Comparative Development and the Evolution of Life History Diversity in Sipuncula

Michael J. Boyle[1]* and Mary E. Rice[2]

ABSTRACT. The biological diversity of marine invertebrate animals is exemplified by variation in the forms and functions they display during early development. Here, we utilize compound and confocal microscopy to investigate variation in developmental morphology among three sipunculan species with contrasting life history patterns: *Phascolion cryptum* develops directly from an embryo to the vermiform stage; *Themiste alutacea* develops indirectly through lecithotrophic trochophore and pelagosphera larvae, and *Nephasoma pellucidum* develops indirectly through a lecithotrophic trochophore and a planktotrophic pelagosphera larva. Their respective embryos and larvae differ in size, rate of development, timing and formation of muscle fibers and gut compartments, and the presence or absence of an apical tuft, prototroch, metatroch, terminal organ, and other larval organs. The amount of embryonic yolk provisioned within species-specific prototroch cells and body regions appears to correlate with life history pattern. Different rates of development observed among these species indicate shifts from an ancestral, indirect planktotrophic life history toward a more rapid development through direct and indirect lecithotrophy. According to modern molecular phylogenetic and phylogenomic relationships in Sipuncula, our observations implicate extensive developmental diversification and heterochrony within the largest sipunculan family, Golfingiidae. We discuss our observations in the context of habitat distributions, developmental priorities, character relationships, and the direction of evolutionary transitions between life history patterns. Moving forward, we have established working protocols for gene expression patterns, have sequenced and assembled developmental transcriptomes, and will pursue cellular fate mapping and genome sequencing to promote sipunculans for comparative evolutionary developmental biology.

INTRODUCTION

Within the metazoan tree of life, most marine species develop through an indirect biphasic life cycle that includes a free-living planktonic larval stage (Thorson, 1950), considered "the most common developmental pathway in the animal kingdom" (Young, 2002:1). Indirect development is typically observed among marine invertebrates, where sexual reproduction leads to motile embryos and larvae with functions and ecologies that are quite different from their respective juvenile and adult forms (Scheltema, 1968; Jägersten, 1972; Freeman and Lundelius, 1992; Strathmann, 1993; McEdward, 2000; Raff, 2008; Page, 2009; Freeman, 2015). Within some clades (e.g., annelids, mollusks), one or more larval stages may exhibit transient structures that facilitate swimming, navigation, sensation, and possibly feeding during formation of the adult body plan (Anderson, 1973; Boyle and Seaver, 2009; Page, 2009). In other groups (e.g., echinoderms, nemerteans), larvae may represent distinct body plans with little or no resemblance to the adult (Raff, 2008; Maslakova, 2010). In either case, production of a ciliated, swimming larva is in high contrast to direct development, which does not produce a recognizable larval stage. There is very little debate about whether numerous records of

[1] Life Histories Program, Smithsonian Marine Station at Fort Pierce, 701 Seaway Drive, Fort Pierce, Florida 34949, USA.

[2] Senior Research Scientist Emeritus, Life Histories Program, Smithsonian Marine Station at Fort Pierce, 701 Seaway Drive, Fort Pierce, Florida 34949, USA.

* Correspondence: boylem@si.edu

feeding (planktotrophic) and nonfeeding (lecithotrophic) larval types from extant and extinct clades across the Metazoa point to an ancient origin of indirect pelagobenthic life history patterns (Jägersten, 1972; Strathmann, 1985, 1993; Westheide, 1997; Wray, 2000a; Peterson, 2005; Raff, 2008; Freeman, 2015). There is plenty of debate about whether direct or indirect development is more primitive among marine invertebrates, including correlated problems about the origin or loss of feeding larvae in different animal groups (Jägersten, 1972; Strathmann, 1985; Hazprunar et al., 1995; Rouse, 2000; Sly et al., 2003; Peterson, 2005; Runnegar, 2007; Raff, 2008; Nielsen, 2009; Page, 2009; Freeman, 2015). Accordingly, many important questions remain unanswered. How did biphasic life cycles first arise? Which molecular, genetic, and cellular mechanisms cause a transition from direct to indirect development or in the opposite direction? Why do highly contrasting life histories evolve within a single genus or family? To better understand the evolution and development of life history patterns, we focus on a particular group of animals for which most, if not all, developmental patterns are known and characterized.

Sipuncula is a distinct clade of exclusively marine, soft-bodied coelomate worms with an unsegmented body plan and global distribution. They have colonized a diversity of benthic substrates within polar, temperate, and tropical environments (Murina, 1971; Amor, 1975; Cutler, 1975, 1977, 1994; Haldar, 1975; Murina and Holodov, 1985). In protected bays, inlets, and littoral zones of the open coast, adult worms may be found in mud, sand, and gravel, in beds of seagrass, mussels, and oysters, inside sedimentary and coral reefs, within gastropod shells and porous rubble, under rocks, and among fouling communities on artificial structures (Fisher, 1952; Stephen and Edmonds, 1972; Rice, 1975, 1988; Cutler, 1994; Rice et al., 1995; Schulze et al., 2005). They also have been retrieved from bathyal and abyssal ocean depths by sampling wood, mixed sediments, and hydrocarbon seeps and by deployment of artificial collection devices (Murina, 1957, 1971; Cutler, 1977; Tarifeño and Rojas, 1978; Rice, 1985; Saiz-Salinas, 2007; Shields and Kedra, 2009; Young et al., 2012; Rubin-Blum et al., 2014; Johnson et al., 2015). Within each habitat, adult sipunculans are cryptic and rarely visible without removal from their substrata. Upon removal and general examination, they are readily distinguishable from other animals. The adult body plan consists of a relatively large posterior trunk region and a narrow retractable introvert with a mouth and tentacles on its anterior end. However, the adult body is macroscopic and represents only one stage within a complex species-specific life history. Early sipunculan development typically includes a series of embryonic and larval stages that are microscopic. As observed in the life cycles of many marine invertebrates, these "other body plans" are the molecular, cellular, and morphological templates from which juvenile and adult forms are ultimately constructed (Wray, 2000b; Raff, 2008; Page, 2009; Freeman, 2015). Historically, very few studies were focused on the early stages of sipunculan development, most notably the works by Emil Selenka (1875), Berthold Hatschek

(1883), and John Gerould (1906). More recently, Bertil Åkesson (Åkesson, 1958) and Mary Rice (Rice, 1967, 1973, 1975) have described fundamental changes between embryonic and larval stages in different organ systems and species, which have subsequently inspired modern research efforts to revisit particular aspects of embryogenesis, tissue and organ development, and larval formation (Adrianov et al., 2008, 2011; Kristof et al., 2008; Schulze and Rice, 2009a, 2009b; Boyle and Seaver, 2010; Boyle and Rice, 2014). Yet, throughout this period, with little exception (Rice, 1981, 1985), there have not been any detailed comparative studies attempting to correlate developmental phenomena with the evolution and divergence of sipunculan life history patterns (Boyle and Rice, 2014).

Thus far, developmental life history patterns have been described for 23 species (Rice, 1985, 1989; Rice et al., 1995), spanning all six families in the clade (Kawauchi et al., 2012; Lemer et al., 2015). Within Sipuncula, there are four recognized developmental patterns (Figure 1): (I) direct development from a fertilized egg to crawling juvenile worm; (II) indirect development with a trochophore larva; (III) indirect development with a trochophore and a lecithotrophic pelagosphera larva; and (IV) indirect development with a trochophore, and a planktotrophic pelagosphera larva. All sipunculan trochophore larvae are lecithotrophic, with morphological characteristics that are similar among polychaetes, echiurans, and mollusks, including a top-shaped body plan with an apical tuft of cilia, a pair of pigmented eye spots, and a prototrochal band of compound cilia that separates the anterior pretrochal episphere from the stomodeum and posttrochal hyposphere (Rice, 1985; Rouse, 1999). Unlike the prototrochs in most types of trochophore larvae, sipunculan prototroch cells are conspicuously large and provide a nutritive function. The pelagosphera larva is distinct from the trochophore and unique among all metazoan clades. Pelagosphera body plans are divided along the anteroposterior axis into head, thorax and trunk regions (Rice, 1985; Rice et al., this volume). Distinct character traits include extensive ciliation of the ventral head surface, a postoral metatroch with exclusive locomotory function, U-shaped digestive architecture within a spacious coelom, a terminal organ with dedicated retractor muscles, a collagenous cuticle enclosing the trunk, and the absence of any segmental rudiments during larval

DIRECT DEVELOPMENT *lecithotrophic*
(I) Egg ⟶ Worm

INDIRECT DEVELOPMENT *lecithotrophic*
(II) Egg ⟶ Trochophore ⟶ Worm
(III) Egg ⟶ Trochophore ⟶ Pelagosphera ⟶ Worm

INDIRECT DEVELOPMENT *lecithotrophic/planktotrophic*
(IV) Egg ⟶ Trochophore *lecitho.* ⟶ Pelagosphera *plankto.* ⟶ Worm

FIGURE 1. Developmental life history patterns in the Sipuncula. Roman numerals (I, II, III, IV) designate four recognized patterns of development. Modified from Rice (1981).

or postlarval development. Internal organs include a centralized nervous system with anterior ganglia connected to a median ventral cord, a single pair of metanephridia, a series of circular muscle bands along the trunk, and two pairs of ventral and dorsal retractor muscles. The retractor muscles enable full retraction of the head and metatroch within the anterior body. In planktotrophic larvae, a functional gut is open at both ends, consisting of a stomodeum, an esophagus with protrusible buccal organ and lip glands, a stomach, a recurved intestine, and an anus on the dorsal side of the trunk. With the exception of buccal, terminal, and external ciliated organs, most of the larval organ systems that are built from muscle, gut, and nervous tissue are retained within the adult body (Rice, 1978, 1985).

On the basis of laboratory observations of early development, the pelagic stage of sipunculan trochophore larvae may last from 2 to 10 days, depending upon species and environment (Rice, 1967, 1985). Similar observations show that pelagosphera larvae may also be relatively short-lived lecithotrophic (days) or planktotrophic (weeks) forms (see Rice, 1985: table 1). However, both laboratory observations and field collections indicate there are relatively long lived (months) teleplanic pelagosphera larvae (Hall and Scheltema, 1975; Rice, 1975, 1981, 1988; Schulze and Rice, 2009b; Adrianov and Maiorova, 2010). Little is known about the dispersal patterns of short-lived forms; however, teleplanic pelagosphera larvae have been collected from many of the major surface currents across Atlantic, Pacific, and Indian Ocean basins (Scheltema and Hall, 1975; Rice, 1981; Scheltema and Rice, 1990; Staton and Rice, 1999; Rice et al., this volume; Schulze et al., this volume). Although there are several documented exceptions (Levin, 2006; Shanks, 2009), such widespread dispersal is thought to enable a natural distribution of marine species lineages on both local and regional scales (Scheltema, 1968; Carlon and Olson, 1993; Shanks and Eckert, 2005; Shanks, 2009; Young et al., 2012) as well as the possible establishment of new species (Thornhill et al., 2008; Kawauchi and Giribet, 2010; Schulze et al., 2012). Estimated correlations between larval duration and dispersal distance for marine larvae in general, and teleplanic pelagosphera larvae in particular, suggest there is potential for genetic connectivity between widely separated species populations (Scheltema, 1968, 1975; Shanks, 2009).

For Sipuncula at least, a relatively recent, comprehensive systematic revision implied that such potential was underestimated. With Cutler's (1994) revision, he significantly reduced the number of previous species designations presented in the monograph of Stephen and Edmonds (1972) and indirectly suggested there is morphological evidence for many cosmopolitan or circumtropical species within the clade (Cutler, 1994). Yet a growing number of molecular studies indicate that several of Cutler's (1994) synonymies may not be supported because similarities based on morphology are incongruent with dissimilarities revealed by DNA analyses testing for cosmopolitan species (Du et al., 2009; Kawauchi and Giribet, 2010, 2013; Schulze et al., 2012). Since Cutler's (1994) revision, there are new species (Kawauchi and Rice, 2009) and new examples of conflicting

records of species (Adrianov and Maiorova, 2012). Of special interest to our work, the developmental pattern of at least one species appears to be quite different among populations previously considered one lineage on opposite sides of the Pacific Ocean (Adrianov et al., 2008; Schulze et al., 2012). These problems have arisen in part from the low number of unambiguous diagnostic characters available among adult worms and, importantly, from limited attempts to utilize embryos, larvae, genes, and proteins to expand the total number of characters available for species identification.

Thus, the timing is now more appropriate than ever for a modern interdisciplinary approach (i.e., developmental biology, life history patterns, traditional morphology, DNA barcoding, and genomics) to sipunculan biodiversity research. Not too long ago, Sipuncula was recognized as a distinct phylum of worms (Clark, 1969; Rice, 1985; Freeman and Lundelius, 1992; Cutler, 1994; Boore and Staton, 2002). Today, they are considered an in-group of the annelid radiation (Struck et al., 2007; Dordel et al., 2010) and one of several divergent lineages near the base of the annelid tree (Struck et al., 2011; Weigert et al., 2014). However, there is unambiguous evidence of sipunculan body fossils in lower Cambrian rocks of China (Huang et al., 2004), either predating or coeval with the oldest known stem group polychaete fossils from Greenland (Conway-Morris and Peel, 2008; Vinther et al., 2011). Therefore, fossil data imply that sipunculans have maintained an unsegmented vermiform body plan for the past 520 million years and further suggest that secondary loss of a segmented body (Purschke et al., 2014), based almost exclusively on molecular hypotheses, may be an oversimplification. Alternative analyses of both molecular and fossil data suggest that sipunculans may actually be the sister group of Annelida (Sperling et al., 2009; Eibye-Jacobsen and Vinther, 2012; Parry et al., 2014). We have not yet ruled out the remote possibility that fossil data, development biology, and life history patterns may once again align with earlier biochemical and morphological analyses that suggest sipunculans have "diverged from the preannelid stock" (Clark, 1969:15), with segmented annelids evolving along a separate lineage from the unsegmented sipunculans. This scenario has a number of implications not only for the direction of life history evolution within the clade but also for the radiation of sipunculans within Spiralia and the Metazoa (Boyle and Rice, 2014).

Currently, four, perhaps all five, of the basal annelid branches contain species with planktotrophic larvae (Jägersten, 1972; Weigert et al., 2014). Recent molecular phylogenetic studies imply that planktotrophy represents the ancestral life history pattern within Sipuncula (Schulze et al., 2007; Kawauchi et al., 2012; Lemer et al., 2015), with clear patterns of both family-specific conservation and divergence. Remarkably, this hypothesis was initially proposed by Rice (1985) prior to the technological revolution of molecular phylogenetics. When such patterns are combined with early developmental characteristics (e.g., egg size and shape, unequal cleavage, yolk content, micromere-macromere size relationships, and the presence/absence of a metatroch, functional gut, terminal organ, and specific musculature), there

appear to be traceable developmental priorities among the four recognized life history patterns. Here, we are in search of evidence that may reveal how direct and indirect lecithotrophic and planktotrophic developmental life history patterns diverged within Sipuncula and hopefully will reemphasize why past and present generations of natural historians continue to explore the elusive, ancient origins of animal body plans.

MATERIALS AND METHODS

The adult sipunculan worms for this investigation were collected from field sites within 20 km of the Smithsonian Marine Station at Fort Pierce, Florida, USA. *Phascolion cryptum* Hendrix, 1975 inhabits small, discarded mollusk shells within sandy seagrass beds of the Indian River Lagoon. Adult worms were extracted from shells with a small benchtop vise and forceps. *Themiste alutacea* Grübe and Oersted, 1858 is found in burrows and crevices within coquina reef substrata of intertidal and subtidal zones along the coast. Coquina rock was removed from the reef, and adults were extracted with a rock hammer and steel chisel. *Nephasoma pellucidum* (Keferstein, 1865) lives within mixed porous rubble on the seafloor and is found at depths below 10 m along the coast of Fort Pierce. Rubble was dredged from the seafloor, and the worms were extracted with a rock hammer and steel chisel.

Adult specimens of each species were placed into bowls of filtered seawater (FSW) at room temperature in the laboratory and maintained with daily FSW exchanges. After spawning events, fertilized eggs were moved into gelatin-coated plastic petri dishes for development. Embryonic and larval development was cultured in antibiotic-treated FSW, and selected stages were isolated, anesthetized (relaxed), and fixed with 4% paraformaldehyde (pfa) by applying stage- or species-specific adjustments to established protocols (Boyle and Seaver, 2010). For postgastrula, trochophore-like, trochophore, and pelagosphera stages, relaxation treatments required the serial addition of 0.5 M $MgCl_2$, 0.25% bupivacaine hydrochloride, and 70% ethanol to FSW until muscle activity was no longer observable.

Each species reacts differently to a series of treatments and was monitored separately for inactivity. When morphology appeared to be natural and the muscles were inactive, fixation was performed by removing most of the relaxation treatment and pouring on 4% pfa in FSW. Specimens were fixed overnight at 4°C for light microscopy and 1–2 hours at room temperature for confocal microscopy. Fixed specimens were rinsed with multiple exchanges of phosphate-buffered saline (PBS) at pH 7.4 to remove pfa. For light microscopy, specimens were moved stepwise (10%, 20%, 40%) into glycerol (40% glycerol, 10% 10× PBS, 50% deionized H_2O) for a minimum of 24 hours, mounted on Rainex-coated glass slides, and imaged with a stem-mounted Nikon Coolpix 4500 digital camera through differential interference contrast (DIC) optics on a Nikon Eclipse E800 compound microscope. Adult specimens were imaged live with a Nikon Coolpix 4500 digital camera through one ocular of a Wild M5 stereomicroscope. For confocal microscopy, specimens in PBS were transferred into phosphate-buffered saline with 0.1% Triton X-100 (PBT). These specimens were then pretreated with RNase A at 1.0 mg/mL PBT for 1.0 hour at 37°C, washed in PBT, and then labeled with a combination of propidium iodide (Sigma) at 5 μg/mL PBT and 1:200 BODIPY FL-phallacidin overnight at 4°C.

After labeling, treated specimens were rinsed in PBS and mounted for imaging. Mounting involved attachment of specimens to poly-L-lysine-coated glass slides, transfers through an isopropanol dehydration series (35%, 50%, 70%, 85%, 95%, 100%), and immersion and mounting in Murray Clear (2:1 benzyl benzoate:benzyl alcohol), with a coverslip sealed on all sides with clear nail polish or topcoat hardener. These slides typically included 10 to 20 specimens each, which were scanned and imaged with a Zeiss LSM510 laser scanning confocal microscope with Zen imaging software. Confocal z-stacks were analyzed, sectioned, and rendered in ImageJ (https://imagej.nih.gov). Compound micrographs with DIC optics were rendered from multiple focal planes with Helicon Focus (Helicon Soft). All images were edited with Adobe Photoshop CS3; all figure plates were prepared with Adobe Illustrator CS3.

