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

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Running headline
Host nutrition alters Plasmodium virulence
ABSTRACT

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

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

3. Food supplementation was effective since supplemented hosts gained body mass during a 15 day period that preceded the infection. Host nutrition had strong effects on infection dynamics and parasite virulence. Overall parasites were more successful in control non-supplemented birds, reaching larger population sizes and producing more sexual (transmissible) stages. However, supplemented hosts paid a higher cost of infection, and when keeping parasitaemia constant had lower haematocrit than control hosts.
4. Parasites grown on control hosts were better able to exploit the subsequent hosts since they reached higher parasitaemia than parasites originating from supplemented hosts. They were also more virulent since they induced higher mass and haematocrit loss.

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

KEY-WORDS

Avian malaria, environmental variation, host-parasite interaction, nutrition, pathogen, Plasmodium relictum, virulence
INTRODUCTION

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

Changes in host nutritional status, both in terms of quantity and quality, can have profound repercussions on the dynamics of infectious diseases (Humphrey 2009; Kau et al. 2011). For a long time, nutrition has been identified as a key environmental factor shaping immune defences and host susceptibility to infection (Scrimshaw, Taylor & Gordon 1959). Malnutrition in terms of insufficient protein intake is associated with an impairment of cell-mediated immunity, phagocyte function, complement system, and cytokine production in humans (Chandra 1996). Deficiency in micronutrients has profound consequences for immune functioning and susceptibility to infection. Carotenoids, vitamins A, C, E, selenium and zinc have immune modulatory effects and dietary manipulations of these micronutrients alter immune functioning in model and non-model systems (Bendich 2001; McGraw & Ardia...
Environmental changes that impoverish food availability in quantity and/or quality might therefore make host populations more prone to infectious diseases. The relationship between nutritional status and infectious diseases is however not so straightforward. For some pathogens, disease severity (the host damage induced by the infection) arises from an over-reacting immune response, rather than direct parasite exploitation (Graham, Allen & Read 2005a; Sorci & Faivre 2009; Long & Graham 2011). In this case, undernourished hosts with an impaired immune response might actually fare better than well-nourished hosts. Finally, the nutritional status of hosts can directly affect pathogen survival and reproduction because well fed hosts can provide more resources to developing parasites (Pulkkinen & Ebert 2004).

A handful of studies have explored the consequences of food availability and quality on the dynamics and the cost of infection (Brown, Loosli & Schmid-Hempel 2000; Pulkkinen & Ebert 2004; Tseng 2006; Tschirren et al. 2007; Bize et al. 2008; Seppälä et al. 2008; Vale et al. 2011). For instance, Hall et al. (2009) studied the association between the fungal pathogen Metschnikowia bicuspidate and the crustacean Daphnia dentifera. Infected host fed with high quality diet produced more parasites and died sooner than hosts fed on poor quality diet, suggesting that increased resource acquisition by well-fed daphnia allowed a better growth of the pathogen population. Similar results have been obtained by manipulating food quantity. Bedhomme et al. (2004) found in the association between the mosquito Aedes aegypti and the microsporidian parasite Vavraia culicis that the production of parasite spores was positively correlated with host food availability. This couple of examples therefore suggest that parasite’s within-host growth can be limited by the amount of resources provided by host. Other examples, however, show that hosts in poor nutritional status might actually provide more favourable environments to their parasites. The flea
*Xenopsylla ramesis* produces more eggs when feeding on undernourished hosts (the rodent *Meriones crassus*), and egg survival was much higher for fleas parasitizing underfed hosts (Krasnov *et al.* 2005).

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

A few studies have used serial passage experiments to investigate parasite adaptation to host nutritional status (Beck, Handy & Levander 2004; Tseng 2006; Little *et al.* 2007). Tseng (2006) performed an experiment where *Ascogregarina* parasites (Apicomplexa) were raised in *Aedes albopictus* mosquitoes kept under two food regimes. The parasites originating from well-fed and poorly-fed hosts were then transferred to new hosts following a factorial design. *Ascogregarina* parasites originating from well-fed hosts were more virulent to subsequent hosts compared to parasites that were grown on poorly-fed hosts,
especially if the new hosts were currently reared under low food levels. This study therefore suggests a complex pattern where previous and current host nutritional status interact to determine parasite virulence. A more detailed example of rapid shift of virulence induced by the host diet has been provided for coxsackievirus B3 infecting mice. Coxsackievirus B3 (CVB3) can induce a myocarditis (an inflammatory heart disease) while some other viral strains (CVB3/0) do not cause the disease. Nevertheless, mice fed a selenium-deficient diet and inoculated with the avirulent strain (CVB3/0) do develop the disease. The viruses grown in selenium-deficient hosts were then passaged to control mice, and interestingly they again appeared to induce the disease. The strongest evidence that evolving in selenium-deficient mice selected for higher virulence was provided by the sequencing of the viral genomic RNA that showed that the avirulent strain had acquired six mutations that reverted it into the virulent strain (Beck et al. 2004) and references therein).

