COMPARATIVE ANALYSIS OF CELL PROLIFERATION PATTERNS IN CILIATED PLANKTOTROPHIC LARVAE OF MARINE INVERTEBRATES

by

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THESIS ABSTRACT

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Title: Comparative Analysis of Cell Proliferation Patterns in Ciliated Planktotrophic Larvae of Marine Invertebrates

Most benthic marine invertebrates have long-lived planktonic ciliated larvae that must feed and grow to reach metamorphosis. Because ciliated cells in animals are unable to divide it is of considerable interest how ciliated larvae are able to grow. To understand how ciliated larvae grow I compared cell proliferation patterns in several species with planktotrophic larvae from five different phyla (Nemertea, Mollusca, Phoronida, Echinodermata, and Annelida). Cell proliferation events were detected using anti-phosphohistone antibody labeling, BrdU assays, and confocal microscopy. Studied larvae included some with monociliated epithelia (pluteus, bipinnaria, actinotroch, and mitraria) and others with multiciliated epithelia (metatrochophore, pilidium, and veliger). Dividing cells were detected in all studied larvae, but the pattern of dividing cells varied among types and correlated with the kind of epithelium (mono- vs. multiciliated) and phylogeny (e.g. protostome vs. deuterostome).

Running z-projection movies of actinotroch, mitraria, veliger and pilidium are included as supplemental files.

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This thesis is dedicated to my family. To Byron who has been a stable source of encouraging words and to Kaydyn and Alric who are so contagiously excited about learning.

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CHAPTER I

INTRODUCTION

Many marine invertebrates have biphasic life cycles with ciliated planktotrophic larvae. These larvae develop from small eggs and may spend considerable time feeding and growing in the plankton in order to reach metamorphosis (e.g. Strathmann 1985, Emlet et al. 1987, McDonald and Grünbam 2010). Many published studies detail feeding and growth in ciliated larvae (Hart 1991, Strathmann and Bone 1997, Reitzel et al. 2004, Milonas et al. 2010), but few studies detail *how* larvae grow (Seaver et al. 2005, Kristof 2011). Are cells in the larval body simply stretching to accommodate an increase in size, or are cells dividing to allow for growth? If cells are dividing, where does cell proliferation take place in the larval body? Are mitotic cells dispersed throughout the larval body or are they restricted to certain regions? Do patterns of cell proliferation vary in different kinds of larvae? In order to address these questions, I compared patterns of cell division in planktotrophic ciliated larvae from several phyla of marine invertebrates. I chose larvae that differ not only in the overall body plan, but also in the type of epithelia they possess (monociliated vs. multiciliated). These include the **bipinnaria** of *Pisaster ochraceus* and *Patiria miniata* (Asteroidea, Echinodermata), the **pluteus** of *Dendraster excentricus* (Echinoidea, Echinodermata), the **actinotroch** of *Phoronopsis harmeri* (Phoronida), the **mitraria** of *Owenia collaris* (Polychaeta, Annelida), the **metatrochophore** of *Serpula columbiana (*Polychaeta, Annelida*),* the **veliger** of *Caesia fossatus (*Gastropoda, Mollusca*),* and the **pilidium** of *Micrura alaskensis (*Heteronemertea, Pilidiophora, Nemertea*)*.

Patterns of growth in the various larval forms may vary because of differences in the overall body plan and ancestry, as well as the type of larval epithelium. Almost no examples exist where ciliated animal cells enter mitosis (Plotnikova et al. 2008), and ciliated cells that do undergo mitosis must first lose their cilium (Helvestine 1921). The cilium then re-grows post-mitotically (Irigoin and Badano 2011).

This apparent inability of animal cells to divide in the presence of cilia, and conversely, loss of the primary cilium by cancerous cells, suggests a dependence or a restrictive effect of intact cilia (or their basal bodies) on the cell cycle (Plotnikova et al. 2008). When a centriole anchors and nucleates a cilium or flagellum, it is referred to as a basal body. Centrioles, linked in pairs, organize microtubules of the mitotic spindle during cell division (Dirksen 1991). A cell with one cilium may divide by degenerating its cilium and use the basal body as a centriole for mitosis (Irigoin and Badano 2011). A multiciliated cell has many basal bodies and if those basal bodies were used as microtubule organizing centers (MTOC) simultaneously this would lead to multiple cleavage furrows initiating at once, and mistakes in segregating the chromosomes to daughter cells, as, for example, often happens in polyspermic eggs (Schatten et al. 1991).

Regardless of the underlying reasons multiciliated cells in animals, apparently, do not divide (Margulis 1981, Buss 1987). This presents interesting questions: how do larvae with multiciliated epithelial cells grow? Do ciliated cells simply stretch or do some of the cells remain non-ciliated (or have a single cilium) and therefore are able to divide? If so, are dividing cells dispersed throughout the larval body (one extreme in the universe

of possibilities) or are they localized in few specific regions (another extreme)? One might expect an intermediate state between these two extremes.

I will now outline my reasons for choosing particular larval types for this study.

Bipinnaria and pluteus

Echinoderm larvae, such as the bipinnaria (Fig. 1a) and the pluteus (Fig. 1b), possess monociliated epithelia, which is believed to be an ancestral feature of deuterostomes, and bilaterians, in general (Nielsen 2001). Because each epithelial cell could potentially loose its cilium, divide and re-grow the cilium, one might expect to find a dispersed pattern of growth in such larvae. Arms of the pluteus larva grow proportionally longer in response to low food concentration (Boidron-Metairon 1988, Strathmann et al. 1993, Miner 2007). Do the arms of the pluteus grow via growth zones at the tips or the base of each arm or is cell division dispersed throughout the entire arm?

Actinotroch

Like the deuterostomes, the phoronid adults and their actinotroch larvae (Fig. 1c) possess monociliated epithelia (Temereva and Malakhov 2010). However, the actinotroch larva has an entirely different body plan than either the pluteus or the bipinnaria. Moreover, the current view of bilaterian phylogeny suggests that phoronids are lophotrochozoan protostomes (Dunn et al. 2008, Santagata and Cohen 2009), which generally have multiciliated epithelia (Nielsen 2001). Therefore, one might expect the

growth pattern of actinotroch larvae to be different from that of echinoderm larvae.

Metatrochophore and mitraria

Mitraria larvae (Fig. 1d) of the polychaete genus *Owenia* are unusual in having a large episphere, a convoluted ciliary band, a juvenile body that develops tucked inside the larval hyposphere, and, uniquely for the phylum, a monociliated epithelium (Smith et al. 1987, Emlet and Strathmann 1994, Smart and von Dassow 2009). The pattern of cell proliferation in the mitraria larva may be restricted as in its recent annelid ancestors, or dispersed due to the relaxation of constraints on cell division with reversal to a monociliated epithelium.

The trochophore and metatrochophore larvae (Fig. 1e) of *Serpula columbiana* represent the typical annelid larva with multiciliated epithelium and, possibly, a restricted proliferating region (reviewed in Anderson 1966) such as a posterior growth zone or two lateral growth zones (Seaver et al. 2005, Kristof 2011).

Veliger and pilidium

The molluscan veliger (Fig. 1f) and nemertean pilidium (Fig. 1e) possess multiciliated epidermis, as is characteristic for their phyla, in general (Nielsen 1987). For that reason, growth in these larvae may be expected to be restricted to certain zones or proliferative regions. However, growth patterns may differ between the pilidium and the veliger due to the difference in body plan and development.

Study design

To reveal patterns of cell proliferation for all larval types, I examined the distribution of mitotic cells using anti-phosphohistone antibody in preserved larvae. This antibody labels the DNA of cells that are in the process of mitosis at the time of fixation. Phosphorylation of serine 10 of histone H3 occurs prior to the initiation of mitosis. The histone protein is a highly conserved protein. An antibody generated against a phosphorylated histone is a certain way to visualize mitotic cells in a wide range of organisms (Hendzel et al. 1997).

While the anti-phosphohistone antibody labels cells which are in the process of division at the time of fixation, the bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) assay was used *in vivo* to label cells undergoing cell division during a particular time interval. This assay allows detection of cell division even when such events are rare.

BrdU is a synthetic thymidine analog incorporated into newly synthesized DNA of replicating cells, specifically at the S phase of the cell cycle (Newmark and Alvarado 2000). I used two types of BrdU assays: the so-called "pulse" and "pulse-chase." In a "pulse" the specimens are incubated with BrdU for a period of time and fixed immediately afterward. If the desire is to visualize the progeny of cells dividing during a certain period of time, the live specimens are transferred back to sea water after incubation with BrdU (pulse) and cultured for a desired period of time (chase), then fixed and assayed using an anti-BrdU antibody.

CHAPTER II

METHODS

Procuring gametes and culturing larvae

All of the species used in this study are intertidal invertebrates common in vicinity of the Oregon Institute of Marine Biology in Charleston, OR, except for the asteroid *Patiria miniata* (see below).

Pisaster ochraceus and *Patiria miniata* (Asteroidea, Echinodermata)

Adults of the starfish *Pisaster ochraceus* were collected in the intertidal from South Cove, Cape Arago, near Charleston, OR and kept in sea tables at the OIMB and fed *Mytilus californianus* at least monthly. *Pisaster ochraceus* is reproductive during Spring and the early Summer months in the field, but may be held in ripe condition as late as October in flowing sea tables in the laboratory. Gametes were obtained by cutting off one arm and removing the gonads. Dissected ovaries and testes can be kept in the refrigerator for up to three days, and used as needed. To obtain oocytes for *in vitro* fertilization dissected ovaries were incubated in filtered sea water (0.45 μm) with 1 μM 1-methyladenine for 30-45 min (Schuetz 1969, Shroeder 1981, Nemoto 1982) until they matured (underwent ovulation and germinal vesicle break down). Testes obtained by dissection as described above. The sperm were transferred to filtered sea water for dilution. Mature oocytes were fertilized in 1 μM 1-methyladenine by a dilute suspension of sperm.

The starfish *Patiria miniata* occur in Oregon, but are not common in the intertidal.

Adults used in this study were collected in Southern California **(**supplied by Marinus Scientific of Long Beach, CA**)**. Adults were kept in quarantine (in recirculating sea water aquaria) at the OIMB and unwanted embryonic and larval cultures disposed of in a way that would prevent them from reaching the local waters alive. *Patiria miniata* is ripe almost all year round. Gametes were obtained as described above for *P. ochraceus.*

Dendraster excentricus (Echinoidea, Echinodermata)

The sand dollar *Dendraster excentricus* adults were collected by dredge within a mile of shore just north of the entrance to Coos Bay. The trawl started at 43°22' 80''N, 124° 20' 91''W in 45 feet of water and headed NE to end in about 20 feet of water. Adults were kept at OIMB submerged in sand in the sea tables with flowing sea water, and used as needed. *Dendraster excentricus* was found to be reproductive April through September. To obtain gametes for *in vitro* fertilization, adults were injected with 1ml 0.5M KCl into the oral membrane, and eggs were collected in a beaker filled with filtered sea water. Concentrated sperm was collected into 1.5 ml microcentrifuge tubes and stored in refrigerator for up to two days.

Phoronopsis harmeri (Phoronida)

The adults of phoronid *Phoronopsis harmeri* were collected as described above for *Micrura alaskensis*. Reproductive adults of *P. harmeri* were encountered May through August. The worms were gently removed from their tubes, and zygotes obtained by dissecting the ampulla region. In this species, fertilization is internal, and dissected

zygotes initiate development upon contact with sea water (Zimmer 1987).

Owenia collaris and *Serpula columbiana* (Polychaeta, Annelida)

The adults of *Owenia collaris* were collected as described above for *Micrura alaskensis*. *Owenia collaris* is locally reproductive March through August (Smart 2008). Gametes for *in vitro* fertilization were obtained by removing adults from their tubes and dissecting them. Dissected primary oocytes were promptly pipetted to a bowl with filtered sea water, allowed to round up and undergo germinal vesicle breakdown (about 30 min upon contact with sea water), and fertilized by adding a few drops of dilute sperm suspension.

The adults of *Serpula columbiana* were collected from the docks in the Charleston Harbor, Charleston, Oregon. Reproductive adults of *S. columbiana* were collected from April to June in 2012. Adults, when ripe, readily shed gametes as they are removed from their tubes. Oocytes were transferred to a bowl with filtered sea water, allowed to round up and undergo germinal vesicle breakdown (about 45-60 min after contact with seawater) and then fertilized by adding a few drops of dilute sperm suspension.

Caesia fossatus (Gastropoda, Mollusca)

The adults of the gastropod *Caesia fossatus* occasionally came up in the dredge while we were collecting sand dollars. These scavenger snails were kept at the OIMB in flowing sea tables and occasionally fed smoked tuna. They deposited their egg masses close to the surface of the water on PVC pipes, the walls of the sea table, Tygon tubing

and other substrata. Egg cases were found March through August. Egg capsules for experiments were transferred to 150 ml glass bowls filled with sea water no higher than one centimeter above the egg cases, and the water was aerated with an air stone before filling the glass bowls. The water was changed three times each week. Planktotrophic veligers hatched from the egg masses within one month after deposition. In some cases, developing veligers were released from 1-3 capsules manually after the veligers developed pigmentation on the velum and were freely moving around in the egg case (about 2 weeks after deposition).

