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Rotifer muscles as revealed by phalloidin-TRITC staining and confocal scanning laser microscopy

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Abstract

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By combining phalloidin-TRITC staining with confocal scanning laser microscopy (CSLM), the pattern of the musculature in two species of Rotifera, *Euchlanis dilatata unisetata* and *Brachionus quadridentatus* is revealed. The same general muscle pattern prevails in both species. The major components of the body wall musculature are: 1. retractor muscles (5 pairs in *E. dilatata unisetata* and 3 pairs in *B. quadridentatus*); 2. Two pairs of dorso-ventral muscles; 3. Two pairs of perpendicular muscles (in *E. dilatata unisetata*); 4. retractors of the corona (median, lateral and ventral); 5. Foot retractors. In addition, three pairs of cutaneo-visceral muscles and visceral muscles (including mastax muscles) are described. The sphincter of the corona was found only in *B. quadridentatus*. The high degree of muscle differentiation points to a high level of development of rotifer muscular system.

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Introduction

Rotifers are aquatic free-living animals, microscopic in size. They rarely exceed 0.5 mm. Rotifers swim with the aid of locomotory cilia located at the anterior end of the animal, the corona. The body is usually elongated with an elongate trunk, and a foot with toes. The trunk has a hard encasement, the lorica, formed by intracellular strengthening elements within the epidermal cells. The muscular pharynx (mastax) is provided with internal jaws (trophi). Rotifers lack distinct layers of body wall muscles, instead they display an array of single muscles. To date, the muscular system in rotifers has mostly been studied in representatives of the Pseudotrocha and only by means of classical histological methods. The morphological data obtained from studying serial sections (Martini, 1912; Nachtwey, 1925; Peters, 1931; Stoßberg, 1932; Dehl, 1934) have been reviewed and summarized by Remane (1929–33). The ultrastructure of the cross-striated, obliquely striated and smooth muscle types has been described on serial sections in *Trichocera rattus* (Amsellem and Clément, 1977, 1988; Clément and Amsellem, 1989). The authors list the innervation and function (withdrawal, escape, foot movement and the control of ciliary beat) of each muscle. Recently, the phalloidin histochemical method which permits the visualization of F-actin in muscle fibres, has been applied in morphological investigations of flatworm muscles (Rieger *et al.*, 1994; Czubaj and Niewiadomska, 1997; Wahlberg, 1998; Hooge and Tyler, 1999). Fluorescence microscopy of whole mount preparations treated with TRITC-conjugated phalloidin, enables one to see the precise arrangement of major and minor muscles. The use of confocal scanning laser microscopy (CSLM) gives additional advantages, permitting not only optical sectioning of the whole-mount preparation, but also flat projections of these sections (max-projection option) and stereo pictures of the preparations.

Materials and Methods

The study was made on two species of Rotifera from the family Brachionidae: *Euchlanis dilatata unisetata* Leydig, 1854, and *Brachionus quadridentatus* Hermann, 1783. The rotifers were collected in pools in the Yaroslavl district of Russia. The animals were fixed in Stephanini fixative (2% paraformaldehyde

and 15% picric acid in 0.1 M Na-phosphate buffer) at pH 7.6, stored for several weeks in fixative at +4 °C, and rinsed for 24–48 h in 0.1 M Na-phosphate buffer (pH 7.6) containing 10–20% sucrose. The rotifers were handled as whole mounts on poly L-lysine coated glass slides. Prior to staining, the animals were immersed in phosphate-buffer saline (PBS) containing 0.2% Triton X-100 (PBS-T) and 2% bovine serum albumin (BSA). Staining of the F-actin fibres with TRITC-labelled phalloidin (Sigma) (1 : 200) was performed in darkness for 20 min at 4 °C (Wahlberg, 1998). After staining, the animals were washed in PBS, mounted with 50% glycerol in PBS and examined in confocal scanning laser microscope (LEICA TCS 4D). The scanning step size was usually about 0.5 µm and never more than 2 µm. The number of optical sections in a series ranged from 15 to 25, depending on the size of the specimen. The max-projection option was used to obtain reconstructions from a series of optical sections. The files with initial resolution 72 pixels per inch (ppi) were processed with Adobe Photoshop 4.0. Only the commands 'levels of grey', 'brightness' and 'contrast' were used, to avoid any distortion of the information content of the images.

Results

The rotifer musculature can be subdivided into body wall muscles, visceral and cutaneo-visceral muscles. The longitudinal muscles of the body wall act on the head and foot as retractors. The proximal parts of main retractor muscles are attached to the body wall by a pair of common roots.

