

# Short communication: first report of nematodes parasitizing the four-eyed-fish, *Anableps anableps* (Pisces, Cyprinodontiformes)

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**Abstract** The genus *Anableps* is composed of species *Anableps anableps*, *Anableps dowi*, and *Anableps microlepis*. These fishes are tropical and usually live on the surface of brackish water, being popularly known as four-eyed-fishes due to the presence of prominent eyes and a pupil split horizontally. *A. anableps* and *A. microlepis* are considered as sister species that live in sympatry in South America. *A. dowi*, however, is restricted to the Pacific Ocean (Central America) and is considered the most primitive species of this genus. The aims of this study were to investigate the presence of endoparasites in *A. anableps* from the Parnaíba's Delta and characterize them morphologically. During the necropsy, larvae of *Contraecaecum* sp. in the third larval stage (L3) were collected from the pancreas of *A. anableps*, but no endoparasites were observed in other organs. The worms had a cuticular tooth and excretory pore located at the anterior end, a thread like body, whitish color, and without distinction of sex. The length of the ventricular appendix of the larvae was much greater than in other studies. This is the first report of endoparasitism in *A. anableps* and the first report of nematodes in four-eyed-fishes.

**Key-words** *Anableps* · *Contraecaecum* · Four-eyed-fish · Larvae · Nematode

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## Introduction

The largest viviparous fish of the order Cyprinodontiformes are represented by species of the genus *Anableps*. They are popularly known as four-eyed fish due to the presence of prominent eyes with pupils split horizontally. These features allow the monitoring of both water and air environments simultaneously and the efficient exploitation of shallow environments (Miller 1979; Santos 1987; Bone et al. 1995). Three species are allocated in the genus *Anableps* Scopoli, 1777: *Anableps anableps* (Linnaeus, 1758), *Anableps microlepis* Müller and Troschel, 1844, and *Anableps dowi* Gill, 1861. Among these, the first two are considered sister species and live in sympatry in northern part of South America. However, *A. dowi* occurs only in the Pacific Ocean (Central America), and it is considered the most primitive species of the genus (Ghedotti 2003). According to Cervigón et al. (1993), the distribution of *A. anableps* extends from the Gulf of Paria in Venezuela to the Amazon River Delta, Brazil. *A. microlepis* can be found in South America, on Trinidad island and coastal basins from Venezuela to the Delta of the Amazon River, Brazil (Reis et al. 2003). According to published reports, observations from fish collections revealed the presence of *A. anableps* and *A. microlepis* at the delta of the Parnaíba River, in the state of Piauí, Brazil. *Anableps* spp. are omnivorous fish with a diet consisting of macroalgae, insects, small crustaceans, and suspended particulate matter (Brenner and Krumme 2007).

Studies on parasitic fauna of four-eyed-fish are extremely scarce. Only one species of endoparasitic helminth has been reported to date, *Amapacanthus amazonicus* Salgado-Maldonado & Santos, 2000 (Acanthocephala), which parasitizes *A. microlepis* from the Maracá island in the state of Roraima, Brazil (Salgado-Maldonado and Santos 2000). In addition, there is only one report of ectoparasitism by

Gnathiidae praniza larvae in *A. anableps* from the city of Bragança, state of Pará, Brazil (Diniz et al. 2008).

## Materials and methods

### Sampling and handling

Twenty-eight *A. anableps* were captured in the Parnaíba River delta, located in the city of Luis Correia, State of Piauí, Brazil, during the night, when the fish are less active. The fish were caught by local fishermen on the banks of the mangrove channels using circular fishing net (flue net type) with 20-mm mesh. Shortly after collection, the fish were packed in insulated boxes containing ice. Later, still in the collection site, the fish were fixed in 10% formalin and stored in plastic containers for subsequent analysis. At the laboratory, the fish were identified by morphological characters, biometric data were analyzed (weight, total length, and standard length), and the sex of each individual was determined. All data were recorded in autopsy forms.

Necropsy procedures, collection, fixation, conservation, and clarification of the parasites were performed according to Amato et al. (1991) and Eiras et al. (2000). Internal organs were placed in Petri dishes, separately, with 0.65% saline solution, dissected, and observed by stereomicroscopy. After removal of the viscera, the coelomic cavity was washed with distilled water. The wash water was passed through a 0.25-mm-mesh sieve. The retained material was collected and placed in a Petri dish containing 0.65% saline solution and examined by stereomicroscopy. Parasite indices (prevalence, mean intensity, and mean abundance) were calculated as described by Bush et al. (1997).

