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POPULATION STRUCTURE OF THE NEMATODE *Contracaecum pelagicum* JOHNSTON & MAWSON, 1942 DURING THE WINTER MIGRATION OF THE MAGELLANIC PENGUIN *Spheniscus magellanicus* (FORSTER, 1781) IN SOUTHERN BRAZIL

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ABSTRACT

Magellanic penguins, *Spheniscus magellanicus*, are frequently found along the coast of Brazil during the winter migration. However, there are very few ecological studies of the parasite fauna of these birds, especially of the adults, which are found dead on Brazilian beaches. Moreover, no studies of parasites of the Magellanic penguins found on the Brazilian coast have evaluated adult or larval nematodes during winter migration so far. Thus, this work is the first one to investigate the parasitism by anisakid nematodes comparatively between young and adult specimens of *S. magellanicus* in southern Brazil. Parasitological indexes were compared in relation to host gender, parasite development stage and gender, and sites of infection. The gastrointestinal tract of 36 specimens of Magellanic penguins found dead along the coast of Paraná and Rio Grande do Sul states, were analyzed. The nematode fauna was composed of a single species, the anisakid, *Contracaecum pelagicum.* In Rio Grande do Sul, the young specimens of *S. magellanicus* were more intensely and abundantly parasitized by *C. pelagicum* than the adult ones. The stomach and the intestine were the organs that showed highest mean density of infection by adult and larval nematodes, respectively, in the hosts collected in both locations. A high prevalence was observed in both areas, although no differences in the parasitism between male and female penguins were detected. The young Magellanic penguins found in Paraná were on average less parasitized than the ones found in Rio Grande do Sul. These results, together with data available in the literature, suggest a latitudinal variation in the parasitological indexes of *C. pelagicum,* which are related to changes in the diet of the Magellanic penguins during their winter migration in the Southwestern Atlantic Ocean.

Keywords: Anisakidae; helminths; larvae; marine birds, parasite.

INTRODUCTION

In the marine environment, nematodes of the genus *Contracaecum* Railliet & Henry, 1912 (Ascaridida, Anisakidae) parasitize several fish species during the larval stage, and marine mammals and piscivorous birds are the definitive hosts (Vicente *et al*. 1995, Torres *et al.* 2000, Nadler *et al.* 2005). *Contracaecum pelagicum* Johnston & Mawson, 1942 (Ascaridida, Anisakidae) was first described as a parasite of the black-browed albatross, *Thalassarche melanophris* (Temminck, 1828), and of the Atlantic yellow-nosed albatross, *T. chlororhynchos* (Gmelin, 1789) (Procellariiformes: Diomedeidae) in Australia (Johnston & Mawson 1942). Since then, this parasite has been reported in several species of seabirds, including penguins (Lent & Freitas 1948, Santos 1984, Silva *et al*. 2005, Fredes *et al*. 2006, Garbin *et al*. 2007, González-Acuña *et al*. 2008, Diaz *et al*. 2010, Fonteneau *et al*. 2011, Campos *et al*. 2013). Garbin *et al.* (2013) described the third larval stage of this nematode as a parasite of the Argentine anchovy, *Engraulis anchoita* Hubbs & Marini, 1935 (Clupeiformes, Engraulidae). This fish species is very important in the diet of Magellanic penguins, *Spheniscus magellanicus* (Forster, 1781**)**

(Sphenisciformes, Spheniscidae), during both breeding and post-breeding seasons, in the Southwestern Atlantic Ocean (SWA) (Frere *et al.* 1996).

The main breeding colonies of *S. magellanicus* in the SWA are found from Cape Horn to latitude 42ºS, including the Falkland Islands (Williams 1995). After the breeding and molting season (September – April), the Magellanic penguins migrate northwards during the austral winter following the seasonal movements of Argentine anchovy up to the coast of southeastern Brazil (Dantas *et al*. 2013, Stokes *et al*. 2014). However, in atypical years, the species can reach northeastern Brazil (ca. 4ºS) (Dantas *et al*. 2013). *Contracaecum pelagicum* was the only nematode observed parasitizing the gastrointestinal tract of the Magellanic penguins found dead on the beaches of the southeastern Brazil so far (Santos 1984, Ederli *et al*. 2009, Prado *et al*. 2011, Rezende *et al*. 2013, Borges *et al*. 2014).

