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REGULAR ARTICLE

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The embryonic development of the temnocephalid flatworms *Craspedella pedum* and *Diceratocephala boschmai*

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Abstract We have analyzed the embryonic development of the temnocephalid flatworms *Craspedella pedum* and *Diceratocephala boschmai*, using a combination of fuchsin-labeled whole-mount preparation, histology, and transmission electron microscopy. Following the staging system recently introduced for another flatworm species (*Mesostoma lingua*), we can distinguish eight morphologically defined stages. Temnocephalids produce eggs of the neoophoran type in which a small oocyte is surrounded by a layer of yolk cells. Cleavage takes place in the center of the yolk mass (stages 1–2) and results in an irregular, multilayered disc of mesenchymal cells that moves to the future ventral egg pole (stage 3). Organ primordia, including those of the brain, pharynx, male genital apparatus, sucker, and epidermis "crystallize" within this disc without undergoing gastrulation movements (stage 4). An invagination of the epidermal primordium pushes the embryo back into the center of the yolk ("embryonic invagination"). As a result, organogenesis begins while the embryo is invaginated (stage 5). The brain differentiates into an outer cortex of cell bodies that surround a central neuropile. Precursor cells of the epidermis, pharynx, and protonephridia become organized into epithelia. During stage 6, the embryonic primordium everts back to the surface, where organogenesis and cell differentiation continues. Epidermal cells fuse into a syncytium that expands around the yolk. Myoblasts initially do not spread out in the way epidermal cells do; they remain concentrated in two narrow, longitudinal bands that extend along the sides of the embryo. Three pairs of axon tracts extending posteriorly from the brain follow the bands of myoblasts. Stages 7 and 8 are characterized by the appearance of eye pigmentation, brain condensation, and the formation of tentacles and a sucker that bud out from the epidermis of the anterior and posterior end, respectively. Comparison of morphogenesis in temnocephalids with observations in other flatworm taxa suggests a phylotypic stage for this phylum of invertebrates.

Keywords Platyhelminth · Temnocephalid · Embryo · Development · Morphogenesis · Organogenesis · Differentiation · *Craspedella pedum*, *Diceratocephala boschmai* (Plathelminthes)

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Introduction

Classical comparative morphology has placed Platyhelminthes at a basal position within the spiralian group of bilaterian animals (Ehlers 1985; Ax 1996). Several of the characteristics of flatworms, including a single gut opening, ciliated epidermis, and anteriorly located central nervous system have been considered as plesiomorphies inherited from the ancestral organism that gave rise to both protostomes and deuterostomes. Whereas some molecular phylogenetic data support the basal position of flatworms (Carranza et al. 1997), other studies place them into a more advanced position among the spiralians (Adoutte et al. 1999; Ruiz-Trillo et al. 1999). This makes the platyhelminths a critically important system for the study of developmental processes. Embryogenesis of different taxa of platyhelminths was studied in the early part of the twentieth century by Bresslau (1904, 1909), Haswell (1909), Hallez (1909), Ball (1916), Surface (1908), and Kato (1940). In more recent times, only a few general descriptions of flatworm embryogenesis have been added (proseriates: Giesa 1966; acoels: Apelt 1969; polyclads: Anderson 1977; lecithoepitheliates: Reisinger et al. 1974a, 1974b; prolecithophorans: Newton 1970; triclads: Bennazzi and Gremigni 1982; Baguna and Boyer 1990; well reviewed by Thomas, 1986). Experimental data, addressing the problems of cell lineage and cell fate, have been gathered for representatives of acoels (Boyer 1971; Henry et al. 2000) and polyclads (Boyer et

al. 1996, 1998). Thorough studies of the formation of epidermis (Williams 1977; Tyler 1981; Tyler and Tyler 1997) and body wall muscles (Reiter et al. 1996) have been carried out. However, much remains to be learned about flatworm development, in particular in respect to morphogenesis and organogenesis.

We have recently analyzed embryogenesis in the rhabdocoels *Mesostoma lingua* and *Gieysztoria superba*, and the polyclad *Imogine mcgrathi*, paying particular attention to morphogenetic movements during early development, the formation of epithelia, and the origin of the nervous system (Hartenstein and Ehlers 2000; Younossi-Hartenstein and Hartenstein 2000a, 2000b; Younossi-Hartenstein et al. 2000). Rhabdocoels show a peculiar mode of yolk formation that has a profound effect on early embryogenesis. They produce small oocytes that become invested by a multilayer of yolk cells formed by yolk glands or vitellaria. Cleavage of the oocyte starts in the center of the egg and often does not follow the spiral pattern described for flatworm taxa lacking external yolk cells (archoophora). Gastrulation in the sense of a separation of different germ layers does not take place. Thus, at a stage when several hundreds of blastomeres have been formed, these cells are arranged in a solid aggregate with no apparent subdivision into distinct germ layers. Different organ primordia, including those of the brain, pharynx, and protonephridia, emerge in situ in the deep layers of the embryonic mass. Only after these inner primordia have appeared does the superficial layer of cells on one side of the embryonic mass become organized into an epithelium that will give rise to the epidermis. Initially restricted to a ventral position, the epidermal epithelium expands, mainly by flattening of the individual cells, and eventually encloses the entire yolk.

In this paper we describe the embryonic development of *Craspedella pedum* and *Diceratocephala boschmai*, two species representing another taxon of rhabdocoel flatworms, the Temnocephalida. The phylogenetic position of this clade among the rhabdocoel flatworms is not yet clear; a recent study by Joffe et al. (1998) gives evidence for autapomorphic characters unifying the temnocephalids. Temnocephalids exhibit morphological characteristics and a lifestyle that are shared in part with the free-living "turbellarians" and in part with the parasitic monogeneans. Thus, similar to monogeneans, most temnocephalids have a posterior sucker, and a nonciliated epidermis; they move by muscular contraction similar to leeches (which led the first observers to classify them as small leeches; reviewed by Williams, 1981, 1986). Temnocephalids permanently live as ectocommensals or ectoparasites on crustaceans. *Diceratocephala* sp. and *Craspedella* sp., whose sensory nervous systems were recently investigated by Joffe and Cannon (1998), are found on the freshwater crayfish *Cherax quadricarinatus*. With free-living rhabdocoel flatworms, temnocephalids share numerous characteristics of their reproductive and digestive system, and current taxonomists consider temnocephalids as a taxon of the rhabdocoels with as yet cladistically undefined relationships to the dalyellids and typhloplanoids (Ehlers 1985; Ax 1996).

Using a combination of fuchsin-labeled whole-mount preparation, histology, and transmission electron microscopy, we analyzed the morphogenetic events shaping the temnocephalid embryo. We discuss developmental strategies found in different flatworm taxa and define a "phylotypic stage" that unifies this group of animals.

Material and methods

Animals

C. pedum and *D. boschmai* of all ages live as commensals on the carapace of the crayfish *Cherax quadricarinatus*. They deposit eggs at characteristic locations of the crayfish, typically close to the gills and at joints. Eggs have a thick, brown semitransparent shell that allow one to distinguish key morphological features used for staging (see Results) in the living embryo. Embryos were transferred for fixation to small dishes containing 4% formaldehyde in 0.1 M phosphate-buffered saline (PBS: 0.125 M NaCl; 16.5 mM Na_2HPO_4; 8.5 mM NaH_2PO_4), or 2.5% glutaraldehyde in PBS, respectively. Following fixation, embryos were individually freed of the surrounding membrane with sharpened tungsten pins and transferred to wire-mesh baskets.

Electron microscopy and histology

Embryos were postfixed for 10 min in a mixture of 1% osmium tetroxide and 2% glutaraldehyde in 0.15 M cacodylate buffer (on ice). Specimens were washed several times in PBS and dehydrated in graded ethanol and acetone (all steps on ice). Preparations were left overnight in a 1:1 mixture of Epon and acetone and then for 5–10 h in unpolymerized Epon. They 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. Alternate 1-µm semi-thin sections and sets of 80-nm (silver) ultrathin sections were taken. Ultrathin sections were mounted on net grids (Ted Pella) and treated with uranyl acetate and lead citrate.

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 Ashburner (1989). 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 2 *N* 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, transferred to Epon, and individually mounted on slides.

Immunohistochemistry

To visualize neurons in whole-mount preparations of embryos, a monoclonal antibody against acetylated tubulin (acTub; Sigma) was used. Embryos ranging in age between stage 4 and 8 (stages after Hartenstein and Ehlers 2000) that had been fixed in 4% formaldehyde were washed in PBT (PBS plus 0.3% Triton X-100; for washing, PBT solution was changed three to five times over a 10-min period) and incubated overnight in PBT containing the antibody at 1:1000 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.

Results

Structure of juvenile *Craspedella* sp

C. pedum adults are 2–3 mm in length when extended. They bear five tentacles at the anterior end, right in front of the mouth opening. *C. pedum* are further characterized by two large eye spots and a posterior sucker, by which they attach intermittently to the substrate during their leech-like locomotion. *C. pedum* juveniles resemble miniature adults; they measure approximately 0.3 mm upon hatching.

