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Avian malaria infection intensity influences mosquito feeding patterns

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ABSTRACT

Pathogen-induced host phenotypic changes are widespread phenomena that can dramatically influence host–vector interactions. Enhanced vector attraction to infected hosts has been reported in a variety of host–pathogen systems, and has given rise to the parasite manipulation hypothesis whereby pathogens may adaptively modify host phenotypes to increase transmission from host to host. However, host phenotypic changes do not always favour the transmission of pathogens, as random host choice, reduced host attractiveness and even host avoidance after infection have also been reported. Thus, the effects of hosts' parasitic infections on vector feeding behaviour and on the likelihood of parasite transmission remain unclear. Here, we experimentally tested how host infection status and infection intensity with avian *Plasmodium* affect mosquito feeding patterns in house sparrows (*Passer domesticus*). In separate experiments, mosquitoes were allowed to bite pairs containing (i) one infected and one uninfected bird and (ii) two infected birds, one of which treated with the antimalarial drug, primaquine. We found that mosquitoes fed randomly when exposed to both infected and uninfected birds. However, when mosquitoes were exposed only to infected individuals, they preferred to bite the non-treated birds. These results suggest that the malarial parasite load rather than the infection itself plays a key role in mosquito attraction. Our findings partially support the parasite manipulation hypothesis, which probably operates via a reduction in defensive behaviour, and highlights the importance of considering parasite load in studies on host–vector–pathogen interactions.

1. Introduction

Pathogen-induced phenotypic changes in host morphology, behaviour and physiology may greatly affect interactions between hosts and insect vectors, and may in turn have an impact on the transmission dynamics of vector-borne pathogens (Hurd, 2003; Libersat et al., 2009; Poulin, 2010; Lafferty and Kuris, 2012). Despite not having a full understanding of its underlying mechanisms, the parasite manipulation hypothesis (Poulin, 1995; Hurd, 2003) has received increasing attention during the last decade (e.g. Lefèvre and Thomas, 2008; Lefèvre et al., 2009). This hypothesis proposes that pathogens manipulate a host's phenotype to increase host–vector contact rates, thereby enhancing both the probability of pathogen acquisition and the transmission to a new host (Lefèvre et al., 2006; Lefèvre and Thomas, 2008; Mauck et al., 2010, 2012). Indeed, the enhanced attractiveness of infected hosts to vectors has been reported in plants (Eigenbrode et al., 2002; Shapiro et al., 2012), invertebrates (Stafford et al., 2011) and vertebrates (O'Shea et al., 2002; Cornet et al., 2013a; De Moraes et al., 2014) including humans (Lacroix et al., 2005; Batista et al., 2014).

Malaria parasites of the genus *Plasmodium* are vector-borne pathogens that require the bite of a competent mosquito to spread from an infected to a new host (Valkiūnas, 2005). A number of studies have reported vector preference for mammalian hosts already infected by malaria parasites. For example, in humans, children harbouring *Plasmodium falciparum* parasites in transmissible stages (i.e. gametocytes) were more attractive to mosquitoes (measured as a reaction to odours) than those harbouring parasites in non-transmissible stages (i.e. trophozoites) or uninfected children (Lacroix et al., 2005). Similarly, Day and Edman (1983) found that mosquitoes fed almost exclusively on malaria-infected mice when both infected and uninfected individuals were made available. However, whether host infection affects vector feeding behaviour remains an open question since contrasting results were also reported. For instance, mosquitoes preferred to feed on bats

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infected with the mildest stages of the malaria-like parasite *Polychromophilus murinus* (Witsenburg et al., 2014) or even preferred to feed on uninfected hosts to the detriment of their infected counterparts (Daugherty et al., 2011).

Avian malaria parasites have recently been used to test the parasite manipulation hypothesis since they may alter host behaviour (e.g. reduced activity, Cauchard et al., 2016) and physiology (e.g. anaemia and enlargement of the liver and spleen, Valkiūnas, 2005), which could potentially affect mosquito attraction. Nonetheless, contradictory trends have been also reported and, for instance, Cornet et al. (2013a) found that birds chronically infected by *Plasmodium relictum* were bitten more frequently by the avian malaria vector *Culex pipiens* than their uninfected or acutely infected counterparts. This finding would support the parasite manipulation hypothesis, as this behaviour would increase the fitness of parasites (transmission success) (Poulin, 1995). On the other hand, Lalubin et al. (2012) reported that *Cx. pipiens* were more attracted to uninfected birds than to *Plasmodium*-infected birds, a finding that cannot be explained by parasitic manipulation but rather by the hypothesis of vector adaptive avoidance (Hart, 1990; Martínez-de la Puente et al., 2009; Lalubin et al., 2012). This latter hypothesis is based on the costs induced by parasites in their vectors, such as decreased fecundity (Vézilier et al., 2012) and survival (Ferguson and Read, 2002; Lalubin et al., 2012). However, Cornet et al. (2013a) used birds infected in the laboratory that were deprived of movement, which may not reflect the situation that occurs in the field. The study by Lalubin et al. (2012) suffers from the technical problem when using olfactometers, i.e. the lack of physical interaction between birds and mosquitoes (e.g. feeding attempts and hosts' defence). Therefore, the actual effects of parasitic infection on vectors' feeding patterns remain to be clarified.

