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An Increase in the Level of Intracellular Serotonin in Blastomeres Leads to the Disruption in the Spiral Cleavage Pattern in the Mollusc *Lymnaea stagnalis*

A. I. Bogomolov^{a, b} and E. E. Voronezhskaya^{b, *}

^a Department of Embryology, Faculty of Biology, Moscow State University, Moscow, 119934 Russia

^b Koltzov Institute of Developmental Biology, Russian Academy of Sciences, Moscow, 119334 Russia

*e-mail: elena.voronezhskaya@idbras.ru

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Abstract—Serotonin (5-HT) is known as a substance with a wide range of physiological effects. At the same time, its presence in the cells of the developing embryo is already shown from the earliest stages of development. However, the effect of increasing the level of intracellular serotonin on the processes of cleavage in representatives of Spiralia has not been studied in detail. For the first time, we investigated changes in the pattern of spiral cleavage in the freshwater mollusk *Lymnaea stagnalis* after incubation of eggs (during 24 h, from the stage of zygote/2 blastomeres) in a solution with serotonin—5-HTP precursor. During cleavage, the mutual arrangement of blastomeres was disrupted in all experimental embryos, starting from the apical rosette stage until the early gastrula. The delay in cytotomy of the blastomeres of the A and C quadrants led to the displacement of blastomeres in all quadrants, including B and D, as a result of which the blastomeres acquired contacts that are unusual for them normally. Subsequently, 80% of the embryos of the experimental group had irreversible impairment of gastrulation and the formation of exogastrula. In embryos that successfully passed gastrulation, malformations of the eyes and shell were observed in 10–15% of cases. Our results show that an increase in the level of intracellular serotonin leads to a disturbance of the mutual arrangement of blastomeres in a representative of Spiralia and may also lead to disorders of further development.

Keywords: intracellular serotonin, a pattern of cleavage, developmental abnormalities, cyclopia, shell malformations, mollusks, Gastropoda, Spiralia

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INTRODUCTION

Serotonin (5-HT) is a widespread monoamine that is usually associated with signal transmission between nerve cells. In addition, 5-HT is a hormone that modulates neurogenesis in vertebrates, starting with the differentiation of nerve cells and until the formation of cytoarchitectonics of the mature brain (Vitalis et al., 2003; Whitaker-Azmitia, 2010; Farrelly et al., 2019). However, serotonin is detected in the embryo already at the earliest, prenervous stages, and the effects of modulation of its activity affect various processes in development (Buznikov et al., 2001; Buznikov, 2007). The serotonin enters the egg, zygote, and blastomeres from the mother's body due to the activity of membrane transporters (Murphy et al., 2004; Cote et al., 2007). Inside blastomeres, 5-HT is found in both ver-

tebrates and invertebrates and is detected both in the cytoplasm and in the nuclei of embryonic cells (Ivashkin et al., 2019). In the cells of an adult organism, serotonin can serve as a substrate for transglutaminases, enzymes that help to provide serotonylation, a specific modification of proteins (Walther et al., 2011).

Serotonylation of proteins is essential for the regulation of long-term, delayed, or cyclic processes (Bader, 2019). In the development of *Lymnaea stagnalis*, serotonylation of proteins in embryos at the stages of early cleavage led to a change in the locomotor activity of developing embryos and juvenile individuals and, under certain conditions, to the occurrence of irreversible gastrulation impairments (Ivashkin et al., 2015). The events in the development of the great pond snail, preceding the impairment of gastrulation in the case of an increase in serotonin levels in blasto-

meres, remained unexplained. In particular, the changes occurring in the pattern of spiral cleavage were not described.

In our work, we used the early development of the great pond snail *Limnaea stagnalis* as a model for analyzing the effect of intracellular serotonin on the spiral cleavage pattern. The great pond snail is a classic model object of embryology and developmental biology and its normal development is well studied. In our experiments, we increased the level of intracellular serotonin at the early stages of cleavage by incubating eggs in the immediate precursor of serotonin, 5-HTP. The location of the blastomeres at the animal pole was mapped in detail, their mutual location in the embryos of the control and experimental groups was tracked. The process of embryo development was traced until the completion of metamorphosis, special attention was paid to the processes of gastrulation, the formation of the shell, and eyes.

