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1 RH: LORENTI ET AL.- LIFE CYCLE OF *PROFILICOLLIS CHASMAGNATHI*
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, bulbous

26 proboscis, double-walled proboscis receptacle, and usually 4 to 8 tubular cement glands (see
27 Nickol et al., 1999; García-Varela et al., 2011, 2013). The genus *Profilicollis* Meyer, 1931
28 was considered as a sub-genus of *Polymorphus* Lühe 1911 until Nickol et al. (1999), based on
29 ecological characters, ranked *Profilicollis* as a genus level. According to Nickol et al. (1999),
30 all species of *Profilicollis* use decapods as an intermediate host, whereas *Polymorphus* use
31 amphipods. Recent phylogenetic analysis based on molecular evidence suggests that
32 *Polymorphus* is paraphyletic and *Profilicollis* is monophyletic (García-Varela and Pérez-
33 Ponce de León, 2008). Amin (2013) recognized 9 species of *Profilicollis*: the type species of
34 the genus *Profilicollis botulus* (Van Cleave, 1916), *Profilicollis altmani* (Perry, 1942) (=
35 *Profilicollis bullocki*, *Profilicollis kenti*, and *Profilicollis texensis*), *Profilicollis antarcticus*
36 Zdzitowiecki, 1985, *Profilicollis arcticus* (Van Cleave, 1920), *Profilicollis chasmagnathi*
37 (Holcman-Spector, Mañé-Garzón and Dei-Cas, 1977), *Profilicollis formosus* (Schmidt and
38 Kuntz, 1967), *Profilicollis major* (Lundström, 1942), *Profilicollis novaezelandensis*
39 Brockerhoff and Smales, 2002, and *Profilicollis sphaerocephalus* (Bremser in Rudolphi,
40 1819) (Amin, 2013; Goulding and Cohen, 2014; Rodríguez et al., 2016). Recently, the
41 validity of *P. antarcticus* was questioned by Rodríguez et al. (2017) who suggested it might
42 be a junior synonym of *P. chasmagnathi*.

43 All members of the genus *Profilicollis* infect mainly waterfowl as adults and use
44 decapods as intermediate hosts (Zdzitowiecki, 1985; Nickol et al., 1999; Rodríguez et al.,
45 2016). Along the southwestern Atlantic coast, only adults of *P. chasmagnathi* have been
46 reported, from the gut of several bird species in the estuaries of Buenos Aires Province
47 (Martorelli, 1989; Vizcaíno, 1989; La Sala et al., 2013), and from that of the kelp gull *Larus*
48 *dominicanus* (Lichtenstein) (Aves, Laridae) on the coast of Chubut Province (Diaz et al.,
49 2011). In contrast, cystacanths of 2 species of *Profilicollis* have been reported on the
50 southwestern Atlantic coast: *P. chasmagnathi* parasitizes different crab species from estuarine

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

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

65 **MATERIALS AND METHODS**

66 **Sampling**

67 Mature acanthocephalan specimens were obtained from a total of 89 kelp gulls, *L.*
68 *dominicanus*, out of which 29 were collected along the coast of Península Valdés and adjacent
69 areas (42°05' to 42°53'S, 64°21' to 65°04'W), Chubut Province, Argentina (see Diaz et al.,
70 2011). The remaining 60 gulls were obtained from the same area between 2012 and 2015
71 while conducting a project aimed to mitigate the interaction between kelp gulls and southern
72 right whales developed by the Ministerio de Ambiente y Control del Desarrollo Sustentable,
73 Chubut and the CCT CONICET- Centro Nacional Patagónico (Decree 1106/12). Some hosts
74 were dissected and the viscera fixed in 10% formalin. Other hosts were immediately dissected
75 or frozen at -20 C until further analysis. In the laboratory, viscera were inspected under a

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 (42°46'S, 65°29'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 *Proflicollis* from kelp gulls *L. dominicanus*, and 3
111 individuals of *Proflicollis* 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 *Proflicollis* 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.

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 (r_s) 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

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 *Profilocollis* 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 *Profilocollis* spp.

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

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