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The life cycle of *Ascocotyle* (*Phagicola*) *longa* (Digenea: Heterophyidae), a causative agent of fish-borne trematodosis

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ABSTRACT

The complete life cycle of the trematode *Ascocotyle* (*Phagicola*) *longa* (Digenea: Heterophyidae) is elucidated by natural observation validated by experimental infections. The natural first intermediate host of *A.* (*P.*) *longa*, an agent of human heterophyiasis in Brazil, is the cochliopid snail *Heleobia australis* (new first intermediate host). Metacercariae were found encysted in the body musculature, heart, stomach, liver, kidney, spleen, gonads and mesentery of mullets *Mugil liza*. Hamsters *Mesocricetus auratus* were experimentally infected with metacercariae of *A.* (*P.*) *longa* obtained from the mullets, and the adults recovered were used to infect the snails *H. australis*. Rediae and cercariae of *A.* (*P.*) *longa* are described for the first time. The ultrastructure of the tegument of *A.* (*P.*) *longa* shows a change in spination pattern from the cercaria with single-pointed spines to the metacercaria and adult with multipointed, brush-shaped spines. The life cycle of *A.* (*P.*) *longa* is related to estuaries and coastal lagoons where the recruitment of mugilid juveniles occurs. The high prevalence (100%) of *A.* (*P.*) *longa* encysted in the mullets examined within the urban area of Rio de Janeiro indicates the potentially great public health impact of the consumption of raw mullets.

1. Introduction

Ascocotyle (*Phagicola*) *longa* Ransom, 1920 (Digenea: Heterophyidae) is a widespread parasite recorded from the Americas, Europe, Africa and the Middle East (Scholz, 1999). It is considered to be one of the causative agents of heterophyiasis, an emerging fish-borne disease of humans (Muller, 2001; Scholz et al., 2001; Fried et al., 2004). The adult parasites are found in the intestine of fish-eating birds and mammals, and the metacercariae are found mainly in mullets (*Mugil* spp.) (Scholz, 1999). Several experimental infections have previously been performed in attempts to obtain adults by feeding laboratory animals, such as dogs, cats and hamsters, with the flesh of mullets (Barros and Amato, 1995a,b, 1996; Scholz et al., 2001). However, the first intermediate host of *A.* (*P.*) *longa* and its intramolluscan stages and cercariae have remained unknown.

In São Paulo, Brazil, Chieffi et al. (1990, 1992) reported human heterophyiasis caused by *Ascocotyle* (*Phagicola*) (=*Phagicola*) and commented that it was probably *A.* (*P.*) *longa*. Antunes and Almeida-Dias (1994) reported 10 positive cases of human heterophyiasis caused by *A.* (*P.*) *longa* among 102 patients that had eaten raw mullets in the previous four months. In Brazil, the metacercariae of *A.* (*P.*) *longa* in mullets have been recorded from São Paulo, Rio de Janeiro and Belém (Almeida-Dias and Woiciechovski, 1994; Knoff et al., 1997; Conceição et al., 2000; Oliveira et al., 2007). Because of its medical importance and worldwide distribution, it is important that the complete life cycle of this species is elucidated.

The present work describes for the first time the natural molluscan first intermediate host of *A.* (*P.*) *longa* and adds new data on its life cycle on the basis of experimental infections, with the first descriptions of rediae and cercariae based on both light and scanning electron microscopy.

2. Material and methods

2.1. Collection of snails and fishes

A total of 1000 snails *Heleobia australis* (d'Orbigny, 1835) (Cochliopidae) were collected (500 snails in June 2008 and 500 in January 2009) at the edge of the Rodrigo de Freitas lagoon, Rio de Janeiro, Brazil (22°57′2″S, 43°11′9″W) and examined for cercariae. In the same area, 21 mullets *Mugil liza* (total length 30–34 cm), 50 guppies *Poecilia vivipara* (4–6 cm), 50 *Phalloptychus januarius* (4–5 cm) and 50 *Jenynsia multidentata* (5–7 cm) were collected and examined for metacercariae. *P. vivipara* and *H. australis* were reared in the laboratory in filtered lagoon water to provide specimens devoid of infection.

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2.2. Experimental infections

Nine hamsters *Mesocricetus auratus* were fed with the muscle tissue of naturally infected *M. liza*. On the seventh day post infection (dpi) the hamsters were examined for ovigerous adults of *A.* (*P.*) *longa*.

Adults of *A.* (*P.*) *longa*, obtained experimentally from hamsters, were used to infect two uninfected stocks, each containing 10 laboratory-reared snails, by placing eggs of *A.* (*P.*) *longa* in their aquaria in order to obtain rediae and cercariae.

Two groups of four laboratory-reared *P. vivipara* each were exposed to 30 cercariae and 50 cercariae, respectively, obtained from experimentally infected *H. australis*. A third group of four laboratory-reared *P. vivipara* was maintained with a single infected mollusc in their aquarium for three days. The fish groups were dissected at 14, 21 and 30 dpi, respectively.

