Chronic malaria infections increase family inequalities and reduce parental fitness: experimental evidence from a wild bird population

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Abstract

Avian malaria parasites (*Plasmodium*) occur commonly in wild birds and are an increasingly popular model system for understanding host–parasite co-evolution. However, whether these parasites have fitness consequences for hosts in endemic areas is much debated, particularly since wild-caught individuals almost always harbour chronic infections of very low parasite density. We used the anti-malarial drug Malarone™ to test experimentally for fitness effects of chronic malaria infection in a wild population of breeding blue tits (*Cyanistes caeruleus*). Medication caused a pronounced reduction in *Plasmodium* infection intensity, usually resulting in complete clearance of these parasites from the blood, as revealed by quantitative PCR. Positive effects of medication on malaria-infected birds were found at multiple stages during breeding, with medicated females showing higher hatching success, provisioning rates and fledging success compared to controls. Most strikingly, we found that treatment of maternal malaria infections strongly altered within-family differences, with reduced inequality in hatching probability and fledging mass within broods reared by medicated females. These within-brood effects appear to explain higher fledging success among medicated females and are consistent with a model of parental optimism in which smaller (marginal) offspring can be successfully raised to independence if additional resources become available during the breeding attempt. Overall, these results demonstrate that chronic avian malaria infections, far from being benign, can have significant effects on host fitness and may thus constitute an important selection pressure in wild bird populations.

Introduction

Parasitic organisms typically reduce the fitness of their hosts and can thus constitute a powerful selective force operating within natural populations (Poulin, 2007). Both theoretical and empirical studies indicate that parasite-related reductions in fitness can have dramatic consequences for host population dynamics (Hudson et al., 1998; Tompkins et al., 2002) and life-history evolution (Sheldon & Verhulst, 1996; Agnew et al., 2000). The magnitude of such fitness costs, as well as which particular fitness components are affected, will have important consequences for how such processes occur. For example, theoretical studies have shown that the extent to which parasites reduce host fecundity, as compared to survival, influences the extent to which they can drive cyclical host population dynamics (Dobson & Hudson, 1992; Smith et al., 2008). Similarly, the type of parasite defence mechanisms that hosts evolve will depend on which fitness components parasite reduce, and at which point during infection, or during the host’s life history, this occurs. Thus, in order to predict the influence of parasites on host evolution, understanding how, when and by how much parasites reduce host fitness under natural conditions is essential.

Since Hamilton & Zuk (1982) used avian blood parasites (largely Haemosporidia belonging to the genera...
Plasmodium, Haemoproteus and Leucocytozoon) to test their theory of parasite-mediated sexual selection, these parasites have become increasingly popular as a model to examine how parasites shape various aspects of host biology, from mate choice (Read, 1990) to life-history trade-offs (Gustafsson et al., 1994; Sheldon & Verhulst, 1996; Knowles et al., 2009). With the development of molecular tools for characterizing haemosporidian diversity (Bensch et al., 2000; Hellgren et al., 2004; Waldenström et al., 2004) publications using these parasites to investigate questions of parasite community ecology, phylogeny, phylogeography and evolution have also dramatically increased in number (e.g. Fallon et al., 2005; Pérez-Tris & Bensch, 2005; Ricklefs et al., 2005; Hellgren et al., 2007). Despite this, whether these parasites have significant fitness effects in populations where transmission is endemic, and how and when such effects may arise, remains poorly understood.

Avian haemosporidia can have pronounced detrimental effects in domestic birds (Atkinson & van Riper, 1991; Williams, 2005) and in naïve host populations where these parasites have been accidentally introduced (van Riper et al., 1986; Atkinson et al., 2000). However, their fitness effects in hosts with which they have had a longer evolutionary association remain uncertain. Observational studies on the relationship between haemosporidian infection and fitness traits in wild populations have yielded inconclusive, or negative, results (Korpimäki et al., 1993; Dawson & Bortolotti, 2000; Sanz et al., 2001a,b; Bensch et al., 2007; Marzal et al., 2008). One difficulty associated with detecting fitness effects of these parasites is that these may vary during the course of an infection. During the brief acute stage of a haemosporidian infection, parasites usually appear in the blood at high density and hosts can suffer marked mortality (Atkinson & van Riper, 1991; Atkinson et al., 2000; Valkiūnas, 2005). However, in individuals that survive the acute stage, long-term chronic infections develop, in which parasites persist at low density and are thought to be controlled by acquired immunity (Atkinson & van Riper, 1991; Atkinson et al., 2001; Sol et al., 2003). The vast majority of wild-caught infected birds harbour such chronic infections, and one reason why costs of infection are rarely detected in wild birds may be that during this stage hosts experience few, if any, effects of infection (Valkiūnas, 2005; Bensch et al., 2007). Even in species where acute infections cause high rates of mortality, such as Hawaii amakihi (Hemignathus virens) infected with Plasmodium relictum (Atkinson et al., 2000), no associations are detectable between chronic infection status and measures of fitness (Kilpatrick et al., 2006). However, inference of fitness effects based on such correlational data is also inherently problematic, as the direction of causality for any association is usually unclear (Blanchet et al., 2009a,b) and one cannot control for the possibility of selective mortality of those individuals most severely affected by parasites. To test rigorously for fitness effects of parasitic infection, an experimental approach is desirable, in which the performance of hosts with parasites either present or experimentally removed can be compared. Several recent studies have used medication to experimentally manipulate haemosporidian infections within wild bird populations (Merino et al., 2000; Marzal et al., 2005; Tomas et al., 2005, 2007). These experiments have shown that medication with primaquine, which reduces Haemoproteus (and sometimes Leucocytozoon) parasite density within the blood (parasitaemia), can lead to significant increases in reproductive success at various stages including egg-laying, hatching and fledging (Merino et al., 2000; Marzal et al., 2005). Such data highlight the possibility that while observational studies may or may not suggest fitness costs of infection, experimental tests can reveal surprisingly large effects. Whether similar effects exist for chronic Plasmodium (malaria) infections, in which parasitaemia is usually far lower than for either Haemoproteus or Leucocytozoon (Valkiūnas, 2005), has yet to be addressed experimentally.

