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CONFERENCE MATERIALS

Heterochronies in the Formation of the Nervous and Digestive Systems in Early Postlarval Development of Opisthobranch Mollusks: Organization of Major Organ Systems of the Arctic Dorid *Cadlina laevis*

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Abstract—For the first time using laser confocal microscopy and histochemical and immunocytochemical methods (detection of F-actine, catecholamines, acetylcholintransferase, substance P and FMRFamide) in combination with classical histological methods and electron microscopy of whole-mount preparations, the structure and patterns of formation of the nervous, muscular, and digestive systems in early postlarval development (from 2 days to 4 months) in the opisthobranch mollusk *Cadlina laevis* were studied. Heterochronies manifested in positive allometry of the sensory organs, ganglia of the central nervous system, and the pharyngeal region of the digestive system in relation to overall body size in juvenile specimens compared to adult animals were detected.

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INTRODUCTION

Both classical and modern "evo-devo" works (e.g. Yablokov and Yusufov, 1976; Carroll, 2008) interpreted evolution as modification of ontogeny. Heterochronies are a key phenomenon linking individual development and evolution (Webster and Zelditch, 2005). Despite the considerable progress in understanding the ontogenetic foundations of the evolutionary process, there is still no synthesis between the diversity of organisms (taxonomic aspect) and their ontogeny (Martynov, 2012). Meanwhile, the absence of information on individual development can seriously affect the quality of classification systems and phylogenetic reconstructions (Martynov and Schrödl, 2011).

Opisthobranch mollusks (Opisthobranchia) is evolutionary very flexible and promising model group (Wägele et al., 2013). However, most studies on opisthobranchs and other gastropods have been focused either on the study of the specific features of their larval development (Thompson, 1967; Perron and Turner, 1977; Bickell and Kempf, 1983; Voronezhskaya et al., 1999; Wollesen et al., 2007, 2008) or on demonstration of the structural–functional organization of their adult representatives (Zaitseva, 1978, 2000a, 2000b; Boyd et al., 1986; Chase, 1986; Zaitseva, 1997; Croll, 2001; etc.). There are extremely limited data on the general patterns and specific features of formation and differentiation of the main organs and organ systems in mollusks after metamorphosis in the course of early juvenile development (Marois and Carew, 1990; Croll et al., 1999; Wanninger, 2008, 2009; Kristof and Klussmann-Kilb, 2010).

The phenomena of heterochrony and allometry and their role in the ontogeny of mollusks remain largely unexplored, especially at early stages of their postlarval (juvenile) development.

The purpose of this work is the integration of the morphological study of the structure and patterns of formation of the main organs and organ systems (nervous, muscular, and digestive) during the early postlarval development of the common species of Arctic opisthobranch mollusks *Cadlina laevis*.

MATERIALS AND METHODS

Collection and maintenance. Egg masses of mollusks and adult specimens of *C. laevis* (L., 1767) were collected in mid-July of 2013 in the White Sea in the area of the biological station of the Zoological Institute (White Sea Biological Station, Zoological Institute of the Russian Academy of Sciences, Cape Kartesh) at a depth of 17–20 m. Egg masses were kept under laboratory conditions ~1.5 months at 5–10°C until hatching. Studies were performed on animals at the age of 2 days to 4 months. During this time mollusks grew from 400 μm to 1 mm under laboratory

conditions. Juvenile *C. laevis* were kept at 5–7°C in artificial seawater that was regularly taken from a sea aquarium with fish and invertebrates: soft corals, sea anemones, mollusks, and other sea animals. Thus, the cultivated mollusks received nutrients and diverse small size food intended for sea animals.

It is known that adult specimens of *C. laervis* feed on the sponge *Halisarca dujardini* (Barbour, 1979); however, in our laboratory culture, during the early postlarval stages mollusks did not eat the sponge offered to them, which was apparently related to their incompletely formed digestive system and radula. It was shown previously that juvenile dorids *Doridella obscura* do not feed on food typical for adult individuals—zooids of bryozoans. Planktotrophic larvae of *D. obscura* settle on bryozoans and after metamorphosis feed on small algae on the surface of the bryozoans until they reach later stages of development, when they become capable of attacking living zooids (Perron and Turner, 1977).

For comparison, to reveal heterochronies and allometries in the development of mollusks, adult individuals of *C. laevis* with a body length of 1 to 2.5 cm were used. In total 70 juvenile and 10 adult animals were studied. All comparisons of body sizes and individual organs of juvenile and adult specimens were performed on fixed material.