RESULTS

In this study, we present descriptions of particular morphological features that were observed during the development of three species of sipunculan worms: *P. cryptum*, *T. alutacea*, and *N. pellucidum* (Figure 2). Each of these species exhibits a distinct developmental life history pattern, which together represent three of the four life history patterns recognized within Sipuncula (Figure 1). Our observations of comparative developmental morphology include recent observations from analyses of compound light and laser scanning confocal microscopy, which were performed over a 3-year period from 2010 through 2013 and previous descriptions made over an extended period of time spanning approximately 40 years by researchers in the Life Histories Program at the Smithsonian Marine Station. Species-specific observations on the size and yolk content of eggs, morphological features of early cleavage stage embryos, and descriptions of developmental life history patterns have been previously published for each of the study species. Therefore, we build upon previous studies that provide a more comprehensive background for our recent observations and establish an important framework for making inferences regarding the evolution and diversification of sipunculan life history patterns discussed in this chapter.

Spawning and Early Development

In the laboratory, temporal patterns of spawning and early development were consistent with known reproductive periods for each respective species. The body wall around the adult trunk
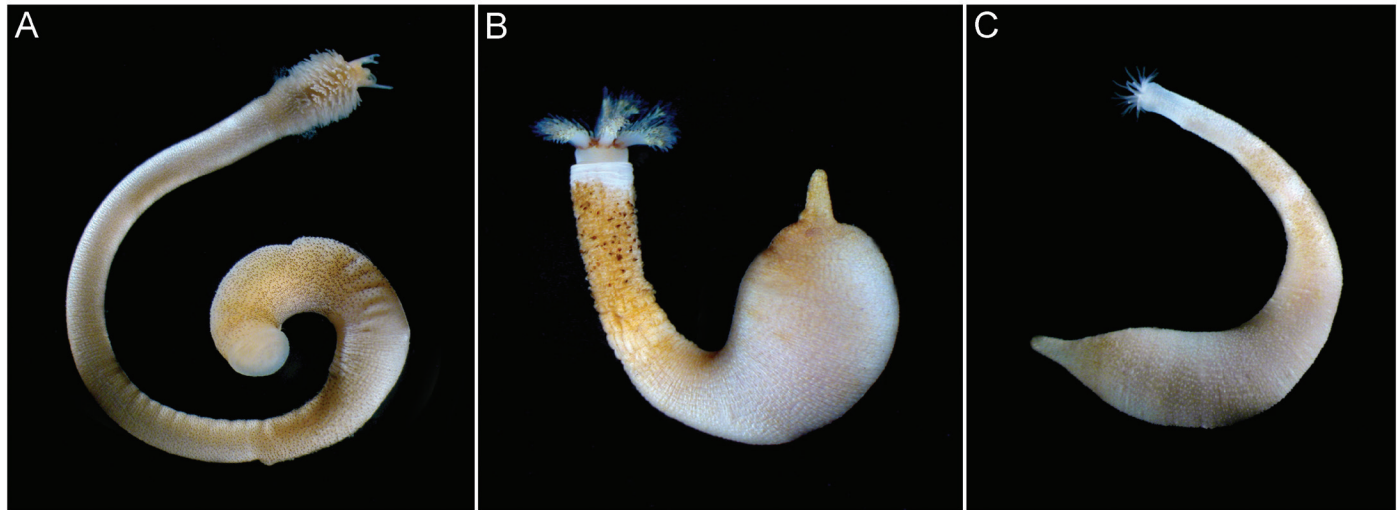
FIGURE 2. Adult sipunculan taxa with contrasting developmental life history patterns. (A) *Phascolion cryptum* Hendrix, 1975. (B) *Themiste alutacea* Grübe and Oersted, 1858. (C) *Nephasoma pellucidum* Keferstein, 1865. *Phascolion cryptum* exhibits direct lecithotrophic development (I). *Themiste alutacea* develops indirectly through lecithotrophic trochophore and pelagosphera larvae (III). *Nephasoma pellucidum* develops indirectly through a lecithotrophic trochophore larva and a planktotrophic pelagosphera larva (IV). Roman numerals designate one of the four recognized developmental life history patterns in the Sipuncula (see Figure 1).

of *P. cryptum* is semitransparent, which enabled the detection of female or male gametes within the coelom and delegation of male and female worms to "spawning" bowls. Spawning events occurred within 1 to 2 weeks after extraction of adult worms from mollusk shells and most often took place during early morning hours from 2:00 to 9:00 AM. The eggs of *P. cryptum* are high in yolk content, spherical in shape, and relatively large (135–137 µm). Fertilized eggs developed on the bottom surfaces of glass or plastic containers, as there are no larval stages with ciliated trochal bands that facilitate swimming behavior. Hatching from the egg envelope was observed within 32 to 34 hours after fertilization, and crawling worms were observed within 44 to 48 hours.

For the adults of *T. alutacea*, it was not possible to detect gametes by visualization through the body wall. Bowls containing a predicted mixture of male and female worms of this species typically spawned synchronously, within 2 to 3 weeks after extraction from coquina reef rubble. The eggs of *T. alutacea* were also noticeably high in yolk content, spherical in shape, and relatively large (137–149 µm). After fertilization, development was rapid, with the formation of a swimming trochophore larva within 18 to 20 hours and a crawling or swimming pelagosphera larva within 28 to 32 hours.

Within the adults of *N. pellucidum*, gametes were not visible, and therefore, male and female worms could not be distinguished. This species reliably spawned within 2 to 3 days after extraction from mixed porous rubble. In contrast to the other two species, the spherical eggs of *N. pellucidum* were relatively small (103–105 µm) and contained visibly lower yolk reserves. In this species, swimming blastulae were observed within 14 to

15 hours of fertilization, swimming trochophore larvae were observed between 45 and 48 hours, and pelagosphera larvae were observed to be swimming and feeding after approximately 68 to 72 hours of development. In all three species, spawning continued over a period of several days to 1 week, and sometimes longer, with some variability in the amount of spawning and number of viable embryos between spawning seasons and field collections for each species-specific developmental program. We did not routinely follow or describe development beyond 3 days of fertilization for each species in this study; normal development was observed for a minimum of 1 week after fertilization.

In *P. cryptum*, *T. alutacea,* and *N. pellucidum*, fertilization initiated a process of unequal, unipolar holoblastic spiral cleavage. The first embryonic cell division divided the fertilized egg into two blastomeres of unequal size, a smaller AB cell and a larger CD cell, with these and subsequent letter designations following the conventional nomenclature established by Edwin Conklin (1897). The second cycle of embryonic cell divisions produced three relatively equal sized blastomeres and a larger D blastomere, which enabled the identification of four distinct quadrants (A, B, C, D) in each of these spiralian embryos. During third cleavage, each of the four blastomeres divided again, producing an 8-cell embryo with four macromeres at the vegetal pole and four micromeres at the animal pole. In the embryo of *P. cryptum*, the micromeres of the A, B, and C quadrants are larger than their respective macromeres, with the D macromere (1D) being the largest cell, followed by the d micromere (1d) being larger than all other blastomeres (Rice, 1975). The 8-cell embryos of *T. alutacea* have not been examined for respective

blastomere sizes but may exhibit a relationship similar to that recorded for two congeneric species within the same life history category (Rice, 1985). Micromeres and macromeres in the A, B, and C quadrants were approximately equal in size within the 8-cell embryo of *N. pellucidum*.

For all three species, features of development associated with blastula and gastrula stages were examined, and those embryonic stages were utilized for molecular research applications, but they are not described in this study. Information is available with more comprehensive descriptions of embryonic cleavage, early development, and metamorphosis of *P. cryptum* and *T. alutacea* (Rice, 1975) and *N. pellucidum* (Schulze and Rice, 2009b).

COMPARATIVE DEVELOPMENTAL MORPHOLOGY: 28 TO 30 HOURS

Phascolion cryptum

At approximately 28 to 30 hours of development, the trochophore-like stage of *P. cryptum* is oval shaped along the anterior-posterior (A/P) axis, with a double row of disproportionately large cells that encircle the anterior hemisphere (Figure 3A). These prototroch cells are nonciliated and contain numerous yolk granules. The content of yolk is also noticeably high within cellular components of all three germ layers. There is no apical tuft or any form of ciliation on the exterior. Internally, distinct bands of circular muscle fibers are detectable and have not yet expanded outward to meet the developing body wall. Circular muscle fibers are connected along the midline in both anterior and posterior hemispheres. Two paired sets of ventral and dorsal retractor

muscle fibers extend from the lateral margins of the stomodeum toward the posterior end and are located medial to the circular muscles for most of their length. Both sets of retractor and circular muscles are bilaterally symmetric along the A/P axis and appear to converge in an anterior arc of fibers between the brain and forget regions. Specimens are able to contract in an A/P direction. The developing brain shows a localized grouping of numerous cell nuclei and has begun to form bilateral lobes. On the ventral side within the circlet of prototroch cells, there is a centralized cluster of filamentous actin (F-actin) protein fibers surrounded by a ring of cell nuclei, which marks the position of the stomodeum and developing esophagus. Large nuclei are visible in the region of the gut but do not appear to be organized. Numerous cell nuclei are located within the posterior hemisphere below the level of prototroch cells and follow the contour of the larval-like body, although they do not exhibit a particular arrangement. This stage of development is within 4 to 6 hours of the process of elongation and hatching from the egg envelope.

Themiste alutacea

Themiste alutacea exhibits the hallmark features of a trochophore larva at this stage. There is a double row of ciliated prototroch cells encircling the anterior hemisphere, with each cell bearing a single large nucleus. A ciliated apical tuft extends from the anterior-dorsal end of the A/P axis to the outside of the larva. Yolk granules are abundant within the prototroch cells and are observed throughout in all other regions. Circular muscle fibers are detectable and interwoven with multiple accessory fibers and
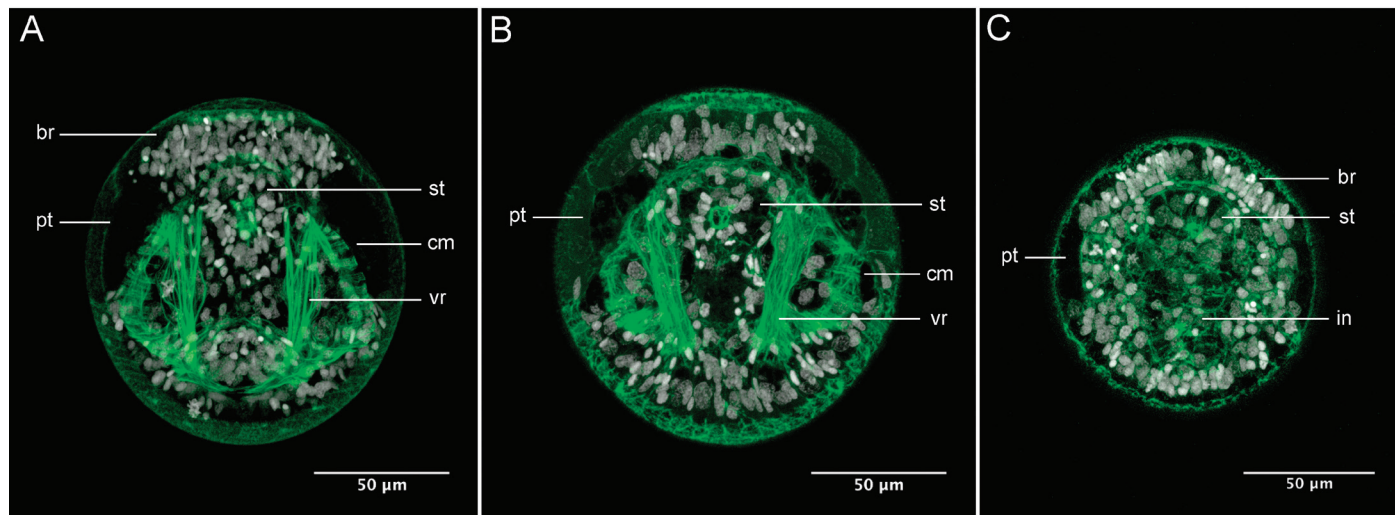

FIGURE 3. Laser scanning confocal micrographs of postembryonic stages of (A) *Phascolion cryptum*, (B) *Themiste alutacea*, and (C) *Nephasoma pellucidum*. Shown are z-stack projections; each specimen is a ventral view with anterior to the top. Specimens were labeled with phallacidin (green, F-actin) to visualize the muscular system and propidium iodide (gray, DNA) to visualize the position of individual cells during development. Time of development is approximately 28–30 hours for each species. Abbreviations are as follows: br, brain; cm, circular muscle fibers; in, intestine precursor cells; pt, prototroch cells; st, stomodeum; vr, ventral retractor muscle fibers.

do not yet show a discrete banding pattern (Figure 3B). Circular muscle fibers do not connect along the midline toward the posterior end. Two pairs of ventral and dorsal retractor muscles are visible, although when compared with the retractors in *P. cryptum*, individual retractor muscle fibers are not well defined. This larva does not exhibit contractile movements. The stomodeum and esophageal canal are lined F-actin fibers, showing a tubular configuration surrounded by cell nuclei. Relatively large nuclei are visible at the site of the gut and the positions of the bilateral mesodermal bands. Actin filaments are also concentrated into a thin band within the central midgut where the intestine is developing. Cell nuclei are assembling into two lobes and a central commissure in the region of the brain. Numerous cell nuclei follow the contour of the larva's posterior end, where they are elongate in shape and oriented more or less along the A/P axis. This stage of development is within 10 to 12 hours of metamorphoses, which will produce a lecithotrophic pelagosphera larva.

Nephasoma pellucidum

At this stage of development, *N. pellucidum* is spherical and swimming. There are a double row of ciliated prototroch cells and an apical tuft of cilia. When compared with *P. cryptum* and *T. alutacea*, the prototroch cells of *N. pellucidum* are small and contain relatively low levels of yolk granules. In addition to the abundant F-actin proteins that were labeled and visualized in epidermal cells (e.g., prototroch and body ectoderm), which present a common pattern in all three species, high levels of F-actin in *N. pellucidum* were also localized to cytoskeletal outlines of cells within bilateral mesodermal domains and developing epithelia of the esophagus and gut endoderm (Figure 3C). Circular muscle fibers were not detected at this stage. Ventral and dorsal retractor muscle fibers were not detected. Cell nuclei of the brain are organized into bilateral lobes. There is a relatively distinct semicircular arc of cell nuclei lining the posterior margin of this early trochophore. A stomodeal invagination is evident, and the developing esophagus exhibits a circular arrangement of epithelial cell nuclei surrounding a centralized lumen. The labeling of cell nuclei in the gut also reveals the alignment of endodermal cells along the apparent precursor of an intestinal lumen. Numerous smaller nuclei are concentrated on lateral margins of the developing intestine. This larval stage precedes the formation of a feeding planktotrophic pelagosphera larva by approximately 40 to 42 hours.

Comparative Developmental Morphology: 3 Days

Phascolion cryptum

The most active and motile stage of development in each species was observed at approximately 72 hours after fertilization (Figure 4A). The vermiform stage of *P. cryptum* was actively crawling along the bottom surfaces of glass and plastic containers and was highly flexible along its dorsal-ventral (D/V) and A/P

axes. *Phascolion cryptum* was able to bend its body in multiple directions, contract its body along the A/P axis, and extend itself to almost twice its "resting" length. However, it was never observed to fully extend its head outside of the body cavity. Yolk could be seen within the digestive organ system and streaming throughout the coelomic cavity from the brain to the posteriormost site of attachment of the developing ventral nerve cord. Cilia are active on the anteriormost end upon a conical-shaped protrusion that extends from the brain to the outside of the body. No mouth or anus is open to the outside of the body. There is no prototroch, metatroch, or terminal organ present. There are typically between 6 and 10 distinct papillae extending from the body on the trunk. Laser scanning confocal micrographs show a series of relatively thick circular muscle bands that are evenly spaced along the A/P axis, and both ventral and dorsal pairs of retractor muscles extend from anterior to posterior along most of the 3-day vermiform (Figure 5A). At this stage, cell nuclei are observed in a continuous narrow band along the ventral midline, extending from the ventral side of the brain to the posterior end of the worm. Cell nuclei are densely concentrated at the dorsal-anterior end of the body, forming a dense circular cluster enclosing a central space, which is void of nuclei, and extend into the conical-shaped protrusion outside the worm on the anterior end of the body. From the surface at the anterior end, between the developing brain and ventral nerve cord, cell nuclei of the developing esophagus extend toward the posterior. The esophagus connects with a narrow cylinder of nuclei that continues more than two-thirds the length of the body in a posterior direction, which then loops toward the anterior and terminates on the dorsal side of the body at the level of a constriction between the anterior and posterior body regions.