Here, we conducted two separate experiments to determine the effects of avian *Plasmodium* infection on the feeding behaviour of the avian malaria vector *Cx. pipiens*. Firstly, we exposed naturally infected and uninfected house sparrows (*Passer domesticus*) to mosquitoes to assess the effect of birds' infection status on mosquito biting rates. Secondly, we assessed the effect of host parasite load on the probability of mosquito bites by treating half of the *Plasmodium*-infected birds (hereafter, 'treated' in this experiment) with an antimalarial drug and then exposing both infected (hereafter, 'control' in this experiment) and treated birds to mosquito bites. In both cases, the pairs of birds representing dual conditions of malaria infection (i.e. infected versus uninfected or control versus treated) were exposed simultaneously to mosquitoes to simulate a common situation of making choices as faced by mosquitoes in the field. In addition, birds were allowed to move freely in their cages to avoid hampering anti-mosquito behaviour that could greatly affect the feeding success of mosquitoes (Darbro and Harrington, 2007). According to the parasite manipulation hypothesis, we predicted that *Plasmodium*-infected control (non-treated) birds would be bitten more often than uninfected and treated individuals, respectively, as parasite-induced changes (e.g. hosts' odours, anti-mosquito behaviours) would facilitate mosquito bites (Day and Edman, 1983; De Moraes et al., 2014). Alternatively, and according to the adaptive avoidance hypothesis, we predicted that mosquitoes would bite infected control birds less often than uninfected and treated birds, since mosquitoes may adaptively select uninfected birds or those with less intense infections to avoid the costs of infection.

2. Materials and methods

2.1. Mosquito and bird collection and rearing

Mosquito larvae were collected in the Cañada de los Pájaros nature reserve (37°14′03″ N, 6°07′50″ W, Seville, Spain) during the summer of 2014 and then transported to the laboratory, where they were supplied with shrimp food (Mikrozell 20 ml/22 g; Dohse Aquaristik GmbH & Co. KG, D-53501, Gelsdorf, Germany) and maintained under controlled conditions (65–70% relative humidity (RH), 27 ± 1 °C and a light (L): dark (D) cycle of 12:12 h). Emerged mosquitoes were anaesthetised with diethyl ether (Lipnick, 1991), sexed and identified following Schaffner et al. (2001). Female *Cx. pipiens* were maintained in insect rearing cages (BugDorm-43030F, 32.5 × 32.5 × 32.5 cm) with ad libitum access to 1% sucrose solution. Mosquitoes were deprived of sucrose solution 24 h before the experiment took place and henceforth only had access to water.

In July 2014, 78 juvenile house sparrows were captured using mist nets in Huelva province (southern Spain). Birds were ringed upon capture and their body mass and wing length were measured using a digital scale (Pesola-MS500) and a 30 cm end-stop ruler, respectively. A blood sample was obtained for further molecular analyses (see Section 2.3). Birds were transferred to the Unit of Animal Experimentation at Estación Biológica de Doñana-Consejo Superior de Investigaciones Científicas (EBD-CSIC), Spain where they were maintained in pairs in cages (58.5 × 25×36 cm) within a vector-free room programmed with a photoperiod cycle of 12:12 h L:D at 22 ± 1 °C. Birds were housed for 1 week before the start of the experiments and had ad libitum access to fresh water and a standard mixed diet for seed and insect-eater birds (KIKI; GZM S.L., Alicante, Spain). Birds were released at their capture site 2–5 days after the completion of the experiments. All experimental procedures were approved by the CSIC Ethics Committee and Animal Health authorities as per Spanish legislation (CEBA-EBD-12-40).