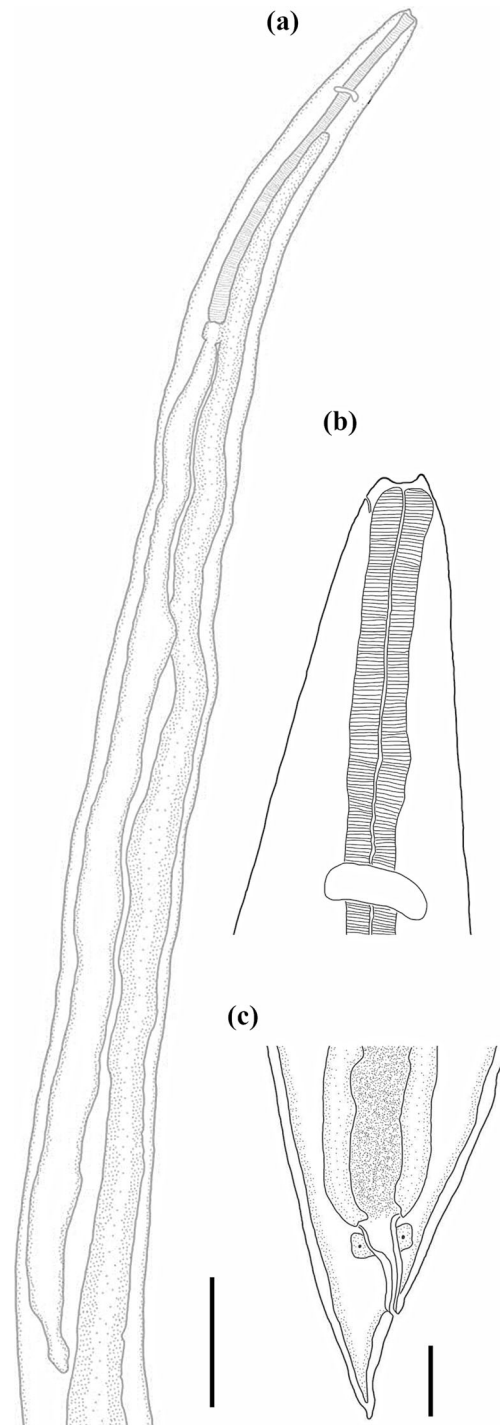
### Light microscopy

A portion of the collected nematode larvae was preserved in 70% ethanol, and then clarified in lactophenol between a slide and coverslip.

Measurements were taken to the nearest micrometer (range (mean  $\pm$  S.D.)) of six third larval stage specimens isolated from different fishes. Measurements were performed with an Axioplan Zeiss light microscope (Carl Zeiss, Germany) equipped with a Canon Power-Shot A640 digital camera (Canon, China) and Zeiss AxionVision Sample Images Software (Carl Zeiss, Germany) for image analysis. Drawings of the parasites were performed with the aid of an Axioplan Zeiss light microscope (Carl Zeiss, Germany) equipped with a camera lucida and were digitized using Adobe Photoshop Elements 8.0 software with the aid of an Intuos4 Wacon® pen tablet (Wacon Co. Ltd., Japan).

### Scanning electron microscopy

Nematode larvae were fixed in 2.5% glutaraldehyde, 4% freshly prepared paraformaldehyde, 5 mM calcium chloride in 0.1 M cacodylate buffer, pH 7.2 and postfixed in 2% osmium tetroxide in 0.1 M cacodylate buffer. The



**Fig. 1** Hand drawing of third stage larvae of *Contracaecum* sp. collected from four-eyed-fishes, *A. anableps*. **a** Anterior region. Bar 425  $\mu$ m. **b** Cephalic end. Bar 100  $\mu$ m. **c** Posterior end. Bar 130  $\mu$ m

samples were dehydrated in an acetone series, critical point dried with CO<sub>2</sub>, sputter-coated with gold, and examined in a Zeiss EVO MA 10 scanning electron microscope (SEM) operating at 15 kV.

Representative specimens were deposited in the Helminthological Collection of the Instituto Oswaldo Cruz (CHIOC), Rio de Janeiro, RJ, under record number CHIOC no 38546.

## Results

Third larval stage (L3) parasites were encysted in the pancreas of 17 fish, of which nine were females and eight were males. During the necropsies, a total of 52 larval specimens were collected, of which 36 were collected from females and 16 from males. A prevalence of 61% of parasitism was observed in the analyzed fishes, with a mean abundance of 3.06 and mean intensity of 1.86 parasites per fish.