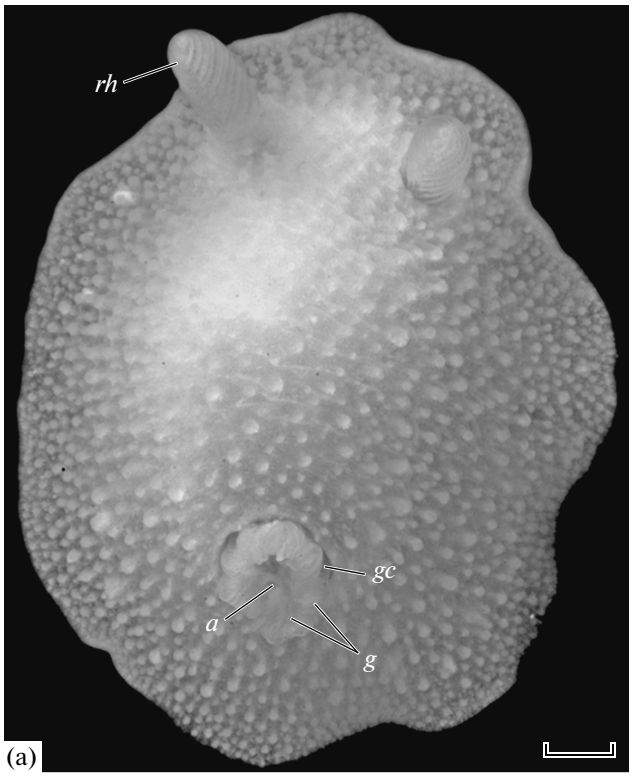
Histological studies. Adult mollusks were fixed for 12 h at 4°C in Bouin's fluid (Romeis, 1948, 1953) or in 4% paraformaldehyde (PFA) P6148 (Sigma-Aldrich, United States) in 0.01 M phosphate buffered saline (PBS) at pH 7.4. Juvenile animals were fixed in 2% PFA for 30 min at room temperature or 2 h at 4°C. After fixation in PFA, mollusks were washed with PBS (3 × 10 min for juvenile specimens and 3 × 30 min for adult specimens), and after fixation in Bouin's fluid molluscs were washed with 70% ethanol until the yellow color disappeared. After the washing the molluscs were passed through a series of alcohols of an increasing concentration, then through isobutanol, benzene and finally they were embedded into paraffin. From the paraffin blocks, series of sections (5–7 μm thick) were made, mounted on slides and stained either with Heidenhain iron hematoxylin and eosine or Heidenhain's AZAN trichrome stain (Romeis, 1948, 1953).

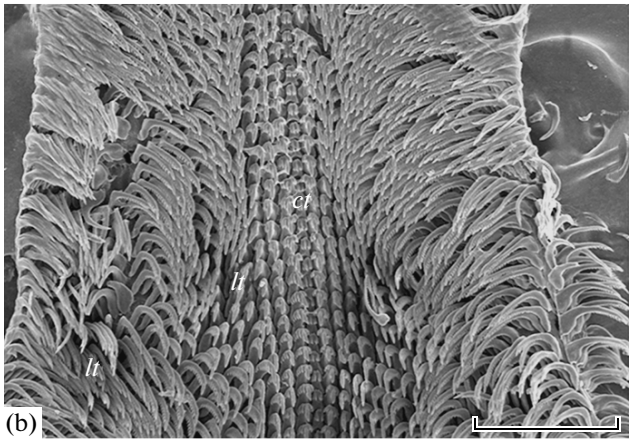
Scanning electron microscopy. Animals were fixed in 4% PFA or 2% glutaraldehyde G5882 (Sigma-Aldrich) in PBS for 2–12 h (depending on the size of animals). Preparations of entire animals and isolated radulae were studied. To reveal the ultrastructure of the surface epithelium, mucus was removed by washing (Zaitseva and Bocharova, 1981). For this purpose fixed mollusks were placed overnight in 16% glycerol in distilled water and then for 6–12 h into 20% ethanol. After dehydration in alcohols and acetone, the material was treated using method of critical point drying. The dried specimens were platinum sprayed under conditions of forvacuum and studied at a magnification from 600 to 15000 using the FEI Quanta 250 electron microscope at the "Taxon" Resource Center of the Zoological Institute of the Russian Academy of Sciences.

Histochemical detection of catecholamines. To stain entire juvenile mollusks, the formaldehyde–glutaraldehyde fluorescence-histochemical method (FaGlu) of Furness et al. (1977) in the modification of Voronezhskaya et al. (1999) was used. Mollusks were incubated for 2 h at room temperature in a fixing solution consisting of 4% PFA and 0.5% of glutaraldehyde in 0.01 M PBS, pH 7.4. Then the mollusks were placed on a slide, dried for 1 h at room temperature under a ventilator, and mounted in 80% glycerol in PBS. Study of the preparations was performed on a Leica TCS SP5 confocal laser microscope at the "Taxon" Resource Center with excitation of fluorescence by a 405-nanometer laser.

The modified method of induced fluorescence of monoamines using glyoxylic acid (GIF) was also applied (De la Torre and Surgeon, 1976). For this purpose, juvenile mollusks were incubated for 1 h at 4°C in a freshly prepared working solution of glyoxylic acid, then placed on slides, and dried first for 30 min at room temperature under a ventilator and then for 30 min in a thermostat at 60°C. The dried objects were mounted in mineral oil. The working solution of glyoxylic acid was prepared according to the protocol: 92 mg of glyoxylic acid G10601 (Sigma-Aldrich) were dissolved in 1 mL of distilled water (final concentration 1 M), then 92 mg of $NaHCO_3$ were added; after neutralization the solution was buffered with HEPES (final concentration 0.1 M) and 100 mg of sucrose (final concentration 300 mM) was added. Studies of the preparations were performed in the same way as in the case of applying the FaGlu method.

