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Five invaginations and shedding of the larval epidermis during development of the hoplonemertean *Pantinonemertes californiensis* (Nemertea: Hoplonemertea)

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We describe the planuliform larval development of the high intertidal hoplonemertean *Pantinonemertes californiensis* using confocal microscopy. We discovered that the newly hatched planuliform larva has two pairs of epidermal invaginations that disappear later in development. The anterior pair of invaginations has an apparent counterpart in other planuliform nemertean larvae; they give rise to the cerebral organs in at least two other hoplonemertean species. Although the adult *P. californiensis* possesses cerebral organs, we were not able to distinguish the anterior invaginations after 3 days of development. The developmental fate and homology of the posterior pair of invaginations is uncertain. We show that the proboscis develops in newly hatched larvae as a fifth distinct invagination just ventral of the apical plate. *P. californiensis* possesses a transitory larval epidermis, composed of approximately 80 multiciliated cells, which is normally shed at 3–4 days of development at 12–16°C.

Keywords: Nemertea; *Pantinonemertes californiensis*; planuliform larva; transitory epidermis; organogenesis

Introduction

Nemerteans, a phylum of lophotrochozoan worms, display a diversity of developmental modes, the most familiar being the long-lived planktotrophic pilidium larva, which uses a specialized ciliated band to feed on microscopic particles (e.g. Salensky 1886, 1912; Dawydoff 1940; Cantell 1966a, 1969; Lacalli and West 1985; Henry and Martindale 1998). Over the course of planktonic development the hat-shaped pilidium larva develops three pairs of ectodermal invaginations, called imaginal disks, which fuse to form the juvenile worm inside the larva. During a catastrophic metamorphosis, the juvenile breaks free of the pilidium, often devouring the larval enclosure in the process (Cantell 1966b). Until recently, it was believed that the pilidium larva is ancestral to the phylum because it is found in heteronemerteans and in some palaeonemerteans (genus *Hubrechtella*) (Cantell 1969). Recent molecular phylogenetic analysis (Thollesson and Norenburg 2003) demonstrated that heteronemerteans and *Hubrechtella* form a monophyletic clade (the Pilidiophora), characterized by the pilidium larva, suggesting that pilidial development is derived in nemerteans.

The palaeonemerteans (excluding *Hubrechtella* and its allies) form a basal paraphyletic assemblage within the phylum (Thollesson and Norenburg 2003).

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Development in the palaeonemerteans is often regarded as 'direct', meaning that the larval body plan does not differ significantly from the juvenile or adult (as opposed to the 'indirect' development of the pilidiophorans). All studied palaeonemerteans have a planktonic stage in their life history, represented by a uniformly ciliated, planula-like larva. This so-called "planuliform" larva has a mouth derived directly from the blastopore, which leads into the blind gut (Smith 1935; Iwata 1960; Maslakova et al. 2004a, 2004b). All evidence suggests that most palaeonemertean planuliform larvae are obligatory planktotrophs (Smith 1935, p. 370; Jägersten 1972, pp. 89–91) and have been observed to feed on relatively large particles offered as food in the laboratory, such as protists (Coe 1943, p. 232), bivalve veligers (G. Gavelis, personal observation) or dissociated blastomeres of purple sea urchins (S.A. Maslakova, personal observation), although the preferred food is yet to be identified for any given species. Maslakova et al. (2004a, 2004b) demonstrated that the planuliform larva of the palaeonemertean *Carinoma tremaphoros* possesses a vestigal prototroch, composed of cleavage-arrested cells derived from the trochoblast lineage. This suggests that palaeonemertean planuliform larvae are modified trochophores (Maslakova et al. 2004b), and further supports the notion that the pilidium is derived within nemerteans.

Hoplonemerteans, the sister clade to the pilidiophorans, have either encapsulated development or lecithotrophic planuliform larvae that are different from the planuliform larvae of palaeonemerteans. Similar to the palaeonemerteans, the hoplonemertean larvae represent a blueprint of the adult and develop into juveniles without a conspicuous metamorphosis or a drastic change in body plan. However, the hoplonemertean planuliform larvae do not have a vestigial prototroch. Instead, many species possess a transitory epidermis of ciliated, cleavage-arrested cells that is shed or gradually resorbed during embryonic or larval development (e.g. Delsman 1915; Reinhardt 1941; Hickman 1963; Maslakova and Malakhov 1999; Maslakova and von Döhren 2009). Some authors described this loss of embryonic or larval epidermis as being homologous to the metamorphic loss of pilidial tissues, suggesting that the pilidial-type development was ancestral to the phylum (Jägersten, 1972; Maslakova and Malakhov 1999). As the palaeonemerteans have been shown to have a modified trochophore larva and to lack any traces of pilidial-type metamorphosis even in vestigial form (Maslakova et al. 2004a, 2004b), it appears increasingly unlikely that the pilidial-type development is ancestral to the phylum. However, it remains an open question whether the hoplonemertean transitory larval (or embryonic, in the case of encapsulated development) epidermis represents a remnant or a precursor of the pilidium. In this scenario, pilidial-type development evolved before the divergence between the hoplonemerteans and pilidiophorans, and was either abbreviated in hoplonemerteans, or further elaborated in pilidiophorans. An alternative would be that the hoplonemertean larvae have nothing in common with the pilidium and have evolved independently from the modified trochophore of basal nemerteans (such as that found in *Carinoma*).

Detailed comparative analysis of development in hoplonemerteans is necessary to differentiate between the hypotheses outlined above, including studies of organogenesis, cell lineage and the ultimate fate of various larval structures and rudiments. Very little is known about morphogenesis in hoplonemertean planuliform larvae, and recent studies are limited to two species *Tetrastemma candidum* (Maslakova and Malakhov 1999) and *Paranemertes peregrina* (Maslakova and von Döhren 2009). The latter study, conducted with the aid of fluorescent confocal microscopy, disagrees in many respects

with the classical reports on the development of other hoplonemertean species (based on histology and light microscopy), which in turn disagree with each other, in their accounts of the development of major organ systems (reviewed in Friedrich 1979). Although one may be tempted to disregard the older literature, we show here that these disagreements may reflect true developmental variation in hoplonemerteans.

Development with a planktonic planuliform larva has been previously documented for *Pantinonemertes californiensis* (Roe 1993). Larval anatomy and shedding of a transitory larval epidermis are documented here for the first time. We describe early development and organogenesis using light and fluorescent confocal microscopy. We report two pairs of transitory epidermal invaginations: an anterior pair that may correspond to the cerebral organ rudiments described in the hoplonemertean *Paranemertes peregrina* (Maslakova and von Döhren 2009) and a posterior pair of undetermined significance. We discuss potential homology of the larval epidermis and these invaginations, with implications for evolution of nemertean larval development.