In southern Brazil, the region where the highest mortality of *S. magellanicus* occurs, studies of their nematode parasites are inexistent. In this context, the aim of this study was to characterize, for the first time, the infrapopulations of *C. pelagicum* from the gastrointestinal tract of young and adult specimens of *S. magellanicus* found in southern Brazil during their winter migration. Comparisons between larval and adult stages of the nematode in each infection site were also evaluated.

MATERIAL AND METHODS

The gastrointestinal tracts of 11 *S. magellanicus* specimens found dead on beaches in Pontal do Paraná (25º34'S; 48º20'W) and Matinhos (25º50'S; 48º29'W) on the coast of Paraná state (PR), Brazil, as well as of 25 specimens found from Itapeva (municipality of Torres, 29°21'S; 49°44'W) to Dunas Altas (municipality of Palmares do Sul, 30°24'S; 50°17'W) in Rio Grande do Sul state (RS), Brazil, were evaluated. The collections were carried out from April to November 2012 and only complete specimens in *rigor mortis*, indicating recent death, were collected.

Before the necropsy, every animal was measured in centimeters with a measuring tape, and the body mass was measured in grams on an analytical scale. The sex was identified by macroscopic observation of the gonads according to Aire (2007) and Jacob & Bakst (2007). The sexual maturity was determined according to the color pattern of the feathers: juvenile plumage of young specimens and adult after first complete molt (Williams 1995).

The gastrointestinal tract (esophagus, stomach and intestine) of each specimen was removed, frozen and subsequently analyzed in the Laboratory for Parasite Ecology of Aquatic Organisms (LEPOA) at the Londrina State University (UEL), Londrina, Paraná state, Brazil. The intestines were cut in sections of 30 cm in length from the anterior to the posterior portion and analyzed separately under a stereoscopic microscope.

The nematodes were collected, most of them were fixed in 4% formaldehyde, preserved in 70% ethanol, cleared in glycerin or Amann lactophenol, mounted temporarily on histological slides and analyzed under an optical microscope (Amato *et al.* 1991). The adult specimens were identified according to Santos (1984), Vicente *et al.* (1995), Anderson (2000), Diaz (2006), and the larvae according to Garbin *et al.* (2007). Subsequently, the nematodes were separated and counted according to their sex and development stage (larvae or adults). In order to characterize the morphological structures and confirm the species by scanning electronic microscopy, some specimens were fixed in 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, rinsed in buffer, post-fixed in a solution of buffered 1% osmium tetroxide and once more rinsed in phosphate solution. The samples were dehydrated through a graded alcohol series and then dried using a CPD 030 Critical Point Dryer (BAL-TEC, Balzers, Lichtenstein). The parasites were mounted on stubs and coated with a 25 nm layer of gold in a sputter coater (SCD 050 – BAL-TEC). The specimens were examined using an FEI Quanta 200 scanning electron microscope in the Microanalysis and Electron Microscopy Laboratory of the Londrina State University.

Prevalence (P%), mean intensity (MI) and mean abundance (MA) of the nematode *C. pelagicum* were calculated with their respective standard deviations (Bush *et al.* 1997) for Paraná and for Rio Grande do Sul samples of Magellanic penguins. The mean densities (MD) of male and female adults and larvae of *C.*

pelagicum were also calculated for each infection site of the gastrointestinal tract of the penguins.

Prevalence rates were compared between males and females, and between young and adult penguins found on Rio Grande do Sul and Paraná coasts using the Fisher's exact test. Mean intensity, mean abundance and mean density were compared in relation to host gender, host maturity, infection site and location using the Mann-Whitney U nonparametric test, because data were not normally distributed, according to results obtained using the KS Normality Test. All parameter estimates and statistical analyses were done using the GraphPad Instat Software, version 3.05 and the significance level used was of 5%. Voucher specimens were deposited in the Helminthological Collection of the Oswaldo Cruz Institute (CHIOC), Rio de Janeiro, Brazil (CHIOC 38359-38363).

