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Ascocotyle patagoniensis (Digenea: Heterophyidae) from the silverside *Odontesthes argentinensis* (Atherinopsidae). The bulbus arteriosus as an optimal ecological niche for this parasite species

Ascocotyle patagoniensis (Digenea: Heterophyidae) del pejerrey *Odontesthes argentinensis* (Atherinopsidae). El bulbo arterial como nicho ecológico óptimo para esta especie parásita

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ABSTRACT: Several biological and morphological features of the parasite and their hosts may be determinants for the dimensions of the helminth niche. Helminths cause tissue damage to their hosts by either mechanical action or secretion of toxic substances. Metacercariae of the *Ascocotyle* genus encyst in different tissues on their fish hosts. In this work, metacercariae present inside the bulbus arteriosus from the silverside *Odontesthes argentinensis* on the coast of Bahia Blanca, Buenos Aires province, were identified as *Ascocotyle* (*A.*) *patagoniensis* based on morphological and molecular analyses. In addition, parasitic indices are related to the fish condition factor, and the host inflammatory response to the presence of metacercariae is described. The prevalence of *A. (A.) patagoniensis* in male silversides was 100% (n= 15), and 92% (n= 25) in females. Most of bulbs presented between 20 and 40 cysts. Six individuals harbored more than 120 parasites and the maximum recorded value was 226 metacercariae in a single bulb. All metacercariae were alive and encysted at the study time. Each cyst contained only one metacercariae. The bulb distal area towards ventral aorta was the most densely parasitized and probably the site where infective cercariae first arrive. A discrete or almost inapparent cellular inflammatory infiltrate surrounds the metacercariae forming a thin connective tissue envelope weakly supporting cysts adhered to the internal tunic of the bulb. The absence of coronary irrigation both in the tunica media and the internal trabecular lamina of the bulb could explain the low fish immune reaction. It is suggested that the silverside bulbus arteriosus represents an optimal ecological niche for *A.* (*A.*) *patagoniensis* metacercariae.

Keywords: fish parasites, taxonomy, 28S rRNA, pathology, immunity

RESUMEN: Varios rasgos de la biología y morfología de los parásitos y sus hospedadores pueden ser determinantes de las dimensiones del nicho de los helmintos. Los helmintos causan daños en los tejidos de sus hospedadores por acción mecánica o a partir de la secreción de sustancias tóxicas. Las metacercarias del género *Ascocotyle* se enquistan en diferentes tejidos de sus peces hospedadores. En este trabajo, a partir de análisis morfológicos y moleculares, se identificaron como *Ascocotyle (A.) patagoniensis* las metacercarias presentes en el bulbo arterial del pejerrey *Odontesthes argentinensis* en la costa de Bahía Blanca, provincia de Buenos Aires. Además, se relacionan los índices parasitarios con el factor de condición del pez, y se describe la respuesta inflamatoria del hospedador a la presencia de las metacercarias. La prevalencia de *A. (A.) patagoniensis* en los pejerreyes machos fue del 100% (n = 15) y en las hembras 92% (n = 25). La mayoría de los bulbos arteriales presentaron entre 20 y 40 quistes. Seis individuos albergaron más de 120 parásitos y el máximo registrado fue de 226 metacercarias en un solo bulbo. Todas las metacercarias estaban vivas y enquistadas al momento del estudio. Cada quiste contenía solo una metacercaria. El área distal del bulbo, hacia la aorta ventral, fue la más densamente parasitada y probablemente es el sitio donde primero arriban las cercarias infectivas. Un infiltrado inflamatorio celular discreto o casi inaparente rodea a las metacercarias conformando una delgada envoltura de tejido conectivo, sosteniendo débilmente a los quistes adheridos a la túnica interna del bulbo. La ausencia de irrigación coronaria, tanto en la túnica media como en la lámina interna trabecular del bulbo, podrían explicar la baja reacción inmune del pez. Se sugiere que el bulbo arterial del pejerrey representa un nicho ecológico óptimo para las metacercarias de *A. (A.) patagoniensis.*

