



A new morphological analysis of eggs and miracidia of *Paratanaisia bragai* (Digenea: Eucotylidae)

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Abstract

Paratanaisia bragai is a trematode that reaches sexual maturity in the kidney collecting ducts of domestic and wild birds, while the gastropods *Subulina octona* and *Leptinaria unilamellata* serve as its intermediate hosts. The morphology of *P. bragai* eggs is described here for the first time, using light, scanning and transmission electron microscopy. Embryonic eggs, obtained from adult worms collected from naturally infected definitive hosts, appeared elliptical, with an evident operculum, by all three microscopic techniques employed. Scanning electron microscopy revealed that the abopercular region presents a prominent U-shaped knob, which stands out from the egg surface. The thickness of the eggs, measured from ultrathin sections, were $0.34 \pm 0.06 \mu\text{m}$ (0.29–0.50) at the ends and $0.31 \pm 0.05 \mu\text{m}$ (0.24–0.36) in the middle region. The internal shell layer was more electron-dense and thinner than the middle layer, which comprised nearly the entire shell, while the external layer was less electron-dense and thinner than the internal layer. Rupture of the eggs, by the coverslip pressure, revealed the miracidia of *P. bragai* that was characterized by light and transmission electron microscopy.

Keywords *Paratanaisia bragai* · Eggs · Microscopy · Miracidium · Larval trematode · Ultrastructure

Introduction

Paratanaisia bragai (Santos, 1934) Freitas, 1959 is a trematode that reaches sexual maturity in the kidney collecting ducts of domestic and wild birds. For larval development, it mainly uses the snail *Subulina octona* (Bruguière, 1798), although *Leptinaria unilamellata* (d'Orbigny, 1837) (Mollusca, Gastropoda, Pulmonata) has also been reported as an intermediate host in Brazil (Brandolini et al. 1997).

The embryonated eggs are eliminated with the excretion products of the definitive hosts and the snail becomes passively infected. After the miracidia hatching, two generations of sporocysts develop inside the snail. The cercariae and metacercariae begin to be visualized 32 days after infection. The definitive host becomes infected by ingesting the parasitized snail (Maldonado 1945; Keller and Araujo 1992; Brandolini and Amato 2006).

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Infection by *P. bragai* is common in domestic birds in the state of Rio de Janeiro (Gomes et al. 2005). However, Unwin et al. (2013) recorded the infection by this parasite in birds from South, Central and North America, Philippines, and India. Wild birds infected with *P. bragai* probably act as reservoirs, because they often come into proximity by eating the same food as domestic birds kept outdoors (Gomes et al. 2005; Luppi et al. 2007). Migration of wild birds infected by trematodes and animal trafficking can also spread helminths to new areas, representing a large risk of native hosts being exposed to infection (Huffman 2008).

There is a controversy about the pathogenicity of *P. bragai*. There are records of infection in birds *Columba livia* Gmelin, 1789, and native birds *Columbina talpacoti* (Temminck, 1811) and *Ara ararauna* (Linnaeus, 1758), resulting in pathological alterations varied ranging from low, medium and high pathogenicity with pathological lesions (Pinto et al. 2004; Xavier et al. 2015; Silva et al. 2016). A high parasite load can cause development of renal monostomosis with mucoid, blood, diarrhea, even death in pigeon (*Columba inornata wetmorei*) (Arnizaut et al. 1992). Thus, this parasite has veterinary and economic importance for wild and domestic birds, due to its great geographic distribution and to serious lesions or death to the host.

Brandolini and Amato (2006; 2007) performed histological analyzes of the larval stages of *P. bragai* and characterized the external surface of adult worm by scanning electron microscopy. D'ávila et al. (2010, 2017) analyzed the anatomy of the musculature, reproductive system and morphology of adults of *P. bragai* through a confocal scanning laser microscope. However, studies of *P. bragai* eggs and miracidia are still scarce, particularly by electron microscopy techniques. Due to the lack of detailed descriptions of the morphological aspects of *P. bragai* eggs, this article described important structural aspects characteristic of these eggs and miracidia based on different microscopic techniques.

