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Morphological characterization of *Eustrongylides* sp. larvae (Nematoda, Dioctophymatoidea) parasite of *Rhinella marina* (Amphibia: Bufonidae) from Eastern Amazonia

Caracterização morfológica de larva de *Eustrongylides* sp. (Nematoda, Dioctophymatoidea) parasita de *Rhinella marina* (Amphibia: Bufonidae) da Amazônia Oriental Brasileira

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Abstract

Eustrongylides spp. nematodes have birds as final hosts and uses other vertebrates as intermediate/paratenic host (fish, amphibians and reptiles) and have zoonotic potential. In amphibians, the larvae may be located in the subcutaneous tissues, liver and mesentery, between the muscle fibres, especially in the lower limbs. *Rhinella marina*, which is widely observed in Brazil, has exhibited complex diversity in its helminth fauna, reflecting the unique habitat of the Amazon biome. For the first time, this study describes the morphological aspects of third-stage larvae of *Eustrongylides* sp. in *Rhinella marina* from Santa Cruz do Arari, Marajó Archipelago, Eastern Amazonia, using light and scanning electron microscopy.

Keywords: Wild life parasites, helminth of amphibians, nematode larva, *Eustrongylides*.

Resumo

Os nematoides do gênero *Eustrongylides* tem as aves como seus hospedeiros definitivos e utilizam outros vertebrados como hospedeiros intermediários/paratênicos (peixes, anfíbios, répteis), além de apresentar potencial zoonótico. Em anfíbios, as larvas podem estar localizadas em tecidos subcutâneos, fígado, mesentério e entre fibras musculares de membros inferiores. *Rhinella marina*, anfíbio o qual é distribuído amplamente no Brasil, apresenta uma complexa diversidade em sua helmintofauna, refletindo o seu hábitat no bioma amazônico. O presente estudo descreve, pela primeira vez, os aspectos morfológicos das larvas de terceiro estágio de *Eustrongylides* sp. em *R. marina* de Santa Cruz do Arari, Arquipélago do Marajó, Amazônia Oriental Brasileira, utilizando-se microscopia de luz e microscopia eletrônica de varredura.

Palavras-chave: Parasitas e animais silvestres, helmintos de anfíbios, larva de nematoide, *Eustrongylides*.

The genus *Eustrongylides* Jägerskiöld, 1909 consists of three species: *Eustrongylides tubifex* Jägerskiöld, 1909, *Eustrongylides ignotus* Jägerskiöld, 1909 and *Eustrongylides excisus* Jägerskiöld, 1909, which are commonly found parasitizing birds, and uses fish, amphibians and reptiles as intermediate/paratenic hosts. These parasites also occasionally parasitise man and thus have zoonotic potential (XIONG et al., 2009). According to Xiong et al.

(2009), the life cycle of these parasites remains scarcely known. Nevertheless, studies have shown that these helminths use piscivorous birds as definitive hosts, oligochaetes as first intermediate hosts (L1 to L3 larval stage) and usually fish as second intermediate hosts (L4 larval stage), and also possibly amphibians and reptiles as second intermediate hosts or paratenic hosts (MORAVEC, 1994; LEZAMA & SARABIA, 2002; FRIEND & FRANSON, 1999).

Rhinella marina Linnaeus, 1758 is an amphibian commonly found in Brazil and presents a complex helminth fauna that reflects a unique diversity, especially in the Amazonia Biome. In the last six years, new taxa have been reported from this host (SANTOS et al., 2008, 2011; MELO et al., 2011; GOMES et al. 2013) together

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with the first report of *Ortleppascaris* (SILVA et al., 2013a, b). This study adds yet another component to this helminth fauna, for the first time describing morphological aspects of second-stage larvae of *Eustrongylides* in *R. marina* from the Marajó Archipelago, Eastern Amazonia.

