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SCIENTIFIC NOTE

Morphological and morphometric study of pre-ovigerous and post-ovigerous adults of *Tanaisia (Paratanaisia) bragai* (Santos, 1934) (Digenea, Eucotylidae)

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Abstract. The aim of this study was to obtain data on the morphology and morphometry of pre-ovigerous and post-ovigerous adults of the species *Tanaisia (Paratanaisia) bragai*, using confocal laser scanning microscopy to obtain tomographic images of the suckers and tegument. For morphometric analysis, 45 specimens (30 pre-ovigerous adults and 15 post-ovigerous adults) were measured with the aid of an ocular micrometer coupled to the objective of a photonic microscope. Pre-ovigerous and post-ovigerous adult individuals, stained with Mair carmalumen and mounted in permanent preparations, were analyzed by means of confocal laser scanning microscopy. Positive correlation was detected between the body length and ovary length of post-ovigerous adults (rs: 0.774; $p<0.01$), as well as between the body length and testes (rs: 0.604 and 0.659; $p< 0.05$), the body length and the length of uterus (rs: 0.839; $p< 0,01$) and between the ovary width and egg length (rs: 0.777; $p<0.01$). Morphological study of the pre-ovigerous adults demonstrated that the ovary and testes develop simultaneously before the development of the uterus and vitelline glands. The acetabulum was detected in pre-ovigerous adults stained with hematoxilin and observed using light microscopy. In these specimens, the acetabulum measured 36.7 ± 6.9 µm (25-50 µm) in width and 39.91 ± 6.8 µm (25-55 µm) in length. The acetabulum was not detected in post-ovigerous adults observed with light microscopy. However, this structure was detected using confocal miscrocopy. In the post-ovigerous specimens, the acetabulum presented a reduced size compared to the pre-ovigerous adults. This may imply that this structure has more functional significance in the larval and pre-ovigerous stages.

Keywords: *Tanaisia*, confocal microscopy, pre-ovigerous adults, post-ovigerous adults.

The digenetic trematode *Tanaisia (Paratanaisia) bragai* is a parasite of domestic and wild birds and occurs in North, Central, and South America, as well as Oceania (Byrd and Denton 1950; Freitas 1951; Franco 1965; Federman *et al.* 1973; Carneiro *et al.* 1975; Keller and Araújo 1992).

The birds acquire the infection by eating land snails infected with metacercariae, which break out of their cysts in the duodenum of the definitive host, migrate to the cloaca and urinary tract, and reach the collector ducts of the kidneys, their final habitat (Maldonado 1945; Keller and Araújo 1992).

In Brazil, studies concerning this trematode have focused on taxonomy, life cycle, pathology in the definitive host, occurrence and prevalence. *Tanaisia bragai* was reported infecting *Columba livia* Gmelin, 1789; *Gallus gallus domesticus* (Linnaeus, 1758); *Odontophorus capueira* Cory, 1915; *Meleagris gallopavo* Linnaeus, 1758 and *Columbina talpacoti* Temmink, 1810 (Santos, 1934; Pinto *et al.* 2004; Franco 1965)

Keller and Araújo (1992) studied the life cycle of *T. bragai*, under laboratory conditions, infecting the land snail *Leptinaria unilamellata* (d'Orbigny, 1835), and Brandolini *et al.* (1997) carried out experimental infection of *Subulina octona* (Bruguière, 1789), the intermediate host first reported by Maldonado (1945).

Portugal *et al.* (1972), as well as Pinto *et al.* (2004), described the pathology caused by *T. bragai* in *C. livia* and *C. talpacoti,* respectively. Freitas (1951) and Franco (1965) published taxonomic studies on this species, and Silva *et al*. (1990) registered the prevalence of *T. bragai* in *C. livia*.

Tanaisia (Paratanaisia) bragai was described by Santos (1934) from specimens recovered from *C. livia* and *G. domesticus*. The life cycle of this species was elucidated approximately ten years later by Maldonado (1945), who described the morphology of all life cycle stages and observed, for the first time, the acetabulum in adult individuals.

The acetabulum is barely detectable in ovigerous individuals, but the presence of this structure in the early developmental stages, as described by Maldonado (1945), led to discussions concerning the taxonomic and phylogenetic position of the eucotylids as well as the validity of the Orders Monostomata and Distomata (Stunkard, 1946).