MATERIALS AND METHODS

Obtaining and Cultivation of Embryos

The embryos of a great pond snail *Limnaea stagnalis* from a laboratory culture were used in the work. The maintenance of animals and the obtaining of eggs was carried out according to the described method (Ivashkin et al., 2015). To synchronize the stages of embryonic development, induced spawning was carried out by transferring mature individuals from compartments with an excess of food (lettuce) into clean standing water. After 5 h of being in clean water, mollusks usually laid egg clutches almost simultaneously, the difference in development between different clutches was no more than 30 min. The stages of embryo development were determined according to the table of development for the great pond snail, Meshcheryakov (1990).

Experimental Increase of Serotonin Level

The eggs were removed from the mucous cocoon and incubated for 24 h in 2 mL of the corresponding freshly prepared solutions. All solutions were prepared based on tap water, which was boiled and filtered through a paper filter. The control group was incubated in 0.1 mM ascorbic-acid solution, and the experimental group was incubated in a solution containing 0.1 mM ascorbic acid and 1 mM serotonin precursor (5-HTP). Ascorbic acid is necessary to prevent oxidation of 5-HTP (Voronezhskaya et al., 2004). After 24 h of incubation at 25°C, the eggs were thoroughly washed with boiled filtered tap water. For subsequent analysis, embryos of three early stages of development: the stage of formation of the apical rosette, flat blastula, and early gastrula (stages 13, 15⁺⁺, and 16 according to Meshcheryakov, respectively) were used. The stage of late gastrula (stage 18) and the stage of completion of metamorphosis (stage 27) were also distinguished.

Immunochemical and Histochemical Labeling

For immunochemical and histochemical labeling, part of the control and experimental embryos that reached the appropriate stages of development (stages 13–18) were extracted from egg capsules by squeezing between two glass slides and subsequent washing with a phosphate buffer (PBS 0.01 M, pH 7.4) through 100 µm mesh. Embryos were fixed in 4% paraformaldehyde at 0.01 M PBS overnight at 4°C and then washed several times with 5% Triton X-100 solution at 0.01 M PBS. The remaining embryos were grown up to juvenile stages. The morphology of embryos that went through metamorphosis was studied under a binocular microscope (Olympus, SZ 60), and developmental abnormalities were documented using an ocular camera (DCM 500, China).

An increase in the intracellular level of 5-HT was controlled by staining the embryos with serotonin antibodies (rabbit polyclonal antibody against 5-HT, Immunostar, Hudson, WI, #20080, dilution 1 : 1000), with subsequent detection by secondary antibodies (goat antirabbit Alexa 488 conjugated IgG, Molecular Probes, 1 : 800). All antibodies were diluted in a blocking solution containing 0.01 M PBS, 5% BSA, and 0.5% Triton X-100. Cell boundaries were labeled with phalloidin (phalloidin-Alexa 488 conjugated, Sigma). The cell nuclei were additionally stained with DAPI. Total preparations of embryos of different developmental stages were clarified in 80% glycerol and then mounted on slides in 80% glycerin.

Analysis of Samples

The analysis of samples was carried out using a confocal microscope (Leica TCS SP5). We used the obtained series of optical sections to construct 3D images using the microscope software. We constructed 2D images of maximum projections using the ImageJ image analysis program (v 1.53) and the GIMP (v 2.10.18) graphic editor, while the layout of the drawings was carried out in Adobe Photoshop CS 8. The positions of the blastomeres were described in accordance with the tables of normal development of *L. stagnalis* (Meshcheryakov, 1990).

At least 25 control and 30 experimental embryos were analyzed at each stage of early development, and 27 control and 32 experimental embryos were analyzed at the gastrula stage. We monitored 220 experimental embryos from 18 clutches up to the juvenile stage. The figures show the most characteristic embryos of each stage of development for the control and experimental groups.

RESULTS AND DISCUSSION

Distribution of Intracellular Serotonin

Incubation with 5-HTP resulted in a uniformed increase in serotonin levels in all blastomeres of early

embryos of the great pond snail. The brightness of antibody staining to serotonin was the same in macro- and micromeres, there was no concentration of a positive reaction in any zones of blastomeres (Fig. 1). These data are different from the previously obtained results on the incubation of embryos of the marine gastropod mollusk *Tritonia* (*Tritonia diomedea*) in 5-HTP. In *Tritonia* embryos, under similar incubation conditions, 5-HT was detected at the animal pole at stage 1–8 blastomeres and in micromeres of the animal pole at the morula stage (Buznikov et al., 2003).

