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The embryonic development of the bodywall and nervous system of the cestode flatworm *Hymenolepis diminuta*

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Abstract Cestodes (tapeworms) are a derived, parasitic clade of the phylum Platyhelminthes (flatworms). The cestode body wall represents an adaptation to its endoparasitic lifestyle. The epidermis forms a non-ciliated syncytium, and both muscular and nervous system are reduced. Morphological differences between cestodes and free-living flatworms become apparent already during early embryogenesis. Cestodes have a complex life cycle that begins with an infectious larva, called the oncosphere. In regard to cell number, cestode oncospheres are among the simplest multicellular organisms, containing in the order of 50–100 cells. As part of our continuing effort to analyze embryonic development in flatworms, we describe here the staining pattern obtained with acTub in embryos and larvae of the cestode *Hymenolepis diminuta* and, briefly, the monogenean *Neoheterocotyle rhinobatidis*. In addition, we labeled the embryonic musculature of *Hymenolepis* with phalloidin. In *Hymenolepis* embryos, two different cell types that we interpret as neurons and epidermal gland cells express acTub. There exist only two neurons that develop close to the midline at the anterior pole of the embryo. The axons of these two neurons project posteriorly into the center of the oncosphere, where they innervate the complex of muscles that is attached to the hooklets. In addition to neurons, acTub labels a small and invariant set of epidermal gland cells that develop at superficial positions, anteriorly adjacent to the neurons, in the dorsal midline, and around the posteriorly located hooklets. During late

stages of embryogenesis they spread and form a complete covering of the embryo. We discuss these data in the broader context of platyhelminth embryology.

Keywords Embryo · Development · Morphogenesis · Organogenesis · Neurogenesis · *Hymenolepis diminuta* (Platyhelminthes, Cestodes)

Introduction

Cestodes are members of the phylum Platyhelminthes with numerous highly derived characteristics, most of which have probably evolved in adaptation to their endoparasitic lifestyle. The cestode bodywall, an unciliated syncytium called the neodermis, represents the defining morphological criterion of the clade Neodermata that contains the cestodes and most of the other parasitic flatworms (Ehlers 1985; Ax 1996). It has been shown that, ontogenetically, the neodermis is formed secondarily underneath an earlier developing “primary” epidermis that is typically ciliated (Grammeltvedt 1973; discussed in Ehlers 1985). Other specializations of the cestodes are the absence of a gut, and the existence of a complex life cycle that often involves several larval stages inhabiting different animal hosts.

Among different subtaxa of cestodes, the morphology of the first larval stage is quite diverse. The gyrocotylideans and amphilinidians, groups that are considered to be more primitive among the cestodes and that parasitize marine fishes, produce lycophora larvae which still exhibit many characteristics of juveniles or larvae of free-living platyhelminths (Xylander 1986, 1987; Rohde and Georgi 1983). As “active” larvae that have to find and invade the primary host (crustacean), they have an elongated body, a ciliated epidermis, an anterior cerebral ganglion, and a paired protonephridial system. Posteriorly they possess an array of ten hooklets that serve as an organ of attachment to the primary host. The first larval stage of “higher” cestodes (cestoidea) that parasitize aquatic and terrestrial vertebrates is structurally simpli-

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fied. It is a “passive” larva, called a coracidium (in aquatic tapeworms) or oncosphere (in terrestrial tapeworms), that enters the primary host by being ingested. Oncospheres and coracidia are minute spherical organisms, measuring less than 20 μm in diameter and containing fewer than 100 cells (reviewed in Rybicka 1966; Ubelaker 1980). Embryonic cells are surrounded by several protecting envelopes. The inner envelope, called the embryophore, has been homologized to the primary, ciliated epidermis of other cestode larvae. In a coracidium larva this layer still retains its ciliation, whereas no cilia are formed by the inner envelope in an oncosphere. No protonephridial system is present. According to the older literature (reviewed in Rybicka 1966; Ubelaker 1980), even a nervous system is absent. Upon hatching in the gut of the primary host (an insect in the case of the rat tapeworm *Hymenolepis diminuta*), the oncosphere sheds its protective envelopes and penetrates through the intestinal wall into the body cavity of the host. It does this by muscle-powered, coordinated “chewing” movements of the posterior hooklets. Once inside the body cavity, the oncosphere metamorphoses into a secondary larva, called the cysticeroid.

The fact that oncospheres can generate coordinated movement of muscles makes it highly unlikely that they do not possess a nervous system. Cells with secretory vesicles and synapse-like specializations have indeed been observed electron microscopically (Fairweather and Threadgold 1981), but the pattern and development of nerve cells remain unknown. In this paper we have analyzed embryogenesis in the cestode *Hymenolepis diminuta*, employing an antibody against acetylated tubulin (acTub) that labels neurons and ciliated cells in embryos of many different species. We utilized this marker in the past to follow the embryonic development of the nervous system of several flatworm species, including *Mesostoma lingua* (typhloplanoid; Younossi-Hartenstein et al. 2000), *Imogine mcgrathi* (polyclad; Younossi-Hartenstein and Hartenstein 2000), and *Craspedella pedum* (temnocephalid; Younossi-Hartenstein et al. 2001). Our results indicate that acTub labels two anteriorly located nerve cells, beside a set of external cells that we interpret as gland cells. Given that in free-living flatworms, gland cells have not been previously observed as targets of acTub, we investigated the acTub-labeling pattern in embryos of the monogenean *Neoheterocotyle rhinobatidis*. Monogeneans are ectoparasitic flatworms which still show many similarities to free-living flatworms, and whose larval morphology, including the different types of gland cells, has been well described (Chisholm and Whittington 1996). We can show that a prominent type of monogenean embryonic gland cells is labeled by acTub. Our results enable us to draw comparisons between the early body plan of highly derived, parasitic flatworms and the typical body plan of free-living flatworms.

