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Molecular epidemiology of malaria prevalence and parasitaemia in a wild bird population

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Abstract

Avian malaria (*Plasmodium* spp.) and other blood parasitic infections of birds constitute increasingly popular model systems in ecological and evolutionary host–parasite studies. Field studies of these parasites commonly use two traits in hypothesis testing: infection status (or prevalence at the population level) and parasitaemia, yet the causes of variation in these traits remain poorly understood. Here, we use quantitative PCR to investigate fine-scale environmental and host predictors of malaria infection status and parasitaemia in a large 4-year data set from a well-characterized population of blue tits (*Cyanistes caeruleus*). We also examine the temporal dynamics of both traits within individuals. Both infection status and parasitaemia showed marked temporal and spatial variation within this population. However, spatiotemporal patterns of prevalence and parasitaemia were non-parallel, suggesting that different biological processes underpin variation in these two traits at this scale. Infection probability and parasitaemia both increased with host age, and parasitaemia was higher in individuals investing more in reproduction (those with larger clutch sizes). Several local environmental characteristics predicted parasitaemia, including food availability, altitude, and distance from the woodland edge. Although infection status and parasitaemia were somewhat repeatable within individuals, infections were clearly dynamic: patent infections frequently disappeared from the bloodstream, with up to 26% being lost between years, and parasitaemia also fluctuated within individuals across years in a pattern that mirrored annual population-level changes. Overall, these findings highlight the ecological complexity of avian malaria infections in natural populations, while providing valuable insight into the fundamental biology of this system that will increase its utility as a model host–parasite system.

Keywords: blue tit, haemosporidian, host–parasite interactions, *Plasmodium*, qPCR

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Introduction

Understanding the causes of host variation in susceptibility to infectious disease is central to understanding and predicting disease dynamics and host–parasite evolution. Yet for many diseases of wild animal populations, such fundamental knowledge remains limited. Whether an individual becomes infected by a given parasite will depend on environmental factors that influence exposure, such as climatic effects on vector or parasite development (Rogers & Randolph 2006), as well as intrinsic host factors such as genetics (Kaslow *et al*. 2008), permanent environmental effects (Monaghan 2008) or effects of host age on immunity (Palacios *et al*. 2007). Thus, the distribution of parasites among hosts is likely to be shaped by a combination of abiotic and biotic factors, and so understanding their relative contributions is an important prerequisite for predicting the evolution of host–parasite relationships. From a

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practical perspective, understanding the sources of variation in parasitological traits is also important so that hypotheses can be framed and tested appropriately. For instance, if parasite prevalence shows strong temporal or spatial variation within populations, conclusions from cross-population comparisons may be strongly influenced by chance decisions about when or where to sample.

Since the development of molecular screening techniques for avian haemosporidia, these parasites have become an increasingly popular model system for testing hypotheses about host–parasite interactions in wild birds (Bensch *et al.* 2009). Numerous studies have documented extensive and complex variation in the prevalence and diversity of these common vector-borne parasites in both vertebrate hosts and mosquitoes, between geographically separate host populations and across biogeographical regions (Bensch & Åkesson 2003; Santiago-Alarcon *et al.* 2008; Chasar *et al.* 2009; Njabo *et al.* 2011). Within-population variation in prevalence according to various abiotic and host factors has also been reported, if less frequently (Bensch *et al.* 2007; Wood *et al.* 2007). However, the biological processes that underpin such prevalence variation remain poorly understood. Moreover, although prevalence (or infection status at the individual level) can reveal whether an organism has become infected or not, this trait offers little insight into host–parasite interactions after the point of infection. Parasitaemia (the density of parasites within infected hosts), although less often measured, may be more informative in this respect: whereas infection status may be determined by the degree of exposure to infective vectors (Sol *et al.* 2000) or intrinsic host factors such as genetic resistance (Westerdahl 2007), parasitaemia may reflect hosts' ability to control established infections, or variation in parasite replication rate in response to a variable host environment (Reece *et al.* 2009). Furthermore, as parasitaemia often influences the probability of detecting infections (Bentz *et al.* 2006; Fallon & Ricklefs 2008), the extent to which documented patterns of prevalence variation may be driven by variation in parasitaemia is currently unclear. Thus, studies that tease apart these two traits in wild populations are critical to further our understanding of avian malaria ecology. For true avian malaria parasites (*Plasmodium*), quantitative data on parasitaemia from field studies are scarce. A major reason for this is the difficulty of measuring this trait, as in *Plasmodium* infections of wild-caught birds, parasite densities are often too low for accurate microscopic quantification (Fallon & Ricklefs 2008). However, with the adoption of quantitative PCR for measuring parasitaemia, the first data on this trait from wild hosts are now emerging (Bentz *et al.* 2006; Zehtindjiev *et al.* 2008).

A further limitation to our understanding of avian malaria ecology is the paucity of longitudinal data on within-individual infection dynamics, particularly in natural settings. As wild birds are rarely resampled, little is known about how temporally stable or dynamic chronic infections are over time. It has been assumed that infection status measured at a single time point can reflect host resistance or susceptibility to malaria, and associations have been sought between infection status and MHC genotype as well as overall genetic diversity on this basis (Ortego *et al.* 2007; Westerdahl 2007; Loiseau *et al.* 2008). However, the value of such an approach will depend on the temporal stability of individual infection status and how this relates to genetic resistance, as well as the extent to which the compared hosts have been equally exposed to infection. Longitudinal data can also provide insight into the biological processes that give rise to observed cross-sectional patterns in parasitological traits. For example, a frequently reported cross-sectional pattern among studies of avian blood parasites is an increase in prevalence across host age cohorts, which is thought to reflect an increased duration of exposure to parasites among older individuals, and maintenance of chronic infections (Stjernman 2004; Wood *et al.* 2007). However, several non-exclusive processes, including age-specific probabilities of infection gain or loss and infection-related mortality could be involved, and without longitudinal analyses these cannot be teased apart (Sol *et al.* 2003).

Here, we present results from a 4-year longitudinal study on the epidemiology of *Plasmodium* infections in a woodland population of blue tits (*Cyanistes caeruleus*). Using a newly developed qPCR assay to measure parasitaemia and a combination of cross-sectional and longitudinal approaches, we conduct a detailed analysis of environmental and host factors predicting variation in both infection status and parasitaemia in this population, and the variability of these traits within individuals over time.

