Muscular Pattern in Three Species of *Macrostomum* (Platyhelminthes, Macrostomorpha)

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ABSTRACT Previous studies demonstrated complex architecture of the muscular system of *Macrostomum* species, especially in the rostrum area and the pharynx. However, little is known about the differences in muscular pattern between species of the genus. This study examines and compares the muscular systems of specimens belonging to three freshwater *Macrostomum* species (*M. quiritium*, *M. tuba* and *M. velastylum*), labeled with phalloidin-rhodamine and studied by confocal microscopy. Our results agree with the previous descriptions, confirming that the muscular patterns for the body wall, rostrum area, pharynx and caudal region differ among species. The muscles of the body wall follow the typical architecture, but the number of fibers in the species analyzed varies between dorsal and ventral surfaces, ranging from 80 to 100 fibers, this record being higher than previous observations. The arrangement of the fibers in the rostrum is complex, especially in the brain area. *Macrostomum tuba* and *M. quiritium* have a set of two muscles crossing at brain level and forming an "X," which is not evident in *M. velastylum*. We identified five different sets of fibers associated to the pharynx and mouth at ventral, medium and deep levels. These different sets are present in all three species studied. The caudal plate in *M. tuba* has an additional layer of diagonal fibers in the body wall, which is not evident in the other two species. The muscles of the reproductive system are independent of the body wall musculature in the species analyzed, but connected to the intestinal wall by specific fibers that may serve as an anchor. J. Morphol. 000:000–000, 2016.

KEY WORDS: Macrostomidae; muscle; pharynx; genital system

RESEARCH HIGHLIGHTS

The muscular systems of three freshwater *Macrostomum* species differ among them. The muscles of the reproductive system are independent of the body wall musculature but connected to the intestinal wall that may serve as an anchor.

INTRODUCTION

The family Macrostomidae van Beneden, 1870 (Platyhelminthes, Macrostomorpha) is a group of cosmopolitan free-living flatworms occurring in a wide range of habitats, including freshwater, brackish and marine environments. The genus *Macrostomum* O. Schmidt, 1848, has approximately 160 known species (Tyler et al., 2006−2016), with only 19 recorded for South America. Of the total, only three freshwater and brackish species have been mentioned for Argentina (*Macrostomum velastylum* Brusa, 2006; *M. puntapiedrensis* Brusa, 2006; *M. platensis* Adami et al., 2012). Furthermore, presence of specimens similar to *M. vejdovskyi* Ferguson, 1940 and *M. viride* van Beneden, 1870 (Brusa, 2006) has been reported.

The morphology of the copulatory stylet is considered to be a significant feature for the diagnosis of *Macrostomum* species (Ferguson, 1954; Rieger, 1977; Rieger et al., 1994). In several cases, evidence of this structure is not conclusive, making it necessary to search for other morphological features, for example, sperm morphology associated with mating behavior to diagnose the species precisely (Schärer et al., 2011). Recently, Adami et al. (2012) analyzed the spatial arrangement of the musculature as a potentially valid taxonomic feature.

The muscular system of flatworms is formed by a network of muscles of the body wall, parenchyma, and those associated with the gut and genital system (Rieger et al., 1991; Tyler and Hooge, 2004). The rostrum can move in all directions while the animal glides over the substrate. The mouth and pharynx are capable of great distension, enabling the animal to capture and ingest algae or even small animals (Jennings, 1957). Confocal Laser Scanning Microscopy (CLSM) combined with phalloidin staining, which

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marks F-actin in muscle cells with high specificity, allows the muscular systems to be viewed accurately in 3D for analysis. This method has been used in small invertebrates such as acoels, rotifers, trematode larvae and nemerteans (e.g., Hooge, 2001; Hooge and Tyler, 2005; Leasi et al., 2012; Petrov and Zaitseva, 2012; Krupenko and Dobrovolskij, 2015) in which the three-dimensional muscle arrangement has emerged as a useful morphological character for taxonomic and phylogenetic analyses. In particular, in free-living flatworms, the muscle system pattern acquired importance in studies on development, regeneration, taxonomy and evolution (Salvenmoser et al., 2001; Morris et al., 2004; Adami et al., 2012).

Several studies focus on the muscular systems of flatworms due to its potential as additional phylogenetic information. In Acoela, details of the arrangement of muscle fibers in the body wall and genital system are considered as synapomorphies at different levels (Jondelius et al., 2011). Within Platyhelminthes, the value of the muscular arrangement in the phylogeny has not yet been evaluated, but appears to be a promising approach. For a comparative analysis, it is important to investigate representatives of all phylogenetic groups, following an approach initiated by Hooge (2001). It is also necessary to analyze the muscular arrangement of representatives of one monophyletic group to test the variability of these characters.

Although there are numerous *Macrostomum* species, the 3D muscular architecture has only been studied in three of them: *M. hystricinum marinum* Rieger, 1977, *M. lignano* Ladurner et al., 2005 and *M. platensis* (Rieger et al., 1991, 1994; Ladurner et al., 2005; Morris et al., 2007; Adami et al., 2012). These studies showed similarities in muscle arrangement, especially in the body wall. Beyond this basic similar pattern, detailed analysis showed differences between species (i.e., number of fibers in the body wall, the great complexity in the anterior region and the arrangement of muscles associated to genital pores).

This study analyzes and compares muscle arrangement in three freshwater species of the genus *Macrostomum* (*M. quiritium* Beklemischev, 1951; *M. tuba* von Graff, 1882 and *M. velastylum* Brusa, 2006), focusing on the 3D muscular structure of the body wall, rostrum, pharynx, caudal region and genital organs. The study of three species of the genus allows a comparative analysis of muscle fiber arrangement and tests its variability.

MATERIAL AND METHODS

Flatworms (*Macrostomum quiritium* Beklemischev, 1951; *M. tuba* von Graff, 1882; *M. velastylum* Brusa, 2006) were collected from their natural habitats from September to December 2014 using a 125 μm mesh net. Only mature specimens were analyzed. *Macrostomum quiritium* specimens were collected at two localities: a vegetated pond located near the Rio de la Plata (34°53′16″S, 57°49′43″W), dominated by *Pistia stratiotes* and the riverside of Paraná River (33°40′16″S, 59°39′ 33″W), dominated by *Azolla* sp. and *P. stratiotes*. Seven specimens of this species were studied alive and three were labeled with phalloidin-rhodamine. *M. tuba* was collected at Río Ceballos (31°10′53″S, 64°19′27″W), Córdoba, from an artificial pond dominated by *Myriophylum aquaticum* and *Eichhornia* sp. Four specimens were studied alive and two with phalloidin-rhodamine. *M. velastylum* was collected near Río de la Plata (35°0′56″S, 57°31′60″W). Two specimens were examined alive and two with phalloidin-rhodamine labeling. The diagnostic structures were studied through the progressive squash method. The species were selected due to their availability in the field, and the number of individuals was limited due to their low density (2–3 specimens/1,000 cm^3).

To analyze muscle arrangement, specimens were fixed in formaldehyde-phosphate buffered saline (PBS, 4%) for 12 h, washed in PBS-Tween (0.05%, PBS-T) and permeabilized in Triton X-100 (1%) for 24 h at 4°C. Then, they were incubated overnight at 4°C with phalloidin-rhodamine solution (1/1,000, Sigma–Aldrich; Adami et al., 2012). Finally, they were mounted in Vectashield medium. The resulting material was observed using a Leica LAS AF Lite Laser Scanning Confocal Microscope. The step size in the confocal stacks was variable: 5 μm for *M. quiritium*, 3 μm for *M. tuba* and 3.5 μm for *M. velastylum*. The images and 3D-projections were finally analyzed with Leica LAS AF Lite Image Examiner software.

To analyze the muscle arrangement in the body wall, the number of fibers was recorded in the middle region of the body along a 100 μm long line perpendicular to fiber orientation (fiber number/100 μm; see Figs. 4, 8 and 12, black lines on the figures; see also Supporting Information S1), on both the ventral and dorsal surfaces. Pharyngeal musculature architecture was analyzed at three different levels (mouth, medium and deepest pharyngeal levels). The deepest pharyngeal level could not be analyzed in *M. velastylum*.

Specimens mounted in polyvinyl–lactophenol and those processed to study the muscular system were deposited in the Invertebrate Collection of Museo de La Plata (FCNyM-UNLP), MLP-He 7025-7031.

RESULTS

The morphological features used to identify the specimens and the remarks about their systematic position are provided below. To describe the muscle pattern of each species, the body was analyzed considering: 1) Pattern of the body wall, described in the middle region and at the caudal plate; 2) the anterior region comprising the rostrum and the pharynx; and 3) the caudal region including the reproductive organs.

Specimen Identification

Macrostomum quiritium. Living mature individuals about 1,700 μm long, with maximum width 730 μm (Fig. 1A). Average fixed mature specimens approximately 1,000 μm long and 486 μm wide. Two black eyes about 13 μm in diameter. Gut with folded edges. Needle-shaped copulatory stylet 113 μm to 178 μm long (mean 140 μm, $n = 7$; Fig. 1B,C). Stylet narrows gradually toward the distal bevelled end (Fig. 1D); mean width of proximal end 23 μm ($n = 7$); average width of distal end 6 μm ($n = 7$).

Remarks: Genital system morphology agrees with the original description by Beklemischev (1951) for *M.*

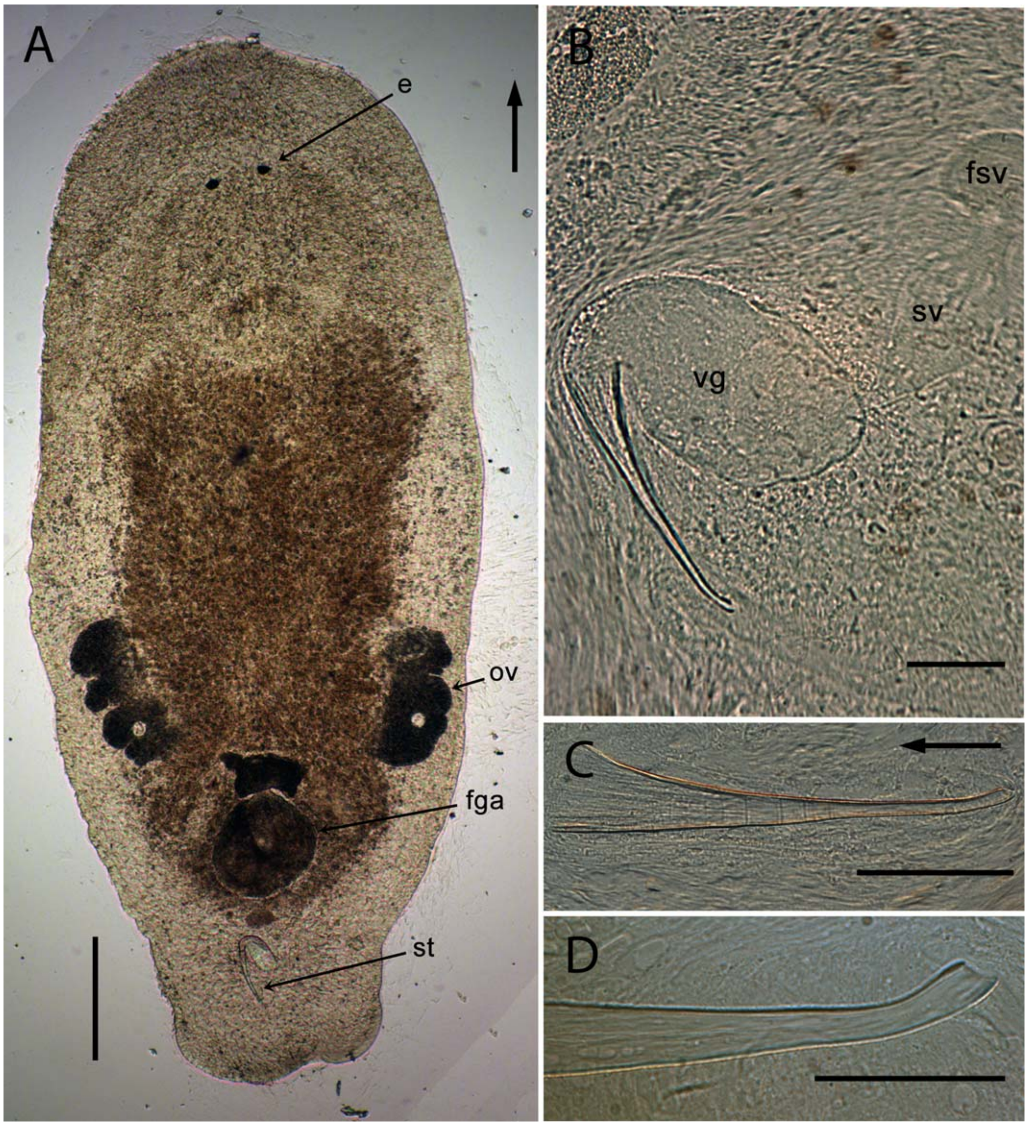

Fig. 1. *Macrostomum quiritium*. **A)** General view under squash. **B)** Male reproductive system. **C)** Stylet. **D)** Detail of distal end of stylet. **e**, eye; **fga**, female genital atrium; **fsv**, false seminal vesicle; **ov**, ovary; **st**, stylet; **sv**, seminal vesicle; **vg**, vesicula granulorum. Black arrows on **A** and **C** indicate the anterior-posterior axis. Scales, **A**: 200 μm; **B**, **C**: 50 μm; **D**: 25 μm.

