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3 **IMPACT OF HOST NUTRITIONAL STATUS ON INFECTION DYNAMICS AND**
4 **PARASITE VIRULENCE IN A BIRD-MALARIA SYSTEM**

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23

24 *Running headline*

25 Host nutrition alters *Plasmodium* virulence

26

27 **ABSTRACT**

28 1. Host resources can drive the optimal parasite exploitation strategy by offering a good or a
29 poor environment to pathogens. Hosts living in resource-rich habitats might offer a
30 favourable environment to developing parasites because they provide a wealth of
31 resources. However, hosts living in resource-rich habitats might afford a higher
32 investment into costly immune defences providing an effective barrier against
33 infection. Understanding how parasites can adapt to hosts living in habitats of
34 different quality is a major challenge in the light of the current human-driven
35 environmental changes.

36 2. We studied the role of nutritional resources as a source of phenotypic variation in host
37 exploitation by the avian malaria parasite *Plasmodium relictum*. We investigated how
38 the nutritional status of birds altered parasite within-host dynamics and virulence,
39 and how the interaction between past and current environments experienced by the
40 parasite accounts for the variation in the infection dynamics. Experimentally-infected
41 canaries were allocated to control or supplemented diets. *Plasmodium* parasites
42 experiencing the two different environments were subsequently transmitted in a full-
43 factorial design to new hosts reared under similar control or supplemented diets.

44 3. Food supplementation was effective since supplemented hosts gained body mass during a
45 15 day period that preceded the infection. Host nutrition had strong effects on
46 infection dynamics and parasite virulence. Overall parasites were more successful in
47 control non-supplemented birds, reaching larger population sizes and producing
48 more sexual (transmissible) stages. However, supplemented hosts paid a higher cost
49 of infection, and when keeping parasitaemia constant had lower haematocrit than
50 control hosts.

51 4. Parasites grown on control hosts were better able to exploit the subsequent hosts since
52 they reached higher parasitaemia than parasites originating from supplemented
53 hosts. They were also more virulent since they induced higher mass and haematocrit
54 loss.

55 5. Our study highlights that parasite virulence can be shaped by the host nutritional status
56 and that parasite can adapt to the environment provided by their hosts, possibly
57 through genetic selection.

58

59

60 **KEY-WORDS**

61 Avian malaria, environmental variation, host-parasite interaction, nutrition, pathogen,
62 *Plasmodium relictum*, virulence

63

64 **INTRODUCTION**

65 In addition to host and parasite genetics, environmental conditions have been recognized as
66 being key to the dynamics of infectious diseases, affecting host defences, parasite
67 transmission and virulence (Lazzaro & Little 2009; Wolinska & King 2009). The environment
68 varies in space and time and many facets of this variation can shape the outcome of the
69 infection (Mostowy & Engelstadter 2011). In recent years much effort has been devoted to
70 the study of the effect of global change on the spread of infectious diseases and the risk of
71 emergence of more virulent parasite strains (Daszak, Cunningham & Hyatt 2000; Jones *et al.*
72 2008; Sorci, Cornet & Faivre 2013). Climate change can directly shape the spread of
73 infectious diseases by speeding up the growth of thermally-sensitive parasites and vectors
74 (Paaijmans *et al.* 2010). However, changes in temperature can also have profound and
75 complex ecological feedbacks on the dynamics of infectious diseases by altering, for
76 instance, the availability and the quality of nutritional resources for the host (Cahill *et al.*
77 2013).

78 Changes in host nutritional status, both in terms of quantity and quality, can have
79 profound repercussions on the dynamics of infectious diseases (Humphrey 2009; Kau *et al.*
80 2011). For a long time, nutrition has been identified as a key environmental factor shaping
81 immune defences and host susceptibility to infection (Scrimshaw, Taylor & Gordon 1959).
82 Malnutrition in terms of insufficient protein intake is associated with an impairment of cell-
83 mediated immunity, phagocyte function, complement system, and cytokine production in
84 humans (Chandra 1996). Deficiency in micronutrients has profound consequences for
85 immune functioning and susceptibility to infection. Carotenoids, vitamins A, C, E, selenium
86 and zinc have immune modulatory effects and dietary manipulations of these micronutrients
87 alter immune functioning in model and non-model systems (Bendich 2001; McGraw & Ardia

88 2003; Mora, Iwata & von Andrian 2008; Cha *et al.* 2010). Environmental changes that
89 impoverish food availability in quantity and/or quality might therefore make host
90 populations more prone to infectious diseases. The relationship between nutritional status
91 and infectious diseases is however not so straightforward. For some pathogens, disease
92 severity (the host damage induced by the infection) arises from an over-reacting immune
93 response, rather than direct parasite exploitation (Graham, Allen & Read 2005a; Sorci &
94 Faivre 2009; Long & Graham 2011). In this case, undernourished hosts with an impaired
95 immune response might actually fare better than well-nourished hosts. Finally, the
96 nutritional status of hosts can directly affect pathogen survival and reproduction because
97 well fed hosts can provide more resources to developing parasites (Pulkinen & Ebert 2004).

