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ON THE LIFE CYCLE OF *CUCULLANUS PINNAI PINNAI* (TRAVASSOS, ARTIGAS & PEREIRA, 1928) (NEMATODA: CUCULLANIDAE) PARASITE OF *RHAMDIA QUELEN* (QUOY & GAIMARD, 1824) (SILURIFORMES) FROM ITS SOUTHERN LOCALITIES

ACERCA DEL CICLO DE VIDA DE *CUCULLANUS PINNAI PINNAI* (TRAVASSOS, ARTIGAS & PEREIRA, 1928) (NEMATODA: CUCULLANIDAE) PARÁSITO DE *RHAMDIA QUELEN* (QUOY & GAIMARD, 1824) (SILURIFORMES) EN SU DISTRIBUCIÓN MÁS MERIDIONAL

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ABSTRACT

Despite the ubiquity of *Cucullanus* spp. in aquatic ecosystems around the world, their embryonic and post-embryonic development, as well as transmission patterns, remain little known and in some cases controversial. In the present study, comparative and embryological data of *Cucullanus pinnai pinnai* (Travassos, Artigas & Pereira, 1928) are provided. Adults from *Rhamdia quelen* (Quoy & Gaimard, 1824) (Heptapteridae) are described and illustrated by scanning electron microphotography. Eggs were obtained from gravid females and embryonic and larval development was followed *in vitro*. The L₂ and L₃ stages are described and illustrated for the first time. The embryonic development of *C. pinnai pinnai* goes very fast at 20-22°C. In five days, hatching takes place and the free L₂ remain alive for less than two weeks. A heteroxenic pattern of transmission is hypothesized involving *R. quelen* as definitive host and *Bryconamericus iheringii* (Boulenger, 1887) (Characidae) as the intermediate host.

Keywords: Cucullanus pinnai – freshwater fishes – life cycle – Nematoda – Rhamdia quelen

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RESUMEN

A pesar de la ubicuidad de *Cucullanus* spp. en los ecosistemas acuáticos de todo el mundo, su desarrollo embionario y post-embrionario, así como los patrones de transmisión, siguen siendo poco conocidos y, en algunos casos, controvertidos. En el presente estudio se proporcionan datos comparativos y embriológicos de *Cucullanus pinnai pinnai* (Travassos, Artigas & Pereira, 1928). Los nematodes adultos provenientes de *Rhamdia quelen* (Quoy & Gaimard, 1824) (Heptapteridae) se describen e ilustran mediante microfotografía electrónica de barrido. Se obtuvieron huevos de hembras grávidas y se siguió el desarrollo embrionario y larvario *in vitro*. Las etapas L_2 y L_3 se describen e ilustran por primera vez. El desarrollo embrionario de *C. pinnai pinnai* es muy rápido a 20-22°C. En cinco días tiene lugar la eclosión y las L_2 libres permanecen vivas menos de dos semanas. Se plantea la hipótesis de un patrón de transmisión heteroxeno que involucra a *R. quelen* como hospedador definitivo y *Bryconamericus iheringii* (Boulenger, 1887) (Characidae) como hospedador intermediario.

Palabras clave: Cucullanus pinnai - peces de agua dulce - ciclo de vida - Nematoda - Rhamdia quelen

INTRODUCTION

The family Cucullanidae Cobbold, 1864 (Ascaridida: Seuratoidea) includes parasitic nematodes from the digestive tract of marine, estuarine and freshwater teleosts, with some representatives in cyclostomes, elasmobranchs and occasionally in aquatic turtles (Anderson et al., 2009; Moravec & Justine, 2011). Cucullanus Müller, 1777, the type-genus, is the richest and most diverse in the family, with more than 100 species described worldwide, of which at least 32 parasitize marine and freshwater fish in the Neotropical region (Luque et al., 2011; Vieira et al., 2015). Despite their ubiquity in aquatic ecosystems around the world, their embryonic and post-embryonic development, as well as transmission patterns, remain little known and in some cases controversial (Moravec, 1979; Anderson, 2000). Valovaya (1978) conducted a detailed study of the embryonic development of Cucullanus cirratus Müller, 1777. Since then heteroxenous patterns have been experimentally demonstrated, involving an intermediate or paratenic host, or monoxenic mechanisms, in which the larvae undergo histotropic phase, in a single host (Moravec, 1979; Baker, 1984; Anderson, 2000; Choudhury & Cole, 2019). To date two records of Cucullanus sp. larvae, without descriptions, were recorded from the Neotropical region, parasitizing the gut of Hemibrycon *surinamensis* Géry, 1962 (Teleostei: Characidae)(Hoshino *et al.*, 2014) and the body cavity of *Bryconamericus iheringii* (Boulenger, 1887) (Teleostei: Characidae)(Tanzola *et al.*, 2019).

