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LETTER

Malaria infection increases bird attractiveness to uninfected mosquitoes

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Abstract

The epidemiology of vector-borne pathogens is largely determined by the host-choice behaviour of their vectors. Here, we investigate whether a *Plasmodium* infection renders the host more attractive to host-seeking mosquitoes. For this purpose, we work on a novel experimental system: the avian malaria parasite *Plasmodium relictum*, and its natural vector, the mosquito *Culex pipiens*. We provide uninfected mosquitoes with a choice between an uninfected bird and a bird undergoing either an acute or a chronic *Plasmodium* infection. Mosquito choice is assessed by microsatellite typing of the ingested blood. We show that chronically infected birds attract significantly more vectors than either uninfected or acutely infected birds. Our results suggest that malaria parasites manipulate the behaviour of uninfected vectors to increase their transmission. We discuss the underlying mechanisms driving this behavioural manipulation, as well as the broader implications of these effects for the epidemiology of malaria.

Keywords

Culex mosquito, experimental infections, malaria transmission, parasite manipulation, *Plasmodium relictum*, vector blood feeding.

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INTRODUCTION

Pathogen evolution is driven by the necessity to exploit its host efficiently and to get access to new hosts. Many vector-borne pathogens have evolved the ability to manipulate the behaviour of their vectors to enhance their own transmission (Hurd 2003; Lefèvre & Thomas 2008). *Plasmodium*-infected mosquitoes (Koella *et al.* 1998; Anderson *et al.* 1999), *Leishmania*-infected sand flies (Rogers & Bates 2007), *Trypanosoma*-infected tse-tse flies (Van Den Abbeele *et al.* 2010) and plague-infected fleas (Eisen & Gage 2012) have all been shown to have higher host probing and/or biting rates than their uninfected counterparts. For parasites, however, manipulating the biting rate of the infected vector is just one of the possible ways of going about maximising transmission. Pathogens that are able to manipulate their hosts to make them more attractive to uninfected vectors should also be strongly favoured by natural selection.

Increased attractiveness of infected hosts has been reported in various animal (Mahon & Gibbs 1982; Turell *et al.* 1984; Scott *et al.* 1990; O'Shea *et al.* 2002) and plant systems (Eigenbrode *et al.* 2002; Mauck *et al.* 2010; Ingwell *et al.* 2012; Mann *et al.* 2012). When working with *Plasmodium,* several authors have noted that uninfected mosquitoes behave differently when faced with an infected, as opposed to an uninfected, host. Ferguson *et al.* (2003) and Ferguson & Read (2004) observed that mosquitoes had a higher probability of engorgement when feeding from an infected host, and Taylor & Hurd (2001) documented larger blood meals from infected hosts. In addition, Rossignol *et al.* (1985) observed that the median duration of probing by mosquitoes exposed to infected mice was 50% lower than that of mosquitoes exposed to uninfected mice. None of these studies, however, presented infected and uninfected hosts simultaneously to the vectors, and therefore did not address the question of mosquito choice: do mosquitoes prefer to feed on *Plasmodium*-infected or uninfected hosts?

Mosquito host-choice behaviour in relation to a *Plasmodium* infection has only been monitored in a handful of studies. Day *et al.* (1983) showed that mosquitoes released into a cage containing one infected and one uninfected mouse engorged almost exclusively on the infected animal, but this result was attributed to a reduction in the anti-mosquito defensive behaviour of the host, rather than a higher attraction towards the infected mouse. Lacroix *et al.* (2005) showed that children infected with the transmissible stages of *Plasmodium falciparum* (gametocytes) were marginally more attractive to uninfected mosquitoes than uninfected children or children infected with the non-transmissible (trophozoite) stages of the malaria parasite, but Lalubin *et al.* (2012) obtained the opposite results on avian malaria: *Cx. pipiens* mosquitoes were significantly more attracted to uninfected wild great tits than to birds infected with an unidentified *Plasmodium* parasite. Both of these studies, however, had drawbacks that reflect the technical (and in the case of human malaria, ethical) limitations of working with wild *Plasmodium* infections. They both relied on natural and, consequently, uncontrolled *Plasmodium* infections. In Lacroix *et al.* (2005), differences in the intrinsic attractiveness of the host in the absence of an infection was controlled for by treating the infected children with the antimalarial drug Fansidar®, a protocol that has been criticised by Bousema & Sauerwein (2005), as was the possible presence of false negatives amongst the uninfected and trophozoite-infected children (parasites were detected microscopically, but submicroscopic levels of sexual and asexual parasites are common in the area, Bousema & Sauerwein 2005). Lalubin *et al.* (2012), on the other hand, did not monitor the change of attractiveness after parasite clearance and thus cannot account for intrinsic between-host differences in attractiveness. In addition, the increased attractiveness of the uninfected hosts was based on a limited number of uninfected birds (five), which questions the generality of their results (Lalubin *et al.* 2012). Here, we contend that because of these issues, the increased attractiveness of *Plasmodium*-infected hosts is still an open question and that the unambiguous demonstration that the host-choice biting behaviour of vectors is altered by the infection requires a more stringent experimental set-up.

In this study, we investigate whether infected hosts are more attractive to mosquitoes using the avian malaria parasite *Plasmodium relictum* (lineage SGS1) and its natural vector, the mosquito *Culex pipiens* (Valkiūnas 2005). *Plasmodium relictum* (SGS1) is a generalist parasite and the most prevalent form of avian malaria in Europe, infecting over 30 birds species in the order Passeriformes (Valkiūnas 2005; Farajollahi *et al.* 2011). Our strain was isolated from house sparrows (*Passer domesticus*) and transferred to uninfected canaries (*Serinus canaria*) by intraperitoneal injection. Mosquitoes of the *Cx. pipiens* complex are the main vectors of *P. relictum* in the field (Glaizot *et al.* 2012), and in a recent survey, up to 90% of *Plasmodium*-infected *Cx. pipiens* were found to carry the SGS1 lineage (F. Zélé, J. Vézilier, G. L'Ambert, S. Gandon, A. Rivero, O. Durond, unpublished).

