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Confocal Microscopy Study of the Embryonic Development of the Viviparous Nemertean Prosorhochmus americanus Reveals Larval Features Supporting Indirect Development In Hoplonemerteans

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A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

by

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Recent studies of hoplonenemertean planuliform larvae have clarified their development and provided insight into larval evolution within the phylum. However, an assessment of viviparous development using modern techniques is lacking. To help facilitate a

comprehensive comparative evaluation of developmental diversity within hoplonemerteans, we have conducted a confocal laser scanning microscopy investigation of the development in *Prosorhochmus americanus*, one of the few viviparous hoplonemertean species. Phalloidin staining provides evidence of a modified transitory larval epidermis, and reveals that the foregut, midgut, proboscis, central nervous system, and body wall musculature form early in development, consistent with observations for planktonic and encapsulated hoplonemertean larvae. However, invaginations characteristic of these larvae were not observed. Acetylated tubulin labeling and light microscopy shows that embryos are uniformly ciliated, and some specimens possess a caudal ciliary cirrus and/or apical tuft which are characteristic of planktonic larvae. These are interpreted as vestigial structures in the non-swimming *P. americanus* embryos. The findings provide additional evidence that hoplonemerteans exhibit a form of metamorphosis in their life history and thus exhibit indirect development. However, a comparative assessment of larval features in *P. americanus* suggests an evolutionary trend towards direct development in this species.

Definition of Anatomical Terms

Apical plate: anterior epidermal invagination bearing elongated cilia

Apical tuft: grouping of conspicuously long cilia extending from the apical plate; sensory function in planktonic developing species

Posterior cirrus: grouping of cilia extending from the caudal end of planuliform larvae; shorter than cilia of the apical tuft; sensory function in planktonic developing species

Cerebral ganglia: paired aggregations of neurons in the anterior region; the nemertean "brain"

Cerebral organs: paired blind, cell-lined canals at the anterior end; chemosensory

Lecithotrophic larva: non-feeding larval stage relying on yolk stores for nutrition

Nerve commissures: nerve axons linking the cerebral ganglia

Ocelli: aggregations of photoreceptors; "eyespots"

Proboscis: eversible tubular organ used in prey capture

Proboscis retractor muscle: muscle attaching posterior end of the proboscis to the posterior end of the rhynchocoel; used to pull proboscis into rhynchocoel after eversion

Rhynchocoel: fluid-filled cavity housing the proboscis

Rhynchodaeum: tubular chamber immediately proximal to the rhynchostomopore; Site of proboscis insertion. Houses opening of digestive tract

Rhynchostomopore: the common mouth/proboscis pore in adult nemerteans

Introduction

The phylum Nemertea includes 1,275 species (Kajihara et al. 2008) unsegmented worms and forms a subclade of the coelomate Lophotrochozoa (see Dunn *et al.* 2008). The primary distinguishing character of these worms is an eversible proboscis used primarily for capturing prey. Most species occupy benthic marine habitats, though pelagic marine, freshwater, and terrestrial species also occur. While a few species are suspension feeders, most are ambush or sit-and-wait predators that hunt with the aid of chemosensors and attack prey with their rapidly eversible proboscis, which, in many species, delivers potent immobilizing neurotoxins (see Thiel and Kruse, 2001, Caplins et al. 2012).

Predatory nemerteans have been shown to be important predators in both soft-bottom and hard bottom communities with the potential to exert influence over the species composition and emigration rates of their prey species (Theil and Kruse, 2001; Wang *et al*, 2008; Caplins et al., 2012). Others are of economic significance. For example, ovivorous species can significantly increase the egg mortality of commercially important crab species (Wickham, 1986), whereas another species has been implicated in negatively affecting soft-shelled clam fisheries (Bourque *et al.*, 1999)

The most comprehensive nemertean phylogeny published to date weakly supports a basal Palaeonemertea (minus Hubrechtella) which is sister to Hoplonemertea + Plilidiophora when data are analyzed using maximum likelihood and Bayesian analyses (Andrade *et al* . 2012). Pilidiophorans are indirect developers with either a planktotrophic

pilidium larva or planktotrophic or lecithotrophic derivatives thereof (Turbeville 2001, see Maslakova and von Döhren, 2009). These larvae are characterized by a complex metamorphosis involving the formation of imaginal discs from the larval epidermis, that coalesce to form the juvenile rudiment within the larval body and ending with the juvenile departing or eating the enclosing larval epidermis. Palaeonemerteans and hoplonemerteans, in contrast, exhibit larvae that develop directly into a juvenile without distinct morphological transitions (see Maslakova and von Döhren, 2009).

In the case of the direct-developing hoplonemerteans, most have a uniformly ciliated, planktonic lecithotrophic planuliform larva (Maslakova and von Döhren, 2009; Hiebert *et al*, 2010), but in other species embryos develop within egg capsules (extra-embryonic chorions) into juveniles (Hickman, 1963; Maslakova and von Döhren, 2009) and, in a minority of species, embryos are gestated in ovaries or ovotestis of the adults to the juvenile stage (viviparous; e. g., *P. americanus*; Gibson *et al*. 1986).

Recent studies suggest that the planuliform and encapsulated larvae exhibit morphogenetic changes that could be equated with larval metamorphosis, calling into question the historical designation "direct development." The development of these larvae reveals two conspicuous features: the presence of up to five ectodermal invaginations and a transitory larval epidermis (Hiebert *et al*, 2010). The ectodermal invaginations have been shown to give rise to the cerebral organs and proboscis and are a major element of a proposed scheme of developmental stages in hoplonemerteans (Hiebert *et al.*, 2010). Other authors, including Iwata (1985) also noted these

invaginations. Only three invaginations (two cerebral organ rudiments and one proboscis rudiment) were described in in *Prosorhochmus viviparous* (Salensky, 1909, 1914), so whether the proposed developmental stages are the rule for all hoplonemerteans is not yet clear.

The transitory larval epidermis has been described in 12 hoplonemertean species. In most of these species it is described as a set of 80-100 conspicuously large, cleavage arrested cells surrounding the larval body (e.g., Maslakova and von Döhren 2008, Hiebert et al. 2010). Ultrastructural research on the tissue reveals large amounts of yolk and other electron-dense material, as well as abnormal nucleoli in these cells (Magarlamov and Chernyshev, 2009). The transitory epidermis envelops the larvae during early organogenesis, before being shed or gradually replaced by the smaller cells of the definitive epidermis. The nondescript external appearance during organogenesis has led some researchers to describe this form of development as a "hidden larvae" (Maslakova and Malakhov, 1999).

The timing of the replacement of the transitory epidermis varies among species (Magarlamov and Chernyshev, 2009). It has been proposed that this is an ancestral trait related to the metamorphosis of the pilidium larvae of the pilidiophoran clade (Hiebert *et al*, 2010). Whether this is an evolutionary vestige of the pilidium or a precursor to indirect development is unclear. Although this feature is likely a characteristic of the

majority of hoplonemerteans, including those with encapsulated non-swimming larvae[1] (Maslakova and Von Dohren, 2009; Magarlamov and Chernyshev, 2009), the presence of the transitory epidermis has not been definitively demonstrated in the development of viviparous species (compare Salensky 1884, 1919 and Bürger, 1895). The aforementioned larval morphogenetic events (invaginations, shedding of larval epidermis) have not been demonstrated in direct-developing viviparous species, which are considered to lack a larval stage entirely, and their presence or absence in a viviparous direct developer will have implications for larval evolution within nemerteans.

P. americanus is a marine, shallow-water hoplonemertean species inhabiting the fouling communities of coastal Virginia, South Carolina, and Florida (Turbeville and Caplins, 2010). These worms are typically found in aggregates beneath the valves of oysters, in the byssal threads of mussels, or associated with barnacles and feeds on co-occurring species of amphipod crustaceans (Caplins and Turbeville, 2011). *P. americanus* is one of only five hoplonemerteans to possess a combination of hermaphroditism and viviparity (Gibson *et al*, 1986, Crandall et al. 1988). The offspring develop asynchronously, thus embryos and juveniles at various stages of development are typically present in a given adult (Gibson *et al*, 1986; Maslakova and Norenburg, 2008, personal observations). Developmental data are scarce for viviparous nemerteans with studies limited to the light histological analyses of Salensky (1884, 1914), Bürger (1895),

[1] A notable exception being *Carcinonemertes epialti,* a planktonic-developing parasitic hoplonemertean (Stricker and Reed, 1981).

Coe (1904), Crandall *et al*. (1988) and a single ultrastructural study of the formation of the circulatory system (Turbeville, 1986).

The aims of this study are to employ light and confocal laser-scanning microscopy (CLSM) to elucidate the larval development of the major body systems of *P. americanus*, determine whether features typical of planktonic larval species (transitory epidermis, apical organ, ciliary tufts) are present, and investigate the presence or absence of the five invaginations and, if present, trace their fates. This information will both provide insight into larval and developmental evolution in Hoplonemertea, will facilitate a comparative evaluation of developmental diversity within the order, and should have implications for the hypothesis that all hoplonemerteans exhibit a form of indirect rather than direct development. .

Materials and Methods

Specimen Collection, Care, and Maintenance

Specimens were collected from north side of the 1st Street jetty in Rudee Inlet, Virginia Beach, Virginia. Individuals were isolated by collecting oysters from the jetty, then allowing them to foul in glass dishes in a lab setting. The specimens were then collected as they migrated out of the fouling oysters. The specimens were stored in petri dishes with 31 ppt Instant Ocean artificial seawater, which was changed 1-2 times per week, and fed local amphipod species (*Corophium* cf. *insidiosum*, *Jassa falcata*) once per week, when available. Adults were set aside for at least 2 weeks to allow for healing after dissection.