2.3. Light microscopy

All larval stages and adults were studied live, with or without vital staining. Specimens were fixed in 70% alcohol or hot 4% formaldehyde solution, stained with Gomori's trichrome or alcoholic chloridic carmine, cleared in clove oil and mounted in Canada balsam. Illustrations were made with the aid of a drawing attachment on a Leica DM LS2 microscope. Measurements are presented in micrometers as the range, with the mean in parentheses.

2.4. Scanning electron microscopy (SEM)

Specimens were fixed with 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer for 1 h at 4 °C. After washing in the same buffer, the specimens were post-fixed for 3 h at room temperature in 1% osmium tetroxide in 0.1 M Na cacodylate buffer. Then the material was dehydrated in an ascending acetone series, dried using the critical point method with CO_2, mounted using silver cellotape on aluminum stubs and sputter-coated with a 20 nm-thick layer of gold. The samples were examined using a Jeol JSM-6791F microscope at an accelerating voltage of 15 kV.

3. Results

3.1. Natural and experimental infections

Guppies *Poecilia vivipara*, *Phalloptychus januarius* and *Jenynsia multidentata* from the Rodrigo de Freitas lagoon were not found naturally infected with metacercariae of *A.* (*P.*) *longa* and experimental infections of laboratory-reared *P. vivipara* were unsuccessful.

All mullets *M. liza* examined were naturally infected with metacercariae of *A.* (*P.*) *longa*. Nine experimental infections of hamsters fed with muscle tissue of naturally infected *M. liza* were successful in obtaining ovigerous adults at 7 dpi.

Forty-eight (9.6%) of 500 *H. australis* snails examined were naturally infected with cercariae of *A.* (*P.*) *longa* during June 2008 (dry winter), compared with 17 (3.4%) of 500 snails studied during January 2009 (rainy summer).

No hatching miracidia were observed. During the experimental infections, two groups of 10 snails ingested eggs, and the shedding of cercariae started at 35 dpi and 42 dpi, respectively. Cercarial release continued for 23 months (end of experiment).

3.2. Morphology of parasites

(A) *Sporocyst*. No sporocysts were observed in the snail host.

(B) *Redia* (measurements based on 10 specimens from the digestive gland and gonads of experimentally infected snails *H. australis*, new host record). Body a thin-walled sac without appendages, measuring 470–500 × 140–160 (480 × 149), filled with germinal balls and 1–6 developing cercariae (Fig. 1A). Pharynx 20–30 × 15–25 (25 × 20) opens to short cecum. Birth pore situated at level of pharynx.

(C) *Cercaria* (measurements based on 10 fully-developed specimens shed from experimentally infected snail, heat-killed in 4% formaldehyde solution). Cercaria pleurolophocercous. Body trapezoid, 110–125 × 60–95 (119 × 81) (Figs. 1B and 2A). Tegument spinose, with simple-pointed spines (Fig. 2A, C, E). Brown pigment scattered throughout body. Tail longer than body, 170–185 (179) long, deeply inserted into tail socket (Figs. 1B and 2A, E); its margins annulated and with small ventro-terminal finfold and small spine-like structure at caudal extremity (Figs. 1B and 2A, F). Oral sucker spherical, 20–32.5 × 20–30 (26.7 × 25) (Fig. 1B, C), with 16 acicular spines on its anterior border and 6 small papillae on its posterior border (Fig. 2C–D). Several sensory papillae symmetrically arranged around and immediately posterior to oral sucker (Fig. 2B–D). Pair of large pigmented eyespots just posterior to oral sucker (Fig. 1B). Seven pairs (3 + 4) of unicellular penetration glands present at mid-body; their ducts open via four groups of pores (3–4–4–3) at anterior extremity (Fig. 1B, C). Esophagus and ceca not differentiated. Flame-cell formula 2[(2 + 2) + (2 + 2)] = 16. Excretory vesicle large, with thick epithelial wall; its narrow stem bifurcates in base of tail and open as lateral excretory pores (Fig. 1B). Cercariae very motile, positively phototatic, alternating periods of swimming and resting phase. Swimming movements start on bottom, where tail extends and bends, causing whip-like circular movements of body to ascends to surface; after a short resting phase near surface with tail extended, cercaria sinks with tail uppermost.