Micrura alaskensis (Pilidiophora, Nemertea)

Adults of nemertean *Micrura alaskensis* were collected at various Coos Bay mudflats near the Oregon Institute of Marine Biology (OIMB) on negative low tides. Specimens were collected by digging up the upper 20-30 cm of sand with a shovel. Reproductive adults were found July through September. Gametes for *in vitro* fertilization were obtained by dissecting adults. Dissected primary oocytes were promptly pipetted to filtered sea water, allowed to round up and undergo germinal vesicle breakdown (about 30-40 min after contact with sea water), and fertilized by adding a few drops of dilute sperm suspension (Maslakova 2010).

Larval culturing

Larvae were cultured at a concentration of 0.2-1.0 larva ml^{-1} in 0.45 μm filtered seawater, at ambient sea temperature. Cultures were stirred continuously with plexiglass

paddles, and the water was changed twice a week. Larvae were fed microscopic algae *Rhodomonas lens* at about 10^4 cells ml^{-1}.

Antibody labeling

Larvae were briefly relaxed, about 5 min, in 0.37 M $MgCl_2$ (except *Serpula columbiana* and *Caesia fossatus* which were relaxed up to 30 min on ice with at least 3 changes in $MgCl_2$). All larvae were fixed in 4% paraformaldehyde in filtered sea water for 30-60 min. The larvae were then washed (one short rinse followed by three 10 min washes) in phosphate buffered saline (PBS) and stored at 4°C, or immediately permeabilized with PBS with 0.1 % Triton X-100 (PBT). Larvae of *S. columbiana* were additionally treated using 0.01 mg ml^{-1} proteinase K for 2 min then rinsed with 2mg ml^{-1} glycine for 20 min, to help permeabilize the cuticle. Nonspecific labeling was blocked by incubating samples in 5-10% normal goat serum in PBT with 0.1% bovine serum albumin (PBT/BSA) for 2 h at room temperature. Larvae were then incubated with anti-phosphohistone H3 (Ser10) antibody (rabbit monoclonal, Cell Signaling Cat# 9706 or rabbit polyclonal, Upstate (Millipore) 06-570) diluted 1:500 in PBT/BSA at room temperature for at least 4 hours or overnight at 4°C. Larvae were washed three times (two five min washes followed by three 10 min washes) in PBT/BSA and incubated with the secondary antibody (Alexa Fluor 488 or Alexa Fluor 555 goat anti-rabbit) diluted 1:1000 in PBT/BSA for 2 h at room temperature or overnight at 4°C. To visualize the cell nuclei and the overall morphology, the larvae were additionally labeled with 500 µM Hoechst 33342 and/or BODIPY-FL phallacidin at 1U per 200 µl, (Molecular Probes, Inc.) or

Rhodamine phalloidin at 1U per 200 µl (Sigma) in PBT at room temperature for 30-60 min. After three 10 min washes in PBS, larvae were mounted in Vectashield (Vector Laboratories) or 75% glycerol in PBS on cover slips coated with poly-L-lysine, and preps were sealed with nail polish. Prepared slides were viewed using the following lenses: 20X NA=.75; 20X oil NA=.85; 40X oil NA=1.30; or 60X oil, NA=1.40 on an Olympus IX81 inverted microscope associated with Olympus FluoView 1000 laser scanning confocal system equipped with 405 nm, 488 nm, or 543 nm lines. Confocal stacks were imported into Image J version 1.45s (National Institutes of Health, Washington, D.C.) and Adobe Photoshop© for image and video processing.

BrdU assay

Larvae were incubated in 0.1mg/ml BrdU (Sigma, B5002**)** in filtered sea water. After BrdU incorporation, larvae were relaxed in 0.37 M $MgCl_2$ as described above for antibody labeling and fixed in 4% paraformaldehyde in filtered sea water for 30-45 min (for a BrdU pulse) or raised in 3L glass jars as described above for varied periods of time and then fixed (for a BrdU pulse-chase). Fixed larvae were washed (two short rinses and three 10 min washes) in PBS. *Serpula columbiana* larvae were additionally treated with proteinase K as described above. Larvae were incubated in ~2.0N HCl for about 15-25 min to denature DNA, and neutralized using 0.1M $Na_2B_4O_7$ (several rinses over 20 min). Following permeabilization in PBT (three washes), nonspecific binding sites were blocked with 5-10% normal goat serum in PBT with 0.1% albumin bovine (PBT/BSA) for 2 h at room temperature. Larvae were incubated with an anti-BrdU mouse

monoclonal antibody (Becton Dickinson, Franklin Lakes, NJ) (1:100) with 5% goat serum in PBT/BSA overnight at 4°C. Larvae were washed four times (one rinse and three 5 min washes) in PBT/BSA and exposed to the secondary antibody, Alexa Fluor 488 goat- anti-mouse (1:500) in PBT for 2 h at room temperature. Larvae were additionally labeled with Hoechst 33342 (500 μM) in PBT at room temperature for 30-40 min. After the larvae were washed three times with PBS, they were mounted on cover glasses coated with poly-L-lysine in Vectashield (Vector Laboratories) or 75% glycerol in PBS and preps were sealed with nail polish. Prepared slides were viewed with an Olympus FluoView 1000 confocal system (as described above).

Microinjection of fluorescent markers in *Micrura alaskensis*

Injection of fluorescent markers (such as FITC dextran and Ensconsin-3XGFP mRNA) into live zygotes of *Micrura alaskensis*, and subsequent culturing of larvae also allows one to distinguish among body regions with varying rates of cell proliferation. These markers become progressively more diluted in cells that undergo multiple cell divisions than in cells that undergo fewer cell divisions.

Recently fertilized eggs of *Micrura alaskensis* were injected with one of the following fluorescent markers: Fluoro-Emerald (FE; Invitrogen, cat D1820) or polyadenylated mRNA encoding 3X-GFP-Ensconsin microtubule binding domain (3X-GFP-Ens). Labeled zygotes were raised for up to six weeks and imaged live with a confocal microscope. The fluorescent molecules are progressively diluted in the dividing cells. This effect is more pronounced in FE-labeled larvae compared to those labeled

with 3X-GFP-Ens (because it cannot be replenished). Nevertheless, injection of either fluorescent marker results in brighter and dimmer patches within the labeled larva. The brighter patches correspond to cells that have undergone fewer cell divisions, and, respectively, have a higher concentration of fluorescent marker. The darker areas correspond to regions with cells that have undergone many cell divisions, diluting the marker. The RNA constructs were prepared using PCS2+ vectors. These vectors allow for RNA sythesis. PCS2+ 3XGFP-Ens were made in the laboratory of Dr. William Bement (U Wisconsin, Madison) as described by von Dassow et al. (2009). The 3X-GFP-Ens mRNA was transcribed *in vitro* using mMessage mMachine SP6 kit (Ambion) and adenylated using Poly(A) Tailing kit (Ambion) by Dr. George von Dassow as previously described (von Dassow et al. 2009). The 3X-GFP-Ens mRNA was diluted to a final concentration of 0.14 $\mu g\ \mu l^{-1}$ i in water. FE were injected at 5 $\mu g\ \mu l^{-1}$ in aspartate injection buffer (100mM potassium aspartate, 50mM potassium chloride, 10 MM HEPES at pH 7.4)

Eggs of *Micrura alaskensis* lack an egg chorion, but are surrounded by a layer of egg jelly, which must be removed prior to injection. Eggs were de-jellied by moving the eggs in and out of the pipette repeatedly until the eggs moved through the pipette easily. The de-jellied zygotes of *M. alaskensis* were injected with 3X-GFP-Ens mRNA or FE roughly equal to 1-2% of the egg volume using glass needles made from 1mm OD filament-containing aluminosilicate glass capillaries (Sutter Instrument) on a micropipette puller (P-97; Sutter Instrument Co.). Injections were carried out in 4 ml cover-glass-bottom plastic Petri-dishes using an inverted microscope **(**Leica DMIL) and a

micromanipulator (Narishige). After injection, zygotes were kept in injection dishes and returned to the sea table for culturing. Development was monitored in injected eggs and eggs not injected (as control).

Pilidia, at various stages of development (ranging from four-day old to six-week old), were mounted on slides in filtered sea water with 0.1% sodium azide (which slows down ciliary beat and allows one to image live larvae), and preps sealed with petroleum jelly. Images of live pilidia were taken using a confocal microscope and processed as described above.

CHAPTER III

RESULTS

Pisaster ochraceus and *Patiria miniata* (Asteroidea, Echinodermata)

Both asteroid species produce a typical planktotrophic bipinnaria larva (Fig. 1a). *Pisaster ochraceus* eggs are 150-160 μm in diameter, and larvae are approximately 390 μm in length five days after fertilization when raised at approximately 12°C. Larvae of *P. ochraceus* take 76-228 days to reach metamorphosis when reared at approximately 12°C and grow considerably during this time. *Patiria miniata* eggs are 172-197 μm in diameter (Strathmann 1987). Larvae may metamorphose 45-66 days after fertilization and similarly to *P. ochraceus* grow considerably during this time (Cameron and Holland 1983). Cell proliferation pattern was assessed using anti-phosphohistone antibody labeling in two-day old (n~15) and three-week-old (n~15) bipinnariae of *Pisaster ochraceus* and in five-day-old (n~15), seven-day-old (n~10, not shown), and two-week-old (n~10, not shown) larvae of *Patiria miniata* (Figs. 2, 3). In larvae of both species, regardless of their age, anti-phosphohistone antibody labeling revealed that proliferating cells are dispersed throughout the larval body, including epidermis, larval ciliary band, coelomic lining, and throughout the lining of the gut. I found no evidence of proliferative centers or distinct regions with a higher proportion of cells undergoing cell division. This overall pattern was confirmed using a nine-hour BrdU pulse (Fig. 4a,b) in seven-day-old larvae of *P. ochraceus* and a twenty-four-hour BrdU pulse in three-week-old larvae of *P. miniata* (Fig. 4c,d).

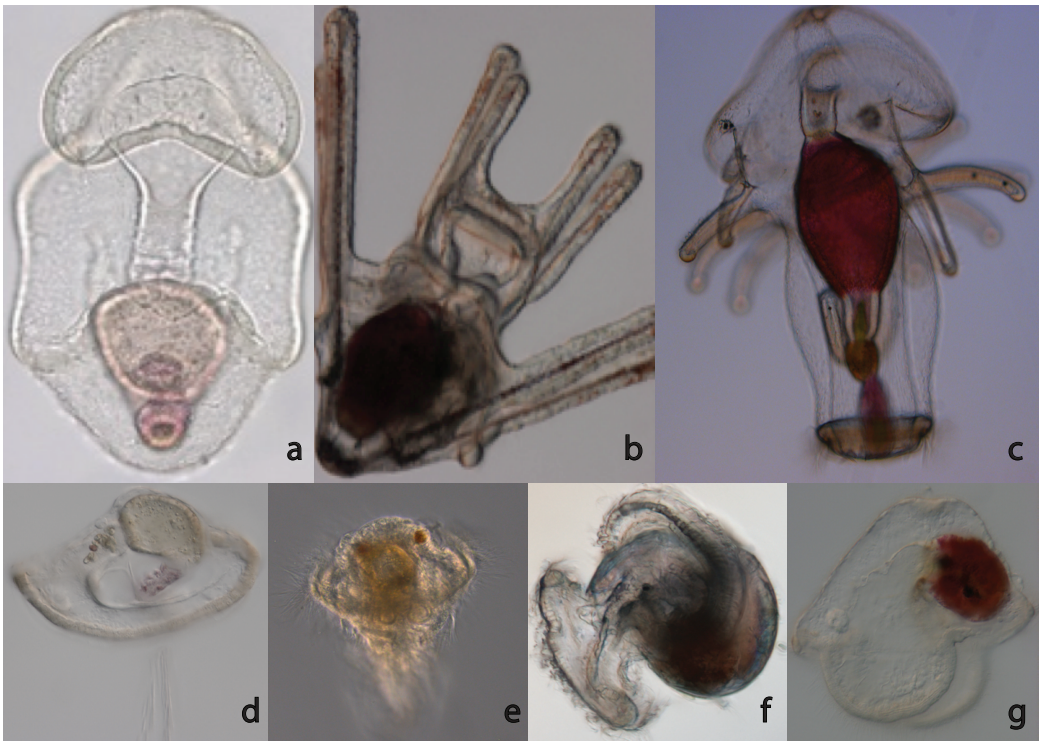

Figure 1. Light microscopy images of marine invertebrate larvae. (a) bipinnaria larva of *Pisaster ochraceus,* ~400 µm long from the anterior to the posterior (b) pluteus of *Dendraster excentricus,* ~500 µm long from the anterior to the posterior (c) actinotroch of *Phoronopsis harmeri,* ~800 µm from the anterior to the posterior (d) mitraria of *Owenia collaris,* ~300 µm dorso-ventral diameter (e) metatrochophore of *Serpula columbiana,* ~300 µm from anterior to posterior (f) veliger of *Caesia fossatus,* ~300 µm velum span (g) pilidium of *Micrura alaskensis,* ~450 µm from apical organ to apex of lappet.