Euchlanis dilatata unisetata (Figs 1, 2, 3, 6A–C, 7, 8)

Five pairs of retractor muscles represent the major part of body wall musculature. They are: 1. anterior-ventral retractors (**avr**); 2. posterior-ventral retractors (**pvr**); 3. lateral retractors (**lr**); 4. anterior-dorsal retractors (**adr**); 5. posterior-dorsal retractors (**pdr**). The first four pairs of retractors originate from common paired roots, i.e. main roots of retractor muscles (**mrr**). The **mrr**'s lie on the lateral sides of the upper border of the mastax and are attached to the body wall near the lateral grooves of the lorica (Figs 1, 2, 3, 6A–B, 7A). The **mrr**'s are connected to each other by a transverse muscle (**tm**), lying above the mastax (Figs 1, 2, 6C, 7B). The retractors differ from each other both in length and in thickness. The short **avr**'s reach down to half of the body length, while the longer **pvr**'s and **lr**'s reach down to two-thirds of the body length (Figs 2, 3, 6A–C, 7A–C, 8C). The shortest pair originating from the same roots is **adr** (Figs 1, 3, 6C, 8C), reaching down to the lower border of the mastax. The **pdr**'s originate at a level near the middle of the **adr** (Figs 1, 3 6C, 7D, 8C). The proximal ends of **pdr**'s closely adhere to the distal ends of the **adr**'s and run parallel to them. The distal parts of **pdr**'s are attached to the dorsal plate of the lorica just above the base of the foot

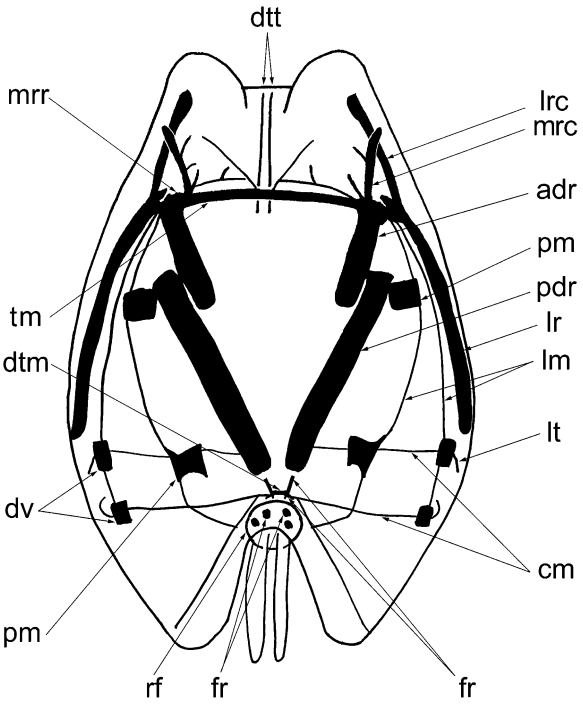

Fig. 1—Schematic drawing of the body wall musculature of *Euchlanis dilatata unisetata* as revealed by phalloidin-TRITC staining. View from the dorsal side.

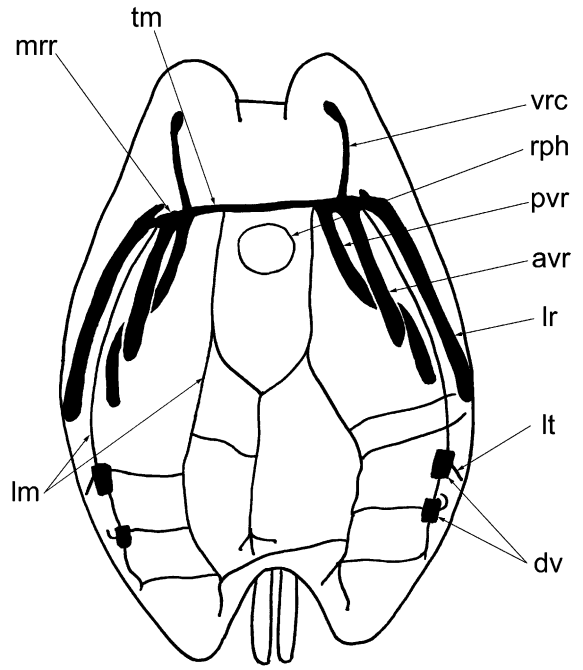

Fig. 2—Schematic drawing of the body wall musculature of *Euchlanis dilatata unisetata* as revealed by phalloidin-TRITC staining. View from the ventral side.

