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1 # Hemoparasites and immunological parameters in Snow
2 # Bunting (*Plectrophenax nivalis*) nestlings

3

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Abstract

Knowledge on hemoparasites and immunological parameters in wild birds with Arctic distribution is limited. In this study, we chose the Snow Bunting (*Plectrophenax nivalis*) as model species to address this issue. Using nestlings, we aimed at (i) detecting hemoparasites with vector-borne transmission (via microscopic and molecular methods) and nest-dwelling ectoparasites and at (ii) exploring the relationship between several immunological parameters and parasitism. Nestlings were infected by *Lankesterella* parasites but hemoparasites of the genera *Plasmodium*, *Haemoproteus*, *Leucocytozoon*, *Trypanosoma*, *Hepatozoon* or *Babesia* were not found. This result may indicate the lack of suitable dipteran vectors or the inability of haemosporidians to reproduce in the Arctic region. Inflammation in response to the phytohaemagglutinin (PHA) injection was negatively related to infection by *Lankesterella* and positively related to weight gain in nestlings. The number of leukocytes and IgG level were not related to infection or PHA response, although the relationship between IgG level and PHA immune response was marginally significant. Besides, nestlings reared in mite-infected nests showed higher IgG level than those reared in mite-free nests. Taken together, (i) the positive relationship between PHA response / weight gain and PHA response / IgG level could reflect the nestlings' nutritional status; and (ii) the higher IgG level in nestlings reared in mite-infected nests may be the result of a specific immune response to mite antigens. Different parasites may alter distinct immunological parameters in birds breeding under extreme weather conditions.

Key words: *Plectrophenax nivalis,* Snow Bunting, *Lankesterella,* phytohaemagglutinin, immune system, hemoparasites, mites

42 ## Introduction

43 Species inhabiting polar ecosystems are generally infected by fewer parasites
44 than species from other geographical areas (Rohde 1999; Nunn et al. 2005;
45 Lindenfors et al. 2007; Barbosa and Palacios 2009). Specifically, the Arctic has
46 been reported as an environment where blood parasites are rare or absent
47 (Laird 1961; Earle and Underhill 1993; Wojczulanis-Jakubas et al. 2010). In
48 addition to this, some studies suggest that species subjected to less parasitic
49 pressure have less reactive immune systems (Barbosa et al. 2007) due to
50 energetic (Martin et al. 2003) and/or autoimmune disease associated costs
51 (Raberg et al. 1998).

52 The Snow Bunting (*Plectrophenax nivalis*) is the only passerine species
53 breeding regularly in high Arctic ecosystems, surviving at temperatures around
54 0°C. In the current climate conditions, the only limit to the Northern distribution
55 of this species is land availability, although its reproduction also needs an
56 appropriate photoperiod (outside the polar day zone they do not enter in
57 reproductive activity) and average temperatures below 10ºC in July
58 (Ryzhanovsky 2015). Studies on this bird species have focused on its
59 distribution in different geographical regions (Bryan et al. 1978; Marzocchi 1978;
60 Campbell and Vanderraay 1985; Jia-Wei et al. 2014), phenology (Ryzhanovsky
61 2015), breeding success (Hoset et al. 2004; Fossøy et al. 2015), different
62 aspects of its behaviour (Hofstad et al. 2002; Hoset et al. 2009) or physiological
63 characterization (i.e. hormone profiles, Romero et al. 1998). However, studies
64 on the parasitofauna of buntings are rare. To date, some studies have detected
65 infections by the intestinal coccidian *Isospora plectrophenaxia* (Dolnik and
66 Loonen 2007) and *Dermanyssus hirundinis* mites (Gwiazdowicz et al. 2012) in
67 other bird populations inhabiting the Svalbard archipelago. In addition,
68 *Lasioseius berlesei* ticks were identified in material collected in the Bering
69 Island in Russia (Voltsit 1997). Notwithstanding, there are no studies on the
70 presence of hemoparasites in the Snow Bunting. Different genera of
71 hemoparasites have been detected in many species of birds in areas located at
72 lower latitudes, where more benign climate favours the development of
73 arthropod vectors (Valkiunas 2004). Among them, the more prevalent are
74 haemosporidia (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*), but

75 piroplams (*Babesia*), hemococcidians (*Lankesterella* and *Hepatozoon*) and
76 *Trypanosoma* have also been detected (Criado et al. 2006; Merino et al. 2006;
77 Badás et al. 2017). All of them have heteroxenous life cycles that require
78 transmission to the vertebrate host through arthropod vectors. Due to the close
79 relationship between temperature and the development of hemoparasites within
80 vectors (Lapointe et al. 2010), the transmission of vector-borne hemoparasites
81 to vertebrate hosts in a particular environment may be used as a biomarker of
82 the effects of temperature increase due to climate change. For this reason, the
83 Snow Bunting could be a good model species to explore this possibility, since
84 weather conditions in its current distribution, both in summer and winter (North
85 of Europe and America), are less favourable for the development of arthropod
86 vectors.

87 One of the main problems to measure immunocompetence in wild animals is
88 the development of adequate tests for this task. Several immunological
89 parameters are described in the literature when trying to establish the
90 competence of the immune system (Calder 2007). Among them, the
91 phytohemagglutinin test (PHA), based on the cellular response mediated by T
92 cells, is one of the more frequently used technique in the past few years (Goto
93 et al. 1978; Smits et al. 1999; Pigeon et al. 2013). This preference is probably
94 driven by its low economic cost, its simplicity and by the fact that the reaction
95 has minor physiological consequences in birds (Merino et al. 1999). Some
96 studies indicate that the PHA test is useful to determine the status of
97 immunocompetence of individuals and it has been used in several species of
98 wild birds in studies related to behaviour, population dynamics, toxicology, or
99 parasitism (Smits and Williams 1999; Brommer et al. 2011; Pap et al. 2011;
100 Podmokla et al. 2014). This technique is performed *in vivo* and it consists of a
101 subcutaneous injection of the mitogen PHA in the membrane of the wing
102 patagium of birds. After 24 hours from injection, the inflammation caused by the
103 proliferation and recruitment of T cells in the area is measured (Goto et al.
104 1978). Individuals who mount greater responses are thought to have increased
105 immunocompetence, although this interpretation is not exempt from criticism
106 (Kennedy and Nager 2006; Owen and Clayton 2007). In fact, some authors
107 advise using this method with caution, since the immunological competence of

108 each species is very distinctive and a single measure of the immune system is
109 not adequate to determine whether a particular individual is immunosuppressed
110 (Calder 2007). Thus, combining different proxies for immune competence is
111 recommended. In this sense, quantification of IgG level (humoral response) and
112 white blood cells counts are two of the most used immunological parameters
113 since only a small quantity of blood is required. IgG level can be determined as
114 total IgG (Johnsen and Zuk 1999; Szép and Møller 1999; Martinez et al. 2003)
115 or specific IgG as immunological response against inert antigens (Deerenberg
116 et al. 1997; Råberg et al. 2000). An increase in these immunological parameters
117 in blood is usually indicative of the presence of an infection.

118 Here, we aimed at detecting the presence of different genera of blood parasites
119 (by microscopy and molecular methods) and nest-dwelling ectoparasites in
120 Snow Bunting nestlings. We also aimed to explore several immunological
121 parameters (response to phytohemagglutinin, serum IgG level, and number of
122 white blood cells) and their relationship with parasitism and nestling body mass.
123 We predict (i) a low prevalence, if any, of hemoparasites transmitted by dipteran
124 since temperatures below 13°C prevent hemosporidian development (Lapointe
125 et al. 2010); (ii) a positive relationship between PHA response and weight gain
126 (Lochmiller et al. 1993) and (iii) parasitized individuals should exhibit lower PHA
127 response and higher IgG level and leukocyte counting as indicated by Johnsen
128 and Zuk (1999) and Szép and Møller (1999).