japonicum quiritium from Russia, and the general morphology agrees with later observation by Kolasa (1973), who described *M. quiritium* specimens from Poland. Beklemischev (1951) schematically drew the vesicula granulorum, seminal vesicle and false seminal vesicle in a row (Beklemischev, 1951: fig. 47). This arrangement is not evident when specimens are observed under light squash, but when squeezed under greater pressure, the three structures appear arranged as described by Beklemischev (Fig. 1B).

Schärer et al. (2011) studied specimens from Russia, Poland and Switzerland from artificial ponds with aquatic vegetation from tropical regions of the world. They therefore considered the origin of the species uncertain. We found this species in two natural sites: a pond connected to Río de la Plata River and the bank of Paraná River. Indeed, this is the first mention of this species in the Neotropical Region.

Macrostomum tuba. Average body length of fixed mature specimens 1,355 μm ($n = 4$) and mean width 759 μm ($n = 4$; Fig. 2A), while live mature specimens reach a maximum body length of 2,400 μm. Common vas deferens expanded in a vesicle of variable shape and size (false seminal vesicle), connected by a narrow duct to the muscular seminal vesicle, which leads into the vesicula granulorum. Vesicula granulorum fusiform, with several glands surrounding its proximal region, discharging through several ducts. Distal end of vesicula granulorum narrows and contacts proximal end of stylet (Fig. 2B). Copulatory stylet straight to slightly curved, with a distal bulbar end (Fig. 2C). Stylet

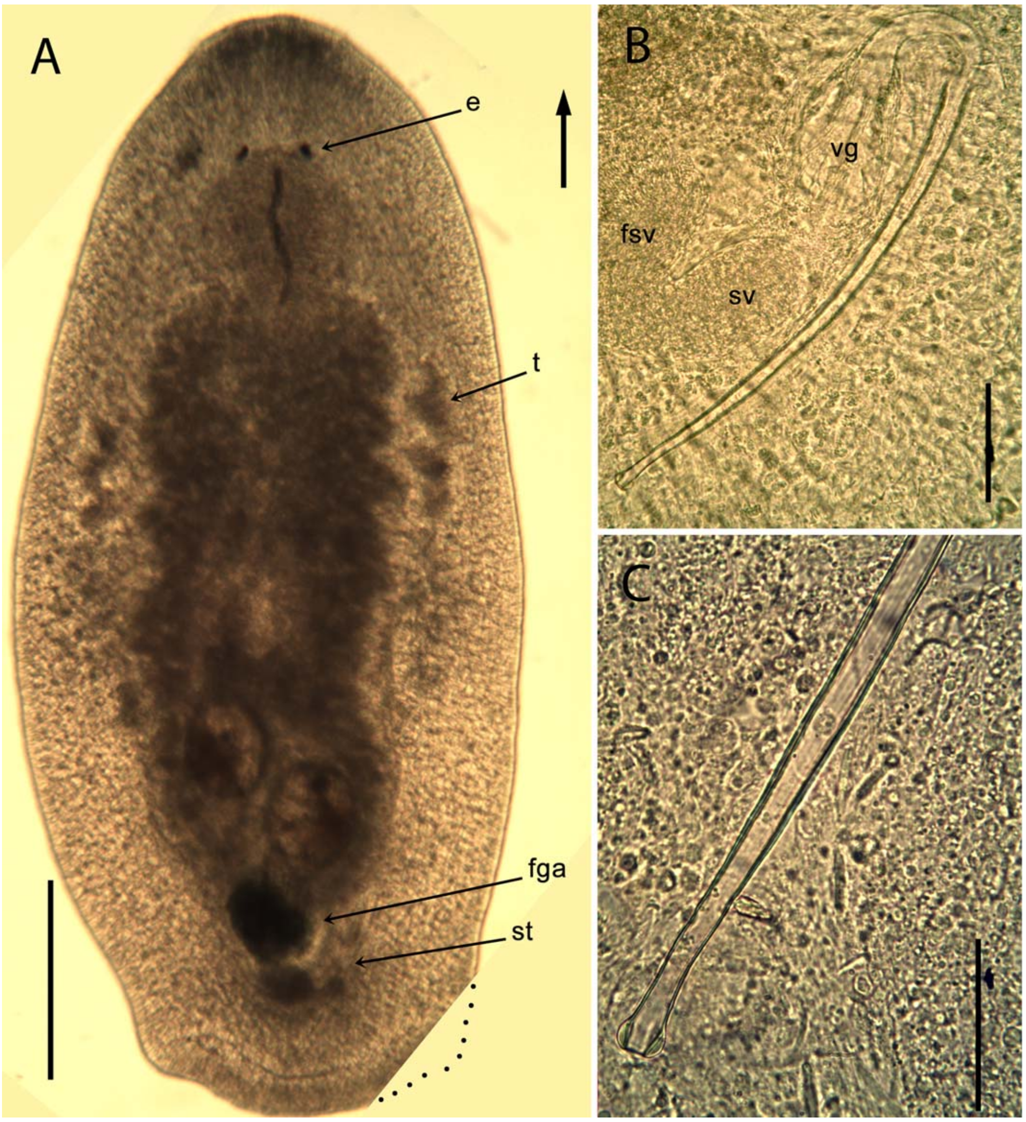

Fig. 2. *Macrostomum tuba*. **A)** General view under squash. **B)** Male reproductive system. **C)** Detail of distal end of stylet. **e**, eye; **fga**, female genital atrium; **fsv**, false seminal vesicle; **st**, stylet; **sv**, seminal vesicle; **t**, testicle; **vg**, vesicula granulorum. Black arrow on **A** indicates the anterior-posterior axis. Scales, **A**: 200 μm; **B**, **C**: 100 μm.

length ranging from 275 μm to 449 μm (mean= 322 μm, $n = 4$). Proximal end width 17 μm on average, with a ring-shaped thickened distal opening 9 μm on average.

Remarks: This is the first mention of this species for Argentina. In the Neotropical Region it was found previously in Brazil (Marcus, 1946 listed it as *Macrostomum gigas* Okugawa, 1930; Gamo and Leal-Zanchet, 2004) and Venezuela (Hyman, 1955).

Macrostomum velastylum. Mature live individuals about 1,600 μm long ($n = 6$), and 420 μm ($n = 6$) maximum width (Fig. 3A). Mean body length of fixed mature specimens 1,100 μm ($n = 2$) and width 550 μm ($n = 2$). Vesicula granulorum surrounded by a very strong muscular sheath (Fig. 3B,C). Copulatory stylet strongly curved, with three lamellae. Total length 145 μm and proximal end 37.6 μm wide (Fig. 3C).

Remarks: The morphology of the reproductive system agrees the original description of the species (Brusa, 2006). The distinctive morphology of the stylet and the strong musculature of the vesicula granulorum allow accurate identification. This species has been frequently observed growing in aquaria.

Muscular Pattern

General pattern. The body wall muscles form a grid beneath the epidermis, comprising outer circular and inner longitudinal fibers and between them, diagonal fibers running in two directions. This pattern is recognized in the mid-region of the body, posterior to the mouth and anterior to the

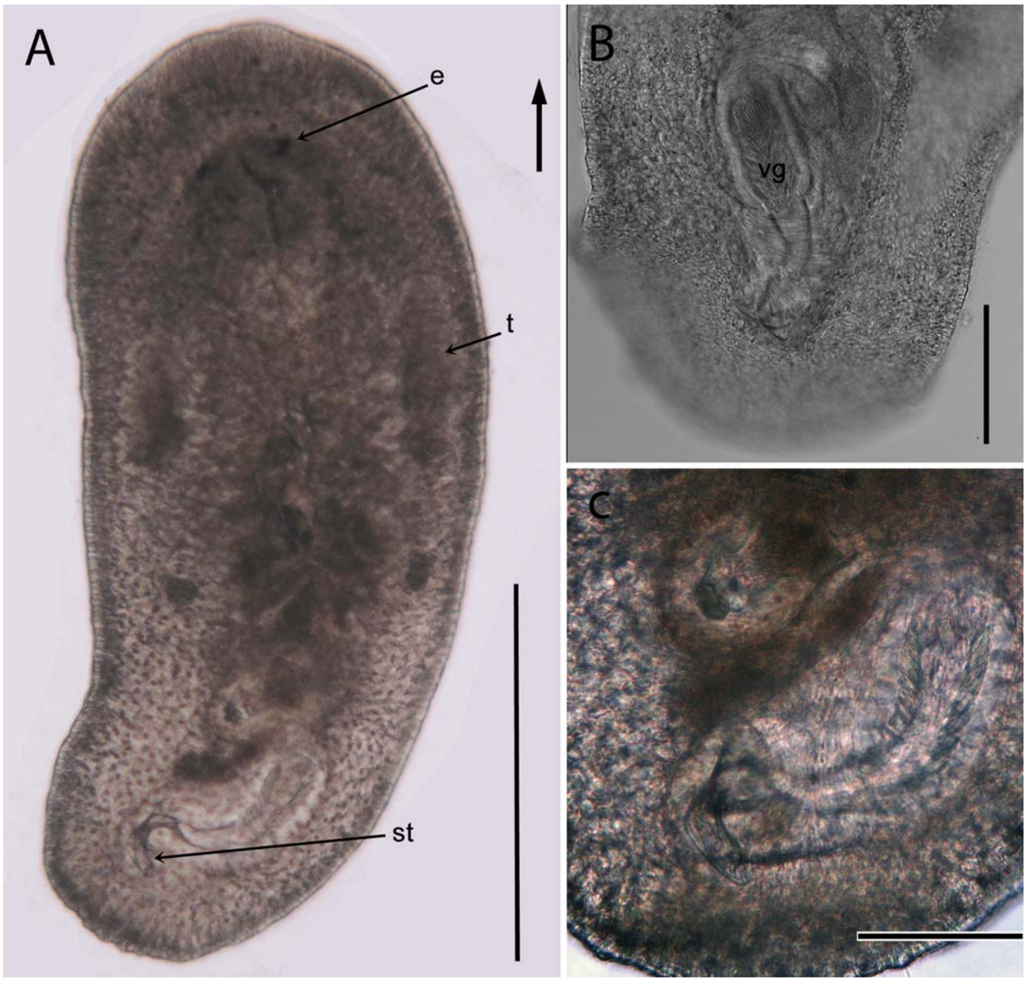

Fig. 3. *Macrostomum velastylum*. **A)** General view under squash. **B)** Detail of the male reproductive system. **C)** Stylet. **e**, eye; **st**, stylet; **t**, testicle; **vg**, vesicula granulorum. Black arrow on **A** indicates the anterior-posterior axis. Scales, **A**: 500 μm; **B**, **C**: 100 μm.