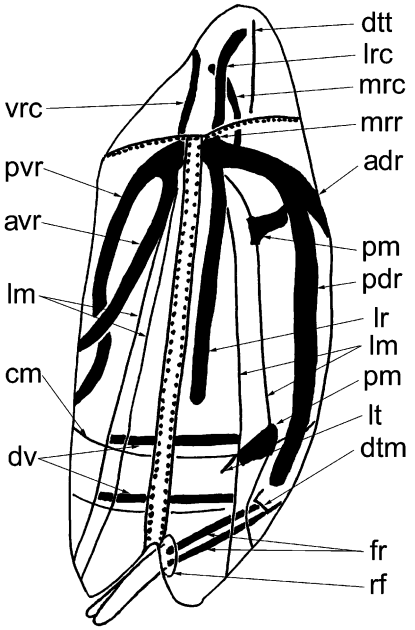

Fig. 3—Schematic reconstruction of the body wall musculature of *Euchlanis dilatata unisetata* seen from the lateral side. The borders of the ventral and the dorsal plates of the lorica are shown as lines with dots.

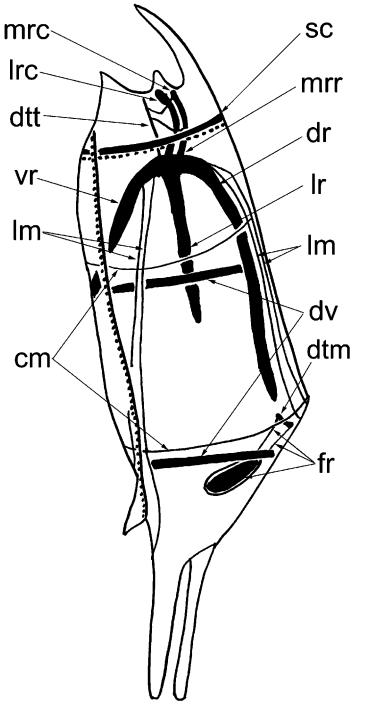

Fig. 5—Schematic reconstruction of the body wall musculature of *Brachionus quadridentatus* seen from the lateral side. The borders of the ventral and the dorsal plates of the lorica are shown as lines with dots.

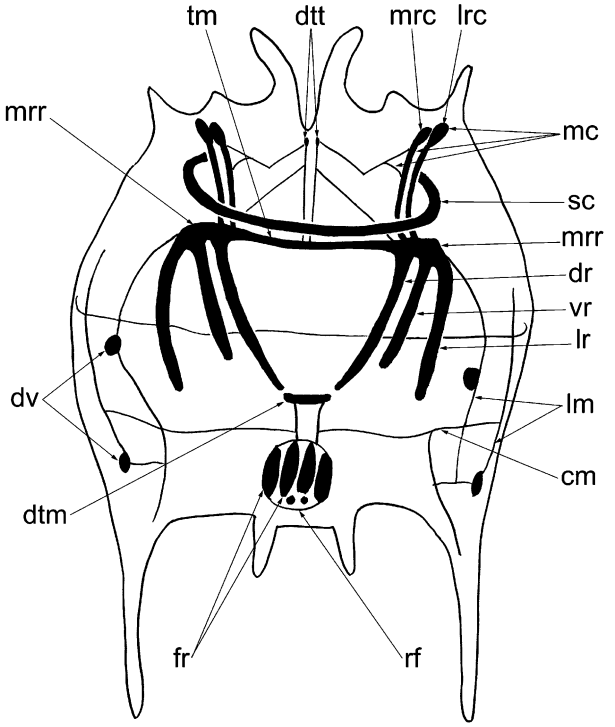

Fig. 4—Schematic drawing of the body wall musculature of *Brachionus quadridentatus* as revealed by phalloidin-TRITC staining.

(Figs 1, 3, 6B,C; 7D, 8C–D). The **pdr**'s are thick and include at least 6 muscle ribbons.

Two pairs of strong short perpendicular muscle bundles (**pm**) were observed to the lateral sides of the **pdr**'s (Figs 1, 3, 6C, 8C). The anterior pair of **pm**'s adjoins the anterior parts of the **pdr**'s. The posterior pair of **pm**'s lies near the posterior parts of the **pdr**'s and is spanned by a circular muscle (**cm**) (Figs 1, 3, 6C, 8C).

Four pairs of longitudinal muscles (**lm**) originate at the **mrr**'s, and run in posterior direction, two on the dorsal side and two on the ventral side of the body, interconnecting different muscle fibres (Figs 1, 2, 3, 6A–C, 8C).

In the posterior part of the body, two pairs of dorso-ventral muscles (**dv**) were detected (Figs 1, 2, 3, 6A–C, 7B–D, 8A–B,D). A network of thin fibres surrounds the posterior part of the body (Figs 1, 2, 6A–C, 7A–C, 8A). Distinct muscular rings encircle the pharynx (**rph**) and the foot (**rf**) (Figs 1, 2, 3, 6A, 8B).