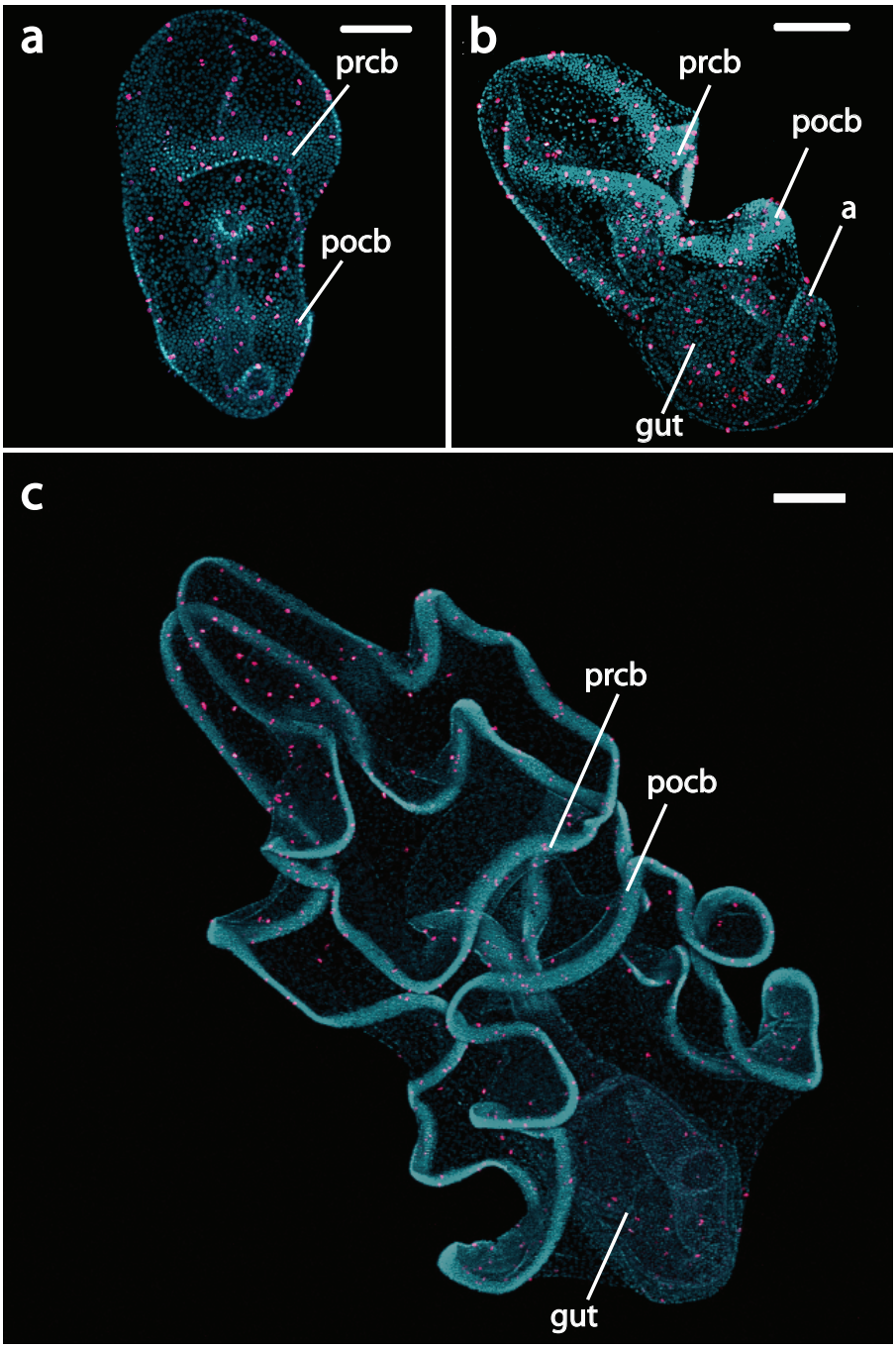

Figure 2. Confocal projections of starfish bipinnaria larvae labeled with anti-phosphohistone antibody. Hoechst (cyan), anti-phosphohistone antibody (pink). Mitotic cells are dispersed throughout the larval body, including the ciliary band. (a) Dorsal view of a two-day-old *Pisaster ochraceus* larva, anterior up. (b) The right lateral view of a five-day-old *Patiria miniata* larva, anterior upper left. (c) A ventro-lateral view of a three-week-old *P. ochraceus* larva. Scale bars = 75 µm. pre-oral ciliary band (prcb), post-oral ciliary band (pocb), anus (a).

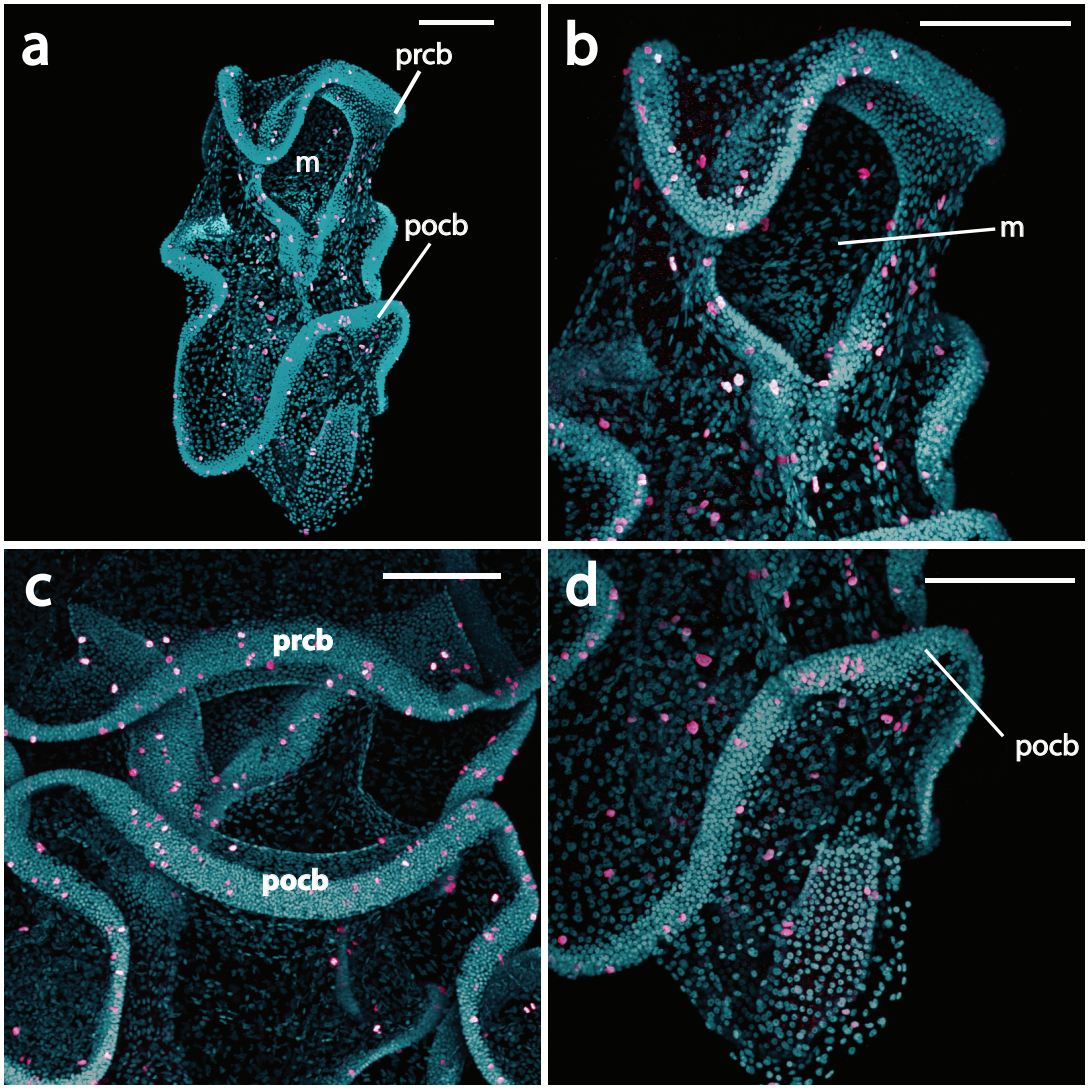

Figure 3. Confocal projections of anti-phosphohistone antibody labeling in *Pisaster ochraceus* larvae, detailed view. Hoechst (cyan), anti-phosphohistone (pink). Mitotic cells are dispersed throughout the larval body, including within the ciliary band. (a) The ventral view of a two-week-old bipinnaria larva, anterior up. (b and d) close up views of larva in (a). (b) The oral view of (a). (c) The oral view of a three-week-old bipinnaria, anterior up. (d) The aboral view of (a). Scale bars = 75 µm. pre-oral ciliary band (prcb), post-oral ciliary band (pocb), anus (a), mouth (m).

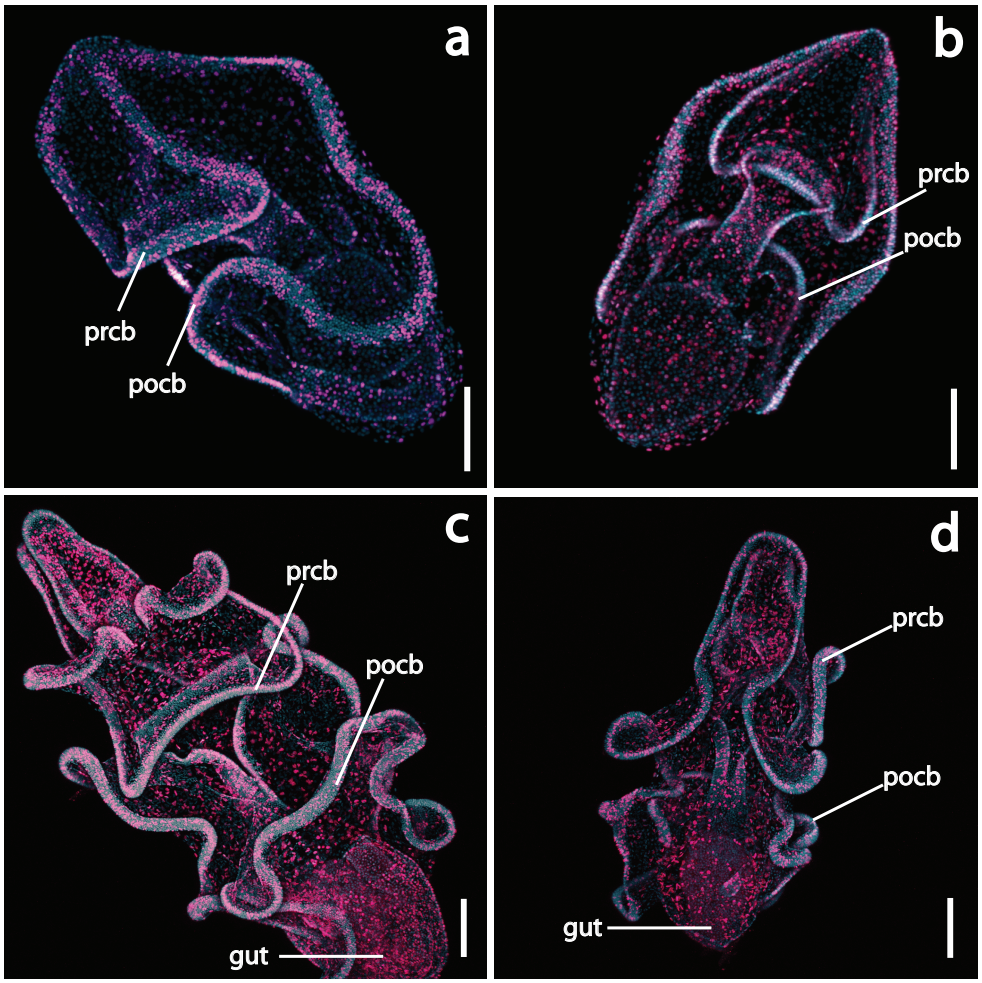

Figure 4. Confocal projections of nine-hour (a,b) and 24-hour (c,d) BrdU pulse in starfish larvae. BrdU (pink), Hoechst (cyan). Mitotic cells are located throughout the larval body with no evidence of a proliferative center. (a) The left lateral view of a seven-day-old larva of *Pisaster ochraceus,* anterior upper left (b) A dorsal view of a seven-day-old larva of *P. ochraceus*, anterior upper right. (c) A ventral view of a four-week-old bipinnaria larva of *Patiria miniata,* anterior up. (d) A dorsal view of a four-week-old larva of *Patiria miniata,* anterior up. Scale bars = 75 µm. pre-oral ciliary band (prcb), post-oral ciliary band (pocb).

