in PTw and an incubation in 100% pre-hybridisation buffer overnight at 65 °C. Antisense and sense digoxigenin-labelled riboprobes were generated with a RNA labeling kit (SP6/T7; Roche; Copenhagen, Denmark), and used at a working concentration of 1 ng/µl for *Tp-mhc* and *Tp-actin* (fragment sizes ca. 700 and 500 bp, respectively). Riboprobes were warmed up in hybridisation buffer (50% formamide, 5x SSC, 0.1% Tween 20) for 10 min at 70 °C. Afterwards, specimens were added to that solution and incubated for 72 hr at 65 °C. After hybridisation, probes were recovered and specimens were washed at 65 °C (30 min in 100% hybridisation buffer, in 75% hybridisation buffer and 75% 2x SSC, in 50% hybridisation buffer and 50% 2x SSC, in 25% hybridisation buffer and 25% 2x SSC, 2 x 15 min in 2x SSC, and final 2 x 15 min washed in 0.2x SSC). Afterwards, specimens were washed two times 5 min in maleic buffer (MABTw; 100 mM maleic acid, 150 mM NaCl, 0.1% Tween 20, pH 7.5), blocked for
1 hr in 1% blocking solution (Roche) and incubated overnight with anti-digoxigenin alkaline phosphatase (AP) conjugated antibody (Roche; 1:200 dilution) in blocking solution at 4 °C. Specimens were washed at least four times 15 min in MABTw and two times 5 min in AP buffer (0.1M NaCl, 0.1M Tris pH 9.5, 0.05M MgCl₂, 0.5% Tween 20) and developed with NBT/BCIP (Roche) in the dark. The reactions were stopped with PTw, specimens were fixed in 3.7% PFA overnight at 4 °C and stored in 80% glycerol in PBS at -20°C. Specimens were analysed and photographed using DIC optics on a Zeiss Axiophot microscope (Zeiss, Jena, Germany) in conjunction with a Leica DFC300FX digital camera (Leica, Microsystems, Wetzlar, Germany).

Results
In the course of the present PhD study, three different sipunculan species, which cover three out of six currently recognised families, were investigated. *Phascolosoma agassizii* was collected and reared at the Friday Harbor Laboratories on the San Juan Island (Washington, USA) whereas *Themiste pyroides* and *Thysanocardia nigra* were acquired at the Vostok-Marine-Station (Vladivostok, Russia). The muscle and nervous system development is described for all investigated species, the distribution of proliferating cells for *T. pyroides* and *T. nigra*, and cloning of homeobox genes as well as the establishment of an in situ hybridisation protocol for *T. Pyroides*. The main findings are summarised in the following section. Further details are given in the chapters II – IV.

Cloning of homeobox genes in *Themiste pyroides*
With the pair of degenerate primers CT77 and CT78, which were designed to the conserved regions within the homeobox, the orthologues for the *hox1* (labial), *hox3*, *hox5* (sex combs reduced), *hox5* (fushi tarazu), *hox8* (abdominal-A), even-skipped, *lox2*, caudal, *xlox*, and *not* were isolated. The isolated gene fragments of *Themiste pyroides* cDNA were generated from mixed embryonic and larval stages; their lengths were between 135 and 271 bp (Table 3). Unfortunately, gene specific primers and RACE PCRs were not able to extent the recovered fragments neither in 5’ nor in 3’ direction. The only exceptions were the genes *hox1* (labial) (47 bp) and *hox8* (abdominal-A) (73 bp), which included the stop codon and a poly-A tail, but the
obtained fragments were too short to generate riboprobes for successful in situ hybridisation experiments.

**Establishment of an in situ hybridisation protocol**

Using a set of degenerate primers (Table 2), gene fragments of 700 bp of *myosin heavy chain* (*Tp-mhc*) and 500 bp of *beta-actin* (*Tp-actin*) were recovered by PCR.

*Tp-mhc* is expressed from early trophophore stages (ca. 2 days after fertilisation (dpf)) onwards in the four developing retractor muscles (Fig. 3A-C). First *Tp-actin* expression is also in the early trophophore larvae in a prominent U-shaped band in the area of the forming retractor muscles (Fig. 3D). In pelagosphera larvae the *Tp-actin* is expressed in distinct areas of the paired dorsal and ventral retractor muscles (Fig. 3E, F).
Results

Figure 3. Expression of myosin heavy chain (Tp-mhc) (A-C) and beta-actin (Tp-actin) (D-F) during larval stages of Themiste pyroides. Anterior is to the top in all panels and scale bars equal 50 µm. Black represents areas of gene expression – Tp-mhc in A-C and Tp-actin in D-F, respectively. (A) Dorsal view of an early trochophore larva showing Tp-mhc expression (arrowheads) in the anlagen of the retractor muscles. (B) Dorsal view of a late trochophore larva. Tp-mhc expression is restricted to the retractor muscles. Note that only three out of four are in focus; mt marks the ciliated metatroch. (C) Lateral view, ventral to the right, pelagosphera larva with Tp-mhc expression in the retractor muscles; pt marks the ciliated prototroch. (D) Dorsal view of an early trochophore larva with Tp-actin expression in the area of the retractor muscles (arrows). (E) Dorsolateral view of an early pelagosphera larva, ventral to the left, showing Tp-actin expression in the retractor muscles. (F) Late pelagosphera larva, dorsal view with strong Tp-actin expression in the retractor muscles.
Serotonergic and FMRFamidergic nervous system development in sipunculan worms (chapters I – III)

In the planktotrophic species *Phascolosoma agassizii*, the first detectable serotonergic and FMRFamidergic nervous structures are visible in the anterior region of the trochophore larvae. A pair of neurites emerging from the apical organ contributes to the ventral nerve cords. During further development, interconnecting commissures and pairs of perikarya (four serotonergic and one pair of FMRFamidergic perikarya) appear subsequently along the ventral nerve cords from anterior to posterior, resulting in a rope-ladder like ventral nervous system. Due to migration of serotonergic perikarya and fusion of serotonergic ventral nerve cords this metameric arrangement is lost towards metamorphosis, and gives rise to the single non-metameric adult ventral nervous system (chapters II and III).