Cucullanus pinnai pinnai (Travassos, Artigas & Pereira, 1928) was described by Travassos et al. (1928) from Synodontis clarias (Linnaeus, 1758)(= Pimelodus clarias) on the Mogi-Guazú river, Sao Paulo state, Brazil. To date it is the taxon with the highest number of records in neotropical freshwater fishes, having been cited in 20 host species, mostly siluriforms of the family Pimelodidae (Moravec, 1998; Soares de Oliveira et al., 2015, Vieira et al., 2015, Chemes & Takemoto, 2020). The southernmost record of this cucullanid is the Napostá Grande stream, southwest of Buenos Aires province, parasitizing Rhamdia quelen (Quoy & Gaimard, 1824) (Heptapteridae) (Tanzola et al., 2009). Given the lack of morphological information of the adults from R. quelen as well as details of their embryonic development and possible patterns of transmission, the aim of this study was to provide comparative and development data of C. pinnai pinnai in the southernmost dispersion area. Also a description of a third stage larvae of *Cucullanus* sp. in the body cavity of *B. iheringii* (Characidae), a fish prey of *R*. quelen from the same habitat, is given and compared to the L_2 experimentally obtained.

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Study area, hosts sampling and dates

Three adults *R. quelen* (Siluriformes: Heptapteridae), 45 *B. iheringii* (Characiformes: Characidae) from Napostá Grande stream ($38^{\circ}42'39.37"S 62^{\circ}15'16.80"$ W) and 45 *B. iheringii* from Sauce Chico river ($38^{\circ}42'06.06"S$ $62^{\circ}27'28.11$ W), were caught with line and hook (*R. quelen*) and trawl net (*B. iheringii*). They were caught during late summer 2018 and late spring 2019. The hosts were transported alive to the laboratory and euthanized by overdose of an ethanolic benzocaine solution ($1g \cdot 30mL^{-1}$) (Gilderhus, 1990). The taxonomic status of the fishes follows Froese & Pauly (2019).

Collection of adult nematodes

The fish were dissected and the visceral mass extracted in NaCl saline solution. The intestine were dissected and the worms collected alive under stereoscopic microscope. They were washed in distilled water and fixed in 10% formaldehyde (at room temperature 20°C) or in hot 70% ethanol. Two males and two females were examined ultrastructurally by scanning electron microscopy (SEM). They were dehydrated in acetone, dried in a CO_2 critical point, metallized in gold, observed and photographied in a JEOL JSM T-100 scanning microscope at 15kv, at Museo de La Plata (Argentina). The measurements are given in μm , unless otherwise is stated, as a mean and range between parentheses. The nomenclature for the male caudal papillae follows Vieira et al. (2015). Voucher specimens (3 males, 3 females) of C. pinnai pinnai were deposited at the Helminthological Collection of the División Zoología Invertebrados del Museo de La Plata, accesion number MLP-He 7691.

Collection of eggs and monitoring of embryonic devolpment

Two gravid females were maintained at room temperature (20°C) in a Petri dish containing distilled water to allow their spontaneous oviposition (Moravec, 1979). Once the eggs were laid, they were monitored every 8-10 h under light microscope and photographied during the process, until the hatching of larvae. Larvae were fixed in 70% ethanol, measured and photographied. Ten larvae were collected in 96% ethanol to future molecular studies.

Collection of L₃ from *Bryconamericus iheringii*

Third stage larvae of a *Cucullanus* sp. were collected alive in the body cavity of "tetras" *B. iheringii*. They were extracted from the peritoneal tissue by mean of dissection needles, washed in saline solution and fixed in 70% ethanol. L_3 were measured and photographied using a Motic BA microscope.

Quantitative data

Parasitic indicators (prevalence, intensity and abundance) were calculated following Bush *et al.* (1997). Parasitic prevalences between sites were compared using the Z statistic for samples ≤ 100 hosts (Morales & Pino, 1987). Parasitic abundances between sites were tested using the U-Mann Whitney test (IBM SPSS Statistic 23).

Ethic aspects: The authors declare that they have no conflict of interest, that no experimental animals have been used and that the research did not produce negative impacts on people or the environment.

