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The ultrastructural features of embryonic and early larval development in Yesso scallop, *Mizuhopecten yessoensis*

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ABSTRACT

Fine structural features of *Mizuhopecten yessoensis* in different embryonic and larval developmental stages from oocyte to early veliger have been studied. Spermatozoa have a conical head, consisting of a short acrosome, nucleus, and 4 mitochondria in the middle piece. The flagellum is approximately 40 μm in length. Oocyte cytoplasm contains numerous yolk granules, which are gradually expended during the larval development and disappear at the early veliger stage. Gastrula has two invaginations: shell gland anlage on the dorsal side and ventral side archenteron. Prototroch of a trochophore consists of two ciliary rings. Telotroch is absent. Myoblasts containing bundles of myofilaments are found in the blastocoel of trochophore. Velum and shell begin forming at the late trochophore stage. Cells of gut epithelium form numerous microvilli, organized into a brush border structure on the surface of enterocytes. A suggestion is made that larvae at the late trochophore stage are capable of digesting food and feeding. The structure of a *M. yessoensis* veliger is typical for pectinids. The velar retractor muscles have cross striation with a period of 1 μm. The digestive system of an 8-day-old veliger consists of the esophagus, stomach (with subdivided gastric shield and style sac), and short intestine. The digestive gland (with no discernible loops at this stage) is a part of the stomach epithelium with two types of digestive enzyme-producing cells.

1. Introduction

Development of aquaculture is of great importance due to the depletion of natural stocks of aquatic species caused by their overexploitation. The necessity to increase the productivity of aquaculture facilities leads to both the intensification of studies of the cultivated organisms and to the development of new approaches to rearing marine species (Moyano et al., 2015; Rurangwa and Verdegem, 2015; Yue and Wang, 2017). The most popular groups of organisms used in aquaculture are fish, crustaceans, mollusks, and holothurians. Bivalves gain the most attention among mollusks due to fairly simple culturing techniques and high taste qualities (Lacoste and Gaertner-Mazouni, 2014; Hassan et al., 2015; Jiang et al., 2016; Kovalev et al., 2016; Galley et al., 2017). The larval development, the formation of the nervous and muscular systems, and other biological features in these animals are a subject of active research (Alatalo et al., 1984; Kakoi et al., 2008; Saucedo and Southgate, 2008; Dyachuk and Odintsova, 2009; Dyachuk et al., 2012; Wassnig and Southgate, 2012; Liu et al., 2015; He et al., 2016; Rusk et al., 2017; Pavlicek et al., 2018).

When studying bivalves, major attention is paid to the mechanisms of shell formation. Until recently, it has been believed that shell develops in a similar way among all mollusks (Morse and Zardus, 1997; Casse et al., 1998). At the trochophore stage, cells of the shell field sink deep into larva, forming an invagination. After that, they begin secreting an organic matter, from which periostracum is formed. However, it turned out that in the venerids *Chione cancellata* and *Ruditapes decussates* no invagination of the shell field cells occurs, and the shell formation has a different mechanism (Mouëza et al., 2006; Aranda-Burgos et al., 2014). Since only two venerid species have been studied to date, it is not clear how unique this mechanism can be.

Pectinids (scallops) are a group of bivalve mollusks widely distributed around the world. Thanks to their relatively large size and high taste qualities, these bivalves are an important food item in human diet in a variety of countries. The high economic value as a sea food explains the increased interest in them among scientists and aquaculture specialists (Shumway and Jay, 2006).

The embryonic and larval development of pectinids has been well studied from many aspects. The features of cleavage, morphology and anatomy of larval and juvenile stages of development in many members of this family were comprehensively considered (Cragg, 2006). The

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main methods in these studies are light and scanning electron microscopy (Sastry, 1965; Culliney, 1974; Tanaka, 1984; Hodgson and Burke, 1988; Bellolio et al., 1993; Beninger et al., 1994; Cragg, 2006). At the same time, transmission electron microscopy is not commonly used in the bivalve embryonic development research. This method was only applied in a few studies that considered mostly the late developmental stages (Cragg and Nott, 1977; Elston, 1980; Cragg, 1985, 2006; Casse et al., 1998; Mouëza et al., 2006; Aranda-Burgos et al., 2014). Thus, the fine structure of larval bivalve mollusks has not been sufficiently studied, particularly at their early development stages.