The aim of the present article was to test the effect of dietary status of hosts on the dynamics of infection and the adaptation of avian malaria parasites. Avian malaria parasites (in particular, *Plasmodium sp.* and *Haemoproteus sp.*) are highly prevalent in wild passerines (Cosgrove et al. 2008; Loiseau et al. 2011; Glaizot et al. 2012) and infection has been shown to affect bird fitness, both in natural and captive populations of hosts (Van Riper III et al. 1986; Atkinson et al. 2000; Williams 2005; Palinauskas et al. 2008; Zehtindjie ć et al. 2008), even when at low chronic levels (Knowles, Palinauskas & Sheldon 2009; Lachish et al. 2011). Environmental conditions have been shown to determine prevalence and intensity of avian malaria at different spatial scales (Wood et al. 2007; Loiseau et al. 2011; Szöllösi et al. 2011). Recently, a large survey of house sparrow (*Passer domesticus*) populations infected with *Plasmodium relictum* has provided evidence for a tight association between prevalence of infection and temperature variables (Loiseau et al. 2013). Under the current situation of
raising temperature potentially affecting the availability of trophic resources, it is crucial to
better understand the relationship between nutritional status and malaria dynamics and
adaptation.

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

Bird maintenance and food treatments

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

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

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

Parasites and experimental infections

We used the avian malaria parasite Plasmodium relictum (lineage SGS1) originally obtained from a natural population of house sparrows, and cross-transferred to naive canaries. Infected blood was cryopreserved and stored at -80°C (see details in Bichet et al. 2012). For the purpose of the present experiment, cryopreserved blood was thawed (Bichet et al. 2012)
and transferred intraperitoneally to 5 domestic canaries. Eleven days post-infection (dpi), parasitaemia was evaluated from thin blood smears (absolute methanol fixation, 10% Giemsa staining, observation of 10,000 erythrocytes). Blood was collected from donors to prepare a stock suspension diluted in PBS containing the desired number of parasites per inoculum (1 x 10⁶ asexual parasites) that served to infect birds of experiment 1 (see below).

A similar procedure was used to infect birds in experiment 2.

Experimental design

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

In experiment 1, two groups of birds in the control (n = 14 birds) or supplemented (n = 15 birds) diets were inoculated intraperitoneally with 1 x 10⁶ P. relictum parasites.

In experiment 2, parasites originating from control (C) and supplemented (S) birds were used to infect birds raised under similar control and supplemented diets. The parasitaemia at 10 dpi of infected birds in experiment 1 was estimated from blood smears.

Suspensions of infected red blood cells were prepared from the donor birds of the control (PC) (n = 9) and supplemented (PS) (n = 10) groups to infect a new set of birds raised under control (DC) and supplemented (DS) diets. Blood of birds with high parasitemia was diluted as to ensure that each donor contributed a similar number of parasites to the suspension.

Mixing parasites from multiple donors has previously been used in other studies where parasites were experimentally transmitted between groups of hosts (Yourth & Schmid-Hempel 2006; Kubinak et al. 2012). The same suspension of infected blood was used to infect all the birds in each treatment with the same inoculum size (1 x 10⁶). To summarize,
birds raised under the control diet (D\textsuperscript{C}) received either parasites originated from control hosts (D\textsuperscript{CP\textsubscript{C}}, n = 15 birds) or supplemented hosts (D\textsuperscript{CP\textsubscript{S}}, n = 15 birds); similarly, birds raised under the supplemented diet (D\textsuperscript{S}) received either parasites originated from control hosts (D\textsuperscript{SP\textsubscript{C}}, n = 15 birds) or the supplemented hosts (D\textsuperscript{SP\textsubscript{S}}, n = 15 birds).