RESULTS

Eighteen of the 25 Magellanic penguins collected in Rio Grande do Sul were categorized as young, of which seven were females, seven were males and four could not have their gender identified. Among the seven adults, five were females and two were males. On the Paraná coast, all eleven specimens analyzed were young, of which eight were females, two were males and one could not be identified. The total length and body mass of the Magellanic penguins analyzed in this study varied between 47.6 and 68.5 cm and 1.8 and 3.0 kg, respectively (Table 1). The largest total length for a young specimen was 64.0 cm and body mass was 3.0 kg (Table 1).

Table 1. Total length average (TL) in centimeters and mean body mass (BM) in kilograms with their respective standard deviations (SD) and minimum - maximum values (min.- max.) of young and adult specimens of *Spheniscus magellanicus* found dead on Rio Grande do Sul and Paraná beaches, southern Brazil, from April to November of 2012. N=number of hosts.

Body size	Rio Grande do Sul		Paraná
	Young (N=18)	Adults (N=7)	Young (N=11)
TL ± SD	56.5±4.4	63.5±3.9	57.0±3.7
(min.-max.)	(49.5-64.0)	(59.0-68.5)	(47.6-61.0)
BM ± SD	2.1±0.2	2.7±0.5	2.5±0.4
(min.-max.)	(1.8-2.5)	(2.0-3.0)	(1.8-3.0)

The larval and adult specimens of the nematodes found in the gastrointestinal tract of Magellanic penguins were confirmed as *C. pelagicum* by scanning electron microscopy (Figure 1), mainly by the morphological features mentioned by Garbin *et al*. (2007). The main characteristics of adults were: body entirely transversally striated, conspicuous cephalic collar with concentric anteriorly directed free edges and V-shaped lateral region without striations; three bifurcated interlabia and lips longer than interlabia with three apical notches; lips with two auricles, each with lateral directed pointed processes on anterior corners; deirids nonconspicuous and rounded shape; male with conical caudal extremity, bearing 25-31 pairs of subventral precloacal papillae, first 18 proximal pairs conspicuous; three pairs of precloacal papillae and seven pairs of postcloacal papillae; spicule distal tip extended and pointed; length of free distal end longer than spicule width; spicule wings slope distally toward shaft and inserted at different points; female with vulva in anterior half of body. For larvae the main characteristics were: oral opening with three well-developed lips, with small winglike processes on their anterior corners.

All Magellanic penguins found on Paraná beaches were infected (P=100%) and they had 16 to 596 specimens of *C. pelagicum* in their gastrointestinal tract. However, the specimens from Paraná had significantly lower parasite abundance than those found on Rio Grande do Sul beaches (p=0.03) (Table 2). Male and female penguins did not show significant differences in the parasitological parameters in Paraná (p=0.30).

Among the adult specimens collected in Rio Grande do Sul, 57.1% were infected with *C. pelagicum*. On the other hand, a higher prevalence (P=94.4%) and a significantly higher abundance were found in young penguins (p=0.02) (Table 2). There was no significant difference in the parasitological rates observed in young male and female penguins found in Rio Grande do Sul (p=0.09). Among the adults, the fact that only one male was found parasitized prevented statistical comparison with the females. However, it is noteworthy that this single male had the largest intensity of nematodes found in this study (Table 2).