2.2. Experimental procedure

Before performing the experiments, birds were molecularly sexed (see Section 2.3) and their infection status with blood parasites (i.e. *Plasmodium*, *Haemoproteus* and *Leucocytozoon*) was determined using primer pairs HaemNF1/HaemNR3 and HaemF/HaemR2 following Hellgren et al. (2004). Their infection status with blood parasites was determined again after completion of the experiments. The presence of amplicons was verified in 1.8% agarose gels. Positive amplifications were sequenced using the BigDye technology (Applied Biosystems, USA) or by the Macrogen sequencing service (Macrogen Inc., The Netherlands). Sequences were edited using the software Sequencher™ v 4.9 (Gene Codes Corp. © 1991–2009, Ann Arbor, MI, USA 48108) and assigned to parasite lineages/morphospecies after comparisons with sequences in GenBank (National Center for Biotechnology Information, (NCBI), USA). Birds infected with *Haemoproteus* or *Leucocytozoon* were not included in this study. In the first experiment, 20 pairs of birds consisting of a *Plasmodium*-infected (10 males and 10 females) and an uninfected bird (10 males and 10 females) were exposed to unfed female *Cx. pipiens* (mean number = 172, range = 156–183). In this experiment, a *Plasmodium*-infected bird was also co-infected with *Haemoproteus* as determined by sequencing of blood after the completion of the experiment. Besides this co-infected bird, 15 individuals were infected with the lineages Rinshi-1 (*P. relictum*), three infected with Rinshi-7 (*P. relictum*), and one bird co-infected with both Rinshi-1 and Donana07 (*Plasmodium* spp.). Each pair contained one male and one female bird, with one infected and one uninfected individual. Eight to 18 day old female mosquitoes were used in all experiments to reduce the potential effect of mosquito age on host location capacity (Bohbot et al., 2013). In the second experiment carried out 15 days after completing the first experiment, only *Plasmodium*-infected birds were used; 16 infected birds (including the above-mentioned co-infected bird) from the first experiment were

re-used to minimise the number of animals employed, nine of which were assigned to the non-treated control group and seven to the treated group (including the above-mentioned co-infected bird). Seven days before exposure to mosquitoes, birds were randomly assigned to either the treated or control group. Besides the *Plasmodium* and *Haemoproteus* co-infected bird, the lineages infecting medicated birds in experiment 2 were Rinshi-1 (14 individuals), Rinshi-7 (1), Rinshi-8 (*P. relictum,* 2) and co-infection by Rinshi-1 and Donana07 (1). In the case of control birds, nine individuals were infected with Rinshi-1, four with Rinshi-7, one with Rinshi-8, one with Donana07, two individuals were co-infected with Rinshi-1 and Donana07, one with Rinshi-7 and PADOM1 (*Plasmodium* spp.) and one with Rinshi-7 and Donana07. Treated birds were s.c. injected with 0.1 mg of primaquine (Sigma, St. Louis, MO, USA) diluted in 0.1 ml of saline solution, while control birds were injected with the same volume of saline solution (see Merino et al., 2000; Tomás et al., 2007 for a similar procedure). Primaquine is a chemical compound that can bind and modify malaria parasites' DNA (López-Antuñano, 1999), as well as disrupt malaria parasites' mitochondrial membranes (Baird and Rieckmann, 2003), thereby effectively reducing the blood parasite load in birds such as house sparrows (Merino et al., 2004; Marzal et al., 2005; Tomás et al., 2007; Martínez-de la Puente et al., 2010). In humans, the effect of a single and low dose of primaquine can clear most of the malaria gametocytes 7 days after treatment (Burgess and Bray, 1961). The biological half-life of primaquine in plasma is approximately 4–9 h (Baird and Hoffman, 2004), hence this drug was not likely to have any direct effect on behaviour 1 week post treatment (Cauchard et al., 2016). Seven days later, 19 treated birds (12 males and seven females) and 19 control birds (10 males and nine females) were exposed to unfed female *Cx. pipiens* (mean number = 151, range = 136–171). Sixteen pairs consisted of one male and one female bird, and three pairs of two males. In both experiments, pairs were maintained in a cage (38.5 × 26 × 25.5 cm) within an insect-rearing tent (BugDorm-3120, white, 60 × 60 × 60 cm). Birds were able to move freely and mosquitoes within each tent were similarly able to freely enter the birds' cage. Trials were carried out under dark conditions from 20:00 h until 08:00 h during the peak mosquito activity period. The following morning, blood-fed mosquitoes were collected, counted and stored at −20 °C; all the birds were immediately blood-sampled again and then released into the field 1–2 days after exposure during the second experiment. In the second experiment, blood smears were used to estimate the intensity of infection with blood parasites. The number of infected cells per 10,000 red blood cells (RBCs) was estimated in visual fields under 10,000× magnification (Carl Zeiss-Imager A1).

2.3. Molecular analyses

Genomic DNA from bird blood samples was isolated using a Maxwell® 16LEV Blood DNA Kit (Gutiérrez-López et al., 2015). Birds were molecularly sexed using primers P2 (5′-TCTGCATCGC TAAATCCTTT-3′) and P8 (5′-CTCCCAAGGATGAGRAAYTG-3′) following Ellegren (1996) and Griffiths et al. (1998). PCR amplifications were conducted with a total volume of 25 μL in thermal cyclers (Agilent sure cycler 8800, USA and BIO-RAD T100, USA). The cycle temperatures and other reaction conditions were as given in Griffiths et al. (1998); the positive amplifications were resolved in 3% agarose gels (TBE (Tris/Borate/EDTA buffer solution) 1×, 110 V, 45 min). Eight different primer pairs targeting different microsatellite fragments were used to genotype birds (Table 1; Garnier et al., 2009). The amplification of each sample was carried out in a total volume of 20 μL containing 13.54 μL of H_2O, 2 μL of extracted DNA, 2 μL of PCR buffer (10×), 0.6 μL of $MgCl_2$ (50 mM), 0.16 μL of total dNTPs (25 mM), 0.1 μL of Taq and 0.8 μL of primer for each of the two DNA strands. To identify homozygous (one band) and heterozygous (two bands) individuals for each microsatellite, positive amplifications were resolved in 3% agarose gels (TBE 1×, 110 V, 60 min) and the amplification pattern was compared between birds from the same trial.

