Comparative approach to developmental analysis: the case of the dalyellid flatworm, *Gieysztoria superba*

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ABSTRACT  Dalyellida represents a taxon of small rhabdocoel flatworms that occur in freshwater habitats all over the world. Combining histology and electron microscopy we have analyzed the embryonic development of a new dalyellid species, *Gieysztoria superba*, in order to obtain more comparative data pertaining to morphogenesis and organogenesis in flatworms. We have used a morphological staging system that we recently introduced for another rhabdocoel, *Mesostoma lingua* (Younossi-Hartenstein et al., 2000). Our data show that in many fundamental respects, such as the irregular cleavage, mesenchymal embryonic primordium, and lack of gastrulation movements, *Gieysztoria* is highly similar to *Mesostoma*. During cleavage (stages 1 and 2) the embryo is located in the center of the egg where it is surrounded by a layer of yolk cells. Cleavage leads up to a solid, disc shaped cell cluster. During stage 3, the embryo migrates to the ventral side of the egg and acquires bilateral symmetry. Stages 4/5 sees the emergence of the first organ primordia, the brain, epidermis and pharynx. A peculiar invagination of the epidermal layer pushes the embryo back into the center of the yolk ("embryonic invagination"). Organogenesis takes place during stages 5 and 6 while the embryo is invaginated. A junctional complex, consisting initially of small septate junctions, followed later by a more apically located zonula adherens, is formed in all epithelial tissues, including epidermis, protonephridia, and pharynx. During late stages (6-8), *Gieysztoria* embryos evert back to the surface where the epidermal primordium expands and grows around the yolk to close dorsally. During this phase of development cytodifferentiation of the different organ systems takes place. Stage 7 is characterized by the appearance of eye pigmentation, brain condensation and spindle shaped myocytes. Stage 8 describes the fully dorsally closed and differentiated embryo. In comparison to other rhabdocoels, including *Mesostoma, Gieysztoria* exhibits a precocious differentiation of an intestinal epithelium and male genital apparatus. In *Mesostoma*, these structures are formed post hatching.

KEY WORDS: Platyhelminth, embryo, development, morphogenesis, organogenesis, differentiation.

Introduction

In recent years the comparative approach to developmental analysis has received a renewed interest, coming with the realization that molecular mechanism controlling development are highly conserved among animals. Thus, by comparing the expression pattern and, where possible, loss or gain of function phenotypes of homologous genes in diverse species, one gains important new information that helps to decipher the function of those genes. One obstacle to comparative analysis is the paucity of detailed embryological data for most invertebrates, in particular when it comes to the analysis of morphogenesis and organ differentiation. The embryonic development of different taxa of platyhelminths has been studied in the early part of our century. These studies, among them the detailed accounts by Bresslau on rhabdocoels (1904) and acoels (1909), Ball (1916) on rhabdocoels, and Surface (1908) and Kato (1940) on polyclads, focus mainly on early developmental stages, in particular cleavage. We have initiated a series of studies on embryonic development of various representatives of the phylum platyhelminthes (flatworms). According to phylogenetic systematic analysis, flatworms have retained a number of fundamental structural characteristics, such as the ciliated epidermis, a single gut opening, and an anterior located central nervous system, from the hypothetical bilaterian ancestor (Ax, 1995; Westheide and Rieger, 1996). 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 (Ruiz-Trillo et al.,...
In this paper we present a light and electron microscopic study on the development of the dalyellid species *Gieysztoria superba* (Hartenstein and Dwine, 2000). “Dalyelloidea” represents a paraphyletic group of the rhabdocoel flatworms (Luther, 1955; Ehlers, 1985). As such, they possess a highly evolved pharynx (“pharynx doliiformis”) and have developed a peculiar mechanism of adding yolk to their eggs. Thus, instead of dumping yolk into the oocyte itself, they invest the oocyte with a thick layer of specialized yolk cells. This “ectolecithal” pattern of yolk distribution has a strong impact on early embryonic development, as shown for a number of different rhabdocoels (for review and discussion, see Thomas, 1986; Hartenstein and Ehlers, 2000). For example, cleavage of the small oocyte appears to be quite irregular, resulting in a mesenchymal mass of cells (“embryonic primordium”) in the center of the egg. During subsequent stages, the embryonic primordium moves to the periphery of the egg and initiates organogenesis, without undergoing any overt gastrulation movements. Thus, within a short period of time, local condensations of postmitotic cells demarcate the formation of the primordia of the brain, pharynx, and other internal structures. Only when tissue differentiation sets in does the outer layer of cells become organized into an epithelium that will give rise to the epidermis.

