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1 *Short Communication*

2

3 **Sex-specific effects of experimental ectoparasite infestation on telomere**
4 **length in great tit nestlings**

5 Barbara Tschirren, Ana Angela Romero-Haro, Sandrine Zahn, François Criscuolo*

6 * There is a spelling error on the publisher's web site ("Cricuolo")

7 **Abstract**

8 Telomere length is a biomarker of biological ageing and lifespan in various
9 vertebrate taxa. Evidence is accumulating that telomeres shorten more rapidly when
10 an individual is exposed to environmental stressors. Parasites are potent selective
11 agents that can cause physiological stress directly or indirectly through the activation
12 of the host's immune system. Yet to date, empirical evidence for a role of parasites
13 in telomere dynamics in natural populations is limited.

14 Here we show experimentally that exposure to ectoparasitic hen fleas (*Ceratophyllus*
15 *gallinae*) during growth results in shorter telomeres in female, but not male, great tit
16 (*Parus major*) nestlings. Females had significantly longer telomeres than males
17 when growing up in experimentally deparasitized nests but because of the sex-
18 specific effects of parasitism on telomere shortening, this sexual dimorphism was
19 absent in birds growing up in experimentally infested nests. Our results provide the
20 first experimental evidence for a role of ectoparasitism in telomere dynamics in a
21 natural vertebrate population, and suggest that the costs of infection manifest in sex-
22 specific ways.

23

24 **Keywords:** biological ageing; life history evolution; host-parasite interactions; sex
25 differences; senescence in wildlife populations; telomere length; telomere
26 shortening; costs of infection; sexual dimorphism; telomere dynamics

27 **Introduction**

28 Evidence is accumulating that the length of telomeres, the protective caps at the end
29 of chromosomes, is a predictor of ageing and lifespan across vertebrate taxa
30 (Wilbourn et al. 2018). Telomeres have been found to shorten more rapidly when an
31 individual is exposed to environmental stressors, such as harsh abiotic conditions or
32 food limitation (Chatelain et al. 2020; Epel et al. 2004). Parasites are particularly
33 potent selective agents that can induce physiological stress through direct damage
34 (e.g. by consuming host resources (Tripet & Richner 1997)) or indirectly through the
35 activation of the host's immune system (Costantini & Møller 2009). Yet to date,
36 empirical evidence for an effect of parasitism on telomere attrition in natural
37 populations is limited.

38 Accelerated telomere shortening in response to experimental pathogen infection has
39 been demonstrated in captive house mice (*Mus musculus*) (Ilmonen et al. 2008), and
40 a correlation between pathogen infection and subsequent telomere attrition is also
41 observed in humans (Asghar et al. 2018). Furthermore, correlative and experimental
42 work in avian malaria host-pathogen systems has demonstrated accelerated
43 telomere shortening in *Plasmodium*-infected birds (Asghar et al. 2015; Asghar et al.
44 2016; Karell et al. 2017), and recent findings suggest that the costs of avian malaria
45 infection in terms of accelerated telomere attrition might be host sex-specific (Sudyka
46 et al. 2019). Yet, given the lack of studies in other natural host-parasite systems, the
47 generality of these findings is currently unclear.

48 Hematophagous hen fleas (*Ceratophyllus gallinae*) are common, nest-based
49 ectoparasites of cavity nesting birds (Tripet & Richner 1997). We have previously
50 shown that exposure to hen fleas during growth has sex-specific consequences for
51 great tit (*Parus major*) nestlings (Tschirren et al. 2003). Male nestlings exposed to