Double fluorescent histochemical and immunocytochemical detection of muscular and nervous elements. Juvenile mollusks were fixed in 2% PFA for 30 min at room temperature, then washed, and permeabilized in three changes of 0.3% Triton X-100 in PBS (PBS/Triton), 20 min each. After this, the mollusks were incubated for 30 min at room temperature in 2.5% bovine serum albumin (BSA) in PBS/Triton (PBS/Triton/BSA) for blocking nonspecific binding of antibodies. As the primary antibodies, antibodies to acetylcholine transferase AB-N34 (Advanced Targeting Systems, United States) in the dilution 1 : 200 and antibodies to neuropeptides–substance P 20064 (Immunostar, United States) and to FMRFamide 20091 (Immunostar) in the dilution 1 : 600 were used. Incubation of the mollusks was performed in one of the listed primary antibodies diluted in PBS/Triton/BSA at room temperature for 20–30 h on an orbital shaker. Further, the mollusks were washed in PBS/Triton/BSA (3 × 30 min) at room temperature and transferred to diluted 1 : 500 in PBS/Triton/BSA secondary antibodies conjugated either with Chromeo 488 ab60314 (Abcam, Great Britain) or with Alexa Fluor 633 A-21071 (dilution 1 : 250) (Life Technologies, United States). Incuba-





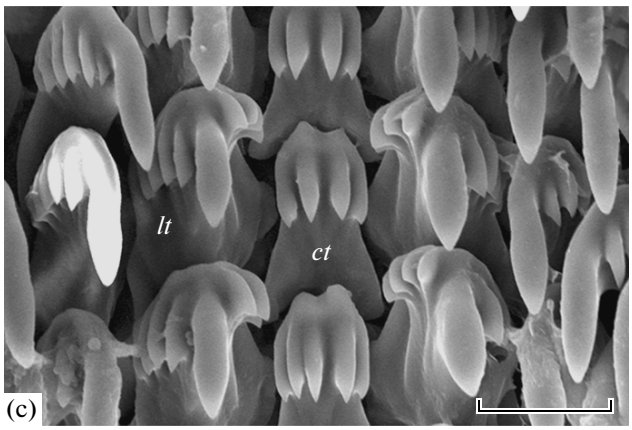


Fig. 1. Adults of *C. laevis*. (a) Dorsal view of living specimens; (b) and (c) multiserial radula and its central part, respectively. Scanning electron microscopy. *a* Anus, *ct* central teeth, *g* gills, *gc* gill cavity, *lt* inner lateral teeth, *rh* rhinophores. Scale: (a) 1 mm, (b) 200 μm, (c) 20 μm.

tion in secondary antibodies was performed for 24 h on an orbital shaker at room temperature. To reveal muscular elements (F-actin), the mollusks were transferred from secondary antibodies into a solution of phalloidin conjugated with Alexa Fluor 546 A22283 (Life Technologies) and diluted in PBS 1 : 300 (up to a concentration of phalloidin ~22 nM). Incubation in phalloidin was performed for 2.5 h on a shaker at room temperature. After washing in PBS (2 × 15 min), the mollusks were mounted on slides in 80% glycerol in PBS.

The specificity of revealing nervous elements was checked by excluding the stage of incubation in primary antibodies and exclusion of antibodies from reaction solutions. The preparations were studied using a Leica TCS SP5 confocal microscope ("Taxon" Resource center).

RESULTS AND DISCUSSION

Studies demonstrated that juvenile specimens of *C. laevis* during the first months after hatching have several considerable morphological differences in the organization of individual organs, their proportions, and location compared to adult animals. Juvenile individuals have not yet formed the gills and gill cavity, and the anus remains on the ventral side under the notum (Figs. 1, 2). The main sensory organs for orientation in nudibranchs as in other gastropods (Bullock and Horridge, 1965; Chase, 1986) are paired head tentacles—rhinophores, also called head tentacles or ommatophores in gastropods from other taxonomic groups. Using their rhinophores, mollusks can orient according to chemical gradients and water or air flows depending on the habitat. The sensory surface of rhinophores of adult specimens of *C. laevis* is considerably increased due to numerous folds (Fig. 1a). In case of danger, rhinophores are drawn with muscular retractors into special sheaths formed by the mantle fold growing around rhinophores (Zaitseva, 2000a, 2000b, 2002; Martynov, 2011). In newly hatched mollusks, rhinophores are smooth, without folds. They are joined together, located at the anterior end of the body along the center, and have not yet formed sheaths (Figs. 2a, 2b). In the first month of life of mollusks, rhinophores come apart, displacing laterally, and form separated sheaths at the anterior edges of the notum (mantle); however, on the surface of rhinophores, folds do not form even in animals at the age of four months (Figs. 2d, 2f). The body of early postlarval specimens does not yet have distinct spicules as in later stages and adult mollusks, but it is covered by a simple ciliary epithelium (Figs. 1a, 2g).