Materials and methods

Animals

Hymenolepis diminuta adults parasitize the intestinal tract of inoculated laboratory rats. Tapeworms were dissected in saline. Tapeworm segments containing eggs of all stages of development were agitated with glass beads in insect saline (0.4% NaCl) for 5 min to break the eggshell. After this, they were incubated for 15 min in 1% trypsin in insect saline to digest the shells. Eggs were fixed in 4% formaldehyde in 0.1 phosphate-buffered saline (PBS: 0.125 M NaCl; 16.5 mM Na_2HPO_4 ; 8.5 mM NaH_2PO_4). The monogenean *Neoheterocotyle rhinobatidis* parasitizes the gills of rays of the *Rhinobatos* group common around Heron Island, Australia. Adult worms and eggs of different stages were collected with fine needles from dissected gill lamellae of *Rhinobatos* specimens. Eggs were fixed in 4% formaldehyde in PBS.

Fuchsin labeling of whole-mounts

The whole-mount technique, which has been extensively used by us and others to label whole embryos of insects and other invertebrates, was adapted from Zalokar and Erk (1977). Briefly, following fixation in 4% PBS-buffered formaldehyde, embryos, contained within small wire mesh baskets holding 20–50 specimens, were washed in 70% ethanol (three changes of 5 min each) and distilled water (5 min). They were placed in 2N HCl (10 min) at 60°C for DNA denaturation. Following one wash in distilled water (5 min) and two washes in 5% acetic acid, embryos were stained for 15 min in 2% solution of filtered basic fuchsin (in 5% acetic acid). Embryos were washed in 5% acetic acid until cytoplasmic fuchsin labeling was removed, dehydrated in graded ethanol, and transferred to Epon, and individually mounted on slides.

Immunohistochemistry

Embryos that had been fixed in 4% formaldehyde (see above) were washed in PBT (PBS plus 0.3% Triton X-100; for washing, PBT solution was changed 3–5 times over a 10-min period) and incubated overnight in PBT containing anti-acetylated tubulin antibody (acTub; Sigma) at 1:1,000 dilution. After another washing step in PBT the preparations were incubated for 4 h in PBT containing the secondary antibody (peroxidase-conjugated rabbit anti-mouse immunoglobulin, Jackson Labs.) at a dilution of 1:800. The preparations were washed and incubated with diaminobenzidine (DAB, Sigma) at 0.1% in 0.1 M phosphate buffer (pH 7.3) containing 0.006% hydrogen peroxide. The reaction was stopped after 5–10 min by diluting the substrate with 0.1 M phosphate buffer. Preparations were dehydrated in graded ethanol (70%, 90%, 95%, 5 min each; 100%, 15 min) and acetone (5 min) and left overnight in a mixture of Epon and acetone (1:1). They were then mounted in a drop of fresh Epon and coverslipped. Preparations were analyzed and photographed with a Zeiss Axiophot photomicroscope.

Histology

Representative acTub-labeled embryos were transferred to molds, oriented, and placed at 60°C for 24 h to permit polymerization of the Epon. Blocks were sectioned with an LKB Ultratome into 1- μm semithin sections. Sections were counterstained with toluidine blue/methylene blue (Ashburner 1989).

Phalloidin labeling

Embryos fixed in PBS-buffered 4% formaldehyde (see above) were incubated for 1 h in rhodamine-conjugated phalloidin in PBS (Sigma). After washing in PBS, clusters of embryos were

transferred to glycerol, coverslipped and viewed under fluorescent light (Zeiss Axiophot photomicroscope, $\times 100$ lens, rhodamine filter set). Some phalloidin-labeled embryos were incubated in a solution of diaminobenzidine (DAB; Sigma; 2 mg/ml) in 100 mM TRIS-HCl (pH 7.4) for photoconversion (Bossing et al. 1996). A drop of DAB solution containing multiple embryos was transferred to the slide and illuminated for 30–45 min with a mercury lamp through a rhodamine filter set and a $\times 40$ lens. The reaction was stopped when the brown color of embryos was detectable under the microscope. Photoconverted embryos were dehydrated, mounted in Epon, and coverslipped.