98 A handful of studies have explored the consequences of food availability and quality
99 on the dynamics and the cost of infection (Brown, Loosli & Schmid-Hempel 2000; Pulkinen
100 & Ebert 2004; Tseng 2006; Tschirren *et al.* 2007; Bize *et al.* 2008; Seppälä *et al.* 2008; Vale *et*
101 *al.* 2011). For instance, Hall *et al.* (2009) studied the association between the fungal
102 pathogen *Metschnikowia bicuspidate* and the crustacean *Daphnia dentifera*. Infected host
103 fed with high quality diet produced more parasites and died sooner than hosts fed on poor
104 quality diet, suggesting that increased resource acquisition by well-fed daphnia allowed a
105 better growth of the pathogen population. Similar results have been obtained by
106 manipulating food quantity. Bedhomme *et al.* (2004) found in the association between the
107 mosquito *Aedes aegypti* and the microsporidian parasite *Vavraia culicis* that the production
108 of parasite spores was positively correlated with host food availability. This couple of
109 examples therefore suggest that parasite's within-host growth can be limited by the amount
110 of resources provided by host. Other examples, however, show that hosts in poor nutritional
111 status might actually provide more favourable environments to their parasites. The flea

112 *Xenopsylla ramesis* produces more eggs when feeding on undernourished hosts (the rodent
113 *Meriones crassus*), and egg survival was much higher for fleas parasitizing underfed hosts
114 (Krasnov *et al.* 2005).

115 An even less explored topic is how pathogens adapt to well-nourished or underfed
116 hosts. As mentioned before, depending on the specific host-parasite association the
117 nutritional status of the host can exert antagonistic effects on parasite fitness. For instance,
118 if starvation increases host background mortality, pathogens are predicted to evolve
119 towards higher virulence because a 'prudent' parasite that manages its host will not be
120 rewarded (Williams & Day 2001). Well-fed hosts might also better tolerate the infection if
121 the amount of energy that is diverted by parasite multiplication is compensated by the
122 acquisition of extra resources in food-rich habitats (Vale *et al.* 2011). In this case also,
123 parasites should evolve towards higher virulence levels, because pathogens exploiting
124 tolerant hosts do not pay the cost of virulence. However, the outcome of pathogen
125 evolution may not be easily predictable as it strongly depends on the relationship between
126 within-host parasite growth rates and host tolerance (Miller, White & Boots 2006; Vale *et al.*
127 2011).

128 A few studies have used serial passage experiments to investigate parasite
129 adaptation to host nutritional status (Beck, Handy & Levander 2004; Tseng 2006; Little *et al.*
130 2007). Tseng (2006) performed an experiment where *Ascogregarina* parasites (Apicomplexa)
131 were raised in *Aedes albopictus* mosquitoes kept under two food regimes. The parasites
132 originating from well-fed and poorly-fed hosts were then transferred to new hosts following
133 a factorial design. *Ascogregarina* parasites originating from well-fed hosts were more
134 virulent to subsequent hosts compared to parasites that were grown on poorly-fed hosts,

135 especially if the new hosts were currently reared under low food levels. This study therefore
136 suggests a complex pattern where previous and current host nutritional status interact to
137 determine parasite virulence. A more detailed example of rapid shift of virulence induced by
138 the host diet has been provided for coxsackievirus B3 infecting mice. Coxsackievirus B3
139 (CVB3) can induce a myocarditis (an inflammatory heart disease) while some other viral
140 strains (CVB3/0) do not cause the disease. Nevertheless, mice fed a selenium-deficient diet
141 and inoculated with the avirulent strain (CVB3/0) do develop the disease. The viruses grown
142 in selenium-deficient hosts were then passaged to control mice, and interestingly they again
143 appeared to induce the disease. The strongest evidence that evolving in selenium-deficient
144 mice selected for higher virulence was provided by the sequencing of the viral genomic RNA
145 that showed that the avirulent strain had acquired six mutations that reverted it into the
146 virulent strain (Beck *et al.* 2004) and references therein).

147 The aim of the present article was to test the effect of dietary status of hosts on the
148 dynamics of infection and the adaptation of avian malaria parasites. Avian malaria parasites
149 (in particular, *Plasmodium sp.* and *Haemoproteus sp.*) are highly prevalent in wild passerines
150 (Cosgrove *et al.* 2008; Loiseau *et al.* 2011; Glaizot *et al.* 2012) and infection has been shown
151 to affect bird fitness, both in natural and captive populations of hosts (Van Riper III *et al.*
152 1986; Atkinson *et al.* 2000; Williams 2005; Palinauskas *et al.* 2008; Zehtindjiev *et al.* 2008),
153 even when at low chronic levels (Knowles, Palinauskas & Sheldon 2009; Lachish *et al.* 2011).
154 Environmental conditions have been shown to determine prevalence and intensity of avian
155 malaria at different spatial scales (Wood *et al.* 2007; Loiseau *et al.* 2011; Szöllösi *et al.* 2011).
156 Recently, a large survey of house sparrow (*Passer domesticus*) populations infected with
157 *Plasmodium relictum* has provided evidence for a tight association between prevalence of
158 infection and temperature variables (Loiseau *et al.* 2013). Under the current situation of

159 raising temperature potentially affecting the availability of trophic resources, it is crucial to
160 better understand the relationship between nutritional status and malaria dynamics and
161 adaptation.

162 We performed experimental infections of domestic canaries (*Serinus canaria*) with
163 the avian malaria parasite *Plasmodium relictum* (lineage SGS1). Hosts were maintained
164 under either a supplemented or a control diet. Although both groups were fed *ad libitum*,
165 the supplemented group received a protein- and vitamin-richer diet than controls. A similar
166 diet manipulation (protein-rich vs protein-poor diet) has been used by Gonzalez *et al.* (1999)
167 in the house sparrow and showed that birds in the protein-rich group mounted a stronger
168 cellular immune response to a novel antigen but also a weaker antibody mediated response.
169 Parasites experimentally infecting hosts in the food-supplemented or control group were
170 then transferred to new hosts that were also experiencing either a supplemented or a
171 control diet. This experimental design allowed us to investigate (i) the effect of host
172 nutritional condition on parasite dynamics (parasitaemia) and virulence (reduction in host
173 body mass and haematocrit, see Mackinnon & Read 2003); (ii) the effect of past and current
174 environments on parasite dynamics and virulence; (iii) the adaptation of malaria parasites to
175 host nutritional conditions. Based on Gonzalez *et al.* (1999) results, we predicted that
176 supplemented-birds should be better able to control parasite multiplication resulting in
177 lower parasitaemia and suffering less from the infection. If parasites adapt to host
178 nutritional status adopting a plastic adjustment of their multiplication rate, we should
179 expect parasitaemia to be mostly affected by the current environmental conditions. On the
180 contrary, if parasites adapt through genetic selection we should expect the former
181 environmental conditions to be the major determinant of parasitaemia (or perhaps the
182 interaction between previous and current environments).