In order to compare this peculiar pattern of development noted previously for *Mesostoma* with that of other rhabdocoels, we have investigated embryogenesis in the dalyellid *Gieysztoria*. Our data show that in many respects, such as the irregular cleavage, mesenchymal embryonic primordium, and lack of gastrulation, *Gieysztoria* embryos resemble *Mesostoma*. During mid stages of embryogenesis, after the embryonic primordium has already migrated to the egg periphery, a peculiar invagination of the epidermal layer pushes the embryo back into the yolk (“embryonic invagination”). During late stages, *Gieysztoria* embryos evert. The epidermal primordium expands and grows around the yolk to close dorsally. An anterior brain and pharynx become fully differentiated. In comparison to other rhabdocoels, such as *Mesostoma*, there is a precocious differentiation of an intestinal epithelium and male genital apparatus, structures that are formed only postembryonically in *Mesostoma*.

**Results**

In a recent investigation of embryogenesis of the rhabdocoel *Mesostoma lingua* we have introduced a system of stages, based upon morphological criteria that can be easily distinguished in live and wholemount material and that represent major developmental steps (Younossi-Hartenstein *et al.*, 2000a). We will use in the following a system of stages defined by similar criteria than those recognized in *Mesostoma*. Development is considerably faster in the dalyellid *Gieysztoria* than in *Mesostoma* (2-3 days instead of 8 days), but it is possible to recognize large scale morphogenetic events that are similar and may be considered homologous. By trying to establish a unified staging system across the boundaries of different flatworm taxa we want to help creating a nomenclature...
that will facilitate future experimental studies of flatworm development.

**Early stages: Cleavage and the formation of the embryonic primordium**

*Gieysztoria* eggs are oval shaped and measure approximately 100 µm in length and 75 µm in width. The small oocyte is located in the center of the yolk mass and can not be seen in the living embryo during the first cleavage divisions (stage 1; approximately first 10 hrs of development). Cleavage leads to the formation of an apparently irregular, mesenchymal cluster of cells, the embryonic primordium. This term was coined for the similarly structured *Mesostoma* embryo by Bresslau (1904). The embryonic primordium in living material forms a barely visible, hyaline disc, surrounded by granular yolk, in the center of the egg (Fig. 1B; stage 2; second half of first day of development). In sectioned material (Fig. 2A-E) the embryonic primordium is seen to contain flattened, irregularly shaped cells with abundant mitotic figures. Surrounding the embryo are more irregularly shaped nuclei of yolk cells (yk). Some cells contain several small, spherical vesicles (arrows in Fig. 2E) that probably represent an early stage in nuclear membrane formation. Despite close contact of the cleaving cells, cellular contacts such as adherens junctions are sparse, if present at all (Fig. 2D).

By the end of the first day of development the embryonic primordium migrates to one surface which thereby is defined as the future ventral side of the embryo (stage 3; Fig. 2E). This movement of the embryonic primordium from the center to the ventral side appears to be typical for rhabdocoel development; it has been also described in *Mesostoma* (Bresslau, 1904; Hartenstein and Ehlers, 2000) and other groups (rev. by Thomas, 1986). The disc shaped embryonic primordium is now approximately 5-7 cell diameters in thickness (dorso-ventrally) and approximately 15 cells in diameter. Histologically, cells appear homogenous, and mitotic activity is scattered throughout the primordium.

