1   **Experimental inhibition of nitric oxide increases *Plasmodium relictum* (lineage SGS1)**

2   **parasitaemia**

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Abstract

Malaria is a widespread vector-borne disease infecting a wide range of terrestrial vertebrates including reptiles, birds and mammals. In addition to being one of the most deadly infectious diseases for humans, malaria is a threat to wildlife. The host immune system represents the main defence against malaria parasites. Identifying the immune effectors involved in malaria resistance has therefore become a major focus of research. However, this has mostly involved humans and animal models (rodents) and how the immune system regulates malaria progression in non-model organisms has been largely ignored. The aim of the present study was to investigate the role of nitric oxide (NO) as an immune effector contributing to the control of the acute phase of infection with the avian malaria agent *Plasmodium relictum*. We used experimental infections of domestic canaries in conjunction with the inhibition of the enzyme inducible nitric oxide synthase (iNOS) to assess the protective function of NO during the infection, and the physiological costs paid by the host in the absence of an effective NO response. Our results show that birds treated with the iNOS inhibitor suffered from a higher parasitaemia, but did not pay a higher cost of infection (anaemia). While these findings confirm that NO contributes to the resistance to avian malaria during the acute phase of the infection, they also suggest that parasitaemia and costs of infection can be decoupled.

47

1. Introduction

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Malaria protozoa still severely threaten the health of human populations, causing around 700,000 of deaths worldwide in 2010 (World Health Organization, 2011). The negative health implications of *Plasmodium* infections have stimulated considerable attention on the study of immunity to malaria (see for instance Doolan et al., 2009; Langhorne et al., 2008), in both humans, who are obviously less amenable to experimental approaches, and in animal models (Artavanis-Tsakonas et al., 2003). Thus, the current knowledge of the immunological pathways involved in resistance/tolerance of malaria infection comes mainly from rodent malaria models, *Plasmodium chabaudii, P. berghei* and *P. yoelii* (Good and Doolan, 1999; Langhorne et al., 2004; Roetynck et al., 2006).

Plasmodium parasites have a complex life cycle involving a mosquito vector, in which sexual reproduction of the pathogen occurs, and a vertebrate host in which the parasite reproduces asexually. Invertebrates and vertebrates differ in many aspects of their immune system which might impose a challenge to the parasite in terms of its ability to adapt to different immunological environments (Hammerschmidt and Kurtz, 2005). However, both hosts share some immunological pathways, in particular the innate arm of the immune system where cytotoxic compounds are released shortly after the infection (i.e. nitric oxide).

Immunity of vertebrate hosts to malaria involves a complex network of immunological effectors. The control of the acute phase of the infection (peak parasitaemia being reached between 8 and 16 days post-infection, depending on the model system considered) depends on the activation of a helper T-cell 1 (Th1) response (Taylor-Robinson et al., 1993). Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), are produced and released with the further activation of macrophages that release cytotoxic

72 compounds. The acute phase of the infection is then followed by a chronic phase with very

73 low parasitaemia eventually leading to recurrent relapses (Huldén et al., 2008). The shift from

74 acute to chronic infection is paralleled by a shift from a Th1 to a helper T-cell 2 (Th2)

75 response, with the production of immunoglobulins (IgGs) specific to the current parasite

76 strains (Taylor-Robinson and Looker, 1998; Taylor-Robinson et al., 1993). Multiple

77 exposures to malaria parasites therefore lead to a partial immunity that has been called

78 premunition (Soe et al., 2001), whereas total immunity is probably prevented by the antigenic

79 variation of malaria parasites producing variants that escape the pre-existing antibody

80 repertoire (Newbold, 1999).

