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

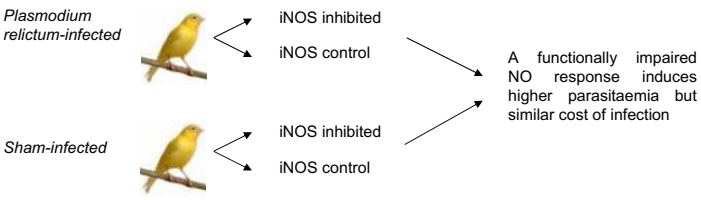
Coraline Bichet [a,*], Stéphane Cornet [b,c], Stephen Larcombe [d], Gabriele Sorci [a]

[a] BioGéosciences, UMR CNRS 5561, Université de Bourgogne, 6 Boulevard Gabriel, 21000 Dijon, France
[b] MIVEGEC, UMR CNRS 5290-IRD 224-UM1-UM2, Institut de Recherche pour le Développement, 911 avenue Agropolis, 34394 Montpellier, France
[c] Centre d'Ecologie Fonctionnelle et Evolutive, UMR CNRS 5175, 1919 route de Mende, 34293 Montpellier, France
[d] Edward Grey Institute, Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, United Kingdom

HIGHLIGHTS

- The immune effectors involved in the resistance to avian malaria are not well known.
- We suppress the production of NO in canaries infected with *Plasmodium relictum*.
- Birds NO-inhibited show a higher parasitaemia, but not pay a higher cost of infection.
- NO contributes to the resistance to avian malaria.
- Parasitaemia and cost of infection can be decoupled.

GRAPHICAL ABSTRACT



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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.

1. Introduction

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, Plasmodium*

* Corresponding author.
E-mail address: coraline.bichet@u-bourgogne.fr (C. Bichet).

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berghei and *Plasmodium 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 compounds. The acute phase of the infection is then followed by a chronic phase with very low parasitaemia eventually leading to recurrent relapses (Huldén et al., 2008). The shift from acute to chronic infection is paralleled by a shift from a Th1 to a helper T-cell 2 (Th2) response, with the production of immunoglobulins (IgGs) specific to the current parasite strains (Taylor-Robinson and Looker, 1998; Taylor-Robinson et al., 1993). Multiple exposures to malaria parasites therefore lead to a partial immunity that has been called premunition (Soe et al., 2001), whereas total immunity is probably prevented by the antigenic variation of malaria parasites producing variants that escape the pre-existing antibody repertoire (Newbold, 1999).

In addition to humans (and non-human primates) and rodents, malarial parasites are widespread pathogens of birds and reptiles (Valkiūnas, 2005), principally in tropical and temperate areas. Avian malaria is thought to be non-lethal in hosts that have a long co-evolutionary relationship with the parasite (Fallis and Desser, 1977). Nevertheless, recent experimental infections have also shown that avian malaria parasites can be costly and substantially reduce host fitness (Knowles et al., 2010; Palinauskas et al., 2008, 2009; Zehtindjiev et al., 2008). Moreover, immunologically naïve and domestic populations can have large significant negative consequences of infection in terms of mortality, raising both conservation and economic concerns (Atkinson, 1999; Atkinson and Van Riper, 1991; Cellier-Holzem et al., 2010; Van Riper et al., 1986; Williams, 2005). The introduction of malaria parasites to the Hawaii archipelago is the classical example of the impact of *Plasmodium* on natural populations of birds that have no co-evolutionary history with the parasite. Upon introduction of the mosquito vector, endemic bird species became infected with *Plasmodium relictum* and experienced a dramatic decline in number, due to high infection-induced mortality (Atkinson et al., 1995; Van Riper et al., 1986). Interestingly, a few years after the introduction of the pathogen, local bird populations now seem better able to tolerate the infection while paying much smaller costs (Woodworth et al., 2005). Similarly, infection of domestic birds (i.e., chickens) with *Plasmodium gallinaceum* can lead to very high mortality depending on the age of the host (young chicks may suffer up to 80% mortality) and the inoculum size (Williams, 2005).

In spite of the importance of avian malaria for the functioning of natural populations and domestic animals, immunity to avian malaria has been poorly studied. In the last decade, a few studies have reported a number of associations between major histocompatibility complex (MHC) alleles and *Plasmodium* prevalence and parasitaemia (Bonneaud et al., 2006; Loiseau et al., 2008, 2011; Westerdahl et al., 2005, 2012). However, experimental approaches are mostly lacking.