Analysis of the Pattern of Cleavage at Successive Stages of Development

When analyzing the pattern of blastomere location at the animal pole, repeated deviations in the pattern of blastomere location were found in the embryos of the experimental group compared with the control group. The differences began to manifest themselves at the stage of formation of the apical cells of the rosette (stage 13). When comparing the pattern of blastomere arrangement, it was found that blastomeres $1c^{121}$, $1a^{121}$, $1d^{11}$, and $1a^{11}$ do not undergo cytotomy in experimental embryos, which leads to displacement of blastomere c^{111} . As a result, c^{111} begins to contact $1b^{112}$, which is not observed in the control (Figs. 2a, 2a'). The difference in the location of the blastomeres increases when the flat blastula stage is reached (stage 15^{++}). In experimental embryos, cytotomy of blastomere $1a^{11}$ does not occur yet. As a result, blastomeres $1a^{12112}$ and $1a^{12111}$ shift to the prototroch cell $1a^{21}$, and blastomere $1c^{12112}$ begins to contact $1b^{1211}$ (Fig. 2b'). Such contacts between the described blastomeres were never observed in the control group (Fig. 2b). The most noticeable changes occur at the stage of early gastrula (stage 16). At this stage, a number of cell displacements are observed in the embryos of the experimental group; it leads to a change in contacts within the quadrants. Thus, the blastomere $1c^{12112}$ shifts to the region of the descendants of the B quadrant and begins to contact $1a^{1121}$. At the same time, the blastomere $1b^{1121}$ cannot contact blastomere $1a^{1121}$ (normally $1b^{1121}$ always contacts $1a^{1121}$); and blastomere $1a^{1122}$ contacts $1a^{21}$, the prototroch cell, which is not found in the control (Figs. 2c, 2c'). After the early gastrula stage (stage 16), the displacement of blastomeres may vary due to the increasing number of cells. At the same time, in all cases, delays in cytotomy that occurred earlier in quadrants C and A (blastomeres $1c^{121}$ and $1a^{121}$), due to which individual blastomeres are displaced, finally lead to a significant disturbance of the relative position of blastomeres in all quadrants at the animal pole in the embryos of the experimental group.

The spiral cleavage pattern is conservative, deterministic, and stable. Both the positions of each new blastomere and its contacts with neighboring cells are clearly defined. Such interaction in the case of spiral

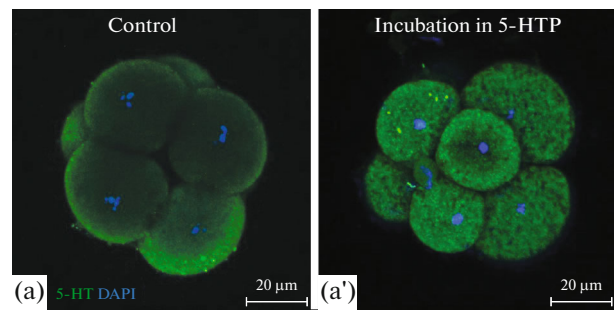


Fig. 1. Detection of intracellular serotonin in the embryos of a great pond snail at the stage of 8 blastomeres. (a) The positive reaction of immunochemical staining with serotonin antibodies in all blastomeres in the control embryo. (a') Uniformed increase in the brightness of immunochemical staining after incubation of the egg with serotonin precursors (5-HTP, 1 mM). Green: antibodies to 5-HT; blue: DAPI. The scale ruler is 20 µm.

cleavage is fundamental since changes in the contacts or mutual arrangement of blastomeres at the stages of cleavage lead to various changes or disturbances in the further development of the gastropod, including the great pond snail (Arnolds et al., 1983). A striking example of the influence of the location of blastomeres on further development is the change in the genetically determined direction of the swirling of the shell in a great pond snail. By mechanical shifting of the rotation axis of micromeres relative to macromeres after the third cleavage division, it is possible to obtain left-twisted snails in a genetically right-twisted line. Similar impairments are also observed with changes in the expression of maternal effect genes, such as *Lsdia1* and *Lsdia2*. It has been proven that the *Lsdia1* gene is associated with the restructuring of the actin cytoskeleton (Kuroda and Abe, 2020). In turn, an increase in the level of intracellular serotonin can lead to long-term changes in the structure of the actin cytoskeleton, which has been shown for smooth muscle cells (Watts et al., 2009). Also, a high level of serotonin affects the properties of the extracellular matrix (Hummerich and Schloss, 2010) and the density of contacts between cells (Li et al., 2016). Changes in the state of the cytoskeleton of blastomeres and the density of contacts between embryo cells when exposed to serotonin may be one of the likely mechanisms underlying the disruption of blastomere movements during great pond snail cleavage. However, this assumption requires additional experimental studies.