81   In addition to humans (and non-human primates) and rodents, malarial parasites are

82 widespread pathogens of birds and reptiles (Valkiūnas, 2005), principally in tropical and

83 temperate areas. Avian malaria is thought to be non-lethal in hosts that have a long co-

84 evolutionary relationship with the parasite (Fallis and Desser, 1977). Nevertheless, recent

85 experimental infections have also shown that avian malaria parasites can be costly and

86 substantially reduce host fitness (Knowles et al., 2010; Palinauskas et al., 2008, 2009;

87 Zehtindjiev et al., 2008). Moreover, immunologically naïve and domestic populations can

88 have large significant negative consequences of infection in terms of mortality, raising both

89 conservation and economic concerns (Atkinson, 1999; Atkinson and Van Riper III, 1991;

90 Cellier-Holzem et al., 2010; Van Riper et al., 1986; Williams, 2005). The introduction of

91 malaria parasites to the Hawaii archipelago is the classical example of the impact of

92 *Plasmodium* on natural populations of birds that have no co-evolutionary history with the

93 parasite. Upon introduction of the mosquito vector, endemic bird species became infected

94 with *Plasmodium relictum* and experienced a dramatic decline in number, due to high

95 infection-induced mortality (Atkinson et al., 1995; Van Riper et al., 1986). Interestingly, a

96 few years after the introduction of the pathogen, local bird populations now seem better able

97 to tolerate the infection while paying much smaller costs (Woodworth et al., 2005). Similarly,

98 infection of domestic birds (i.e., chickens) with *Plasmodium gallinaceum* can lead to very

99 high mortality depending on the age of the host (young chicks may suffer up to 80%

100 mortality) and the inoculum size (Williams, 2005).

101   In spite of the importance of avian malaria for the functioning of natural populations

102 and domestic animals, immunity to avian malaria has been poorly studied. In the last decade,

103 a few studies have reported a number of associations between major histocompatibility

104 complex (MHC) alleles and *Plasmodium* prevalence and parasitaemia (Bonneaud et al., 2006;

105 Loiseau et al., 2008, 2011; Westerdahl, et al., 2005, 2012). However, experimental

106 approaches are mostly lacking.

107   Among the possible effectors that might contribute to the control of the acute phase of

108 the infection, nitric oxide is a very good candidate. Nitric oxide (NO) is a highly reactive and

109 unstable free-radical gas that is produced by the oxidation of L-arginine to citrulline by the

110 enzyme inducible NO synthase (iNOS) (Vincendeau et al., 2003). iNOS is rapidly synthesized

111 by a wide array of cells and tissues in response to pro-inflammatory cytokines produced

112 during the infection (Rivero, 2006). NO has both suppressive and stimulatory functions: it

113 inhibits and promotes cell proliferation, it modulates the production of cytokines, chemokines

114 and growth factors, and it directly acts as a non-specific cytotoxic effector molecule (Bogdan

115 et al., 2000). Previous work has shown that NO has a cytostatic (cessation of growth) and

116 cytotoxic effect on different *Plasmodium* species both *in vitro* and *in vivo*) (Taylor-Robinson,

117 1997; Taylor-Robinson and Looker, 1998; Taylor-Robinson and Smith, 1999). Interestingly,

118 NO is an immune effector shared by both vectors and vertebrate hosts (Rivero, 2006).

119   Epidemiological studies have also reported negative correlations between severity of

120 malaria infection in children and iNOS expression (Anstey et al., 1996). Similarly, in the

121 mosquito vector, induction of iNOS expression contributes to control infection with

122 *Plasmodium* parasites (Luckhart et al., 1998; Peterson et al., 2007). Evidence for avian hosts

123 is, however, restricted to a single study where *in vivo* NO production by macrophages isolated

124 from chickens infected with *Plasmodium gallinaceum* was positively correlated with

125 parasitaemia (Macchi et al., 2010).

126   In this article, we wished to explore experimentally the role plaid by NO in the

127 regulation of the acute phase of the infection with *Plasmodium relictum* in domestic canaries

128 (*Serinus canaria*). This was achieved by using the specific iNOS inhibitor aminoguanidine

129 (AG) (Allen, 1997; Wideman et al., 2006). In addition to assessing parasitaemia in AG-

130 treated and control hosts, we also measured the cost of infection in the absence of a functional

131 NO response.

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133   **2. Material and methods**

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135   2.1 Bird husbandry

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137   The experiment was conducted during the autumn of 2009. Birds were kept in

138 individual cages (0.6 x 0.4 x 0.4 m), with food (commercial seed mix, Versele-Laga,

139 Belgium), grit and water provided *ad libitum*. The temperature was kept constant (21 ± 1°C),

140 under a controlled daily light cycles (LD 13:11 h). The birds originated from a bird breeder

141 and were kept under the above conditions three weeks before the start of the experiment.

