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Research paper

New insights into the genital musculature of *Macrostomum johni* (Platyhelminthes, Macrostomorpha), revealed with CLSM

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ABSTRACT

The genital system is the most thoroughly studied morphological feature in the genus *Macrostomum* (Platyhelminthes, Macrostomorpha), for two main reasons: 1) taxonomic identification and 2) understanding different mating strategies. In the present study the musculature of the genital system of the freshwater flatworm *Macrostomum johni* was visualized using phalloidin-linked fluorescent dye by confocal laser scanning microscopy (CLSM), and a 3D model was provided. In the female antrum, two muscular chambers are observed, both attached to the muscular body wall by muscle fibers. In the male system, three muscle sets associated with the stylet are recognized. Finally, we discuss about the interspecific variability of sexual musculature arrangement within *Macrostomun* and other related genus, and speculate whether there is a common pattern in the musculature of genital organs related to different mating-behavior.

1. Introduction

The genus *Macrostomum* (Platyhelminthes: Macrostomorpha) is a group of cosmopolitan free-living flatworms that occur in a wide range of habitats, including limnic, brackish and marine environments (http://macrostomorpha.info/). They are present both in clean and eutrophic waters (Sun et al., 2015).

Species of *Macrostomum* are hermaphrodites, and internal fertilization occurs by means of two types of copulatory behavior: hypodermic insemination and reciprocal mating syndrome. There is an evolutionary link between mating-behavior, genital morphology, and sperm type. So species in which the stylets carry blunt distal thickenings, have stiff lateral bristles in the sperm, a thickened epithelium in the sperm-receiving organ and reciprocal mating-behavior. On the contrary, simple sperm and female genital morphology is present in species with needle stylets and hypodermic insemination (Schärer et al., 2011). Genital morphology is considered a significant trait for species identification (Ferguson, 1954; Rieger, 1977; Rieger et al., 1994) and to understand the different mating-behavior strategies in these flatworms (Schärer et al., 2011; Vizoso et al., 2010). Among the genital features used to identify species of the genus, the penis stylet is the most important because it is relatively constant in shape. Despite the importance of genital morphology, the description of *M. johni* Young, 1972a,b do not include detailed images, making difficult to interpret the morphology and make comparisons among the species.

The use of phalloidin-linked fluorescent dye together with confocal microscopy to describe the muscular system of flatworms has acquired importance in studies on development, regeneration, feeding behavior, taxonomy and evolution (Adami et al., 2012, 2017; Hooge, 2001; Krupenko and Dobrovolskij, 2015; Morris et al., 2004, 2007; Reiter et al., 1996; Rieger et al., 1994; Salvenmoser et al., 2001; Smith et al., 2015). The muscular system is involved in all essential activities such as locomotion, feeding and reproduction; so, the specific arrangement of the genital muscular architecture is probably associated with different mating-behaviors. Nevertheless, few studies include comparative analyses of the copulatory organ musculature (Adami et al., 2017; Doe, 1982; Raikova et al., 2006).

This study describes in detail the genital morphology and muscle arrangement of the freshwater flatworm *Macrostomum johni* Young, 1972a,b through the use of confocal microscopy and 3D reconstruction. The integration of the genital muscular traits to other morphological features could provide insights into the evolution of the reproductive system in Macrostomorpha.

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2. Material and methods

Macrostomum johni specimens were collected from marsh vegetation dominated by *Azolla* sp. and *Pistia stratiotes* in the Sagastume stream (33°38′34″S, 58°49′63″W), Entre Ríos Province, Argentina, during 2015. This is the first record of the species in Argentina.

Fifteen specimens were examined alive. Diagnostic characters were studied through the progressive squash method (Noreña et al., 2016) under a compound microscope. Interference contrast illumination was used for photomicrographs of diagnostic characters. Polyvinyl–lactophenol whole mounts were prepared to study stylet morphology. We enhance the contrast of Fig. 1C to be the bristles easier to see. Hard and soft sexual structures were measured: a- maximum diameter of female antrum (measured over the body transverse axis), b- maximum diameter of vesicula granulorum, c- maximum diameter of seminal vesicle, d- length of the stylet (distance from proximal opening to distal tip), e- proximal and distal opening of the stylet (d and e following Gelhen and Lochs, 1990).