**Embryonic invagination and organogenesis**

The mid stages of *Gieysztoria* development are characterized by a large scale movement, embryonic invagination, that may be typical for dalyellids and temnocephalids (Haswell, 1909; Younossi-Hartenstein et al., 2000b), but that is absent in *Mesostoma*. As the embryonic primordium expands, it invaginates into the interior of the embryo, forming a deep furrow along the antero-posterior axis (Fig. 3). Primordia of the main organ systems, in particular the brain and pharynx, appear as local condensation of cells in the anterior wall of this invagination (stage 4/5; second day of development). There is no gastrulation in the sense that three tissue layers, an outer ectoderm surrounding an inner mesoderm and endoderm, are generated first, and organ primordia develop subsequently from these layers. Instead, the surface layer of the embryonic primordium, facing the lumen of the invagination, reorganizes into a monolayered epithelium that will give rise to the epidermis. The remaining "deep" cells of the primordium sort out into different lineages giving rise to the nervous system, pharynx, somatic musculature, gut, and gonads. The first distinct structure that becomes visible light microscopically is the primordium of the brain, a condensation of "deep" cells at the anterior of the embryo. Differentiation of nerve cells proceeds very fast and results in the formation of a two layered brain, consisting of an outer cortex of small, round neurons that surround an inner neuropile (Fig. 3A,B). As tissues further grow and differentiate, the embryo evaginates to cover the ventral surface of the egg (Fig. 4A,B). The epidermis becomes very flat, yet maintains its cellular structure, as opposed to temnocephalids where epidermal cells fuse into a syncytial layer (Younossi-Hartenstein et al., 2000b). At the basal surface of the epidermis spindle shaped muscle precursors appear. The basement membrane that separates these two layers is visible as a light zone light microscopically from stage 6 onward. It grows to an enormous thickness towards the end of embryogenesis (Fig. 4E). Starting during stage 5, and definitively at stage 6, one can recognize oscillating movement in the center of the embryo, probably corresponding to myogenic twitching of somatic and/or pharyngeal musculature.

The primordia of the brain and pharynx, the latter developing right next to the brain, occupy the anterior half of the embryo. As described for the rhabdocoel *Mesostoma*, the pharynx epithelium does not arise by invagination from the outer ectoderm; instead, a group of "deep" cells reorganize into an epithelium surrounding an inner lumen (Fig. 4A,D). Initially, this emergent pharynx lumen has no connection to the outside. The definitive mouth is formed during later stages.

Pigmentation of the eyes that develop in the anterior cortex of the brain demarcates the onset of stage 7/8 (middle of third day of development; Fig. 4C). The epidermis now has grown around the

![Fig. 3. Embryonic invagination. (A,B) Sections of a stage 5 Gieysztoria embryo. (C) Diagrammatic representation of a sagittal section of an embryo at that stage. Straight lines in C indicate the plane of section shown in A and B. Cells of the embryonic primordium have segregated into a surface epithelium that constitutes the primordium of the epidermis (ep) and a deeper mesenchymal layer that contains the precursors of the musculature (mp) and brain (br). In the brain, neural differentiation has set in, resulting in an outer cortex surrounding a central neuropile (np). The epidermal primordium has undergone invagination (ein) into the center of the egg. Bar: 20 µm](Image 368x158 to 494x418)
yolk and the embryo shows rapid muscular movement in the egg. The brain, and in particular the neuropile core, has grown to an enormous size, compared to the overall size of the embryo (Fig. 4C). The pharynx has elongated and fills almost the entire embryo ventrally and posterior to the brain (Fig. 4E). A population of “deep” cells becomes organized into a loose epithelial layer that surrounds the remaining yolk mass and forms a rudimentary gut (int in Fig. 4 D,F). A small group of posteriorly located deep cells generates the primordium of the reproductive system. It consists of an outer layer of muscle that surround an inner group of large cells that differentiate into one of the male reproductive structures, the cuticular organ (gp in Fig. 4 E,F; see below). Both the generation of a more or less complete gut and of a reproductive structure distinguishes the embryo of Gieysztoria from that one of Mesostoma, whereas cells set aside for the gut and reproductive system can be recognized in late embryos, but do not differentiate until postembryonic stages.