Themiste alutacea

The 3-day pelagosphera larva of *T. alutacea* is also highly flexible along it D/V and A/P axes, and its head is typically extended outside the body (Figure 4B). This larva is able to constrict its body, which greatly extends its overall length, and may also contract both its head and/or its posterior end completely within the mid-body section. The digestive system and coelomic cavity have an abundance of yolk streaming within each of those respective regions. There is no mouth or anus at this stage and no indication of feeding behavior. A pair of larval eyes is visible on the dorsal anterior side of the head. A band of metatrochal cilia is active between the head and posterior body region, which enables the larva to swim in all directions, along substrates or within the water column. The ventral surface of the head is ciliated, and a distinct band of cilia forms an arc around two-thirds of the head on the dorsal anterior side. A larval cuticle is visible on the surface of the body, from the metatroch to the posteriormost end of the developing trunk. There is no terminal organ. Similar to *P. cryptum*, in *T. alutacea* there are well-developed circular muscle bands and distinct pairs of ventral and dorsal retractor muscles, with each type of muscle group extending along
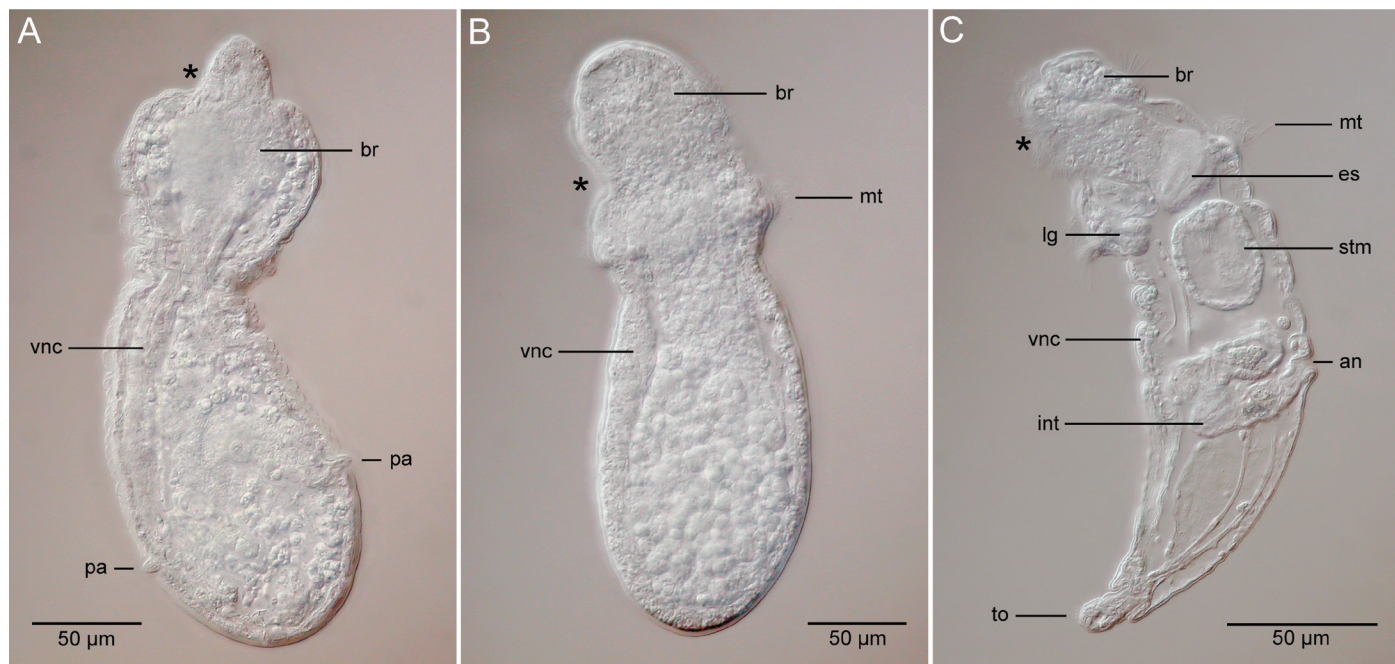
FIGURE 4. Compound light micrographs of prominent dispersive life history stages of *Phascolion cryptum*, *Themiste alutacea*, and *Nephasoma pellucidum*. Multifocal plane stacks are shown; each specimen is oriented with anterior to the top and ventral to the left side. (A) Lecithotrophic crawling vermiform stage of *P. cryptum*. (B) Lecithotrophic crawling-swimming pelagosphera larva of *T. alutacea*. (C) Swimming planktotrophic pelagosphera larva of *N. pellucidum*. Time of development is approximately 3 days for each specimen. Asterisks mark the ventral-anterior end of a nonfunctional esophagus (*P. cryptum*, *T. alutacea*) or mouth (*N. pellucidum*). Abbreviations are as follows: an, anus; br, brain; es, esophagus; int, intestine; lg, lip gland; mt, metatroch; pa, papilla; stm, stomach; to, terminal organ; vnc, ventral nerve cord. Light micrographs were imaged with transmitted light illumination through differential interference contrast optics.

most of the A/P axis (Figure 5B). However, unlike *P. Cryptum*, the retractor muscles are visible beyond the anterior end of the trunk body, where they attach to the posterior side of the brain. Cell nuclei form a continuous band along the ventral midline at the position of the ventral nerve cord, and a concentration of nuclei in the head marks the position of the developing brain, consisting of a cerebral ganglion and commissure, which is void of labeled cell nuclei. Cell nuclei also show the position of a developing esophagus between the brain and ventral nerve cord. The esophagus is continuous with a cylinder-shaped group of cell nuclei extending in a posterior direction and representing the developing intestine of the gut. The gut bends toward the dorsal surface of the trunk, posterior to the anterior body constriction, at a distance of approximately one-third the length of the trunk, and more posterior along the A/P axis than observed in *P. cryptum*.

Nephasoma pellucidum

The pelagosphera larva of *N. pellucidum* was able to extend its body along the A/P axis and retract its head within the anterior body region but does not contract its posterior body to the extent observed in the other two species. At 3 days of development in the laboratory, this larva is typically swimming off the bottom of its container. It may also attach to the bottom or to other larvae by its terminal organ. When attached to the bottom of a dish, the larva will bend along its D/V axis in a ventral direction and contact the dish surface with the ventral side of its head. The ventral surface of the head is ciliated; there is a prototrochal arc of cilia around lateral and dorsal sides of the head, and there is a metatrochal band of cilia (Figure 4C). The cilia are active in each of these regions. This larva does not contain visible yolk resources in the coelom or gut. The mouth and anus are open to the exterior of the larva. The digestive system is functional and is subdivided into a ciliated esophagus and stomach and an intestine that descends from the stomach in a posterior direction and then loops back anteriorly to its connection with the anus on the dorsal posterior body, midway between the metatroch and terminal organ (Figure 4). The digestive system of *N. pellucidum* at this stage also includes a buccal organ and lip gland. The brain is visible at the anterior end on the dorsal anterior side of the esophagus, and there are visible cell clusters marking the position of the ventral nerve cord along the anterior two-thirds of the trunk body. Circular muscle bands are detectable along the A/P
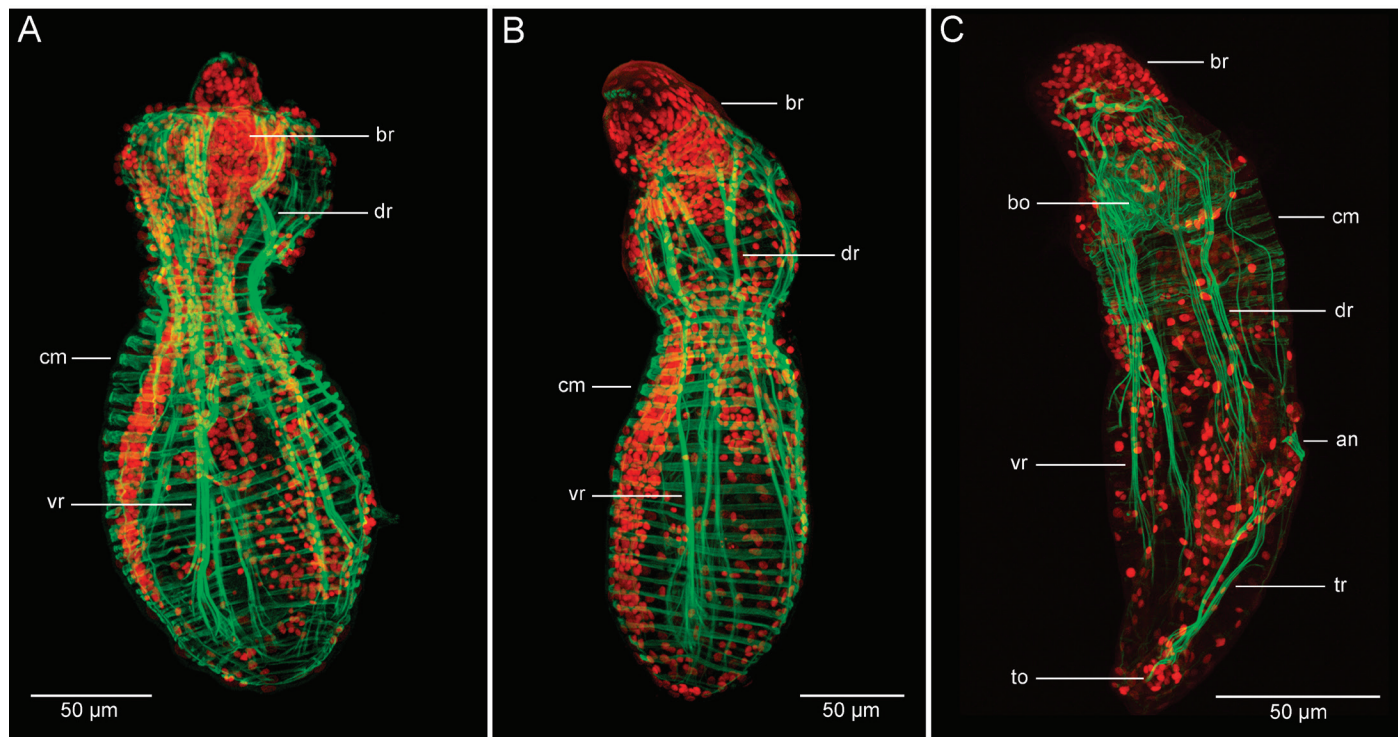
FIGURE 5. Laser scanning confocal micrographs of prominent dispersive life history stages of *Phascolion cryptum*, *Themiste alutacea*, and *Nephasoma pellucidum*. Shown are z-stack projections; each specimen is oriented with anterior to the top and ventral to the left side. Specimens were labeled with phallacidin (green, F-actin) to visualize the muscular system and propidium iodide (red, DNA) to visualize the position of individual cells during development. (A) Lecithotrophic crawling vermiform stage of *P. cryptum*. (B) Lecithotrophic crawling-swimming pelagosphera larva of *T. alutacea*. (C) Swimming planktotrophic pelagosphera larva of *N. pellucidum*. Time of development is approximately 3 days for each species. Abbreviations are as follows: an, anus; bo, buccal organ; br, brain; cm, circular muscle fibers; dr, dorsal retractor muscle fibers; to, terminal organ; tr, terminal retractor muscle fibers; vr, ventral retractor muscle fibers.

axis, where they are relatively thin and primarily visible along the anterior end of the body, posterior to the metatroch (Figure 5). Two pairs of ventral and dorsal retractor muscles extend from their connection with the brain to their respective attachment sites on the body wall, posterior to the level of the anus. The retractor muscles of *N. pellucidum* do not extend as far posteriorly as the retractor muscles in *P. cryptum* or *T. alutacea* (Figure 5C). Additional musculature includes a pair of terminal organ retractor muscles, a series of contractile rings surrounding the anus, and a complex system of fibers supporting the buccal organ and esophagus. There is also a small, distinct ring of muscle fibers located between the esophagus and stomach.

DISCUSSION

LIFE HISTORY PATTERNS AND HABITAT DISTRIBUTION

We are in search of evidence that may reveal how direct and indirect lecithotrophic and planktotrophic developmental life history patterns diverged within Sipuncula. The models investigated in this study include *P. cryptum*, which develops directly from an embryo to the vermiform stage; *T. alutacea*, which develops indirectly through lecithotrophic trochophore and pelagosphera larval forms; and *N. pellucidum*, which develops indirectly through a lecithotrophic trochophore and a planktotrophic pelagosphera larva (see Figure 1). Although each one of these species exhibits a distinct life history pattern, the distribution of their adult forms overlap within some regions of the Indian River Lagoon (IRL) estuary. However, each species was found to be individually more abundant within the relatively distinct habitat substrate from which it was collected, either within or outside the estuary.

Phascolion cryptum is the most abundant and widely distributed sipunculan within the IRL (Rice et al., 1983, 1995). This abundance could suggest that in this particular shallow sand and seagrass environment, direct development has an ecological advantage. Outside of the IRL within the wave-swept intertidal and subtidal coquina reef substrates along the coast, *T. alutacea* is one of the more common sipunculan species. The presence

of a relatively short-lived, nonfeeding pelagosphera larva may indicate a more adaptive distribution and recruitment strategy in that environment, although other species with planktotrophic pelagosphera co-occur within the same reef structure. Offshore, *N. pellucidum* is found subtidally in deeper water within mixed rubble, along with other sipunculans that exhibit a category IV life history pattern. We did not observe *T. alutacea* in the mixed rubble habitat where we found *N. pellucidum*, and we did not observe *N. pellucidum* within nearshore coquina substrates, further suggesting that conditions within the offshore benthos may be less accommodating or accessible to the short-lived pelagosphera stage of category III development. These general observations also indicate that nutritional resources several kilometers offshore of Florida's southeast coast may not be optimal for lecithotrophic oogenesis and larval development within Sipuncula. Additionally, during this study we never observed *P. cryptum* in the same substrate types where we found the other two species, which may suggest that direct lecithotrophy with the absence of any swimming stage represents a reproductive and/or developmental pattern that has not become adapted to wave-swept environments along the coast or the deeper bottom-dwelling ecology offshore of the IRL. In several locations within the IRL, typically near inlets from the coastal ocean where natural and man-made hard substrates are found, we collected low numbers of both *T. alutacea* and *N. pellucidum*, supporting previous observations (Rice et al., 1995). The presence of these two species near inlets demonstrates that larval stages likely facilitate movement into or out of the estuary and that the lack of a larval form may prevent the dispersal of a species out of the estuary to colonize offshore habitats, as in the case of *P. cryptum*. Although not quantitative, these observations do provide a basic framework for future studies that would attempt to measure and identify correlations between life history patterns and species distributions among local or regional habitat types or between climate zones.

A review of the relatively small number of sipunculan species for which development has been studied shows that some general habitat-associated trends are apparent. Among the six recognized sipunculan families (Kawauchi et al., 2012), the most common life history pattern is category IV, with a planktotrophic pelagosphera larva (Figures 4C,5C). This pattern includes both a relatively short-lived pelagosphera, as indicated by laboratory records (Rice, 1985), and teleplanic pelagosphera larvae with a potential for long-distance dispersal, as inferred from oceanographic plankton tows (Rice, 1981; Scheltema and Hall, 1975; Scheltema and Rice, 1990). The diversity of species that exhibit category IV development is globally distributed within sand and mud flats, intertidal boulder fields, mixed reef structures along coastlines, and muds of the deep sea. Adult worms of category III species are often found with worms from category IV, where they co-occur within and between coral rubble, mollusk shells, compacted sediments of nearshore reefs, and deeper structures from temperate to tropical climate zones (Stephen and Edmonds, 1972; Rice, 1975; Cutler, 1994; Rice et al., 1995). The only species described with category II development are apparently restricted to

temperate or polar marine climates (Cutler, 1994; Rice, 1985). Species that exhibit category I development have been collected subtidally from gastropod shells in soft sand and mud sediments (Åkesson, 1958; Rice et al., 1983) and from mixed sands under boulders and sediment-filled crevices of the intertidal zone (Rice, 1967; Gibbs, 1975) of temperate and subtropical regions. In summary, planktotrophic pelagosphera consistently develop from the larger sand and mud-burrowing species; planktotrophic and lecithotrophic pelagosphera typically develop from species that inhabit hard substrates, and direct development without any larval stage is found in species that occupy relatively calm inlets or bays that are protected from surf zones with high wave action.

Although there are many informative studies of sipunculan development (Gerould, 1903, 1906; Åkesson, 1958, 1961; Rice, 1967, 1973, 1975; Pilger, 1987; Schulze and Rice, 2009b; Boyle and Seaver, 2010; Kristof et al., 2011) and numerous records of sipunculan species distributions (Stephen and Edmonds, 1972; Cutler, 1994), specific correlations between developmental pattern and adult habitat remain elusive. Furthermore, contrasting life histories often overlap spatially where multiple sipunculan taxa coexist, irrespective of the habitat, ecosystem, or climate where such patterns are found. Because of this, life history patterns alone do not provide obvious signatures of environmental selection for the presence or absence of those patterns. Alternatively, heritable developmental modifications during oogenesis (e.g., yolk production level), embryonic cleavage (e.g., micromere-macromere size relationship), and organ formation (e.g., the presence or absence of ciliated bands) may provide more realistic indicators of how particular life history patterns originally diverged from one another and became the four established categories we recognize among different sipunculan species today.

LIFE HISTORY PATTERNS REFLECT DEVELOPMENTAL PRIORITIES

Within Sipuncula, category-specific life history patterns (e.g., direct development, indirect lecithotrophy, indirect planktotrophy) exhibit morphologically distinct developmental characters. Therefore, the presence, absence, and/or degree of morphogenesis of different characters such as ciliation, musculature, and a functional gut may indicate there are "priorities" during development that enable essential life history behaviors such as swimming, crawling, and feeding, respectively. In this context, one priority of direct lecithotrophic development is to build the functional musculature of a benthic crawling juvenile worm. In contrast, indirect planktotrophic development would emphasize the relatively rapid formation of a metatroch, alimentary canal, and the integrated central and peripheral nervous systems for the survival of larvae in a pelagic environment. Is it reasonable to think that such priorities are developmental signatures of evolutionary changes that occurred during the divergence of different life history patterns within Sipuncula? Direct laboratory observations combined with compound light and confocal laser scanning microscopy suggest this may be a valid interpretation.

TABLE 1. Developmental characteristics of species-specific life history patterns in Sipuncula. Characteristics are from laboratory observations. Abbreviations: A/P, anterior-posterior axis; dia, diameter; dpf, days postfertilization; hpf, hours postfertilization.

Characteristic	Direct development (I), *Phascolion cryptum*	Indirect lecithotrophy (III), *Themiste alutacea*	Indirect planktotrophy (IV), *Nephasoma pellucidum*
Egg size (dia)	136 μm[a]	138 μm[a]	105 μm[b]
8-cell blastomeres (A, B, C quadrants)	micromeres > macromeres[a]	micromeres > macromeres	micromeres = macromeres[c]
Yolk reserves	high, cellular and coelomic	high, cellular and coelomic	moderate, primarily cellular
Prototroch cells	large, non-ciliated	large, ciliated	small, ciliated
Trochophore larva	no trochophore	present, ~20 hpf [a]	present, ~48 hpf[c]
Apical tuft	absent	present	present
Circular muscles ~30 hpf	distinct circular muscle bands	indistinct circular muscle bands	circular muscle bands not detected
Retractor muscles ~30 hpf	distinct ventral and dorsal fibers	diffuse ventral and dorsal fibers	retractor muscles not detected
Pelagosphera larva with metatroch	no pelagosphera, crawling worm	lecithotrophic, ~30 hpf crawl-swim larva[a]	planktotrophic, ~72 hpf swimming larva[a]
Terminal organ	absent	absent[a]	present[c]
Circular muscles ~3.0 dpf	thick bands of A/P	thick bands of A/P	thin bands at anterior
Retractor muscles ~3.0 dpf	extend length of A/P	extend length of A/P	extend 2/3 of A/P, with additional musculature
Functional gut	1 week[a]	2 weeks[a]	3 days[c]
Juvenile worm	1 week[a]	4 weeks[a]	~6 weeks[c]

[a] Rice (1975).
[b] Åkesson (1958).
[c] Schulze and Rice (2009).

The observed relationship between larger egg sizes and higher yolk content among lecithotrophic life history patterns in categories I, II, and III may indicate a temporal shift away from relatively rapid formation of a functional gut in planktotrophic larvae toward an earlier development of juvenile-specific structures (Anderson, 1973; Havenhand, 1993; Smith et al., 2007; Page, 2009; Pernet and McHugh, 2010). This shift is most obvious where micromeres of the A, B, and C quadrants at the 8-cell stage of lecithotrophic species are larger than their respective macromeres (Table 1). These micromeres contribute to characteristically large prototroch cells (Figure 3), which eventually degenerate to introduce a substantial source of yolk nutrition into the coelom during extension and retraction movements associated with metamorphosis (Gerould, 1906).