Among the possible effectors that might contribute to the control of the acute phase of the infection, nitric oxide is a very good candidate. Nitric oxide (NO) is a highly reactive and unstable free-radical gas that is produced by the oxidation of L-arginine to citrulline by the enzyme inducible NO synthase (iNOS) (Vincendeau et al., 2003). iNOS is rapidly synthesized by a wide array of cells and tissues in response to pro-inflammatory cytokines produced during the infection (Rivero, 2006). NO has both suppressive and stimulatory functions: it inhibits and promotes cell proliferation, it modulates the production of cytokines, chemokines and growth factors, and it directly acts as a non-specific cytotoxic effector molecule (Bogdan et al., 2000). Previous work has shown that NO has a cytostatic (cessation of growth) and cytotoxic effect on different *Plasmodium* species both *in vitro* and *in vivo*) (Taylor-Robinson, 1997; Taylor-Robinson and Looker, 1998; Taylor-Robinson and Smith, 1999). Interestingly, NO is an immune effector shared by both vectors and vertebrate hosts (Rivero, 2006).

Epidemiological studies have also reported negative correlations between severity of malaria infection in children and iNOS expression (Anstey et al., 1996). Similarly, in the mosquito vector, induction of iNOS expression contributes to control infection with *Plasmodium* parasites (Luckhart et al., 1998; Peterson et al., 2007). Evidence for avian hosts is, however, restricted to a single study where *in vivo* NO production by macrophages isolated from chickens infected with *P. gallinaceum* was positively correlated with parasitaemia (Macchi et al., 2010).

In this article, we wished to explore experimentally the role plaid by NO in the regulation of the acute phase of the infection with *P. relictum* in domestic canaries (*Serinus canaria*). This was achieved by using the specific iNOS inhibitor aminoguanidine (AG) (Allen, 1997; Wideman et al., 2006). In addition to assessing parasitaemia in AG-treated and control hosts, we also measured the cost of infection in the absence of a functional NO response.

2. Materials and methods

2.1. Bird husbandry

The experiment was conducted during the autumn of 2009. Birds were kept in individual cages (0.6 × 0.4 × 0.4 m), with food (commercial seed mix, Versele-Laga, Belgium), grit and water provided *ad libitum*. The temperature was kept constant (21 ± 1 °C), under a controlled daily light cycles (LD 13:11 h). The birds originated from a bird breeder and were kept under the above conditions 3 weeks before the start of the experiment.

The experiments were performed under the licence #21-CAE-085 delivered by the departmental veterinary service.

2.2. Experiment 1: experimental inhibition of the NO response

To check whether AG has an inhibitory effect on the iNOS as reported for chickens, we performed an experiment where non-infected domestic canaries were either treated with AG [intraperitoneal injection of 1 mg AG dissolved in 100 μL of phosphate buffer saline (PBS)] or kept as control and injected with the same volume of PBS. Within each of these treatments, half of the birds had their inflammatory response stimulated by an intraperitoneal injection of *Escherichia coli* lipopolysaccharide (LPS) (0.02 mg dissolved in 100 μL of PBS), whereas the other half received a same volume PBS injection. Each experimental group contained 9 birds. We took a blood sample (ca. 100 μL) from the brachial vein before the treatment started (h0) and after 9 hours (h9) to measure plasmatic concentrations of NO. Nine hours post-challenge corresponds to the peak of LPS-induced NO production (Takahashi et al., 1999).

NO production was indirectly measured using the Griess reaction. Because of the very short half life of nitric oxide (few seconds) in biological tissues, NO was measured by quantifying nitrates (NO_3^-) and nitrites (NO_2^-) (NOx). NOx were produced during the reaction with different oxygen species (Sild and Horak, 2009). First, $ZnSO_4$ and NaOH solutions were added to deproteinize the plasma. The supernatant produced by this reaction was recovered and a glycine buffer was added. In the second step, nitrate was reduced to nitrite by using cadmium granules, activated with sulfuric acid and $CuSO_4$ solutions. The last step consists in the Griess reaction, in which plasma products were put in a microplate and Griess reagent (sulphanilamide and *N*-naphtylethylene-diamine) was added. The microplate was placed into a spectrophotometer at 25 °C, under shaker. Spectrophotometric measurements were done at 540 nm and measures were taken every 5 min for 30 min (SPECTRAMaxPLUS384, Molecular Devices). We used the optical density (OD) values at 30 min. NOx concentrations were determined using a standard curve of known nitrate concentration. Standards were obtained by successive dilutions of a $NO3^-$ solution at 100 μM. A full description of the method can be found in Sild and Horak (2009).