In the lecithotrophic species *Themiste pyroides* and *Thysanocardia nigra*, neurogenesis is similar to *Phascolosoma agassizii* (Figs. 4-6). The first serotonergic and FMRFamidergic cells are in the apical organ and a pair of neurites contributes to the ventral nerve cords (Figs. 4A, 6A). In contrast to *P. agassizii*, a first, second, and a third pair of serotonergic perikarya appear already in the trochophore larvae of *T. pyroides* and *T. nigra* in an anterior to posterior progression, while later the fourth pair appears in the pelagosphera larva (Figs. 4A-D, 5E, H).
Figure 4. Confocal micrographs of the serotonergic nervous system in trochophore larvae of *Themiste pyroides*. A and C are in ventrolateral left view, whereas B and D are in ventral view. Anterior faces upwards and scale bars equal 50 µm in all aspects except the insert in B where it is 10 µm. Age of larvae is given in days post fertilisation (dpf). (A) Early trochophore larva (2 dpf) showing an apical organ comprising three cells (arrowheads), two neurites of the developing ventral nervous system (vn) that is interconnected by a commissure (arrow), and a pair of perikarya (asterisks). (B) Slightly later (2 dpf), at the base of the apical organ (ao), the first cells of the adult cerebral organ are formed (cg), the two ventral neurites are stronger developed than in the stage before, and the first perikarya of the second pair appears. Insert is a magnification of the apical pole of B showing two flask-shaped and two round cells in the apical organ. (C) Trochophore larva (2.5 dpf) with two pairs of perikarya associated with the ventral neurite bundles and the first perikarya of the third pair already formed. (D) Slightly later (2.5 dpf) the second perikarya of the third pair is established posteriorly to the second pair. Note that the cerebral ganglion is more elaborated than in the stage before.

During subsequent development a second pair of FMRFamidergic perikarya appears along the ventral nervous system in *T. pyroides* and *T. nigra*, while there is only one in *P. agassizii* (Fig. 6D). All three species show four serotonergic and two to three FMRFamidergic flask-shaped cells in their apical organ, as well as a serotonergic nerve ring underlying the ciliated metatroch (Figs. 4A, B, Insert, 5A, B,
E, F, I-L). It has to be noted that in *T. pyroides* and *T. nigra* the serotonergic metatrochal nerve ring is considerably weaker than that in *P. agassizii* (Fig. 5A, E, I-L).

**Figure 5.** Confocal micrographs of the serotonergic nervous system in pelagosphera larvae of *Themiste pyroides*. All aspects in ventral view except I, K, J and L, which are in ventro lateral left view. Anterior faces upwards and scale bars represent 50 µm in A, E, I, K, J and L, while they equal 10 µm in B, C, D, F, G and H. A, E, I, and K are color-coded confocal micrographs, where blue and purple colors are
positioned to the ventral, colors of green and yellow in between, whereas orange and red colors are to the dorsal side. Age of larvae is given in days post fertilisation (dpf). (A) Early pelagosphera larva (3 dpf) showing a weakly stained metatrochal neurite (mtn), ventral neurite bundles (vn) that are interconnected by commissures (arrows) always anteriorly positioned to a pair of perikarya (asterisks); cg marks the cerebral ganglion. (B) Dorsal portion of the same specimen as in A; magnification of the apical pole showing four cells (arrowheads) in the apical organ. Note that two cells are flask-shaped. (C) Ventral portion of the same specimen as in A; magnification of the apical pole showing numerous cells associated with the neuropile of the developing adult cerebral ganglion. (D) Magnification of the ventral trunk area of an early pelagosphera larva (3 dpf) with three pairs of metamERICally arranged perikarya associated with the ventral neural bundles. (E) Slightly older pelagosphera (4 dpf) as in A with stronger developed ventral neural bundles that bear four pairs of perikarya and a more elaborate cerebral ganglion. (F) Dorsal portion of the same specimen as in E; magnification of the apical pole showing five cells in the apical organ. (G) Ventral portion of the same specimen as in E, magnification of the apical pole showing numerous cells and a neuropile in the cerebral ganglion. (H) Same specimen as in E. Magnification of the ventral trunk area showing the ventral nervous system with four pairs of perikarya and three commissures interconnecting the median with the two outer neural bundles. (I) Magnification of the anterior region of a pelagosphera larva (8 dpf) showing a complex central nervous system that comprises the cerebral ganglion with numerous cells, the apical organ with its cells and own neuropile (double arrows), peripheral cells (double arrowheads), metatrochal neurite, peripheral neurites (pn), and the ventral neural bundle. Note that the ventral neural bundle has retained its paired character only in the most anterior part. (J) Dorsal portion of the same specimen as in I showing the apical organ with its cells and neuropile. Note that two of the cells are flask-shaped. (K) Magnification of the anterior region of a late pelagosphera larva (14 dpf) with a prominent cerebral ganglion, apical organ with two flask-shaped cells, peripheral cells and neurites, a prominent metatrochal neurite ring, and ventral neural bundles. (L) Dorsal portion of the same specimen as in K showing the apical organ that comprises less cells than in the previous stage. Three out of five cells disappeared while only the two flask-shaped cells and a part of the neuropile remain visible.

In addition to the loss of the metameric arrangement in the ventral nervous system towards metamorphosis, less serotonergic and FMRFamidergic apical cells are detectable in all of the investigated species, while the adult cerebral ganglion becomes more elaborate (Figs. 5I-L, 6D).
Figure 6. Confocal micrographs of FMRFamidergic nervous system in pelagosphera larvae of *Thysanocardiida nigra*. All aspects in ventral view except B and C which are in ventrolateral left view. Anterior faces upwards and scale bars represent 50 µm except the insert in A where it equals 30 µm. Age of larvae is given in days post fertilisation (dpf). (A) Early pelagosphera larva (3 dpf) with two flask-shaped cells in the apical organ (arrowheads), the *anlage* of the adult cerebral ganglion (cg), and a pair of ventral neurites (vn). Insert shows a slightly older stage (3 dpf), with the first interconnecting commissure (arrow) and pair of perikarya (asterisks) associated with the ventral neural bundles. Note the presence of a median ventral neurite. (B) Pelagosphera larva (4 dpf) showing a more elaborate cerebral ganglion and ventral nervous system that now has a second commissure that interconnects the ventral neurites; pn marks peripheral neurite. (C) Slightly older pelagosphera larva (5 dpf) with a well
developed cerebral ganglion below the apical cells and ventral nervous system. (D) Late pelagosphera larva (11 dpf) with numerous peripheral neurites in the trunk area as well as in the ventral nervous system. Note the second pair of perikaya along the ventral neurites. No FMRF-positive cells appear in the apical organ.

**Myogenesis and cell proliferation during sipunculan development (chapter IV)**

Myogenesis in all investigated species in the present PhD thesis display a similar mode of muscle system development.