The musculature of the corona (**mc**) is rather complex (Figs 1, 2, 3, 6A–C, 7A,C–D, 8B–D). The corona has three main pairs of retractors: medial retractors (**mrc**), lateral retractors (**lrc**) and ventral retractors (**vrc**). Numerous smaller fibres are intercalated between the retractors going to the sides of the corona on ventral and dorsal sides. They are spanned by transversal fibres originating at the proximal regions of **mrc**'s (Figs 1, 6B, 8B–D). Two thin muscular fibres (**dtt**) run to the

anterior dorsal tubular tentacle (Figs 1, 3, 6B). The lateral tentacles have only one fibre each (**lt**) (Figs 1, 2, 3, 6A–B).

The musculature of the foot comprises two pairs of foot retractors (**fr**) that are attached to the dorsal plate of the lorica, posterior to the short dorsal transverse muscle (**dtm**) (Figs 1, 3, 6B–C, 7C–D, 8). In the fixed rotifers the almost fully retracted foot lies perpendicular to the body axis, pointing toward the ventral side.

Mastax muscles (**mm**) constitute the major part of the visceral musculature (Figs 6A,C, 7A–B, 8A–B). The unpaired fulcrum has two muscles running parallel on its sides. The paired mastax sclerites (unci, rami and manubrii) have a pair of muscles each. The internal organs of the posterior part of the body demonstrate a network of visceral muscles (**vm**) (Figs 6A–C, 7C, 8A).

The cutaneo-visceral musculature (**cv**) is represented by cutaneo-pharyngeal muscles, muscles adjoining the base of the foot and muscles lying near the dorsal groove of the lorica (Figs 6A–B, 7C–D, 8B,D).

Brachionus quadridentatus (Figs 4, 5, 6D, 9)

The musculature of this species follows the same general pattern as that of *Euchlanis dilatata unisetata.* Paired dorsal (**dr**), lateral (**lr**) and ventral (**vr**) retractor muscles composed of numerous muscle ribbons are attached by a pair of common roots (**mrr**) to the body wall (Figs 4, 5, 6D, 9A–B). From these roots, two pairs of muscles, lateral (**lrc**) and medial (**mrc**) retractors of the corona run forward, ending in apical thickenings at the sides of the corona (Figs 4, 5, 6D, 9B–D). Two muscles (**dtt**) run through the corona to the dorsal tentacle (Figs 4, 5, 6D, 9A). The middle parts of the corona display a complex pattern of longitudinal and transversal muscles (Figs 4, 5, 6D, 9B–D). The sphincter of the corona (**sc**) is well developed (Figs 4, 5, 6D). Two pairs of dorso-ventral muscles (**dv**) lie in the posterior part of the body (Figs 4, 5, 6D, 9C–D). The foot is surrounded by a muscular ring (**rf**) and provided by 4 strong foot retractors (**fr**) and 2 weakly developed muscle ribbons (Figs 4, 6D, 9C-D). Visceral musculature (**vm**) is mostly represented by mastax muscles (**mm**) (Fig. 9A,C–D).

Discussion

The combination of phalloidin-TRITC staining and CSLM permitted us to reveal the pattern of musculature on whole-mount preparations of two rotifers. All the main elements in the musculature of the body wall, visceral and cutaneo-visceral musculature, described earlier from the series of sections in the genera *Brachionus* and *Euchlanis* by Stoßberg (1932), were detected by the phalloidin-TRITC method. In addition to the muscles, described by Stoßberg (1932) in *E. pellucida*, we have detected in *E. dilatata unisetata* a pair of anterior-ventral retractors and a transversal muscle, linking the main roots of retractor muscles. Two new thin muscle ribbons running along the foot of *Brachionus quadridentatus* were revealed with the phalloidin-TRITC method.

The posterior-dorsal retractors are called 'central' in *Euchlanis pellucida* (Stoßberg, 1932). In the case of *E. dilatata unisetata* these muscles lie close to the dorsal side of the body, and have therefore been renamed accordingly.

The two closely related species, *Brachionus quadridentatus* and *Euchlanis dilatata unisetata* displayed the same general muscle pattern. It is characterized by the common root of attachment of the proximal parts of all the retractor muscles, with the exception of posterior-dorsal ones.

A pair of major attachment spots of rotifer musculature (main roots of retractor muscles) was singled out in this study. The main muscles are connected with these roots, demonstrating the unity of the muscular system. The functional significance of this observation is yet unclear. Further research is needed to reveal if there is any evidence of specific innervation of those spots.