(D) *Metacercaria* (measurements based on 10 specimens from body musculature of naturally infected *Mugil liza*). Metacercariae encysted in body musculature, heart, stomach, liver, kidney, spleen, gonads and mesentery. Cyst oval, thin-walled, translucent, 225–240 × 215–235 (232 × 229) (Fig. 1D). Body of excysted metacercaria pyriform, 400–490 × 120–150 (443 × 142), with tegument entirely covered with spines (Figs. 1E and 3A). Pre-oral lobe triangular, 10–15 (14.2) long. Oral sucker 20–30 × 40–50 (26 × 48), surrounded by a single row of 16 circumoral spines measuring 13–18 × 2.5–5 (15 × 3.5) (n = 20) (Figs. 2B, C and 3B, C), with well-developed posterior appendage, 75–100 (85) long (Fig. 1E). Ventral sucker 25–35 × 25–35 (30 × 30), enclosed within ventrogenital sac. The oral/ventral sucker width ratio 1:0.55–0.75 (1:0.63). Prepharynx 100–150 (126) long; pharynx 30–50 × 25–30 (40 × 26). Cercarial eyespot pigments scattered at posterior level of pharynx. Esophagus 15–30 (21) long; intestinal ceca reach back to testicular level, filled with platelets. Testes symmetrical, close to posterior extremity; left testis 23–38 × 35–58 (31 × 49), right testis 25–38 × 40–60 (32 × 53). Ventrogenital sac contains bipartite gonotyl composed of two pad-like lobes, 13–15 × 38–50 (14 × 41), each containing 8–13 refractile pockets (Figs. 1E and 3D). Ovary dextral, 15–18 × 25–30 (16 × 27). Flame-cell formula 2[(2 + 2) + (2 + 2)] = 16. Excretory vesicle X-shaped, with lateral arms anterior and posterior to testes, filled with dark granules; excretory pore terminal (Fig. 1E).

(E) *Adult* (measurements based on 10 specimens from experimentally infected hamsters, *Mesocricetus auratus*). Body pyriform, 480–720 × 190–250 (574 × 224) (Figs. 1G and 4A). Pre-oral lobe 13–18 (15) long; oral sucker 25–45 × 45–60 (28 × 52), surrounded by single row of 16 circumoral spines, 15–18 × 2.5–5 (16 × 3) (n = 20) (Figs. 1F and 4B, C). Unicilliate papillae protrude over pre-oral lobe and between tegumental spines (Fig. 4B, C). Posterior appendage of oral sucker well developed, 75–170 (107) long (Fig. 1F). Ventral sucker 30–45 × 30–50 (39 × 37),