In *Phascolosoma agassizii*, *Themiste pyroides* and *Thysanocardia nigra*, the rudiments of the four longitudinal retractor muscles appear at the same time as the first circular body wall muscles. Throughout subsequent development, novel circular body wall muscles appear along the entire anterior-posterior axis and seem to be formed by fission from existing myocytes. In addition, all three sipunculans show loosely arranged longitudinal body wall muscles, except in the vicinity of the prominent retractor muscles. In contrast to *P. agassizii*, *T. pyroides* and *T. nigra* have no terminal organ and, therefore, no respective retractor muscle. Furthermore, the *anlagen* of the buccal musculature appear much earlier in *P. agassizii* (trochophore larva) than in *T. pyroides* and *T. nigra* (late pelagosphera, shortly before the onset of metamorphosis). However, in none of these species does myogenesis follow an annelid-like metameric development.

Cell proliferation was described in *Themiste pyroides* and *Thysanocardia nigra* during development from early embryos until early juvenile stages by visualisation of the nucleotide analogue 5-ethynyl-2’-deoxyuridine (EdU) that is incorporated during the synthesis phase (S-phase) of the cell cycle. There is a similar distribution of S-phase cells during the development of both investigated species. First, the proliferating cells are scattered throughout the ectoderm. At the beginning of anterior-posterior axis elongation, proliferating cells are distributed in two lateral bands around the eyes, around the mouth, and the ventral trunk region. Slightly later, in the early pelagosphera larvae most S-phase cells appear in the ventral nerve cord and the rudiments of the digestive system. Interestingly, in the pelagosphera stage the proliferation is only present in the posterior tip of the ventral trunk area and in the venral nerve cord. EdU-positive cells are arranged in units and by intensity loss it seems as if the direction of proliferation is from posterior to anterior. As the larvae grow, more S-phase cells appear in the digestive system, around the anus, and in the
area of the developing tentacles of post metamorphic stages. However, no S-phase cells can be detected in the posterior tip of the ventral trunk region anymore.

**General discussion**

Immunocytochemistry and F-actin labelling in conjunction with confocal microscopy and 3D reconstruction software has proven to be an excellent tool to characterise the neuromuscular system as well as the expression pattern of certain neurotransmitters, thus allowing for comparative analyses of neural and muscular characters (Wanninger 2009, Richter et al. 2010). Using these methods, the main goal of the present PhD thesis was to characterise nervous and muscle system formation as well as the distribution of proliferating cells during sipunculan development. Additional studies on myo- and neurogenesis in other sipunculans supplement this comparative approach (Wanninger et al. 2005a, Schulze and Rice 2009). So far, eight species representing four families and three different developmental modes have been investigated by the above mentioned methods.

**Early neurogenesis in Trochozoa**

In all investigated sipunculan species neurogenesis begins in the trochophore larvae at the apical pole, from which two neurites grow posteriorly, forming a scaffold for the future ventral nervous system (Wanninger et al. 2005a; chapters I and II herein). Moreover, the investigated species herein, *Phascolosoma agassizii*, *Themiste pyroides*, and *Thysanocardia nigra*, all show initially two and later up to four serotonergic and FMRFamidergic flask-shaped cells within the apical organ (chapters I-III), whereas *Phascolion strombi* lacks serotonergic apical cells (Wanninger et al. 2005a). The most common developmental pathway in sipunculans is via a lecithotrophic trochophore and a planktotrophic pelagosphera (Fig. 1), which might remain pelagic for several weeks or even months (Scheltema and Hall 1975, Rice 1981, 1985). Compared to other sipunculans, *Phascolion strombi* has a relatively short larval development (approx. 3 days) (Åkesson 1958, Wanninger et al. 2005a). Accordingly, the lack of serotonergic expression in the apical organ might be a secondary condition. However, the above described pattern of early neurogenesis found in sipunculans is remarkably similar to that reported for the early trochophore larvae of the polychaetes *Polygordius lacteus*, *Pomatoceros lamarkckii*, *Sabellaria*...

Establishment and loss of segmentation during sipunculan neurogenesis
Adult sipunculans exhibit a single ventral neurite bundle that lacks ganglia. However, in all investigated sipunculans to date, a paired serotonin- and FMRFamide-positive ventral neurite bundle with interconnecting commissures appears during ontogeny (Wanninger et al. 2005a; chapters I-III herein). Furthermore, four pairs of serotonergic perikarya associated with the ventral neurite bundles and three serotonergic and FMRFamidergic commissures appear subsequently in a strict anterior-posterior progression during sipunculan ontogeny, leading to a rope ladder-like ventral nervous system (chapters I-III). Interestingly, the FMRFamidergic ventral nervous system exhibits a median neurite bundle between the two ventral neurites and one (Phascolosoma agassizii) or two perikarya (Themiste pyroides, Thysanocardia nigra), which appear in the same manner as the serotonergic ones subsequently on the outer ventral neurites (chapters I and II). A similar condition with a median neurite bundle in the ventral nervous system has been described for a number of polychaete, myzostomid, echiuran, as well as hirudinean larvae and adults (Sawyer 1986, Müller and Westheide 2000, 2002, Eeckhaut et al. 2003, Orrhage and Müller 2005, McDougall et al. 2006, Hessling 2002, Hessling and Westheide 2002, Hessling 2003). Accordingly, the segmental mode of neurogenesis, which indicates the existence of a posterior growth zone, and a median neurite bundle clearly link sipunculans to annelids, thus strongly supporting a segmented ancestry of both taxa.

Unique to sipunculans, the metameric arrangement of the ventral nervous system is lost towards metamorphosis (chapters I-III). The serotonergic ventral neurite bundles fuse, their commissures disappear, and the metameric arrangement of the perikarya is lost by the time a fifth pair appears (chapters I-III). The two FMRFamidergic ventral neurite bundles that form the ventral nervous system come to lie closer to each other prior to metamorphosis, and more neurites appear between them, resulting in a single, non-metameric ventral nerve cord in the adult stage.
Accordingly, this is the first report of the ontogenetic establishment and loss of a metamerically arranged organ system in the nervous system of a metazoan animal.

**Comparative trochozoan neurogenesis**

An early neurogenesis that starts with a paired ventral neurite is common for all annelids (i.e., including myzostomids, sipunculans and echiurans *sensu* Mwinyi et al. 2009 and Struck et al. 2011) as well as many lophotrochozoans, suggesting two ventral neurites to be part of the body plan of the last common ancestor of Lophotrochozoa (Wanninger 2009, Chernyshev and Magarlamov 2010, Fischer 2010; chapters I, and II herein).