The phylogenetic position of the Rotifera is still under discussion. According to Ivanov (1975), rotifers have evolved from some archaic bilaterians, ancestors to Platyhelminthes, Nemathelminthes and Protocoelomata. Flatworms were often supposed to be the ancestors of rotifers (Beklemischev, 1952; Beauchamp, 1965; Kutikova, 1970; Clément, 1987; Clément and Wurdak, 1991; Malakhov, 1994; Kotikova, 1995). However, rotifers have much more specialized muscles than those characteristic of Platyhelminthes (see Rieger *et al.*, 1994; Hooge and Tyler, 1999). Detailed phylogenetic analysis recently published by Ahlrichs (1995, 1997) led to the recognition that the Rotifera together with Gnathostomulida and Acanthocephala belong to a new monophylum named Gnathifera Aldrich 1995. This taxon was founded on the basis of a special ultrastructure of the pharyngeal hard-parts and a special protonephridial canal structure. According to morphological and molecular data, the Rotifera constitute a sister group to Acanthocephala (Garey *et al.*, 1998) and both constitute a sister group to Gnathostomulida (Rieger and Tyler, 1995). The Acanthocephala have two layers of muscles under their epidermis: outer circular and inner longitudinal muscles. The differentiation of strong retractor muscles composed of many muscular ribbons, points to the higher level of organization of rotifer musculature in comparison with that of acanthocephalans.

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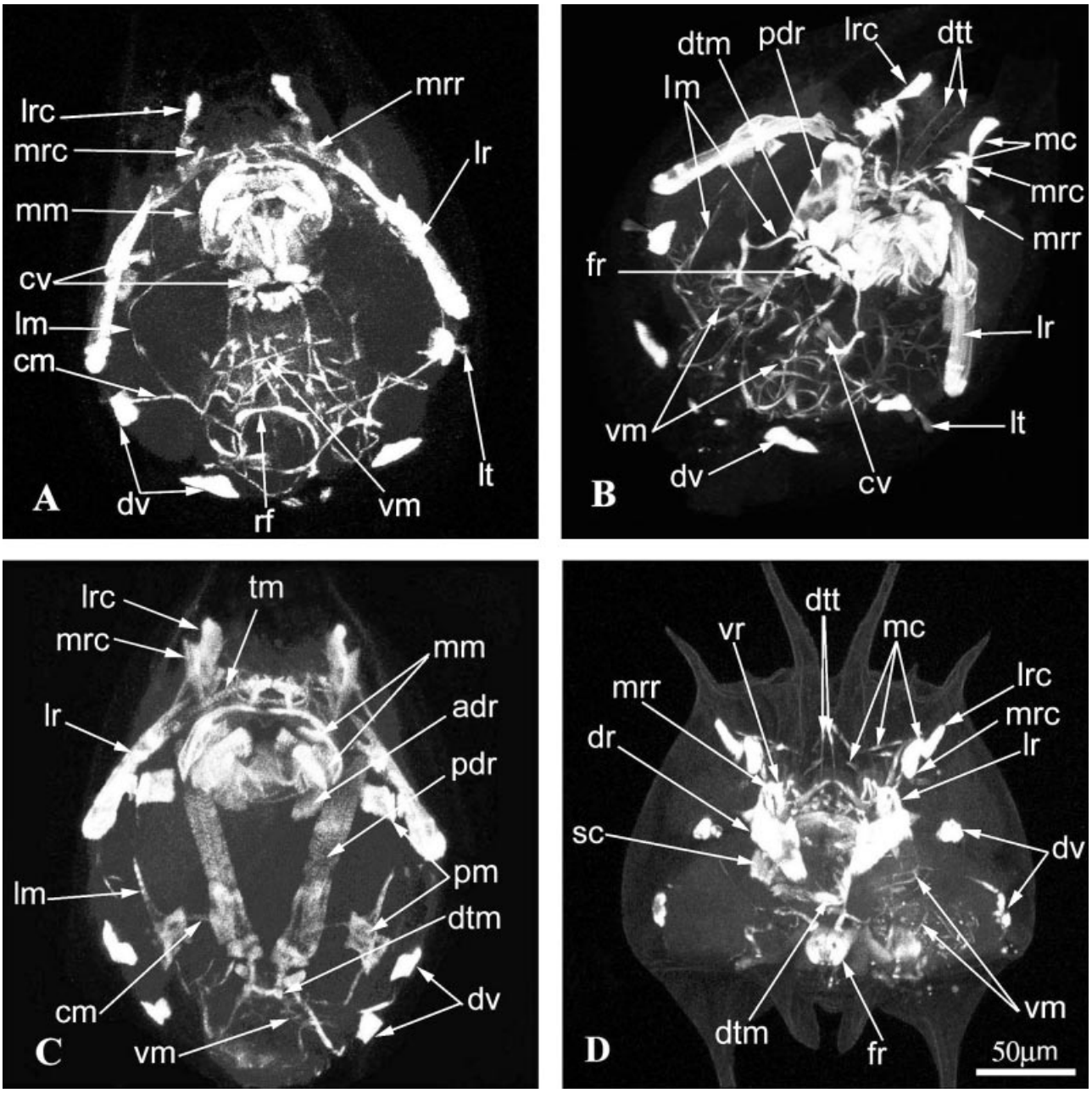