183

184 **MATERIALS AND METHODS**

185 *Bird maintenance and food treatments*

186 Experiments were carried out using (1-year old) domestic canaries originating from different
187 breeders and obtained from a bird provider. Birds were kept in individual cages (0.6 x 0.4 x
188 0.4 m) at constant room temperature ($21 \pm 1^\circ\text{C}$) and under a controlled daily light cycle (LD
189 13:11 h). Prior to the experiments, we used diagnostic PCRs (Waldenström *et al.* 2004) to
190 ensure that the canaries were not infected with haemosporidian parasites.

191 Birds in the control food group received a commercial mixture of seeds for canaries
192 (Versele-Laga, Belgium) provided *ad libitum*. Birds assigned to the supplemented group
193 received the same mixture of seeds plus, every 2 days, a quarter of hard-boiled egg, apple
194 and lettuce, which increased the proportion of dietary protein, vitamins and minerals. All
195 birds had water provided *ad libitum*. Birds were maintained under their food regime (control
196 or supplemented) from 15 days prior to the parasite infection until the end of the
197 experiment (17 days post infection).

198 The experiment was conducted during autumn 2009 and performed under the
199 licence # 21-CAE-085 delivered by the departmental veterinary service.

200

201 *Parasites and experimental infections*

202 We used the avian malaria parasite *Plasmodium relictum* (lineage SGS1) originally obtained
203 from a natural population of house sparrows, and cross-transferred to naive canaries.
204 Infected blood was cryopreserved and stored at -80°C (see details in Bichet *et al.* 2012). For
205 the purpose of the present experiment, cryopreserved blood was thawed (Bichet *et al.* 2012)

206 and transferred intraperitoneally to 5 domestic canaries. Eleven days post-infection (dpi),
207 parasitaemia was evaluated from thin blood smears (absolute methanol fixation, 10%
208 Giemsa staining, observation of 10,000 erythrocytes). Blood was collected from donors to
209 prepare a stock suspension diluted in PBS containing the desired number of parasites per
210 inoculum (1×10^6 asexual parasites) that served to infect birds of experiment 1 (see below).
211 A similar procedure was used to infect birds in experiment 2.

212

213 *Experimental design*

214 A full-factorial design with host diet (control or supplemented) and parasite origin
215 (previously reared in control or supplemented birds) was used to test the effects of previous
216 and current environments on parasitaemia and virulence.

217 In experiment 1, two groups of birds in the control (n = 14 birds) or supplemented (n
218 = 15 birds) diets were inoculated intraperitoneally with 1×10^6 *P. relictum* parasites.

219 In experiment 2, parasites originating from control (C) and supplemented (S) birds
220 were used to infect birds raised under similar control and supplemented diets. The
221 parasitaemia at 10 dpi of infected birds in experiment 1 was estimated from blood smears.
222 Suspensions of infected red blood cells were prepared from the donor birds of the control
223 (P^C) (n = 9) and supplemented (P^S) (n = 10) groups to infect a new set of birds raised under
224 control (D^C) and supplemented (D^S) diets. Blood of birds with high parasitemia was diluted as
225 to ensure that each donor contributed a similar number of parasites to the suspension.
226 Mixing parasites from multiple donors has previously been used in other studies where
227 parasites were experimentally transmitted between groups of hosts (Yourth & Schmid-
228 Hempel 2006; Kubinak *et al.* 2012). The same suspension of infected blood was used to
229 infect all the birds in each treatment with the same inoculum size (1×10^6). To summarize,

230 birds raised under the control diet (D^C) received either parasites originated from control
231 hosts (D^{CP^C} , n = 15 birds) or supplemented hosts (D^{CP^S} , n = 15 birds); similarly, birds raised
232 under the supplemented diet (D^S) received either parasites originated from control hosts
233 (D^{SP^C} , n = 15 birds) or the supplemented hosts (D^{SP^S} , n = 15 birds).

234 All birds were monitored at 5, 8, 10, 14 and 17 dpi, when we recorded body mass to
235 the nearest 0.1 g and collected a small amount of blood by puncturing the left brachial vein
236 for haematocrit measurement (around 20 μ L, centrifugation 10,000 rpm for 5 min) and
237 molecular analysis (around 20 μ L flushed with 500 μ L Queen Lysis Buffer).