To analyze muscle arrangement, two specimens were fixed in formaldehyde (4%, w/v in PBS) for 12 h, washed in PBS-Tween (0.05%) (PBS-T) and permeabilized in Triton X-100 (1%) for 24 h at 4 °C. They were incubated overnight at 4 °C with phalloidin-

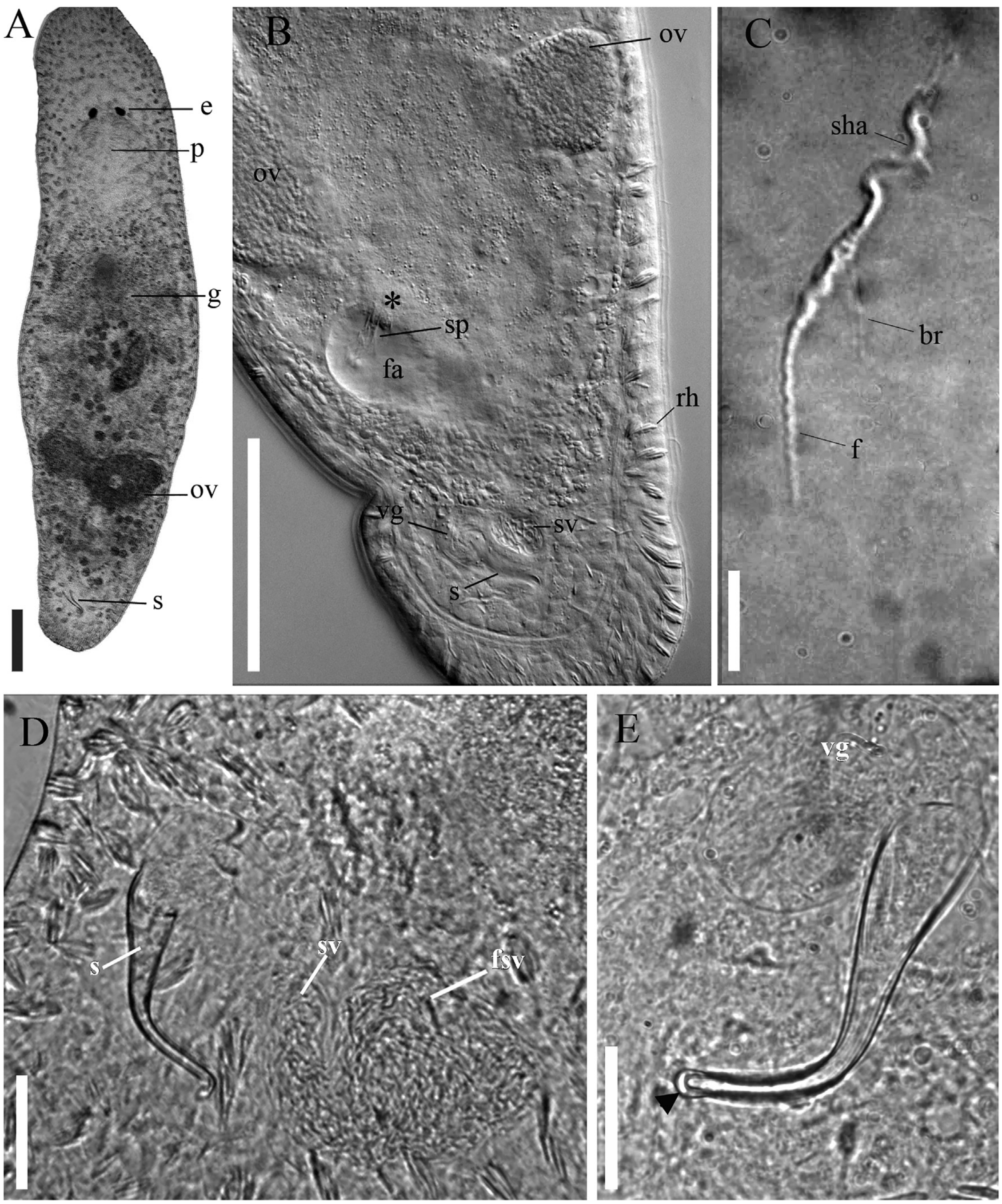

Fig. 1. *Macrostomum johni*. (A) General view in vivo. (B) Caudal region showing female and male genital system (the asterisk shows the cellular valve in the female antrum). (C) Sperm with bristles. (D) Stylet, seminal vesicle and false seminal vesicle. (E) Vesicula granulorum and stylet (arrowhead: distal end of the stylet). (B and E) are interference contrast micrographs.