The sipunculan species investigated herein all have a serotonergic and FMRFamidergic neurite that underlies the metatrochal ciliary bands, which constitute the primary locomotory organ of the larvae (chapters I-III). However, it has to be noted that the immunoreactivity against both neuronal markers of the metatrochal neurite in *Themiste pyroides* and *Thysanocardia nigra* is much weaker than in *Phascolosoma agassizii*, and even absent in *Phascolion strombi* (Wanninger et al. 2005a; chapters I-III herein). This is probably due to the different life history patterns, since the pelagosphera larvae of *P. agassizii* are the ones that feed and have the longest development in the plankton. This might explain why their locomotory organ is well developed and innervated. *T. pyroides* and *T. nigra* both have lecithotrophic, short-lived pelagosphera stages (10-14 days at 17-19 °C), while this is even more reduced in *P. strombi* (pelagosphera stage for approx. 12 to 24 hours at 12-16 °C). However, a neurite underlying a ciliated locomotory organ is also found in other trochozoan larvae including polychaetes (Hay-Schmidt 1995, Voronezhskaya et al. 2003, McDougall et al. 2006, Brinkmann and Wanninger 2008, 2009, Fischer et al. 2010), mollusks (Friedrich et al. 2002, Croll et al. 1997, Kempf et al. 1997), nemerteans (Lacalli and West 1985, Maslakova 2010), ectoprocts (Wanninger et al. 2005b, Gruhl 2009), phoronids (Hay-Schmidt 1990a, b), and various deuterostomes (Nielsen and Hay-Schmidt 2007), although the expression of neural markers in this neurite varies. Hence, this condition might be an adaptation of marine invertebrate larvae to swimming and feeding during their planktonic dispersal phase.

A possible apomorphy of Lophotrochozoa might be an apical organ containing serotonergic and/or FMRFamidergic flask-shaped cells (Wanninger 2009). Similar to
the sipunculan species investigated herein, the polychaete *Phyllodoce maculate* exhibits four flask-shaped serotonergic and FMRFamidergic cells within the apical organ (Voronezhskaya et al. 2003; chapters I-III herein). In addition, some mollusks (but not the comparatively basal Polyplacophora with 8-10 flask-shaped apical cells), euctoprops, nemerteans, and brachiopods share this feature of up to four immunoreactive flask-shaped cells within the apical organ (Hinman et al. 2003, Croll and Dickinson 2004, LaForge and Page 2007, Voronezhskaya et al. 2008, Dyachuk and Odintsova 2009, Gruhl 2009, Wanninger 2009, Altenburger and Wanninger 2010, Chernyshev and Magarlamov 2010, Kristof and Klussmann-Kolb 2010), thus indicating that the last common lophotrochozoan ancestor probably had an apical organ comprising approximately four serotonergic flask-shaped apical cells.

Taken together, sipunculan neurogenesis shares numerous features with annelid larvae. These include early neurogenesis with an apical organ from which two neurite bundles are formed in posterior direction, giving rise to the ventral nervous system as well as the metatrochal and oral nerve ring (chapters I-III). Moreover, the formation of the ventral nerve cord follows a segmental pattern, whereby perikarya and commissures associated with the ventral nerve cord are formed one after another in an anterior to posterior progression (chapters I-III). This supports recent molecular phylogenetic analyses and a segmental ancestry of Sipuncula (chapters I-III).

**Sipunculan growth zone(s)**

Annelid segment formation is defined by the presence of a posterior growth zone from which the segmentally arranged organs are progressively budded off, leading to the anterior-posterior progression of developing organ systems such as the nervous and muscle system (Iwanonff 1928, Anderson 1966, 1973, Weisblatt et al. 1988).

Interestingly, the sipunculans *Themiste pyroides* and *Thysanocardia nigra* show a metameric pattern of mitotic cells in their development that originates from the ventral posterior trunk area just dorsal to the roots of the retractor muscles, and seem to progress from posterior to anterior (chapter IV). Congruently with sipunculan neurogenesis (chapters I-III), this metameric pattern disappears in metamorphic competent larvae (chapter IV). Furthermore, these findings are strikingly similar to the description of a growth zone in sipunculan larvae that only remains for a short period of time (Åkesson 1958).
While Åkesson (1958) described the embryology and neurogenesis in *Phascolion strombi*, he also reported a growth zone that is situated anterior to the retractor roots. In addition, he showed that with the exception of *P. strombi* the growth zone in sipunculan larvae remains in this anterior position for only a part of their life cycle. However, in *P. strombi* the introvert retractors are attached at the very posterior end of the body. Moreover, during the juvenile stages the left nephridium degenerates and the ventral and dorsal retractors fuse, with the latter becoming more prominent, while the ventral retractor often degenerates (Åkesson 1958). *P. strombi* has a short larval development as well as fusion and degeneration processes in the first juvenile stages. Accordingly, similar to Åkesson’s description, a putative growth zone in *T. pyroides* and *T. nigra* is only present in their pelagosphera larvae prior to metamorphosis (chapter IV). However, the question remains whether and how long such a putative growth zone may exist during ontogeny of *P. strombi* or *Phascolosoma agassizii*, since both display different developmental pathways than *T. pyroides* and *T. nigra*.

Recently, ontogenetic studies showed that segmentation in annelids is more variable than previously thought (Seaver et al. 2005, Brinkmann and Wanninger 2008, 2010b). For instance, the segments in the polychaetes *Capitella* and *Hydroides* are formed from a ventrolateral region of the body, while a posterior growth zone is only evident in post-metamorphic stages (Seaver et al. 2005). This corroborates the data on another polychaete, *Sabellaria alveolata*, where no larval posterior growth zone appears (Brinkmann and Wanninger 2010b). Moreover, this variability of segment formation is also mirrored in the nervous and muscle system formation in *Sabellaria alveolata*, where certain parts develop synchronously and others subsequently in an anterior to posterior manner (Brinkmann and Wanninger 2008, 2010b). Clearly, more ontogenetic studies, preferably on supposedly basal polychaetes such as Dinophilidae, Oweniidae, and Protodrilida, are needed to shed more light on segment formation in Annelida (including Sipuncula).