Specimen Preparation, Fixation, and Labeling

Adult *P. americanus* were anesthetized with magnesium chloride isotonic to seawater on standard microscope slides. Slides were transferred to a Nikon SMZ stereoscope and embryos and developing juveniles were dissected out of adult specimens using insect pins. For imaging of live specimens, anesthetized embryos were placed in a depression slide with a few drops of artificial seawater, transferred one at a time to a slide with magnesium chloride, compressed with a cover slip and imaged using bright field or interference contrast microscopy on a Nikon Eclipse E600. Images and video were captured using Nikon Elements, version 3.10. The total number of live specimens imaged was 36.

For confocal microscopy, embryos obtained as above were transferred to a 2 ml centrifuge tubes and fixed in 4% paraformaldehyde in PBS buffer for 30-60 minutes at room temperature then washed twice in PBS containing 0.1% Tween (PBT) for 15 minutes each to permeabilize the cells. Specimens were then bathed for 1 hour in 1:49 phalloidin (Alexa Fluor 488, Invitrogen) /PBT solution, washed three times for 15 minutes each with 10% PBT, and mounted on standard glass slides in Fluormount-G (SouthernBiotech).

For acetylated tubulin labeling, the specimens were fixed using the above protocol, then placed in a 1:500 primary antibody solution in 10% PBT and left on a shaker at room temperature for 3 hours. Specimens were then washed three times in 10% PBT and placed in a 1:500 secondary antibody (goat-anti-mouse IGG, AlexaFluor 568, Invitrogen) solution in 10% PBT and left on a shaker at room temperature for another 3 hours. The specimens were then washed twice in 10% PBT and once in 10% PBS for 15 minutes each and subsequently mounted on glass slides in Fluormount-G (SouthernBiotech)

Confocal Imaging

All CLSM images were captured in z-stacks using the Nikon EZ-C1 imaging program, version 3.80. Settings were as follows: 1024x1024 resolution, medium pinhole setting, step size=0.5-1.0 µm, gain=4.25-6.30, offset=127. Light microscope images were captured using Nikon Elements ver. 3.10. Images were rendered and manipulated using Nikon Elements ver. 3.10, ImageJ ver. 1.44o, and Adobe Photoshop CS5. All z-

projections are maximum intensity projections. The total number of specimens imaged was 142.

Results

Overview of embryonic development[2]

Because of the asynchronous development of *P. americanus* embryos and the difficulty of observing them within the ovaries of adult worms, staging has been approximated using a combination of embryo size and morphology (Table 1). The earliest stage observed was spherical and measured (105-135 μm in diameter) It is characterized by relatively large epidermal cells (20-30 μm across) with smaller cells (<10 μm across) interspersed between (Fig. 2A). An embryo at a possibly later stage exhibited many additional smaller cells and fewer large cells. These larger cells begin to disappear at this later stage (~130 μm, Fig. 2B). These early stages exhibit little internal differentiation but phalloidin labeling reveals a diffuse complex to moderately organized actin-rich strands that stretch across the interior. It is likely that these are forming muscle and nerves (Fig. 2).

In what were inferred to be later-stage embryos (118-150 μm), the body axis is clearly defined. The yolk is localized in the developing midgut situated in posterior half of the embryo (Fig. 2C), but whether yolk formation is autosynthetic or heterosynthetic is unclear. Yolk granules are enclosed in the large globular cells of the midgut. The size of the granules (diameter = 4 - 6 μm) appears to diminish over time, with the yolk apparently replenished periodically through much of development (an advanced, but not fully developed [703 μm] juvenile was observed to possess yolk granules throughout the

[2] See Fig. 1 for an overview of hoplonemertean larval anatomy.

midgut, with no gut diverticula). Embryos in this size range are uniformly ciliated (Fig. 7). One anteriorly situated pair of ocelli were observed in embryos of approximately 140 μm (Fig. 2D) and were well-formed at around 180 μm (Fig. 2E). The proboscis is also visible using light microscopy at this stage (Fig. 2F).

The body begins to elongate, first into a typical elliptical planuliform shape (200-300 μm, fig. 2E, 2F), then into a larger "teardrop" shape (approx 300 μm; (Fig 2G). At this stage, the embryonic proboscis appears to reach its fixed location towards the anterior end of the embryo. Growth and elongation of the embryo body continues, while the relative size of the proboscis decreases (Fig 2H). The characteristic second pair of ocelli is not apparent until the crawling vermiform stage at lengths of greater than 635 μm.

Transitory Larval Epidermis

Phalloidin labeling suggests a process of epidermal cell replacement early in development consistent with the presence of a transitory larval epidermis that characterizes the larval stage of other hoplonemerteans (Fig. 3). Large epidermal cells (11-37μm across) of the early embryos appear to be gradually replaced by smaller cells (4-5 μm across) that will presumably become the definitive epidermis (Fig. 9). This state was observed in two embryos. At the time of organogenesis, the epidermal cells are numerous, of relatively uniform size (6-15 μm) and not particularly large.

Musculature

The muscles of the body wall form early in development (106-121 μm). The circular and longitudinal muscles form first, followed shortly thereafter by the diagonal muscles. The layering of the muscles is as follows (distal to proximal): circular, diagonal, longitudinal. All muscles layers follow a similar growth pattern, wherein they exist early on as individual, widely-spaced strands, then multiply outward from the original strands. The circular and longitudinal muscles proliferate as the embryos mature and become densely packed, forming nearly uniform sheets in juveniles. The diagonal muscles also proliferate as the embryos develop but remain more widely spaced than their circular and longitudinal counterparts.

Other major muscles, such as the proboscis retractor, lateral proboscis muscles, proboscis insertion muscles, and nerve cord muscles also form early in development (~121 μm), closely correlated with development of the posterior and lateral proboscis, and longitudinal nerve cords, respectively (Fig. 4).

Proboscis

The proboscis rudiment forms early in development, appearing in early-stage embryos (as early as 106 μm) as an ovoid group of cells containing a lumen near the anterior end and 40 μm in length; it is one of the first clearly discernable structures inside of the embryo. It is dorsally situated and does not appear to be anchored by proboscis insertion muscles at this early stage (Fig. 5A). It also does not appear that the

rhynchodaeum is formed at this early stage. An ectodermal invagination leading to the formation of the proboscis rudiment was not observed in any of the embryos examined.

At the 125-130 μm stage, the proboscis is muscularized by widely spaced circular and longitudinal muscles, and the proboscis retractor muscle and proboscis insertion muscles are also apparent (Fig. 5C). At this stage, the proboscis is bipartite, divided into a posterior "bulb" which may differentiate into the middle and posterior proboscis and an anterior portion that appears to be open-ended and attached to the rhynchodaeum at the apical end of the embryo. The organ can be seen extending through the center of the ring formed by the early cerebral ganglia and dorsal and ventral commissures. The proboscis insertion muscles can be distinguished from the rest of the proboscis at this stage, as left and right outward extensions from the anterior lateral proboscis rudiment to left and right longitudinal muscles of the body wall. The proboscis retractor muscle extends from the posteriormost point of the proboscis bulb and attaches dorsally near the caudal end of the embryo. At this point, the bulbous posterior end of the proboscis is still closely associated with the body wall on the dorsal side, but it soon migrates to a more proximal location and becomes parallel to the anterior-posterior axis of the larva (239 μm). The size of the proboscis is fairly large relative to the rest of the embryo at early embryonic stages (<250 μm), but its growth rate decreases as the rest of the juvenile body matures and the anterior end of the organ constricts (Fig. 4). It is unclear when the stylet apparatus differentiates, but the proboscis is fully formed and functional immediately after birth. The rhynchocoel rudiment was not observed in this study.

Digestive Tract

Though the foregut should be formed during gastrulation, it is not clearly visible in live specimens and only in phalloidin labeled specimens at later stages (earliest: 121 μm). Acetylated tubulin reveals that the foregut is heavily ciliated (Fig. 6). Post-gastrulation a diffuse midgut area that is composed of large yolk-containing cells is discernable via brightfield microscopy (Fig. 2C). As the embryo develops, the size of the yolk granules decreases resulting in cells with lower yolk density.

The anus is present early in development and has been observed in embryos as small as 106 μm (Fig. 10). The stomodeum and proboscis pore were visible in early-stage embryos (as small as 121 μm; Figs. 2B, 10), and these will eventually fuse into the common mouth/proboscis pore (rhynchostomopore). It is unclear at which size (stage) this fusion occurs.

Nervous System and Cerebral Organs

Based on phalloidin labeling and positional data from published hoplonemertean studies, the central nervous system, which consists of the dorsal and ventral cerebral ganglia, ventral and dorsal nerve commissures linking the ganglia, and lateral nerve cords, forms early in development (106 μm; Fig. 5). All four components of the central nervous system are visible as early as 131 μm, although the ganglia have expanded only minimally (Fig. 6). The cerebral ganglia and linking commissures encircle the anterior end of the proboscis rudiment. It appears that the nerve cord neurons form before the

nerve-cord muscles, which run through the lateral nerve cords (Fig. 5A). All major components of the central nervous system are completely formed before the embryo reaches the planuliform stage (200 μm).

The apical plate is present in early stage embryos (125μm) and persists through much of development, though its size gradually diminishes. Acetylated tubulin labeling reveals a group of neurons radiating out of the apical plate (Fig 7). The apical plate is generally not visible by the end of the planuliform stage (300 μm).