In its histological structure, *Craspedella* sp. shares most characteristics with other free-living rhabdocoel flatworms, but also shows some features typical for the parasitic neodermatids, in particular the monogeneans. Most importantly, the epidermis is formed by a thin syncytial layer lacking cilia, except for the specialized ciliary endings of sensory neurons (Fig. 1). Histological sections of a juvenile *Craspedella* sp. specimen showed an epidermis that was 2–3 µm in diameter and contained a surprisingly small number of flattened nuclei. A typical cross section of the trunk contained only five to eight nuclei (Fig. 1C); the epidermis of one entire tentacle housed less than ten nuclei. Apically, the epidermal membrane was folded into microvilli and contained rhabdites. Separating the epidermis from the underlying body-wall musculature was a thick basement membrane. Electron-microscopically it contained multiple systems of interlaced fibrils (Fig. 1D, G). Inserting at the basement membrane, the massive body musculature filled most of the internal spaces not taken up by the brain, digestive tract, and gonads. There were circular, oblique, and longitudinal fibers measuring 4–6 µm in diameter underneath the epidermis (Fig. 1B, D), as well as palisades of dorsoventral fibers arranged along the lateral edges of the entire trunk, and in between brain and pharynx (Fig. 1C, E). Muscles also filled the interior of the tentacles and sucker. Muscle cell bodies containing nuclei were clustered at a few locations, in particular anterior to the brain (tentacular muscles), between brain and pharynx (Fig. 1C), and along the lateral edges of the trunk (Fig. 1E). By contrast, the subepidermal muscular layers, tentacles, and sucker contained only myofibril-filled muscle processes and no nuclei.

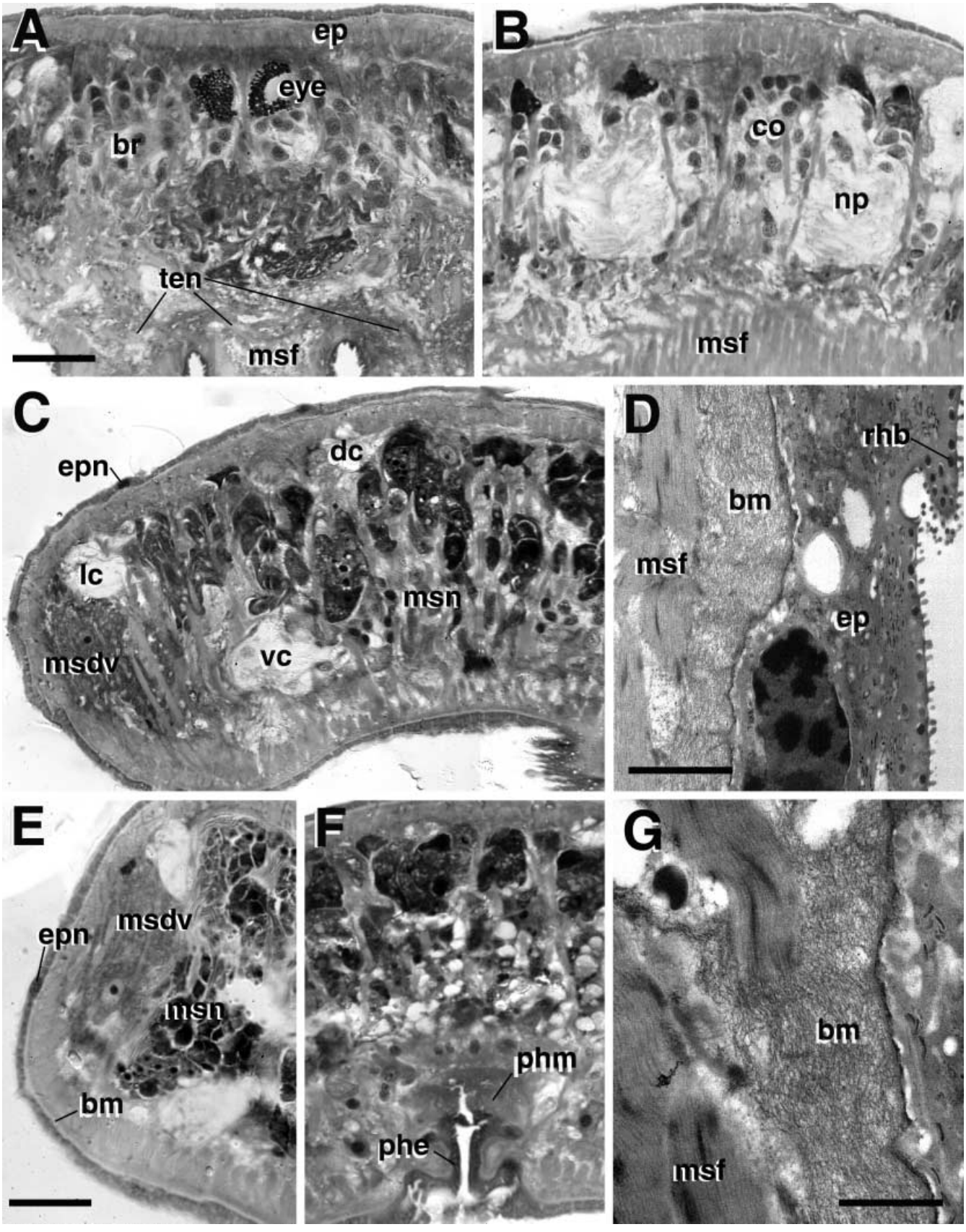
The brain filled most of the anterior one-third of the juvenile body. It was formed by a cortex of ovoid cell bodies, 3–5 cell diameters in thickness, that surrounded a central neuropil (Fig. 1A, B). Structure and development of the *C. pedum* nervous system will be described in more detail in another paper (Younossi-Hartenstein et al. 2001). The brain contains almost all of the nerve cells, including sensory neurons, that innervate the body. Three massive connectives issued from the posterior surface of the brain and traveled at a ventral, lateral, and dorsal level to the posterior end of the animal (Fig. 1C). Brain and connectives were not surrounded by glial sheaths, but flanked by processes of gland cells and muscle fibers. Close to the dorsal midline, the anterior cortex of the brain contained the two cup-shaped, pigmented eyes (Fig. 1A).

The digestive system consisted of a muscular pharynx that opened anteriorly and ventrally behind the brain of the animal (Fig. 1F). The pharynx of the juvenile was rather small. It was lined by a thin, nonciliated epithelium similar in appearance to the epidermis (Fig. 1F) and was surrounded by a basement membrane and layers of radial and circular muscle fibers. Posteriorly, the pharynx abutted the rudimentary gut, an irregularly shaped, spongy mass of cells, intermingled with remnants of yolk, lacking a regular epithelial lining. Many large, mesenchymal cells were scattered around the gut, as well as other locations of the juvenile worm. These cells probably comprised the neoblasts that give rise to the intestinal lining, as well as other structures during postembryonic growth.

The excretory, protonephridial system was formed by an extensively branched tube running along either side of the body and terminating in the lateral head, close to the brain. The main tube was quite large in diameter (Fig. 2B) and lined by a thin epithelial cell. Scattered endings of protonephridial branches (flame cells) were recognizable by bundles of cilia; they occurred scattered throughout the body directly underneath the muscle layer. Close to its exterior opening, the main tube widened into a bulbous ampulla (Fig. 2A), whose wall was surrounded by muscle cells.

The reproductive system of juvenile *C. pedum* was predominantly male and consisted of packets of testis cells aligned on both sides in the posterior half of the animal, and of the penis apparatus located posteriorly. The testes contained masses of polygonal spermatogonia and swirls of differentiated sperm (Fig. 2B, C). The penis apparatus was formed by a roughly transversally oriented, hollow cylinder with a thick, muscular wall (Fig. 2C). Enclosed within this muscular sheath was the penis, a conical, hollow structure made of sclerotized, extracellular matrix material. Surrounding the penis apparatus were several glandular tubes containing granular secretions.

The posterior tip of the juvenile *C. pedum* bore the massive sucker, shaped as a muscle-filled, cylindrical process recessed in a circular fold of the body wall (Fig. 2C). The musculature was formed by multiple layers of obliquely oriented fiber bundles that in a spiraling fashion converged into the sucker.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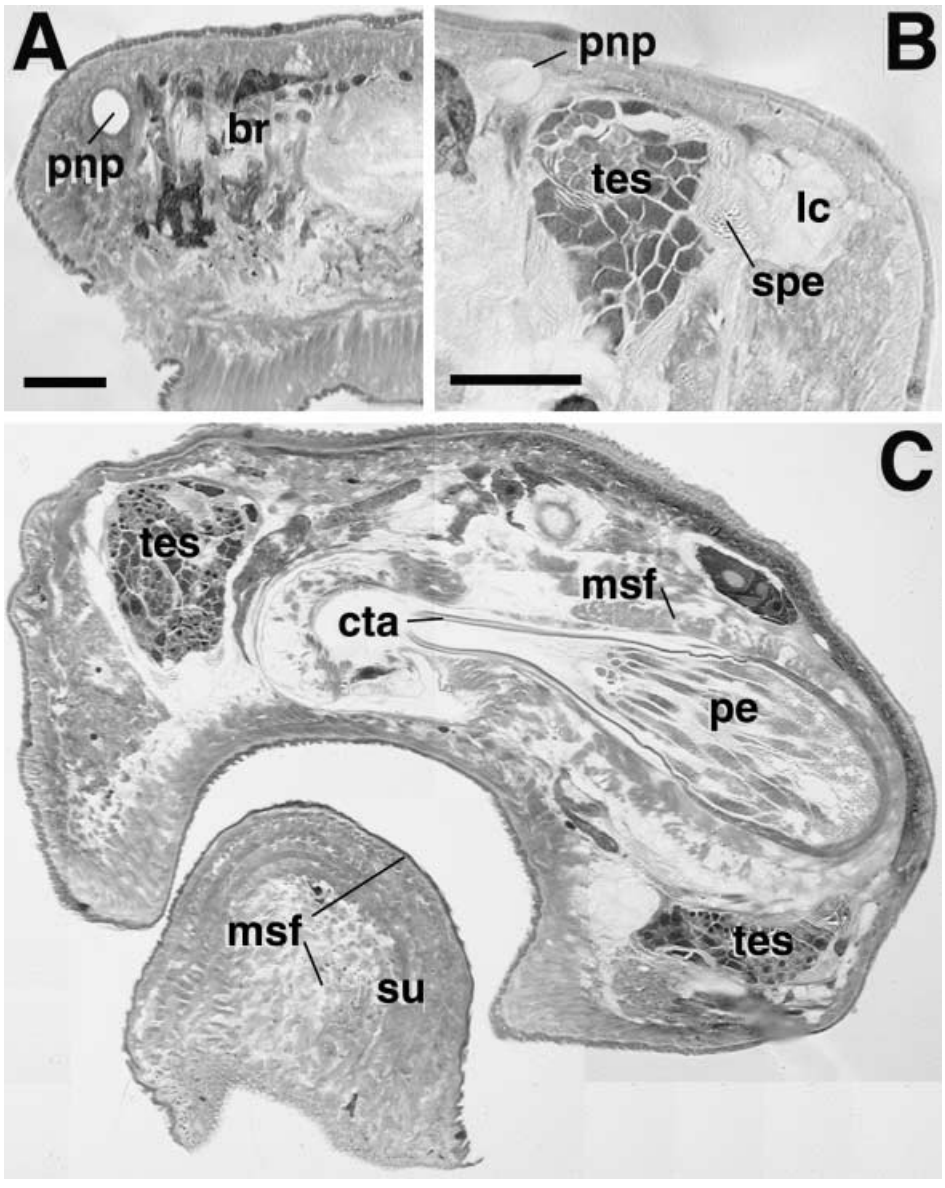