We monitored the choice of uninfected *Cx. pipiens* mosquitoes by releasing them into a cage containing one experimentally infected and one uninfected bird and quantifying *a posteriori* the number of mosquitoes that fed on each host using genetic (microsatellite) analyses of the blood meal. We contend that this system has the advantage of eliminating one pervasive technical difficulty encountered with the use of olfactometers, namely that the majority of mosquitoes do not react to the olfactory stimuli provided (see e.g. Lacroix *et al.* 2005; Lalubin *et al.* 2012), and gives a more biologically relevant measure of host choice because it captures the whole behavioural sequence from the detection of the odour to the decision to bite.

Like many other vector-borne diseases, primary *Plasmodium* infections go through an initial (acute) phase, characterised by very high parasitaemias, followed by a low level (chronic) phase which can last for months or even years (Roper *et al.* 1996; Snounou *et al.* 2000; Valkiūnas 2005; Asghar *et al.* 2012). It is currently unknown whether these chronic infections result from the reactivation of phanerozoites (exoerythrocytic dormant stage of the parasite), the replication of erythrocytic asexual parasites or a continuous interchange between exoerythrocytic and erythrocytic forms. The establishment of such chronic infections is thought to be an adaptive strategy of parasites enabling parasite survival during the non-transmission season and maximising opportunities for transmission (Reece *et al.* 2009). In natural populations, these long-lasting chronic infections offer enormous potential for selection to operate on. Despite this, most published *Plasmodium* studies concern primary acute stage infections. To account for the existence of these two very different phases of infection related to the dynamics of the malaria parasite, birds were assessed during the acute and chronic phases of infection. Bird pairs were also assessed before the infection to control for intrinsic difference in attractiveness between the birds.

The specific aims of this study are to determine: (1) whether hosts that are infected by *Plasmodium* are more attractive to mosquitoes than their uninfected counterparts and (2) whether there is a temporal variability in mosquito choice during the course of the infection, specifically, whether there are differences between early (acute) infections and late (chronic) infections in bird attractiveness to the mosquitoes.

MATERIALS AND METHODS

Experimental system

Malaria parasite

Plasmodium relictum (lineage SGS1) is the aetiological agent of the most prevalent form of avian malaria in Europe (Valkiūnas 2005). This generalist *Plasmodium* parasite lineage was originally isolated from house sparrows caught in the region of Dijon (France) in 2009 (Cellier-Holzem *et al.* 2010) and subsequently transferred to naïve canaries (*Serinus canaria)* by intraperitoneal injection. In our experiment, bird infections took place by intraperitoneal injection of *ca.* 50–100 μL of blood from our infected bird stock to limit the variation in the infection dose.

Birds

Experiments were carried out using (1-year old) domestic canaries (*Serinus canaria*) Prior to the mosquito host-choice experiments, the birds were weighed, and a small amount (20–30 μL) of blood was taken from the brachial vein via a small puncture. Genomic DNA was extracted from blood using the procedure outlined in the DNeasy Blood & Tissue Kit (Qiagen). We carried out a series of PCRs to establish the sex (Griffiths *et al.* 1998) and genotype the birds at the microsatellite Cuμ28 locus (see below). We also carried out five consecutive diagnostic PCRs (Waldenström *et al.* 2004) to ensure that the canaries were not already infected with haemosporidian parasites.

Mosquitoes

Experiments were conducted with a laboratory strain of *Culex pipiens* (SLAB, Berticat *et al.* 2002). Mosquitoes were reared under standard conditions (for details see, Vézilier *et al.* 2010). We used females 7–10 days after emergence that had had no prior access to blood, had been maintained on glucose solution (10%) since their emergence, and had been starved (but provided with water) for 6 h before the experiment.

EXPERIMENTAL PROCEDURE

Bird movements and defensive behaviours can alter the feeding process and bias the choice of *Cx. pipiens* mosquitoes (Day *et al.* 1983; Darbro *et al.* 2007). We chose to minimise this effect by immobilizing the birds in a specially designed PVC tube that rendered their legs accessible to the mosquitoes while protecting the rest of the body (Fig. S1). Birds were assorted into 25 different pairs making sure that birds within a pair had different microsatellite profiles. To avoid undesirable bias, birds within pairs were matched for sex and, as far as possible, for body mass. Each bird pair was placed inside a cage (dimensions L80 × W30 × H30 cm, see Fig. S1) with 70 haphazardly chosen female mosquitoes for 2 h (from 8 to 10 pm to coincide with the peak of *Cx. pipiens* activity in the field). Choice experiments were conducted in the dark. For each bird pair, mosquito choice was assessed three times. To account for an intrinsic difference in attractiveness between the birds, bird pairs were first assessed few days before the infection (henceforth 't0'). Then, one bird from each pair was randomly infected with *P. relictum* (all birds were infected by intraperitoneal injection on the same day, see above) and the pairs were assessed a second time 10–13 days post-infection (dpi) to coincide with the acute phase of the infection ('t10'). The peak of parasitaemia is often reached around 10 dpi (but parasitaemia does not vary significantly between days 10 and 13 post-infection, see below). Finally, a third test was carried 24–26 dpi, to coincide with the chronic stage of the infection ('t24'). As some bird mortality occurred during the final stages of the experiment, the third and last time point (t24) was carried out using only 16 bird pairs. Each test (t0, t10 and t24) was carried out using a different batch of mosquitoes (reared in identical conditions)

so that all mosquitoes were of identical age and physiological state at the time of the experiments. For clarity, in the remainder of the article we refer to the bird that was randomly chosen to be infected within each pair as the 'focal bird'.