The origin of the cerebral organs is unclear. No paired anterior invaginations were identified in the early post-gastrula period. The cerebral organ canals are well formed by later stages, however (Fig 11).

Ciliary Tufts

Embryos possess an apical plate, but it generally lacks conspicuously long motile cilia. Of 36 observed only a singe 236.4 μm individual was identified with a long (26.6 μm average length) apical tuft composed of multiple motile cilia (Fig. 8A). Acetylated tubulin labeling and light microscopy show that some embryos (8/36) possess a caudal tuft composed of cilia slightly longer than those that uniformly cover the embryonic body, which are 4-7 μm long (Figs. 6, 8B). Light microscopical investigation also suggests that the posterior cirrus is most common in embryos between 197-240 μm (n=5/36), although outliers have been found on both sides of this range (n=3/36). Tufts are composed of 4-5 motile cilia that are generally 2-4 times longer than epidermal cilia. The tufts eventually

disappear as development continues, as none were observed in late stage embryos or in juveniles (Fig. 8C).

Discussion

Transitory epidermis

The transitory larval epidermis has been described in twelve planktonic and encapsulated developing hoplonemerteans (Maslakova and von Döhren, 2009). It consists of a collection of 80-100 large, multiciliated, cleavage-arrested cells, generally with a dense, yolky cytoplasm, distinct nucleus, and an underdeveloped or absent basal lamina. The size of these cells varies, ranging from 20-75 μm in diameter, depending on the species. Some studies (Hickman, 1963; Hiebert *et al*, 2010) suggest that the cells function in yolk storage for the lecithotrophic larvae. For example, in *A. australiensis* (Hickman, 1963) these nonciliated cells contain a considerable amount of yolk. Maslakova and von Döhren (2009) also posit that the function may be locomotory in planktonic larvae, citing the multiciliated cells and the lessened developmental strain caused by having a tissue dedicated to the temporary swimming function while the definitive epidermis differentiates.

The planuliform larva is also referred to as a "hidden larva," (Maslakova and Malakhov, 1999) due to the fact that the internal organs develop while being obscured by the transitory epidermis. The transitory epidermis is lost, either by shedding or gradual replacement by the cells of the definitive epidermis as the cleavage-arrested cells cytolyze (Maslakova and Von Döhern, 2009). There may be a correlation between developmental mode and the process by which the transitory epidermis is replaced, with encapsulated developers exhibiting gradual cell replacement and those with swimming

larvae shedding the tissue (Hiebert *et al*, 2010). The developmental timing of this epidermal transition varies among species. In the planktonic developing *Quasitetrastemma stimpsoni* (Magarlamov, 2008) and encapsulated *Pantinonemertes californiensis* (Hiebert et al, 2010), the epidermal "exchange," as it is described, occurs within two to three days after fertilization, whereas in the encapsulated larva of *Paranemertes peregrina*, it takes place at around 10 days past fertilization (Maslakova and von Dohren, 2009). No clear relationship has been established between larval developmental mode and retention of the transitory epidermis. There is also some variation among species in the composition and morphology of the transitory epidermis, generally related to the amount of yolk in the cells (Magarlamov, 2008). Maslakova and von Döhren (2009) suggest that the that the transitory epidermis is common to all hoplonemerteans, although it has not yet been identified in the viviparous *Notagaeanemertes folzae* (Crandall et al, 1998) and there is some doubt over its presence in the development of the crab symbiont/egg predator *Carcinonemertes epialti* (Stricker and Reed, 1981; Magarlamov, 2008) and in the viviparous *Geonemertes agricola* (Coe, 1904) and *Prosorhochmus claparedii* (Bürger 1895). Based on observations of histological sections Bürger (1895) suggested that a transitory epidermis might be present in this species, but would not confirm it without investigation of additional specimens. In the *P. viviparous* no transitory epidermis was observed (Salensky, 1884).

The presence of the transitory epidermis in the development of *P. americanus* is highly probable, albeit in a modified state. There are relatively large cells interspersed

with smaller ones present in early stage embryos of *P. americanus*, which may be cleavage-arrested. These cells appear to be replaced by smaller cells as has been observed in *Argonemertes australiensis* (Hickman, 1963), *Antarctonemertes phyllospadicola* (Maslakova and von Döhren, 2009), *Paranemertes peregrina* (Maslakova and von Döhren, 2009), and *Tetrastemma candidum* (Maslakova and Malakhov, 1999) and likely represent cells of a transitory epidermis. Interestingly, the large cells in question appear to degenerate much earlier in development than any other described hoplonemertean. Chernyshev and Magarlamov (2010) observed that the transitory epidermis is gone within 50 hours in *Quasitetrastemma stimpsoni*, but provides no information on the state of organogenesis, making comparisons with *P. americanus* difficult. They also suggest an accelerated transitory epidermis is the case for *Carcinonemertes epialti* (Stricker and Reed, 1981), but it has not been clearly demonstrated. When these putative larval epidermal cells are present in *P. americanus*, there are no organs apparent, only actin-rich structures that may be forming muscles and nerves. This contrasts the "hidden larva" description of the transitory epidermis for other hoplonemerteans (Maslakova and Malakhov, 1999). At this phase, there are no clearly discernable organs in the interior of the embryo, only actin-rich structures that may be forming muscles and nerves. In other species, including ones whose development has been investigated using similar methods, internal organs, such as the proboscis, musculature, nervous system, and digestive tract have formed or have begun to form while the larva is still enclosed in the transitory epidermis. Even in the earliest stages of organogenesis in *P. americanus*, the cells of the

epidermis are uniformly sized, numerous, and not particularly large. These cells also lack the thickness of the transitory epidermal cells described by Malakhov (2008) (31.1±3.3), Magarlamov and Chernyshev (2010) (~30 μm), and Maslakova and von Döhren (2009) (approximately half the larval diameter).

Assuming one or both of the posited functions of the transitory epidermis are accurate, a larval epidermis would not be a necessity *P. americanus* larvae. Larvae of this species, unlike those of planktonic planuliform larvae, have no need to swim for extended periods of time (they are born crawling) and their yolk is localized in the midgut cells. Given the putative functions of the transitory epidermis in planuliform larvae, a larval epidermis could be interpreted as a vestigial trait in this viviparous species.
The acceleration of this process relative to planuliform and encapsulated hoplonemertean larvae may be correlated with this loss of function in the viviparous species.

Although limited data are suggestive of a transitory epidermis in *P. americanus*, CLSM data from additional specimens and ultrastructural research of the larval epidermis in will be required to definitively confirm its presence. Such studies will also be necessary to determine if the putative transitory epidermal cells are ciliated or yolk-filled.

Musculature

The muscular development of *P. americanus* is consistent with what has been described for other hoplonemerteans using CLSM. Hiebert et al. (2010) describe the body wall muscles, as well as most other muscle groups (longitudinal/circular proboscis

muscles, proboscis insertion muscles, and nerve cord muscles) in three-day-old *Pantinonemertes californiensis* embryos as "well-developed." The musculature of four-day-old and thirteen-day-old larvae are "nearly identical." Likewise, in *P. americanus*, all major muscles form relatively early in development, and fibers of all layers proliferate substantially during embryonic development. Hiebert et al (2010) make no mention of such expansion of the body wall muscles, and it is not readily apparent from their figures that any significant proliferation occurred up to four days past fertilization (dpf). Although precise stage comparisons between these species cannot be made, it is possible that early proliferation of the body wall muscles is unique to viviparous development, as muscular locomotion on hard substrates is necessary immediately after juvenile emergence, whereas muscles are presumably necessary only for steering in planktonic larvae.

Maslakova and von Döhren (2009) noted that the longitudinal and circular muscles of the body wall of *Paranemertes peregrina* form between two and four dpf, with diagonal muscles forming shortly thereafter and clearly visible five dpf. They also observed that the nerve muscles are visible as early as five dpf, while the proboscis insertion muscles are formed by eight dpf. While the timing cannot be precisley compared, formation of the body wall musculature of *P. americanus* follows a similar trajectory. Early-stage embryos have been visualized with only circular and longitudinal muscles, as well as embryos with a full compliment of body wall muscles and no

proboscis insertion or nerve muscles. In some embryos, it was difficult to conclusively determine the presence of certain muscle groups.

Chernyshev's (2010) CLSM-based study on the musculature of adult nemerteans, revealed that an anterior-posterior gradient exists in the density of the diagonal muscles, with a greater concentration of muscle fibers at the anterior end of the animal. This is also the case in *P. americanus* and becomes noticeable in later stages (405 μm). He also noted that the adult proboscis consists of five muscle layers, which is a characteristic that presumably develops later in embryonic development, as the embryos visualized in this study only have two (circular and longitudinal) muscle layers in the early proboscis. It is likely that the musculature proliferates closer to birth in *P. americanus* embryos, after the density of the body wall muscles makes it difficult to distinguish all layers with for phalloidin labeling. Additionally, the gonadal musculature of mature *P. americanus* forms and "irregular meshwork" corresponding to Chernyshev's (2010) description for other hoplonemerteans (personal observations, not pictured).

Proboscis

In hoplonemerteans, the proboscis forms from an ectodermal invagination on the anterior end. Though the description of the invagination's location differs slightly in the literature, it appears that this is a result of word choice and that the location is the same across the species studied. The timing of the rudiment's formation varies widely between species, however. In *E. gracile* (Iwata, 1985), it is reported at 9 hpf, whereas in *P.*

peregrina (Maslakova and von Dohren, 2009), it was not observed until 5 dpf. The description of early proboscis formation is relatively consistent. It is described as either a small spherical or tubular mass with a small lumen. As the proboscis develops, it becomes bipartite, with a bulbous posterior end and a tubular anterior end. Attachment to the rhynchodeum varies in its timing as well, being reported as early as 47 hpf (Iwata, 1985) and greater than 5 dpf (Hiebert et al., 2010). In embryos of *P. americanus* the primary mouth opens to the outside independently of the proboscis pore (Fig. 10), whereas in *P. viviparous* the mouth has no connection to the outside independent of the proboscis pore during development (Salensky, 1909, 1914).