Fig. 6 — Max-projections of whole mount preparations of *Euchlanis dilatata unisetata* (**A–C**) and *Brachionus quadridentatus* (**D**) with different degrees of contraction of the retractor muscles. Phalloidin-TRITC staining. **A**, **B** — thick optical sections passing through the middle of the animal; **C**, **D** — thick optical sections of the dorsal side of the animal.

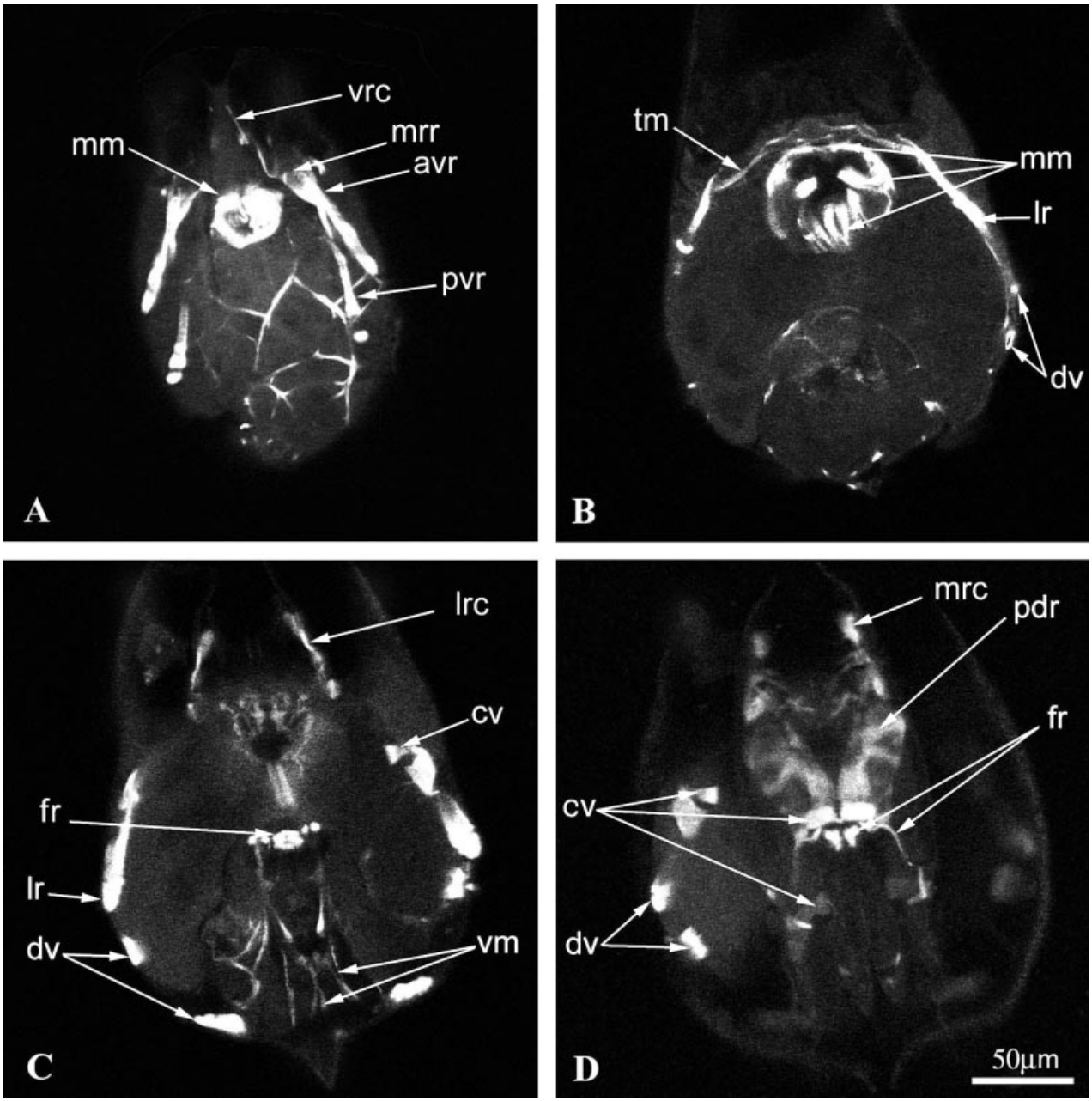

Fig. 7—Serial optical frontal sections of a whole mount preparation of Euchlanis *dilatata unisetata*, starting from the ventral side (**A**) and ending at the dorsal side (**D**). Phalloidin-TRITC staining.

