

Sipuncula: an emerging model of spiralian development and evolution

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ABSTRACT Sipuncula is an ancient clade of unsegmented marine worms that develop through a conserved pattern of unequal quartet spiral cleavage. They exhibit putative character modifications, including conspicuously large first-quartet micromeres and prototroch cells, postoral metatroch with exclusive locomotory function, paired retractor muscles and terminal organ system, and a U-shaped digestive architecture with left-right asymmetric development. Four developmental life history patterns are recognized, and they have evolved a unique metazoan larval type, the pelagosphaera. When compared with other quartet spiral-cleaving models, sipunculan development is understudied, challenging and typically absent from evolutionary interpretations of spiralian larval and adult body plan diversity. If spiral cleavage is appropriately viewed as a flexible character complex, then understudied clades and characters should be investigated. We are pursuing sipunculan models for modern molecular, genetic and cellular research on evolution of spiralian development. Protocols for whole mount gene expression studies are established in four species. Molecular labeling and confocal imaging techniques are operative from embryogenesis through larval development. Next-generation sequencing of developmental transcriptomes has been completed for two species with highly contrasting life history patterns, *Phascolion cryptum* (direct development) and *Nephasoma pellucidum* (indirect planktotrophy). Looking forward, we will attempt intracellular lineage tracing and fate-mapping studies in a proposed model sipunculan, *Themiste lageniformis*. Importantly, with the unsegmented Sipuncula now repositioned within the segmented Annelida, sipunculan worms have become timely and appropriate models for investigating the potential for flexibility in spiralian development, including segmentation. We briefly review previous studies, and discuss new observations on the spiralian character complex within Sipuncula.

KEY WORDS: *pelagosphaera*, *metatroch*, *unequal*, *U-shaped*, *ectomesoderm*

Ancient worms with a new status

Sipuncula is a clade of unsegmented, coelomate marine worms. They have colonized benthic habitats worldwide from intertidal zones to abyssal plains in polar, temperate and tropical environments, and they have evolved a larval form that is unique among all metazoans, the pelagosphaera (Mueller, 1850; Hatschek, 1883; Gerould, 1906; Rice, 1967). The adult body plan consists of a relatively large posterior trunk region with a single undivided coelomic cavity, and a retractable introvert with an array of tentacles on the anterior end that typically surrounds the mouth. The mouth leads to a U-shaped digestive system that terminates with an anus on the dorsal-anterior side of the trunk. Adult sipunculans have a centralized nervous system with a single, median, unsegmented

ventral nerve cord. There is comprehensive information on their reproductive biology (Rice, 1975b; Rice, 1989), anatomy (Rice, 1973, 1993), taxonomy (Stephen and Edmonds, 1972), systematics (Cutler, 1994) and evolutionary relationships (Rice, 1985; Schulze *et al.*, 2007; Kawauchi *et al.*, 2012). There are extensive records of sipunculan species diversity around the world (Stephen and Edmonds, 1972; Cutler, 1994), and their developmental life history patterns are well-characterized (Rice, 1967, 1975a, 1988). However, classical descriptions of spiralian embryology and comparative development were primarily focused on annelids,

Abbreviations used in this paper: 1q, first quartet micromere; D/V, dorsal-ventral axis; DIC, differential interference contrast; foxA, Forkhead box A transcription factor.

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mollusks, and some echiurans, with very few studies dedicated to sipunculan embryos.

Some of the earliest investigations of sipunculan development were conducted by Selenka (1875) and Hatschek (1883), prior to the use of nomenclature that Conklin (1897) established for designated blastomeres in the equal-cleaving gastropod embryos of *Crepidula*. Selenka (1875) made a concise study of embryonic cleavage and development of a pelagosphera larva. Hatschek (1883) produced the first detailed descriptions of sipunculan development in the large mud-dwelling species, *Sipunculus nudus*. The earliest cleavage stages that he examined were most likely after the sixth round of embryonic cell divisions (Hatschek, 1883). To date, the most comprehensive study of sipunculan embryology was performed by John H. Gerould (Gerould, 1906). In that work, Gerould (1906) employed “Conklin’s modification of Wilson’s plan” for blastomere nomenclature, and meticulously described egg maturation, polarity, fertilization and quartet spiral cleavage through gastrulation and formation of both trochophore and pelagosphera larval stages in two species (Gerould, 1906). Another notable body of work came from Bertil Åkesson (1958), with an extensive multi-species comparison of sipunculan nervous systems, and other organ systems, although spiral cleavage was not described. From that time forward, modern molecular investigations of spiralian development expanded to include not only new models from the annelids, mollusks and echiurans, but also nemerteans and both acoel and polyclad turbellarian flatworms. Yet again, when compared with other spiralian taxa regarding molecular, genetic or cellular aspects of embryology and development, sipunculan worms, which clearly exhibit hallmark characteristics of the quartet spiral cleavage program, were noticeably absent from the list. Is there important biology missing from our collective knowledge of spiralian development?

Looking back at the turn of the 19th century, and for much of the 20th century, including relatively recent studies (Clark, 1969; Stephen and Edmonds, 1972; Rice, 1985; Freeman and Lundelius, 1992; Cutler, 1994; Valentine, 1997), Sipuncula was recognized as a distinct clade of animals with phylum status. However, a series of molecular phylogenetic hypotheses have been supporting an inference that echiurans, siboglinids, and now sipunculans, are members of the annelid radiation (McHugh, 1997; Boore and Staton, 2002; Struck *et al.*, 2007; Dordel *et al.*, 2010; Struck *et al.*, 2011). With changes in the status and position of Sipuncula

as a new and distinct clade of annelids, there come a number of provocative implications. First, as predicted by Clark (1969), Annelida has become a heterogeneous group, as well as one of the most biologically diverse and internally unresolved clades within Bilateria, if not Metazoa. Thus far, there are no obvious molecular or morphological affinities between Sipuncula and particular members of the paraphyletic Polychaeta (Struck *et al.*, 2011). Second, sipunculans are an ancient clade of animals with unambiguous representation in Lower Cambrian rocks, suggesting the “evolutionary stasis” of a non-segmented annelid body plan for over 520 million years (Huang *et al.*, 2004). And with some evidence for pushing back quartet spiral cleavage to well before the Cambrian (Chen *et al.*, 2006), the evolution and divergence of Sipuncula within Annelida is presumed to be much older than their fossil record. Third, recent molecular analyses of sipunculan relationships (Schulze *et al.*, 2007; Kawauchi *et al.*, 2012) uphold an earlier proposition that their ancestral life history pattern includes a planktotrophic larval stage (Rice, 1985), which may be contrary, in part, to a suggestion that “lecithotrophy is the plesiomorphic condition for polychaetes” (Rouse, 2000). Planktotrophy could have evolved independently within the sipunculan stem lineage, yet based on the most recent phylogenomic analysis of Annelida (Weigert *et al.*, 2014), four, and perhaps all five, of the basal annelid groups, including Sipuncula, are known to have planktotrophic larval stages. Fourth, sipunculans exhibit a number of developmental characteristics that are not typically shared by other annelids, as exemplified by persistent cleavage asymmetries, enormous prototroch cells, a novel terminal organ system, locomotory metatrochal ciliary band, U-shaped gut and a unique metazoan larval form. Accordingly, sipunculans, which are no longer an outgroup (Rouse and Fauchald, 1997), bring new and distinctive character modifications into the branches of the annelid tree. Several of those characters (e.g. terminal organ, pelagosphera) likely represent evolutionary novelties within Sipuncula, however, with sipunculans as an annelid ingroup, other sipunculan traits that are shared by echiurans, siboglinids and many polychaetes (e.g. collagenous cuticle) but absent in other metazoans, now become an apomorphy of Annelida, and thus it is perhaps time to reconsider some features of the annelid ground pattern (Purschke, 2002).

Evidence of these and other character modifications may be more common than would be expected from an otherwise conserved, stereotypic developmental program (Martindale and Henry, 1995;

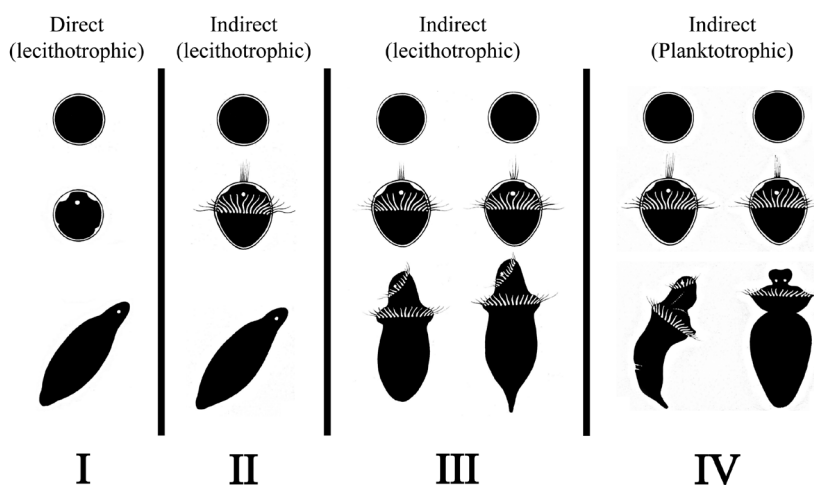


Fig. 1. Schematic of the four recognized developmental life history patterns in Sipuncula. (I) Direct development: embryogenesis leads directly to the production of a crawling vermiform stage. (II) Indirect lecithotrophic development: embryogenesis produces a swimming trochophore larva followed by metamorphosis into a juvenile worm. (III) Indirect lecithotrophic development: embryogenesis produces a swimming trochophore larva with metamorphosis into a swimming lecithotrophic pelagosphera larva, with or without a terminal organ, followed by the second metamorphosis into a juvenile worm. (IV) Indirect planktotrophic development: a swimming trochophore larva undergoes metamorphosis into a swimming planktotrophic pelagosphera larva, followed by the second metamorphosis into a juvenile worm. Planktotrophic pelagosphera larvae may be either short-lived (weeks) forms, or a larger long-lived teleplanic larva (months). All sipunculan trochophore larvae are lecithotrophic.