Table 1
Primers used in this study for genotyping house sparrows. Adapted from Garnier et al. (2009).

Locus name	Primer sequence (5′–3′)	GenBank Accession no.
PdoA08[a]	AGCTTTTCAGGTCTCCTTCT[b]VIC CTACACCAGCAAGATCCATT	FJ422589
PdoB01[a]	GCCTGCTTAAACTATCTTGG[b]PET GATATAGGGAGCAGAGTTCTTG	FJ422590
PdoB04	ATTTGGGTGGTTAGTTCAAA[b]FAM CAAATACAGTGCATCTACAACC	FJ422591
PdoC11	GCAGCATGTCATAATAGCAG[b]FAM TTTTCCTTTGCATACACCA	FJ422592
PdoD09[a]	CTCTCCTGCTATGCTTCCT[b]PET CTTGGGATATGATGGAAATG	FJ422593
PdoE09	TGACTAAAATAGATCAAGGCTTTT[b]FAM TGCAAAGATACCAGAACTCAT	FJ422594
PdoF05	GCATATTTCTGGCATTCTTC[b]VIC TCAAATAAAGTGCTCCACAA	FJ422595
PdoF09[a]	CACGGGTGGTATTTTATATG[b]NED ATGTTGCAGATTGAAAAGTG	FJ422596

[a] Primers used in DNA sequencing.
[b] Primer labelled with FAM, VIC, NED or PET.

To identify the origins of mosquitoes' blood meals, we isolated DNA from engorged mosquitoes using the HotSHOT procedure (see Alcaide et al., 2009; Martínez-de la Puente et al., 2013 for further details). In those cases where less than 30 engorged mosquitoes were obtained, we isolated the DNA from all engorged mosquitoes. When there were more engorged mosquitoes, we isolated DNA from 30 randomly selected individuals. Overall, an average of 20.2 ± 1.68 (mean ± S.E.) (range = 4–30) and 27.2 ± 1.29 (range = 9–30) engorged mosquitoes per pair were selected from the first and second experiments, respectively. The abdomen of each mosquito was separated from the head and thorax using sterile tips on chilled Petri dishes. One negative control of DNA extraction (i.e. without any tissue) was included for each plate. We applied the molecular sexing protocol detailed above for mosquito blood meals to partially identify the hosts of mosquitoes for those trials containing a male and a female bird. One-band amplifications were identified as male-derived blood meals. Given that samples with the amplification of two bands could be derived from a blood meal from a female bird or a mixed blood meal from both a male and female bird, these blood meals were processed by further analyses. Mosquitoes from trials containing two males (see results) were processed as follows: after genotyping birds, we analysed mosquitoes' blood meals using microsatellite primer pairs that had mutually exclusive amplification patterns for each bird within a pair, that is, bird A in a pair had a one-band amplification for one microsatellite but two bands for the other microsatellite, whereas bird B in that pair displayed the opposite pattern. Thus, samples with one-band amplification for either of the pair of primers were identified as blood meals from either one of the pair of birds, while those with two-banded amplifications for the two microsatellites were identified as mixed-blood meals. When birds showed a similar amplification pattern, four different microsatellites (Pdo A08, B01, D09 and F09; see also Table 1) from bird blood samples and mosquito blood meals were sequenced using the 3130*xl* ABI Genetic Analyzer (Applied Biosystems, USA) and the alleles were scored using GENEMAPPER v3.7 (Applied Biosystems). We managed to identify the origins of these remaining samples by comparing the sizes of alleles in birds and in mosquitoes' blood meals.

