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4   **Does avian malaria reduce fledging success: An experimental test of the**

5   **selection hypothesis**

6   Sarah A. Knutie[1,2], Jessica L. Waite[1,2,3], Dale H. Clayton[1]

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9   [1]Biology Department, University of Utah, Salt Lake City, Utah, 84112 USA

10   [2]SAK and JLW contributed equally to this work.

11   [3]Corresponding author: jessi.waite@gmail.com

12   Telephone: (801) 585-9742

13   Fax: (801) 581-4668

14

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24 **Abstract**

25   Like many parasites, avian haematozoa are often found at lower infection intensities in

26 older birds than young birds. One explanation, known as the "selection" hypothesis, is that

27 infected young birds die before reaching adulthood, thus removing the highest infection

28 intensities from the host population. We tested this hypothesis in the field by experimentally

29 infecting nestling rock pigeons *(Columba livia)* with the malaria parasite *Haemoproteus*

30 *columbae*. We compared the condition and fledging success of infected nestlings to that of

31 uninfected controls. There was no significant difference in the body mass, fledging success, age

32 at fledging, or post-fledging survival of experimental versus control birds. These results were

33 unexpected, given that long-term studies of older pigeons have demonstrated chronic effects of

34 *H. columbae*. We conclude that *H. columbae* has little impact on nestling pigeons, even when

35 they are directly infected with the parasite. Our study provides no support for the selection

36 hypothesis that older birds have lower parasite loads because parasites are removed from the

37 population by infected nestlings dying. To our knowledge, this is the first study to test the

38 impact of avian malaria using experimental inoculations under natural conditions.

39

Introduction

Parasites influence fundamental aspects of the evolutionary ecology of their hosts, such as population dynamics (Anderson and May 1978; Anderson 1979) and life history evolution (Hochberg et al. 1992). The impact of parasites on host fitness depends partly on the age at which hosts become infected. A common pattern in host-parasite interactions is that younger individuals have higher parasite loads than adults (Gregory et al. 1992; Hudson and Dobson 1997). Sol et al. (2003) considered three hypotheses to explain this pattern. The "selection" hypothesis suggests that highly parasitized juvenile hosts die before they reach adulthood, removing large numbers of parasites from the population. The "immunity" hypothesis suggests that the developing immune system of juveniles is not yet capable of killing parasites, while adults are much more effective at reducing parasite intensity. The "vector exposure" hypothesis suggests that adult behavior reduces their exposure to infected vectors, and thus parasites, compared to juveniles.

Sol et al. (2003) evaluated these hypotheses using data from a study of feral rock pigeons *(Columba livia)* infected with malaria parasites *(Haemoproteus columbae)* vectored by pigeon louse flies *(*Hippoboscoidea: *Pseudolynchia canariensis)*. The authors rejected the vector exposure hypothesis because they found that adult pigeons (> 6 months old) are not, in fact, exposed to fewer vectors than juvenile pigeons (Sol et al. 2000). Although the authors reported higher rates of juvenile mortality (61%) compared to adult mortality (33%), consistent with the selection hypothesis, selection in their study was not strong enough to explain the lower number of parasites observed in adult birds. The youngest birds in Sol et al.'s study had already fledged from the nest; however, the greatest impact of *H. columbae* on pigeons may occur while birds are still in the nest. We conducted a study to test the impact of *H. columbae* on the condition and

62   fledging success of younger, nestling rock pigeons. We used an experimental approach in which

63   we compared nestlings injected with *H. columbae* to control birds not injected with the parasite.

64   At least 200 species of *Haemoproteus* are known to infect birds worldwide (Martinsen et

65   al. 2008). Perez-Tris et al. (2005) classified *Haemoproteus* as an avian malaria parasite because

66   members of the genus were nested phylogenetically within the genus *Plasmodium*. *H. columbae*

67   is a parasite of pigeons and doves that uses blood-feeding pigeon flies as vectors (Valkiūnas

68   2005). The parasite enters a feeding fly and reproduces in its midgut, where *H. columbae*

69   oocysts attach to the gut wall. Once mature, the oocysts burst and release infective sporozoites

70   that migrate from the fly's gut into its salivary glands. The fly then injects these sporozoites into

71   a pigeon when it feeds. *H. columbae* reproduces asexually in the lungs of the pigeon, then

72   invades and matures in the red blood cells (Ahmed and Mohammed 1978).

73   *Haemoproteus* species can have several negative effects on host fitness. These effects

74   include reductions in host body condition (Merino et al. 2000), lower reproductive success

75   (Marzal et al. 2004; Tomas et al. 2007), and even death (Atkinson and Forrester 1988; Sol et al.