In addition to the question of whether or not parasites reduce host fitness, knowing which fitness components are affected is important for understanding the way in which parasites impose selection on hosts. Infection may reduce adult survival, or may affect the number, or the quality, of offspring produced. Moreover, if parental infection adversely affects dependent offspring (Merino et al., 2000), these negative effects may not be distributed equally among offspring. For example, offspring may vary in their sensitivity to changes in parental condition as a result of hatching asynchrony, differential allocation of resources to offspring, or variation in offspring nutritional requirements or competitive ability. In birds, late-hatched, smaller offspring often display higher variance in survival, indicating a greater sensitivity to prevailing conditions than early-hatched offspring (e.g. Forbes et al., 2002; Forbes, 2009). Thus, if parasitic infection influences parental ability to raise a brood, we may predict late-hatched or ‘marginal’ brood members to be more adversely affected than others, and hence to benefit if parental infections are treated.

In this study, we conducted an anti-malarial medication experiment in a wild population of blue tits (Cyanistes caeruleus) infected by Plasmodium parasites. The aims of this study were two-fold: first, to test experimentally for fitness effects of chronic Plasmodium infection in a wild bird population where these parasites are endemic, and second, to determine how any fitness effects detected are manifest.

**Methods**

**Field experimental procedures**

The experiment was conducted in 2008, under UK Home Office licence, in a nestbox-breeding population.
of blue tits occupying a 193-ha area of Bagley Woods (51°42′N, 5°37′W) near Oxford, UK. Nestboxes were monitored periodically to determine reproductive parameters such as lay date, clutch size, and hatch date (first day of egg-hatching). Females were first captured on the nest mid-incubation and were ringed for identification purposes, weighed, and aged according to plumage characteristics (Svensson, 1992). A pretreatment blood sample was taken by brachial venepuncture, and stored in SET buffer (0.015 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 8.0). Each female was then randomly allocated to one of four treatments, all of which were administered orally to the bill: a low (0.07 mg), medium (0.21 mg) or high (0.49 mg) dose of Malarone™ (Atovaquone and Proguanil Hydrochloride; GlaxoSmithKline, UK) dissolved in 20 μL phosphate-buffered saline (PBS), or a control treatment of 20 μL PBS alone. Malaria is a highly effective anti-malarial drug used for prophylactic and curative treatment of Plasmodium falciparum infections in humans, in which it has few side-effects (Looareesuwan et al., 1992). The efficacy of this drug for clearing blood stage (but not tissue stage) Plasmodium infections in several passerine species has also been recently demonstrated (Palinuaskas et al., 2009). Around day 0 (the first day of egg-hatching), females were captured on the nest again and given the same dose of medication or control treatment they received mid-incubation. Around day 8 of the nestling stage, both parents were captured whilst feeding nestlings and a passive transponder (pit tag) fixed to a plastic colour ring was fitted to the tarsus in order to measure parental care behaviour. Females were blood sampled again (post-treatment sample) and a third identical dose of medication or PBS alone was administered. In total, n = 111 females received an initial drug or control treatment, with the number remaining in the experiment by the final blood sampling occasion reduced to n = 91 as a result of nest failure or desertion at various stages. On day 13, at a subset of nests, an antenna connected to a data logger (Francis Scientific Instruments, UK) was fitted to the nest box entrance to monitor each parent’s provisioning rate and roost time. Provisioning rate was estimated as the number of minutes in which an individual was recorded at the nest box entrance between 06:30 and 12:30 hours on day 14 (parents are only recorded upon entry to or exit from the nest, and not whilst in the nest), and roost time was calculated as the number of minutes between an individual’s last visit on day 13 and first visit on day 14. On day 14, nestlings were ringed and nestling mass and tarsus length were measured to the nearest 0.1 g and 0.1 mm respectively. Unhatched eggs were counted and the number of parents alarm calling during ringing of nestlings was recorded as a measure of whether one or two parents were present at this stage. After the breeding season, all nests were inspected to determine which nestlings had successfully fledged.