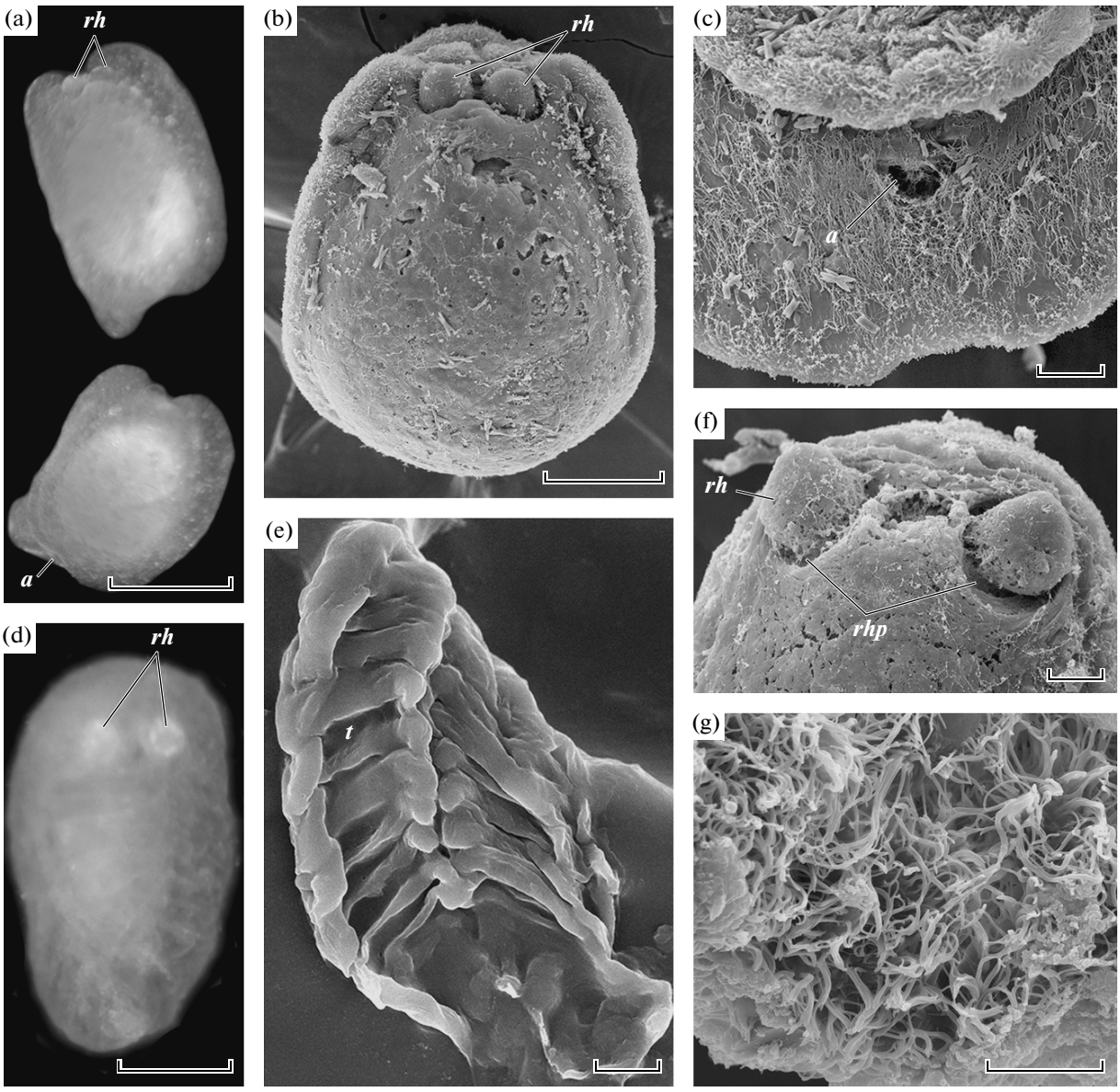


Fig. 2. Postlarval development of *C. laevis*. (a–c) 2–4 days after hatching; (d–g) 2–4 months after hatching, (a, d) general dorsal view of mollusks; (b) joined together rhinophores, sheaths of rhinophores not formed, anus and gills on the dorsal side of the body absent; (c) anus, ventral side of the posterior side of the body; (e) general view of oligoserial radula; (f) dorsal view of the anterior part of the mollusk, large rhinophores have already drawn apart and have separated sheaths; (g) ciliated epithelium covering notum. (a, d) living specimens; (b, c, e, f, g) scanning electron microscopy. *rhp* Sheath of the rhinophore; *t* juvenile teeth of radula; the remaining designations are the same as in Fig. 1. Scale: (a, d) 200 μm; (b) 100 μm; (c, f) 30 μm; (e, g) 10 μm.