238

239 *Estimation of infection intensity by quantitative PCR*

240 Infection intensity was assessed using the quantitative PCR assay as described by Cellier-
241 Holzem *et al.* (2010). For each individual, two qPCR reactions in the same run were
242 conducted: one targeting the nuclear 18s rDNA gene of *Plasmodium* (Primers 18sPlasm7 5'-
243 AGC CTG AGA AAT AGC TAC CAC ATC TA-3', 18sPlasm8 5'-TGT TAT TTC TTG TCA CTA CCT CTC
244 TTC TTT-3', and fluorescent probe Plasm Hyb2 5'-6FAM-CAG CAG GCG CGT AAA TTA CCC AAT
245 TC-BHQ1-3') and the other targeting the 18s rDNA gene of bird (Primers 18sAv7 5'-GAA ACT
246 CGC AAT GGC TCA TTA AAT C-3', 18sAv8 5'-TAT TAG CTC TAG AAT TAC CAC AGT TAT CCA-3'
247 and fluorescent probe 18sAv Hyb 5'-VIC-TAT GGT TCC TTT GGT CGC TC-BHQ1-3'). Parasite
248 intensity was calculated as a relative quantification value RQ ($2^{-(Ct_{18s Plasmodium} - Ct_{18s Bird})}$) using
249 the software SDS 2.2 (Applied Biosystem). Ct is the number of PCR cycles at which
250 fluorescence is first detected as statistically significant above the baseline. RQ can be seen as
251 the fold-amount of the target gene (*Plasmodium* 18s rDNA) with respect to the amount of
252 the reference gene (host 18s rDNA). All qPCR reactions were carried out using an ABI Prism
253 7900 cycler (Applied Biosystem).

254

255 *Statistical analyses*

256 The statistical analyses were run using the R software (v. 2.14.0).

257 Variation in body mass, haematocrit and parasite intensity (RQ, log-transformed) was
258 analyzed using linear mixed-effect models (*lme* function, *nlme* package) with bird as a
259 random effect to overcome the pseudo-replication due to the repeated sampling of
260 individual hosts. In addition, models were also implemented with a temporal auto-
261 correlation structure (*corAR1*) within the random effect structure (Pollitt *et al.* 2012). A
262 squared time term (time^2) was included to account for non-linear effects in analyses
263 involving haematocrit and parasite intensity. Mortality was analyzed using a logistic
264 regression. Models were simplified by sequentially eliminating the least non-significant term
265 to obtain minimal adequate models using a standard procedure of likelihood comparison
266 (using the function *anova.lme* specifying a marginal type test). Significant *P* values in the text
267 are for the minimal models whereas non-significant values refer to those obtained before
268 the deletion of the term from the model.

269

270

271 **RESULTS**

272 During the course of the experiment four birds died during or soon after manipulation.
273 These were not included in the further analyses. Neither diet nor parasite origin treatments
274 accounted for differences in mortality among groups (experiment 1: $n_{D^C} = 2$, $n_{D^S} = 0$, diet
275 $\chi^2_1 = 3.07$, $P = 0.0792$; experiment 2: $n_{D^C P^C} = 3$, $n_{D^C P^S} = 1$, $n_{D^S P^C} = 3$, $n_{D^S P^S} = 1$, diet $\chi^2_1 =$
276 0.04 , $P = 0.8443$; parasite origin $\chi^2_1 = 2.28$, $P = 0.1313$).

277

278 *Experiment 1*

279 Although birds were randomly assigned to the diet treatment, supplemented birds ($22.5 \pm$
280 0.59 g) had initially slightly larger body mass than control birds (20.9 ± 0.48 g; $F_{1, 28} = 4.54$, P
281 $= 0.0421$). Nevertheless, supplemented birds gained in body mass over the 15-day period of
282 supplementation whereas body mass of controls remained constant (time*diet $F_{1, 28} = 8.52$,
283 $P = 0.0069$; Fig. 1B). Haematocrit also increased during the 15 days that preceded the
284 infection ($F_{1, 29} = 32.93$, $P < 0.0001$; Fig. 1C) but this was unrelated to the diet treatment ($F_{1,$
285 $_{28} = 0.26$, $P = 0.6116$).

286 In the following sections, we will only consider the variation in the traits of interest
287 that happened during the infection period (0-17 dpi).

288 Parasitaemia followed a bell-shaped relationship, typical of an acute *Plasmodium*
289 infection (time $F_{1, 140} = 23.81$, $P < 0.0001$; Fig. 1A), but the pattern markedly differed
290 between the two diet treatments (time*diet $F_{1, 140} = 11.54$, $P = 0.0009$). Overall,
291 parasitaemia was lower in supplemented birds and the peak of infection was reached earlier
292 in supplemented hosts (8 vs 14 dpi, Fig. 1A), suggesting that control birds were less able to
293 keep parasite multiplication under control.

294 Following infection, only birds that had received the control diet experienced a
295 reduction in body mass, whereas supplemented hosts maintained similar body mass along
296 the experiment (time*diet $F_{1, 141} = 11.87$, $P = 0.0008$; Fig 1B).

297 Infected birds were anaemic and haematocrit declined as the infection progressed,
298 reaching the minimum values around 10-14 dpi before recovering to values similar to the
299 pre-infection ones (time $F_{1, 127} = 8.10$, $P = 0.0052$; time² $F_{1, 127} = 7.82$, $P = 0.0060$; Fig. 1C).

300 Whereas birds fed with a control diet showed a moderate reduction in haematocrit,
301 haematocrit of supplemented birds was critically reduced across the course of the infection
302 (time*diet $F_{1, 127} = 7.77$, $P = 0.0061$; time²*diet $F_{1, 127} = 4.51$, $P = 0.0356$; Fig 1C). In addition,
303 variation in haematocrit was affected by a diet*parasitaemia interaction (parasitaemia $F_{1, 127}$
304 = 12.82, $P = 0.0005$, diet*parasitaemia $F_{1, 127} = 6.69$, $P = 0.0108$). This last result shows that,
305 even though control birds were more heavily parasitized, for a constant parasite load they
306 paid a smaller cost of infection in terms of haematocrit reduction (Fig. 2).

307

308 *Experiment 2*

309 Again, birds fed with the supplemented diet achieved higher body mass at the end of the
310 pre-infection period, contrary to control birds (time*diet $F_{1, 57} = 56.75$, $P < 0.0001$; Fig. 3B).