Results

Morphology of the mature oncosphere

The minuscule oncosphere of *Hymenolepis* consists of less than 50 cells. The oncosphere is enclosed within an outer and inner envelope, syncytial structures that originate during early cleavage from the macromeres (Rybicka 1966; Ubelaker 1980; Fig. 1; see below). The most prominent features of the larva are three pairs of hooklets, one pair on each side and one at the posterior tip (Figs. 1, 2). Hooklets are anchored in the bodywall by a thickened collar. A ventrally curved blade extends outward from the collar; a conical shank ending in a rounded knob reaches inward. The bodywall comprises a thin syncytial layer, the embryonic epithelium, which encloses a system of gland cells (Fig. 2A–C). According to Rybicka (1973), the embryonic epithelium is formed by processes of a binucleated cell whose cell body is buried deep in the center of the oncosphere. The embryonic epithelium corresponds to the syncytial, non-ciliated neodermis that forms the integument of cestodes. The system of gland cells underlying the embryonic epithelium seems to be more complex than previously reported (Reid 1948; Ogren 1955; Rybicka 1966; Ubelaker 1980). The so-called penetration gland is formed by a syncytium of granule-packed cells arranged in the shape of a “U,” with

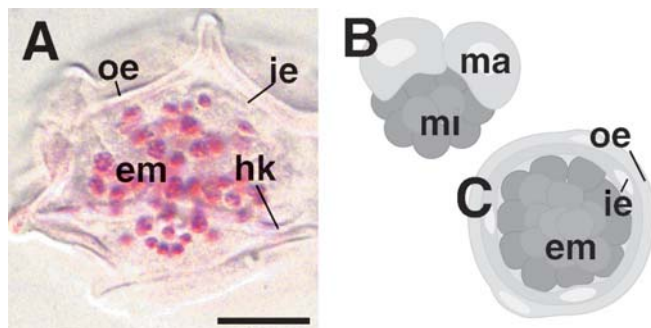


Fig. 1A–C *Hymenolepis* oncosphere. **A** Mature oncosphere labeled with fuchsin. Dorsal view, anterior is to the top. The embryo (em) is composed of approximately 50 cells whose nuclei are visualized by fuchsin (red). Hooklets (hk) and envelopes (ie inner envelope or embryophore; oe outer envelope) can be seen in interference contrast. **B**, **C** Schematic representation of embryo during early cleavage stage (**B**) and at onset of morphogenesis (**C**), depicting derivation of the envelopes from macromeres (ma) and the embryo proper from microcomeres (mi). Bar 10 μ m

the base of the U located anteriorly, and the two arms extending posteriorly on the right and left side of the larval body, respectively (Fig. 2A–C). In addition to the penetration gland, which is not labeled by the acTub antibody, there is an acTub-positive layer of flat, fibrillar cells that almost completely surround the oncosphere (Fig. 2B, C). We assume that these cells, which we refer to as superficial epidermal glands, constitute gland cells,

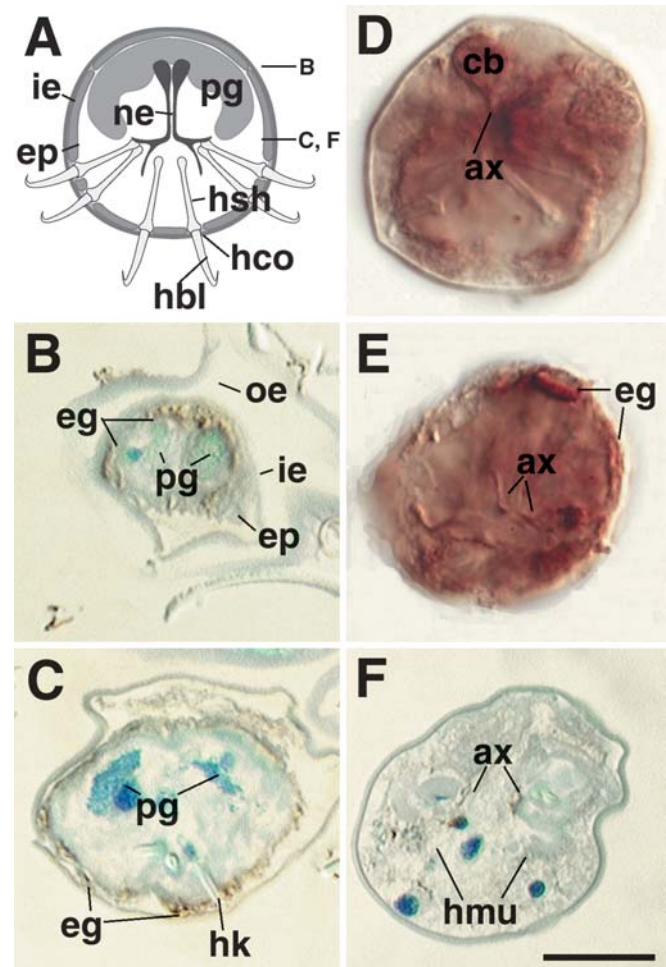


Fig. 2A–F Labeling of *Hymenolepis* oncosphere with acTub. Control experiments using only secondary antibody did not show any staining of specific cells (data not shown). **A** Schematic depiction of mature oncosphere, ventral view, anterior to the top. Illustrated in different shades of gray are the inner envelope (ie embryophore), syncytial epidermis (ep), penetration gland (pg), hooklets (hbl hooklet blade, hco hooklet collar, hsh hooklet shank) and neurons (ne). Horizontal lines indicate the plane of sections shown in **B**, **C** and **F**. **B**, **C** Sections of oncosphere labeled with acTub and counterstained with toluidine blue/methylene blue. AcTub decorates in a fibrillar pattern a thin cellular layer underneath the epidermal syncytium that we interpret as epidermal gland (eg). Other structures are the penetration gland (pg), hooklets (hk), outer envelope (oe) and inner envelope (ie). **D**, **E** Deep (**D**) and superficial (**E**) focal plane of whole-mount of oncosphere labeled with acTub, showing epidermal gland (eg) and neurons (cb cell body, ax axons). **F** Section of oncosphere labeled with acTub. In this preparation, epidermal gland staining shows up only weakly. The two axons (ax) branching out towards the hooklet musculature (hmu) can be seen in the center of the embryo. Bar 10 μ m