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

**Estimation of infection intensity by quantitative PCR**

Infection intensity was assessed using the quantitative PCR assay as described by Cellier-Holzem et al. (2010). For each individual, two qPCR reactions in the same run were conducted: 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 CCG CGT AAA TTA CCC AAT TC-BHQ1-3’ and the other targeting the 18s rDNA gene of bird (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 GCG TC-BHQ1-3’). Parasite intensity was calculated as a relative quantification value RQ (2\textsuperscript{-(Ct 18s Plasmodium – Ct 18s Bird)}) using the software SDS 2.2 (Applied Biosystem). Ct is the number of PCR cycles at which fluorescence is first detected as statistically significant above the baseline. RQ can be seen as the fold-amount of the target gene (*Plasmodium* 18s rDNA) with respect to the amount of the reference gene (host 18s rDNA). All qPCR reactions were carried out using an ABI Prism 7900 cycler (Applied Biosystem).
Statistical analyses

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

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

RESULTS

During the course of the experiment four birds died during or soon after manipulation. These were not included in the further analyses. Neither diet nor parasite origin treatments accounted for differences in mortality among groups (experiment 1: n D⁰C = 2, n D⁰S = 0, diet \( \chi^2_1 = 3.07, P = 0.0792 \); experiment 2: n D⁰P⁰C = 3, n D⁰P⁰S = 1, n D⁵P⁰C = 3, n D⁵P⁰S = 1, diet \( \chi^2_1 = 0.04, P = 0.8443 \); parasite origin \( \chi^2_1 = 2.28, P = 0.1313 \)).
Experiment 1

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

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

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

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

Infected birds were anaemic and haematocrit declined as the infection progressed, reaching the minimum values around 10-14 dpi before recovering to values similar to the pre-infection ones (time \( F_{1, 127} = 8.10, P = 0.0052 \); time\(^2\) \( F_{1, 127} = 7.82, P = 0.0060 \); Fig. 1C).
Whereas birds fed with a control diet showed a moderate reduction in haematocrit, haematocrit of supplemented birds was critically reduced across the course of the infection (time*diet $F_{1, 127} = 7.77, P = 0.0061$; time$^2$*diet $F_{1, 127} = 4.51, P = 0.0356$; Fig 1C). In addition, variation in haematocrit was affected by a diet*parasitaemia interaction (parasitaemia $F_{1, 127} = 12.82, P = 0.0005$, diet*parasitaemia $F_{1, 127} = 6.69, P = 0.0108$). This last result shows that, even though control birds were more heavily parasitized, for a constant parasite load they paid a smaller cost of infection in terms of haematocrit reduction (Fig. 2).

**Experiment 2**

Again, birds fed with the supplemented diet achieved higher body mass at the end of the pre-infection period, contrary to control birds (time*diet $F_{1, 57} = 56.75, P < 0.0001$; Fig. 3B). The current diet did not affect parasitaemia either on its own ($F_{1, 55} = 0.34, P = 0.8542$) or in interaction with other factors (all $P$-values $> 0.39$). Parasitaemia varied according to the origin of the parasites (parasite*time$^2$ $F_{1, 261} = 6.93, P = 0.0090$; Fig. 3A, 4A) with birds suffering higher parasite load when infected by parasites previously grown on control hosts (PC).

The reduction in body mass during the infection (time $F_{1, 268} = 83.67, P < 0.0001$) was affected by both the current diet and the parasite origin (see below). The 3-way diet*parasite*time interaction term was not significant ($F_{1, 267} = 2.41, P = 0.1220$). However, both treatments (current diet and parasite origin) had an effect in interaction with time. Overall, as in experiment 1, control birds lost more weight than did supplemented birds (diet*time $F_{1, 268} = 8.46, P = 0.0039$; Fig. 3B, 4B), and birds infected with parasites originating from
control hosts (independently of the current diet in experiment 2) lost more body mass (time*parasite $F_{1, 268} = 11.14, P = 0.0009$; Fig. 3B, 4B).

Changes in haematocrit during the course of the infection (time $F_{1, 265} = 85.66, P < 0.0001$; time$^2 F_{1, 265} = 91.18, P < 0.0001$; Fig. 3C) were influenced by both the current diet and the parasite origin (significant 3-way interaction term, time*diet*parasite $F_{1, 265} = 5.15, P = 0.0240$; Fig. 3C). To better describe this complex pattern, we computed the maximum percent reduction in haematocrit for each of the four treatment groups. Parasites that were grown in hosts with a control diet induced the largest haematocrit reduction and hosts with a supplemented diet had a larger haematocrit reduction compared to control hosts (Fig. 4B).