Abbreviations: br, bristles; e, eyes; f, feeler; fa, female antrum; fsv, false seminal vesicle; g, gut; ov, oocyte; p, pharynx; rh, rhabdites; s, stylet; sha, shaft; sp, sperm; sv, seminal vesicle; vg, vesicula granulorum. Scale bars: A, B = 100 μm; C = 10 μm; D = 50 μm; E = 20 μm.

solution (Sigma–Aldrich) (1/1000) and mounted in Vectashield medium (Adami et al., 2012). Specimens were observed in a Leica LAS AF Lite Confocal Laser Scanning Microscope. The step size in the confocal stacks was 2 μm along the Z-axis. The images and 3D projections were analyzed with Leica LAS AF Lite Image Examiner software.

The image stack was reduced in size, converted to gray-scales and subsequently imported into the visualization and reconstruction software Amira 4.1. The muscles associated with the different structures were labeled manually, mainly using the brush tool. Afterward, a surface for each structure was generated using the Surface-Gen module, which was optimized by subsequent steps of triangle reduction and smoothing. Snapshots and videos were recorded with the Amira software 4.1.

For the description of the different arrangements of muscle fibers the following terms were used, a) circular fibers as those that surrounds a structure or organ and are arranged at an angle of 90° to the main axis of the organ, the circular fibers can adopt a ring-shape and form a sphincter; b) longitudinal fibers as those that run parallel to the main axis of the structure or organ; c) diagonal fibers as those that run obliquely in relation to the main axis of the structure or organ; d) helical fibers those curved around a structure in the form of a spiral; e) radial fibers those that spread out with respect to an axis or a reference point.

Two specimens mounted in polyvinyl–lactophenol, five fixed in ethanol (100%), and those processed to study the muscular system were deposited in the Invertebrate Collection of the Museo de La Plata (FCNyM-UNLP), MLP-He 7360.

3. Results

Macrostomum johni Young, 1972a.

3.1. External morphology

Mature individuals are about 900 μm long and 300 μm maximum wide. Fixed mature specimens are about 500 μm long and maximum 250 μm wide. Two black eyes measure about 17 μm in diameter (Fig. 1A), and the rhabdites are 10 μm long, forming bundles of four to nine (Fig. 1B and D).

3.2. Male genital system

Paired lateral testes are in the central region of the body, anterior to the ovaries, though usually inconspicuous. A false seminal vesicle, with walls lacking muscle fibers, was observed in two specimens (Fig. 1D). Bands of circular muscle fibers enclose a duct that connects the false seminal vesicle to the seminal vesicle (Figs. 2D cmd and 4A and C lilac). The seminal vesicle shows a thick wall of muscle fibers irregularly arranged (Figs. 2C-E sv, and 3A and C). A short muscular intervesicular duct connects the seminal vesicle with the vesicula granulorum (Figs. 2D di, 3C and 4C; see 3D model 1 and 2). In dorsal view, the vesicula granulorum has coarse diagonal muscle bands (Figs. 2F dmvg and 4C). Some of these bands (located near the distal part of the vesicula granulorum) extend in a helical pattern and run over to the proximal part of the stylet. Probably they are related to the body wall musculature, acting as protractor muscles (Figs. 2C-D hm, E arrowhead, and 4C). In addition, two of these fibers crisscross over the muscle fibers that surround the stylet (Fig. 2D arrow). The mean diameter of seminal vesicle and vesicula granulorum is 28.8 ± 4.4 μm (n = 7) and 36.6 ± 2.5 μm (n = 5), respectively.

The stylet is funnel-shaped, curved in two planes, although this depends on the squashing degree. The distal opening is subterminal (Fig. 1D and E arrowhead). The stylet is 40 μm–65 μm long (mean 56 ± 2 μm, n = 12). The diameter of the proximal end is 12.5 μm–15 μm (mean 13.7 ± 0.4 μm, n = 7), while the distal end is 2.5 μm–4.3 μm wide (mean 3.6 ± 0.3 μm, n = 7). The stylet is surrounded by circular musculature along its length with exception of the proximal portion, where such fibers are not observed (Figs. 2C–E cm, 3C and 4C beige). In ventral view, fine outer longitudinal muscle fibers are arranged over the circular fibers (Figs. 2C lm and 3C yellow; see 3D model 1 and 2).