2.3. Experiment 2: effects of aminoguanidine on parasitaemia and cost of infection

Parasites used for the experimental infections were obtained from a natural population of house sparrows (*Passer domesticus*) in Dijon, France and cryopreserved at −80 °C. Blood (ca. 200 μL) of SGS1 infected house sparrows [as detected by a nested PCR method (Waldenström et al., 2004) that amplifies a section of the mitochondrial cytochrome b gene and sequencing of the PCR products] was intraperitoneally injected into domestic canaries. At day 10 post-infection, blood of infected canaries was cryopreserved using the protocol described in Diggs et al. (1975). Briefly, fresh infected heparinized blood was centrifuged at 800*g* for 5 min and the supernatant removed. A cryopreserving solution (6.2 M glycerol + 0.14 M Na lactate + 0.0005 M KCl + PBS to 500 ml) was added dropwise with gentle vortexing to packed red blood cells at 4 to 1 volumes. Blood was then stored at −80 °C. For the present experiment, cryopreserved blood was thawed at 37° without agitation for 2 min. We then added 0.2 volume of 12% NaCl (dropwise with gentle vortexing), allowed to stand for 5 min and added 9 volumes of 1.6% NaCl dropwise as above. Blood was then centrifuged for 5 min at 650*g* and the pellet resuspended in PBS.

Thawed blood was directly transferred intraperitoneally into five domestic canaries, using 0.5 ml insulin syringes, in order to increase parasite intensity. Eleven days after infection, we measured the haematocrit of these five birds and prepared blood smears for microscopic examination. Smears were made by spreading a drop of blood from each bird on a glass slide, fixing with absolute methanol and then staining with 10% Giemsa solution (Sigma–Aldrich). We counted the number of asexual infectious stages of the parasite observed in a total of 10,000 erythrocytes. Parasite intensities and haematocrit allowed us to evaluate the number of parasites per μL of blood for each bird (a haematocrit of 50% corresponded approximately to 5,000,000 erythrocytes per microliter of blood). We collected blood from donors, which was subsequently diluted in 0.9% saline solution to obtain the desired number of parasites per inoculum.

In a 2-way factorial design, we investigated the effects of AG, and hence the effect of NO synthesis inhibition, on parasitaemia of *P. relictum*. For this purpose, 60 non-infected canaries were randomly distributed among four experimental groups ($n = 15$ per group). At day 0, the first group was intraperitoneally inoculated with a dose of 1×10^6 parasites (lineage SGS1) and received a daily injection of 1 mg of AG (in 100 μL of PBS) until day 15 post-infection (AG^+/P^+). The second group was infected with the same sized parasite inoculum but only received a daily injection of 100 μL of PBS (AG^-/P^+). The third group was sham-infected and received the same daily injection of AG as group 1 (AG^+/P^-). The final group served as a double negative control since birds were sham infected and received a daily injection of PBS (AG^-/P^-). Sham infection was performed by injecting a volume of PBS (50 μL) corresponding to the volume of parasite inoculum. Previous work has shown that injecting PBS represents an appropriate control similar to injecting non-infected blood (Cellier-Holzem et al., 2010).

Birds were monitored at day 5, 8, 10, 14 and 17 post-infection. At each of these time points, we recorded body mass to the nearest 0.1 g and we collected a blood sample from the left brachial vein using heparinized capillaries. Twenty microliters were used to assess haematocrit after centrifugation for 5 min at 10,000 rpm; 20 μL were flushed with 500 μL of Queen Lysis Buffer for parasite quantification.