Materials and methods

Adult birds (*C. livia*) were collected in the Irajá district of the city of Rio de Janeiro (22°49'51" S and 43°20'17" W) and were transported to the Helminth Biology and Ecology Laboratory of the Animal Biology Department, UFRRJ, in Seropédica city. Birds were subjected to fecal examination by Ritchie's method, in which the feces were homogenized in 10% formaldehyde, in a Borel bottle, strained, centrifuged (500 × g, 10 min) with disposal of supernatant. The clean pellet is resuspended in ether and shaken vigorously. The content is centrifuged the sediment containing the eggs was analyzed to detect infection of pigeons (De Carli 1994). Three birds (two infected and one uninfected) were euthanized in a CO₂ chamber and kidneys removed. Kidneys were placed in

Petri dishes containing saline solution (0.85% NaCl) and were sectioned using scalpel. After kidney sectioning, adult helminths were recovered and transferred to another Petri dish containing saline solution, where they remained for 24 hours at room temperature for eggs release. As the number of spontaneously eggs release was small, some parasites were opened to release uterine eggs (Brandolini and Amato 2006). The embryonated eggs containing the miracidium inside were fixed with 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) at 4 °C for 24 hours.

Light microscopy (LM) The embryonated *P. bragai* eggs, fixed as described above, were mounted between slides and cover-slips using the fixation agent as mounting medium. Eggs were observed under an Olympus BX51 light microscope using bright field and differential interference contrast (DIC). Images were captured with an Olympus DP 12 digital camera, using iTEM software, for Windows. Eggs were drawn under a stereomicroscope coupled to a bright-field chamber, and their dimensions (length and width) were measured in the drawings ($n = 90$ eggs). Results are given as means and standard deviation with maximum and minimum values recorded.

Scanning electron microscopy (SEM) Fixed embryonated eggs were washed with cacodylate buffer, post-fixed in 1.0% osmium tetroxide and 0.8% potassium ferrocyanide in cacodylate buffer for 1 hour and washed again. Eggs were dehydrated in an ascending ethanol series (30–100%) for 1 hour in each solution and dried in a critical point chamber using CO₂ (Baltec CPD). The processed eggs were then mounted on metallic stubs and gold-coated 50 seconds to obtain a 20 nm thick layer for observation under a Quanta FEI 250 scanning electron microscope operating at 20 kV (Pinheiro et al. 2004).

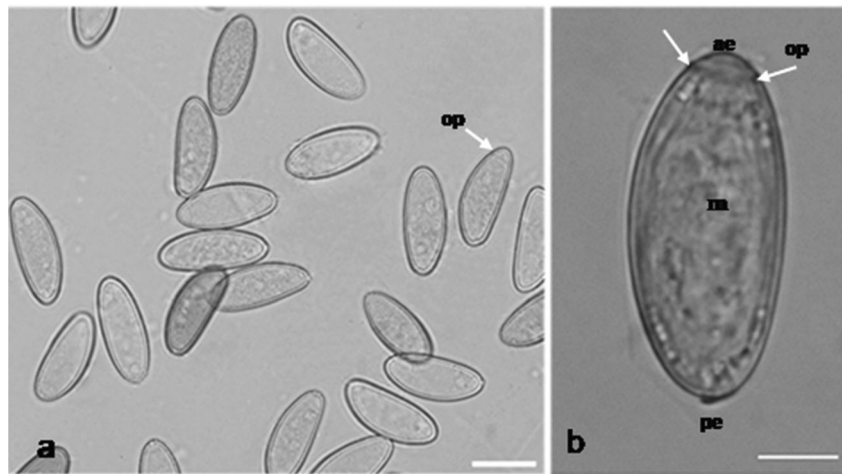
Transmission electron microscopy (TEM) Embryonated eggs were fixed and post-fixed as described above. Eggs were dehydrated in an ascending acetone series and embedded in epoxy resin (Embed 812). Ultrathin sections were obtained with a Leica Reichert Ultracut microtome and contrasted with 5% uranyl acetate and 1% lead citrate, for observation under a Zeiss 1200 transmission electron microscope operating at 80 kV. The images were obtained using the iTEM software, Olympus (De Souza 2007).

Results

Eggs

Light microscopy Eggs are elliptical with coloring varying from pale yellow to dark brown. It was possible to observe the fully developed miracidium inside the egg, and the operculum, at the lowest and highest magnifications (Figs. 1a, b).