2.4. Statistical analyses

Generalised Linear Mixed Models (GLMMs) with binomial error and logit link function were used to assess the effect of bird infection status (i.e. infected versus uninfected birds) and intensity (i.e. control versus treated birds) on mosquito biting rates. The dependent variable 'biting rate' was incorporated into models as the number of mosquitoes that fed on one bird with respect to the number of mosquitoes that fed on the other bird within a pair (including mixed blood meals) using the *cbind* function. In any given trial, the number of engorged mosquitoes on a particular bird was analysed as a binomial variable with the total number of engorged mosquitoes as the binomial denominator. Bird body mass and log-transformed parasite load (only available for the second experiment) were included as covariates. We ran alternative models using body condition, estimated as the standardised residuals of linear regressions of body mass on wing length fitted separately for males and females (to control sexual dimorphism in wing length), instead of body mass. As the results did not change qualitatively, we only present the results for the models fitted with body mass. Bird sex and infection status (infected versus uninfected) or intensity (control versus treated) were included as fixed factors, while bird pair and bird identity were included as random terms. No evidence of collinearity between the two continuous independent variables included in the models was found, as the generalised variance inflation factors (gVIFs) were <4 (O'Brien, 2007). Model selection was based on the second order Akaike's Information Criteria (AICc). Delta AICc(ΔAICc) was calculated as the difference in AICc between the model with the lowest AICc and the other models. Model averaging of all models with ΔAICc < 2 was performed following the zero method in Grueber et al. (2011). The variance explained for each model was calculated by conditional R^2 (Nakagawa and Schielzeth, 2013). Paired *t*-tests were used to compare the mean infection intensity between control and treated birds (Experiment 2). To account for any potential effect of the mixed blood meals, we repeated the same analyses by excluding these data from the calculation of biting rates. As results were qualitatively the same (data not shown), hereafter we only show the results including data of mixed blood meals. To account for any potential effects of outliers, we excluded a trial that contained a bird with a very high parasite load in the second experiment and repeated the above-mentioned analyses. As results remained qualitatively the same, we only present the results without the outlier. In addition, to account for any potential effect of co-infection by *Plasmodium* and *Haemoproteus*, we repeated the analyses without the trial that contained the co-infected bird in experiment 1, and results showed no qualitative change (data not shown); in experiment 2 the co-infected bird was assigned to the treated group and treated with an anti-malarial drug before exposure to mosquitoes. Hence, the results we present below include the co-infected bird. All analyses were performed in R 3.2.5 (R Core Development Team, 2016. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria) using the packages *arm* (Gelman, A., Su, Y.-S., Yajima, M., Hill, J., Pittau, M.G., Kerman, J., Zheng, T., Dorie, V., 2009. arm: Data analysis using regression and multilevel/hierarchical models. R package, version 9.01), *lme4* (Bates, D., Maechler, M., Bolker, B., Walker, S., 2014. lme4: Linear mixed-effects models using Eigen and S4. R package, version 1) and *MuMIn* (Bartoń, K., 2013. MuMIn: multi-model inference. R package, version 1).

3. Results

The percentage of engorged mosquitoes from the total mosquitoes introduced in each trial varied from 3.45% to 37.6% (mean = 14.2% ± 1.92% S.E.) for the first experiment and from 13.8% to 71.0% (mean = 38.2% ± 4.06% S.E.) for the second experiment. In total, the bloodmeal origin for each of 403 and 508 mosquitoes for the first and second experiment, respectively, was identified to the individual bird level. The mean number of engorged mosquitoes with known bloodmeal origin was 11.0 ± 1.02 S.E. per individual for the first experiment and 15.3 ± 1.46 S.E. per individual for the second experiment. The average number of mixed bloodmeals was 1.85 per trial in the first experiment and 2.44 in the second experiment.

In the first experiment, the mean biting rate was 0.52 ± 0.06 S.E. (range = 0.05–1.00) and 0.60 ± 0.06 S.E. (range = 0.05–0.95) for *Plasmodium*-infected and uninfected groups, respectively. Three GLMMs analysing the variation in biting rates between *Plasmodium*-infected and uninfected birds were selected based on AICc criteria (Table 2). However, none of the explanatory variables significantly affected the biting rate, as indicated by 95% confidence intervals (CIs), which included zero in all cases (Table 3). We did not find any significant relationship between *Plasmodium* infection status and biting rate (Table 3; Fig. 1A).

In the second experiment, the mean biting rate was 0.65 ± 0.07 S.E. (range = 0.10–1.00) and 0.44 ± 0.07 S.E. (range = 0.00–0.93) for control and treated groups respectively. The infected birds treated with primaquine had significantly lower parasitaemia levels than the controls (t = 2.14, d.f. = 17, P = 0.046; Fig. 2). Based on AICc criteria, three different models were selected to explain the variation in mosquito biting rate (Table 4). The variance explained was 30.0% for the first model, 22.7% for the second model, and 6.56% for the third model. The averaged model indicated that the biting rate was lower in treated than in control birds (Fig. 1B), lower in males than in females, and positively correlated to log-parasitaemia (Table 5). The relative importance of the independent variables

Table 2
Results of Generalised Linear Mixed Models analysing the variation in mosquito biting rate in relation to bird sex, infection status (uninfected or infected) and body mass, and the interaction between sex and infection status. Individual and pair identities were included as random terms. $\triangle i$ (AICc) = [AICci − min AICc]; ω_i (AICc) = the rounded second-order Akaike weights. Models were ranked by AICc values. Crosses indicate variables included in each model. Bold indicates top models ($\triangle i$ (AICc) ≤ 2).