The Yesso scallop *Mizuhopecten (Patinopecten) yessoensis* (Jay, 1856) (Pectinidae) is distributed from the northern part of Korean Peninsula in the south to the South-Kuril Shallow in the north and also to the coast of Japan. Since the Stone Age, it has been harvested by the peoples inhabiting the coasts of the Far East (Krasnov et al., 1977). The popularity of *M. yessoensis* as a sea food is growing steadily thanks to its high taste qualities and large size (the largest among pectinids). Farms for artificial breeding this species appeared in Japan over one century ago (Motavkin, 1986). Currently, *M. yessoensis* aquaculture is most developed in China and Japan (these countries account for up to 9/10 of production), but Russia and South Korea also possess a potential to build up this industry (FAO, 2018).

The high economic value of *M. yessoensis* increases the interest in this bivalve among researchers. In the last century, the anatomy, physiology, embryonic, and larval development of this species have been studied (Malakhov and Medvedeva, 1986; Motavkin, 1986; Bower and Meyer, 1990). The efficient methods of rearing and increasing the productivity of Yesso scallop using thermal stress have been developed (Motavkin, 1986). Presently, *M. yessoensis* has become even more important subject of biochemical (Nishita et al., 1997) and genetic research (Bao et al., 2015; Dou et al., 2016; Hou et al., 2016; Li et al., 2016; Sun et al., 2016). Methods of obtaining the biologically active substances and medicines from tissues of this scallop are being developed (Jeong et al., 2007).

However, some aspects of *M. yessoensis* biology remain poorly understood. There are few publications on its embryonic development (Malakhov and Medvedeva, 1986; Bower and Meyer, 1990). Moreover, descriptions of the early larval stages from blastula to veliger have been performed using only light microscopy and, therefore, can give only a general view about the larval morphology. The fine structure of *M. yessoensis* larvae is presently unknown. Therefore, we have attempted to study the fine structure of Yesso scallop larvae in the early developmental stages.

2. Materials and methods

Works on cultivation of *Mizuhopecten (Patinopecten) yessoensis* (Jay, 1856) were conducted at the Research Module for Growing of Larvae of Commercial Species of Marine Invertebrates at the Zapad Station, National Scientific Center of Marine Biology (NSCMB), FEB RAS. Adult specimens with a shell diameter of 7–8 cm and weighing 100–120 g were collected in Peter the Great Bay (Sea of Japan, Russia) from April to May and kept in 3 m^3 tanks with flowing aerated sea water. Spawners were fed with cultures of *Nannochloris* sp. and *Phaeodactilum triakantum* twice a day. Spawning was induced by thermal stress according to Motavkin (1986). Spawners were exposed to air at a temperature of 17 °C for 2–2.5 h. Then females and males were placed in separate 30-liter containers with sea water having the same temperature. The water was quickly cooled to 13 °C by adding ice made from sea water. This thermal stress induced a gamete release. The water containing oocytes was screened through a nylon sieve with a mesh size of 100 × 100 μm, then most of the water was gently poured off, and freshly sterilized sea water added. The washing procedure was repeated three times. Then, the oocytes were placed into 30-liter containers. The concentration of oocytes was adjusted to 90 cells/mL. For fertilization, 130 mL of spermatozoa suspension with a concentration of 10^3 cells/mL were added to each container. The initial stages of development took place in the same tanks.