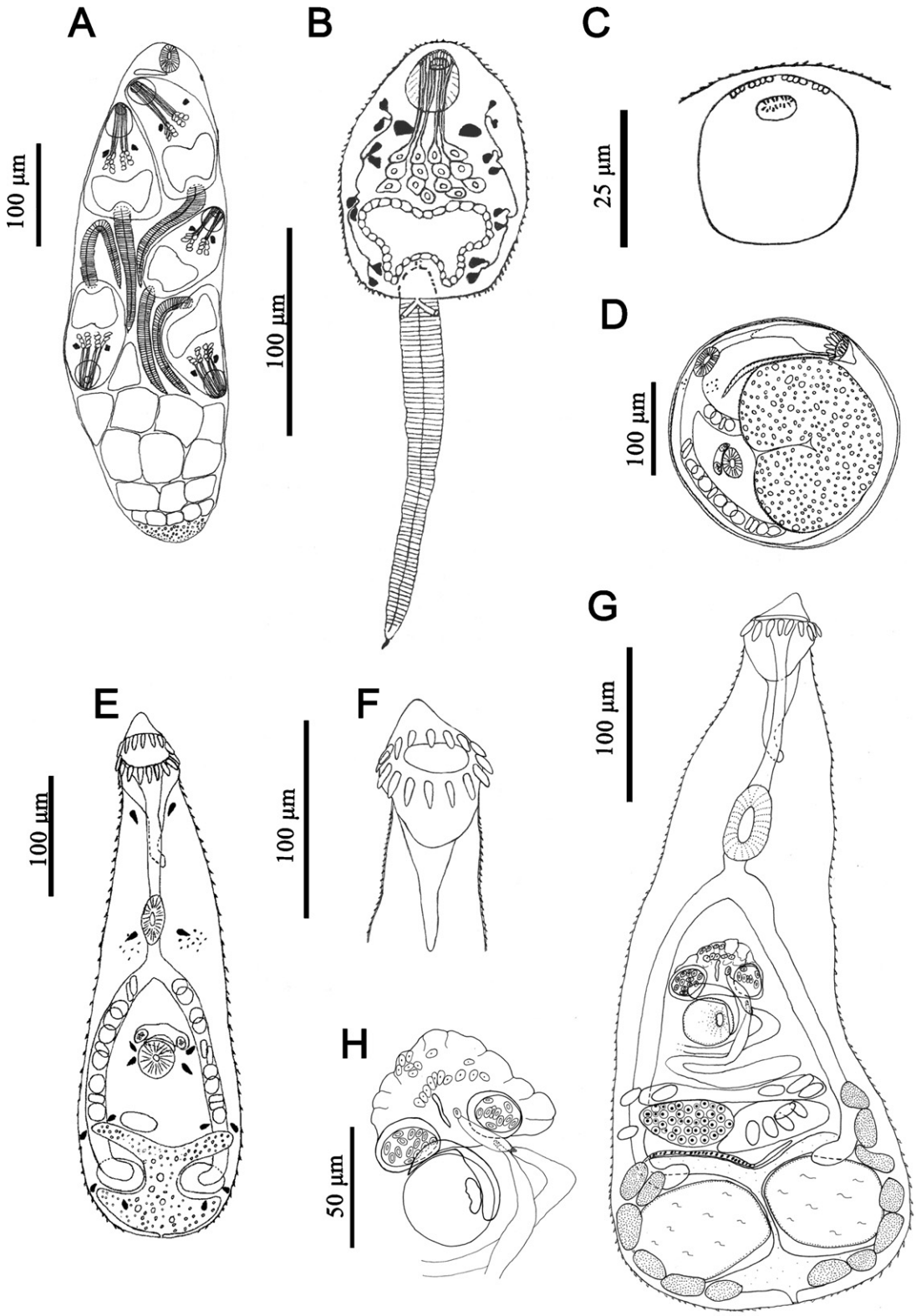

Fig. 1. *Ascocotyle* (*Phagicola*) *longa*, light microscopy. (A) Redia from experimentally infected *Heleobia australis*; (B) cercaria shed from experimentally infected *H. australis*, ventral view; (C) detail of oral sucker of cercaria with pores of penetration glands and acicular spines; (D) encysted metacercaria; (E) ventral view of metacercaria from naturally infected *Mugil liza*; (F) detail of pre-oral lobe of adult surrounded by circumoral spines and posterior appendage of oral sucker; (G) adult from experimentally infected hamster, ventral view; (H) detail of ventrogenital sac of adult.

enclosed within ventrogenital sac (Fig. 1H). Oral/ventral sucker width ratio 1:0.54–0.88 (1:0.73). Prepharynx 90–175 (125) long; pharynx 40–50 × 25–40 (48 × 34.5); esophagus 15–50 (23.5) long. Intestinal ceca with curved posterior ends. Testes symmetrical, close to posterior end of body; left testis 50–70 × 70–105 (61 × 81); right testis 50–80 × 75–110 (67 × 88). Seminal vesicle sigmoid, posterosinistral to ventral sucker; ejaculatory duct opens into ventrogenital sac.