In June 2009, 14 specimens of *R. marina* were obtained from a peridomestic area in the municipality of Santa Cruz do Arari (0° 39' 48" S 49° 10' 30" W), Marajó Archipelago, State of Pará, eastern Brazilian Amazon. Nematodes recovered from cysts on the serosa of the stomach and intestines were fixed in AFA (95% alcohol, 3% formaldehyde and 2% glacial acetic acid), dehydrated in an ascending series of ethyl alcohol, cleared in Aman's lactophenol and analysed under an Olympus BX41 light microscope coupled with a drawing tube. Measurements are given in millimetres, unless otherwise noted, and they are represented as mean and standard deviation with the range in parenthesis. Some nematode samples fixed in AFA were post-fixed in 1% osmium tetroxide, dehydrated to the CO₂ critical point, sputtered with gold and examined in a LEO 1459 scanning electron microscope from the René Rachou Research Institute, Oswaldo Cruz Foundation - Minas Gerais, Brazil.

Eustrongylides sp. (Second-stage Larvae)

Description (based on 10 specimens): Cylindrical body, surface with delicate transverse striations (Figures 1 and 2). Total length 8.68 mm ± 0.52 (7.46-8.46); total width at the end of the oesophagus 0.09 ± 0.004 mm (0.08-0.09). Cephalic extremity

presenting 15 sub-spherical papillae, arranged in two circles (an inner circle near the oral opening with three pairs of small papillae and an outer circle with four pairs of bulky papillae). The inner circle papillae, near the oral opening distributed into pairs of two lateral, two sub-ventral and two sub-dorsal. Four lateral, two sub-ventral and two sub-dorsal papillae in the external circle (Figures 1B, 2A-B). An additional papilla found between the two pairs of sub-ventral papillae of the outer circle (Figure 2B). Buccal capsule 0.52 mm ± 0.07 (0.42-0.66) × 0.18 mm ± 0.20 (12 to 5.5) followed by filarioid oesophagus 1.55 mm ± 0.14 (1.22-1.73) × 0.09 mm ± 0.09 (0.05-0.07) (Figure 1A-C). Nerve ring 0.80 mm ± 0.10 (0.54-0.91) from the anterior extremity. Excretory pore not observed. Terminal anus with rectal ampulla 1.65 mm ± 0.19 (1.28-2) in length (Figures 1C-D and 2C-D). Genital primordium, not observed.

Taxonomic Summary

Superfamily: Dioctophymatoidea Railliet 1915 (Roman 1965).

Family: Dioctophymatidae (Railliet, 1915).

Eustrongylides Jägerskiöld, 1909.

Eustrongylides sp. (Larvae).

Host: *Rhinella marina* (Amphibia, Bufonidae).

Infection site: Encysted on the serosa of stomach and intestines.

Location: Santa Cruz do Arari, Pará, Brazil (0° 39' 48" S 49° 10' 30" W).

Prevalence: 21.43% (3 of 14 hosts infected).