In category IV, the only developmental category that generates a feeding pelagosphera, micromeres and macromeres in the A–C quadrants of 8-cell embryos are approximately equal in size. The mechanism most likely responsible for this variation in blastomere size relationships and the associated partitioning of yolk reserves to prototroch cell precursors is a shift in the relative positions of cleavage spindles between the animal and vegetal poles in each blastomere of the four-cell embryo. A shift has been documented in the position of metaphase chromosomes during unequal cleavage in two-cell sipunculan embryos (Boyle

and Rice, 2014) but not yet along the animal-vegetal axis. Characterization of how and when such a mitotic shift takes place in sipunculan embryos will require a series of in-depth molecular labeling experiments. Importantly, the observed differences in maternal yolk investment and asymmetric cell mitosis between blastomeres and between species may represent early "signatures" of the subsequent presence or absence of trochoblast ciliation and a swimming trochophore or whether a feeding or nonfeeding pelagosphera is formed and therefore which life history stages may be expected in a particular sipunculan species (Table 1). One probable cause for these differences among sipunculan embryos could be a genetically regulated increase in yolk protein production and its allocation to larger eggs. Although mechanistic differences in yolk production have not been experimentally determined in our study, it would not be an unreasonable hypothesis for sipunculans, considering previous work in other marine invertebrates (Vance, 1973; Strathmann, 1977, 1985; Levin, 1984; Rice, 1985; McEdward and Morgan, 2001; Pernet and McHugh, 2010).

To date, modern cell lineage and cell fate studies have not been performed in sipunculan embryos. In the absence of such studies, there is a noticeable gap in comparative data among spiralian clades. In *P. cryptum*, *T. alutacea,* and *N. pellucidum*, we have observed that the fundamental topology of spiral cleavage

is conserved from first cleavage through the blastula stage but also recognize that there must be changes in cell sizes, the timing of quadrant-specific cell divisions, and cell fate specification events that differ among their life history patterns. Furthermore, our expectation of such changes suggests there are sipunculan-specific modifications to the "stereotypic" spiralian fate map relative to other lophotrochozoan groups (Martindale and Henry, 1995; Boyer et al., 1998; Henry and Martindale, 1999; Hejnol et al., 2007; Meyer et al., 2010; Boyle and Rice, 2014). Not only do sipunculans produce the unique pelagosphera larval type within the Metazoa, but larval precursor stages also begin to build novel structures such as a buccal organ, lip gland, terminal organ, metatroch, paired sets of retractor muscles, and a distinct U-shaped gut configuration. Individually or collectively, these structures present interesting research problems for comparison with suites of very different, but presumably homologous, structures in the embryos and larvae of closely related spiralian taxa (Gerould, 1906; Åkesson, 1958; Rice, 1985; Purschke et al., 1997; Rouse, 1999; Tzetlin and Purschke, 2006; Henry et al., 2007; Schulze et al., 2007). Moreover, the presence or absence of such structures currently guides our ability to distinguish particular life history patterns among postgastrula stage embryos and larvae within Sipuncula. Thus, developmental morphology

that is directly related to motility or feeding, including trochal, muscular, and digestive organ systems, indicates that there is traceable variation in developmental priorities between life history categories (Figures 3, 4, 5).

When comparing direct development with indirect planktotrophy at ~24 hours after fertilization, we noticed an emphasis on the formation of particular organ systems in each of the two species. In *P. cryptum*, mesodermal linings of coelomic compartments are not yet defined, the esophagus is developing from the stomodeum but shows no sign of a lumen or tubular architecture, and the endoderm does not show any indication of epithelial organization in the region of the intestine (Figure 6A). In contrast, the ciliated, swimming 24-hour embryos of *N. pellucidum* have an esophagus with a lumen that extends in a dorsal direction to its connection with the stomach, there are epithelia forming around the esophageal tube and the stomach, and the founder cells of visceral mesoderm and mesoderm-associated cavities mark the positions of both lateral and anterior coeloms (Figure 6B). In the embryos of *P. cryptum* at ~28–30 hours of development, there are well-defined circular muscles and longitudinal retractor muscle fibers, and the embryo has an ability to contract (Figure 3A). At a similar time of development in the swimming embryos of *T. alutacea*, the circular and retractor muscle groups are visible but not
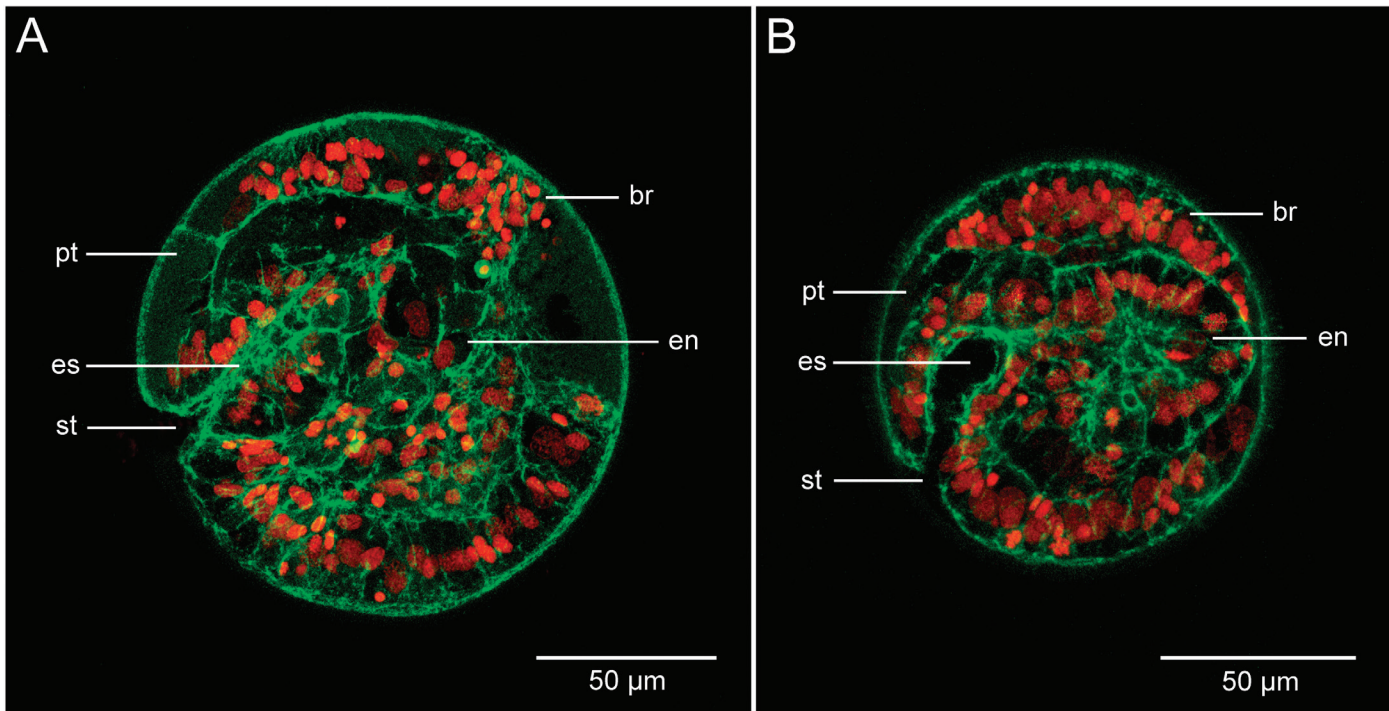

FIGURE 6. Laser scanning confocal micrographs of the postgastrula stage embryos of (A) *Phascolion cryptum* and (B) *Nephasoma pellucidum*. Medial z-stack projections are shown; each specimen is oriented in lateral view with anterior to the top. Both specimens were labeled with phallacidin (green, F-actin) to visualize cell and tissue margins and propidium iodide (red, DNA) to visualize the position of individual cells during development. Time of development is approximately 22 to 24 hours in each species. Abbreviations are as follows: br, brain; en, endoderm; es, esophagus; pt, prototroch cells; st, stomodeum.

as well organized as they are in *P. cryptum*, and no circular or retractor muscle fibers are detectable in *N. pellucidum* at that time. However, epithelia are forming around margins of the esophagus and intestine of *N. pellucidum*, whereas no corresponding epithelia are distinguishable in either one of the lecithotrophic species. Again, organogenesis within the category IV embryo appears to be prioritized for building a functional larval gut that will soon be required to feed on exogenous nutrients, whereas the musculature is already contractile and at a more advanced stage during early development of the crawling vermiform in category I and the crawling-swimming pelagosphera larva of category III, respectively (see Figure 1).

Prioritized differences in organ formation are even more pronounced in the most prominent dispersive stages at 3 days of development (Figures 4, 5). In the 72-hour pelagosphera larva of *N. pellucidum*, different arrangements of cells, tissues, and complex musculature delineate the buccal organ, esophagus, stomach, intestine, and anus of a functional digestive system, and there is a pair of terminal organ retractors that are indirectly involved in feeding but are not found at any stage of development in the other two species. Comparatively thick bands of circular muscles and extensive retractor muscle fibers in *P. cryptum* and *T. alutacea* are observed to facilitate crawling and elongation behavior on and within substrates in the laboratory, yet neither one of their respective digestive systems are complete, nor will they be functional for another 6 to 7 days and 10 to 11 days, respectively (Rice, 1975). During that time, endogenous yolk resources sustain their development through metamorphosis into a juvenile worm. An overall comparison between the most contrasting developmental patterns is revealing: within a few days of fertilization, planktotrophic larvae develop a nervous system for navigation, ciliation for swimming, musculature to obtain and handle food, and a functional gut for digestion, yet from the moment of fertilization in species with direct development, embryogenesis constructs the tissues, organ systems, and body plan of a juvenile worm. Thus, sipunculan life history patterns are developmentally prioritized. This hypothesis implicates a history of shifts in the timing of gene regulation, morphogenesis, and character loss, from egg production through organ system development, during evolutionary transitions between planktotrophy and lecithotrophy (Raff and Wray, 1989; Smith, 2003; Pernet and McHugh, 2010), as discussed further below.

Life History Patterns and Characters Are Asymmetrically Distributed

The two most comprehensive monographs on sipunculan systematics, including detailed taxonomic descriptions, identification keys, and distribution records, estimate the number of species at ~320 (Stephen and Edmonds, 1972) and 149 (Cutler, 1994). However, recent measurements of genetic connectivity between distant, yet potentially overlapping populations, and thus evidence for either cryptic speciation or cosmopolitanism, suggest the true number of valid species is somewhere between those two estimates (Kawauchi and Giribet, 2010, 2013; Schulze et al., 2012; Hsu et al., 2013; Johnson et al., 2015). The number and naming of sipunculan families has also "progressed" over time from several nondistinct group names (Baird, 1868; Pickford, 1947; Åkesson, 1958) to four family designations, Sipunculidae, Golfingiidae, Phascolosomatidae, and Aspidosiphonidae (Stephen and Edmonds, 1972), then six family designations with the addition of Themistidae and Phascolionidae (Cutler, 1994; Maxmen et al., 2003; Schulze et al., 2007). These designations were followed by molecular phylogenetic analyses with a revised proposal for the following six families: Sipunculidae, Golfingiidae, Siphonosomatidae, Antillesomatidae, Phascolosomatidae, and Aspidosiphonidae (Kawauchi et al., 2012). The most recent study of relationships by Lemer et al. (2015), which reevaluated the families with comparative transcriptomic data, independent of all molecular characters previously analyzed for Sipuncula, provided strong support for the six families that were proposed by Kawauchi et al. (2012). When combined with information from multiple related studies of sipunculan development (Rice, 1985, 1989; Rice et al., this volume; Schulze et al., this volume) these phylogenetic analyses have collectively enabled us to assign one or more of the four recognized developmental life history patterns (Figure 1) to each of six sipunculan families. From this assignment process we have discovered that all four developmental life history patterns (I, II, III, IV) are exhibited by species in the family Golfingiidae (Figure 7). Notably, each one of the species examined in our study, *P. cryptum*, *T. alutacea*, and *N. pellucidum*, belongs to one of the sipunculan genera within Golfingiidae. With one recorded exception (Rice, 1970), indirect planktotrophic development in category IV is the only life history pattern observed within each of the five remaining sipunculan families (Figure 7).

On the basis of this correlation of life history pattern with family-level assignment, a variety of modifications to developmental characters must have occurred on the evolutionary branch leading to Golfingiidae. Our observations suggest those modifications may have included variations in yolk production, changes in egg size, asymmetries in the positions and/or the orientations of macromere cleavage spindles, alternate patterns and locations of ciliation, temporal changes in the formation of different organ systems, and the reduction or complete loss of a larval life history stage (Table 1). Accordingly, as mentioned previously, although the nature of spiral cleavage is conserved on multiple levels and among multiple animal clades (Henry and Martindale, 1999), many important studies indicate there is considerable flexibility in the spiralian developmental program (Freeman and Lundelius, 1992; Boyer et al., 1998; Henry and Martindale, 1999; Lambert, 2010), including patterns of blastomere cleavage (Costello and Henley, 1976; Henry, 1986; Render, 1989), determination of embryonic quadrants and axes (Lambert and Nagy, 2003; Henry et al., 2006; Lambert, 2007; Henry and Perry, 2008), cell fate specification (Martindale and Henry, 1995; Hejnol et al., 2007; Lambert, 2007; Meyer and Seaver, 2010; Meyer et al., 2010), and organ formation (Render,
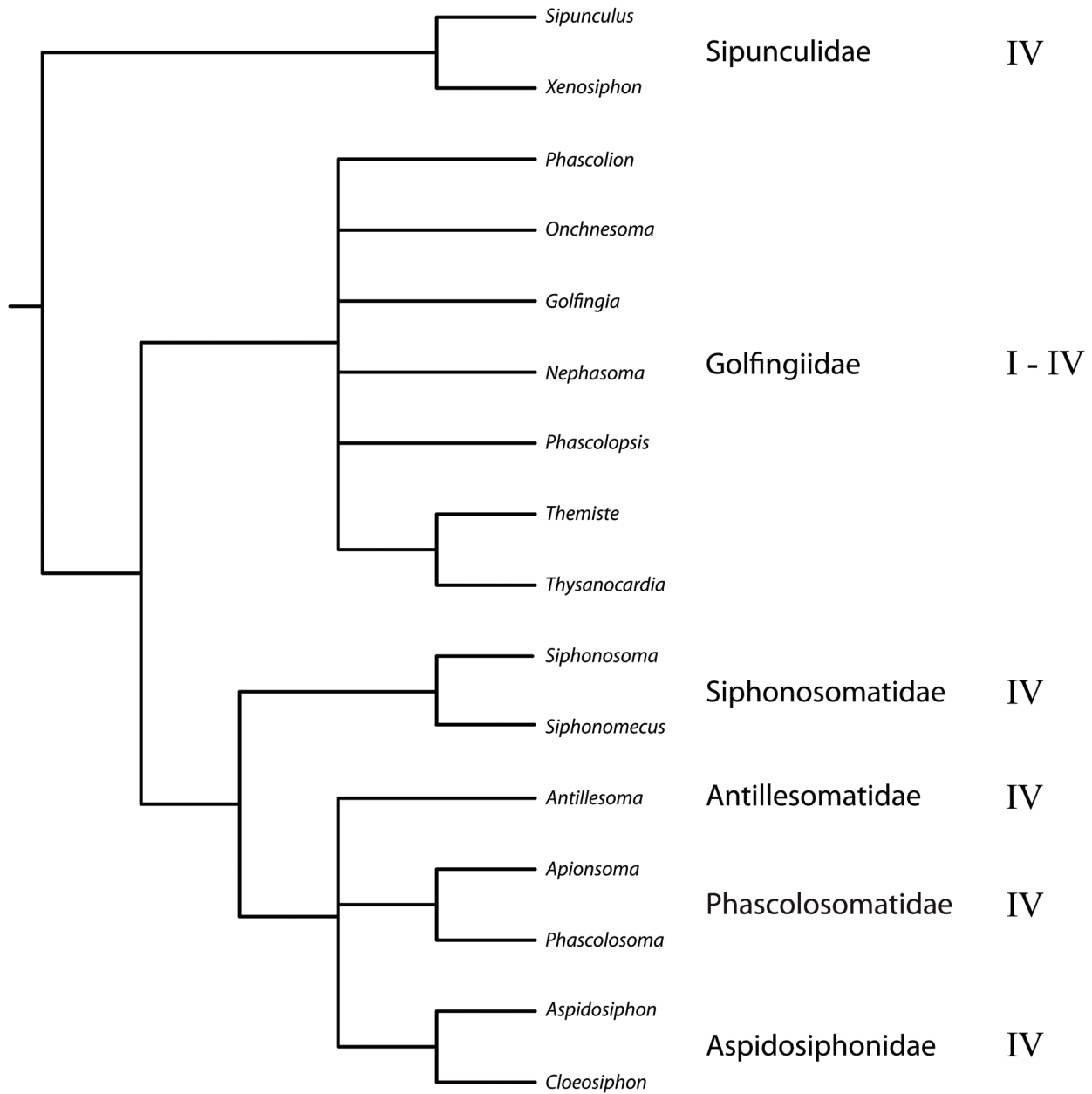
FIGURE 7. Correspondence of developmental life history patterns to a hypothesis of evolutionary relationships within Sipuncula. Genera are located on the branch tips of the cladogram. Five sipunculan families (Sipunculidae, Golfingiidae, Siphonosomatidae, Phascolosomatidae, and Aspidosiphonidae) are located to the right side of individual clades of genera; one family (Antillesomatidae) is placed adjacent to the only recognized genus within its family. Roman numerals designate the predominant developmental life history patterns (see Figure 1) observed within each family. The cladogram is modified from Kawauchi et al. (2012).

1983; Henry, 1989; Maslakova et al., 2004a, 2004b; Henry et al., 2007). If true, such flexibility would suggest there may have also been considerable morphogenetic potential for the evolution of distinct life history patterns, not only within the embryos of ancestral spiralians but also after individual spiralian lineages diverged, such as the branch leading to Sipuncula. We find it very interesting that in every species of sipunculan worm where early development has been examined, embryos undergo a pattern of unequal, holoblastic spiral cleavage (Rice, 1985, 1988). Compared with other spiralian groups that have equal and unequal cleavage (e.g., mollusks, polychaetes) or only equal-cleaving embryos (e.g., nemerteans, platyhelminths), unequal cleavage is conserved across Sipuncula regardless of which developmental life history pattern a particular species exhibits. It is not yet clear how unequal cleavage became the established program for sipunculans worldwide; however, the implications are provocative.