Boyer and Henry, 1998; Hejnal, 2010; Lambert, 2010). And new forms of evidence from understudied taxa will likely confirm why spiral cleavage is viewed as a flexible character complex that builds diverse larval and adult body plans (Henry and Martindale, 1999; Hejnal, 2010). Therefore, when considering the diversity of extant spiralian, and the ability to infer homology, or secondary loss, of cell types, tissues and organ systems among those animals, it would be advantageous to investigate as many spiralian clades, and characters, as possible in an effort to better understand the potential for flexibility in spiralian development. We think that sipunculans are an appropriate choice for this purpose, especially if one of the overarching aims in choosing non-model organisms includes the potential for finding “unique effects of development” on the evolution of animal body plans (Jenner and Wills, 2007). Here, we present a brief review of studies on sipunculan development and life history evolution, combined with new observations from modern imaging techniques that build upon more than 40 years of research in the Life Histories Program at the Smithsonian Marine Station. We introduce and discuss particular attributes and potential novelties of the spiralian character complex within this group of worms. And, we share recent projects and progress in the laboratory toward establishing sipunculan models for comparative research on spiralian development and evolution.

Overview of developmental life history patterns within Sipuncula

Beginning in 1972, the Life Histories Program has been investigating the reproduction, development, systematics and life history patterns among a broad diversity of marine invertebrates. Throughout this period, one particular group of animals has been given considerable focus: the non-segmented, exclusively marine sipunculan worms. Sipunculan life history patterns have been characterized for more than twenty species (Rice, 1967, 1975a, 1981, 1985, 1988), and they are classified into four categories, including direct development, and three patterns of indirect development through distinct lecithotrophic and planktotrophic larval forms (Fig. 1). Direct developing species are lecithotrophic and develop through a non-ciliated trochophore-like stage without any larval form. Indirect development includes a trochophore stage. All sipunculan trochophore larvae are lecithotrophic, and swim by means of a prototrochal band of cilia that encircles the episphere. Depending on the life history pattern, a trochophore larva will metamorphose into a crawling vermiform stage, a lecithotrophic pelagosphere larva, or a planktotrophic pelagosphere capable of feeding for several weeks, or potentially for many months, before undergoing metamorphosis into a juvenile worm. Teleplanic pelagosphere larvae have been collected from surface currents along transects within tropical and sub-tropical Pacific, Indo-Pacific, Indian and North Atlantic Ocean basins, with indications that many sipunculans have the capacity for dispersal over long distances (Scheltema and Hall, 1975; Rice, 1981; Scheltema and Rice, 1990). The prominent organ of locomotion in all pelagosphere larvae is the metatroch. During metamorphosis from trochophore to pelagosphere, the metatroch becomes active, and the prototroch is reduced to a small band of cilia on the dorsal head, or is lost altogether.

Planktotrophic development occurs in each of the six recognized sipunculan families (Kawauchi *et al.*, 2012). Interestingly, in only one of those six families, Golfingiidae, we find all four of the

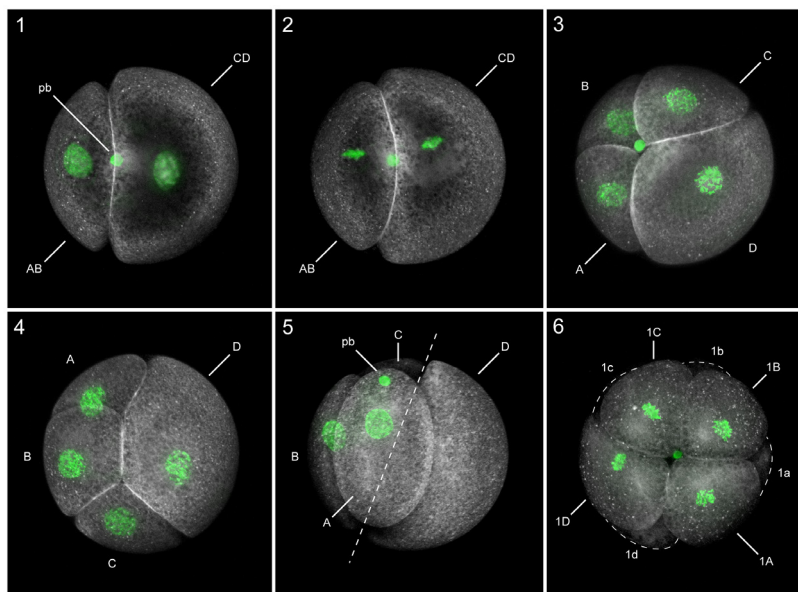
recognized life history patterns (Fig. 1). However, with few exceptions (see below) planktotrophy is the exclusive developmental life history pattern in each of the other five families. Because planktotrophic development is widespread among all sipunculan families, and the only pattern observed within the most basal family, Sipunculidae (Schulze *et al.*, 2007; Kawauchi *et al.*, 2012), we infer planktotrophic development to represent the primitive life history pattern for Sipuncula. From this primitive pattern, life histories are presumed to have evolved in one direction toward lecithotrophy with an increase in yolk, a reduction in pelagic larval stages, and direct development, and in another direction toward a reduction in yolk and development of teleplanic pelagosphere larvae of the open ocean (Rice, 1985). Importantly, contrasts between lecithotrophic and planktotrophic patterns include structures such as the metatroch and terminal organ, and formation of trochophore and pelagosphere larval stages, all of which typically develop in planktotrophic life histories but are absent in direct development, and also absent in several indirect lecithotrophic patterns as well (Rice 1967, 1975a, 1976). However, within a particular habitat (e.g. intertidal reefs), or similar environment (e.g. subtropical) very different sipunculan life history patterns are often observed together (Rice, 1975a), suggesting that divergent developmental modes may be adapted to similar conditions, although with different patterns of feeding, reproduction and dispersal. This scenario provides important natural experiments on sipunculan evolution, and spiralian biodiversity in general, especially when sipunculans, polychaete annelids, nemerteans, polyclad flatworms, and both gastropod and bivalve mollusks typically co-occur on and within substrates of the same habitat, and where they collectively exhibit a wide variety of body plans and life histories. Our most recent investigations have therefore focused on comparing morphological and molecular features of development among three sipunculan species with highly contrasting life history patterns: *Phascolion cryptum* (direct development), *Themiste alutacea* (indirect lecithotrophy), and *Nephasoma pellucidum* (indirect planktotrophy), representing three genera within Golfingiidae.

Observations on sipunculan development

Diocious sexual reproduction is pervasive in Sipuncula (Rice, 1989). There is one species that may reproduce asexually by budding (Rice, 1970), one hermaphroditic species (Åkesson, 1958), and one species capable of parthenogenesis (Pilger, 1987). Mature spherical or elliptical-shaped eggs are spawned through nephridiopores into seawater, and contain yolk reserves in amounts that typically correlate with lecithotrophic or planktotrophic development (Rice, 1975b). Sipunculan eggs have a distinctive thick, perforated multi-layered egg envelope (Rice, 1975b; Rice, 1989). In every sipunculan where early development has been observed, cleavage is unequal, holoblastic and spiral. First cleavage divides the zygote into two cells of unequal size, a large CD and smaller AB cell (Fig. 2). Second cleavage results in a relatively large D cell, with A, B and C blastomeres having similar dimensions. The cleavage plane between C and D is shifted ~30 degrees relative to the animal-vegetal axis (Fig. 2.5). At some time between 2nd and 3rd cell divisions, cleavage spindles move toward the vegetal pole resulting in asymmetric division of the A, B, and C blastomeres to produce 1A, 1B and 1C macromeres that are smaller than (lecithotrophy), or similar in size to (planktotrophy) their respective

Fig. 2. Unequal, holoblastic spiral cleavage in parthenogenetically activated 2-cell, 4-cell, and 8-cell embryos of the sipunculan, *Themiste lageniformis*.

(1) View from the animal pole at the end of first cleavage. (2) View from the animal pole during metaphase in the AB and CD blastomeres prior to second cleavage. Metaphase chromosomes in the CD blastomere are asymmetrically shifted for unequal cell division. (3) Animal pole during prophase of a 4-cell embryo showing the comparatively larger D blastomere. (4) Vegetal pole of a 4-cell embryo showing the large D blastomere and crossfurrow between D and B. (5) View of a 4-cell embryo from the A' quadrant. The A and B blastomeres are noticeably smaller than D. The cleavage plane between C and D is offset ~30 degrees relative to the animal-vegetal axis (dashed line). Polar body is showing through the animal end of the A blastomere. (6) View from the vegetal pole of an 8-cell embryo. The 1D macromere is larger than all other macromeres, and the 1a, 1b, 1c and 1d micromeres, which are larger than their respective macromeres with the exception of 1D, are shifted in a dextral pattern around the embryonic axis, relative to an orientation from the animal pole (sinistral as viewed here from the vegetal pole). Green, DNA in cell nuclei and polar bodies (pb). Laser scanning confocal micrographs. Green, CYTOX® Green labeling of DNA; grayscale, BODIPY® FL phalloidin labeling of F-actin.