Palabras clave: ictioparásitos, taxonomía, 28S rRNA, patología, inmunidad

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INTRODUCTION

Regarding the parasitic microhabitat, most fish respond to the presence of helminths by mean of cellular immune reactions, particularly those with an extra-intestinal microhabitat. Helminths cause mechanical damage to the host tissues when they attach or release toxic substances interfering with the rate of nutrient absorption and metabolism (Prasanna Vankara and Chikkam, 2013). The damage degree depends on the parasitic burden, the affected tissue, and the host body condition. A working hypothesis can be postulated regarding the involved tissue: if the infection site has a low blood supply (e. g. the bulbus arteriosus), impeding the quickly mobilization of cellular and humoral effectors into the tissues, a weak immune response is expected and the infected tissue may be an optimal site for parasite survival as a result.

Tissue reactions caused by metacercariae vary depending on the trematode species and host microhabitat. These factors can be very serious such as those reported on salmonids infected by *Ichtyocotylurus erraticus* (Rudolphi, 1809) (Strigeidae) that causes mass mortalities (Orecka-Grabda, 1991). Also, *Apatemon gracilis* (Rudolphi, 1819) (Strigeidae) induces both a negative effect on the body condition and a reduction on 20-40% of the cardiac output in *Oncorhynchus mykiss* Walbaum (Watson *et al*., 1992). However, Hicks and Steele (2003) concluded that with high prevalences and infection intensities of *Ascocotyle tenuicollis* Price, 1935 (Heterophyidae) inside the heart of a small fish *Fundulus heteroclitus* (L.), effects on the cardiac function and host health were minimal. Metacercariae of *A. (Ascocotyle) patagoniensis* were described from the bulbus arteriosus on the silverside *Odontesthes argentinensis* (Valenciennes) and *O. smitti* (Lahille) from the San Matias Gulf, Patagonia, Argentina, (Hernández-Orts *et al*., 2019). Levy *et al*. (2019) recorded three species of *Ascocotyle* Looss, 1899 in *O. argentinensis* from several localities from the Argentinian shelf; *Ascocotyle (Phagicola) longa* Ransom, 1920 and *A. (Phagicola) diminuta* (Stunkard & Haviland, 1924), both in the gills, and *A. (Ascocotyle) patagoniensis* in the heart. *Ascocotyle (P.) longa* was found encysted in several tissues of fish (gills, body musculature, stomach, heart, liver, spleen, kidney, gonads, and mesentery) (Emerique Simões *et al*., 2010, Montes *et al*., 2013). *Ascocotyle (Ascocotyle) patagoniensis* has not been found in another niche other than the bulbus arteriosus up to date. For this reason, it would be of interest to evaluate whether the bulbus can be an optimal ecological niche for this species. Some traits of host physiology, ecology or behavior may be markedly different in both sexes, and indirectly affect both the population and community parameters of their parasitic helminths (Santoro *et al*., 2014, Abdelkhalik *et al*., 2021).

The aims of this paper are i) to identify the Heterophyidae metacercariae parasitizing the bulbus arteriosus of the silverside *Odontesthes argentinensis* (Atherinopsidae) from the Buenos Aires coast using both morphological and molecular methods; ii) to analyze the relation of parasitic parameters with both sex and fish condition factor; iii) to evaluate the inflammatory response caused by these larvae, and; iv) to discuss the bulbus arteriosus as an optimal ecological niche for the mentioned metacercariae.

MATERIALS AND METHODS

Samples collection

A total of 40 silverside *Odontesthes argentinensis* were collected in Bahía Blanca estuary (38°57′43 ″ S; 61°54′47 ″ W) Buenos Aires, Argentina between May 2012 and July 2018. Fish were collected by angling and kept fresh at 4°C. For each specimen, the sex, length (L), weight (w), eviscerated weight (W) and gonadal weight were recorded. Fish were dissected and the bulbus arteriosus (n = 40) were examined for metacercariae using a stereoscopic microscope. The worms were released using dissection needles. The cysts were easily detached from the inner wall while the fishes were dissected fresh.

