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2   **LIFE CYCLE OF THE PARASITE *PROFILICOLLIS CHASMAGNATHI***

3   **(ACANTHOCEPHALA) ON THE PATAGONIAN COAST OF ARGENTINA BASED**

4   **ON MORPHOLOGICAL AND MOLECULAR DATA**

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17   Abstract: This study verifies the identity of adult specimens of the parasite *Profilicollis*

18   *chasmagnathi* (Acanthocephala, Polymorphidae) recovered from kelp gulls *Larus*

19   *dominicanus* (Aves, Laridae), and cystacanths found in crabs *Cyrtograpsus altimanus*

20   (Crustacea, Decapoda) from the southwestern Atlantic coast. The life cycle of this parasite is

21   elucidated in the intertidal zone of Patagonia, Argentina, based on morphological and

22   molecular data. Preferences by size and sex of the intermediate host and seasonal variation of

23   this parasite are provided, contributing to the knowledge of this host-parasite association.

24   Adult members of the family Polymorphidae are endoparasites of marine mammals,

25   waterfowl, and fish-eating birds. They are diagnosed by having a spinose trunk, bulbose

proboscis, double-walled proboscis receptacle, and usually 4 to 8 tubular cement glands (see Nickol et al., 1999; García-Varela et al., 2011, 2013). The genus *Profilicollis* Meyer, 1931 was considered as a sub-genus of *Polymorphus* Lühe 1911 until Nickol et al. (1999), based on ecological characters, ranked *Profilicollis* as a genus level. According to Nickol et al. (1999), all species of *Profilicollis* use decapods as an intermediate host, whereas *Polymorphus* use amphipods. Recent phylogenetic analysis based on molecular evidence suggests that *Polymorphus* is paraphyletic and *Profilicollis* is monophyletic (García-Varela and Pérez-Ponce de León, 2008). Amin (2013) recognized 9 species of *Profilicollis*: the type species of the genus *Profilicollis botulus* (Van Cleave, 1916), *Profilicollis altmani* (Perry, 1942) (= *Profilicollis bullocki*, *Profilicollis kenti*, and *Profilicollis texensis*), *Profilicollis antarcticus* Zdzitowiecki, 1985, *Profilicollis arcticus* (Van Cleave, 1920), *Profilicollis chasmagnathi* (Holcman-Spector, Mañé-Garzón and Dei-Cas, 1977), *Profilicollis formosus* (Schmidt and Kuntz, 1967), *Profilicollis major* (Lundström, 1942), *Profilicollis novaezelandensis* Brockerhoff and Smales, 2002, and *Profilicollis sphaerocephalus* (Bremser in Rudolphi, 1819) (Amin, 2013; Goulding and Cohen, 2014; Rodríguez et al., 2016). Recently, the validity of *P. antarcticus* was questioned by Rodriguez et al. (2017) who suggested it might be a junior synonym of *P. chasmagnathi*.

All members of the genus *Profilicollis* infect mainly waterfowl as adults and use decapods as intermediate hosts (Zdzitowiecki, 1985; Nickol et al., 1999; Rodríguez et al., 2016). Along the southwestern Atlantic coast, only adults of *P. chasmagnathi* have been reported, from the gut of several bird species in the estuaries of Buenos Aires Province (Martorelli, 1989; Vizcaíno, 1989; La Sala et al., 2013), and from that of the kelp gull *Larus dominicanus* (Lichtenstein) (Aves, Laridae) on the coast of Chubut Province (Diaz et al., 2011). In contrast, cystacanths of 2 species of *Profilicollis* have been reported on the southwestern Atlantic coast: *P. chasmagnathi* parasitizes different crab species from estuarine

and rocky intertidal habitats in Uruguay and Argentina (Holcman-Spector et al., 1977a; Martorelli, 1989; La Sala et al., 2012; Rodríguez et al., 2017), while *P. altmani* parasitizes the mole crab *Emerita brasiliensis* (Schimitt) on sandy beaches along the Uruguayan coast (Rodríguez and D'Elía, 2016; Rodríguez et al., 2016).

Closely related species of *Profilicollis* are difficult to distinguish based on their phenotype. Moreover, there is limited knowledge about their degree of geographic variation (Near et al., 1998; Balboa et al., 2009), and the identity of some populations of *Profilicollis*, mostly of their immature stages, remains unclear (Rodríguez et al., 2016). One goal of this study was to test the relationship between the adult specimens of *Profilicollis* recovered from the kelp gull *L. dominicanus*, and that of cystacanths found in the crab *Cyrtograpsus altimanus* Rathbun (Crustacea, Decapoda) using morphological and molecular evidence. Additionally, seasonal variation of this parasite and its preferences for size and sex of the intermediate host and was studied. These investigations contribute to the knowledge of life-cycles and host-parasite interactions in the intertidal zone of Patagonia, Argentina.