Methods

Field methods

The study was conducted in Wytham Woods (51°46′N, 1°20′W), a 385-ha mixed deciduous woodland near Oxford, UK, where 250–450 pairs of blue tits breed in nestboxes every year (Perrins 1979). Each breeding season (April–June), all 1205 nestboxes at the site are monitored at regular intervals, so that lay date (date on which the first egg was laid; LD), hatch date (date on which the first egg hatched; HD) and clutch size (CS) were determined for all reproductive attempts. The analyses conducted here involve samples collected from

breeding adults between 2005 and 2008. Adults were captured and blood-sampled when their broods were between 6 and 15 days post-hatch. In this way, all hosts were sampled at a standardized point in their annual cycle, thus controlling for previously documented seasonal variation in parasite prevalence at this location (Cosgrove *et al.* 2008). All blood samples were taken by brachial or jugular venepucture, under Home Office licence. In 2006 and 2007 as part of another experiment (Knowles *et al.* 2010a), a subset of females were additionally sampled around egg-hatching, thus providing two samples per year approximately 2 weeks apart for these individuals. Host sex was determined based on the presence (female) or absence (male) of a brood patch, and age (first year or older) according to plumage characteristics (Svensson 1992). For 75% of the individuals in this study, an exact age could be assigned, because they were either first captured as yearlings, or born locally and ringed as nestlings. For non-local birds first captured with adult plumage, a minimum age of two was assigned. Thus, all birds in the population could be assigned a minimum age at each capture.

Avian malaria diagnosis

We used quantitative (q)PCR for detecting and quantifying *Plasmodium* parasites, using the primers L9 5′-AAACAATTCCTAACAAAACAGC-3′ and NewR 5′-ACATCCAATCCATAATAAAGCA-3′, which target a 188-bp region of the mitochondrial cytochrome *b* gene. Genomic DNA was extracted from blood samples, using DNeasy extraction kits (Qiagen) for samples from 2005, and a standard ammonium acetate method for samples from 2006 to 2008. DNA concentration was measured using a Picogreen assay (Quant-iT Picogreen dsDNA Assay Kit, Invitrogen) and samples were diluted to a working concentration of 2 ng/μL prior to qPCR. Standard curves were created using a full-length cyt *b* PCR product from *P. relictum* (lineage pSGS1) amplified using the protocol of Perkins & Schall (2002). qPCR reactions were performed exactly as described in Knowles *et al.* (2010b). For the purpose of analysing within-individual changes in parasitaemia, all samples for a given individual were run on the same qPCR plate, to eliminate the possibility of artefactual effects arising because samples were tested in different batches.

Nested PCR (Waldenström *et al.* 2004) was also performed on samples from 2005 to 2007 as described elsewhere (Wood *et al.* 2007), allowing us to compare the detection sensitivity of our qPCR assay with that of a commonly used non-quantitative PCR assay. In addition, nested PCR sequence data was used to identify a consistent difference in qPCR product melting temperature that could be used in *Plasmodium* species diagnosis. Two divergent cyt *b* clades were regularly detected in this population (comprising 98.2% all infections), which correspond to two well-defined morphospecies, *Plasmodium relictum* (lineages pSGS1 and pGRW11) and *P. circumflexum* (lineages pTURDUS1 and pBT7; Palinauskas *et al.* 2007). For simplicity, we refer to them by their morphospecies classification from here on. Because qPCR products from *P. relictum* melted around 1 °C above those from *P. circumflexum* (approximately 75.2 °C vs. 74.2 °C), we used qPCR product melting temperature to diagnose *Plasmodium* species (see Data S1 in Supporting information for further details).

To estimate the repeatability of qPCR parasitaemia estimates, DNA was re-extracted from 35 blood samples from 2008 and qPCR repeated as described earlier. ln(1 + *Plasmodium* DNA copies), our measure of parasitaemia in analyses, was highly repeatable between extractions, with $r = 0.80$ among samples where at least one tested positive ($n = 28$). For a larger number of samples ($n = 360$), qPCR was repeated on the same DNA extraction and revealed similar levels of repeatability ($r = 0.78$; $n = 193$); this data set was also used to ascertain the probability of non-detection for samples known to be positive, and the rate at which prevalence increases when the same samples are tested repeatedly (see Results).

Measurement of breeding location and environmental variables

To investigate broad spatial trends in prevalence and parasitaemia across the study site, we used GIS-derived measures of the position of each nestbox where a blue tit was captured and sampled. The x and y coordinates of all nestboxes in Wytham were known (±3 m), as these were digitally mapped using differential GPS in 2004–2005 (Wilkin *et al.* 2007a). These coordinates and their interaction term were entered in cross-sectional analyses of infection status and parasitaemia to test for linear effects of nestbox location ('latitude' and 'longitude') as well as broad patterns of spatial clustering for these traits within the woodland. As breeding tits are territorial and forage in the immediate vicinity of the nest (Stauss *et al.* 2005), these coordinates give an accurate description of individuals' location at this time of year. As nestbox coordinates predicted both prevalence and parasitaemia, we created interpolated maps in MapInfo professional v8.5 to visualize spatial variation in these traits with greater resolution. Interpolations were created using inverse distance weighting (IDW), which uses a moving point average to interpolate pixel values and estimate local trends in spatially variable data. Each pixel value is calculated by averaging the

weighted sums of all data points within a user-defined search area, such that points farther away influence the pixel value less than those that are close (decay exponent = 2). The radii of the search and display areas used were four times the average point density (400 m) as in Wood *et al.* (2007). Environmental predictors of spatial variation in prevalence in this population have been documented previously (Wood *et al.* 2007), and so we do not explore these further here. However, we explored possible environmental drivers of spatial variation in parasitaemia, using four variables that describe the local environment of a nestbox, derived using GIS software (MapInfo Professional v7.8 & Vertical Mapper v.3):

1. *Territory size.* Tessellations (Thiessen polygons) were formed around nestboxes by placing boundary lines equidistant between occupied nestboxes in each year. Polygon area (ha) is inversely related to breeding density and provides an individual-specific measure of territory size (Wilkin *et al.* 2006). As great tit territories are interspersed with those of blue tits and these species share similar breeding ecology and are known to compete (Minot & Perrins 1986), we included great tit breeding events when calculating territory size. As the distribution of territory sizes was heavily right-skewed, we used a log-transformation in analyses (which was approximately normally distributed) to reduce the potential for extreme values to heavily influence results.
2. *Oak tree abundance.* In addition to nestboxes, the locations of all mature pedunculate and sessile oak trees (*Quercus robur* and *Q. petraea*) in Wytham were mapped due to their importance for tit breeding ecology; caterpillars of species such as the winter moth (*Operophtera brumata*) constitute a primary food source for tits during the breeding season (Wilkin *et al.* 2009) and occur most abundantly on newly emerged oak leaves (Feeny 1970). We therefore used the number of oaks within a 50 m radius of each nestbox (Oaks50) as a measure of local food availability (Wilkin *et al.* 2009).
3. *Proximity to the woodland edge.* We used an edge distance index (EDI; defined in Wilkin *et al.* 2007b) as a measure of how peripheral a nestbox is at the study site. EDI accounts for both the edge distance and the number and layout of edges near a nestbox. Environmental quality is expected to increase with EDI (Wilkin *et al.* 2007b).
4. *Altitude.* As Wytham covers a low-lying hill, the altitude of nestboxes ranges from approximately 60 to 160 m and is inversely correlated with edge distance (Wilkin *et al.* 2007b). Because several potentially relevant habitat features vary altitudinally (e.g. temperature, soil type and water content) and in order to distinguish between edge and altitude effects, we included nestbox altitude as well as an interaction between altitude and EDI in environmental analyses.