Maldonado (1945) pointed out that basing the taxonomy entirely in the adult characteristics creates a difficulty for the elucidation of the systematic position of the eucotylid species. Therefore, studies concerning the morphology of the various developmental stages of the eucotylid species are highly desirable, contributing to the understanding of their systematic position and relationships with other digenetic families.

The aim of this study was to describe the morphology and morphometry of pre-ovigerous and post-ovigerous adults of the species *Tanaisia*

(Paratanaisia) bragai, using confocal laser scanning microscopy to obtain tomographic images of the suckers and tegument.

For the morphometric analysis, 45 specimens (30 pre-ovigerous adults and 15 post-ovigerous adults) were collected from an infrapopulation obtained from *C. livia*, naturally infected, in the municipality of Juiz de Fora, Minas Gerais, Brazil. The terminology adopted to assign the two life stages studied was that used by SEBELOVÁ *et al.* (2004).

The parasites were recovered from the kidney collector ducts of *C. livia*. Following necropsy of the host, the specimens were collected and prepared according to the conventional helminthological techniques, following the protocols proposed by AMATO *et al.* (1991). The helminths were stained with Mayer's carmalumen according to the regressive technique proposed by AMATO *et al.* (1991) and mounted on permanent slides. These slides were then observed through a light field microscope and through a confocal scanning laser microscope (LSM 510 META - laser He/Ne 543, filter LP560, Zeiss) at the Pathology Laboratory of Oswaldo Cruz Institute. Spearman correlation coefficient ($p<0.05$) was calculated to determine the relationships between the morphometric data of the structures analyzed.

The data obtained from morphometric analysis of the pre-ovigerous and post-ovigerous adults are presented in the Tables 1-2. Positive correlation was detected between the body length and the length of the ovary (rs: 0.774), testes (rs: 0.604 and 0.659) and the uterus (rs: 0.839) of the post-ovigerous adults. There was a significant positive relationship (rs: 0.777; $p<0.01$) between the ovary width and the egg length.

Table 1. Morphometry of pre-ovigerous adults of the species Tanaisia (Paratanaisia) bragai (Santos, 1934).

	Amplitude	Mean ± standard deviation
Body width	91.6 - 215µm	131.81 ± 27.9 µm
Body length	530 - 1000µm	736 ± 119.5 µm
Oral sucker width	70 - 130µm	90.91 ± 13.17µm
Oral sucker length	50 - 625µm	95 ± 101µm
Acetabulum width	25 - 50µm	36.7 ± 6.9µm
Acetabulum length	25 +/- 55µm	39.91 ± 6.8µm
Pharynx width	30 - 125µm	52.67 ± 20.51µm
Pharynx length	27.5 - 75µm	41.63 ± 11.3µm
Intestinal caeca width	10 - 100µm	23.31 ± 19.4µm
Intestinal caeca length	187.5 - 1450µm	1048.9 ± 320.1µm
Ovary width	30 - 105µm	55.9 ± 18.5µm
Ovary length	32.5 - 175µm	100.4 ± 32.8µm
Right testicle width	25 +/- 55µm	35.1 ± 9.5µm
Right testicle length	25 - 75µm	49.1 ± 17.2µm

Table 1. Continuation.

Left testicle width	22.5 - 55µm	35.05 ± 9.8µm
Left testicle length	25 - 110µm	51.04 ± 20.4µm
Distance between intestinal caeca and posterior margin of the body	72.5 +/- 182.5µm	108.17 ± 24.8µm
Excretory canal length	12.5 +/- 55µm	28.2 ± 14.2µm
Uterus width	15 +/- 30µm	22.58 ± 4.3µm
Uterus length	35 +/- 400µm	196.5 ± 105.3µm

Table 2. Morphometry of post-ovigerous adults of the species *Tanaisia (Paratanaisia) bragai* (Santos, 1934).