**Myogenesis in sipunculans and annelids**

In all sipunculans investigated so far, myogenesis follows a similar pattern. Four introvert retractor muscles begin to develop together with a considerable number of circular muscles (Wanninger et al. 2005a, Schulze and Rice 2009; chapter IV herein). The planktotrophic species *Phascolosoma agassizii* and *Nephasoma pellucidum*...
exhibit an additional retractor muscle for the terminal organ and the anlagen of the buccal musculature (Schulze and Rice 2009; chapter IV herein). This is probably due to the longer planktotrophic stage, since all other investigated species have either direct or indirect lecithotrophic development, hence develop the buccal organ considerably later (Wanninger et al. 2005a, Schulze and Rice 2009; chapter IV herein). However, the simultaneous establishment of circular body wall muscles along the entire anterior-posterior axis throughout sipunculan ontogeny seems to be independent from the life cycle pathway, suggesting that this mode of myogenesis may be ancestral to sipunculans (Wanninger et al. 2005a, Schulze and Rice 2009, Wanninger 2009; chapter IV herein). By contrast, the circular body wall muscles in annelids develop in a segmental manner from anterior to posterior, although circular muscles are not always present in polychaetes (Hill and Boyer 2001, Seaver et al. 2005, Tzetlin and Filippova 2005, Purschke and Müller 2006, Bergter et al. 2007, Hunnekuhl et al. 2009, Wanninger 2009, Fillipova et al. 2010). Furthermore, comparative analyses on polychaete larvae suggest that the body wall of the last common annelid ancestor probably had four longitudinal muscle strands that develop from anterior to posterior (Purschke and Müller 2006, Bergter et al. 2008, Brinkmann and Wanninger 2010b).

From early development onwards, sipunculans exhibit four longitudinal retractor muscles (Wanninger et al. 2005a, Schulze and Rice 2009; chapter IV herein), which in post-metamorphic stages might fuse (Åkesson 1958). Moreover, longitudinal body wall muscles are always densely arranged in the areas of the retractor muscles and more loose towards the mid-body, thus suggesting that the retractor muscles evolved from fused longitudinal body wall muscles (chapter IV). Interestingly, one dorsal and one ventral pair of longitudinal body wall muscles appear first in polychaetes and oligochaetes, which develop then in anterior to posterior direction (McDougall et al. 2006, Bergter et al. 2007, 2008, Brinkmann and Wanninger 2010b, Fischer et al. 2010). Accordingly, sipunculan and annelid larvae exhibit both four separate longitudinal muscle strands that develop from anterior to posterior, and are considered having been present in their last common ancestor (e.g., Cutler 1994, Schulze and Rice 2009, Brinkmann and Wanninger 2010b). This again supports the recent phylogenetic analyses that consider the sipunculans as in-group annelids (e.g., Zrzavy et al. 2009, Struck et al. 2011).
Conclusions and future perspectives

Despite the fact that circular body wall muscles do not develop in a segmental manner, the data presented herein document for the first time traits of segmentation in Sipuncula. For a short period of time during sipunculan ontogeny, a repeated pattern is generated from the posterior pole of the larvae in their nervous system, which resembles the annelid-like mode of segmentation. In agreement with recent molecular phylogenetic analyses, this strongly argues for a segmented sipunculan and annelid last common ancestor, which had four longitudinal body wall muscles that developed from anterior to posterior. However, the absence of circular body wall muscles in a number of adult polychaetes and their larvae casts doubt on the assumption that ring muscles were part of the annelid ground pattern, although multiple, independent loss of these muscles in the respective lineages are also possible. Accordingly, the different pathways of body segmentation in annelids (including sipunculans, echiurans, and myzostomids) might be due to adaptations to different modes of life, thus indicating that segmentation is more complex as well as labile to evolutionary changes than previously assumed. The fact that a segmented body is established and later secondarily lost during ontogeny renders sipunculans an ideal group to study the ontogeny and evolution of segmentation, in particular in comparison to the well studied annelid, arthropod and chordate model organisms. This might help to shed light on the contradicting conclusions on morphological urbilaterian characteristics (i.e., a complex, segmented versus a simple, non-segmented species at the base of the bilaterian tree of life).

In this context, one primary aim of future studies would be to deepen the knowledge on the “segmentation” process in Sipuncula from a molecular perspective. The way segments are specified in insects, especially *Drosophila*, is different from the way segments are controlled in vertebrates or annelids (Chipman 2010). Therefore, knowing which genes are involved in sipunculan segmentation, which unlike the above mentioned model organisms looses its segmentation during ontogeny, can be a good objective for understanding the evolution of segmental specification and loss.

Modern high-throughput sequencing technologies such as 454 reads or *illumina* are able to generate a large EST dataset of almost all genes expressed during the development of an animal. Accordingly, the analysis of the EST dataset and the gene expression of so-called “segmentation” genes (i.e., *hairy, notch, delta, wingless,*
engrailed, even-skipped) will provide important insights into the molecular processes that underlie the establishment and loss of segmentation during sipunculan ontogeny. The developmental-morphological study herein provides an anatomical framework that enables detailed interpretation of gene expression patterns in sipunculans. As shown in the present thesis, the sipunculan Themiste pyroides is ideally suited as a model sipunculan for these kinds of studies in the future.

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References


Chapter II

Segmental Mode of Neural Patterning in Sipuncula

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Summary

Recent molecular phylogenetic analyses suggest a close relationship between two worm-shaped phyla, the nonsegmented Sipuncula (peanut worms) and the segmented Annelida (e.g., earthworms and polychaetes) [1–5]. The striking differences in their bodyplans are exemplified by the annelids’ paired, ladder-like ventral nervous system, which contains segmentally arranged ganglia, and the sipunculans’ single ventral nerve cord (VNC), which is devoid of any segmental structures [6, 7]. Investigating central nervous system (CNS) formation with serotonin and FMRFamide labeling in a representative sipunculan, Phascolosoma agassizii, we found that neurogenesis initially follows a segmental pattern similar to that of annelids. Starting out with paired FMRFamidergic and serotonergic axons, four pairs of associated serotonergic perikarya and interconnecting commissures form one after another in an anterior-posterior progression. In late-stage larvae, the two serotonergic axons of the VNCs fuse, the commissures disappear, and one additional pair of perikarya is formed. These cells (ten in total) migrate toward one another, eventually forming two clusters of five cells each. These neural-remodeling processes result in the single nonmetameric CNS of the adult sipunculan. Our data confirm the segmental ancestry of Sipuncula and render Phascolosoma a textbook example for the Haeckelian hypothesis of ontogenetic recapitulation of the evolutionary history of a species [8].

