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Some Details on the Morphological Structure of Planarian Musculature Identified by Fluorescent and Confocal Laser-Scanning Microscopy

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Abstract—The details of the morphological organization of the body musculature in the planarians *Girardia tigrina* and *Polycelis tenuis* were investigated by histochemical staining of actin filaments with fluorescently labeled fluorescent. The whole mount preparations and frozen tissue sections of planarians were analyzed by fluorescent and confocal laser scanning microscopy. The results indicate that the muscle system is well differentiated in both planarian species and is represented by the somatic musculature of the body wall, the musculature of the digestive tract, and the musculature of the reproductive system organs in *P. tenuis*, which reproduces sexually. The differences and similarities between the two species in the morphological characters of the musculature, which are the size and density of myofibrils in different muscle layers, were described. The results present the basis for further studies on the regulation of muscle function in planarians.

Keywords: planarians, musculature, fluorescent microscopy, confocal laser scanning microscopy, phalloidin
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Free-living flatworms, planarians, belong to the class Turbellria, type Platyhelminthes, and are used as a model for studies on evolutionary aspects of nervous system formation [1, 2], regeneration [3, 4], asexual reproduction [5, 6], and stem cell proliferation [7, 8], as well as the effects of physical [9–11] and chemical factors on morphogenetic processes [12, 13].

Planarian musculature is involved in a variety of motion activities: locomotion, food uptake, and both asexual and sexual reproduction. Since these organisms have no skeleton, their musculature also has a body-support function. Planarians use their musculature for search and retention of prey and for food uptake with the muscular pharynx, which is capable of moving outwards. Muscle contraction in a caudal region seems to facilitate successful detachment of a caudal zooid during planarian asexual reproduction. In species that reproduce sexually the musculature is a prerequisite for searching for a partner and reproductive behavior.

The planarian musculature was previously studied at the ultrastructural [14, 15] and microscopic levels using tissue sections [16–18]. This approach is based on a time-consuming procedure with serial sections and requires miniscule microscopic analysis with further reconstruction and interpretation. Development of a histochemical procedure for identification of actin filaments using the phalloidin toxin from *Amanita phalloides*, which can irreversibly bind with fibrillar actin from muscle cells, was progressive in the sense of studies on muscle cell cytoskeleton [19, 20]. Phalloidin–fluorochrome conjugates, for example TRITC and FITC, made the study of stained tissues by fluorescent microscopy possible. The presence of actin was shown in 1992 [21]; this was the basis for this approach to planarian studies. Thus, confocal laser-scanning microscopy of whole mount samples allowed study of morphological characters of the musculature in flatworms at the modern level. Knowledge on the planarian musculature is the important prerequisite for studies on muscle contraction at the earliest stages of animal evolution.

The object of this study was the structure of the musculature of two fresh water and free-living planarian species: *Girardia tigrina* (Turbellaria, Dugesiidae) and *Polycelis tenuis* (Turbellaria, Planariidae). Whole mount preparations, frozen sections, phalloidin fluorescent tags, and confocal laser scanning microscopy were used for this purpose.

MATERIALS AND METHODS

Planarian culture. The asexual laboratory race *G. tigrina* (Fig. 1a) was cultivated in large glass aquariums filled with tap and distilled water (2 : 1) at

Abbreviation: PBS, phosphate buffered saline.