Morphological identification of metacercariae

When the hosts were processed all of the metacercariae examined were alive. Ten excysted metacercariae were fixed in 5% formalin and mounted unstained. Measurements were taken using an Olympus CH light microscope with the aid of an ocular micrometer. All measurements as the mean and range, in parentheses, were indicated in micrometers (µm) unless otherwise indicated. Voucher specimens were deposited in Helminthological Collection from Museo de La Plata MLP-He 7766.

Molecular data

Total genomic DNA was extracted from a single ethanol-fixed metacercaria using the automated MagnaPure Compact system (Roche), with a previous lysis step performed with 400 µL of MagNA Pure DNA Tissue Lysis Buffer (Roche) and 20 µL of proteinase K. Partial fragments of the large subunit (28S) of the ribosomal RNA gene (domains D1–D3) were amplified using the primers: LSU-5 (5'-TAG GTC GAC CCG CTG AAY TTA AGC A-3'; Littlewood *et al*., 2000) and 1500R (5'-GCT ATC CTG AGG GAA ACT TCG-3'; Tkach *et al*., 2003). The expected product size was 1250 bp. Amplification was performed using the HotStarTaq Plus Master Mix Kit (Quiagen), with a final reaction mixture concentration of 1X, 0.5-5ng DNA and 0.5 µM of each primer, in a final volume of 25µL. The PCR was performed on a Veriti thermocycler (Applied Biosystems) under the following cycling conditions: 95 °C for 5 min; 30 cycles

of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; and 72 °C for 10 min. PCR products were purified with QIAquick PCR purification kit (Qiagen) and quantified with a NanoDrop 1000 spectrophotometer (Thermo Scientific). PCR amplicons were cycle-sequenced from both strands using the PCR primers and two internal primers: 300F (5'-CAA GTA CCG TGA GGG AAA GTT G-3') and ECD2 (5'-CTT GGT CCG TGT TGT AAG GG-3') (Tkach *et al*., 2003) using a BigDye Terminator v1.1 kit (AppliedBiosystem) according to manufacturer protocol on a Genetic Analyzer ABI 3500 (AppliedBiosystem). Contiguous sequences were assembled and base-calling differences resolved using SeqScape v.3. The consensus sequence obtained was compared with those available in GenBank database using Nucleotide BLAST tool.

Quantitative aspects

Prevalence (P), mean intensity (MI) and mean abundance (MA) were calculated according to Bush *et al*. (1997). The dispersion coefficient was calculated following Morales and Pino (1987) and the Clarke condition factor (KC) of the fish, $KC = (W / L^3) \times 100$. Comparison of prevalences between sexes was made through the Z statistic (Morales and Pino, 1987). Comparison of parasitic abundances between sexes was evaluated by the non-parametric Mann-Whitney U test and the respective correlations between the condition factor (KC) and the parasitic burden using Spearman's non-parametric correlation coefficient, r_s. Likewise, with this last coefficient, the effect of size on the accumulation of parasites was evaluated. The non-parametric methods were carried out with the help of the statistical package SPSS version 23.0. For all analyses the level of statistical significance was $\alpha = 0.05$.

Histopathological studies

The hearts of eight silversides were used for pathological investigation. Fresh hearts were fixed in 10% buffered formalin, dehydrated in ethyl alcohol, embedded in Paraplast©, sectioned at 5–7 µm, and stained with hematoxylin and eosin. Normal heart histology was performed with orcein stain in a hematoxylin background. Microphotographs were obtained using a Motic BA200 microscope.

RESULTS

From the total collected silversides, fifteen were males [total length 20.02 (17-24.5) cm; total weight 61.72 (33-109) g], and 25 were females [total length 28.25 (15.6-38) cm; total weight 202.24 (27-420) g]. Thirty-four cysts were isolated and measured.