311 The current diet did not affect parasitaemia either on its own ($F_{1, 55} = 0.34$, $P =$
312 0.8542) or in interaction with other factors (all P -values > 0.39). Parasitaemia varied
313 according to the origin of the parasites (parasite*time² $F_{1, 261} = 6.93$, $P = 0.0090$; Fig. 3A, 4A)
314 with birds suffering higher parasite load when infected by parasites previously grown on
315 control hosts (P^c).

316 The reduction in body mass during the infection (time $F_{1, 268} = 83.67$, $P < 0.0001$) was
317 affected by both the current diet and the parasite origin (see below). The 3-way
318 diet*parasite*time interaction term was not significant ($F_{1, 267} = 2.41$, $P = 0.1220$). However,
319 both treatments (current diet and parasite origin) had an effect in interaction with time.
320 Overall, as in experiment 1, control birds lost more weight than did supplemented birds (diet
321 *time $F_{1, 268} = 8.46$, $P = 0.0039$; Fig. 3B, 4B), and birds infected with parasites originating from

322 control hosts (independently of the current diet in experiment 2) lost more body mass
323 (time*parasite $F_{1, 268} = 11.14$, $P = 0.0009$; Fig. 3B, 4B).

324 Changes in haematocrit during the course of the infection (time $F_{1, 265} = 85.66$, $P <$
325 0.0001 ; time² $F_{1, 265} = 91.18$, $P < 0.0001$; Fig. 3C) were influenced by both the current diet
326 and the parasite origin (significant 3-way interaction term, time*diet*parasite $F_{1, 265} = 5.15$, P
327 $= 0.0240$; Fig. 3C). To better describe this complex pattern, we computed the maximum
328 percent reduction in haematocrit for each of the four treatment groups. Parasites that were
329 grown in hosts with a control diet induced the largest haematocrit reduction and hosts with
330 a supplemented diet had a larger haematocrit reduction compared to control hosts (Fig. 4B).
331 In addition, maximal reduction in haematocrit was reached 3 days earlier in birds with
332 control diet (D^C) than those under supplemented diet (D^S) (Fig. 3C). It is worth mentioning
333 that similar results (not shown) were obtained when the analysis was performed on the 0-10
334 dpi period.

335 Parasitaemia negatively affected haematocrit ($F_{1, 256} = 5.58$, $P = 0.0189$) but the
336 relationship was influenced by the current host diet and the parasite origin
337 (parasitaemia*diet*parasite $F_{1, 256} = 4.03$, $P = 0.0449$). The inspection of the data, however,
338 suggested that this significant 3-way interaction might be due to a single point with very
339 high parasitaemia in the $D^S P^S$ group. When removing this single point from the analysis, the
340 3-way interaction was indeed far from reaching the significance threshold ($F_{1, 255} = 0.31$, $P =$
341 0.5779). Simplifying the model by removing non-significant terms showed that haematocrit
342 was negatively correlated with parasitaemia ($F_{1, 258} = 14.78$, $P = 0.0002$) and that birds with
343 the supplemented diet had a lower haematocrit than controls ($F_{1, 56} = 10.27$, $P = 0.0022$),

344 whereas parasite origin did not reach the significance threshold ($F_{1,56} = 3.58$, $P = 0.0635$) (Fig.
345 5).

346

347

348 **DISCUSSION**

349 The rapid human-driven environmental changes that we are currently facing pose a major
350 threat to population and species persistence, and modification of food availability with
351 raising temperature has been identified as the most widespread mechanism underlying local
352 extinction (Cahill *et al.* 2013). In addition to the obvious effect of starvation on reproductive
353 output and survival, changes in diet composition (food quantity and quality) may also have
354 more subtle effect on the susceptibility to infectious diseases and on the evolution of
355 parasite virulence. Here, we investigated how food quality affected the dynamics of the
356 avian malaria agent *Plasmodium relictum*, the cost paid by the host and the parasite
357 adaptation to the nutritional status of the host.

358 We found that hosts with a supplemented diet in terms of protein and vitamin
359 contents were better able to control parasite multiplication. This result might therefore
360 suggest that avian populations exposed to reduced food availability and/or poorer food
361 quality are more susceptible to malaria parasites, possibly because of less effective immune
362 defences. Interestingly however, for a given parasite load, supplemented birds paid a much
363 higher cost of infection, at least as measured in terms of increased anaemia. This finding
364 shows that while effective immune defences are needed to control parasite growth, they
365 might also induce substantial fitness costs (Graham *et al.* 2005a; Sorci & Faivre 2009).
366 Anaemia is primarily the consequence of the disruption of infected red blood cells and it
367 often correlates with parasitaemia (Cellier-Holzem *et al.* 2010), as also found in the present

368 study. Anaemia might also be a host response to control the infection by increasing the
369 clearance and the destruction of both parasitized and non-parasitized red blood cells
370 (Lamikanra *et al.* 2007; Metcalf *et al.* 2011; Percario *et al.* 2012). Overreacting immune and
371 inflammatory responses of supplemented hosts could have produced higher clearance of red
372 blood cells whether they were infected or not, inducing an immunopathological damage
373 (Graham *et al.* 2005a; Day, Graham & Read 2007; Long & Graham 2011). Previous work with
374 mammalian and avian malaria has indeed suggested that a substantial fraction of the
375 *Plasmodium*-induced cost of infection has an immunological origin (Graham *et al.* 2005b;
376 Bichet *et al.* 2012), the most striking example being cerebral malaria in humans where high
377 fatality is due to brain inflammation (Grau & Craig 2012).