These nestbox-specific measures of territory size, oak abundance, altitude and EDI are all associated with either life-history traits or measures of reproductive success for great tits at the study site (Wilkin *et al.* 2006, 2007a, 2009), which are similar in their breeding ecology to blue tits, and therefore reflect important components of the local environment during breeding. Because *Plasmodium* infections seem to be acquired in the Summer/Autumn after birds have settled on their breeding territories in this population (Cosgrove *et al.* 2008; S. Knowles unpublished data), these measures provide a description of individuals' local environment around the time of malaria transmission as well as during breeding.

Statistical analyses

Cross-sectional analyses of infection status. Infection status was modelled using binomial generalized linear models (GLMs) with a logit link, both for pooled *Plasmodium* infections as well as *P. relictum* and *P. circumflexum* separately. These analyses included only samples collected from breeding blue tits 6–15 days after their first egg hatched. Where multiple years' data were available for an individual, all but one was randomly excluded from analysis. We tested for effects of five host factors on infection status: minimum age, a quadratic age term (minimum age^2), sex and two reproductive parameters—clutch size (CS) and standardized hatch date (HD_{std}). HD_{std} measures an individual's relative timing of breeding (and therefore also sampling) within a given year, and is calculated as (HD—annual mean HD)/annual HD standard deviation. To test for spatial variation in infection probability, nestbox x and y coordinates were included as well as their interaction term. Because several effects on infection status appeared to differ between *Plasmodium* species, we then used a binomial generalized linear mixed model (GLMM) to test directly for species differences in infection status predictors. In this model, each sample was represented by two data points: one diagnosis for *P. relictum* and one for *P. circumflexum*. Multiple samples per individual (where available) were included in this analysis, which allowed us to test the robustness of results from previous analyses that excluded all but one datapoint per individual. All effects that predicted infection status for either species were included in the starting model, as

well as all two-way interactions with species. To control for pseudoreplication arising from this approach (each sample represented twice, and some individuals multiple times), we included sample identity nested within individual identity as a random effect in this analysis.

Longitudinal analyses of infection status. To investigate the temporal stability of infection status, we examined three parameters, across three time intervals. These parameters were (i) diagnostic agreement rate (the proportion of identical diagnoses) (ii) the probability of infection loss for individuals or samples first scored as *Plasmodium*-positive; (iii) the probability of infection gain for individuals or samples first scored as *Plasmodium*-negative. The first time interval relates to the same DNA extraction being tested twice ($n = 358$ samples) and serves as a control to estimate apparent loss and gain because of diagnostic error. The second interval concerns females tested approximately 2 weeks apart during the same breeding season, once around hatching and once whilst feeding young ($n = 227$ females, mean = 13.0 ± 0.14 days interval, range 5–22 days). The third interval concerns individuals tested whilst feeding young in separate years ($n = 238$ individuals, including both males and females). For between-year analyses, only the first pair of years where the given parameter could be estimated were used for each individual; in most cases, this related to a 1-year interval (e.g. in the diagnostic agreement rate analysis, 1-year interval $n = 224$, 2-year $n = 32$, 3-year $n = 5$). By comparing loss and gain rates across these intervals, one can test for real loss and gain of infection above detection failure and assess the timeframe over which such processes occur. Infection gains and losses were estimated both for pooled *Plasmodium* and on a species-specific basis; thus, a sample that switched *Plasmodium* species between time points was treated as maintenance of overall *Plasmodium* infection in the pooled analysis, but as a loss of one species and gain of another in species-specific analyses.

Parasitaemia analyses. As the distribution *Plasmodium* cyt *b* copy number was heavily right-skewed, to meet GLM assumptions we modelled parasitaemia as ln(1 + *Plasmodium* copies), which was approximately normally distributed. Parasitaemia was modelled using GLMs with a normal error structure and an identity link function, including in the starting model the same predictor variables tested for infection status, together with all 2-way interactions with *Plasmodium* species. For individuals present multiple times in this data set, all but one entry was randomly excluded. To explore spatial effects on parasitaemia further, we tested for effects of territory size, EDI, altitude and Oaks50 (see above) on parasitaemia in a further GLM, including all significant effects from the original model for parasitaemia, except nestbox coordinates. Repeatability of parasitaemia measurements was estimated using data from individuals *Plasmodium*-positive more than once, across the same three time intervals as described earlier.

All models were simplified by backwards stepwise elimination, sequentially removing terms for which $P > 0.1$ and finally those with $P > 0.05$ to leave the minimal model. GLMs were performed in JMP v.6 (SAS Institute, 2005), and GLMMs using the GLIMMIX platform in SAS v9.2 (SAS Institute, Cary, NC), using the Kenward-Roger approximation for degrees of freedom.

Results

Assay sensitivity and comparison of nested and qPCR

Agreement in diagnosis by qPCR and nested PCR was relatively high, with infection status by one method strongly predicting status by the other (agreement rate = 78%, $\chi^2_1 = 36.80 = 428.44$, $P < 0.001$, $n = 1254$). However, the sensitivity of qPCR detection was significantly higher (Table S1 in Supporting information). Among samples positive by qPCR, parasitaemia positively predicted detection probability by nested PCR ($\chi^2_1 = 191.42$, $P < 0.001$, $n = 548$). Similarly, among samples tested twice by qPCR, the probability of detection on the second test was positively predicted by initial parasitaemia ($\chi^2_1 = 36.80$, $P < 0.001$, $n = 161$). Thus, for both methods, samples with higher parasitaemia were more likely to be detected. All results presented from here on concern qPCR diagnoses only. (See Data S1 and Fig. S1 in Supporting information for further details of assay sensitivity and parasite diversity detected by nested PCR).