Fig. 2A–C Anatomy of juvenile *C. pedum*. Light-microscopic cross sections at level of brain (**A**), pharynx (**B**), and tail end (**C**). **A** Ampulla of protonephridium (*pnp*) laterally adjacent to brain (*br*). **B** Spatial relationship between longitudinal trunk of protonephridium (*pnp*), testes (*tes*), and lateral connective (*lc*; *spe* spermatozoa). **C** Section of testes (*tes*), male reproductive system (penis, *pe*, with cuticular apparatus, *cta*, and muscular wall, *msf*). Tucked underneath the ventral surface of the body wall is the sucker (*su*), filled with multiple layers of spiraling muscle fibers (*msf*). *Bars* **A, C** 50 µm; **B** 50 µm

◀ **Fig. 1A–G** Anatomy of juvenile *Craspedella pedum*. Light-microscopic (**A–C, E, F**) and electron-microscopic (**D, G**) cross sections of a hatched juvenile in anterior to posterior order. **A** Level of brain (*br*), with eyes (*eye*), tentacles (*ten*), and syncytial epidermis (*ep*). **B** Posterior brain with cortex (*co*) and neuropil (*np*). Body wall and tentacles in **A** are equipped with thick bundles of muscle fibers (*msf*). **C** Level posterior of brain, showing ventral (*vc*), lateral (*lc*), and dorsal (*dc*) connectives, respectively. The syncytial epidermis forms a thin layer with sparse nuclei (*epn*). Cell bodies of muscle cells are clustered at this level, in between brain and pharynx (*msn* muscle cell nuclei). Lateral edges of body are filled with packets of dorsoventral muscle fibers (*msdv*). **D** Electron micrograph of epidermal cell (*ep*) with rhabdites (*rhb*), basement membrane (*bm*), and subepidermal muscle fiber (*msf*). **E** Lateral body wall with epidermal layer and epidermal nuclei (*epn*), basement membrane (*bm*), dorsoventral muscles (*msdv*), and muscle nuclei (*msn*). **F** Level of pharynx, showing thin epithelial layer (*phe*) and muscle layer (*phm*). **G** Electron micrograph of basement membrane (*bm*) and inserting muscle fiber (*msf*). *Bars* **A–C** 50 µm; **E, F** 25 µm; **D** 2 µm; **G** 1 µm

Embryogenesis of *Craspedella* sp.: staging system

In previous studies of the embryonic development of the rhabdocoel flatworms *M. lingua* and *G. superba*, we have introduced a system of stages that can be easily distinguished in live and fixed wholemount material and that represent major morphogenetic events (Hartenstein and Ehlers 2000; Younossi-Hartenstein et al. 2000). In the following, we will adapt these stages for the temnocephalids *Craspedella* sp. and *Diceratocephala* sp. Key morphogenetic events that define the stages are graphically represented in Fig. 3. Although quite different in size, embryos of *C. pedum* and *D. boschmai* develop in a very similar way. The size difference usually reflects cell size rather than cell number. Thus, where cell counts were made on representative histological section and whole-mounts, figures were similar in both temnocephalid species. Cells and nuclei of *Diceratocephala* sp.

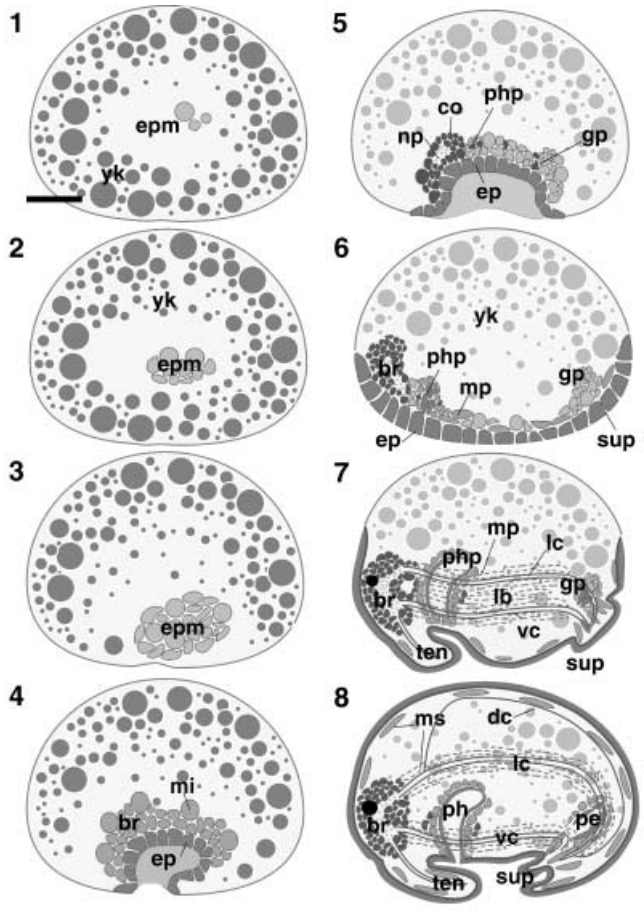


Fig. 3 Stages of *C. pedum* development. Schematic drawings of sagittal sections of embryos of increasing age, showing the major events of temnocephalid embryogenesis. Anterior is to the *left* and dorsal to the *top*. The *number at the top left* of each drawing indicates the embryonic stage as defined in the text. Cleavage of the embryo primordium (*epm*) that is located in the center of the egg and surrounded by yolk cells (*yk*) takes place during stages *1* and *2*. At stage *3*, the embryonic primordium shifts toward the ventral surface of the egg. Stage *4* is characterized by embryonic invagination. The surface layer of the embryonic primordium starts forming an epithelium, the epidermal primordium (*ep*), which invaginates back into the embryo. Deep mesenchymal cells lining the basal surface of the epidermal primordium form local densities that contain the precursors of the pharynx and brain (*br*). Mitotic figures (*mi*) are still abundant in the deep layers. By stage *5*, neurons of the brain have formed a central neuropil (*np*), surrounded by a dense cell body layer (cortex, *co*). The primordia of the pharynx (*php*) and male genitalia (*gp*) form marked cellular conglomerations in the deep layer of the embryonic primordium. During stage *6*, epidermal primordium everts back to the surface and starts to grow dorsally around the yolk. Muscle precursors (*mp*) differentiate in the subepidermal layer and form a system of circular and radial fibers in the pharynx wall. Stage *7* is characterized by the darkening of eye pigmentation (the eyespots had become visible in whole-mounted specimens by the end of stage 6), brain condensation, and near complete enclosure of the yolk by epidermis. Ventral and posterior to the brain, tentacle buds (*ten*) grow out. At the posterior end of the embryo, the circular primordium of the sucker (*sup*) takes shape. Two thick pairs of neuronal connectives (*lc* lateral connective, *vc* ventral connective) grow out of the brain. Precursors of musculature (*mp*) and protonephridia are clustered along the connectives. Axons and surrounding muscle/nephridial precursors form distinctive "lateral bands"(*lb*). Stage *8* describes the fully dorsally closed and differentiated embryo. Muscle contraction moves the body in the egg shell (*dc* dorsal connective, *ms* muscle cells, *pe* penis, *ph* pharynx). *Bar* 25 µm

were 2–3 times larger in diameter than cells of *Craspedella* sp. The other main structural difference between the two genera was the absence of a sucker from *Diceratocephala* sp., and, ultrastructurally, the formation of a sparse ciliation of the epidermis in *Diceratocephala* sp., absent from *Craspedella* sp. The following description of development refers to both species, unless differences are explicitly denoted.

Cleavage and the formation of the embryonic primordium (stages 1–3)

During early development, the embryo of temnocephalids form a dividing mass of cells in the center of the egg. This embryonic primordium was surrounded by a layer of 200–300 yolk cells (Figs. 3, 4). At some early developmental stage, the cytoplasm of yolk cells fused, forming a multinucleate syncytium. Most yolk nuclei initially formed a peripheral layer underneath the egg surface, leaving a 5- to 8-µm-wide cytoplasmic rim outside the nuclear layer (Fig. 4A). At later stages, yolk nuclei became distributed more evenly throughout the egg (Fig. 4B, C). Yolk cells did not appear to divide; from early to late stages, approximately the same number of nuclei were distinguished in fuchsin-stained whole mount preparations.