In addition, maximal reduction in haematocrit was reached 3 days earlier in birds with control diet ($D^C$) than those under supplemented diet ($D^S$) (Fig. 3C). It is worth mentioning that similar results (not shown) were obtained when the analysis was performed on the 0-10 dpi period.

Parasitaemia negatively affected haematocrit ($F_{1, 256} = 5.58, P = 0.0189$) but the relationship was influenced by the current host diet and the parasite origin (parasitaemia*diet*parasite $F_{1, 256} = 4.03, P = 0.0449$). The inspection of the data, however, suggested that this significant 3-way interaction might be due to a single point with very high parasitaemia in the $D^SP^S$ group. When removing this single point from the analysis, the 3-way interaction was indeed far from reaching the significance threshold ($F_{1, 255} = 0.31, P = 0.5779$). Simplifying the model by removing non-significant terms showed that haematocrit was negatively correlated with parasitaemia ($F_{1, 258} = 14.78, P = 0.0002$) and that birds with the supplemented diet had a lower haematocrit than controls ($F_{1, 56} = 10.27, P = 0.0022$),
whereas parasite origin did not reach the significance threshold ($F_{1,56} = 3.58, P = 0.0635$) (Fig. 5).

### DISCUSSION

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

We found that hosts with a supplemented diet in terms of protein and vitamin contents were better able to control parasite multiplication. This result might therefore suggest that avian populations exposed to reduced food availability and/or poorer food quality are more susceptible to malaria parasites, possibly because of less effective immune defences. Interestingly however, for a given parasite load, supplemented birds paid a much higher cost of infection, at least as measured in terms of increased anaemia. This finding shows that while effective immune defences are needed to control parasite growth, they might also induce substantial fitness costs (Graham et al. 2005a; Sorci & Faivre 2009). Anaemia is primarily the consequence of the disruption of infected red blood cells and it often correlates with parasitaemia (Cellier-Holzem et al. 2010), as also found in the present
Anaemia might also be a host response to control the infection by increasing the clearance and the destruction of both parasitized and non-parasitized red blood cells (Lamikanra et al. 2007; Metcalf et al. 2011; Percario et al. 2012). Overreacting immune and inflammatory responses of supplemented hosts could have produced higher clearance of red blood cells whether they were infected or not, inducing an immunopathological damage (Graham et al. 2005a; Day, Graham & Read 2007; Long & Graham 2011). Previous work with mammalian and avian malaria has indeed suggested that a substantial fraction of the Plasmodium-induced cost of infection has an immunological origin (Graham et al. 2005b; Bichet et al. 2012), the most striking example being cerebral malaria in humans where high fatality is due to brain inflammation (Grau & Craig 2012).

The comparison of the infection cost when keeping constant the intensity of the infection gives us an interesting insight into a possible tolerance-resistance trade-off. Resistance refers to the capacity of the host to control and clear the parasite, whereas tolerance indicates the capacity of the host to withstand the infection with minimal fitness costs (Råberg, Sim & Read 2007; Råberg, Graham & Read 2009; Ayres & Schneider 2012; Medzhitov, Schneider & Soares 2012). Our results suggest that these two mechanisms of defence might show a phenotypic trade-off. Indeed, while supplemented birds were able to better resist to the infection they also appeared to less tolerate it. Beyond, anaemia and the inflammatory response triggered by infection, malaria pathogenesis also arises from the release of toxic hemes in the blood stream following the hemolysis of infected erythocytes. It has been shown that the expression of heme oxygenase-1 (HO-1) prevents the onset of severe cerebral malaria or hepatic failure in rodents through its antioxidant activity (Pamplona et al. 2007; Seixas et al. 2009). The greater tolerance observed in birds fed with a control diet may have been due to similar mechanisms. Alternatively, control birds may have
also been better at controlling the damage of the inflammatory response, hence decreasing the associated costs (Sears et al. 2011). As mentioned above the cause of this trade-off could come from the dual effect of infection-induced immune activation. Evidence for such trade-offs between tolerance and resistance is still rare (Read, Graham & Råberg 2008) but, in future studies, it will be essential to take into account these two processes if we want to better understand the effect of environmental changes on the ecology of host-parasite interactions.