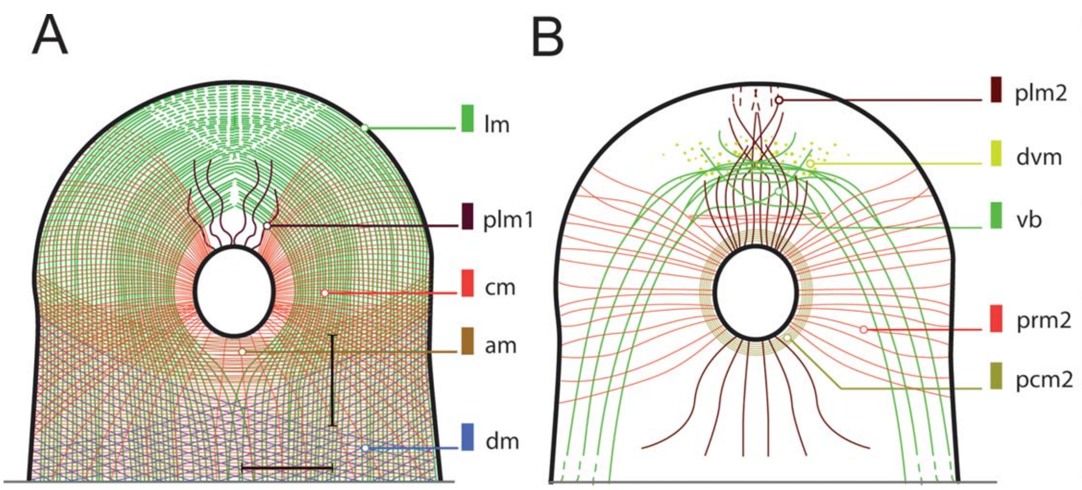

Fig. 4. *Macrostomum quiritium*. Diagrams showing the muscular pattern in the anterior region. **A)** Ventral surface and **B)** Mid-pharyngeal level. Diagrams based on the specimens shown in Figures 5 and 6. **am**, additional muscles; **cm**, circular muscles; **dm**, diagonal muscles; **dvm**, dorsoventral muscles; **lm**, longitudinal muscles; **pcm2**, pharyngeal circular muscles; **plm1**, pharyngeal longitudinal muscles at ventral surface; **plm2**, pharyngeal longitudinal muscles at mid-level; **prm2**, pharyngeal radial muscles at mid-level; **vb**, ventral brain muscles. Black lines on **A** indicate the area where body wall fibers were counted. Scale: 100 μm.

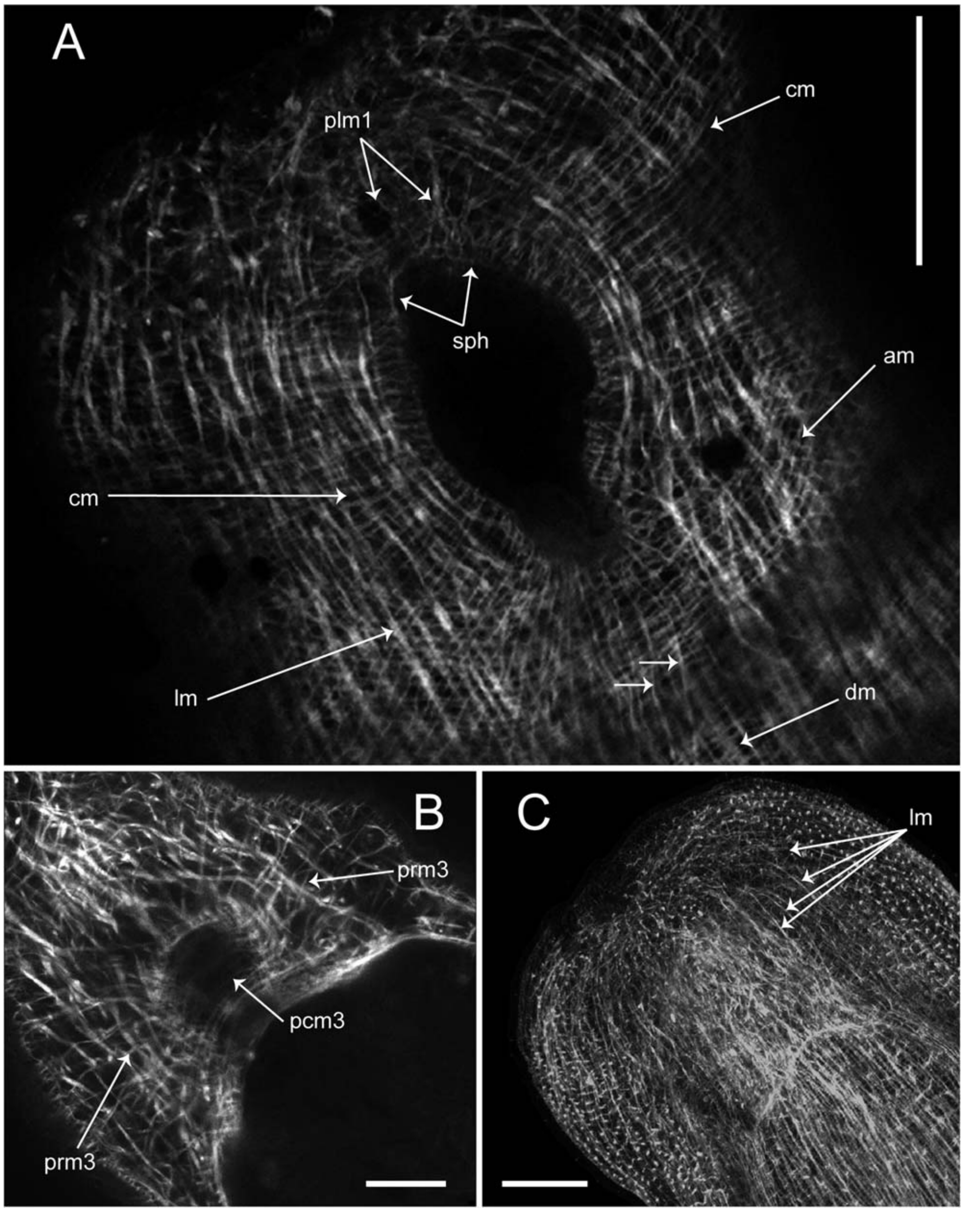

Fig. 5. *Macrostomum quiritium*. Anterior region at different levels. **A)** Detail of mouth region at a ventral surface. **B)** Deepest level of the pharynx. **C**) Dorsal Z-projection showing longitudinal fibers of the body wall that sink into brain zone. **am**, additional muscle fibers posterior to the mouth; **cm**, circular muscles of the body wall; **dm**, diagonal muscles of the body wall; **lm**, longitudinal muscles of the body wall; **pcm3**, pharyngeal circular muscles at the deepest level; **plm1**, anterior pharyngeal longitudinal muscles at ventral surface; **prm3**, pharyngeal radial muscles at the deepest level; **sph**, sphincter; **Arrows**, longitudinal fibers running toward the posterior region. Scales, **A**: 100 μm; B, **C**: 50 μm.

genital pore, while it is modified in the anterior region due to the presence of the mouth, pharynx and rostrum and in the posterior region due to the presence of the adhesive region and the copulatory apparatus.

Macrostomum quiritium. *Musculature of the body wall.* The arrangement of the body wall muscles follows the general pattern (Figs. 4A and 5A; Supporting Information S2). Circular fibers are more abundant on the dorsal surface, while diagonal and longitudinal fibers are more abundant on the ventral surface (Table 1). On the dorsal surface, the diagonal fibers run along the whole length, while on the ventral surface they are evident only posteriorly to the mouth (Figs. 4A and 5A). Circular, diagonal and longitudinal fibers have different thickness (Figs. 5A and 6A).

Rostrum. The general pattern of body wall musculature becomes more complex in the rostrum. In a dorsal view, several longitudinal muscles of the body wall change their parallel arrangement. Some of these dorsal longitudinal fibers sink in and run into the

TABLE 1. Number of pairs of fibers described for Macrostomum quiritium, M. tuba and M. velastylum

	M. quiritium		*M. tuba*		*M. velastylum*	
	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral
Body-wall						
Circular (cm)	25, 35[a]	20, 26[a]	28	19	26	22
Diagonal (dm)	6	8, 10[a]	12	7	11	17
Longitudinal (lm)	10, 18[a]	18, 20[a]	11	13	9	13
Pharynx						
Ventral Level						
Circular of body wall (cm)	52		45		41	
Longitudinal pharyngeal (plm 1)	3		1		1	
Medium level						
Pharyngeal radials 2 (prm 2)	14, 16[a]		15		30	
Longitudinal pharyngeal (plm 2)	6, 7[a]		3		2, 3[a]	
Dorsal level						
Pharyngeal radials 3 (prm 3)	9, 14[a]		5, 9[a]		?	

Fibers associated with the pharynx were counted on the right side and classified according to the level of the confocal sections.
? = no data.
[a]More than one value indicates that two specimens were analyzed.

rostrum region (Figs. 5C and 6A,B). Other longitudinal fibers at each side of the mouth reach the brain area and cross over in the sagittal plane (Fig. 6C,D). These cerebral fibers form a crescent-shaped arch around the brain (Figs. 4B and 6C,D), with two of them forming an X-like structure (Figs. 4B and 6E, vb). The region next to crescent-shaped arch is abundant in dorsoventral fibers (Figs. 4B and 6D–F).

Pharynx region. At the level of the mouth, several circular fibers of the body wall reach the mouth and form part of the longitudinal muscles of the pharynx (Table 1; Figs. 4A and 5A, cm); at the posterior edge of the mouth, six pairs of these fibers run almost longitudinally toward the posterior region of the body (Figs. 4A and 5A, arrows). Three pairs of longitudinal fibers reach the mouth from the anterior region, also forming the longitudinal muscles of pharynx (Table 1; Figs. 4A and 5A, plm1). A delicate sphincter formed of 2–3 fibers surrounds the mouth (Fig. 5A, sph). Next to the posterior edge of the mouth, groups of additional fibers are evident, bending up to each lateral side of the body crossing the circular fibers (Figs. 4A and 5A, am).

At a mid-level, radial muscle fibers link the pharynx and the body wall (Table 1; Figs. 4B and 6D,E, prm2) also forming part of the longitudinal muscles of the pharynx. On this plane, some fibers run longitudinally along the anterior and posterior edges of the pharynx (Table 1; Fig. 6D,E, plm2). The anterior fibers cross the cerebral muscles, whilst those of the posterior edge reach the intestinal musculature (Figs. 4B and 6D,E, plm2 and vb). Circular fibers of the pharynx are closely grouped and due to their arrangement, they were not quantified (Fig. 6D, pcm). At the most dorsal level, radial fibers connect the pharynx with the body wall edge (Table 1; Fig. 5B, prm3).

Caudal region. On the dorsal surface, the diagonal fibers of the body wall are abundant and cross at the middle axis, running close to each other (Fig. 7C, white arrowheads). At the caudal adhesive plate, fibers running in dorsoventral direction and connecting both surfaces are evident. In a more detailed view, they seem to form groups of two or three fibers each (Fig. 7A,B, dvm).

Male genital system. In the specimens analyzed, the false seminal vesicle lacks muscles. The walls of the seminal vesicle have strong, irregularly distributed muscles (Fig. 7A), finally reaching the large vesicula granulorum (approximately 50 μm long in labeled specimens), which also has strong muscular walls. A small number of longitudinal fibers link the vesicula granulorum to the body wall (Fig. 7C–D, asterisks). The walls of the seminal vesicle and the vesicula granulorum are closely connected, the seminal vesicle being ventral to the vesicula granulorum (Fig. 7A–C). The vesicula granulorum opens at the base of the stylet forming an acute angle (Fig. 7C). A thin muscular sheath containing circular and longitudinal fibers encircles the stylet, running along it up to the gonopore (Fig. 7C).