Stage 1 of development corresponded to early cleavage divisions. The embryonic primordium was too small to be perceived through the egg shell in living material. In fuchsin-stained material, the embryo formed an irregular cluster of cells (Figs. 3, 4A). Embryonic nuclei were slightly smaller and denser than yolk nuclei. No clear spiral pattern of cleavage was discerned; however, markers that stain structures of dividing cells more specifically need to be employed to analyze in detail the pattern of early cleavage divisions.

Stage 2 was reached during later cleavage, when the embryonic primordium contained approximately 50–150 cells. These cells were arranged in the center of the egg as an ovoid cluster of approximately 6–8 cells in diameter and 3–4 cells in thickness (Fig. 4B). Mitotic activity was scattered throughout the disc. Bilateral symmetry was generated when, during stage 3 (approximately 200–600 cells), the embryonic primordium adopted a "butterfly"-like shape, with the two symmetrically arranged "wings" forming the left and right side of the embryo, respectively (Figs. 3, 4C).

Early phase of organogenesis (stage 4–5)

In temnocephalids, as in other members of the rhabdocoel flatworms, no gastrulation in the sense of a separation of different germ layers takes place. Organ primordia, in particular the two brain hemispheres located anteriolaterally, appear directly in situ within the mesenchymal mass of cells that constitute the embryonic primordium. This event demarcates stage 4 of development

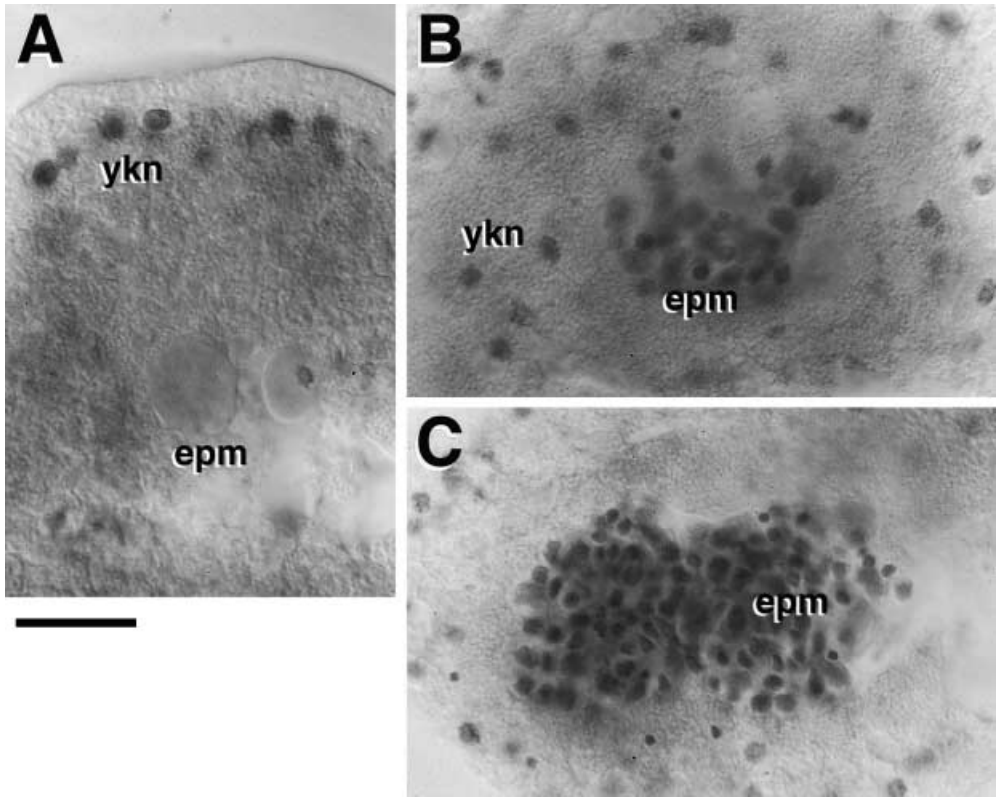


Fig. 4A–C Early stages of *C. pedum* development. Whole-mounts of embryos stained with basic fuchsin in ventral view. **A** Stage 1. Embryonic cells (*epm*) are fixed during early cleavage. Nuclei of yolk cells (*ykn*) are arranged around the periphery of the egg. **B** Stage 2, late cleavage. Embyo is formed from an irregularly shaped disc, the embryonic primordium (*epm*), which is several layers thick and located in the center of the egg. Yolk nuclei (*ykn*) are distributed evenly throughout egg. **C** Stage 3. Embryonic primordium (*epm*) has grown and contains approximately 400–600 cells. It is bilaterally symmetric and resembles a butterfly in shape. Mitoses are scattered throughout the embryonic primordium. *Bar* 20 µm

(Figs. 3, 5A). At the same time, when the densely clustered, postmitotic cells of the brain became distinct, a small group of cells located more medially and posteriorly than the brain became organized into a cup-shaped epithelium that formed the early primordium of the epidermis (Figs. 5A, B, 6A, C, D). The epidermal primordium existed as an invaginated structure, with the apical surface of the cuboidal epidermal cells facing toward the inner lumen of the primordium. We called this process by which the embryonic primordium was folded interiorly "embryonic invagination."

During stage 5, with the epidermal primordium still invaginated, differentiation of the epidermis and the nervous system began. Epidermal cells fused into a syncytial layer. The irregularly spaced nuclei of the epidermal syncytium formed irregularly scattered "bumps" (Fig. 6C–E). Differentiating neurons formed a neuropil in the center of the brain (Figs. 5E, 6C). Beside the nervous system, other organs started to appear. In between the posterior aspects of the brain hemispheres and immediately in front of the epidermal invagination, the pharynx formed. It comprised a small, central group of cuboidal cells that represented the pharynx epithelium, surrounded by a ring of small pharyngeal myoblasts (Figs. 5E, 6B, C). Connecting the pharynx to the brain on either side were groups of cells which probably represented precursors of neurons innervating the pharynx. Cells on either side of the epidermal invagination became arranged in a palisade-like manner and formed the myoblasts of the body-wall musculature (Figs. 5D, 6B, D). Cells located posterior to the epidermal invagination condensed and formed the primordium of the male genitalia and testes (Fig. 5D). A circular arrangement of epidermal cells at the posterior tip of the embryo defined the primordium of the sucker in *C. pedum* (Fig. 5D). Mitotic activity all but ceased with the end of stage 5.

Late organogenesis and cytodifferentiation (stages 6–8)

During stage 6 the formerly invaginated epidermal primordium everted and covered the ventral aspect of the egg (Figs. 3, 7, 8). Subsequently, the epidermis gradually stretched over the entire yolk. Since no cell division occurred, epidermal cells increased in surface area and became extremely thin. By contrast, myoblasts did not spread out in the way epidermal cells did; initially they remained concentrated in two longitudinal bands that extended along the sides of the embryo (Figs. 7A, 8C). Electron-microscopically, the first myofibrils were recognized. A basement membrane formed underneath the syncytial epidermal layer. The brain became compacted into a dumbbell-shaped mass of densely packed neurons surrounding a central neuropil (Figs. 7B, 8B, C). On either side, bundles of axons grew out posteriorly, following the bands of myoblasts (Figs. 7A, C, 8C). These axons formed the connectives that extended along the body wall and converged posteriorly to terminate among the myoblasts of the sucker (Fig. 7E). Antiacetylated tubulin-labeled preparations also showed the beginning of the pro-