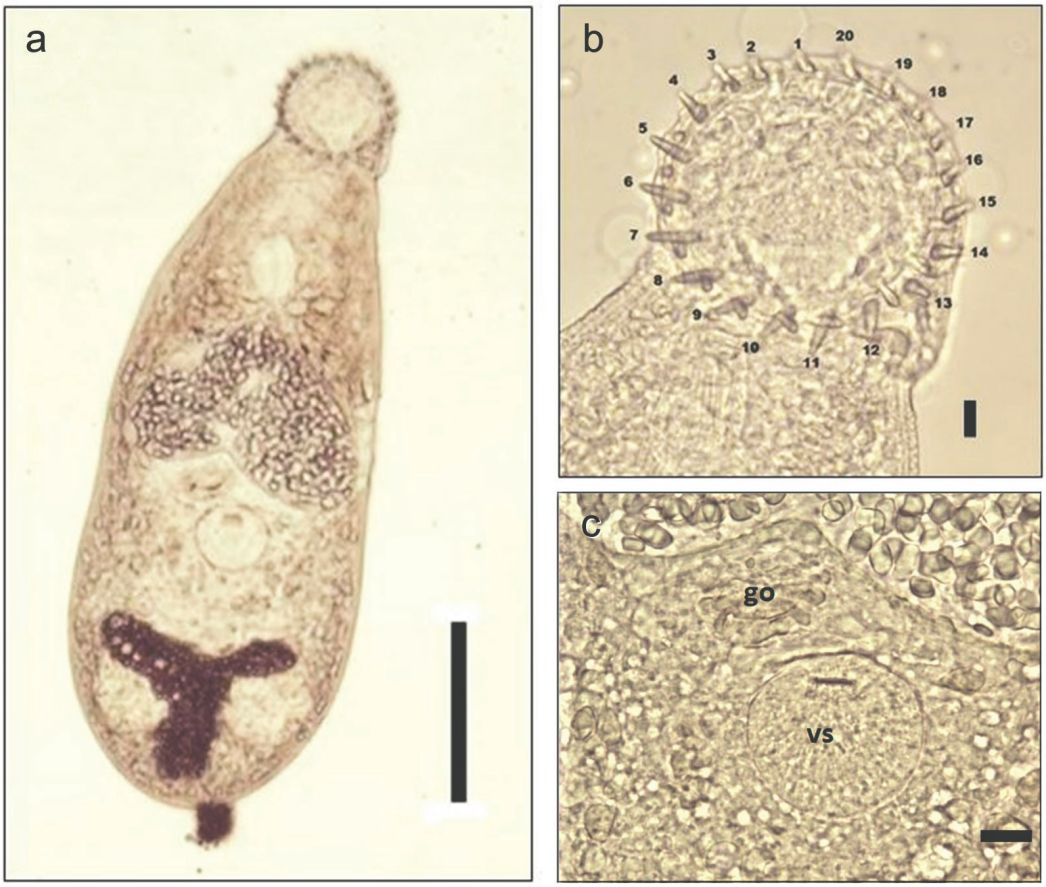

Figure 1. *Ascocotyle (A.) patagoniensis*, metacercaria, fresh. a) Metacercaria *in toto*. Scale bar= 0.25 mm. b) Oral sucker showing the double spiny crown, fresh. Scale bar= 0.01mm. c) Gonotyl (go) and ventral sucker (vs). Scale bar= 0.01mm

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Morphological description of *Ascocotyle* (*A.*) *patagoniensis* metacercaria (Fig. 1 a-c)