Cross-sectional analyses of infection status

Overall *Plasmodium* prevalence was 42.0%, with 22.6% of individuals infected by *P. relictum* and 19.1% by *P. circumflexum*. Results from cross-sectional analyses of factors predicting infection status are shown in Tables 1 and S2 (Supporting information). Prevalence varied markedly across the 4 years studied, and these annual fluctuations differed between *Plasmodium* species, with *P. relictum* exhibiting more pronounced annual fluctuations and *P. circumflexum* prevalence more stable across years [Tables 1 and S2 (Supporting information); Fig. 1a, b]. When only yearling birds were considered (i.e. only individuals that could have acquired infection within the preceding year), prevalence patterns for both species closely matched those including all individuals (Fig. 1a, b). Nestbox location also strongly predicted

Table 1 Factors predicting malaria infection status (prevalence) in the blue tit population of Wytham Woods

		All *Plasmodium* (n = 1499)			*P. relictum* (n = 1496)			*P. circumflexum* (n = 1496)		
Predictor	df	χ^2	P	r	χ^2	P	r	χ^2	P	r
Minimum age	1	16.78	**<0.001**	**0.106**	6.05	**0.014**	**0.064**	4.81	**0.028**	**0.057**
Minimum age^2	1	4.44	**0.035**	**−0.054**	1.76	0.184	−0.034	0.73	0.394	−0.022
Year	3	25.04	**<0.001**		66.67	**<0.001**		11.02	**0.012**	
x	1	13.22	**<0.001**	**−0.094**	0.14	0.711	0.010	15.61	**<0.001**	**−0.102**
y	1	3.44	**0.064**	**0.048**	31.13	**<0.001**	**−0.144**	75.09	**<0.001**	**0.224**
x*y	1	6.63	**0.010**	**−0.067**	0.34	0.560	0.015	3.28	0.070	−0.047
Sex (F)	1	0.73	0.394	0.022	4.21	**0.040**	**0.053**	0.37	0.544	−0.016
Clutch size	1	1.80	0.1780	0.035	0.83	0.363	0.024	0.36	0.552	0.015
HD_{std}	1	0.62	0.431	0.020	0.81	0.369	0.023	1.39	0.239	−0.031

All variables in the starting model are listed, with effects in the minimal model in bold. For significant effects ($P < 0.05$), statistics are from the minimal model; for non-significant effects, statistics are presented from the last model that included this term. Effect sizes (Pearson's r) are given for all effects with a single degree of freedom.
HD_{std}, standardized hatch date, calculated as described in methods; x and y are the geographical coordinates of an individual's nestbox.

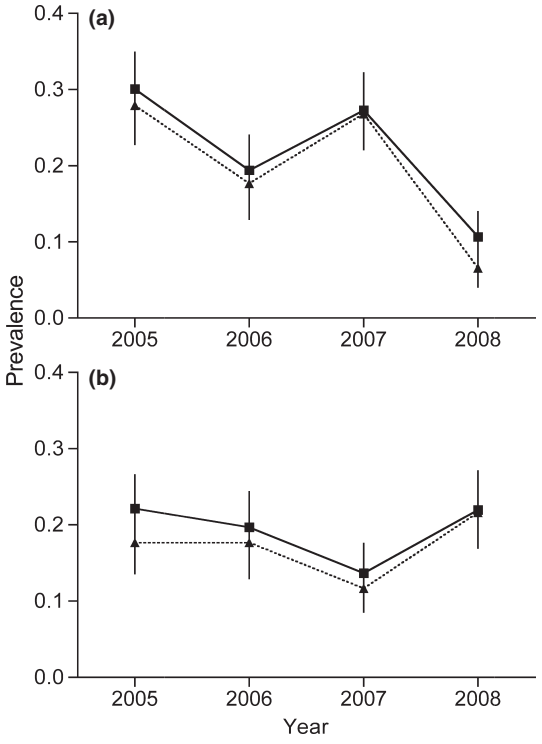

Fig. 1 Annual changes in prevalence for (a) *Plasmodium relictum* and (b) *P. circumflexum*, for all birds (squares) and yearlings only (triangles). Means and 95% confidence intervals are plotted using raw data.

infection status, and again patterns were species-specific. Although latitude (y coordinate) predicted infection status for both species, the effects were in opposite directions, with *P. circumflexum* more prevalent in the north and *P. relictum* in the south of the woodland; in addition, whilst *P. circumflexum* was more prevalent in the west, there was no significant effect of longitude for *P. relictum* [Tables 1 and S2 (Supporting information); Fig. 3a–c]. To explore whether these spatial patterns were broadly stable across years, we tested for year interaction terms with x, y or x*y in the minimal model for each species. None of these year interaction terms were significant (*P. relictum*: year*y $\chi^2_1 = 1.71$ $P = 0.634$; *P. circumflexum*: year*x $\chi^2_1 = 0.59$, $P = 0.899$, year*y $\chi^2_1 = 5.20$, $P = 0.158$, year*x*y $\chi^2_1 = 1.45$, $P = 0.766$), suggesting a degree of spatial stability in the infection patterns. For *P. relictum* (but not *P. circumflexum*), we found some evidence that females were more likely to be infected [Tables 1 and S2 (Supporting information)]. Neither clutch size nor hatch date (HD_{std}) predicted infection status for either species (Table 1).

Malaria prevalence showed a significant quadratic relationship with host age, increasing initially but declining among older hosts (Table 1, Fig. 2a), a pattern that was similar for both *Plasmodium* species (Table S2 in Supporting information). To examine which processes might underlie this pattern, we calculated the expected age-prevalence pattern under a simple set of assumptions (scenario 1). These assumptions were (i) all infections are maintained and cannot be lost; (ii) the risk of becoming infected is constant (e.g. regardless of age or year) and equals the prevalence among yearling birds; (iii) mortality, recapture probability and dispersal of birds into or out of the population are all independent of infection status and age. The observed age-prevalence pattern clearly differs from this scenario (Fig. 1a) and a goodness of fit test, in which the age distribution of infected individuals is

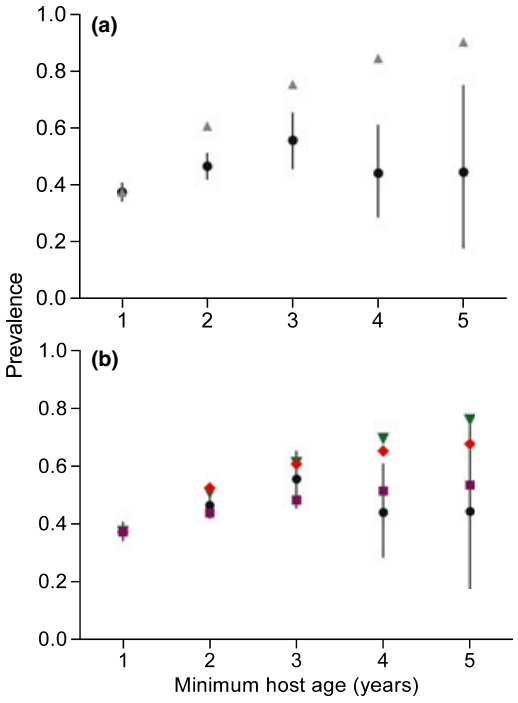

Fig. 2 (a) Effect of minimum host age on prevalence for pooled *Plasmodium* species (circles), and the age-prevalence pattern expected assuming constant infection rates with age, no infection loss and infection-independent mortality (scenario 1, outlined in Results; triangles). (b) Age-prevalence patterns expected under scenarios 2 (triangles), 3 (diamonds) and 4 (squares), as outlined in Results, shown alongside the observed age-prevalence pattern (circles). Means and 95% confidence intervals are plotted using raw data.

compared to that expected under scenario 1, indicates that at least one of the assumptions is violated ($\chi^2_5 = 25.52$, $P < 0.001$). In subsequent longitudinal analyses, we explored the evidence for two of the above assumptions, specifically whether (i) all infections are maintained and (ii) the risk of infection is age-independent; testing the other assumption(s) is beyond the scope of this paper.