Juvenile specimens already have well-formed nervous and digestive systems, as well as all the sensory organs of adult mollusks: besides rhinophores they have paired eyes and statocysts (Figs. 3, 4). Eyes in gastropods are usually connected anatomically with the head tentacles and in representatives of different taxonomic groups are located in a perioptic sinus under a surface epithelium at the base or top of the tentacles (Bullock and Horridge, 1965). Unlike other gastropods, in adult nudibranchs, the eyes are at the level of ganglia of the central nervous system (CNS) not far from the area of entry into them of tentacular nerves near statocysts (Zaitseva, 1978, 1999). *C. laevis* is not an exception in this respect (Fig. 3). It is of interest, however, that eyes in *C. laevis* are formed, as in other gastropods, in head tentacles and only later in the course of ontogeny become displaced to the brain. In the first months after hatching of *C. laevis*, their eyes, although located already beyond rhinophores, nevertheless remain localized in front of the cerebral ganglia far from statocysts (Figs. 4, 5b).

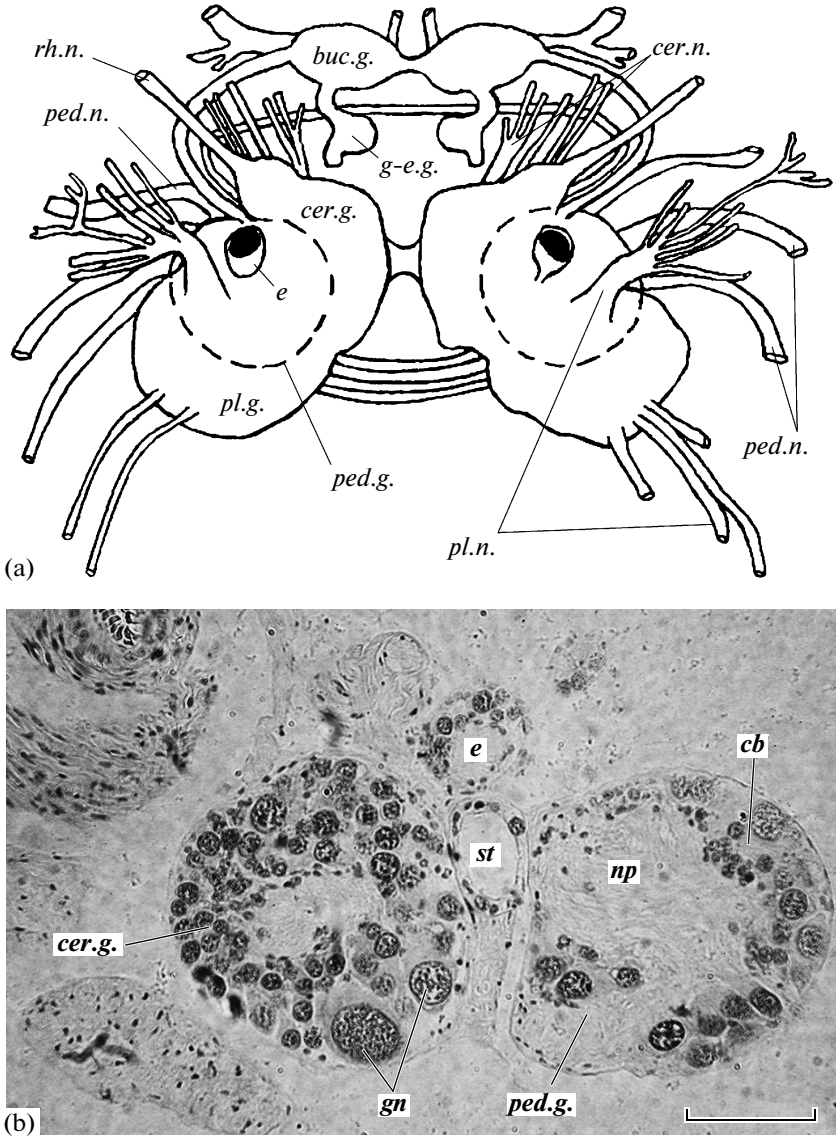


Fig. 3. The central nervous system (CNS) of adult specimen of *C. laevis*: (a) general scheme of organization; (b) saggital section through the CNS. Hematoxylin and eosine staining. *cer.g., pl.g, ped.g. buc.g, g-e.g* Paired cerebral, pleural, pedal, buccal, and gastro-esophagal ganglia, respectively; *cer.n., pl.n., ped.n.* cerebral, pleural, pedal nerves, respectively; *cb* region of cellular bodies; *e* eye; *gn* giant neurons; *np* neuropil; *rh.n.* rhinophoral nerve of cerebral ganglia; *st* statocyst. Scale: 100 μm.

The early postlarval formation of *C. laevis* demonstrates distinct heterochronies in the development of nervous and digestive systems, as well as of all main sensory organs (all ganglia, eyes, rhinophores, statocysts, and pharynx) in relation to the overall body size compared to adult animals. The brain of 2- to 3-month-old mollusks, as in adult animals, is already represented by four pairs of main (cerebral, pleural, pedal, and buccal) and one pair of small additional (gastroesophagal) ganglia forming a compact nervous ring at the pharynx around oesophagus (Figs. 3, 4). All the main nerves typical of adult animals are already present. The active growth of bodies of giant neurons, characteristic of adult gastropods, begins in ganglia: among the total mass of neurons with a diameter of 5–7 μm, specimen cells with a diameter of 15 μm appear (Fig. 4a; Fig. 5a). In 2- to 4-month-old mollusks, the brain occupies the entire central part of the body by its width (Fig. 4a) and height (Fig. 4b). Although all ganglia and the brain as a whole in *C. laevis* increase with age, the ratio of sizes of ganglia of the CNS to body sizes considerably decreases. For instance, the ratio of the diameter of pedal ganglia to the body length in 4-month-old animals averages 1 : 5, and in 8-mm adult animals it is 1 : 19. The diameter of the pedal ganglion of 4-month-old mollusks averages 60–70 μm, and that of the adult animal has a length of 8 mm—260–280 μm.