Materials and methods

Adult *Pantinonemertes californiensis* were collected from "Glasgow Beach" behind the Glasgow Store in the small unincorporated community of Glasgow just north of the McCullough Bridge in North Bend, Oregon in early September 2008 and in August and September 2009. Worms aggregated under rocks partially embedded in gravel just below the high tide mark. Reproductive males and females were kept in 0.5-litre plastic containers with screw caps with a few drops of seawater to maintain humidity. Worms often spawned on the day of or within a few days of collection. The pinkish eggs were deposited as primary oocytes and completed maturation (underwent germinal vesicle break down) upon contact with seawater. Eggs were placed into 150-ml glass custard dish with 0.45 μm-filtered seawater and fertilized with a dilute suspension of sperm. Embryos and larvae of *P. californiensis* were cultured in glass custard dish in 0.45 μm-filtered seawater changed by reverse filtration every other day. Cultures were kept in a flowing seawater table at ambient sea temperature (12–16°C). Images of embryos and larvae were obtained using a Sony DXC-C33 3CCD colour video camera or Leica DFC400 digital colour camera mounted on an Olympus BX51 DIC microscope.

Embryos and larvae were fixed for confocal microscopy at various times after fertilization. Unhatched embryos were manually dechorionated using sharp forceps and immediately fixed. Occasionally, normally developing embryos lack extraembryonic envelopes and can be fixed and processed at "prehatching" stages without dechorionation. Larvae that developed muscles and became contractile were relaxed in a 1 : 1 mix of 0.33 M $MgCl_2$ with filtered seawater for 10 minutes before fixation. Embryos and larvae were fixed for 30 minutes in 4% paraformaldehyde (Electron Microscopy Sciences) in seawater, washed three times for 10 minutes in phosphate-buffered saline (PBS) and stored at 4°C until staining, or were permeabilized, immediately after fixation, in three 10-minute washes in PBS with 0.1% Triton X-100 (PBT) and incubated with Bodipy FL phalloidin (Invitrogen) or rhodamine phalloidin (Sigma) at 1 U per 200 μl PBT with 1 μg/ml Hoechst 33342 (Sigma). To visualize the surface cell outlines stained specimens were rinsed three times for 10 minutes in PBS, mounted in PBS or Vectashield (Vector Laboratories) on poly-L-lysine-coated coverslips. For reconstructions of internal anatomy, stained larvae were rinsed in PBS, mounted on

poly-L-lysine-coated coverslips, quickly dehydrated through a series of isopropyl alcohol (not to exceed 4 minutes in total), cleared and mounted in Murray Clear (1 : 2 benzyl alcohol : benzyl benzoate). Samples were viewed using an Olympus Fluoview 1000 laser scanning confocal microscope using either a Plan Apo 60 × 1.4-NA oil or Plan Apo 60 × 1.2-NA water lens. Images were processed in IMAGEJ (Wayne Rasband, National Institute of Health, Bethesda, MD, USA). Cell surface counts for phalloidin-labelled larvae were performed with the help of VOXX (Indiana University) as previously described (Maslakova and von Döhren 2009).

Live labelling of transitory epidermal cells was accomplished by bathing larvae in a ~2 μg/ml solution of fluorescein diacetate in filtered seawater for 15–20 min. Larvae were then transferred through several changes of filtered seawater, and observed either with an epifluorescence stereomicroscope (Leica MZ10F) or by confocal microscopy.

Results

Spawning and early development

Ovaries filled with pinkish oocytes show through the body wall of ripe females. Ripe males appear whitish. Spawning was triggered in the ripest individuals by handling during collecting. Worms were often in dry tubes when spawning occurred. Many specimens spawned in the laboratory within days of collecting. On several occasions spawning was apparently induced by handling or exposure to light. Most laboratory spawning events nearly or completely emptied the animal of visible gametes. Females free-spawned thousands to tens of thousands of pinkish oocytes surrounded by egg envelopes. Oocytes from three different females had an average diameter of 97 μm ($n = 30$). The egg envelope consists of an outer chorion 282 μm in diameter ($n = 30$), filled with two layers of gelatinous matrix, and the thin innermost vitelline envelope that becomes visible after fertilization and traps the polar bodies at the animal pole of the egg. Some oocytes had a thin stalk at the vegetal pole that penetrated the egg envelopes and reached out to a small vegetal pore in the egg chorion (Figure 1A). Spermatozoa in this species have a slightly elongated headpiece.

At 13.5°C the first polar body formed at 50 minutes and the second polar body at 1 hour 15 minutes after insemination. Cleavage is equal and holoblastic, as is typical for nemerteans (Figure 1A–H). First cleavage was observed at 2.5 hours, with second and third cell divisions at approximately 3.5 hours and 4.5 hours after insemination. Cilia were first observed circumferentially and at the vegetal pole of 20-hour-old embryos. We also observed a little dimple at the vegetal pole (probably the blastopore). By 22 hours, embryos rotated inside the egg envelopes in a clockwise direction (viewed from the animal pole), were slightly elongated, and were equipped with a short apical tuft at the animal pole. We also observed two small indentations near the animal pole.

Larvae hatched at 30 hours after insemination at 12–13°C, and at 27 hours at 15–16°C. Newly hatched larvae are uniformly ciliated, possess a conspicuous apical tuft and a short posterior cirrus (Figure 2A). Subsequent development, as detailed below, may be summarized by three stages: five invaginations and the mouth form (invagination stage, Figure 2A); rudiments of adult organs, muscles and nervous system emerge (rudiment stage, Figure 2B); and the larva acquires worm-like habits and behaviours (vermicular stage).

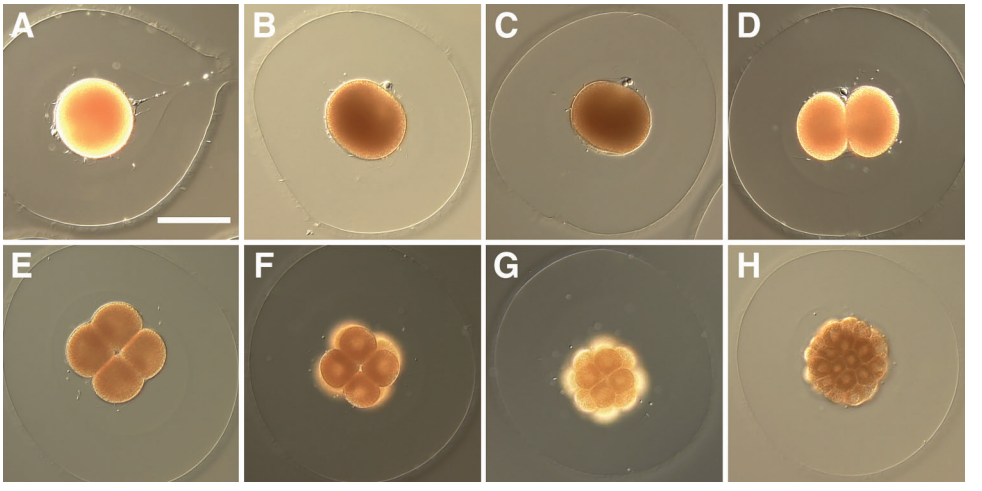

Figure 1. Cleavage in *Pantinonemertes californiensis*. (A) Fertilized egg in egg envelopes; (B) first polar body formation; (C) second polar body formation; (D) two-cell stage; (E) four-cell stage; (F) eight-cell stage; (G) 16-cell stage; (H) 32-cell stage. Scale bar 100 μm.