Fig. 1 Light microscopy of the eggs of *Paratanaisia bragai*. **a** General view of the eggs and opercular region (op). Scale bar 20 μm . **b** Egg with miracidium (m) inside, showing the anterior end (ae), opercular region (op) and posterior end (pe). The insertion point of the operculum in the egg is indicated by the arrows. Scale bar 7 μm



The eggs had mean length about twice as large as the width (1:2.28), evidencing the elliptical shape. The mean length of the operculum was nearly the same as the width, resulting in a semi-spherical shape (Table 1).

Scanning electron microscopy – A cluster of eggs were observed (Fig. 2a) in one specimen that broke during processing, allowing their observation while still inside the adult parasite’s body. Individual eggs were also examined to allow a better description of their morphological features. By SEM it was possible to confirm that the eggs are elliptical (Fig. 2b). In the opercular region, the line between the operculum and eggs body is easily seen (Figs. 2c, d), with the operculum considered to be the anterior end of the egg. It was also possible to observe the abopercular region at the posterior end. In the abopercular region there is a prominent U-shaped knob that stands out from the egg surface (Figs. 2d, e). It was also possible to observe that the *P. bragai* eggs have a rough surface (Fig. 2f). Figure 2g shows an egg without the operculum, while still inside the adult parasite’s body.

Transmission electron microscopy Observation of ultrathin sections of *P. bragai* eggs revealed the insertion line of the operculum and eggs body, which is very thick, measuring $0.34 \pm 0.06 \mu\text{m}$ (0.29–0.50) at the ends and $0.31 \pm 0.05 \mu\text{m}$

(0.24–0.36) in the middle region. Figure 3a confirms the semi spherical shape of the egg. The operculum is located at the anterior end of the egg, measuring 1.13 μm height from the suture line to the top.

The eggshell has three layers: a thinner and more electron-dense inner layer with thickness of $0.03 \pm 0.01 \mu\text{m}$ (0.05–0.01) at the ends and $0.02 \pm 0.01 \mu\text{m}$ (0.05–0.01) in the middle region; the middle layer is less electron-dense than the inner layer, composing most of the eggshell, measuring $0.24 \pm 0.04 \mu\text{m}$ (0.35–0.19) at the ends and $0.21 \pm 0.05 \mu\text{m}$ (0.29–0.12) in the middle region; and the outer layer is less electron-dense than the inner layer, with thickness of $0.09 \pm 0.03 \mu\text{m}$ (0.15–0.06) on the lateral region near to the ends and $0.08 \pm 0.02 \mu\text{m}$ (0.13–0.04) in the middle region (Fig. 3a; Table 2).

Observation of ultrathin sections of the adult worm enabled visualizing the egg attached to a structure, possibly the ootype, due to the position of the section in the parasite (Figs. 3b–e). Figure 3e shows the positioning of the egg with the anterior end, where an operculum is seen, and the posterior end region inserted in the adult worm body, reinforcing that this is the ootype region.