To avoid interference, each pair of birds was assessed in a separate controlled temperature room (4 different CT rooms, temperature: 25 ± 2 °C, relative humidity: 75 ± 5%). Each run of the experiment (t0, t10 and t24) was spread over 3 or 4 consecutive evenings. After each run, all engorged mosquitoes were taken out of the cage and stored at −80 °C. Forty-five engorged females from each cage were haphazardly chosen for DNA extraction and microsatellite genotyping.

All the host-choice experiments were blind with regards to the infection status of the birds. For each of the three time-steps (t0, t10 and t24), the experiments were carried out in the evenings. In the morning prior to each experiment, all birds were weighed and bled to get a small blood sample for the quantification of the haematocrit (volume of red blood cells per total volume of blood in the capillary after centrifugation 5 min at 10 000 rpm, measured using a graduated ruler), and parasitaemia (see below).

Molecular analyses

Quantification of bird parasitaemia

The quantification of bird parasitaemia was carried out using a quantitative PCR (qPCR) protocol adapted from Cellier-Holzem *et al.* (2010). Briefly, for each individual, we conducted two qPCRs in the same run: one targeting the nuclear 18s rDNA gene of *Plasmodium* (Primers 18sPlasm7 5'-AGCCTGAGAAATAGCTACCACATCTA-3', 18sPlasm8 5'-TGTTATTTCTTGTCACTACCTCTCTTTCTTT-3') and the other targeting the 18s rDNA gene of the bird (Primers 18sAv7 5'-GAAACTCGCAATGGCTCATTAAATC-3', 18sAv8 5'-TATTAGCTCTAGAATTACCACAGTTATCCA-3'). All qPCRs were run on an ABI 7900HT real-time PCR system (Applied Biosystems).

Parasite intensities were calculated as relative quantification values (RQ). RQ can be interpreted as the fold-amount of target gene (*Plasmodium* 18s rDNA) with respect to the amount of the reference gene (Bird18s rDNA) and are calculated as $2^{-(Ct18s\ Plasmodium\ -\ Ct18s\ Bird)}$ For this purpose, all samples were run in triplicate and their mean was used to calculate the threshold C*t* value (the number of PCR cycles at which fluorescence is first detected, which is inversely correlated with the initial amount of DNA in a sample) using the software Light Cycler 480 (Roche). For convenience, RQ values were standardised by $\times 10^4$ factor and log-transformed. These log-transformed RQ values allowed the monitoring of parasite load inside infected birds over the duration of the infection.

Microsatellite genotyping

Blood ingested by mosquitoes was genotyped at the bird genome locus Cuμ28 (Melo & Hansson 2006) to establish which bird the mosquitoes had fed on. PCR amplifications, using the primers Cuμ28-F (5'-6-FAM-GAGGCACAGAAATGTGAATT-3') and Cuμ28-R (5'-TAAGTAGAAGGACTTGATGGCT-3') were performed in a total volume of 10 μL, containing 5 μL Qiagen multiplex mix, 1 μL of each primer (2 μM), 2 μL H_2O and 1 μL DNA (50–100 ng μL^{-1}). The thermal profile of both PCRs started with 15 min of denaturation at 94 °C, followed by 35 cycles at 94 °C for 30 s, 58 °C for 1 min 30 s, 72 °C for 1 min, and ended with an elongation step at 60 °C for 10 min. PCR amplifications were visualised using an automated sequencer Applied ABI Prism 3130 XL (Applied Biosystems). Preliminary assays performed with varying ratios (ranging from 1 : 9 to 1 : 1) of blood from 2 birds confirmed that multi-host feeding could be easily detected at low concentration.

Statistics

The results were analysed using generalised linear mixed models (lmer function, lme4 package) available in the R statistical package (v. 2.14.0). The binomially distributed response variables were as follows: the proportion of mosquitoes that took a blood meal (blood feeding success), the proportion of mosquitoes that fed on both birds (multi-host feeding) and the proportion of mosquitoes that bit the focal bird (uninfected at t0, infected at t10 and t24) relative to the total number of blood-fed mosquitoes (excluding multi-host feeders). Models were fitted by specifying infection treatment (t0, t10 and t24) and Δ haematocrit (the difference in haematocrit between the focal and the control bird) as fixed effects. To account for the repeated measurements, the bird pair identity was treated as a random effect (Crawley 2007). Maximal models were simplified by sequentially eliminating non-significant terms and interactions ($P > 0.05$) to obtain minimal models following standard stepwise deletion procedures. The significance of the explanatory variables was established using a χ^2 test. When appropriate, a posteriori contrasts were carried out by aggregating factor levels that did not significantly differ from each other and by testing the fit of the simplified model (Crawley 2007). One pervasive problem of statistical models with binomial error structure is the existence of overdispersion. The best way to deal with overdispersion is to fit the model using quasibinomial error distributions (Crawley 2007). However, to date, it is not currently possible to account for quasibinomial distributions within a mixed model lmer procedure. When overdispersion was present, we verified the validity of our results by (1) transforming the response variable (arcsine transformation, Sokal & Rohlf 1995) and refitting the mixed model using normal errors (lme procedure) and (2) using a replicated G-test of goodness of fit (Sokal & Rohlf 1995).

The effect of the infection on bird condition (body mass, haematocrit, log-transformed parasitaemia) was investigated using linear mixed models (lme function, nlme package) using the same procedure as above. Here, the significance of explanatory variables was established using a likelihood ratio test.

Ethics statement

Animal experiments were carried out in strict accordance with the 'National Charter on the Ethics of Animal Experimentation' of the French Government, and all efforts were made to minimise suffering. Experiments were approved by the Ethical Committee for Animal Experimentation established by the authors' institution (CNRS) under the auspices of the French Ministry of Education and Research (permit number CEEA- LR-1051).