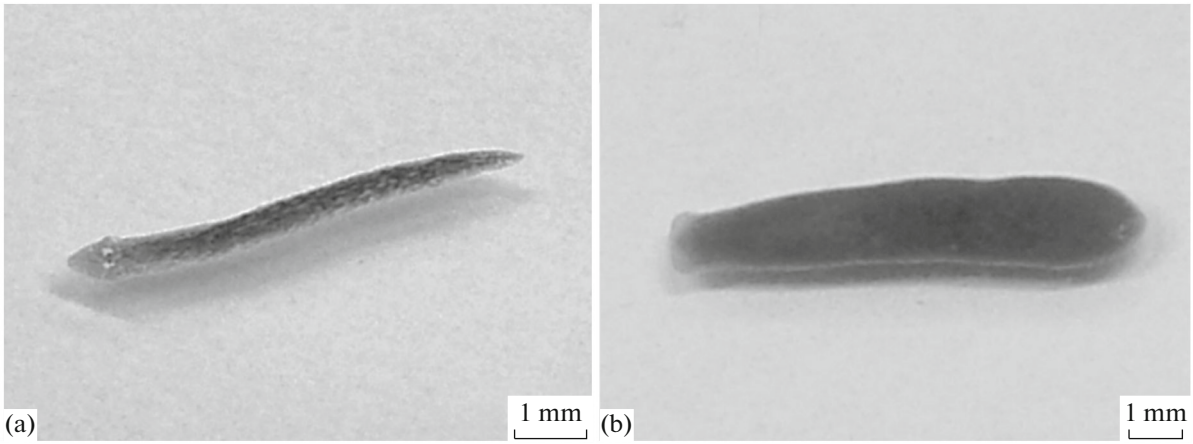

Fig. 1. Planarian species belonging to the asexual laboratory race *G. tigrina* (a) and free-living *P. tenuis* that inhabit reservoirs of the Oka River basin (b).

20 ± 1°C. Wild *P. tenuis* individuals were captured in lakes that belong to the Oka River basin near the town of Pushchino (Moscow Region). Animals were kept for 2 to 3 months and fed with blood worms or earthworms once or twice a week.

For histochemical staining, frozen sections of *P. tenuis*) and whole mount samples of *G. tigrina*) were prepared. Fluorescent phalloidin staining made visualization of myofilaments of the planarian body, pharynx, intestine, and reproductive organs possible.

Histochemistry. *P. tenuis* individuals were fixed with 4% paraformaldehyde (MP Biomedicals, United States) in 0.1 M phosphate buffer saline (PBS; pH 7.4; Helicon, Russia) for 4 h at room temperature. The samples were transferred to 1.5 mL Eppendorf tubes filled with a fresh paraformaldehyde solution and incubated for 6 h at 4°C. The samples were placed in PBS buffered 10% sucrose (Helicon, Russia) and incubated for 3–5 days at 4°C. The samples were then mounted with Tissue Tek compound (Tissue Tek, United States), and longitudinal sections of 12–17 μm were prepared with a Shandon Cryomatrix microtome (Termoelectron Corporation, United States) at –18 to –20°C. The sections were placed on sample glasses treated with poly-L-lysine (Polysine, Menzel-Glaser, Germany), dried for 1 h, and stored at –20°C until they were stained. Just before staining the sections were thawed at room temperature for 10–15 min and washed three times for 10–15 min in a PBST solution (PBS with 0.3% triton X-100; Sigma, United States; 0.1% sodium azide; Helicon, Russia; and 0.1% bovine serum albumin; Amresco, United States). The sections were placed strictly horizontally in a wet chamber and stained with TRITC-tagged phalloidin (Sigma, United States) diluted 1 : 200 for 4–6 h at 4°C. The samples were washed three times in PBS for 5 min, placed in 90% gycerol in PBS (Helicon, Russia), covered by cover glasses, and analyzed with a fluorescent microscope.

To prepare whole mount *G. tigrina* samples individuals (8–9 mm) were fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) under a cover glass for 4 h at room temperature. The samples were incubated in the PBST buffer for 24 h at 4°C, washed several times with PBS, and placed in the TRITC- or FITC-tagged phalloidin solution for 12 h at 4°C. The prepared whole mount samples were placed on sample glasses in a drop of 75% glycerin in PBS and covered by cover glasses.