378 The comparison of the infection cost when keeping constant the intensity of the
379 infection gives us an interesting insight into a possible tolerance-resistance trade-off.
380 Resistance refers to the capacity of the host to control and clear the parasite, whereas
381 tolerance indicates the capacity of the host to withstand the infection with minimal fitness
382 costs (Råberg, Sim & Read 2007; Råberg, Graham & Read 2009; Ayres & Schneider 2012;
383 Medzhitov, Schneider & Soares 2012). Our results suggest that these two mechanisms of
384 defence might show a phenotypic trade-off. Indeed, while supplemented birds were able to
385 better resist to the infection they also appeared to less tolerate it. Beyond, anaemia and the
386 inflammatory response triggered by infection, malaria pathogenesis also arises from the
387 release of toxic hemes in the blood stream following the hemolysis of infected erythrocytes.
388 It has been shown that the expression of heme oxygenase-1 (HO-1) prevents the onset of
389 severe cerebral malaria or hepatic failure in rodents through its antioxidant activity
390 (Pamplona *et al.* 2007; Seixas *et al.* 2009). The greater tolerance observed in birds fed with a
391 control diet may have been due to similar mechanisms. Alternatively, control birds may have

392 also been better at controlling the damage of the inflammatory response, hence decreasing
393 the associated costs (Sears *et al.* 2011). As mentioned above the cause of this trade-off could
394 come from the dual effect of infection-induced immune activation. Evidence for such trade-
395 offs between tolerance and resistance is still rare (Read, Graham & Råberg 2008) but, in
396 future studies, it will be essential to take into account these two processes if we want to
397 better understand the effect of environmental changes on the ecology of host-parasite
398 interactions.

399 Our study is essentially based on the assumption that the experimental manipulation
400 of the diet affected the expression of the immune response against *Plasmodium*. Reliable
401 assessment of immune effectors that are functionally involved in parasite resistance has
402 proved difficult for non-model organisms (Adamo 2004). Nevertheless, there is a wealth of
403 literature on humans and animal models unambiguously showing that both food quality and
404 quantity do shape immune responsiveness and susceptibility to infectious diseases
405 (Gonzalez *et al.* 1999; Siva-Jothy & Thompson 2002; Smith *et al.* 2007; Kau *et al.* 2011; Hoke,
406 Kuzawa & McDade 2012). Undernutrition is probably the most prevalent environmental
407 factors inducing immune suppression and risk of infection in human populations. The
408 availability of microelements acquired with the diet (e.g. vitamins, zinc, selenium) can also
409 have profound effects on the expression of the immune response. With this respect our
410 treatment effectively induced a gain in body mass by increasing the availability of both
411 protein (through the provisioning of hard-boiled eggs) and micronutrients (through the
412 provisioning of apples and lettuce). For instance, apples contain micronutrients such as zinc,
413 selenium, β -carotene, vitamins E and C, which are known to have an immunomodulatory
414 effect.

415 Little information is available on the immune effectors involved in the resistance to
416 avian malaria. Previous work has focused on immune genes (Westerdahl *et al.* 2005; Loiseau
417 *et al.* 2008; Loiseau *et al.* 2011), whereas work conducted in the first half of the 20th century
418 and in the last decade suggests that both innate, inflammatory effectors, and antibody
419 mediated responses contribute to control parasite growth within the bird host (Sergent &
420 Sergent 1952; Cellier-Holzem *et al.* 2010; Bichet *et al.* 2012). For instance, Cellier-Holzem *et*
421 *al.* (2010) showed that parasitaemia of canaries that were re-exposed to the same *P.*
422 *relictum* strain (SGS1) was much lower compared to a primary infection, strongly suggesting
423 that immunological memory confers a partial protection towards re-infection (see also
424 Buckling & Read 2001). The acute phase of the infection should however elicit more the
425 innate inflammatory response. Recently, Bichet *et al.* (2012) showed that canaries whose
426 inducible nitric oxide synthase was experimentally inhibited had higher parasitaemia
427 compared to control suggesting a role for nitric oxide in the control of *Plasmodium* growth.

428 In addition to assessing the role of host nutritional status on parasite dynamics and
429 cost of infection, we also wished to investigate the potential for parasite adaptation to hosts
430 experiencing specific diets. This is particularly relevant because if environmental variation
431 alters the availability of food resources this might produce an evolutionary shift in the
432 parasite population. As for free organisms, parasites can adapt to their environment
433 adopting a plastic adjustment of their phenotypic traits or by genetic selection.
434 Disentangling these two processes might prove very difficult because a final demonstration
435 of genetic selection would require keeping track of genetic variants across generations. Here
436 we adopted an indirect, statistical, approach to infer whether adaptation occurred via a
437 plastic response or a genetic selection. We used parasitaemia (within-host parasite
438 multiplication) as a measure of parasite fitness (but we are aware that this might be an

439 oversimplification given that we did not take into account transmission to the vector host)
440 and predicted that if *Plasmodium* adapts through phenotypic plasticity, the major
441 determinant of parasite replication should be the quality of the current environment. On the
442 contrary if adaptation proceeds through a genetic selection we should expect that major
443 determinant of parasite success to be the quality of the previous environment (or the
444 interaction between the two). In agreement with the latter prediction we found that
445 parasitaemia of passaged parasites was only affected by the diet treatment of their previous
446 hosts, with parasites coming from control hosts reaching higher parasitaemia in their
447 subsequent hosts. The inspection of figure 3B suggests that this pattern is mostly driven by
448 the D^CP^C group (parasites coming from control hosts and infecting birds in control diet). It
449 could be that we lacked statistical power to detect a significant interaction here, but even a
450 statistical significant interaction between the previous and current environments would
451 rather suggest a process of adaptation through genetic selection. This argument might also
452 explain the apparent discrepancy between the results of experiment 1 and 2 in terms of the
453 effect of the current environment on parasitaemia. Indeed, in experiment 1 we found that
454 supplemented hosts harboured a significantly lower parasitaemia whereas in experiment 2
455 the current diet of the host did not affect parasitaemia. The pattern we found is consistent
456 with the idea that selection operated on the parasites infecting supplemented and control
457 hosts of experiment 1, subsequently masking the effect of the current environment. Serial
458 passage experiments are well known to induce rapid evolution of pathogens favouring the
459 genotypes with the highest multiplication rate (Ebert 1998). In agreement with this, we
460 indeed found that parasitaemia reached in the experiment 2 was much higher than in
461 experiment 1 ($\chi^2_1 = 18.70$, $P < 0.0001$). Since our inoculum was certainly formed by a mix of
462 different genotypes/clones (the parasite was isolated from naturally infected house