RESULTS

Description of adults *Cucullanus pinnai pinnai* Travassos, Artigas & Pereira, 1928

Male (n=5): Total length 7,254 (6,650-7,750) mm. Maximum width 240 (200-270). Oral opening dorso-ventral elongated with a cuticular collarete surrounding a border of about 80 denticules (Fig. 1a). Two submedian pairs of cephalic papillae. Small pair of lateral amphids between them. Oesophagus length 774 (730-850). Distance of the nervous ring to cephalic end 300 (300-300). Distance of deirids to the cephalic end 442 (390-510). Excretory pore was not observed. Tail 170 (150-190), curved ventrally. Precloacal sucker well developed, notorious in translucent specimens but inapparent under SEM images. Spicules equal 687,5 (570-800) semicircular in cross section, with distal end pointed and bent (Fig. 1b). Gubernaculum present, about 60 in length. Ten pair of caudal papillae arranged as follows: Precloacal five pairs, first one (sv1) in the frontal border of precloacal sucker (Su), second (sv2) in the

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posterior limit of the sucker, third (sv3) in midpoint between sv2 and cloacal opening, fourth (sv4) and fifth (sv5) close to cloaca. A little adcloacal subventral pair (ad). Four postcloacal pairs, two subventral (sv6, sv7) and two lateral (l1, l2). A little pair of phasmids (f) between to the last postcloacal papillae (l2 and sv7) slightly behind them. <u>Female (n=4)</u>: Total length 7,53 (6,35-8,82) mm. Maximum width 257,5 (220-280). Oesophagus length 827,5 (810-870). Distance of the nervous ring to cephalic end 302,5 (280-320). Distance of deirids to the cephalic end 550-550. Excretory pore not observed. Vulva postequatorial, distance to the anterior end 4,522 (3,400-5,770) mm, in the midventral line, slightly elevated (Fig. 1c). Tail 225,6 (212-230). Eggs in uterus 30 x 37,5-45, eggs, 32 x 55 once laid.



Figure 1. *Cucullanus pinnai pinnai* Travassos, Artigas & Pereira, 1928 adults from *Rhamdia quelen* (SEM). a- Oral end in frontal view (female), a= amphids, p= cephalic papillae; b= caudal end (male), f= phasmid, Su= precloacal sucker, svd= subventral right papillae, svi= subventral left papillae, ld= lateral right papillae, li= lateral left papillae; c= posterior region (female), vulva (white arrow), anus (black arrow), inbox: detail of vulva. Scale bars: 1a=0,05mm, 1b=0,10mm, 1c=0,20mm.



Figure 2. *Cucullanus pinnai pinnai*, embryonic and postembryonic development. a- unsegmented egg at oviposition; b= twocells embryo (Day 1); c= 16-cells morula (Day 2); d= L1 in egg (*: micropyle)(Day 4); e= newly hatched L₂ (inbox: detail of M1 (arrow))(Day 5); f: *Cucullanus* sp. L₃ from the body cavity of *Bryconamericus iheringii* (inbox: detail of the caudal end). Scalebars: Figs. 2a-e=0,05mm; 2f=0,10mm (inbox=0,05).

Remarks: The three hosts were parasitized by *C. pinnai pinnai* (with 20, 37 and 131 worms, respectively). This fact allowed us to hypothesize that it is a common parasite of *R. quelen* in the area.

Observations on the embryonic development and free larvae

Gravid females spontaneously began to lay eggs immediately placed in distilled water. The eggs were laid in the one- or two-cell stage, mostly in small groups of 4-6 or individually (Figs. 2a, 2b). They were oval, $45-55 \times 30-32,5$, thin shelled with a visible mycropile at one of the poles. Apparently, a thin vitelline cover is present and holds the eggs together or allows them to adhere to solid substrates (i.e. the glass of a Petri dish or perhaps the leaves of submerged vegetation in the natural environment). The observation of two cellular size in the 2-cell stage (S1 and P1) leads us to think that the cleavage model responds to that typical of Secernentea, that is, of the determinated type. Cleavage splits happen quickly and about 24 h a 16-blastomers morula is formed (Fig. 2c). The gastrula was not observed but in 72-96 h the L₁ stage is visible inside the egg and showed slow movements (Fig. 2d). A molt could not be observed in this stage but at day 5 the larvae began to hatch and move actively and twisted. These larvae are covered by a thin envelope considered as a moult M1 (Fig. 2e). So, the larvae are in the L2 stage when hatch. They measured 211-275 in total length and 10-12,5 in maximum width. The oesophagus occupies about 40% of the total length and the intestinal joining is visible. They could not be observed neither the nervous ring nor the excretory pore. The intestine is loaded with a granular content and the anus is a conspicuous knob that marks the origin of the pointed tail, about 26-33 in length. Second stage larvae remained active till day 12-14 and then all died. No growth in body mass was detected during the free stage. It seems that L_2 doesn't feed in free stage and depends on the embryonic nutritional reserves.

