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Short communication

Morphological and molecular identification of the fish-borne metacercaria of *Ascocotyle* (*Phagicola*) *longa* Ransom, 1920 in *Mugil liza* from Argentina

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ABSTRACT

This is the first report of *Ascocotyle* (*Phagicola*) *longa* Ransom, 1920 (Digenea: Heterophyidae) in Argentina confirmed by morphological and molecular studies. The metacercaria was found encysted in myotomal musculature, heart and mesentery of the mullet *Mugil liza* (Pisces: Mugilidae) from Samborombon bay. We provide a morphological description of the metacercaria which we identified using species-specific primers for *A.* (*Phagicola*) *longa* and nucleotid sequence. This worldwide parasite has been reported as one of the causative agents of heterophyiosis, an emerging fish-borne disease of humans, contracted by the consumption of raw mullet. The discovery of *A.* (*Phagicola*) *longa* in Argentina represents a warning of the potentially great impact of this parasite on public health.

In a survey on parasites of fishes in mixohaline environments of Argentina, a heterophid metacercaria was found encysted in juveniles and adults of *Mugil liza*, the only species of mullet in Argentina. This species is commercially exploited by artisanal fishermen in Río de La Plata estuary, Samborombon bay, and Bahía Blanca estuary. The larval stage found shows morphological features consistent with the subgenus *Phagicola* that had been reported in human infections (Chieffi et al., 1990, 1992; Antunes and Almeida-Dias, 1994; Luque, 2004). The main objective of this study was to identify the parasite found, and verify whether it belongs to the zoonotic species *Ascocotyle* (*Phagicola*) *longa* Ransom, 1920.

Mullet were collected from 2009 to 2010 in Salado river (35°50′S, 57°25′W; $N = 130$) and Ajo river (36°20′S, 56°54′W; $N = 148$), both rivers entering Samborombon bay, Argentina. Samborombon bay is an extensive mixohaline wetland of Argentina ranging over 244,000 Ha. Along its coast, artisanal fishing ports are located mainly along the Salado river, San Clemente and General Lavalle fishing ports.

Fish captured were transported alive to the laboratory in plastic bags filled with water and suffused with oxygen. In the laboratory, they were kept alive in aerated aquaria until their examination for parasites. Fish were sacrificed by cervical dislocation. Collected metacercariae were studied alive and using stained wholemount specimens. Each individual parasite was heat-killed under cover slide without press, fixed with AFA, stained in Semichon's acetocarmine, cleared in clove oil and mounted in Canadian Balsam. The drawing was made with the aid of a camera lucida. Measurements are given in μm, as the means with the range in parentheses. Morphological identification was done according to Scholz (1999) and Simões et al. (2010).

For molecular identification, individual metacercariae were preserved in 100 μl of 96% molecular grade ethanol at −20 °C. Metacercariae of *Microphallus szidati* collected from

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the crab *Cyrtograpsus angulatus* in Mar Chiquita coastal lagoon (37°32′S, 57°19′W) were used as a control in molecular determination. We used species-specific primers for *A.* (*Phagicola*) *longa* according to Dzikowski et al. (2004): Pha 1463F (5′-ACT CGT GCG GGT GGC GGT ATT CT-3′) and Het 11824R (5′-AAT CGG TAG TAG CGA CGG GCG GT-3′).

Prior to DNA extraction, each individual metacercaria was crushed under binocular dissecting microscope using small needles, and pipetted into a 1.5 ml microtube for drying until total ethanol evaporation in a Speedvac evaporator centrifuge. DNA was extracted from individual parasites using a DNeasy Tissue Kit (Quiagen) with some minor modifications in relation to the small size of the parasites, following Webster (2009). Steps 1–3 were modified, adding 45 μl of ATL buffer instead 180 μl, and 5 μl of proteinase K instead of 20 μl. After 1 h of 55 °C incubation, 50 μl of AL Buffer and 50 μl of ethanol were added (instead of 200 μl of ATL buffer and 200 μl of ethanol). Steps 5–8 were performed according to the Kit protocol.