***Dendraster excentricus* (Echinoidea, Echinodermata)**

The sand dollar *Dendraster excentricus* has a typical planktotrophic pluteus larva (Fig. 1b). *Dendraster excentricus* eggs are approximately 130 µm in diameter, and the

larvae are approximately 390 µm from anterior to posterior five days after fertilization when raised at approximately 12°C. Larvae of *D. excentricus* are about 750 µm from anterior to posterior when they reach metamorphosis (Strathmann 1987). Anti-phosphohistone antibody labeling revealed many mitotic cells which were more or less evenly dispersed throughout the larval body in both two-armed (n~10), six-armed (n~15, not shown), and eight-armed (n~15) plutei (Fig. 5). BrdU labeling was not attempted on the pluteus larvae, because plenty of mitotic cells are detected with the anti-phosphohistone labeling, and also because the HCl treatment would result in the dissolution of larval skeleton, and poor overall morphology.

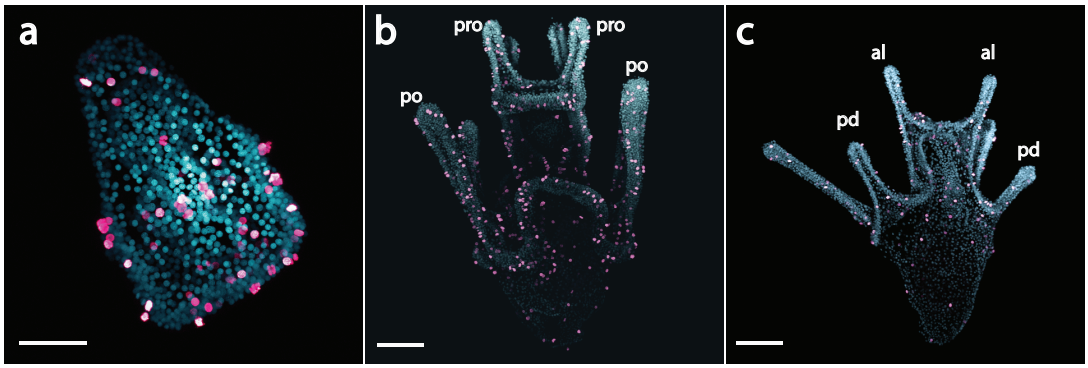

Figure 5. Confocal projections of anti-phosphohistone antibody labeling in plutei of *Dendraster excentricus.* Hoechst (cyan), anti-phosphohistone (pink). Mitotic cells are located throughout the larval body including the ciliated band with no evidence of discrete proliferative regions.(a) The dorsal view of a two-armed pluteus; anterior down and right. Scale bar = 40 µm (b) A ventral view of an eight-armed pluteus; anterior up. Scale bar = 75 µm (c) A dorsal view of a eight-armed pluteus, anterior up. Scale bar = 75 µm post-oral arms (po) pre-oral arm (pro), antero-lateral arm (al), postero-dorsal (pd).

***Phoronopsis harmeri* (Phoronida)**

Fertilized oocytes of *Phoronopsis harmeri* are 90-100 µm in diameter. Once

released into sea water, zygotes of *P. harmeri* develop into typical planktotrophic actinotroch larvae (Fig. 1c). Competent *Phoronopsis harmeri* larvae are 1200-1500 μm from the top of preoral hood to the telotroch and possess 12 pairs of tentacles formed progressively from the mid-dorsal region of the larval body (Temereva and Malakhov 2007). Cell proliferation patterns were assessed with anti-phosphohistone antibody in seven-day-old (n~15, not shown), 4.5-week-old (n~15) and 5.5-week-old (n~15) larvae (Fig. 8; movie 1). Mitotic cells were evident throughout the larval body in all studied larvae. Dividing cells were located in the larval epidermis, gut, metasomal sack, and the coelomic lining. A somewhat higher proportion of mitotic cells was found in the pre-oral hood, at the base of the tentacles, and throughout the telotroch (Fig. 6); however, no attempt was made to quantify this difference. A five-hour BrdU pulse (Fig. 7) in 15-day-old larvae revealed a conspicuous pattern to the distribution of BrdU-positive cells. A noticeably larger proportion of BrdU-positive cells was found at the base of the larval tentacles (e.g. as compared to the tips of the tentacles). A particularly dense aggregation of labeled cells was found in the newly formed tentacles and tentacle buds - near the dorsal midline (Fig. 7b).

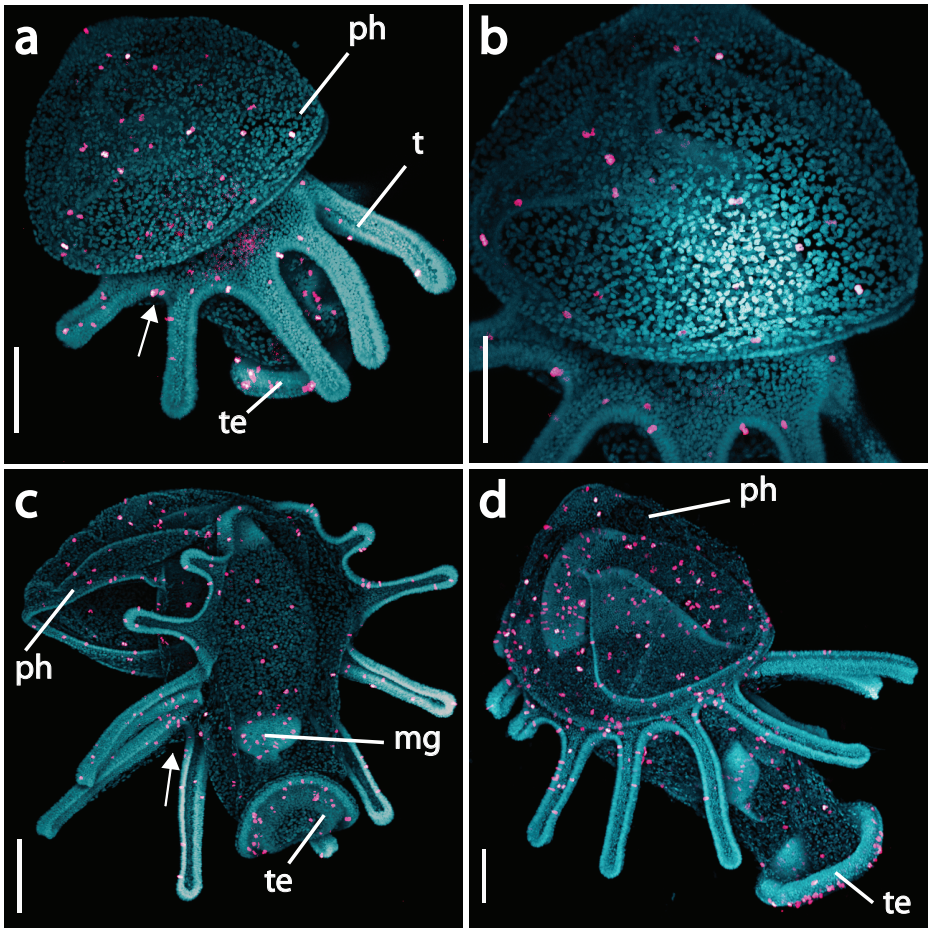

Figure 6. Confocal projections of anti-phosphohistone antibody labeling in actinotroch larvae of *Phoronopsis harmeri*. Hoechst (cyan), anti-phosphohistone (pink). Mitotic cells are clearly seen throughout the larval body, with a higher proportion of cell divisions at the base of tentacles, and the telotroch. (a) The antero-ventral view of an eleven-day-old actinotroch larva, anterior upper left. (b) A close up view of the larva in (a). (c) Left latero-dorsal view of a 4.5-week-old actinotroch larva, anterior up. (d) The right antero-lateral view of a 5.5-week-old actinotroch larva, anterior upper left. Scale bars = 75 μm. pre-oral hood (ph), tentacle (t), midgut (mg), telotroch (te).

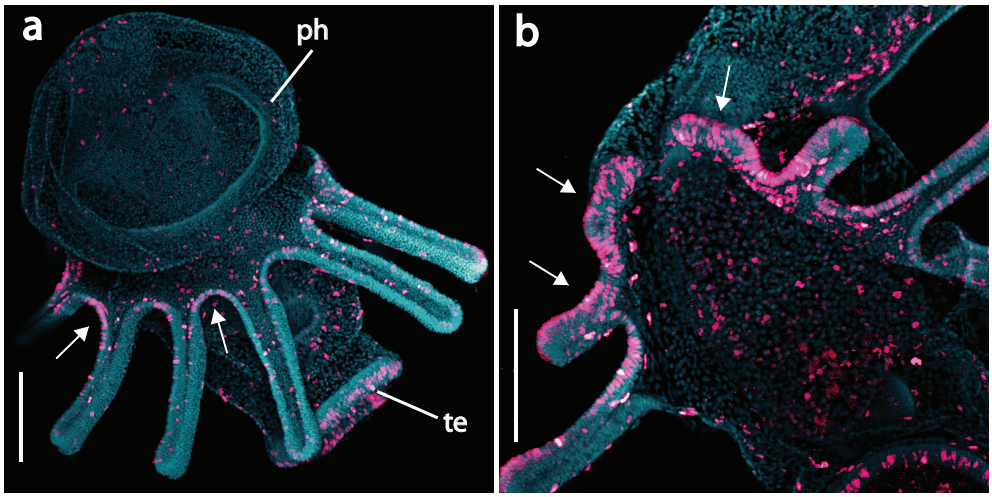

Figure 7. Confocal projections of five hour BrdU pulse in 15-day-old actinotroch larvae of *Phoronopsis harmeri*. Hoechst (cyan), BrdU (pink). BrdU-positive cells are located throughout the larval body with a somewhat higher proportion of labeled cells at the base of tentacles (arrows), telotroch, and in the hood. (a) A ventral view of the actinotroch larva, anterior upper left. (b) A right dorso-lateral view of an actinotroch larva, anterior up. Scale bars = 75 µm. pre-oral hood (ph), telotroch (te).

***Owenia collaris* (Polychaeta, Annelida)**

Owenia collaris possesses a unique kind of planktotrophic larva called the mitraria (Fig. 1d). *Owenia collaris* eggs are approximately 70-80 µm in diameter. Larvae of *O. collaris* metamorphose when the dorso-ventral diameter reaches approximately 470 µm (Smart and von Dassow 2009). Cell division pattern was first assessed in three-day-old (n~10, not shown), nine-day-old (n~10), 12-day old (n~15) and 4.5-week-old mitrariae (n~15, not shown) using anti-phosphohistone antibody (Fig. 8a,b; movie 2). Dividing cells were found dispersed throughout the larval body, but not uniformly. The number and distribution of mitotic cells varied among larvae of different ages. In the nine-day-old mitrariae, a higher proportion of dividing cells was located in the juvenile rudiment invagination and in the larval esophagus. Few dividing cells were seen in the midgut, in

the larval epidermis near the apical organ, and the primary ciliated band. An occasional mitotic cell could be seen in the larval epidermis outside the ciliated band and in the secondary ciliated band. Twelve-day-old mitrariae possessed a higher proportion of dividing cells in the midgut, hindgut, and primary ciliated band, compared to the larval esophagus and the juvenile rudiment invagination. Similar to the nine-day-old larvae, few dividing cells were located in the larval epidermis associated with the apical organ. Certain regions of the larval ciliated band seemed to possess a higher proportion of dividing cells (Figs 8c, d; movie 2**)**, but no attempt was made to quantify the difference**.** There are very few, if any, dividing cells observed in the larval epidermis outside the ciliated band or the region of the apical organ.

A five-hour BrdU pulse (Fig. 9) was carried out on 12-day-old larvae (n~15). BrdU-positive cells were detected in the esophagus, midgut, hindgut, juvenile rudiment invagination and primary ciliated band. Occasionally, there were BrdU-positive cells in the epidermis near the apical organ. Particularly dense aggregations of BrdU-positive cells were located in the midgut, hindgut (Fig. 9a), and primary ciliated band (Fig. 9b). BrdU labeling further highlighted the regional differences in the distribution of proliferating cells within the larval ciliated band (Fig 9b).