More larvae were collected from female fish (average intensity of  $4 \pm 4.85$ , ranging from 1 to 16 larvae), than male fish (average intensity of  $2 \pm 0.93$ , ranging from 1 to 3 larvae per fish).

According to the morphological characters, the larvae were identified as *Contracaecum* sp. (Figs. 1, 2, and 3). They possessed an intestinal cecum directed to the anterior end, and excretory pore located laterally, next to the mouth opening, prior to the nerve ring (Figs. 1 b and 3b).

The larvae were classified as third larval stage (L3) due the absence of a developed reproductive tract. Larvae present a filiform body, whitish in color, with tiny cuticular teeth at the

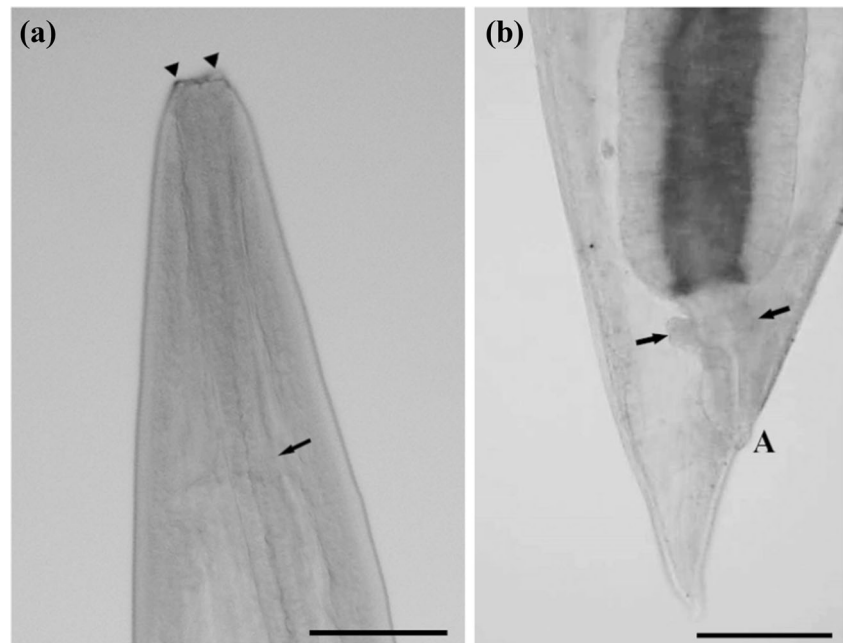
anterior end, with a delicate contour and triangular shape (Figs. 1b, 2a, and 3). Total length ranged from 9496.6 to 12,717.6 ( $11,065.5 \pm 1261.6$ ); greater body width was observed at the posterior third ranging from 327.4 to 547.9 ( $425.2 \pm 68.0$ ); distance from the nerve ring to anterior end ranging from 310.0 to 328.9 ( $314.7 \pm 11.0$ ). Muscular esophagus measured 1079.7 to 1571.9 ( $1214.2 \pm 190.9$ ); intestinal caeca directed to the anterior end, measuring 586.5 to 849.6 ( $717.9 \pm 109.5$ ); presence of a long ventricular appendix measuring 3221.7 to 4001.2 ( $3116.1 \pm 321.1$ ). Intestinal caeca: ventricular appendix ratio about 1:5 (Fig. 1a). Tail with conical end without caudal spine (Fig. 1c) measuring 130.5 to 174.3 ( $158.1 \pm 17.2$ ). Body width at anus level ranging from 79.4 to 111.8 ( $96.9 \pm 11.1$ ).