Miracidium

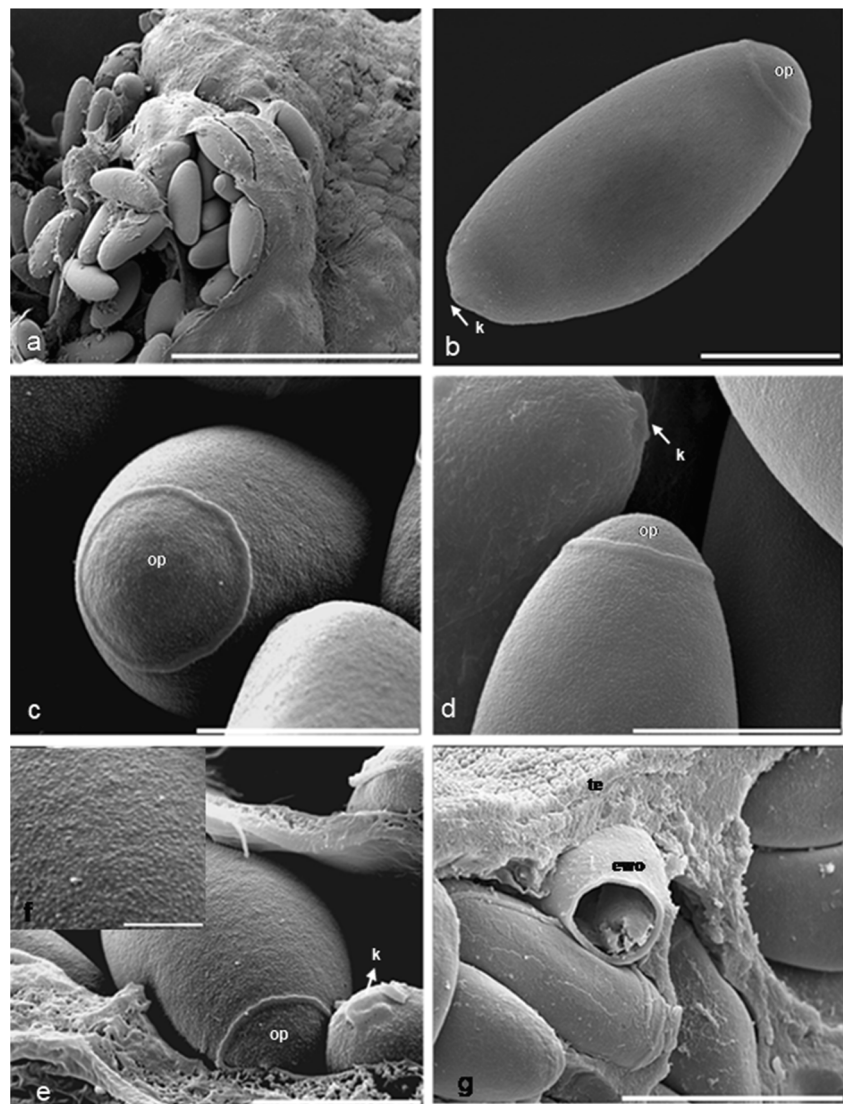
Light microscopy – It was possible to observe that the miracidium is elongated, with length of 29.0 μm , width of 3.9 μm at the anterior end, 12.8 μm in the middle region and 5.8 μm at the posterior end of the body. With the rupture of the eggshell, produced by the slipcover pressure, it was possible to observe the miracidium still inside the egg (Fig. 4a). Employing DIC it was possible to visualize the anterior end with the presence of an extroverted terebratorium without cilia and a clearly line dividing the terebratorium region from the ciliated body of the larvae (Fig. 4b). The posterior end of the body is more rounded, without an elliptical shape as those of the anterior end, and the larval body was covered by cilia (Fig. 4b). The observation

Table 1 Measurements (mean \pm standard deviation), of the egg and operculum of *Paratanaisia bragai*.

	Egg	Operculum
Length (μm)	41.83 ± 2.74 (35.06–49.06)	7.49 ± 0.75 (6.59–8.24)
Width (μm)	18.33 ± 1.55 (13.79–22.49)	7.11 ± 0.90 (5.76–7.80)

Numbers in parentheses represent the minimum and maximum values observed

Fig. 2 Eggs of *Paratanaisia bragai* observed by scanning electron microscopy. **a** General view of a set of eggs inside the adult parasite after rupture. Scale bar 100 μ m. **b** Opercular region (op) and abopercular region with a knob (k). Scale bar 10 μ m. **c** Top view of the opercular region (op). Scale bar 10 μ m. **d** Side view of the opercular region (op) and knob (k) of the abopercular region. Scale bar 10 μ m. **e** Side view of the opercular region (op) and top view of the knob (k) in the abopercular region. Scale bar 10 μ m. **f** Detail of the egg surface, showing the rough appearance. Scale bar 10 μ m. **g** Eggs inside the parasite, with detail of tegument (te), showing an egg without operculum (ewo). Scale bar 20 μ m