463 sparrows and passaged using a mix of blood from different infected birds), high genetic
464 diversity allowed selection to produce a very rapid shift. A possible caveat of mixing
465 parasites from multiple donors to infect subsequent hosts may have been that a few
466 parasite strains/genotypes were overrepresented in the inoculum because of their
467 competitive advantage. However, we made sure that each donor contributed a similar
468 number of parasites to the inoculum which should reduce the risk of such competitive
469 asymmetry among parasite strains/genotypes. As mentioned above, mixing parasites from
470 multiple donors, however, probably allows maintaining a large amount of genetic variation
471 within the parasite population and more closely matches the natural situation where each
472 vertebrate host is bitten by multiple vectors.

473 In addition to being more adapted to non-supplemented hosts, parasites grown in
474 such hosts induced higher costs when passaged to novel hosts. We used two proxies of
475 parasite virulence (reduction in body mass and haematocrit) and with respect to both
476 variables, parasites coming from control, non-supplemented hosts, appeared to become
477 more virulent. The evolution of higher virulence in hosts fed with poor diets raises a concern
478 if human-driven environmental changes induce an impoverishment of food quality and
479 quantity. Interestingly, these results parallel those obtained with the avirulent coxsackievirus
480 B3/0 where a single passage in mice kept on selenium-deficient diet restored the virulence
481 usually observed for the B3 strain (Beck *et al.* 2004), even though the mechanisms in play
482 are likely to be different. The generality of this result has, however, yet to be established,
483 since other studies involving invertebrate hosts have rather suggested that evolution to
484 higher virulence might be driven by well-fed hosts (Tseng 2006; Little *et al.* 2007).

485 Avian malaria are common parasites of many bird species. The spread of the
486 pathogen has threatened the persistence of immunologically naïve host populations, as
487 witnessed by the case study of Hawaiian birds (Atkinson *et al.* 1995). Recently, a concern has
488 arisen on the possible consequences of climate change on the spread of the disease
489 (Garamszegi 2011; Loiseau *et al.* 2013). If changes in temperature affect the availability of
490 food quantity and quality, our study shows that malaria parasites might become a more
491 serious concern for their avian hosts. Finally, host nutrition is also likely to modify host
492 attractiveness and palatability to vectors, affecting malaria transmission. Indeed, infected
493 birds are more attractive to vectors than uninfected birds and bird haematocrit has been
494 shown to influence host choice behaviour of mosquito vectors in this avian malaria system
495 (Cornet *et al.* 2013). Much work remains to be done to fully understand the effects of
496 resource availability on host-vector-malaria interactions and to clarify the role and
497 importance of vectors in the evolution of parasite virulence.