Microscopy. The stained sections were examined with the use of a fluorescent microscope Leica DM600 (Leica, Germany) equipped with a digital camera DC300F (Leica, Germany). For FITC (fluorescein isothiocyanate), a fluorescent filter I3 (excitation spectrum 450–490 nm; emission spectrum 515 nm) was used, while TRITC (tetramethylrhodamine isothiocyanate) was detected with a N2.1 filter (excitation spectrum 515–560 nm; emission spectrum 590 nm). The images were saved in the TIFF format at high resolution.

The whole mount planarian specimens were analyzed with a Leica TCS SP5 confocal laser scanning microscope (Leica, Germany). The microphotographs were represented as a total of 8 to 32 consequent optical sections. The sections were obtained by sample scanning (20–80 μm sample thickness) and the images were reconstructed, if needed, at the maximum fluorescence intensity. The computer program used was the one that was included in the microscope equipment. The analysis of each variant was performed in 5–7 replicas (samples). The morphological measurements were carried out with fluorescent and confocal scanning microscopes and the ImageJ program (National Institutes of Health, United States) in the Java programming language.

RESULTS

The organization of body musculature in *G. tigrina*. Phalloidin staining revealed a well-developed muscle system consisting of numerous tightly packed myofilaments in the *G. tigrina* body. The body wall muscles are divided in three layers: external, internal, and diagonal fibers. The external layer is represented by regularly located circular fibers; the internal layer consists of thicker longitudinal muscle fibers while the diagonal layer includes rare diagonal fibers placed between the longitudinal and circular muscle layers of the body wall (Figs. 2a, 2b). This structure is typical for a variety of body regions ranging from the cephalic to caudal end (Figs. 2b, 2d). Both the thin (1.8–2.4 μm) and thick (3.1–4.3 μm) bundles of the circular fibers were observed immediately beneath an external epithelial cell layer with 2.5–3.0 μm distance between them, sometimes up to 5 μm (Figs. 2a, 2c). The diagonal muscle filaments of the body wall (0.8–1.6 μm) are oriented in two intersecting directions (Figs. 2a, 2c). The fibers are loosely packed at a distance of 7–14 μm between them (Fig. 2c). The internal longitudinal layer consists of thick (5–12 μm) bundles of tightly packed muscle fibrils with the distance of 2.5–3.0 μm (scarcely up to 8 μm) between them (Figs. 2b, 2c). Dorsoventral muscle fibrils connect the upper and lower sides of the body wall. They are more or less evenly distributed and they traverse the entire animal body. Thin transverse fibers connecting two lateral body sides were also observed.

The pharynx, which is located in the body center, has a tube-like musculature (Fig. 2e). The external pharynx muscle layer consists of blocks of longitudinal fibers and circular fibers located underneath (Figs. 2f, 2e). The internal pharynx muscle layer contains circular, longitudinal (Fig. 2f), and diagonal fibers. The phalloidine staining was observed in pharyngeal short radial fibers connecting the external and internal layers of the pharynx wall (Fig. 2f). The phalloidin staining was intensive in so-called anchoring muscles, which attach the pharynx to the planarian body at the pharynx base. The musculature of the mouth opening is situated on the ventral side and includes both circular and radial muscle fibers, thus forming the sphincter, which the pharynx moves forward through to pump food in the intestine (Fig. 2g). In *G. tigrina*, intestinal lumen are surrounded by irregular oriented and scarce circular and diagonal muscles (Fig. 2h).

The body wall muscles of *Polycelis tenuis*. The staining with TRITC-tagged phalloidin revealed the developed body musculature in *P. tenuis*. Numerous small "eyes" (photoreceptors) located on the anterior end of the worm are surrounded by very thin muscle filaments (Figs. 3a, 3b). Similar to *G. tigrina*, the body wall of *P. tenuis* includes three layers of muscle fibers: circular, longitudinal, and diagonal layers (Figs. 3b, 3d). A thin layer of circular fibers is located immediately beneath the epithelium and the basal membrane (Figs. 3c, 3d). The thicker longitudinal fibers are regularly packed. This muscle layer is organized in 3–5 μm bundles consisting of two to four muscle fibrils with 4–5 μm distance among them (Fig. 3d). The circular and diagonal layers normally consist of single or double muscle fibers of 1.3–2.1 μm thickness. The phalloidin tag is visible in the fibers that connect the dorsal and ventral surfaces of the *P. tenuis* body (dorsoventral fibrils). These have a rather regular orientation (Fig. 3b), and some of them penetrate the spongy tissue of the cerebral nervous ganglia. At a distance of 50–60 μm, dense dorsoventral bundles (10–20 μm) consisting of several muscle fibers were revealed in the lateral region of the planarian body (Fig. 3c).