142   The experiments were performed under the licence # 21-CAE-085 delivered by the

143 departmental veterinary service.

144

145   2.2 Experiment 1: Experimental inhibition of the NO response

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147   To check whether AG has an inhibitory effect on the iNOS as reported for chickens,

148   we performed an experiment where non-infected domestic canaries were either treated with

149   AG [intraperitoneal injection of 1mg AG dissolved in 100 µL of phosphate buffer saline

150   (PBS)] or kept as control and injected with the same volume of PBS. Within each of these

151   treatments, half of the birds had their inflammatory response stimulated by an intraperitoneal

152   injection of *Escherichia coli* lipopolysaccharide (LPS) (0.02mg dissolved in 100 µL of PBS),

153   whereas the other half received a same volume PBS injection. Each experimental group

154   contained 9 birds. We took a blood sample (ca 100 µL) from the brachial vein before the

155   treatment started (h0) and after nine hours (h9) to measure plasmatic concentrations of NO.

156   Nine hours post-challenge corresponds to the peak of LPS-induced NO production (Takahashi

157   et al., 1999).

158   NO production was indirectly measured using the Griess reaction. Because of the very

159   short half life of nitric oxide (few seconds) in biological tissues, NO was measured by

160   quantifying nitrates (NO_3^-) and nitrites (NO_2^-) (NOx). NOx were produced during the reaction

161   with different oxygen species (Sild and Horak, 2009). First, $ZnSO_4$ and NaOH solutions were

162   added to deproteinize the plasma. The supernatant produced by this reaction was recovered

163   and a glycine buffer was added. In the second step, nitrate was reduced to nitrite by using

164   cadmium granules, activated with sulfuric acid and $CuSO_4$ solutions. The last step consists in

165   the Griess reaction, in which plasma products were put in a microplate and Griess reagent

166   (sulphanilamide and *N*-naphtylethylene-diamine) was added. The microplate was placed into

167   a spectrophotometer at 25°C, under shaker. Spectrophotometric measurements were done at

168   540nm and measures were taken every 5 minutes for 30 minutes (SPECTRAMaxPLUS384,

169   Molecular Devices). We used the optical density (OD) values at 30 minutes. NOx

170   concentrations were determined using a standard curve of known nitrate concentration.

171 Standards were obtained by successive dilutions of a NO_3^- solution at 100µM. A full

172 description of the method can be found in Sild and Horak (2009).

173

174   2.3 Experiment 2: Effects of aminoguanidine on parasitaemia and cost of infection

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176   Parasites used for the experimental infections were obtained from a natural population

177 of house sparrows (*Passer domesticus*) in Dijon, France and cryopreserved at -80°C. Blood

178 (ca. 200 µl) of SGS1 infected house sparrows [as detected by a nested PCR method

179 (Waldenström et al., 2004) that amplifies a section of the mitochondrial cytochrome b gene

180 and sequencing of the PCR products] was intraperitoneally injected into domestic canaries. At

181 day 10 post-infection, blood of infected canaries was cryopreserved using the protocol

182 described in Diggs et al. (1975). Briefly, fresh infected heparinized blood was centrifuged at

183 800g for 5 minutes and the supernatant removed. A cryopreserving solution (6.2M glycerol +

184 0.14M Na lactate + 0.0005M KCl + PBS to 500ml) was added dropwise with gentle vortexing

185 to packed red blood cells at 4 to 1 volumes. Blood was then stored at -80° C. For the present

186 experiment, cryopreserved blood was thawed at 37° without agitation for 2 minutes. We then

187 added 0.2 volume of 12% NaCl (dropwise with gentle vortexing), allowed to stand for 5

188 minutes and added 9 volumes of 1.6% NaCl dropwise as above. Blood was then centrifuged

189 for 5 minutes at 650g and the pellet resuspended in PBS.