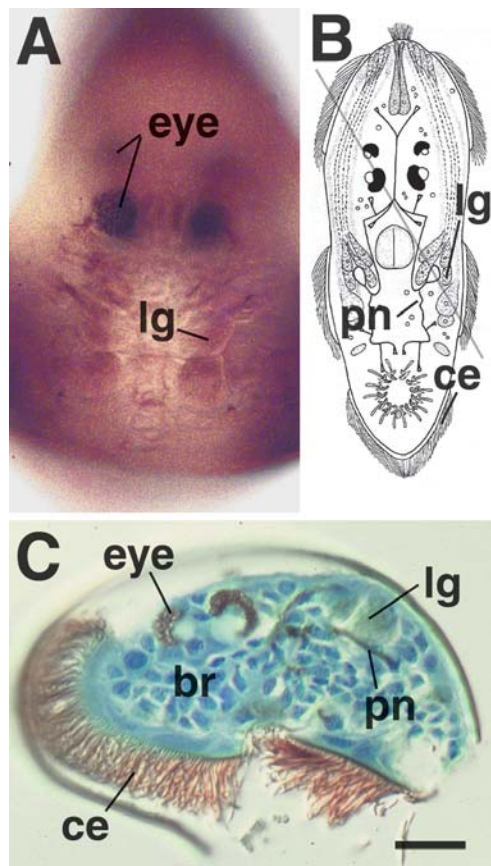


Fig. 3A–C AcTub labels subset of glands in late embryo of the monogenean *Neoheterocotyle*. **A** Whole-mount of late *Neoheterocotyle* embryo labeled with acTub. Dorsal view, anterior is to the top. **B** Schematic depiction of early *Neoheterocotyle* larva (after Chisholm and Whittington 1996). **C** Oblique section of late *Neoheterocotyle* embryo labeled with acTub and counterstained with methylene blue/toluidine blue. Plane of sectioning is shown by gray line in **B**. AcTub labels patches of ciliated epidermis (*ce*), protonephridial tubes (*pn*) and large cell bodies belonging to lateral glands (*lg*) (*br* brain, *eye* cup eyes). Bar 10 μ m

given that acTub strongly labels this type of cell in other flatworm species, in particular the embryos and larvae of monogeneans (Fig. 3). Here, beside the patches of ciliated epidermis (typical for monogeneans; Lyons 1973), protonephridia, and a subset of central nerve cells, the large cell bodies representing the lateral glands are acTub positive. Lateral glands have a characteristic location behind the brain and close to the dorsal midline. Other types of glands described for monogeneans do not express the acTub epitope.

In the *Hymenolepis* oncosphere, a complex system of muscle fibers underlies the superficial epidermal glands (Fig. 4). Three main groups of fibers can be distinguished (nomenclature after Ubelaker 1980). Capping the anterior part of the larva are the outer longitudinal and inner transverse epidermal muscles (EL, ET in Fig. 4), which are connected to the epidermal epithelium or superficial epidermal glands, respectively. In the posterior larva, a superficial system of muscles inserting at the collar

region, and a deep system inserting at the base of the shank, are responsible for hooklet movements.

The musculature is innervated by a pair of neurons that stain with anti-acTub antibody (Fig. 2A, D–F). The cell bodies lie in the midline near the anterior tip of the larva, underneath the base of the penetration gland. Axons extend posteriorly and branch profusely as they enter the tightly packed bundles of muscles attached to the shanks of the hooklets (Fig. 2E, F). Branches grow along the deep muscle fibers. Our preparations did not permit us to determine whether superficial muscles also received neurite branches.

Morphogenesis of the *Hymenolepis* embryo

The beginning of cleavage and concomitant formation of embryonic envelopes has been followed in several cestode species (reviewed in Rybicka 1966; Ubelaker 1980). By contrast, morphogenesis is largely unknown, with the exception of the formation of the hooklets (Swiderski 1973). The labeling of epidermal gland and neural precursors in the present study provides a step towards understanding morphogenesis in the highly derived cestode embryo.

According to the classical literature, the oocyte of *Hymenolepis* cleaves totally and unequally, giving rise to several large macromeres and micromeres during the first few divisions (Fig. 1A). The macromeres fuse into a syncytium that grows around the cleaving mass of micromeres and develops into the outer and inner envelope. The so-called embryophore, a tough membrane around the embryo, is laid down within the cytoplasm of the inner envelope, which represents the homolog of the primary epidermis.

Surrounded by the envelopes, the postcleavage embryo forms a bilaterally symmetric mesenchyme of approximately 50 cells. Before the onset of cellular differentiation, no organization into germ layers can be observed. Thus, as has been described for the free-living neophoran platyhelminths (Hartenstein and Ehlers 2000), gastrulation is absent in cestode embryogenesis. Differentiation sets in with the formation of the hooklets, a process that has been well described light and electron microscopically (Swiderski 1973; Fig. 5). Hooklets are secreted intracellularly within six large cells, called oncoblasts, that are aligned around the posterior surface of the embryo. The growth and shape changes of the hooklets provide easily detectable criteria based on which the morphogenetic phase of cestode embryogenesis was subdivided into five stages (Swiderski 1973). Stage 1 is defined by the first appearance of the hooklet primordia adjacent to the large ovoid nuclei of the oncoblasts. Hooklets are 50–75% in length of the oncoblast during stage 2, reach the oncoblast membrane by stage 3, and break through the outer oncoblast membrane during stage 4. Hooklets reach their final size and structural differentiation into blade, collar and shank during stage 5.