Additionally, coarse diagonal fibers of the body wall are evident and crisscross at the male gonopore area (Fig. 2B dm).

Spermatids are approximately 65 μm long, with two bristles and a 15 μm feeler (Fig. 1C and Video 1 in Supplementary materials).

3.3. Female genital system

Developing oocytes are lateral, posterior to the testes (Fig. 1B ov). The oocytes move toward the antrum located on middle axis of the body on the caudal end of the gut (Video 2 in Supplementary materials). Phalloidin preparations show the female antrum partially divided by a constriction into two sections or chambers (Figs. 2A, B fa, C, 3B and 4B). The anterior chamber shows thick circular and few longitudinal muscle fibers (Figs. 3B and 4B green, white and 3D model 1). In the most rostral region of the anterior chamber, the muscle fibers show a ring pattern, giving the appearance of a sphincter (Fig. 2B asterisk). The posterior chamber of the antrum shows thinner circular and longitudinal muscles (Figs. 2A cm, lm and 3B) and has a sphincter to connect to the vagina (Fig. 2B sp). The mean diameter of female antrum is 57.8 ± 12.5 μm, (n = 5). The vagina is surrounded by cement glands and leads to the female gonopore (Fig. 2A v, cg and Video 2 in Supplementary material). Radial fibers run from the wall of the female antrum toward the body wall. These fibers are most abundant in the posterior chamber, act as dilator muscles and anchor the structure (Figs. 2A-C rm, 3A, B blue and 3D model 2). A sphincter is observed around the female gonopore (Figs. 2A fg and 3D model 1). The egg is 96 μm in diameter.

4. Discussion

4.1. Taxonomic and geographic notes

The specimens studied herein share the shape of the stylet and other general morphological features with *Macrostomum johni*. Under slight squash preparations, the stylet is curved in two planes and the distal opening is subterminal. However, mean stylet length was shorter (56 μm) than the range (80–98 μm) observed by Young (1972a) for *M. johni*. Another difference is the diameter of the proximal opening of the stylet; in the specimens from Argentina it is 12.5 to 15 μm, smaller than specimens from United Kingdom described by Young (1972a) (13–33 μm). It should be noted that the method used to measure the stylets length used by Young (1972a) was different to the method used by us (following Gelhen and Lochs, 1990). Using Youngś method, the mean stylet length of Argentina specimens was 45 ± 1.6 μm (n = 12), similar to the stylet length reported by Gamo and Leal Zanchet (2004) for specimens from southern Brazil (40–45 μm). Also, the lengths of mature specimens from Argentina (0.9 mm) and Brazil (0.6–0.7 mm) are shorter than those described by Young (1972a) (1.3 mm).

Three other species resemble to *M. johni*. *M. silesiacum* Kolasa, 1973 is the most similar, with a slightly twisted stylet of 42–56 μm long and a proximal openning of 24–32 μm (Kolasa, 1973). Both species have a thickened distal end and subterminal opening. *M. lutheri* Beklemischev, 1927 and *M. mediterraneum* Ax, 1956 are similar to *M. johni* because the length of their stylets is similar to the length recorded in the original description of *M. johni*, and the distal end of the stylet is similar in all three species.