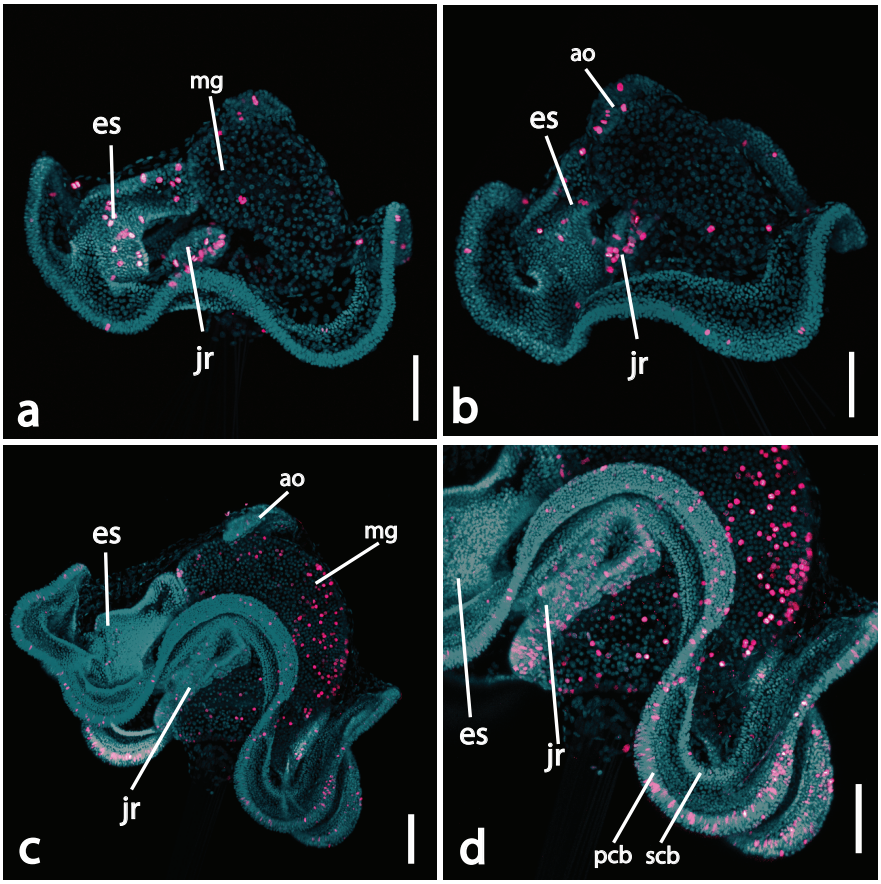

Figure 8. Confocal projections of anti-phosphohistone antibody labeling in nine-day-old (a,b) and 12-day-old (c,d) mitraria larvae of *Owenia collaris*. Hoechst (cyan), anti-phosphohistone (pink). Left lateral view, apical plate up. (a, b) Nine-day-old mitraria larva. A higher proportion of dividing cells is found in the juvenile rudiment invagination and the larval esophagus, a few dividing cells are seen in the midgut, the epidermis tissue associated with the apical organ, the primary ciliated band, and, occasionally, a cell in the larval epidermis outside the ciliated band. (c) Twelve-day-old mitraria larva. A higher proportion of dividing cells is found in the juvenile rudiment invagination, midgut, hindgut and primary ciliated band. Very few dividing cells are found in the larval epidermis associated with the apical organ. (d) A detailed view of the larva in (c). Scale bars = 50 µm. esophagus (es), midgut (mg), juvenile rudiment invagination (jr), apical organ (ao), primary ciliary band (pcb), secondary ciliary band (scb).

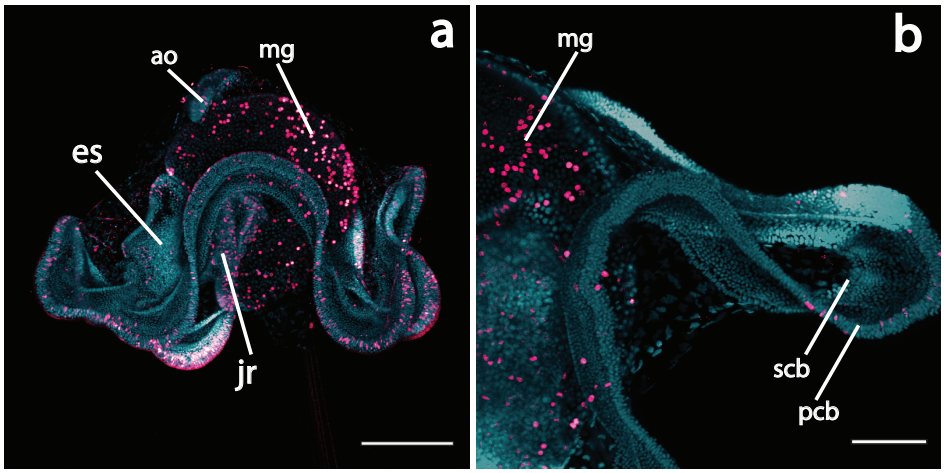

Figure 9. Confocal projections of five-hour BrdU pulse (pink) in 12-day-old mitraria larvae of *Owenia collaris.* Hoechst (cyan), BrdU (pink). (a) A left lateral view, apical up. A higher proportion of BrdU-positive cells is found in the juvenile rudiment invagination, midgut, hindgut, chaetal sac, and primary ciliated band. Very few dividing cells are seen in the larval epidermis near the apical organ. (b) A close up view of the posterior region of the larval ciliated band. Scale bars = 50 µm. esophagus (es), midgut (mg), juvenile rudiment invagination (jr), apical organ (ao), primary ciliary band (pcb), secondary ciliary band (scb).

***Serpula columbiana* (Polychaeta, Annelida)**

Serpula columbiana has a classical feeding trochophore larva (Fig. 1e) with a prototroch and metatroch. Eggs of *S. columbiana* are approximately 60 µm in diameter. According to Young and Chia (1982) larvae of this species are competent for metamorphosis at 41-50 days; however, I noticed juveniles in culture as early as 24 days after fertilization. At about three weeks post fertilization, larvae were about 300 µm from anterior to posterior. Anti-phosphohistone antibody labeling was attempted in seven-day-old and three-week-old larvae but was not successful, possibly due to the presence of the cuticle which may have prevented antibody from penetrating the tissues. Treatment of preserved larvae with proteinase K improved antibody penetration, but dramatically

decreased morphological preservation of specimens (n~30, data not shown) which made results difficult to interpret. A 20-hour BrdU pulse in 15-day-old (n~15) and three-week-old (n~15, not shown) larvae pre-treated with proteinase K revealed several discrete regions of dividing cells in the larval body. Larval apical organ was consistently labeled (Fig. 10a,b). BrdU-positive cells were also found in post-trochal epidermis and in the gut, although poor morphological preservation and non-specific staining made it difficult to assess the pattern. No BrdU-positive cells were found within the prototroch (Figure 10).

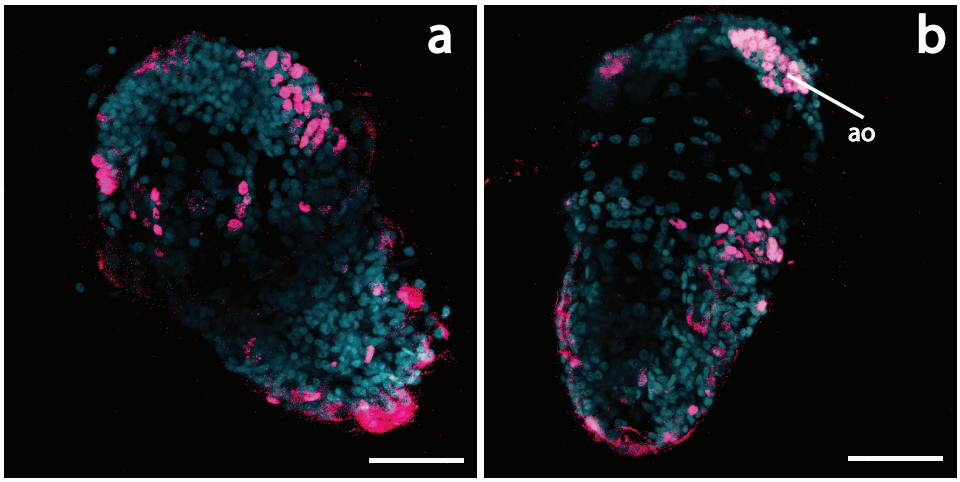

Figure 10. Confocal projections of twenty-hour BrdU pulse in 15-day-old metatrochophore larvae of *Serpula columbiana.* Hoechst (cyan), BrdU (pink). BrdU-positive cells are concentrated at discrete areas of the larva: in the apical organ, and certain locations in the post-trochal region. Occasionally, isolated dividing cells are found throughout the larval post-trochal epidermis and in the gut. No BrdU-positive cells were visible within the prototroch. (a) anterior upper left. (b) anterior upper right. Scale bars = 40 μm. apical organ (ao).

***Caesia (Nassarius) fossatus* (Gastropoda, Mollusca)**

Caesia fossatus has a classical feeding veliger larva (Fig. 1f) with prototrochal and metatrochal ciliary bands spanning the velum. Eggs of *C. fossatus* are approximately 150

µm in diameter. In larvae of *C.* fossatus, the velar lobes may reach 300 µm across at about one month of age. Anti-phosphohistone antibody labeling was carried out in three-week-old (n~20, not shown) and one-month-old (n~15) larvae of *C. fossatus*. Very few mitotic cells were detected using this method (1-5 per specimen). There was variation in location of dividing cells between specimens. Labeled cells were found in the post-trochal epidermis of the velum, the pre-trochal epidermis in association with the tentacles and apical ganglia, the visceral mass (Fig. 11a), the epidermal lining of the esophagus, and the food groove (Fig. 16b, movie 3). Cell proliferation patterns were also assessed using 12-hour and 24-hour BrdU pulse in one-month-old veligers (n~20). More dividing cells were revealed with BrdU labeling than with the anti-phosphohistone antibody. BrdU-positive cells were found in the visceral mass, as well as the pretrochal and post-trochal epidermis of the velum, the tentacles, and the apical ganglia (Fig. 12a,c). Labeled cells were also found in the foot (Fig. 12c). No dividing cells were labeled in the prototroch except in the small region adjacent to the mouth (Fig. 12b). All the labeled larvae except one larva possessed a discrete group of BrdU-positive cells in the pre-oral region of the prototrochal ciliary band.

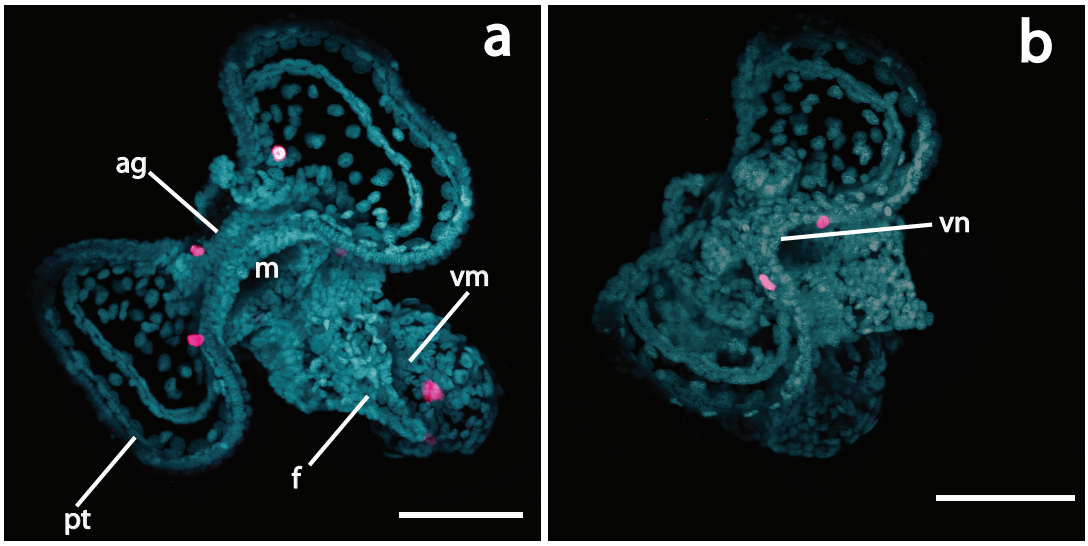

Figure 11. Confocal projections of anti-phosphohistone antibody labeling in veliger larvae of *Caesia fossatus*. Hoechst (cyan), anti-phosphohistone (pink). (a) Antero-ventral view of a one-month old veliger larva. Labeled cells are found in the food groove, visceral mass, post-trochal epidermis, the pre-trochal region (apical ganglia in particular) and the hyposphere in proximity to the esophagus (b) Antero-ventral view of a one-month-old veliger larva. Two labeled cells are visible in pre-trochal epidermis adjacent to the prototroch near ventral notch of the velum, and in the esophageal lining. Scale bars = 75 µm. apical ganglia region (ag), mouth (m), visceral mass (vm), foot (f), prototroch (pt), velar notch (vn).