Equal cleavage is thought to be the ancestral or primitive mechanism of D quadrant specification and establishment of the D/V axis in spiralian lophotrochozoans (Freeman and Lundelius, 1992; Lambert, 2010). Unequal cleavage is associated with precocious specification of the D/V axis and the asymmetric distribution of cytoplasmic materials among blastomeres of the early embryo (Freeman and Lundelius, 1992; Boyer and Henry, 1998). Spiralian groups with unequal cleavage tend to have many derived characters, as exemplified by annelid taxa such as oligochaetes and leeches that "display highly modified forms of development" (Henry and Martindale, 1999:262). Freeman and Lundelius (1992) also point out that when the D quadrant is specified early in unequally cleaving spiralians, the first functional stages are observed among life history patterns with direct development or advanced larval forms such as the setigers of polychaetes, veligers of mollusks, and pelagosphera larvae of sipunculans.

The actual timing and mechanism of D quadrant identity or establishment of the D/V axis have not yet been examined in Sipuncula, although with unequal cleavage being conserved across the clade, we assume that these events would be similar to observations in other spiralians (Costello and Henley, 1976; Freeman and Lundelius, 1992; Henry and Martindale, 1999). There is a notable correlation between cleavage pattern and egg size among polychaete annelids (Anderson, 1973; Schroeder and Hermans, 1975), where unequal cleavage is typically associated with larger eggs (≥100 μm). Thorson (1950) found that benthic invertebrate species that develop from small eggs generally have a planktonic larval phase, whereas species with larger eggs (>180 μm) develop directly into juveniles without a larval stage in their life cycle. Jägersten (1972) extends that trend to life history nutritional modes, where a low level of egg yolk is associated with planktotrophy and an abundance of yolk is associated with lecithotrophy. Thus far, every sipunculan species examined has an egg with at least one dimension exceeding 100 μm in diameter, and among species, there is a consistent trend showing that smaller eggs are associated with planktotrophic species, whereas larger eggs are found among a variety of lecithotrophic patterns (Rice, 1985, 1989).

Taken together, egg size, yolk content, cleavage pattern, timing of D quadrant specification, and life history all appear to be correlated. These relationships are clearly demonstrated within Sipuncula (Table 1), where large yolky eggs exhibit unequal spiral cleavage and develop through direct and indirect lecithotrophic life history patterns (Rice, 1967, 1975, 1985, 1989). Such relationships are also consistent among the three species in this study, where contrasting egg sizes, nutritional modes, and priorities in organ development vary with life history pattern: larger eggs, higher yolk content, larger micromeres, and a nonfunctional gut during early development in lecithotrophic species and smaller eggs, lower yolk content, smaller micromeres, and a functional gut in planktotrophic species. However, it is important to recognize that a gradient of egg sizes and intermediate larval types are found in several marine invertebrate groups across the Metazoa, including the occurrence of "facultative feeding" (Allen and Pernet, 2007; Pernet and McHugh, 2010). Although we have not found cases of facultative planktotrophy or lecithotrophy among sipunculan larvae, they may exist. Therefore, a clear planktotrophy-lecithotrophy dichotomy based on larval development, morphology, and food requirements may be somewhat misleading, especially when used to interpret the direction of evolutionary transitions between life histories (Allen and Pernet, 2007).

The Evolution of Developmental Life History Patterns within Sipuncula

The ancestral pattern of development from which all sipunculans are derived was first hypothesized to include yolk-rich eggs and swimming lecithotrophic larvae (Åkesson, 1958; Gerould, 1906; Rice, 1967). The genus *Golfingia* represented that pattern, which included species thought to exhibit the most primitive forms of development and adult morphology (see Rice, 1967). From those ancestors, sipunculans would evolve in one direction toward species with direct development and in another direction toward a life cycle with planktotrophic larvae (Rice, 1975:157, fig. 45). Then, in 1985, Rice proposed a new hypothetical scheme for the evolution of sipunculan developmental patterns (Rice, 1985:291, fig. 18.5). In that scheme, a spiralian stem group taxon with a feeding trochophore gave rise to planktotrophic modes of development in category IV, followed by subsequent evolution of all other life history patterns (Figure 8). Therefore, the inferred diversification of life histories within Sipuncula is from an ancestral pattern of indirect development with a planktotrophic pelagosphera larva toward an oceanic pelagosphera in one direction and, in another direction, toward different forms of indirect lecithotrophic development with a nonfeeding pelagosphera, followed by, or in parallel with, transitions toward direct development, including the eventual reduction and loss of all larval stages. As previously mentioned, all four categories of extant life history patterns are found within Golfingiidae (Figure 7), a single large family that contains approximately one-half of the number of currently recognized species (Cutler, 1994; Kawauchi et al., 2012). The revised hypothetical scheme (Rice,
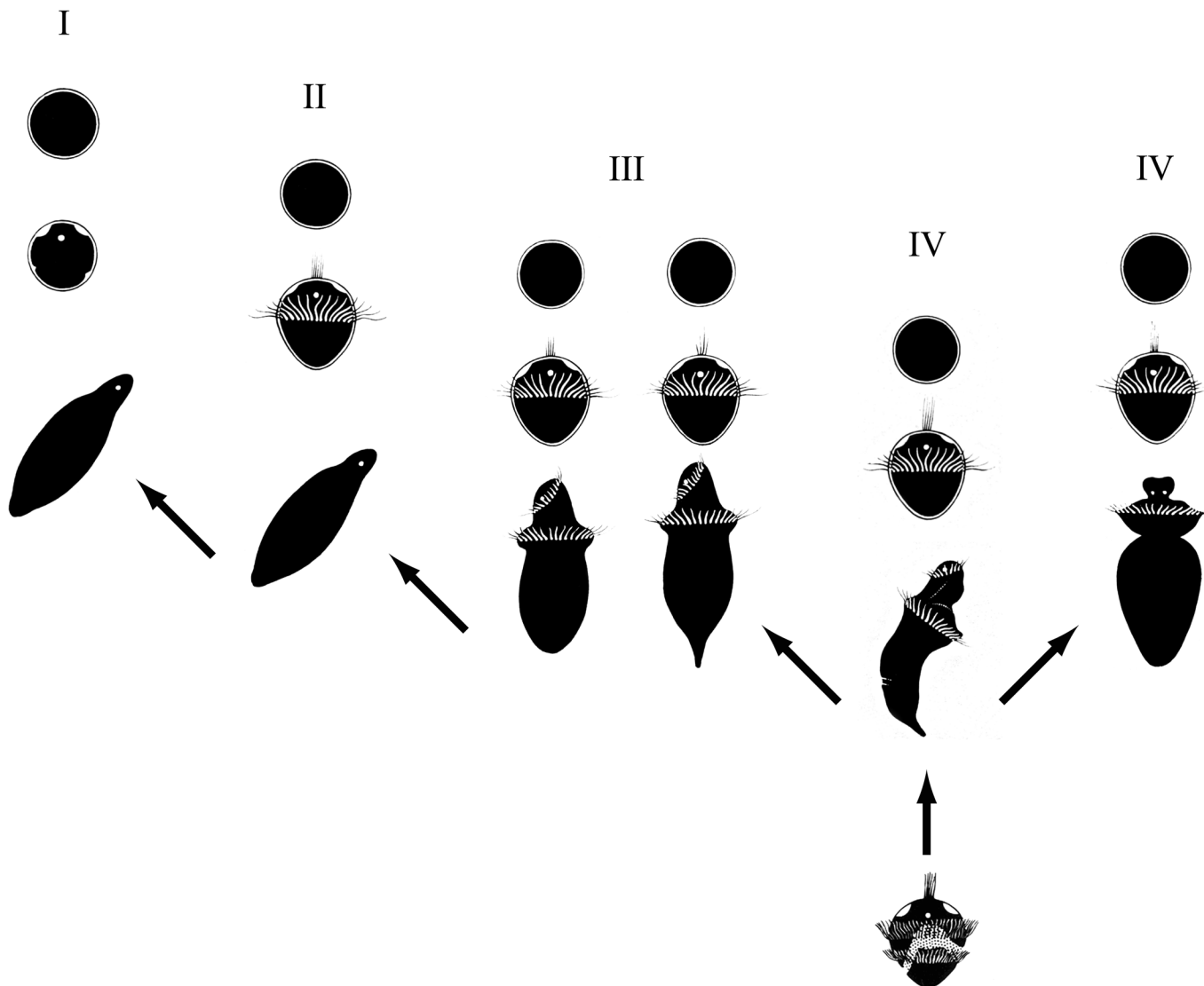
FIGURE 8. Schematic representation of a hypothesis for the evolution of developmental life history patterns within Sipuncula. Roman numerals designate the four recognized life history patterns: (I) direct development, (II) indirect development without a pelagosphera larva, (III) indirect development with a lecithotrophic pelagosphera larva, and (IV) indirect development with a planktotrophic pelagosphera larva. In each of the indirect developing patterns (II, III, IV), the first larval stage is a nonfeeding, lecithotrophic trochophore with a prototrochal band of cilia as the primary organ of locomotion. Indirect patterns with a pelagosphera larva (III, IV) show a metatrochal band of cilia as the primary organ of locomotion. The ancestral larval form for sipunculan life history evolution is inferred to be a feeding trochophore larva, shown at the bottom of this schematic. Modified from Rice (1985).

1985), which designated planktotrophy as part of the ancestral life cycle, was based on development, histology, and morphology. That hypothesis is now generally supported by a series of molecular hypotheses of sipunculan relationships (Schulze et al., 2007; Kawauchi et al., 2012; Lemer et al., 2015).

Additional information that is not depicted in the revised scheme or any of the molecular trees should also be considered. First, all sipunculan trochophore larvae are lecithotrophic, and they are observed in three of the four developmental categories (II, III, IV). Thus, the first larval form of indirect lecithotrophic and indirect planktotrophic development is nonfeeding. Second, gene expression and confocal imaging experiments show that direct development and a unique parthenogenic pattern pass through trochophore-like and elongation stages with gut, musculature, nervous system, and prototroch cells having positions and morphologies similar to their homologous organs in the swimming trochophore and first metamorphic stages of categories II, III, and IV (Boyle and Seaver, 2010; Boyle and Rice,

2014). Such similarities likely reflect a shared history of developmental transitions from the ancestral pattern. Third, among planktotrophic species, there is a broad diversity of both short-lived (Rice, 1985; Schulze and Rice, 2009b) and long-lived pelagosphera larvae (Rice, 1981; Scheltema and Rice, 1990), which may have evolved independently or through speciation in several of the families with exclusively category IV development (Rice, 1985, 1988; Kawauchi et al., 2012). Either developmental categories I, II, and III, all observed within Golfingiidae and found among *Phascolion*, *Golfingia*, *Phascolopis*, *Themiste,* and *Thysanocardia*, have lost planktotrophy, or a feeding larva was lost in their common ancestor, although *Nephasoma* has retained the ancestral category IV pattern (Figure 7). Relationships among genera within Golfingiidae are unresolved and will be the next important target of phylogenetic analysis (Lemer et al., 2015). Fourth, a single case of lecithotrophic parthenogenesis, *Themiste lageniformis* (Pilger, 1987); one hermaphroditic species, *Nephasoma minuta* (Gibbs, 1975); and one with asexual budding, *Aspidosiphon elegans* (Rice, 1970), exist. Apart from budding, the other two examples of asexual reproduction belong to species within Golfingiidae, further suggesting it is the most developmentally diverse family. Moreover, there are definitive fossil sipunculans in lower Cambrian rocks, *Archaeogolfingia* and *Cambrosipunculus*, which are "strikingly similar to the modern golfingiid sipunculans" (Huang et al., 2004:1673). Evidence of "golfingiid-like" body plans from ~520 mya, the earliest known sipunculan records, implies that extant life history patterns (I, II, III, IV) represent a very ancient, yet asymmetric, diversification of developmental phenomena and associated genera within Sipuncula (Figures 7, 8).

Currently, there is no straightforward explanation for how or why direct and indirect lecithotrophic development evolved from an ancestral lineage in which embryogenesis led to a swimming, feeding pelagosphera larva. The presence of intermediate larvae with facultative feeding would help guide an explanation (Allen and Pernet, 2007), although none have been identified. However, among the first three life history categories (I, II, III), the early appearance of juvenile structures (e.g., intestine, introvert retractors, nephridia, tentacles) is prevalent. For example, relative to the development of *N. pellucidum* (IV), which produces smaller eggs with lower yolk content and a functional larval gut, the nonfeeding lecithotrophic larva of *T. alutacea* (III) and direct development in *P. cryptum* (I) reach their respective juvenile stages much earlier. The observed contrast may reflect lineage-specific adaptations to expedite the colonization of available parental habitats (e.g., gastropod shells, reef structure). Within these and other habitats, particular sipunculan species are common (Rice, 1975; Rice et al., 1983, 1995; Pilger, 1987). The lack of a larval stage or having short-duration nonfeeding larval stages would limit dispersal distance and might help retain offspring locally in subsequent generations, especially if there is active selection for adult habitats as observed in other marine worms (Levin, 1984; Grassle et al., 1992; Qian, 1999; Snelgrove et al., 1999; Pernet, 2003). The wide distribution and abundance of *P. cryptum* in assorted gastropod shells of seagrass beds along the IRL and *T. alutacea* within coquina reefs along the outer coast are examples of populations that likely benefited from evolutionary transformations to lecithotrophy (Figures 7, 8). In these environments, accelerated development of juvenile worms would shorten the overall length of time from fertilization to metamorphosis and settlement. Therefore, within Sipuncula, direct and indirect lecithotrophic life histories may imply adaptive developmental strategies. This implication is supported by evidence for the direction of evolution from an ancestral pattern (explained above), a situation in contrast to most molluscan life histories for which an adaptive explanation is apparently not required (discussed below), and also by the observed contrasts in egg size, yolk content, and delay in timing of a functional gut relative to planktotrophy (*N. pellucidum*), which are consistent with similar hypotheses for lecithotrophy in other spiralian taxa (Strathmann, 1978, 1985; Freeman and Lundelius, 1992; Schneider et al., 1992; Pernet, 2003; Pernet and McHugh, 2010). In a more simplistic and primarily correlative view (Thorson, 1950; Jägersten, 1972; Anderson, 1973; Schroeder and Hermans, 1975; Rice, 1985, 1989; Strathmann, 1985), larger relative egg sizes from larger maternal investments of yolk have led to lecithotrophy and direct development. Thus far, no definitive molecular or genetic mechanisms have explained this correlation (Thorson, 1950; Strathmann, 1985).

Our observations have been focused primarily on morphological similarities and differences between the most contrasting life history patterns: direct and indirect planktotrophic development of *P. cryptum* and *N. pellucidum*, respectively. Compared with embryonic stages, as previously described (Figure 6), developmental priorities in organ system formation are even more pronounced in subsequent stages (Figure 9). At ~56 to 58 hours of development in *N. pellucidum*, circular and retractor muscles are active; musculature of the buccal organ, esophagus, stomach, and intestine is well developed; a ciliated track joins major subregions of the digestive system; and the terminal organ is almost functional. The brain and ventral nerve cord are morphologically distinct and tethered by muscle fibers, ciliated metatroch cells are in place, ciliated prototroch cells are relatively small, and coelomic and digestive compartments are almost depleted in yolk (Schulze and Rice, 2009b), although nephridia are not yet organized. In contrast, at ~33 to 35 hours of development in *P. cryptum*, although circular and retractor muscles are active and well developed, the gut is not differentiated into epithelia or lined with cilia, there is no buccal or terminal organ, the brain and nerve cord are not well organized, there are no ciliated bands, the prototroch cells are large with yolk, and the nephridia show distinct morphology. Comparatively, both species are ~10 to 12 hours away from producing their most prominent dispersal stages: the planktotrophic pelagosphera of *N. pellucidum* and the crawling vermiform of *P. cryptum* (Figures 4, 5). Although some organs are present in one species and not the other, we can assume that shared developmental characters are homologous and that both species pass through a similar stage, for
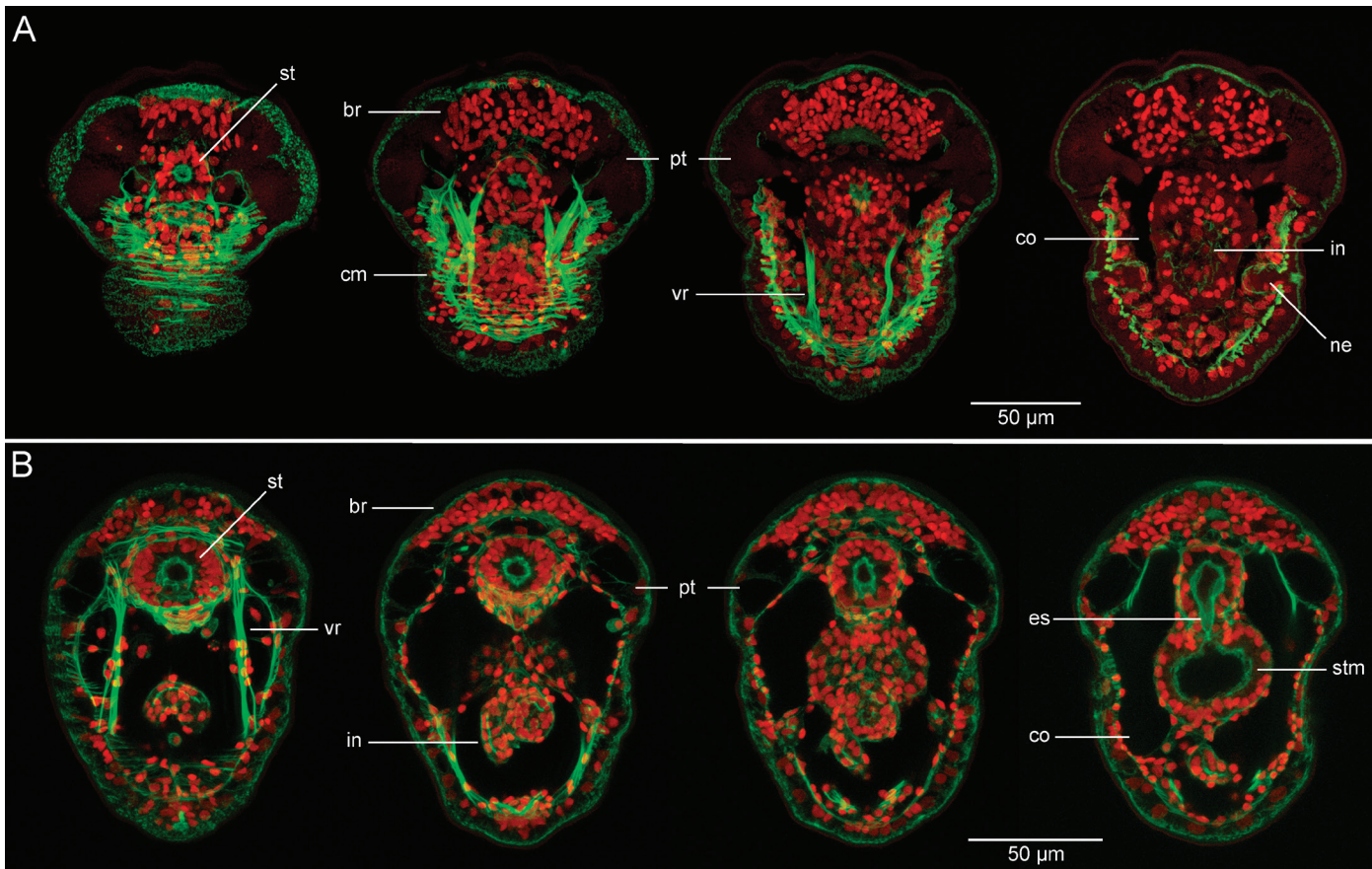
FIGURE 9. Laser scanning confocal micrographs of the elongation stages of (A) *Phascolion cryptum* and (B) *Nephasoma pellucidum*. Each series of images shows a single specimen viewed in four progressively deeper z-stack projections through the medial region from left to right. Each image is a ventral view with anterior to the top. Both specimens were labeled with phallacidin (green, F-actin) to visualize tissue margins, musculature, and the digestive system and propidium iodide (red, DNA) to visualize the position of individual cells during development. Time of development in each series of confocal micrographs is approximately 35 hours for *P. cryptum* and 58 hours for *N. pellucidum*. Abbreviations are as follows: br, brain; cm, circular muscle fibers; co, coelom; es, esophagus; in, intestine; ne, nephridium; pt, prototroch cells; st, stomodeum; stm, stomach; vr, ventral retractor muscle fibers.

example, a ciliated prototroch in the swimming trochophore of *N. pellucidum* and an unciliated prototroch in the nonswimming trochophore-like stage of *P. cryptum* (Figure 3).