Development of quantitative PCR assay for quantification of Plasmodium parasites

Because microscopic examination of blood smears has a low sensitivity for detecting very light infections (< 0.001% infected cells), which are common among chronic Plasmodium infections, we developed a quantitative polymerase chain reaction (qPCR)-based assay for detecting and quantifying malaria infections. Previous work has shown that the diversity of haemosporidian cytochrome b (cyt b) lineages in Bagley Woods is very similar to that of a nearby blue tit population at Wytham Woods (Wood et al., 2007), but with a relatively higher prevalence of lineage pSGS1; Haemoproteus parasites have not been detected in this population (S. Knowles, unpublished data). To design genus-specific primers, we aligned full-length cyt b sequences from GenBank for all Plasmodium lineages detected in blue tits from both Bagley Woods and Wytham Woods, as well as Haemoproteus and Leucocytozoon sequences, in Sequencer v4.2 (GeneCodes). From this alignment, we designed primers L9 5′-AAA-CAATTCCTAAAAAAACGGC-3′ and NewR 5′-ACATCCATCCATAAAGCA-3′, which target a 188-bp region of this gene. As Leucocytozoon parasites are known to be present at high prevalence in both Bagley Woods and Wytham Woods tit populations (S. Knowles, unpublished data) and we required a Plasmodium-specific assay, the primer-binding region was chosen to be conserved among all Plasmodium lineages but divergent across Plasmodium and Leucocytozoon lineages. To confirm assay specificity for Plasmodium, we tested these primers on a randomly selected set of 28 blue tit samples from Wytham Woods that had been diagnosed as positive or negative for both Plasmodium and Leucocytozoon using the protocol of Beadell & Fleischer (2005). Positive amplification using primers L9 and NewR was strongly associated with prior Plasmodium diagnosis (Fisher’s exact test P = 0.000) but showed no association with prior Leucocytozoon diagnosis (Fisher’s exact test P = 0.149; if anything, this reflected a tendency for Leucocytozoon-positive samples to be qPCR-negative).

Application of the qPCR assay

Genomic DNA was extracted from all blood samples collected in this experiment using a standard ammonium acetate method and total DNA concentration was measured using a Picogreen assay (Quant-iT Picogreen dsDNA Assay Kit; Invitrogen). All samples were diluted to a standard working concentration of 2 ng μL⁻¹ prior to qPCR. To create material for a standard curve, the full-length cyt b gene of Plasmodium lineage pSGS1 was amplified using the protocol of Perkins & Schall (2002) and purified using a QiaVac Multiwell vacuum manifold. Total DNA concentration of this PCR product was determined using the Picogreen assay and molecular conversion calculations, based on the size and base...
composition of the DNA fragment, were used to estimate DNA copy number in this solution. Five serial dilutions of this PCR product were then used on each qPCR plate as a standard curve, covering the range 32–20,000 estimated *Plasmodium* DNA copies. qPCR reactions were performed on an Mx3000P machine (Stratagene) with SYBR-green-based detection. We used a UDG/dUTP-containing mix (Platinum SYBR Green qPCR SuperMix-UDG; Invitrogen) and a 2-min pre-incubation at 50 °C to avoid PCR product contamination and included multiple negative controls on each plate. Reactions were run in volumes of 25 μL containing: 10 ng DNA, 12.5 μL of SuperMix and 0.2 μM of each primer. The temperature profile (after the 50 °C pre-incubation and 2-min denaturation at 95 °C) consisted of 43 cycles of 95 °C for 15 s, 56 °C for 30 s and 72 °C for 30 s. Each sample was run in triplicate and *Plasmodium* DNA copy number was scored as the average across all three wells. For each sample, the melt curve was inspected to confirm that only *Plasmodium*-specific products, which melted between 73.6 and 75.3 °C, had been amplified. To confirm repeatability of qPCR parasitaemia estimates from blood, DNA was re-extracted from 35 blood samples from 2008 (from qPCR parasitaemia estimates from blood, DNA was extracted positive (√22) and √r = 0.80 among samples where at least one tested positive (n = 28). To obtain the cyt b sequence of parasites detected by qPCR, neat DNA extractions from all qPCR-positive samples were screened using the nested PCR protocol of Waldenström et al. (2004), and the purified products directly sequenced. In addition, in order to test whether medication had any effect on *Leucocytozoon* parasites, we screened pre- and post-treatment samples for the presence of *Leucocytozoon*, using the protocol of Hellgren et al. (2004); see Supporting Information for further details).