Longitudinal analyses of infection status

The rate of agreement in infection status was high for the same sample tested twice (range 85-97%) and decreased as samples from an individual were taken at longer intervals (Table 2). We found clear evidence that infections could be lost over time: considering pooled *Plasmodium* infections, the probability of infection loss across both years and weeks was significantly higher than that seen when the same sample was tested twice (years: $\chi^2_1 = 14.96$, $P < 0.001$, $n = 128$; weeks: $\chi^2_1 = 4.87$, $P = 0.027$, $n = 102$). However, the patterns of loss appeared to differ for the two species, whereas the proportion of *P. relictum* infections lost across both weeks and years was significantly higher than seen with repeat sample testing (years: $\chi^2_1 = 27.11$, $P < 0.001$, $n = 86$; weeks: $\chi^2_1 = 10.78$, $P = 0.001$, $n = 46$), for *P. circumflexum* there was only evidence for infection loss across years but not weeks (years: $\chi^2_1 = 5.46$, $P = 0.019$, $n = 49$; weeks: $\chi^2_1 = 1.37$, $P = 0.242$, $n = 54$; Fig. 4a). By subtracting the rate of infection loss seen with repeat testing from that observed across years, one can estimate the probability of real (biologically meaningful) infection loss across years. For all *Plasmodium* pooled, this was 13.4%, and was 26.3% for *P. relictum* and 13.8% for *P. circumflexum*.

The probability of infection gain across weeks did not differ significantly from that seen when testing the same sample twice (*P. relictum*: $\chi^2_1 = 0.10$, $P = 0.909$, $n = 173$; *P. circumflexum:* $\chi^2_1 = 0.01$, $P = 0.927$, $n = 181$). However, the rate of infection gain across years was significantly higher than that seen with repeat sample testing, for both *Plasmodium* species (*P. relictum*: $\chi^2_1 = 7.02$, $P = 0.008$, $n = 161$; *P. circumflexum:* $\chi^2_1 = 5.43$, $P = 0.020$, $n = 161$; Fig. 4b). Among individuals that maintained overall *Plasmodium* infection across two time points, there were also cases of species switches. This occurred in 5% of cases when the same sample was tested twice or across weeks (7/136 and 4/75, respectively) and in 13% of cases (11/83) across years. The probability of species switching across years was significantly higher than that for repeat sample testing ($\chi^2_1 = 8.07$, $P = 0.005$) and across weeks ($\chi^2_1 = 7.53$, $P = 0.006$).

We also found evidence that infection risk was age dependent. The risk of infection during the first year of life was taken as the prevalence among yearling birds, whilst the risk of infection between years for 1-, 2- or 3-year-old hosts was determined using longitudinal data from exactly aged birds. We found a marked reduction in infection risk between the first and second years of life, from 0.372 to 0.214 (Fig. 4c; posthoc test of 1st vs. 2nd year infection gain probability, $\chi^2_1 = 10.43$, $P = 0.0012$, all other comparisons $P > 0.20$). When considering *Plasmodium* species separately, infection gain probability differed between the 1st and 2nd year of life for *P. circumflexum* ($\chi^2_1 = 10.56$, $P = 0.001$), whereas this difference was not significant for *P. relictum* ($\chi^2_1 = 1.91$, $P = 0.167$). Equivalent analyses revealed no evidence for age dependency in the probability of infection loss (all comparisons $P > 0.30$).

To test how age dependency of infection and/or loss of infection between years might influence the cross-sectional age-prevalence pattern observed, we calculated the expected age-prevalence pattern under three further scenarios. Scenario 2 included age dependency of infection risk, scenario 3 incorporated a con-

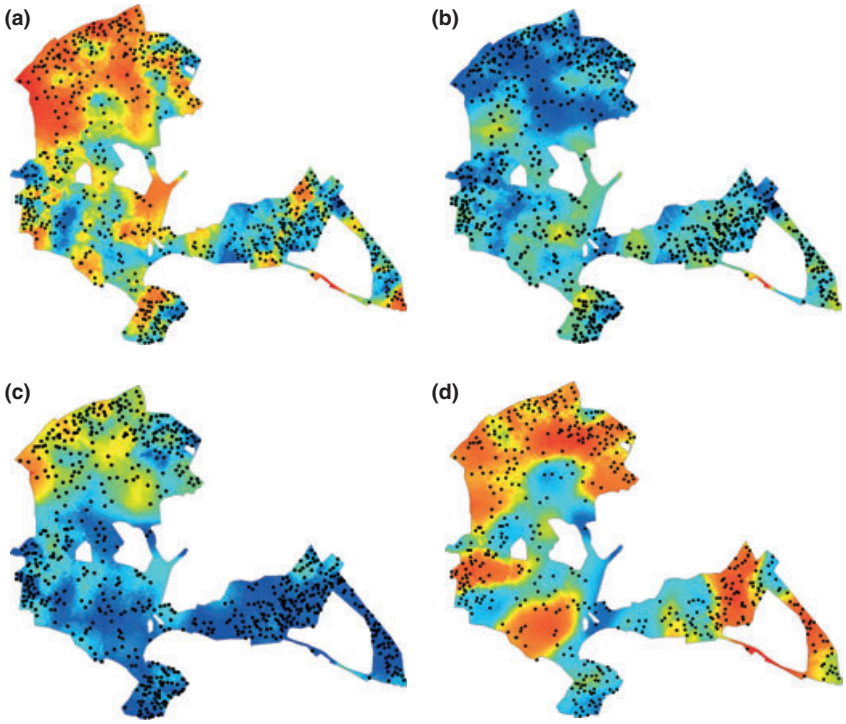

Fig. 3 Interpolations of (a) pooled *Plasmodium* prevalence (b) *P. relictum* prevalence (c) *P. circumflexum* prevalence and (d) parasitaemia among infected individuals (ln(1 + *Plasmodium* DNA copies), as detected by qPCR in the Wytham Woods blue tit population between 2005 and 2008. Areas of high prevalence or parasitaemia appear as red, with orange, yellow, green, pale blue and dark blue indicating areas of decreasing prevalence or parasitaemia.

stant rate of infection loss between years, and scenario 4 incorporated both age-dependent infection risk and infection loss between years. In scenarios incorporating age dependency of infection risk, this was assumed to be 0.372 in the first year of life and 0.214 subsequently, based on longitudinal data. Where applicable, infection loss was included at a rate of 13.4% per year, again based on findings from longitudinal data. Scenarios 2 and 3 both show improved fit to observed data compared to scenario 1, although there is still some evidence for lack of fit under scenario 2 (Fig. 2b; goodness of fit tests: scenario 2 $\chi^2_5 = 10.61$, $P = 0.058$, scenario 3 $\chi^2_5 = 7.93$, $P = 0.160$). Under scenario 4, where both age dependency of infection and infection loss are incorporated, there is no significant discrepancy with observed data (Fig. 2b; $\chi^2_5 = 4.27$, $P = 0.512$).