Our study is essentially based on the assumption that the experimental manipulation of the diet affected the expression of the immune response against Plasmodium. Reliable assessment of immune effectors that are functionally involved in parasite resistance has proved difficult for non-model organisms (Adamo 2004). Nevertheless, there is a wealth of literature on humans and animal models unambiguously showing that both food quality and quantity do shape immune responsiveness and susceptibility to infectious diseases (Gonzalez et al. 1999; Siva-Jothy & Thompson 2002; Smith et al. 2007; Kau et al. 2011; Hoke, Kuzawa & McDade 2012). Undernutrition is probably the most prevalent environmental factors inducing immune suppression and risk of infection in human populations. The availability of microelements acquired with the diet (e.g. vitamins, zinc, selenium) can also have profound effects on the expression of the immune response. With this respect our treatment effectively induced a gain in body mass by increasing the availability of both protein (through the provisioning of hard-boiled eggs) and micronutrients (through the provisioning of apples and lettuce). For instance, apples contain micronutrients such as zinc, selenium, β-carotene, vitamins E and C, which are known to have an immunomodulatory effect.
Little information is available on the immune effectors involved in the resistance to avian malaria. Previous work has focused on immune genes (Westerdahl et al. 2005; Loiseau et al. 2008; Loiseau et al. 2011), whereas work conducted in the first half of the 20th century and in the last decade suggests that both innate, inflammatory effectors, and antibody mediated responses contribute to control parasite growth within the bird host (Sergent & Sergent 1952; Cellier-Holzem et al. 2010; Bichet et al. 2012). For instance, Cellier-Holzem et al. (2010) showed that parasitaemia of canaries that were re-exposed to the same *P. relictum* strain (SGS1) was much lower compared to a primary infection, strongly suggesting that immunological memory confers a partial protection towards re-infection (see also Buckling & Read 2001). The acute phase of the infection should however elicit more the innate inflammatory response. Recently, Bichet et al. (2012) showed that canaries whose inducible nitric oxide synthase was experimentally inhibited had higher parasitaemia compared to control suggesting a role for nitric oxide in the control of *Plasmodium* growth.

In addition to assessing the role of host nutritional status on parasite dynamics and cost of infection, we also wished to investigate the potential for parasite adaptation to hosts experiencing specific diets. This is particularly relevant because if environmental variation alters the availability of food resources this might produce an evolutionary shift in the parasite population. As for free organisms, parasites can adapt to their environment adopting a plastic adjustment of their phenotypic traits or by genetic selection. Disentangling these two processes might prove very difficult because a final demonstration of genetic selection would require keeping track of genetic variants across generations. Here we adopted an indirect, statistical, approach to infer whether adaptation occurred via a plastic response or a genetic selection. We used parasitaemia (within-host parasite multiplication) as a measure of parasite fitness (but we are aware that this might be an
oversimplification given that we did not take into account transmission to the vector host) and predicted that if *Plasmodium* adapts through phenotypic plasticity, the major determinant of parasite replication should be the quality of the current environment. On the contrary if adaptation proceeds through a genetic selection we should expect that major determinant of parasite success to be the quality of the previous environment (or the interaction between the two). In agreement with the latter prediction we found that parasitaemia of passaged parasites was only affected by the diet treatment of their previous hosts, with parasites coming from control hosts reaching higher parasitaemia in their subsequent hosts. The inspection of figure 3B suggests that this pattern is mostly driven by the D\textsuperscript{CP}C group (parasites coming from control hosts and infecting birds in control diet). It could be that we lacked statistical power to detect a significant interaction here, but even a statistical significant interaction between the previous and current environments would rather suggest a process of adaptation through genetic selection. This argument might also explain the apparent discrepancy between the results of experiment 1 and 2 in terms of the effect of the current environment on parasitaemia. Indeed, in experiment 1 we found that supplemented hosts harboured a significantly lower parasitaemia whereas in experiment 2 the current diet of the host did not affect parasitaemia. The pattern we found is consistent with the idea that selection operated on the parasites infecting supplemented and control hosts of experiment 1, subsequently masking the effect of the current environment. Serial passage experiments are well known to induce rapid evolution of pathogens favouring the genotypes with the highest multiplication rate (Ebert 1998). In agreement with this, we indeed found that parasitaemia reached in the experiment 2 was much higher than in experiment 1 ($\chi^2_{1} = 18.70, P < 0.0001$). Since our inoculum was certainly formed by a mix of different genotypes/clones (the parasite was isolated from naturally infected house
sparrows and passaged using a mix of blood from different infected birds), high genetic
diversity allowed selection to produce a very rapid shift. A possible caveat of mixing
parasites from multiple donors to infect subsequent hosts may have been that a few
parasite strains/genotypes were overrepresented in the inoculum because of their
competitive advantage. However, we made sure that each donor contributed a similar
number of parasites to the inoculum which should reduce the risk of such competitive
asymmetry among parasite strains/genotypes. As mentioned above, mixing parasites from
multiple donors, however, probably allows maintaining a large amount of genetic variation
within the parasite population and more closely matches the natural situation where each
vertebrate host is bitten by multiple vectors.