Histology and ultrastructure of juvenile Gieysztoria

The body wall of Gieysztoria juveniles is formed by a flat, cellular epidermal layer, a massive basement membrane and a double layer of circular and longitudinal muscle fibers (Figs. 5,6). Epidermal cells have a distinctive apical cytoplasmic cortex that appears light and devoid of organelles, as opposed to the more electron dense basal cytostoplasm that contains ribosomes and all other organelles (Fig. 6A). Rhabdites and cilia are a characteristic specialization of the flatworm epidermis. A large junctional complex interconnects neighboring epidermal cells (Fig. 6B). This complex is comprised of two components, an apically located, wide zonula adherens and a more basal belt of septate junctions. The basement membrane equals in diameter the epidermal or muscular layer (Figs. 5B, 6E). It is composed of systems of fibers, presumably collagen, that are embedded in a dense matrix (Fig. 6E). The muscle layer at most levels consists only of thin processes containing myofilaments. Cell bodies of muscle cells are grouped together in a few clusters located in the head and midbody (Fig. 6C). Embedded in the muscle layer are the tubules that form the protonephridial system. They have a thin wall and are filled with parallel arrays of cilia (Fig. 6A).

The anterior of the body is filled with the brain and pharynx. The brain is formed by small, round and electron dense neurons surrounding a massive neuropile (Fig. 5A). At some places the neuropile directly abuts the musculature of the pharynx (Figs. 5B, 6H). Embedded in the anterior cortex of the brain are the eyes (Figs. 5C, 6F). They are formed by a cup shaped pigment cell, enclosing a dense array of microvilli that are formed by the photoreceptor neuron and that contain the photopigment. Axons leave the brain at its posterior surface and form a pair of dorsal and ventral connectives (Figs. 5B, 6G) that are accompanied by branches of the protonephridial system.

The pharynx starts ventrally and anteriorly of the brain as a kind of atrium, called pharyngeal pocket (Fig. 5A). The pharyngeal pocket continues into the massive pharynx doliiformis characteristic of the dalyellids (Luther, 1955; Ehlers, 1985). A thin epithelial lining bearing a dense array of apical microvilli (Figs. 5D, 7C) is surrounded by systems of circular and radial muscle fibers that make up the muscular wall of the pharynx. Posterior of the pharynx is the gut, a loosely organized layer of flattened cells that are devoid of apical cilia and a prominent junctional complex (Figs. 5E, 7A). In the tail of the juvenile, the developing male reproductive system forms a characteristic structure (Figs. 5F, 7D). Surrounded by scattered muscle fibers, it has an electron dense core made up of one or a few cells. Between this core and the muscle fibers is a cylindrical cell whose inner membrane (facing the core) is rifled into approximately 40 parallel ridges that correspond to the future “spikes” of the cuticular apparatus typical for the adult.

Discussion

In this paper we have described key morphogenetic events in the developing dalyellid, Gieysztoria superba. In our analysis we have employed a morphological staging system recently introduced for the rhabdocoel species Mesostoma lingua. In the following, the basis of the staging system and its utility in comparing different species will be briefly discussed.