Explanatory variables				Criterion		
Sex	Status	Body mass	Sex × Status	AICc	$\triangle i$ (AICc)	ωi AICc
				238.4	**0.00**	**0.324**
	+			**239.4**	**1.00**	**0.196**
		+		**240.1**	**1.72**	**0.137**
+				240.6	2.18	0.109
	+	+		241.0	2.56	0.090
+	+			241.8	3.40	0.059
+		+		242.7	4.25	0.039
+	+	+		243.7	5.31	0.023
+	+		+	244.5	6.03	0.016
+	+	+	+	246.2	7.78	0.007

Table 3
Summary of the averaged model derived from the top model set explaining the variation in mosquito biting rates in relation to bird infection status (infected versus uninfected birds). Model-averaged coefficients (conditional average) ± S.E., 95% Confidence intervals, *z* value and *P* values of the averaged model are shown.

Explanatory variables	Estimate	S.E.	95% CI		*z* value	*P*
Intercept	0.240	0.200	−0.165	0.645	1.163	0.245
Infection status	0.484	0.393	−0.312	1.280	1.191	0.234
Body mass	0.349	0.400	−0.462	1.160	0.842	0.400

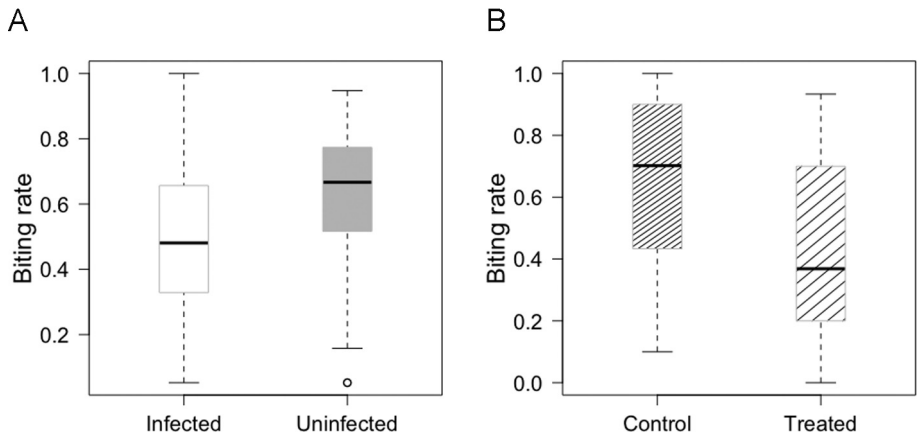

Fig. 1. Comparison of biting rate between (A) *Plasmodium*-infected and uninfected birds and (B) control (non-treated) and treated birds. The line within each box indicates the median and the edges of each box the first (Q1) and third (Q3) quartiles; the whiskers extend over 1.5 times the interquartile range.

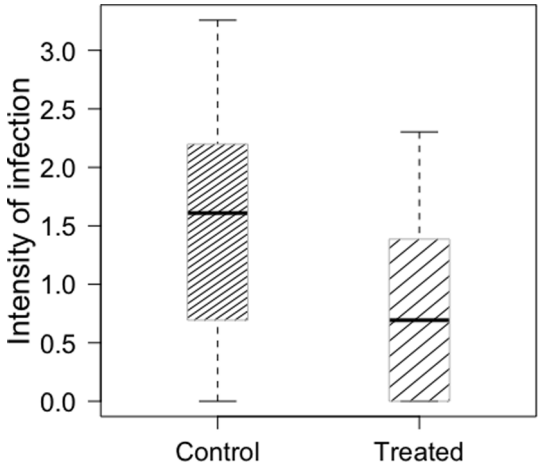

Fig. 2. Intensity of infection (log-transformed) with *Plasmodium* in control (non-treated) and treated birds. The intensity of infection was measured as the number of infected cells per 10,000 red blood cells. The line within each box indicates the median and the edges of each box the first (Q1) and third (Q3) quartiles; the whiskers extend over 1.5 times the interquartile range.

was 1.0 for medication treatment, 0.25 for log-parasitaemia and 0.21 for sex. The 95% CI included zero for the variables log-parasitaemia and sex, thus indicating that treatment was the only explanatory variable that significantly affected the mosquito biting rate (Table 5).

4. Discussion

We combined two experiments to assess the role of *Plasmodium* infection prevalence and intensity in mosquito feeding patterns. Contrary to our predictions derived from the parasite manipulation and the adaptive avoidance hypotheses, in the first experiment we found that mosquitoes bit uninfected and infected birds with a similar probability. However, in the second experiment, mosquitoes were found to feed predominantly on control (infected) individuals when birds with high and experimentally reduced infection intensities were exposed simultaneously. These results highlight the need to perform experimental manipulations of the parasite load when attempting to assess the impact of parasite infection levels on host selection by mosquitoes.