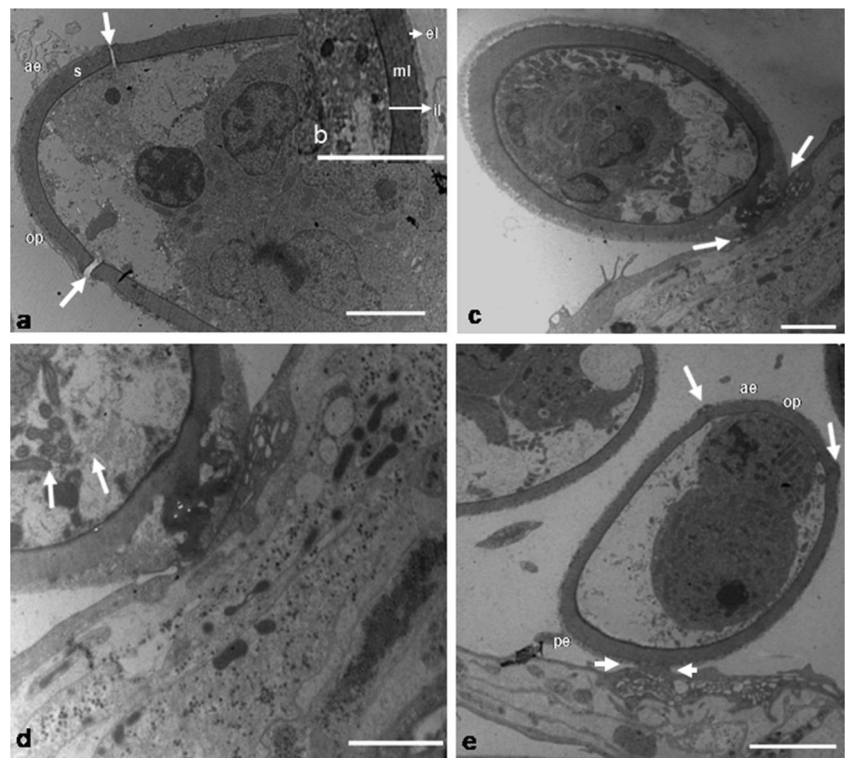
of miracidia using DIC did not evidence the presence of eyespots. Transmission electron microscopy allowed visualization of the germinal cells of the miracidium inside the egg (Figs. 4c, d).

Discussion

Veterinary importance of *P. bragai* result from the economic losses imposed by infection may results in serious lesions or death of the host (Unwin et al. 2013; Bunbury et al. 2008; Arnizaut et al. 1992). There are few reports in the literature regarding the eggs of *P. bragai*, especially those using electron microscopic techniques (Brandolini and Amato 2006). Therefore, this paper presents, by the first time, morphological details of this parasite's eggs and miracidium.

The difference in color of the eggs when observed by light microscopy is related to the tanning process of the egg proteins, since phenolic compounds are oxidized to quinones and transformed into scleroproteins, process that begins in the anterior region of the uterus and along the passage of the egg through the uterus, its shell hardens and darkens (Stephenson 1947; Smyth and Clegg 1959; Rey 2008). In this way the darker colored eggs are eggs in a more advanced stage of development and are fulfilled in the terminal portions of the uterus, while the pale yellow eggs are found in the initial regions of the uterus. Scanning electron microscopy enabled observation of a prominent U-shaped knob on the egg surface in the abopercular region, located at the posterior end of the egg, i.e., the opposite region of the operculum. Krejci and Fried (1994) described the abopercular region, where they proposed that the knob at the abopercular end of the eggs of *Echinostoma Rudolphi*, 1809 is a structure

Fig. 3 Eggs of *Paratanaisia bragai*. **a** Egg showing its shell (s) and anterior end (ae) and opercular region (op). The insertion point of the operculum in the egg is indicated by the arrows. Scale bar 1 μ m. **b** Detail of the egg shell layers. External layer (el), middle layer (ml), which is thicker, and internal layer (il). Scale bar 2 μ m. **c** Egg fixed in the adult tissue, indicated by the arrows. Scale bar 2 μ m. **d** Detail of two fixation points, indicated by the arrows. Scale bar 2 μ m. **e** Egg showing the opercular region (op) and insertion point of the operculum in the egg (arrows) in the anterior end (ae). Posterior end (pe) region with a fixation point in the tissue, indicated by the small arrows. Scale bar 5 μ m



related to the attachment point of the developing egg to the adult worm's ootype.

In the present study, when examining ultrathin sections from the parasite's posterior region, eggs were attached by their posterior end to a structure that appeared to be the ootype. According to Freitas (1951), the ovary of *P. bragai* is pre-testicular and shifted laterally; in addition, it is located in the middle third of the body. The testicles are located in the middle of the parasite's body and the acetabulum is situated in the middle third of the body (Byrd and Denton 1950). When the eggs leave the ovary, they pass through the oviduct to the ootype and then go to the uterus (Rey 2008). The oviduct originates dorsally of the ovary, passing poster-laterally to form the ootype (Stunkard 1945; Byrd and Denton 1950). Therefore, the sections of the parasite's hindbody, just after

the acetabulum, include regions of the male and female genital apparatus of *P. bragai*. From these observations, it can be assumed that the structure in which the eggs are attached is the ootype, where, according to Rey (2008), the eggs are shaped and receive yolk cells.