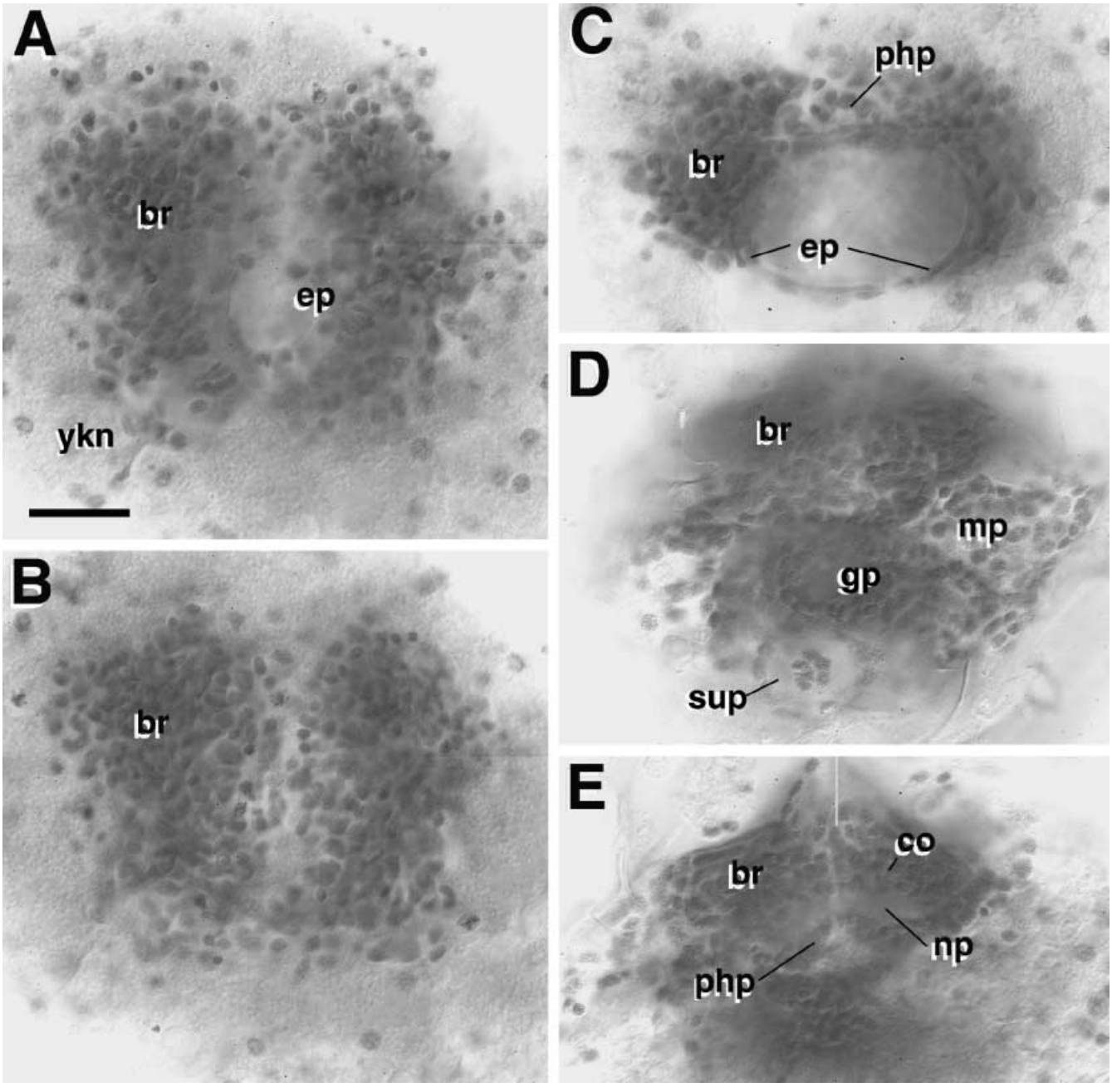


Fig. 5A–E Early organogenesis in *C. pedum*. Whole-mounts of embryos stained with basic fuchsin in ventral view. **A, B** Deep (**A**) and superficial (**B**) focal plane of stage 4 embryo. In the anterior of the embryonic primordium, the bilaterally symmetric brain hemispheres (*br*) have crystallized. Superficial cells in the center of the embryonic primordium have been epithelialized and represent the epidermal primordium (*ep*). Along with the primordia of brain and inner organs, the epidermal primordium has invaginated into the interior of the embryo. Yolk nuclei (*ykn*) are still visible throughout the egg. **C** Ventroanterior view of stage 4 embryo. The invaginated epidermal primordium (*ep*) has widened and deepened. The small pharynx primordium (*php*) is visible in the midline of the embryo, slightly posterior to the brain hemispheres. **D, E** Ventral view of stage 5 embryo; deep focal plane (**D**) and superficial focal plane (**E**). Neurons have started to differentiate and form a superficial cortex (*co*) surrounding the central neuropil (*np*). The center of the embryonic primordium is dominated by a large concentration of cells that form the primordium of the male genitalia (*gp*). Concentrically arranged cells at the tail end form the primordium of the sucker (*sup*). Myoblasts (*mp*) and progenitors of the protonephridia are concentrated in two longitudinal bands along the lateral edges of the embryonic primordium. *Bar* 20 µm

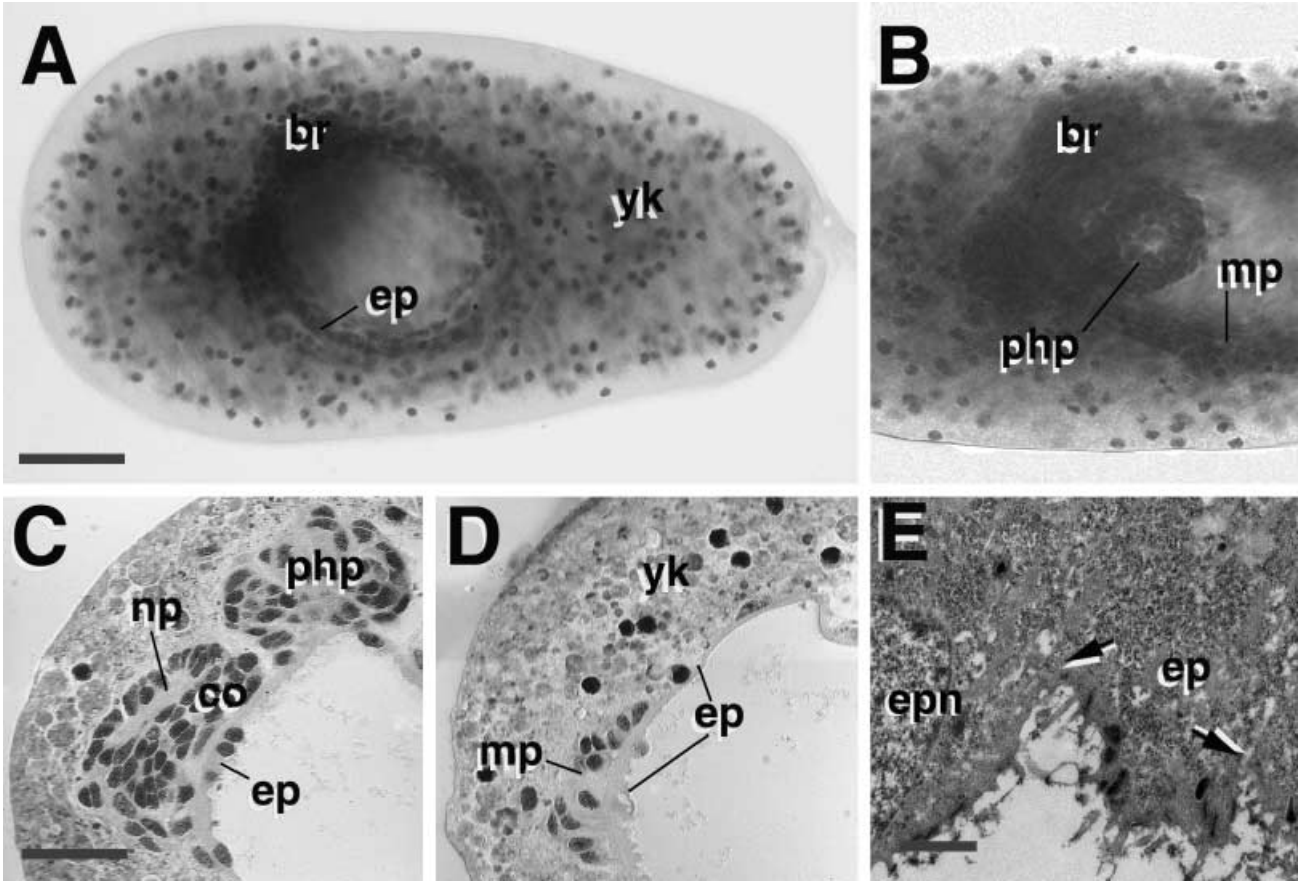


Fig. 6A–E Early organogenesis in *Diceratocephala boschmai*. **A, B** Fuchsin-stained whole-mount of stage 5 embryo in ventral view; superficial focal plane (**A**) and deep focal plane (**B**). The invagination of the epidermal primordium (*ep*) is much more pronounced than in *C. pedum* (see Fig. 5). The brain hemispheres (*br*) and pharynx primordium (*php*) are attached to the anterior wall of the invaginated epidermis. Muscle precursors (*mp*) are concentrated in two longitudinal bands running alongside the lateral walls of the epidermal invagination. **C, D** Cross sections of stage 5 embryo: level of brain (**C**) and center of epidermal invagination (**D**). Only the left half of the embryo is shown. The epidermal primordium forms a thin syncytial layer. In **C**, primordium of brain (*co* cortex; *np* neuropile) and pharynx (*php*) is visible. A photograph of the posterior section in **C** reveals the bands of muscle precursors (*mp*) in the lateral wall of epidermal invagination. **E** Electron micrograph of epidermal primordium (*ep*) of stage 5 embryo. Nuclei (*epn*) protrude into the lumen of the epidermal invagination. Apical membrane invaginations between epidermal cells are still visible (*arrows*), but complete membranes in between cells are absent. *Bars* **A, B** 50 µm; **C, D** 25 µm; **E** 2 µm

tonephridial system in the form of an irregular row of 12–15 scattered flame cells distributed lateral to the myoblast bands (Fig. 7C). Unlike the staining pattern which Hartenstein and Ehlers (2000) observed in the typhloplanoid *Mesostoma lingua*, where the ciliated channel cells forming the longitudinal trunks of the protonephridial system were strongly labeled with acTub, these trunks did not stain at any stage in *Craspedella* sp. embryos.

Stage 7 was defined by the appearance of pigmented eyes in the anterior cortex of the brain (Figs. 3, 7D). During this stage, a row of five transversally oriented, hemispherical buds located ventrally and posteriorly to the brain initiated the development of the tentacles (Fig. 7E). From the beginning, the tentacular buds were covered with a thin, syncytial epidermis and were filled with muscle and gland cell processes, as well as sensory axons that grew out of neurons located within the brain. The sucker differentiated in a similar fashion. The tail end of the embryo bent over ventrally and anterior. As a result, the tips of the growing tentacles lay next to the tail of the embryo during late stage 7 and stage 8 (Figs. 3, 9D).

The pharynx of a stage 7 *C. pedum* and *D. boschmai* embryo is comprised of four regular, concentrically arranged layers. In the center, the pharyngeal epithelium has formed. Two small groups of cells, one ventrally and the other more dorsally, were assigned to the pharynx epithelium in fuchsin whole-mount preparations and sections (Figs. 7D, 8C). As the overlying epidermis sank inward, the pharyngeal epithelium became continuous with the epidermal epithelium. The epithelium was surrounded by an inner belt of myoblasts that gave rise to the circular muscles of the pharynx, and an outer belt of myoblasts that formed the longitudinal and/or obliquely organized musculature that was visible underneath the circular muscles in the juvenile worm. The outermost layer of the embryonic pharynx contained more irregularly arranged, flattened cells whose later fate was not be followed.