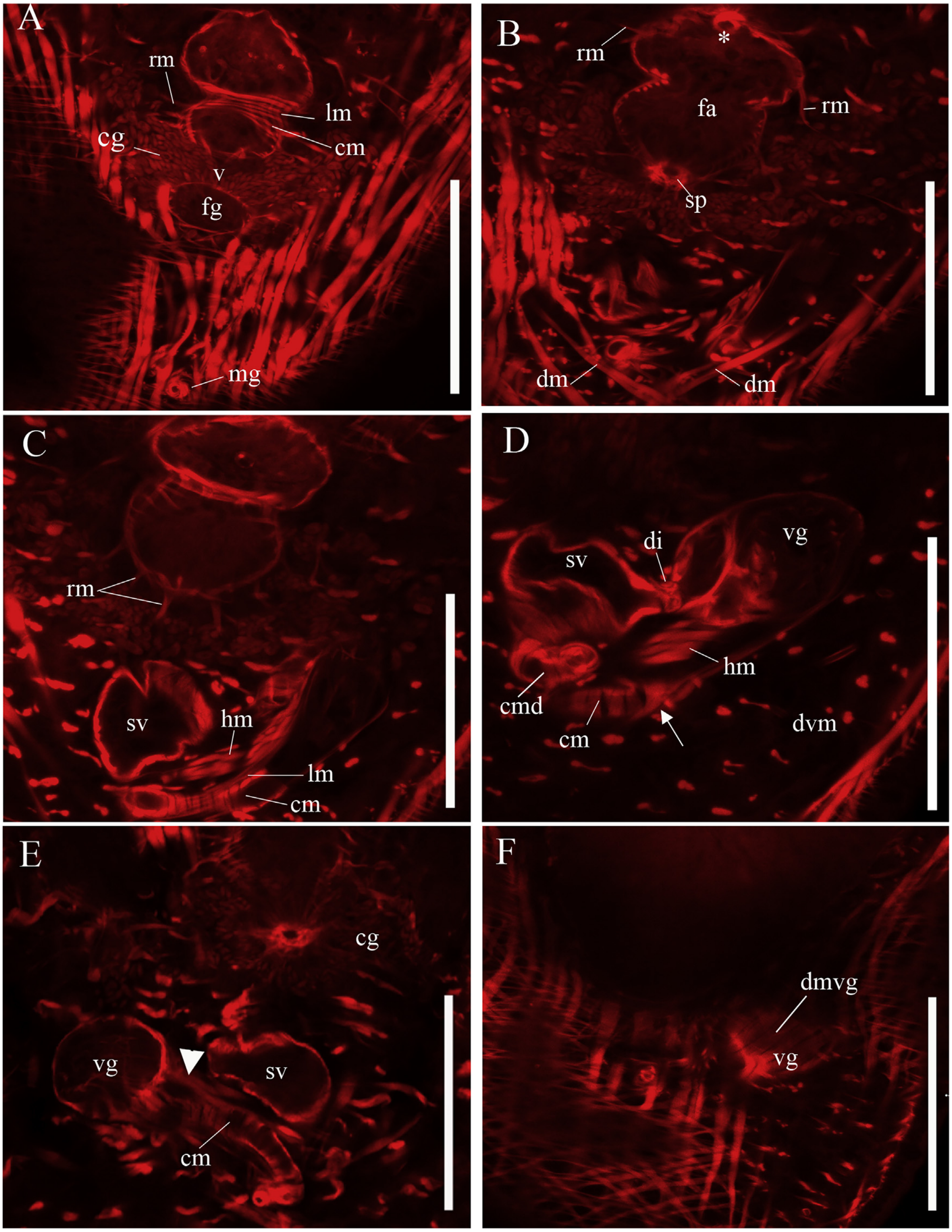

Fig. 2. Muscular pattern of the genital system of *M. johni*. (A, B, D and E) Ventral and deep ventral planes of genital system. (C and F) Deep dorsal and dorsal plane of genital system. (A–D and F) correspond to specimen 1, while (E) corresponds to specimen 2. A high-resolution version of CLSM stacks for use with the Virtual Microscope is available as eSlide: VM04871.
Abbreviations: cm: circular muscles; cmd: circular muscles of the duct that connects the false seminal vesicle to the seminal vesicle; cg: cement gland; di: intervesicular duct; dm: body wall diagonal fibers; dmvg: diagonal muscles of the vesicula granulorum; dvm: dorso-ventral muscles; fa: female antrum; fg: female gonopore; hm: helical muscles; lm; longitudinal muscles; mg: male gonopore; rm: radial muscles; sp: sphincter; sv, seminal vesicle; v: vagina; vg, vesicula granulorum. Arrow: two helical fibers crossing on the stylet zone; arrowhead: helical muscles related with vesicula granulorum; asterisk: ring muscle in the rostral tip of the female antrum; Scale bars = 50 μm.