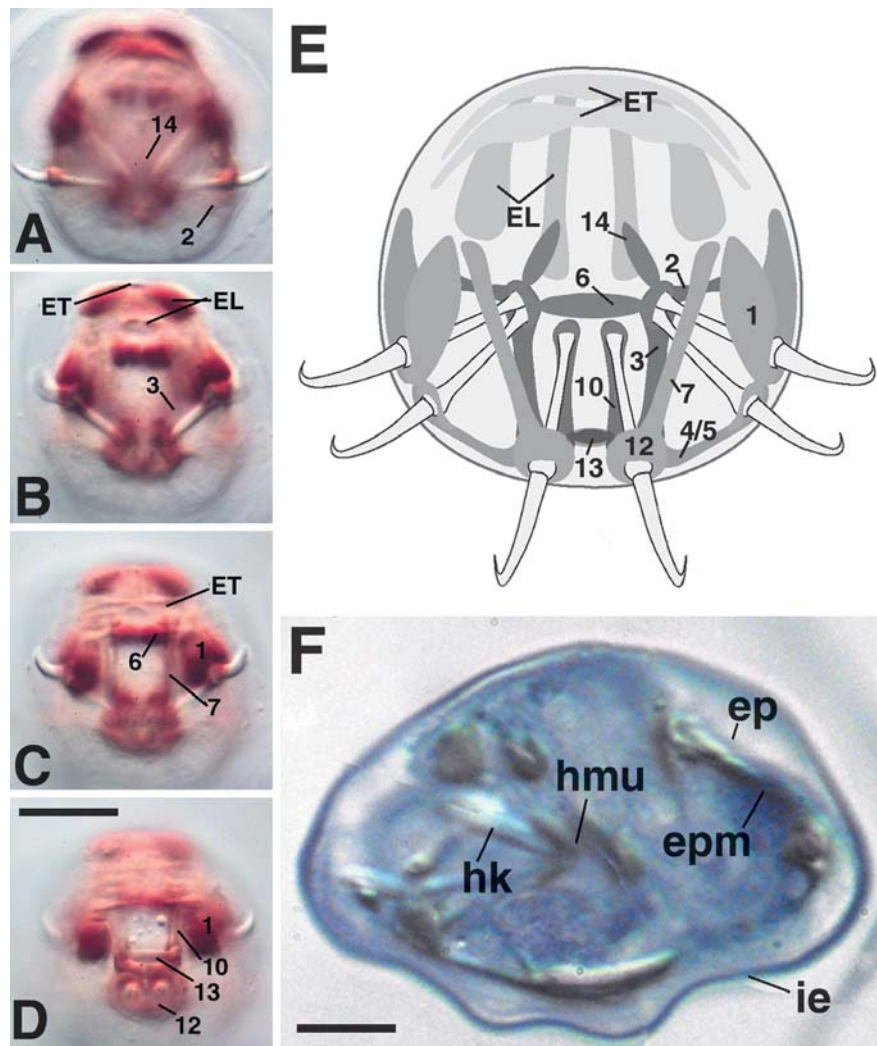


Fig. 4A–F Labeling of *Hymenolepis* oncosphere with phalloidin. **A–D** Micrographs of oncosphere labeled with phalloidin, taken at different focal planes (**A** close to anterior pole, **D** close to posterior pole). Phalloidin labels set of muscle cells associated with the epidermis (**ET** transverse fibers, **EL** longitudinal fibers) and hooklets. *Numbers* refer to hooklet muscle nomenclature introduced by Ubelaker (1980). **E** Schematic diagram of oncosphere, ventral view, anterior to the top. Muscle fibers are indicated and identified by *numbers and letters* (after Ubelaker 1980). Transverse and longitudinal fibers (**ET**, **EL**; light gray) underlie the epidermis of