RESULTS

Blood feeding

The proportion of mosquitoes that took a blood meal (blood feeding success) was upwards of 0.9, irrespective of the infection time

(Fig. S2a). However, significantly fewer blood-fed females were retrieved during the acute (t10) phase of the infection ($\chi^2_2 = 18.47$, $P < 0.0001$). The majority of mosquitoes fed on only one of the two birds (focal or control). The proportion of mosquitoes that fed on both birds (multi-host feeding) was very low at all time intervals (mean ± SEM, t0 = 0.032 ± 0.007, t10 = 0.056 ± 0.009, t24 = 0.050 ± 0.008, Fig. S2b). Infection had an effect on the rate of multi-host feeding ($\chi^2_2 = 8.12$, $P = 0.0172$) so that a higher proportion of mosquitoes took a mixed blood meal when there was an infected bird in the trial (contrast t0 vs. t10 and t24: $\chi^2_1 = 7.63$, $P = 0.0057$).

Body condition differences between focal and control birds

We first compared focal and control birds for two body condition parameters that may potentially affect mosquito choice: body mass and haematocrit (a proxy for anaemia). The body mass of all birds fluctuated over time ($\chi^2_2 = 6.72$, $P = 0.0348$). This fluctuation was, however, similar in focal and control birds ($\chi^2_2 = 0.22$, $P = 0.8946$, Fig. 1a). As a result, the difference in body mass between focal and control birds within each pair stayed constant across the three time points ($\chi^2_2 = 0.23$, $P = 0.8907$).

The haematocrit of focal and control birds behaved very differently across time ($\chi^2_2 = 33.72$, $P < 0.0001$, Fig. 1b). At t0 (when both focal and control birds are uninfected), and t24 (when the focal bird is in a chronic infection) there was no significant difference between the haematocrit of these two groups (t0: $F_{1,48} = 3.49$, $P = 0.0678$, t24: $F_{1,30} = 3.55$, $P = 0.0692$). However, during the acute phase of the infection (t10), the haematocrit of the focal bird dropped drastically ($F_{1,48} = 21.28$, $P < 0.0001$) as a result of the high blood parasitaemia (Fig. 1c). Hence, the difference in haematocrit between birds within a pair (Δ haematocrit) also varied with time, being higher during this acute phase ($\chi^2_2 = 41.56$, $P < 0.0001$). Globally, the haematocrit of the infected birds (t10 and t24 only) was negatively correlated with the intensity of parasitaemia in the blood ($r = -0.41$, $F_{1,39} = 8.06$, $P = 0.0071$). Parasitaemia did not differ between the 2–4 consecutive days around the peak of infection (no significant difference in parasitaemia between days 10, 11, 12 and 13, $F_{3,21} = 0.83$, $P = 0.4917$) nor during the chronic phase of infection (no difference between days 24, 25 and 26, $F_{2,13} = 0.46$, $P = 0.6425$).

Effect of *Plasmodium* infection on bird attractiveness/vector preference

In general, mosquitoes preferred birds with the highest haematocrit ($\chi^2_1 = 3.97$, $P = 0.0463$), an effect that was consistent across the three time points (the interaction term was not statistically significant, $\chi^2_2 = 4.95$, $P = 0.0841$; Fig. 2b). *Plasmodium* infection had a drastic effect on bird attractiveness, although the effect varied across the three different time points ($\chi^2_2 = 17.88$, $P = 0.0001$, Fig. 2a, b). As expected, at t0 (before infection) mosquitoes did not show an overall preference for either bird (proportion of choosing the focal bird as estimated by the statistical models = 0.50, $P = 0.9646$). During the acute phase of the infection (t10), the probability of choosing the focal (infected) bird increased (proportion = 0.54) but remained statistically undistinguishable from 0.50 ($P = 0.2137$). Contrast analyses revealed that there were indeed no significant differences in mosquito preference between t0 and t10 (contrast t0 vs. t10: $\chi^2_1 = 1.47$, $P = 0.2252$). In contrast, the probability of feeding on the focal (infected) bird increased markedly during the chronic phase of the infection (proportion = 0.62, $P < 0.0001$). This increase in mosquito preference for the infected bird at t24 relative to t0 and t10 was highly statistically significant ($\chi^2_1 = 15.28$, $P < 0.0001$). We used two complementary approaches to verify the validity of the increased preference for chronically infected birds. A replicated *G*-test of goodness-of-fit, confirmed a statistically significant departure between the observed (choice at t24) and the expected (choice at t0) frequencies (total-$G = 158.41$, 16 *df*, $P < 0.0001$; pooled-$G = 23.55$, 1 *df*, $P < 0.0001$), albeit with a significant heterogeneity between the replicates (heterogeneity-$G = 134.86$, 15 *df*, $P < 0.0001$). In addition, the reanalysis of the data using the arcsin-transformed values and normal errors confirmed that host-choice behaviour is influenced by both host haematocrit ($\chi^2_1 = 4.49$, $P = 0.0349$) and the infected status of the host ($\chi^2_1 = 5.55$, $P = 0.0185$).