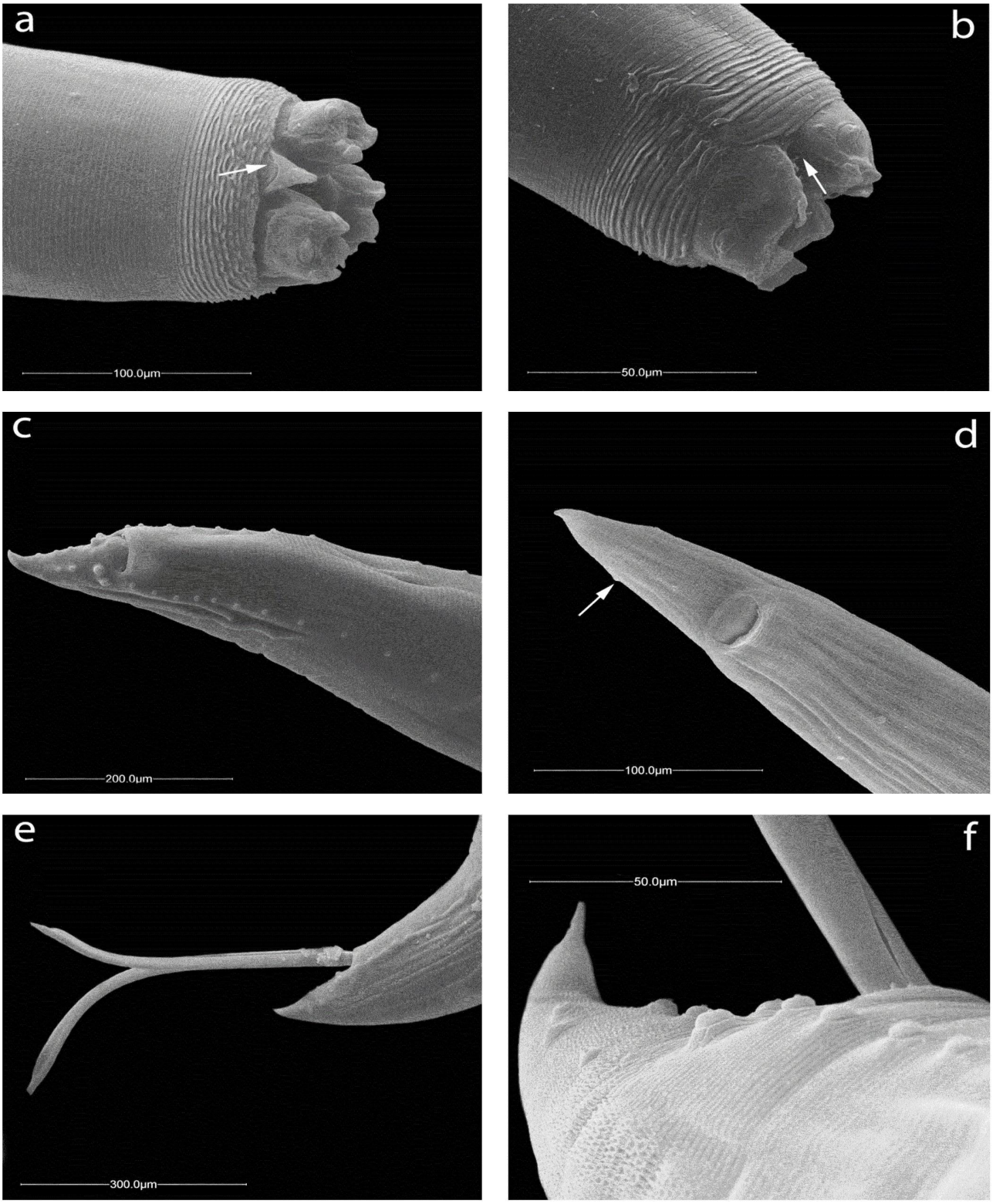

Figure 1. Electron micrograph of adult and L4 larvae of *Contracaecum pelagicum* specimen. a) Detail of an adult male's anterior end; the arrow points to the interlabium. b) Detail of an L4 larva's anterior end. The arrow indicates absence of interlabium separating the lips. c) Adult male's posterior end showing precloacal papillae and the postcloacal major papillae. d) Larva's posterior end. The arrow indicates the well-defined deirids. e) Male posterior end showing the spicules. f) Male posterior end showing two ventral and three lateral postcloacal papillae.