The formation of the proboscis in *P. americanus* matches closely the process described for other hoplonemerteans investigated with CLSM, though no ectodermal invagination was clearly identified. Maslakova and von Döhren (2009) also failed to detect an invagination and speculated that the invagination likely occurs in pre-hatching stages. It is unclear when and how the mid proboscis forms or when the armature becomes present. It appears that the anterior tubular portion elongates somewhat and it is plausible to assume the proboscis grows in an anterior direction. It is unclear when the rhynchodaeum forms, and therefore when it fuses with the stomodaeum. The bulbous posterior region also has similar morphology to the portion of the adult proboscis that houses the stylet. The only mention of stylet formation is in relation to the apparently slow-developing *P. peregrina* and occurs at 3 weeks (Maslakova and von Döhren, 2009).

Digestive Tract

There exists quite a bit of variability in the development of the hoplonemertean digestive tract (see Iwata, 1985). The major similarities among the described species are that the midgut is formed after the closure of the blastopore (1-2dpf), while the foregut forms from a separate ventral invagination. The lumens mid- and foregut are separate until a later point in development. Maslakova and von Döhren (2009) noted a small invagination marking the putative anus, but the formation of the anus is not mentioned in many studies of hoplonemertean development, (Iwata, 1985; Hiebert *et al*, 2010) suggesting it generally forms later in development.

The development of the digestive tract of *P. americanus* appears to match the more general aspects described in the literature. Though we were unable to track the closure and fate of the blastopore, the midgut is present in the earliest post-gastrula individuals surveyed (106 μm). A clear foregut can be identified in a slightly later stage individual (121 μm). Acetylated tubulin labeling also reveals that the foregut is more heavily ciliated than the midgut, corroborating the observations of Stricker and Reed (1981). The two portions of the digestive tracts do indeed appear to be separate in early stages. The fusion of the mid and foregut, but they do appear to be fused by the time the larva begins to elongate (372 μm). A band of circular muscles at the posterior end in early planuliform embryos forms a sphincter at marks the site of the anus.

Nervous System and Cerebral Organs

Little information exists in the literature on the development of the nervous system in hoplonemertean planuliform larvae. Chernyshev and Margarlamov (2010) state that the encapsulated larva of *Quasitetrastemma stimpsoni* possesses a "larval brain," consisting of 3 groups of neurons at emergence, 12 hours after fertilization. The general consensus is that the major organs of the system (cerebral ganglia, longitudinal nerve cords, and dorsal/ventral commissures) appear relatively early in development (2-4 days old), forming a ring of nervous tissue around the proboscis, and become more prominent through larval development (Chernyshev and Margarlamov, 2010; Hiebert et al, 2010; Maslakova and von Döhren, 2009). Salensky (1909) observed a nervous system rudiment in *Prosorhochmus viviparous* using classical light histology that he described as an "ectodermal thickening" and the brain and lateral nerve cords were apparent in early embryos. This is consistent with observations for *P. americanus.* Enervation of the ocelli and ciliary tufts (and presumably, the apical plate) was also noted in planuliform larvae (Stricker and Reed, 1981).

The development of the cerebral organs remains unclear. Maslakova and von Döhren (2009) state that the cerebral organ rudiments are visible at 2 days old, appearing as paired anteriolateral invaginations, then are open to the external environment at 4-5 days old, and label prominently with phallacidin at 8 days old. Hiebert et al. (2010) are more cautious, stating that the cerebral organs *may* originate from paired anterior invaginations, but it is likely that they develop fully later in the larval stage.

The development of major structures of the nervous system in *P. americanus* appears to corroborate previous observations, though no rudiment was identified. Though the presence of a larval brain (*sensu* Chernyshev and Margarlamov, 2009) cannot be verified, the central nervous system was observed in some of the earliest embryos surveyed. Enervation of the apical and caudal ciliary tufts was also confirmed using acetylated tubulin labeling.

The developmental origin of the cerebral organs was not identified in this study. The paired anterior invaginations mentioned elsewhere were not seen in early embryos of *P. americanus*. Additionally, the cerebral organs could not be definitively and consistently identified until the larvae had reached later stages (>275 μm). This may be attributed to limitations of the visualization and mounting methods employed.

The apical plate is visible early in development, though its formation appears to lag slightly behind the major organs of the nervous system. The apical plate may have a sensory function in swimming larvae (Richter *et al*., 2010) and, may be a vestigial structure in *P. americanus* as they do not swim. It has been suggested that the apical organ develops into the adult chemoreceptive frontal organ in adults (Chernyshev and Margarlamov, 2009). It is also possible that the apical plate, in conjunction with the ciliary tufts, has evolved a new function in this viviparous species, for example sensing conditions in the ovotestis to facilitate embryo movement to optimize physiological exchange during development.

Ciliary Tufts

Most previous research on hoplonemertean larvae indicate that the planktonic planuliform larvae possess apical and caudal tufts of elongated cilia (e.g., Hiebert *et al*, 2010; Iwata, 1985; Chernyshev and Magarlamov, 2009 Maslakova and von Döhren, 2009; Stricker and Reed, 1981), but these structures are not reported for species lacking nonpelagic stages, including some encapsulated developers and viviparous species (see Friedrich, 1979). It should be noted that no mention of ciliary tufts was made in the investigation of the viviparous *Notogaeanemertes folzae*, although the focus of the research was gonadogenesis and embryogenesis in the adult worm, and the description of the larva is very limited and tangential to the study (Crandall et al., 1998). Both the apical tuft and posterior cirrus are innervated and assumed to have a sensory function. (Stricker and Reed, 1981, Chernyshev and Magarlamov, 2009).

Using light microscopy to investigate a set of *P. americanus* embryos and larvae (n = 36) at varying stages, it was observed that 22% of individuals possessed a posterior cirrus. Most occurrences were within an embryo size range of 197-260 μm, though there were cirri in both earlier and later stages. The mean length of the posterior cirri was 16.5 μm, with a range of 10.8 μm (n = 7), noticeably longer than the cilia covering the epidermis, which had a mean length of 6 μm in these individuals. No movement of the posterior cirrus was observed. A single individual (2.7% of those sampled) possessed an apical tuft, which had a length of 26.6 μm. It was composed of 4-5 cilia that were observed moving slowly back and forth. The actual movement pattern and speed of the

apical tuft was not discernable, since the specimen was compressed under a cover slip, and its function remains unclear. The exaggerated size of the apical tuft relative to the posterior cirrus matches all previous descriptions of planuliform larvae.

A recent phylogeny of the Nemertea based on several molecular markers suggests that the planktonic larvae of hoplonemerteans is likely ancestral to the clade (Andrade et al., 2012). Because the viviparous *P. americanus* lacks a swimming larval or juvenile stage, it is possible that this trait is being selected against and that the observation of cirri in only a proportion of the individuals sampled may reflect the occasional expression of the ancestral trait (atavism). We also cannot rule out the possibility that these features are expressed transiently in all embryos and represent a vestigial trait.

Conclusions & Future directions

This investigation of viviparous development in nemerteans has filled a major void in our knowledge of hoplonemertean development, providing insight into the evolution of direct development and further demonstrating the uncritical use of the term for hoplonemerteans. Most importantly, it has revealed three larval characters: an apical plate, apical and posterior ciliary tufts, and a putative transitory epidermis. The loss of these larval features suggests a metamorphosis like that of non-viviparous hoplonemerteans and provides additional support for the hypothesis that hoplonemerteans are indirect developers possessing a "hidden larva" (see Maslakova and Malakhov 1999; Maslakova and von Döhren, 2008). The acceleration of the epidermal transition and the possible

vestigiality of the apical plate and ciliary tufts in *P. americanus* suggest that larval features are being selected against and that this viviparous species is following an evolutionary transition away from indirect development.

Further examination of the development is warranted. Additional insight into the transitory larval epidermis will require supplemental observations of phalloidin-labeled embryos at the appropriate stages. Vibratome sectioning of agarose-embedded specimens along the longitudinal plane followed by phalloidin labeling should increase the number of embryos that can be observed in a single individual and will obviate potential damage to early-stage embryos that can occur during dissection and mounting. Given the fragile nature of early-stage embryos, this should allow more reliable observation of the developmental stages relevant to the identification of the transitory epidermis. A combination of histological sectioning and fluorescent labeling/immunohistochemistry will also serve to increase the level of detail and understanding of organogenesis in this nemertean.