52 hen fleas have a significantly lower body mass and smaller body size compared to
53 male nestlings growing up in a flea-free nest, whereas no significant negative effect
54 of hen flea exposure on the growth of female nestlings is observed (Tschirren et al.
55 2003). Thus, at first sight, males appear to be more susceptible to hen fleas than
56 females. However, individuals exposed to parasites (or other environmental
57 stressors) are predicted to prioritise resource allocation to key fitness-related traits
58 and functions, at the expense of traits and functions less closely related to fitness, in
59 order to minimize the negative impact of parasitism on fitness (Boonekamp et al.
60 2018; Vedder et al. 2017; Waddington 1942). If selection is acting in a sex-specific
61 way, these allocation priorities will differ between males and females. As a
62 consequence, the costs of parasitism might manifest differently in the two sexes.
63 Here we experimentally quantify the (sex-specific) effect of ectoparasite infestation
64 on telomere length (TL) in a natural bird population. We show that exposure to hen
65 fleas during growth negatively affects TL of female, but not male, great tit nestlings,
66 demonstrating a sex-specific role of ectoparasitism in telomere dynamics.

67

68 **Materials & Methods**

69 *Experimental procedures*

70 The study was performed in a great tit population breeding in nest boxes in a forest
71 near Bern, Switzerland (46°54'N 7°17'E/46°57'N 7°21'E). Nest boxes were visited
72 regularly from the beginning of the breeding season onwards to determine the start
73 of nest building and egg laying. One day after hatching, nestlings from broods with
74 the same hatching date and a similar brood size were partially exchanged ('cross-
75 fostering'). Nestlings were marked individually by clipping down feathers, ranked in
76 their original nest according to body mass and then alternately assigned to stay in

77 their original nest or to be moved to the foster nest. During cross-fostering, the
78 nesting material of all nests was heat-treated in a microwave oven to kill hen fleas
79 naturally present in the nest (Richner et al. 1993). One randomly chosen nest of
80 each cross-fostering pair was then assigned to be experimentally re-infested with 40
81 female and 20 male hen fleas, whereas no fleas were added to the other nest
82 (hereafter referred to as 'uninfested') (Richner et al. 1993). Note that although fleas
83 can naturally immigrate into 'deparasitized' nests, these numbers are small
84 compared to the flea load of experimentally infested nests (Heeb et al. 1996). This
85 experimental design ensured that for each family, half of the siblings were exposed
86 to ectoparasitic hen flea during growth, whereas the other half grew up in a nest with
87 no (or very few) fleas present. The hen fleas used for the experimental re-infestation
88 were obtained from old nests collected in the study area at the start of the breeding
89 season. Eight days post-hatching, nestling were weighed and a small blood sample
90 (approx. 20 μ l) was taken for molecular sex determination (as described in (Tschirren
91 et al. 2003)) and TL analysis. Because blood was only sampled at one time point, we
92 inferred telomere shortening by comparing nest mates and biological siblings that
93 were randomly assigned to the two flea treatment groups, rather than based on
94 within-individual telomere dynamics.

95 All procedures comply with the current laws of Switzerland and were approved by
96 the Animal Experimentation Board of the Cantonal Veterinary Office of Berne, Berne,
97 Switzerland (license 16/02) and the Federal Office for the Environment, Berne,
98 Switzerland (license 2200). The municipality of Berne approved field work in their
99 forests.

100

101

102 *Telomere length analysis*

103 Telomere length (TL) was quantified in whole blood of a randomly selected subset of
104 159 nestlings from 62 nests (39 males and 43 females from flea-free nests and 42
105 males and 35 females from flea-infested nests) using real-time qPCR. In short,
106 relative individual telomere length was calculated as the ratio (T/S ratio) of the
107 quantity of telomere repeated sequences (T) relative to the DNA quantity of a
108 reference gene (S) (see (Stier et al. 2016) and the electronic supplementary
109 materials ESM1 for a detailed description). Telomere measurements were
110 standardised (z-score) for statistical analysis.