Results and Discussion

Ontogenetic Establishment of the Segmented Sipunculan CNS

Despite their proposed close phylogenetic relationship [1–5], annelids and sipunculans differ significantly from each other in that most annelids exhibit a typical segmented body with metamERICally arranged appendages, commissures interconnecting two ventral nerve cords (VNCs), and paired sets of ganglia, whereas sipunculans lack any trace of metamerism and have only a single VNC. Using antibodies against the neurotransmitters serotonin and FMRFamide, we found that neurogenesis in Phascolosoma starts in the anterior region with the larval apical organ and the subsequently developing paired serotonergic and FMRFamidergic axons of the VNC. Slightly later, one pair of serotonergic perikarya associated with the serotonergic portion of the VNCs appears (Figure 1A). As development and growth along the anterior-posterior axis of the animal proceeds, a second pair of ventral perikarya arises (Figure 1B). In the following stages, the entire neural architecture of Phascolosoma is elaborated, resulting in a complex anterior nervous system that shares numerous features with annelid larvae. These include a (larval) serotonergic prototroch nerve ring, a (larval) apical organ comprising several serotonergic cells, the early anlage of the adult brain, and an oral nerve ring (Figures 1C and 1D). Most notable and important, however, is the successive formation of two additional pairs of serotonergic perikarya associated with the serotonergic axons of the VNCs, together with three serotonergic and FMRFamidergic commissures that interconnect the respective axons of the VNC (Figure 1B, inset, and Figures 1C–1F). This results in a segmental central nervous system (CNS) comprising four metamERICally arranged pairs of serotonergic perikarya along the VNCs of the late-stage Phascolosoma larva (Figure 2A and Figure 3). Interestingly, only one pair of FMRFamidergic perikarya is found to be associated with the FMRFamidergic VNC (Figure 1F).

Annelid segmentation is not typified simply by the repetitive arrangement of organs along the longitudinal body axis but, more specifically, by their successive formation mode in a strict anterior-posterior progression; this indicates the presence of a posterior growth zone from which the segmentally arranged organs are budded off [9–13]. With the increasing molecular evidence for an annelid-sipunculan assemblage [1–5], the question of whether the last common ancestor (LCA) of both taxa was segmented or not is still open because within Lophotrochozoa (animals with a ciliated larval stage in their life cycle), a segmented nervous system had been known only in annelids, including echiurans (spoon worms, a group of marine worms believed to nest within the annelid phylum) [1–5, 12, 14, 15]. Our findings fill this gap in knowledge by providing the hitherto-lacking proof of a segmented ancestry of the sipunculan nervous system [16], thus strongly arguing in favor of a segmented annelid-echiuran-sipunculan LCA.

Ontogenetic and Evolutionary Loss of the Segmental Sipunculan CNS

The fully developed segmented nervous system of the larva of Phascolosoma agassizii comprises four pairs of serotonergic perikarya and three serotonergic and FMRFamidergic commissures interconnecting the VNCs (Figures 1D, IF, 2A, and 3D). As the larva approaches metamorphic competence, the two serotonergic axons of the VNCs fuse, the serotonergic commissures disappear, and a fifth pair of serotonergic perikarya is formed (Figure 2B). Together with subsequent cellular migratory processes, this eventually results in a CNS consisting of a single serotonergic VNC, which only retains its paired character in the anterior-most region where it connects to the brain, as well as two neural cell clusters comprising five perikarya each (Figures 2C and 3E). Accordingly, the segmental serotonergic neural architecture is lost prior to the onset of metamorphosis of the Phascolosoma larva. At this stage, the three FMRFamidergic commissures are still discernable, although the two FMRFamidergic axons of the VNCs lie in much closer proximity to each other than in the previous larval stages (Figure 1F).

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Initially paired ventral serotonergic nerves that fuse during development have recently been described for another sipunculan, *Phascolion strombi*. Although this species did not show unambiguous signs of segmentation during its ontogeny (a fact that is probably due to its shortened larval phase), the transitional occurrence of three FMRFamidergic commissures in late *P. strombi* larvae indicates cryptic remnants of a once-segmented CNS in this species as well [17].

The findings of earlier morphological and developmental studies proposing a taxon comprising Sipuncula and Mollusca [18] have recently been rejected by a number of molecular analyses that clearly argue for an annelid-echiuran-sipunculan clade [1–5, 16]. Accordingly, novel data on CNS formation in these groups cast new light on the evolution of annelid segmentation. Although the architecture of the adult annelid CNS seems to be quite plastic, ranging from one to five VNCs [19], neurogenesis consistently starts with an anterior neuropil (the predecessor of the brain) and one pair of serotonergic and FMRFamidergic axons in the VNCs, irrespective of the number of VNCs that are present in the adult stage [20–23]. This is congruent with the findings on echiuran and sipunculan neurogenesis and suggests that a paired ventral CNS was also present in the LCA of these three groups. However, various kinds of independent fusion and duplication events have taken place during evolution, accounting for the phenotypic plasticity of the adult CNS that is encountered in these worm-shaped animals today.

In many annelids, ganglia and commissures are commonly formed after the establishment of the first rudiments of the paired VNC. Development of these commissures and ganglia is strictly directional and follows an anterior-posterior progression, a fact that is typically assigned to the presence of a posterior growth zone [9, 10, 24, 25], and that eventually leads to the typical ladder-like metameric annelid ventral CNS. Although rather obvious in “typical” (e.g., polychaete) annelids, indications for the segmental ancestry of the echinurans are much more subtle and were only revealed recently by studies of their neural development [12, 14, 15]. As such, echinurans exhibit a double-stranded VNC in early larval stages similar to the annelids [12]. Although these nerve cords fuse during subsequent development, the associated perikarya form in anterior-posterior succession until the juvenile neural bodyplan is established [12, 14, 15]. Thus, although modified, the segmental origin of the echinuran CNS can still be recognized in the adult stage by the metameric arrangement of the perikarya associated with the VNC. Moreover, cell-proliferation markers indicate an annelid-like posterior growth zone in *Bonella viridis*, thus further strengthening the evidence for the segmented origin of echinurans [15]. In sipunculans, reduction of neural segmentation traits has progressed even further than in echinurans, resulting in a CNS that is devoid of any metameric structures in the adult stage (Figures 2C and 3E).
To our knowledge, this work for the first time documents ontogenetic establishment and loss of CNS segmentation in the life cycle of a recent metazoan and demonstrates the segmental origin of Sipuncula. Accordingly, *Phascolosoma* confirms the Haeckelian notion that cryptic character states, which have been lost during evolution in the adult bodyplan, may have been conserved during ontogeny [8], thus stressing the importance of developmental studies for the reconstruction of ancestral bodyplan features of metazoan clades.