1a, 1b, and 1c micromeres, as seen in nemertean embryos, but unlike most spiralian that undergo quartet spiral cleavage. There is no recorded instance of a tier of 'small' 1st quartet micromeres. In all 8-cell embryos observed thus far, the 1D macromere is the largest cell, followed by the 1d micromere. The relatively large 1st quartet micromeres contribute to conspicuously large sipunculan prototroch cells, which are considered to play an important nutritive role later in development (Gerould, 1906). The 2d somatoblast is the largest cell at the 16-cell stage and gives rise to the somatic plate. The 4d cell divides to produce a pair of teloblasts that will form bands of mesoderm (Hatschek, 1883; Gerould, 1906), presumably homologous to endomesoderm in other spiralian where cell lineage/fate studies have been performed. A polar lobe has not been detected, and it is not known when or how the D quadrant is established or if there is a sipunculan organizer, although a combination of unequal cleavage and conspicuously large D macromere suggest the mechanism may be similar to what is implicated in mollusk and polychaete embryos (Clement, 1962; Henry and Martindale, 1999; Henry, 2002; Lambert, 2007, 2010).

No cell-lineage experiments by injection of intracellular tracers have been performed in a sipunculan embryo, and therefore direct cell-fate characterizations in embryos or later stages are unavailable. Gastrulation in sipunculans occurs by epiboly with lecithotrophic development, and by invagination and/or epiboly in species with planktotrophic development (Hatschek, 1883; Åkesson, 1958; Rice, 1967; Rice, 1975b).

In postgastrula embryos, marginal cells of the apical plate become detached from the egg envelope, and form an 'apical groove' encircling the anterior end (Fig. 3). According to Gerould (1906) and Åkesson (1958), these are ectomesoblast cells that elongate and sink beneath the surface of the apical plate, become "spindle-shaped fibers" and give rise to prominent components of larval musculature – two pairs of ventral and dorsal longitudinal retractors that develop in trochophore larvae, and function in both pelagospheara and adult stages (Fig. 4). Åkesson (1958) observed that the anterior ectomesoderm cells connect with posterior ectomesoderm cells that grow in an anterior direction from lateral sides of the larval body wall, posterior to the site where the metatroch will form (but

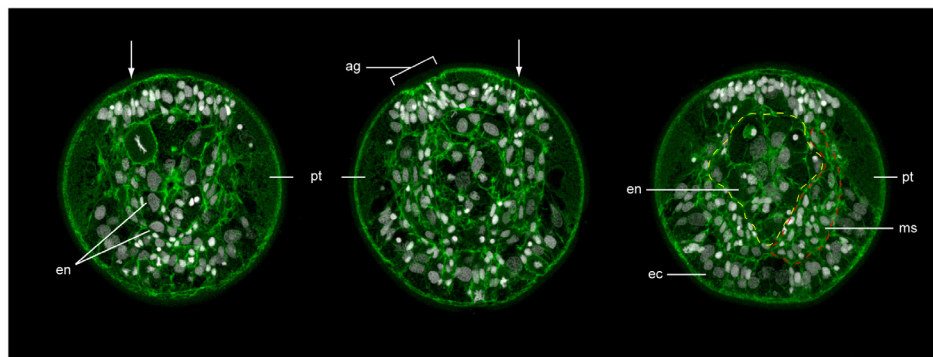


Fig. 3. Postgastrula embryo at the 'apical groove' stage in a direct-developing sipunculan, *Phascolion cryptum*. Left-to-right: laser scanning confocal micrograph series of progressively deeper z-stacks through the medial plane of the same embryo. Ventral views with anterior to the top. Putative 'ectomesoblast' cells of the apical plate migrate internally (arrows) to form the introvert retractor muscles (Gerould, 1906; Åkesson, 1958). Marginal cells of the apical plate move inward and away from the egg membrane of the embryo, forming a circular groove around the apical plate (ag-bracket). A band of large,

non-ciliated prototroch cells encircle the anterior hemisphere. Precursor cells of posterior trunk ectoderm, endomesoderm (red dashed outline) of the primary coelom, and endoderm (yellow dashed outline) of the digestive system are detectable. Grayscale, propidium iodide labeling of DNA in cell nuclei; green, BODIPY® FL phalloidin labeling of F-actin. ag, apical groove; ec, ectoderm; en, endoderm; ms, mesoderm; pt, prototroch cells.

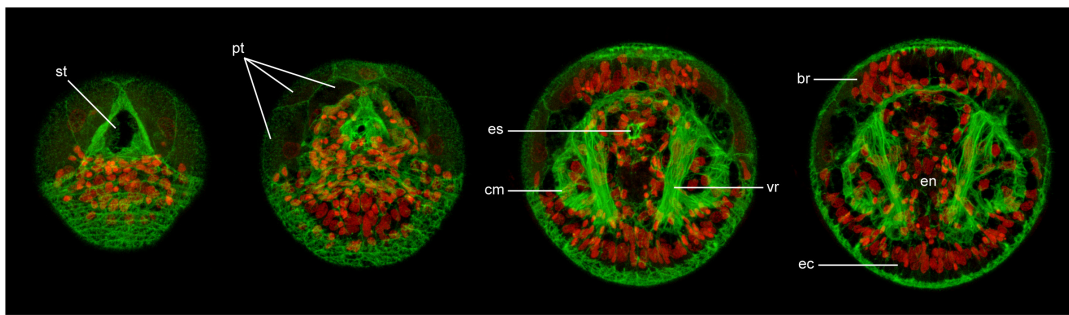


Fig. 4. Anatomy of a lecithotrophic trochophore larva of the sipunculan, *Themiste alutacea*.

Left-to-right: laser scanning confocal micrograph series of progressively deeper z-stacks through the same larval specimen at ~28 hours of development. Ventral views with anterior to the top. A double row of large, ciliated

prototroch cells encircles the anterior hemisphere. The stomodeum is positioned on the mid-ventral side of the episphere between a medial pair of small prototroch cells of the bottom row. The stomodeum is closed to the outside of the larva at this stage and extends dorsally into the lumen of the developing esophagus. Bands of circular muscles are growing toward the body wall. A ventral pair of longitudinal retractor muscles is visible on left and right sides of the developing esophagus and midgut endoderm. The brain is located at the larva's anterior end, bilaterally symmetric, and encircled on its posterior side by the top row of prototroch cells. Cell nuclei of trunk ectoderm are positioned along the internal margin of the hyosphere, posterior to the prototroch. Larval eyes are present (not shown). *br*, brain; *cm*, circular muscles; *ec*, ectoderm; *en*, endoderm; *es*, esophagus; *pt*, prototroch cells; *st*, stomodeum; *vr*, ventral retractor muscles. Red, propidium iodide labeling of DNA in cell nuclei; green, BODIPY® FL phalloidin labeling of F-actin.

see Rice, 1973). Thus, marginal apical plate cells represent one source of ectomesoderm that contributes specifically to introvert retractor muscles, however there is another source of ectomesoderm. The region between the posterior retractor myoblasts and the metatroch is an area of cell proliferation, which is considered the "main center of the longitudinal growth of the larva" and a source of ectomesoderm for the formation of circular muscles (Åkesson, 1958). This would indicate that an annelid-like posterior growth zone is absent during larval development and metamorphosis from a trochophore to the pelagosphaera. Furthermore, this also implies that sipunculan circular muscles originate from ectomesoderm of the trunk body wall, and not a pair of mesotoloblast cells that are typically associated with a 4d mesentoblast origin, typical of annelids and mollusks. Both Gerould (1906) and Åkesson (1958) noted that retractor muscles, and circular muscles that originate in the region of the middle sphincter adjacent to posterior attachment sites of the retractor muscles, are not only of ectomesodermal origin, but are differentiated when the coelomesoblast (4d) is still solid and undifferentiated, prior to formation of the coelom. Longitudinal musculature of the body wall and coelomic linings of the trunk and introvert initiate their development later than retractor and circular

musculature, and are derived from bands of endomesoderm produced by a bilateral division of the 4d mesentoblast.

The stomodeum is formed by an invagination of ectoderm at the site of a completely closed blastopore, and develops on the midventral side, subsurface to a bilaterally symmetric pair of prototroch cells (Figs. 4, 5a, 5b). The prototroch is ciliated in trochophore larvae of indirect developers, or unciliated in direct developing species, and typically consists of a double row of 16-19 large cells encircling the episphere (Figs. 3, 4, 5). Trochophore larvae are capable of swimming and also have an apical tuft of cilia. All sipunculans develop a U-shaped digestive system that is subregionalized into a mouth, esophagus, descending and ascending loops of the intestine, a rectum and anus (Fig. 6). Both the rectum and anus are formed by an invagination of ectoderm on the dorsal side of the trunk sometime after elongation of the trochophore or trochophore-like stage. In trochophore larvae of species with a planktotrophic life history, such as *Nephasoma pellucidum*, the stomodeum, esophagus and midgut undergo relatively rapid development prior to the formation of longitudinal retractor or circular muscles (Fig. 5). It appears that gut formation is a developmental priority in planktotrophic species, in contrast to lecithotrophic development where retractor and circular

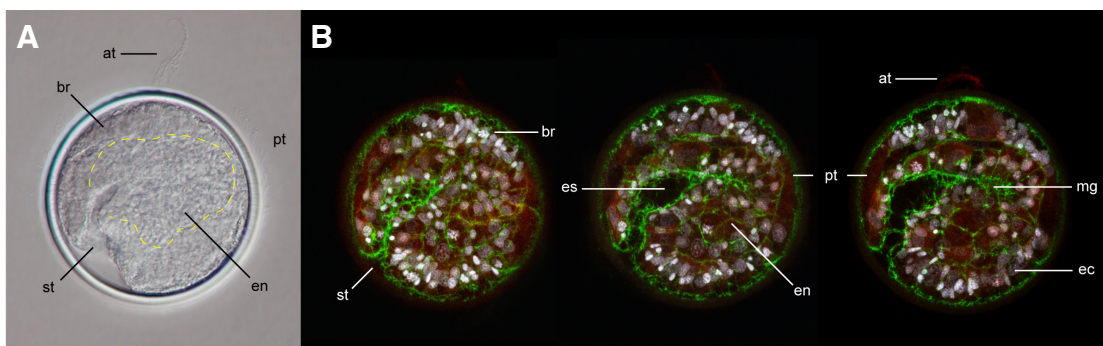


Fig. 5. Early gut formation in the lecithotrophic trochophore larva of the sipunculan, *Nephasoma pellucidum*.