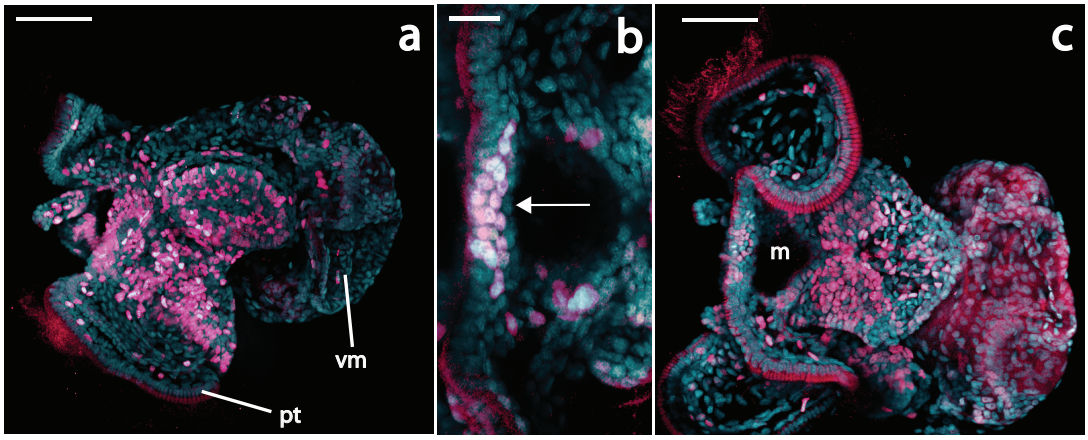

Figure 12. Confocal projections of BrdU assays in veligers of *Caesia fossatus.* Hoechst (cyan), anti-BrdU (pink). One month-old veliger larvae labeled with a 12-hour (a, c) or 24 hour (b) BrdU pulse. Scale bar = 75 µm **(a)** The left lateral view with BrdU labeled cells in the visceral mass, the foot, the tentacles and the post-trochal epidermis **(b)** The oral view of a one-month-old larva. Note a distinct region with BrdU-positive cells in the prototrochal ciliary band immediately adjacent to the mouth. Scale bar = 25 µm **(c)** ventral view of veliger with BrdU labeled cells in the visceral mass, the foot, the tentacles, the pre-trochal epidermis, the post-trochal epidermis and in the prototrochal ciliary band immediately adjacent to the mouth. Scale bar = 75 µm (pt), visceral mass (vm), mouth (m).

***Micrura alaskensis* (Pilidiophora, Nemertea)**

Micrura alaskensis has a classical pilidium larva (Fig. 1g). Eggs are approximately 76 µm in diameter. Larvae of this species typically take 6-7 weeks to reach metamorphosis when raised at 11-14°C (Maslakova 2010). Pilidia are about 450 µm from the apical organ to the tip of the lateral lappets when larvae approach competency to metamorphose. Zygotes were injected with Fluoro-Emerald (FE) or polyadenylated mRNA encoding 3X-GFP-Ensconsin microtubule binding domain (3X-GFP-Ens), raised for up to six weeks, and imaged live with a confocal microscope (Figs. 13, 14 and 15).

Four-day-old *M. alaskensis* labeled with 3X-GFP-Ensconsin (n~3) showed mitotic

figures in the epidermis at the junctions between the anterior and posterior lobes and the lateral lappets (Fig. 13); these four regions I subsequently refer to as the "axils" (Latin for "pit"). I have not observed mitotic figures anywhere else in the larval body using these labels.

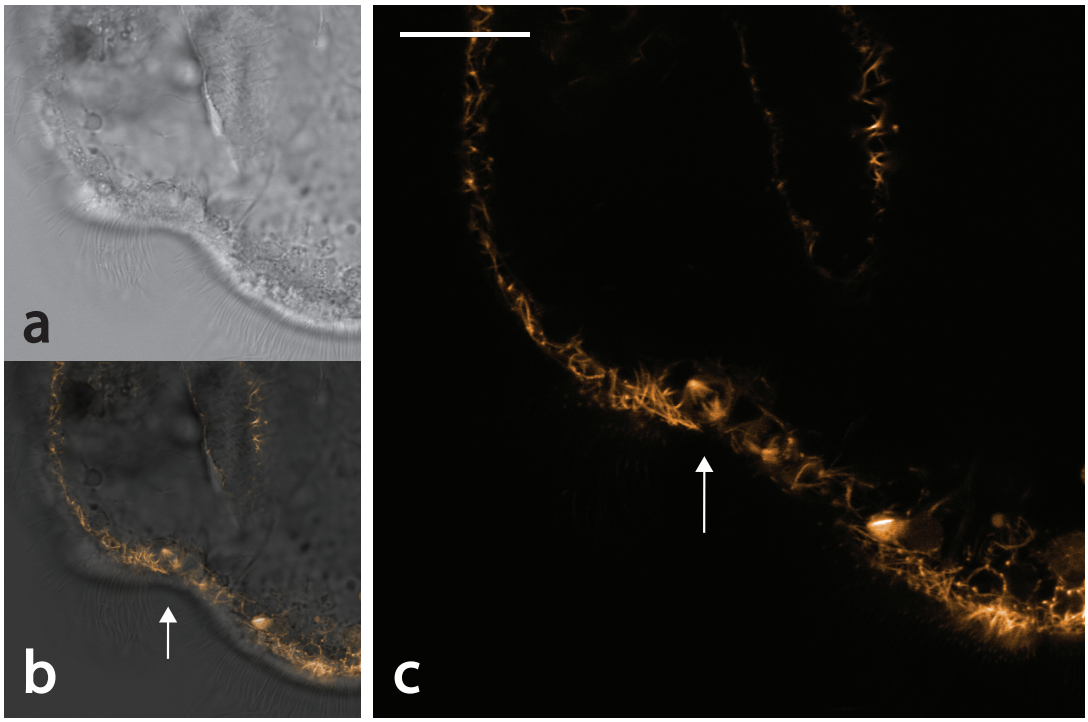

Figure 13. Confocal projections of the posterior right axil region in a four-day-old pilidium larva of *Micrura alaskensis* injected with 3X-GFP-Ens mRNA as a zygote. Transmitted light image (a), fluorescent image (c). The overlay of the two (b) shows a mitotic figure at the axil (arrow). Scale bars = 20 µm.

The eight-day-old pilidia injected with FE (n~3) show label in almost the entire larval body except for the axils where the dye seems to be diluted (Fig. 14b,c; arrowheads). The presence of darker patches is also apparent in larvae injected as zygotes with 3X-GFP-Ens (Fig. 15). The darker regions progressively occupy a larger and larger area of the larval body, e.g. compare 10-day-old pilidia (n~3, Fig. 15a), 20-day-old larvae

(n~3, Fig. 15b), and the 4-week old larvae injected with 3X-GFP-Ens (n~3, Fig. 15c). The majority of the larval body in six-week-old pilidia labeled with 3X-GFP-Ens is dark. Small patches of label remain in the larval epidermis at the distal-most portion of anterior and posterior lobes and lateral lappets, and in a region of epidermis surrounding the apical organ. Additionally, label is incorporated into the juvenile rudiment (n~3, Fig. 15d).

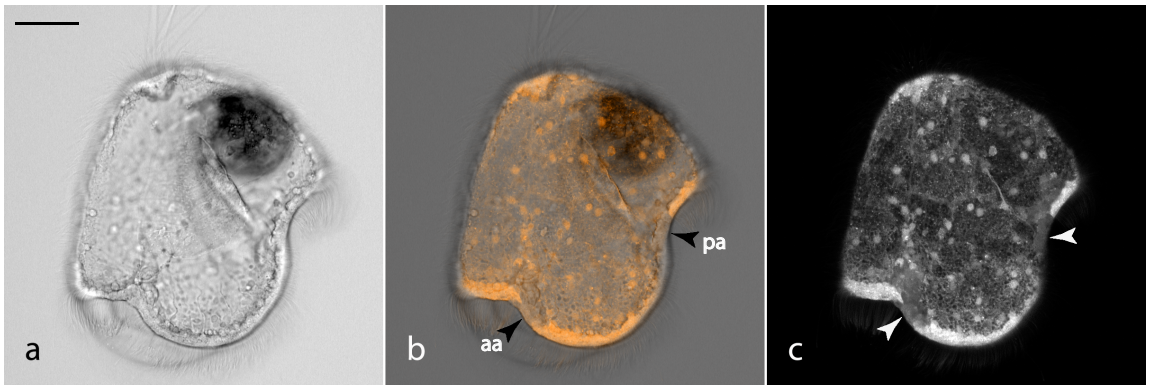

Figure 14. Confocal projections of eight-day-old pilidium larva of *Micrura alaskensis* injected with FE as a zygote. Oriented to show left lateral side, apical organ is up. Transmitted light image (a), fluorescent image (c). The overlay of the two (b) shows reduced fluorescence in the axils (arrowheads). Scale bars = 30 μm. anterior axil (aa), posterior axil (pa).

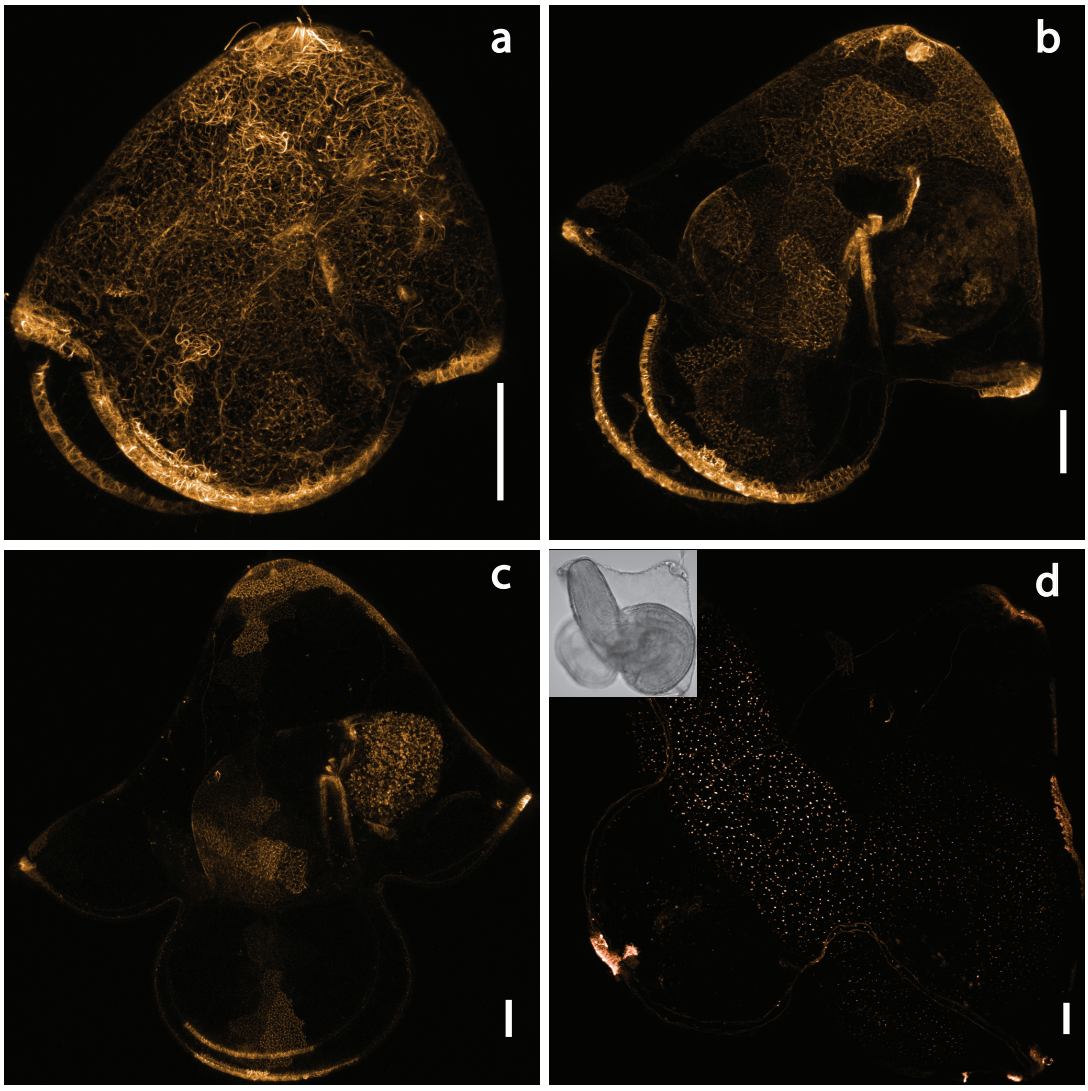

Figure 15. Confocal projections of variation in cell division rates in pilidium larvae of *Micrura alaskensis* injected with 3X-GFP-Ens mRNA as zygotes. Left lateral view, apical organ up. (a) Ten-day-old pilidium. The majority of the larval body is labeled, but the axils are darker. (b) 20-day-old pilidium. The axils and a portion of the larval body adjacent to the axils are dark. (c) Four-week-old pilidium. A larger region of the larval body is dark. (d) Six-week-old pilidium with a fully developed juvenile inside (the inset shows a light micrograph of the larva in the same orientation). The majority of the larval body is dark except at the apex of the anterior and posterior lobes and lateral lappets, and a region of epidermis surrounding the apical organ. Additionally, label is evident in the juvenile rudiment. Scale bars = 40 μm.