The present record of *M. johni* is the first for Argentina and the third in the Neotropical Region. This species was previously known from its type locality (Llyn Cwellyn, North Wales, United Kingdom; Young 1972a,b), Brazil (Santo Antônio da Patrulha, Rio Grande do Sul; Gamo and Leal Zanchet, 2004) and Peru (Pacaya-Samiria National Reserve, Loreto; Noreña et al., 2006). The distribution of these records raises certain questions. The dispersal capacities of microturbellaria and the processes that guide their current distribution are not clear. On the other hand, cryptic species are frequently recognized within several micrometazoan linages where

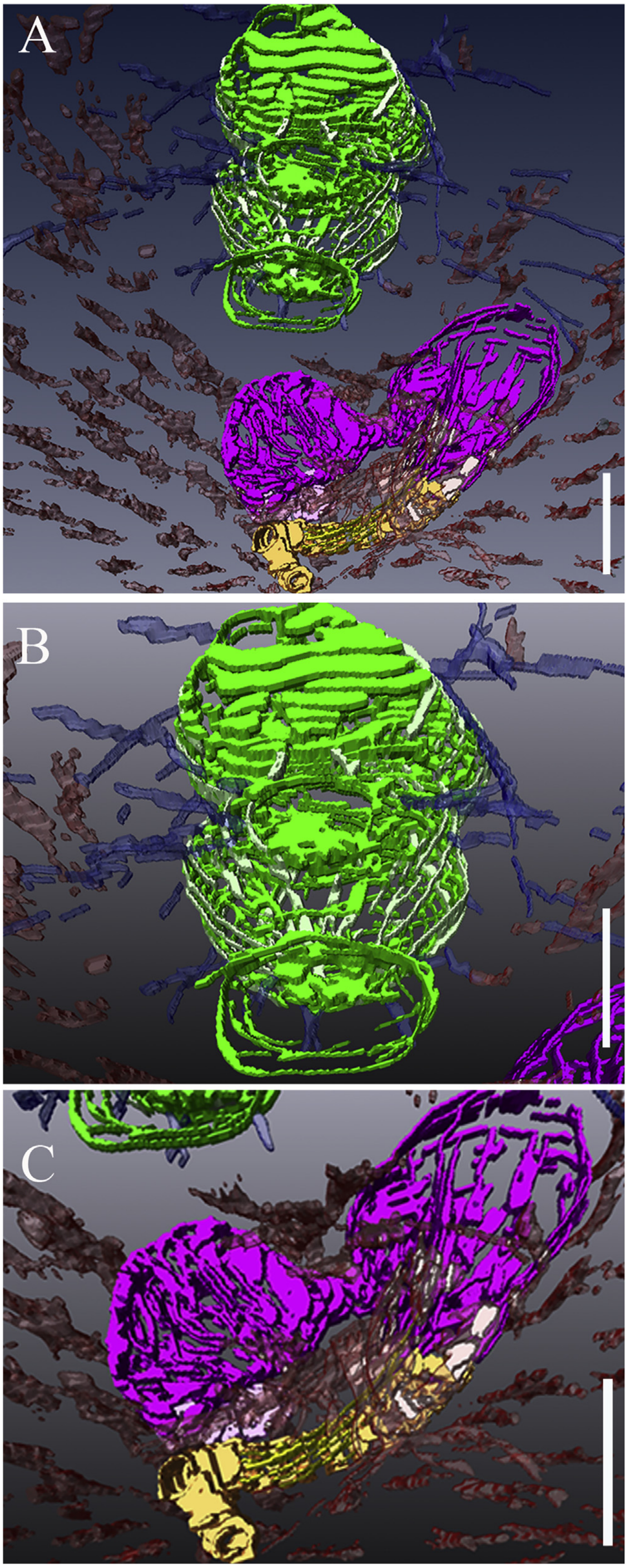

Fig. 3. Genital system 3D-reconstruction based on serial optical sections of a whole mount specimen at ventral view (A) General view, (B) detail of the female organs, (C) detail of the male organs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).
Color codes: reddish gray: dorso-ventral muscles of the body wall; blue: radial muscle fibers associated with the antrum and diagonal fibers of the body wall; green: circular muscles of the antrum; white: longitudinal muscles of the antrum; magenta: seminal vesicle and vesicula granulorum; beige: circular fibers of the stylet; yellow: longitudinal fibers of the stylet. Some transverse fibers and longitudinal muscles of the body wall are omitted for clarity. This reconstruction was made from specimen 1 of Fig. 2; Scale bars = 20 μm.