**Supplemental Data**

Supplemental Data include Supplemental Experimental Procedures and two movies and can be found with this article online at [http://www.current-biology.com/cgi/content/full/18/15/1129/DC1/](http://www.current-biology.com/cgi/content/full/18/15/1129/DC1/).

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Chapter III

 Comparative molecular, developmental and morphogenetic analyses show that the three major segmented animal groups—Lophotrochozoa, Ecdysozoa and Vertebrata—use a wide range of ontogenetic pathways to establish metameric body organization. Even in the life history of a single specimen, different mechanisms may act on the level of gene expression, cell proliferation, tissue differentiation and organ system formation in individual segments. Accordingly, in some polychaete annelids the first three pairs of segmental peripheral neurons arise synchronously, while the metameric commissures of the ventral nervous system form in anterior-posterior progression. Contrary to traditional belief, loss of segmentation may have occurred more often than commonly assumed, as exemplified in the sipunculans, which show remnants of segmentation in larval stages but are unsegmented as adults. The developmental plasticity and potential evolutionary lability of segmentation nourishes the controversy of a segmented bilaterian ancestor versus multiple independent evolution of segmentation in respective metazoan lineages.

Ontogeny and Functional Implications of Segmentation

The evolution of a segmented bodyplan is often considered a crucial metazoan innovation because it allows the subdivision and specialization of individual body regions along the anterior-posterior axis of an animal.1,2 This partitioning typically involves both the ectodermal and the endodermal germ layers and often results in metameric ectodermal appendages (parapodia) and segmentally arranged, paired mesodermal body cavities (coelomic sacs). Traditionally, a condition where all segments have the same type of parapodia and house identical sets of internal organs such as ganglia, nephridia, gonads and muscles has been regarded as basal for annelids and arthropods (homonomic segmentation).1,2 From this basal (plesiomorphic) condition, concentration of individual organ systems into segments of specific body regions combined with reductions of organs in other segments are thought to have occurred multiple times within various lineages, and eventually led to morphologically distinct segments along the anterior-posterior body axis (heteronomous segmentation).1,2

While often considered an important hint towards ancestral segmentation of a species, serial repetition of organs along the anterior-posterior axis alone is not decisive for a segmental evolutionary history (cf., e.g., the multiple ring muscles in the non-segmented platyhelminths). On the cellular and organ system level, segmentation can only be proven with the aid of developmental studies, because segmented animals typically exhibit a posterior growth zone from which all segments are progressively budded off.3-7 Accordingly, ontogenetically older segments—and thus also the organs associated with them—are found anterior to the younger segments, a fact that is illustrated by the gradual decrease of the degree of organ system differentiation from anterior to posterior (Fig. 1).8 This makes the pattern of organogenesis an ideal marker to test for the segmental ancestry of worm-shaped lophotrochozoan taxa.8-12

Coelomic compartmentalization of a cylindrical body has frequently been proposed to be of selective advantage due to the fact that these animals are able to regulate the hemolympthic pressure in each compartment (segment) individually. The interplay of coelomic pressure and the contractile ring and longitudinal muscles of the body wall enable direct and independent control over the diameter of the body in each individual segment, thus allowing for a diversity of complex movement patterns.2 However, while coelomic segmentation has often (but not always) been retained in large, burrowing annelids (e.g., earthworms), secondary loss is often observed in non-benthic free-living (e.g., leeches), interstitial (e.g., Protodrilus), or sessile forms (e.g., tube worms).

Loss of Segmentation

Despite the loss of segmentation in various annelid taxa, ontogenetic remnants of their segmented ancestry are present in a number of annelids that do not show obvious segmental features in the adult body (e.g., leeches).3,4 Recent developmental studies have shown that this holds also true for representatives of the Sipuncula (peanut worms), unsegmented lophotrochozoans that are regarded either as derived ingroup annelids or as a direct annelid sister clade.13-17 Interestingly, however, segmental traits in the sipunculans are restricted to the nervous system but have been completely lost on the level of coelom organisation.18 Accordingly, larvae of Phascolosoma agassizii develop four pairs of perikarya that are associated with the paired ventral nerve cords and express the common neurotransmitter serotonin (Fig. 2A). These paired perikarya form, together with commissures that interconnect the ventral nerve cords, in a typical annelid-like anterior-posterior progression, thus demonstrating the segmental ancestry of Sipuncula. During subsequent larval
perikarya are formed in a discrete anterior-posterior progression.9-11 Noticeably, within Sipuncula the degree of preservation of neural segmentation appears to be dependent on the duration of the larval phase and is thus directly correlated with the basal versus the derived mode of sipunculan development. The segmented nervous system in Phascolosoma is expressed in the so-called pelagosphera larva. This larval stage is a defining (apomorph) character for Sipuncula and is thus considered part of the ancestral sipunculan life cycle.19 Neurogenesis in another sipunculans species that lacks the pelagosphera stage, Phascolion strombi, shows that the remnants of the metameric nervous system have been reduced even further. As such, while also having a primarily paired ventral nerve cord, this species lacks the associated perikarya and only has retained three transitional ventral commissures as the sole remnants of the ancestral segmented neural bodyplan.20 Cryptic segmentation in taxa with close annelid affinities seems to be more common than previously assumed. The echiurans (spoon worms), now considered as clustering within Annelida,13,16,17 do not show any segmental traits in their adult gross morphology. However, neurogenesis revealed the same ontogenetic mechanisms as found in annelids and sipunculans, namely that paired sets of perikarya are formed in a discrete anterior-posterior progression.9-11 In contrast to the sipunculans and similar to the condition found in “typical” annelids, this metameric organization of the nervous system persists in the adult echiurans. Accordingly, the annelid-echiuran-sipunculan lineage shows a gradual decrease of preservation of nervous system segmentation, a notion that is further supported by patterns of myogenesis. Hereby, annelids exhibit the typical anterior-posterior progression of ring and dorsoventral muscle formation, while sipunculan myogenesis starts with synchronous formation of early ring muscle rudiments, followed by the emergence of additional ring muscles along the entire anterior-posterior axis by fission from already existing myocytes.8,20 Accordingly, Sipuncula represents a developmental mosaic of segmental and non-segmental bodyplan patterning mechanisms, whereby the ectoderm-derived nervous system has to some degree maintained its segmental ancestry while the mesodermal musculature is formed entirely non-metamerically.