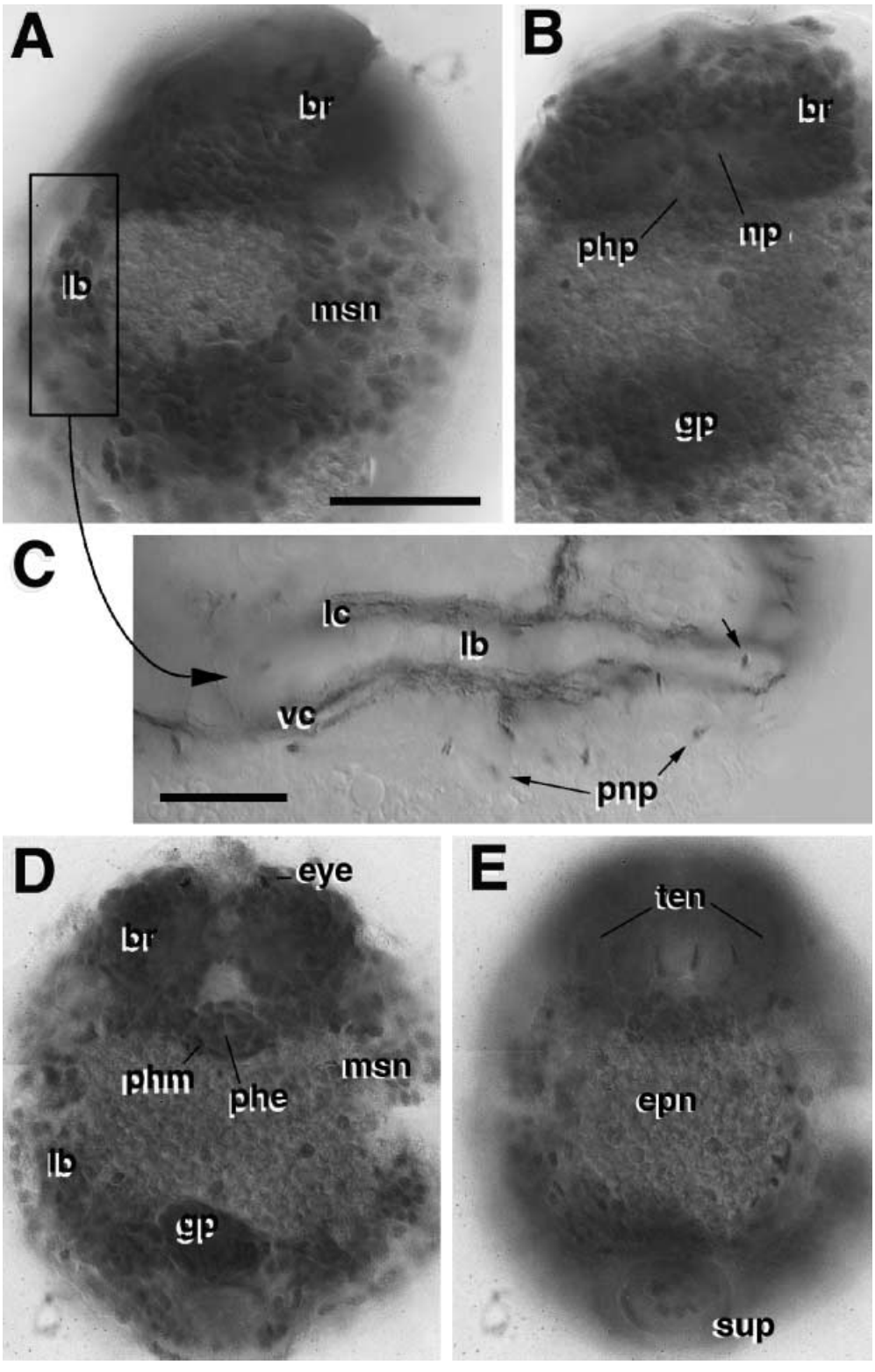


Fig. 7A–E Late organogenesis in *C. pedum*. **A, B** Fuchsin-stained whole-mounts of stage 6 embryo in ventral view. The superficial focal plane (**A**) shows condensations of muscle precursors (*msn*) that form longitudinal bands (*lb*). Brain (*br, np* neuropil), pharynx primordium (*php*), and male genital primordium (*gp*) are visible in the deep focal plane (**B**). **C** Lateral part of stage 6 embryo (outlined by *box* in **A**) labeled with antibody against acetylated tubulin to visualize axons and protonephridial cells (*brown product*). Axons form a ventral (*vc*) and lateral connective (*lc*) that run alongside longitudinal band (*lb*) formed by muscle precursors (not stained). Of the protonephridial system, only precursors of flame cells (*pnp*) are labeled. These cells are aligned in several irregular rows along the lateral edge of the longitudinal band. **D, E** Fuchsin-stained whole-mounts of deep focal plane (**D**), and superficial focal plane (**E**) of stage 7 embryo; ventral view. Stage 7 is characterized by the condensation of the brain (*br*) and pigmentation of the eyes (*eye*). The pharynx primordium exhibits inner epithelial layer (*phe*) surrounded by pharynx muscle precursors (*phm*). The epidermis (*epn* epidermal nuclei) forms a thin syncytial layer covering the ventral half of the embryo. In front of the pharynx primordium, the epidermis has folded into 5 tentacle buds (*ten*). Genital primordium (*gp*) and sucker primordium (*sup*) have moved posteriorly. *Bars* **A, B, D, E** 50 µm; **C** 10 µm

The primordium of the penis apparatus adopted a multilayered appearance during stage 7 (Fig. 7D). In its center a nuclear-free ovoid space formed, indicating the initial stage in the formation of the cuticular apparatus of the penis. Surrounding this structure were three layers of cells that corresponded to the epithelial lining and the muscular wall of the penis apparatus. On either side of the penis apparatus and more anteriorly, irregular clusters of cells, located deeper than the body-wall myoblasts, were thought to constitute primordia of the testes.

Stage 8 was characterized by the condensation of the brain that brought the two eye spots, which were now