Over the course of the experiment, we lost several bird pairs due to bird mortality (6 infected and 3 uninfected, each in a different

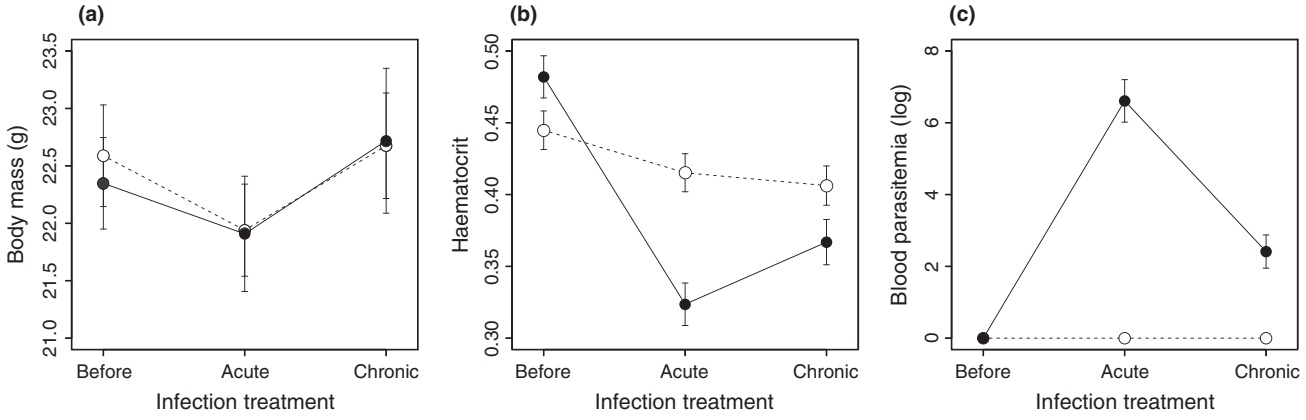

Figure 1 Variation (mean ± SEM) in (a) body mass (in grams), (b) haematocrit (ratio between red blood cells volume and total blood volume) and (c) blood parasitaemia (number of parasite gene copies relative to the number of host gene copies, log transformation) for control birds (open circle, dash line) and focal birds (dark circle, solid line) at three different stages of the infection: before infection (t0), acute infection (t10) and chronic infection (t24). Focal birds refer to the birds randomly chosen to be infected by *Plasmodium relictum* (uninfected at t0, infected at t10 and t24).

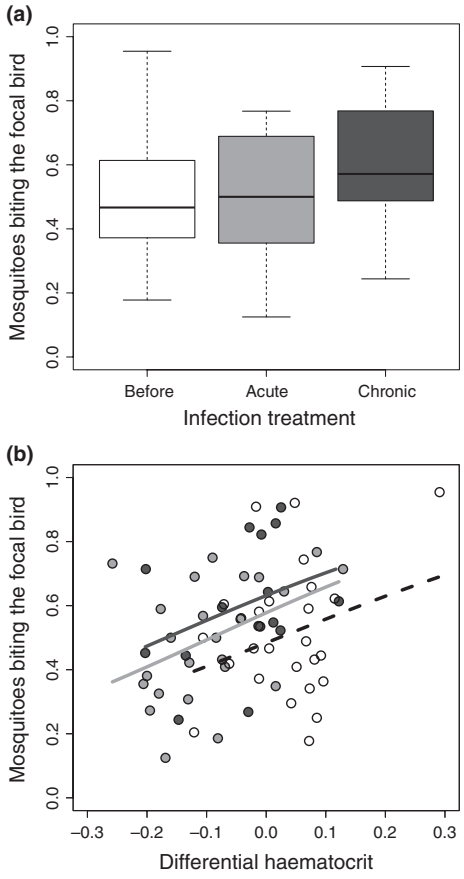

Figure 2 Effect of infection by *Plasmodium relictum* on bird attractiveness. Infection treatment: before infection (t0), acute infection (t10) and chronic infection (t24). Bird attractiveness refers to the proportion of uninfected *Culex pipiens* mosquitoes that bit the focal bird (uninfected at t0 but infected at t10 and t24) relative to the number of blood-fed mosquitoes. (a) Raw data. Boxes are interquartile ranges, thick lines are medians and bars enclose 90% of the distribution. (b) Relationships between bird attractiveness and differential haematocrit, which refers to the difference in haematocrit between the focal bird and the control bird within pairs. Legend: before infection: white symbol, dashed line; acute infection: light grey, light grey line; chronic infection: dark grey, dark grey line. Lines are the fits of GLMM models.

pair), so that at t24 we had only 16 replications instead of the 25 that we started with. The infected birds that died in these pairs had heavier parasitaemias at t10 ($F_{1,23}$ = 16.96, P = 0.0004) and lost significantly more body mass ($F_{1,23}$ = 8.42, P = 0.0080) and haematocrit ($F_{1,23}$ = 5.76, P = 0.0249) between t0 and t10 than the other infected birds. To check the robustness of our results we repeated the analyses eliminating these nine pairs from all the time-steps. The statistical results were qualitatively identical (Fig. S3), showing that the effect of *Plasmodium* infection on mosquito behaviour was not affected by this potential mortality bias.

DISCUSSION

Our results show that uninfected *Cx. pipiens* mosquitoes feed preferentially on *P. relictum*-infected birds. We see three potential explanations for these results. First, the behaviour could be adaptive for the mosquitoes. Indeed, Day *et al.* (1983) have shown that infected mouse is lethargic and therefore less likely to kill the mosquito during the blood feeding event. Although our birds were immobilised to prevent any anti-mosquito defensive behaviours, the cues used by mosquitoes to detect (and prefer) infected hosts may have still been in place (e.g. odours). We have never, however, observed any such lethargy in chronically infected birds. In addition, feeding on infected birds has been shown to carry significant costs for the mosquito in terms of fecundity (the number of eggs is reduced by c.a. 30%, Vézilier *et al.* 2012) and longevity (albeit in a less consistent way, Vézilier *et al.* 2012). An alternative, and in our opinion, more likely explanation is that, as suggested by Lacroix *et al.* (2005), the preference of mosquitoes for infected hosts is the result of a parasite-driven manipulation of the host aimed at maximising its own transmission. The third explanation, that the behaviour is a mechanistic by-product of the infection of no adaptive value for either the mosquito or the parasite is, in our opinion, a red herring: parasite-associated illness symptoms (e.g. fever, sweat, CO2, odours) that allow the mosquito to efficiently locate an infected host thereby increasing parasite fitness, would strongly select for parasites able to manipulate these traits.

