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Demonstration of the preoral coelom in the brachiopod *Lingula anatina* with consideration of its phylogenetic significance

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ABSTRACT

The origin of the Bilateria and reconstruction of the last common bilaterian ancestor (LCA) are fundamental problems in zoology, including the question whether the LCA was a coelomic creature or not? Insight into the nature of the LCA might be obtained by investigating the coelomic system of poorly studied bilaterians. The Brachiopoda is a relict group of marine invertebrates whose anatomy has been seldom studied with modern methods. For most brachiopods, the coelomic system has been described as bipartite, i.e., as consisting of two parts: the lophophore coelom and trunk coelom. In the present report, a tripartite coelomic system is described for the first time in adult brachiopods, the linguliform *Lingula anatina*. In addition to a lophophoral and trunk coelom, *L. anatina* has a preoral coelom. The protocoel is located at the base of the epistome and has its own lining, which consists of non-muscular monociliated epithelial cells connected by desmosomes and tight junctions. Among brachiopods there are two types of the coelom: bipartite and tripartite. The same is known in phoronids and bryozoans (=ectoprocts). These three phyla – brachiopoda, phoronida, and bryozoa – are traditionally united into clade called lophophorata. An analysis of coelomic system organization revealed that the trimeric coelom is plesiomorphic for the lophophorates. The trimeric coelom is typical for the most of deuterostomes. The presence of the trimeric coelom in two main lineages of the bilateria allows to suggest that the LCA may have had a tripartite coelom and strongly specialized two the most anterior parts of the body – the prosome and the mesosome. This suggestion is consistent with published gene expression studies, in which *Hox* genes are never expressed in the two first segments of the body, whereas *Otx* genes are expressed in the most anterior segments of some bilaterians.

1. Introduction

Brachiopoda is a phylum of relict marine invertebrate species, which were extremely abundant in the palaeozic but which have mostly gone extinct (Zezina, 1979). Typically, based on a limited number of morphological and embryological characters brachiopods together with other lophophorates have been regarded as the closest relatives of the deuterostomia. This inference was based on similarities of the coelom organization in the lophophorates and deuterostomes. According to current molecular phylogenies, however, the lophophorates are protostome animals, which together with trochozoa form a clade called the lophotrochozoa (Halanych et al., 1995). Although the monophyly of lophophorata was rejected in early and more recent papers (Halanych et al., 1995; Paps et al., 2009), it was again supported in a recent paper (Nesnidal et al., 2013).

According to the traditional view and the latter paper, the lophophorates comprise the bryozoa, phoronida, and brachiopoda (Nesnidal et al., 2013). All of these animals have a lophophore, which is a tentacle-bearing, specialized part of the mesosome. The epistome (or brachial fold) is the uppermost part of the body, which can be found in some phylactolaemate bryozoans, phoronids, and brachiopods. Among both phoronids and bryozoans, there are species that have a true coelomic cavity in the epistome (Gruhl et al., 2005; Temereva and Malakhov, 2011a; Temereva, 2015). In phoronids, the protocoel occupies the epistome, the mesocoel is located at the lophophore base and in tentacles, and the metacoel is the largest coelom of the trunk. This division of the phoronid coelom into three compartments that appears similarly to the tripartite coelomic cavities of ambulacral deuterostomes was the basis of this homology inference of the unity of the lophophor-

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ata and deuterostomia, which form a clade called archicoelomata (Ulrich, 1951). Adult brachiopods seem to lack this compartmentalization. According to previous reports, they have only two coeloms: trunk and lophophoral (Williams et al., 2007). At the same time, some data indicate the presence of additional cavities in the brachiopod lophophore, but the nature of these cavities has not been clearly established (Pross, 1980; Kuzmina and Malakhov, 2011).

Detailed investigation of the coelom in brachiopods may help clarifying the relationships between the different lophophorate phyla, thereby shedding light on the development and the organization of the coelomic system in the last common ancestor of bilateria (Balavoine, 1998). To increase our understanding of the origin of the bilateria, the current study provides new information on organization of the coelomic system in the brachiopod *Lingula anatina* (Lamark, 1801).

2. Materials and methods

Adults of *L. anatina* (Lamark, 1801) were collected from September–October 2012, in Nhatrang Bay South China Sea Vietnam. The current research focused on the lophophores of *L. anatina*. Specimens were dissected at the anterior end to obtain a lophophore with mouth, tentacles, and epistome. Whole lophophores were fixed for histology, semi-thin sectioning, and scanning electron microscopy (SEM). Small parts of the lophophore were fixed for transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM).

For histology, a 4% paraformaldehyde solution in filtered sea water was used as a fixative. Whole lophophores were rinsed in distilled water, dehydrated in ethanol, and embedded in Paraplast Regular (Sigma). Cross sections (5 μm thick) of whole lophophores of five specimens were made with a Leica rotary microtome (Leica RM 2125). The sections were stained with Caracci hematoxylin, examined with a Zeiss Axioplan2 microscope, and photographed with an AxioCam HRm camera. Three-dimensional models of the coelomic system were constructed using Imaris ver. 7.1.1 software (Bitplane, Zurich, Switzerland).