Parasitaemia was assessed using a quantitative PCR, following the protocol described in Cellier-Holzem et al. (2010). For each individual we conducted two qPCR reactions in the same run: one targeting the nuclear 18s rDNA gene of *Plasmodium* (Primers 18sPlasm7 (5′-AGC CTG AGA AAT AGC TAC CAC ATC TA-3′), 18sPlasm8 (5′-TGT TAT TTC TTG TCA CTA CCT CTC TTC TTT-3′), and fluorescent probe Plasm Hyb2 (5′-6FAM-CAG CAG GCG CGT AAA TTA CCC AAT TC-BHQ1-3′)); and the other targeting the 18s rDNA gene of birds (Primers 18sAv7 (5′-GAA ACT CGC AAT GGC TCA TTA AAT C-3′), 18sAv8 (5′-TAT TAG CTC TAG AAT TAC CAC AGT TAT CCA-3′) and fluorescent probe 18sAv Hyb (5′-VIC-TAT GGT TCC TTT GGT CGC TC-BHQ1-3′)). Parasite intensities were calculated as relative quantification values (RQ) as $2^{-(\text{Ct 18s } \textit{Plasmodium} - \text{Ct 18s Bird})}$ using the software SDS 2.2 (Applied Biosystem). Ct represents the number of PCR cycles at which fluorescence is first detected as statistically significant above the baseline and RQ can be interpreted as the fold-amount of target gene (*Plasmodium* 18s rDNA) with respect to the amount of the reference gene (host 18s rDNA). All qPCR reactions were carried out in an ABI Prism 7900 cycler (Applied Biosystem).

2.4. Statistical analyses

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

The effect of treatments on NOx concentration (log-transformed) at h0 and h9 was investigated using a Kruskal–Wallis test.

2.4.2. Experiment 2: effects of aminoguanidine on parasitaemia and cost of infection

Changes in log-transformed parasitaemia were modelled using a Generalized Linear Mixed Model (GLMM) with a beta distribution of errors (Duerr et al., 2004). Time post-infection, squared time post-infection, treatment (AG vs control) and the two-way interactions (time × treatment, squared time × treatment) were included as fixed factors. Individual identity was declared as a random factor to take into account the repeated measures of individuals. Degrees of freedom were corrected using the Satterthwaite method. Obviously, this analysis only concerned experimentally infected birds.

The physiological cost of infection was assessed by changes in body mass and haematocrit during the course of the experiment. For our measures of parasitaemia, we used GLMMs with a normal distribution of errors and Satterthwaite correction for degrees of freedom. The models included time post-infection, squared time post-infection, treatment (AG vs. control), infectious status (infected vs non-infected), the two- and three-way interactions as fixed effects. Individual identity was declared as a random effect.

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

3. Results

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

At time h0, NOx concentration did not differ among the four groups ($X^2_3 = 3.21$, $P = 0.36$). At time h9, the AG^-/LPS^+ group had a statistically significant higher NOx concentration than the AG^+/LPS^+ group ($X^2_3 = 8.57$, $P = 0.036$) (Fig. 1).

3.2. Experiment 2: effects of aminoguanidine on parasitaemia and cost of infection

Parasitaemia of experimentally infected birds not treated with AG showed the expected bell-shaped variation with time, reaching a peak at day 14 pi. Parasitaemia of AG-treated birds, however showed a steady increase (with the exception of day 11 pi), with peak parasitaemia being reached at day 17 pi. This resulted in a statistically significant interaction between squared time and treatment (Table 1, Fig. 2).

Infection was costly in terms of haematocrit. Infected birds suffered a clear drop in haematocrit with minimum values reached at day 11 pi, whereas the haematocrit level of non-infected birds remained constant through the experimental period (Fig. 3). This resulted in a statistically significant interaction between squared time and infectious status (Table 2). Interestingly, however, variation in haematocrit did not depend on the aminoguanidine treatment (Table 2), in spite of infected, AG-treated birds having higher parasitaemia.

Body mass was not affected by either infectious status or AG treatment (Table 3).

4. Discussion

The aim of this study was to experimentally assess the contribution of a specific immunological pathway (the NO response) to the control of *P. relictum* (lineage SGS1) parasitaemia in domestic canaries. Even though aminoguanidine has already been shown to be an effective inhibitor of iNOS in chickens (Wideman et al., 2006), we first wished to check whether its inhibitory function was preserved in domestic canaries. In agreement with the results reported for chickens, we found that LPS injected birds had a smaller NO response when simultaneously injected with AG. It is worthwhile to note that the inhibitory effect was not total and AG-treated birds did produce some NO upon stimulation with LPS.