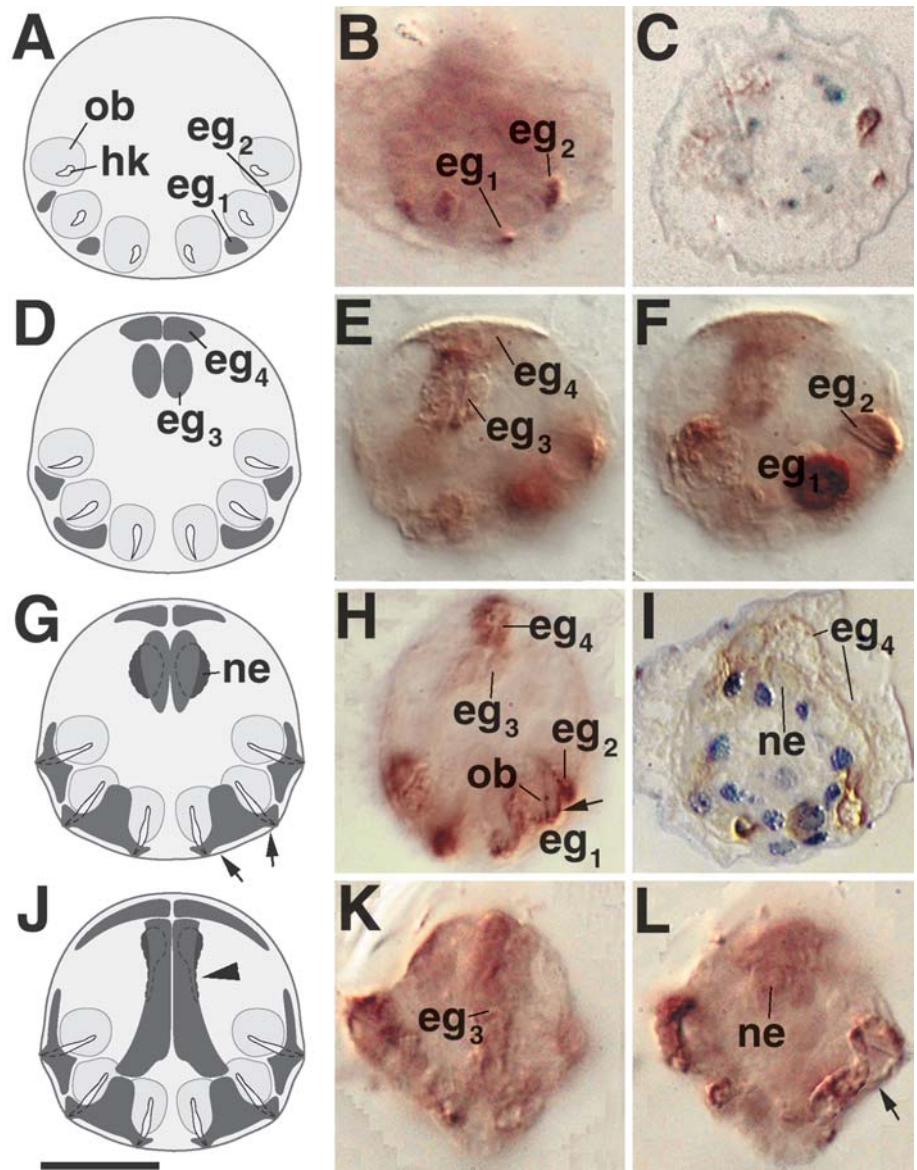
the anterior oncosphere. At the posterior pole, superficial muscles (*medium gray*; 1, 4/5, 7, 12, 13) insert at hooklet collars and epidermis, and deep muscles (*dark gray*; 2, 3, 6, 10, 14) stretch out in between hooklet shanks and epidermis. **F** Sagittal section of oncosphere labeled with phalloidin and counterstained with toluidine blue/methylene blue, showing superficial epidermal muscles (*epm*) at anterior pole and hooklet musculature (*hmu*) in center and posterior pole (*ep* epidermal epithelium, *hk* hooklet, *ie* inner envelope). Bars 10 μm (**A–D**), 5 μm (**F**)

The acTub antibody labels two groups of cells, one superficial group that we interpret as the precursors of the superficial epidermal glands, and a deep pair of cells that give rise to the nervous system of the oncosphere. Epidermal gland precursors start out as two pairs of superficially located cells at the posterior pole of the embryo during stage 1 (Fig. 5A–C). At this early stage, only small cap-shaped patches of staining adjacent to the lateral pairs of oncoblasts are labeled. During stage 2, the whole cells can be seen; they are located between the oncoblasts (Fig. 5D–F). In many preparations, it appears that one of the oncoblasts (typically only on one side of the embryo) expresses acTub as well (Fig. 5F). Given

that, at later stages, oncoblasts themselves are never labeled, we assume that this early occasional oncoblast staining represents an artifact, related to the close association of the labeled epidermal gland precursor with the oncoblast. An additional pair of acTub-positive epidermal gland precursors located anterior-medially joins the pattern during stage 2, followed by another dorsomedially located, elongated pair of cells slightly during stage 2 (Fig. 5D, E).

Epidermal gland precursors flatten and spread around the surface of the embryo during stages 3–5. The posterior cells start out by sending out darkly labeled “tentacle-like” processes around the tips of the hooklets as these

Fig. 5A–L Development of the nervous system and epidermal glands. *Panels of the left column (A, D, G, J)* show schematic representations of *Hymenolepis* embryos at stages 1, 2, 3 and 4, respectively (staging based on hooklet morphogenesis, after Swiderski 1973; see text). Oncoblasts (*ob*) and hooklets (*hk*) are rendered in light gray; epidermal gland cells (*eg*₁–*eg*₄) are shown in medium gray, neurons (*ne*) in dark gray. *Panels of intermediate and right columns* show micrographs of whole-mounts (**B, E, F, H, K, L**) and sections (**C, I**; counterstained with methylene blue) of acTub-labeled embryos at consecutive stages. **A–C** Stage 1. AcTub labeling appears in apices of cells *eg*₁ and *eg*₂ which are intercalated between oncoblasts. **D–F** Stage 2. Labeling of *eg*₁ and *eg*₂ has expanded. Two cells located at anterior pole, *eg*₃ and *eg*₄, commence acTub expression. **G–I** Stage 3. Epidermal gland cells *eg*₁ and *eg*₂ send processes around the collar region of adjacent hooklets (*arrows*). Pair of neurons (*ne*) starts expressing acTub. **J–L** Stage 4. Epidermal gland cells spread around surface of embryo. Neurons send axons posteriorly (*arrowhead*). Bar 10 μ m



penetrate the oncoblast membrane (Fig. 5G–I). Subsequently, flat processes of the posterior epidermal gland precursors grow anteriorly to meet similar processes of the anterior cells. At some point individual cells seem to fuse and form a syncytium, although this process would require electron-microscopic confirmation. By stage 5 (i.e., the mature oncosphere), a thin acTub-positive sheath almost completely surrounds the embryo (Fig. 2C).