Table

Stage	*Embryo Size (μm)*	*Structures formed*
Early spherical	80-115	Definitive epidermis, internal organs not discernable
Late spherical	112-140	Proboscis rudiment, midgut
Early planuliform	106-134	Ovoid proboscis, proboscis circular/longitudinal muscles, cerebral ganglia, longitudinal nerve cords, nerve commissures, foregut, body wall muscles (circular, longitudinal, diagonal), anus, apical plate
Late planuliform	138-285	Bipartite proboscis, nerve muscles, proboscis retractor muscle, lateral proboscis muscles, expanded cerebral ganglia
Early teardrop	270-397	Fused foregut/midgut
Late teardrop	400-635	Well-formed cerebral organs and central nervous system

Table 1: Staging of embryonic development is based on external morphology and state of internal organs

Figures

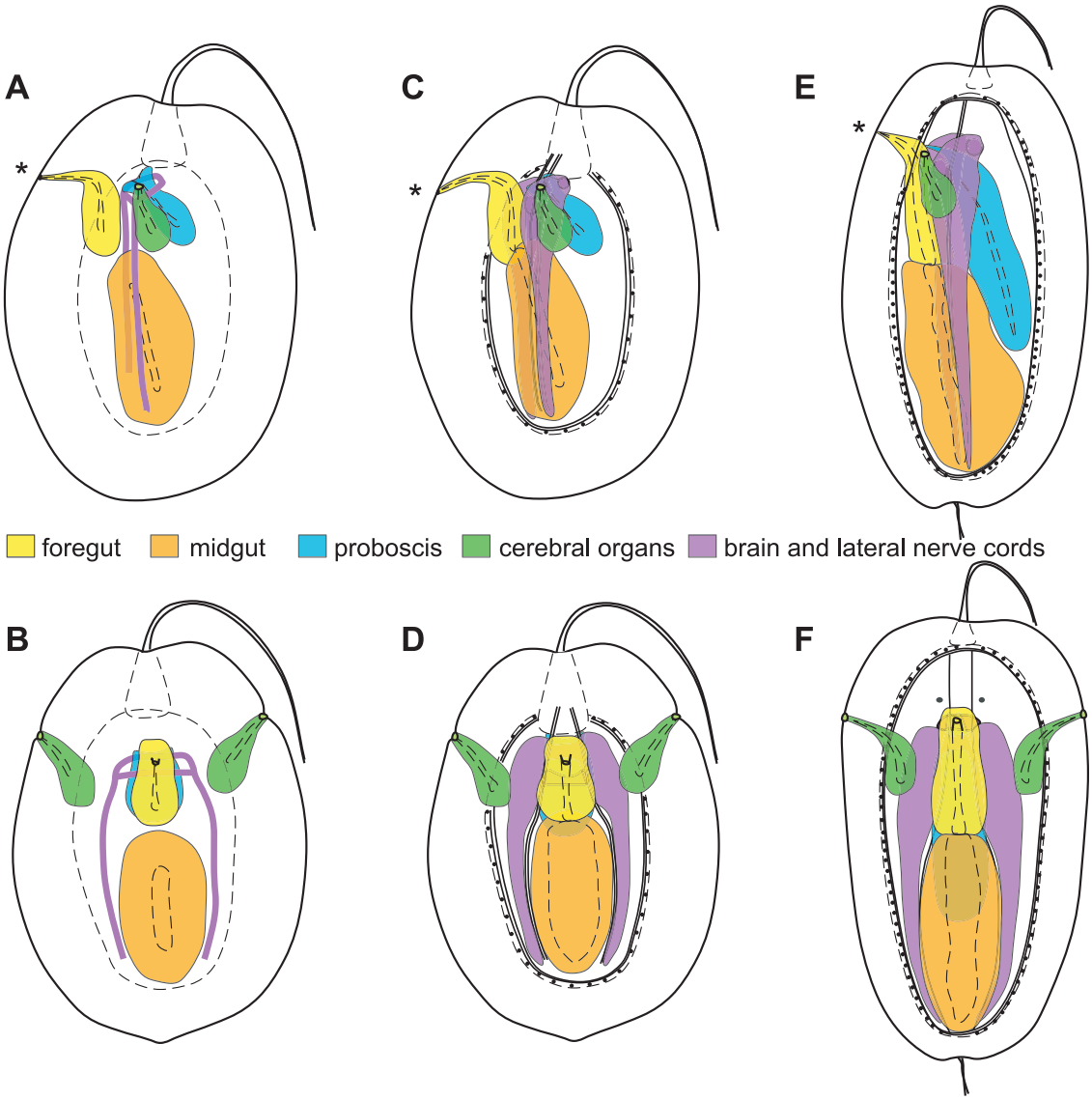

Figure 1: Schematic overview of the development of the major organs in the planktonic (planuliform) larva of the hoplonemertean *Paranemertes peregrina*. From Maslakova and von Döhren, 2009. Abbrevations in this thesis: foregut=fgt, midgut=mgt, proboscis=pb, cerebral organs=co, brain (cerebral ganglia)=cg, longitudinal nerve cords=lnc

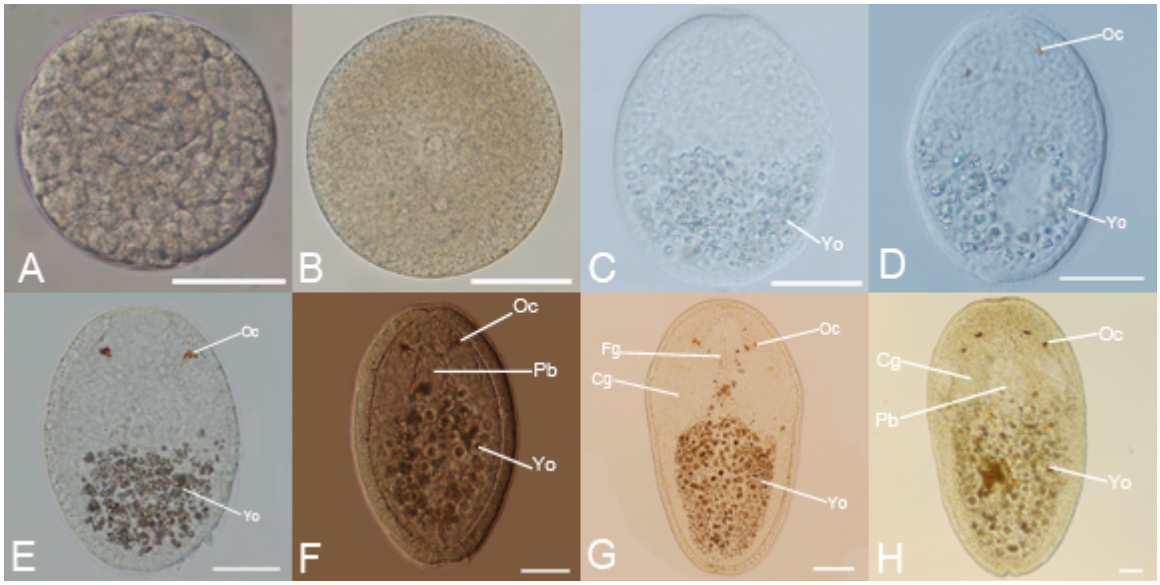

Figure 2: Light micrographs showing early development in *P. americanus* (dorsal view). (A) Early spherical stage with mostly large cells interspersed with smaller ones. (B) Late spherical stage with larger cells replaced with apparently uniformly-sized smaller ones. (C) Early planuliform stage with deposited yolk (Yo). Clear anterior-posterior differentiation exists, but major organs are not noticeable using this visualization method. (D) Early planulfirm stage with ocelli (Oc) developing. (E) Well-formed ocelli in a late planuliform embryo. (F) Late planuliform stage, proboscis (Pb) visible. (G) Early teardrop stage; proboscis and cerebral ganglia (Cg) visible. (H) Late teardrop stage approaching crawling vermiform stage. Apical is up, all scale bars=50 µm.