190   Thawed blood was directly transferred intraperitoneally into five domestic canaries,

191 using 0.5 ml insulin syringes, in order to increase parasite intensity. Eleven days after

192 infection, we measured the haematocrit of these five birds and prepared blood smears for

193 microscopic examination. Smears were made by spreading a drop of blood from each bird on

194 a glass slide, fixing with absolute methanol and then staining with 10% Giemsa solution

195 (Sigma–Aldrich). We counted the number of asexual infectious stages of the parasite

196   observed in a total of 10,000 erythrocytes. Parasite intensities and haematocrit allowed us to

197   evaluate the number of parasites per µl of blood for each bird (a haematocrit of 50%

198   corresponded approximately to 5,000,000 erythrocytes per microliter of blood). We collected

199   blood from donors, which was subsequently diluted in 0.9% saline solution to obtain the

200   desired number of parasites per inoculum.

201   In a 2-way factorial design, we investigated the effects of AG, and hence the effect of

202   NO synthesis inhibition, on parasitaemia of *P. relictum*. For this purpose, 60 non-infected

203   canaries were randomly distributed among four experimental groups (n = 15 per group). At

204   day 0, the first group was intraperitoneally inoculated with a dose of 1 x 10^6 parasites (lineage

205   SGS1) and received a daily injection of 1mg of AG (in 100 µl of PBS) until day 15 post-

206   infection (AG^+/P^+). The second group was infected with the same sized parasite inoculum but

207   only received a daily injection of 100 µl of PBS (AG^-/P^+). The third group was sham-infected

208   and received the same daily injection of AG as group 1 (AG^+/P^-). The final group served as a

209   double negative control since birds were sham infected and received a daily injection of PBS

210   (AG^-/P^-). Sham infection was performed by injecting a volume of PBS (50 µl) corresponding

211   to the volume of parasite inoculum. Previous work has shown that injecting PBS represents an

212   appropriate control similar to injecting non-infected blood (Cellier-Holzem et al., 2010).

213   Birds were monitored at day 5, 8, 10, 14 and 17 post-infection. At each of these time

214   points, we recorded body mass to the nearest 0.1 g and we collected a blood sample from the

215   left brachial vein using heparinized capillaries. Twenty microliters were used to assess

216   haematocrit after centrifugation for 5 min at 10,000 rpm; 20µl were flushed with 500 µl of

217   Queen Lysis Buffer for parasite quantification.

218   Parasitaemia was assessed using a quantitative PCR, following the protocol described

219   in Cellier-Holzem et al. (2010). For each individual we conducted two qPCR reactions in the

220   same run: one targeting the nuclear 18s rDNA gene of *Plasmodium* (Primers 18sPlasm7 (5'-

221   AGC CTG AGA AAT AGC TAC CAC ATC TA-3'), 18sPlasm8 (5'-TGT TAT TTC TTG

222   TCA CTA CCT CTC TTC TTT-3'), and fluorescent probe Plasm Hyb2 (5'-6FAM-CAG

223   CAG GCG CGT AAA TTA CCC AAT TC-BHQ1-3')); and the other targeting the 18s rDNA

224   gene of birds (Primers 18sAv7 (5'-GAA ACT CGC AAT GGC TCA TTA AAT C-3'),

225   18sAv8 (5'-TAT TAG CTC TAG AAT TAC CAC AGT TAT CCA-3') and fluorescent probe

226   18sAv Hyb (5'-VIC-TAT GGT TCC TTT GGT CGC TC-BHQ1-3')). Parasite intensities

227   were calculated as relative quantification values (RQ) as $2^{-(Ct\ 18s\textit{Plasmodium}\ -\ Ct\ 18s\ Bird)}$ using the