Cysts slightly oval 200 (125–260) in diameter, thin-walled (5–8 thick), smooth and weakly adhered to the surrounding tissue. Body piriform covered by minute tegumentary spines (Fig. 1 a). Total length 780 (690-800), maximum width 210 (195-230) at level of the hindbody. Oral sucker rounded, 85 (74-90) in length by 87.5 (85-92.5) in width, projects a digitiform expansion in the shape of a dorsal lip and a conical posterior appendage 230 (200-350) in length. It has a double crown of 40 spines of different sizes, grouping into 20 spines in each circle. The spines are blunt with sharp free end. Those in the anterior circle measured 13.0-16.25 in length, while the posterior ones were slightly shorter, 10-13 in length (Fig. 1 b). Muscular and robust pharynx, 45 (40-50) in length by 55 (48-60) in width and two wide and short intestinal ceca, loaded with refracting discoid crystals in fresh. Ventral sucker smaller than the oral one, 63 (55-65) in length by 60 (55-75) in width. At its anterior edge opens the common genital pore connected to a simple and large gonotyl, without refractile bodies and with five to seven folds on posterior margin (Fig. 1 c). Two testes in parallel situated near posterior end, similar in size, 70-110 in diameter, separated by a large excretory vesicle Y shaped. In fresh, ovary was not clearly observed.

Identification at molecular level

The consensus sequence obtained (671 bp.) was deposited in GenBank under the accession number ON357880. It was compared with those available in GenBank (15 sequences) obtaining a 97.17% identity with *A. (A.) patagoniensis* (MK359081.1 – Query Cover 100%– and MK359082.1– Query Cover 100%), and 90.18% with *Ascocotyle cameliae* Hernández-Orts, Georgieva, Landete and Scholz, 2019 (MK359080.1 – Query Cover 97%).

Quantitative aspects

Two out of 40 bulbus arteriosus were free of parasites (P = 95%). The prevalence of *A.* (*A.*) *patagoniensis* in silverside males was 100% (n=15), and 92% (n=25) in females. No significant differences were found on prevalence between males and females (Z = 1.85 p > 0.05). Most of bulbus arteriosus harbored between 20 and 40 cysts with 6 hosts hosting more than 120 parasites (maximum range of 226). The mean intensity was 68.2 metacercariae per host and the mean

Figure 2. Normal histology of a non-parasitized heart in sagittal section: a= atrium, ba= bulbus arteriosus, v= ventricle. Hematoxylin and eosin.

abundance was 64.8. The dispersion index on the total sample was 56.2 determining an aggregate dispersion pattern. Mean intensities were 51 metacercariae in males and 79.3 in females whereas mean abundances were 51 and 73 metacercariae per bulbus in males and females, respectively. No significant differences were observed in MI (U Mann-Whitney= 123.5 p>0.05), nor in MA (U Mann-Whitney= 153.5 p>0.05) between sexes. No statistically significant effect of the parasite burden on KC was observed (r_s = 0.146 p >0.05), and neither the host size effect on parasite accumulation (r_s = 0.158 p>0.05).

Normal heart histology of *Odontesthes argentinensis*

The bulbus arteriosus is a thick-walled chamber which contains no cardiac muscle and functions as a passive elastic reservoir during the cardiac cycle (Fig. 2). The bulbus surface is an adventitia which includes densely packed collagen fibers and circulatory elements such as coronary vessels supplying blood to the underlying tissues. The media layer is the most developed, composed by elastic fibers and a smooth muscle arranged into circumferential and longitudinal bundles. This organization allows the bulbus mechanical function which can distend and recoil elastically to assist blood flow into the ventral aorta. The innermost layer consists of a complex trabecular endothelial tissue continuous with the ventricular endocardium. No coronary irrigation on the media layer or the inner wall is present.

Histopathological effects of metacercariae of *A.* (*A.*) *argentinensis* in the bulbus arteriosus of *O. argentinensis*

All metacercariae were alive and encysted into the inner wall from the bulbus arteriosus. Each cyst contained only one parasite. The bulb distal area was the most densely parasitized site towards the ventral aorta, and probably the site where the infectious cercariae first arrived (Fig. 3). A protective protein cover of 5-7.5 thickness surrounds the metacercaria, and a discrete or almost unapparent infiltrate of inflammatory cells encloses the cyst (Fig. 4). Both the tissue debris and eosinophilic amorphous fluid complete the inflammatory zone. This conforms a loose envelope of connective tissue holding the cysts.