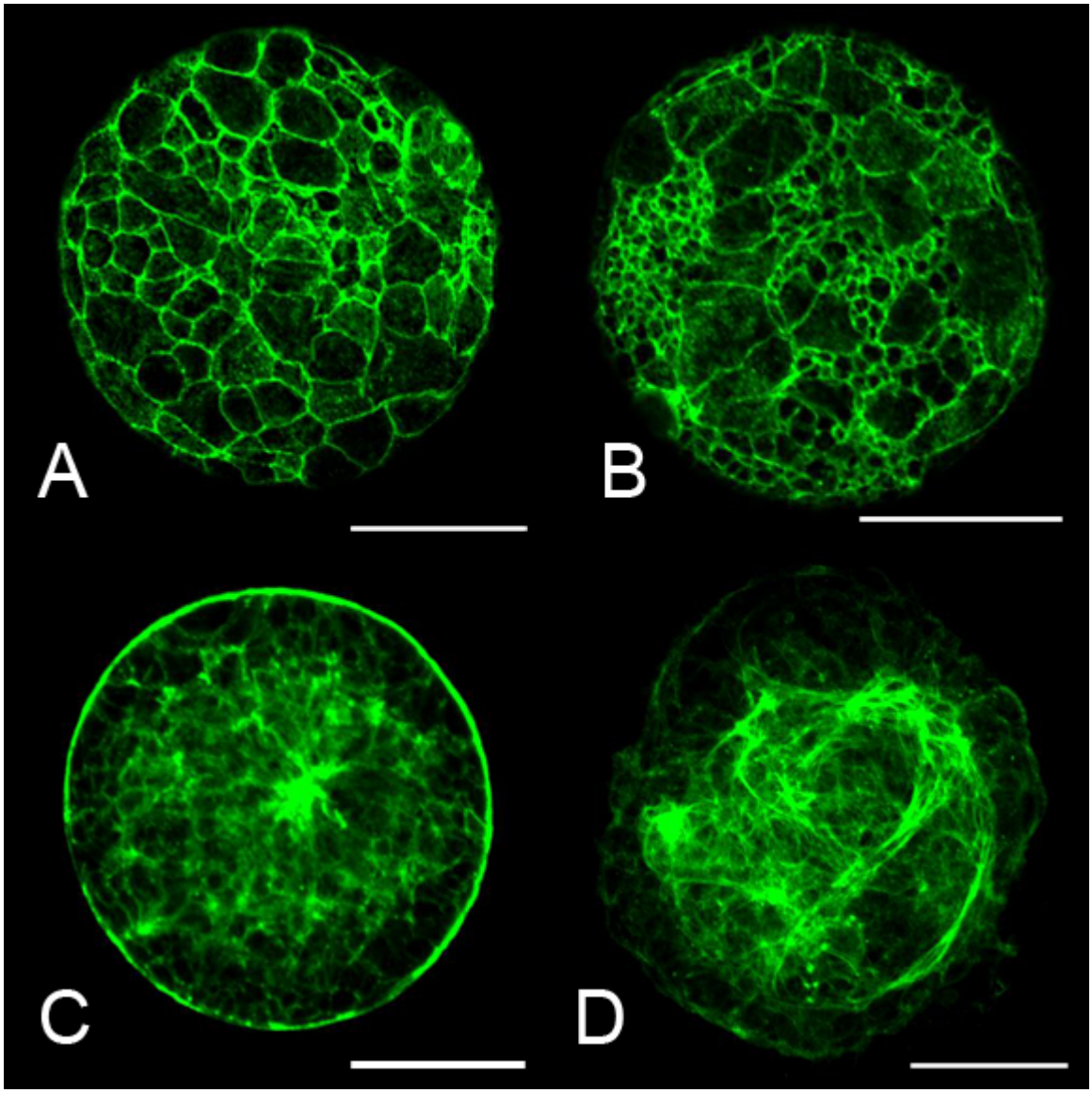

Figure 3: Phalloidin-labeled spherical stage *P. americanus* embryos. (A) In the early spherical stage, cells are uniformly large and are gradually replaced by smaller cells pictured in the late spherical embryo in (B). (C) and (D): Early –stage embryos possess actin-containing structures, presumably muscles and and nervous system components lacking clear organization. Z-projections: (A): 4 sections; (B): 3 sections; (C): 11 sections; (D): 12 sections. Scale bars=50 µm.

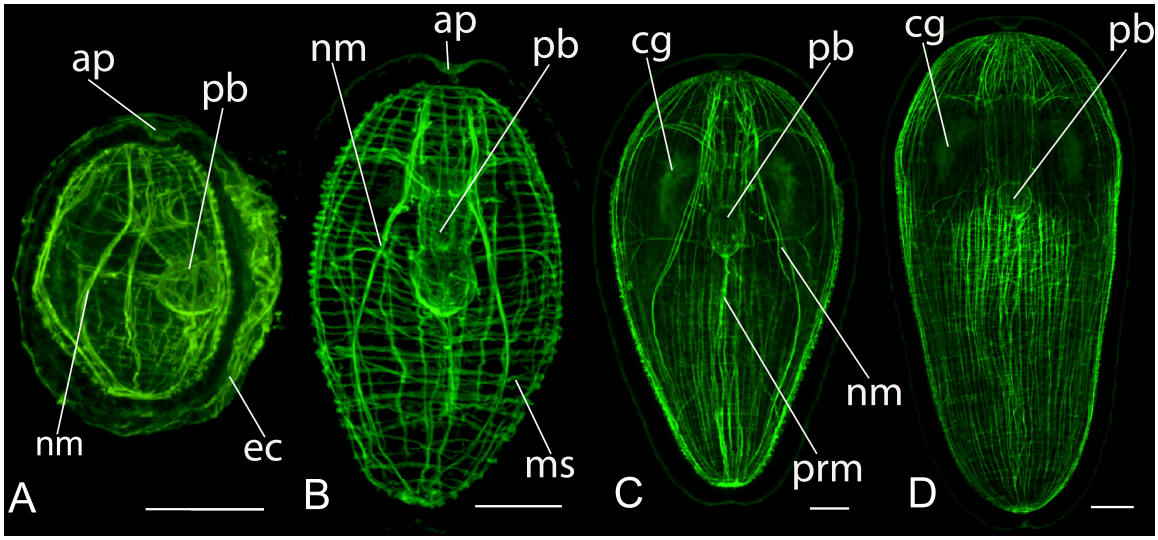

Figure 4: Confocal projectios of phalloidin-labeled embryos demonstrating general growth and development through embryonic development. (A) Sagittal view of early planuliform stage. (B) Coronal view of late planuliform stage. (C) Coronal view of early teardrop stage. (D) Coronal view of late teardrop stage. Note the expansion of the body wall muscles to cover the interior of the body wall and the shrinkage of the proboscis bulb relative to the size of the rest of the body in (B)-(D). Abbreviations: ap=apical plate, pb=proboscis, nm=nerve muscle, ec=chorion, ms=body wall muscles, cg=cerebral ganglia, prm=proboscis retractor muscle. Z-projections: (A): 15 sections; (B): 12 sections; (C): 12 sections; (D): 14 sections. Scale bars=50µm.

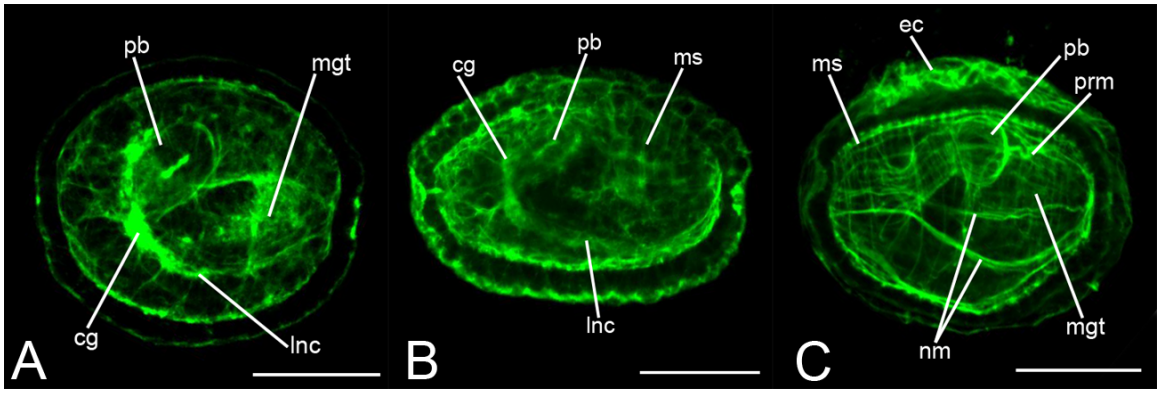

Figure 5: Confocal projections of early post-gastrula development in *P. americanus*. (A) The proboscis (pb) rudiment consists of a mass of cells situated anteriorly. The midgut (mgt) and nervous rudiments of the cerebral ganglia (cg) and longitudinal nerve cords (lnc) have also formed. The body-wall muscles have not yet formed. (B) Later stage revealing body-wall muscles (ms) form shortly thereafter. (C) Later stage showing well-developed muscle groups, including, the circular and longitudinal proboscis muscles of the proboscis, proboscis retractor muscle (prm), and nerve muscles (nm), which run through the longitudinal nerve cords and longitudinal and circular body-wall muscles, are present at this stage. The embryo is enclosed in the chorion (ec). Z-projections: (A): 5 sections; (B): 7 sections; (C): 15 sections. Scale bars=50 µm.