Each image in (A,B) is a lateral view with ventral to the left side, anterior to the top. (A) DIC composite micrograph of the trochophore near the medial plane. This non-feeding larval stage has an apical tuft of cilia, a broad prototrochal region typical of sipunculan

trochophores, and is capable of directional swimming. (B) Left-to-right: laser scanning confocal micrograph series of progressively deeper z-stacks through the same larva at ~30 hours of development. Epithelia are formed along margins of the stomodeum, esophagus and stomach of the midgut, which are connected by the developing gut canal. Ciliated prototroch cells encircle most of the episphere and are relatively low in yolk content. Groups of cell nuclei distinguish precursor regions of the brain, midgut and trunk in a succeeding larval stage, the pelagosphaera. Grayscale, propidium iodide labeling of DNA in cell nuclei; green, BODIPY® FL phalloidin labeling of F-actin; red, antibody labeling of acetylated α -tubulin. *at*, apical tuft; *br*, brain; *ec*, ectoderm of the trunk; *en*, endoderm; *es*, esophagus; *mg*, midgut/stomach; *pt*, prototroch; *st*, stomodeum.

muscle fibers are differentiated well before gut epithelia or distinct subregions of the digestive system (Figs 4, 5). Additionally, in mid-to-late trochophore stages, bilateral clusters of cell nuclei at the larva's anterior end indicate formation of a cerebral ganglion, and a linear non-metameric assemblage of cell nuclei along the ventral midline mark the position of the ventral nerve cord (Figs. 4, 5, 6). The brain and ventral nerve cord appear to be at more advanced stages of development in planktotrophic species when compared with lecithotrophic modes at a similar time point, which is implied by visualization of more structured aggregations of cell nuclei in each respective region of the central nervous system. Most likely, this difference relates to rapid development of a functional larval nervous system involved with swimming and feeding behaviors compared with slower development of juvenile and adult counterparts that are not required until after metamorphosis. During the first metamorphosis from a trochophore to a pelagosphaera larva, retractor, longitudinal, and circular muscles initiate an elongation of the trochophore by introversion and contraction along the anterior-posterior axis (Fig. 6). As metamorphosis proceeds in both lecithotrophic and planktotrophic species, the anterior or pre-trochal portion of the egg envelope may be discarded entirely or in part, and typically the posterior or post-trochal envelope will be transformed into larval cuticle of the trunk region. Shedding or transformation of the egg envelope does not appear to correlate with specific developmental patterns or the amount of yolk present during metamorphosis (Rice, 1985). Additional metamorphic changes that accompany an overall elongation of the posttrochal body region include a reduction of the prototroch, and an expansion of the main coelomic compartment of the trunk.

In pelagosphaera larvae there are several specialized organs. A metatroch is present in all indirect modes of development where a pelagosphaera is observed, and consists of a postoral band of cells with compound cilia that beat in an anterior-to-posterior direction, and musculature enabling extension and contraction of the ciliary

band (Fig. 7). The metatroch becomes the prominent locomotory organ and is not utilized in feeding, unlike metatrochal bands in some polychaete and mollusk larvae (Strathmann, 1978; Rouse, 1999, 2000). When the metatroch becomes functional, part of the stomodeum is everted to form a ciliated field on the ventral surface of the head. A remnant of the prototroch forms a "horseshoe-shaped" ciliated band on the dorsal side of the head, with an undermined function. At the posterior end of the larva, there is typically a retractable terminal organ enabling attachment of the larva to various substrata (Figs. 6, 7). In all pelagosphaera larvae, ventral and dorsal pairs of retractor muscles are completely functional and facilitate contraction of the anterior head and metatroch, and in some species, contraction of the larva's posterior end, within the central body cavity. In all life history patterns, a pair of metanephridia will develop for excretion of coelomic materials, and the sorting, collection and spawning of gametes in adult worms. Pelagosphaera of planktotrophic species have additional organs associated with feeding, including a functional stomach, with a muscular sphincter between the esophagus and stomach. In these larvae there is a ventral ciliated groove extending from the anterior end of the head to the mouth, a buccal organ that is protrusible through the mouth, and a large ventral lip extending posteriorly from the mouth with a lip gland and external lip pore that together serve a secretory function (Fig. 7). Extensive musculature is associated with the buccal organ, esophagus, sphincter, terminal organ, and anal canal (Fig. 7A). And finally, there is a conserved left-right (L/R) asymmetry in the digestive system, whereby the intestine is connected to the stomach on its right-posterior end (Fig. 8). From this junction, the intestine descends toward the posterior of the larva, turns in a dorsal direction, and then ascends anteriorly toward its connection with the anus on the dorsal body wall of the trunk. This pattern of L/R asymmetry is detectable in all of the planktotrophic larval forms examined in the laboratory thus far, and may be the directional source of helical gut coiling observed in the majority of

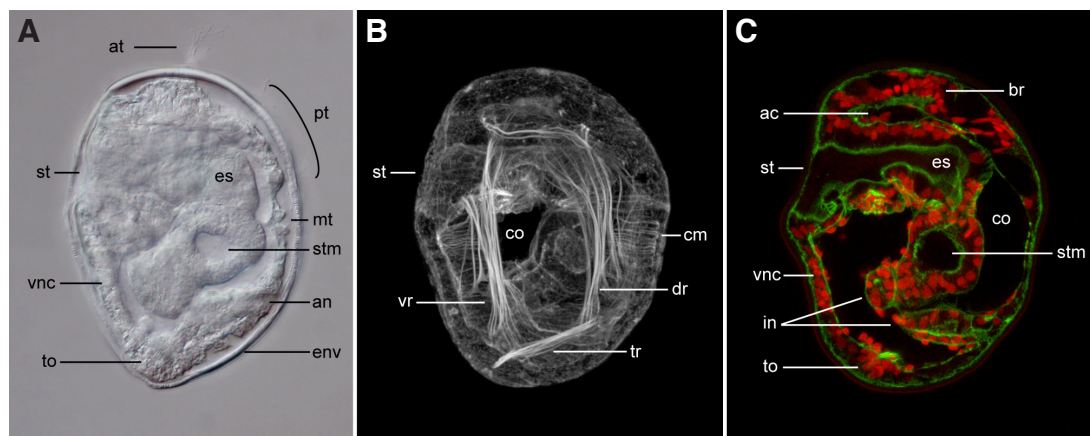


Fig. 6. The elongation-stage of metamorphosis from a trochophore larva to a pelagosphaera larva of the sipunculid, *Nephasoma pellucidum*. Each image is a lateral view with ventral to the left side, anterior to the top. **(A)** DIC composite micrograph showing an elongation of the post-trochal hemisphere during the first metamorphosis at ~58-60 hours of development. The gut

is subregionalized into the stomodeum, esophagus, stomach, intestine and anus. The prototroch is undergoing a reduction in size and change in position, and several new larval organ systems are functional, including the metatroch, terminal organ, ventral nerve cord and musculature. **(B,C)** Laser scanning confocal micrographs of the same specimen at ~58-60 hours showing a large z-stack projection of the muscular system and tissue margins **(B)**, and a small z-stack projection through the medial plane outlining the digestive organ system **(C)**. The lumen of the gut canal is lined by Actin filaments and extends from a stomodeum on ventral-anterior to a terminus of the intestine on the dorsal-posterior body wall. There is a large trunk coelom surrounding the gut, and a conspicuous cavity between the brain and esophagus that may represent development of the tentacular coelom. Red, propidium iodide labeling of DNA in cell nuclei; grayscale **(B)** and green **(C)**, BODIPY® FL phalloidin labeling of F-actin. ac, anterior coelom; an, anus; at, apical tuft; br, brain; cm, circular muscles; co, coelom; dr, dorsal retractor muscles; env, egg envelope; es, esophagus; in, intestine; mt, metatroch; pt, prototroch; st, stomodeum; stm, stomach; to, terminal organ; tr, terminal organ retractor muscles; vnc, ventral nerve cord; vr, ventral retractor muscles.