MATERIALS AND METHODS

Sampling

Mature acanthocephalan specimens were obtained from a total of 89 kelp gulls, *L. dominicanus*, out of which 29 were collected along the coast of Península Valdés and adjacent areas (42°05' to 42°53'S, 64°21' to 65º04'W), Chubut Province, Argentina (see Diaz et al., 2011). The remaining 60 gulls were obtained from the same area between 2012 and 2015 while conducting a project aimed to mitigate the interaction between kelp gulls and southern right whales developed by the Ministerio de Ambiente y Control del Desarrollo Sustentable, Chubut and the CCT CONICET- Centro Nacional Patagónico (Decree 1106/12). Some hosts were dissected and the viscera fixed in 10% formalin. Other hosts were immediately dissected or frozen at -20 C until further analysis. In the laboratory, viscera were inspected under a

3

76 stereomicroscope and acanthocephalans collected from the gut. Some parasite specimens were

77 fixed in 10% formalin and preserved in 70% ethanol for morphological analyses. Specimens

78 recovered from the fresh and frozen hosts were fixed and stored in 96% ethanol for

79 subsequent DNA extraction.

80 Specimens of larval acanthocephalan were obtained following dissection of 94

81 specimens of *C. altimanus*. Crabs were collected by hand in the intertidal zone of Punta

82 Cuevas, Puerto Madryn (42º46'S, 65º29'W), Chubut Province, between 2007 and 2016, during

83 all seasons. Crabs were transported alive to the laboratory, measured (carapace width in mm)

84 and separated into three size intervals (S): S1, 4.1 – 10 mm; S2, 10.1 – 16 mm and S3, 16.1 –

85 22 mm). Size intervals were determined by dividing the total size range (22 mm maximum

86 size – 4.1 mm minimum size) into 3 equal size classes, and the crab frequency in each size

87 interval was computed. Crabs were dissected, sexed, and larvae removed from the hemocoel

88 under a stereomicroscope. Most larvae were placed in small Petri dishes containing

89 physiological solution and incubated at 39 C. They were observed at different time intervals

90 to study the evagination of the proboscis. They were then fixed in 10% formalin and

91 preserved in 70% for morphological analysis. Some specimens were fixed and stored in 96%

92 ethanol for subsequent DNA extraction.

93 **Morphological identification**

94 Specimens were studied in temporary mounts of lactophenol or eugenol using an

95 Olympus BX51® microscope (OM) (Olympus, Tokyo, Japan). Several specimens were

96 dehydrated in a graded ethanol series, dried using the critical point method (Hayat, 1973),

97 coated with gold, examined by scanning electron microscopy (SEM) (Jeol 6360LV®, JEOL,

98 Tokyo, Japan), and photographed. Measurements, given in micrometers unless otherwise

99 indicated, are provided as the mean followed by the range in parentheses. Eggs were

100 measured through the body wall. Acanthocephalans were identified following specific

101 bibliography (Holcman-Spector et al., 1977a, 1977b; Zdzitowiecki, 1985; Vizcaíno, 1989;

102 Nickol et al., 1999; Amin, 2013). Scientific names of hosts are according to WoRMS (2017).

103 Voucher specimens were deposited in the Helminthological Collection of the Museo de La

104 Plata, Buenos Aires, Argentina (MLP 6312; 7249) and in the Parasitological Collection of the

105 Instituto de Biología de Organismos Marinos (CCT CONICET-CENPAT), Puerto Madryn,

106 Chubut province, Argentina (CNP-Par 18; 137).

107 **Molecular data and phylogenetic analysis**

108 Genetic comparisons and phylogenetic analyses were based on a fragment of 578 base

109 pairs of the mitochondrial gene cytochrome oxidase I (hereafter *COI*). The Chubut sample is

110 comprised of sequences of 2 individuals of *Profilicollis* from kelp gulls *L. dominicanus*, and 3

111 individuals of *Profilicollis* from the crab *C. altimanus*; the latter 3 sequences were generated

112 by Rodríguez et al. (2017) and downloaded from GenBank. The 2 new sequences were

113 generated from DNA extracted using a commercial kit (Wizard® Genomic DNA Purification

114 Kit, Promega, Madison, Wisconsin) and amplified using the primers detailed by Folmer et al.

115 (1994), following the protocol of Rodríguez and D'Elía (2016). Amplicons were sequenced

116 using an external sequencing service (Macrogen Inc., Seoul, South Korea); DNA sequences

117 were edited using Codon-Code (Codon Code Aligner, Dedham, Massachusetts) and deposited

118 in GenBank (MG859265; MG859266).

