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Molecular identification and larval morphological description of *Contracaecum pelagicum* (Nematoda: Anisakidae) from the anchovy *Engraulis anchoita* (Engraulidae) and fish-eating birds from the Argentine North Patagonian Sea

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ABSTRACT

Anisakids use invertebrates as paratenic and/or intermediate hosts as a basic feature of larval transmission. The third-stage larva usually develops in invertebrates which are prey items of finfish paratenic hosts. *Contracaecum* larvae molt twice inside the egg and hatch as free third-stage larvae ensheathed in the second-stage larval cuticle. Copepods act as paratenic or obligatory hosts, usually ingesting these free L3 larvae, and fish act as intermediate/paratenic or metaparatenic hosts preying on infected copepods. Fish-eating birds acquire L3 larvae by ingesting infected fish where they develop into the fourth-stage larvae and adults. Objectives of this work were to establish the specific correspondence between *Contracaecum pelagicum* L3 larvae parasitizing the anchovy *Engraulis anchoita*, and the adults parasitizing the Magellanic penguin *Spheniscus magellanicus* and the Imperial shag *Phalacrocorax atriceps* through the use of molecular markers; and, to evaluate the anisakid L3 larval recruitment and infection caused by ingestion of anchovy by *S. magellanicus*. Sixteen specimens of *Contracaecum* L3 larvae were analyzed from *E. anchoita* from Bahía Engaño, Chubut, eight adult nematodes from *S. magellanicus* and six adult specimens from *P. atriceps* both from the Valdés Peninsula, Chubut. All nematodes were sequenced for three genes: mitochondrial cytochrome oxidase 2 (mtDNA *cox2*), mitochondrial ribosomal RNA (*rrnS*), and the internal transcribed spacers (ITS-1 and ITS-2) of the nuclear ribosomal DNA region. Phylogenetic analyses were performed by using Maximum Parsimony (MP) analysis by PAUP. In addition, studies under SEM and LM were carried out on L3 larvae. All L3 individuals from *E. anchoita*, adults from *S. magellanicus*, and *P. atriceps* clustered in the same clade, well supported in the MP tree inferred from the mtDNA *cox2*, and *rrnS* gene sequences analyses. Further, the sequence alignments of L3 larvae and adults of *C. pelagicum* here obtained at the ITS-1 and ITS-2 regions of the rDNA matched the sequences of *C. pelagicum* previously deposited by us in GenBank. Nematode recruitment (R_o) was equal to 33.07 (7.20–91.14) L3 larvae for *C. pelagicum* in each penguin's meal of anchovy. The MP tree topologies obtained from mtDNA *cox2* and *rrnS* genes demonstrated that specimens of *Contracaecum* L3 larvae from *E. anchoita* and *C. pelagicum* from *S. magellanicus* as well as from *P. atriceps* constitute a unique clade, well-distinct and supported from all the others formed by the *Contracaecum* spp. sequenced so far for these genes. Molecular markers are considered to be an effective tool to elucidate larval transmission. The *Contracaecum* L3 larval recruitment value showed that many worms fail to establish in the bird digestive tract, probably because they are below a critical size. Further work is needed to elucidate other factors (e.g., physiological, immunological) that control nematode populations in the penguin digestive tract.

1. Introduction

In most anisakid species, the use of invertebrates as paratenic and/or intermediate hosts is a basic feature of larval transmission. The infective stage (third-stage larva L3) usually develops in invertebrates which are eaten by vertebrate (mainly fish) intermediate/paratenic hosts. The larva generally remains as L3 in the new host but, occasionally, development to the fourth-stage has been observed. The L3 can also increase in size and show genital primordium development — "precocial phenomenon" [1,2,5,6]. This process can occur also in the invertebrate intermediate host when teleost fish are definitive hosts.

Contracaecum pelagicum Johnston and Mawson, 1942, probably has a cycle with invertebrate paratenic hosts such as copepods, euphausids and amphipods, and vertebrate intermediate/paratenic hosts such as teleost fish (e.g., *Engraulis anchoita* and *Merluccius hubbsi*). These are

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prey items of the Magellanic penguin *Spheniscus magellanicus* Foster, and the Imperial shag *Phalacrocorax atriceps* King — definitive hosts of *C. pelagicum* (Fig. 1) [3,4].

The *C. pelagicum* life cycle develops as suggested by Moravec [7] who described the transmission of *Contracaecum rudolphii* (=*spiculigerum*) (Hartwich, 1964) through several fish species and the Great Cormorant *Phalacrocorax carbo sinensis* (Blumenbach). Thus, embryonated eggs are expelled with fish-eating bird feces, falling into the sea and developing into the first (L1), second (L2) and third-stage (L3) larvae. The latter hatches ensheathed in the L2 cuticle and attaches itself to the seabed (Fig. 1) [7, Garbin et al., unpublished results]. Unknown copepods would act as invertebrate paratenic hosts and ingest the L3 which escapes from the L2 cuticle within the new host. The L3 does not grow within the invertebrate [7, Garbin et al., unpublished results].

The present work starts with the hypothesis that *E. anchoita* would be the vertebrate intermediate/paratenic (metaparatenic) host acquiring L3 larvae by ingesting these planktonic copepods. The L3 larva increases in size inside the fish which would be the link for piscivorous birds to acquire and develop L4 larvae and adults of this species (Fig. 1) [7, Garbin et al., unpublished results].

