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Metamorphosis in the craniiform brachiopod *Novocrania anomala*

Running title: Metamorphosis in *Novocrania*

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Abstract

Keywords

ontogeny, development, craniiforma, phylogeny, morphology
Introduction

The Brachiopoda is a marine animal phylum comprising 370 recent species divided into three clades, Craniiformea, Rhynchonelliformea, and Linguliformea (Williams et al., 1996; Zezina, 2008). Each clade has a different typical larva. The larvae of Craniiformea are lecithotrophic, bilobed, with three pairs of dorsal setal bundles on the posterior mantle lobe; the larvae of rhynchonelliformea are lecithotrophic, have three lobes and four setal bundles at the mantle lobe; and the larvae of Linguliformea are planktotrophic, swimming juveniles without a true metamorphosis (Nielsen, 2005). Herein we describe the development and metamorphosis of the larva of *Novocrania anomala*, a member of the Craniiformea (Lee and Brunton, 2001). The cleavage pattern of the *N. anomala* eggs is holoblastic and radial, a character that was among others used to consider brachiopods to be a sister group to the deuterostomes (Freeman, 2000; Nielsen, 1991, 2005). This traditional view that was based on morphological characters has been challenged by molecular studies which consistently find Brachiopoda to be a member of the superclade Lophotrochozoa (Hausdorf et al., 2010; Hejnol et al., 2009; Mallatt et al., 2010; Paps et al., 2009; Podsiadlowski et al., 2009; Sperling et al., 2011).

The metamorphosis of *N. anomala* attracted much interest in recent years. The finding, that both valves of the juveniles are secreted by mantle epithelia that originates at the dorsal side of the posterior lobe led to extensive conclusions about a folding event during brachiopod evolution (Cohen et al., 2003; Freeman, 2000; Malakhov and Kuzmina, 2006; Nielsen, 1991). This brachiopod fold hypothesis was supported by the existence of fossils that are worm like with shells on the dorsal side of the anterior and posterior end of the body, such as the halkieriid *Halkieria evangelista* (Conway Morris and Peel, 1995). The hypothesis that *Halkieria evangelista* belongs within the
brachiopod stem, has since been questioned by several authors and other Lower Cambrian fossils (e.g. the tommotiid *Micrina*) with a different reconstructed body plan have been put forward as potential brachiopod stem groups (Holmer et al., 2008; Vinther and Nielsen, 2005; Williams and Holmer, 2002).

Nielsen described the metamorphosis of *N. anomala* in detail and observed that prior to metamorphosis the ‘larvae become inactive and lie curled together on the bottom with the pair of ventral muscles maximally contracted’ (Nielsen, 1991). Our observations of the metamorphosis of *N. anomala* draws a slightly different picture as curled larvae seem not to metamorphose and larvae that metamorphose remain with an extended anterior-posterior body axis.

**Materials and Methods**

Collection of embryos

Adults of *Novocrania anomala* were dredged in the vicinity of the Sven Lovén Centre for Marine Sciences, Gullmarsfjord, Sweden (58°15’921”N, 11°25’103”E) in September 2008. The rocks with *N. anomala* were kept in running seawater at the ambient seawater temperature of 14°C. Adults were removed from the stones and dissected. Eggs and sperm were removed with pulled glass pipettes, washed and left in filtered seawater in separate beaker glasses. Eggs were regularly checked for germinal vesicle breakdown. Sperm cells were checked for motility under a compound microscope. When eggs were ready for fertilization (approximately 12 hours after dissection), 2 ml of a highly diluted sperm suspension were added to the eggs. Embryos and larvae were fixed at various stages from 4 hours after fertilization (hpf) to 17 days after settlement in 4% paraformaldehyde in 0.1M phosphate buffer.
Thereafter, larvae were washed three times for 15 min each in 0.1M PB and finally stored in 0.1M PB containing 0.1% NaN₃ at 4°C.

**Scanning electron microscopy**

Specimens have been sputter coated with gold and investigated with a Zeiss Supra 35VP scanning electron microscope.