The topography of the eggshell of *Echinostoma caproni* Richard, 1964 and *Echinostoma trivolvis* (Cort, 1914) were compared by Krejci and Fried (1994). The authors stated that the knob of *E. trivolvis* eggs contains deep invaginations with folds in the shell, while the eggshell of *E. caproni* only has superficial folds.

Fujino et al. (2000) reported similar characteristics for *E. caproni* eggs, in abopercular region. According to Fujino et al. (2000), in *Echinostoma paraensei* Lie & Basch, 1967 the abopercular region of the egg has deep wrinkles, while Pinheiro (2003) found in the same region a knob, folds and invaginations. However, no folds in the knob at the abopercular of the *P. bragai* eggs were observed. There is a report of enlargement of the abopercular region in the eggs of the trematodes *Paragonimus westermani* Kerbert, 1878) and *Paragonimus uterobilateralis* Voelker and Voget, 1965 (Acha and Szyres 2003).

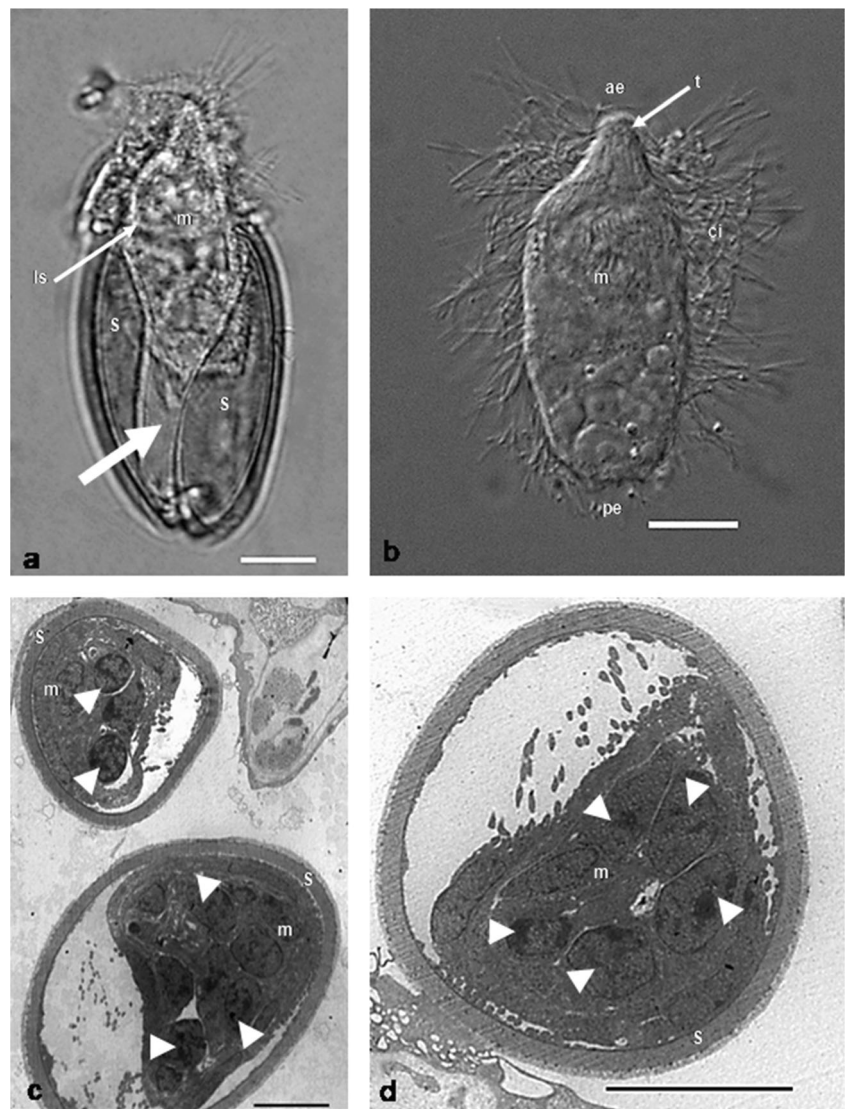
The *P. bragai* eggs are elliptical, with a width/length ratio of 1:2.28. In the review of Freitas (1951), the author reported various egg measurements with width/length ratios greater than 1:2, with an average of 1:2.31. Furthermore, according to this author, the eggs have length ranging from 15 to 34 μ m and width of 15 to 19 μ m. Santos (1934) reported