228   software SDS 2.2 (Applied Biosystem). Ct represents the number of PCR cycles at which

229   fluorescence is first detected as statistically significant above the baseline and RQ can be

230   interpreted as the fold-amount of target gene (*Plasmodium* 18s rDNA) with respect to the

231   amount of the reference gene (host 18s rDNA). All qPCR reactions were carried out in an

232   ABI Prism 7900 cycler (Applied Biosystem).

233

234   2.4 Statistical analyses

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236   2.4.1   Experiment 1: experimental inhibition of the NO response by aminoguanidine

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238   The effect of treatments on NOx concentration (log-transformed) at h0 and h9 was

239   investigated using a Kruskal-Wallis test.

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241   2.4.2   Experiment 2: Effects of aminoguanidine on parasitaemia and cost of infection

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243   Changes in log-transformed parasitaemia were modelled using a Generalized Linear

244   Mixed Model (GLMM) with a beta distribution of errors (Duerr et al., 2004). Time post-

245   infection, squared time post-infection, treatment (AG vs control) and the two-way interactions

246   (time * treatment, squared time * treatment) were included as fixed factors. Individual

247   identity was declared as a random factor to take into account the repeated measures of

248   individuals. Degrees of freedom were corrected using the Satterthwaite method. Obviously,

249   this analysis only concerned experimentally infected birds.

250   The physiological cost of infection was assessed by changes in body mass and

251   haematocrit during the course of the experiment. For our measures of parasitaemia, we used

252   GLMMs with a normal distribution of errors and Satterthwaite correction for degrees of

253   freedom. The models included time post-infection, squared time post-infection, treatment

254   (AG vs. control), infectious status (infected vs non-infected), the two- and three-way

255   interactions as fixed effects. Individual identity was declared as a random effect.

256   All tests were performed using SAS v.9.2 (SAS 2002)

257

258   **3. Results**

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260   3.1 Experiment 1: experimental inhibition of the NO response by aminoguanidine

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262   At time h0, NOx concentration did not differ among the four groups (X^2_3=3.21,

263   P=0.36). At time h9, the AG$^-$/LPS$^+$ group had a statistically significant higher NOx

264   concentration than the AG$^+$/LPS$^+$ group (X^2_3=8.57, P=0.036) (Fig.1).

265

266   3.2 Experiment 2: Effects of aminoguanidine on parasitaemia and cost of infection

267

268   Parasitaemia of experimentally infected birds not treated with AG showed the

269   expected bell-shaped variation with time, reaching a peak at day 14 pi. Parasitaemia of AG-

270   treated birds, however showed a steady increase (with the exception of day 11 pi), with peak

271 parasitaemia being reached at day 17 pi. This resulted in a statistically significant interaction

272 between squared time and treatment (Table 1, Fig. 2).

273   Infection was costly in terms of haematocrit. Infected birds suffered a clear drop in

274 haematocrit with minimum values reached at day 11 pi, whereas the haematocrit level of non-

275 infected birds remained constant through the experimental period (Fig. 3). This resulted in a

276 statistically significant interaction between squared time and infectious status (Table 2).

277 Interestingly, however, variation in haematocrit did not depend on the aminoguanidine

278 treatment (Table 2), in spite of infected, AG-treated birds having higher parasitaemia.

279   Body mass was not affected by either infectious status nor AG treatment (Table 3).

280

281   **4. Discussion**

282

283   The aim of this study was to experimentally assess the contribution of a specific

284 immunological pathway (the NO response) to the control of *Plasmodium relictum* (lineage

285 SGS1) parasitaemia in domestic canaries. Even though aminoguanidine has already been

286 shown to be an effective inhibitor of iNOS in chickens (Wideman et al., 2006), we first

287 wished to check whether its inhibitory function was preserved in domestic canaries. In

288 agreement with the results reported for chickens, we found that LPS injected birds had a

289 smaller NO response when simultaneously injected with AG. It is worthwhile to note that the

290 inhibitory effect was not total and AG-treated birds did produce some NO upon stimulation

291 with LPS.