Disturbances of Gastrulation and Malformations

Upon reaching the gastrula stage, 80% of the embryos of the experimental groups have an irreversible lethal developmental abnormality. At this stage, a two-layer gastrula with a well-defined blastopore is formed in control embryos (Fig. 3a). At the same time, the experimental embryos consist of two dense

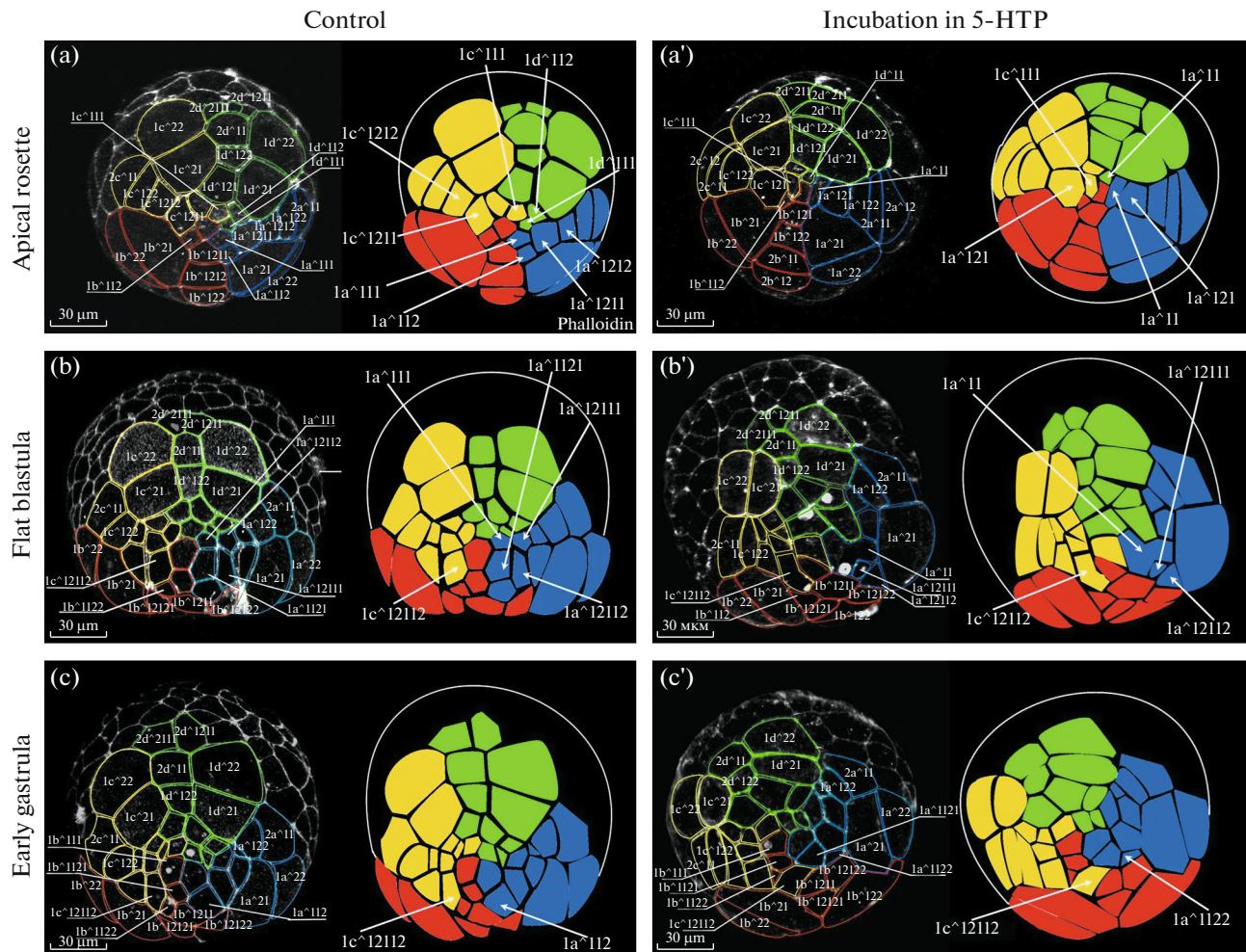


Fig. 2. With an increase in intracellular serotonin level, the mutual arrangement of blastomeres is disrupted during the spiral cleavage of *Lymnaea stagnalis*. All embryos are shown from the animal pole, and cell boundaries are stained with phalloidin (white). Blue drawing is for Quadrant A, red drawing is for Quadrant B, yellow drawing is for Quadrant C, green drawing is for Quadrant D. (a, a') The disturbance in the cleavage pattern begins at the stage of formation of the apical rosette cells (stage 13). The arrows indicate the following: delay in cytotomy of blastomeres $1a^{121}$ and $1a^{11}$ (in quadrant A); nondivided blastomere $1c^{121}$ and displacement of the position of blastomere c^{111} (in quadrant C); blastomere $1d^{11}$ delayed in cytotomy (in quadrant D). (b, b') Disturbances in the arrangement of blastomeres are more clearly visible at the stage of a flat blastula (stage 15^{++}). The arrows indicate the following: blastomere $1a^{11}$ that is still not divided; $1a^{12112}$ and $1a^{12111}$, shifted to the prototroch cell $1a^{21}$; blastomere $1c^{12112}$, shifting towards the quadrant B. (c, c') Pronounced disturbances of the pattern of the arrangement of the animal pole cells at the stage of early gastrula (stage 16). The arrows indicate the following: blastomere $1a^{1122}$, which is displaced and contacts with the prototroch cell $1a^{21}$; blastomere $1c^{12112}$, embedded in the quadrant B. Scale ruler is 30 μ m.

spherical cell masses: ectoderm and endoderm that are bonded together in the area of blastopore formation (Fig. 3a'). Such an abnormality of development in a large pond snail was previously described as an exogastrula (Raven, 1966). The formation of such developmental disorders has been described during short-term incubation of embryos at the stages of the zygote or 2 blastomeres in LiCl (Holland et al., 2005) or azakenpaullone solutions (Kunick et al., 2003). In both cases, the authors assume the involvement of the canonical Wnt signaling pathway in the occurrence of this irreversible developmental abnormality. The question of