129

130 Materials and methods

131 *Study species*

132 The Snow Bunting (*Plectrophenax nivalis*, Linnaeus 1758) is a passerine bird
133 that inhabits the colder regions of the planet such as the tundra at the Arctic
134 Circle (Nethersole-Thompson 1966). During winter, they migrate to the North of
135 Europe, Canada, USA, Russian steppes of the Caspian Sea and Kazakhstan,
136 where temperatures are higher, forming large flocks. In April, they return to the
137 Arctic Circle where minimum and maximum mean temperature are still -17°C
138 and -9°C, respectively, and snow is still abundant. Males return to the breeding

139 area one month before females in order to choose and defend a nesting place.
140 Bulky nests are built with branches and straw close to the ground and they are
141 usually placed among rocks to protect it from predators (Hoset et al. 2009).
142 Eggs or nestlings are insulated by the use of feathers in the nest. Clutches
143 contain between two and seven eggs. During incubation, the female does not
144 leave the nest, being fed by the male on seeds, sprouts leaves and insects.
145 Nestlings are fed only with invertebrates (arthropods and worms, see Rising et
146 al. 2011). Thus, the presence of some dipteran species has been reported
147 during the breeding season in Svalbard (Hågvar et al. 2007). As females begin
148 incubation before the last egg is laid, hatching is asynchronous (Hussell 1985;
149 Lyon and Montgomerie 1985). However, asynchrony was not checked in the
150 present study. After about two weeks, nestlings leave the nest joining adult
151 members in flocks (see Encyclopedia of life 2018). In this study, the mean
152 weight (g) of the nestlings was 25.48 ± 3.45 (mean ± SD) on day 8 post-
153 hatching (males 26.44 g ± 3.61 and females 24.36 g ± 2.90).

154 Field methods

155 The study was conducted in 2011 during the Snow Bunting breeding season
156 (June and July) in the vicinity of Longyearbyen (78º 13' N 15º 39' E; Spitsbergen
157 Island, Svalbard archipelago, Norway; Figure 1), where the largest population of
158 Snow Bunting is found (Gwiazdowicz et al. 2012). Due to its location in the
159 Arctic Ocean, climate in this region is relatively mild for this latitude due to the
160 influence of the Gulf Stream. Therefore, the annual average temperature is -
161 6.7°C, with barely four months of summer where the temperature can reach up
162 to 5°C in average (Norwegian Meteorological Institute 2012).

163 In the study area, nests were actively and systematically searched for following
164 the activity of adult birds. Snow Buntings usually breed in artificial nest-boxes (n
165 = 21) situated in human structures but we also checked the nests in some
166 natural cavities (n = 11). Nest boxes are cleaned every year to avoid the
167 occurrence of ectoparasites at the beginning of the breeding season. Nests
168 were inspected every 3 days to determine laying date. Day 0 of the study was
169 hatching date in each nest. At day 8 nestlings were weighed to the nearest 0.25
170 gram with a spring balance, checked for presence of mites (all nestlings from

171 mite-infested nests were considered infested), injected in the wing patagium
172 with phytohemagglutinin (PHA) and a blood sample was obtained from the
173 brachial vein with a sterilized needle using a heparinized micro capillary
174 (BRAND, micro-haematocrit tubes, 75×1.1mm, Na-heparinized). A drop of blood
175 was then smeared on a slide, air dried, and later fixed with 96º ethanol and
176 stained with Giemsa (1:10, v/v) for 40 min (Merino et al. 1997). Another drop of
177 blood was transferred to FTA classic cards (GE Healthcare Life Sciences). The
178 remaining blood sample was centrifuged (2000×g, 5 min) with a portable
179 centrifuge (Labnet, Mini Centrifuge, cat. no. 1201-220V, Woodbridge, NJ, USA).
180 Serum fractions were separated and maintained below 15°C before being
181 frozen on the same day for later analysis. On day 9 nestlings were weighed
182 again to calculate the change in body mass and the inflammation produced by
183 PHA was measured (see below). A total of 127 nestlings from 32 nests were
184 sampled for hemoparasite screening. However, 8 nests were excluded from
185 experiment since the exact age of nestlings was unknown. These nestlings
186 were not injected with PHA nor weighted. Thus, only 24 nests (90 nestlings)
187 were used for statistical analyses.

188 *Phytohaemagglutinin Test (PHA)*

189 Only nests with known hatching date were used in the experiment (i.e., 24
190 nests). To reduce handling time per nest, only half of nestlings of each nest (45
191 nestlings) were injected with PHA (0.2 mg PHA/ 40µL, Sigma L-8754) in the
192 patagium of the wing that had not been used for blood sampling (see Smits et
193 al., 1999). The rest of the nestlings (45) were used as controls and they were
194 injected with a sterile physiological solution. Nestlings injected with PHA were
195 chosen randomly. The potential inflammation caused by the PHA or
196 physiological solution was measured in triplicate with a spessimeter (Mitutoyo
197 7/547, Tokyo, Japan) at 24 hours post-injection. The mean value in millimetres
198 was used for statistical analysis.

199 *Total IgG quantification*

200 The IgG level was determined from blood plasma by direct ELISA using an anti-
201 chicken IgG antibody (Sigma A-9046) conjugated with peroxidase (Martínez et

202 al. 2003). IgG level was measured using a plate spectrophotometer at λ = 405
203 nm and expressed as unit of absorbance.

204 *Microscopic screening of hemoparasites and quantification of leukocytes*

205 Blood parasites were searched using a microscope (Olympus model SC30)
206 under both 400x and 1000x magnification (Merino et al. 1997). Total leukocytes
207 counts were carried out in 10 fields with 400x magnification. The fields that were
208 used for counting had a monolayer distribution of cells with adequate
209 erythrocyte abundance to carry out the quantification. Following this, 100 white
210 cells were counted under 1000x magnification in order to determine the relative
211 proportion of each type of leukocytes.

212 *Sex determination*

213 Following Griffiths et al. (1998) protocol for sex determination, we amplified the
214 chromo-helicase-DNA-binding (CHD) genes using PCR. In birds, these genes
215 are located on W and Z chromosomes. The set of primers (P1 and P8) amplifies
216 homologous sections of both genes and incorporates introns whose lengths
217 usually differ. Therefore, females (ZW) render two bands and males (ZZ) only
218 one. PCRs were performed in a 10 μL reaction volume containing between 20–
219 100 ng template DNA, 0.25 μM of each primer, and Supreme NZYTaq 2x Green
220 Master Mix (NZYTech, Lda. - Genes and Enzymes, Portugal). PCR conditions
221 were as follows: 95 °C for 10 min (polymerase activation), 40 cycles at 95 °C for
222 30 s, annealing temperature 45 °C for 30 s, extension temperature 72 °C for 30
223 s, and a final extension at 72 °C for 10 min. PCR assays were checked using
224 agarose gel (1.5%) electrophoresis.

225 *Molecular screening of hemoparasites*

226 Genomic DNA from samples stored in FTA cards was extracted following
227 Martínez et al. (2009). Next, the DNA solution was purified using the
228 commercial kit NZYGelpure (NZYTech, Lda. - Genes and Enzymes, Lisbon,
229 Portugal). PCR reactions consisted of 10 μL reaction volumes containing
230 between 20 and 100 ng of template DNA, 0.25 μM of each primer and SYBR®
231 Select Master Mix (Applied Biosystems, Foster City, CA, USA). The reactions
232 were cycled using StepOnePlus Real-Time PCR System (Applied Biosystems).