The complex life cycles of anisakids make larval transmission experiments difficult. Such work requires handling intermediate and paratenic hosts and the identification of third stage larval species that lack characteristic features such as caudal papillae, lips and spicules. Thus, there are few studies on larval transmission based on experimental infections [8–12]. Mature eggs from *C. rudolphii* parasitizing *Phalacrocorax* spp. were incubated under different laboratory conditions and hatched L2 larvae were used to infect copepods which were then offered as food to several fish species. Then, developed L3 larvae were found encapsulated in host mesenteries [8]. Semenova [10] studied the life cycle of *Contracaecum micropapillatum* (Stossich, 1890) and was able to infect experimentally several copepod species with L3 larvae which then were used to infect frogs and fish. Third stage-larvae migrated to the body cavity of these hosts, encapsulated and showed to be infective to pelicans, in which developed the adult stage. Due to the logistics required to reproduce the entire anisakid life cycle, in addition to the difficulty of identifying larval species, molecular genetic studies could facilitate studies of larval transmission. For example, Shamsi et al. [13] described and characterized genetically different morphotypes of *Contracaecum* larvae using sequence data of internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA, and assigned them to specified *Contracaecum* adults in definitive hosts.

A further question is to ascertain how many anisakid larvae can be acquired by fish-eating birds during feeding. We only know that Magellanic penguin stomach contents average 430 g with a range from 80 to 1183 g. [14]. Penguins can also eat several anchovy individuals at each feeding. But, the number of ingested L3 larvae remains uncertain. As yet, no studies have been done on this.

Therefore, the main aims of this work were: i) to establish larval transmission and the specific correspondence – identification of specimens as the same species – between L3 larvae parasitizing anchovy, and the fourth-stage larvae and adults parasitizing *S. magellanicus* and *P. atriceps*, and ii) to evaluate anisakid L3 larval recruitment in *S. magellanicus* by quantifying the infection caused by a typical meal of anchovy.

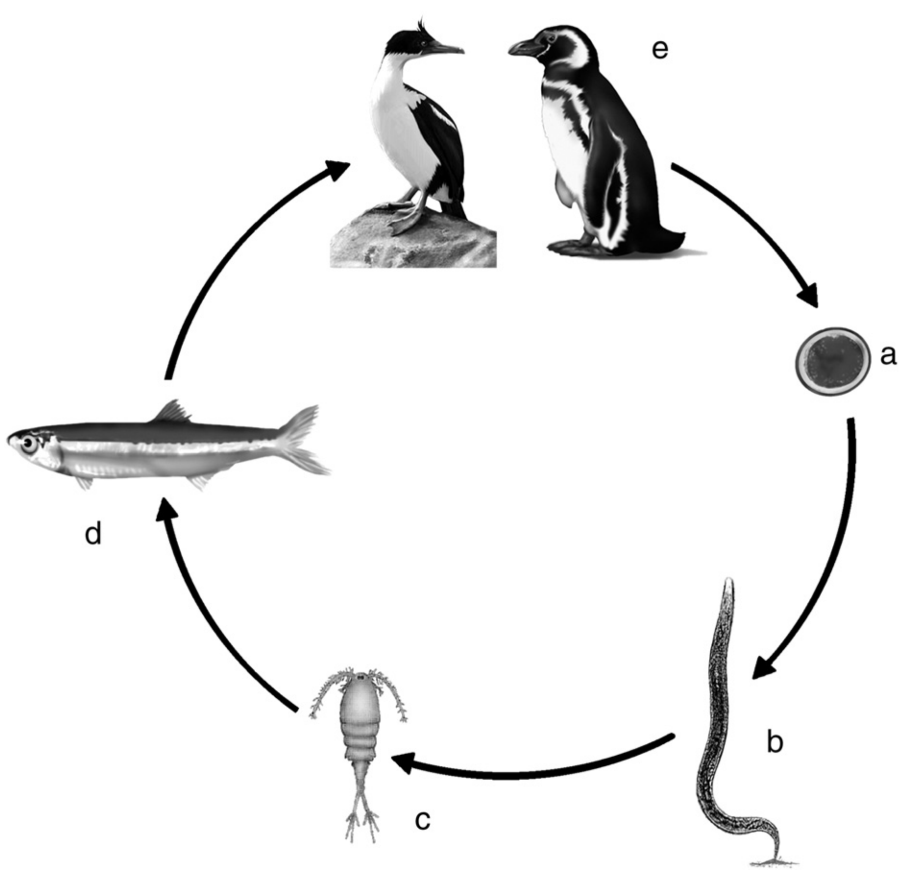

Fig. 1. Hypothetical life cycle of *Contracaecum pelagicum*. a) Developing egg(s) containing first, second and third-stage larvae; b) third-stage larvae attached to the substratum and ensheathed in the L2 cuticle; c) crustacean (copepod), first invertebrate paratenic (or obligatory?) host, ingesting later L3 larvae; d) *Engraulis anchoita*, second vertebrate intermediate/paratenic (metaparatenic) host ingesting infected crustacean; e) *Spheniscus magellanicus* and *Phalacrocorax atriceps*, definitive hosts acquiring L3 larvae by eating anchovy.