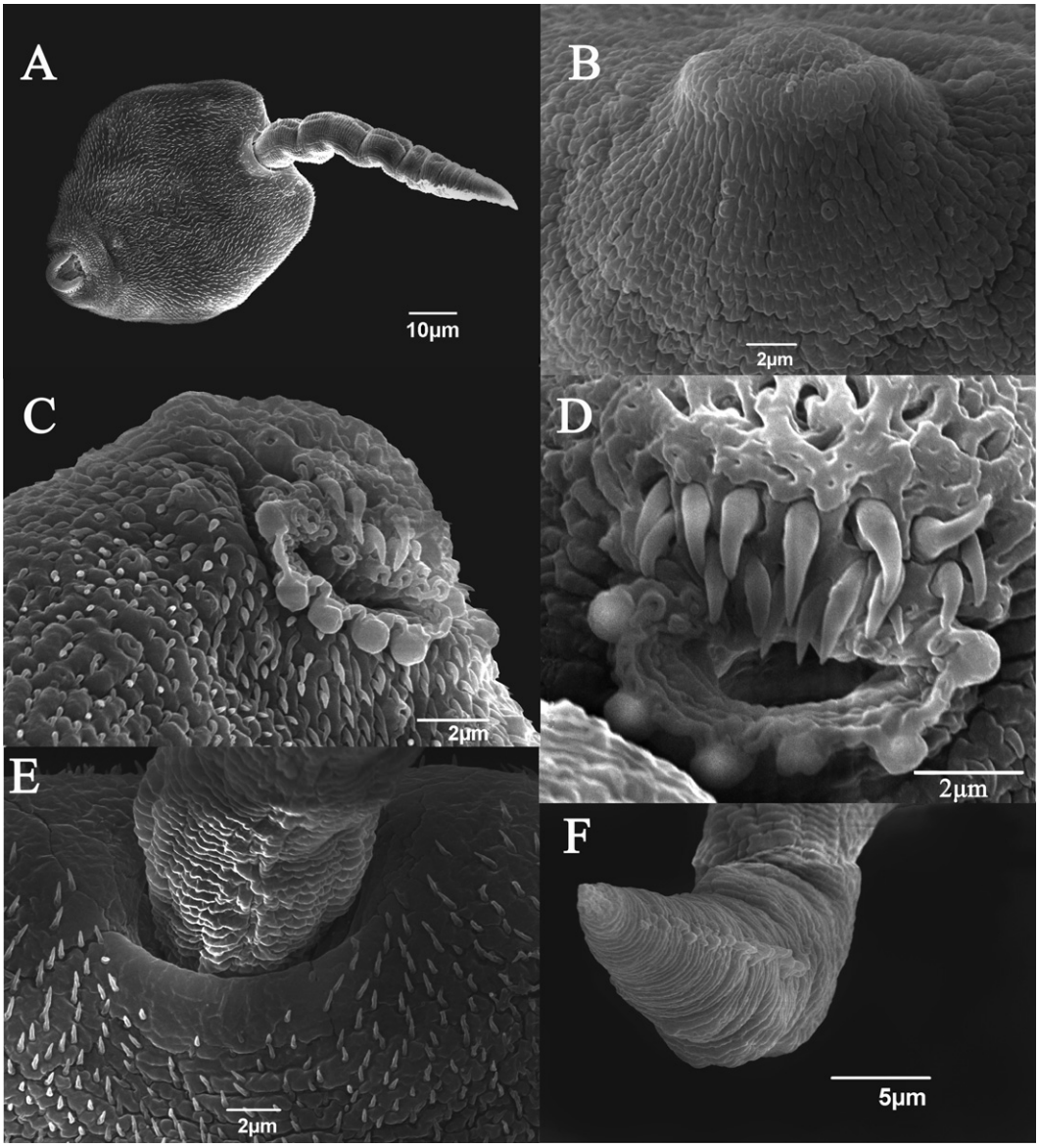

Fig. 2. Cercaria of *Ascocotyle* (*Phagicola*) *longa*, SEM micrographs. A: body with long tail, ventral view; (B) dorsal view of anterior end with sensory papillae; (C) anterior end with oral sucker, acicular spines, papillae and spined tegument; (D) detail of acicular spines arranged in two rows and papillae at posterior border of oral sucker; (E) area of tail insertion at posterior extremity of body; (F) terminal region of tail with small alae.

Ventrogenital sac with slit-like aperture (Fig. 4E), containing bipartite gonotyl composed of two pad-like lobes, 20–50 × 58–120 (28 × 71), containing 5–9 refractile pockets (Fig. 1H). Ovary 30–75 × 50–75 (41 × 63), anterodextral to right testis. Seminal receptacle 30–50 × 50–80 (40 × 58) (Fig. 1G). Vitellarium composed of 7–9 follicles on posterolateral sides of body between ventral sucker and posterior extremity. Uterus sinuous, extending from pretesticular level to ventrogenital sac. Eggs operculate, 17.5–22.5 × 10–12.5 (20 × 10) (n = 27). Excretory vesicle X-shaped, with lateral arms anterior and posterior to testes (Fig. 1G); excretory pore terminal (Fig. 4F).

3.3. Life history

Ascocotyle (*Phagicola*) *longa* has a three-host life cycle with a snail first intermediate host (*Heleobia australis*), a mugilid fish (*Mugil liza*) as the second intermediate host and a mammal (indicated by successful experiments with hamsters), bird and occasionally man as the definitive host. The eggs of *A.* (*P.*) *longa* are released within the feces of the definitive host and fall directly into, or are carried into, the water. These eggs are ingested by the snail *H. australis* and the miracidia are likely transformed into sporocysts. Another generation of larvae, rediae, develops in the hepatopancreas, and produces pleurolophocercariae. Cercariae are released into the water via the birth pore. These cercariae infect the second intermediate host, a mugilid fish, and metacercariae encyst in the body musculature, heart, stomach, liver, kidney, spleen, gonads and mesenteries. Mammals and fish-eating birds are infected by feeding fish with metacercariae and trematodes develop to the adult stage in their intestine.

4. Discussion

The life cycle of the heterophyid *Ascocotyle* (*Phagicola*) *longa* is completed for the first time and the cochliopid snail *Heleobia australis* is found to serve as its first intermediate host.

Experimental infections were previously performed to elucidate the life cycle of *A.* (*P.*) *longa*, but the mollusc first intermedi-