**Confocal laser scanning microscopy**

The larval musculature was stained with phalloidin. Prior to staining, fixed larvae were washed in phosphate buffer (PB) that was changed every 15 min three times. Then the larvae were incubated for 1 h in PB containing 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) to permeabilize the tissue, left overnight at 4°C in 0.1M PB containing 0.2% Triton X-100 and 1:40 diluted Alexa Fluor 488 phalloidin (Invitrogen, Molecular Probes, Eugene, OR, USA), and subsequently washed in 0.1M PB that was changed three times every 15 min. Finally, the specimens were embedded in Fluoromount G (Southern Biotech, Birmingham, AL, USA) on glass slides. Negative controls omitting the phalloidin dye were performed in order to test for autofluorescence and rendered no signal. The stained larvae and juveniles were analyzed with a Leica DM RXE 6 TL fluorescence microscope equipped with a TCS SP2 AOBs laser scanning device (Leica Microsystems, Wetzlar, Germany). Animals were scanned with 0.16 µm - 0.49 µm step size, and the resulting image stacks were merged into maximum projection images. Light micrographs were recorded to allow overlay with the CLSM images for exact orientation and localization of the muscle systems within the animals.
Schematic drawing and figure plates

Drawings and micrograph figures were assembled with Photoshop CS3 and Illustrator CS3 software (Adobe, San Jose, CA, USA).

**Results**

General larval development

Cleavage is radial and the first two divisions are holoblastic (Fig. 1B, 2A-B). The blastula is round and cells start to invaginate at around 18 hours after fertilization (hpf) (Fig. 2C). The gastrula is first spherical and invagination takes place at the vegetal pole of the larva. The archenteron cells come to lie opposite of the ectoderm, and the blastocoel disappears completely (Fig. 1C, 2D). Later in development the gastrula elongates and the blastopore comes to lie at the postero-ventral side of the swimming larva (Fig. 1D, 2E). The elongated gastrula subsequently differentiates into two larval lobes, an apical lobe and a posterior mantle lobe (Fig. 1E-F, 2F-G). Larval development completes with the growth of three pairs of dorsal setal bundles on the mantle lobe (Fig. 1G, 2H).

Metamorphosis

At a water temperature of 14 °C, metamorphosis takes place around six to ten days after fertilization (dpf). During metamorphosis the larva attaches to the substrate, secretes the shell, and retains its larval lobes, which are subsequently rebuild to form the lophophore and other adult organs (Figs. 1H, 2I). Prior to settlement, the larva swims along the bottom of the culture dish, probably in order to test if the substrate is suitable for settlement. In contrast to the descriptions by Nielsen (1991), the larvae do not curl before metamorphosis. Although we also found commonly curled larvae are in the culture dishes (Fig. 3A), those curled larvae
seemed to be unable to metamorphose as their musculature is completely contracted (Fig. 3D). Larvae that settle attach themselves on the substrate with the dorsal part of the ventral mantle lobe and excrete the dorsal valve (Fig. 3B). The larval lobes remain elongated (Fig. 3C), which can also be seen in the remaining larval musculature in the settled juveniles that remains relaxed (Fig. 3E). Some days after settlement the larval lobes are rebuild into the adult tissues and the lophophore (Fig. 3F).

**Discussion and Conclusions**

Development of *Novocrania anomala* and regional specification during embryogenesis has been described previously (Nielsen 1991; Freeman 2000). Our results are congruent with these data. However, the two authors disagree about the development of the coelom and the formation of the mesoderm. According to Nielsen, the sheet of cells that invaginates during gastrulation is composed of two cell populations, endoderm and mesoderm, whereas Freeman states that the mesoderm is formed by individual cells which immigrate from the endodermal cell layer after invagination has been completed (Nielsen 1991; Freeman 2000). Nielsen describes the coelom as consisting of an anterior coelomic pouch in the apical lobe and three pairs of coelomic cavities in the posterior lobe of the larva, whereas Freeman denies the existence of larval coelomic structures and states that the coelom develops after the larvae have undergone metamorphosis (Nielsen 1991; Freeman 2000). The methods used here do not allow a conclusive statement concerning coelom and mesoderm formation in larvae of *N. anomalae*, there is more work needed to resolve the controversies on an ultrastructural level.

The former descriptions of the metamorphosis in *N. anomalae* are in large parts congruent (Freeman, 2000; Nielsen, 1991). Prior to metamorphosis the apical lobe
functions as locomotory organ for the larvae. Once the larva has found a suitable place for settlement it attaches itself to the substrate with the posterior part of the mantle lobe. A distinguishable domain of cells in between the setal bundles on the dorsal side of the mantle lobe participates in the formation of the mantle that will secrete the shell after metamorphosis (Freeman, 2000; Nielsen, 1991).