233 The diagnosis was performed by visualizing the melting curve of the amplicons.
234 Sequences of the primers, size of the amplicons, and PCR conditions are
235 shown in the table (Online Resource 1). After screening, positive samples were
236 amplified again to obtain larger amplicons that facilitate the identification of
237 haplotypes and the phylogenetic analysis. PCR reaction volume (20 µL)
238 contained between 20 and 100 ng of template DNA, 0.25 M of each primer, and
239 Supreme NZYTaq 2x Green Master Mix (NZYTech, Lda. - Genes and Enzymes,
240 Lisbon, Portugal). Data on primers and PCR conditions are detailed in the table
241 (Online Resource 2). All amplicons were recovered from agarose gels and
242 subjected to direct sequencing using an ABI 3730 XL automated sequencer
243 (Applied Biosystems). To prevent from contamination, we used different sets of
244 pipettes and filter tips for extraction, PCR set up and downstream fragment
245 analyses. DNA extraction and PCR set up were always performed in different
246 laminar flow cabinets. We never detected amplicons in negative controls added
247 in each PCR batch. A positive control for each pair of primers was routinely
248 used.

249 *Phylogenetic analysis*

250 The three DNA sequences (18S rRNA) obtained in the present study were
251 aligned together with other 97 sequences obtained from GenBank. The
252 alignment was performed using PROBCONS
253 (http://toolkit.tuebingen.mpg.de/probcons). Poorly aligned positions and
254 divergent regions of the alignment were suppressed using GBlocks program
255 (Talavera and Castresana 2007) selecting the following options: "Minimum
256 Number of Sequences for a Conserved Position" to 51, "Minimum Number of
257 Sequences for a Flank Position" to 51, "Maximum Number of Contiguous
258 Nonconserved Positions" to 8, "Minimum Length of a Block" to 5, and "Allowed
259 Gap Positions" to "With Half". The final alignment contained 1453 positions and
260 100 sequences. The substitution model GTR+I+G was selected using
261 jModeltest 2.1.4 (Darriba et al. 2012) to perform the Bayesian analysis with
262 MrBayes 3.2.3 software. This analysis consisted of 2 runs of 4 chains each, with
263 3,000,000 generations per run and a burn-in of 750,000 generations (45,000
264 trees for consensus tree). The final standard deviation of the split frequencies
265 was lower than 0.01.

266 *Statistical analysis*

267 All statistical analyses were performed in R (R Core Team 2015) using package
268 'lme4' (Bates et al. 2015). To explore the relationship between parasite
269 infections and other variables prior to PHA-treatment, we performed three linear
270 mixed models using leukocytes, weight gain or IgG level as dependent
271 variables (linear mixed models, LMM). The total IgG level deviated from the
272 normal distribution and was normalized with a Johnson transformation prior to
273 analysis. A total of 90 nestlings from 24 nests were included in this analysis. We
274 included sex, infection by *Lankesterella*, mites, IgG level (excluded when acting
275 as dependent variable), weight gain (excluded when acting as dependent
276 variable) and leukocytes (excluded when acting as dependent variable) as fixed
277 effects, and the nest ID as random effect. We used restricted maximum
278 likelihood (REML) parameter estimation for LMM to obtain unbiased estimates
279 of variance components, and likelihood ratio test statistics to test if variances
280 significantly differed from zero (Verbeke and Molenberghs 2000). Statistics for
281 the fixed effects were calculated on the basis of conditional Wald tests with the
282 residual degree of freedom numerically derived (not necessarily integers;
283 Kenward and Roger 1997).

284 The nestlings' response to the PHA treatment and other physiological traits
285 were analysed with linear mixed models (LMM). We included sex, mites
286 (presence / absence), PHA treatment, infection by *Lankesterella* (presence /
287 absence), total IgG, leukocytes number, weight increment and their two-way
288 interactions as fixed effects, and we also included the nest ID as random effect.
289 The total IgG level deviated from the normal distribution and was normalized
290 with a Johnson transformation prior to analysis. The most parsimonious model
291 was selected on the basis of AIC (Akaike Information Criterion) (Burnham et al.
292 2011) via its corrected version for small sample size (AICc, Sugiura 1978).
293 Because of our original hypothesis, model selection was performed among a
294 set of models that included the above-mentioned fixed effects and the two-way
295 interactions between PHA-treatment and *Lankesterella* infection, PHA-treatment
296 and weight increment, and PHA-treatment and IgG level. Alternative models
297 were those that sequentially eliminated each two-way interaction and the
298 corresponding fixed effect, except treatment and sex, which were always kept in

299 the model. We chose a set of models that would allow meaningful conclusions
300 (Table 1). When the difference in AICc between two or more models was less
301 than 10 units (ΔAICc<10), all of these models were considered (as opposed to
302 selecting one single model) because these are thought to be reasonably well-
303 fitted models (Bolker et al. 2009). In order to quantify the relative importance of
304 individual variables we calculated model weights (Johnson and Omland 2004)
305 from all models tested in the analyses (including those with a difference in AICc
306 higher than ten units; see Table 1). We obtained robust parameter estimates
307 and significance values for each variable through model averaging using the
308 models that fulfilled these two conditions: (i) models that had ΔAICc<10
309 (Burnham and Anderson 2002), and, in order to further confirm our results, (ii)
310 models that were significantly different from the null model after parametric
311 bootstrapping (all χ^2>16.158, all P<0.01, number of simulations = 1000
312 iterations), following Badás et al. (2018). The R package 'pbkrtest' (Halekoh and
313 Højsgaard 2014) was used for bootstrapping and 'MuMIn' (Barton 2016) was
314 used for model averaging. Because model averaging and IT-AIC methods
315 (Information Theory-AIC) do not perform well when missing data are present in
316 a dataset, we used the complete-case approach as suggested by other authors
317 (Nakagawa and Freckleton 2011). Thus, a total of 88 nestlings were included in
318 this analysis.

319 In addition, the relationship between mite-infected and *Lankesterella*-infected
320 nests was evaluated with the Fisher's exact test. The sample size was 32 nests.

321 Results

322 *Microscopic and molecular detection of hemoparasites*

323 Hemoparasites from the genera *Plasmodium*, *Haemoproteus*, *Leucocytozoon*,
324 *Trypanosoma*, *Babesia* and *Lankesterella* were not detected by microscopy.
325 The presence of hemoparasites was detected only at the molecular level using
326 primers hep900F/EimRodR (see Online Resource 1). A larger fragment of 18S
327 rDNA gene was amplified from positive samples (see Online Resource 2).
328 Sequencing of these amplicons (1567 base pairs) revealed the presence of
329 three haplotypes with genetic identities between them of 99.9%. Subsequent

330 phylogenetic analyses grouped these three haplotypes with two sequences of
331 the genus *Lankesterella* previously deposited in GenBank, *L. minima* and *L.*
332 *valsainensis*. The clade formed by these two species and the three haplotypes
333 obtained in the present study was grouped with high statistical support (100%,
334 Figure 2). The prevalence of this hemococcidian was 20% (25 of 127 nestlings)
335 and was only detected in 8 of the 32 nests studied. The prevalence within nests
336 ranged from 25% to 100% (mean prevalence 71.25%). We did not find
337 molecular evidence of other hemoparasites infecting Snow bunting nestlings.

338 In addition, we only detected mites infesting 8 of the 32 nests inspected. The
339 occurrence of mites in the nests is not significantly related to *Lankesterella*
340 infection (Fisher's exact test, $n = 32$, $p > 0.99$).

341 *Effect of parasite infections on nestlings before PHA injection*

342 *Lankesterella* infections were not significantly related to IgG levels, leukocyte
343 numbers, or nestling weight gain (models for leukocytes and weight gain are not
344 shown: Likelihood Ratio tests, all $\chi^2<4.5$, all $p>0.05$). However, nestlings reared
345 on mite-infected nests showed higher IgG level than those reared on uninfected
346 nests (Table 2 and Figure 3). The sample size was 90 nestlings from 24 nests.