Range of infection: 3-7

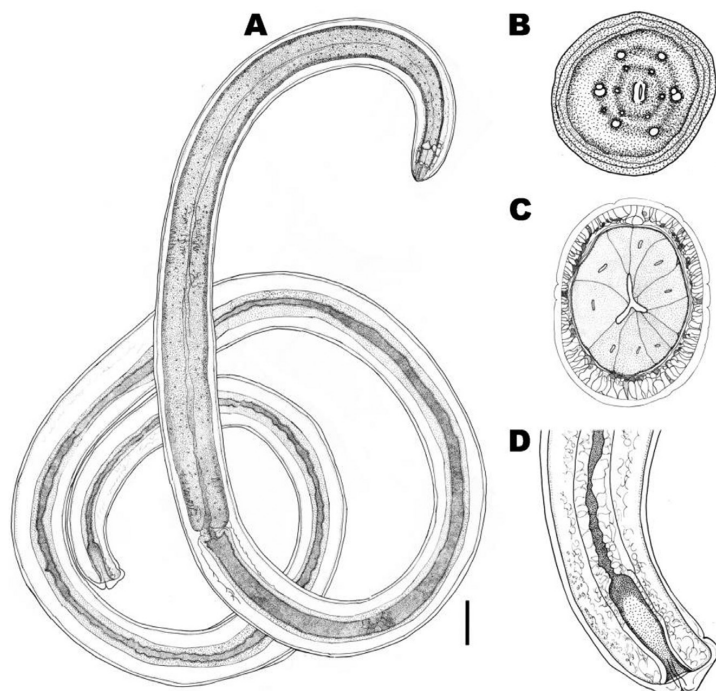


Figure 1. Line drawings of *Eustrongylides* sp. larvae found parasitizing *Rhinella marina* from Eastern Brazilian Amazon. (A) Nematode general view. Bar = 100 µm; (B) Detail of apical section, showing two small lips and the presence of two papillae circles: an inner circle and an outer circle. Bar = 50 µm; (C) Transverse section at oesophagus region, showing details of the lumen. Bar = 50 µm; (D) Posterior region, showing the terminal anus. Bar = 200 µm.