the effect of serotonin on the Wnt cascade remains open at the moment. The model proposed by us may be useful for studying the possible interaction between these two regulatory pathways in the development process.

After the gastrula stage, the embryos go through the veliger and velikonkha stages in development, undergo metamorphosis, and spend some more time in the egg before the hatching stage. After metamorphosis, the embryo resembles a miniature adult snail: in control embryos, a head with tentacles and paired dark eyes is formed, a foot is clearly distinguished, and

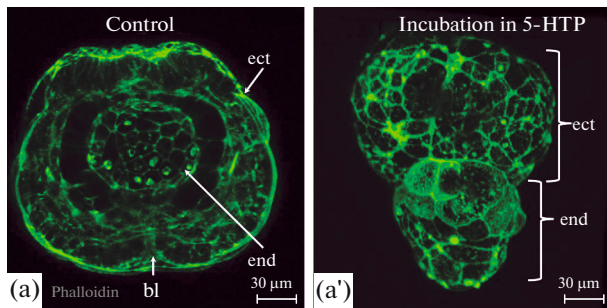


Fig. 3. Embryos of the control and experimental groups at the stage of the late gastrula. The cell boundaries are marked with phalloidin (green). (a) 36 h after the appearance of the first furrow of the cleavage divisions, the embryos of the control group pass the stage of late gastrula with a well-pronounced blastopore. (a') At the same stage of development, the embryos of the experimental group represent in two dense spherical cell masses bonded together in the area of blastopore formation. Designations: bl—blastopore; end—endoderm; ect—ectoderm. The scale ruler is 20 μm .

the formed organs of the visceral complex are covered by the whorls of the shell (Fig. 4a). In 10–15% of the embryos of the experimental group, which continued their development and successfully underwent gastrulation, malformations of two types were observed. In the first case, malformations are associated with eye formation. The formation of one asymmetric eye

(cyclopia) (Fig. 4d), and the appearance of unpaired eyes (Fig. 4e), or multiple eyes on only one side of the head (Fig. 4f) are possible. The second type of malformations was a change in the shape of the shell. The forming shell can lose whorls; the shape of the whorl can be both elongated (Fig. 4b) or wide (Fig. 4c). There was also a divergence between the whorls of the shell with each other, while the normal number of whorls was maintained. It should be noted that malformations of the eyes and the shell can occur both independently and in the same embryo.