76   2003). Studies of the impact of malaria on juvenile birds have consisted of observational studies

77   in the field (Sol et al. 2003), and experimental studies using captive birds (Yorinks and Atkinson

78   2000; Garvin et al. 2003). The goal of our study was to use an experimental approach under

79   field conditions. We infected nestling birds with malaria parasites to test the impact on body

80   mass, fledging success, age at fledging, and post-fledging survival of experimental versus control

81   birds.   Studies with captive birds suggest that the most pathogenic phase of the *Haemoproteus*

82   life cycle occurs when parasites enter red blood cells to mature (Atkinson and Forrester 1988;

83   Atkinson and van Riper 1991). In the case of *H. columbae* this takes place about 24-37 days

84   after infection (Ahmed and Mohammed 1978). Since pigeons fledge at about 32 days of age, it

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85   is not possible to be sure that fledglings are infected with malaria parasites, short of

86   experimentally infecting them. Experimental manipulation is the most powerful approach for

87   testing the impact of parasites on hosts in any case (McCallum and Dobson 1995). To our

88   knowledge, this is the first study to test the impact of avian malaria parasites using experimental

89   inoculation under natural conditions.

90

91   **Materials and Methods**

92   We experimentally manipulated *H. columbae* in nestling rock pigeons. The study took

93   place August-November 2009 under a highway overpass in Draper, Utah, USA (40°31'36" N,

94   111°53'28" W). We visited the field site every 2-3 days throughout the study period. Nestlings

95   were weighed at each visit to the nearest 1.0 g with a pesola scale. Our experiment was

96   restricted to nests with two nestlings, the normal number for rock pigeons. Nests were

97   sequentially assigned to one of three treatment groups: experimental (n = 12 nests), control (n =

98   13), or background (n = 12). When nestlings were 4 - 7 days old (50 - 150 g), those at

99   experimental nests were injected with a suspension of *P. canariensis* flies infected with *H.*

100   *columbae* (Ahmed and Mohammed 1978). We created the infected fly suspension by feeding

101   flies (bred from wild stock) on heavily infected captive birds. Following 10-12 days on a bird,

102   flies were placed in vials and taken to the field site, where batches of ten live flies were

103   macerated in 1000 μL of phosphate buffered saline for three minutes. Experimental nestlings

104   were injected intraperitoneally with 500 μL of the infected fly suspension using a 0.5 cc syringe.

105   Control birds were injected with 500 μL of another suspension made using uninfected flies.

106   Background birds were handled but not injected.

107   Prior to the field experiment, we conducted a test of the inoculation method using 27 wild

108   trapped, captive rock pigeons. After blocking by capture date and site, 13 randomly chosen birds

109   were injected with a suspension of infected flies, as described above. Fourteen control birds

110   were injected with a suspension of uninfected flies. At 25, 35, and 42 days post injection, blood

111   samples were taken from all birds and smears were prepared for examination. Each smear was

112   carefully examined under oil immersion at 1000x for 10 minutes; if parasites were detected, then

113   the number of parasites was quantified in 25 microscope fields per bird. All 13 experimental

114   birds were infected with *H. columbae*, while none of the 14 control birds was infected.

115   When nestlings were approximately ten days old they were fitted with a numbered

116   aluminum band and three plastic color bands. To score fledging success we observed and

117   identified birds after they left the nest on the basis of their color band combinations. We

118   conducted a thorough census of all birds at the bridge during each visit to the field site. We also

119   searched for banded birds at other bridges within 8 km of the study site in order to determine

120   whether newly fledged birds were dispersing from the natal site.

121   We continued to monitor birds at the bridge for 50 days post injection (ca. 25 days post

122   fledging) because peak parasitemia can be delayed for this long after injection (extrapolated from

123   Ahmed and Mohammed (1978)). To confirm experimental infections, we examined the blood of

124   birds after they fledged. We used walk-in traps to capture pigeons from 30-50 days post

125   injection. Blood samples were taken and birds immediately released. Blood smears were

126   prepared and examined back in the lab.

127   Data were analyzed using Prism® v.5.0b (GraphPad Software, Inc.). Power analyses

128   were conducted in G*Power 3 with an error probability set at 0.05 (Buchner et al. 1997). Where

129   necessary, data were log transformed for normalization. To avoid pseudoreplication (Hurlbert

130   1984) we averaged values for nestlings within each nest. We used one-way ANOVAs to

131   compare parasite abundance and host age and mass at fledging among treatments. A repeated-

132   measures ANOVA was used to compare the number of birds per nest at hatching, fledging, and

133   one, two, and three weeks post-fledging.