347 In addition, there were no differences in the mites' prevalence between nest
348 boxes and natural nests (Fisher's exact test, $n = 32$, $p = 0.2$).

349 *Response to the phytohaemagglutinin (PHA) injection*

350 The results of the implemented linear mixed model appear in Table 1. The
351 sample size was 88 nestlings from 24 nests. As expected, the treatment had a
352 significant effect on the inflammation of the wing patagium and nestlings
353 injected with PHA experienced more swelling in the patagium than nestlings
354 from the control group. In addition, (i) nestlings infected by hemoparasites
355 (*Lankesterella*) had weaker inflammation responses to PHA (Figure 4 and Table
356 3), and (ii) nestlings with increasing weight gain developed a more intense
357 inflammation response after the PHA injection (Figure 5 and Table 3). The rest
358 of variables were not significantly related to PHA response.

359 When we tested all relationships using the ratio heterophyles / lymphocytes
360 instead of total leukocytes, the same results were reached. However, to avoid
361 colinearity, we did not include the ratio heterophyles / lymphocytes as covariate
362 in the analyses because these were positively and significantly correlated to the
363 total leukocytes count (Pearson correlation, r = 0.244, p = 0.035).

364 Discussion

365 The results obtained in this study indicate that infections by blood parasites are
366 scarce in Snow Bunting nestlings, as shown by the fact that we were unable to
367 detect infections by hemosporidians (*Plasmodium*, *Haemoproteus* and
368 *Leucocytozoon*), *Trypanosoma*, *Hepatozoon* or *Babesia*. The first four genera
369 are transmitted by dipterans and the last two genera by ticks. The absence of
370 hemosporidians and *Trypanosoma* in this Arctic bird species was foreseeable,
371 since there is only one species of biting Diptera in the area, the mosquito *Aedes*
372 *nigripes* (Coulson 2007) and in most years their emergence seems to be later
373 than the birds' breeding season. Besides, the climatic conditions of the area of
374 study are not favourable for the reproduction of hemoparasites during much of
375 the birds' breeding season. In fact, the time required by some species of
376 haemosporidia to reach the infective form in its vector can be extended up to 28
377 days at low temperatures and below 13°C some species do not reproduce at all
378 (Lapointe et al. 2010). Blood parasites were also absent in other bird species
379 such as the little auk (*Alle alle*) in the same region (Wojczulanis-Jakubas et al.
380 2010).

381 However, we were able to detect a haemococcidian parasite within the genus
382 *Lankesterella*. The presence of this parasite in the Snow Bunting nestlings is
383 probably related to the physiological peculiarities of mites acting as vectors. Bird
384 mites are ectoparasites with direct transmission that find suitable conditions for
385 reproduction amongst bird feathers, and therefore, they are less dependent on
386 weather conditions. In addition to this, some mite species have developed
387 physiological adaptations to survive at low temperatures when they leave their
388 hosts (Gwiazdowicz et al. 2012). The presence of the intestinal coccidian
389 *Isospora plectrophenaxia* (Dolnik and Loonen 2007) in this bird species could
390 also be explained by resilience to harsh conditions. In this case, the parasite life

391 cycle does not depend on vectors and its transmission is directly done through
392 the ingestion of oocysts, a form of resistance that remains viable under adverse
393 weather events.

394 *Lankesterella* is a coccidian genus that remains elusive in birds and, in fact, it
395 was misidentified within the genus *Hepatozoon* until recently (Merino et al.
396 2006). The life cycle of this parasite is heteroxenous and, as discussed above,
397 different species of mites can act as vectors. Unfortunately, in this study, we
398 were not able to identify mite species infecting Snow Bunting nests. However,
399 previous studies in the same area confirmed the presence of *Dermanyssus*
400 *hirundinis* mites in nests (Gwiazdowicz et al. 2012). This mite species was
401 introduced in the archipelago of Svalbard by Snow Buntings (Gwiazdowicz et al.
402 2012). *D. hirundinis* is a nidicolous ectoparasite that spends most of their life
403 cycle in the nest of its host, where its reproduction also takes place. It is
404 possible that *D. hirundinis* acted as vector of *Lankesterella* in this region, since
405 it presents a great tolerance to low temperatures (Gwiazdowicz et al. 2012).
406 However, the detection of mites in nests was not related to infection by
407 *Lankesterella* in nestlings. An explanation for this could be related to the
408 prepatence time of the blood parasite (i.e., the period between infection with a
409 parasite and its detection in blood). Since blood extractions were performed
410 when nestlings were nine days old, it is possible that only those individuals that
411 were infected very soon after hatching developed parasite forms in blood that
412 could be detected by molecular methods later on. It is also important to note
413 that we could not detect the parasite in blood smears, which may be indicative
414 of very low levels of parasitaemia. This also reinforces the hypothesis that
415 longer prepatent periods may explain the lack of relationship between the mite's
416 presence in the nest and *Lankesterella* infection in nestlings. In any case, the
417 nestlings' infections found in this study provide clear evidence of transmission of
418 a blood parasite, *Lankesterella*, in Svaldbard.

419 A longer prepatent time could also explain the absence of other blood parasites
420 in Snow Bunting nestlings like hemosporidians and *Trypanosoma*. The lack of
421 time coincidence between chick growth and mosquito emergence could also
422 explain these results. In addition, the extreme climatic conditions in the area,
423 which make the reproduction of blood parasites difficult, should also be

424 considered. Since Snow Buntings also winter in regions of lower latitude with
425 milder climates, adults could be infected with other hemoparasites. However,
426 the average temperature in wintering regions is still too low to favour the
427 reproduction of hemoparasites in their dipteran vectors. However, we cannot
428 discard hemoparasite transmission during its return to the breeding areas. In
429 the present study, Snow Bunting adults were not captured due to the high
430 susceptibility of nest desertion after capture and handling during incubation and
431 the high difficulty of catching during nestling development (Moksnes A, pers
432 comm 2011). Therefore, in order to confirm that other hemoparasites are absent
433 in this species, future studies should aim at capturing adults outside the
434 breeding season. Moreover, yearly screenings of hemoparasites transmitted by
435 dipteran vectors in Snow Bunting nestlings could be used to study the effects of
436 climatic change. For example, the increase in temperature could favour the
437 introduction of new dipteran vectors and the reproduction of haemosporidian
438 species in the region. However, the consequences of this scenario on bird
439 populations are unknown. On the one hand, the introduction of a novel parasite
440 could be detrimental to Snow Buntings because this species has not coevolved
441 with the parasite (Liao et al. 2017). On the other hand, an increase in vectors'
442 abundance could provide more food resources (i.e., arthropods) for nestling
443 provisioning (Serrano-Davies and Sanz 2017).

444 Snow Bunting nestlings that experienced a greater increase in weight or were
445 not parasitized by *Lankesterella* developed a greater response to PHA injection.
446 The relationship between PHA response and weight or increase in weight had
447 already been described in other species, and regarded as an indicator of quality
448 associated with immunocompetence of individuals and nutritional status
449 (Lochmiller et al. 1993; Merino et al. 2000; Alonso and Tella 2001, Barbosa and
450 Moreno 2004). In fact, nutritional status during bird development is essential for
451 the correct formation of lymphoid organs, which may atrophy when there is
452 protein restriction in the diet (Lochmiller et al. 1993). Therefore, the relationship
453 between weight gain and PHA response could be related to
454 immunosuppression, which in turn may be associated with malnutrition, as
455 previously described in other organisms (Powell et al. 2000; Katona and
456 Katona-Apte 2008). However, the use of PHA response as an indicator of a

457 competent immune system must be taken with caution due to the fact that some
458 components of the immune system present threshold values, and any change
459 above this threshold may not be biologically significant (Adamo 2004).