	Amplitude	Mean ± standard deviation
Body width	191.6 - 350µm	275.7 ± 49 µm
Body length	1675 - 2250 µm	1879 ± 186 µm
Oral sucker width	77.5 - 190 µm	145.7 ± 30.8 µm
Oral sucker length	75 - 155 µm	137 ± 23.59 µm
Pharynx width	55 - 85 µm	71 ± 11 µm
Pharynx length	40 - 75 µm	57.7 ± 11.3 µm
Ovary width	85 - 220 µm	138.4 ± 41.9 µm
Ovary length	165 - 210 µm	183.18 ± 14.87 µm
Right testicle width	50 - 125 µm	81.5 ± 22.9 µm
Right testicle length	65 - 190 µm	125 ± 30.9 µm
Left testicle width	50 - 115 µm	70.3 ± 20.9 µm
Left testicle length	75 - 180 µm	124.3 ± 31 µm
Egg width	16.1 - 20 µm	18.72 ± 1.23 µm
Egg length	31.7 - 37.5 µm	34.72 ± 1.5 µm
Rigth vitelline gland width	15 - 47.5 µm	30.6 ± 9.8 µm
Rigth vitelline gland length	365 - 1225 µm	882 ± 241.8 µm
Left vitelline gland width	15 - 45 µm	30.6 ± 8.9 µm
Left vitelline gland length	360 - 1126 µm	888.1 ± 229.7 µm
Distance between intestinal caeca and posterior margin of the body	200 - 360 µm	252.1 ± 56.1 µm
Uterus width	175 - 263.3 µm	216.6 ± 28.9 µm
Uterus length	1225 - 1975 µm	1599 ± 212.6 µm

Figure 1-A shows the arrangement of the organs in the reproductive tract of post-ovigerous adult. In this stage, the uterus is totally formed and full of eggs. The vitelline glands are also totally formed and the ovary and testes are segregated. Study of the pre-ovigerous adult morphology demonstrated that the ovary and testes develop simultaneously before the development of the uterus and vitelline glands (Figure 1-B). The acetabulum was detected in