Table 2. Prevalence (P%), mean intensity (MI) and mean abundance (MA) with their respective standard deviations (SD) and minimum-maximum values (min.ï max.) of *Contracaecum pelagicum* parasitizing young and adults, males, females and undetermined sex, and the total of *Spheniscus magellanicus* specimens found dead on beaches in Paraná and Rio Grande do Sul, southern Brazil, from April to November of 2012. ni=number of infected hosts. N=number of hosts.

Sexual Maturity	Sex	Paraná			Rio Grande do Sul		
		P% (ni/N)	MI±SD (min.-max.)	MA±SD (min.-max.)	P% (ni/N)	MI±SD (min.-max.)	MA±SD (min.-max.)
Young	Males	100.0 (2/2)	361.0±332.3 (126-596)	361.0±332.3 (196-596)	100.0 (7/7)	385.1±186.2 (160-675)	385.1±186.2 (160-675)
	Females	100.0 (8/8)	208.1±168.2 (17-510)	208.1±168.2 (17-510)	100.0 (7/7)	426.4±209.6 (273-779)	426.4±209.6 (237-779)
	Undetermined	100.0 (1/1)	16.0 -	16.0 -	75.0 (3/4)	304.0±154.3 (127-410)	228.0±197.4 (0-410)
	Total	100.0 (11/11)	218.5±197.7 (16-596)	218.5±197.7 (16-596)	94.4 (17/18)	387.8±185.5 (127-779)	366.3±201.9 (0-779)
Adults	Males	-	-	-	50.0 (1/2)	1033.0 (1033)	516.5±730.4 (0-1033)
	Females	-	-	-	60.0 (3/5)	105.3±152.2 (15-281)	63.2±122.1 (0-281)
	Undetermined	-	-	-	-	-	-
	Total	-	-	-	57.1 (4/7)	337.3±480.2 (15-1033)	12.7±384.4 (0-1033)

The largest mean density of adult parasites was found in the stomach in both study locations ($p<0.0001$). On the other hand, larval stages (L4) were equally distributed in the stomach and intestine ($p=0.92$ and $p=0.13$ for PR and RS, respectively) (Table 3). In the esophagus, adult and larval nematodes were found only in penguins collected in Paraná (Table 3).

DISCUSSION

Contracaecum pelagicum was the only nematode species found in the gastrointestinal tract of *S. magellanicus* specimens collected on beaches in Paraná and Rio Grande do Sul states, southern Brazil, during the winter migration. Although *C. plagiaticium* Lent & Freitas, 1948 and *Cosmocephalus obvelatus* (Creplin, 1825) have also been reported in Magellanic penguins from other regions (Pazos *et al.* 2003, Campos *et al.* 2013), *C. pelagicum* is the most common nematode parasite reported for this species (*e.g.*, Pazos *et al.* 2003, Ederli *et al.* 2009, Diaz *et al.* 2010, Prado *et al.* 2011, Rezende *et al.* 2013).

Table 3. Mean density (MD) of infection with its respective standard deviations (SD) of male and female adults and larvae of *Contracaecum pelagicum* specimens by infection site in the gastrointestinal tract of Magellanic penguins, *Spheniscus magellanicus*, found dead on the coast of Rio Grande do Sul (RS) and Paraná (PR), southern Brazil, from April to November of 2012.

Locations	*Contracaecum pelagicum* development stages	Esophagus MD ± SD	Stomach MD ± SD	Intestine MD ± SD
RS	Adult Males	----	89.4 ± 74.9	1.6 ± 0.9
	Adult Females	----	168.3 ± 138.1	5.5 ± 6.9
	Larvae	----	93.3 ± 58.9	63.1 ± 83.5
PR	Adult Males	14.0 ± 19.8	59.6 ± 45.5	6.2 ± 9.9
	Adult Females	17.6 ± 23.8	79.2 ± 61.5	8.0 ± 12.5
	Larvae	21.0 ± 22.8	41.8 ± 30.4	48.9 ± 58.6

Table 4. Comparison of prevalence index (P%), mean intensity (MI) and mean abundance (MA) of *Contracaecum pelagicum*, a parasite of young (Y) and adult (A) *Spheniscus magellanicus* specimens found in the literature during the reproductive period in Península Valdés, Argentina (ARG) (1, 2 and 3) and Mar del Plata (ARG) (3), and during the migratory period in Brazil, including Rio Grande do Sul (RS) and Paraná (PR) (4 - this study), São Paulo (SP) (5 and 6), Rio de Janeiro (RJ) (6) and Espírito Santo (ES) (7).