Cross-sectional analyses of parasitaemia

Mean parasitaemia (among infected individuals only) varied markedly between years (Fig. 5a, Table 3). These temporal fluctuations did not differ between *Plasmodium* species (Table 3) and did not mirror annual changes in prevalence (cf. Figs 1a, b and 5a). For both species, parasitaemia was positively predicted by host age (Fig. 5b, Table 3). Clutch size also predicted para-

Table 2 Proportional rates of agreement in malaria infection status, infection loss and infection gain across three different timescales: when the same sample was tested twice, and when individuals were tested approximately 2 weeks apart or across years

Parasite	Same Sample	2 weeks apart	Across years
All *Plasmodium*			
Agreement rate	0.85	0.81	0.73
Loss	0.16 (0.11, 0.23)	0.25 (0.17, 0.34)	0.30 (0.23, 0.38)
Gain	0.14 (0.10, 0.20)	0.15 (0.10, 0.22)	0.24 (0.18, 0.31)
P. relictum			
Agreement	0.87	0.82	0.73
Loss	0.24 (0.18, 0.30)	0.44 (0.32, 0.58)	0.50 (0.40, 0.60)
Gain	0.09 (0.07, 0.12)	0.09 (0.06, 0.15)	0.16 (0.11, 0.22)
P. circumflexum			
Agreement	0.94	0.95	0.88
Loss	0.17 (0.12, 0.24)	0.11 (0.05, 0.23)	0.31 (0.20, 0.45)
Gain	0.04 (0.03, 0.06)	0.04 (0.02, 0.08)	0.08 (0.05, 0.13)

Agreement rate is given by the proportion of diagnoses that were identical. Rates of loss and gain are given with 95% confidence intervals.

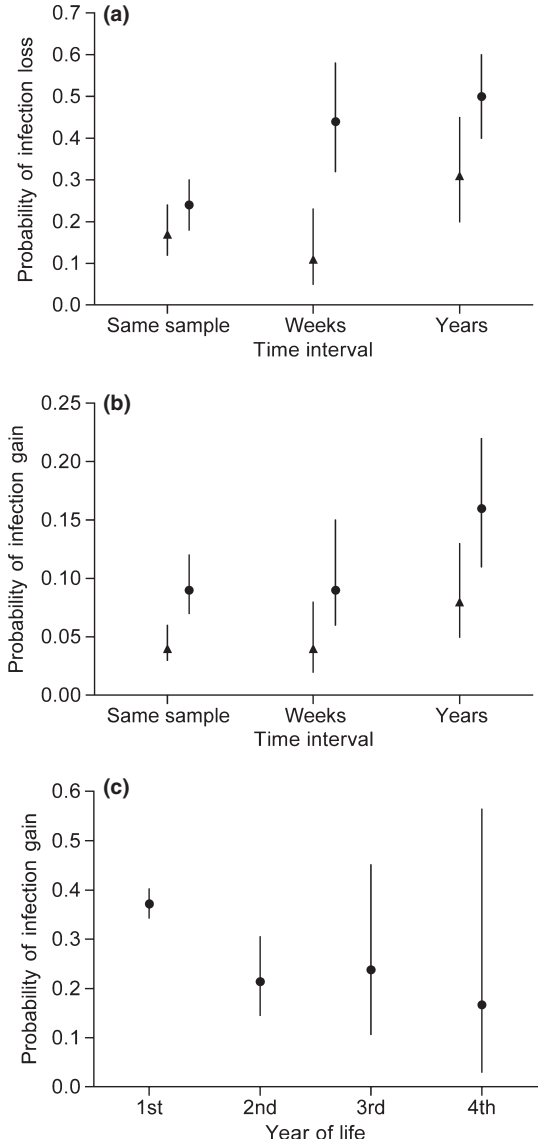

Fig. 4 Probability of *Plasmodium* infection (a) loss and (b) gain across different temporal timescales: when the same blood sample was tested twice, or when birds were tested across either an approximately 2-week period or across years. Mean probabilities are plotted with 95% confidence intervals for *Plasmodium relictum* (circles) and *P. circumflexum* (triangles). Symbols for the two species are offset for clarity. (c) Probability of infection gain according to host age.

sitaemia, such that birds with a larger clutch size tended to have higher infection intensity (Fig. 5c; Table 3). A significant effect of nestbox y coordinate indicated the presence of broad-scale spatial variation in parasitaemia, with infection intensity tending to be higher in the north of the woodland (Table 3). However, an interpolated map of parasitaemia indicated a heterogeneous distribution of heavily infected birds, rather than any consistent spatial trend (Fig. 3d). Spatial variation in parasitaemia did not obviously relate to spatial variation in prevalence, as mean parasitaemia did not predict prevalence across arbitrarily defined 0.25 km^2 grid squares ($F_{1,24} = 0.26$, $P = 0.613$, $r = 0.104$; analysis weighted by N individuals in each grid square, including only grids with ≥10 individuals). However, several local environmental characteristics predicted parasitaemia, including the number of oaks near a nestbox (Oaks50), as well as the interaction between EDI and altitude; birds breeding in oak-rich territories had lower parasitaemia (Fig. 5d, Table 4), and the interaction between EDI and altitude indicated that whereas near the woodland edge altitude had little effect on parasitaemia (which may be because there is little altitudinal variation here), in the centre of the woodland birds breeding at higher altitude had lower parasitaemia (Table 4). All interactions between these environmental variables and *Plasmodium* species were non-significant (Oaks50*Species $F_{1,601} = 0.63$, $P = 0.427$, EDI*Species $F_{1,601} = 1.02$, $P = 0.312$, Altitude*Species $F_{1,601} = 1.77$, $P = 0.184$, EDI*Altitude*Species $F_{1,600} = 0.00$, $P = 0.969$). Because tits at the edge of this woodland are more likely to be immigrants (Wilkin *et al.* 2007a), we tested whether immigrant status (whether a bird was born and ringed in a local nestbox) could explain the edge effect detected. However, immigrant status did not predict parasitaemia ($F_{1,588} = 1.84$, $P = 0.176$) and its inclusion did not influence the edge effect.