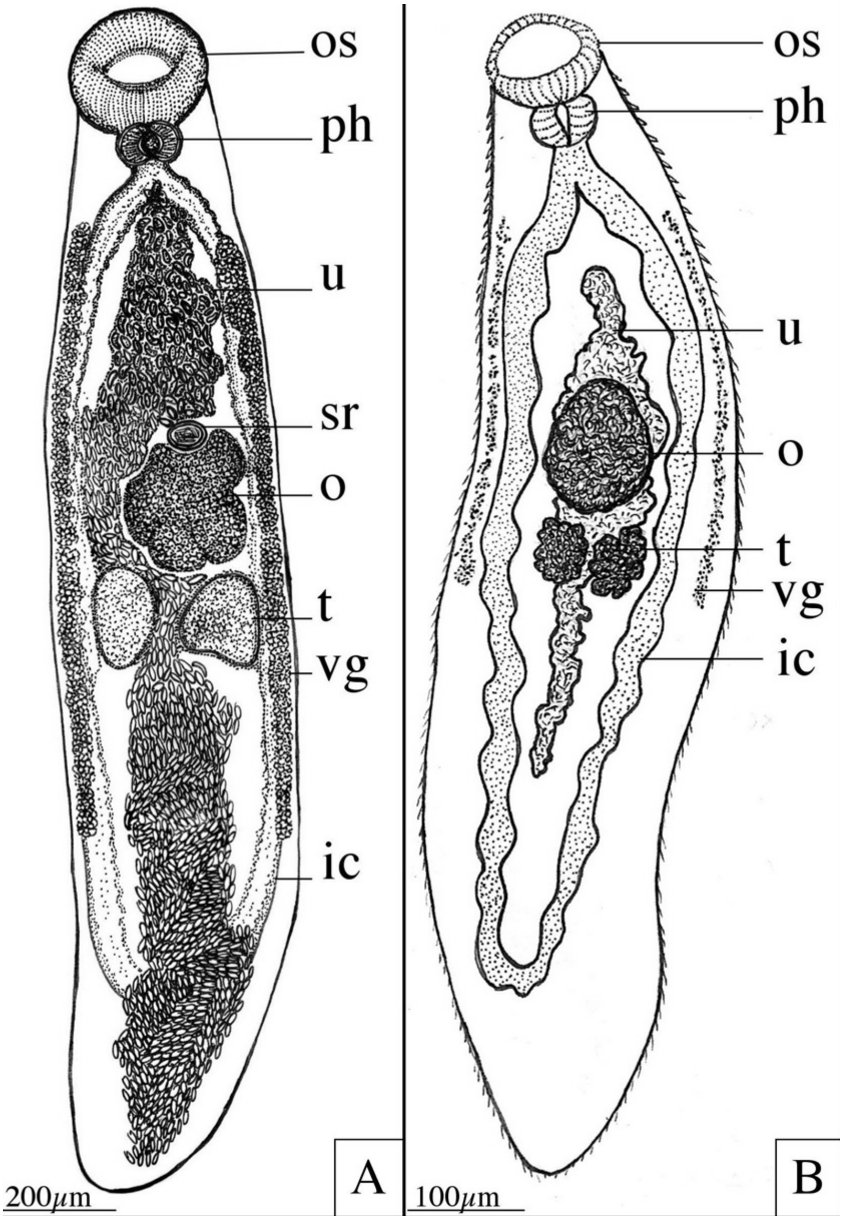

Figure 1. Post-ovigerous (A) and pre-ovigerous adults (B) of the trematode species *Tanaisia (Paratanaisia) bragai* (Santos, 1934) (Digenea, Eucotylidae). os: oral sucker; ph: pharynx; u: uterus; sr: seminal receptacle; o: ovary; t: testis; vg: vitelline gland; ic: intestinal caeca.

pre-ovigerous adults observed by means of light microscopy. In these specimens, the acetabulum measured 36.7 ± 6.9 µm (25-50 µm) in width and 39.91 ± 6.8 µm (25-55 µm) in length. Although the acetabulum was not observed in post-ovigerous adults using light microscopy, it was detected using confocal microscopy. In the post-ovigerous specimens, the acetabulum presented a reduced size, when compared to the pre-ovigerous adults. The tomographic images obtained are shown in Figure 2.