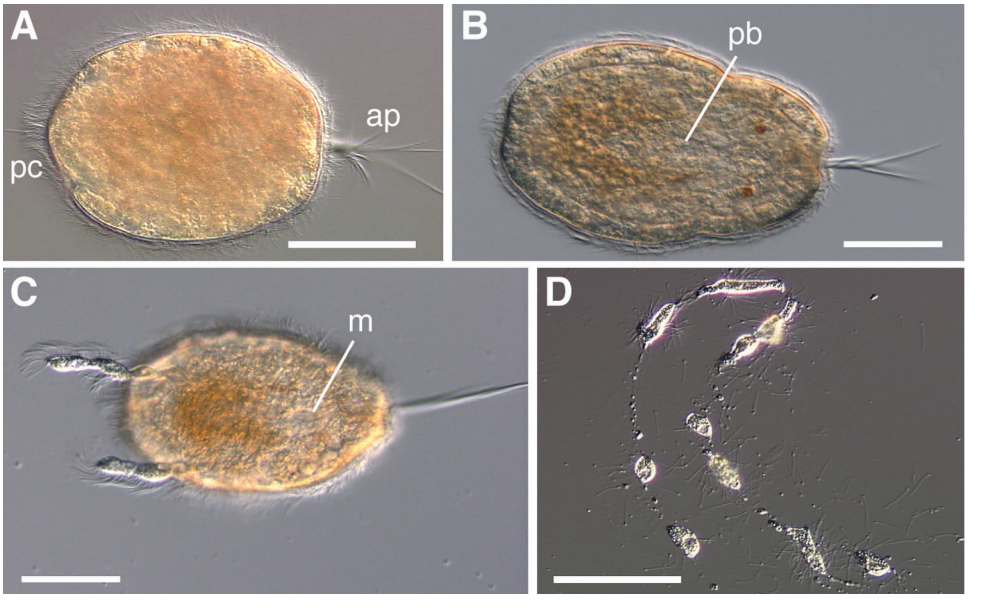

Figure 2. Planuliform larva of *Pantinonemertes californiensis*. (A) Newly-hatched invagination stage larva of *P. californiensis* is uniformly ciliated, has an apical tuft (ap) and posterior cirrus (pc); (B) late rudiment stage larva (3 days old at 15–16°C) has a distinct proboscis rudiment (pb) and two ocelli; (C) late rudiment stage larva in the process of shedding ciliated cells of its larval epidermis from around the mouth (m); (D) a chain of ciliated larval epidermal cells shed and left behind by a larva of *P. californiensis*. Scale bars 50 μm.

The five invaginations

The most conspicuous anatomical features highlighted by phalloidin in the prehatching stage embryos (28.5 hours at 12–13°C) include the apical plate, the posterior cirrus, five narrow epidermal invaginations and the ventral stomodeum (Figure 3A, B). The deepest pair of invaginations (about 15–20 μm long) is located at the anterior end, on each side of the apical plate at approximately 11 o'clock and 1 o'clock. In 46-hour-old

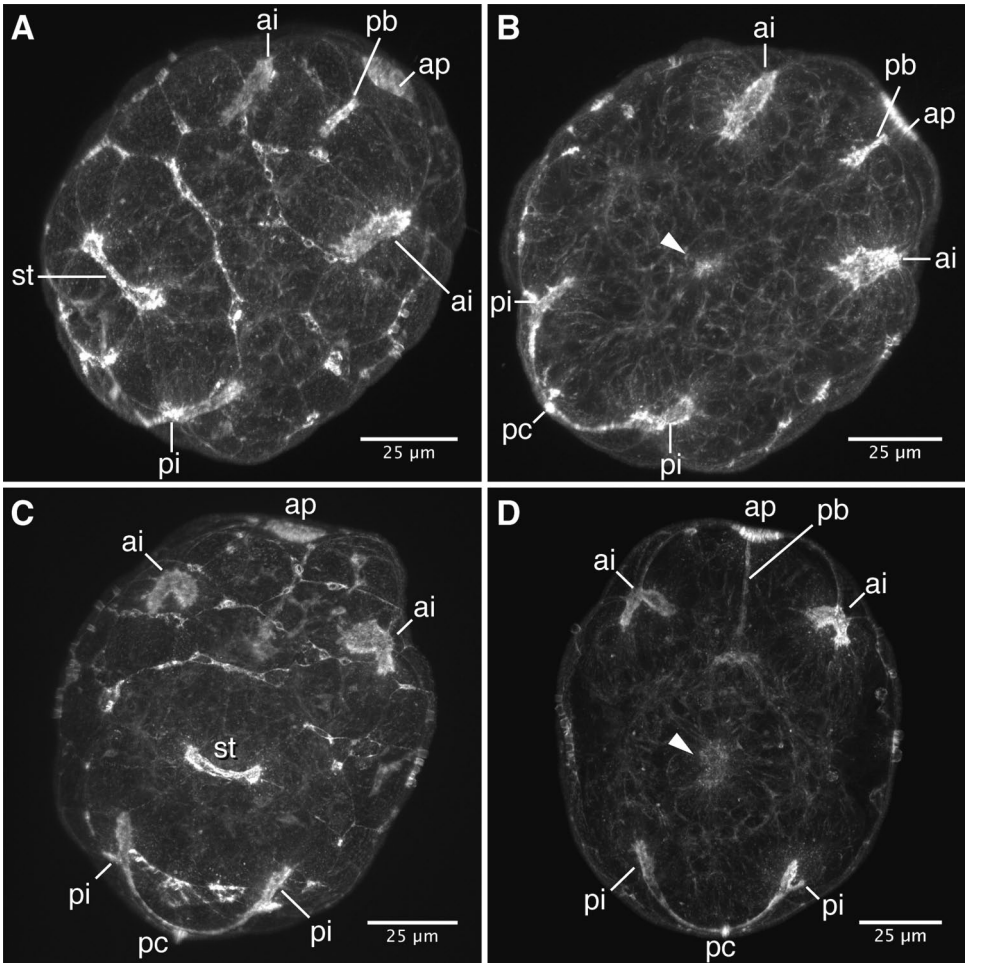

Figure 3. Confocal micrographs of phalloidin-labelled early (A, B) and late (C, D) invagination stage larvae of *Pantinonemertes californiensis*. (A, B) A 28.5-hour-old prehatched embryo (12–13°C); (C, D) a 46-hour-old (12–13°C) larva; apical pole marked by the apical plate (ap) is up or upper right. (A) Confocal Z-projection (ventral view) showing stomodeum (st), proboscis rudiment (pb), paired anterior (ai) and posterior (pi) invaginations; (B) a 30-μm thick sub-stack of frontal sections showing the posterior cirrus (pc), paired anterior and posterior invaginations, proboscis and midgut lumen (arrowhead); (C) confocal Z-projection (ventral view), showing bifurcated anterior and posterior invaginations and the stomodeum (st); (D) a 29-μm thick sub-stack of frontal sections showing the five invaginations and midgut lumen (arrowhead).