Longitudinal analyses of parasitaemia

Repeatability of parasitaemia decreased as the time interval between tests increased, with $r = 0.83$ for the same DNA extraction tested twice, $r = 0.51$ among infected samples from females taken approximately 2 weeks apart, and $r = 0.39$ for samples taken from the same individual across one or more years. Among individuals infected in multiple years, we performed a variance component analysis to examine the relative importance of individual identity and year in explaining parasitaemia variation. Significance for both random effects was assessed using log-likelihood ratio tests, that is, by testing the change in deviance when each random effect was excluded from the full model against the chi-squared distribution. Whereas year explained a relatively small proportion of the variance in parasitaemia (2.9%; $\chi^2_1 = 3.73$, $P = 0.053$), individual identity explained over a third of the variance in this trait (38.3%; $\chi^2_1 = 21.59$, $P < 0.001$).

To assess whether within-individual increases in parasitaemia could underlie the cross-sectional pattern of increasing parasitaemia across age groups, we tested the effect of minimum host age in a mixed model containing individual identity as a random effect, including

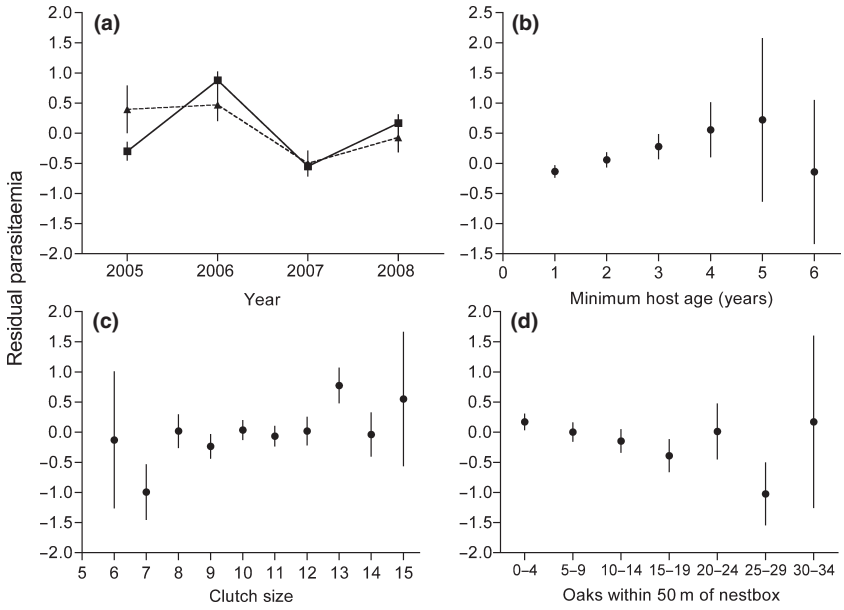

Fig. 5 Predictors of parasitaemia among *Plasmodium*-infected blue tits at Wytham Woods: (a) Year (b) Minimum host age (c) Clutch size and (d) Oak richness of breeding location, measured as the number of oaks within a 50 m radius of the nestbox. In (a), the within-individual pattern of annual changes in parasitaemia (triangles) is shown alongside the cross-sectional pattern (squares). In all plots, means and standard errors are plotted and data are residuals from the appropriate minimal model excluding only the factor of interest (see Tables 3 and 4 for details of covariates). Data for longitudinal patterns in (a) were derived from a model including minimum host age as a fixed effect and individual identity as a random effect, amongst all individuals infected on more than one occasion.

data from individuals with parasitaemia estimates from at least two years (n = 191 samples from 90 individuals). Minimum age was significant in this model ($F_{1,121}$ = 4.21, P = 0.043), suggesting parasitaemia increases within individuals as they age. The magnitude of this within-individual age effect was similar to that seen for host age in cross-sectional analysis (r = 0.132 vs. r = 0.101 respectively). Although it is theoretically possible that such an effect could arise from age-related selective appearance or disappearance of individuals with high or low parasitaemia (van de Pol & Verhulst 2006), we found no convincing evidence this was the case (see Data S1 in Supporting information). This model also retained a significant effect of year ($F_{3,150}$ = 4.03, P = 0.009) indicating that parasitaemia fluctuated within individuals across years, in a pattern very similar to annual differences in parasitaemia observed in cross-sectional analyses (Fig. 5a).

Discussion

Analysis of individual variation in avian malaria infection status and parasitaemia within a single wild bird population revealed a complex set of environmental and host influences underlying these traits. Longitudinal analyses further showed that these infections, although thought to be chronic and long lasting, show marked temporal dynamics within naturally infected wild hosts.

Spatiotemporal variation in prevalence and parasitaemia

Annual changes in prevalence were pronounced in this population, and were mirrored by those occurring in yearlings, suggesting that annual differences in parasite transmission drive these effects. However, both temporal as well as spatial prevalence patterns differed notably for two common species of *Plasmodium*, with *P. relictum* showing marked annual fluctuations and a southerly distribution, whilst *P. circumflexum* exhibited more stable prevalence across years and a northerly distribution (Figs 1a, b and 3). Parasitaemia also showed marked interannual and spatial variation within the population, yet these patterns did not obviously parallel those seen for prevalence (cf. Figs 1a, b, 5a and 3a, d). Thus, although it has been shown previously (Bentz *et al*. 2006), and in the present study, that parasitaemia affects the probability of detecting *Plasmodium* infections, this effect appears not to be sufficient to drive patterns of prevalence in this population, at least when employing the relatively sensitive diagnostic method of qPCR. Indeed, the differences in temporal and spatial patterns of prevalence and parasitaemia found here,

Table 3 Factors predicting parasitaemia among *Plasmodium*-infected individuals in the population (n = 640)

Predictor	df	F	P	r
Minimum age	1	6.14	**0.014**	0.098
Minimum age^2	1	0.02	0.901	−0.006
Year	3	12.31	**<0.001**	
Clutch size	1	7.63	**0.006**	**0.109**
HD_{std}	1	0.54	0.464	−0.029
Sex (female)	1	0.17	0.680	−0.016
Species (*P. relictum*)	1	0.17	0.684	0.016
x	1	2.83	0.093	0.067
y	1	5.10	**0.024**	**0.089**
x*y	1	0.00	0.954	0.002
Minimum age*Species	1	0.00	0.951	0.003
Minimum age^2 *Species	1	0.82	0.366	0.037
Year*Species	3	1.84	0.139	
HD_{std}*Species	1	0.37	0.543	−0.024
Sex*Species	1	0.67	0.413	−0.033
x*Species	1	0.16	0.686	−0.016
Clutch size*Species	1	0.70	0.402	−0.034
y*Species	1	1.81	0.179	−0.054
x*y*Species	1	1.38	0.240	0.047

All variables in the starting model are listed, with effects in the minimal model in bold. For significant effects (P < 0.05), statistics are from the minimal model; for non-significant effects, statistics are presented from the last model that included this term. Effect sizes (Pearson's r) are given for all effects with a single degree of freedom.
HD_{std}: standardized hatch date, calculated as described in methods; x and y are the geographical coordinates of an individual's nestbox.