Blastula stage larvae were transferred to 370-liter cylindrical tanks with aerated sea water at a temperature of 13–14 °C and a salinity of 33‰. No antibiotics were used throughout the development period. The sea water used for cultivation was filtered through a 1-μm Geyser cartridge filter and then UV treated (Resun Algae Gone UV Clarifier, 13 W). Two-thirds of the water volume was replaced with fresh sea water daily. Starting from the trochophore stage (at 50–55 h post fertilization), larvae were fed with a 1 : 1 mixture of cultures of the microalgae *P. triakantum* and *Nannochloris* sp. A 50 mL portion of a mixture with a concentration of 5×10^5 cells/mL was added to each tank three times a day. On day 4 post fertilization (veliger stage), the mixture of *P. triakantum* and *Nannochloris* sp. was supplemented with an equal volume of *Chaetoceros mulleri* at the same concentration. Live larvae were photographed using Axio imager. Z2 and Axiovert 200 M microscopes prior to fixation. The larvae were collected approximately every 10–12 h starting from 24 h post fertilization.

For scanning electron microscopy analysis, samples were fixed with 2.5% glutaraldehyde in 0.05 M cacodylate buffer at pH 7.6 and 4 °C; then they were dehydrated through increasing concentrations of ethanol and acetone and dried under CO_2. The dried specimens were mounted on SEM pin stubs, coated with carbon, and analyzed using an EVO 40 scanning electron microscope.

For transmission electron microscopy analysis, samples were fixed with the same fixative for 1–7 days at 4 °C. After rinsing in the same buffer, the material was postfixed with a 1% solution of OsO_4 in cacodylate buffer for 1 h, decalcified with 2% ascorbic acid and 0.3 M sodium chloride solution for one week, dehydrated using increasing concentrations of ethanol and acetone, and embedded in Araldite. Sections were cut with glass knives on Ultracut E (Reichert) and UC6 (Leica) ultratomes. Semithin (0.7 μm) sections were stained in a 1% solution of methylene blue in 1% aqueous sodium borate. The sections were then examined and photographed using a Jenamed 2 light microscope (Carl Zeiss Jena) equipped with a Nikon D1x digital camera, and Axio Imager. Z2. Ultrathin (50–70 nm) sections were contrasted with uranyl acetate and lead citrate and examined using Carl Zeiss Libra 120 and Carl Zeiss Libra 200 transmission electron microscopes.

To identify nerve cells, veliger-stage larvae were fixed with 4% paraformaldehyde prepared on phosphate-buffered saline (PBS), pH 7.5, during 8 h. Then they were rinsed in several portions of PBS for 24 h. After that the material was placed in 1% bovine serum albumin (BSA) solution in PBS supplemented with 0.5% Triton X-100 for 3 h and then transferred into a mixture of antibodies. For this, we used anti-serotonin mouse monoclonal antibodies (diluted to 1:200) and anti-acetylated α-tubulin rabbit polyclonal antibodies (Abcam, USA), diluted to 1:400 in the solution of 1% BSA in PBS with the addition of 0.02% TWEEN 20. The incubation time was 12 h at 4 °C. After rinsing in PBS supplemented with 0.02% TWEEN 20, the material was incubated with secondary anti-species antibodies, tagged with Alexa 488 and 546 (Invitrogen, USA), diluted to 1:1000 in 1% BSA in PBS; the incubation time was 1.5 h at room temperature. After rinsing in PBS, sections were placed in the Vectashield medium (Vector Laboratories, USA) supplemented with DAPI for staining the nuclear DNA. The material was analyzed using a confocal laser scanning microscope LSM 780 (Carl Zeiss, Germany).

Processing of the material, cutting of semi-thin sections, and 3D reconstruction of veligers were carried out as previously described (Dolmatov et al., 2016). Semithin sections (1 μm) were cut with glass knives on Ultracut E (Reichert) and UC6 (Leica) ultratomes. Complete series of sections were collected on slides and stained with 1% methylene blue in 1% aqueous sodium borate. For 3D reconstruction, every semithin section was photographed under an Axio Imager Z2 (Carl Zeiss, Jena) light microscope equipped with an Axio Cam HRC digital camera to generate a stack of images. Alignment, tracing of contours of interest, and 3D surface representations were performed