We found an important difference between the previous described metamorphosis and our observations that has an impact on the hypothesis that proposes a brachiopod fold event during brachiopod evolution. It was previously described that larvae curl during settlement and the anterior part of the head and the posterior part of the body are in close proximity in contact with the substratum (Nielsen, 1991). The curling is caused by maximal contraction of the lateral musculature in the larval body. Even so, we also observed curled larvae, those larvae seem to be arrested in their development and do not metamorphose. This can be clearly seen in the differences of the musculature of curled and settled larvae. Curled larvae have absolutely contracted muscles (Fig. 3D) whereas settled juveniles remain relaxed lateral muscles (Fig. 3E and see also Altenburger and Wanninger, 2010 for a general description of the musculature in *N. anomala*).

So the larvae do not fold together before and during metamorphosis, thus hypothesis arguing for a brachiopod fold event during brachiopod evolution should are not supported by the larval development and metamorphosis of *Novocrania anomala*.

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anomala. Gary Wife (University of Uppsala) is thanked for assistance with the scanning electron microscope.

References


Assessing the root of bilaterian animals with scalable phylogenomic methods.


**Figures**

Fig. 1. Drawings of developmental stages of *Novocrania anomala* at a water temperature of 14 °C. Numbers indicate the age in hours after fertilization for all
stages except for H where it is hours after the onset of metamorphosis. Size of all stages is around 130 µm in diameter. Anterior is oriented upwards. Cilia have been omitted for clarity. A, unfertilized oocyte (black) with egg shell (grey). B, apical view of a four cell stage with the egg shell. C, frontal view of a gastrula with blastopore (asterisk), ectoderm (ec), and endoderm (en). The gastrula starts to swim at this point of development. D, lateral view of an elongated gastrula with ectoderm (ec) and endoderm (en). The blastopore (asterisk) is situated on the posterior end of the gastrula. E, dorsal view of an elongated gastrula with almost distinct apical lobe (AL). F, ventral view of an early two-lobed larva with apical lobe (AL) and posterior lobe (PL). The blastopore is closed and larval setae (se) start to grow on the posterior side. G, dorsal view of a fully developed larva with apical lobe (AL), posterior lobe (PL), and three pairs of dorsal setae bundles (se). H, ventral view of a juvenile after metamorphosis. The larval apical lobe (AL) and pedicle lobe (PL) are still visible. The juvenile shell (s) is formed on the dorsal side with larval setae (se) extending from it.

Fig. 2. Scanning electron micrographs of Novocrania anomala developmental stages. Anterior is oriented upwards, scale bars equal 25µm, numbers in the upper right corner of each specimens indicates the developmental time in hours after fertilization (A-H) or hours after settlement (I). A, four cell stage. B, eight cell stage with polar bodies, and clear visibility of the radial cleavage pattern. C, blastula stage. D, round shaped gastrula, with blastopore (arrowhead). E, wedge shaped gastrula with blastopore (arrowhead). F, wedge shaped gastrula with first signs of the anterior lobe (AL) and almost closed blastopore (arrowhead). G, early larva with anterior lobe (AL), posterior lobe (PL), and three sets of larval setal bundles (se) start to grow on the
dorsal side of the posterior lobe. H, ventral view of a fully developed larva with anterior lobe (AL), posterior lobe (PL), and three pairs of setae bundles (se). I, ventral view of a settled juvenile with the larval shell (s), anterior lobe (AL), posterior lobe (PL), and the attachment area on the substrate (arrow).

Fig. 3. The musculature in larvae of *Novocrania anomala* before and after metamorphosis. Anterior is up in all micrographs and scale bars equal 25µm. A-C, scanning electron micrographs. A, dorsal view of a folded larva as described by Nielsen 1991 with anterior lobe (AL), posterior lobe (PL), and three pairs of setae bundles (se). This folded larva can probably not metamorphose. B, dorsal view of a settled juvenile with shell (s) and old larval setae (se). C, ventral view of a settled juvenile with shell (s), anterior lobe (AL), posterior lobe (PL), and attachment area on the substrate at the posterior lobe (arrow). D-E, confocal laser scanning microscopic micrographs of a larva (D) and a juvenile (E) corresponding to the stages shown in micrograph A and B. Muscles were stained with phalloidin and appear red in the micrographs. D, folded larva corresponding to the stage shown in A, with anterior lobe (AL), posterior lobe (PL), and strongly condensed musculature (empty arrow). Normally, the larval musculature is elongated between anterior and posterior lobe. E, settled juvenile with shell (s), elongated larval longitudinal musculature (empty arrows), and juvenile anterior aductor muscles (aad). The larval lobes, anterior lobe (AL) and posterior lobe (PL) are still recognizable under the shell. F, light micrograph of a settled juvenile with remaining larval setae (se), shell (s), anterior lobe that is rebuilt into the lophophore (AL/Lo), and remnants of the posterior lobe (PL).