Female genital system. The genital antrum is an ovoid muscular sac, with crossed fibers. The dorsal side of the antrum has the highest number of fibers (Fig. 7E). The antrum of the studied specimen is dilated, filling almost the entire thickness of the animal. The muscle wall of the antrum is associated to the muscles of the body wall, especially in the equatorial region (Fig. 7E,F, asterisks). The female gonopore has circular muscle fibers forming a sphincter (Fig. 7F, sph).

Macrostomum tuba. *Musculature of the body wall.* The three-layered muscular organization of the body wall is conserved (Figs. 8A, 9A,B and 10A; Supporting Information S3), with abundant circular and diagonal fibers on the dorsal surface, while longitudinal fibers seem to prevail on the ventral surface (Table 1). Furthermore, on the ventral side there are diagonal fibers located posterior

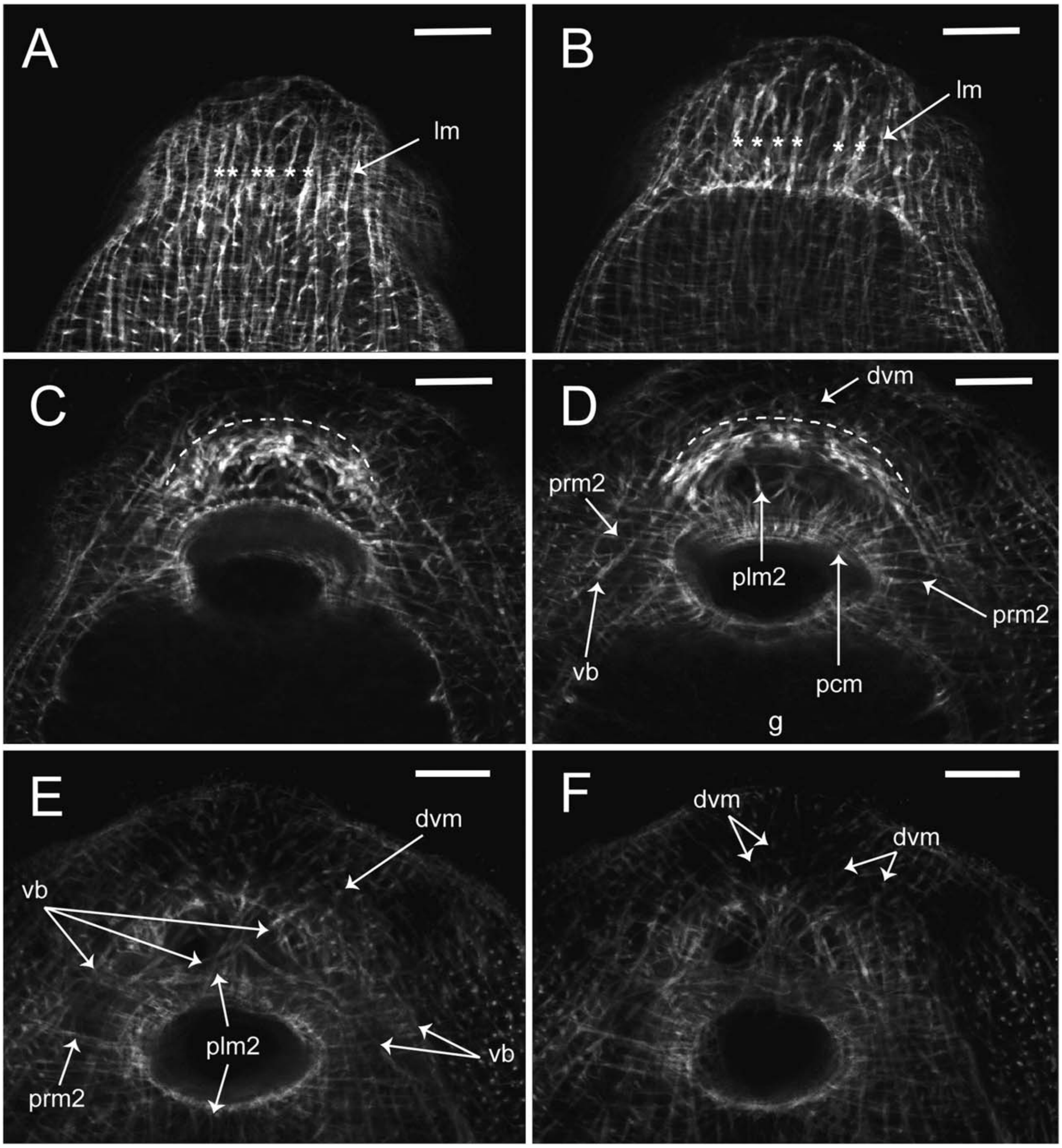

Fig. 6. *Macrostomum quiritium*. Anterior region showing fibers associated to brain and pharynx, at different optical sections. **A** and **B)** Dorsal view at two different levels. **C** and **D)** Mid-level showing arch in the brain zone and pharyngeal muscles associated with the brain. **E** and **F)** Two planes of the deepest level of the pharynx, showing the associations between brain fibers that cross on the sagittal plane and pharyngeal longitudinal fibers. **dvm**, dorsoventral muscles; **g**, gut; **lm**, longitudinal muscles in the body wall; **pcm**, pharyngeal circular muscles at mid-level; **plm2**, pharyngeal longitudinal muscles at mid-level; **prm2**, pharyngeal radial muscles at mid-level; **vb**, ventral brain fibers; **asterisks**, longitudinal body wall fibers that descend to brain zone; **dashed line** indicates brain region. Scales, 50 μm.

to the mouth, increasing the number of muscles in the proximity of the mouth (Figs. 8A and 9A, dm).

Rostrum. On the ventral surface, the longitudinal fibers of the body wall change their parallel trajectory, converging to the sagittal plane. At a deeper level, 1 to 3 pairs of fibers on each side of the brain branch to form a semicircle. Another pair of longitudinal fibers runs in opposite direction (Fig. 8A, dark green fibers). Some of these fibers diverge and connect to circular and dorsoventral fibers. The outermost pair of fibers contacts one longitudinal fiber (Figs. 8A and 9C, asterisks and dotted line). Next to the anterior edge of the mouth, another longitudinal fiber

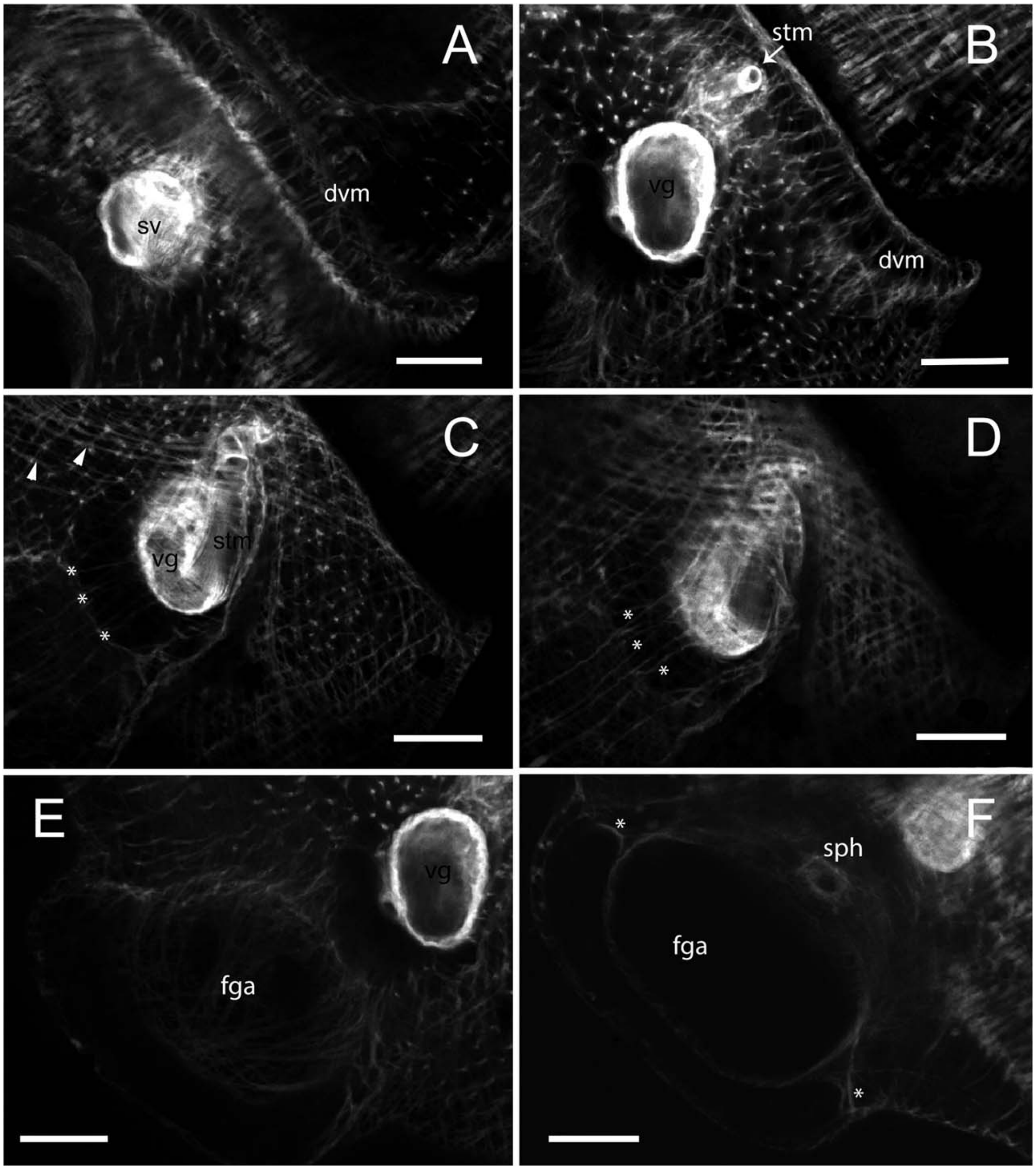

Fig. 7. *Macrostomum quiritium*. Musculature of caudal end and genital system. **A–D**) Different planes from ventral to dorsal level. **A** and **B**) Different planes at ventral level. **C**) Mid-level. **D**) Dorsal level. **E** and **F**) Details of female genital system. Note that the posterior region of the animal is bent. **dvm**, dorsoventral muscles; **fga**, female genital antrum muscles; **sph**, sphincter; **stm**, stylet musculature; **sv**, seminal vesicle muscles; **vg**, vesicula granulorum muscles; **arrowheads**, diagonal fibers of the body wall; **asterisks**, fibers making contact with the walls of the vesicula granulorum and with female antrum. Scales: 50 μm.

bends in diagonal direction toward the anterolateral region of the body (Figs. 8A and 9C, white arrowhead). Furthermore, other longitudinal fibers bend toward a deeper plane at the anterior edge of the mouth (Figs. 8A and 9C). At mid-level, a set formed by 6 pairs of longitudinal brain fibers run laterally to the pharynx, reaching the brain zone (Figs. 8B and 9D,E, vb). These fibers coming from both sides cross over each other at the sagittal plane, forming a conspicuous cephalic arch. One of