Description of Cucullanus sp. L_3 from Bryconamericus iheringii

A sample of *B. iheringii* from Napostá Grande stream and Sauce Chico river presented in the body cavity early third-stage larvae completely enclosed in a sheat, the moult of the L_2 (Fig. 2f). From the following combination of characters, they were considered to belong to *Cucullanus* sp. Total length

1,100-1,259 mm and 40-45 in maximum width. At the cephalic end they showed a mouth opening as a dorso-ventral groove surrounded by a cuticular collarete in formation. Oesophagus about 330 in length, sligthly club-shaped, with a dense esophageal gland near the oral opening. Nervous ring approximately 130 to the anterior end. Excretory pore immediately behind the nervous ring level. Tail conical elongated, 130-140 in length. They were found moving freely between the perivisceral peritoneum with no reactive capsule from the host.

Quantitative data of *Cucullanus* sp. L₃ from *Bryconamericus iheringii*

Prevalence 48,7% (Naposta Grande stream, n=45) and 35,5% (Sauce Chico river, n=45)(Z= 1,023 p>0,05). Mean abundance 2,24 (Naposta Grande stream, n=45) and 1,40 (Sauce Chico river, n=45)(U Mann Whitney= 7,00 p>0,05). This L_3 was a component species of the helminth assemblages of *B. iheringii* from both environments.

DISCUSSION

Moreira et al. (2014) highlighted the low host specificity of C. pinnai pinnai having been recorded from 20 host species. Previous findings in R. quelen, without descriptions, are those of Tanzola et al. (2009), Venancio et al. (2010) and Moreira et al. (2014). Values of prevalence and abundance were similar in the first two references and higher in Moreira et al. (op.cit.). Albuquerque et al. (2008) collected C. pinnai as adults in the stomach, intestine and larvae in the body cavity of Pimelodus maculatus Lacépède, 1803 (Pimelodidae) from Rio Guandu basin, Rio de Janeiro, Brazil, suggesting that under certain environmental conditions, it could experiment a histotropic phase as observed in other *Cucullanus* spp. (Anderson, 2000). No descriptions of C.pinnai larvae are available to date.

The final host, *R. quelen* was considered as an omnivorous fish with tendency to carnivorism (Ringuelet, 1975; Brazil-Souza *et al.*, 2009). López-Cazorla *et al.* (2003) categorized this catfish as euriphagous because 24 food items were

registered in Sauce Grande river, from that basin of Napostá Grande and Sauce Chico river belong. A gastropod was the dominat food item in that study and may be due to the abundance of this mollusk in the Sauce Grande river. There R. quelen actually behaves like an opportunistic predator. On the other hand, B. iheringii inhabits calm and vegetated waters as the streams of Southwest Buenos Aires province. It is considered as omnivorous or invertebrate-predator which feeds mostly on aquatic insects (Escalante, 1987; López-Cazorla et al., 2003). Grossman et al. (1996) concluded that B. iheringii is a species related to the phytoplancton-periphyton in a pond from centre of Buenos Aires province. The possibility that R. quelen predates on B. iheringii is not ruled out, and more data are needed to confirm this hypothesis. The L_2 experimentally obtained from gravid females of C. pinnai pinnai have a significant similarity with L₃ collected in *B. iheringii*. Future molecular approaches will demonstrate their taxonomic correspondence and the consequent heteroxenous pattern of transmission.

The embryonic development of C. pinnai pinnai goes very fast. In less than five days from laid, the L_2 hatches and must find the right host in less than two weeks or die. This shortened transmission model matches that of C. chabaudi which hatches in 5-6 days post-oviposition, but follows a histotropic phase of development in the liver of Pangasius pangasius (Hamilton, 1822), a freshwater fish from Southeastern Asia (Anderson, 2000). Janiszewska (1938) failed to try to infect several crustacean with eggs and larvae of C. minutus. Most attempts to infect invertebrates with eggs or larvae of another cucullanid genera (Truttaedacnitis Petter, 1974, Dichelyne Jägerskiöld, 1902) gave negative results (Gibson, 1972, Kuzia, 1978- in Anderson, 2000). It seems to be that life cycle of C. pinnai pinnai requires at least the presence of a fish as intermediate or paratenic host. This constitutes the first description of the embryonic and post-embryonic development of C. pinnai pinnai.

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