Localities (South to North)	Number of Hosts			*Contracaecum pelagicum*								
	Y	A	Total	Adults			Larvae			Adults and Larvae		
				P%	MI	MA	P%	MI	MA	P%	MI	MA
ARG[1]	4	5	9	56.0	161.0	89.0	100.0	520.0	520.0	-	-	-
ARG[2]	-	-	31	66.7	190.0	127.0	100.0	377.0	377.0	-	-	-
ARG[3a]	-	-	20	40.0	149.0	60	100.0	283.0	283.0	-	-	-
ARG[3b]	-	-	4	100.0	-	-	-	-	-	-	-	-
RS[4]	18	7	25	84.0	259.7	218.2	76.0	121.6	92.4	84.0	378.2	317.7
PR[4]	11	-	11	100.0	141.5	141.5	81.8	100.0	82.8	100.0	218.5	218.5
SP[5]	28	-	28	-	-	-	-	-	-	96.4	126.5	121.6
SP/RJ[6]	237	-	237	-	-	-	-	-	-	49.4	46.6	23.0
ES[7]	-	-	1	100.0	48.0	48.0	-	-	-	-	-	-

Pazos *et al.* 2003 (1); Diaz 2006 (2); Garbin *et al.* 2007 (3a and 3b); Present study (4); Prado *et al.* 2011 (5); Rezende *et al.* 2013 (6); Ederli *et al.* 2009 (7).

Although the parasitological parameters obtained for *C. pelagicum* larvae were higher than for adults in our study, higher abundance and intensity of *C. pelagicum* larvae were found in penguins from northern Patagonia, Argentina, during the reproductive period (austral summer) (Pazos *et al*. 2003, Diaz 2006) (Table 4). In that location, the Argentine anchovy constitutes the most common prey consumed by the Magellanic penguins (Frere *et al*. 1996). Timi *et al*. (2001) studied the parasite community of *E. anchoita* in Argentina and found this species parasitized by L3 larvae of the genus *Contracaecum*. The nematode species was later confirmed as *C. pelagicum* through molecular studies by Garbin *et al*. (2013). Thus, *E. anchoita* is considered to be the intermediate host of *C. pelagicum* (Garbin *et al*. 2013).

Therefore, there is a shift in the ratio between larvae and adults of *C. pelagicum* in the digestive tract of Magellanic penguins during the winter migration in the SWA. The highest values of the parasitological indexes (prevalence, mean intensity and mean abundancy) for the larvae of *C. pelagicum* found in Argentina (Table 4), which indicates a recent infection, is probably related to the fact that *E. anchoita* is the most important prey in the diet of the penguins in the colonies of northern Patagonia (Scolaro & Badano 1996, Scolaro *et al*. 1999). On the other hand, the higher values of mean intensity for adult specimens of *C. pelagicum* in Magellanic penguins found in Rio Grande do Sul compared to the ones from northern Patagonia can be explained by the development of the larvae that were acquired in higher latitudes prior to migration of these birds to the Brazilian coast. The Argentine anchovy is one of the most abundant pelagic fishes of the southern and southeastern coasts of Brazil and two stocks of the species have been identified in this region. The southern stock ("bonaerensis") from southern Brazil extends to Uruguay and Argentina and exhibits migratory behavior (Carvalho & Castello 2013). This stock moves from Patagonian coastal waters in spring and summer, and northward to the Brazilian coast in winter (Castello 2005, 2007). This event broadly coincides with the Magellanic penguin's migration (Stokes *et al*. 2014). A second stock of Argentine anchovy seems to occur in south and southeastern Brazil, from Paraná to Rio de Janeiro states, and apparently does not exhibit migratory behavior (Carvalho & Castello 2013).