larvae at 12–13°C the anterior invaginations appear bifurcated (Figure 3C, D). By 2 days at 15–16°C, or 3 days at 12–13°C of development, these invaginations seem to evaginate, so that the two forks of each invagination are but shallow dimples (about 5 μm deep) on the surface, separated by a distance of about 20 μm (Figure 4A, B). We were not able to distinguish anterior invaginations with phalloidin labelling by 3 days at 15–16°C, or 4 days at 12–13°C of development (Figure 5).

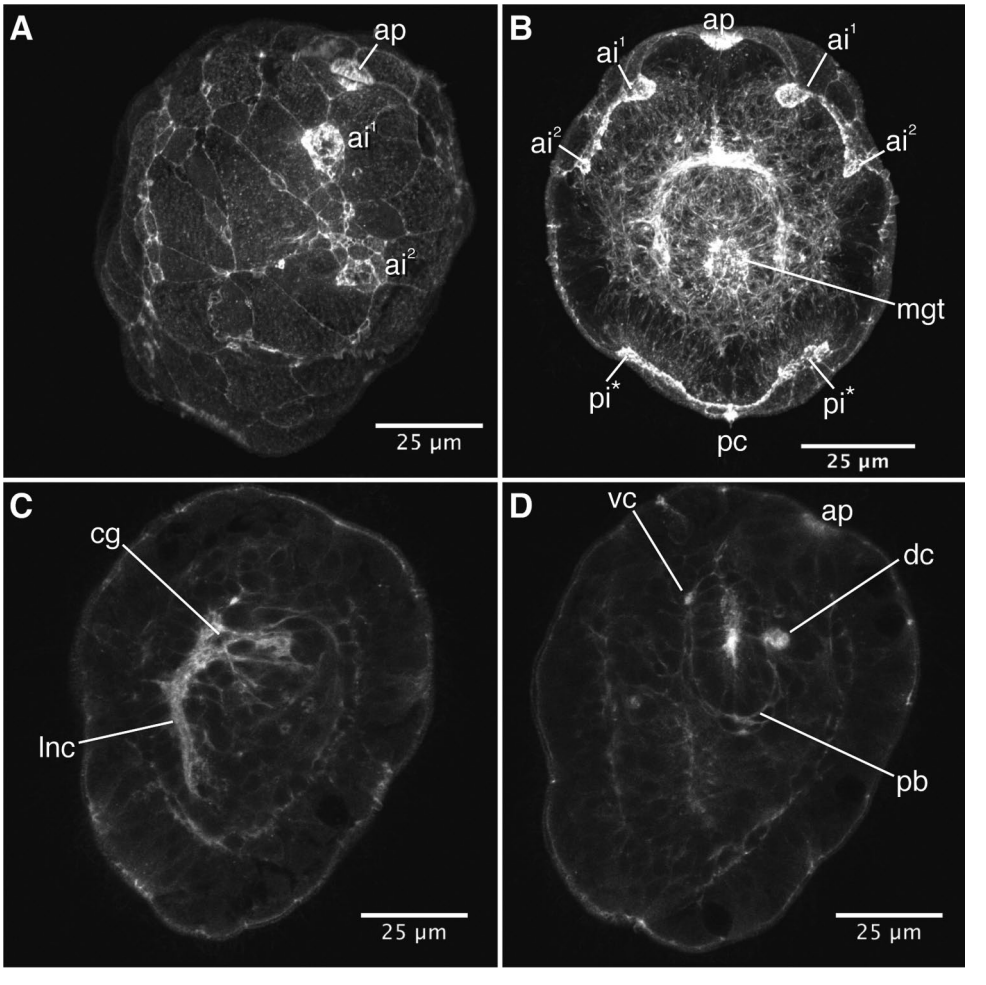

Figure 4. Confocal micrographs of phalloidin-labelled early rudiment stage *Pantinonemertes californiensis* larvae. (A, C, D) Three-day-old larvae (12–13°C); (B) 2-day-old larva (15–16°C); apical pole up, ventral to the left in (A, C, D). (A) Lateral view, showing apical plate (ap) and the two shallow dimples at the anterior end – remnants of one of the anterior invaginations (ai^1 and ai^2); (B) a 15-μm frontal sub-stack showing the two forks of anterior invaginations (ai^1 and ai^2), the remnants of posterior invaginations (pi^*), posterior cirrus (pc) and midgut lumen (arrowhead); (C) a single sagittal 1-μm section showing the cerebral ganglia (cg) and lateral nerve cord (lnc); (D) same embryo as in (C), different 1-μm section showing the proboscis (pb), dorsal commissure (dc), and ventral commissure (vc) of the brain.

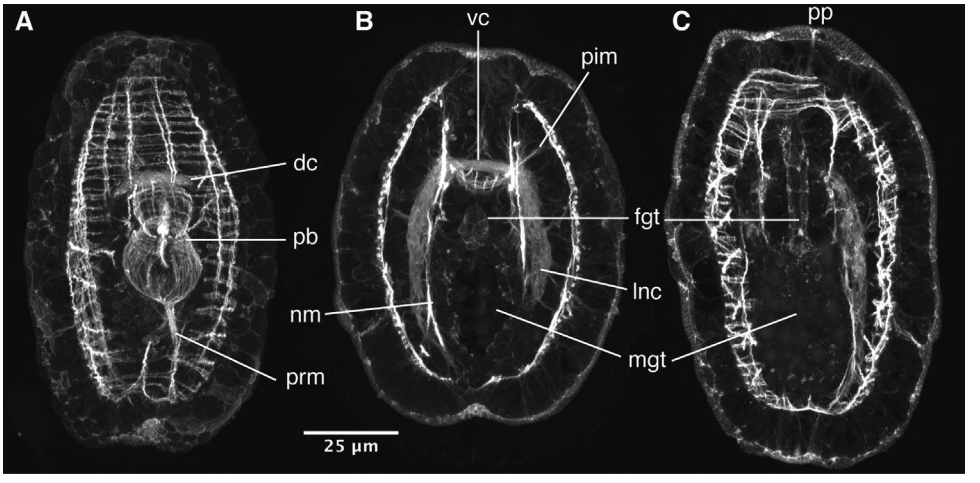

Figure 5. Confocal micrographs of the late rudiment stage larvae of *Pantinonemertes californiensis* (3-day-old at 15–16°C) labelled with phalloidin. This is the stage at which the larvae shed larval epidermal cells; apical is up. (A) A 10-μm sub-stack of frontal sections showing well-developed body wall muscles, the bipartite proboscis (pb) equipped with a muscle retractor (prm) and the dorsal commissure of the brain (dc); (B) a 5-μm sub-stack of frontal sections of a different larva showing the ventral commissure of the brain (vc), the lateral nerve cords (lnc) with the associated nerve cord muscles (nm), and proboscis insertion muscles (pim); (C) a 6.5-μm sub-stack of frontal sections of yet another larva showing the fused foregut (fgt) and midgut (mgt), and the proboscis pore (pp).