As a prerequisite for many different aspects of experimental work, it is necessary to gage the developmental stage a given...
embryo is at. One way to define the stage is to indicate the time interval that has elapsed between fertilization, or egg deposition, and a particular developmental event to be considered. Several problems are attached to this method. One is that developmental time depends on temperature, which is sometimes difficult to hold constant or set to a standard value if working with different species. Moreover, the starting point zero of embryogenesis, i.e., fertilization, is often difficult to determine, in particular when considering embryos that develop in the body of their mother or embryos of species that are “harvested” from nature, instead of being grown in a controlled laboratory environment. A preferable alternative to indicate developmental time is to base staging on morphological criteria, as currently done for all “model” embryos, such as Drosophila (Campos-Ortega and Hartenstein, 1985), C. elegans ( Sulston et al., 1983) or Xenopus (Nieuwkoop and Faber, 1956). The main requirements for morphological criteria used to define developmental stages is that they should be easy to identify, ideally in the living embryo. For flatworms, there has so far been no staging system in place, with the exception of one proposed by Benazzi and Gremigni (1982) for triclads. In our recent study of Mesostoma lingua we have introduced a system of eight easily identifiable stages (Younossi-Hartenstein et al., 2000b; Hartenstein and Ehlers, 2000) that we hope can be extended to other species as well.

The eight stages defined for Mesostoma can be easily recognized in Gieysztoria (Fig. 8). Both species produce ectolecithal eggs with small oocytes surrounded by yolk cells. Oocytes are small and undergo an irregular type of cleavage in the center of the yolk (stage 1 and 2). Subsequently, the mesenchymal embryonic primordium moves to one side of the egg (stage 3). The driving force behind this movement is enigmatic, given that it takes place at a time in development before any cell of the embryo has differentiated to form contractile filaments or cilia. It is possible that cells within the yolk layer are contractile and push the embryonic primordium to the periphery.

As described for Mesostoma, organogenesis in Gieysztoria commences in the absence of prior gastrulation. Within different strata of the embryonic cell mass, local condensations of cells define the early primordia of the brain, pharynx, genital apparatus, and epidermis (stage 4). It is noteworthy that the pharynx primordium appears further anterior, closer to the brain, in Gieysztoria compared to Mesostoma. This corresponds to the anterior position of the pharynx in the mature dalyellid, a condition not typical for most platyhelminths, and indicates that no migration of precursor cells is involved in positioning the pharynx at the anterior pole.

The epidermal primordium reorganizes into an epithelial layer (stage 5), the first one in the embryo. As the epidermal layer forms, it undergoes invagination, pulling the other embryonic primordia that are attached its basal surface back into the center of the egg. This movement, for which we propose the term “embryonic invagination” has been observed for several other rhabdocoel embryos, in particular temnocephalids (Haswell, 1909), but is not found in Mesostoma, where the epidermis during stage 5 resembles a simple shield topping the ventral surface of the egg.

While invaginated, the epidermal primordium of Gieysztoria grows considerably and differentiation of cells of the brain and pharynx take place (stage 6). Despite the different topology of organ primordia, in the center of the egg in Gieysztoria, and close to the surface in Mesostoma, the comparison between the two species is easy. In the brain, a central clear domain representing the neuropile is formed; at its posterior surface, bundles of axons forming the dorsal and ventral connectives issue from the brain. In fixed material, a subepidermal layer of spindle-shaped myocytes with myofibrils differentiates. Between myocytes and epidermis, a basement membrane is laid down. This basement membrane increases in diameter and fibrillar complexity to a much higher degree in Gieysztoria than Mesostoma; it becomes clearly visible in light microscopic sections as a clear hyaline layer of almost 1 µm. A similarly massive development of the basement membrane has been observed in the parasitic dalyellids, Paravortex (MacKinnon, 1981) and Kronborgia (Koie and Bresciani, 1973). The pharynx wall becomes divided into three layers, an inner epithelium, a thick layer of radially oriented myoblasts, and an outer layer of circular myoblasts. Interestingly, the Gieysztoria embryo, and in particular the pharynx, becomes motile at stage 6, as evidenced by fast, oscillating movement visible in the center of the pharynx.