111

112 *Statistical analysis*

113 We used a linear mixed model to test for sex-specific effects of ectoparasite
114 exposure during growth on nestling TL using the *R* package *lme4* (Bates et al. 2011).
115 Nestling sex, flea treatment and their interaction were included as fixed effects. Nest
116 of origin and nest of growth were included as random effects to account for the non-
117 independence of biological siblings and nestlings growing up in the same nest,
118 respectively. We also ran a second model, including the factors described above and
119 body mass and the interaction between body mass and sex or flea treatment as
120 additional factors. Significance of predictors was evaluated using Satterthwaite's
121 degree of freedom method in *lmerTest* (Kuznetsova et al. 2017). Pairwise posthoc
122 comparisons were performed for significant outcomes in the main models using
123 *lsmeans* (Lenth 2016). All statistical analyses were performed in *R* version 4.0.2. (R
124 Core Team 2017). Normality of the residuals was confirmed by visual inspection and
125 Shapiro-Wilk test.

126

127 **Results**

128 We observed a significant interaction effect between nestling sex and experimental
129 flea infestation on nestling TL ($t = -2.05$, $df = 130.1$, $P = 0.042$; Fig. 1; ESM3).
130 Posthoc contrasts revealed that flea-exposed female nestlings (mean \pm 1SD: $1.263 \pm$
131 0.277) had significantly shorter telomeres than female nestlings growing up in an
132 uninfested (mean \pm 1SD: 1.409 ± 0.326) nest ($t = 2.103$, $df = 79.8$, $P = 0.039$; Fig. 1),
133 whereas the TL of male nestlings in flea-infested (mean \pm 1SD: 1.189 ± 0.286) and
134 uninfested (mean \pm 1SD: 1.135 ± 0.246) nests did not differ significantly ($t = -0.686$,
135 $df = 67.5$, $P = 0.495$; Fig. 1). Consequently, a significant sex difference in TL was
136 observed in flea-free nests, with female nestlings having significantly longer
137 telomeres than male nestlings ($t = -4.194$, $df = 152$, $P < 0.001$; Fig. 1), whereas in
138 flea-infested nests no significant sex difference in TL was observed ($t = -1.332$, $df =$
139 152 , $P = 0.185$; Fig. 1).
140 No significant association between body mass and TL was found (body mass: $t =$
141 0.90 , $df = 133.3$, $P = 0.370$; sex x body mass: $t = -0.55$, $df = 147.6$, $P = 0.585$; flea
142 treatment x body mass: $t = -1.56$, $df = 106.4$, $P = 0.121$). Furthermore, including body
143 mass as a covariate in the model did not change the sex x flea interaction effect on
144 TL ($t = -2.01$, $df = 129.2$, $P = 0.047$). Full model outputs are presented in the
145 electronic supplementary materials ESM3.

146

147 **Discussion**

148 This study provides the first experimental evidence for a (sex-specific) role of
149 ectoparasite infestation in telomere dynamics, adding to the growing evidence that
150 environmental stressors accelerate telomere shortening in natural populations
151 (Chatelain et al. 2020).

152 Thus far, evidence for parasite-induced telomere attrition in wild vertebrates was
153 limited to avian malaria host-pathogen systems, where accelerated telomere
154 shortening in response to chronic infection was observed (Asghar et al. 2015;
155 Asghar et al. 2016; Karell et al. 2017). In contrast, the effect of ectoparasites on
156 nestling telomere shortening presented here was acute, occurring after only eight
157 days of exposure. This finding is in line with previous studies that demonstrated
158 particularly rapid telomere shortening in response to environmental stressors during
159 the juvenile period (Salomons et al. 2009). Importantly, early life telomere length
160 (Eastwood et al. 2019; van Lieshout et al. 2019) and rate of telomere attrition
161 (Boonekamp et al. 2014) appear to be a particularly strong fitness predictors.
162 Currently it is unclear if the observed effect of hen flea exposure on host telomere
163 shortening is caused by direct blood loss and subsequently enhanced blood cell
164 division (Tripet & Richner 1997), the transfer of flea-borne pathogens (Aivelo &
165 Tschirren 2020), or the activation of the nestlings' immune system (Costantini &
166 Møller 2009).