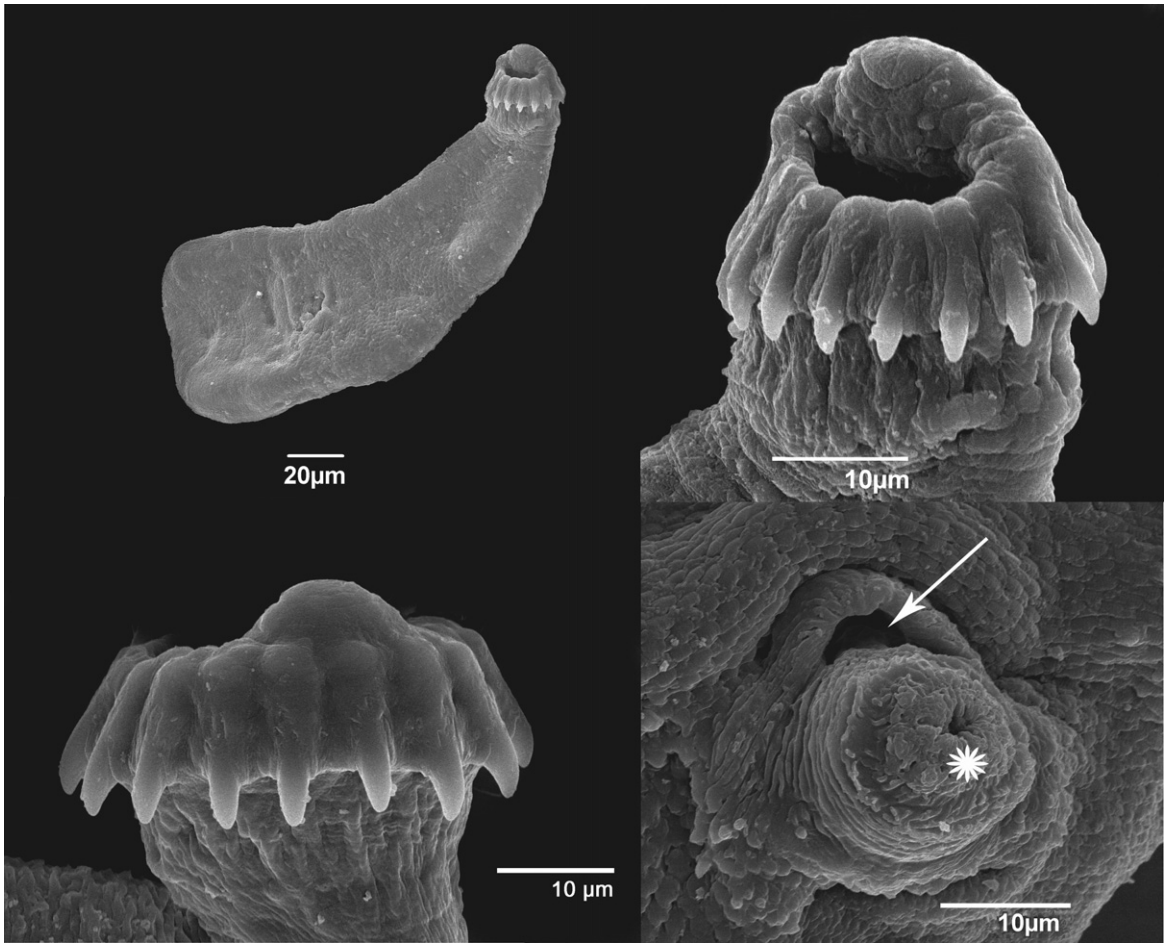

Fig. 3. Metacercaria of *Ascocotyle* (*Phagicola*) *longa*, SEM micrographs. A: body pyriform with row of spines on anterior region, ventral view; (B) ventral view of anterior end showing pre-oral lobe and circumoral spines; (C) dorsal view of oral sucker with pre-oral lobe and row of spines; (D) detail of aperture of gonotyl (arrow) and ventral sucker everted (star) from ventrogenital sac.

ate host was not properly identified and intramolluscan stages, including cercariae, remained undescribed (Almeida-Dias and Woiciechovski, 1994; Barros and Amato, 1995a,b, 1996; Scholz et al., 2001; Dzikowski et al., 2003, 2004). Hutton and Sogandares-Bernal (1959) reported an experimental infection of mullets with pleurolophocercous cercariae naturally shed by the snail *Cerithium muscarum* Say, 1822 (Cerithiidae) from Florida, suggesting that the cercaria could be related to *A.* (*P.*) *longa* based only on the flame-cell formula. However, the hamster fed with these experimentally infected young mullets did not reveal adults of *A.* (*P.*) *longa*. Yamaguti (1971, 1975) confirmed the absence of a detailed morphological description of the cercaria, adding that the flame-cell formula was similar to that of *Ascocotyle* (*Phagicola*) *minuta* (Looss, 1899) (=*Phagicola minutus*). Similarly, Castro et al. (2006) commented that an oculocercaria from *H. australis* in Uruguay could be related to *A.* (*P.*) *longa*. However, it was only briefly reported in a congress, without the formal publication of a detailed morphometrical description of the cercaria. Etchegoin (1997) also cited the presence of heterophyid cercariae in *H. australis* in Argentina without a specific identification. In the present study, we show, from natural infections validated by experimental infections, that *A.* (*P.*) *longa* utilizes the cochliopid snail *H. australis* as its first intermediate host in Brazil.

Cochliopid snails are generally reported to be suitable intermediate hosts for heterophyid digeneans (Ostrowski de Núñez, 1993, 1998) and, in Rio de Janeiro, we previously reported *H. australis* as the first intermediate host of *Pygidiopsis macrostomum* Travasssos, 1928 and the cryptogonimid *Acanthocollaritrema umbilicatum* Travassos, Freitas and Bührnheim, 1965 (Simões et al., 2008, 2009).

The complete life cycles of *Ascocotyle* (*Phagicola*) species already elucidated were restricted to *Ascocotyle* (*Phagicola*) *diminuta* (Stunkard & Haviland, 1924) and *Ascocotyle* (*Phagicola*) *angeloi* Travassos, 1928, which use the cochliopid snails *Heleobia parchappei* (d'Orbigny, 1835) and *Heleobia castellanosae* (Gaillard, 1974) as intermediate hosts (Ostrowski de Núñez, 1993, 1998). As fish hosts, they use poecilid, cyprinodontid and cichlids, but whereas the metacercariae of *A.* (*P.*) *diminuta* are found exclusively in the gills, those of *A.* (*P.*) *angeloi* are found in the muscles, body cavity, internal organs and gill chamber. In the life cycle of *A.* (*P.*) *longa* the metacercariae were found in the body musculature and internal organs, except for the gill or gill chamber, of mullets *Mugil liza*. The poecilid fish *Poecilia vivipara* and *Phalloptychus januarius*, and the anablepid *Jenynsia multidentata*, were not naturally infected and experimental infections were unsuccessful. Additional differences, including the morphology of larval stages, are discussed below.