Neural precursors appear during stage 2 as a tight pair of small, ovoid cells located interiorly near the anterior tip of the embryo. They are adjacent to the pair of anterior-medial epidermal gland precursors described above. During stage 4, neural precursors grow in size and send out short processes posteriorly. At that point myoblasts differentiate to form muscle fibers around the hooklets and bodywall (Fig. 6). As the deep system of muscle fibers inserting at the hooklet shanks grows interiorly, axons establish contact with them and branch into

numerous processes. Coordinated movement of hooklets arises by late stage 5.

Discussion

Despite its extreme reduction in overall cell number and different cell types, the oncosphere larva of cestodes exhibits several fundamental similarities with larvae or juveniles of other flatworms, both in regard to its morphology and development. Using an antibody against acTub, we show that an anterior “brain,” possibly consisting of only two neurons, is present in the oncosphere. The same marker also provides further insight into the formation of the secondary epidermal bodywall.

The early development of various species of cestodes has been described by several authors (reviewed in

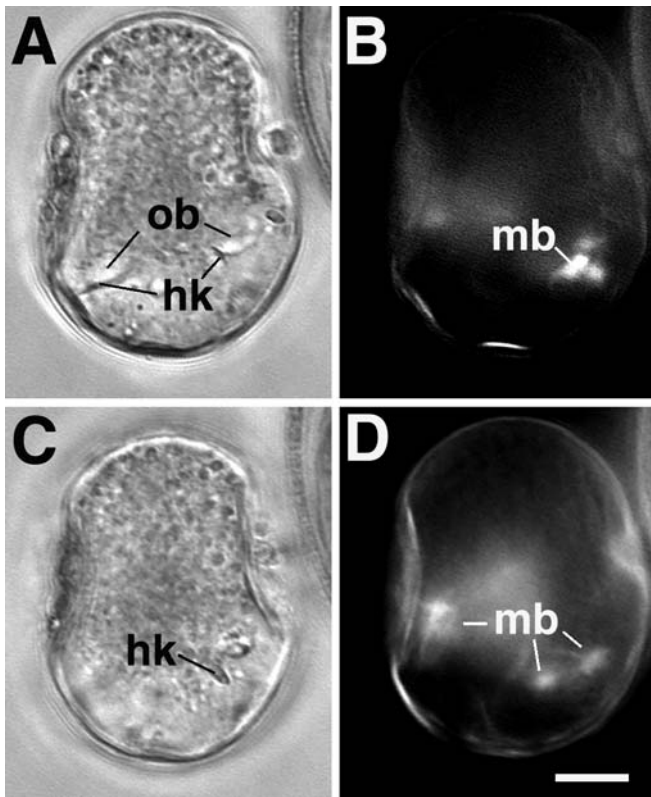


Fig. 6A–D Development of the musculature. **A–D** Two focal planes (**A, B** dorsoanterior; **C, D** ventroposterior) of phalloidin-labeled whole-mount of stage 3 embryo. **A** and **B** show outline of embryo, hooklet rudiments (*hk*) and onco blasts (*ob*) in Nomarski optics. **C** and **D** are fluorescence-microscopic images of the same confocal planes, showing first accumulation of phalloidin-positive actin bundles in rounded myoblasts (*mb*) located adjacent to the onco blasts in periphery of embryo. Bar 5 μ m

Rybicka 1966; Ubelaker 1980). The oocyte, surrounded by a hard outer capsule formed within the ovary, divides totally and unequally. Similar to what has been described for embryos of many rhabdocoel flatworms, no spiral cleavage pattern is apparent. During the early cleavage divisions, five large macromeres separate from the remaining cells, called micromeres, and give rise to two syncytial sheaths surrounding the embryo. The first two macromeres form the outer envelope, the remaining three macromeres/mesomeres the inner envelope. The latter is considered as the primary epidermis; in coracidium larvae of aquatic groups of cestodes (pseudophyllidea; Rybicka 1966) this layer becomes ciliated. The inner envelope does not form cilia in the oncosphere of *Hymenolepis*.

Surrounded by the two envelopes, the micromeres that form the embryo proper undergo several more divisions that increase the overall number of cells to approximately 50. These cells form a bilaterally symmetric, mesenchymal mass. Within this mass, without any prior morphogenetic movements that resemble gastrulation, primordia of organs, or rather “organules” (since they are each composed of only a few cells), crystallize. Organules comprise the hooklets, formed by six onco blasts at the

posterior pole, a set of muscle cells located close to the onco blasts, a simple central nervous system formed by two anteriorly located neurons, a system of gland cells, and the secondary epidermis. In addition, a small number of undifferentiated progenitor cells that will give rise to all tissues of later developmental stages are located at the anterior end of the larva (Ubelaker 1980).