292   AG-treated canaries were less able to control the acute phase of infection with

293 *Plasmodium relictum* compared to control animals, suggesting that NO contributes to the

294 immunological defences deployed during the infection with avian malaria. This is in

295 agreement with previous results involving other malaria parasites infecting mammalian hosts

296 (see Taylor-Robinson, 2010 for a recent review). Our results therefore corroborate the well-

297 established idea that NO has important anti-parasitic properties. Several studies have used

298 similar experimental approaches to manipulate the NO response in insects and vertebrates.

299 For instance, in the mosquito *Anopheles gambiae*, the inhibition of nitric oxide with inert L-

300 arginine leads to a decrease in the ability of mosquitos to kill *Escherichia coli* bacteria

301 (Hillyer and Estevez-Lao, 2010). In another mosquito species, *Anopheles stephensi*, a

302 provision of the NOS substrate, L-ARGININE, reduced *Plasmodium* infections, whereas a

303 dietary provision of the NO inhibitor L-NAME significantly increased parasite burden

304 (Luckhart et al., 1998). Moreover, nitric oxide is associated with *Plasmodium* ookinete lysis

305 (Peterson et al., 2007). In vertebrates, the role of NO as an effective immune effector against

306 malaria has only been explored in humans (Anstey et al., 1996; Hobbs et al., 2002) and mice

307 (Taylor-Robinson, 2010). Wang et al. (2009) showed that mice experimentally infected with

308 *Plasmodium yoelii* exhibit an increase in NO which coincides with a decrease in parasitaemia.

309 Some authors have even suggested that supplementation with the nitric oxide synthetic

310 metabolite (S-nitrate) could be used as a therapy against *Plasmodium* infection (Nahrevanian

311 et al., 2008).

312   Although our finding that AG-treated birds suffered from increased parasitaemia

313 strongly suggests a role for NO in the control of the acute phase of the infection with

314 *Plasmodium relictum*, a definitive conclusion cannot be drawn without measuring NO

315 production following the experimental infection. We did not have a clear prediction on when

316 would be the best time to measure NO during the infection period. Ideally, we would have

317 measured NO on a daily basis but daily blood sampling would have certainly induced too

318 much stress. Future work should nevertheless be directed towards establishing the link

319 between NO production and parasitaemia in this system.

320   In spite of their increased parasitaemia, AG-treated birds did not seem to pay a higher

321   cost of infection compared to control individuals. Haematocrit level, body mass and mortality

322   (only 4 birds died during the experiment, one in AG^+/P^- group, one in AG^+/P^+ group, and two

323   in AG^-/P^+ group) did not differ between AG-treated and control hosts. A visual inspection of

324   figure 3 suggests that AG-treated birds have a lower haematocrit especially at day 14 and 17

325   p.i., that is when parasitaemia reaches its maximum values. However, there is no statistical

326   support to the idea that AG-treated birds had a lower haematocrit compared to control

327   individuals. Even when restricting the analysis to infected birds, the difference in haematocrit

328   between AG^- and AG^+ individuals was very far from reaching the significance threshold (time

329   p.i. * AG treatment, $F_{1,131} = 0.11$, $p = 0.740$; squared time p.i. * AG treatment, $F_{1,130} = 0.33$, p

330    $= 0.564$). These results might appear somewhat puzzling because infection does incur costs in

331   our model system, especially in terms of reduction in haematocrit level (Cellier-Holzem et al.,

332   2010; the present study). A reduction in haematocrit is partly the direct consequence of the

333   asexual reproduction of the parasites within the red blood cells and the subsequent lysis and

334   release of merozoites in the blood stream. Since haematocrit levels and parasitaemia are

335   usually negatively correlated (the more parasites, the more lysis of red blood cells takes place)

336   we should have expected that AG-treated birds paid a higher cost of infection. Anaemia and

337   haematocrit reduction could also partly arise as a consequence of immune responsiveness,

338   with immune effectors targeting infected red blood cells. Indeed, in a rodent malaria system,

339   it has been estimated that 10% of anaemia is due to an over-reacting immune response

340   (Graham et al., 2005b). We might then speculate that inhibiting the NO response produced

341   two counter-balancing effects: increased parasitaemia enhances the cost of infection, but

342   reduced NO production also reduces the costs of the immune defence.