Interestingly, we found that the modification of vector choice varies throughout the course of the infection. Like most other *Plasmodium* infections, primary *P. relictum* infections go through an initial *acute* phase, followed by a low level but long-lasting *chronic* phase (Asghar *et al.* 2012). During the acute phase, no mosquito preference for either the infected or the uninfected bird was detected. During the chronic phase (24 dpi), however, mosquitoes showed a very clear preference for the infected birds. Three mechanisms can be invoked to explain this pattern. First, gametocytes (the transmissible stages of the parasite) may not have been present at the time the acute phase trials were carried out. Lacroix *et al.* (2005) indeed found that *P. falciparum*-infected children bearing no gametocytes were no more attractive to mosquitoes than uninfected children. However, in contrast with *P. falciparum, P. relictum* is an asynchronous parasite: gametocytes are present in high numbers as soon as the erythrocytic forms of the parasite reach the blood (pers. obs., Valkiūnas 2005). Second, there may be a physiological constraint such that the build up of the phenotype used by the mosquito to make a choice, whatever it might be (see below), takes some time. A third explanation is that the observed behaviour is the result of two antagonistic effects: the attraction induced by the manipulating parasite and the repellence induced by an altered blood meal. The physiological and immunological conditions of the host are indeed known to be altered during the acute phase of a *Plasmodium* infection. One striking effect of acute infections is a drastic drop in red blood cell counts (Fig. 1b). Red blood cells represent the main source of proteins for egg production in mosquitoes, which probably explains why, in the absence of the infection, mosquitoes strongly prefer the bird with the highest haematocrit (Fig. 2b). In other words, under this scenario, the parasites may well have had an effect early in the infection, but this effect may have been counteracted by the low quality of the host for blood meal purposes at that point in time. The balance only tips in favour of the parasite once the physiological state of the bird recovers during the chronic phase of the infection (Fig. 1b, 2b).

Beyond the explanation for the time dependence of the choice, these results raise questions as to the cues used by mosquitoes to detect the infected hosts and which may be the target of parasite manipulation. Our experimental protocol ruled out the use of visual

(it was done in the dark) and behavioural cues (the birds were immobilised, preventing anti-mosquito defensive behaviour). We can also rule out fever as previous studies have failed to detect changes in body temperature associated with *P. relictum* SGS1 infections (Palinauskas *et al.* 2008). This leaves olfactory cues as the most likely mechanism. In humans, there is plenty of evidence that the odours emanating from gland secretions and the skin microflora play an important role in mosquito attraction (Verhulst *et al.* 2009, 2010; Smallegange *et al.* 2011). In birds, the uropygial glands are a key source of avian chemical substances and produce large amounts of volatile and non-volatile substances in the form of waxy fluids that are spread on the bird's plumage through preening (Hagelin & Jones 2007). Birds host a diversity of microbes on feathers and skin, which may also be involved in the production of chemical substances. Syed & Leal (2009) have recently identified the aldehyde *nonanal*, as a strong attractant for *Culex* mosquitoes, which could explain the ornitophilic nature of these mosquitoes (birds produce more nonanal than other vertebrate hosts, Syed & Leal 2009). A difference in the chemical signatures may account for the differences in intrinsic attractiveness between uninfected birds we observed (Fig. 2a, b).

Many diseases are associated with changes in the odour profile of the host (Prugnolle *et al.* 2009). There is, however, no direct experimental evidence that *Plasmodium* parasites are able to manipulate the chemical signature of its vertebrate hosts. Mauck *et al.* (2010) have convincingly argued that such a manipulation must necessarily involve the elevation or exaggeration of the usual cues used by vectors for host location [what Dawkins & Krebs (1979) called a 'supernormal stimuli']. Evolutionarily speaking, deceptive signals must mimic reliable signals because the fitness costs of ignoring such signals for the receiver, the mosquito in this case, are large. The overproduction of a normal stimulus is therefore a good way to prevent the evolution of resistance against the attractant in the mosquito population when, as is the case for *Plasmodium,* the parasite reduces the fitness of the vector.

In conclusion, the use of the novel (albeit natural) *P. relictum* - *Cx. pipiens* model system provides a powerful tool to investigate the infection-induced changes in host attractiveness following a *Plasmodium* infection and opens up new avenues of research into the mechanistic and adaptive bases of this manipulation. One obvious avenue for future research is the examination of the odorant profiles of *Plasmodium*-infected and uninfected hosts to detect quantitative differences in compounds with known attractant properties ('supernormal stimuli'). The identification of such compounds will provide a useful target for understanding and controlling the transmission of malaria (Syed & Leal 2009; Carey *et al.* 2010). Another important perspective to this work is the behaviour of the infected vectors: would mosquito choice have been any different had the mosquito been infected? Despite the importance of this question for parasite transmission dynamics, the host-choice behaviour of infected vectors has attracted very little attention (but see Ingwell *et al.* 2012; Mann *et al.* 2012). Answering this question is key to determine the importance of behavioural manipulation of vector choice over the whole malaria cycle. We believe that animal models that associate vector-*Plasmodium* combinations with a long, common evolutionary history, such as the avian malaria system, will play an important role in addressing these pending issues. This could help improve our understanding of human malaria epidemiology and produce more effective health policies.

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AUTHORSHIP

SC, AR and SG designed research; SC performed research and analysed data; AN performed qPCR assays and preliminary work on microsatellites, and SC, AR and SG wrote the manuscript.