A pair of posterior invaginations, about 10 μm deep, is located on each side of the posterior cirrus at approximately 5 o'clock and 7 o'clock in the prehatching stage described above (Figure 3A, B). They become even more conspicuous in the 46-hour-old larvae at 12–13°C and appear to be connected to each other by a thin furrow along the posterodorsal surface of the larva (Figure 3C, D). By 3 days at 12–13°C or 2 days at 15–16°C the lumens of the posterior invaginations can no longer be distinguished with phalloidin labelling, but there is a patch of small epidermal cells in a broad depression marking the spot (Figures 4B and 7B), suggesting that the posterior invaginations also evaginate.

The fifth invagination is the rudiment of the proboscis. It appears as a slender approximately 15 μm deep unpaired epidermal invagination, just ventral to the apical plate in 28.5-hour-old 12–13°C embryos (Figure 3A, B). The proboscis rudiment is ~30 μm long by 46 hours at 12–13°C (Figure 3D). By 3 days at 12–13°C the proboscis rudiment appears as a tubular organ about 50 μm long, comprised of a monolayered columnar epithelium, surrounded by a thin layer of squamous cells, which may represent a rudiment of the rhynchocoel (Figure 4D). By 4 days at 12–13°C, or 3 days at 15–16°C, the proboscis is about 75 μm long, bipartite and has a retractor muscle (Figure 5A). At this stage the proboscis is still connected to the surface via a small proboscis pore located subterminally on the ventral side about 20 μm anterior to the mouth (stomodeum) (Figure 5C). In 5-day-old larvae at 12–13°C the mouth leads into a definite foregut lumen. No connection to the wall of this lumen from the proboscis can be observed. A strand of tissue extends from the proboscis, which leads to the

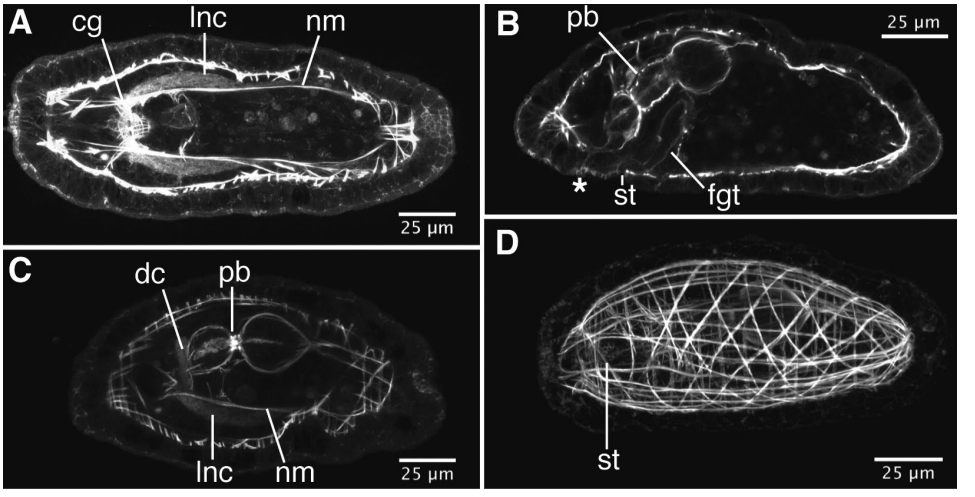

Figure 6. Confocal micrographs of phalloidin-labelled vermicular stage larvae of *Pantinonemertes californiensis*; anterior is to the left. (A–C) Five-day-old larvae (12–13°C). (A) An 8-µm sub-stack of frontal sections, showing cerebral ganglia (cg), lateral nerve cords (lnc) and nerve cord muscles (nm); (B) a 1-µm sagittal section, showing the proboscis (pb), foregut (fgt) and stomodeal opening (st). The position of the original proboscis pore (which is no longer apparent) is marked with an asterisk; (C) a 10-µm sub-stack of sagittal sections, illustrating the relative position of the dorsal commissure (dc) and lateral nerve cords with respect to proboscis; (D) confocal Z-projection (ventral view) of a 13-day-old larva, showing the well-developed circular, longitudinal and diagonal muscles of the body wall as well as the small cells that surround the stomodeum.

surface anterior to the mouth. Although no pore is evident at this stage, this may represent the original site of the proboscis invagination (Figure 6B).

Digestive system

After the blastopore closes, the mouth (stomodeum) forms separately on the ventral side of the embryo. In 28.5-hour-old embryos at 12–13°C the mouth appears as a shallow transverse indentation on the ventral surface surrounded by a characteristic rosette of large cells (Figure 3A). At this time, the midgut rudiment is an internal mass of cells with a small lumen (Figure 3B). The gut lumen is more distinct in 46-hour-old larvae at 12–13°C, but it is still disconnected from the foregut. By 3 days of development at 15–16°C, the foregut fuses with the midgut (Figure 5B, C).

Nervous system

Bundles of nerve fibres are enriched with filamentous actin so phalloidin labels the axons of major nerves with this fixation and staining procedure. The rudiments of the juvenile brain and the lateral nerve cords are apparent in 2-day-old larvae at 15–16°C and 3-day-old larvae at 12–13°C (Figure 4C, D) and become even more prominent in later stages (Figures 5B, and 6A, C, D). The dorsal and ventral brain commissures connect the cerebral ganglia forming a ring around the proboscis rudiment (Figures 4C, D and 6C).