Many authors believe that the search for the Argentine anchovy is the main motivation for the movement of these birds during the migratory period (Fonseca *et al.* 2001, Pinto *et al.* 2007, Baldassin *et al.* 2010). In fact, the parasitism of the Magellanic penguins by L4 larvae of *C. pelagicum* in Rio Grande do Sul and Paraná suggests the consumption of *E. anchoita* in this region, although cephalopods are known to be the main food item of *S. magellanicus* in Brazilian waters (*e.g.*, Pinto *et al.* 2007, Baldassin *et al.* 2010).

Regarding the preferred sites of *C. pelagicum*, the higher density of adult nematodes in the stomach of Magellanic penguins in the present study corroborates the findings of Garbin *et al.* (2007) and Prado *et al.* (2011). On the other hand, the presence of *C. pelagicum* specimens in the esophagus and intestine can be explained by the fact that these parasites show great vagility and they tend to migrate from the infection site to other organs when the host dies. However, according to the results of the present study, it seems that the anterior intestine is the most common site of the larvae.

As verified in this study, the higher values of mean intensity and abundance of *C. pelagicum* found in young penguins compared to those found in adults appear to occur for all helminths in every species of Sphenisciformes (Clarke & Kerry 1993). The difference in mean intensity and mean abundance between young and adult penguins can also be due to the fact that the immunity of young penguins is not well developed, and also to the passive transfer of infective larva or adult nematodes from the stomach of the parents while feeding the chicks in the colonies. The hypothesis of passive transfer was also postulated to explain the higher prevalence in chicks than in adults of another nematode species, *Stegophorus macronectes* (Johnston & Mawson, 1942) (Rhabditida, Acuariidae), in the chinstrap penguin, *Pygoscelis antarctica* (Forster, 1781) (Sphenisciformes, Spheniscidae) (Vidal *et al.* 2012). In addition, the young birds, when leaving their nests, typically face stressful conditions, such as competition for food, which can lead to the depletion of their reserves during migration, resulting in nutritional deficiency, making them more susceptible to parasitism (Mäder *et al.* 2010) and possibly to death. In fact, the vast majority of Magellanic penguins found dead along the Brazilian coast are young specimens (*e.g.* García-Borboroglu *et al.* 2010). Therefore, we expect the strongest, healthiest, and possibly less infected penguins to have a greater chance to reach the adult phase.

A study of the diet of Magellanic penguins with stable isotopes showed that there were no differences between males and females diets during the reproductive period. On the other hand, differences in foraging methods were identified between males and females at the beginning of the post-reproductive period (Silva *et al.* 2014). Nevertheless, these diet discrepancies do not seem to be completely reflected in the parasitism by *C. pelagicum* between male and female Magellanic penguins, especially among the young. Therefore, other factors such as the number of ingested prey, aggregate distribution of parasites and host susceptibility to parasites may also be involved.

Finally, the present study suggests the occurrence of a latitudinal gradient in the infection levels of *C. pelagicum* in Magellanic penguins along the Brazilian coast. In this sense, the values of mean intensity and mean abundance of total infection (adults + larvae) of this nematode seem to decrease toward lower latitudes from Rio Grande do Sul to Espírito Santo states (Table 4). The possible differences in the parasitic fauna between the two different stocks of *E. anchoita* and the changes in the diet of Magellanic penguins during their migration could contribute to this pattern. In this context, recent stable isotope analyses of Magellanic penguins from Brazil showed that the Brazilian sardinella, *Sardinella brasiliensis* (Steindachner, 1879) (Clupeiformes, Clupeidae), is another important fish prey consumed by the penguins in southeastern Brazil (Di Beneditto *et al.* 2015). Therefore, the role of this fish species in the diet of *S. magellanicus*, as well as of the cephalopods (Pinto *et al.* 2007, Baldassin *et al.* 2010), can contribute to a decrease in the parasitism by *C. pelagicum* in southern Brazil. Nevertheless, further studies counting both larval and adult specimens of *C. pelagicum* in young and adult Magellanic penguins, especially along the southeastern Brazilian coast, are required for a full comprehension of the dynamic of this parasite-host interaction.

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