species identification is based only on morphology (e.g., Casu and Curini-Galletti, 2006; Meyer-Wachsmuth et al., 2014; Reyes and Brusa, 2017; Van Steenkiste et al., 2018). Even though the diagnostic characters of *M. johni* are present in the specimens studied here, the morphological differences in the genital system could indicate cryptic species. Similar hypotheses have been suggested for other groups of microturbellarians (e.g., Casu and Curini-Galletti, 2006; Reyes and Brusa, 2017).

4.2. *Genital morphology*

The morphology of the male copulatory organ remains the most important character in the current system of *Macrostomum* (Fang et al., 2016; Schärer et al., 2011; Sun et al., 2015). Although the significance of stylet variability is not yet known, stylet morphology could suggests the mating-behavior (Schärer et al., 2011). This study shows new features of *M. johni*, with emphasis on the muscular arrangement of the genital system. Pictures and a 3D model provide for the first time detailed information about the muscles of the female antrum, false seminal vesicle, seminal vesicle, vesicula granulorum and the sperm of squashed animals. Although copulation has not been described in *M. johni*, reciprocal mating-behavior can be inferred due to the morphology of the sperm and the stylet.

We identify that two different sets of muscles are associated with the stylet: ventral longitudinal muscles and circular muscles. A third set of helical muscles, related with the muscles of the prostatic vesicle, cover the proximal part of the stylet and could be responsible for the movement of the stylet. A recent study reported that the muscular organization in the stylet differs among three *Macrostomum* species regarding the presence and arrangement of different fiber sets (Adami et al., 2017). Circular and longitudinal muscles have been found in all species analyzed (*M. velastylum* Brusa 2006, *M. quiritium* Beklemischev, 1951 and *M. tuba* Graff, 1882); however, the third set of helical muscles, have only been observed in *M. velastylum* (Adami et al., 2017). Based on this interspecific variability in stylet muscular pattern, we hypothesize that the muscular pattern, in addition to stylet morphology, could be involved in different mating strategies.

Papi (1951) described the musculature of the *Axia gieysztori* (Ferguson, 1939) genital system and analyzed its specific function during copulation, recognizing five different sets of muscles associated with the stylet (ring muscles, depressor muscles, protractor muscles, retractor muscles and flexor muscles). One of these sets, the protractor muscles, has also been reported for *Macrostomum sensitivum* Silliman, 1884, *M. axi* Papi, 1959 and *M. dorsiforum* Beltagi, 1972 (Beltagi, 1972; Papi, 1951, 1959), among other species. In our study, the longitudinal and helical muscles associated with the stylet are similar to the retractor and protractor muscles reported by Papi (1951: fig. 52-53) whose function is to retain and move back the male channel; they also have the function of extending the copulatory organ increasing the angle between the stylet and the longitudinal axis of the vesicula granulorum. In *M. johni*, the musculature of the duct connecting the seminal vesicle to the false seminal vesicle shows circular muscular fibers similar to those described by Papi (1951) for *M. retortum* Papi, 1951.

The rostral chamber observed in the female antrum of *M. johni* has the cellular valve, where sperm anchor in the epithelium (Ladurner et al., 2005; Vizoso et al., 2010). Both chambers of the female antrum have radial muscular fibers associated with the body wall musculature being dilator muscles, also described in *M. hamatum* (Luther, 1947). These dilator muscle fibers probably play a