460 In order to reach firm conclusions on nestling immunocompetence it would be
461 necessary to test whether individuals with lower response to PHA are more
462 likely to be infected. However, experimental infection of wild birds is not
463 desirable for ethical reasons. Nonetheless, our results confirm that nestlings
464 infected by *Lankesterella* showed lower responses to PHA injection as
465 compared to uninfected individuals. Several mechanisms could explain this
466 result: lower response may be due (i) to previous immunodepression, (ii) to
467 immunodepression provoked by infection or (iii) it may be a consequence of the
468 activation of specific effector mechanisms in order to fight this hemoparasite.
469 Indeed, we should take into account the fact that defensive mechanisms
470 triggered by an infection could be specific (Adamo 2004) and thus, changes
471 induced in an immunological parameter could be balanced by variation in
472 another parameter in order to maintain an efficient response (Calder 2007). This
473 is in accordance with the lack of relationship between infection and IgG level or
474 total leukocyte counts. If a lower response to PHA in infected individuals is
475 related with immunosuppression this could probably be reflected in nestling
476 condition. However, in the present study the relationship between weight
477 increase and infection in Snow Bunting nestlings was not significant. Besides,
478 as the IgG level was positively associated with PHA response in this study
479 (although marginally) and the IgG level has been positively related to nutritional
480 status in other passerine species (Moreno et al. 2008), it would be possible that
481 only nestlings in good condition (i.e., more IgG level and weight increment) can
482 afford to mount a suitable cellular immune response (Merino et al. 1996).

483 Many factors may be involved in modulating an immune response (Pigeon et al.
484 2013), for example increased physical activity (Owen and Moore 2008),
485 emotional stress (Shao et al. 2003), contaminants (de Swart et al. 1996) or
486 temperature (Hu et al. 2016). Therefore, a lower response to the PHA test in
487 infected individuals could be an indirect consequence of an immune response
488 aimed at fighting off the infection without further consequences on the
489 individuals' immunocompetence. However, because infections by

490 hemoparasites in breeding birds tend to be chronic (Valkiunas 2004), further
491 effects on immunocompetence may appear in the longer term.

492 In addition to this, we explored the relationship between *Lankesterella* infection
493 and other variables before the PHA-treatment. IgG level, weight increase or
494 leukocytes were not affected by *Lankesterella* infection. However, nestlings that
495 were reared in mite-infected nests showed higher IgG level than those reared in
496 uninfected nests. Experimental studies in domestic fowl infected with
497 *Dermanyssus gallinae* (Harrington et al. 2009; Harrington et al. 2010) have
498 shown that mites or mite antigens can raise IgG levels, but other cellular
499 immune responses such as Th1 and Th2 cytokine mRNA expression in
500 peripheral mononuclear blood cells remained unaltered (Harrington et al. 2010).
501 Thus, parasitic infections may affect only certain immune parameters (i.e., the
502 immune response could be parasite specific). In fact, in Snow Bunting nestlings,
503 the mites' presence in the nest was related to the nestling's IgG level but not to
504 their response to PHA, while the opposite was seen with respect to
505 *Lankesterella* infections.

506 In summary, we have reported infections by the blood parasite *Lankesterella*
507 sp., in Snow Bunting nestlings for the first time in the Arctic. Infected individuals
508 or those with lower body mass gain showed a weaker PHA response, as
509 expected. Moreover, Snow Bunting nestlings reared in mite-infested nests
510 showed higher IgG level that those reared in mite-free nests. These results
511 highlight the need for further research on host-parasite interactions and immune
512 responses in polar organisms in order to understand the mechanisms
513 underlying these relationships in extreme environments.

514

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516

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525
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Table legends

Table 1. Model selection explaining PHA-induced response in snow buntings. Variables included in each model were marked with “X”. A total of 90 nestlings from 24 nests were included in this analysis. The most parsimonious models are highlighted in bold. Models that were included in model averaging were further confirmed by parametric bootstrapping (all P<0.0001, see main text). Codes: AICc=corrected AIC, ΔAICc=increment in AICc between the most parsimonious models, AICcw =AICc weights, Treat=Treatment, Lank=Lankesterella infected, Weight= weight gain, IgG=total immunoglobulin, Leuko=leucocytes.

Table 2. Results of the linear mixed model for immunoglobulins (IgGs). Effect sizes for each variable are presented as Eta-squared and partial Eta-squared (Eta^2 and $pEta_2$). The df_{num} and df_{den} refer to the numerator and denominator degrees of freedom in the F-test for each effect. The sample size was 90 nestlings from 24 nests.

Table 3. Results for the model averaging for the PHA response. Parameter estimates and standard error (SE) are shown. AICc weights ($AICc_w$) refers to the variable importance within the set of 15 models run (see the main text for further details). The sample size was 88 nestlings from 24 nests (45 PHA-injected and 43 control). Treat=PHA Treatment.

Figure legends

Figure 1. Map of the study area showing the sampling site (i.e., Longyearbyen) in Spitsbergen Island, Svalbard archipelago, Norway.

Figure 2. Phylogenetic tree obtained by Bayesian inference. Sequences obtained in the present study are marked in bold.

Figure 3. Graphic representation of the relationship between IgG level and mite infestation in Snow Bunting nestlings. The sample size was 90 nestlings from 24 nests. Bars represent error standard.

807 Figure 4. Graphic representation of the interaction between treatment (PHA
808 injection) and *Lankesterella* infection in Snow Bunting nestlings. The sample
809 size was 90 nestlings from 24 nests. Bars represent error standard.
810 Figure 5. Graphic representation of the interaction between treatment (PHA
811 injection) and weight gain in Snow Bunting nestlings. The sample size was 90
812 nestlings from 24 nests.
813