Table 2 Eggshell and operculum size, (mean \pm standard deviation) of *Paratanaisia bragai* eggs

Measurements (μ m)	<i>Paratanaisia bragai</i> eggshell thickness (μ m)			
	All layers	Inner layer	Middle layer	Outer layer
Ends	0.34 \pm 0.06 (0.29–0.50)	0.03 \pm 0.01 (0.05–0.01)	0.24 \pm 0.04 (0.35–0.19)	0.09 \pm 0.03 (0.15–0.06)
Middle region	0.31 \pm 0.05 (0.24–0.36)	0.02 \pm 0.01 (0.05–0.01)	0.21 \pm 0.05 (0.29–0.12)	0.08 \pm 0.02 (0.13–0.04)

Numbers in parentheses represent the minimum and maximum values observed

Fig. 4 Miracidium of *Paratanaisia bragai*. **a** Egg with suture line (sl) of the operculum (thin arrow) and opening of the egg shell (s) (broad arrow), caused by the pressure of the cover slip, showing the miracidium (m), observed by bright-fieldlight microscopy. Scale bar 7 μ m. **b** Miracidium (m), showing the anterior end (ae), with the terebratorium (t), posterior end (pe) and cilia (ci), observed by differential interference contrast (DIC) microscopy. Scale bar 7 μ m. **c** and **d** Miracidium (m) inside the egg, evidenced by its shell (s), and germinative cells, indicated by the arrowheads, observed by transmission electron microscopy. Scale bar 2 μ m



egg measurements of 31 by 13 μ m, a width/length ratio greater than 1:2, as did Tubangui and Masiluñgan (1941), with length of 30 to 33 μ m and width of 12 to 13 μ m, and Byrd and Denton (1950), who reported length ranging from 30 to 34 μ m and width from 16 to 22 μ m. Therefore, our findings are in accordance with the measurements reported by Freitas (1951).

Scanning electron microscopy also revealed that the eggs of *P. bragai* have a rough surface, a result that differs from those reported by Freitas (1951) that observed smooth egg surfaces. This divergence can be related to the resolution power used to examine the eggs. Freitas (1951) did not report the magnification used. Besides a general view of the eggs, we also presented an image of the shell with 15.000 \times magnification.

This article presents the first microscopic images of the miracidium of *P. bragai*. Maldonado (1945) studied the life

cycle of *Tamerlania bragai* (= *P. bragai*), but only presented a diagram of the miracidium. The length of the miracidium of *P. bragai* reported here (29 μ m) is approximately the same as that found by Maldonado (1945), that reported 30 μ m, the only measurement given in this article.

According to Roberts and Janovy (2009), the miracidia of digenetic trematodes are very small with piriform appearance, and the anterior end of the body contains a retractable apical papilla. Rey (2008) stated that the anterior end of the miracidium of *Schistosoma mansoni* Sambon, 1907 projects outward like a small cone, as also observed here. The apical papilla can also be called the terebratorium. Pinheiro et al. (2005) showed the terebratorium region of *E. paraensei* with many folding in the tegument, which can be formed into small suckers, besides the intense activity of the secretory cells of the miracidium reinforce the general idea that the terebratorium region has an opening for the release of enzymes and other products that

contribute to the penetration process. The terebratorium region was observed at the *P. bragai* miracidium, however it was not possible to detail it.

In the present study, it was possible observe the miracidial body covered by many long cilia, but it was not possible to observe the organization of these structure in epidermal ciliated plates as Pinheiro et al. (2004) showed for *E. paraensei* miracidia. The surface of the miracidium body covered by cilia is reported by Roberts and Janovy (2009) as characteristic of the miracidia of digenetic trematodes, as this larva must actively swims in the external aquatic medium to find the snail first intermediate host. In *P. bragai* the infection passively occurs and after an hour of ingestion the eggs reach the snail's digestive tract (Brandolini and Amato 2006). The miracidium is released in the digestive tract and penetrates the tissue, and the larvae must migrate, until it reaches the site where it begin its development and asexual reproduction.

The eyespots in *P. bragai* miracidia were not observed with the techniques used here. The pattern of its life cycle supports our finding, once the miracidium hatching occurs inside the digestive system of the snail host. According to Galaktionov and Dobrovolskij (2003), the passive life cycle influences the organization of the sensory and nervous structures of the miracidium, which seems to support the view of the extreme reduction of these structures. In addition, these same authors report that the miracidium in a passive life cycle does not need environmental information, as it is inside the egg, that is, there is no possibility of a response regarding its behavior.