DISCUSSION

The trematode life cycles of the Heterophyidae family complex involve aquatic snails and fish as intermediate hosts, and mammals or piscivorous

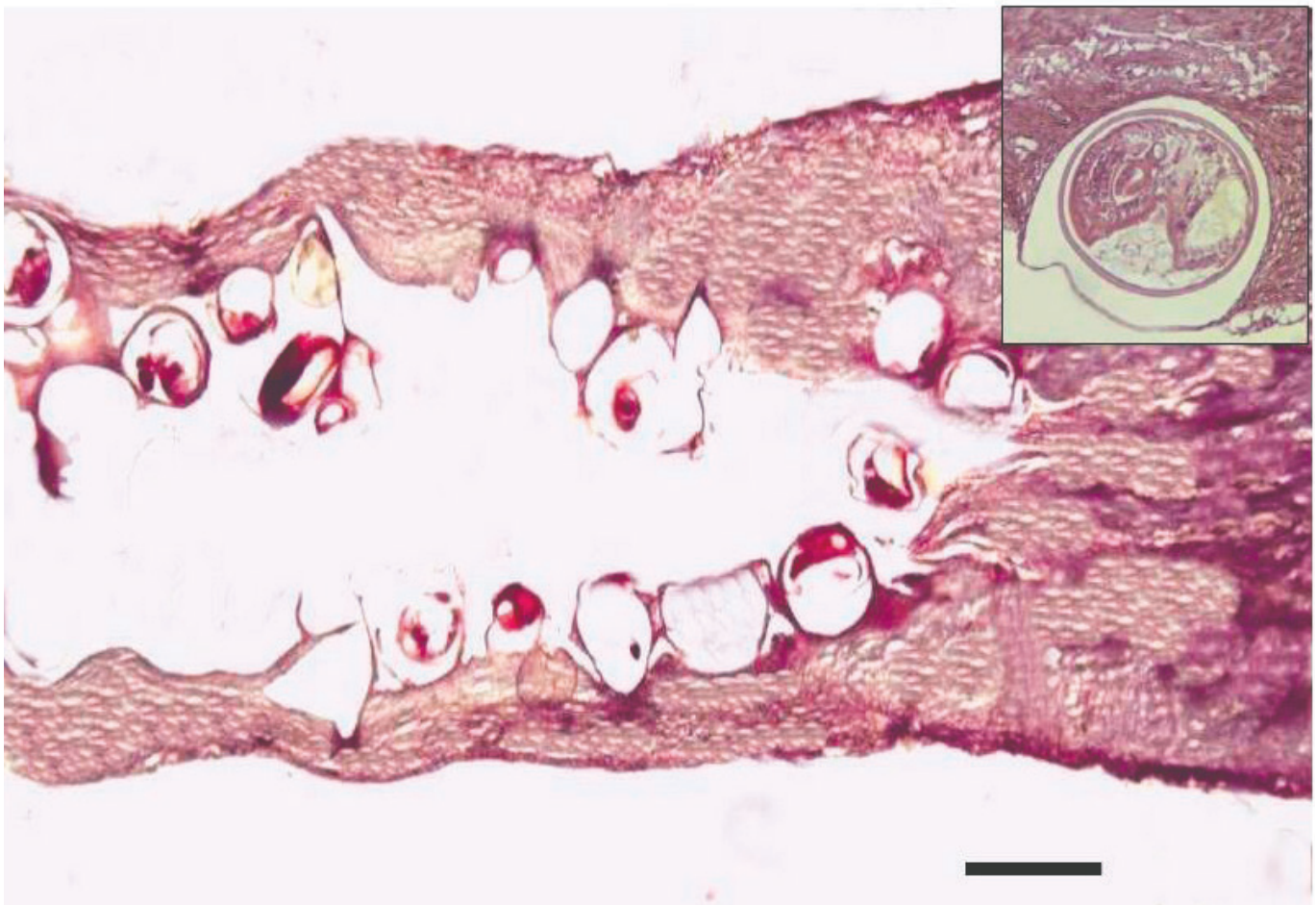

Figure 3. Bulbus arteriosus parasitized by *Ascocotyle* (*A.*) *patagoniensis* metacercariae in sagittal section showing the loose envelope. Scale bar= 0.25mm. Detail of a cyst and the loose envelope (upper right box).