Figure 1

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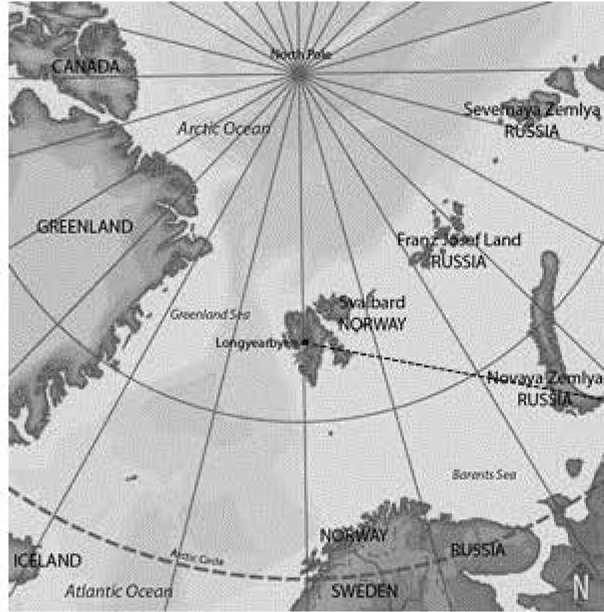
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North Pole
Arctic Ocean
Severnaya Zemlya
RUSSIA
GREENLAND
Franz Josef Land
RUSSIA
Svalbard
NORWAY
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Novaya Zemlya
RUSSIA
Barents Sea
NORWAY
RUSSIA
ICELAND
Arctic Circle
Atlantic Ocean
SWEDEN
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Sjuøyene
Kvitøya
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Orvin land
Storøya
Verlegenhuken
Vestfonna
Nordaustlandet
Hinlopenstretet
Wahlenbergfjorden
Widjefjorden
Woodfjorden
Ny
Frisland
Gustav
Adolf
land
Austfonna
Hartogbukta
Albert I
land
Andrée
land
Kapp
Mohn
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Wahlbergøya
Abeløya
Håkon VII
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Kapp
Mitra
Erik Eriksenstredet
Kong Karls Land
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James I
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Olav V
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Kapp
Payer
Svenskøya
Forlandsundet
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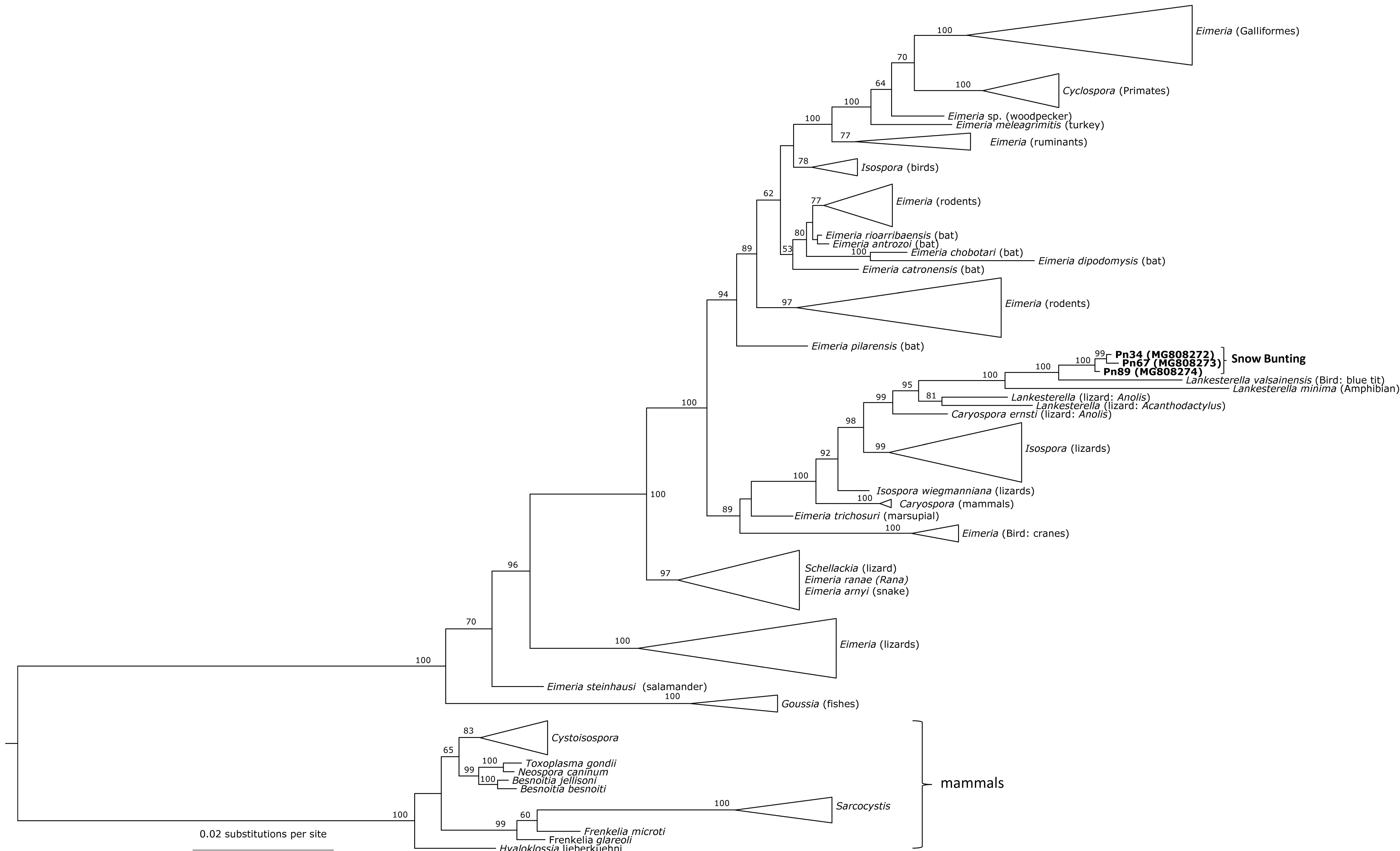
land
Barentsøya
Olgastretet
Nordfjorden
Billefj.
SPITSBERGEN
Sabine
land
Freemansundet
Isfjorden
Longyearbyen
Barentsburg
Agardh-
bukta
Edge-
jøkulen
Nordenskiöld
land
Sveagruva
Van Mijenfj.
Edgeøya
Heer
land
Storfjorden
Bellsundet
Nathorst
land
578
Tjuvfjorden
1431
Hornsundtind
Kval-
vågen
Wedel
Jarlsberg
land
Negerpynten
Torell
land
Hopen
Hornsund
Barentshavet
Sørkapp
land
0
50
100 km
Sørkapp

Figure 2

Click here to download Figure Figure 2.tif



Eimeria (Galliformes)
Cyclospora (Primates)
Eimeria sp. (woodpecker)
Eimeria meleagrimitis (turkey)
Eimeria (ruminants)
Isospora (birds)
Eimeria (rodents)
Eimeria rioarribaensis (bat)
Eimeria antrozoi (bat)
Eimeria chobotari (bat)
Eimeria dipodomysis (bat)
Eimeria catronensis (bat)
Eimeria (rodents)
Eimeria pilarensis (bat)
Pn34 (MG808272)
Pn67 (MG808273)
Pn89 (MG808274)
Snow Bunting
Lankesterella valsainensis (Bird: blue tit)
Lankesterella minima (Amphibian)
Lankesterella (lizard: Anolis)
Lankesterella (lizard: Acanthodactylus)
Caryospora ernsti (lizard: Anolis)
Isospora (lizards)
Isospora wiegmanniana (lizards)
Caryospora (mammals)
Eimeria trichosuri (marsupial)
Eimeria (Bird: cranes)
Schellackia (lizard)
Eimeria ranae (Rana)
Eimeria arnyi (snake)
Eimeria (lizards)
Eimeria steinhausi (salamander)
Goussia (fishes)
Cystoisospora
Toxoplasma gondii
Neospora caninum
Besnoitia jellisoni
Besnoitia besnoiti
mammals
Sarcocystis
Frenkelia microti
Frenkelia glareoli
Hyaloklossia lieberkuehni
0.02 substitutions per site