Classical accounts of cestode embryogenesis were unable to recognize nerve cells and by inference must have assumed that the intricate movement patterns of the hooklets by which oncospheres penetrate the gut epithelium of their intermediate hosts are controlled by a myogenic mechanism. Fairweather and Threadgold (1981) recognized neurosecretory granules and synapse-like cell-cell junctions in their electron-microscopic study of the *Hymenolepis* oncosphere. They concluded that the oncosphere did possess a simple nervous system, which appears very plausible in light of the fact that highly coordinated hooklet movements are required for penetrating the host intestinal wall. Employing a paraldehyde-fuchsin stain for neurosecretory material, they obtained further light-microscopic evidence for the existence of neurons, but they were unable to locate them properly. In the present report, we provide further evidence for a simple nervous system, formed by two anteriorly located neurons that send posterior processes into the center of the oncosphere, where they branch and terminate on hooklet muscle fibers. The evidence that these cells are neurons is based on their characteristic shape and location and their expression of acTub. As reported for the embryos of free-living rhabdocoel flatworms, precursors of neurons develop “in situ” within the deep layer of the embryo (Younossi-Hartenstein et al. 2000, 2001). Thus, following cleavage, the embryo of free-living rhabdocoel flatworms forms an undifferentiated mesenchyme, the embryonic primordium, that becomes localized at the ventral side of the egg. Without any foregoing gastrulation movements and formation of epithelial germ layers, organ primordia differentiate. The outer (ventral) cells become the epithelial epidermal primordium. Deep cells near the anterior pole give rise to the brain; those located in the posterior half of the embryonic primordium form muscle, gonads, glands, and protonephridia. We conclude that this general mode of flatworm development can still be discerned in the highly derived *Hymenolepis*.

In addition to neurons, a set of flat, superficially located cells, which we interpret as epidermal gland cells, are labeled with acTub. Acetylated tubulin is laid down in stabilized microtubules typically found in cilia, axons, and other elongated processes. In previous studies on flatworm development in which acTub was utilized, ciliated cells of the epidermis, digestive tract and protonephridia were recognized in a wide range of different species (Hartenstein and Ehlers 2000; Younossi-Hartenstein and Hartenstein 2000, 2001; Younossi-Hartenstein et al. 2001). As shown in this study, the same cell types are labeled by acTub in embryos of the monogenean *Neoheterocotyle* which in development and overall bodyplan closely resemble the embryos of free-

living rhabdocoels (Kearn 1963a, 1963b; Llewellyn 1963; Lyons 1973). In addition to epidermis and protonephridia, a group of gland cells, identifiable by their typical shape and location posterior to the brain, pick up the label. In *Hymenolepis*, protonephridia and digestive cells do not exist. It is therefore most likely that the labeled superficial cells correspond to gland cells. Previous depictions of the oncosphere glandular system are sketchy. Histological and histochemical analyses show a voluminous, U-shaped, syncytial gland underlying the anterior pole of the oncosphere. This gland, called the penetration gland (Reid 1948; Rybicka 1966), is also recognized in the present study; it does not label with acTub. The older light-microscopic literature (e.g., Ogren 1955, 1958) describes a system of supposed gland cells which seem to be more superficial and spread out than the penetration gland, and which were called epidermal glands. These cells could correspond to the acTub-positive epidermal gland cells described in this study. The fact that they form intimate contacts to the collar of the hooklets is yet another argument for the glandular nature of the cells. Since the oncosphere invades the host tissue with the hooklets first, secretory products used for digesting the gut epithelium should be most effective if released near the hooklets. It had puzzled previous authors (e.g., Fairweather and Threadgold 1981) that ducts of the penetration gland proper open only at the anterior side of the oncosphere (which is the trailing side during invasion). In the light of old observations, as well as the findings of this study, it seems most likely that the penetration gland forms only one part of the glandular system, the second part being represented by the epidermal gland cells which are primarily located at the posterior (leading) pole of the oncosphere.

The alternative interpretation of the superficial acTub-positive cells is that they represent epidermal cells. The epidermis of parasitic flatworm is formed by a syncytial epithelium. During early development of these animals, a primary epidermis, often composed of ciliated cells just as in free-living flatworms, forms the outer covering of the embryo. Later, secondary epidermal precursors, located deep inside the animal underneath the muscular layer, send processes towards the surface, where they spread out and eventually replace the primary epidermis (discussed in Ehlers 1985). In *Hymenolepis* and other cestodes, the inner embryonic envelope is supposed to represent the primary epidermis. Previous reports (Rybicka 1973) had suggested that the secondary epidermis is formed by a large binucleated cell located in the center of the oncosphere, the so-called "medullary center," which sends out thin processes that spread as a complete envelope around the surface. It is possible that such internal epidermal precursors do indeed exist and form a thin syncytial envelope located superficially to the acTub-positive epidermal gland cells. Based on Rybicka's description (1973), this syncytial layer would be too thin to be resolved light microscopically. However, it is also possible that the deep medullary cells identified by Rybicka (1973) form only part of the epidermal syncy-

tium, and that the acTub-positive superficial cells represent a second population contributing to this syncytium. We consider this scenario less likely, given that the epidermal syncytium in cestodes is generally formed by deep cells, and that so far no other cases have been reported where acTub labels non-ciliated epidermis.

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