Sporocysts of *A.* (*P.*) *longa* were not observed in either naturally or experimentally infected *H. australis*. This stage is generally difficult to detect and remains the least studied in the life cycle of trematodes (Galaktionov and Dobrovolskij, 2003; Ostrowski de Núñez, 1993, 1998). Morphological and biological organization of sporocysts of trematodes with passively infecting miracidia is reported to be diverse and a complete reduction of the soma at their early stages may occur and as a result, only a small aggregation of

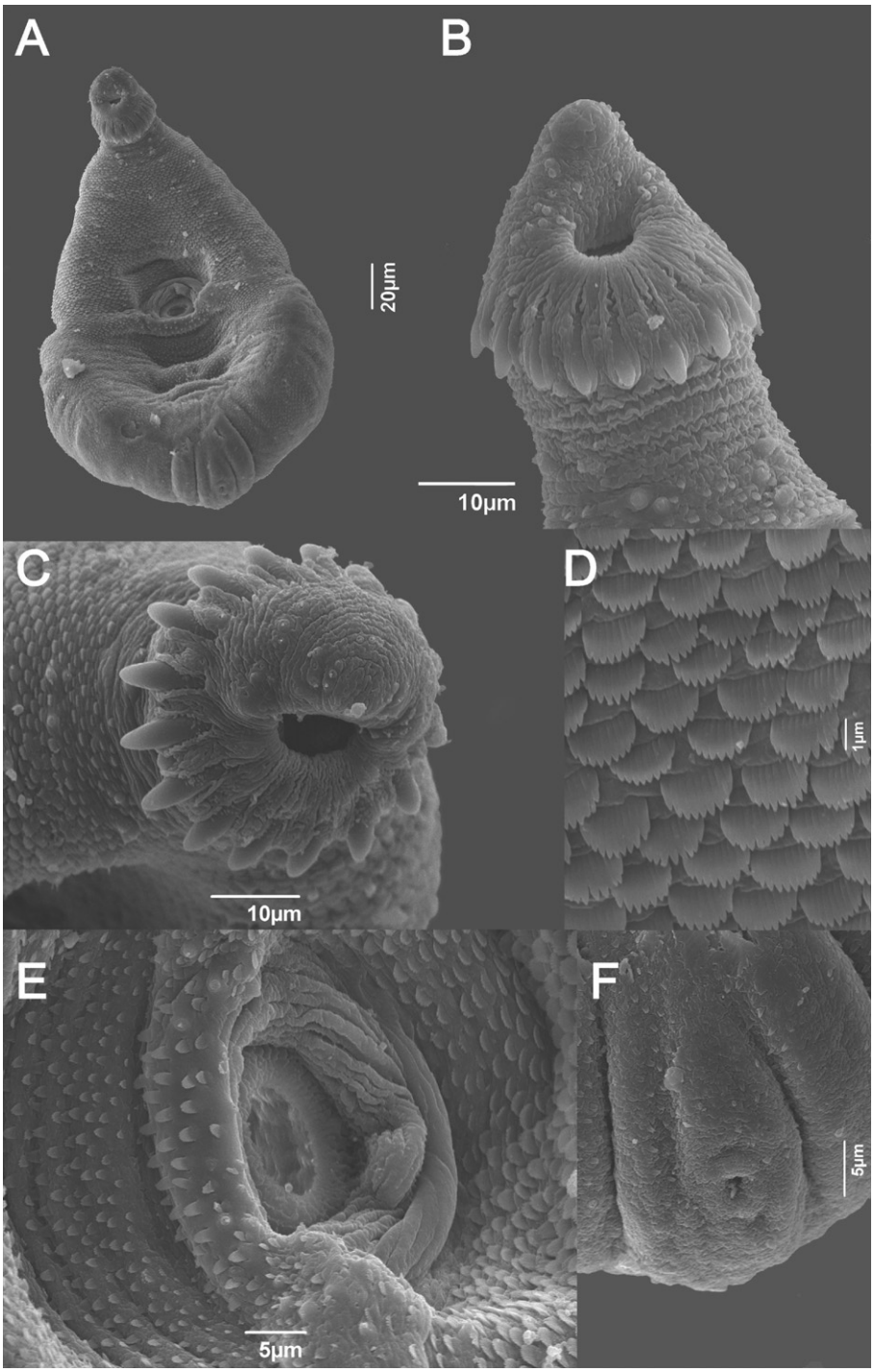

Fig. 4. Adult of *Ascocotyle* (*Phagicola*) *longa*, SEM micrographs. A: body pyriform row with of spines on anterior region, ventral view; (B) ventral view of anterior end showing pre-oral lobe with papillae, large circumoral spines and spined tegument; (C) dorso-lateral view of oral sucker with papillate pre-oral lobe and row of 16 spines; (D) detail of tegument with brush-bordered spines; (E) ventral sucker enclosed in the ventrogenital sac; (F) detail of excretory pore.

cells is localized directly at the site of penetration of the miracidium (Galaktionov and Dobrovolskij, 2003). This may be the case of *A.* (*P.*) *longa* sporocysts and further histological studies are needed to elucidate it.