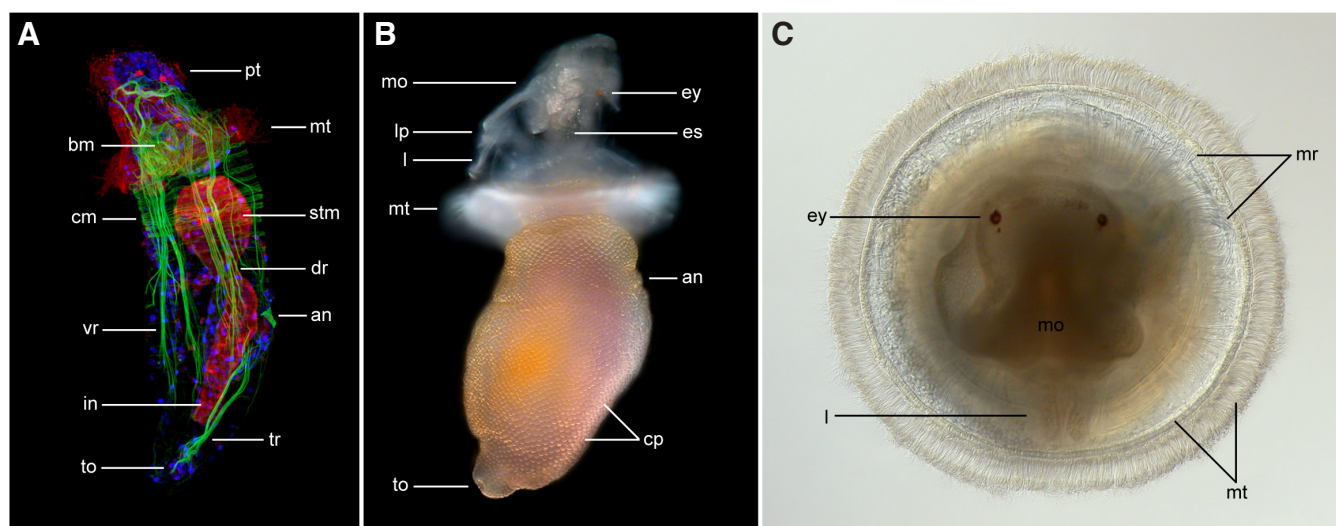


Fig. 7. Anatomical features of planktotrophic pelagosphera larval forms within Sipuncula. (A) Laser scanning confocal micrograph of the planktotrophic pelagosphera larva of *Nephasoma pellucidum* at ~3 days of development. The image is a z-stack projection in lateral view, with anterior to the top. This specimen is approximately 280 micrometers in length along the anterior-posterior axis. Green, labeling of F-actin showing circular muscle bands, pairs of longitudinal and terminal organ retractor muscles, musculature of the buccal organ and esophagus, and contractile ring muscles encircling the anus. Red, labeling of acetylated α -tubulin shows the larval eyes, ciliation on the ventral side of the head and mouth region, prototrochal and metatrochal cilia, and cellular elements in the stomach and intestine. Blue, propidium iodide labeling of DNA in cell nuclei of the brain, terminal organ and throughout the trunk. (B) DIC fiber-optic light micrograph composite of a teleplanic pelagosphera larva from the Florida current of the Gulf Stream. Lateral view with anterior to the top. Specimen is approximately 1.0 mm in length. The lower lip, lip pore and ventrally grooved mouth are part of a complex of anterior feeding structures. The anus is positioned on the anterior-dorsal side of the trunk. (C) DIC micrograph composite of a teleplanic pelagosphera larva from an anterior view, with its dorsal side to the top. This view shows the metatrochal band of compound cilia, fully extended and integrated by a complex system of metatrochal retractor muscles. The metatroch is the primary organ of locomotion in the water column by each form of pelagosphera larva, which is a unique type of larva within Metazoa. an, anus; bm, buccal organ musculature; cm, circular muscles; dr, dorsal retractor muscles; ep, epidermal papillae; es, esophagus; ey, larval eye; in, intestine; mo, mouth; mt, metatroch; pt, prototroch; stm, stomach; to, terminal organ; tr, terminal organ retractor muscles; vr, ventral retractor muscles.

adult sipunculan species. When reviewing all of the larval organ systems, individually or combined, there does not appear to be any definitive morphological evidence of metamerism, or distinctly organized boundaries between cells in endodermal, mesodermal or ectodermal tissues to suggest cellular rudiments of segmentation.

Brief discussion of sipunculan characters

Recognized features of a spiral-cleavage character complex include the conserved orientations of cleavage spindles and micromeres around the animal-vegetal axis, and blastomere fates in embryonic and later stages of development (Wilson, 1892; Costello and Henley, 1976; Henry and Martindale, 1999; Hejnal, 2010). Each set of characters is more or less directly comparable among spiralian taxa that exhibit quartet spiral cleavage. Yet, not only are there variations among the 'conserved' features of cell division and cell fate, but also putative modifications to these and other characters that must have contributed to the diversity of extant spiralian body plans. It would be helpful to broaden the range of characters to include cleavage asymmetries, alternative cell functions, novel organs, life history patterns, and more. These and other characters may provide only indirect comparisons if they are found to be sipunculan novelties derived from an ancestral polychaete lineage, and it therefore becomes difficult to reconstruct the developmental origins of such characters, especially when the inferred polychaete sister taxon is quite different (Weigert *et al.*, 2014). And in likely cases of secondary loss or major modifications

in the sipunculan stem lineage, such as the absence of a posterior growth zone, terminal anus or segmentation, morphology becomes misleading and of limited relevance in phylogenetic hypotheses based exclusively on molecular characters. Nevertheless, sipunculans possess a number of putative character modifications that may be indicators of developmental events that influence the production of contrasting morphologies not only within Annelida, but also among different spiralian models.

Freeman and Lundelius (1992) considered the symmetry of first cleavage to be an important character. Observations from quartet spiral-cleaving embryos show that both equal and unequal cleavage patterns are found among mollusks and annelids (see Lambert, 2010). However, equal cleavage is the only pattern observed in the embryos of polyclad turbellarians (Surface, 1907; Boyer and Henry, 1998; Rawlinson, 2010), nemertean (Martindale and Henry, 1995; Henry and Martindale, 1998; Maslakova *et al.*, 2004), and echiurans (Torrey, 1903; Newby, 1932). This shows that equal cleavage appears to be common, as well as a 'fixed' pattern in three distinct spiralian lineages, and perhaps more. The more widespread observation of equal cleavage and its 'primitive' cell-fate induction mechanism suggest that equal quartet spiral cleavage is the ancestral form of development for spiralian lophotrochozoans (Freeman and Lundelius, 1992; van den Biggelaar *et al.*, 1997; Henry and Martindale, 1999). As previously mentioned, every sipunculan embryo examined thus far exhibits unequal quartet spiral cleavage (Fig. 2). Unequal cleavage is thought to be a derived condition that correlates with early determination of

the D-quadrant, and precocious specification of the dorsal-ventral (D/V) axis (Freeman and Lundelius, 1992). Henry and Martindale (1999) noted that unequal cleavage is associated with “a multitude of derived characters” in several groups of mollusks, and the “highly modified forms of development” displayed by clitellate annelids. The Echiura and Sipuncula are now considered part of the annelid radiation (Struck *et al.*, 2007; Dunn *et al.*, 2008; Struck *et al.*, 2011), where equal and unequal cleavage patterns are scattered among the branches. The equal-cleaving echiurans may have evolved among the unequal-cleaving polychaetes of Capitellida (Struck *et al.*, 2007). However, with the exclusively unequal-cleaving sipunculans currently placed near the base of the annelid tree along with Chaetopteridae (Dordel *et al.*, 2010; Struck *et al.*, 2011), which also exhibit unequal cleavage and multiple character modifications (Irvine *et al.*, 1999), we now have new and intriguing problems for the evolution and development of annelid biodiversity. If the relative positions of sipunculans and chaetopterids receive subsequent support, as implied by a recent analysis of basal annelid taxa (Weigert *et al.*, 2014), it would suggest that the derived form of spiral cleavage (e.g. unequal cleavage, precocious D/V specification) was already present early in the annelid radiation.

Associated with unequal cleavage, sipunculans consistently generate a tier of large micromeres during the third round of embryonic cell division. In all developmental modes, first quartet micromeres eventually contribute to a double row of 16–19 cleavage-arrested prototroch cells with a notably high yolk content in lecithotrophic trochophore and trochophore-like (direct development) stages (Fig. 4). According to Gerould (1906), internal degeneration of the large prototroch cells during metamorphosis enables “the entire substance of the prototroch” to enter into the coelom, which provides substantial nutrition during further development. Nemerteans also produce conspicuously large 1q micromeres and prototroch cells, as shown by the pattern of equal cleavage in *Carinoma tremaphoros*, which leads to the production of ~40 prototroch cells (Maslakova *et al.*, 2004). There are ~25 prototroch cells in the trochophore larvae of the polychaetes, *Amphitrite* and *Clymenella* (Mead, 1897), ~32 prototroch cells in the gastropod trochophore of *Patella vulgata* (Dictus and Damen, 1997), and hundreds of prototroch cells in the polychaete, *Capitella teleta*, which in contrast to the other species listed here, likely do not become mitotically arrested during cleavage and therefore produce many more prototroch cells (Meyer *et al.*, 2010). It is not clear whether prototroch degeneration provides

energy resources during development in any of those species. What is known is that in lecithotrophic sipunculan embryos, yolk reserves are sequestered into enormous trochoblast cells for energy utilization during the development of pelagosphaera larvae and their subsequent metamorphosis (Gerould, 1906; Åkesson, 1958; Rice, 1967; Rice, 1975b, a, 1985). This may represent either an understated role, or a novel cellular function in spiralian development.

Another feature of sipunculan development is the apical groove (Fig. 3). The apical groove is a transient morphogenetic signature of 1q ectomesoderm ingression in sipunculan embryos. This character is very pronounced on the anterior end in all embryos, regardless of life history pattern. According to Gerould (1906), there is a “diamond-shaped rosette” of four cells in the center of the apical plate corresponding to the apical organ. Marginal cells of the apical plate sink beneath the surface, pulling the plate margins away from the egg envelope to form a circular apical groove around the center of the plate. The cells that sink inward are first quartet (1q) ectomesoblasts that form the larval retractor muscles. Thus, sipunculan retractor muscles develop from 1q micromeres (Gerould, 1906). Larval retractors and circular muscles are retained in adult stages, with retractors providing the mechanism of introvert retraction throughout the clade. Thus far, a 1q source of ectomesoderm is described in one echiuran (Torrey, 1903), and a leech (Huang *et al.*, 2002). There are no records from intracellular lineage-tracing studies of ectomesoderm being derived from 1q micromeres in nemerteans, polyclad turbellarians, mollusks or polychaete annelids (Hejnl *et al.*, 2007; Meyer *et al.*, 2010), and thus appears to be a relatively rare modification of 1q fate among spiralian.