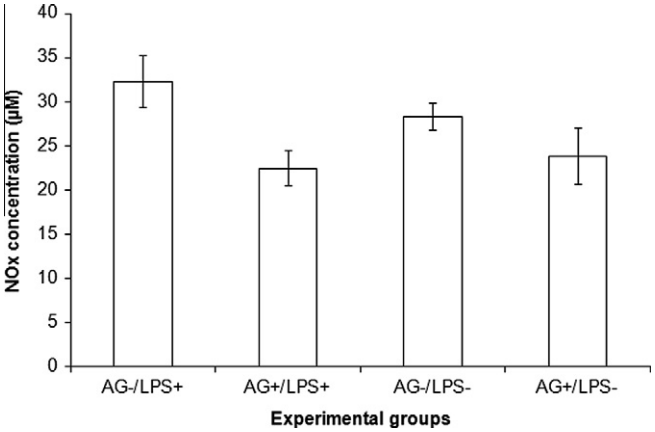

Fig. 1. Mean (±SE) NOx concentration in domestic canaries that were injected with LPS (or PBS as a control) and with aminoguanidine (AG) (or PBS as a control), giving rise to four experimental groups (AG^-/LPS^+, AG^+/LPS^+, AG^-/LPS^-, AG^+/LPS^-). NOx was measured 9 h post-challenge.

Table 1
Generalized linear mixed model showing the effect of the iNOS inhibitor aminoguanidine on *Plasmodium relictum* parasitaemia. Time refers to days post-infection. Bird identity was declared as a random factor.

Source of variation	DF	*F*	*P*
Time	180.1	4.29	0.042
Squared time	180.09	2.86	0.095
Aminoguanidine treatment (AG)	184.67	4.32	0.041
Time × AG	180.1	4.68	0.034
Squared time × AG	180.09	5.16	0.026

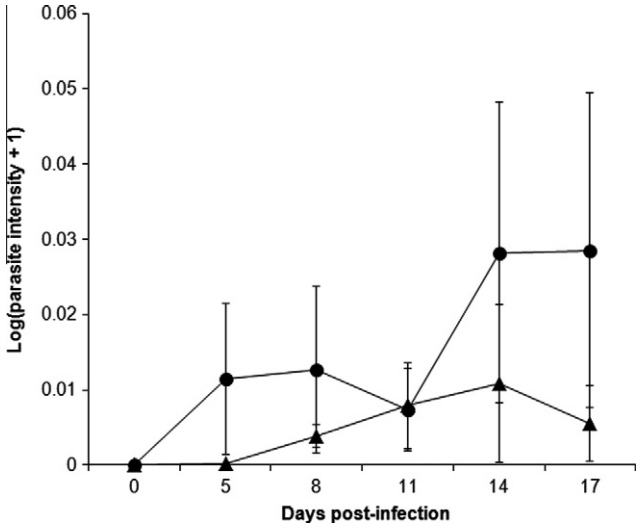

Fig. 2. Changes in parasitaemia (mean ± SE) during the course of the experiment. Triangles represent birds in the AG^-/P^+ group, and dots birds in the AG^+/P^+ group.

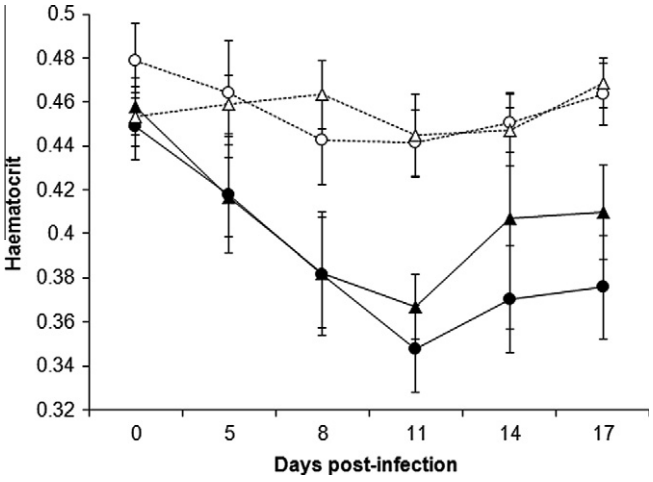

Fig. 3. Variation in haematocrit (mean ± SE) during *Plasmodium relictum* infection. Solid lines and black triangles represent birds in the AG^-/P^+ group, dashed lines and white triangles birds in the AG^-/P^- group, solid lines and black dots birds in the AG^+/P^+ group, and dashed lines and white dots birds in the AG^+/P^- group.