134

135   **Results**

136   Three times as many experimental birds were infected as control or background birds

137   (Fig. 1a); the three groups also differed in parasite abundance (Fig. 1b; ANOVA $F_{2,17} = 4.25$, $P <$

138   0.05). Dunnett's post hoc comparisons confirmed that experimental birds had significantly more

139   parasites than controls ($P < 0.05$), while control and background birds did not differ significantly

140   ($P > 0.05$).

141   There was no significant difference in the age of birds at fledging, nor body mass prior to

142   fledging (see Table 1). There was no significant difference in the proportion of nests that

143   fledged at least one offspring ($\chi^2 = 0.005$, $P = 0.99$). There was also no significant effect of

144   treatment on the mean number of birds fledged per nest, nor the number of birds observed after

145   fledging (Fig. 2; repeated measures ANOVA, treatment $F_{2,34} = 0.64$, $P = 0.53$). There was a

146   significant effect of time (Fig. 2; time, $F_{4,136} = 43.32$, $P < 0.0001$), but no significant interaction

147   between time and treatment (time*treatment, $F_{8,136} = 0.49$, $P = 0.86$).

148   We reanalyzed the data after excluding naturally infected control and background birds,

149   as well as experimental birds for which we could not confirm infection. We still found no

150   significant difference in age at fledging ($F_{2,31} = 0.53$, $P = 0.60$) mass at fledging ($F_{2,31} = 1.01$, $P =$

151   0.38), or the proportion of nests that fledged at least one offspring ($\chi^2 = 0.01$, $P = 0.99$).

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152   Our experiment had considerable power (1.0) to detect the level of juvenile mortality

153   (61%) reported by Sol et al. (2003); we had power of 0.8 to detect mortality of at least 30%

154   (effect size of $f = 0.55$).

155

156   **Discussion**

157   Our goal was to experimentally test the "selection" hypothesis. This hypothesis,

158   reviewed by Gregory et al. (1992), states that lower parasite loads of adults, compared to

159   juveniles, are the result of heavily infected juveniles dying before adulthood, removing parasites

160   from the population. Previous tests of this hypothesis involving avian malaria have focused on

161   juvenile (fledged) birds and relied on observational data (Sol et al. 2003; van Oers et al. 2010).

162   These studies provided some support for the selection hypothesis, but the intensity of selection

163   measured could not fully explain differences in juvenile and adult parasite loads. It was

164   conceivable, therefore, that the greatest impact of *H. columbae* on pigeons takes place while they

165   are still in the nest.

166   Our results provided no support for the selection hypothesis because there was no impact

167   of malaria on any of the components of host fitness we measured. Specifically, there was no

168   significant difference in the body mass, fledging success, age at fledging, or post-fledging

169   survival of experimental versus control birds. We are confident that our measures of post-

170   fledging survival were accurate because none of the birds from our study were observed at other

171   bridges (see methods). Young pigeons do not normally disperse until three months of age, in any

172   case (Johnston and Janiga 1995).

173   The results of our study were unexpected, given that Sol et al.'s longer-term study

174   demonstrated that *H. columbae* has a significant negative impact on pigeon fitness. The fact that

175 malaria had no detectable impact on fledging success in our study was not due to unusually low

176 rates of fledging in both experimental and control birds. Fledging success was 73% (Fig. 2),

177 similar to that in other studies of feral pigeons (reviewed by Johnston and Janiga (1995), Table

178 18.4 (values adjusted for hatching rates)). Similarly, the fact that malaria had no detectable

179 impact on fledging was not due to methodological problems with the creation of experimental

180 infections. The malaria parasite levels in our study were comparable to those observed in other

181 studies of naturally infected pigeons (Kartman 1949; Klei and DeGuisti 1975; Paperna and

182 Smallridge 2002). However, *H. columbae* may affect hosts only at levels higher than what we

183 observed (Earle et al. 1993; Paperna and Smallridge 2002). For example, the *H. columbae* levels

184 in Sol et al.'s (2003) study were among the highest ever recorded for feral rock pigeons.

185   Another factor that could conceivably contribute to why the birds in our study did not

186 appear to be affected by *H. columbae*, compared to the reduction in survival shown for older

187 birds by Sol et al. (2003), is that nestling pigeons could have higher tolerance to parasites than

188 older birds. Nestlings are fed a rich diet of crop milk by both parents. The milk, which consists

189 of the sloughed lining of the parents' crop, is very high in fat and protein (Johnston and Janiga

190 1995). It would be interesting to test the impact of *H. columbae* on nestlings fed a less nutritious

191 diet.