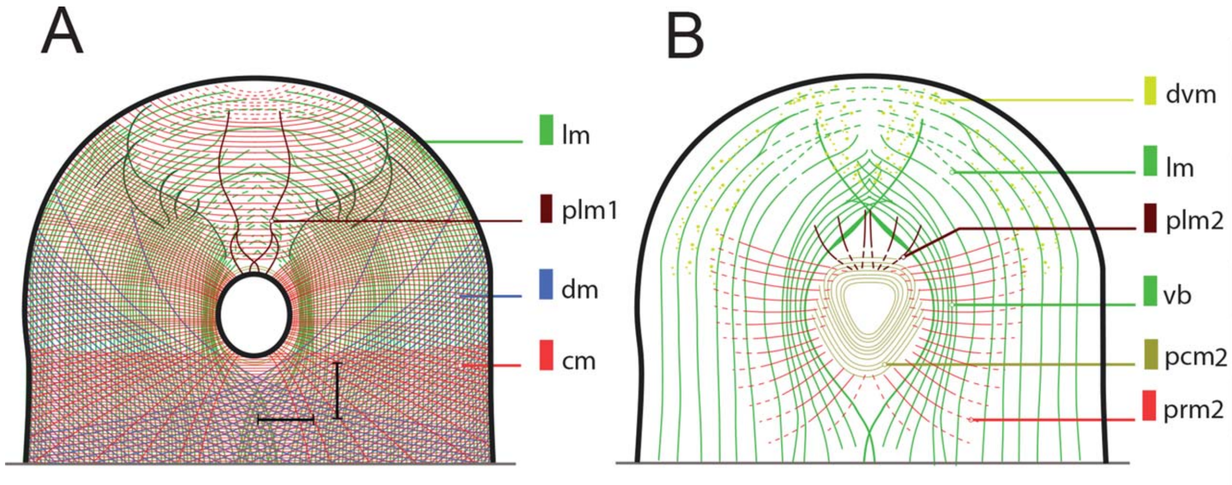

Fig. 8. *Macrostomum tuba*. Diagrams showing muscular pattern in anterior region. **A)** Ventral surface. **B)** Mid-pharyngeal level. Diagrams based on the specimen shown in Figure 9. **cm**, circular muscles; **dm**, diagonal muscles; **dvm**, dorsoventral muscles; **lm**, longitudinal muscles; **pcm2**, pharyngeal circular muscles; **plm1**, pharyngeal longitudinal muscles at ventral surface; **plm2**, pharyngeal longitudinal muscles at mid-level; **prm2**, pharyngeal radial muscles at mid-level; **vb**, ventral brain muscles. In dark green longitudinal fibers at each side of the brain forming a semicircle. Black lines on A indicate the area where body wall fibers were counted. Scale: 100 μm.

these pairs of brain fibers cross at the brain level (Figs. 8B and 9E, arch).

Pharynx region. A fine sphincter formed by three muscle fibers surrounds the mouth (Fig. 9B). Circular fibers of the body wall reach the mouth to form part of the longitudinal muscles of the pharynx (Figs. 8A and 9A, cm). At the posterior edge of the mouth, five pairs of these muscle fibers radiate, acquiring longitudinal direction (Figs. 8A and 9A, arrow). There are also longitudinal fibers of the body wall crossing with those of the opposite side (Figs. 8A and 9B, black arrowheads). Two longitudinal fibers run close to the sagittal plane across the rostrum area to the anterior rim of the mouth, joining the longitudinal muscles of the pharynx (Figs. 8A and 9C, plm1).

At a deeper level, a set of radial fibers on each side of the pharynx coming from the longitudinal pharyngeal muscles diverge to the body wall (Table 1; Figs. 8B and 9D, prm2). The circular pharyngeal fibers are evident at this level, but they were not quantified (Fig. 9E,F, pcm2 and pcm3).

Three pairs of fibers extend in an anterior direction connect the pharynx to the brain fibers (Figs. 8B and 9D,E plm2). Deeper down, the set of longitudinal fibers that form the pharynx tube are evident, crossing with the circular pharyngeal fibers to form a net (Fig. 9F, pcm3 and plm3). At the deepest level, radial fibers also connect the pharynx to the body wall (Table 1; Fig. 9F, prm3).

Caudal region. Close to the posterior end, diagonal muscles form a conspicuous set of fibers on both dorsal and ventral sides of the body (Figs. 10A and 11C, dm). At mid-level, a set of diagonal fibers located posteriorly to the stylet cross and dorsoventral fibers are observed (Fig. 10D, dm2 and dvm).

The number of dorsal longitudinal fibers is lower (7/100 μm) than the number of ventral fibers (23/100 μm; Fig. 10B,C). Ventral fibers are grouped, forming bands composed of 2–3 fibers. At the posterior edge of the adhesive tip, these bands branch to form single fibers (Fig. 10C, arrowheads).

Male genital system. The images show the presence of an oval seminal vesicle and a piriform vesicula granulorum. These structures are aligned beside the wall of the intestine on the left side of the animal (Fig. 11A), showing circular and longitudinal fibers (Fig. 11B). A set of fibers coming both from the body and the intestinal walls is associated with those of the seminal vesicle. The deferent duct opens in the anterior region of the seminal vesicle.

The musculature of the seminal vesicle remains independent from that of the vesicula granulorum (Fig. 11A,B). The duct between them is narrow, surrounded by a delicate group of fibers running longitudinally. The vesicula granulorum is larger and has a conspicuous wall associated with the muscle fibers of the body and the intestine. The muscular wall is thickest in the region where the necks of the prostatic glands converge. The distal portion may act as a storage area for the prostatic secretion.

The most distal region of the vesicula granulorum bends to join the proximal region of the stylet. It has weak musculature (Fig. 11A). The stylet is surrounded by outer longitudinal and inner circular fibers, more conspicuous distally (Fig. 12A,B). From the middle to the distal end of the stylet, groups of diagonal fibers of the body wall link to the stylet fibers, following its trajectory (Figs. 10D, dm2 and 11A, B).

Female genital system. The female antrum has a muscular wall, constituted of a grid of circular and

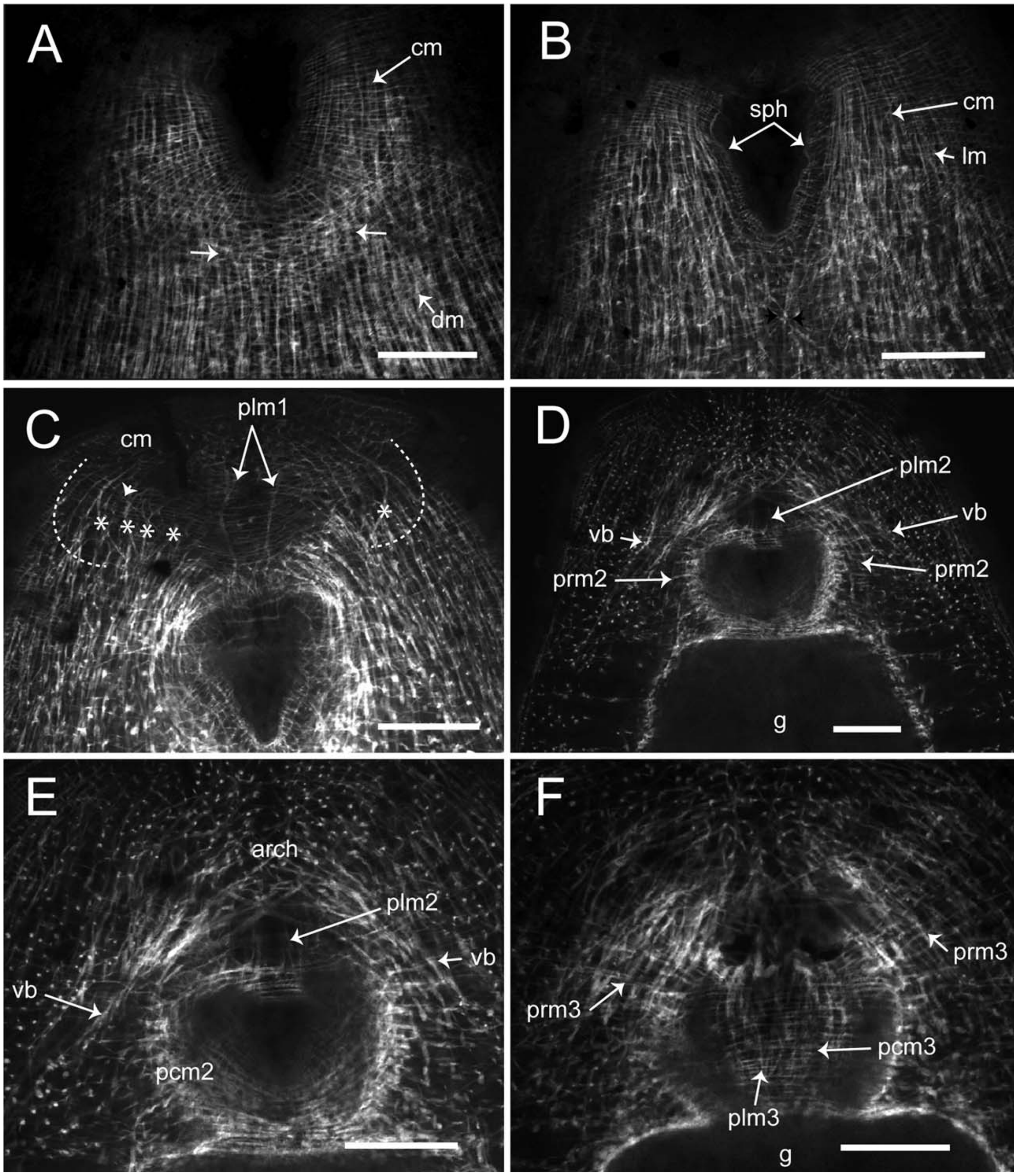

Fig. 9. *Macrostomum tuba*. Anterior region. **A–C)** Ventral surface. **D** and **E)** General view and a detail of pharynx and rostrum at a deeper ventral level. **F)** Deepest pharynx level. **arch**, cerebral arch anterior to the brain; **cm**, circular muscles; **dm**, diagonal fibers; **g**, gut; **lm**, longitudinal muscles; **pcm2**, pharyngeal circular muscles; **plm1**, pharyngeal longitudinal muscles at ventral surface; **plm2**, pharyngeal longitudinal muscles at mid-level; **plm3**, pharyngeal longitudinal muscles at deep level; **prm2**, pharyngeal radial muscles at mid-level; **prm3**, pharyngeal radial muscles at deep level; **sph**, sphincter; **vb**, ventral brain muscles; **asterisk**, branched fibers at each side of the brain forming a semicircle; **black arrowheads**, longitudinal fibers crossing posterior to the mouth; **arrows**, muscle fibers acquiring longitudinal direction; **white arrowhead**, longitudinal fiber bending diagonally toward anterolateral region; **dotted line**, semicircle formed by branched longitudinal fibers. Scales: 100 μm.