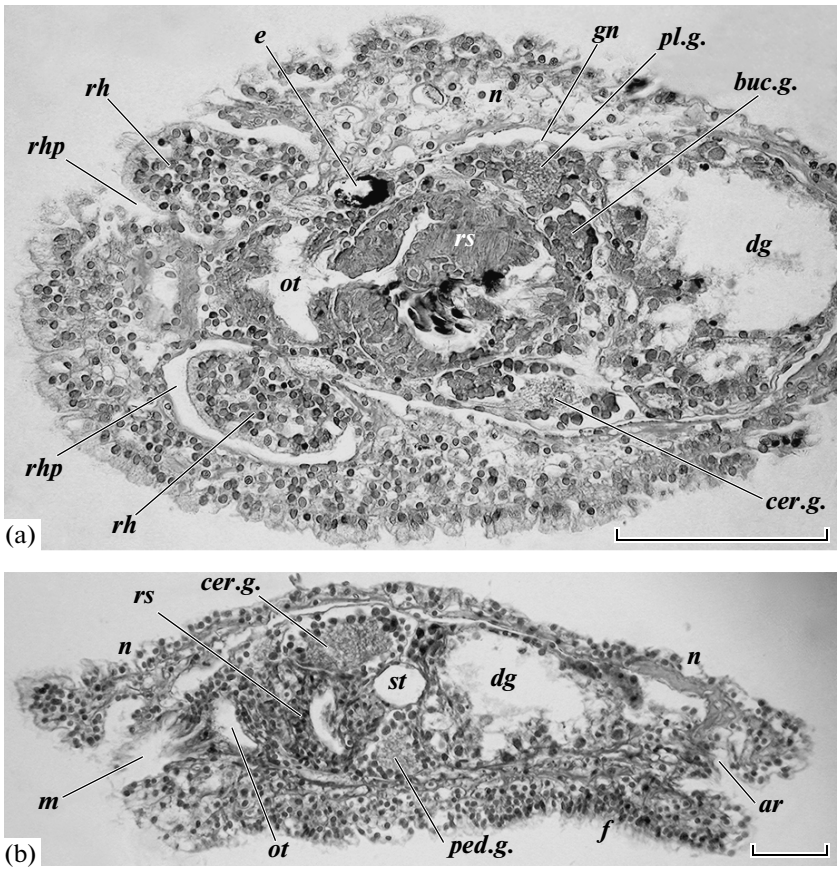


Fig. 4. Structural organization of juvenile specimen of *C. laevis* (4 months after hatching). (a) Frontal section, (b) saggital section. Heidehain's AZAN staining. *ar* Area of anus, *dg* digestive gland, *f* foot, *gn* giant neuron, *m* mouth, *n* notum, *ot* oral tube, *rs* radular sac; the remaining designations are the same as in Figs. 1–3. Scale: 100 μm.

The digestive system of juvenile *C. laevis* is represented, as in adult animals, by a muscular pharynx with a radula and jaws, oesophagus, stomach, digestive gland, and hindgut with anus. The pharynx of juvenile specimens with respect to body sizes has considerably larger sizes than in adult animals; its length can reach 280 μm (Fig. 4). The ratio of the body length (400–500 μm without the notum) to the length of the pharynx in 4-month-old animals averages 1.4 : 1, and in adult animals with a size of 8 mm, it is 3.2 : 1. Despite the considerable size and well defined musculature, the pharyngeal apparatus of juvenile specimens, probably cannot as yet function in full because the radula at this stage only starts to differentiate. The juvenile radula is oligoserial and contains ~10 transverse rows of teeth, in each of which there is only two teeth (Fig. 2e), while the radula of adult animals is multiserial and contains up to 80 transverse rows of teeth, in each of which there are up to 60 teeth (Figs. 1b, 1c). In addition, juvenile teeth are barely differentiated, strongly differ by shape from definitive teeth, and have no denticles, characteristic for adult animals.

This study using modern histochemical and immunocytochemical methods with confocal laser microscopy made it possible to show details and common patterns of the nervous and muscular systems of the mollusk *C. laevis*. Until recently, studies of these systems in juvenile gastropods after metamorphosis have been scarce. There are only a few papers in which the initial stages of formation of nervous and muscular systems in several species of gastropods up to an age of 2–3 days after metamorphosis are shown (Marois and Carew, 1990; Croll et al., 1999; Kristof and Klussmann-Kolb, 2010).