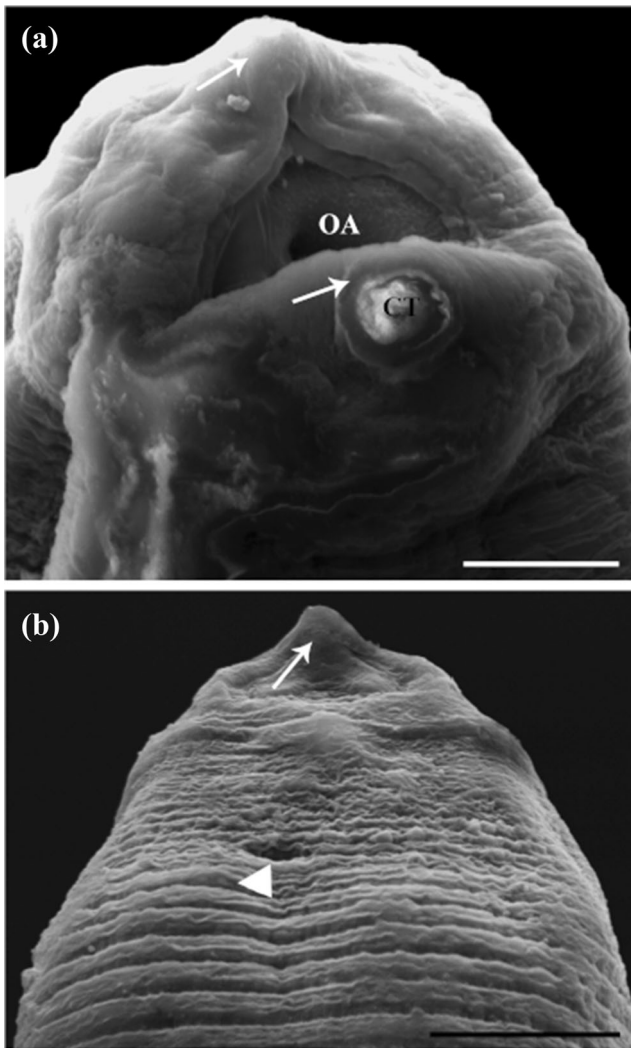
## Discussion

This study is the first report of endoparasites in *A. anableps*, and is also the first report of nematodes parasitizing fish of the genus *Anableps*. Previous studies only described ectoparasites isolated from this same fish species (Diniz et al. 2008; Martins et al. 2005) and a species of Acanthocephala in *A. microlepis* (Salgado-Maldonado & Santos, 2000). Thus far, there has been no report of parasitism in *A. dowi*.

The nematode larvae of *Contracaecum* are similar to those from *Hysterothylacium* by having the intestinal cecum directed to anterior end and a well-developed ventricular appendix. However, these two genera can be differentiated by the location of the excretory pore: in *Contracaecum* spp. it is located at the anterior end, near the oral aperture, following the cuticular tooth; in *Hysterothylacium* spp. the excretory pore is

**Fig. 2** Light microscopy of third stage larvae of *Contracaecum* sp. collected from four-eyed-fishes, *A. anableps*. **a** Anterior end lips (arrow heads) and nerve ring (arrow). Bar 100  $\mu$ m. **b** Posterior end showing rectal glands (arrows) and anus (A). Bar 150  $\mu$ m





**Fig. 3** Scanning electron microscopy of anterior end of third stage larvae of *Contracaecum* sp. collected from four-eyed-fishes, *A. anableps*. **a** Anterior extremity showing lips (arrows) and oral aperture (OA). Bar 10 μm. **b** Ventral view of anterior region, showing lip (arrow) and aperture of excretory pore (arrow head). Bar 20 μm

located in the esophagus region, at the level of nerve ring (Køie and Fagerholm 1995). According to these authors, it can be inferred that the larvae collected in this study belong to the genus *Contracaecum*. In addition to the morphological characteristics common to these two genera, the larvae observed in the present study have the excretory pore in the anterior region, which can be confirmed both by the scanning electron microscopy (Fig. 3b) and light microscopy (Fig. 1b).

Morphological analysis of larvae collected in this study did not present a developed reproductive system. For this reason, these parasites can be considered as third larval stage (L3). Moreover, according to Huizinga (1967), the second larval stage (L2) are thin, elongated, possess a cuticular tooth present on the ventral side of the buccal opening, have an undeveloped intestinal cecum, and esophagus, ventricle, and nerve ring with incomplete development, being difficult to be

visualized with an magnification of  $\times 400$ . In addition, L2 possess a thin intestine containing granular material inside.

The identification of the collected larvae at the species level was not possible, since the specific identification of nematodes is performed based on a set of morphological characters of the reproductive system, together with the morphometry of male and female specimens (Barson 2004). Moreover, there is a lack of studies describing the morphology and ultrastructure of nematode larvae. To identify L3 larvae, molecular biological studies are required, but in this case were not available.

The length of the ventricular appendix of the larvae of *Contracaecum* that were collected in this study was much greater than that of other parasites of the same genus isolated from different fish species (Yamaguti 1954; Martins et al. 2005; Pardo et al. 2009; Shamsi and Aghazadeh-Meshgi 2011). The only exception to this are larvae collected from *Otolithes* sp. (Bloch & Schneider, 1801) which presented a length ranging from 3000 to 4600 μm (Yamaguti 1954) very close to that found in our study (3221.7 to 4001.2 μm) (Table 1).