119 The 5 sequences of *Profilicollis* from Chubut Province (see below) were assembled in

120 a matrix with other sequences downloaded from GenBank. It included 16 sequences of *P.*

121 *chasmagnathi* retrieved from definitive and intermediate hosts from the southwestern Atlantic

122 (Uruguay) and Pacific (Chile) coasts generated by Rodríguez et al. (2016, 2017). A total of 21

123 sequences of *P. chasmagnathi* were analyzed. The matrix also included sequences of *P.*

124 *altmani*, *Polymorphus brevis* (Van Cleave, 1916), *Polymorphus minutus* Goeze, 1782*, and *P.*

125 *botulus*, which were used to form the outgroup.

5

126   Sequences were aligned in Clustal using MEGA 7 software (Tamura et al., 2013)

127   using default parameter values. Observed genetic p-distances (p) between haplotype and

128   sample pairs were calculated in MEGA 7. Phylogenetic relationships were inferred via

129   Maximum Likelihood analysis (ML) conducted using IQ-TREE (Nguyen et al., 2015), and the

130   online implementation W-IQ-TREE (http:/iqtree.cibiv.univie.ac.at; Trifinopoulos et al., 2016).

131   The IQ-TREE software was also used to select the model of nucleotide substitution

132   (TPM3u+G4). Support for clades found in the most likely tree was calculated via the SH-

133   aLRT test (Guindon et al., 2010) and with 1,000 pseudoreplicates of ultrafast bootstrap (BL).

134   **Ecological parameters**

135   Prevalence (P), mean intensity (MI) and mean abundance (MA) were calculated

136   following Bush et al. (1997). The seasonal distribution of adult acanthocephalans was based

137   on counts of the kelp gulls made by Diaz et al (2011). For data analysis, Spearman's rank-

138   order coefficient (rs) was used to establish the relationship between crab size and season vs. P

139   and MI. An unequal variance t-test was used to establish statistical differences in size between

140   male and female crabs. Probability (p) values <0.05 were considered significant. The Chi-

141   square, Fisher´s test, and unconditional test were applied to test differences between P values;

142   MI differences were estimated by bootstrap tests, and p values <0.05 were considered

143   significant, using Quantitative Parasitology 3.0 Budapest software (Rózsa et al., 2000).

144   **RESULTS**

145   **General morphology**

146   *Adult (based on 10 males and 5 females) (Fig. 1 A-F):* Body divided into 3 sections:

147   proboscis, neck, and trunk. The proboscis has a spheroid shape, armed with 18-22

148   longitudinal rows, each one with 7-8 hooks. Apical hooks slightly smaller than basal hooks.

149   Neck long and slender. Trunk long covered with spines anteriorly. Genital spines absent.

150   *Male*: Proboscis 1,150 (900-1,350) in diameter. Apical hooks 43 (30-50), median

151   hooks 47 (30-55), basal hooks 50 (40-65). Proboscis receptacle 5,104 (3,700-7,050) long.

152   Neck 2,577 (1,800-3,500) long, 244 (200-300) wide. Trunk 5,683 (2,200-10,240) long, 1,522

153   (950-2,100) wide. Testes tandem, anterior testis 811 (450-1,100) long, 644 (500-950) wide;

154   posterior testis 789 (500-1,150) long, 582 (400-850) wide. Four tubular cement glands, 4,106

155   (2,500-5,800) long.

156   *Female*: Proboscis 1,133 (1,000-1,300) wide. Apical hooks 51 (45-60), median hooks

157   46 (30-60), basal hooks 54 (45-70) long. Proboscis receptacle 5,800 (5,750-5,850) long. Neck

158   2,917 (1,950-3,700) long, 233 (200-250) wide. Trunk 7,390 (6,200-9,360) long, 1,787 (1,400-

159   2,200) wide. Eggs without polar elongations, 59 long to 21 wide (n = 10).

160   *Cystacanth from crabs (based on 10 specimens with evaginated proboscis) (Fig. 1 G-*

161   *J):* Body divided into 3 sections: proboscis, neck, and trunk. Proboscis oval to spheroid, 595

162   (500-750) long, 435 (400-500) wide, armed with 18 (16-18) longitudinal rows, each with

163   usually 8 (7-8) hooks. Apical hooks 44 (30-55) long, smaller than basal hooks 52 (40-60).