REFERENCES

Anderson, R.A., Koella, J.C. & Hurd, H. (1999). The effect of *Plasmodium yoelii nigeriensis* infection on the feeding persistence of *Anopheles stephensi* Liston throughout the sporogonic cycle. *Proc. R. Soc. Lond. B Biol. Sci.*, 266, 1729–1733.

Asghar, M., Westerdahl, H., Zehtindjiev, P., Ilieva, M., Hasselquist, D. & Bensch, S. (2012). Primary peak and chronic malaria infection levels are correlated in experimentally infected great reed warblers. *Parasitology*, 139, 1246–1252.

Berticat, C., Boquien, G., Raymond, M. & Chevillon, C. (2002). Insecticide resistance genes induce a mating competition cost in *Culex pipiens* mosquitoes. *Genet. Res.*, 79, 41–47.

Bousema, J.T. & Sauerwein, R.W. (2005). Comment on 'Lacroix, R., Mukabana, W. R., Gouagna, L.C. & Koella, J.C. 2005. Malaria infection increases attractiveness of humans to mosquitoes. *PLoS Biol.*, 3, e298. Available at: http://www.plosbiology.org/article/comments/info%3Adoi%2F10.1371%2Fjournal.pbio.0030298. Last accessed 13 April 2012.

Carey, A.F., Wang, G.R., Su, C.Y., Zwiebel, L.J. & Carlson, J.R. (2010). Odorant reception in the malaria mosquito *Anopheles gambiae*. *Nature*, 464, 66–U77.

Cellier-Holzem, E., Esparza-Salas, R., Garnier, S. & Sorci, G. (2010). Effect of repeated exposure to *Plasmodium relictum* (lineage SGS1) on infection dynamics in domestic canaries. *Int. J. Parasitol.*, 40, 1447–1453.

Crawley, M.J. (2007). *The R Book*. John Wiley & Sons, Ltd, England.

Darbro, J.M., Dhondt, A.A., Verrneylen, F.M. & Harrington, L.C. (2007). *Mycoplasma gallisepticum* infection in house finches (*Carpodacus mexicanus*) affects mosquito blood feeding patterns. *Am. J. Trop. Med. Hyg.*, 77, 488–494.

Dawkins, R. & Krebs, J.R. (1979). Arms races between and within species. *Proc. R. Soc. Lond. B Biol. Sci.*, 205, 489–511.

Day, J.F., Ebert, K.M. & Edman, J.D. (1983). Feeding patterns of mosquitoes (Diptera: Culicidae) simultaneously exposed to malarious and healthy mice, including a method for separating blood meals from conspecific hosts. *J. Med. Entomol.*, 20, 120–127.

Eigenbrode, S.D., Ding, H.J., Shiel, P. & Berger, P.H. (2002). Volatiles from potato plants infected with potato leafroll virus attract and arrest the virus vector, *Myzus persicae* (Homoptera: Aphididae). *Proc. R. Soc. Lond. B Biol. Sci.*, 269, 455–460.

Eisen, R.J. & Gage, K.L. (2012). Transmission of flea-borne zoonotic agents. *Annu. Rev. Entomol.*, 57, 61–82.

Farajollahi, A., Fonseca, D.M., Kramer, L.D. & Kilpatrick, A.M. (2011). 'Bird biting' mosquitoes and human disease: a review of the role of *Culex pipiens* complex mosquitoes in epidemiology. *Infect. Genet. Evol.*, 11, 1577–1585.

Ferguson, H.M. & Read, A.F. (2004). Mosquito appetite for blood is stimulated by Plasmodium chabaudi infections in themselves and their vertebrate hosts. *Malaria J.*, 3, 12.

Ferguson, H.M., Rivero, A. & Read, A.F. (2003). The influence of malaria parasite genetic diversity and anaemia on mosquito feeding and fecundity. *Parasitology*, 127, 9–19.

Glaizot, O., Fumagalli, L., Iritano, K., Lalubin, F., Van Rooyen, J. & Christe, P. (2012). High prevalence and lineage diversity of avian malaria in wild populations of Great Tits (*Parus major*) and mosquitoes (*Culex pipiens*). *PLoS ONE*, 7, e34964.

Griffiths, R., Double, M.C., Orr, K. & Dawson, R.J.G. (1998). A DNA test to sex most birds. *Mol. Ecol.*, 7, 1071–1075.

Hagelin, J.C. & Jones, I.L. (2007). Bird odors and other chemical substances: a defense mechanism or overlooked mode of intraspecific communication? *Auk*, 124, 741–761.

Hurd, H. (2003). Manipulation of medically important insect vectors by their parasites. *Annu. Rev. Entomol.*, 48, 141–161.

Ingwell, L.L., Eigenbrode, S.D. & Bosque-Pérez, N.A. (2012). Plant viruses alter insect behavior to enhance their spread. *Sci. Rep.*, 2, 578.

Koella, J.C., Sorensen, F.L. & Anderson, R.A. (1998). The malaria parasite, *Plasmodium falciparum*, increases the frequency of multiple feeding of its mosquito vector, *Anopheles gambiae*. *Proc. R. Soc. Lond. B Biol. Sci.*, 265, 763–768.

Lacroix, R., Mukabana, W.R., Gouagna, L.C. & Koella, J.C. (2005). Malaria infection increases attractiveness of humans to mosquitoes. *PLoS Biol.*, 3, e298.

Lalubin, F., Bize, P., van Rooyen, J., Christe, P. & Glaizot, O. (2012). Potential evidence of parasite avoidance in an avian malaria vector. *Anim. Behav.*, 84, 539–545.

Lefèvre, T. & Thomas, F. (2008). Behind the scene, something else is pulling the strings: emphasizing parasitic manipulation in vector-borne diseases. *Infect. Genet. Evol.*, 8, 504–519.

Mahon, R. & Gibbs, A. (1982). Arbovirus infected hensattract more mosquitoes. In: *Viral Diseases in Southeast Asia and Western Pacific* (ed MacKenzie, J.S.). Academic, Sidney, Australia, pp. 502–505.