343   These results can feed the current debate on the relationship between parasite

344   multiplication and virulence (the trade-off model for the evolution of parasite virulence) and

345   the role played by immunopathology as a major determinant of virulence (Alizon et al., 2009;

346   Day et al., 2007; Graham et al., 2005a; Long and Graham, 2011). Nevertheless, the idea that

347   down-regulating the immune response decouples the cost of infection from parasitaemia

348   undoubtedly requires further work to be fully established.

349   We found no effect of *Plasmodium* infection and AG treatment on body mass. Benign

350   environmental conditions, with *ad libitum* food and water, and constant temperature, might

351   contribute to explain this result. Interestingly, other studies based on experimental infection of

352   European passerines in the lab have reported a similar lack of effect of infection on body mass

353   (Palinauskas et al., 2008, 2011; Zehtindjiev et al., 2008). These results suggest that, in

354   addition to the role plaid by favourable environmental conditions, a lost in body mass is not

355   include in the physiological costs of infection with *Plasmodium relictum* for birds that have

356   coevolved with the parasite. A different picture emerges for host species that did not coevolve

357   with the parasite, as shown by the experimental infection of Hawaiian birds with *Plasmodium*

358   *relictum*. Both *Myadestes obscurus* and *Hemignathus virens* have been shown to suffer from a

359   substantial decrease in body mass following the infection with *Plasmodium relictum*

360   (Atkinson et al., 2000, 2001).

361   Parasitaemia was highly variable among infected birds. Among-individual variation in

362   parasite intensity is a common finding (Cellier-Holzem et al., 2010; Palinauskas et al., 2009;

363   Zehtindjiev et al., 2008), and multiple sources may account for this Individual hosts vary in

364   their genetic background and this can shape their susceptibility to infection (Bonneaud et al.,

365   2006; Loiseau et al., 2008, 2011; Westerdahl, et al., 2005, 2012). In addition host age and sex

366   might also contribute to generate among-individual variation in parasite intensity (McCurdy

367   et al., 1998; Sol et al., 2000; Williams, 2005).

368   Vector borne parasites have to face and adapt to different environments (the vector

369   and the host) to complete their life cycle. Shared immunological pathways between the vector

370 and the host could therefore be the main target of immune-mediated selection acting on the

371 parasite. Nitric oxide is one such shared immunological pathway, and we might expect

372 parasites to evolve strategies to escape the NO produced in response to infection. Indeed, it

373 has been suggested that some protozoa (*Trypanosoma cruzi* and *Leishmania major*) can

374 deplete the substrate of the NOS (L-arginine) by activating arginases (Vincendeau et al.,

375 2003). Whether *Plasmodium* parasites have evolved the same escape strategy remains an open

376 question.

377

382

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567

568 **Figure captions**

569

570 Fig. 1. Mean ($\pm$ SE) NOx concentration in domestic canaries that were injected with LPS (or

571 PBS as a control) and with aminoguanidine (AG) (or PBS as a control), giving rise to four

572 experimental groups (AG^-/LPS^+, AG^+/LPS^+, AG^-/LPS^-, AG^+/LPS^-). NOx was measured 9

573 hours post-challenge.

574

575 Fig. 2. Changes in parasitaemia (mean $\pm$ SE) during the course of the experiment. Triangles

576 represent birds in the AG^-/P^+ group, and dots birds in the AG^+/P^+ group.

577

578 Fig. 3. Variation in haematocrit (mean $\pm$ SE) during *Plasmodium relictum* infection. Solid

579 lines and black triangles represent birds in the AG^-/P^+ group, dashed lines and white triangles

580 birds in the AG^-/P^- group, solid lines and black dots birds in the AG^+/P^+ group, and dashed

581 lines and white dots birds in the AG^+/P^- group.