The ratio between the total length of the larvae of *Contracaecum* found in the present study and the length of their ventricular appendix is greater than that described in other parasites of the same genus isolated from other fish species (Table 1). The larvae studied by Yamaguti (1954) in *Otolithes* sp. displayed a lower ratio between body length and the ventricular appendix than that observed in our study (1:4 and 1:3, respectively). This ratio is the closest to that of the larvae collected from *A. anableps*. Martins et al. (2005) and Pardo et al. (2009) found much lower ratios between the total body length and the length of the ventricular appendix in *Contracaecum* larvae collected from *Hopliuserythrinus unitaeniatus* (Spix & Agassiz, 1829) (1:42) and *Soribim cuspicaudus* Littmann, Burr & Nass (2000) (1:53). Medians proportions of the characteristics described above were reported in larvae collected from *Synagris taeniopterus* (Yamaguti, 1953) (1:17), *Saurida gracilis* (Quoy & Gaimard, 1824) (1:11), *Chorinemus moadetta* Cuvier, 1832 (1:11) (Yamaguti 1954), *Barbus* spp. (Cuvier & Cloquet, 1816) (1:26) (Shamsi and Aghazadeh-Meshgi 2011) when compared to the present study (Table 1). Variability in the length of the ventricular appendix in L3 between species of *Contracaecum* is observable. This characteristic can be used to help identify species of infective larvae. Klöser et al. (1992) distinguished two types of larvae according to total body length and the ratio of the length of the esophagus and intestinal cecum, although no other morphological differences between the two types of larvae were identified.

The body length of L3 of *Contracaecum* sp. isolated from *A. anableps* presented a total length longer than that of parasites of the same genus isolated from other fish species, with exception of larvae collected from *H. unitaeniatus* (Table 1). The total length of the larvae is not a reliable characteristic for

**Table 1** Measurements in microns of main characters of infective larvae of *Contracaecum* spp. from fishes

	<i>Synagris taeniopterus</i> <sup>1</sup>	<i>Saurida gracilis</i> <sup>1</sup>	<i>Chorinemus moadetta</i> <sup>1</sup>	<i>Otolithes</i> sp. <sup>1</sup>	<i>Barbus</i> spp. <sup>2</sup>	<i>Hoplerhythrinus unitaeniatus</i> <sup>3</sup>	<i>Sorubimcuspicaudus</i> <sup>4</sup>	Present study
Body total length (TL)	7300	3300	5350	9800–18,300	19,260	12,000–34,500	17,920	11,379.3 ± 1118.5 (9496.56–12,717.6)
Esophagus	730	750	630	700	1980	1870–2610	1100	1214.2 ± 190.8 (1079.6–1571.9)
Ventriculum	60	75	66	90	110	60–120	90	–
Ventricular appendix (VA)	440	290	500	3000–4600	740	400–720	340	3616.1 ± 321.1 (3221.7–4001.1)
Ratio TL:VA	1:17	1:11	1:11	1:4*	1:26	1:42*	1:53	1:3
Intestinal caecum	420	120	150	120–180	1450	1200–2150	1170	717.9 ± 109.5 (586.5–849.6)
Tail	100	120	190	140–240	120	100–220	150	158.1 ± 17.2 (130.5–174.3)
Caudal spine	–	–	+	–	+	–	–	–
Cuticular tooth	+	+	–	+	–	+	+	+

\*The medians between the minimum and maximum values were used for the ratio of the body total length:ventricular appendix

<sup>1</sup> Yamaguti (1954)

<sup>2</sup> Shamsi and Aghazadeh-Meshgi (2011)

<sup>3</sup> Martins et al. (2005)

<sup>4</sup> Pardo et al. (2009)

distinguish species, since it continues its growth inside the fish, and where it can pass its whole life cycle (Køie and Fagerholm 1995; Garbin et al. 2013).

Worms collected from *A. anableps* presented cuticular teeth, but caudal spines were absent. Yamaguti (1954) and Shamsi and Aghazadeh-Meshgi (2011) described larvae of *Contracaecum moadettae* Yamaguti, 1954, collected from fish of the genus *Barbus* without cuticular tooth (Table 1), but with a visible caudal spine.

The current study has revealed that *A. anableps* participate in the epidemiological chain of at least one species of *Contracaecum*, since this parasite has a heteroxenous life cycle. Further studies are necessary to identify the *Contracaecum* larvae observed in *A. anableps* at the species level.

#### Compliance with ethical standards

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