Our observations reveal changes in the timing of appearance of differentiated cell types, tissues, and organs between similar stages of development in each species. Such changes between closely related species of marine invertebrates with contrasting life histories are considered indicators of a history of heterochronic shifts in development (Jägersten, 1972; Strathmann, 1985; Raff and Wray, 1989; Swalla et al., 1994; Byrne, 1995; Irvine et al., 1999; McEdward, 2000; Wray and Strathmann, 2002; Smith, 2003; Moss, 2007; Raff, 2008; Page, 2009; Pernet and McHugh, 2010). Cautiously, we recognize that heterochrony is only relative to an inferred ancestral pattern of development, which for Sipuncula includes a planktotrophic pelagosphera. Therefore, temporal shifts in embryonic and larval morphogenesis would

implicate not only past changes in the timing of development of homologous characters but also loss of characters (e.g., ciliated bands, buccal organ, terminal organ) during diversification from indirect planktotrophy through lecithotrophy to direct development without an intervening larval stage (Figure 8). As Page (2009) insightfully points out, molluscan life histories essentially undergo direct development of class-specific juvenile bodies, upon which transient larval structures are "superimposed" for a temporary planktonic phase. Because they do not have separate larval and juvenile body plans, "heterochrony is not needed to explain the appearance of juvenile structures prior to loss of the transient larval structures" (Page, 2009:223). However, sipunculan worms with direct development (*P. cryptum*) have lost multiple characters, including whole organs, during their inferred diversification from planktotrophy. Thus, although pelagosphera and juvenile stages share the same body, the pelagosphera is not

simply a transient, swimming, feeding planktonic juvenile with prolonged direct development. A planktotrophic pelagosphera must transform from a trochophore into its unique body plan with many larval-specific characters. Subsequent development entails a complex metamorphosis of anterior organ systems, including formation of a retractable introvert with elaborate tentacular and sensory systems, and with an extended planktonic period, some teleplanic forms may not be able to undergo metamorphosis, thereby swimming and feeding indefinitely as a larva. Heterochrony is most likely part of a broader explanation for the evolution of direct development from indirect planktotrophy in Sipuncula (Figure 8). Relative to the ancestral life history, we hypothesize that there have been multiple adaptive reductions in the amount of developmental time required to build juvenile organs in direct and indirect lecithotrophic species within Golfingiidae (Figure 7).

It will be important to follow this up with an empirical test for "phylogenetic patterns of change in developmental timing" by defining specific features of multiple characters and plotting them onto independent sipunculan phylogenies (Smith, 2003:615). This approach will attempt to evaluate the polarity of developmental changes across life histories within Sipuncula, possibly within accurate evolutionary time frames (Raff and Wray, 1989; Smith, 2003; Kawauchi et al., 2012). Of course, a number of associated challenges need to be to overcome. Schematically, the above hypothesis is oversimplified (Figure 8), as direct and indirect lecithotrophy may have diversified in parallel, through a number of putative intermediate feeding and/or nonfeeding larval stages. Developmentally, the complex process of diversification would require a series of heritable changes leading to the loss or suppression of larval characters (Strathmann, 1978; Freeman and Lundelius, 1992; Rouse, 2000), including changes in the regulation, timing, and location of the expression of transcription factors and signaling proteins within gene networks that specify those characters (Boyle and Seaver, 2010; Boyle et al., 2014; McEdward, 2000; Raff, 2008; Wray, 2007). Additional challenges include resolving the current lack of congruence between classification schemes and between molecular and morphological trees (Stephen and Edmonds, 1972; Gibbs and Cutler, 1987; Cutler, 1994; Jenner, 2004; Bleidorn, 2007; Kawauchi et al., 2012), as well as interpreting primary absence or secondary loss for particular characters and thus the correct direction of character evolution (Purschke et al., 2000, 2014; Jenner, 2004; Bleidorn, 2007).

Across the Metazoa, the loss and/or homoplasy of both molecular and morphological characters appears to be more common than previously thought (Moore and Willmer, 1997; Purschke et al., 2000, 2014; Purschke, 2002; Bleidorn, 2007; Dunn et al., 2014, 2015; Jékely et al., 2015). Within Annelida, there are several hypotheses of morphological character reduction or loss, with examples from coelomic cavities (Smith et al., 1986), nuchal organs (Purschke et al., 2000; Rouse and Pleijel, 2001; Purschke, 2002), eyes (Worsaae, 2005), chaetae (Ax, 1999; Rouse and Pleijel, 2001), and ciliary bands (Rouse, 1999;

Purschke, 2002). Furthermore, some relatively recent studies suggest there has been a loss of segmentation in both the Echiura and Sipuncula (Purschke et al., 2000; Hessling and Westheide, 2002; Bleidorn, 2007; Kristof et al., 2008), although in sipunculans such interpretations are questionable (Åkesson, 1958; Rice, 1985; Wanninger et al., 2005; Boyle and Rice, 2014). These challenges also extend to life histories, where each pattern represents a suite of characters. For example, not long ago, lecithotrophy was considered the ancestral developmental mode for polychaetes, with feeding larvae having evolved several times (Rouse, 2000). Yet among marine invertebrates in general, and several clades in particular, the inferred direction of evolution includes a loss of ancestral planktotrophy and associated feeding structures (Strathmann, 1978; Hart, 1996; Wray, 1996; Pernet, 2003; Nielsen, 2009; Freeman, 2015). Our scheme of sipunculan diversification does not conflict with that trend (Figure 8), but it does contrast with the suggested ancestral mode for polychaetes. However, the unsegmented Sipuncula are now considered members of the segmented Annelida (Struck et al., 2007, 2011; Dunn et al., 2008), with developmental and phylogenetic evidence for planktotrophy as the ancestral pattern. Upon review of the two most recent phylogenomic relationships, sipunculans are hypothesized to be among the basal annelid lineages (Struck et al., 2011; Weigert et al., 2014), which all appear to have feeding larvae. Thus, planktotrophy is most likely a plesiomorphic condition of the annelid radiation, providing a new framework for character reconstruction.

DEEPER QUESTIONS AND FUTURE GOALS OF LIFE HISTORY RESEARCH

Indeed, this is an exciting time to revisit fundamental questions about the origins and evolution of marine invertebrate life histories, including definitions of indirect development. Some invertebrate groups "do not have a larval body separate from the juvenile/adult body" (Page, 2009:223; see also Rawlinson, 2010), in contrast to other groups in which the larval and juvenile bodies are distinct and independent of each other (Zimmer and Woollacott, 1977; Davidson et al., 1995; Maslakova, 2010). Obviously, these groups are not directly comparable. As Page (2009) has suggested for some molluscan taxa, indirect development could be reinterpreted as the temporary planktonic phase of an otherwise direct life cycle. There are also views on the origin of indirect life history patterns in which larval forms represent transitory, feeding or nonfeeding dispersive stages between the embryonic products of adult reproduction and descendants of the next generation. One view suggests that the primitive life cycle of an "ancestral coelomate protostome" would include a bathypelagic larval form (Freeman and Lundelius, 1992:235; Jägersten, 1972). This life history pattern would most likely have been inherited from the protostome-deuterostome ancestor, the eubilaterian stem species. Another view suggests that the ancestral bilaterian, a predecessor of the protostome-deuterostome ancestor, was a direct-developing marine organism resembling

an acoelomorph flatworm (Hejnol and Martindale, 2008; Raff, 2008). Yet the bilaterally symmetric planula larvae of Cnidaria, which branched off from a lineage leading to the Bilateria, imply that indirect development is perhaps the ancient life history pattern for bilaterian taxa, if not Metazoa. These and other larval forms further imply that indirect larval stages were reinstalled in the life cycles of marine organisms leading from the direct-developing Urbilateria to the protostome-deuterostome ancestor. Scientists may never know the correct answer about the original life history pattern of the metazoan stem species, which is likely obscured because of the loss of species through extinction and, with them, the loss of essential clues about patterns and processes of life history evolution. Therefore, we remain cautious about broad statements on the origins of direct versus indirect development in major clades (e.g., Protostomia, Lophotrochozoa). In the case of Sipuncula, our current view is relatively clear: planktotrophy is part of the ancestral developmental life history pattern.

With this investigation, we have shown that there is stark contrast between direct development and indirect planktotrophy in the priority and timing of events that take place during construction of functional organ systems (Figure 9). However, the morphogenesis of complex organ systems, modifications to existing gene networks that specify them, and the loss or gain of cell types, tissues, and organs over time are poorly understood processes. With access to living embryonic and larval resources from contrasting life history patterns in Florida, there is an impetus for applying a more complete set of tools to our life histories research. We have begun to unravel some of the mysteries with experiments on developmental gene expression; we have generated developmental transcriptome catalogs for three species, including *P. cryptum* and *N. pellucidum*, and we will be attempting to establish the first cell lineage and fate map for a member of Sipuncula. With this integrated approach, it will be possible to finally address several outstanding questions for comparison with studies in other spiralian groups: Why are there no sipunculan eggs that undergo equal cleavage? When is the sipunculan D quadrant specified? And why are there no feeding trochophore larvae within this clade? The Sipuncula should be, and will be, pursued as new and complementary nonmodel organisms in the field of evolutionary developmental biology.

ACKNOWLEDGMENTS

REFERENCES

Adrianov, A. V., and A. S. Maiorova. 2010. Reproduction and Development of Common Species of Peanut Worms (Sipuncula) from the Sea of Japan. *Russian Journal of Marine Biology*, 36(1):1–15. https://doi.org/10.1134/S1063074010010013.

Adrianov, A. V., and A. S. Maiorova. 2012. Peanut Worms of the Phylum Sipuncula from the Nha Trang Bay (South China Sea) with a Key to Species. *Zootaxa*, 3166:41–58.

Adrianov, A. V., A. S. Maiorova, and V. V. Malakhov. 2008. Embryonic and Larval Development of the Peanut Worm *Themiste pyroides* (Sipuncula: Sipunculoidea) from the Sea of Japan. *Invertebrate Reproduction and Development*, 52(3):143–151. https://doi.org/10.1080/07924259.2008.9652282

Adrianov, A. V., A. S. Maiorova, and V. V. Malakhov. 2011. Embryonic and Larval Development of the Peanut Worm *Phascolosoma agassizii* (Keferstein 1867) from the Sea of Japan (Sipuncula: Phascolosomatidea). *Invertebrate Reproduction and Development*, 55(1):22–29. https://doi.org/10.1080/07924259.2010.548638.

Åkesson, B. 1958. *A Study of the Nervous System of the Sipunculoideae, with Some Remarks on the Development of the Two Species* Phascolion strombi *Montagu and* Golfingia minuta *Keferstein*. Lund, Sweden: C. W. K. Gleerup.

Åkesson, B. 1961. The Development of *Golfingia elongata* Keferstein (Sipunculidea) with Some Remarks on the Development of Neurosecretory Cells in Sipunculids. *Arkiv för Zoologi*, 13(23):511–531.

Allen, J. D., and B. Pernet. 2007. Intermediate Modes of Larval Development: Bridging the Gap between Planktotrophy and Lecithotrophy. *Evolution and Development*, 9(6):643–653. https://doi.org/10.1111/j.1525-142X.2007.00202.x.

Amor, A. 1975. "On the Zoogeography of the Sipuncula Fauna in South America." In *Proceedings of the International Symposium on the Biology of the Sipuncula and Echiura*, ed. M. E. Rice and M. Todorović, pp. 19–24. Belgrade: Naučno Delo Press.

Anderson, D. T. 1973. *Embryology and Phylogeny in Annelids and Arthropods*. Oxford: Pergamon Press.

Ax, P. 1999. *Das System der Metazoa: Ein Lehrbuch der phylogenetischen Systematik.* Volume 2. Stuttgart: Fischer Verlag.

Baird, W. B. 1868. Monograph on the Species of Worms Belonging to the Subclass Gephyreae with a Notice of Such Species as Contained in the Collection of the British Museum Ann. Des Scie. Nat 3sr. Zool. *Proceedings of the Zoological Society of London*, 77:76–114.

Bleidorn, C. 2007. The Role of Character Loss in Phylogenetic Reconstruction as Exemplified for the Annelida. *Journal of Zoological Systematics and Evolutionary Research*, 45(4):299–307. https://doi.org/10.1111/j.1439-0469.2007.00425.x.

Boore, J. L., and J. L. Staton. 2002. The Mitochondrial Genome of the Sipunculid *Phascolopsis gouldii* Supports Its Association with Annelida Rather Than Mollusca. *Molecular Biology and Evolution*, 19(2):127–137. https://doi.org/10.1093/oxfordjournals.molbev.a004065.

Boyer, B. C., J. J. Henry, and M. Q. Martindale. 1998. The Cell Lineage of a Polyclad Turbellarian Embryo Reveals Close Similarity to Coelomate Spiralians. *Developmental Biology*, 204(1):111–123. https://doi.org/10.1006/dbio.1998.9084.

Boyer, B. C., and J. Q. Henry. 1998. Evolutionary Modifications of the Spiralian Developmental Program. *American Zoologist*, 38(4):621–633. https://doi.org/10.1093/icb/38.4.621.

Boyle, M. J., and M. E. Rice. 2014. Sipuncula: An Emerging Model of Spiralian Development and Evolution. *International Journal of Developmental Biology*, 58(6–8):485–499.

Boyle, M. J., and E. C. Seaver. 2009. "Evidence of a Dorsal Pharynx in the Marine Polychaete *Capitella teleta*." Proceedings of the Ninth International Polychaete Conference, *Zoosymposia*, 2:317–328.

Boyle, M. J., and E. C. Seaver. 2010. Expression of FoxA and GATA Transcription Factors Correlates with Regionalized Gut Development in Two Lophotrochozoan Marine Worms: *Chaetopterus* (Annelida) and *Themiste lageniformis* (Sipuncula). *EvoDevo*, 1:2. https://doi.org/10.1186/2041-9139-1-2.

Boyle, M. J., E. Yamaguchi, and E. C. Seaver. 2014. Molecular Conservation of Metazoan Gut Formation: Evidence from Expression of Endomesoderm Genes in *Capitella teleta* (Annelida). *EvoDevo*, 5:39. https://doi.org/10.1186/2041-9139-5-39.

Byrne, M., 1995. Changes in Larval Morphology in the Evolution of Benthic Development by *Patiriella exigua* (Asteroidea: Asterinidae), a Comparison with the Larvae of *Patiriella* Species with Planktonic Development. *Biological Bulletin*, 188(3):293–305. https://doi.org/10.2307/1542306.

Carlon, D. B., and R. R. Olson. 1993. Larval Dispersal Distance as an Explanation for Adult Spatial Pattern in Two Caribbean Reef Corals. *Journal of Experimental Marine Biology and Ecology*, 173(2):247–263. https://doi.org/10.1016/0022-0981 (93)90056-T.

Clark, R. B. 1969. "Systematics and Phylogeny: Annelida, Echiura, Sipuncula." In *Chemical Zoology*, Volume 4, ed. M. Florkin and B. T. Scheer, pp. 1–62. London: Academic Press.

Conklin, E. G. 1897. The Embryology of *Crepidula*. *Journal of Morphology*, 13:1–226. https://doi.org/10.1002/jmor.1050130102.

Conway-Morris, S., and J. S. Peel. 2008. The Earliest Annelids: Lower Cambrian Polychaetes from the Sirius Passet Lagerstätte, Peary Land, North Greenland. *Acta Palaeontologica Polonica*, 53(1):137–148.

Costello, D. P., and C. Henley. 1976. Spiralian Development: A Perspective. *American Zoologist*, 16:277–291. https://doi.org/10.1093/icb/16.3.277.

Cutler, E. B. 1975. "Distribution of Non-tropical Atlantic Ocean Sipuncula." In *Proceedings of the International Symposium on the Biology of the Sipuncula and Echiura*, ed. M. E. Rice and M. Todorović, pp. 25–33. Belgrade: Naučno Delo Press.

Cutler, E. B. 1977. The Bathyal and Abyssal Sipuncula. *Galathea Report*, 14:135–156.

Cutler, E. B. 1994. *The Sipuncula: Their Systematics, Biology, and Evolution*. Ithaca: Comstock Publishing Associates.

Davidson, E. H., K. J. Peterson, and R. A. Cameron. 1995. Origin of Bilaterian Body Plans: Evolution of Developmental Regulatory Mechanisms. *Science*, 270(5240):1319–1325. https://doi.org/10.1126/science.270.5240.1319.

Dordel, J., F. Fisse, G. Purschke, and T. H. Struck. 2010. Phylogenetic Position of Sipuncula Derived from Multi-gene and Phylogenomic Data and Its Implication for the Evolution of Segmentation. *Journal of Zoological Systematics and Evolutionary Research*, 48(3):197–207. https://doi.org/10.1111/j.1439-0469.2010.00567.x.