The cylindrical muscular pharynx is connected to the body at its basal end with anchoring muscles (Fig. 3e). The pharynx tube consists of two (external and internal) muscle layers that contain circular and longitudinal fibers densely packed in the pharynx wall. The layers are joined with radial fibers. The cavity that encompasses the pharynx tube is situated in the middle of the body. Its walls are formed by the circular, longitudinal, and diagonal muscle fibers, which is similar to the organization of the external musculature of the body wall (Fig. 3e). The intestinal lumen are overlaid with loose and extremely thin muscle filaments. As well, the longitudinal muscle fibers that underlie the intestine lumen were revealed in *P. tenuis*. The *P. tenuis* reproductive system is complex and includes several organs represented by muscle tubes and reservoirs. As an example, the walls of the copulation bursa contain irregularly oriented thin myofilaments (0.8–1.5 μm) located in all directions (Fig. 3f).

DISCUSSION

In this study, the histochemical method was used to characterize the body musculature of the planarian species *G. tigrina* and *P. tenuis* in detail. The quantitative estimation of the muscle fiber size and the distance between them in the different muscle layers of the body wall are presented for the first time. The most important data on the spatial organization of the musculature were obtained from the *G. tigrina* whole mount slides, while only *P. tenuis* frozen sections were used because of the large size and thickness of this planarian.

Comparison of *G. tigrina* and *P. tenuis* showed that both species have many traits in common concerning the structure of the musculature. Microscopic analysis revealed a highly organized body wall in both species. The external circular fibers overlie internal longitudinal filaments that together form tightly packed muscle layers with few diagonal muscles between them. *G. tigrina* has musculature that is less tight; however, the position of the layers is similar to that of *P. tenuis*. The dorsoventral muscle fibers are quite regular along the entire body. In *G. tigrina*, the intestine is surrounded by very thin, infrequent, and irregular muscle