The redia of *A.* (*P.*) *longa*, containing cercariae, is similar to those of the most closely related species, *A.* (*P.*) *diminuta* and *A.* (*P.*) *angeloi* (Ostrowski de Núñez, 1993, 1998), i.e. it is a sac-like stage without locomotory appendages and with a reduced gut, typical for a heterophyid according to Galaktionov and Dobrovolskij (2003).

Cercariae of the three above-mentioned species possess a similar pattern of penetration glands (3 + 4 + 4 + 3) and six papillae on the posterior border of the oral sucker. However, the cercaria of *A.* (*P.*) *longa* can be differentiated from them by its pigmented body, the presence of 16 acicular spines on the oral sucker (*vs*. 5 in *A.* (*P.*) *diminuta* and 11 in *A.* (*P.*) *angeloi*) and the number of flame cells, i.e. 2[(2 + 2) + (2 + 2)] = 16 (*vs.* 24 in *A.* (*P.*) *angeloi*) (Ostrowski de Núñez, 1993, 1998). Cercariae which infect *H. australis* in the same area as *P. macrostomum* can also be differentiated from *A.* (*P.*) *longa* by the absence of oral spines and the flame-cell formula

2[(2+2+2)+(2+2+2)]=24 (Simões et al., 2009), whereas *A. umbilicatum* may be differentiated by the presence of a single row of small spines on oral sucker, a tail with dorsal and ventral finfolds and a flame-cell formula of 2[(2+2)+(2+2)]=16 (Simões et al., 2008).

The ultrastructure of the tegument of *A.* (*P.*) *longa* described here showed a change of spination pattern from a cercaria with single-pointed spines to a metacercaria and adult with multi-pointed brush-bordered spines. According to Køie (1977, 1992) and Chai et al. (2000), these developmental changes are required to help the flukes to penetrate and adapt to their hosts. Within the genus, *Ascocotyle* (*Phagicola*) *pindoramensis* (Travassos, 1928) was the only species in which body spination of metacercariae and adults was compared and, in agreement with the present study, no differences were observed (Simões et al., 2006). However, the cercaria in which a change of spination is expected, is still to be studied.

The wide distribution of intermediate hosts of *A.* (*P.*) *longa*, the cochliopid snail *H. australis* (occurring from Rio de Janeiro (Brazil) to Argentina), and infected mullets (Mugilidae) (De Francesco and Isla, 2004; Armas de Conroy, 1986), may have epidemiological consequences because of the risk of human infection in a large area. The potentially great public health impact is confirmed by the high prevalence (100%) of *A.* (*P.*) *longa* encysted in mullets examined within the urban area of Rio de Janeiro.

Almeida-Dias and Woiciechovski (1994) also investigated the parasitism by *A.* (*P.*) *longa* in juvenile *Mugil platanus* from near São Paulo (Brazil) and reported that the prevalence of infection increased with fish size as follows: 2.4–4 cm = 0%, 10–13 cm = 60% and > 20 cm = 100%. This corresponds to the present data, because adult mugilid fish from the Rodrigo Freitas lagoon, 30–34 cm long, were all infected.

Mugilidae spawn in the open sea and recruitment of juveniles occurs in estuaries and coastal lagoon areas, which provides the necessary conditions for their growth during a large part of their life; when they mature, they migrate back to the sea during spawning period (Vieira, 1991). The life cycle of *A.* (*P.*) *longa* is related to estuaries and coastal lagoons. This euryhaline environment is not only considered a recruitment area for Mugilidae, but also the area preferred by *H. australis* (De Francesco and Isla, 2004). As juvenile fish enter the protected areas they come into increasing contact with the released cercariae as they grow. Furthermore, during the migration period back to the sea there is an increase of fisheries which may influence the transmission of the parasite to human population.

Contrary to reports on literature which indicate that *A.* (*P.*) *longa* rarely occur in body muscles (Yamaguti, 1975), a work in progress in our laboratory is showing a high prevalence of metacercaria in the body musculature of *M. liza*. Considering the large number of oriental restaurants which serve raw fish (sashimi, sushi) in large cities such as Rio de Janeiro and São Paulo and the occurrence of annual Mullet Festivals in several cities along the Brazilian coast, we consider that the public health impact of *A.* (*P.*) *longa*, as a causative agent of fish-borne trematodosis, is underestimated.

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