An extensive study on eyespot in Digenea considered that the pigmentation preservation is erratic and apparently is not related to the group's systematic (Faust 1918). The trematodes, which passively infect the snail intermediate host, did not present miracidia with pigmented eyespots. Though, Maldonado (1945) reported the presence of refractile granules in the miracidia of *T. bragai*, however without using nomenclature 'eyespot'. Conversely, it is possible to find trematodes, whose miracidia carry out active infection and do not have eyespots, as in Schistosomatidae. The absence of eyespots follows the evolution of digenetics and that is not strictly related to the infection pattern used by the miracidia (Maldonado 1945; Galaktionov and Dobrovolskij 2003).

The greatest difficulty to study miracidium in trematodes that infection occurs passively is to obtain the larva without the egg. The processing of the eggs to TEM analysis usually results in poor quality of the miracidium because fixatives and epoxy resin does not penetrate well the eggshell. In the present study, it was possible to obtain well fixed and embedded miracidia inside the eggs, allowing the observation of a great number of germinal cells in the larval body, with cell nucleus containing electron-dense nuclear material. The miracidial body inside the egg, observed in our study, was already coated by cilia, showing that the released eggs are completely embryonated and the miracidia are formed and ready to infect the snail host.

Pan (1980), in a study about the structure of the miracidium of *S. mansoni*, observed about 20 germinal cells located at the level of the third and fourth tiers of epidermal plates, in the posterior region of the larval body. Germinal cells are irregularly shaped, and the nucleus contains a prominent nucleolus, and is poor in heterochromatin. In the present study, the germinal cells are irregularly shaped, with large nucleus, which nearly occupies more than one-half of the cytoplasm, but the nucleolus is not as conspicuous as showed by Pan (1980) in *S. mansoni* miracidia. This difference may reflect the distinct development status once some eggs were obtained from the uterus of dissected adult worms. Thus, being possible that they are not completely mature.

The knowledge about the morphology of larval stages of digenetic trematodes is scarce, some few species, with outstanding medical importance, have more information on this matter. *Paratanaisia bragai* is an important parasites of wild and domestic birds, has a very controversial taxonomy and the taxonomic tools must be improved to understand the distribution and biology of the parasite and the diseases associated to it. As parasite of birds, *P. bragai* has been recorded in the UK (Unwin et al. 2013), the American continent, including Brazil, Philippines and India (Malik et al. 2016), and surely, the distribution of this parasite is underestimated. The intense smuggling of wild birds, can contribute to this scenario, in which Brazil is one of the countries with the most intense illegal trade (trafficking) of wild birds, contributing to the dispersion of parasites associated with the species of smuggled birds (Karesh et al. 2005; CISS 2019), including *P. bragai*. In (Ehlers 1985), using electron microscopy, propose the Neodermata clade, from the characteristics of the integument and its formation pattern. New information on basic morphology from microscopy is indicator to the elucidation of phylogenetic relationships. Since the last century, the use of (light and electron) microscopic characters is used to add information and to improve the taxonomic description of parasites, as for example, Gupta et al. (2017) who recommended that the morphological characteristics, obtained by light and scanning electron microscopy, of Digenea species, must be considered as taxonomic tools in future studies of the species. The present study allow us to observe, for the first time, details of morphology and ultrastructure of eggs and miracidium of *P. bragai*, contributing to an improvement of the biology knowledge about this parasite.

Conclusions

The taxonomy of parasites from *Paratanaisia* genus is controverted and confuse. By this reason, additional studies, which may bring light to a better understanding of the morphology and biology of these trematodes, surely will endow future studies with important information about *P. bragai*.

The present study brings, for the first time, details of the morphology of *P. bragai* eggs using different microscopic techniques and the observation of the morphology of miracidium, both inside and outside the egg, contributing to enrich the knowledge about the biology and morphology of this parasite, to support future studies of its morphology, biology and control.

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Compliance with ethical standards

Ethical approval The experiment was approved by the Ethics Committee for Animal Use (CEUA), Veterinary Institute, UFRRJ (protocol number 186/2011, process number 23083.011596/2011-67).

Conflict of interest The authors declare that they have no conflict of interest.

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