AG-treated canaries were less able to control the acute phase of infection with *P. relictum* compared to control animals, suggesting that NO contributes to the immunological defences deployed during the infection with avian malaria. This is in agreement with previous results involving other malaria parasites infecting mammalian hosts (see Taylor-Robinson, 2010 for a recent review). Our results therefore corroborate the well-established idea that NO has important anti-parasitic properties. Several studies have used similar experimental approaches to manipulate the NO

Table 2
Generalized linear mixed model showing the effect of the iNOS inhibitor aminoguanidine and the infection status with *Plasmodium relictum* on haematocrit. Time refers to days post-infection. Bird identity was declared as a random factor. Table A reports the full model; table B reports the model after removal of non-significant interactions.

Source of variation	DF	*F*	*P*
A			
Time	1263.4	32.98	<0.001
Squared time	1261.9	20.16	<0.001
Infection	1149.3	0.19	0.668
Aminoguanidine treatment (AG)	1149.3	0.03	0.866
Time × AG	1263.4	0.10	0.756
Time × infection	1263.4	11.72	0.001
Squared time × infection	1261.9	5.37	0.021
Squared time × AG	1261.9	0.00	0.954
Infection × AG	1149.3	0.64	0.426
Time × infection × AG	1263.4	1.28	0.260
Squared time × infection × AG	1261.9	1.72	0.191
B			
Time	1267.3	32.96	<0.001
Squared time	1265.8	20.06	<0.001
Infection	1152	0.19	0.664
Aminoguanidine treatment (AG)	157.08	0.14	0.710
Time × infection	1267.4	12.03	0.001
Squared time × infection	1266	5.55	0.019

Table 3
Generalized linear mixed model showing the effect of the iNOS inhibitor aminoguanidine and the infection status with *Plasmodium relictum* on body mass. Time refers to days post-infection. Bird identity was declared as a random factor. Table A reports the full model; table B reports the model after removal of non-significant interactions.

Source of variation	DF	*F*	*P*
A			
Time	1276.9	3.12	0.079
Squared time	1276.6	0.59	0.441
Infection	162.41	0.00	0.988
Aminoguanidine treatment (AG)	162.41	0.13	0.721
Time × AG	1276.9	0.38	0.540
Time × infection	1276.9	2.68	0.103
Squared time × infection	1276.6	2.42	0.121
Squared time × AG	1276.6	0.24	0.627
Infection × AG	162.41	0.34	0.560
Time × infection × AG	1276.9	1.21	0.271
Squared time × infection × AG	1276.6	1.56	0.213
B			
Time	1282.9	3.17	0.076
Squared time	1282.6	0.59	0.442
Infection	157.18	0.10	0.757
Aminoguanidine treatment (AG)	157.18	0.04	0.837

response in insects and vertebrates. For instance, in the mosquito *Anopheles gambiae*, the inhibition of nitric oxide with inert L-arginine leads to a decrease in the ability of mosquitos to kill *E. coli* bacteria (Hillyer and Estevez-Lao, 2010). In another mosquito species, *Anopheles stephensi*, a provision of the NOS substrate, L-arginine, reduced *Plasmodium* infections, whereas a dietary provision of the NO inhibitor L-NAME significantly increased parasite burden (Luckhart et al., 1998). Moreover, nitric oxide is associated with *Plasmodium* ookinete lysis (Peterson et al., 2007). In vertebrates, the role of NO as an effective immune effector against malaria has only been explored in humans (Anstey et al., 1996; Hobbs et al., 2002) and mice (Taylor-Robinson, 2010). Wang et al. (2009) showed that mice experimentally infected with *P. yoelii* exhibit an increase in NO which coincides with a decrease in parasitaemia. Some authors have even suggested that supplementation with the nitric oxide synthetic metabolite (*S*-nitrate) could be used as a therapy against *Plasmodium* infection (Nahrevanian et al., 2008).

Although our finding that AG-treated birds suffered from increased parasitaemia strongly suggests a role for NO in the control of the acute phase of the infection with *P. relictum*, a definitive conclusion cannot be drawn without measuring NO production following the experimental infection. We did not have a clear prediction on when would be the best time to measure NO during the infection period. Ideally, we would have measured NO on a daily basis but daily blood sampling would have certainly induced too much stress. Future work should nevertheless be directed towards establishing the link between NO production and parasitaemia in this system.