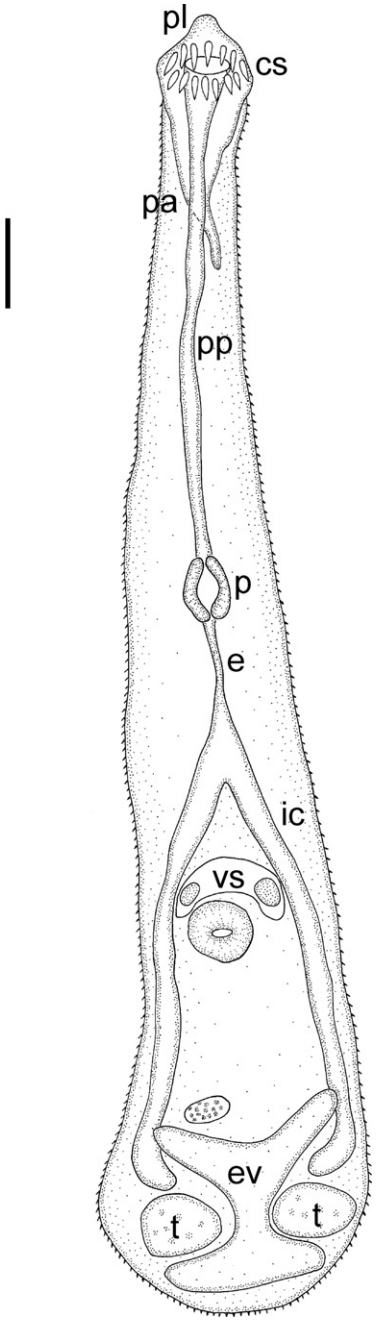

Fig. 1. Excysted metacercaria of *Ascocotyle* (*Phagicola*) *longa* showing preoral lobe (pl), circumoral spines (cs), posterior appendage of oral sucker (pa), prepharynx (pp), pharynx (p), esophagus (e), intestinal ceca (ic), testes (t), ventrogenital sac (vs), and excretory vesicle (ev). Scale = 50 μm.

Table 1
Mean abundance ± standard deviation, mean intensity ± standard deviation, and prevalence of *Ascocotyle* (*Phagicola*) *longa* in *Mugil liza* from Salado river (SR; *N* = 130) and Ajo river (AR; *N* = 148), during 2009 and 2010.

	Mean abundance	Mean intensity	Prevalence
AR 2009	3.85 ± 10.92	10.88 ± 16.28	0.35
AR 2010	26.4 ± 78.69	93.06 ± 127.89	0.28
SR 2009	4.46 ± 12.86	14.5 ± 20.09	0.31
SR 2010	5.38 ± 31.79	38.88 ± 81.35	0.14

PCR was performed in 25 μl volumes using: 12.5 μl of Go Taq Green Master Mix (Promega®), 0.5 μl of each primer, 9.5 μl of free nuclease water, and 3 μl of DNA template. The cycling conditions used were: initial denaturation at 94 °C for 3 min, 3 step cycling (34 cycles) of denaturation at 94 °C for 60 s, annealing at 65 °C for 45 s, and extension at 72 °C for 60 s, followed by a final extension step of 72 °C for 5 min. Final amplicons were separated by 2% TBE agarose gel electrophoresis stained with ethidium bromide, visualized under ultraviolet light, and photographed using a Doc-it Image System and an acquisition software (UVP Bioimaging Systems). The PCR products were purified according to the manufacturer's protocols, using Wizard SV Gel and PCR Clean-Up system (Promega®, Madison, WI, USA). The sequences of each product were sequenced directly by the ABI3130XL Sequencer Genetic (Applied Biosystems, USA), Unidad Genómica, INTA Castelar, Argentina.

We found metacercariae of *A.* (*Phagicola*) *longa* parasitizing myotomal musculature, heart and mesentery of *M. liza*. Table 1 shows prevalence, mean intensity and mean

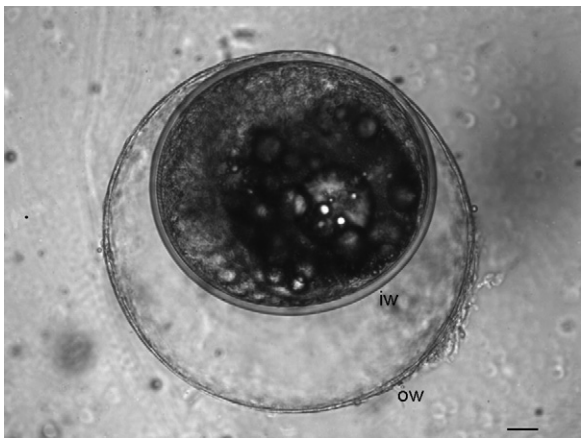

Fig. 2. Cyst of *Ascocotyle* (*Phagicola*) *longa* in gill muscle of *Mugil liza* showing translucent outer wall (ow) and thin inner wall (iw). Scale = 30 μm.