Musculature

Longitudinal, circular and diagonal body wall muscles are well developed in 3-day-old larvae at 15–16°C (Figures 5A and 7D). At this stage longitudinal and circular proboscis muscles and proboscis insertion muscles are also well developed. Each of the lateral nerve cords is accompanied by a strand of nerve cord muscles, which

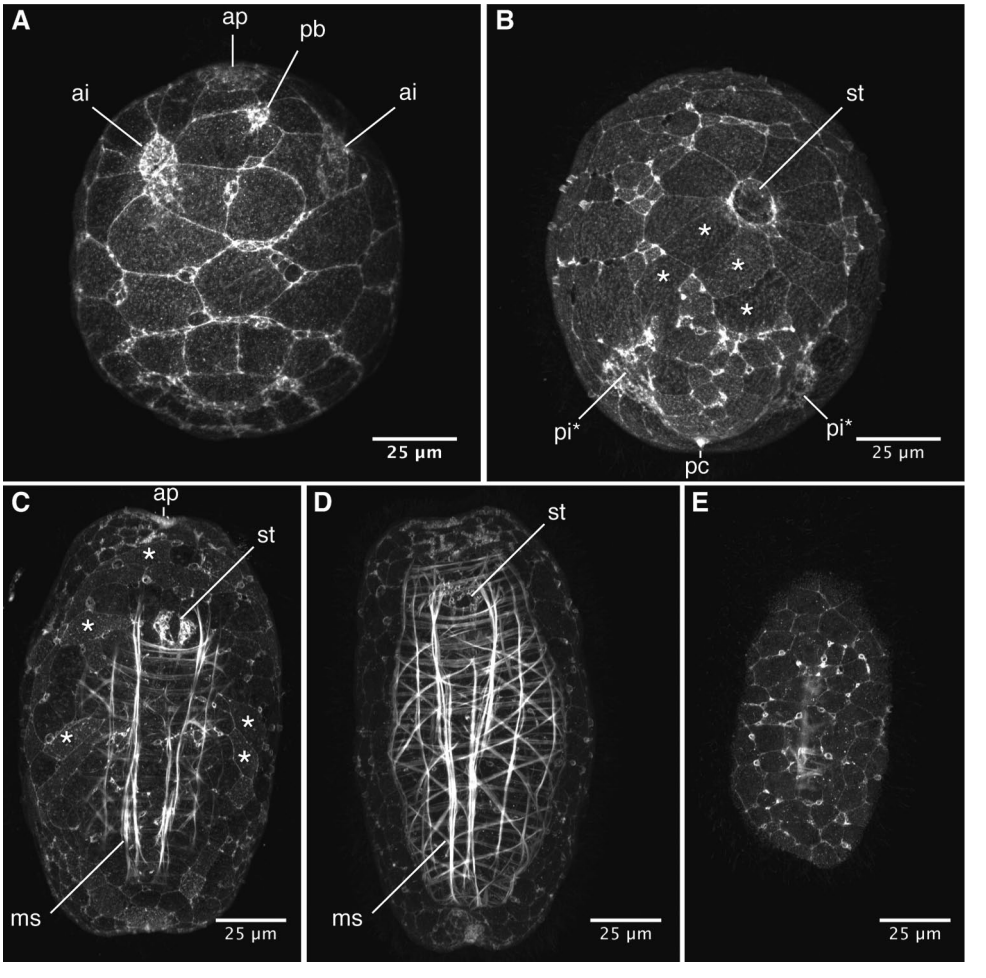

Figure 7. Confocal projections of phalloidin-labelled larvae of *Pantinonemertes californiensis* showing surface cell outlines in the early invagination stage (A), early rudiment stage (B), late rudiment stage, which is contemporary with shedding of the larval epidermis (C), and the vermicular stage (D, E). At 15–16°C these stages correspond to 23.5 hours (A), 2 days (B), 3 days (C) and 4 days (D, E) of development; ventral view, apical up. (E) An 11.5-µm sub-stack from the same stack as (D) shows the cell outlines without the interference of body wall muscles. (A) Mounted in Vectashield and scanned using 60 × Oil lens (NA 1.4); (B–E) mounted in phosphate-buffered saline and scanned using 60 × water lens (NA 1.2). Apical plate (ap), proboscis (pb), stomodeum (st), anterior invaginations (ai), remnants of posterior invaginations (pi*), muscles (ms). Asterisks mark several provisional larval epidermis cells.

extend into the pre-cerebral region (Figure 5B, C). Four-day-old larvae at 15–16°C (Figure 7B) exhibit nearly the same muscular anatomy as the 13-day-old larvae (Figure 6D).

Characteristic developmental stages

Because the timing of developmental events strongly depends on the ambient temperature, we propose a staging scheme based on the characteristic morphology, as described above, rather than the absolute age. The first morphologically characteristic stage described here is the "invagination stage" at which point one can distinguish the five invaginations and the stomodeum (Figures 3A, B and 7A). The late invagination stage is characterized by the forked anterior invaginations (Figure 3C, D). Next, we recognize the "rudiment stage", in which the rudiments of the proboscis, the brain and the lateral nerve cords become apparent. In the early rudiment stage the muscles are not yet well developed, and one can distinguish the remnants of the anterior and posterior invaginations on the surface (Figures 4A and 7B). In the late rudiment stage, the proboscis appears bipartite (Figure 5A), the muscles are well developed, the remnants of anterior and posterior invaginations can no longer be distinguished (Figures 5 and 7C), and the larvae shed chains of ciliated epidermal cells (Figure 2C, D). Soon after, the larvae become more elongated, strongly contractile and acquire worm-like behaviours so we propose to call these later stages "vermicular" (Figures 6 and 7D).

Transitory larval epidermis

The surface of early invagination stage embryos is covered by approximately 80 (76–80, $n = 3$) large ciliated apparently cleavage-arrested cells (Figures 7A and 8). The nuclei of these cells, as visualized by Hoechst staining, are distinctive – larger than others and containing numerous condensed regions and vesicles (data not shown). In the epidermis of the early rudiment stage one can distinguish cells of two different

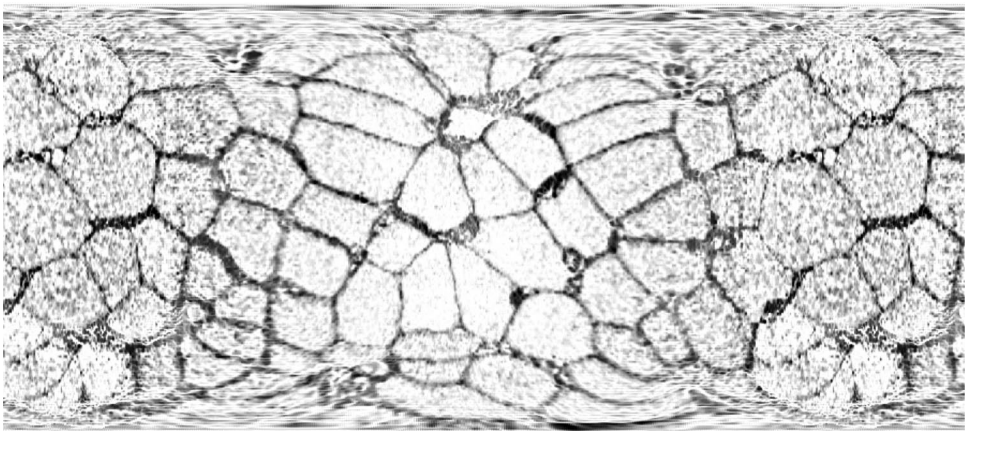

Figure 8. Roll-out epidermal map of phalloidin-labelled early invagination embryo (23.5-hour-old at 15–16°C) of *Pantinonemertes californiensis*, showing cell outlines (a total of 80 large cells).

types: the large cells, like those surrounding the stomodeum, and the intercalating groups of smaller cells (Figure 7B).