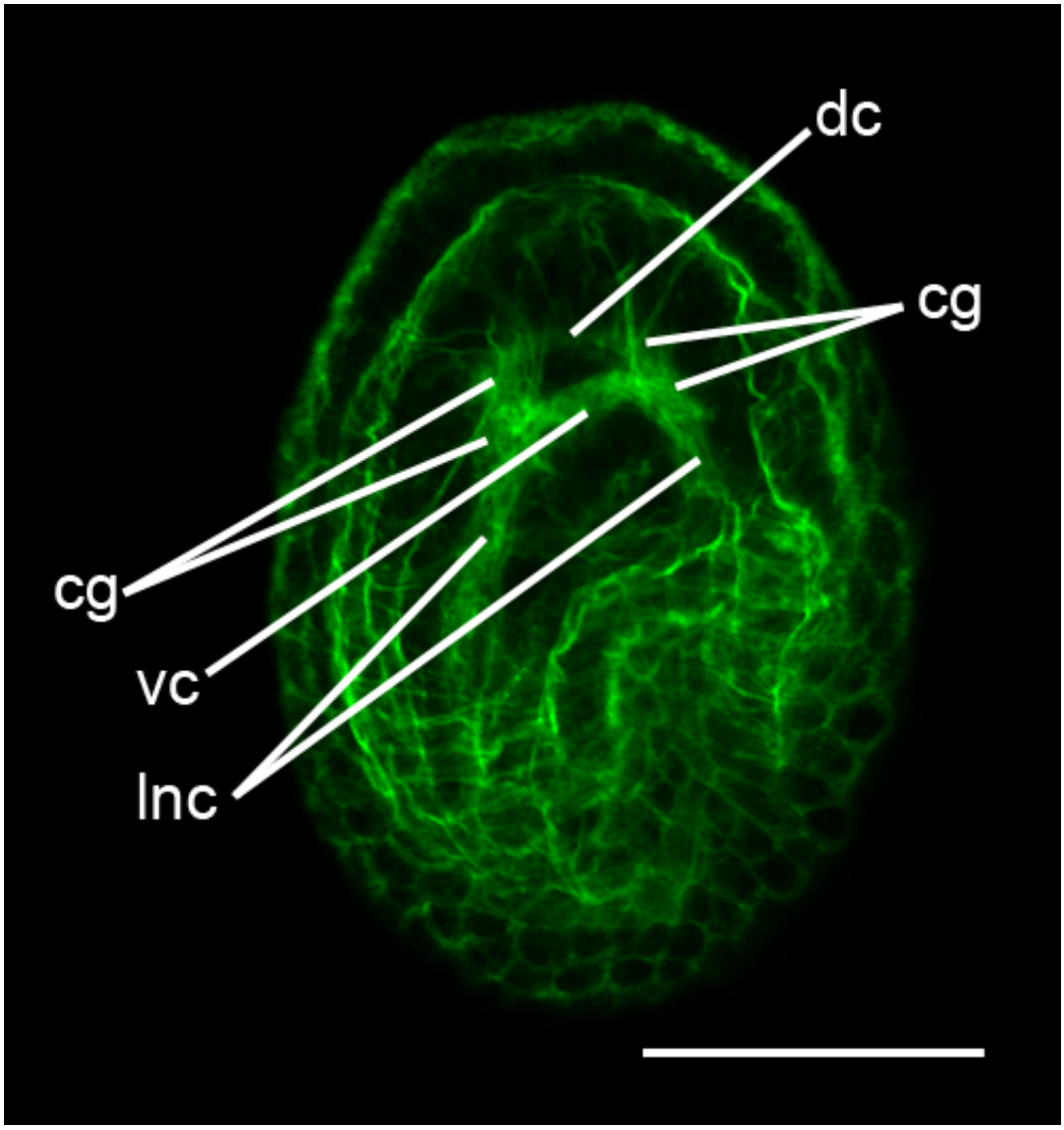

Figure 6: Early nervous system development (coronal section). All major components of the central nervous system are formed relatively early in development. The cerebral ganglia (cg) and dorsal/ventral commissures (dc/vc) are fused in a ring-like formation, with the longitudinal nerve cords (lnc) extending posteriorly. Z-projection: 31 sections. Scale bar=50 µm

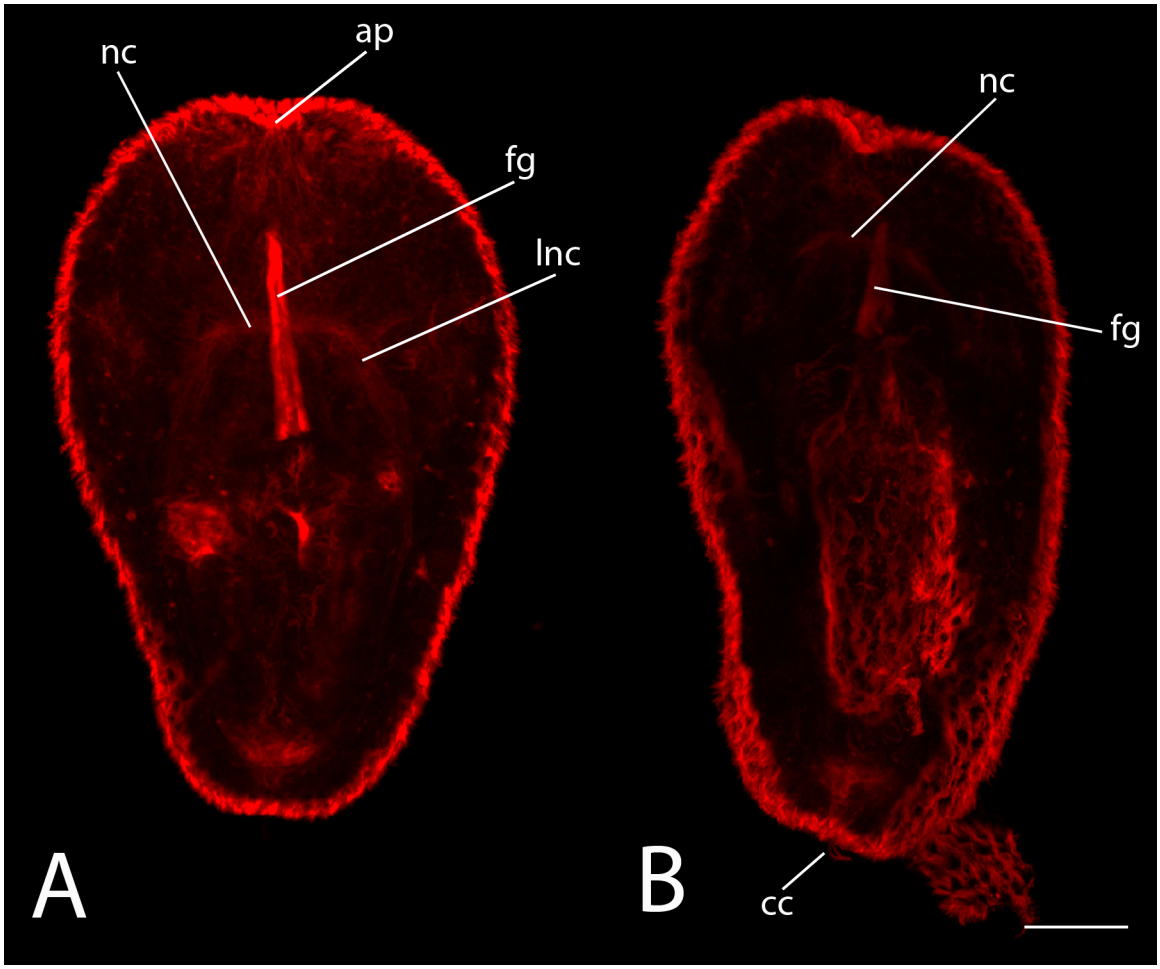

Figure 7: Confocal projections (coronal view) of acetylated tubulin-labeled *P. americanus* embryos. Labeling indicates uniform ciliation of the epidermis, and a heavily ciliated foregut or pharynx (fg). An outgrowth of nerve fibers extending from the putative apical plate (ap) is also visible as is a posterior cirrus (cc), which is likely vestigial. nc=nerve commissure; lnc=longitudinal nerve cord. Z-projections: (A): 11 sections; (B): 18 sections. Scale bar=50 µm.

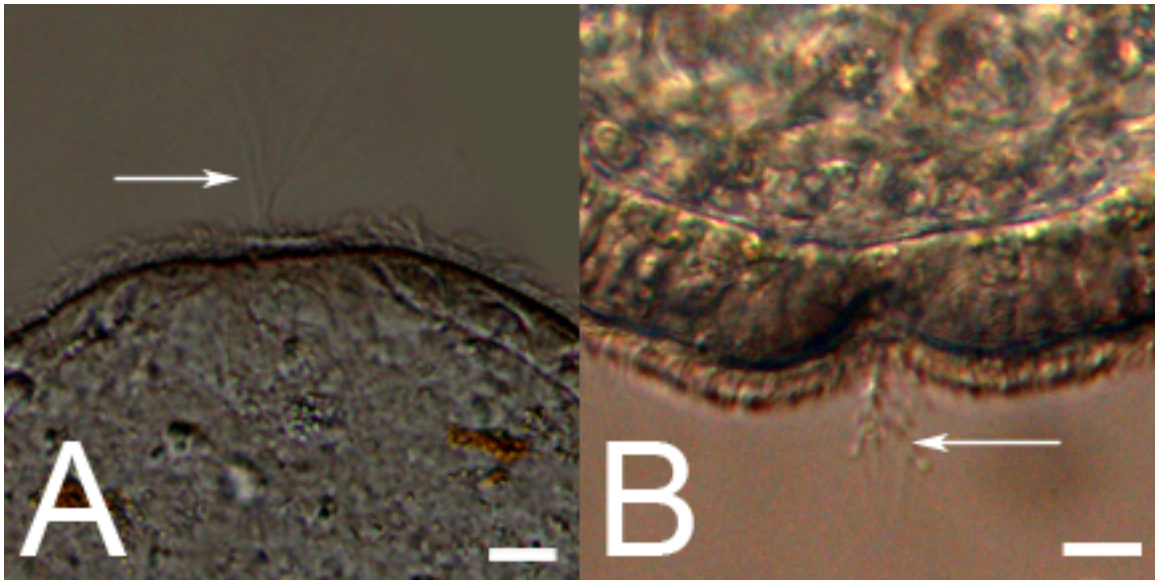

Figure 8: Light micrographs of cilary tufts and cirri in developing embryos. (A) An apical tuft was only identified in one individual. It is conspicuously long and is motile. (B) Posterior cirrus (C) The posterior cirrus. Scale bars=10 µm.