As Gerould (1906) has inferred, and Åkesson (1958) has carefully determined, there is a second source of ectomesoderm that contributes to posterior rudiments of introvert retractors, and the circular muscles in larvae and juvenile worms. This second source of ectomesoderm comes from a postoral region on mid-lateral sides of trochophore larvae, most likely from descendants of second (2q) or third quartet (3q) micromeres where ectomesoderm is typically derived in mollusk and annelid embryos (Anderson, 1973; Hejnl *et al.*, 2007; Meyer and Seaver, 2010). This postoral, mid-lateral body wall of sipunculan trochophore larvae is also a region of “rapid cleavage activity” (Åkesson, 1958). In addition to ectomesoblast differentiation during circular muscle development, cell proliferation in this region becomes the main center for longitudinal growth of

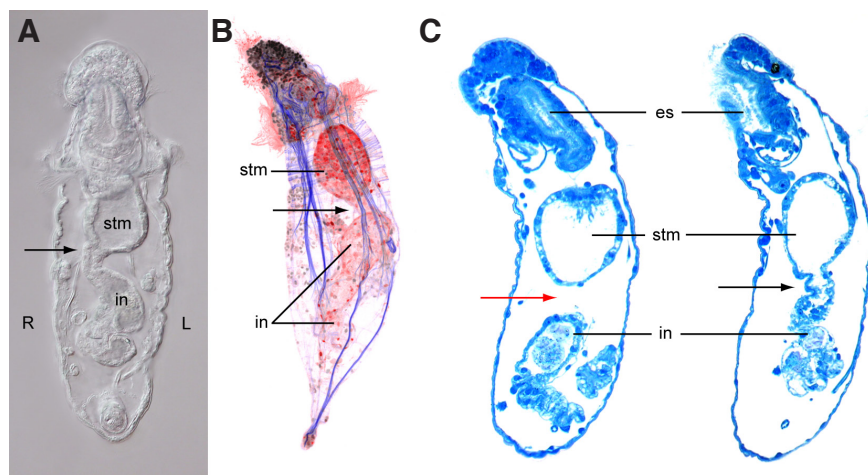


Fig. 8. Left-right asymmetry during development of the digestive organ system in the sipunculan, *Nephrosoma pellucidum*. (A) DIC micrograph composite of a planktotrophic pelagosphaera larva at ~3 days of development. Ventral view with anterior to the top. The intestine is connected to the right-posterior side of the stomach (black arrow). (B) Laser scanning confocal z-stack projection of a planktotrophic pelagosphaera larva at ~7 days of development. Left lateral view with anterior to the top. The intestine extends from the stomach on the right side (far side) of the larvae (black arrow). (C) 1.0 micrometer thin sections through the stomach of a planktotrophic pelagosphaera larva at ~7 days of development. Lateral views with anterior to the top. There is no connection between the stomach and intestine on the left side of the larva (red arrow); however, their respective lumens are connected on the right side (black arrow). es, esophagus; in, intestine; L, left; R, right; stm, stomach.

the larva, and dominates as a growth zone during postlarval and juvenile development in the sipunculan, *Phascolion strombus* (Åkesson, 1958). In the direct-developing sipunculan, *Gofingia minuta*, longitudinal growth is transferred to a more posterior region over time, but is never terminal. Lateral proliferation of ectoderm and mesoderm was described in a polychaete prior to segment addition from a posterior growth zone (Seaver *et al.*, 2005). Yet, in contrast to the prepygidial region of typical segment formation in many of the late larval and juvenile stages of polychaete annelids (Anderson, 1973; Wanninger *et al.*, 2005; Kristof *et al.*, 2011), there is no clear evidence of a definitive posterior growth zone in Sipuncula.

There are two distinct larval stages observed among sipunculan life history patterns. Sipunculan trochophore larvae are always lecithotrophic, with a ciliated prototroch and apical tuft. In many species, the trochophore will metamorphose into a pelagosphaera larva (Selenka, 1875; Gerould, 1906; Rice, 1976, 1981, 1985). The pelagosphaera is unique among all metazoans (Mueller, 1850; Scheltema and Hall, 1975; Rice, 1985), and considered an advanced larval form that was modified from the trochophore (Jägersten, 1972; Freeman and Lundelius, 1992). All pelagosphaera larvae have a postoral circling of cilia used exclusively for locomotion, the metatroch. Compound cilia of the pelagosphaera metatroch beat downward in a posterior direction and have not been observed to collect or move particles toward the mouth, in contrast to the function of metatrochs in the opposed-band feeding systems of other spiralian larval types (Strathmann, 1978; Rouse, 1999; Nielsen, 2012). Only one cell-lineage of a polychaete embryo indicates that the metatroch is formed from 3c and 3d micromere descendants (Woltereck, 1904). In two other polychaetes, cell-lineage studies show that 3c and 3d micromeres contribute to mesoderm, although neither one of those species has a metatroch (Treadwell, 1901; Meyer *et al.*, 2010). An embryonic origin of the sipunculan metatroch has not been determined, and thus with regard to its cellular or functional homology among spiralian, it remains a mystery.

Planktotrophic larvae have additional structures associated with feeding, including a protrusible buccal organ, ciliated lip, lip gland, lip pore and a functional U-shaped digestive system with a bulbous stomach (Rice, 1967, 1976, 1993). The anus is located on the dorsal body wall in larvae and adults of all species, and there is no evidence that the anus derives from the blastopore, or rotates from the blastoporal region during development. During formation of the anus, a proctodeum develops internally from endodermal cells, which grow in a dorsal direction to meet a "cluster of ectoderm cells" on the dorsal side of the larval body that eventually invaginate to form the anus (Gerould, 1906). During metamorphosis of the pelagosphaera, anterior larval feeding structures are lost, and the mouth is repositioned from the ventral side of the larval head to the anterior end of the juvenile worm where an array of tentacles develops. The digestive system proper (mouth, esophagus, intestine, rectum, anus) is retained in juvenile and adult worms. And with the exception of ciliary bands, most other larval organs are retained as well, including the central nervous system, one or two nephridia, and the circular, longitudinal, and retractor musculature.

Finally, pelagosphaera larvae develop a unique organ, the terminal organ. Ruppert and Rice (1983) characterized the ultrastructure and function of the terminal organ, observing its properties for adhesive attachment of larvae to substrates, the secretion of mucus involved in larval motility, and a possible sensory function for site selection during settlement and metamorphosis. The terminal

organ has a dedicated pair of retractor muscles, which enable full retraction and manipulation of the terminal bulb position when in contact with sediments, buoyant materials in the water column, and other sipunculan larvae. The terminal organ is thought to have independently evolved as a unique sipunculan organ system.

Summary of an extended sipunculan character complex

- Unequal cleavage in all species
- Micromeres \geq Macromeres in 8-cell embryos
- Large prototroch cells with nutritive function
- 1q ectomesoblast origin of introvert retractor muscles
- Longitudinal growth by mid-body cell proliferation
- Trochophore larva is always lecithotrophic
- Pelagosphaera larva unique among metazoans
- Postoral metatroch with exclusive locomotory function
- U-shaped digestive system with mid-dorsal anus
- Terminal organ system with retractor muscles
- No segmental rudiments

Toward a sipunculan model of spiralian development and evolution

As previously mentioned, there is a history of fundamental studies on sipunculan reproduction, embryogenesis, organ systems and life history patterns. And when combined with related work on ecology and metamorphosis of larval forms, there is a comprehensive body of literature on the developmental biology of Sipuncula. Most of that research involved basic descriptive studies with live specimens, and/or histological preparations for imaging and analysis with electron and compound light microscopes. However, in the broader context of comparative spiralian development, that work has been surpassed by no less than two decades of modern molecular, genetic and cellular investigations with spiralian representatives from the annelids, mollusks, nemertean, and both polyclad and acoel flatworms. Only in recent years has there been some progress toward utilizing sipunculan taxa for modern comparative studies of animal development. Several projects have published information on particular structures of the nervous and muscular organ systems (Wanninger *et al.*, 2005; Kristof *et al.*, 2008; Schulze and Rice, 2009a; Kristof *et al.*, 2011). Unfortunately, those investigations were initiated in trochophore or later stages of development, and not when genetic or morphogenetic mechanisms are thought to specify blastomeres, determine embryonic axes, segregate germ layers, or regulate other critical events that pattern the sipunculan body plan. To find out how cell types and organ systems develop and evolve, including the suite of characters we have described above, a sipunculan model should be established where workers would have relatively reliable access to the eggs, embryos and larvae of reproductive adult worms. Based on some recent progress in the laboratory, we are building a case for including at least one sipunculan species, among others, as an important comparative model of the spiralian developmental program.