In late rudiment stage the epidermis is a patchwork of more brightly labelled slightly larger cells (which we suspect correspond to the larval epidermis) and slightly smaller cells, which are likely to be the cells of the definitive epidermis (Figure 7C). Because the size difference and the intensity of staining are subtle, we do not make an attempt to count the numbers of cells of each type. At this stage (3 days at 15–16°C) we noticed that larvae compressed slightly between a slide and coverslip would begin to shed large epidermal cells (Figure 2C). Cells are shed in a connected mass or chain, their cilia continuing to beat as the larva swims away (Figure 2D). Younger and older larvae did not shed cells when trapped. The distribution of the larger brighter cells in our phalloidin-labelled larvae appeared to correspond with the distribution of the cells that we observed being shed by live larvae. In early vermicular stage (4 days at 15–16°C) the epidermis looks more homogeneous, because, we suspect that the cells of the larval epidermis have been shed (Figure 7D, E).

To determine whether these shed cells are in fact a distinctive transitory epidermis, we labelled live larvae with fluorescein diacetate (FDA), a cell-permeant fluorophore, which is retained in cells after enzymatic cleavage. In late rudiment stage larvae, FDA brightly labels numerous large cells on the surface (Figure 9B). Upon slight compression, FDA-labelled larvae were observed to shed several of these cells within minutes (9 of 13 larvae shed 7–12 cells in connected chains within 10 minutes on a slide). Although FDA labelling does not survive fixation and mounting, by perfusing fixative under the coverslip we were able to catch several larvae in the act of shedding; all shed cells were large and brightly fluorescent (Figure 9). To determine whether

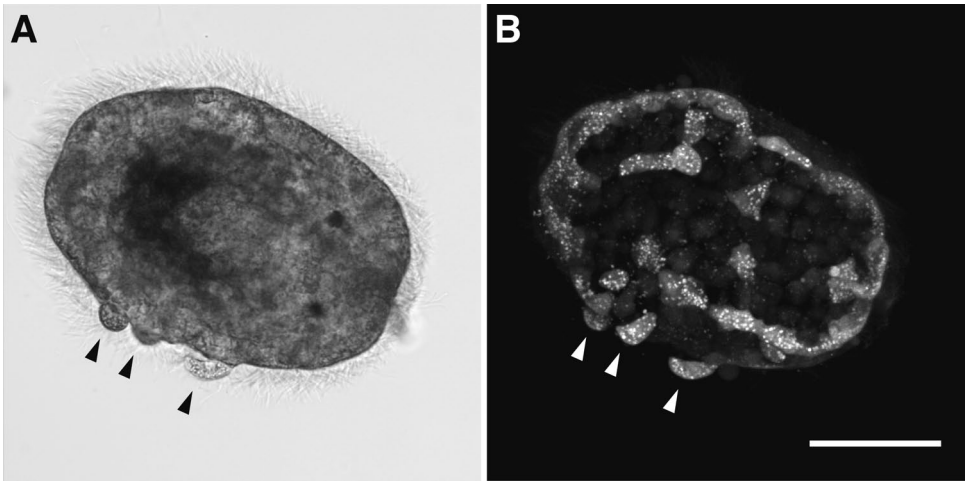

Figure 9. Fluorescein diacetate (FDA)-stained late rudiment stage larva of *Pantinonemertes californiensis* in the act of shedding cells of the larval epidermis. (A) Bright-field view; (B) a confocal projection of 21 1-μm sections of the same larva, showing the brightly-fluorescent FDA-labelled cells of the transitory epidermis. Anterior is to the right. The larva was trapped on a slide, fixed by flowing formaldehyde in seawater under the coverslip, and the image was collected immediately. Arrows highlight the same shedding cells in both images, which are brightly labelled with FDA.

shedding would occur without trapping, we placed isolated FDA-labelled late rudiment stage larvae into 5 μl of particle-free seawater in sealed coverslip chambers of ~1 mm depth; these larvae were completely free to swim. None had shed any cells within 8 hours. After 24 hours, seven of these isolated larval preparations had partially succumbed to bacterial infestation, and these were discounted. The remaining five chambers contained actively swimming, morphologically normal larvae, with few or no brightly labelled cells on the larval epidermis, and dozens of brightly labelled cells within the chamber, some scattered and most in a large connected cluster. We therefore conclude that the larval epidermis is normally shed in late rudiment–early vermicular stage larvae (between 3 and 4 days of development at 15°C).

Discussion

Transitory epidermis in hoplonemertean development

The presence of transitory epidermis has been previously documented in at least 11 species of hoplonemerteans with both planktonic planuliform larvae and encapsulated development. These include *Tetrastemma vermiculus* and *Drepanophorus spectabilis* (Lebedinsky 1898), *Emplectonema gracile* (Delsman 1915), *Prostoma graecense* (Reinhardt 1941), *Argonemertes australiensis* (Hickman 1963), *Tetrastemma candidum* (Maslakova and Malakhov 1999), *Prosadenoporus floridensis* (Maslakova and Norenburg 2008), *Quasitetrastemma stimpsoni* (Magarlamov and Chernyshev 2009), *Paranemeretes peregrina*, *Antarctonemertes phyllospadicola* and *Oerstedia dorsalis* (Maslakova and von Döhren 2009). The description here of *Pantinonemertes californiensis* brings this total to 12. Because these species represent a broad taxonomic sampling across the Hoplonemertea (including a reptant polystiliferan species, *D. spectabilis*, which is only distantly related to the rest) it is most parsimonious to assume that development with a transitory epidermis is ancestral to hoplonemerteans.

The process of replacement of the transitory epidermis by the definitive epidermis is best documented in planuliform larvae of *Paranemeretes peregrina* (Maslakova and von Döhren 2009). In this species, which has relatively large yolky eggs (240 μm), the approximately 90 cells of the larval epidermis are 50 μm or more across and have distinctive nuclear morphology compared with the much smaller (~5 μm) cells of the definitive epidermis. The cells of larval epidermis become spatially separated from each other by the intercalating cells of the definitive epidermis, and gradually diminish in size, which is interpreted as evidence of cytolysis. The cells of the transitory embryonic epidermis are also very distinct (in being large, yolky and having characteristic nuclei) in other species with large yolky eggs and encapsulated development, e.g. *Argonemertes australiensis* (630–740 μm egg), *Tetrastemma candidum* (220 μm egg) and *Antarctonemertes phyllospadicola* (310–375 μm egg). In all of these, embryonic epidermis appears to gradually resorb (i.e. shedding of the cells has not been observed), although neither cell death nor use of cellular material by other embryonic cells has been directly demonstrated for any of these species. In embryos of *T. candidum* (Maslakova and Malakhov 1999) and *A. australiensis* (Hickman 1963), which have been studied histologically, the cells of the embryonic epidermis are considerably yolkier than the cells of the definitive epidermis or other embryonic tissues (except for the gut). We suggest that this embryonic epidermis may function as a depository of embryonic yolk reserves.