Location of proliferating cells was assessed using anti-phosphohistone antibody (Fig. 16) in four-day-old (n~40, not shown), six-day-old (n~25), 13-day-old (n~20, not shown), 14-day-old (n~30, not shown), and 4.5-week-old (n~20) pilidium larvae. Mitotic cells were found in the epidermis near the apical organ, the ciliated ridges, the four axils (Fig. 16a,b), and the imaginal discs (Fig. 16c). The exact number and position of cells varied among the pilidia examined, ranging from 1-7 in the larval body. Later in the development, the number of cell divisions that could be detected increased due to the division within the imaginal discs.

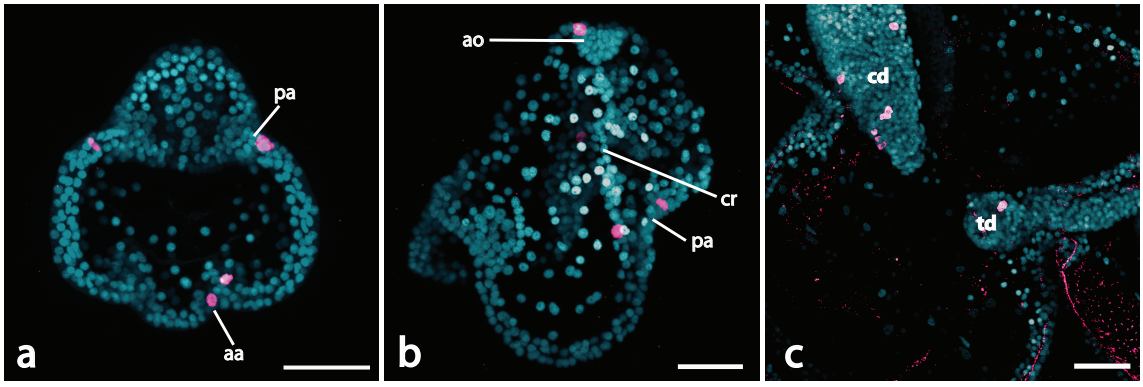

Figure 16. Confocal projections of anti-phosphohistone antibody labeling in pilidia of *Micrura alaskensis*. Hoechst (cyan), anti-phosphohistone (pink). Mitotic cells are limited to a few discrete proliferative centers, including paired anterior and posterior axils. (a) Oral view of a six-day-old pilidium; posterior larval lobe is up. Mitotic cells are found in both of the posterior and the anterior left axils (b) Left lateral view of a six-day-old pilidium, apical organ up. Mitotic cells are found in the larval epidermis associated with the apical organ, ciliated ridges and posterior left axil (c) Left lateral view of a 4.5-week-old pilidium. Mitotic cells are found in the imaginal discs. Scale bars = 30 µm. anterior axil (aa), posterior axil (pa), apical organ (ao), ciliated ridges (cr).

BrdU pulse assays were carried out in five-day-old (n~15), six-day-old (n~50), nine-day-old (n~14), 14-day-old (n~10, not shown), 17-day-old (n~10) and 21-day-old pilidia (n~10). A 24-hour BrdU pulse in five-day-old larvae (Fig. 17; movie 4) revealed

BrdU-positive cells (approximately 45 total) in the ciliated ridges, the gut, the axis, the epidermis associated with the apical organ, and a pair of subepidermal cells in the lateral lappets (Fig. 17). A six-hour pulse in a six-day-old pilidium (Fig. 18a) revealed BrdU-positive cells (approximately 35 total) in the ciliated ridges, the gut, the axils, the epidermis associated with the apical organ, and a pair of subepidermal cells in the lateral lappets. A six-day-old larva exposed to a six-hour BrdU pulse followed by a three-day chase (Fig. 18b) revealed BrdU-positive cells (~ 100 in the visible portion of the larva) in the ciliated ridges, the ciliated band, the axils (although less brightly), the gut, the epidermis associated with the apical organ, the cup of the apical organ and a subepidermal cell in one of the lappets. A six-day-old larva exposed to a six-hour BrdU pulse followed by an 11-day chase (Fig. 18c) revealed BrdU-positive cells (~ 150 in the visible portion of the larva) in the ciliated ridges, the ciliated band, the gut, the epidermis associated with the apical organ, the cup of the apical organ, a subepidermal cell in one of the lappets, and the developing imaginal discs. A six-day-old larva exposed to a six-hour BrdU pulse followed by a 14-day chase (not shown) revealed BrdU-positive cells similar to that of the six-hour BrdU pulse followed by an 11-day chase (above). A six-day-old larva exposed to a six-hour BrdU pulse followed by a 15-day chase (Fig. 18d) revealed BrdU-positive cells (~ 300 in the visible portion of the larva) in the ciliated ridges, the ciliated band, the gut, the epidermis associated with the apical organ, the cup of the apical organ, a subepidermal cell in the lappet, the axils, and the developing imaginal discs.

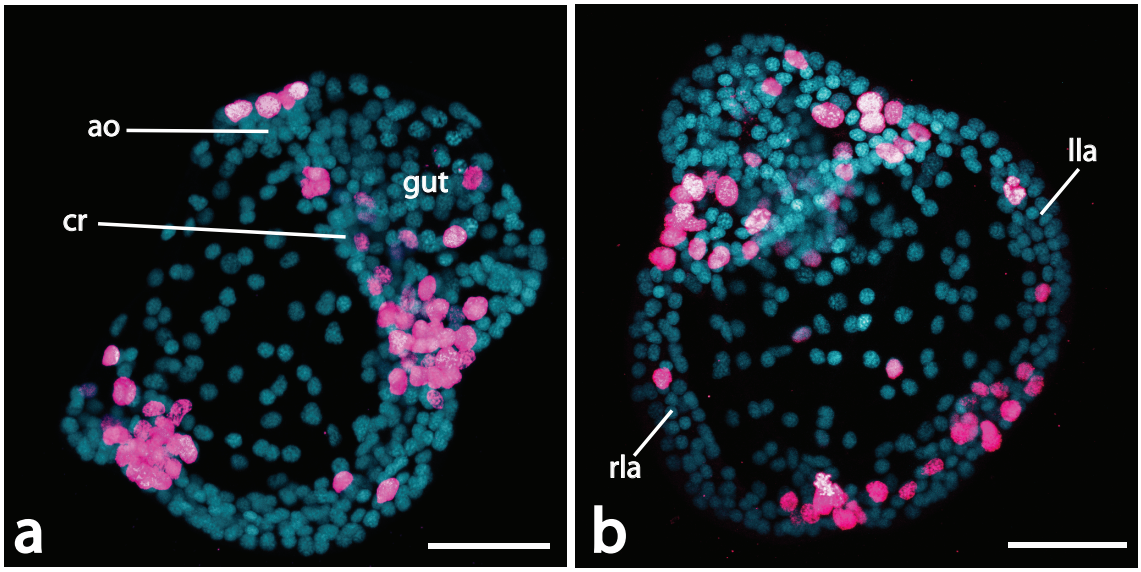

Figure 17. Confocal projections of five-day-old pilidium larvae of *Micrura alaskensis* labeled with a 24 hour pulse of BrdU. Hoechst (cyan), BrdU (pink). (a) The left lateral view of a five-day-old pilidium, apical organ is up. BrdU-positive cells are found in the axils, the larval epidermis associated with the apical organ, and the gut. (b) A view from the hyposphere. Posterior larval lobe is at upper left. BrdU-positive cells are located in the axils and a cell in each lappet located between the anterior and posterior axils. Scale bars = 30 µm. anterior axil (aa), posterior axil (pa), apical organ (ao), ciliated ridges (cr), right lappet (rla), left lappet (lla)

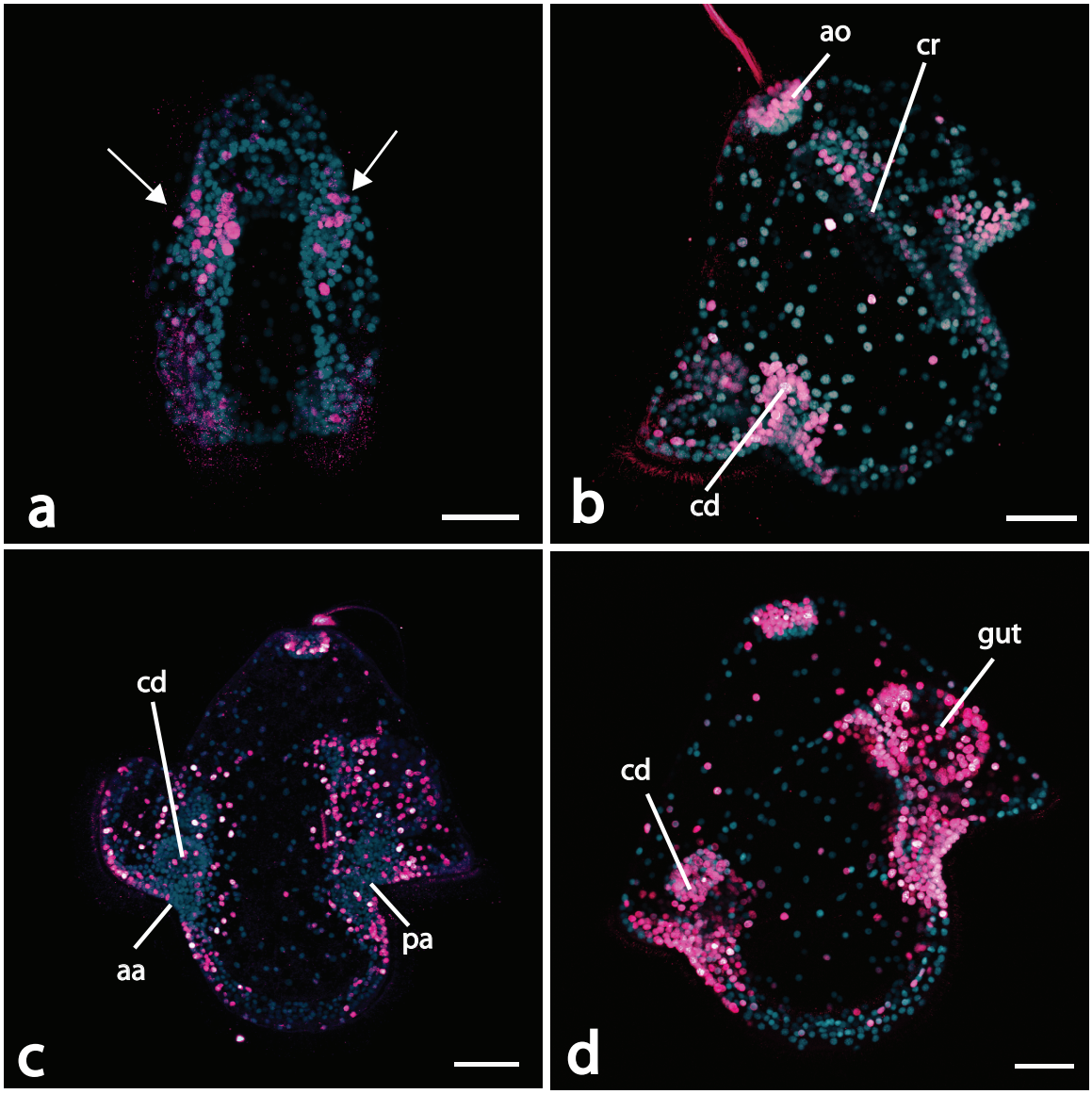

Figure 18. Confocal projections of BrdU pulse and pulse-chase assays with pilidia of *Micrura alaskensis.* Hoechst (cyan), anti-phosphohistone (pink). All pilidia are oriented to show left lateral view except (a) which is an oral view showing the posterior axils. (a) A six-day-old pilidium labeled with a six hour pulse of BrdU. BrdU-positive cells are found in the posterior axils (arrows), the larval epidermis associated with the apical organ, the ciliated band in vicinity of the axils, as well as larval epidermis in vicinity of the axils, and also and the gut (b) A nine-day-old pilidium labeled with a six-hour BrdU pulse followed by a three-day chase. Labeled cells are found in the apical organ, gut, ciliated ridges, ciliary band, around the axils, and the cephalic imaginal discs. (c) A 17-day-old pilidium labeled with six-hour BrdU pulse followed by an 11-day chase. Labeled cells are found in the apical organ, ciliated band, gut, the developing imaginal discs, and epidermis including the ciliated band. (d) A 21-day-old pilidium labeled with a six-hour BrdU pulse followed by a 15-day chase. Labeled cells are found in the apical organ,

ciliated band, gut, imaginal discs, epidermis including the axils and the ciliated band. Scale bars = 30 μm. apical organ (ao), ciliated ridge (cr), cephalic disc (cd), anterior axil (aa), posterior axil (pa).