Statistical analyses

As the distribution of *Plasmodium* DNA copy number was strongly skewed, log(1 + *Plasmodium* DNA copies), which was approximately normally distributed, was used as a measure of parasitaemia in statistical analyses to meet model assumptions. To examine the effect of Malarone on *Plasmodium* parasitaemia, we used a mixed model among females infected by *Plasmodium* prior to the experiment, including female identity as a random effect, and tested specifically for an interaction between drug dose (as a four-level factor) and sampling occasion (pre- or post-treatment). In subsequent analyses, we entered medication as a binary variable (medicated or control) to simplify analyses and to minimize the degrees of freedom used by this term. Changes in female mass in relation to medication were investigated by testing for an interaction between medication and sampling occasion in an analogous mixed model. We used generalized linear models (GLMs) with binomial errors, a logit link and an overdispersion correction where necessary to investigate the effect of medication on reproductive success at four consecutive stages of the breeding attempt. Although this approach involves conducting a number of statistical tests (increasing the risk of Type I error), it permits a dissection of exactly when during the breeding process effects of parasitism are most important. The stages we considered were: (i) whether any eggs hatched (i.e. whether the nest was abandoned prior to hatching); (ii) hatching success (where at least one egg hatched); (iii) the proportion of hatchlings that survived to day 14; and (iv) fledging success of day 14 nestlings. We modelled hatching success and fledging success as binomial responses (i.e. whether all eggs hatched, and whether all day 14 nestlings fledged, respectively).

Any effects of medication on the response variables described above, if due to malaria parasite removal, should only occur in females that were infected with *Plasmodium* prior to the experiment. Therefore, in all analyses we considered whether any effect of medication was found amongst females that were *Plasmodium*-infected. *Plasmodium*-uninfected (hereafter ‘infected’ and ‘uninfected’ respectively) or in all females regardless of infection status. To do this, we included main effects of medication, infection status and their interaction term in the model. 

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all models. In analyses where interaction terms involving medication were already present (analyses of female and nestling condition), we began by conducting separate analyses for infected and uninfected females. If a significant two-way interaction term involving medication was found in such analyses, we then tested whether this differed between infected and uninfected females by examining the significance of the relevant three-way interaction involving infection status.

**Results**

**Pre-experimental conditions**

As treatments were blocked with respect to timing of breeding, medicated and control females did not differ with respect to lay date or hatch date (lay date: $F_{1,109} = 1.49, P = 0.23$; hatch date: $F_{1,95} = 0.27, P = 0.60$), although medicated females had, unexpectedly, larger clutch sizes ($F_{1,109} = 6.88, P = 0.01$; see Effects of medication and clutch size in Results for confirmation that this did not influence conclusions). Medicated and control females did not differ significantly with respect to initial *Plasmodium* infection status ($\chi^2 = 2.12, P = 0.15, n = 108$) or parasitaemia among those that were infected ($F_{1,27} = 0.50, P = 0.49$).

**Efficacy of anti-malarial treatment**

At the start of the experiment, 29.7% ($n = 111$) of incubating females were found to harbour *Plasmodium* infections. Malarone treatment had a highly significant effect on *Plasmodium* parasitaemia among infected females (drug dose × sampling occasion interaction $F_{3,43} = 11.79, P < 0.0001$; Fig. 1); all infected females given a medium or high drug dose showed complete clearance of *Plasmodium* from the blood, and all controls maintained infections while the small number of infected low dose females showed an intermediate response. Very few females gained infection during this experiment; of 61 females uninfected at the start of the experiment, four were positive at the end. Two had received a medium drug dose, one a low dose and one a control; hence medication did not protect against gaining or relapse of *Plasmodium* infection, potentially because of a short half-life of Malarone in the blood, although the pharmacokinetics of this drug in birds are currently unknown. Of the qPCR-positive pretreatment samples for which a cyt $b$ sequence was obtained, 12 were identified as pSGS1 and one as pGRW11. Both lineages have been identified as belonging to the morphospecies *P. relictum* (Palinauskas et al., 2007). Of the four *Plasmodium* infections successfully cleared by Malarone treatment for which cyt $b$ lineage was determined, all were pSGS1.

**Effect of medication on reproductive success at successive stages**

Both medication and *Plasmodium* infection independently increased the likelihood that nests were abandoned before any eggs hatched (infection status $\chi^2 = 10.06, P = 0.001, n = 108$; medication $\chi^2 = 6.10, P = 0.014, n = 108$; Table 1). However, among nests that reached hatching, medication caused an increase in hatching success (the probability that all eggs hatched), although only in females that were infected with *Plasmodium* parasites prior to treatment (medication × infection status interaction $\chi^2 = 4.50, P = 0.034, n = 96$; Fig. 2a, Table 1). Medication had no significant effect on nesting survival to day 14, regardless of female infection status (medication $\chi^2 = 0.31, P = 0.58, n = 94$; medication × infection status interaction $\chi^2 = 0.24, P = 0.63, n = 94$; Table 1), but had a positive effect on fledging success (probability that all day 14 nestlings fledged), although this effect did not differ significantly between *Plasmodium*-infected and -uninfected females (medication $\chi^2 = 5.53, P = 0.012, n = 84$; Fig. 2b, Table 1). There was no evidence that medication influenced change in female mass across the experiment for either *Plasmodium*-infected (medication × sampling occasion interaction $F_{1,45} = 0.20, P = 0.66$) or uninfected (medication × sampling occasion interaction $F_{1,129} = 2.45, P = 0.12$) females.