Although previous studies have assessed the role of the malarial infection status on mosquito feeding patterns, methodological differences complicate comparisons of their conclusions. For instance, Day and Edman (1983) used a rodent malaria model and found that infected individuals suffered more bites from *Anopheles stephensi* mosquitoes than uninfected individuals. A similar pattern is to be expected in birds, as shown by Cornet et al. (2013a), who found that mosquitoes fed more frequently on birds suffering experimental infections with *Plasmodium* than on uninfected individuals. The stage of infection was important as mosquitoes feed preferentially on birds in the chronic phase of infection, in comparison with control uninfected birds and those in the acute phase of infection with *Plasmodium* (Cornet et al., 2013a). The acute phase of infection occurs immediately after infection with a high proliferation of *Plasmodium* in host blood, which lasts for 10–13 days (Cornet et al., 2013a). Given that the birds had been maintained in a mosquito-free room for 13–19 days in experiment 1 and 32–35 days in experiment 2 before mosquito exposure we can assume that all the individuals were in the chronic phase of infection. Cornet et al. (2013a,b), however, immobilised birds to avoid anti-mosquito behaviour, which is known to seriously affect mosquito feeding success (Day and Edman, 1984; Edman and Scott, 1987; Darbro and Harrington, 2007). In addition, the experiments by Cornet et al. (2013a) were performed within a short time span (just 2 h). In our study, however, mosquitoes were allowed to feed on birds for 12 h to reproduce more accurately the natural interactions between mosquito feeding attempts and host defence. Moreover, fitness costs of experimentally infected birds could be milder than those experienced by naturally infected birds due to lower selection pressure (Møller and Nielsen, 2007; Appleby et al., 1999). This probably allowed these birds to tolerate more severe

Table 4
Results of Generalised Linear Mixed Models analysing variation in the biting rate in relation to bird parasitaemia, body mass, sex, treatment (control or treated) and the interaction between sex and treatment (Sex × Treatment). Individual and pair identities were included as random terms. $\triangle i$ (AICc) = [AICci − min AICc]; ω_i (AICc) = the rounded second-order Akaike weights. Models were ranked by AICc values. Crosses indicate variables included in each model. Bold indicates top models ($\triangle i$ (AICc) ≤ 2).

Explanatory variables					Criterion		
log. Parasitaemia	Body mass	Sex	Treatment	Sex × Treatment	AICc	$\triangle i$ (AICc)	ωi AICc
			+		**246.1**	**0.00**	**0.257**
+			**+**		**247.6**	**1.49**	**0.122**
		+	**+**		**247.9**	**1.83**	**0.103**
+					248.2	2.09	0.091
					248.7	2.61	0.070
	+		+		248.8	2.69	0.067
+		+	+		249.7	3.58	0.043
+		+			249.9	3.75	0.039
		+			250.0	3.86	0.037
+	+		+		250.5	4.37	0.029
	+	+	+		250.7	4.61	0.026
		+	+	+	250.8	4.71	0.024
+	+				250.9	4.79	0.023
	+				251.3	5.14	0.020
	+	+			252.4	6.32	0.011
+	+	+			252.5	6.44	0.010
+		+	+	+	252.6	6.46	0.010
+	+	+	+		252.7	6.57	0.010
	+	+	+	+	253.8	7.69	0.005
+	+	+	+	+	255.8	9.67	0.002

Table 5
Summary statistics of the averaged model derived from the top model set, which explains the variation in the mosquito biting rate in relation to bird infection intensity (control non-treated versus treated birds). Model-averaged coefficients (conditional average) ± S.E., 95% Confidence intervals, *z* value and *P* values of the averaged model are shown.

Explanatory variables	Estimate	S.E.	95% CI		*z* value	*P*
Intercept	0.270	0.276	0.942	−0.292	0.832	0.346
Treatment	−1.219	0.568	2.066	−2.376	−0.062	0.039
log.Parasitaemia	0.668	0.598	1.074	−0.552	1.888	0.283
Sex	−0.526	0.552	0.916	−1.651	0.599	0.360