Previously, similar malformations were described after manipulations with micromeres, descendants of the 3D organizer, as well as with changes in the position of blastomeres on the cephalic plate in *Lymnaea stagnalis* (Arnolds et al., 1983; Martindale et al., 1985). The authors link such malformations with disturbances in the differentiation of mesodermal and ectodermal derivatives.

CONCLUSIONS

We have shown that incubation with serotonin precursors from the zygote stage until 24 h of development leads to a uniform increase in the level of intracellular serotonin in all blastomeres in *Lymnaea stagnalis* embryos. At the same time, there is a deviation from the classical pattern of spiral cleavage associated with a delay in cytotomy in some blastomeres and a

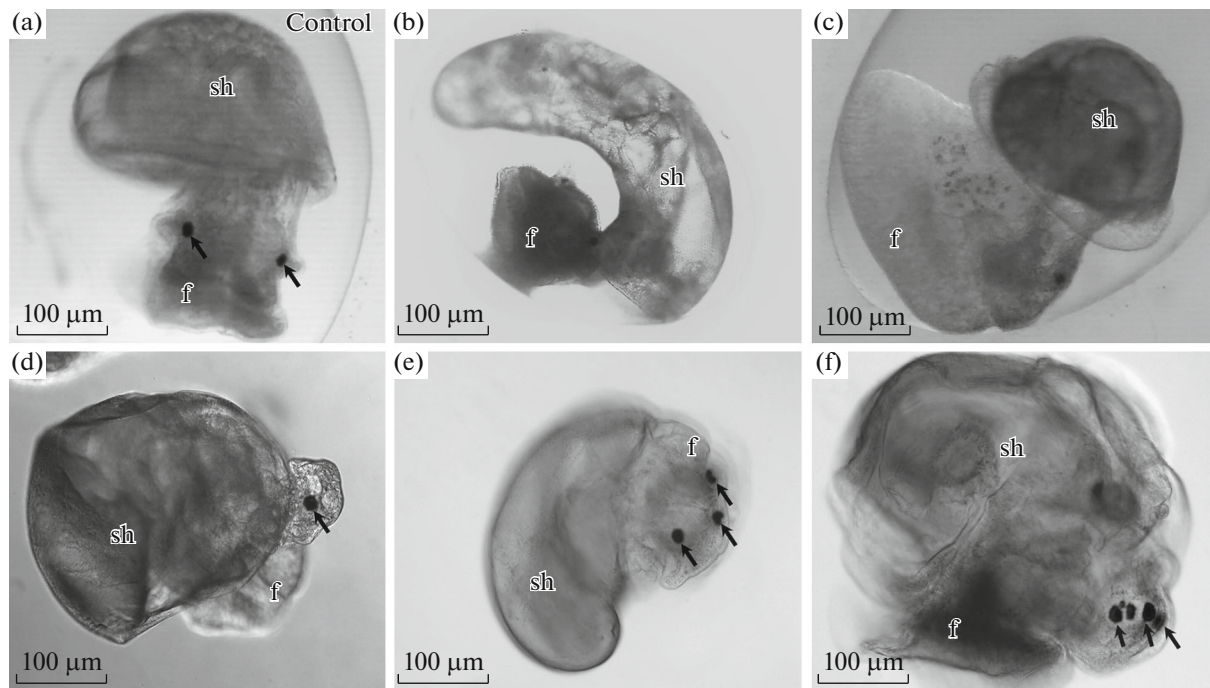


Fig. 4. Examples of malformations that were observed in embryos of the experimental group after the completion of metamorphosis. (a) An embryo of the control group with paired dark eyes and shell whorls. (b, c) Shell malformations: elongated shell without whorls (b), a wide shell without whorls (c). (d–f) Malformations of the eyes: (d) cyclopia, (e) the formation of unpaired eyes, (f) the formation of eyes on one side. The scale ruler is 50 μm . Designations: black arrow—eyes; sh—shell; f—foot.

shift in the relative position of the blastomeres of the animal pole relative to each other. In the subsequent development of some embryos, irreversible impairments of gastrulation, malformation of the eyes, and shell development occur.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

AUTHOR CONTRIBUTIONS

A.I. Bogomolov performed all the experimental work, obtained samples and participated in writing the text of the article. E.E. Voronezhskaya developed the experimental protocol, participated in obtaining the material, and in writing and editing the text of the article.

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