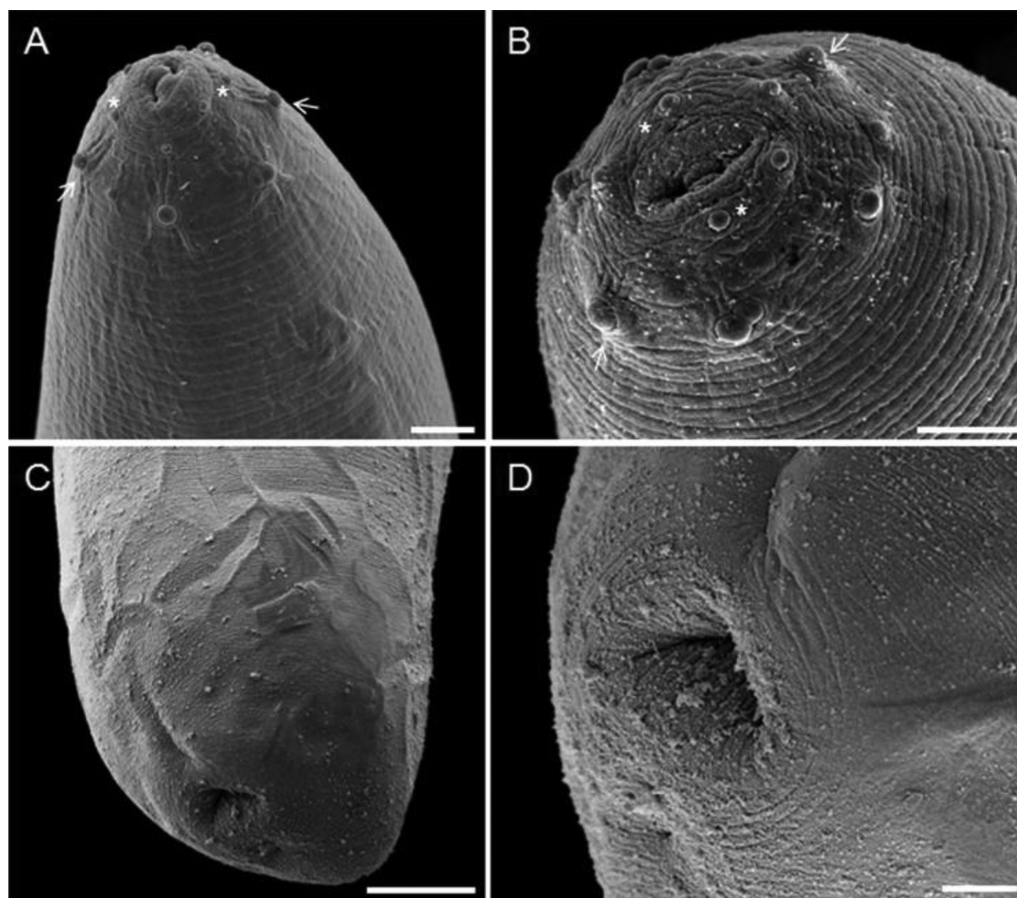


Figure 2. Scanning electron microscopy of *Eustrongylides* sp. larvae found parasitizing *Rhinella marina* from Eastern Brazilian Amazon. (A) Overview of the anterior portion of the nematode showing two small lips and the presence of two papillae circles: an inner circle (*) and an outer circle (arrows). Bar = 10 μ m; (B) Detail of anterior end, showing the papillae arrangement: the inner circle (*) with 6 papillae (3 pairs), consisting of 2 lateral, 2 sub-ventral and 2 sub-dorsal, and the outer circle (arrow) with 4 pairs of papillae, 1 lateral pair, 1 dorsal pair and two sub-ventral pairs, with an odd papilla between the two pairs of sub-ventral papillae. Bar = 5 μ m; (C) Posterior region showing the terminal anus. Bar = 20 μ m; (D) Detail of the terminal anus. Bar = 5 μ m.

The encysted larvae on the serosa of stomach and intestines of *R. marina* from the municipality of Santa Cruz do Arari, eastern Amazonia have characteristics of the genus *Eustrongylides*, including the presence of six cephalic papillae distributed in an inner circle, eight cephalic papillae distributed in an outer circle and a terminal anus. According to Anderson (2000) and Lezama & Sarabia (2002), these characteristics are important for diagnosis of the genus *Eustrongylides*.

The absence of genital primordium in the larvae examined characterises it as in the second developmental stage. According to Lichtenfels & Pilitt (1986), Moravec (1998) and Xiong et al. (2009), second-stage larvae may have remnant cuticle kept from the first moult at the posterior extremity; and absence of genital primordium; third-stage larvae differ from fourth-stage larvae as the latter present a larger genital primordium, different shape of inner papillae circle and differences in morphometry (fourth stage larvae are larger).

The cephalic papillae of both the inner and outer circles of the *Eustrongylides* sp. larvae of *R. marina* presented sub-spherical shapes with clear cuticle delineations. These data are different from those reported by Lichtenfels & Pilitt (1986) and Xiong et al.

(2009), who described cephalic papillae of the inner circle of *Eustrongylides* presenting large pointed apices, and outer-circle papillae with nipplelike apices.

Eustrongylides sp. larvae found in *R. marina* from the eastern Amazonia have larger outer circle cephalic papillae compared to papillae in the inner circle. Thatcher (2006) used the morphology of these papillae to differentiate *E. ignotus* and *E. tubifex* larvae. According to this author, fourth-stage larvae of *E. ignotus* have larger papillae inner circle cephalic papillae than those in the outer circle, the reverse is observed in *E. tubifex*. In addition, third-stage larvae of these two species have opposite characteristics. Thus, *Eustrongylides* sp. larvae, parasites of *R. marina*, are similar to *E. ignotus* larvae; however, they differ both in the shape of the cephalic papillae and their host, which are domesticated birds of the Anseriformes Order in the latter species.

The morphological characteristics described herein suggest that *Eustrongylides* sp. may be a different species from *E. ignotus* and *E. tubifex*; however, species identification involving larval stage comparisons is complex, given the somewhat confusing taxonomic status of species within the genus *Eustrongylides*. Then,

molecular determination of identity is very important as supported by Measures (1988).