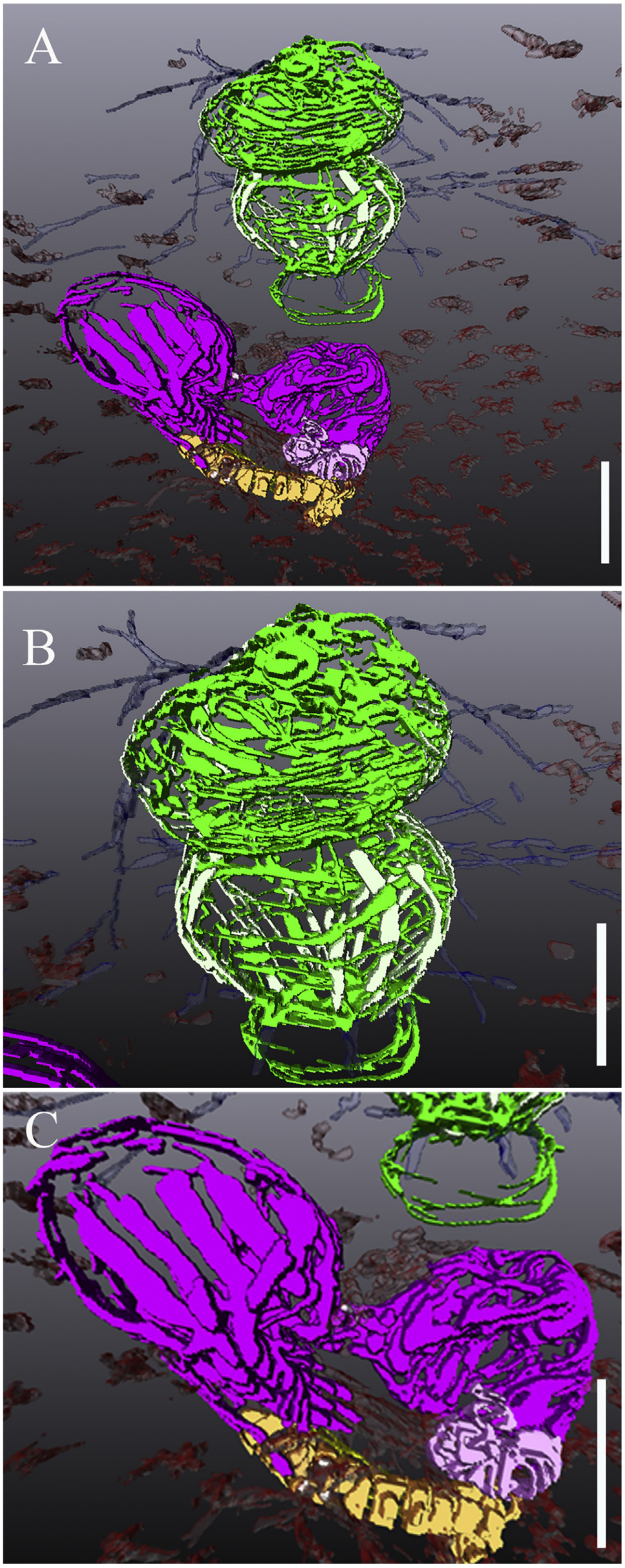

Fig. 4. Genital system 3D-reconstruction based on serial optical sections of a whole mount specimen at dorsal view. (A) General view, (B) detail of the female organs, (C) detail of the male organs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).
Color codes: reddish gray: dorso-ventral muscles of the body wall; blue: radial muscle fibers associated with the antrum and diagonal fibers of the body wall; green: circular muscles of the antrum; white: longitudinal muscles of the antrum; magenta: seminal vesicle and vesicula granulorum; lilac : duct that connect the seminal vesicle with the false seminal vesicle; beige: circular fibers of the stylet; yellow: longitudinal fibers of the stylet. Some transverse fibers and longitudinal muscles of the body wall are omitted for clarity. This reconstruction was made from specimen 1 of Fig. 2; Scale bars = 20 μm.

role in expanding the female antrum while the egg resides in the antrum.

In species of *Macrostomum* and in the free-living flatworms in general, the morphology of the genital organs is considered a key factor, not only for specific identification but also to infer mating-behavior (Schärer et al., 2011). Knowledge of the muscular pattern of the genital system might thus be useful in functional morphology studies, e.g., to analyze mating-behavior (Ramm et al., 2015; Vizoso et al., 2010). However, the muscular pattern of these organs has only been considered occasionally. We therefore believe that further detailed studies of the musculature associated with the genital organs could be consider together with the other genital traits currently considered in phylogenetic analyses (Janssen et al., 2015). The use of confocal microscopy together with 3D model reconstruction shows the detailed architecture of musculature of the genital organs in *Macrostomum johni*. Further information in this regard will provide new insights into the functional role of the genital muscular pattern in species with different mating-behaviors.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcz.2018.04.003.

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