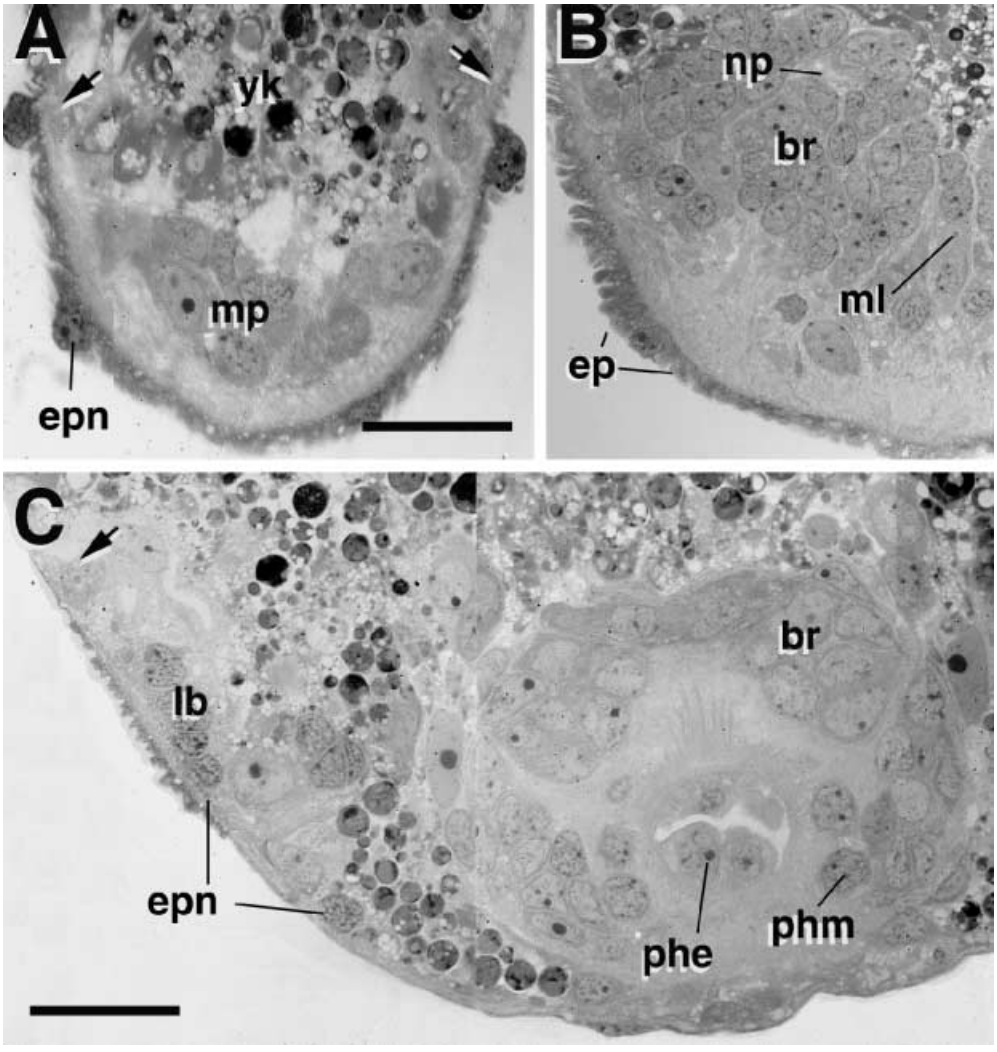


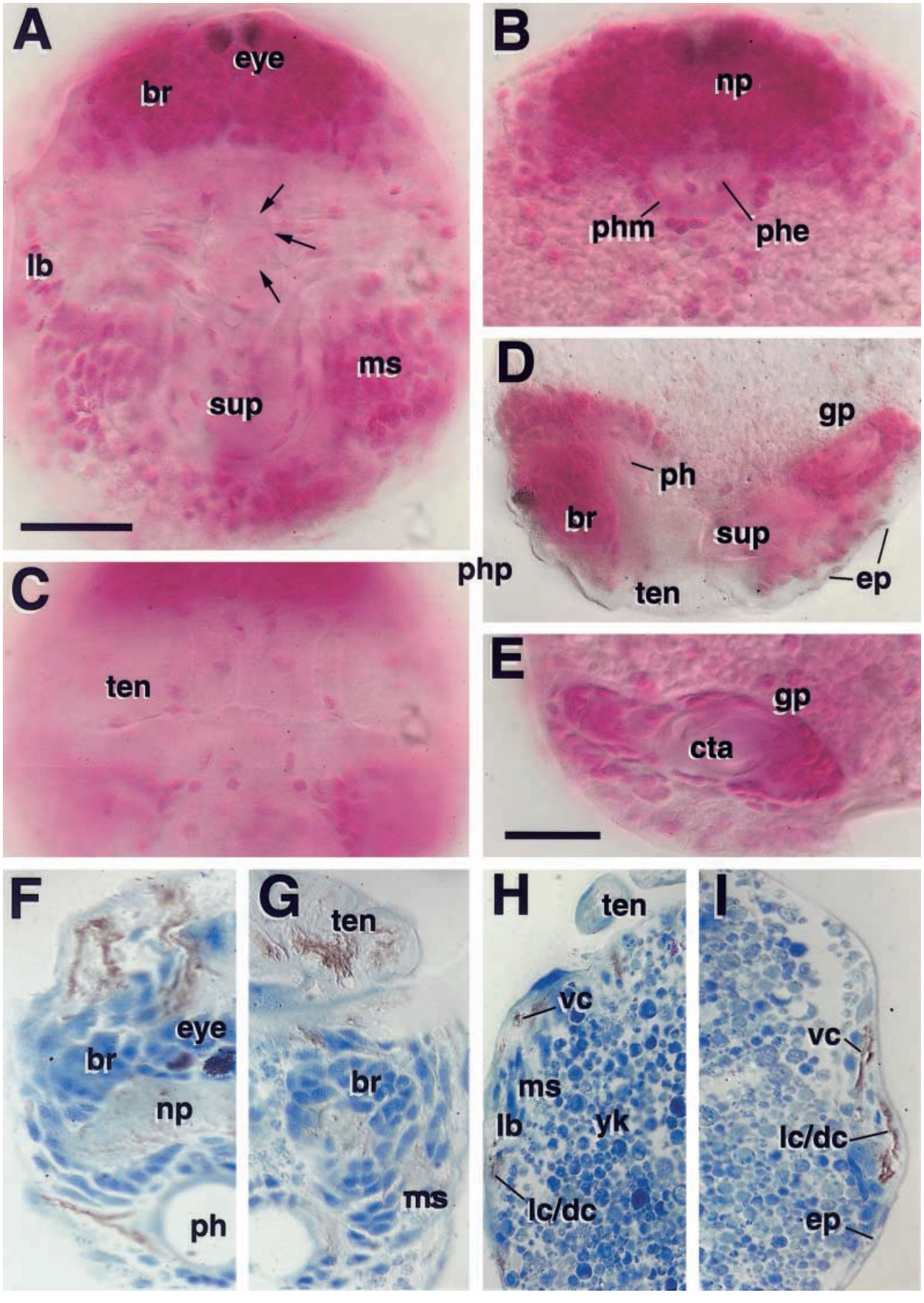
Fig. 8A–C Late organogenesis (stage 7) in *D. boschmai*. Cross sections; ventral is at the *bottom*. **A** Cross section at level anterior of brain. *Arrows* demarcate the lateral edges of the epidermal primordium (*epn* epidermal nuclei). Masses of large cells, most likely the cell bodies of muscle precursors (*mp*) that will form the tentacular muscles, are concentrated at this anterior location. **B** Section at brain level (*br*; only the left half is shown). Note conspicuous septum formed by midline cells (*ml*), neuropil (*np*), and thin epidermal layer (*ep*). **C** Posterior aspect of brain (*br*), pharynx (*phe* pharynx epithelium, *phm* pharyngeal muscles), and begining of longitudinal bands (*lb*). *Bars* **A, B** 50 µm; **C** 25 µm

large and darkly pigmented, next to each other (Figs. 3, 9). Tentacles and posterior sucker were extended. The epidermis had overgrown the yolk as a thin layer. Apart from the epidermis, all other embryonic structures, including brain, pharynx, somatic muscles, tentacles, and sucker were huddled close to each other in a small, ventral domain comprising less than one-quarter of the volume of the egg (Fig. 8D). As a result, the epidermal syncytium covering the ventral body wall, tentacles, and sucker was thrown into numerous longitudinal and transverse folds (Fig. 9A) that later became smooth as these structures enlarged and moved to their final positions. Somatic muscle fibers were still only recognized in a relatively narrow ventrolateral band (Fig. 9F–I); from there they would spread out to form a complete muscular tube underlying the epidermis, as seen in juvenile animals.

The pharynx had differentiated and a cylindrical cavity communicating with the exterior was recognizable (Fig. 9B, D). In whole-mount preparations, a thick nuclear-free layer demarcated the muscle fibers that surrounded the epithelium (Fig. 9B). Muscle cell nuclei formed a ring that had expanded a lot since stage 7; the previously clearcut distinction between three separate layers was no longer visible. The penis apparatus showed an inner lumen, lined by a thin epithelial layer that communicated with the outside through a genital pore. The conical cuticular apparatus of the penis had formed and filled most of the lumen (Fig. 9D, E).

Discussion

In this paper we have described the sequence of morphogenetic events shaping embryonic development of the temnocephalid *C. pedum* and included observations on some selected stages of *Diceratocephala* sp. We have used a morphological staging system introduced in our recent paper on *M. lingua* and extended to other flatworm species (Hartenstein and Ehlers 2000; Younossi-Hartenstein and Hartenstein 2000a, 2000b; Younossi-Hartenstein et al. 2000). The underlying assumption is that the temporal sequence in which homologous morphogenetic events occur, and the spatial framework in which these events unfold, is sufficiently similar be-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tween temnocephalids and other rhabdocoel flatworms, so that corresponding stages can be identified. This assumption turned out to be correct.

The eight stages defined for *Mesostoma* sp. can be recognized easily in *Craspedella* sp. and *Diceratocephala* sp., although we do not know the absolute length of stages, since individual embryos were not followed from beginning to end of development. As members of the neoophoran clade of flatworms, temnocephalids produce ectolecithal eggs, characterized by small oocytes surrounded by a thick layer of yolk cells. Oocytes cleave in a pattern that seems to involve unequal divisions, based on the occurrence of differently sized blastomeres, but that does not correspond to a clearcut spiral pattern. At the beginning of cleavage (stage 1), the small cluster of blastomeres is not visible through the semitransparent eggshell of living embryos. With ongoing cleavage divisions, the embryonic primordium becomes visible as a small "island," more transparent than the surrounding granular yolk, located in the center of the egg (stage 2). Stage 3 is defined by the first appearance of bilateral symmetry. In *Mesostoma* sp., as well as *Diceratocephala* sp., the embryonic primordium moves to the egg periphery during stage 3. This ventral migration is incomplete in *Craspedella* sp. and the temnocephalids investigated by Haswell (1909), where the primordium remains near the egg center. One implication of this finding is that phylogenetically *Diceratocephala* sp. stands more basally among the temnocephalids, and thereby closer to dalyellids; recent cladistic analyses by Cannon and Joffe (2000) support this supposition.

Temnocephalid embryonic stages 4 and 5 are characterized by the first appearance of organ primordia. As described for *M. lingua*, organogenesis in temnocephalids commences in the absence of prior gastrulation. At the anterior side of the embryonic primordium, bilaterally symmetric condensations of postmitotic cells define the primordia of the brain hemispheres (stage 4). The primordium of the pharynx is a small, rosette-shaped assembly of cells in the midline immediately behind the brain primordium. Shortly after these primordia have made their appearance, cells at one of the surfaces of the embryonic primordium reorganize into an epithelium (stage 5), thereby manifesting their fate as epidermal primordium. The position of the epidermal layer defines the ventral pole of the embryonic primordium. As the epidermal layer forms, it buckles in, forming a central cavity that is open ventrally. The apical membrane compartments of the epidermis face toward the lumen of the cavity; the basal surface and organ primordia attached to the basal surface (e.g., brain) face away from it. All primordia of the embryo are affected by this embryonic invagination (Younossi-Hartenstein and Hartenstein 2000a). At later stages, the whole process is reversed; the epidermis everts and comes to lie at the surface of the egg. Embryonic invagination has been observed by Haswell (1909) for the temnocephalid species *Temnocephala fasciata* and recently by us for the dalyellid *G. superba*; it is not found in other rhabdocoels, including *Mesostoma spp.*, where the epidermis from the onset forms a convex shield topping the ventral surface of the egg. Not unlike mass movements that take place during arthropod development, such as germ band extension and retraction, embryonic invagination in rhabdocoels does not leave any permanent traces in the structure of the embryo. The morphogenetic function of embryonic invagination is not obvious.