**Effect of medication on nestling condition and parental care**

We analysed nestling tarsus length (indicative of structural size) and mass on day 14 as indicators of offspring performance in relation to maternal treatment. Evidence that treatment of maternal malaria infections affected the structural size of offspring was equivocal. Among
infected females only, there was a significant interaction between hatch date and medication, suggesting that nestling size decreased with later hatching if infections were treated (hatch date × medication interaction $F_{1,188} = 8.76$, $P = 0.01$; Table 2). However, when all females were considered, the three-way interaction between hatch date, medication and female infection status was not significant ($F_{1,802} = 2.35$, $P = 0.13$), indicating that the evidence for a hatch date-dependent effect of parasite removal on nestling structural size is weak at best.

In contrast, we found strong evidence that treatment of maternal malaria infections influenced nestling mass, but that this effect was unequal among broodmates, and depended upon nestling structural size (tarsus length). In the analysis of nestling mass, the interaction between

Table 1 Results of model selection for factors predicting reproductive success at four consecutive stages of the breeding attempt. For significant terms ($P < 0.05$, shown in bold), statistics are given from the minimal model; for nonsignificant terms, statistics are those at the point that factor left the model. Effect sizes (Pearson’s $r$) are given for all effects.

<table>
<thead>
<tr>
<th>Response variable</th>
<th>Predictors</th>
<th>d.f.</th>
<th>Parameter estimate ± SE</th>
<th>$\chi^2$</th>
<th>$P$-value</th>
<th>$r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any eggs hatched</td>
<td>Medication (control) 1</td>
<td>1</td>
<td>1.09 ± 0.55</td>
<td>6.10</td>
<td>0.014</td>
<td>0.238</td>
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<tr>
<td>(108)</td>
<td>Infection status (uninfected) 1</td>
<td>1</td>
<td>1.05 ± 0.34</td>
<td>10.06</td>
<td>0.002</td>
<td>0.305</td>
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<td></td>
<td>Medication × infection status $^*$</td>
<td>1</td>
<td>$-0.74 ± 0.35$</td>
<td>5.37</td>
<td>0.021</td>
<td>$-0.237$</td>
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<tr>
<td>Hatching success</td>
<td>Medication (control) 1</td>
<td>1</td>
<td>$-0.72 ± 0.34$</td>
<td>0.44</td>
<td>0.506</td>
<td>$-0.068$</td>
</tr>
<tr>
<td>(96)</td>
<td>Infection status (uninfected) 1</td>
<td>1</td>
<td>0.65 ± 0.34</td>
<td>4.50</td>
<td>0.034</td>
<td>0.217</td>
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<tr>
<td></td>
<td>Medication × infection status</td>
<td>1</td>
<td>$-0.09 ± 0.05$</td>
<td>2.63</td>
<td>0.105</td>
<td>$-0.166$</td>
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<td></td>
<td>Hatch date</td>
<td>1</td>
<td>$-0.25 ± 0.14$</td>
<td>3.63</td>
<td>0.057</td>
<td>$-0.194$</td>
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<td>Clutch size</td>
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<td>0.31</td>
<td>0.578</td>
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<td>Hatching survival to day 14</td>
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<td>$-0.15 ± 0.27$</td>
<td>0.29</td>
<td>0.588</td>
<td>0.056</td>
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<td>(94)</td>
<td>Infection status (uninfected) 1</td>
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<td>$-0.14 ± 0.28$</td>
<td>0.24</td>
<td>0.628</td>
<td>$-0.050$</td>
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<td></td>
<td>Medication × infection status</td>
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<td>2.89</td>
<td>0.089</td>
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<td>Fledging success</td>
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<td>$-0.85 ± 0.38$</td>
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<td>(84)</td>
<td>Infection status (uninfected) 1</td>
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<td>Medication × infection status</td>
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<td></td>
<td>Hatch date</td>
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<td>11.81</td>
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<td>$N$ parents present (one)</td>
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<td>$1.24 ± 0.41$</td>
<td>10.82</td>
<td>0.001</td>
<td>$-0.359$</td>
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</table>

$^*$Term excluded since model became unstable upon its inclusion; visual inspection of the data indicated no evidence for an interaction.