Du, X., Z. Chen, Y. Deng, and Q. Wang. 2009. Comparative Analysis of Genetic Diversity and Population Structure of *Sipunculus nudus* as Revealed by Mitochondrial COI Sequences. *Biochemical Genetics*, 47(11–12):884–891. https://doi.org/10.1007/s10528-009-9291-x.

Dunn, C. W., G. Giribet, G. D. Edgecombe, and A. Hejnol. 2014. Animal Phylogeny and Its Evolutionary Implications. *Annual Review of Ecology, Evolution, and Systematics*, 45:371–395. https://doi.org/10.1146/annurev-ecolsys-120213-091627.

Dunn, C. W., A. Hejnol, D. Q. Matus, K. Pang, W. E. Browne, S. A. Smith, E. Seaver, G. W. Rouse, M. Obst, G. D. Edgecombe, M. V. Sorensen, S. H. Haddock, A. Schmidt-Rhaesa, A. Okusu, R. M. Kristensen, W. C. Wheeler, M. Q. Martindale, and G. Giribet. 2008. Broad Phylogenomic Sampling Improves Resolution of the Animal Tree of Life. *Nature*, 452(7188):745–749. https://doi.org/10.1038/nature06614.

Dunn, C. W., S. P. Leys, and S. H. Haddock. 2015. The Hidden Biology of Sponges and Ctenophores. *Trends in Ecology and Evolution*, 30(5):282–291. https://doi.org/10.1016/j.tree.2015.03.003.

Eibye-Jacobsen, D., and J. Vinther. 2012. Reconstructing the Ancestral Annelid. *Journal of Zoological Systematics and Evolutionary Research*, 50(1):85–87. https://doi.org/10.1111/j.1439-0469.2011.00651.x.

Fisher, W. K. 1952. The Sipunculid Worms of California and Baja, California. *Proceedings of the U.S. National Museum*, 102(3306):371–450. https://doi.org/10.5479/si.00963801.102-3306.371.

Freeman, G. 2015. "Phyla, Phylogeny, and Embryonic Body Plans." In *Conceptual Change in Biology: Scientific and Philosophical Perspectives on Evolution and Development*, ed. A. C. Love, pp. 221–241. Boston Studies in the Philosophy and History of Science 307. New York: Springer.

Freeman, G., and J. W. Lundelius. 1992. Evolutionary Implications of the Mode of D Quadrant Specification in Coelomates with Spiral Cleavage. *Journal of Evolutionary Biology*, 5:205–247. https://doi.org/10.1046/j.1420-9101.1992.5020205.x.

Gerould, J. H. 1903. "Studies on the Embryology of the Sipunculidae. I. The Embryonal Envelope and Its Homologue." In *Mark Anniversary Volume*, pp. 437–452. New York: Henry Holt and Company.

Gerould, J. H. 1906. The Development of *Phascolosoma* (Studies on the Embryology of the Sipunculidae Ii). *Zoologische Jahrbücher. Abteilung für Anatomie und Ontogenie der Tiere*, 23:77–162.

Gibbs, P. E. 1975. Gametogenesis and Spawning in a Hermaphroditic Population of *Golfingia minuta* (Sipuncula). *Journal of the Marine Biological Association of the United Kingdom*, 55:69–82. https://doi.org/10.1017/S0025315400015757.

Gibbs, P. E., and E. B. Cutler. 1987. A Classification of the Phylum Sipuncula. *Bulletin of the British Museum* (*Natural History*), *Zoology*, 52(1):43–58.

Grassle, J. P., C. Ann Butman, and S. W. Mills. 1992. Active Habitat Selection by *Capitella* Sp. I Larvae. II. Multiple-Choice Experiments in Still Water and Flume Flows. *Journal of Marine Research*, 50(4):717–743. https://doi.org/10.1357/002224092784797557.

Grübe, E., and A. S. Oersted, 1858. Annulata Oerstediana. Videnskabelige Meddelelser fra Dansk naturhistorisk Forening I Kjøbenhaven, pp. 105–120. Copenhagen.

Haldar, B. P. 1975. "Sipuncula of the Indian Ocean in the Collection of the Zoological Society of India." In *Proceedings of the International Symposium on the Biology of the Sipuncula and Echiura*, ed. M. E. Rice and M. Todorović, pp. 51–92. Belgrade: Naučno Delo Press.

Hall, J. R., and R. S. Scheltema. 1975. "Comparative Morphology of Open-Ocean Pelagosphaera." In *Proceedings of the International Symposium on the Biology of the Sipuncula and Echiura*, ed. M. E. Rice and M. Todorović, pp. 18–25. Belgrade: Naučno Delo Press.

Hart, M. W. 1996. Evolutionary Loss of Larval Feeding: Development, Form and Function in a Facultatively Feeding Larva, *Brisaster latifrons*. *Evolution*:174–187. https://doi.org/10.1111/j.1558-5646.1996.tb04484.x.

Hatschek, B. 1883. Ueber Entwicklung von *Sipunculus nudus*. *Arbeiten aus dem Zoologischen Institute der Universität-Wien und der Zoologischen Station in Triest*, 5:61–140.

Havenhand, J. N. 1993. Egg to Juvenile Period, Generation Time, and the Evolution of Larval Type in Marine Invertebrates. *Marine Ecology Progress Series*, 97:247–260. https://doi.org/10.3354/meps097247.

Hazprunar, G., L. v. Salvini-Plawen, and R. M. Rieger. 1995. Larval Planktotrophy—A Primitive Trait in the Bilateria? *Acta Zoologica*, 76(2):141–154. https://doi.org/10.1111/j.1463-6395.1995.tb00988.x.

Hejnol, A., and M. Q. Martindale. 2008. Acoel Development Supports a Simple Planula-Like Urbilaterian. *Philosophical Transactions of the Royal Society B, Biological Sciences*, 363(1496):1493–1501. https://doi.org/10.1098/rstb.2007.2239.

Hejnol, A., M. Q. Martindale, and J. Q. Henry. 2007. High-Resolution Fate Map of the Snail *Crepidula fornicata*: The Origins of Ciliary Bands, Nervous System, and Muscular Elements. *Developmental Biology*, 305(1):63–76. https://doi.org/10.1016/j.ydbio.2007.01.044.

Hendrix, G. Y. 1975. A Review of the genus *Phascolion* (Sipuncula) with the Descriptions of Two New Species from the Western Atlantic. In *Proceedings of the International Symposium on the Biology of the Sipuncula and Echiura*, ed. M. E. Rice and M. Todorović, pp. 117–140. Belgrade: Naučno Delo Press.

Henry, J. J. 1986. The Role of Unequal Cleavage and the Polar Lobe in the Segregation of Developmental Potential During First Cleavage in the Embryo of *Chaetopterus variopedatus*. *Roux's Archives of Developmental Biology*, 195:103–116. https://doi.org/10.1007/BF00456106.

Henry, J. J. 1989. Removal of the Polar Lobe Leads to the Formation of Functionally Deficient Photocytes in the Annelid *Chaetopterus variopedatus*. *Roux's Archives of Developmental Biology*, 198:129–136. https://doi.org/10.1007/BF02438937.

Henry, J. J., and K. J. Perry. 2008. MAPK Activation and the Specification of the D Quadrant in the Gastropod Mollusc, *Crepidula fornicata*. *Developmental Biology*, 313(1):181–195. https://doi.org/10.1016/j.ydbio.2007.10.019.

Henry, J. Q., A. Hejnol, K. J. Perry, and M. Q. Martindale. 2007. Homology of Ciliary Bands in Spiralian Trochophores. *Integrative and Comparative Biology*, 47(6):865–871. https://doi.org/10.1093/icb/icm035.

Henry, J. Q., and M. Q. Martindale. 1999. Conservation and Innovation in Spiralian Development. *Hydrobiologia*, 402:255–265. https://doi.org/10.1023/A:1003756912738.

Henry, J. Q., K. J. Perry, and M. Q. Martindale. 2006. Cell Specification and the Role of the Polar Lobe in the Gastropod Mollusc *Crepidula fornicata*. *Developmental Biology*, 297(2):295–307. https://doi.org/10.1016/j.ydbio.2006.04.441

Hessling, R., and W. Westheide. 2002. Are Echiura Derived from a Segmented Ancestor? Immunohistochemical Analysis of the Nervous System in Developmental Stages of *Bonellia viridis*. *Journal of Morphology*, 252(2):100–113. https://doi.org/10.1002/jmor.1093.

Hsu, T. H., Y. Ning, J. C. Gwo, and Z. N. Zeng. 2013. DNA Barcoding Reveals Cryptic Diversity in the Peanut Worm *Sipunculus nudus*. *Molecular Ecology Resources*, 13(4):596–606. https://doi.org/10.1111/1755-0998.12097.

Huang, D. Y., J. Y. Chen, J. Vannier, and J. I. Saiz Salinas. 2004. Early Cambrian Sipunculan Worms from Southwest China. *Proceedings of the Royal Society B, Biological Sciences*, 271(1549):1671–1676. https://doi.org/10.1098/rspb.2004.2774.

Irvine, S. Q., O. Chaga, and M. Q. Martindale. 1999. Larval Ontogenetic Stages of *Cheatopterus*: Developmental Heterochrony in the Evolution of Chaetopterid Polychaetes. *Biological Bulletin*, 197(3):319–331. https://doi.org/10.2307/1542786.

Jägersten, G., 1972. *Evolution of the Metazoan Life Cycle*. London: Academic Press.

Jékely, G., J. Paps, and C. Nielsen. 2015. The Phylogenetic Position of Ctenophores and the Origin(s) of Nervous Systems. *EvoDevo*, 6:1. https://doi.org/10.1186/2041-9139-6-1.

Jenner, R. A. 2004. When Molecules and Morphology Clash: Reconciling Conflicting Phylogenies of the Metazoa by Considering Secondary Character Loss. *Evolution and Development*, 6(5):372–378. https://doi.org/10.1111/j.1525-142X.2004.04045.x.

Johnson, N. D., C. Sanders, A. Maiorova, and A. Schulze. 2015. Cryptic Species in Pacific Sipunculans (Sipuncula: Phascolosomatidae): East-West Divergence between Non-sister Taxa. *Zoologica Scripta*, 45(4):455–463. https://doi.org/10.1111/zsc.12158.

Kawauchi, G. Y., and G. Giribet. 2010. Are There True Cosmopolitan Sipunculan Worms? A Genetic Variation Study within *Phascolosoma perlucens* (Sipuncula, Phascolosomatidae). *Marine Biology*, 157(7):1417–1431. https://doi.org/10.1007/s00227-010-1402-z.

Kawauchi, G. Y., and G. Giribet. 2013. *Sipunculus nudus* Linnaeus, 1766 (Sipuncula): Cosmopolitan or a Group of Pseudo-cryptic Species? An Integrated Molecular and Morphological Approach. *Marine Ecology*, 35(4):478–491. https://doi.org/10.1111/maec.12104.

Kawauchi, G. Y., and M. E. Rice. 2009. Two New Species of *Nephasoma* (Sipuncula: Golfingiidae) from the Western Atlantic Ocean. *Proceedings of the Biological Society of Washington*, 122(1):1–13. https://doi.org/10.2988/08-32.1.

Kawauchi, G. Y., P. P. Sharma, and G. Giribet. 2012. Sipunculan Phylogeny Based on Six Genes, with a New Classification and the Descriptions of Two New Families. *Zoologica Scripta*, 41(2):186–210. https://doi.org/10.1111/j.1463-6409.2011.00507.x.

Keferstein, W. 1865. Beiträge zur anatomischen und systematischen Kenntniss der Sipunculiden. Zeitschrift für Wissenschaftliche Zoologie, 15:404–445.

Kristof, A., T. Wollesen, A. S. Maiorova, and A. Wanninger, 2011. Cellular and Muscular Growth Patterns During Sipunculan Development. *Journal of Experimental Zoology B, Molecular and Developmental Evolution*, 316B(3):227–240.

Kristof, A., T. Wollesen, and A. Wanninger. 2008. Segmental Mode of Neural Patterning in Sipuncula. *Current Biology*, 18(15):1129–1132. https://doi.org/10.1016/j.cub.2008.06.066.

Lambert, J. D. 2007. Mesoderm in Spiralians: The Organizer and the 4d Cell. *Journal of Experimental Zoology B, Molecular and Developmental Evolution*, 310(1):15–23.

Lambert, J. D. 2010. Developmental Patterns in Spiralian Embryos. *Current Biology*, 20(2): R72–R77. https://doi.org/10.1016/j.cub.2009.11.041.

Lambert, J. D., and L. M. Nagy. 2003. The Mapk Cascade in Equally Cleaving Spiralian Embryos. *Developmental Biology*, 263(2):231–241. https://doi.org/10.1016/j.ydbio.2003.07.006.

Lemer, S., G. Y. Kawauchi, S. C. Andrade, V. L. Gonzalez, M. J. Boyle, and G. Giribet. 2015. Re-evaluating the Phylogeny of Sipuncula through Transcriptomics. *Molecular Phylogenetics and Evolution*, 83:174–183. https://doi.org/10.1016/j.ympev.2014.10.019.

Levin, L. A. 1984. Multiple Patterns of Development in *Stepblospio benedicti* Webster (Spionidae) from Three Coasts of North America. *Biological Bulletin*, 166(3):494–508. https://doi.org/10.2307/1541157.

Levin, L. A. 2006. Recent Progress in Understanding Larval Dispersal: New Directions and Digressions. *Integrative and Comparative Biology*, 46(3):282–297. https://doi.org/10.1093/icb/icj024.

Martindale, M. Q., and J. Q. Henry. 1995. Modifications of Cell Fate Specification in Equal-Cleaving Nemertean Embryos: Alternate Patterns of Spiralian Development. *Development*, 121(10):3175–3185.

Maslakova, S. A. 2010. Development to Metamorphosis of the Nemertean Pilidium Larva. *Frontiers in Zoology*, 7(1):30. https://doi.org/10.1186/1742-9994-7-30.

Maslakova, S. A., M. Q. Martindale, and J. L. Norenburg. 2004a. Fundamental Properties of the Spiralian Developmental Program Are Displayed by the Basal Nemertean *Carinoma tremaphoros* (Palaeonemertea, Nemertea). *Developmental Biology*, 267(2):342–360. https://doi.org/10.1016/j.ydbio.2003.10.022.

Maslakova, S. A., M. Q. Martindale, and J. L. Norenburg. 2004b. Vestigial Prototroch in a Basal Nemertean, *Carinoma tremaphoros* (Nemertea; Palaeonemertea). *Evolution and Development*, 6(4):219–226. https://doi.org/10.1111/j.1525-142X.2004.04027.x.

Maxmen, A. B., B. F. King, E. B. Cutler, and G. Giribet. 2003. Evolutionary Relationships within the Protostome Phylum Sipuncula: A Molecular Analysis of Ribosomal Genes and Histone H3 Sequence Data. *Molecular Phylogenetics and Evolution*, 27(3):489–503. https://doi.org/10.1016/S1055-7903(02)00443-8.

McEdward, L. R. 2000. Adaptive Evolution of Larvae and Life Cycles. *Seminars in Cellular and Developmental Biology*, 11(6):403–409. https://doi.org/10.1006/scdb.2000.0193.

McEdward, L. R., and K. H. Morgan. 2001. Interspecific Relationships between Egg Size and the Level of Parental Investment Per Offspring in Echinoderms. *Biological Bulletin*, 200(1):33–50. https://doi.org/10.2307/1543083.

Meyer, N. P., M. J. Boyle, M. Q. Martindale, and E. C. Seaver. 2010. A Comprehensive Fate Map by Intracellular Injection of Identified Blastomeres in the Marine Polychaete *Capitella teleta*. *EvoDevo*, 1(1):8. https://doi.org/10.1186/2041-9139-1-8.

Meyer, N. P., and E. C. Seaver. 2010. Cell Lineage and Fate Map of the Primary Somatoblast of the Polychaete Annelid *Capitella teleta*. *Integrative and Comparative Biology*, 50(5):756–767. https://doi.org/10.1093/icb/icq120.

Moore, J., and P. Willmer. 1997. Convergent Evolution in Invertebrates. *Biological Reviews of the Cambridge Philosophical Society*, 72(1):1–60. https://doi.org/10.1017/S0006323196004926.

Moss, E. G. 2007. Heterochronic Genes and the Nature of Developmental Time. *Current Biology*, 17(11): R425–R434. https://doi.org/10.1016/j.cub.2007.03.043.

Murina, V. V. 1957. Abyssal Sipunculids (Genus Phascolion Theel) of the Northwestern Part of the Pacific Collected by "Vitjaz" Expeditions in 1950–1955. *Zoologicheskii Zhurnal*, 36(12):1777–1791.

Murina, V. V. 1971. Geographical Distribution of Marine Worms of the Phylum Sipuncula in the World Ocean. *Zoologicheskii Zhurnal*, 50:184–192.

Murina, V. V., and V. I. Holodov. 1985. "Sipunculids of the Chukchi Sea." In *Ecology of the Ocean Coastal Zone Benthic Fauna and Flora*, ed. P. P. Shirshov, pp. 36–46. Moscow: Academii Nauk SSSR.

Nielsen, C. 2009. How Did Indirect Development with Planktotrophic Larvae Evolve? *Biological Bulletin*, 216(3):203–215. https://doi.org/10.1086/BBLv216n3p203.

Page, L. R. 2009. Molluscan Larvae: Pelagic Juveniles or Slowly Metamorphosing Larvae? *Biological Bulletin*, 216(3):216–225. https://doi.org/10.1086/BBLv216n3p216.

Parry, L., A. Tanner, J. Vinther, and A. Smith. 2014. The Origin of Annelids. *Palaeontology*, 57(6):1091–1103. https://doi.org/10.1111/pala.12129.

Pernet, B. 2003. Persistent Ancestral Feeding Structures in Nonfeeding Annelid Larvae. *Biological Bulletin*, 205(3):295–307. https://doi.org/10.2307/1543293.

Pernet, B., and D. McHugh. 2010. Evolutionary Changes in the Timing of Gut Morphogenesis in Larvae of the Marine Annelid *Streblospio benedicti*. *Evolution and Development*, 12(6):618–627. https://doi.org/10.1111/j.1525-142X.2010.00446.x.

Peterson, K. J. 2005. Macroevolutionary Interplay between Planktic Larvae and Benthic Predators. *Geology*, 33(12):929–932. https://doi.org/10.1130/G21697.1.

Pickford, G. E. 1947. "Sipunculida." In *Encyclopedia Britannica*, Volume 20, pp. 717–718b. Chicago: University of Chicago.

Pilger, J. F. 1987. Reproductive Biology and Development of Themiste Lageniformis, a Parthenogenic Sipunculan. *Bulletin of Marine Science*, 41(1):59–67.

Purschke, G. 2002. On the Ground Pattern of Annelida. *Organisms Diversity and Evolution*, 2:181–196. https://doi.org/10.1078/1439-6092-00042.