Schulze and Rice (2009b) initially proposed *Nephasoma pelucidum* as a "good model species" for research on sipunculan development. Adult worms typically spawn within hours of extraction from rubble, embryos develop rapidly, larvae are transparent, and development includes the ancestral life history pattern of planktotrophy. In spite of these attributes, the feasibility of utilizing *N.*

pellucidum as a model sipunculan is problematic. Field collection and extraction are laborious, very few adults are obtained for the effort, and although they readily spawn in the laboratory, they do not spawn a substantial number of eggs, or spawn over extended time periods, and thus the primary goal – reliable access to eggs, embryos and larvae – is not achieved. We have also assessed the feasibility of utilizing *Themiste alutacea* and *Phascolion cryptum* as experimental models, and found that reliable access to developmental material is also an issue. Yet, when considering questions of life history evolution within Sipuncula, *N. pellucidum*, *T. alutacea* and *P. cryptum* exhibit highly contrasting developmental modes, which is an ideal framework for comparative experiments, and we have established working gene expression protocols in each of them. As an example, we have begun to characterize the expression patterns of regulatory genes that are known to be involved in metazoan gut development (Fig. 9 A-C). The list of candidate genes includes *foxA*, *brachyury*, *wingless*, and the parahox trio, *gsx*, *xlox* and *cdx*. Furthermore, we have produced multiple sets of confocal micrographs for all three species, which enable detailed comparisons of embryonic and larval development, and accurate interpretations of gene expression patterns (Fig. 9D). When comparing preliminary results from our work with some previous studies (Arendt *et al.*, 2001; Martindale *et al.*, 2004; Oliveri *et al.*, 2006; Boyle and Seaver, 2008; Hejnal and Martindale, 2008; Boyle and Seaver, 2010; Boyle *et al.*, 2014), several of the candidate genes show a conserved pattern of transcription along the anterior-posterior axis of the gut, typically associated with particular subregions such as ectoderm of the foregut and

hindgut, and endoderm of a centralized digestive cavity or midgut (Fig. 10). These expression patterns suggest that regardless of the sac-shaped, tube-shaped, or U-shaped gut configurations observed throughout Metazoa, genetic regulation of gut development is most likely an ancient process.

Upon careful consideration of other models, including the species presented in this manuscript, there is one species that exhibits a number of favorable characteristics – *Themiste lageniformis* Baird, 1868. This particular species is established in the Western Pacific from Japan to Australia, including Hawaii, throughout the Indian Ocean, the southern coasts of Africa, and in Cuba and Florida (Cutler and Cutler, 1988). Ecologically, *T. lageniformis* is the most common and most studied species in the genus, and is typically found within oyster beds, coral rubble and soft rock (Pilger, 1987; Cutler and Cutler, 1988; Cutler, 1994). The adults are relatively large worms that are capable of spawning thousands of big yolky eggs (~140 μm) from mid-summer through late fall months (Pilger, 1987). Unique among sipunculans, *T. lageniformis* can also reproduce by parthenogenesis (Pilger, 1987), and their oocytes may be extracted and chemically activated to initiate an unequal, unipolar, holoblastic spiral cleavage program (Fig. 2). Therefore, access to multiple stages of development does not appear to be a barrier to progress with this species. Previously, Boyle and Seaver (2010) generated a developmental timeline and staging schematic with corresponding laser scanning confocal micrographs, and established culturing and fixation methods for embryonic and later stages. The life history pattern is lecithotrophic with a non-motile trochophore-like stage, and a motile pelagosphera larva that un-

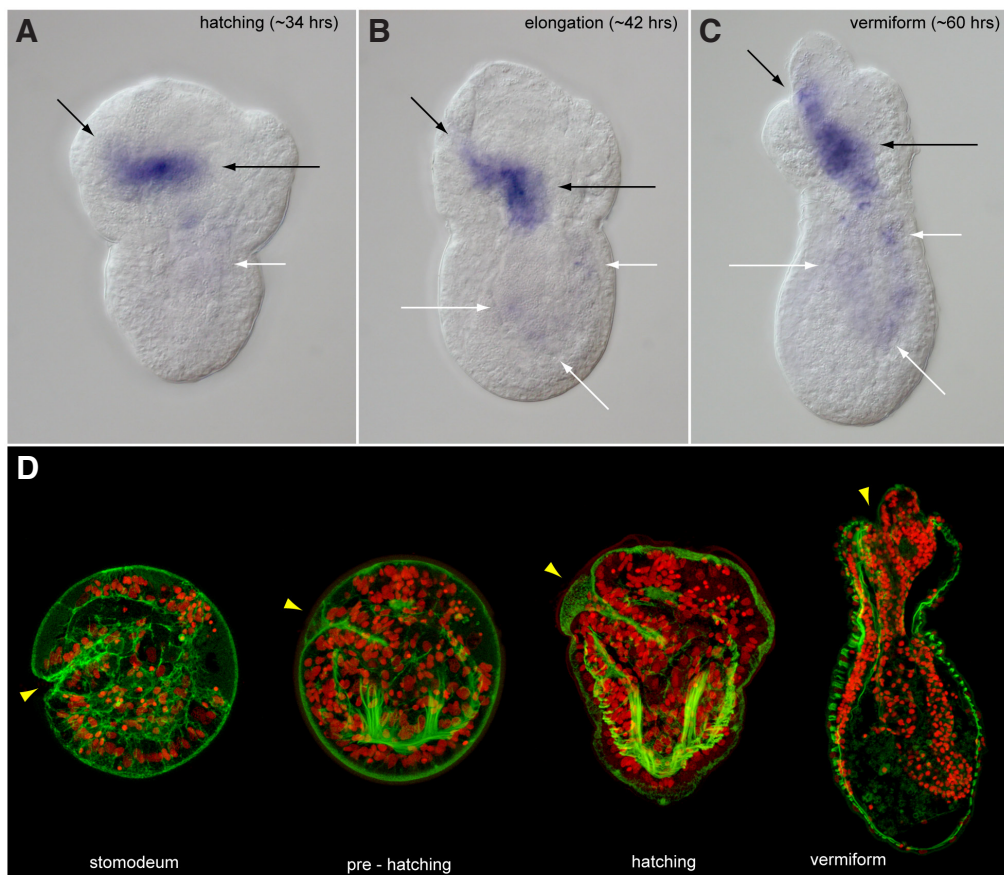


Fig. 9. Expression of the *forkhead* gene (*foxA*) during gut development and morphogenesis in the direct-developing sipunculan, *Phascolion cryptum*. (A-C) DIC micrographs of *foxA* expression by whole-mount in situ hybridization. Lateral views with anterior to the top. Transcripts of the *foxA* gene are detectable within the developing esophagus (black arrows) and endoderm along descending and ascending regions of the U-shaped intestine (white arrows). Expression appears to be stronger in the esophagus, and is persistent during morphogenetic rotation of the esophagus from the ventral side to the anterior end within hatching, elongation and crawling vermiform stages. (D) Laser scanning confocal z-stack projections through the medial planes of selected developmental stages. Each specimen is oriented in lateral view with ventral to the left side, and anterior to the top. These four stages show a morphogenetic rotation of the esophagus in a dorsal direction from ventral to anterior (yellow arrowheads), where a crown of tentacles will subsequently develop around the mouth in the juvenile sipunculan.

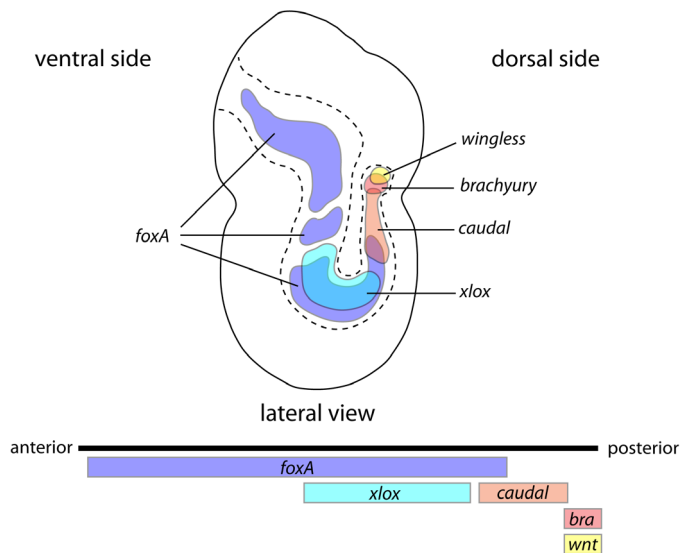


Fig. 10. Schematic of candidate gene expression patterns along the digestive organ system in the direct developing sipunculan, *Phascolion cryptum*. The expression patterns of six candidate genes were analyzed during development in three species of sipunculan worms by whole-mount *in situ* hybridization. Preliminary expression analyses indicate that five of the six genes (*foxA*, *brachyury*, *wingless*, *xlox*, *cdx*) are transcribed within the sipunculan digestive system, from ventral-anterior to dorsal-posterior, following the U-shaped configuration of sipunculan gut morphology. In the direct-developing species, *Phascolion cryptum*, each one of the five genes exhibit expression patterns that are consistent with having roles during gut development within Sipuncula. These results serve to establish working gene expression protocols in multiple sipunculan species with contrasting life history patterns, and represent progress in utilizing this unique group of spiralian animals for studies in the field of evolution and development.

dergoes metamorphosis to a juvenile worm within approximately two weeks of development. Although *T. lageniformis* does not represent the ancestral sipunculan life history pattern, it does exhibit most of the prominent developmental characters described above, including large prototroch cells, distinct metatroch, a terminal organ and a U-shaped digestive system without the specialized feeding structures found in planktotrophic pelagosphaera larvae. Methods for maintaining laboratory cultures of adult worms over extended time periods have not yet been resolved, however the adults are readily obtained from field sites in Florida, may be kept in bowls of filtered seawater for months at a time, and have been successfully shipped to several laboratories without compromising their reproductive or developmental capacity. Most importantly thus far, the first gene expression patterns in a member of Sipuncula were successfully and routinely performed by *in situ* hybridization in *T. lageniformis* (Boyle and Seaver, 2010). In that study, the expression patterns of *foxA* and *GATA* transcription factor genes were described in embryonic and larval stages of development. Since then, additional genes have been cloned and analyzed for expression. However, at this time there are no publically available genomic or transcriptomic data, and no modern cell lineage or embryonic fate map for *T. lageniformis* or any other sipunculan taxa. In order to move this group of spiralian forward as valuable comparative models, we will need to generate such data as demonstrated by intracellular fate-mapping studies (Boyer *et al.*,

1996; Henry and Martindale, 1998; Maslakova *et al.*, 2004; Hejnol *et al.*, 2007; Meyer *et al.*, 2010; Meyer and Seaver, 2010), and several next-generation sequencing projects (Lambert *et al.*, 2010; Riesgo *et al.*, 2012; Simakov *et al.*, 2012; Lapraz *et al.*, 2013). If successful, this would provide the basic information and technology to pursue comparative bioinformatics and functional genomics research (Newmark and Sanchez Alvarado, 2002; Lambert, 2010; Lambert *et al.*, 2010).