In *Pantinonemertes californiensis* the size difference between the approximately 80 cells of the larval epidermis and the cells of the definitive epidermis is not as dramatic as in the four species with yolky eggs mentioned above. Nevertheless, the epidermal cells of these two populations can be distinguished by nuclear morphology. In contrast to *Paranemertes peregrina* and other species with yolky eggs, cells of the transitory epidermis are not resorbed gradually, but are shed by the late-rudiment stage planuliform larvae of *P. californiensis*. The larval epidermis was described and presumed to be shed in a Mediterranean (Naples) population of *Emplectonema gracile* (Delsman 1915). More recently, shedding of larval epidermis was observed by S.A. Maslakova in a northwest Atlantic (Woods Hole) population of *Oerstedia dorsalis* (Maslakova and von Döhren 2009). Coincidentally, in all three species that are reported to shed the larval epidermis, the eggs are relatively small (135–140 μm in *Emplectonema gracile* from Naples, ~150 μm in *O. dorsalis* from Woods Hole, and ~100 μm in *P. californiensis*). Additionally, there are two older reports of uncertain validity of the shedding of the larval epidermis in what are probably species of *Carcinonemertes* (van Beneden 1861, Dieck 1874), which also have small eggs (less than 100 μm). One may speculate that discarding the larval epidermis would be less disadvantageous in species with oligolecithal eggs, compared with those with macrolecithal eggs, if yolk is concentrated in the cells of transitory epidermis.

The report of transitory larval epidermis in a palaeonemertean species (Iwata 1960) have not been confirmed by Maslakova et al. (2004a, 2004b) in their recent studies of development in *Carinoma tremaphoros*. Instead, Maslakova et al. discovered a kind of larval "test" derived from the classical spiralian trochoblast cell lineage, composed of 40 multiciliate cells, which gradually reduced their surface coverage forming a compact preoral belt (vestigial prototroch) before disappearing entirely. In the sister clade to the hoplonemerteans, the Pilidiophora, the entire larval body (the pilidium) is dropped, and, in most cases, devoured by the juvenile, during a catastrophic metamorphosis. The fact that the number of cells in the hoplonemertean larval epidermis is more than twice the number of cells composing the vestigial prototroch in *Carinoma*, and the way in which these cells disappear (a discrete domain in *Carinoma* versus isolated "islands" in hoplonemerteans) suggests that these structures have different developmental and evolutionary origins. It remains a possibility that the hoplonemertean larval epidermis represents a vestige or a precursor of the pilidium larval body. Future cell lineage analysis of the hoplonemertean transitory epidermis will probably help to determine the homology of the transitory epidermis of hoplonemerteans.

Organogenesis

Classical reports on hoplonemertean development, conducted with the aid of paraffin sections and light microscopy, disagree with each other in many respects (reviewed in Friedrich 1979). At this point, it is not entirely clear whether the disagreements are the result of misinterpretation of morphogenetic events by some authors, or whether they reflect the actual developmental diversity in this group. Here we will mostly make the comparison with larval development of *Paranemertes peregrina*, which was recently described using confocal microscopy by Maslakova and von Döhren (2009). The blastopore closes in all previously described species of hoplonemerteans. Similarly, in *P. peregrina* and *Pantinonemertes californiensis*, the stomodeum (mouth) develops

as a separate opening on the ventral side of the larva, and subsequently connects to the primary gut (=midgut).

Similar to *Drepanophorus spectabilis* (Lebedinsky 1898), but unlike most other previously studied species (including *Paranemertes peregrina*), the proboscis rudiment in *Pantinonemertes californiensis* forms as a distinct epidermal invagination, which maintains a narrow lumen connecting it to the subterminal (ventral to the apical plate) proboscis pore throughout larval development. The proboscis pore and the mouth are separated by only about 20 μm in early vermicular stage and, we expect, they eventually fuse to form a common rhynchostomopore.

One of the most striking differences in development of *Pantinonemertes californiensis* and *Paranemertes peregrina* is the fate of the paired anterior invaginations. These undoubtedly give rise to the cerebral organs in *P. peregrina*, but either close or evaginate, and thereby become indistinguishable by the late rudiment stage in *P. californiensis*. Similar epidermal invaginations give rise to the cerebral organs in the embryos of the viviparous hoplonemertean from Bermuda *Prosadenoporus agricola* (Coe 1904). Paired anterolateral invaginations have been described in the development of almost every studied species of hoplonemertean and palaeonemertean, but the interpretations of their fate vary. Transitory anterior epidermal invaginations have been described in larvae of the palaeonemerteans *Cephalothrix rufifrons* (Smith 1935), *Procephalothrix filiformis* and *Procephalothrix simulus* (Iwata 1960), *Carinoma tremaphoros* (Maslakova et al. 2004b) and the commensal hoplonemertean *Malacobdella grossa* (Hammarsten 1918), all of which lack cerebral organs as adults. Smith (1935), Iwata (1960) and Maslakova et al. (2004b) interpreted those as the nervous system rudiments, whereas Hammarsten (1918) suggested that they are vestigial cerebral organ rudiments.

The adult *Pantinonemertes californiensis* possesses small cerebral organs. It is possible that the invaginations in question do give rise to the cerebral organs in *P. californiensis*, but that the lumen is obstructed, and the ciliated canal of the cerebral organs is formed *de novo* later in development. The alternative explanation may be that the transitory invaginations in *P. californiensis* give rise to other structures (e.g. nervous system rudiments), as had been previously suggested for other nemertean species, including *Tubulanus punctatulus* (Iwata 1960), which also has cerebral organs as an adult. In this case, one might expect that the cerebral organs would form later in development. A rather unorthodox hypothesis is that the transitory anterior invaginations in *P. californiensis* and, possibly, in some other nemerteans represent homologues of the cephalic and cerebral organ imaginal discs of pilidiophorans.

Perhaps the most mysterious feature of development in *Pantinonemertes californiensis* is the presence of a pair of transitory posterior epidermal invaginations. These have no obvious counterpart in the development of other hoplonemerteans or palaeonemerteans, but positionally correspond and may be homologous to the trunk imaginal discs of the pilidiophorans. Future cell lineage analysis using injected cell trackers, as well as rudiment-specific genetic markers will help to determine the ultimate fate of the anterior and posterior invaginations.

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