In spite of their increased parasitaemia, AG-treated birds did not seem to pay a higher cost of infection compared to control individuals. Haematocrit level, body mass and mortality (only 4 birds died during the experiment, one in AG^+/P^- group, one in AG^+/P^+ group, and two in AG^-/P^+ group) did not differ between AG-treated and control hosts. A visual inspection of Fig. 3 suggests that AG-treated birds have a lower haematocrit especially at day 14 and 17 pi, that is when parasitaemia reaches its maximum values. However, there is no statistical support to the idea that AG-treated birds had a lower haematocrit compared to control individuals. Even when restricting the analysis to infected birds, the difference in haematocrit between AG^- and AG^+ individuals was very far from reaching the significance threshold (time pi × AG treatment, $F_{1,131} = 0.11$, $P = 0.740$; squared time pi × AG treatment, $F_{1,130} = 0.33$, $P = 0.564$). These results might appear somewhat puzzling because infection does incur costs in our model system, especially in terms of reduction in haematocrit level (Cellier-Holzem et al., 2010; the present study). A reduction in haematocrit is partly the direct consequence of the asexual reproduction of the parasites within the red blood cells and the subsequent lysis and release of merozoites in the blood stream. Since haematocrit levels and parasitaemia are usually negatively correlated (the more parasites, the more lysis of red blood cells takes place) we should have expected that AG-treated birds paid a higher cost of infection. Anaemia and haematocrit reduction could also partly arise as a consequence of immune responsiveness, with immune effectors targeting infected red blood cells. Indeed, in a rodent malaria system, it has been estimated that 10% of anaemia is due to an over-reacting immune response (Graham et al., 2005b). We might then speculate that inhibiting the NO response produced two counter-balancing effects: increased parasitaemia enhances the cost of infection, but reduced NO production also reduces the costs of the immune defence.

These results can feed the current debate on the relationship between parasite multiplication and virulence (the trade-off model for the evolution of parasite virulence) and the role played by immunopathology as a major determinant of virulence (Alizon et al., 2009; Day et al., 2007; Graham et al., 2005a; Long and Graham, 2011). Nevertheless, the idea that down-regulating the immune response decouples the cost of infection from parasitaemia undoubtedly requires further work to be fully established.

We found no effect of *Plasmodium* infection and AG treatment on body mass. Benign environmental conditions, with *ad libitum* food and water, and constant temperature, might contribute to explain this result. Interestingly, other studies based on experimental infection of European passerines in the lab have reported a similar lack of effect of infection on body mass (Palinauskas et al., 2008, 2011; Zehtindjiev et al., 2008). These results suggest that, in addition to the role plaid by favourable environmental conditions, a lost in body mass is not include in the physiological costs of infection with *P. relictum* for birds that have coevolved with the parasite. A different picture emerges for host species that did not coevolve with the parasite, as shown by the experimental infection of Hawaiian birds with *P. relictum*. Both *Myadestes obscurus* and *Hemignathus virens* have been shown to suffer from a substantial decrease in body mass following the infection with *P. relictum* (Atkinson et al., 2000, 2001).

Parasitaemia was highly variable among infected birds. Among-individual variation in parasite intensity is a common finding (Cellier-Holzem et al., 2010; Palinauskas et al., 2009; Zehtindjiev et al., 2008), and multiple sources may account for this individual hosts vary in their genetic background and this can shape their susceptibility to infection (Bonneaud et al., 2006; Loiseau et al., 2008, 2011; Westerdahl et al., 2005, 2012). In addition host age and sex might also contribute to generate among-individual variation in parasite intensity (McCurdy et al., 1998; Sol et al., 2000; Williams, 2005).

Vector borne parasites have to face and adapt to different environments (the vector and the host) to complete their life cycle. Shared immunological pathways between the vector and the host could therefore be the main target of immune-mediated selection acting on the parasite. Nitric oxide is one such shared immunological pathway, and we might expect parasites to evolve strategies to escape the NO produced in response to infection. Indeed, it has been suggested that some protozoa (*Trypanosoma cruzi* and *Leishmania major*) can deplete the substrate of the NOS (L-arginine) by activating arginases (Vincendeau et al., 2003). Whether *Plasmodium* parasites have evolved the same escape strategy remains an open question.

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