Figure 1. Schematic representation of neurogenesis in the polychaete annelid Sabellaria alveolata based on serotonin immunoreactivity, revealing differences in the mode of establishment of metamery in the peripheral segmental neurons and the ventral commissures, respectively. Both aspects are ventral views with anterior facing upwards. Total length of the specimens is approximately 280 μm in (A) and 330 μm in (B). (A) Late larva with synchronously established peripheral segmental neurons (yellow). Ventral commissures and perikarya along the paired ventral nerve cord (vnc) are still lacking. The prototroch nerve ring (pnr) and the nerve ring underlying the telotroch (ttm) constitute subsets of the larval nervous system, while the circumoesophageal commissures (cc) and the longitudinal trunk neurons (ltn) are parts of the adult nervous system. (B) Larva prior to metamorphosis. The ventral commissures (asterisks) of the first five segments have been established progressively, together with the paired, metameric sets of perikarya (red dots) along the ventral nerve cords (vnc). The six pairs of peripheral segmental neurons (yellow) correspond to the segments II–VII, because development of segment I is retarded in this species, resulting in development of the paired peripheral segmental neuron of this segment at a later stage. Note that ontogeny of the peripheral segmental neurons precedes development of the ventral commissures in segments VI and VII. pns – the nerves of the peripheral nervous system.

Plasticity of Segmentation

Despite the long standing definitions concerning the characteristics of a segmented bodyplan (see above), recent data have shown that the ontogenetic establishment of annelid segmentation may follow quite different developmental pathways. Traditionally, it had been proposed that the first three (larval) segments form more or less synchronously by schizocoely from the paired lateral mesodermal band, while the following (adult) segments develop from a pre-anal growth zone.21 Accordingly, one would assume that the organ systems associated with the first three segments also arise synchronously, while only the subsequent segmental organs follow the anterior-posterior differentiation gradient. However, this is only partly true for the polychaete Sabellaria alveolata. While the three larval segments indeed arise synchronously in this species, only the corresponding pairs of peripheral segmental neurons form synchronously, while the ventral commissures develop subsequently one after another (Fig. 1).22 While this may be interpreted as (secondary)
Ancestry of Segmentation

Comparative analyses of the developmental mechanisms that form metameric organs in segmented lophotrochozoan worms demonstrate a high plasticity of ontogenetic patterns that lead to a segmented bodyplan and show that segmentation may be lost during evolution. This raises the question as to what extent such an evolutionary loss has yet remained unrecognized in other phyla, thus reviving the discussion about a possible segmented ancestor of Lophotrochozoa and Bilateria as a whole. Such a scenario has been repeatedly proposed by the advocates of a conserved molecular pathway that is thought to underlie the ontogeny of metazoan segmentation.7,26 However, despite some similarities on the molecular level, there are also significant differences in the way typical “segmentation genes” are expressed, and cellular and tissue differentiation pathways that eventually give rise to individual segments vary between lophotrochozoans, vertebrates and ecdysozoans.25 To complicate matters further, segment formation is highly variable even between phyla within the respective “superclades” Ecdysozoa and Annelida.25 Moreover, morphologically similar segments may follow different ontogenetic pathways even within the same individual, and distinct metamerically arranged subunits of the nervous system may form differently in individual segments of the same animal (e.g., the ventral commissures versus the peripheral segmental neurons in polychaetes; see above). Lastly, a mosaic of segmental and non-segmental modes of organogenesis may occur within an individual, indicating the occurrence of dissociation of organogenesis from the segmentation process in some species (e.g., muscle formation in sipunculans; see above).

Given the incongruencies and the plasticity of the processes involved in the ontogeny of segmentation in the various major bilaterian subgroups, no final statement as to whether or not Urbilateria was segmented can yet be made. In any case, assuming a segmented urbilaterian would imply a wide range of evolutionary modifications of the ancestral segmentation pathway on the molecular, cellular and morphogenetic level, as well as secondary loss of a segmented body in a number of lineages. Both, experimental developmental genetics employing RNAi experiments and comparative morphogenetic analyses provide exciting tools that enable us to directly test for evolutionary hypotheses concerning shared molecular segmentation pathways on the one hand and for cryptic remnants of a segmented bodyplan in seemingly non-segmented recent phyla on the other. This should eventually lead to a sound reconstruction of the ancestry.
of one of the key innovations of metazoan evolution: the origin of segmented bodies.

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References

Chapter IV

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 pelagosphera 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. J. Exp. Zool. (Mol. Dev. Evol.) 314B, 2011. © 2011 Wiley-Liss, Inc.


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 similarities have been found in the foregut of certain sipunculans and polychaetes as well as in their collaginous 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 segregation 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-ethyl-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 4 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,
Corporation, Ottawa, Ontario, Canada). Digital line drawings were created with Corel Draw 11.0 (Corel imaging software Imaris v. 4.1 (Bitplane, Zürich, Switzerland). 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 throughout 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 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 (9–12 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
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 μm in all aspects except for C, in which it equals 10 μm. 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. 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 (vnc). (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. 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.
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 adulthood, 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.

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.

(J) Latest 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–E) 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 (vrm) and dorsal (drm) 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.
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 17–19 °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 Northwest (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 rings of 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 oligocheates). 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 echinurans, 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
Figure 6. Myogenesis from the early trochophore (A) to the pelagosphera stage (D-E) of Themiste pyroides. Anterior faces upwards and scale bars represent 50 μm 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 (2 dpf) showing the anlage of the paired ventral (vrm) and dorsal (drm) longitudinal retractor muscles as well as numerous circular body wall muscles (cm); asterisk marks the mouth opening. (B) Slightly later stage (2.5 dpf) 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 3 dpf), 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 (3.5 dpf) 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 (8 dpf) 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. (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. (H) Schematic reconstruction of (E). Note the strong and solid retractor muscles of the pelagosphera larva.
Hydroidea 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 planktrotrophic 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 (Jaekle 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., 1988; 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 (Tzetlin and Filippova, 2005; Purschke and Müller, 2006). This may be correlated with the lifestyle of many polychaetes 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 musculature.

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 polychaetes, 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 verniform soft-bodied lophotrochozoans, such as annelids, platyhelminthes, nemertines, or basal mollusks (Solengogastes, Caudofoveata, larval Polyclaplacophora), 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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**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.

**LITERATURE CITED**