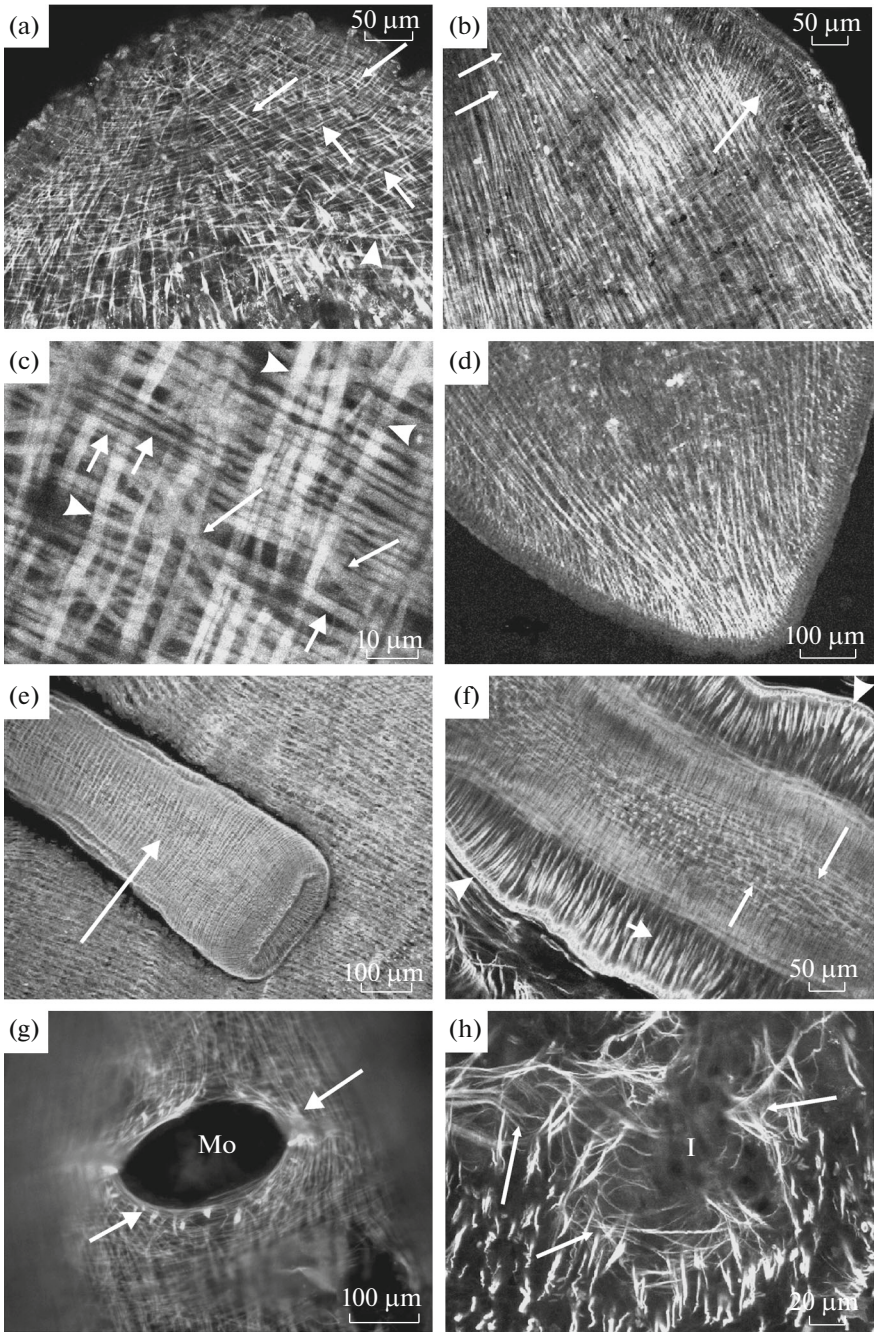

Fig. 2. *G. tigrina* body musculature. The sample was stained with TRITC-tagged phalloidin (gray) and studied under the confocal laser scanning microscope. (a) The anterior body end is represented. The longitudinal, circular, and diagonal muscles are designated by the sharp long arrows, blunt arrows, and anterior end, respectively. (b) The right lateral side of the anterior body end is shown. The dense layer of the longitudinal muscles and muscles in the right lateral auricular region (two of them represented by a pair of sensitive formations situated on the anterior end) are designated by the thin arrows and thick long arrows, respectively. (c) The upper right angle close to the cerebral end is shown with the higher amplification. The longitudinal (arrow heads), circular (blunt arrows), and diagonal (thin long arrows) fibers and fiber bundles of different thickness, belonging to the body wall, are visible. (d) The longitudinal musculature layer of the caudal part of the planarian body is shown. (e) The muscular pharynx in the central body region is represented. As well, the external longitudinal and circular layers of the pharynx wall are visible. (f) Here, the pharynx musculature is represented as an optical cross section of the pharynx central region. The longitudinal and circular fibers of the internal pharynx muscle layer (long arrows) are visible, as well as the radial (short arrows) and circular (arrow heads) fibers of the external pharynx muscle layer. (g) The musculature of the sphincter (arrows) of the mouth opening (Mo) is shown. The pharynx tube is capable of moving forward due to the anchor muscles for food uptake through the mouth opening located on the ventral planarian side. (h) The thin myofibrils surrounding the lumen of the blind intestine (I) can be seen.