498

499

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511 **DATA ACCESSIBILITY**

512 Data for this study are available at Dryad – doi:10.5061/dryad.t3jp4 (provisional)

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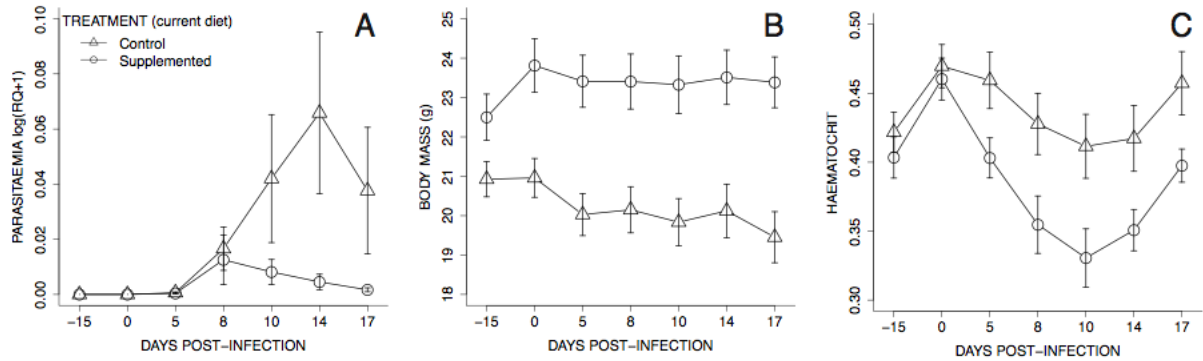
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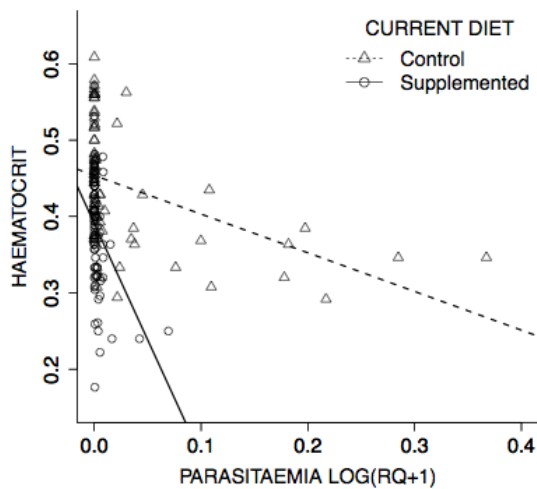
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734 **Figure 1.** Variation (means \pm s.e.) in blood parasitaemia Log(RQ+1) (A), body mass (B), and
 735 haematocrit (C) in birds infected by the avian malaria *Plasmodium relictum* (SGS1) and
 736 maintained under control (triangles) or supplemented diet (circles). Diets were provided
 737 from 15 days before parasite infection until the end of the experiment (17 dpi).
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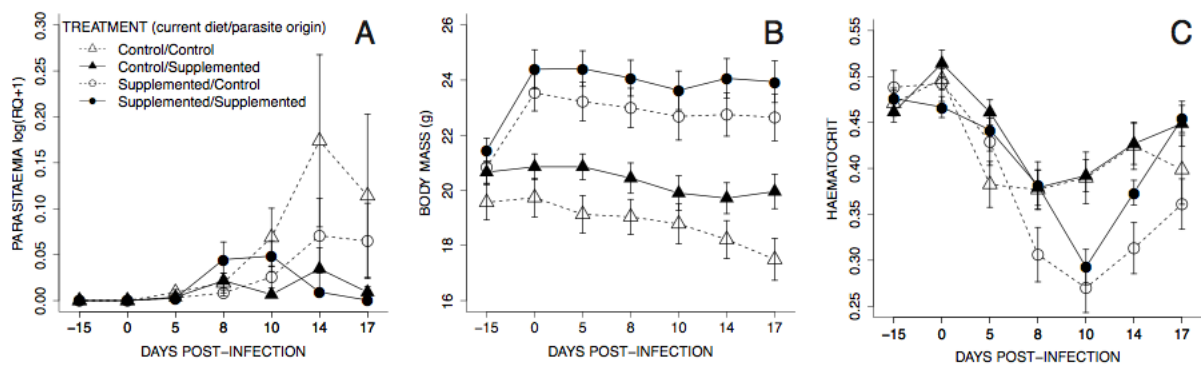
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 742 **Figure 2.** Covariation between haematocrit values and blood parasitaemia Log(RQ+1) of
 743 *Plasmodium relictum* (SGS1) in infected birds reared under control (triangles, dashed line) or
 744 supplemented (circles, solid line) diet (Experiment 1).
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748 **Figure 3.** Variation (means \pm s.e.) in blood parasitaemia Log(RQ+1) (A), body mass (B), and
 749 haematocrit (C) in birds maintained under control (D^C , triangles) or supplemented (D^S ,
 750 circles) diets and infected by *Plasmodium relictum* (SGS1) parasites previously reared in
 751 control (P^C , open symbols) or supplemented hosts (P^S , filled symbols). Diets were provided
 752 from 15 days before parasite infection until the end of the experiment (17 dpi). Legend:
 753 $D^C P^C$: open triangles, $D^C P^S$: filled squares, $D^S P^C$: open circles, $D^S P^S$: filled circles.

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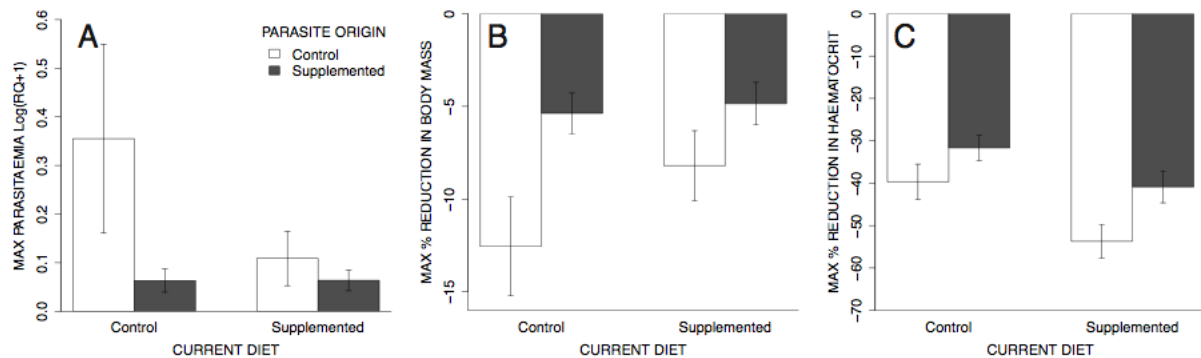
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758 **Figure 4.** Variation (means \pm s.e.) in maximum parasitaemia Log(RQ+1) (A) and maximum
 759 percent reduction in body mass (B) and in haematocrit (C) over the infection period
 760 according to current diet and parasite origin (Experiment 2).

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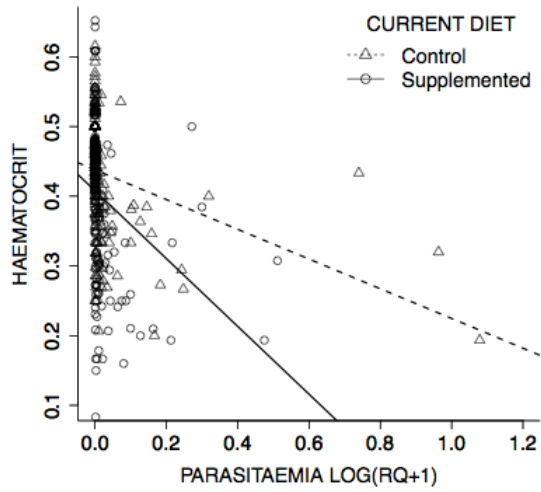


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764 **Figure 5.** Covariation between haematocrit values and blood parasitaemia Log(RQ+1) of
765 *Plasmodium relictum* (SGS1) in infected birds reared under control (triangles, dashed line) or
766 supplemented (circles, solid line) diet (Experiment 2).

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