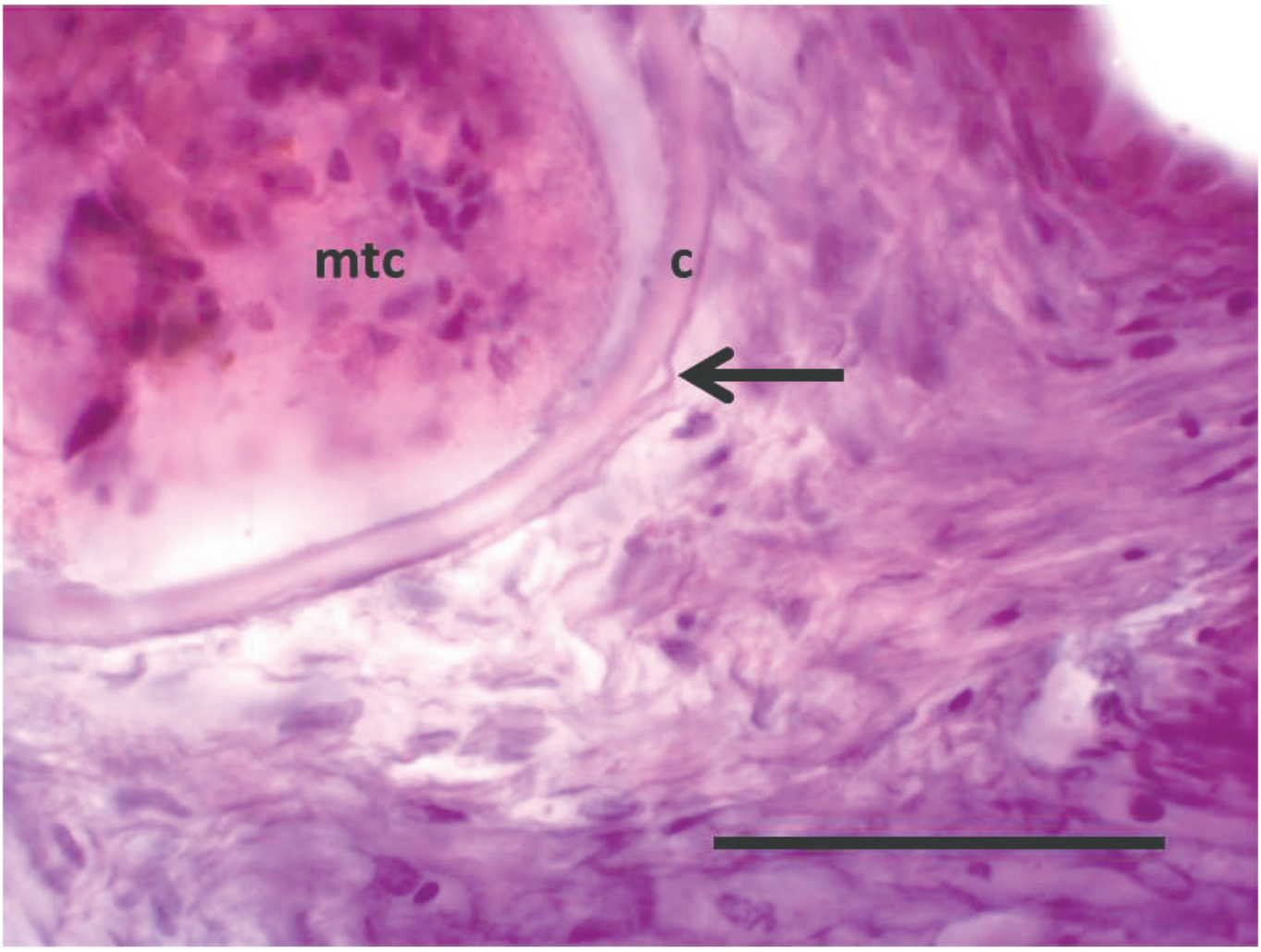

Figure 4. Host-parasite interphase showing the metacercaria (mtc), its cystic wall (c) and the surrounding thin fibrotic layer (arrow). Scale bar= 0.05mm