Figure 3

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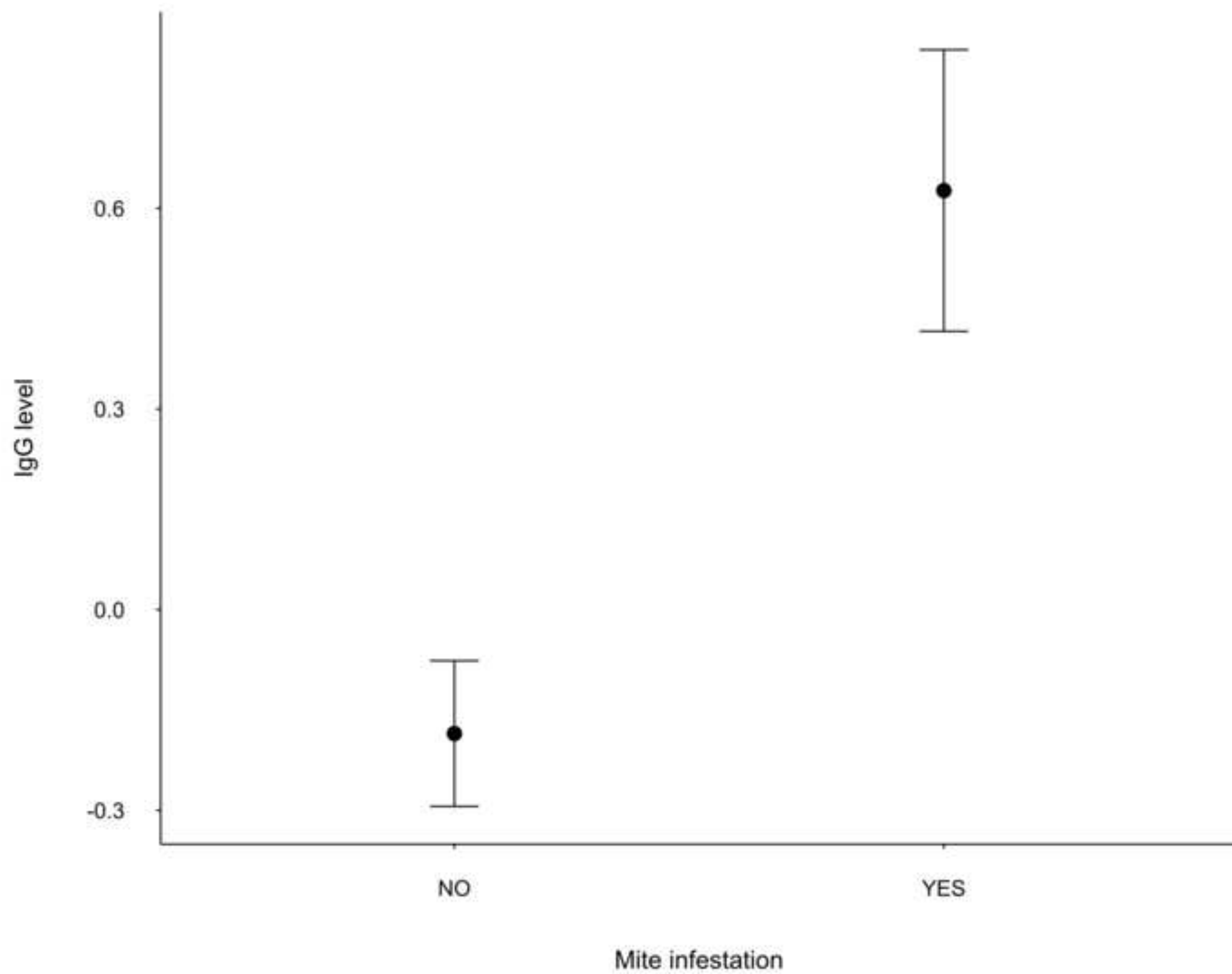