192   A few control and background birds were naturally infected with *H. columbae*. However,

193 infection levels were still significantly higher in the experimental groups than the control or

194 background groups. Even after excluding the naturally infected birds, we did not find that

195 malaria parasites affected age, mass, or fledging success.

196   Since *H. columbae* had no apparent effect on nestling rock pigeons, our study does not

197 provide support for the "selection hypothesis". Sol et al. (2003) reported results that were

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198 consistent with selection hypothesis; however, selection in their study was not strong enough to

199 explain the differences in parasitemia they observed between juvenile and adult pigeons.

200 Because Sol et al. (2000, 2003) reported data ruling out the "vector exposure" hypothesis, they

201 suggested a combination of the selection and immunity hypotheses may explain the fact that

202 juvenile birds have higher parasitemia than adult birds. Our data provide no reason to disagree

203 with this assessment.

204   To our knowledge, this is the first study to test the impact of avian malaria parasites using

205 experimental inoculation under natural conditions. This approach has several advantages. First,

206 like many malaria parasites, *H. columbae* takes several weeks to appear in the peripheral blood

207 after the host is infected. This fact makes early infections difficult to detect without more

208 invasive methods, such as collection of organ tissues (Valkiūnas 2005; Cosgrove et al. 2006).

209 Experimental infections get around this problem. Second, inoculating hosts with parasites has

210 the strong advantage of controlling for factors that could lead to spurious negative correlations

211 between parasite load and host fitness (Hawlena et al. 2006; Blanchet et al. 2009).   The greatest

212 limitation of our study is that the modest sample sizes limit our ability to detect relatively small

213 effect of malaria parasites on birds. For example, to detect a 10% reduction in juvenile survival

214 with a power of 0.8 would require a sample of 93 nests per treatment for a total of 279 nests. A

215 study of this magnitude may be feasible in the future using feral Rock Pigeons and *H. columbae.*

216

217 **Acknowledgements**

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293

293   Table 1. Age of birds at fledging and body mass prior to fledging. Values are grand means

294   (± SE) of the mean value per nest.

	Experimental	Control	Background	Test statistic	P
Age in days	32.3 ± 0.5	31.8 ± 0.6	32.3 ± 0.6	$F = 0.25$	0.78
(number of nests)	(11)	(12)	(11)		
Mass in grams	298 ± 14.9	311.3 ± 12.5	313.1 ± 11.4	$F = 0.35$	0.71
(number of nests)	(11)	(12)	(11)		

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316   **Figure legends**

317

318   Fig. 1. Prevalence (a) and mean abundance (+ SE) (b) of malaria parasites 30-50 days after

319   treatment.

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321   Fig 2. Mean (± SE) offspring observed per nest. The mean (± SE) number of offspring fledged

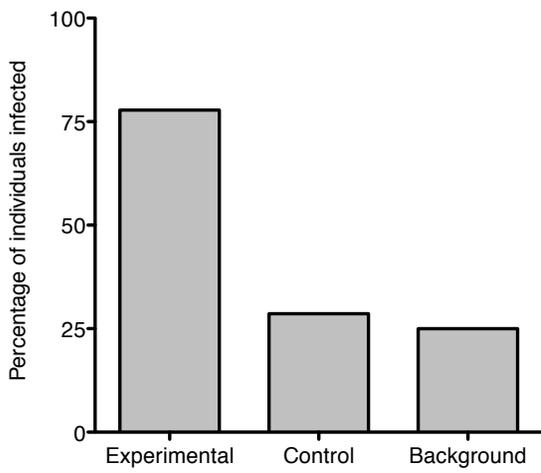322   per nest did not differ significantly among treatments; see text.

Fig 1a
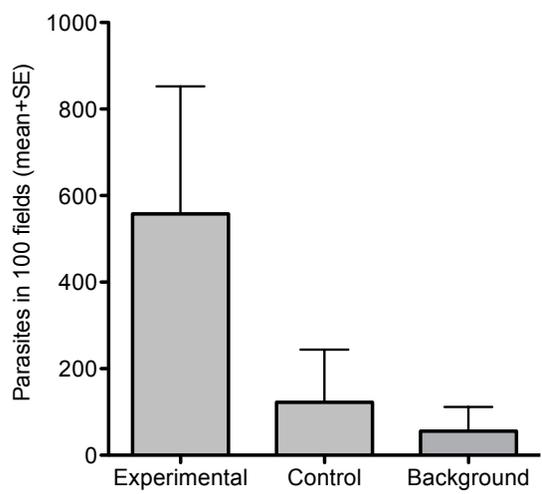
Fig 1b

Fig 2