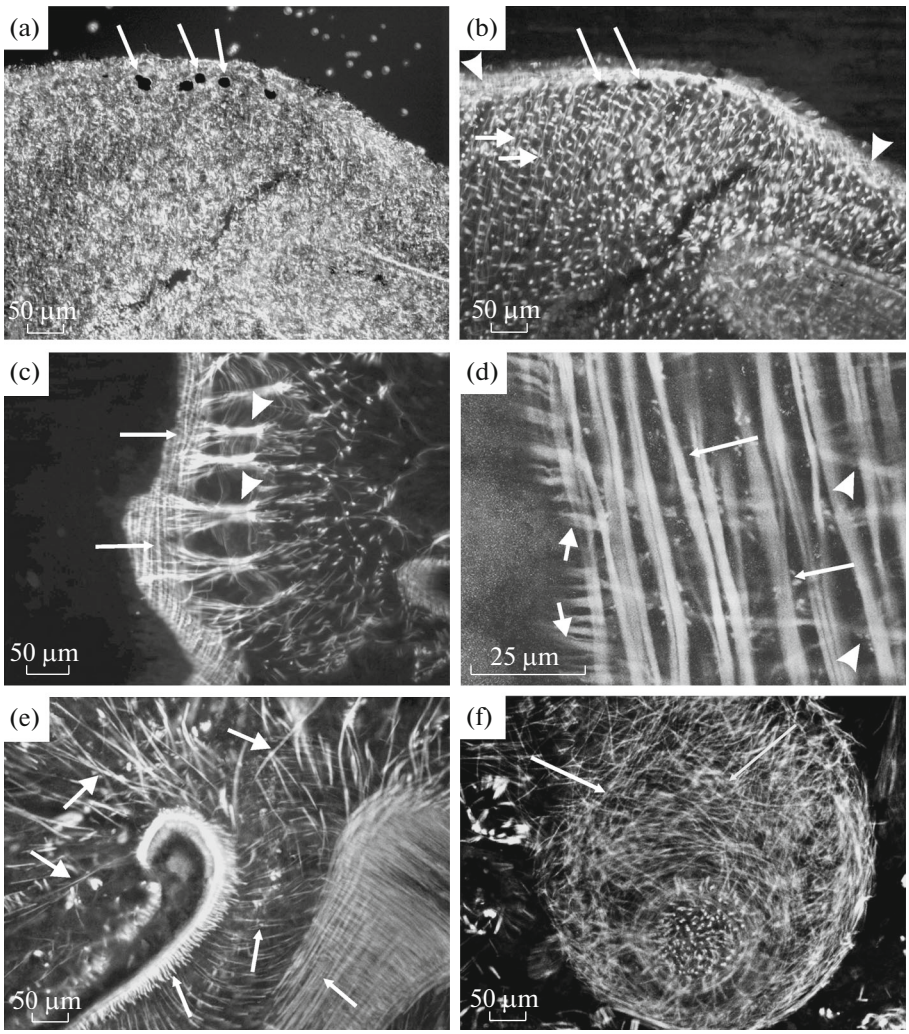

Fig. 3. Frozen tissue sections of *P. tenuis* stained with TRITC-tagged phalloidin. (a) The light microscopy revealed the cerebral end with the photoreceptors (long arrows). The fluorescent microscopy images are represented in (b) through (e). (b) The cerebral end is shown. The circular, diagonal (the short blunt arrows), and longitudinal (arrow heads) muscles of the body wall are represented. In the front line, the photoreceptors surrounded by the thin myofibrils are visible. (c) The longitudinal and diagonal fibers building the wall musculature (long arrows) together with the dense regular bundles connecting the dorsal and ventral sides (located dorsoventrally and designated by arrowheads) are shown. (d) The thin diagonal myofibrils (arrow heads) located between the longitudinal (long arrows) and circular (short arrows) muscle layers are represented. (e) The basal pharynx section is shown. The pharynx myofibrils (thin arrows) and the anchor muscles (thick arrows), which hold the pharynx, are represented. (f) The thin irregular muscle fibers in the planarian reproductive system (long arrows) can be seen.