Future directions

The next major step in sipunculan research includes sequencing populations of mRNA that code for transcription factors, signaling pathway genes, and members of gene families that build embryos, larvae and adult worms. Thus far, we have purified RNA from early stages (cleavage, gastrulation), middle stages (post-gastrula, larva) and the adults of two species with the most contrasting life history patterns: direct lecithotrophic development (*Phascolion cryptum*), and indirect planktotrophic development (*Nephasoma pellucidum*). Assembly and analyses of the six 'developmental transcriptomes' are underway, with expected sequence coverage of ~100 million paired-end reads for each of the six samples. A second phase of RNA-Seq is planned for *Themiste lageniformis*, with RNA targets to include microRNA and maternal transcripts, as well as mRNA pools from different stages of development in an effort to establish *T. lageniformis* as a model sipunculan. The immediate utility of comparative transcriptome inventories is clear. Deep coverage of expressed developmental genes will most likely enable verification of the presence or absence of particular genes and gene family members between developmental stages, between life history patterns, and between sipunculans and other metazoans. Moreover, the time required to move from gene identification and cloning to gene expression by whole mount *in situ* hybridization would be significantly shortened. Currently, without any genomic data, this process requires months of labor-intensive degenerate and RACE PCR methods.

The first bioinformatic and gene expression targets should be informative to many investigators. With an ancient fossil record, unique body plan, and new position within Annelida, sipunculans will likely inspire many important studies on the origin, evolution and/or loss of segmentation in one of only three segmented lineages within Metazoa (Seaver, 2003; Huang *et al.*, 2004; Struck *et al.*, 2011; Hannibal and Patel, 2013). Do the non-segmented sipunculans possess the basic repertoire of 'segmentation' genes found in other annelids, arthropods and vertebrates? If so, when and where are they expressed? Questions will also be aimed at whether the full complement of Hox genes is present in Sipuncula, and if particular members are expressed along the anterior-posterior axis during direct and/or indirect development; Where mesoderm and muscle patterning genes are expressed, especially during the time when 1q ectomesoderm lineages are building sipunculan retractor muscles; Which members of the TGF-beta family are present, and does expression correlate with L/R asymmetry during sipunculan gut development; And, do the expression patterns of neural genes support or refute claims of segmental remnants in the ventral nerve cord during sipunculan development (Kristof *et al.*, 2008; Wanninger *et al.*, 2009). Of course this represents only a partial list of outstanding questions. In the strict context of spiralian embryology, sipunculan research has lagged behind while some

of the most basic and important problems have been worked out by decades of intensive studies on early determination of the D-quadrant, and precocious specification of the dorsal-ventral (D/V) axis, which are both associated with unequal cleavage (Freeman and Lundelius, 1992; Henry and Martindale, 1999). Therefore, future gene expression and bioinformatic experiments must also focus on the transcription factors and signaling pathways that are currently thought to regulate specification of the mesentoblast and the spiralian 'organizer' (Henry, 2002; Lambert, 2007), and other equally fundamental questions regarding sipunculan-specific cleavage asymmetries, and candidate genes that are likely to pattern some of their unique organ systems.

The second major step will include an attempt to produce the very first embryonic fate map for a member of Sipuncula. As mentioned above, reproductive biology in *T. lageniformis* enables access to not only high numbers of eggs, but chemically controlled activation of eggs to obtain sequential batches of embryos. This is a fundamental requirement for intracellular injection and cell-tracing experiments. Sipunculan eggs and embryos currently present a challenge due to their relatively thick egg envelopes, however, we are hopeful that penetration and permeability issues may be chemically or mechanically alleviated. With *T. lageniformis* having ~ 140 µm eggs, unequal cleavage, conspicuously large micromeres, 1q ectomesoderm, a unique larval form and other distinct characters, a sipunculan cell lineage/fate map would likely broaden our knowledge of spiralian character modifications. And, a delivery system would finally be in place in order to perturb gene expression and gene function. This would be a critical advancement in sipunculan developmental biology. Initially, functional experiments would be aimed at single transcripts such as *foxA* (Fig. 9), for which expression patterns have been characterized in many animals, including several annelids (Arenas-Mena, 2006; Boyle and Seaver, 2008, 2010; Christodoulou *et al.*, 2010). It would also be appropriate to develop an antibody against the FoxA protein, and other proteins, in order to show where the final gene products are localized in different cell types and tissues. Ultimately, future functional experiments would focus on gene orthologs with known roles in well-characterized gene regulatory networks that specify endomesoderm (Davidson *et al.*, 2002; Maduro, 2009), endoderm (Maduro and Rothman, 2002; Peter and Davidson, 2011) and other cell layers in both classic model organisms and emerging systems (Röttinger *et al.*, 2012).

Sipuncula once represented a distinct phylum of invertebrates that develops through a recognizable pattern of quartet spiral cleavage along with annelids, echiurans, siboglinids (vestimentiferans, pogonophorans), mollusks, nemerteans and polyclad turbellarians (Gerould, 1906; Costello and Henley, 1976; Rice, 1985; Henry and Martindale, 1999). Now, there are four distinct groups recognized with quartet spiral cleavage – annelids, mollusks, nemerteans and polyclad turbellarians – instead of seven. And let's not forget the highly modified forms (acoels, rotifers, entoprocts, gnathostomulids) and groups where spiral cleavage may have been lost (phoronids, brachiopods, bryozoans) or reduced (Hejnol, 2010; Lambert, 2010). As phylogenetic and phylogenomic analyses likely continue to rearrange animal relationships within and among spiralian lophotrochozoans, attempts to reconstruct patterns of spiralian evolution will also become more challenging. Yet, regardless of where different clades eventually reside within a spiralian-specific phylogeny, modifications that led to a setiger,

veliger, trochophore, pilidium, actinotroch, pelagosphera, and other larval types, represent divergent evolution of spiralian life histories and body plans, and therefore models from each clade should be investigated from the first cleavage through metamorphosis to their respective adult forms.

Materials and Methods

The information in this section is a condensed summary of methods for obtaining the data presented in figures, and is supplementary to specific details listed in each of the figure captions. Live adult sipunculan worms were collected from three field locations within ~ 20 km of the Smithsonian Marine Station, along the southeast coast of Florida, USA. *Phascolion cryptum* was obtained by collecting small mollusk shells from seagrass beds within the Indian River Lagoon estuary. *Themiste alutacea* was collected from coquina reef rock in the wave-swept intertidal zone along the coast. *Nephasoma pellucidum* was collected from mixed porous rubble that was dredged up from the seafloor ~ 4-6 miles offshore of the Fort Pierce Inlet. In each case, adult worms were extracted from their respective hard substrata, placed into bowls of filtered seawater (FSW), and monitored for spawning in the laboratory at room temperature, with daily seawater exchanges.

Fertilized eggs were moved into plastic gelatin-coated petri dishes and cultured for development. Embryonic and larval stages were maintained in antibiotic-treated FSW, and fixed for labeling and imaging on a Zeiss LSM510 laser scanning confocal microscope, or a Nikon E800 compound light microscope. Culturing, fixation and labeling methods for imaging followed previously established protocols (Boyle and Seaver, 2010), with stage-specific and species-specific adjustments made to the anesthetic chemical mixture during relaxation steps. In late trochophores, and all pelagosphera larval stages, relaxation of the musculature required a combination of treatments by the serial addition of 0.5M MgCl₂, 0.25 % bupivacaine hydrochloride, and 70% ethanol to FSW. When muscular activity was no longer observable, fixation was performed by exchanging an anesthetic treatment with 4% paraformaldehyde in FSW. Fixed specimens were washed into phosphate-buffered saline (PBS) for labeling and confocal microscopy, or moved stepwise into 60% glycerol for compound microscopy. Details are available upon request.

We have also presented a subset of *foxA* transcription patterns in *P. cryptum*. The expression patterns were obtained by replicating gene isolation, gene cloning and whole mount *in situ* hybridization protocols for the sipunculan, *Themiste lageniformis* (Boyle and Seaver, 2010). The antisense *Pc-foxA* ribonucleotide probe was transcribed from a 1329 bp RACE fragment, and hybridized *in situ* for 72 hours at 65 °C. Specimens were mounted in 40% glycerol and imaged with a Nikon E800 compound microscope. The *Pc-foxA* expression (Fig. 9) represents a proof of principle for *P. cryptum*, which has also been performed in *T. alutacea* and *N. pellucidum*, extending the *in situ* hybridization protocols to four sipunculan species. Each of the *Pc-foxA* expression micrographs, and all other compound light micrographs, were imaged with DIC optics and rendered from multiple focal planes with Helicon Focus (Helicon Soft Ltd.). All figure images were edited with Adobe® Photoshop® CS3, and all figures plates were prepared with Adobe® Illustrator® CS3.

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