Purschke, G., C. Bleidorn, and T. Struck. 2014. Systematics, Evolution and Phylogeny of Annelida – A Morphological Perspective. *Memoirs of Museum Victoria*, 71:247–269. https://doi.org/10.24199/j.mmv.2014.71.19.

Purschke, G., R. Hessling, and W. Westheide. 2000. The Phylogenetic Position of the Clitellata and the Echiura – On the Problematic Assessment of Absent Characters. *Journal of Zoological Systematics and Evolutionary Research*, 38(3):165–173. https://doi.org/10.1046/j.1439-0469.2000.383146.x.

Purschke, G., F. Wolfrath, and W. Westheide. 1997. Ultrastructure of the Nuchal Organ and Cerebral Organ in *Onchnesoma squamatum* (Sipuncula, Phascolionidae). *Zoomorphology*, 117:23–31. https://doi.org/10.1007/s004350050026.

Qian, P.-Y. 1999. Larval Settlement of Polychaetes. In *Reproductive Strategies and Developmental Patterns in Annelids*, pp. 239–253. Dordrecht: Springer. https://doi.org/10.1007/978-94-017-2887-4_14.

Raff, R. A. 2008. Origins of the Other Metazoan Body Plans: The Evolution of Larval Forms. *Philosophical Transactions of the Royal Society B, Biological Sciences*, 363(1496):1473–1479. https://doi.org/10.1098/rstb.2007.2237.

Raff, R. A., and G. A. Wray. 1989. Heterochrony: Developmental Mechanisms and Evolutionary Results. *Journal of Evolutionary Biology*, 2:409–434. https://doi.org/10.1046/j.1420-9101.1989.2060409.x.

Rawlinson, K. A. 2010. Embryonic and Post-embryonic Development of the Polyclad Flatworm *Maritigrella crozieri*; Implications for the Evolution of Spiralian Life History Traits. *Frontiers in Zoology*, 7:12. https://doi.org/10.1186/1742-9994-7-12.

Render, J. 1983. The Second Polar Lobe of the *Sabellaria cementarium* Embryo Plays an Inhibitory Role in Apical Tuft Formation. *Roux's Archives of Developmental Biology*, 192(3–4):120–129. https://doi.org/10.1007/BF00848680.

Render, J. 1989. Development of *Illyyanassa obsoleta* Embryos after Equal Distribution of Polar Lobe Material at First Cleavage. *Developmental Biology*, 132(1):241–250. https://doi.org/10.1016/0012-1606(89)90220-0.

Rice, M. E. 1967. A Comparative Study of the Development of *Phascolosoma agassizii*, *Golfingia pugettensis*, and *Themiste pyroides* with a Discussion of Developmental Patterns in the Sipuncula. *Ophelia*, 4:143–171. https://doi.org/10.1080/00785326.1967.10409618.

Rice, M. E. 1970. Asexual Reproduction in a Sipunculan Worm. *Science*, 167(3925):1618–1620. https://doi.org/10.1126/science.167.3925.1618.

Rice, M. E. 1973. *Morphology, Behavior, and Histogenesis of the Pelagosphera Larva of* Phascolosoma agassizii *(Sipuncula)*. Washington, D.C.: Smithsonian Institution Press.

Rice, M. E. 1975. "Observations on the Development of Six Species of Caribbean Sipuncula with a Review of Development in the Phylum." In *Proceedings of the International Symposium on the Biology of the Sipuncula and Echiura*, ed. M. E. Rice and M. Todorović, pp. 35–49. Belgrade: Naučno Delo Press.

Rice, M. E. 1978. Morphological and Behavioral Changes at Metamorphosis in the Sipuncula. In *Settlement and Metamorphosis of Marine Invertebrate Larvae*, ed. M. E. Rice and F-S. Chia, pp. 83–102. New York: Elsevier North-Holland Biomedical.

Rice, M. E. 1981. Larvae Adrift: Patterns and Problems in Life Histories of Sipunculans. *American Zoologist*, 21(3):605–619. https://doi.org/10.1093/icb/21.3.605.

Rice, M. E. 1985. Sipuncula: Developmental Evidence for Phylogenetic Inference. In *The Origins and Relationships of Lower Invertebrates*, ed. S. C. Morris, J. D. George, R. Gibson, and H. M. Platt, pp. 274–296. Oxford: Oxford University Press.

Rice, M. E. 1988. Observations on Development and Metamorphosis of *Siphonosoma cumanense* with Comparative Remarks on *Sipunculus nudus* (Sipuncula, Sipunculidae). *Bulletin of Marine Science*, 42(1):1–15.

Rice, M. E. 1989. "Comparative Observations of Gametes, Fertilization, and Maturation in Sipunculans." In *23rd European Marine Biology Symposium*, ed. J. S. Ryland and P. A. Tyler, pp.167–182. Fredensborg, Denmark: Olsen and Olsen.

Rice, M. E., J. Piraino, and H. F. Reichardt. 1983. Observations on the Ecology and Reproduction of the Sipunculan *Phascolion cryptus* in the Indian River Lagoon. *Quarterly Journal of the Florida Academy of Sciences*, 46(3/4):380–396.

Rice, M. E., J. Piraino, and H. F. Reichardt. 1995. A Survey of the Sipuncula of the Indian River Lagoon. *Bulletin of Marine Science*, 57(1):128–135.

Rouse, G. W. 1999. Trochophore Concepts: Ciliary Bands and the Evolution of Larvae in Spiralian Metazoa. *Biological Journal of the Linnean Society*, 66:411–464. https://doi.org/10.1111/j.1095-8312.1999.tb01920.x

Rouse, G. W. 2000. Polychaetes Have Evolved Feeding Larvae Numerous Times. *Bulletin of Marine Science*, 67(1):391–409.

Rouse, G. W., and F. Pleijel. 2001. *Polychaetes*. Oxford: Oxford University Press.

Rubin-Blum, M., E. Shemesh, B. Goodman-Tchernov, D. F. Coleman, Z. Ben-Avraham, and D. Tchernov. 2014. Cold Seep Biogenic Carbonate Crust in the Levantine Basin Is Inhabited by Burrowing *Phascolosoma aff. turnerae*, a Sipunculan Worm Hosting a Distinctive Microbiota. *Deep Sea Research, Part I: Oceanographic Research Papers*, 90:17–26. https://doi.org/10.1016/j.dsr.2014.04.014.

Runnegar, B. 2007. No Evidence for Planktotrophy in Cambrian Molluscs. *Evolution and Development*, 9(4):311–312. https://doi.org/10.1111/j.1525-142X.2007.00165.x.

Saiz-Salinas, J. I. 2007. Sipunculans and Echiurans from the Deep Angola Basin. *Journal of Natural History*, 41(45):2789–2800. https://doi.org/10.1080/00222930701782187.

Scheltema, R. S. 1968. Dispersal of Larvae by Equatorial Ocean Currents and Its Importance to the Zoogeography of Shoal-Water Tropical Species. *Nature*, 217(5134):1159–1162. https://doi.org/10.1038/2171159a0.

Scheltema, R. S. 1975. Relationship of Larval Dispersal, Gene-Flow and Natural Selection to Geographic Variation of Benthic Invertebrates in Estuaries and along Coastal Regions. *Estuarine Research*, 1:372–390.

Scheltema, R. S., and J. R. Hall. 1975. "The Dispersal of Pelagosphaera Larvae by Ocean Currents and the Geographical Distribution of Sipunculans." In *Proceedings of the International Symposium on the Biology of the Sipuncula and Echiura*, ed. M. E. Rice and M. Todorović, pp. 103–115. Belgrade: Naučno Delo Press.

Scheltema, R. S., and M. E. Rice. 1990. Occurrence of Teleplanic Pelagosphera Larvae of Sipunculans in Tropical Regions of the Pacific and Indian Oceans. *Bulletin of Marine Science*, 47(1):159–181.

Schneider, S., A. Fischer, and A. W. Dorresteijn. 1992. A Morphometric Comparison of Dissimilar Early Development in Sibling Species of *Platynereis* (Annelida, Polychaeta). *Roux's Archives of Developmental Biology*, 201:243–256. https://doi.org/10.1007/BF00188755.

Schroeder, P. C., and C. Hermans. 1975. Annelida: Polychaeta. *Reproduction of Marine Invertebrates*, 3:1–213.

Schulze, A., E. B. Cutler, and G. Giribet. 2005. Reconstructing the Phylogeny of the Sipuncula. *Hydrobiologia*, 535/536:277–296. https://doi.org/10.1007/s10750-004-4404-3.

Schulze, A., E. B. Cutler, and G. Giribet. 2007. Phylogeny of Sipunculan Worms: A Combined Analysis of Four Gene Regions and Morphology. *Molecular Phylogenetics and Evolution*, 42(1):171–192. https://doi.org/10.1016/j.ympev.2006.06.012.

Schulze, A., A. Maiorova, L. E. Timm, and M. E. Rice. 2012. Sipunculan Larvae and "Cosmopolitan" Species. *Integrative and Comparative Biology*, 52(4):497–510. https://doi.org/10.1093/icb/ics082.

Schulze, A., and M. E. Rice. 2009a. Musculature in Sipunculan Worms: Ontogeny and Ancestral States. *Evolution and Development*, 11(1):97–108. https://doi.org/10.1111/j.1525-142X.2008.00306.x.

Schulze, A., and M. E. Rice. 2009b. "*Nephasoma pellucidum*: A Model Species for Sipunculan Development." In *Proceedings of the Smithsonian Marine Science Symposium*, ed. M. A. Lang, I. G. Macintyre, and K. Rützler, pp. 209–217. Smithsonian Contributions to Marine Science 38. Washington, D.C.: Smithsonian Institution Scholarly Press.

Selenka, E. 1875. Eifurchung und Larvenbildung von *Phascolosoma elongatum* Kef. *Zeitschrift für wissenschaftliche Zoologie*, 25:442–450.

Shanks, A. L. 2009. Pelagic Larval Duration and Dispersal Distance Revisited. *Biological Bulletin*, 216(3):373–385. https://doi.org/10.1086/BBLv216n3p373.

Shanks, A. L., and G. L. Eckert. 2005. Population Persistence of California Current Fishes and Benthic Crustaceans: A Marine Drift Paradox. *Ecological Monographs*, 75(4):505–524. https://doi.org/10.1890/05-0309.

Shields, M. A., and M. Kedra. 2009. A Deep Burrowing Sipunculan of Ecological and Geochemical Importance. *Deep Sea Research, Part I: Oceanographic Research Papers*, 56(11):2057–2064. https://doi.org/10.1016/j.dsr.2009.07.006.

Sly, B. J., M. S. Snoke, and R. A. Raff. 2003. Who Came First—Larvae or Adults? Origins of Bilaterian Metazoan Larvae. *International Journal of Developmental Biology*, 47(7–8):623–632.

Smith, K. K. 2003. Time's Arrow: Heterochrony and the Evolution of Development. *International Journal of Developmental Biology*, 47(7–8):613–621.

Smith, M. S., K. S. Zigler, and R. A. Raff. 2007. Evolution of Direct-Developing Larvae: Selection vs Loss. *Bioessays*, 29(6):566–571. https://doi.org/10.1002/bies.20579.

Smith, P. R., J. Lombardi, and R. M. Rieger. 1986. Ultrastructure of the Body Cavity Lining in a Secondary Acoelomate, *Microphthalmus* cf. *listensis* Westheide (Polychaeta: Hesionidae). *Journal of Morphology*, 188(3):257–271. https://doi.org/10.1002/jmor.1051880302.

Snelgrove, P. V., J. P. Grassle, J. F. Grassle, R. F. Petrecca, and H. Ma. 1999. In Situ Habitat Selection by Settling Larvae of Marine Soft-Sediment Invertebrates. *Limnology and Oceanography*, 44(5):1341–1347. https://doi.org/10.4319/lo.1999.44.5.1341.

Sperling, E. A., J. Vinther, V. N. Moy, B. M. Wheeler, M. Semon, D. E. Briggs, and K. J. Peterson. 2009. MicroRNAs Resolve an Apparent Conflict between Annelid Systematics and Their Fossil Record. *Proceedings of the Royal Society B, Biological Sciences*, 276(1677):4315–4322. https://doi.org/10.1098/rspb.2009.1340.

Staton, J., and M. E. Rice. 1999. Genetic Differentiation Despite Teleplanic Larval Dispersal: Allozyme Variation in Sipunculans of the *Apionsoma misakianum* Species Complex. *Bulletin of Marine Science*, 65(2):467–480.

Stephen, A. C., and S. J. Edmonds. 1972. *The Phyla Sipuncula and Echiura*. London: Trustees of the British Museum (Natural History).

Strathmann, R. R. 1977. Egg Size, Larval Development, and Juvenile Size in Benthic Marine Invertebrates. *American Naturalist*, 111(978):373–376.

Strathmann, R. R. 1978. The Evolution and Loss of Feeding Larval Stages of Marine Invertebrates. *Evolution*, 32(4):894–906. https://doi.org/10.1111/j.1558-5646.1978.tb04642.x.

Strathmann, R. R. 1985. Feeding and Nonfeeding Larval Development and Life-History Evolution in Marine Invertebrates. *Annual Review of Ecology and Systematics*, 16:339–361. https://doi.org/10.1146/annurev.es.16.110185.002011.

Strathmann, R. R. 1993. Hypotheses on the Origins of Marine Larvae. *Annual Review of Ecology and Systematics*, 24:89–117. https://doi.org/10.1146/annurev.es.24.110193.000513.

Struck, T. H., C. Paul, N. Hill, S. Hartmann, C. Hosel, M. Kube, B. Lieb, A. Meyer, R. Tiedemann, G. Purschke, and C. Bleidorn. 2011. Phylogenomic Analyses Unravel Annelid Evolution. *Nature*, 471(7336):95–113. https://doi.org/10.1038/nature09864.

Struck, T. H., N. Schult, T. Kusen, E. Hickman, C. Bleidorn, D. McHugh, and K. M. Halanych. 2007. Annelid Phylogeny and the Status of Sipuncula and Echiura. *BMC Evolutionary Biology*, 7:57. https://doi.org/10.1186/1471-2148-7-57.

Swalla, B. J., M. E. White, J. Zhou, and W. R. Jeffery. 1994. Heterochronic Expression of an Adult Muscle Actin Gene During Ascidian Larval Development. *Developmental Genetics*, 15(1):51–63. https://doi.org/10.1002/dvg.1020150107.

Tarifeño, E. S., and M. Rojas. 1978. El Phylum Sipuncula en Chile. *Studies on Neotropical Fauna and Environment*, 13:93–134. https://doi.org/10.1080/01650527809360535.

Thornhill, D. J., A. R. Mahon, J. L. Norenburg, and K. M. Halanych. 2008. Open-Ocean Barriers to Dispersal: A Test Case with the Antarctic Polar Front and the Ribbon Worm *Parborlasia corrugatus* (Nemertea: Lineidae). *Molecular Ecology*, 17(23):5104–5117. https://doi.org/10.1111/j.1365-294X.2008.03970.x.

Thorson, G. 1950. Reproductive and Larval Ecology of Marine Bottom Invertebrates. *Biological Review of the Cambridge Philosophical Society*, 25(1):1–45. https://doi.org/10.1111/j.1469-185X.1950.tb00585.x.

Tzetlin, A., and G. Purschke. 2006. Fine Structure of the Pharyngeal Apparatus of the Pelagosphera Larva in *Phascolosoma agassizii* (Sipuncula) and Its Phylogenetic Significance. *Zoomorphology*, 125:109–117. https://doi.org/10.1111/j.1469-185X.1950.tb00585.x.

Vance, R. 1973. On Reproductive Strategies in Marine Benthic Invertebrates. *American Naturalist*, 107(955):339–352. https://doi.org/10.1086/282838.

Vinther, J., D. Eibye-Jacobsen, and D. A. Harper. 2011. An Early Cambrian Stem Polychaete with Pygidial Cirri. *Biological Letters*, 7(6):929–932. https://doi.org/10.1098/rsbl.2011.0592.

Wanninger, A., D. Koop, L. Bromham, E. Noonan, and B. M. Degnan. 2005. Nervous and Muscle System Development in *Phascolion strombus* (Sipuncula). *Development, Genes and Evolution*, 215(10):509–518. https://doi.org/10.1007/s00427-005-0012-0.

Weigert, A., C. Helm, M. Meyer, B. Nickel, D. Arendt, B. Hausdorf, S. R. Santos, K. M. Halanych, G. Purschke, C. Bleidorn, and T. H. Struck. 2014. Illuminating the Base of the Annelid Tree Using Transcriptomics. *Molecular Biology and Evolution*, 31(6):1391–1401. https://doi.org/10.1093/molbev/msu080.

Westheide, W. 1997. The Direction of Evolution within the Polylchaeta. *Journal of Natural History*, 31:1–15. https://doi.org/10.1080/00222939700770011.

Worsaae, K. 2005. Phylogeny of Nerillidae (Polychaeta, Annelida) as Inferred from Combined 18s rDNA and Morphological Data. *Cladistics*, 21(2):143–162. https://doi.org/10.1111/j.1096-0031.2005.00058.x.

Wray, G., and R. Strathmann. 2002. Stasis, Change, and Functional Constraint in the Evolution of Animal Body Plans, Whatever They May Be. *Vie et Milieu*, 52(4):189–199.

Wray, G. A. 1996. Parallel Evolution of Nonfeeding Larvae in Echinoids. *Systematic Biology*, 45(3):308–322.

Wray, G. A. 2000a. The Evolution of Embryonic Patterning Mechanisms in Animals. *Seminars in Cellular and Developmental Biology*, 11(6):385–393. https://doi.org/10.1006/scdb.2000.0191.

Wray, G. A. 2000b. Gene Expression During Echinoderm Metamorphosis. *Zygote*, 8(Suppl. 1):S48–S49.

Wray, G. A. 2007. The Evolutionary Significance of Cis-Regulatory Mutations. *Nature Reviews Genetics*, 8(3):206–216. https://doi.org/10.1038/nrg2063.

Young, C. M. 2002. *Atlas of Marine Invertebrate Larvae*. San Diego: Academic Press.

Young, C. M., R. He, R. B. Emlet, Y. Li, H. Qian, S. M. Arellano, A. Van Gaest, K. C. Bennett, M. Wolf, T. I. Smart, and M. E. Rice. 2012. Dispersal of Deep-Sea Larvae from the Intra-American Seas: Simulations of Trajectories Using Ocean Models. *Integrative and Comparative Biology*, 52(4):483–496. https://doi.org/10.1093/icb/ics090.

Zimmer, R. L., and R. M. Woollacott. 1977. "Metamorphosis, Ancestrulae, and Coloniality in Bryozoan Life Cycles." In *Biology of Bryozoans*, pp. 91–142. New York: Academic Press.