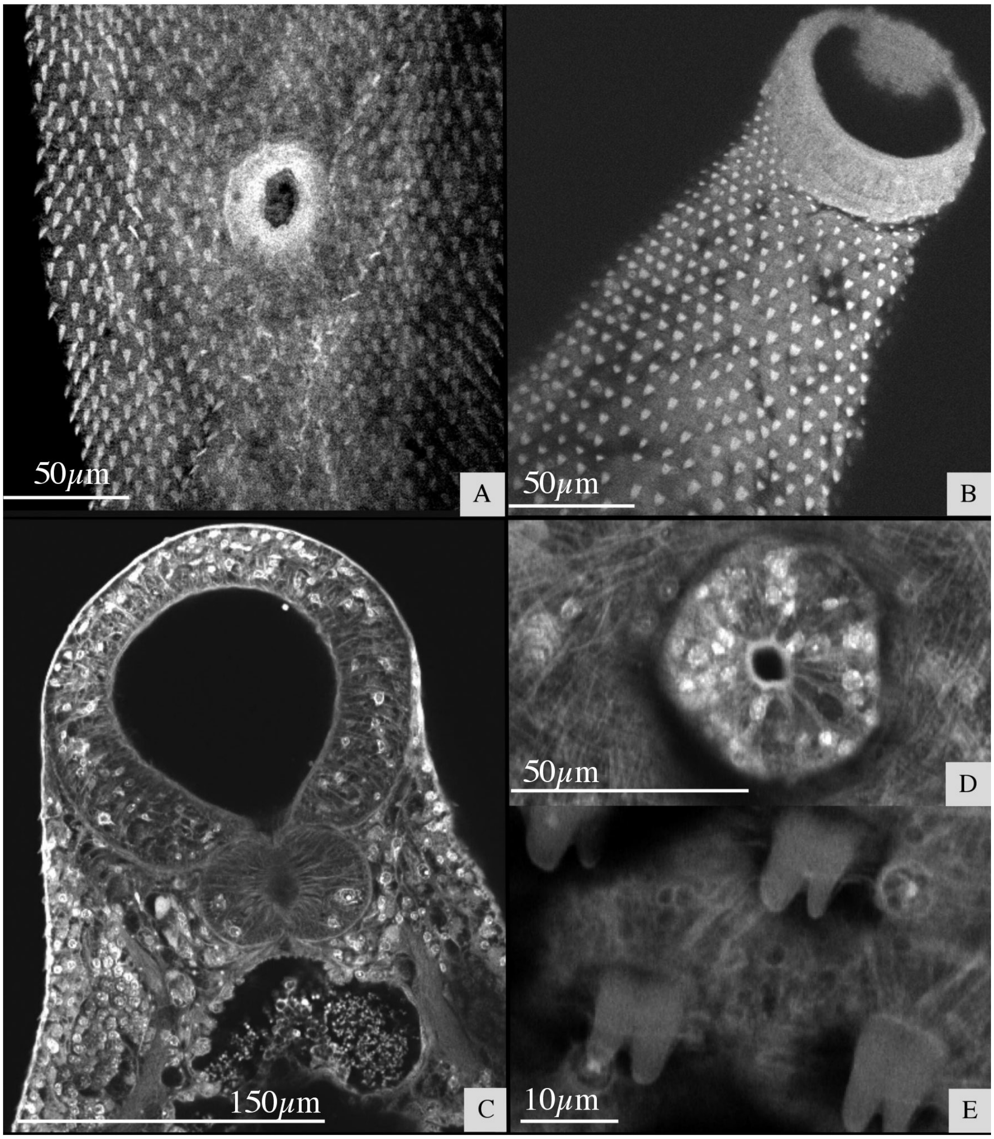

Figure 2. Confocal tomographic images of post-ovigerous and pre-ovigerous adults of the trematode species *Tanaisia (Paratanaisia) bragai* (Santos, 1934) (Digenea, Eucotylidae). A. Tegument covered with scales and acetabulum of pre-ovigerous adults. B. oral sucker of pre-ovigerous adults. C. oral sucker of post-ovigerous adults. D. acetabulum of post-ovigerous adults. E. scales of post-ovigerous adults.

With confocal laser microscopy, it was possible to observe the scales that cover the tegument. Pre-ovigerous adults presented scales with finer distal portion compared to those of post-ovigerous individuals.

In the present study, the positive relationship between body length and the length of the eggs and the organs of the reproductive tract may be a consequence of indeterminate growth, in which the development of the reproductive tract follows the body growth and the consequent increase in space and energetic reserves (Cichon 1999). However, there are no studies concerning growth strategy in digeneans.

The reduced size of the acetabulum observed in the post-ovigerous specimens may imply that this structure has more functional significance in larval and pre-ovigerous stages.

These results agree with the observations of Maldonado (1945) who reported that, during the development of *T. bragai* from metacercaria to the adult stage, growth of the acetabulum does not follow that of the organs and of the body length. In his study on the life cycle of *T. bragai*, Maldonado (1945) observed that the suckers were the first structures to be formed in the cercariae. The acetabulum appears as a prominent structure in the middle of the body.

According to this author, when the cercaria is fully formed, the acetabulum is globular and prominent, measuring 25µm in diameter. In the metacercariae, the acetabulum is globular and strongly muscular, almost as wide as the oral sucker. A compact set of cells located in the same region of the acetabulum constitutes the genital primordium. When *T. bragai* reaches the definitive host, the size of the body and arrangement of the organs are practically identical to that of metacercariae. The first notable structural modifications described by Maldonado (1945) were the segregation of the ovary and, later, the segregation of the testes and appearance of the uterus and vitelline glands.

Maldonado (1945) observed for the first time the presence of acetabulum in larval forms and pre-ovigerous adults of *T. bragai*. This finding was confirmed by Stunkard (1945). Afterward, Dollfus (1946) registered the presence of acetabulum in *T. gallica* Dollfuss, 1946 (= *T. zarudnyi*). According to him, in post-ovigerous adults the acetabulum is undetectable. Dollfus (1946) included *T. bragai* among the species without an acetabulum, but mentioned the studies performed by Maldonado (1945) and Stunkard (1945). The finding of an acetabulum in *T. zarudnyi* (Skrjabin, 1924) and *T. bragai* led to questions regarding the systematic classification of these trematodes as monostomes and to discussions about its phylogenetic position. Byrd and Denton (1950), based on the observations made by Maldonado (1945) and Dollfus (1946), as well as on their own studies, questioned the validity of the classification of the digenetic trematodes in Monostomata and Distomata. They stated that the presence of acetabulum in the adult forms is not sufficient to infer taxonomic relationships.