Besides epidermal cells, other tissues, in particular the brain, pharynx, and nephridial system, also begin to differentiate during stage 5. The dispersion and differentiation of muscle cells begins slightly later, during stage 6. This temporally coordinated onset of differentiation of all major organ systems occurs in exactly the same manner as in *M. lingua* and several other flatworm species, including even polyclads, in which early development follows a pattern quite different from that one observed in rhabdocoels (Surface 1908; Kato 1940; Anderson 1977; Younossi-Hartenstein and Hartenstein 2000b). One might call stage 6 the "phylotypic" stage of flatworms, using the term introduced for arthropods and vertebrates (reviewed by Slack et al., 1993) to denominate the stage where embryos of all members of a phylum bear greatest resemblance to each other. Key elements observed so far in stage 6 embryos of all free-living flatworms are (Fig. 10):

- Ventral epidermal epithelium
- Anterior brain with neuropil and a ventral and lateral pair of connectives
- Pharynx epithelium and muscle (reduced in indirectly developing polyclads) formed in the deep layer of the embryo
- Myoblasts and nephridial cells concentrated in lateral strands that extend at the level of lateral connective from the posterior surface of the brain to the posterior tip of the animal

◀ **Fig. 9A–I** Mature *C. pedum* embryo (stage 8). **A–C** Three focal planes (**A** deep; **B** intermediate; **C** superficial) of fuchsin-labeled whole-mounts in ventral view. The embryo has curled ventrally, bringing the sucker primordium (*sup* in **A**) that marks the tail end in close contact with the anteriorly located tentacles (*ten* in **C**), and causing transverse folds in the ventral epidermis (*arrows* in **A**). Brain (*br*) and central neuropil (*np*) have further condensed, bringing the eyes (*eye*) close to the midline. **D** Fuchsin-stained whole-mount of stage 8 embryo, lateral view, showing primordia of brain (*br*), pharynx (*ph*), tentacles (*ten*), genitalia (*gp*) and sucker (*sup*). **E** High-magnification view of fuchsin-stained genital primordium. Outer muscular layer surrounds central lumen filled by cuticular apparatus (*cta*) of penis. **F–I** Sections of stage embryo labeled with antibody against acetylated tubulin (*brown*). The plane of sectioning is tilted between transverse and horizontal and progresses from anterior-ventral (**F**) to posterior-dorsal (**I**). Left halves and right halves in alternating succession. **F** Left half of section at level of brain (*br*) and pharynx (*ph*). **G** Right half of section at level of brain (*br*) and tentacle base (*ten*). Note thick nerve roots entering tentacles. **H** Left half of section at level of tentacle tips (*ten*) and beginning longitudinal bands (*lb*). Axons growing from the brain into the trunk form three tracts, the ventral (*vc*), lateral (*lc*), and dorsal (*dc*) connective. **I** Right half of section at posterior-dorsal level, showing longitudinal band with connectives and epidermis (*ep*; *ms* muscle cells, *yk* yolk.) *Bars* **A–D, F–I** 25 μm; **E** 10 μm

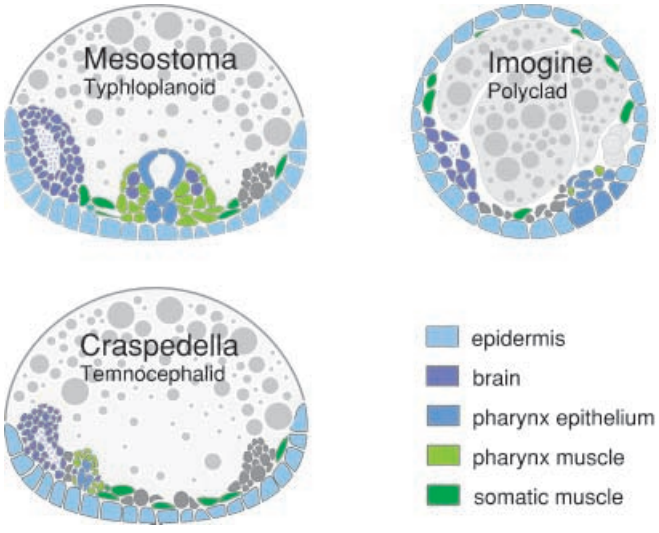

Fig. 10 Phylotypic stage in flatworm embryogenesis. Schematic sagittal sections of stage 6 embryos of representatives of three different taxa. Corresponding tissue primordia are labeled by identical colors. For more details, see Discussion

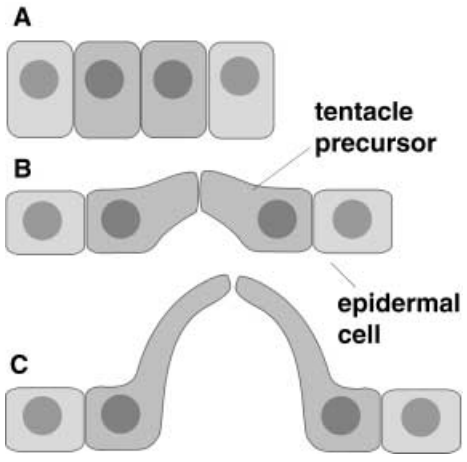

Fig. 11A–C Morphogenesis of tentacles. **A–C** show schematic length sections of tentacle precursors at three successive stages of development. Increase in length of tentacles (*dark gray*) is effected by stretching of cells, as opposed to cell proliferation or convergent extension

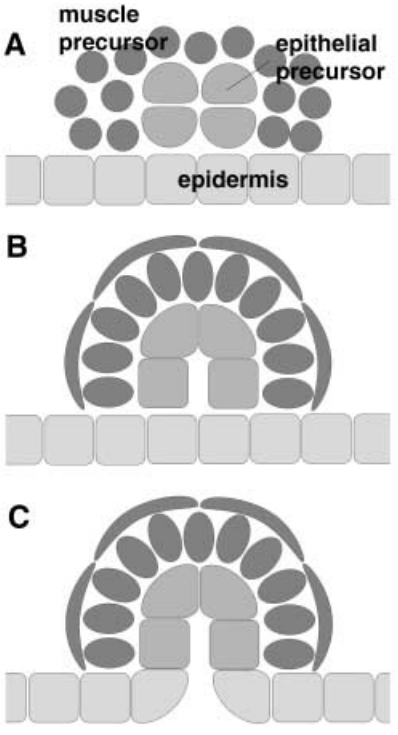

Fig. 12A–C Morphogenesis of epitheliomuscular inner organs, shown for pharynx. Schematic of organization of pharynx primordium at successive stages of development. **A** Initially, precursors of epithelium appear as a group of deep mesenchymal cells, surrounded by precursors of pharynx muscle. **B** Epithelial precursors become organized into an epithelium that has no connection to the epidermis. **C** Pharynx epithelium links up with the epidermis, generating the mouth-opening

During later stages, temnocephalid embryos still bear a fundamental resemblance to embryos of other flatworm species; in addition, the group-specific traits become manifest. As in other rhabdocoels, stage 7 is defined by the appearance of eye pigmentation and the beginning of brain condensation. The brain primordium, which had formed a rather wide, flat structure during stages 5 and 6 increases in the thickness of the cortex and decreases in its transverse extension. The epidermis expands around the yolk. Since no cell division occurs during epidermal expansion, individual cells become large in surface area and thin in apical-basal diameter. We assume that epidermal cells fuse into a syncytial layer, since cells are individualized during stage 6 and syncytial in juveniles.

Buds that will give rise to the five tentacles (anterior) and the sucker (posterior) appear as epidermal specializations during stage 7. These buds are formed by circularly arranged groups of epidermal cells that subsequently grow in length as they form the elongated tentacles and sucker. As stated above for the epidermis in general, there is no cell proliferation and also apparently only little convergent extension of cells involved in this process (Fig. 11). Cells simply fuse into a syncytium that grows in length; nuclei remain at the base of the tentacles.

It is worth mentioning that a common morphogenetic program seems to initiate the development of diverse body structures, including pharynx, reproductive system, and muscular sucker (Fig. 12). These organs all share an epithelial component, as well as a layer of muscles attached to the epithelium. Primordia of these structures “crystallize” in the deep cell layers of the embryo during stages 5 and 6. We emphasize this point because it is in contrast to a mode in which epithelial linings of inner organs such as pharynx and protonephridia would arise by invagination from the epidermal layer. Such invagination is frequently cited or simply assumed in the older literature, but is probably erroneous. Thus, in the rhabdocoel embryos investigated by us, precursors of the pharynx epithelium initially appear as clusters of rather large, deeply located mesenchymal cells, surrounded by small-

er myoblasts that become organized in a concentric fashion around the epithelial precursors. The epithelial precursors undergo a mesenchymal-to-epithelial transition, resulting in the formation of a lumen in the center of the epithelial precursor cluster. Only in late embryos do the epithelial precursors link up with the epidermis, resulting in a mouth that opens into the pharynx lumen.

Stage 8 describes the fully dorsally closed and differentiated embryo. Although the anatomy of the late embryo and hatching juvenile is fundamentally similar between different flatworm taxa, a number of characters that are different in detail deserve mentioning. Most notable among these is the degree of differentiation of the pharynx and gut, and the reproductive system. As described in this study, the muscular pharynx of temnocephalid juveniles is well developed, a characteristic they share with the typhloplanoid *Mesostoma* sp. and dalyellid *Gieysztoria* sp. By contrast, the pharynx of hatching polyclad larvae is very simple, possibly an adaptation to the planktonic lifestyle of these organisms. The gut of most flatworm juveniles, including temnocephalids and typhloplanoids, seems to be ill differentiated. The interior is filled by a spongy mass of cells, intermingled with yolk, and no gut epithelium is discernible. By contrast, both the dalyellid *G. superba* and polyclad *I. mcgrathi* possess a well-developed gut epithelium as late embryos. Male reproductive organs show no signs of differentiation in late embryos of typhloplanoids and polyclads, but are well developed in dalyellids and temnocephalids. It is likely that the different degree to which pharynx, gut, and reproductive system have developed in juveniles of different flatworms are related to the different "lifestyles" in which these organisms have to engage.

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