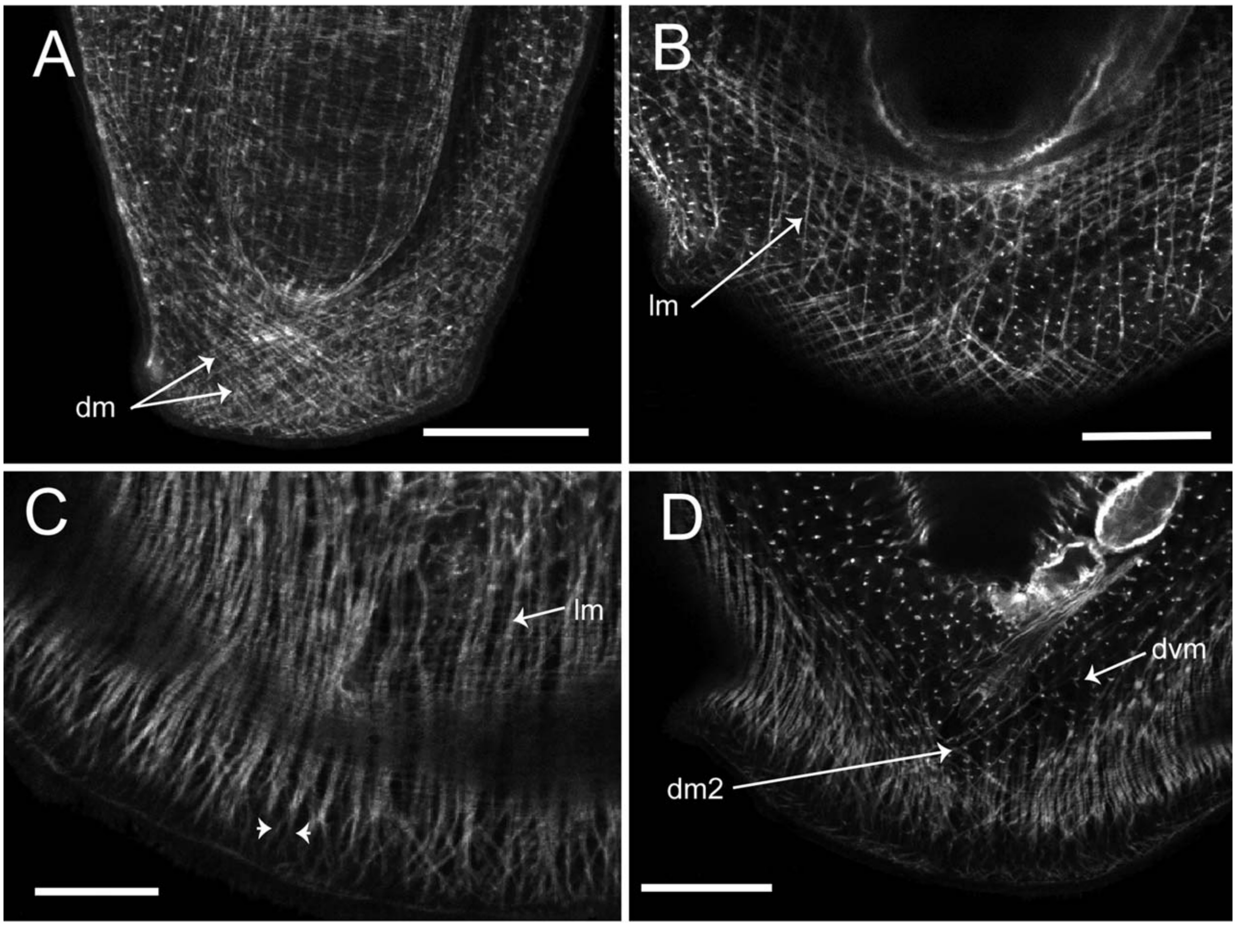

Fig. 10. *Macrostomum tuba*. Musculature of caudal end. **A)** Z-projection of dorsal surface of caudal adhesive plate. **B)** Deeper dorsal level. **C)** Ventral surface of posterior end. **D)** Deeper ventral level. **dm**, diagonal fibers; **dm2**, subset of diagonal fibers; **dvm**; dorsoventral muscles; **lm**, longitudinal muscles; **arrowhead**, split of the band of longitudinal fibers. Scales, **A**, **B**, **D**: 100 µm; **C**: 50 µm.

longitudinal fibers (Fig. 11D, fga). It also has two thin lateral extensions at the level of the ovaries (Fig. 11B, asterisks), which may represent the place where the oocytes reach it. Other groups of fibers connect the antrum wall to the intestinal and body walls (Fig. 11D). In the ventral region, there are 8–10 circular fibers crossed by radial fibers ending at the female gonopore. The last fiber is surrounded by circular fibers that constitute the sphincter (Fig. 11D). A group of circular fibers running from the body wall delimits the gonopore area (Fig. 11C, black arrowheads).

Macrostomum velastylum. *Musculature of the body wall.* Circular fibers are more abundant on the dorsal surface, while longitudinal and diagonal fibers prevail on the ventral surface (Figs. 12A and 13B; Table 1; Supporting Information S4).

Rostrum. On the ventral surface, the longitudinal fibers of the body wall are grouped according to their trajectory. The groups of fibers running on each side of the mouth are more numerous than those running laterally and in the anterior region, sinking to a deeper level (Figs. 12A and 13C, dotted lines). One pair of fibers diverges from its longitudinal direction and crosses over the fibers running laterally (Figs. 12A and 13C, asterisk). At a deeper level, about a dozen longitudinal fibers run laterally to the pharynx, crossing over when they reach the brain level, thus forming an arch (Fig. 13F, vb, arch).

Pharynx region. Two fibers form a delicate sphincter to enclose the mouth (Fig. 13A, sph). Circular fibers of the body wall reach the mouth and run along the pharynx to form part of the longitudinal muscles of this structure (Table 1; Figs. 12A and 13B,C, cm). Three pairs of these fibers at the anterior edge radiate from the mouth and run deeper to the anterolateral edge of the body (Figs. 12A and 13C, arrowheads). At the posterior edge of the mouth, four or five pairs radiate to the posterior-lateral region of the body (Figs. 12A and 13A, black arrowheads). Two longitudinal fibers that run from the anterior end form the longitudinal fibers of the pharynx (Figs. 12A and 13C, plm1).

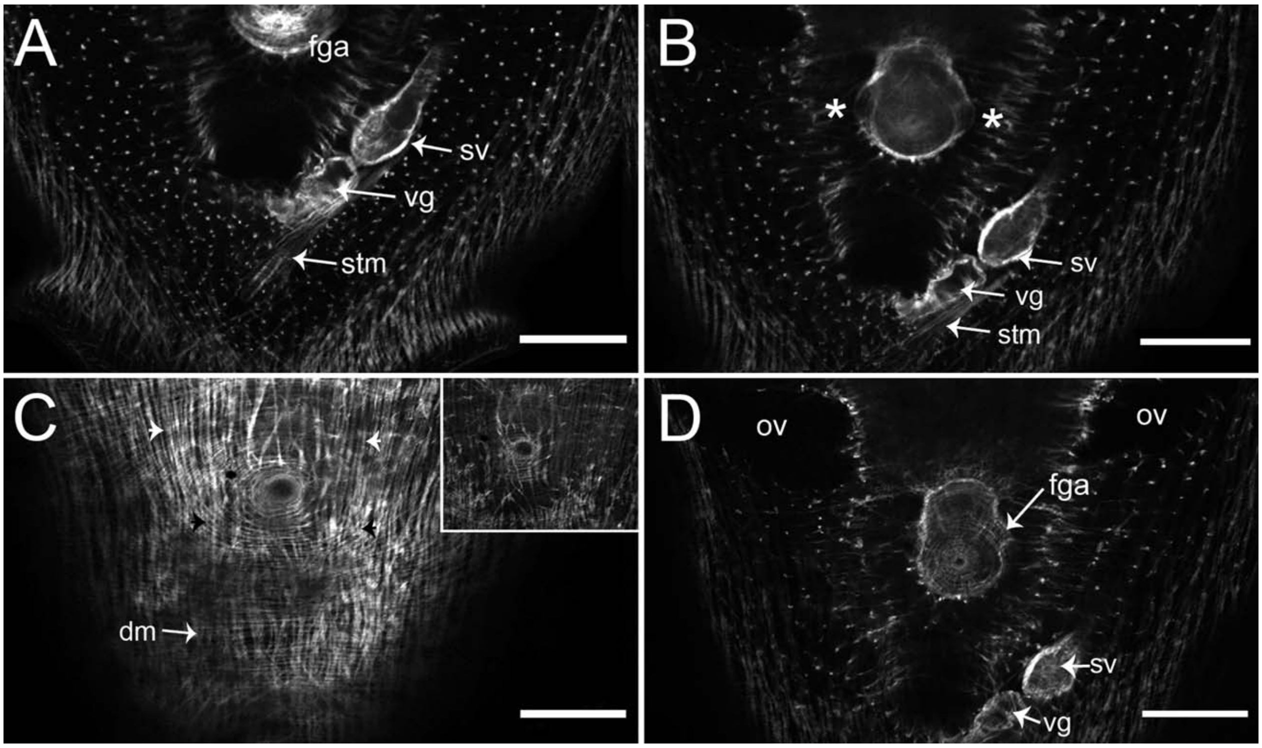

Fig. 11. *Macrostomum tuba*. Musculature of genital system. **A)** Detail of muscles of seminal vesicle and vesicula granulorum, together with musculature associated with stylet. **B)** Muscles of female antrum and probable point of entrance of oocytes into female antrum. **C)** Ventral surface showing longitudinal and circular fibers of body wall associated with female gonopore; gonopore at a deeper level (inset). **D)** Muscular pattern of female antrum and region of ovaries. **dm**, diagonal muscles; **fga**, female genital antrum muscles; **ov**, ovary; **stm**, stylet musculature; **sv**, seminal vesicle; **vg**, vesicula granulorum; **asterisk**, probable point of entrance of oocytes into female antrum; **black arrowheads**, circular muscles associated with female gonopore; **white arrowheads**, longitudinal muscles. Scales: 100 μm.

At mid-level, radial fibers connect the body wall to the pharynx, being added to the longitudinal muscles (Table 1; Figs. 12B and 13E, prm2). At the anterior edge of the pharynx, longitudinal fibers run anteriorly, along the sagittal axis, joining the brain fibers (Table 1; Figs. 12B and 13E, plm2 and vb).

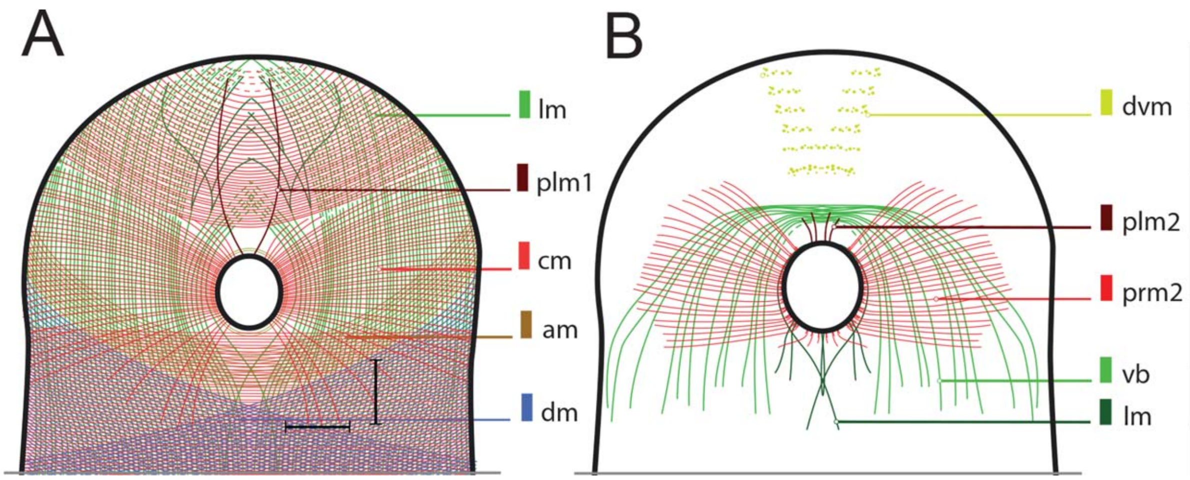

Fig. 12. *Macrostomum velastylum*. Diagrams showing muscular pattern in anterior region. **A)** Ventral surface. **B)** Mid-pharyngeal level. Diagrams based on the specimen shown in Figure 13. **am**, additional muscles; **cm**, circular muscles; **dm**, diagonal muscles; **dvm**, dorsoventral muscles; **lm**, longitudinal muscles; **plm1**, pharyngeal longitudinal muscles at ventral surface; **plm2**, pharyngeal longitudinal muscles at mid-level; **prm2**, pharyngeal radial muscles at mid-level; **vb**, ventral brain muscles. Black lines on A indicate the area where body wall fibers were counted. Scale: 100 μm.