Fig. 2 Effect of Malarone medication on (a) hatching success (the probability of successfully hatching all eggs), controlling for clutch size, (b) fledging success (the probability that all day 14 nestlings fledged) at nests where two parents were present on day 14 and controlling for hatch date, (c) female provisioning rate (number of minutes present at nest between 06:30 and 12:30 on day 14) and (d) the coefficient of variation (CV) for nestling mass within broods. Dark and light grey bars represent Plasmodium-uninfected and infected females respectively and 95% confidence intervals are indicated.
nesticling tarsus and medication was highly significant amongst Plasmodium-infected females, but not among uninfected females (medication × tarsus interaction, infected females: $F_{1,186} = 10.73$, $P = 0.001$; uninfected females: $F_{1,609} = 0.347$, $P = 0.56$; Table 2); when all nests were considered, the three-way interaction term between medication, tarsus and infection status was significant ($F_{1,802} = 9.92$, $P = 0.002$). Examination of this interaction among infected females showed that the slope of nestling tarsus on mass was shallower for medicated females than control females, indicating that relatively small nestlings were heavier for their size when the female had been medicated (Fig. 3). To explore this effect further, we performed two supplementary (and related) analyses. First, we tested (using a GLM with normal error structure) the effect of medication, infection status and hatch date on nestling tarsus (Table 2). Second, we tested this interaction further, we performed two supplementary (and related) analyses. First, we tested (using a GLM with normal error structure) the effect of medication, infection status and hatch date on nestling tarsus (Table 2). Second, we tested this interaction.
status, and their interaction on the within-brood slope of nestling tarsus on mass, and second we tested their effect on the coefficient of variation (CV) in nestling mass across nests; hatch date was also included as a covariate in both analyses. In both models, the interaction term was significant. Among Plasmodium-infected (but not uninfected) females, medication was associated with a shallower slope of nestling tarsus on mass within broods (medication × infection status interaction \( \chi^2 \) \(_1\) = 4.04, \( P = 0.04, n = 82\); Table 2; slopes for uninfected control: 0.75 ± 0.15, uninfected medicated: 0.85 ± 0.07, infected control 1.12 ± 0.15, infected medicated: 0.67 ± 0.18). When four nests where only two or three nestlings remained alive at day 14 (and for which slope estimates are based on very few observations) were excluded from this analysis, the effect was stronger (\( \chi^2 \) \(_1\) = 6.04, \( P = 0.014, n = 78\)). Analysis of the within-brood variation in nestling mass, showed that for Plasmodium-infected (but not uninfected) females, the CV was significantly lower for the broods of medicated compared to control females (medication × infection status interaction \( \chi^2 \) \(_1\) = 4.30, \( P = 0.04, n = 83\); Fig. 2d, Table 2; see Box 1).

To explore the significance of these within-brood effects for fitness, we performed additional GLMs to test whether the within-brood slope of nestling tarsus on mass, or the nestling mass CV, predicted the probability that all day 14 nestlings fledged. Both variables strongly predicted fledging success: the shallower the slope or the lower the CV, the higher the probability that the entire
brood fledged (slope: $\chi^2_1 = 10.48, P = 0.001$; CV: $\chi^2_1 = 19.16, P < 0.001$).

Analyses of parental care data suggested that the within-brood effects of medication detected may be explained by altered female provisioning behaviour; anti-malarial medication led to an increase in female provisioning rate, but only among *Plasmodium*-infected females (medication × infection status interaction $\chi^2_1 = 3.92, P = 0.048, n = 55$; Fig. 2c, Table 2). Total provisioning rate at a nest (summed across parents) negatively predicted both nestling mass CV and the within-brood slope of nestling tarsus on mass (slope: $F_{1.63} = 5.04, P = 0.028$; CV: $F_{1.63} = 13.25, P = 0.001$) and similar but weaker negative relationships were seen when only female provisioning rate was considered (CV: $F_{1.53} = 2.92, P = 0.093$, slope: $F_{1.53} = 0.78, P = 0.381$). Medication had no effect on female roost time, but infection status remained in the minimal model as a main effect: females infected with *Plasmodium* parasites before the experiment had longer roost times than uninfected females ($F_{1.46} = 4.99, P = 0.030$; Table 2).

**Effects of medication and clutch size**

As we found that clutch size unexpectedly covaried with medication treatment (medicated females tending to have larger clutch sizes, see Pre-experimental conditions), and clutch size may be an indicator of female quality, we tested whether this covariance could have influenced our results concerning positive effects of medication. Inclusion of clutch size as a covariate in analyses of the four reproductive success measures considered, as well as nestling mass CV and mass-tarsus slopes (see Tables 1 and 2) showed clutch size to be nonsignificant in all cases (all $P > 0.35$) except for the case of hatching success, where clutch size had a negative effect on the proportion of eggs that hatched, i.e. in the opposite direction to the effect of medication (Table 1). Furthermore, there is no reason to predict that any positive association between natural clutch size and reproductive success would show an interaction with female infection status, as was seen for most effects of medication on measures of reproductive success. Thus, we found no evidence that the effects of medication detected were influenced by clutch size.