Figure 4

Click here to download Figure Figure 4.tif

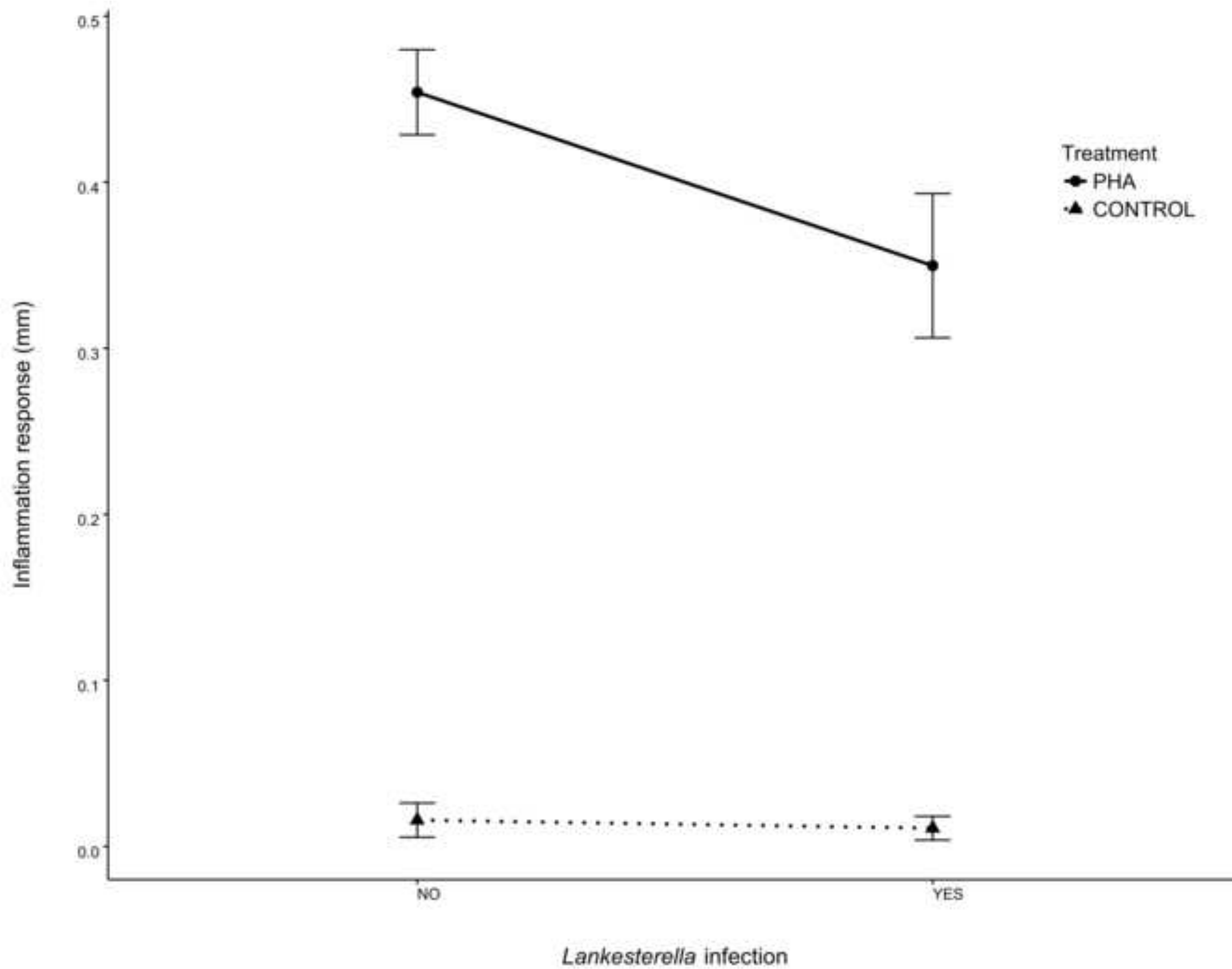

Figure 5

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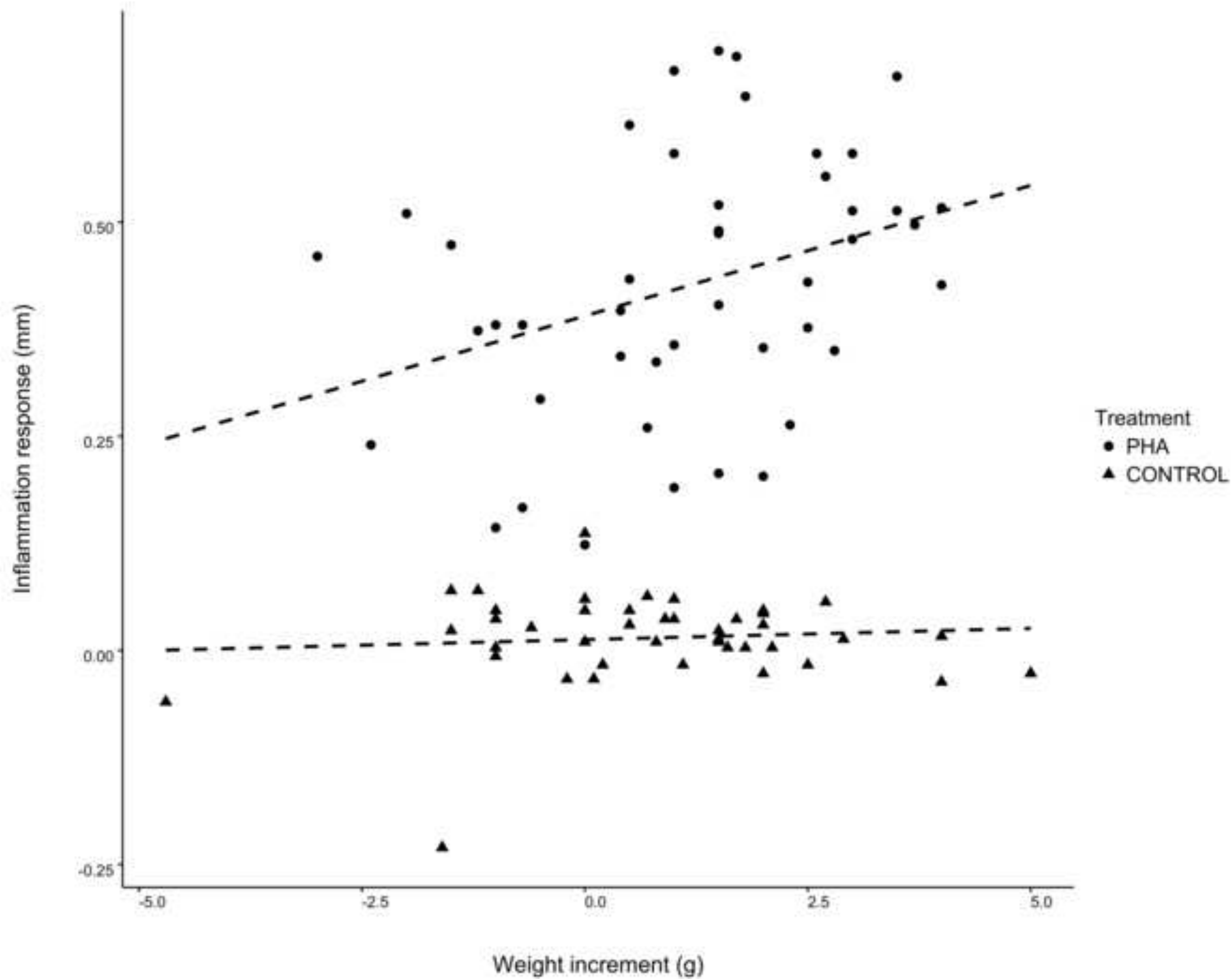

Click here to download Table Table 1.docx

Model	Treat	Sex	Mites	Lank	Weight	IgG	Leuko	Treat*Weight	Treat*IgG	Treat*Lank	Treat*Leuko	AICc	ΔAICc	$AICc_w$
1	x	x	x	x	x	x	x	x	x	x	x	-35.59	35.05	0
2	x	x	x	x	x	x	x	x	x	x		-50.91	19.73	0
3	x	x	x	x	x	x	x	x	x		x	-39.53	31.11	0
4	x	x	x	x	x	x	x	x		x	x	-49.15	21.49	0
5	x	x	x	x	x	x	x		x	x	x	-43.40	27.24	0
6	x	x	x	x	x	x	x	x	x			-54.86	15.78	0
7	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**		**x**		**-63.90**	**6.74**	**0.023**
8	x	x	x	x	x	x	x	x			x	-53.92	16.72	0
9	x	x	x	x	x	x	x		x	x		-59.94	10.7	0.003
10	x	x	x	x	x	x	x		x		x	-55.53	15.11	0
11	x	x	x	x	x	x	x			x	x	-56.11	14.53	0
12	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**				**-68.71**	**1.93**	**0.269**
13	**x**	**x**	**x**	**x**	**x**	**x**	**x**		**x**			**-62.74**	**7.9**	**0.013**
14	**x**	**x**	**x**	**x**	**x**	**x**	**x**			**x**		**-70.64**	**0**	**0.683**
15	x	x	x	x	x	x	x				x	-61.35	9.29	0.007

 Click here to download Table Table 2.docx

Explanatory variable	Estimate	Std. Err.	Eta2	pEta2	SS-efect	F value	DF	DF.res	P-value
Sex	0.052	0.077	0.002	0.007	0.201	0.460	1	66.53	0.500
Lankesterella	0.042	0.247	0.000	0.000	0.012	0.027	1	79.04	0.869
Mites	-0.392	0.177	0.026	0.069	2.134	4.882	1	20.60	**0.039**
Weight gain	0.018	0.050	0.000	0.002	0.052	0.118	1	79.09	0.732
Leukocytes	0.002	0.005	0.001	0.004	0.112	0.256	1	80.80	0.614

Table 3 Click here to download Table Table 3.docx

Source	**Estimate**	**SE**	**Adjusted SE**	**Z value**	**AICc$_w$**	**P-value**
Intercept	0.2571417	0.0399486	0.0405824	6.336	-	<0.001
Sex	0.0172226	0.0120013	0.0121772	1.414	1	0.15726
Weight gain	0.0127563	0.0067736	0.0068805	1.854	1	0.06374
Leukocytes	-0.000551	0.0006525	.00006629	0.831	1	0.40579
IgG	0.0241662	0.0140790	0.0142979	1.690	0.73	0.09099
Mites	-0.0164093	0.0172332	0.0174939	0.938	1	0.34824
Lankesterella	-0.0557088	0.0304481	0.0309072	1.802	0.96	0.07147
Treat	0.2009204	0.0164789	0.0166522	12.066	1	**<0.001**
Treat*Weight gain	0.0129155	0.0063686	0.0064702	1.996	0.45	**0.04592**
Treat*IgG	0.0187595	0.0115820	0.0117625	1.595	0.19	0.11075
Treat**Lankesterella*	-0.1050168	0.0373275	0.0379314	2.769	0.66	**0.00563**

Table ESM_1. Primers used to detect hemoparasites. The primer sets used to detect *Plasmodium* / *Haemoproteus*, *Leucocytozoon* and *Trypanosoma* were designed in a previous study (Martínez et al. 2016). The rest of the primers were designed in the present study.

Primer	sequence 5' → 3'	pb	annealing	extensión	parasite (gen)
PALU-Fq **PALU-R**	caaggtagctctaatcctttagg dggaacaatatgtaraggagt	201	54°C-30s	60°C-30s	*Plasmodium*/*Haemoproteus* (cyt b)
L180 **Leunew1R**	gagaactatggagtggatgg cccagaaactcatttgwcc	221	60°C-30s	60°C-30s	*Leucocytozoon* (cyt b)
TryR **TryF**	atgcactaggcaccgtcg ggagagggagcctgagaaata	121	60°C-30s	60°C-30s	*Trypanosoma* (18S rRNA)
Hep900F **EimRodR**	gtcagaggtgaaattcttagatttg gcatttccctatctctagtcgg	160	58°C-30s	60°C-30s	Coccidios (18S rRNA)
Hep900F **Hep4**	gtcagaggtgaaattcttagatttg taaggtgctgaaggagtcgtttat	188	60°C-30s	60°C-30s	Hepatozoon (18S rRNA)
Hep900F **RLB-R**	gtcagaggtgaaattcttagatttg tcttcgatcccctaactttc	101	60°C-30s	60°C-30s	Babesia (18S rRNA)

Table ESM_2. Primers used to achieve larger amplicons from nestlings infected with *Lankesterella* (Megía et al. 2014). Primer NBA1 also named BT-F1.

primer	secuencia 5' → 3'	annealing	extensión
NBA1	ggttgatcctgccagtagt	58°C-30s	72°C-80s
EimRodR	gcatttccctatctctagtcgg		
Hep900F	gtcagaggtgaaattcttagatttg	58°C-30s	72°C-60s
Hep1615R	aaagggcagggacgtaatc		