This study is the first report of parasitism of second-stage larvae of the genus *Eustrongylides* in amphibians, and for *R. marina* (Bufonidae) from eastern Amazonia. Larvae of this genus have been reported in other amphibian hosts. Goldberg & Bursey (2002), Lezama & Sarabia (2002), Bursey & Brooks (2010) and Yildirimhan et al. (2012) reported parasitism by *Eustrongylides* in amphibians of the Ranidae family (Nearctic - California, U.S.A), and Kuperman et al. (2004) reported parasitism in amphibians of the Pipidae family (Nearctic - California, U.S.A). In Brazil, according to Vicente et al. (1991) *Eustrongylides* larvae were found in *Leptodactylus ocellatus* (Leptodactylidae) in the municipality of Salobras, State of Mato Grosso do Sul, Central-West Region of Brazil, in the Pantanal Biome. In fish hosts, those nematodes were first reported by Travassos et al. (1928) parasitising: *Callichthys callichthys*, *Pimelodus clarias*, *Synbranchus marmoratus*, *Salminus maxillosus*, *Pseudoplatystoma* sp., *Acesthrobranchius* sp., and *Poecilia vivipara*. Later, Rego & Vicente (1988a) reported in *Auchenipterus nigripinnis*, *Serrasalmus nattereri*, *Pirinampus pirinampu*, *Rhaphiodon vulpinus*, *Pseudoplatystoma fasciatum* and *Serrasalmus marmoratus* from Cuiabá River, MT; in *Hoplias malabaricus* from Pirassununga - SP (REGO & VICENTE, 1988b), Eiras & Rego (1988) found in *Paratrygon* sp., *Pygocentrus nattereri* and *P. corruscans* from Cuiabá River - MT; from Paraná river, Martins et al. (2009) reports *Eustrongylides* larvae in *Cichla piquiti*, *Plagioscion squamosissimus* and *Hoplias malabaricus*. In Amazonia, Benigno et al. (2012) described the occurrence of *Eustrongylides* larvae in the fish species: *Hoplerthrinus unitaeniatus*, *H. malabaricus* and *Pygocentrus nattereri* from Lake Arari (Marajó Archipelago, Pará) and Meneguetti et al. (2013) report *Eustrongylides* sp. in *Hoplias malabaricus* from Rondônia.

Eustrongylides larvae can cause a zoonotic infection known as human eustrongylidosis. The transmission occurs through the consumption of raw or undercooked fish meat and clinical presentations in humans may range from stomach inflammation to intestinal perforation requiring surgical removal of helminths (EBERHARD et al., 1989). Thus, records of the occurrence of *Eustrongylides* larvae in Amazon region are important, especially considering that the Eastern Amazon region has an extensive hydrographic basin and a large riverside population that use fish as their main food source.

Measures (1988) stated that the adult stage of nematodes of the genus *Eustrongylides* occurs mainly in Ciconiformes, Podicipediformes, Pelecaniformes, Gaviiformes and Anseriformes birds. Among these orders, *Eustrongylides* parasitism has been reported in Ciconiformes in Brazil; additionally, Vicente et al. (1995), reports some Ciconiformes bird parasitized by *Eustrongylides* in the Marajó Archipelago. Valente et al. (2011) described other piscivorous birds of the Accipitriformes (Pandionidae family) and Charadriiformes (Laridae and Sternidae families) in the same Archipelago. *Phalacrocorax brasilianus brasilianus* was found as a definitive host for *Eustrongylides* nematodes in South of Brazil (MONTEIRO et al., 2011), thus, as it is also a common fish-eating bird species in Amazon region, it should be considered as a potential definitive host for *Eustrongylides* nematodes in that area. These birds, due to their feeding habits, may participate in the biological cycle of *Eustrongylides* in the region. In addition, the

presence of migratory birds may facilitate dispersal among areas of the Amazonia region and to other countries in the Americas.

Then, as crocodiles, alligators, crows, white-faced herons, kites, eagles, bush stone-curlews, tawny frogmouths, water rats, giant white-tailed rats and snakes are natural predators of *Rhinella marina* (ZUG & ZUG, 1979; BRANDT & MAZZOTTI, 2009), we present here for the first time, the marine toad as a intermediate/paratenic host in the biological cycle of *Eustrongylides*. This is the first report of these nematodes in Bufonidae from Marajó Archipelago, eastern Amazonia and adds another species to the known helminth parasites of *R. marina* in Brazil and in the Neotropical Region.

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