CHAPTER IV

DISCUSSION

Overall, the results were consistent between the anti-phosphohistone antibody and the BrdU labeling. Mitotic cells were detected using the anti-phosphohistone antibody in all types of larvae, except the metatrochophores of *Serpula columbiana* (likely due to the issues with antibody penetration through the larval cuticle). As expected, BrdU revealed more dividing cells than the anti-phosphohistone antibody. BrdU was successful in all types of larvae, although the morphology of the veligers and the metatrochophores suffered, likely due to the harsh acid treatment required for denaturation of DNA in this procedure.

Bipinnaria and pluteus

The bipinnariae of *Pisaster ochraceus* and *Patiria miniata* display similar patterns of cell proliferation. Mitotic cells are dispersed throughout the larval body regardless of developmental stage (Fig. 2,3). BrdU assays (Fig. 4) confirm the pattern revealed by the anti-phosphohistone antibody. Although mitotic cells are found throughout the larval body, the distribution is somewhat uneven. The anti-phosphohistone antibody data reveals that certain regions of the larval ciliary band contain a higher proportion of dividing cells than others (Fig. 2a,b; Fig. 3b,c). The BrdU data further highlight this regional variation (Fig. 4a,c). Both the bipinnaria and plutei seem to possess a higher proportion of dividing cells per unit area in the ciliary band when compared to the rest of

the larval epidermis (Fig. 2-4,5). However, this may be due to the fact that there are simply more nuclei per unit area in the ciliary band.

It is conceivable that the proportion of dividing cells in a given area may change throughout development. The ciliary bands of some ciliated invertebrate larvae are known to increase length to improve feeding efficiency under low food conditions (Boidron-Metairon 1988, Strathmann 1987, Hart 1991, Strathmann et al. 1993). The ciliary band may have a proportionally higher cell division rate than the rest of the larval epidermis earlier in development, when it is beneficial for the larva to increase ciliary band length. Later in development, when increasing ciliary band size is no longer a great feeding benefit, it may be more beneficial to increase the proportion of dividing cells in regions such as the adhesive disc for settlement, the brachiolar arms or the juvenile rudiment.

Actinotroch

The anti-phosphohistone antibody label was located throughout the larval body with a somewhat higher proportion of mitotic cells in the pre-oral hood, at the base of the tentacles, and throughout the telotroch. This pattern becomes more obvious in older larvae. The BrdU assay largely confirms this pattern, but reveals an additional aspect: a large proportion of mitotic cells are found at the base of the newly formed tentacles and tentacles buds on the dorsal side of the actinotroch (Fig 7b). This is consistent with the fact that actinotroch tentacles are formed near the dorsal midline (Temereva and

Malkakov 2007). One would expect a higher proportion of dividing cells in a "growth zone."

According to Temereva and Malkakov (2010), the midgut of the *Phoronopsis harmeri* actinotrochs, collected from the Sea of Japan, is composed of biciliated cells. Cell division was detected in the midgut of actinotrochs (Fig. 6c; movie 1). However, it is conceivable that there are isolated monociliated or non-ciliated cells in the midgut, that are able to divide.

Mitraria

Despite the fact that mitraria larvae of *Owenia collaris* possess monociliated epithelia, mitotic cells are not evenly distributed throughout the larval body. There are certain regions where the proportion of dividing cells is higher, which probably reflects actively growing regions. Not surprisingly, one of these regions is the juvenile rudiment. The others include the larval foregut, midgut, hindgut, and the primary ciliated band. Apparently the growth rates of different larval structures vary among developmental stages. The nine-day-old larvae possess higher proportions of dividing cells in the juvenile rudiment invagination and in the larval foregut while the twelve-day-old larvae possess high proportions of dividing cells in the midgut, hindgut, and primary ciliated band. Interestingly, certain regions of the primary ciliary band contain more dividing cells than others. It is possible that these regional differences reflect the ancestry of mitraria (i.e. the fact that monociliated epithelium is derived in this larva from the

multiciliated condition presumed to be ancestral for the Protostomes (Nielsen 2001).

Metatrochophore

Annelid larvae are thought to add segments from a proliferative "growth" zone at the posterior end of the larva. However, this information is primarily derived from studies on Clitellata (reviewed in Seaver et al. 2005). The larvae of polychaete annelids *Hydroides elegans* and *Capitella teleta* possess cell proliferation, not at the posterior, as is expected, but within two bilateral growth zones. The typical posterior growth zone was not visible in larvae of *H. elegans* and *C. teleta*, but was visible in juveniles (Seaver et al. 2005). Additionally, growth in *Sabellaria alveolata* shows no evidence of a posterior growth zone in larvae or later stages (Brinkmann and Wanninger 2010). However, as both Seaver (2005) and Brinkmann and Wanninger (2010) point out, lack of an evident posterior growth zone may be due to mesoteloblasts that bud off cells in the posterior region and subsequently migrate in the anterior direction, making cell proliferation appear as lateral growth zones when a posterior growth zone is in fact the mechanism of growth.

The larvae of *Serpula columbiana* did not label with anti-phosphohistone antibody without special pre-treatment with proteinase K (to improve penetration). Antibody penetration was improved after treatment with proteinase K, but larvae showed poor morphological preservation and non-specific labeling. It may be possible in future studies to arrive at an optimal concentration of proteinase K to balance the penetration

and preservation issues. Non-specific labeling was possibly due to the aged (less than a year old) batch of BrdU, as using a fresh batch improved results. Although it is difficult to assess the pattern using the data at hand, it was evident that no BrdU-positive cells were located in the prototroch, which is expected because the prototroch cells in typical spiralians become multiciliated and cleavage-arrested early in development (Damen and Dictus 1994, Nielsen 2004).

Veliger

It appears that the mitotic cells found in the pre-trochal region are located primarily in vicinity of the tentacles and the apical ganglia (Page and Parries 2000, Dickinson and Croll 2003, and Kempf and Page 2005). The fact that many mitotic cells were found in the visceral mass is not surprising, since those cells are presumably non-ciliated. One of the most interesting observations (revealed by BrdU labeling, Fig. 12) is the presence of a localized proliferative region in the prototroch adjacent to the mouth that contains a particularly dense aggregation of mitotic cells. The molluscan velum is believed to be derived from the prototroch of the trochophore larva. The cells of the prototroch become ciliated and cease to divide. This means that the prototroch may not grow. But, clearly, the molluscan velum grows dramatically over the course of development (Strathmann 1993, Nielsen 2004, Harding 2006). In larvae of *Caesia* fossatus, for example, the velar lobes may reach 300 μm across at about one month of age. The cells within the velar ciliary band are relatively small, suggesting that cell division rather than cell stretching is responsible for increase in the size of the velum.

This suggests that 1) either the trochoblasts in veliger larvae somehow escape the cleavage arrest, or that 2) some other cells whose ability to divide is not restricted contribute to the velum. The fact that I found a distinct ventral proliferative region in the velum suggests that the latter may be correct.

Pilidium

The pilidium, similar to the molluscan veliger, faces a dilemma: its epidermis is composed largely of multiciliated cells (Nielsen 2005); however, it grows considerably. The larval ciliary band of the pilidium, similar to the veliger, is composed of many small cells (e.g. note the density of the nuclei on Fig. 16-18; movie 4**,** which suggest cell division rather than stretching of cells as the mechanism of growth. Therefore the question arises - which cells divide to accommodate the increase in size?

Distribution of fluorescent label in pilidium larvae injected as zygotes (Fig. 15) suggests a higher rate of cell proliferation in certain parts of the larval body, notably the axils and an area surrounding the apical organ (which are darker) compared to the areas where cell division is restricted (the brighter patches). One might say that the bright patches in the epidermis (Fig. 15) represent the "original larval body" while the darker regions represent the new growth.

The anti-phosphohistone antibody and BrdU labeling confirm this observation. Indeed, mitotic cells are clearly unevenly distributed throughout the larval body, and, notably, are concentrated in the axils, as well as near the apical organ. Interestingly, scanning electron microscopy of the pilidium larvae (Maslakova pers. com.) reveals

distinct clusters of small cells in pilidial axils which either lack cilia or possess a single rudimentary (possibly, primary) cilium. Additionally, monociliated cells have been identified in the apical plate of the pilidium belonging to *Lineus bilineatus* (Cantell et al. 1982). These observations combined suggest that the pilidial growth is largely restricted to these localized regions of stem cells. I further tested this hypothesis by using BrdU pulse-chase experiments, which show clearly that the progeny of the mitotic cells in the axils indeed contribute to the growth of the ciliary band.

Moreover, the BrdU pulse-chase experiments demonstrate that the progeny of mitotic cells in the axils also contribute to the juvenile rudiments - the imaginal discs. This aligns nicely with previous observations (Maslakova 2010) that both the cephalic and the trunk discs originate in close proximity to the pilidial axils. However, the third pair of imaginal discs - the cerebral organ discs - do not originate near larval axils; instead, they appear at the end of the esophageal ciliary ridges (Maslakova 2010). One might expect, therefore, to see some mitotic cells in or near the ciliary ridges. Indeed, BrdU labeling supports this hypothesis (Fig 17a, Fig. 18, movie 4); mitotic cells are found not only along the ciliary ridges, but also in the larval gut. Furthermore, expression of the stem-cell marker *piwi* in the pilidium larvae of *M. alaskensis* (L. Hiebert and A. Bird pers. obs.) corresponds to the distribution of mitotically active cells identified by BrdU and anti-phosphohistone antibody labeling (e.g in the axils, ciliary ridges, near the apical organ, and the gut). Another interesting detail revealed by the anti-phosphohistone antibody labeling as well as BrdU labeling is the consistent presence of a pair of labeled cells (one in each lateral lappet subepidermally in the vicinity of the ciliary band).

When a pulse-chase is performed, results should show cells labeled during the pulse and the cells born during the chase. The unincorporated BrdU should be washed out after the pulse and therefore not available for incorporation during the chase. The pilidia which were labeled with a six hour BrdU pulse and followed by a variety of chase lengths generally show subsequent BrdU label dilution in the axils. The exception are the pilidia treated with a six hour pulse followed by a 15 day chase (Fig. 18d) where the labeled cells are found at a higher proportion than expected at the axils, the gut, ciliated ridges and the apical organ. I believe the unincorporated BrdU was not throughly washed out after the six hour pulse and was subsequently incorporated into dividing cells during what should have been the chase.

Many questions remain unanswered. For example, what lineage gives rise to the populations of stem cells in the multiciliated spiralian larvae? Is the cell lineage the same or different in each type of larva?

The presence of distinct populations of stem cells that contribute to the growth of the ciliary bands in the veliger and pilidium also raises interesting questions about the regenerative ability of these larvae. Preliminary observations by George von Dassow (pers. comm.) suggest that pilidium larva is capable of regenerating its lobes and lappets. It is of interest to find out how regeneration would affect cell division rates. Furthermore, one wonders whether the pilidium would maintain its regenerative ability if the axillary regions were removed. Future cell lineage and regeneration studies should be able to answer some of these questions.

CHAPTER V

CONCLUSIONS

This is the first comparative study of cell proliferation patterns in ciliated larvae of marine invertebrates. All seven kinds of ciliated larvae examined here (bipinnaria, pluteus, mitraria, metatrochophore, pilidium, actinotroch, and veliger) clearly possess some dividing cells to allow for larval growth (i.e. larval cells are not simply stretching to accommodate for increase in size). The location of dividing cells varies among larval types, and appears to correlate with the type of larval epidermis and phylogenetic position. The three examined types of larvae with multiciliated epidermis (nemertean pilidium, gastropod veliger, and annelid metatrochphore) possess localized regions of cell proliferation (e.g. the axils in the pilidium, and the preoral region in the prototroch of the gastropod veliger). Larvae with monociliated epidermis, on the other hand, have dividing cells dispersed throughout the body, but not necessarily uniformly. The deuterostomes (bipinnaria and pluteus) have dividing cells dispersed throughout the larval body more or less uniformly. However, the protostomes with monociliated epithelia (represented in this study by the phoronid actinotroch and the annelid mitraria larva) have distinct regional differences in location of dividing cells. Certain regions of the larval body have more dividing cells than others. These differences highlight an interesting variation in how protuberances (lobes, tentacles) of larval ciliary bands may grow in different kinds of larvae. There appears to be three ways by which larvae can make such structures. For example in the annelid mitraria larva, the lobes themselves have many proliferating cells, whereas in the pilidium larva, dividing cells are generally

lacking in the lobes and lappets, and new cells are contributed from the axils (areas that separate the larval lobes and lappets). Similar to the pilidium, actinotroch tentacles appear to be growing by addition of cells at the base of the tentacles rather than at the tip. On the other hand, the arms of the echinoid pluteus larva seem to grow by adding new cells along the entire length of the arm.

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