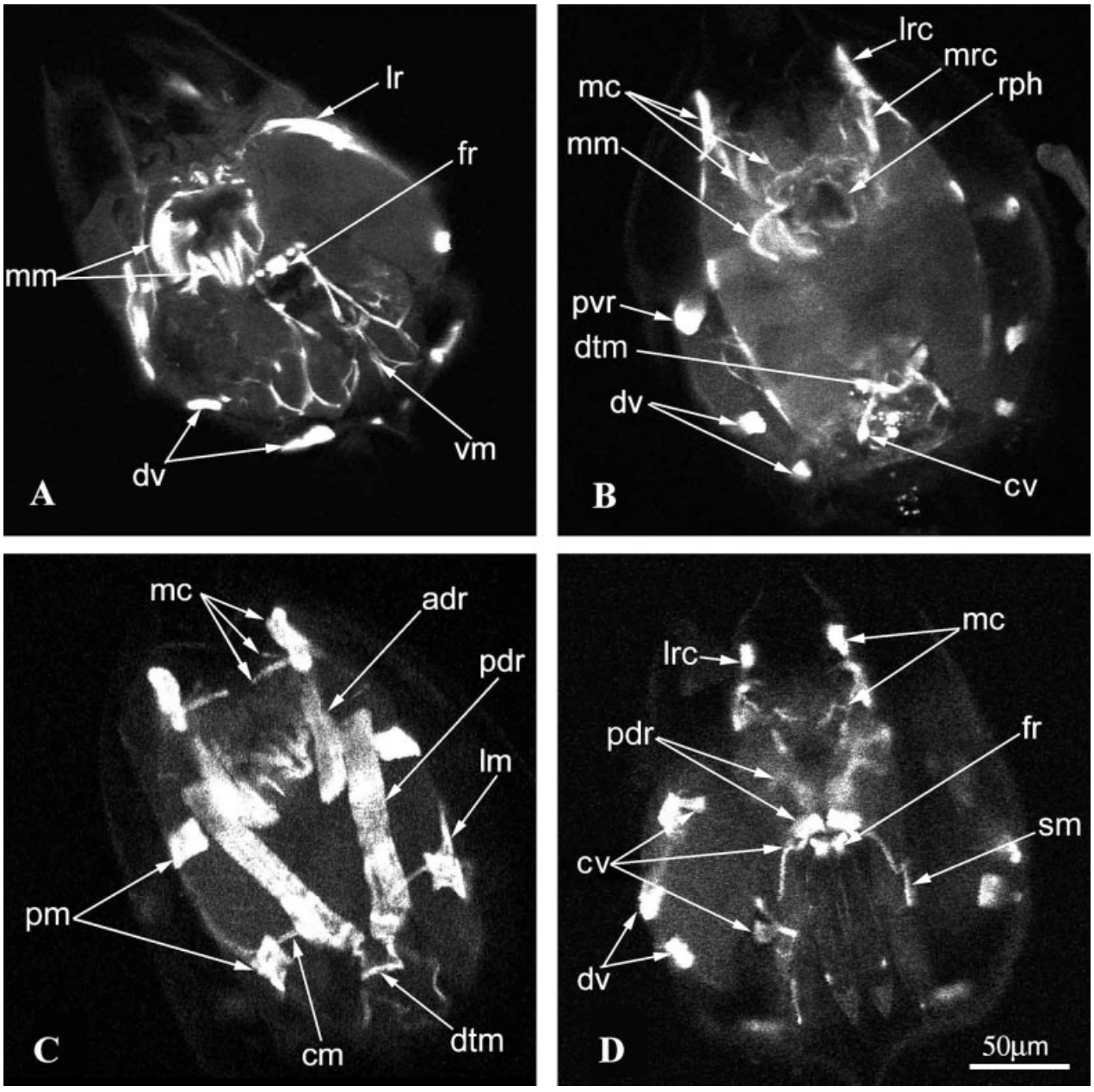

Fig. 8—Optical frontal sections of different specimens of *Euchlanis dilatata unisetata.* Phalloidin-TRITC staining. **A, B** sections through the middle of the animal; **C, D** —sections of the dorsal side of the animal.