**Discussion**

Our results provide the first experimental evidence that chronic avian *Plasmodium* infections can have negative fitness consequences in a wild population where malaria is endemic. Observational studies have provided conflicting results as to whether such infections have appreciable fitness effects in the wild (Davidar & Morton, 1993; Bensch et al., 2007; Marzal et al., 2008) and perhaps this, coupled with the observation of extremely low parasite densities in most chronic infections (Valkiūnas, 2005), is why it has been suggested that they may be relatively benign. However, here we show that even *Plasmodium* infections of very light parasitaemia can reduce fitness in breeding blue tits. Medication of female blue tits using Malarone, which proved highly effective at reducing *Plasmodium* parasitaemia – eliminating parasites from the bloodstream as far as our assay was concerned, had positive effects on both hatching success and fledging success. These effects could largely be interpreted in terms of a reduction in within-brood inequalities among the offspring of medicated females, as shown by an increase in the proportion of eggs that hatched, a reduction in nestling mass variation, and improved condition of the smallest nestlings. We also found that treating *Plasmodium* infections increased the provisioning rate of female parents, suggesting that the experimental effects on offspring may have been mediated by increased parental effort.

Our results complement those of previous experimental studies on related blood parasites, which have detected positive effects of medication on various measures of reproductive success (Merino et al., 2000; Marzal et al., 2005). However, as these studies used primaquine, which apparently acts against both *Haemoproteus* and *Leucocytozoan* parasites (Merino et al., 2000; Tomas et al., 2005), and the prior infection status of females was not controlled for, it was not always possible to determine whether treatment of one or both of these parasite genera, or the drug itself (irrespective of anti-parasite activity), was responsible for the observed effects. In this study, we were able to target *Plasmodium* parasites specifically without any detectable effect on the prevalence of *Leucocytozoan* parasites (see Supporting Information). Moreover, in the majority of analyses we performed, positive effects of medication were only observed in females that harboured malaria parasites before the experiment. To explore whether overall the effect of medication on reproductive success was conditional on individuals being infected with *Plasmodium* before the experiment, we estimated the mean weighted effect size (Cooper & Hedges, 1994) for the effect of medication on the four measures of reproductive success considered here, for *Plasmodium*-infected and -uninfected females separately. Although confidence intervals for these effect sizes are wide as only four data points are used in their estimation, this analysis suggested that across the entire reproductive attempt, positive effects of medication are stronger in females that were *Plasmodium*-infected before the experiment [infected females: mean weighted effect size ($Z^*_r =$ 0.048, 95% CI: $-0.286$ to 0.381; uninfected females: $Z^*_r =$ $-0.020$, 95% CI: $-0.211$ to 0.171]; the same pattern holds when only hatching and later stages of the breeding attempt were included (i.e. excluding the effect of nest abandonment prior to hatching: infected females: $Z^*_r =$ 0.241, 95% CI: $-0.305$ to 0.788; uninfected females: $Z^*_r =$ 0.046, 95% CI: $-0.253$ to 0.346). Hence, the medication-related
increases in reproductive success detected here can be attributed to the removal of these parasites.

Our results show that Malarone medication increased the likelihood of nest abandonment prior to hatching (regardless of female infection status), as did infection with *Plasmodium* parasites. As control females were subject to identical handling, this suggests there were some negative effects of drug itself, that increased the risk of nest desertion. There is therefore a need to optimize a safe and effective dosage regime in future field studies that use this drug, so that side-effects and the risk of drug-associated nest desertion are minimized. Despite this initial negative effect of drug administration, among nests that reached the hatching stage, treatment of malaria infections had positive effects on reproductive success at two stages. First, infected females that were medicated showed a significantly higher hatching success compared to controls. Interestingly, a marked effect on hatching success was also detected by Marzal et al. (2005), in which house martins given primaquine to treat *Haemoproteus prognei* infection experienced a 29% increase in hatching success, constituting a major part of the overall increase in reproductive success detected by this study. Sanz et al. (2001b) also found a negative correlation between trypanosome infection and hatching success. Taken together these results suggest that parasitic infection or physiological differences associated with infection (e.g. an active immune response) may alter the thermoregulatory or incubation behaviour of females, with consequences for hatching success. Second, we found that anti-malarial medication of females caused a significant increase in fledging success, suggesting that females were better able to care for offspring when *Plasmodium* infections were removed. Similarly, Merino et al. (2000) found that female blue tits (infected with *Haemoproteus* and *Leucocytozoon* parasites) treated with primaquine showed increased nestling survival: in the same population, Tomas et al. (2007) also showed that primaquine-treated females increased their provisioning rate more than control females from the early to the late nestling stage. In this study, we also find evidence to suggest that the effect of medication on fledging success is mediated by effects on provisioning rate and nestling condition.