Stage 7 is defined by the appearance of eye pigmentation and the beginning of brain condensation. The brain primordium, that 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. A cleft filled with processes of muscle and...
gland cells appears between brain and epidermis. Stage 8 describes the fully dorsally closed and differentiated embryo. Although the anatomy of the late embryo and hatching juvenile is fundamentally similar between Mesostoma and Gieysztoria, a number of characters that are different in detail deserve mentioning. Among these are the epithelial mouth cavity, a fully epithelialized gut, and a differentiated (male) genital organ that are present in juvenile Gieysztoria, but not yet developed in juvenile Mesostoma. The anterior position of the pharynx, already mentioned above, and the epithelial “mouth cavity” which the pharynx epithelium continues forward to open at the tip of the animal reflect apomorphic traits of the free living dalyellids (Luther, 1955). On the other hand, the basic structure of the gut epithelium and reproductive system is shared between dalyellids and typhloplanoids. The different degree to which gut and reproductive system have developed in juveniles of Gieysztoria and Mesostoma are therefore worth emphasizing and may be related to different (as yet undefined) “lifestyles” in which juveniles of these species have to engage.

Reports on embryogenesis of other dalyellids to which our analysis of Gieysztoria might be compared are rare. Two detailed light microscopic descriptions, both of the genus Paravortex, exist in the classical literature (Ball, 1916; Hallez, 1909). Paravortex is an endoparasite of marine molluscs and produces embryos that develop in the parenchyme of the mother animal. There are marked differences in morphology between Paravortex and free living dalyellids such as Gieysztoria; in particular, the gut and surrounding mesenchyme occupy by far the largest part of the body, and pharynx-brain complex are comparatively small. The parasitic lifestyle, as well as the atypical morphology and mode of development may in part explain the differences that one can observe when comparing early embryogenesis in Paravortex and Gieysztoria. According to Ball (1916), Paravortex development starts with a quite irregular type of cleavage, resulting in a mesenchymal embryonic primordium surrounded by yolk. Subsequently, he observes two waves of superficial cells that he calls primary and secondary endoderm, respectively, on accord of their function...
which is to absorb yolk. These endoderm cells form an irregular layer between the free yolk and the embryonic primordium that, at this stage, still lacks any differentiation into organ primordia. The fate of these primary and secondary endoderm cells is unclear; there is no evidence that they contribute to the final gut of the animal. Apart from the unusual appearance of vitellophagic endoderm cells, the development of other structures, including brain, eyes, and pharynx epithelium seems to resemble closely to what we describe here for Gieysztoria.

Material and Methods

**Animals**

Individuals of Gieysztoria were collected from a small freshwater stream near Brisbane, Queensland. During the summer (January and February) these animals occurred at a high density in the fine grained mud and around algae. Small groups of specimens transferred to watch glasses along with fragments of algae and minute amounts of mud survived several weeks at room temperature (23-25°C) and laid eggs on the glass bottom of the dish. One animal produced 10-20 eggs over a period of several days; these eggs matured sequentially in the ovary and were laid one at a time. Eggs are lentil shaped and approximately 75 by 100 by 75 µm in size. They are surrounded by a brownish, semitransparent shell that just barely allow one to observe some landmark events, such as the ventral migration of the embryonic primordium, the formation of the brain and pharynx, and eye pigmentation in the living embryo (Fig. 1). We were not able to remove this shell from living embryos. For fixation, embryos at different stages were transferred with a forceps into an agar coated dish filled with 4% formaldehyde [in 0.1 phosphate buffered saline (PBS: 0.125 M NaCl; 16.5 mM Na₂HPO₄; pH: 7.2) or 2.5 glutaraldehyde (in PBS), respectively]. Following fixation, the embryos were freed of the surrounding shells with sharpened tungsten needles and transferred to small glass containers. They 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 hrs to permit polymerization of the Epon. Blocks were sectioned with an LKB Ultratome. Alternating 1 mm 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.

Acknowledgements

We would like to thank Dr. Lester Cannon, Kylie Dwine for valuable help in obtaining and identifying the Gieysztoria specimens, and to Dr. David Merritt for generously providing laboratory space to carry out the experimental work. This work was supported by NSF Grant IBN 9600194 to V.H.

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Received: February 2000
Accepted for publication: March 2000