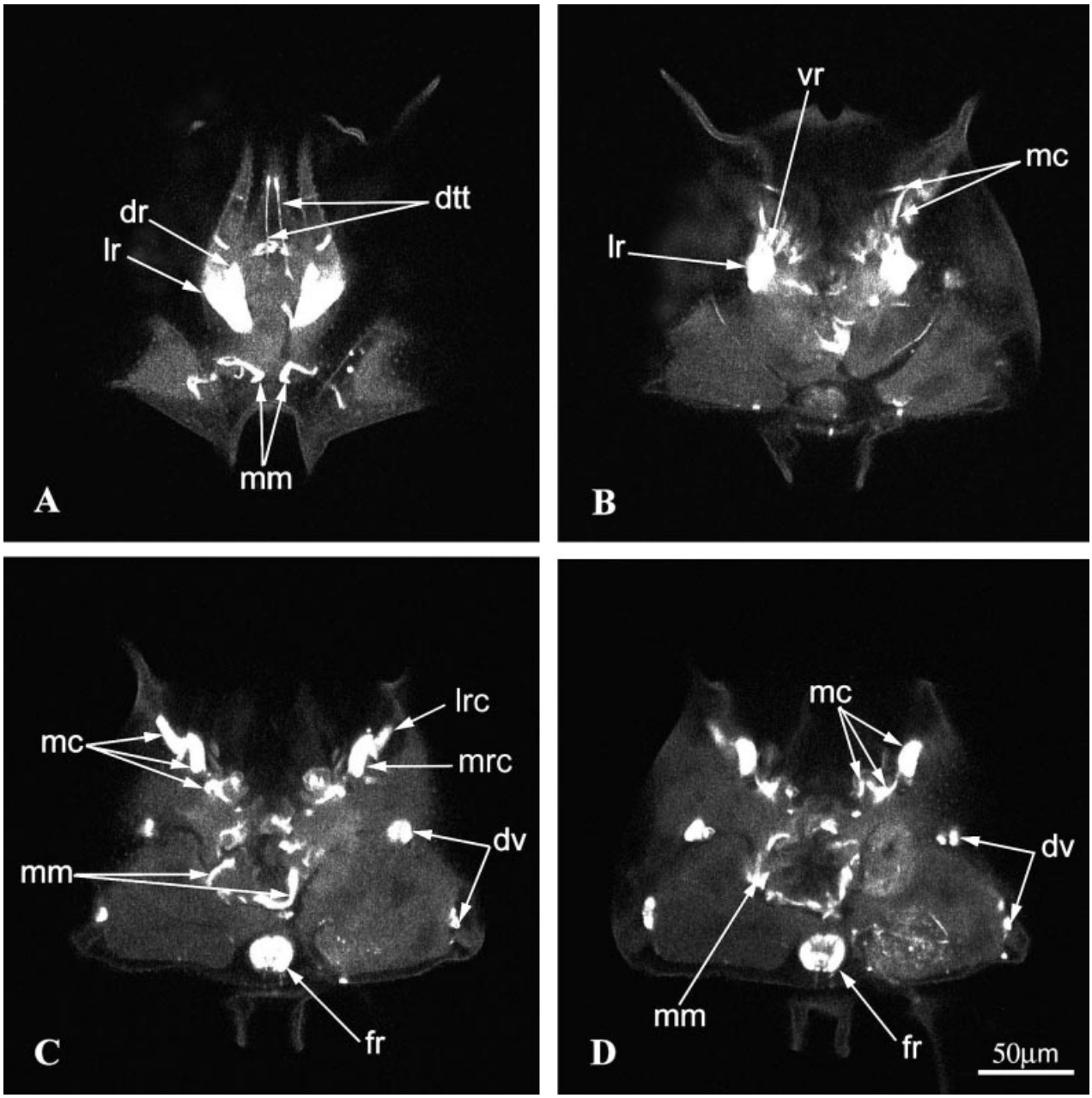

Fig. 9—Serial optical frontal sections of *Brachionus quadridentatus* stained with phalloidin-TRITC, beginning at the dorsal side (**A**) and ending in the middle of the preparation.

Abbreviations

adr – anterior-dorsal retractors
avr – anterior-ventral retractors
cm – circular muscles
cv – cutaneo-visceral muscles
dr – dorsal retractors
dtm – dorsal transverse muscle
dtt – dorsal tubular tentacle muscle
dv – dorso-ventral muscles
fr – foot retractors
lm – longitudinal muscles
lr – lateral retractors
lrc – lateral retractors of the corona
lt – lateral tentacle muscles
mc – muscles of the corona
mm – muscles of the mastax
mrc – medial retractors of the corona
mrr – main roots of retractor muscles
pdr – posterior-dorsal retractors
pm – perpendicular muscles
pvr – posterior ventral retractors
rf – muscle ring of the foot
rph – muscle ring of the pharynx
tm – transverse muscle
sc – sphincter of the corona
vm – visceral muscles
vr – ventral retractors
vrc – ventral retractors of the corona

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