fibers; among these the best distinguished are the diagonal fibers that stretch in two perpendicular directions. In *P. tenuis*, thin longitudinal fibers that underlie the intestinal lumen were revealed (these results, in part, have been represented previously [22]). The pharynx of both species is located in the peripharyngeal space and attached to the body by its basal end with anchor muscles, as was described in our previous study on pharynx regeneration in *Dugesia tigrina* [23]. The external pharynx muscle layer consists of blocks of the longitudinal fibers overlaying the circular ones. The internal pharynx muscle layer consists of circular, longitudinal, and diagonal fibers. The radial muscle fibers connect the external and internal layers.

The two species were found to differ in the size and density of the myofibrils that constitute the body wall. Moreover, in *P. tenuis*, the clearly visible musculature of the reproductive organs, which is absent in *G. tigrina* due to its asexual reproduction, was revealed.

Thus, there are three muscle systems in the planarian organism. The first is the somatic musculature of the body wall, which consists of external circular, internal longitudinal, and intermediate diagonal myofibrils. This system seems also to include the dorso-

ventral and lateral muscle fibers found in *P. tenuis*, thus constituting a framework for organs, tissues, and cells. The second system is the musculature of the digestive tract including the thin fibers surrounding the intestinal lumen (the intestine is blind in planarians) and the well-developed dense musculature of the flexible pharynx. Finally, the third system is the musculature of the reproductive apparatus identified in *P. tenuis*.

There are few data on the structure of the planarian muscle system. This information was obtained with different methods and purposes, and mostly concern of pharyngeal musculature. Planarian muscle cells beside of actin [21] are known to contain myosin [18, 24] and combine the properties of both smooth [14, 15] and striated muscles. The *DjMHC-A* and *DjMHC-B* genes were previously found to encode two types of heavy myosin chains in *D. japonica* [25]. These genes are expressed in pharyngeal and body muscles. As well, pharynx regeneration in this flatworm was studied with TMUS13 antibodies against planarian muscle cells [16]. These antibodies specifically labeled the musculature of the intact and regenerating pharynx. In another study, DjMHC-A antibodies were used to identify the pharynx musculature in *D. japonica* [18]. In *D. tigrina*, cell differentiation of pharyngeal muscles [23] and the planarian anterior end [26] was studied with tagged phalloidin in the regeneration process. The morphological structure of the *D. japonica* musculature was described in detail via immunocytochemical localization of antibodies against the myosin DjMHC-A and DjMHC-B [17] heavy chains.

Currently, ultrastructural studies on the musculature of free-living and parasitic flatworms are possible via laser scanning microscopy and fluorescently tagged phalloidin [27, 28]. A number of parasitic worm species were involved in studies on the musculature of adult individuals and larvae performed at Queen's University of Belfast, UK; Abo Academi University, Finland; and the Severtsov Center of Parasitology, Severtsov Institute of Ecology and Evolution of the Russian Academy of Sciences, Moscow, Russia. The species were *Diphyllobothrium dendriticum* [29], *Fasciola hepatica* [30], *Schistosoma mansoni* [31, 32], *Apatemon cobitidis proterorhini Cotylurus erraticus*, *Bucephaloides gracilescens* [33], *Echinoparyphium aconiatum*, *Cyathocephalus truncates*, and others [34–36]. The comparison of our results obtained on *G. tigrina* and *P. tenuis* with the data on other flatworm representatives [37, 38] showed that the studied features are conserved among Platyhelminthes.

The closest relatives of planarians are parasitic flatworms with complex reproductive cycles that are difficult to reproduce under laboratory conditions. For this reason, planarians, due to their simple cultivation and convenient manipulation, can be used as the model for a search for new drugs that are efficiently targeted to and affect flatworm musculature.

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