infections and led to profound changes in their phenotypes (Medzhitov et al., 2012), while most naturally infected birds, with the exception of some individuals that have strong immune defences, may not be able to survive a severe infection (Appleby et al., 1999; Woodworth et al., 2005; Valkiūnas et al., 2006; Bensch et al., 2007; Møller and Nielsen, 2007). In the study by Cornet et al. (2013a), the naïve birds infected in the laboratory may be sicker than the wild naturally-infected birds we used here, which were in the asymptomatic stage of chronic infection (Zehtindjiev et al., 2008). This difference in infection-induced phenotypic changes could dramatically alter the mosquito feeding patterns. Consequently, we cannot determine whether the differences between our results and those reported by Cornet et al. (2013a) are due to the immobilisation of birds, to differences in the development status of *Plasmodium* in the naturally infected individuals used in our study or to the experimental manipulation of parasite load. The outcome of mosquito feeding patterns could vary greatly owing to different phases of infection or the relative use of host cues by mosquitoes. These factors may even produce contradictory findings since mosquitoes may be more attracted to uninfected than to infected birds (Lalubin et al., 2012). In addition, it is important to highlight the fact that birds included in our study were naturally infected and that their infection status was assessed based on molecular amplification of parasite DNA. The nested-PCR method used here provided positive amplifications in birds with infections corresponding to only one parasite per 100,000 host blood cells (Hellgren et al., 2004). Although we only measured parasite load rather than gametocyte load, recent studies have shown that parasitaemia is positively correlated to gametocytemia in different lineages of avian *Plasmodium* (Pigeault et al., 2015). In addition, parasitaemia is thought to be a better predictor of mosquito infection rather than gametocytemia, due to its correlation with host immunity and metabolic profiles (Pigeault et al., 2015). Parasitaemia has successfully been used as a proxy for the intensity of avian *Plasmodium* infection to assess its effects on mosquito attraction (Cornet et al., 2013a,b). Therefore, our result that mosquitoes more often bit birds with a higher parasite load might suggest a greater chance of pathogen transmission to mosquitoes, which could in turn increase pathogen transmission among hosts. Results from this experiment may, at least partially, support the parasite manipulation hypothesis.

Our study underlines the role of the intensity of infection by *Plasmodium* rather than the infection itself in mosquito feeding preferences. Mosquito feeding behaviour is a complex phenomenon that includes flight activation, attraction to hosts, landing on selected hosts, and biting of specific parts of the hosts' bodies. Host-seeking mosquitoes use visual, thermal and olfactory cues to discriminate different hosts, which also depend on the specific environment in which host–vector interactions occur (Day, 2005; Takken and Verhulst, 2013; Cardé, 2015; van Breugel et al., 2015). In our study, birds and mosquitoes interacted closely with each other (within 60 cm) and it is likely that cues such as moisture and heat acted as signals for host localisation and selection by mosquitoes (Cardé, 2015; van Breugel et al., 2015). However, anti-mosquito behaviour may in fact have ultimately determined the number of bites received by each bird (Day and Edman, 1984; Darbro and Harrington, 2007). Avian malaria can cause high mortality in early stages of infection, which implies that only birds with strong immune systems can survive with chronic infections in the wild (Nordling et al., 1998; Knowles et al., 2009). Wild birds with chronic infections are usually asymptomatic and often only display mild changes in behavioural traits, although olfactory profiles, for example, may be affected (Palinauskas et al., 2008; Cauchard et al., 2016). This could explain

why there were no significant differences in our study on mosquito biting rates between naturally infected birds and uninfected birds. However, bird activity such as anti-mosquito behaviour may vary over time and could be enhanced by the reduction of parasite load induced by the anti-malaria treatment (Cauchard et al., 2016). This could explain why infected birds treated with primaquine in our study were bitten less than infected non-treated birds. The alternative that side effects of primaquine, instead of its effect on parasite load, alter mosquito behaviour is poorly supported by previous studies. Bird activity levels did not differ between uninfected-treated and uninfected-control Great tits (*Parus major*) (Cauchard et al., 2016), suggesting that bird susceptibility to mosquito bites could not be affected by the treatment itself. In addition, the biological half-life of primaquine in plasma is approximately 4–9 h (Baird and Hoffman, 2004). Given that we treated the infected birds with primaquine 7 days prior to mosquito exposure, the potential side effects of anti-malarial treatment on mosquito attraction are poorly supported. Our finding that birds with higher parasite loads were bitten more often was in agreement with Day and Edman (1983), who found that mice infected with *Plasmodium* displayed less anti-mosquito behaviour than uninfected individuals. Similarly, Yorinks and Atkinson (2000) reported that infected birds devoted less time to both locomotory and stationary activities that may contribute to avoiding mosquito bites.

Infection with different *Plasmodium* lineages and mixed infections with multiple lineages are commonly recorded in wild birds (Valkiūnas et al., 2003, 2006; Clark et al., 2016). This is the case in our study, and the birds used in our experiments were infected with different lineages or with double lineages. Unfortunately, the high diversity of lineages presented here does not allow incorporation of this factor into our analyses. Consequently, we cannot exclude the possibility that the diversity of lineages may have added some noise to our results. Thus it is advisable that future studies consider lineage identity as a factor in the experimental design. However, to date and to the best of our knowledge, there is no evidence for lineage-specific effects on mosquito attraction.

To date, many studies have focused on host-parasite interactions, but host–vector interactions may also be important, as enhanced feeding on infected hosts will increase the likelihood of parasite transmission. In conclusion, our results partially support the parasite manipulation hypothesis by way of a quantitative association between biting rate and parasite load rather than qualitative comparison of infection status, that is, the *Plasmodium* load in birds influences blood-feeding patterns of mosquito vectors.

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