164   Neck long and slender, 1,395 (750-2,200) long, 454 (300-750) wide. Trunk 2,378 (1,500-

165   4,150) long, 1019 (900-1,110) wide, covered with spines anteriorly.

166   **Genetic results**

167   The genealogical analysis indicated that sequences of the adults from the kelp gull *L.*

168   *dominicanus* and cystacanths from the crab *C. altimanus* collected on the southwestern

169   Atlantic coast of Argentina are very similar; *p*-distance values for sequence samples pairs

170   ranged between 0.005 and 0.013 (average = 0.009). These sequences are part of a highly

171   supported clade (SH-aLRT = 100; BL = 100) formed by sequences of *P. chasmagnathi* (Fig.

172   2). This clade showed low genetic variation (average = 0.6%, range = 0-0.5%). In addition,

173   the genetic variation of *P. chasmagnathi* is not geographically structured. For example, two

174   cystacanth larvae obtained from *Cyrtograpsus angulatus* (Varunidae) from Uruguay share the

175   same sequence with cystacanth larvae obtained from *Neohelice granulata* and *Hemigrapsus*

176   *crenulatus* (Varunidae) from Uruguay and Chile respectively. In contrast, the most divergent

177   sequences of this clade were found in adults obtained from *L. dominicanus* from Argentina

178   and cystacanth larva obtained from *C. angulatus* from Uruguay.

179   **Ecological analysis**

180   Of the 89 kelp gulls examined, 16 were parasitized (P = 19%); a total of 62 adults were

181   found in the gut (MI = 3.87; MA = 0.73). Male crabs were larger than females (p = 0.01). Of

182   the 94 crabs examined, 25 were parasitized (P = 26.6%); a total of 46 cystacanth larvae were

183   found in the hemocoel (MI = 1.84; MA = 1.49). The number of larvae per crab ranged from 1

184   to 7. The prevalence (P) in male crabs was higher than in females (29% vs. 23%,

185   respectively). In contrast, MI was higher in females than in males (3.1 vs. 1.5, respectively).

186   However, these differences were not statistically significant. The maximum P and MI were

187   found in S2 (37.9% and 2.5%, respectively) (Fig. 3), and were significantly higher than in S1

188   (p = 0.02 and p = 0.04, respectively). Regarding the seasonal distribution of parasites, it was

189   observed that in the intermediate hosts, P and MI were higher in autumn and winter

190   respectively (Fig. 4), whereas in their definitive host they were higher in spring and summer,

191   respectively (Fig. 5), although, these differences were not statistically significant.

192   **DISCUSSION**

193   Measurements of specimens collected in the present study fall within the range

194   provided for *P. chasmagnathi* by previous authors (Martorelli, 1989; Vizcaino, 1989). The

195   molecular characterization indicates that *P. chasmagnathi* in Península Valdés uses the crab

196   *C. altimanus* as the intermediate host and the kelp gull as definitive host, demonstrating a

197   trophic relationship between both host species and link between stages in the life cycle.

198   In the host-parasite system studied here, females of *P. chasmagnathi* infect *L.*

199   *dominicanus* and produce eggs (with acanthor inside) that are released into the environment

8

200   with the feces of the bird host. Shelled acanthors are ingested by the crab *C. altimanus*, in

201   which the acanthor develops into an acanthella in the hemocoel, and then into a cystacanth

202   that infects the gulls when the latter preys upon an infected *C. altimanus* (Fig. 1).

203   The correlation observed between prevalence (P) and crab size could be explained by

204   the fact that larger hosts are older, and therefore exhibit more prolonged exposure to parasites

205   (Poulin, 1997). Also, the difference observed in size between males and females could explain

206   the higher P (although not statistically significant) observed in males than in females. In

207   addition, larger crabs consume more food, and are thus may be more frequently exposed to the

208   shelled acanthors. It was also observed that smaller crabs occupy the spaces made available in

209   the mussel beds, forcing large crabs to migrate to adjacent cobblestone (tidal pools) habitat

210   (Vázquez et al., 2012) where the crabs are in close contact with the eggs released by birds.

211   Considering that the highest P and MI in crabs occur in autumn and winter, and based

212   on the time that larvae require to reach maturity (see Holcman-Spector et al., 1977b), it was

213   also expected that the highest prevalence and intensities in birds would occur after autumn.

214   Data from this study substantiate this trend, but results were not statistically significant.

215   Capasso and Diaz (2016) found immature specimens identified as *Profilicollis* sp.

216   parasitizing *Calidris* spp. (Aves: Scolopacidae) near Península Valdés. Other studies have

217   mentioned immature *P. altmani* parasitizing *Calidris* spp. in different sites of southern Brazil

218   (Buehler et al., 2010). However, the absence of adults in these shorebirds, suggests that

219   *Calidris* spp. would not be involved in the parasite life cycle of *Profilicollis* spp.