167 The effect of ectoparasite exposure on telomere shortening was sex-specific with
168 females showing a significantly accelerated telomere attrition when exposed to fleas,
169 whereas no significant difference in telomere length of males in flea-infested and
170 uninfested nests was observed. Interestingly, the opposite pattern was found in
171 captive house mice (*Mus musculus*) where telomere shortening in response
172 experimental *Salmonella enterica* infection was much more pronounced in males
173 than in females (Ilmonen et al. 2008). It is currently unclear why infection has
174 opposite sex-specific effects on TL in the two systems (see also below).

175 Previously we have shown that male nestlings exposed to hen fleas are significantly
176 smaller and lighter, whereas no significant difference in body size or mass is found in

177 female nestlings from flea-infested and flea-free nests (Tschirren et al. 2003). It
178 suggests that costs of infection manifest in sex-specific ways, with males prioritising
179 telomere maintenance at the expense of early life growth when exposed to
180 ectoparasites, and vice versa in females. We would expect such sex-specific
181 resource allocation priorities if early life telomere attrition and growth, respectively,
182 are differentially associated with fitness in males and females. No sex-specific effect
183 of fledging body mass or size on first year survival (i.e. local recruitment) is found in
184 the study population (Tschirren 2015). However, juvenile body mass or size might
185 affect other fitness components in a sex-specific way, such as reproductive success.
186 Indeed, juvenile mass (but not adult mass) is a significant predictor of clutch size in
187 female great tits (Tilgar et al. 2010).

188 Similarly, the associations between telomere length (or attrition) and fitness might be
189 sex-specific (Olsson et al. 2011). Telomere length predicts local recruitment (i.e. first
190 year survival) in great tits (Salmon et al. 2017), but it is currently unknown if this
191 association is sex-specific. In birds, males typically live longer than females (Che-
192 Castaldo et al. 2019), and accelerated ageing and / or a shorter lifespan might thus
193 have more severe fitness consequences for males than for females (Barrett &
194 Richardson 2011). Yet, associations between sex, telomere length and lifespan
195 appear to be highly taxon specific (Barrett & Richardson 2011; Wilbourn et al. 2018),
196 and further research is required to elucidate the causes and consequences of this
197 taxon-specific variation.

198 Our results demonstrate sex difference in telomere shortening in response to
199 ectoparasitism, but at the same time we also found an absolute sex difference in
200 early life telomere length in the absence of infection, with females having significantly
201 longer telomeres than males. Interestingly, sex differences in telomere length (and

202 attrition) are found in various taxa (e.g. Barrett & Richardson 2011; Gardner et al.
203 2014; Watson et al. 2017), but we currently do not have a good understanding of the
204 causes and consequences of sexual dimorphism in telomere length (or attrition)
205 across species (Barrett & Richardson 2011).

206 In conclusion, our study provides the first experimental evidence for a role of
207 ectoparasitism in telomere dynamics in a natural vertebrate population. Importantly,
208 the consequences of ectoparasite exposure for telomere attrition was sex-specific,
209 with TL of females being more strongly affected than TL of males. This finding
210 contrasts previous observations of ectoparasite-induced growth reduction in male
211 nestlings and suggests that males and females are prioritising different traits and
212 functions when exposed to environmental stressors, such as ectoparasites (i.e. sex-
213 specific canalization). Understanding sex-specific associations between TL and
214 fitness within and across taxa will help to elucidate the causes and consequences of
215 such sexual dimorphism in TL dynamics.

216

217 **Data Accessibility**

218 Data are available from the Dryad Digital Repository: doi: xxx

219

220 **Authors' Contributions**

221 xx conceived and designed the study, performed sample collection and analysed the
222 data. Xx and xx performed telomere length analysis. xx and xx prepared the
223 manuscript. All authors revised the manuscript, gave final approval for publication
224 and agreed to be accountable for all aspects of the work.

225

226 **Competing interests**

227 We declare we have no competing interests.

228

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236

237 **Footnotes**

238 Electronic supplementary material is available online at xxxx

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353

354 Figure legend

355 Fig. 1. Experimental hen flea exposure during growth results in shorter telomeres (z-
356 transformed T/S ratio) in female but not male great tit nestlings. Means \pm 1SE are
357 shown.

358