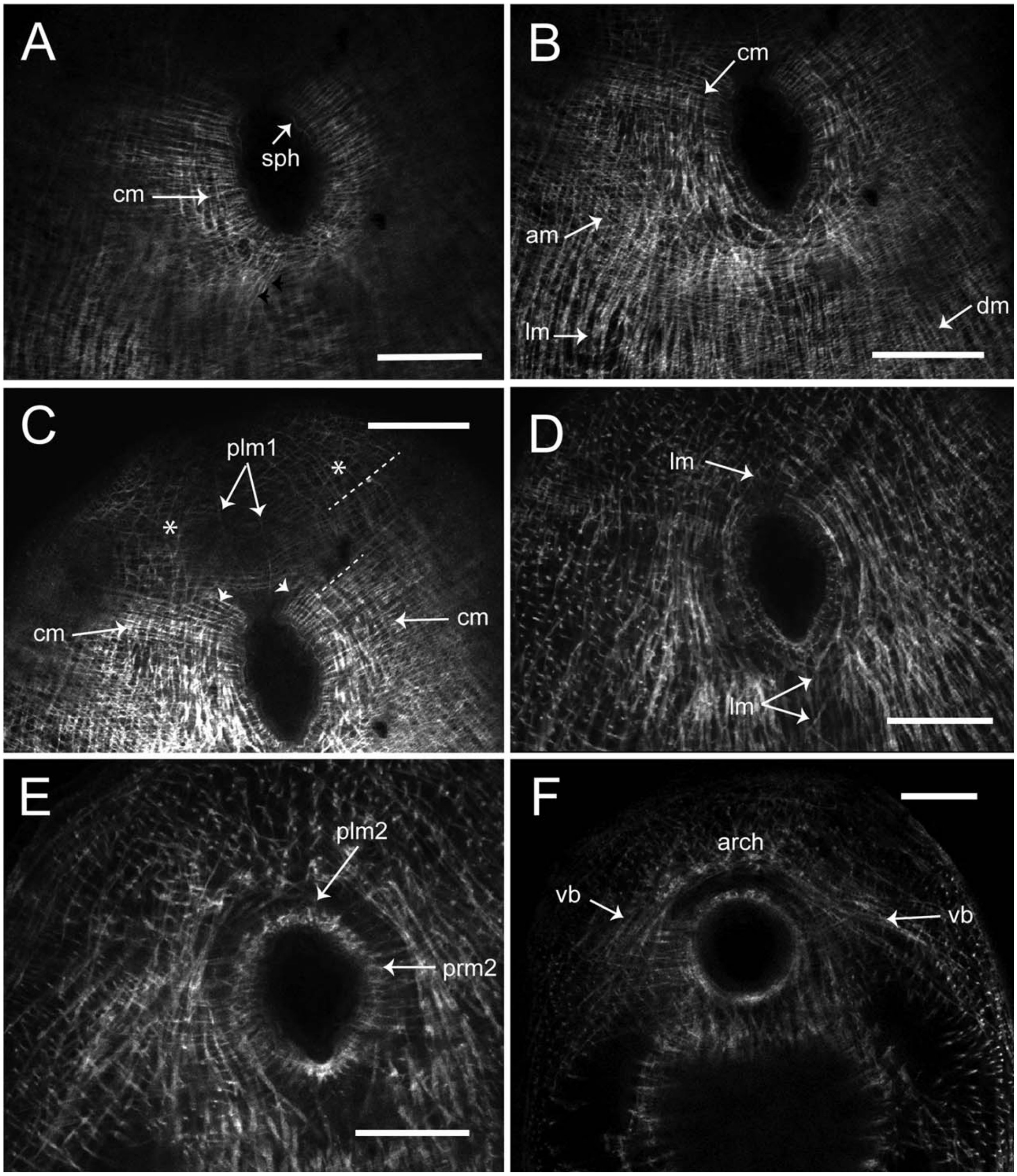

Fig. 13. *Macrostomum velastylum*. Anterior region at different levels. **A–D)** Different ventral planes at body wall level. **E** and **F)** Views of pharynx at different ventral levels. **am**, additional muscles; **arch**, cerebral arch anterior to the brain; **cm**, circular muscles; **dm**, diagonal muscles; **lm**, longitudinal muscles; **plm1**, pharyngeal longitudinal muscles at ventral surface; **plm2**, pharyngeal longitudinal muscles at mid-level; **prm2**, pharyngeal radial muscles at mid-level; **sph**, sphincter; **vb**, ventral brain muscles; **black arrowheads**, fibers radiating from posterior edge of mouth in posterior direction; **white arrowheads**, anterior circular fibers running to a deeper level; **asterisk**, longitudinal fiber that shifts its longitudinal direction; **dotted lines**, longitudinal fibers running to a deeper level. Scales: 100 µm.

The typical arrangement of some of the body wall fibers changes near the mouth. Longitudinal fibers running close to the mouth cross over when they reach its anterior and posterior edges (Figs. 12A and 13D, lm). Also posteriorly to the mouth, a group of additional fibers bends toward the

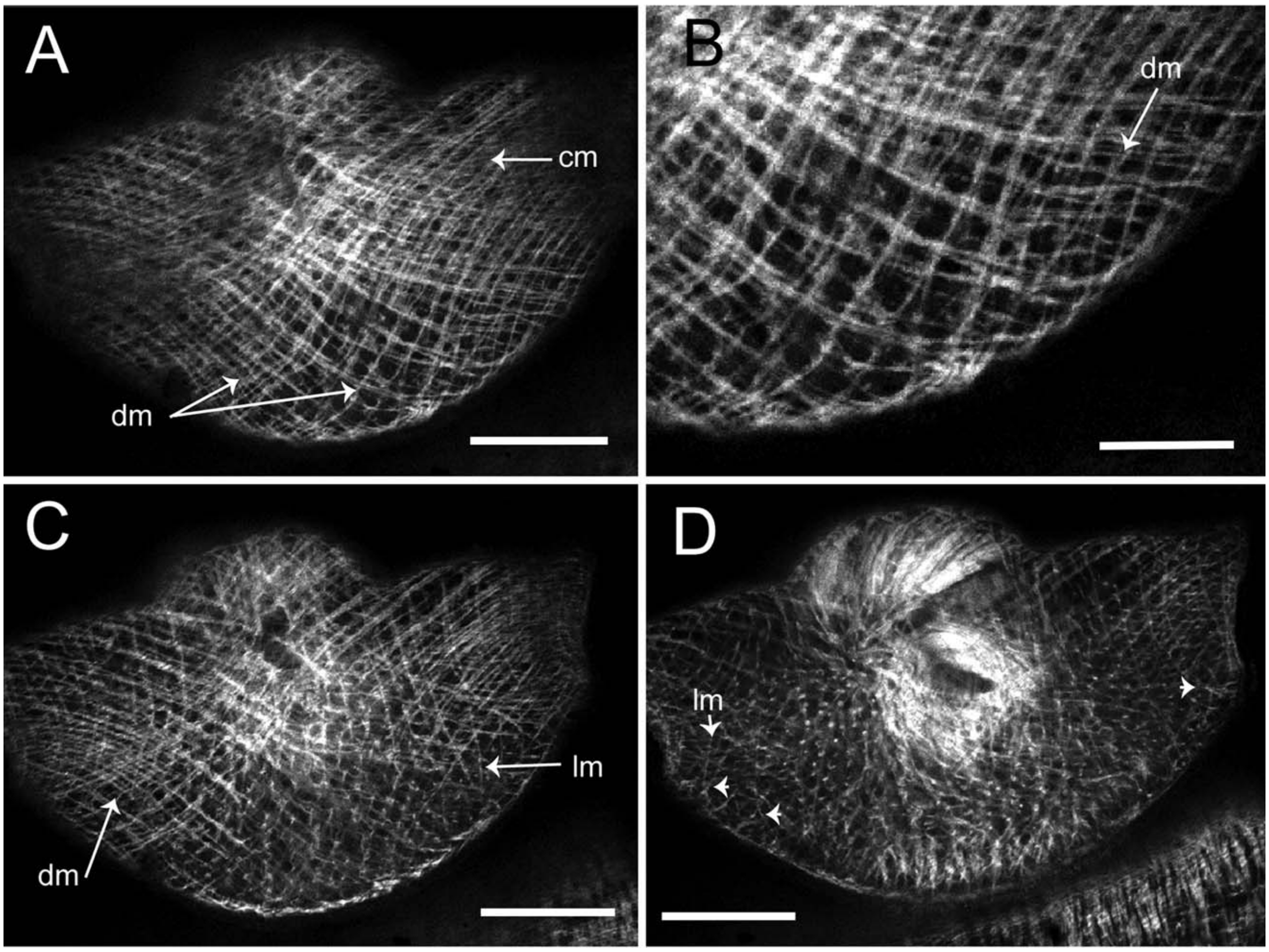

Fig. 14. *Macrostomum velastylum*. Musculature of caudal end. **A)** General dorsal view. **B)** Detail of dorsal surface. **C)** Deeper dorsal level. **D)** Mid-level of body. **cm**, circular muscles; **dm**, diagonal muscles; **lm**, longitudinal muscles; **arrowheads**, longitudinal fibers that shift to diagonal direction. Scales: 100 µm.

marginal region of the body, reaching and surpassing the anterior edge of the mouth, and crosses over the circular fibers (Figs. 12A and 13B, am).

Caudal region. At the dorsal surface, circular and diagonal fibers of the body wall are evident (Fig. 14A). The diagonal fibers are arranged in bands formed of 2 or 3 fibers (Fig. 14B). Near the posterior end, longitudinal lateral fibers change their trajectory, acquiring a diagonal direction and crossing over each other (Fig. 14C,D, arrowheads).

Male genital system. A narrow seminal duct surrounded by a weak muscular layer opens into an elongated seminal vesicle. The vesicle walls are formed of circular fibers that open into the proximal end of the vesicula granulorum through a thin duct with delicate muscular walls (Fig. 15A,B, asterisk). The vesicula granulorum is tubular and large, with strong muscular walls with decussated fibers (Fig. 15B,C, df). Finally, a sheath of helical fibers, more developed in the proximal region, surrounds the stylet (Fig. 15C,D, hf). This muscular stylet sheath also encircles the vesicula granulorum along almost its entire length. The muscular walls of the vesicula granulorum and the stylet can be clearly distinguished (Fig. 15A–C). The male gonopore is inconspicuous.

Female genital system. The female genital antrum is an oval structure formed by radial and circular muscles. In one of the specimens, the muscular wall of the antrum is connected to both the vesicula granulorum (Fig. 15E, arrowheads) and the body wall (Fig. 15F, arrowheads). The gonopore is encircled by several circular fibers.