Treatment of malaria infections seems to have increased the total amount of resources a female blue tit could provide to her brood, as provisioning rate was increased in infected females that were medicated (Fig. 2c). However, it appears these extra resources did not benefit brood mates equally. The smallest chicks on day 14, which are likely to be late-hatched offspring (Magrath et al., 2009), experienced the main benefits of maternal malaria treatment, since treatment led to a reduction in the slope of the relationship between nestling tarsus and mass within broods and a change in the variance (Fig. 2d) rather than the mean nestling mass across broods. Both variables were strongly associated with an increased probability that all nestlings fledged from a breeding attempt. Parental optimism (Mock & Forbes, 1995), where more offspring are produced than can survive the period of parental care, is widespread in nature and is thought to perform multiple functions including allowing parents to track unpredictable resources (i.e. raise extra offspring should circumstances permit) and providing insurance against offspring that die unexpectedly (Lack, 1947; Mock & Forbes, 1995; Forbes et al., 1997; Forbes, 2009). In support of resource-tracking explanations for offspring overproduction, long-term studies of Yellow-headed blackbirds (Forbes et al., 2002) have shown that in good years (when food is abundant), initially optimistic parents can afford to raise marginal, late-hatched offspring which might have perished in bad years: an unexpected food surplus allows parents to devote resources to offspring that otherwise may have been left to starve whilst core offspring were prioritized. Our results are consistent with the idea that reproducitively optimistic parents, which then experienced unexpected extra resources (via relief from parasitic infection) could channel those resources into smaller, late-hatched offspring that might otherwise not fledge (see Fig. 3). In a similar way, our finding that treatment of malaria infections increased the likelihood that all eggs hatched could also be interpreted as a treatment-related reduction in within-family inequalities. These results showing that only some members of a brood benefited from parents receiving anti-parasite treatment are similar to those of Reed et al. (2008), who found that female European shags treated with an anti-helminthic drug showed increased nest provisioning behaviour, but that only male offspring (which are more expensive to rear than females in this species) benefited from this effect through increased survival. That parents may alter their parental care strategy in response to anti-parasite treatment (or any other manipulation that provides them with more resources) in a way that benefits some, but not all, brood members, should be considered in future studies of this type. Such effects will mean that although average brood traits may not be much affected by the treatment, within-brood effects may occur with significant fitness consequences. In addition, these results suggest that unpredictability in resource availability at the level of the individual rather than the population (such as annual food availability) may be important when considering resource-tracking explanations of parental optimism (Amundsen & Slagsvold, 1996).

Positive effects of medication on measures of reproductive success could reflect a release from the drain imposed by the direct costs of parasitism (e.g. red blood cell destruction), or a release from investment in costly immune defence (Svensson et al., 1998; Råberg et al., 2000). Although we cannot distinguish between these two possibilities from our data, we consider the latter more likely, as other passerine species experimentally
infected with the parasite treated in this experiment (cyt b lineage pSG51) that develop chronic infections show no clear signs of anaemia or direct impact of parasites (Pallanauskas et al., 2008). Across studies of wild birds, there is good evidence that artificially increasing the demand for parental care can lead to increases in the intensity of haemosporidian parasitism, as well as to reduced immune responsiveness (Knowles et al., 2009). It is therefore possible that these effects and positive effects of medication such as those detected here reflect the same underlying resource allocation trade-off between parental care and immune defence against parasites. To address more directly why reproductive success increases following medication, further work investigating how parasite removal affects the physiology and immunology of hosts would be useful.

It is important to note that this experiment focused on the fitness effects of only one stage of Plasmodium infection (the chronic stage) and examined how such infections affected reproduction over a single reproductive attempt. As chronic infections may be of long duration (Valkiūnas, 2005), iteroparous or long-lived organisms may experience fitness effects of these infections over a large part of their lifetime, and the cumulative effect could then be quite significant. Other stages of infection, such as the initial acute stage or relapses (Applegate, 1971; Atkinson & van Riper, 1991) may also have fitness consequences that we have not explored here. Hence, the estimates of parasite-induced reductions in reproductive success detected here can be viewed as a minimum cost of Plasmodium infections in wild birds.

Acknowledgments

We are very grateful to O. Hellgren, S. Larcombe, M.J. Wood and S. Straebler for help with fieldwork and S. Bensch for assistance with qPCR development. This work was funded by a NERC studentship to S.C.L.K. and a NERC grant to B.C.S.

References


Supporting information

Additional Supporting Information may be found in the online version of this article:

Data S1 Effect of Malarone treatment on Leucocytozoon parasites.

Figure S1 Effect of Malarone on Leucocytozoon prevalence; pre- and post-treatment prevalence are shown in dark and light grey respectively.

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Received 17 September 2009; revised 17 November 2009; accepted 24 November 2009