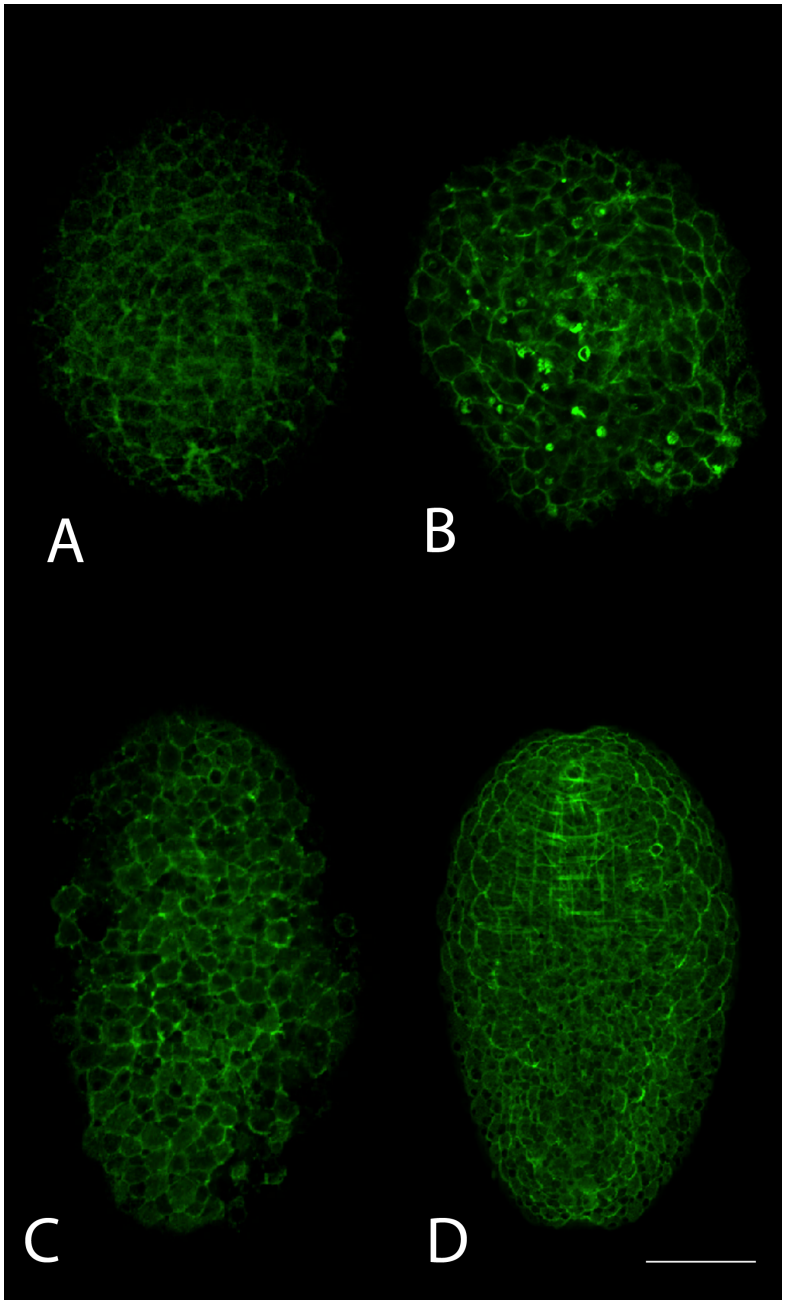

Figure 9: Confocal projections of *P. americanus* embryos of varying size. Epidermal cell size exhibits little variation in embryos sizes above 150 µm in length. Z-projections: (A): 14 sections. (B): 9 sections. (C): 9 sections. (D): 24 sections. Scale bar=50µm

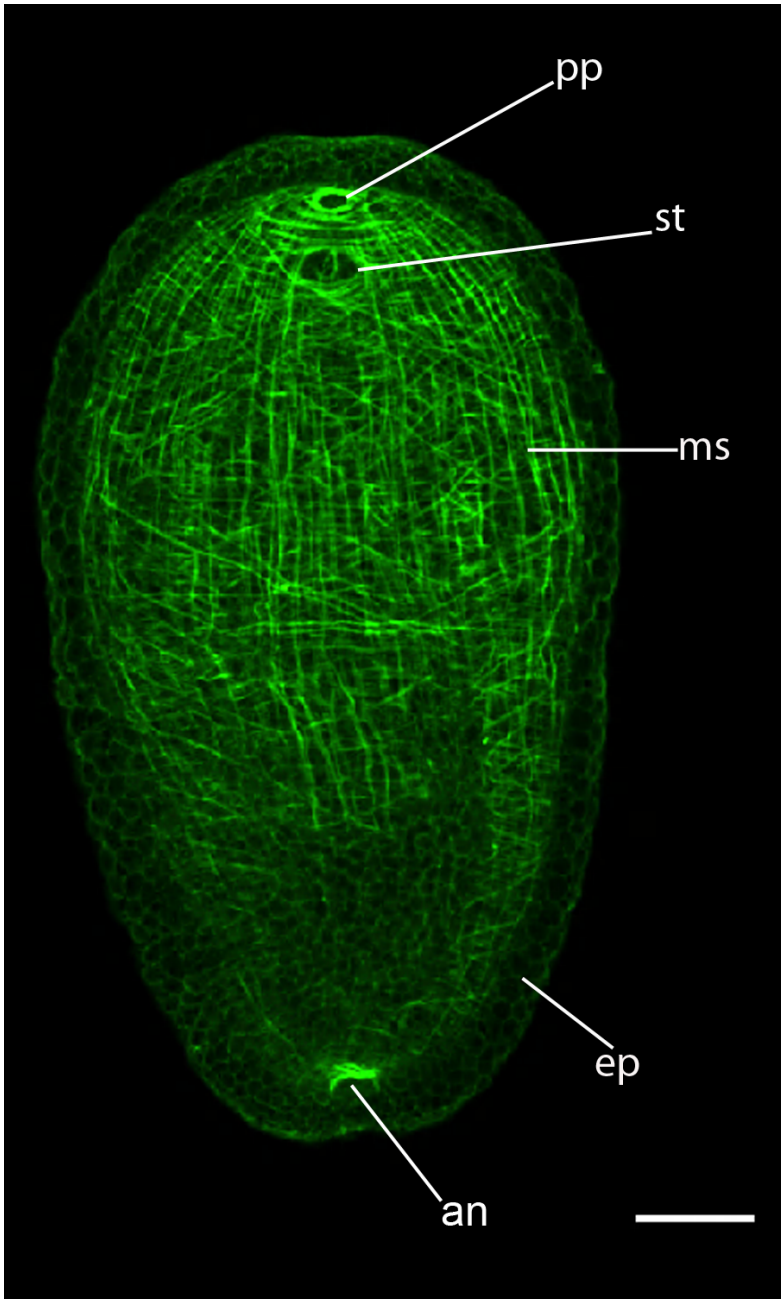

Figure 10: Confocal projections of phalloidin labeled embryo shows the stomodeum (st), proboscis pore (future rhynchostomopore) (pp), and anus (an) of a late-stage embryo. The epidermis (ep) and body wall muscles (ms) are also visible. Z-projection: 28 sections. Scale bar=50µm

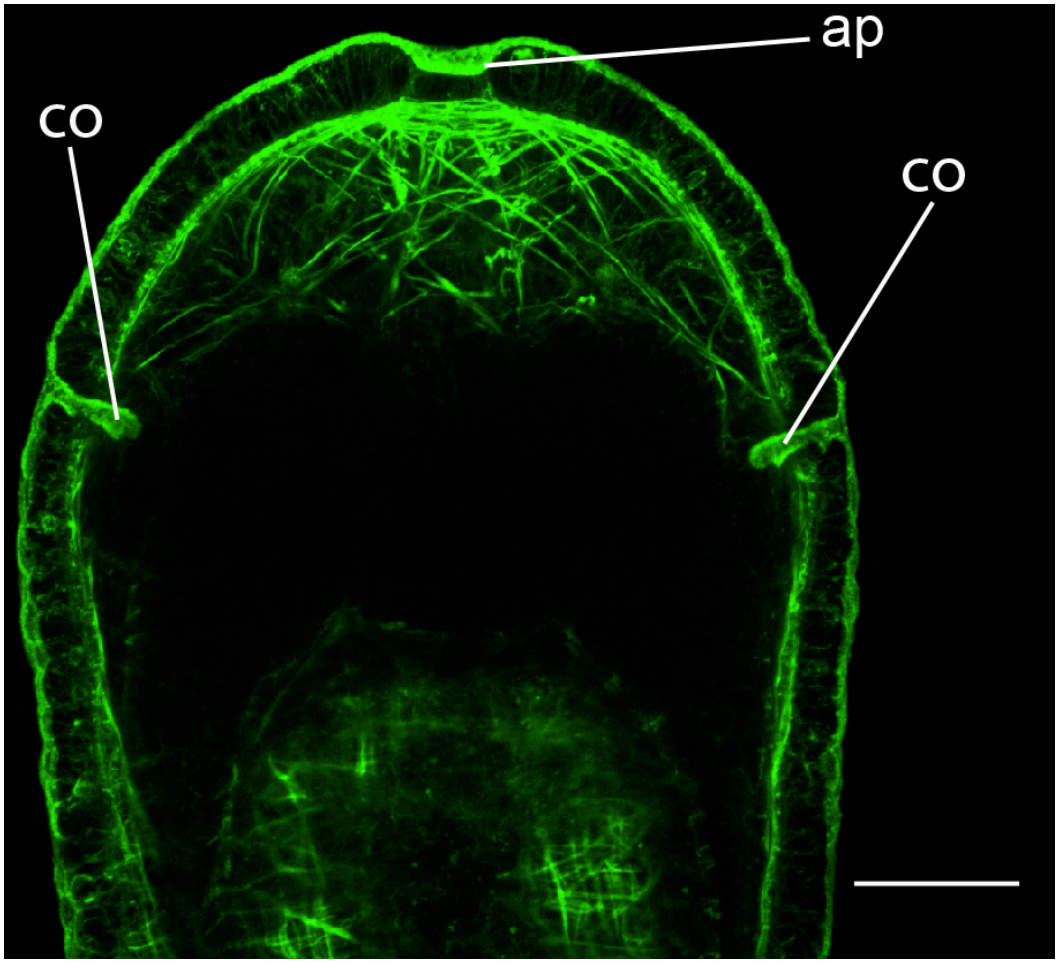

Figure 11: Phalloidin labeling shows well-formed cerebral organs (co) and apical plate (ap) in a late-stage embryo. The musculature of the apical end is also visible. Z-projection: 16 sections. Scale bar=50µm

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Vita

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