Present study demonstrates that in the peripheral and central nervous system of juvenile *C. laevis* there are a great number of catecholaminergic (CA-er) nervous elements (Fig. 5a). Nervous and receptor cells immunoreactive to the topographic marker of cholinergic nervous elements–acetylcholine transferase (ChAT-im), to neuropeptides substance P (SP-im) and FMRFamide (FMRFa-im) are also detected (Fig. 5b). Catecholaminergic neurons are present in all ganglia of the CNS and are located mainly by one, two, or in small groups. The intensive positive response to catelochlamines is observed in neuropil, commissures, connectives, and in all the main nerves of ganglia of the CNS (Fig. 5a). It is determined by localization of a considerable

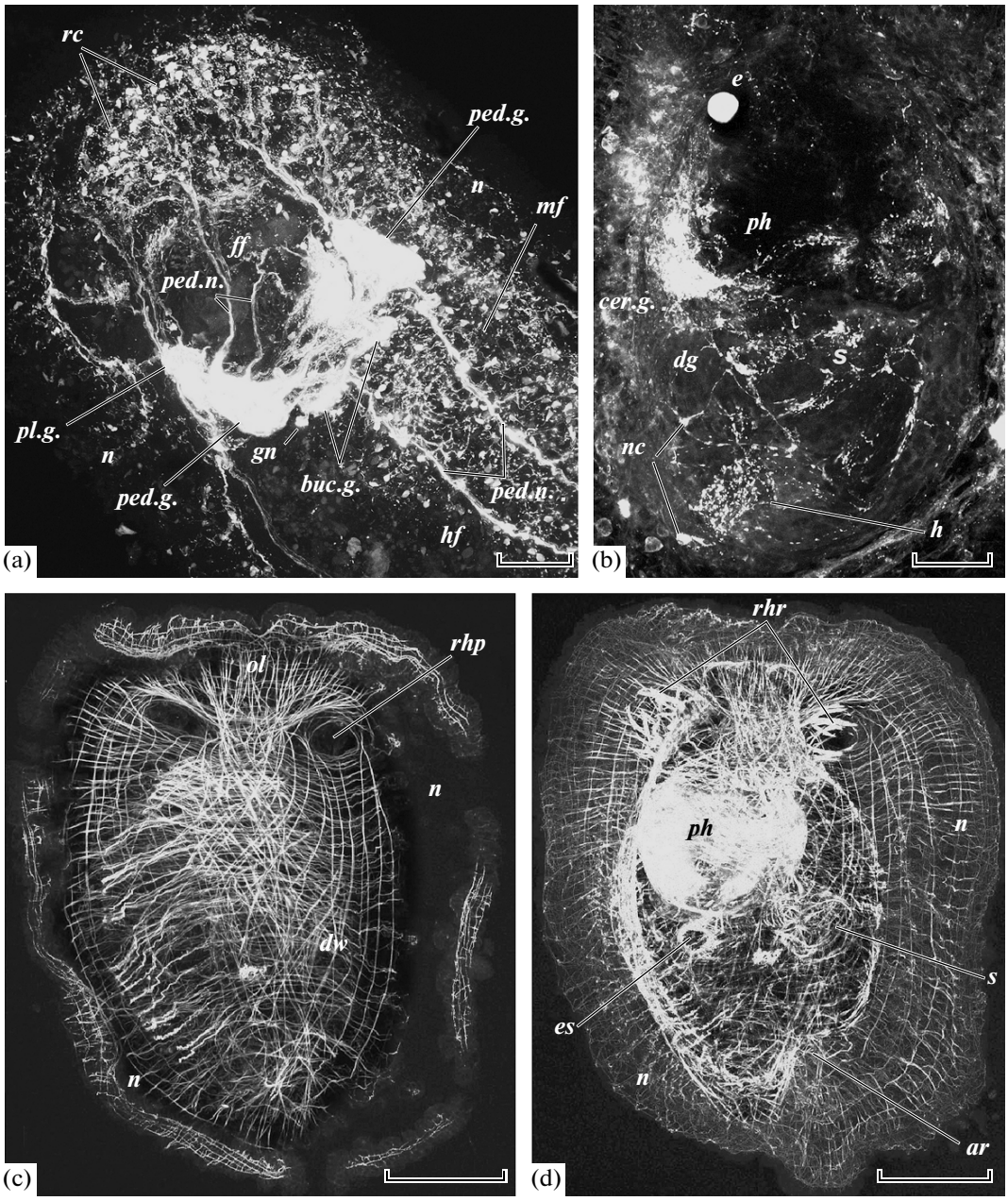


Fig. 5. Nervous system and musculature of a juvenile *C. laevis*. (a, b) Distribution of catecholamine- and FMRFamidergic elements, respectively; (c, d) distribution of muscular elements of the body wall and inner organs, respectively. Reconstructions made at different depth series of confocal optical sections of the entire mollusk. (a) Histochemical method GIF, (b) immunochemical detection of FMRFamide; (c, d) histochemical detection of F-actine with phalloidin. *dw* Dorsal wall of the body, *es* oesophagus, *ff* frontal part of the foot, *h* hindgut, *hf* hind part of the foot, *mf* middle part of the foot, *nc* nerve cells, *ol* oral velum, *ped.n.* pedal nerves, *ph* pharynx, *rc* receptor cells in the sole of the foot, *rhr* retractor of the rhinophore, *s* stomach; the remaining designations are the same as in Figs. 1–4. Scale: 100 μm.

amount of CA-er-fibers. Most of the latter belongs to neurons located in the peripheral neuron system and primarily feeling receptor cells (Fig. 5a). Receptor CA-er-cells are distributed evenly in the body wall, sole of the foot, and notum.