Franco (1965) recorded the occurrence of *T. bragai*, *T. inopina* Freitas, 1951 and *T. augusta* Franco, 1965 in Brazil. He did not observe the presence of acetabulum in these species. Keller and Araújo (1992) described the morphology of larval forms and adults of *T. bragai*, but did not mention the presence of acetabulum in the species. Freitas (1951) observed the acetabulum in *T. bragai*, *T. incerta* Freitas, 1951, *T. minax* Freitas, 1951, *T. magnicola* Freitas, 1951, *T. confusa* Freitas, 1951, and *T. robusta* Freitas, 1951, describing the acetabulum as a superficial, weakly muscular structure, perceptible only in a few species. The diagnosis of Eucotylidae proposed by this author included a new character: "acetabulum present or absent". The diagnosis of the subfamily Tanaisinae included "acetabulum generally present, weakly developed". Freitas (1951) also questioned the inclusion of Eucotylidae in Monostomata, pointing to the lack of information for the elucidation of the phylogenetic affinities between Eucotylidae and the other digenetic trematodes. Morphometric data provided by various authors regarding the acetabulum of the *Tanaisia* species is shown in Table 3. Morphometric data related to *T. bragai* supplied by Santos (1934) is presented in Table 4.

Table 3. Morphometry of acetabulum of Tanaisia species.

	Dimensions of the acetabulum	Autors
T. bragai (Santos, 1934)	30 a 100µm x 30 a 80µm	Freitas (1951)
T. zarudnyi (Skrjabin, 1924)	40 a 44µm in diameter 120 a 127µm in diameter	Byrd and Denton (1950) Dollfus (1946)
T. fedtschenkoi Skrjabin, 1924	54 a 84µm in diameter	Byrd and Denton (1950)
T. incerta Freitas, 1951	29 a 34µm in diameter	Freitas (1951)
T. minax Freitas, 1951	70 a 100µm in diameter	Freitas (1951)
T. magnicolica Freitas, 1951	20 a 40µm X 20 a 50µm	Freitas (1951)
T. confusa Freitas, 1951	50µm in diameter	Freitas (1951)
T. robusta Freitas, 1951	60 a 70µm X 60 a 100µm	Freitas (1951)

Table 4. Morphometry of Tanaisia (Paratanaisia) bragai, given by Santos (1934).

Body width	3.3 mm
Body length	0.66mm
Oral sucker width	0.41 mm
Oral sucker length	0.5mm
Pharynx diameter	0.16 mm
Ovary width	0.15 mm

Table 4. Continuation.

Ovary length	0.22 mm
Right testicle length	0.5 mm
Left testicle width	0.5 mm
Right testicle length	0.35 mm
Left testicle width	0.47 mm
Distance between viteline glands and anterior margin of the body	0.32 mm
Distance between viteline glands and posterior margin of the body	0.32 mm
Egg length and egg width	0.031 x 0.013 mm

In early classifications proposed for Digenea, the attachment apparatus was considered to be an important taxonomic character. However, with the progressive accumulation of studies covering all developmental stages of trematode species, the taxonomic unities based on the attachment structures became obsolete. It became evident that Distomata and Monostomata were polyphyletic groups (Stunkard, 1946).

Currently, the classification of the digenetic trematodes into Monostomata or Distomata is no longer used. As early as the 1940s, Stunkard (1946) pointed to the need for a transition from the artificial classification system, predominant at that time, to a natural system, which would express the phylogenetic affinities between the digenetic trematode species. Recently, morphological characters and nucleotide sequences are used to infer phylogenetic relationships between Digenea and other Platyhelminthes clades, as well as internal relationships in Digenea (Barker *et al*. 1993; Blair 1993; Blair and Barker 1993; Brooks and McLennan 1993; Johnston *et al.* 1993). Despite these efforts, there is insufficient resolution concerning the internal phylogeny affinities of Digenea.

Maldonado (1945) and Byrd and Denton (1950) had pointed out the need for more studies on anatomy and life cycle of *Tanaisia* species. However, little information obtained from modern microscopy techniques had been added until today (D`Ávila *et al*. 2010).

New microscope techniques have made possible great advances in studies of the morphology of various species of digenetic trematodes (Robson and Erasmus, 1970; Mair *et al*., 1998a,b and 2000; Abdul-Salam and Sreelatha, 2000, 2004; Stewart *et al*., 2003; Halton, 2004; Neves *et al*., 2004, 2005). Confocal scanning laser microscopy has become an important tool to clarify the organization of the musculature and innervation, as well as the morphology of the reproductive and alimentary tract of various species (Mair *et al.* 1998a, b, 2000; NEVES *et al.,* 2004, 2005; STEWART *et al*., 2003).

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