Mann, R.S., Ali, J.G., Hermann, S.L., Tiwari, S., Pelz-Stelinski, K.S., Alborn, H. T. *et al.* (2012). Induced release of a plant-defense volatile 'deceptively' attracts insect vectors to plants infected with a bacterial pathogen. *PLoS Pathog.*, 8, e1002610.

Mauck, K.E., De Moraes, C.M. & Mescher, M.C. (2010). Deceptive chemical signals induced by a plant virus attract insect vectors to inferior hosts. *Proc. Natl Acad. Sci. USA*, 107, 3600–3605.

Melo, M. & Hansson, B. (2006). Identification of 15 polymorphic microsatellite loci in the Principe seedeater (*Serinus rufobrunneus*) and assessment of their utility in nine other *Serinus* species (Fringillidae, Aves). *Mol. Ecol. Notes*, 6, 1266–1268.

O'Shea, B., Rebollar-Tellez, E., Ward, R.D., Hamilton, J.G.C., El Naiem, D. & Polwart, A. (2002). Enhanced sandfly attraction to *Leishmania*-infected hosts. *Trans. R. Soc. Trop. Med. Hyg.*, 96, 117–118.

Palinauskas, V., Valkiūnas, G.N., Bolshakov, C.V. & Bensch, S. (2008). *Plasmodium relictum* (lineage P-SGS1): effects on experimentally infected passerine birds. *Exp. Parasitol.*, 120, 372–380.

Prugnolle, F., Lefèvre, T., Renaud, F., Moller, A.P., Misse, D. & Thomas, F. (2009). Infection and body odours: evolutionary and medical perspectives. *Infect. Genet. Evol.*, 9, 1006–1009.

Reece, S.E., Ramiro, R.S. & Nussey, D.H. (2009). Plastic parasites: sophisticated strategies for survival and reproduction? *Evol. Appl.*, 2, 11–23.

Rogers, M.E. & Bates, P.A. (2007). *Leishmania* manipulation of sand fly feeding behavior results in enhanced transmission. *PLoS Pathog.*, 3, e91.

Roper, C., Elhassan, I.M., Hviid, L., Giha, H., Richardson, W., Babiker, H. *et al.* (1996). Detection of very low level *Plasmodium falciparum* infections using the nested polymerase chain reaction and a reassessment of the epidemiology of unstable malaria in Sudan. *Am. J. Trop. Med. Hyg.*, 54, 325–331.

Rossignol, P.A., Ribeiro, J.M.C., Jungery, M., Turell, M.J., Spielman, A. & Bailey, C.M. (1985). Enhanced mosquito blood-feeding success on parasitemic hosts: evidence for vector-parasite mutualism. *Proc. Natl Acad. Sci. USA*, 82, 7725–7727.

Scott, T.W., Lorenz, L.H. & Edman, J.D. (1990). Effects of house sparrow age and arbovirus infection on attraction of mosquitoes. *J. Med. Entomol.*, 27, 856–863.

Smallegange, R.C., Verhulst, N.O. & Takken, W. (2011). Sweaty skin: an invitation to bite? *Trends Parasitol.*, 27, 143–148.

Snounou, G., Jarra, W. & Preiser, P.R. (2000). Malaria multigene families: the price of chronicity. *Parasitol. Today*, 16, 28–30.

Sokal, R.R. & Rohlf, F.J. (1995). *Biometry*, 3rd edn. W.H. Freeman, New York.

Syed, Z. & Leal, W.S. (2009). Acute olfactory response of *Culex* mosquitoes to a human- and bird-derived attractant. *Proc. Natl Acad. Sci. USA*, 106, 18803–18808.

Taylor, P.J. & Hurd, H. (2001). The influence of host haematocrit on the blood feeding success of *Anopheles stephensi*: implications for enhanced malaria transmission. *Parasitology*, 122, 491–496.

Turell, M.J., Bailey, C. & Rossi, C. (1984). Increased mosquito feeding on Rift Valley Fever Virus-infected lambs. *Am. J. Trop. Med. Hyg.*, 33, 1232–1236.

Valkiūnas, G. (2005). *Avian Malaria Parasites and Other Haemosporidia*. CRC Press, Boca Raton, FL, USA.

Van Den Abbeele, J., Caljon, G., De Ridder, K., De Baetselier, P. & Coosemans, M. (2010). *Trypanosoma brucei* modifies the Tsetse salivary composition, altering the fly feeding behavior that favors parasite transmission. *PLoS Pathog.*, 6, e1000926.

Verhulst, N.O., Beijleveld, H., Knols, B.G.J., Takken, W., Schraa, G., Bouwmeester, H.J. *et al.* (2009). Cultured skin microbiota attracts malaria mosquitoes. *Malaria J.*, 8, e302.

Verhulst, N.O., Andriessen, R., Groenhagen, U., Kiss, G.B., Schulz, S., Takken, W. *et al.* (2010). Differential attraction of malaria mosquitoes to volatile blends produced by human skin bacteria. *PLoS ONE*, 5, e15829.

Vézilier, J., Nicot, A., Gandon, S. & Rivero, A. (2010). Insecticide resistance and malaria transmission: infection rate and oocyst burden in *Culex pipiens* mosquitoes infected with *Plasmodium relictum*. *Malaria J.*, 9, 379.

Vézilier, J., Nicot, A., Gandon, S. & Rivero, A. (2012). *Plasmodium* infection decreases fecundity and increases survival of mosquitoes. *Proc. R. Soc. B Biol. Sci.*, 279, 4033–4041.

Waldenström, J., Bensch, S., Hasselquist, D. & Ostman, O. (2004). A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. *J. Parasitol.*, 90, 191–194.

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