In addition to being more adapted to non-supplemented hosts, parasites grown in
such hosts induced higher costs when passaged to novel hosts. We used two proxies of
parasite virulence (reduction in body mass and haematocrit) and with respect to both
variables, parasites coming from control, non-supplemented hosts, appeared to become
more virulent. The evolution of higher virulence in hosts fed with poor diets raises a concern
if human-driven environmental changes induce an impoverishment of food quality and
quantity. Interestingly, these results parallel those obtained with the avirulent coxsackievirus
B3/0 where a single passage in mice kept on selenium-deficient diet restored the virulence
usually observed for the B3 strain (Beck et al. 2004), even though the mechanisms in play
are likely to be different. The generality of this result has, however, yet to be established,
since other studies involving invertebrate hosts have rather suggested that evolution to
higher virulence might be driven by well-fed hosts (Tseng 2006; Little et al. 2007).
Avian malaria are common parasites of many bird species. The spread of the pathogen has threatened the persistence of immunologically naïve host populations, as witnessed by the case study of Hawaiian birds (Atkinson et al. 1995). Recently, a concern has arisen on the possible consequences of climate change on the spread of the disease (Garamszegi 2011; Loiseau et al. 2013). If changes in temperature affect the availability of food quantity and quality, our study shows that malaria parasites might become a more serious concern for their avian hosts. Finally, host nutrition is also likely to modify host attractiveness and palatability to vectors, affecting malaria transmission. Indeed, infected birds are more attractive to vectors than uninfected birds and bird haematocrit has been shown to influence host choice behaviour of mosquito vectors in this avian malaria system (Cornet et al. 2013). Much work remains to be done to fully understand the effects of resource availability on host-vector-malaria interactions and to clarify the role and importance of vectors in the evolution of parasite virulence.

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DATA ACCESSIBILITY

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

REFERENCES


Figure 1. Variation (means ± s.e.) in blood parasitaemia Log(RQ+1) (A), body mass (B), and haematocrit (C) in birds infected by the avian malaria *Plasmodium relictum* (SGS1) and maintained under control (triangles) or supplemented diet (circles). Diets were provided from 15 days before parasite infection until the end of the experiment (17 dpi).

Figure 2. Covariation between haematocrit values and blood parasitaemia Log(RQ+1) of *Plasmodium relictum* (SGS1) in infected birds reared under control (triangles, dashed line) or supplemented (circles, solid line) diet (Experiment 1).
Figure 3. Variation (means ± s.e.) in blood parasitaemia Log(RQ+1) (A), body mass (B), and haematocrit (C) in birds maintained under control (D\textsuperscript{C}, triangles) or supplemented (D\textsuperscript{S}, circles) diets and infected by *Plasmodium relictum* (SGS1) parasites previously reared in control (P\textsuperscript{C}, open symbols) or supplemented hosts (P\textsuperscript{S}, filled symbols). Diets were provided from 15 days before parasite infection until the end of the experiment (17 dpi). Legend: D\textsuperscript{C}P\textsuperscript{C}: open triangles, D\textsuperscript{C}P\textsuperscript{S}: filled squares, D\textsuperscript{S}P\textsuperscript{C}: open circles, D\textsuperscript{S}P\textsuperscript{S}: filled circles.

Figure 4. Variation (means ± s.e.) in maximum parasitaemia Log(RQ+1) (A) and maximum percent reduction in body mass (B) and in haematocrit (C) over the infection period according to current diet and parasite origin (Experiment 2).
Figure 5. Covariation between haematocrit values and blood parasitaemia Log(RQ+1) of *Plasmodium relictum* (SGS1) in infected birds reared under control (triangles, dashed line) or supplemented (circles, solid line) diet (Experiment 2).