birds as definitive hosts (Malek, 1980). Swimming *Ascocotyle* spp. cercariae colonize fish hosts by the respiratory current. Once there they enter into the blood stream by penetrating the vascular tissue and encyst as metacercariae in different organs (e.g. gills, heart, mesenteries, musculature or gut wall) (Scholz *et al*., 2001, Hicks and Steele, 2003). In the present study, we confirmed that the species parasitizing the bulbus arteriosus of *O. argentinensis* from the Bahia Blanca estuary is *A. (A.) patagoniensis*, by using morphological and molecular methods.

Although neither adults nor metacercariae show host specificity, larval stages of many *Ascocotyle* species are located in well-defined microhabitats. *Ascocotyle (A.) tenuicollis* (Price, 1935), *Ascocotyle pachycystis* (Schroeder and Leigh, 1965), and *Ascocotyle (A.) leighi* (Burton, 1956) parasitize the bulbus arteriosus of cyprinodontiform fish (Sogandares-Bernal and Lumsden, 1963; Ostrowski de Núñez, 1974; Coleman and Travis, 1998). Instead, *A. (Phagicola) longa* (Ransom, 1920) encysts in gills, liver, heart, and skeletal musculature of mullets *Mugil* spp. (Scholz, 1999).

Ascocotyle (A.) patagoniensis is distributed in high prevalence -more than 86%- into the heart of both *O. argentinensis* and *O. smitti* (Hernández-Orts *et al*., 2019, Levy *et al*., 2019) indicating that silversides are good intermediate hosts for this parasite species, as reflected in the present work and precedent records.

Silverside females were longer and wider than males. Santoro *et al*. (2014) stated that the larger size of females on the Antarctic fish *Chionodraco hamatus* (Lönnberg) (Channichthyidae) would be related to its heavier parasite burden since a large body host would have more space, more energy flux, and more microhabitats for parasites than a smaller host. Nevertheless, the present study showed that the infection of *A. (A.) patagoniensis* occurs along the size range and without sex preferences. Also, the infection does not affect the body condition of hosts.

Only the inner wall from the bulbus arteriosus was occupied by the encysted metacercariae no affecting other tissues on the examined hosts. Histological sections showed that the metacercaria secretes its own cystic wall and the host connective tissue surrounds the parasite by a thin reaction. Perhaps,

the fish weak inflammatory response coupled with the survival of 100% of the larvae, explains the high concentration of parasites in a space as small as the bulbus arteriosus distal half. In this sense, Hicks and Steele (2003) did not find evidence of tearing or other damage in the bulbus arteriosus wall of *F. heteroclitus* affected by *A. tenuicollis* metacercariae. Likewise, the lack of a coronary supply from both the bulbar media and inner convoluted boundary would explain the low immune capacity of this chamber.

Although there is evidence that metacercariae provoke different degrees of inflammatory reactions in several fish organs (Orecka-Grabda, 1991, Watson *et al*., 1992, Prasanna Vankara and Chikkam, 2013), the present results (i.e. absence of negative effects on the body condition, weak inflammatory process) suggest that the bulbus arteriosus of the silverside *O. argentinesis* represents an optimal ecological niche for *A. (A) patagoniensis* metacercariae.

The high prevalence of helminth infections in both humans and animals reflects their ability to manipulate the host systems inhibiting their capability to reject the infective stages (McSorley and Maizels, 2013). The latter could be a topic for future investigations on digenean parasites of fish.

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