Several receptor cells are revealed near the top of each rhinophore and along the frontal edge of the oral velum. Some CA-er-cells are observed along the digestive tract from the oesophagus to the hindgut. In the sole of the foot, three paired large racemose aggregations of CA-er-neurons (one pair each in the anterior, middle, and posterior parts of the foot) are distinguished. Their axons stretch into the neuropil of pedal ganglia within the respective pedal nerves (Fig. 5a). Nerve CA-er-fibers innervate the musculature of the body wall, foot, and pharynx. It is obvious that in juve-

nile specimens of *C. laevis*, catecholamines can already actively participate in regulation of sensory functions, in the work of musculature and the digestive system, and in feeding behavior, as in the previously studied adult representatives of some species of gastropods (Quinlan et al., 1997; Hernádi and Elekes, 1999; Croll, 2001; Wyeth and Croll, 2011, et al.).

A considerable number of epidermal receptor cells, as well as of nerve fibers innervating the musculature of the body wall and notum of the mollusk, are ChAT-im. Most ChAT-im receptor cells are represented by intraepithelial, slightly oval cells the apical surface of which communicates with the environment. Their axons stretch into the CNS within the respective nerves. Receptor cells similar in shape and manifesting acetylcholine esterase activity (as ChAT, this is a marker of cholinergic nervous elements) were described earlier in skin of polychaetes (Fominykh, 1982). There are data on the important role of acetylcholine as a neurotransmitter in neuromuscular interactions (Lloyd and Church, 1994; Kononenko and Zhukov, 2005), as well as on its possible participation in regulation of digestive functions in gastropods (Zaitseva and Kuznetsova, 2008; Zaitseva and Markosova, 2008). Its presence also was shown in some neurons in larvae of opisthobranch mollusks (Kempf et al., 1992).

Neuropeptides (FMRFamide and substance P) are detected in juvenile *C. laevis* mainly in the digestive system, as well as in ganglia of the CNS (Fig. 5b). A relatively small amount of nerve SP-im- and FMRFa-im-fibers and cells form interweavings in the foot and notum. In addition, they are present in the oral velum, rhinophores, and rhinophoral ganglia, and some of them are represented by primarily feeling receptor cells. FMRFamidergic regulatory elements are widespread in the central and peripheral nervous systems of all gastropods studied to date in this respect (Lehman and Price, 1987; Moroz et al., 1994; Suzuki et al., 1997; Zaitseva, 2004; Kononenko and Zhukov, 2005; et al.) and their larvae (Croll and Voronezhskaya, 1995; Kristof and Klussmann-Kolb, 2010). There is considerably less evidence on the distribution of SP-im-elements and their function in gastropods, although this peptide is well known as a neuromuscular mediator in vertebrates and several invertebrates. It was also revealed in receptor-endocrine cells of the digestive tract of many animals (Punin, 2001; Ovsyannikov, 2003). SP-im-cells and fibers were found in the CNS, digestive tract, skin and musculature of *Helix aspersa* and *Lymnaea stagnalis* (Schot et al., 1981; Boyd et al., 1986).

Unlike the nervous system and receptor organs that in juvenile specimens of *C. laevis* form in the first days after metamorphosis (further only growth of neurons and an increase in the number of nerve and receptor cells continue), the muscular system develops more slowly. First some muscular fibers forming the main body outline, retractors, and future muscular pharyngeal wall appear. During the first four months of postlarval development of *C. laveis*, the number of muscular fibers increases, several groups of muscles and retractors differentiate, the musculature of the digestive tract develops, and a strong two-layer muscular wall of the pharynx, oral and anal sphincters form (Figs. 5c, 5d). Similar processes were shown at the initial stages of formation of postlarval musculature in other species of gastropods (Marois and Carew, 1990; Wanninger, 2008; Kristof and Klussmann-Kolb, 2010).

CONCLUSIONS

Early postlarval *C. laevis* demonstrates distinct heterochronies in the development of the nervous and digestive systems, as well as of the main sensory organs manifested in the positive allometry of a number of organs (first of all, of the entire brain, eyes, rhinophores, statocysts, and pharynx). Rapid differentiation and growth of these organs is in contrast to a relatively slow increase in the body sizes of mollusks and development of the gill apparatus. Differentiation and growth of the nervous and muscular systems occur in parallel and are observed throughout the study period. In the juvenile mollusk, early development and growth of organs and organ systems that provide orientation and basic functionality (i.e., of the sense organs and nervous and digestive systems) are obviously directly related to the efficiency of survival.

This study made it possible to reveal the general patterns of the central and peripheral nervous systems of the mollusk *C. laevis* and to demonstrate the distribution of catecholaminergic, cholinergic, and peptidergic nervous elements, as well as the three-dimensional structure of the body musculature and walls of the inner organs and neuromuscular interactions.

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