DISCUSSION

The muscular arrangement in flatworms is highly variable, and knowledge of it provides relevant information for studies of development, regeneration and evolution (Rieger et al., 1994; Hooge and Tyler, 1999; Ladurner and Rieger, 2000; Hooge, 2001; Salvenmoser et al., 2001; Meyer-Wachsmuth et al., 2013; Krupenko and Dobrovolskij, 2015). The muscular systems in flatworms have different functions; and in

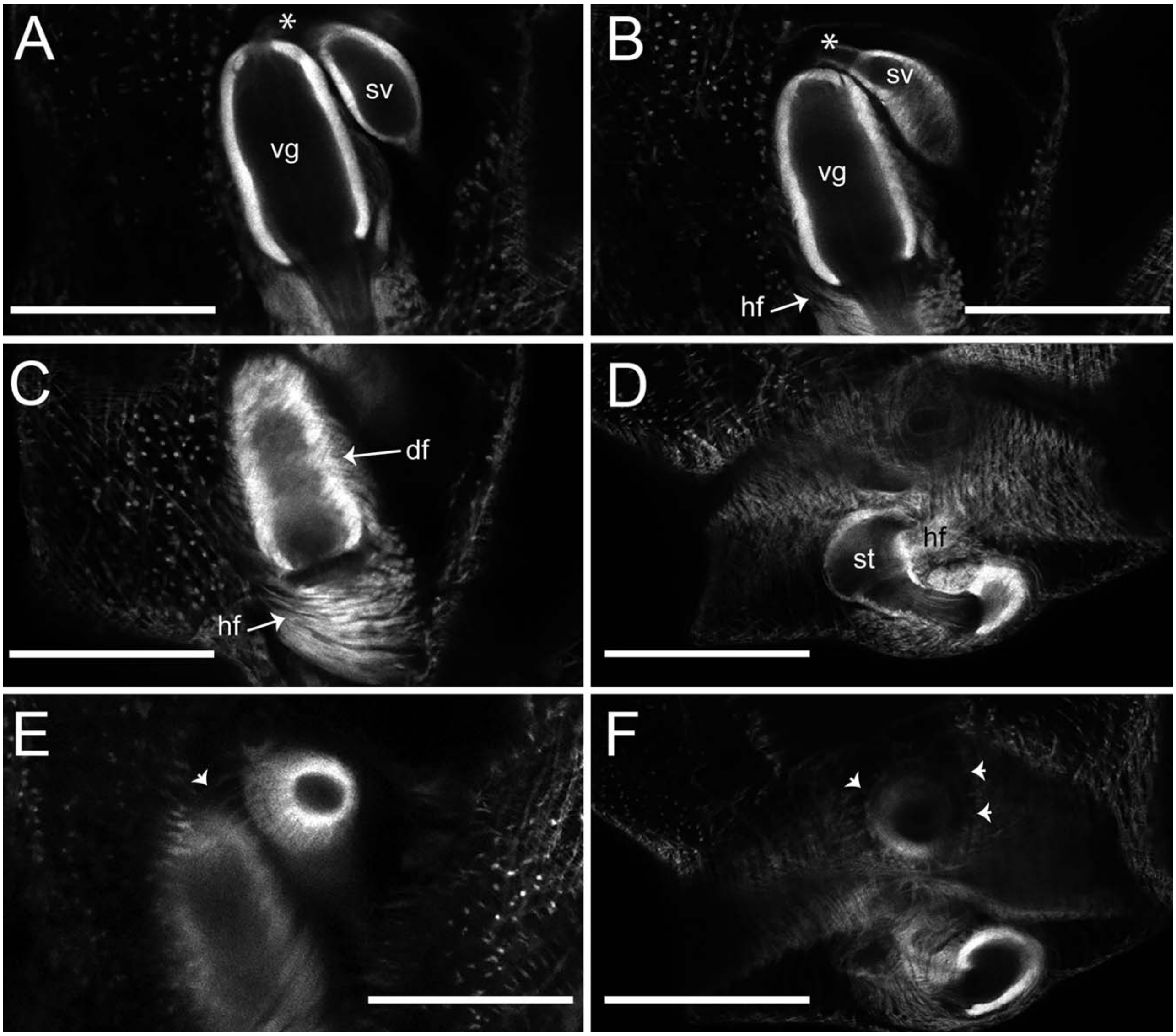

Fig. 15. *Macrostomum velastylum*. Musculature of genital system. **A** and **B)** Detail of musculature of seminal vesicle and vesicula granulorum. **C** and **D)** Detail of external muscles associated with male reproductive system. **E** and **F)** Radial muscles between female gonopore and body wall. **df**, decussated fibers of vesicula granulorum; **hf**, helical fibers of vesicula granulorum, **sv**, seminal vesicle; **vg**, vesicula granulorum; **arrowheads**, radial muscles between ends of female system, near gonopore and body wall; **asterisk**, thin duct connecting seminal vesicle and vesicula granulorum. Scales: 100 µm.

fact, different kinds of habitats, substrates and feeding behaviors may define specific patterns of the muscular fibers (Halton and Maule, 2004). The elucidation of muscular patterns in lower bilaterians has thus been useful for understanding morphological and functional traits (Tyler and Hyra, 1998). In Acoelomorpha (Hooge, 2001), the musculature is of phylogenetic and taxonomic relevance, similarities and differences in muscular pattern provided recognizable taxonomic features, ranging from species to higher taxonomic levels (Tyler and Hyra, 1998; Hooge and Tyler, 2005; Jondelius et al., 2011). Other studies have focused on the muscular system of macrostomids (Rieger et al., 1994; Morris et al., 2007). Our results agree with the general muscular pattern described, although there are differences between *Macrostomum* species.

Musculature of the Body Wall

In agreement with previous studies (Rieger et al., 1994; Hooge, 2001; Tyler and Hooge, 2004), our results show that the typical organization of the body wall musculature described for Rhabditophora (Hooge, 2001) is present in the three species analyzed (i.e., outer circular, innermost longitudinal and diagonal fibers between them). On the ventral surface, the arrangement of the body wall fibers varies due to the presence of the mouth and the genital pores, resulting in complex fiber arrangement. On the dorsal surface, this complexity occurs in the rostrum, where a system of predominantly longitudinal fibers diverges from the body wall into the parenchyma forming a muscular net around the brain. These observations agree with the description of the rostral area for *M. hystricinum marinum* (Rieger et al., 1994), *M. lignano* (Morris et al., 2007) and *M. platensis* (Adami et al., 2012).

Rieger et al. (1994) reported that the circular muscles of the body wall of the adult could form bands enclosing the whole body. These authors also mentioned that each band consists of 1 to 4 fibers, with not all the fibers in a given band surrounding the circumference of the body. Our results also show differences in the number of circular fibers on the dorsal and ventral surfaces.

Several studies analyzed the number of fibers in the body wall. For instance, Ladurner et al. (2005) reported that mature *M. lignano* individuals had up to 50 longitudinal fibers, which was confirmed by studies of the other species in the genus (Rieger et al., 1994; Adami et al., 2012). The total number of fibers in the species analyzed ranged from 80 to 100, a number clearly higher than those reported in previous observations.

Head Region

Previous studies of *M. lignano* described the presence of muscles and glands associated with the brain (Morris et al., 2007). These muscles form a fine meshed system of longitudinal, transverse and vertical fibers surrounding the neuropile. Some groups of fibers penetrate the brain region horizontally and vertically at stable positions (Morris et al., 2007). In a detailed analysis of the brain region, Morris et al. (2007) showed two groups of body wall longitudinal fibers that originate in the dorsal and ventral cerebral fibers surrounding the outer surface of the brain. Adami et al. (2012) described a complex arrangement of fibers in the head region of *M. platensis*, which run into the parenchyma around the brain. Unfortunately, the ventral sets of longitudinal fibers cannot be identified in the available images. However, in agreement with the pattern described by Morris et al. (2007) and Adami et al. (2012), these three species show a set of conspicuous longitudinal fibers running along the side of the pharynx and reaching the brain zone. In addition, at least one pair of longitudinal fibers located on the midline of the body reaches the brain zone in all the species studied. These fiber sets were also described by Rieger et al. (1994) for *M. hystricinum marinum*. In accordance with Morris et al. (2007), *M. tuba* and *M. quiritium* have a set of two muscles crossing at the brain level, forming an "X," which is not evident in *M. velastylum*. Furthermore, despite this structure not being mentioned in the original study (Adami et al., 2012) it was also present in *M. platensis*.

Pharynx Region

It has been proposed that the pharynx morphology may be relevant for taxonomic and phylogenetic studies of flatworms (Rieger et al., 1991). The simplest form is the "pharynx simplex," which has evolved convergently in several taxa, such as Acoela, Catenulida, Macrostomida and Haplopharyngida (Doe, 1981).

The first detailed study of the musculature of *Macrostomum* (Rieger et al., 1994) reported that the pharynx muscles consisted of an inner circular layer (lying next to the pharyngeal epithelium) surrounded by a longitudinal layer. Radial fibers were clearly visible radiating from the dorsal and lateral sides. Furthermore, a set of circular fibers from the body-wall formed the longitudinal muscles of the pharynx (Rieger et al., 1994).

Our study identified five different sets of muscle fibers pertaining to the pharynx and mouth at the ventral, middle and deep levels: 1- Sphincter muscles (sph), 2- pharyngeal circular muscle (pcm), 3- circular body wall muscles (cm), 4- pharyngeal radial muscles (prm), 5- pharyngeal longitudinal muscles (plm). It should be noted that sets 3, 4 and 5 contribute forming the longitudinal muscles of the pharynx. These different sets are present in the three species studied. In agreement with Rieger et al. (1994), these results suggest that the longitudinal muscles of the pharyngeal tube derive from different groups of muscles (i.e., body wall circular, pharyngeal longitudinal and pharyngeal radial muscle fibers).

Doe (1981) and Rieger et al. (1994) reported that the circular fibers of the body wall form part of the longitudinal musculature of the pharynx. Indeed, Doe (1981) emphasized that only the circular muscles constitute the longitudinal muscles of the pharynx. However, in agreement with Rieger et al. (1994), we found at least one pair of longitudinal muscle fibers entering the mouth from the rostrum zone, close to the body sagittal line.

Doe (1981) has also reported a second set of "special body wall circular muscles" forming part of the longitudinal pharyngeal fibers. On the other hand, Rieger et al. (1994) and Adami et al. (2012) reported the presence of "special diagonal muscles" radiating from the pharynx in *M. histricinum marinum* and *M. platensis*, which were not observed in the present study. A set of short radial fibers holding the pharyngeal apparatus described by Rieger et al. (1994) was not recognized in the present study. Interestingly, in the pharynx/gut transition zone, *M. quiritium* and *M. velastylum* showed an additional set of ventral fibers posterior to the mouth, which may act as the "pharynx-holding apparatus" (Rieger et al., 1994).

Jennings (1957) reported that the mouth and the pharynx of *Macrostomum* distends to catch a prey by the action of the radial muscles, with the mucous glands playing a relevant role in food capture. These activities require high coordination among the muscular elements involved. Taxa that share ecological features and/or evolutionary histories may be expected to develop similar arrangements (Leasi et al., 2012). Knowledge of *Macrostomum* feeding behavior and prey preferences is scarce and has not been analyzed in detail. They feed on algae, testate amoeba, ciliates, rotifers, nematodes, annelids, cercariae, water fleas, ostracods, etc. (Marcus, 1946; Jennings, 1957; Holliman and Mecham, 1971; While, 1971; Heitkamp, 1982; Ladurner et al., 2005; Brusa, 2006). Better knowledge of the feeding behavior of *Macrostomum* species from different habitats (marine, freshwater and brackish) and with different food preferences would help to understand the muscular pattern variability of the pharynx.

Musculature of the Posterior Body Region

As previously described by Rieger et al. (1994), the posterior region of the body has a complex arrangement of muscles. In fact, additionally to the general muscular pattern of the body wall, the caudal region has numerous dorsoventral fibers. Rieger et al. (1994) reported that these fibers are forked in *M. h. marinum*, having at least two branches at each end. These muscles keep the tail plate flat, and may facilitate adhesion to the substrate. In *M. quiritium*, the dorsoventral muscles form bands of one to three fibers each. *M. tuba* has an additional, innermost layer of diagonal fibers in the body wall.

The muscles associated to the *reproductive system* appear to be independent of the musculature of the body wall in all three species analyzed. Doe (1981) described the musculature of the copulatory organs in *Macrostomum*, pointing out that the vesicula granulorum and the stylet are enclosed by a muscular sheath, as was also observed in the species studied here.

For the female antrum, Ladurner et al. (2005) mentioned that the arrangement of the muscles may play a role when a ripe egg reaches it. The muscles associated with this structure in the three species form a loose network of fibers without regular arrangement. Although the copulatory apparatus musculature is independent from the body wall, it appears to be closely related with the intestinal wall by means of specific muscle fibers that may serve as an anchor to allow the proper movements of this structure.

The muscular pattern of species studied retains the previously described muscular pattern for the genus. However, differences were detected between species. The number of longitudinal fibers of the body wall was approximately twice as high as reported previously in the literature. Two of the species have an additional set of fibers located posterior to the mouth, which has so far not been recognized in other *Macrostomum* species. Specific differences in the pattern of fibers of the rostrum and associated with the brain were found. Moreover, the number of circular fibers in the body wall associated with the pharynx is variable. Data on more species are needed before any definitive conclusions can be reached, but with our results, the muscular pattern, especially in the body wall and pharynx, is a promising feature for species identification and future phylogenetics studies.

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