220   There are differences in the patterns of host specificity of species of *Profilicollis* in

221   Chile and Argentina. In this context, adults of *P. altmani* in Chile have been reported to infect

222   different gull species, whereas adults of *P. chasmagnathi* only infect *L. dominicanus*

223   (Rodríguez et al., 2017). In contrast, on the Argentinean coast, *P. chasmagnathi* was reported

224   from several bird species (Martorelli, 1989; Vizcaino, 1989; La Sala et al., 2013), and so far

225   this is the only species of *Profilicollis* found in *L. dominicanus*.

226   The differential host distribution of *P. altmani* and *P. chasmagnathi* could be related to

227   the type of habitat frequented by their intermediate and definitive hosts. Rodríguez et al.

228   (2017) reported that intermediate hosts of *P. altmani* inhabit the sandy intertidal zone,

229   whereas those from *P. chasmagnathi* are associated with estuaries and the rocky intertidal.

230   Studies of kelp gulls from Chile included populations that eat decapods from those three

231   different environments (Rodríguez et al., 2016), whereas those from Argentina include birds

232   that prey decapods from estuaries (e.g., Martorelli 1989; Vizcaino, 1989; La Sala et al., 2013)

233   and the rocky intertidal (Diaz et al., 2011; present study).

234   The molecular analysis showed that *P. chasmagnathi* shows low genetic variation that

235   is not structured on the basis of hosts or geography. Recent studies have shown that *P. altmani*

236   also presents low genetic variation lacking geographic structure (Goulding and Cohen, 2014;

237   Rodríguez and D'Elía, 2016; Rodríguez et al., 2016, 2017). This finding may be attributed to

238   the high vagility of their definitive hosts, allowing mixing of acanthocephalan populations and

239   thus resulting in their genetic homogenization. For *P. chasmagnathi,* shorebirds with high

240   dispersal potential, e.g., *L. dominicanus*, *L. atlanticus* and the imperial cormorant *P. atriceps*,

241   have been reported as definitive hosts (Torres et al., 1992; La Sala et al., 2013; Rodríguez et al

242   2016). While bird host vagility could explain the lack of phylogeographic structure, it would

243   not be the cause of the low levels of genetic variation observed. In fact, the processes causing

244   low genetic variation remain unknown. The issue can be addressed by assessing variation in

245   nuclear genes sequences (e.g., *ITS1, ITS2*) recovered from additional host populations and

246   localities, as a way to test whether the observed levels of genetic variation of the

247   mitochondrial DNA, instead of reflecting demographic history (e.g., recent population

248   expansions), are caused by selective sweeps (Nielsen, 2005).

249

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358 Figure 1. Schematic illustration of the life cycle of *Profilicollis chasmagnathi* on the

359 Patagonian coast of Argentina (upper) and scanning electron micrographs of various stages

360 (lower). DH: definitive host, IH: intermediate host. (**A-F**) Adult specimens from *Larus*

361 *dominicanus*. (A-D) Proboscis showing detail of hook distribution. (E, F) Detail of anterior

362 trunk spines. (**G-J**) Cystacanth from *Cyrtograpsus altimanus* (G) Proboscis, apical view

363 showing hook distribution. (H) Proboscis, lateral view showing the number of hooks in each

364 row. (I) Whole cystacanth. (J) Detail of anterior trunk spines. Scale bars: A, E, J = 200 µm; B,

365 C, D, F, G, H = 100 µm; I = 500 µm.

366 Figure 2. Tree showing the relationships of the *COI* gene sequences of individuals of the

367 genus *Profilicollis,* based on maximum likelihood analysis (ln = -2,643.278). Nodal support

368 values > 50, for species and multispecies clades, are consecutively from the the SH-aLRT test

369 and ultrabootstrap analysis. Intermediate and definitive hosts and country (AR, Argentina;

370 CH, Chile; UY, Uruguay) are provided for *Profilicollis chasmagnathi*. Accession numbers are

371 given for sequences downloaded from GenBank.

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372   Figure 3. Prevalence (P) (left Y axis) and mean intensity (MI) (right Y axis) of *Profilicollis*

373   *chasmagnathi* in their hosts. **(A)** Cystacanths from *Cyrtograpsus altimanus* by size intervals

374   (S): S1, 4.1 – 10 mm; S2; 10.1 – 16 mm; S3; 16.1 – 22 mm). **(B)** Cystacanths from *C.*

375   *altimanus* by season. **(C)** Adults from *Larus dominicanus* by season.