For SEM, specimens were fixed in a 4% paraformaldehyde solution as before. The fixed specimens were dissected in 70% ethanol, dehydrated in ethanol followed by an acetone series, critical point dried, and then sputter coated with platinum-palladium alloy. Specimens were examined with a Jeol JSM scanning electron microscope.

For semi-thin sectioning and TEM, specimens were fixed at 4 °C in 2.5% glutaraldehyde in 0.05 M cacodylate buffer containing 21 mg/ml NaCl and were then postfixed in 2% osmium tetroxide in distilled water. Postfixation was followed by *en bloc* staining for 2 h in 1% uranyl acetate in distilled water. The stained specimens were dehydrated in ethanol followed by an acetone series and then embedded in Spurr resin. Semi-thin and thin sections were cut with a Reichert Ultracut E ultramicrotome. Semi-thin sections were stained with methylene blue, observed with a Zeiss Axioplan2 microscope, and photographed with an AxioCam HRm camera. Thin sections were stained with lead citrate and then examined with a JEOL JEM 100B electron microscope.

For cytochemistry, parts of *L. anatina* lophophores were fixed overnight in a 4% paraformaldehyde solution in a filtered sea water and washed two times in phosphate buffer (pH 7.4) (Fisher Scientific, Pittsburgh, PA, USA) with Triton X-100 (0.3%) (Fisher Scientific) for a total of 2 h. Then, the specimens were incubated in a mixture of rhodamine-conjugated phalloidin (1:50) (Fisher Scientific). They were subsequently washed in PBS (three times for 40 min each time) and embedded in Murray Clear. Specimens were examined with a Leica TCS SP5 confocal microscope (IDB, Moscow, Russia). Z-projections were generated using Image J version 1.43 software.

3. Results

L. anatina has a spirolophe-type lophophore. It consists of two symmetric arms with a mouth in between. The distal ends of the brachial axes are twisted into spirals. The lophophore has a brachial fold, i.e., an epistome (Fig. 1A). The brachial fold follows the lophophore and covers the tentacle bases. The food groove is located between the epistome and the tentacle bases and then passes into the mouth. The epistome covers the mouth from the dorsal side.

In cross sections of the lophophore, three large cavities are evident (Figs.1B and C, 2A). The largest cavity is the large coelomic canal of the lophophore. It occupies the central portion of each arm of the lophophore and is surrounded by connective tissue, which is thickest near the mouth. The second cavity is the small canal of the lophophore. It contains the lophophoral blood vessel, and it branches into each tentacle. The third cavity is located at the base of the epistome and contacts a net of smaller cavities, which are located along the inner surface of the epistome.

Three-dimensional reconstruction revealed that all three cavities of the lophophore pass along each arm and repeat the shape of lophophore (Fig. 1E and F). The cavity located at the base of the epistome passes along each arm of the lophophore and above the mouth. Thus, this cavity has one central part and two lateral branches, each of which forms four coils (Fig. 1G). The epistomal cavity does not connect to either the large or small canals of the lophophore (Fig. 1E and F). Among the cavities of the lophophore, the epistomal cavity is the uppermost; it passes above the mouth and can be regarded as the protocoel (Fig. 1F).

TEM images show that the inner lining of the epistome is composed of epithelial cells (Fig. 2B). These are polarized cells interconnected via desmosomes, underlain by ECM, and bearing cilia (one cilium per cell). In the main cavity, which is located at the epistome base, the epithelial cell lining has numerous vacuoles and inclusions (Fig. 2C and D). The lateral walls of the cells form thin projections, which bear desmosomes. One to three desmosomes occur along each projection (Fig. 2C). The basal surfaces of some cells form short protrusions, which contact the thin basal lamina via hemidesmosomes. Some cells have short apical microvilli (Fig. 2D). In general, there are two types of cell contacts, desmosomes and tight-like junctions (Fig. 2F).

The small cavities, which are located along the inner surface of the epistome and contact the main cavity via thin slit-like canals, have a more complex organization of the lining than that of the large cavity at the base of the epistome. The lining of the small cavities are formed by an internal layer of muscle cells and an external layer of epithelial cells (Fig. 2E). Muscle cells lack desmosomes and are attached to the basal lamina via hemidesmosomes. Muscle cells contain myofilaments of different diameters, which are orientated along the epistome. These muscle cells form the complex muscular net of the epistome edge and facilitate its motility (Fig. 1D). Epithelial cells form thin long projections, which connect via desmosomes and cover the muscle cells.

Thus, according to our data, the epistomal cavity in *L. anatina* is closed. This closed cavity has its own lining consisting of muscle and epithelial cells, which interconnect via adherence junctions and attach to the ECM. These cellular features are completely in accordance with those of a true epithelial lining, and therefore, the epistomal cavity can be regarded as a protocoel.

4. Discussion

4.1. Organization of the coelom in brachiopods

Traditionally the trimeric organization is suggested as plesiomorphy of ground pattern of Brachiopoda (Ax, 1989; Nielsen,