Table 4 Local environmental variables predicting parasitaemia among *Plasmodium*-infected blue tits in Wytham Woods (n = 637)

Predictor	df	F	P	r
Year	3	12.95	**<0.001**	
Minimum age	1	6.36	**0.012**	**0.100**
Clutch size	1	7.61	**0.006**	**0.109**
EDI	1	5.97	**0.015**	**0.097**
Altitude	1	5.07	**0.025**	**−0.089**
EDI*Altitude	1	4.18	**0.041**	**−0.081**
Oaks50	1	5.33	**0.021**	**−0.092**
Territory size	1	0.17	0.684	−0.017

All variables in the starting model are listed, with effects in the minimal model in bold. For significant effects (P < 0.05), statistics are from the minimal model; for non-significant effects, statistics are presented from the last model that included this term. Effect sizes (Pearson's r) are given for all effects with a single degree of freedom.

combined with differences in other predictors found for parasitaemia but not prevalence (Tables 1 and 3), suggest quite different biological processes underpin variation in these two traits. The spatial prevalence patterns found here mirror those from a previous study on this population (Wood *et al.* 2007), which showed that for *P. circumflexum* and pooled *Plasmodium* species, prevalence was highest in the northwest of Wytham, closer to the adjacent River Thames (Fig. 3a, c). Recent findings suggest mosquito abundance is highest in this area (R. Alves *et al.* unpublished data), and it seems likely that variable exposure to infective vectors, for example determined by the abundance of preferred larval breeding habitats plays a key role in driving this fine-scale spatial variation in malaria prevalence. If *P. relictum* and *P. circumflexum* are transmitted by vector species that differ in habitat requirements (a possibility supported by laboratory-based vector competency studies of these species; Valkiūnas 2005), this could explain their contrasting spatiotemporal patterns. However, spatial variation in prevalence may not only reflect environmental variation in transmission potential, but also potentially host-driven processes such as non-random dispersal (e.g. Garant *et al.* 2005) with respect to infection status, a possibility that warrants further investigation.

Whereas spatiotemporal prevalence patterns clearly differed between *Plasmodium* species, the factors predicting parasitaemia showed no species-specificity. Thus, whereas parasite-specific processes, such as particulars of transmission biology, appear to influence whether a host becomes infected, individual parasitaemia may depend more on host characteristics such as immunological status or genetic resistance, or environmental changes that affect all hosts (and therefore probably both *Plasmodium* species) approximately equally. Investigations of *Plasmodium* parasitaemia in other populations are needed to assess the generality of this finding.

Several descriptors of the local breeding environment predicted parasitaemia in this population, including the oak richness of an individual's territory, with birds on oak rich territories tending to have lower parasitaemia. Oak richness can be considered a measure of territory quality, as it affects nestling diet quality and fledging mass among tits in Wytham (Wilkin 2006; Wilkin *et al.* 2009). Several processes could conceivably underlie a negative correlation between oak richness and parasitaemia, including direct effects of diet quality on immune function (Lochmiller *et al.* 1993), or improved immune function as a result of reduced energetic expenditure required to provision young in high quality habitats (Stauss *et al.* 2005). Consistent with the latter possibility, we found that parents making a larger reproductive effort (those with larger clutch sizes) had higher *Plasmodium* parasitaemia (Fig. 5c). Furthermore, there is consistent published evidence for causal effects of reproductive effort on blood parasitaemia and immune function (Knowles *et al.* 2009). Although higher intrinsic quality of birds inhabiting oak-rich territories (Przybylo

et al. 2001) could alternatively explain the negative association between oak richness and parasitaemia, several results argue against this: high quality individuals may be expected to breed relatively early, in larger territories and produce large clutch sizes, yet none of these factors negatively predicted parasitaemia in this study (see Tables 3 and 4), and in fact the association with clutch size is positive.

Within-individual parasitaemia variation

Parasitaemia showed significant repeatability within infected hosts, and individual identity explained nearly 40% of the variance in this trait among infected individuals between years. There is therefore clear potential for genetic or permanent environmental effects on this trait. However, parasitaemia was also labile within individuals with respect to host age, as well as fluctuating within individuals across years (Fig. 5a). Thus, it seems parasitaemia is sensitive to changes in either host physiological state and/or environmental conditions, though these effects are subtler than the influence of individual identity. The finding that parasitaemia shows parallel annual dynamics within individuals is intriguing and suggests that annually varying environmental factors influence parasitaemia similarly in all individuals and that there may be 'good' and 'bad' years in terms of parasitaemia. One possibility is that immune suppression of malaria infection competes with other demands on hosts that vary between years, such as the need for thermoregulation or foraging effort.

Plasmodium parasitaemia increased across host age cohorts (Fig. 5b) and longitudinal analyses indicated that this effect was largely due to within-individual increases in parasitaemia as they age. These findings are contrary to documented patterns for *Haemoproteus* infections, in which parasitaemia has been shown to decline as hosts get older (Sol *et al.* 2003; Stjernman 2004). Declining parasitaemia among *Plasmodium*-infected humans is also the norm (e.g. Syafruddin *et al.* 2009). Thus, the age-related increase in parasitaemia reported here, although statistically well supported, is unexpected. It may be that declines in parasitaemia reflecting acquired immunity would be observed on a shorter timescale than across years, or only among individuals that are able to control or eventually clear infections, which appears to be possible (at least for *P. relictum,* see below) on much shorter timescales. The pattern of increasing parasitaemia with age cannot therefore be interpreted as a lack of effective acquired immunity against these parasites. Indeed, clear evidence for development of immunity against avian *Plasmodium* parasites exists in the experimental literature (Atkinson *et al.* 2001; Graczyk *et al.* 1994). One possibility is that an age-related increase in parasitaemia reflects a process of immunosenescence among individuals that maintain chronic infections. Age-related declines in several measures of cell-mediated and humoral immune responsiveness have been documented in birds (Lavoie 2005; Palacios *et al.* 2007).

Within-individual changes in infection status

Through longitudinal analysis, malaria infection status within individuals was shown to be non-static, with infections disappearing from the blood across both short (2 week) and long (across years) timescales. Whilst accounting for detection failure, up to 26% of patent *P. relictum* infections were lost on a fortnightly or between-year basis. If these effects were due simply to parasitaemia fluctuating such that infections sometimes drop below our assay's detection limit, we would expect infections to appear across the same timescales they disappear. However, this was not observed (cf. Fig. 4a, b for *P. relictum* across weeks), suggesting the process of infection disappearance we observe is one-way. Several processes could explain the apparent loss of infections, including (i) clearance of infection, i.e. sterilizing immunity (ii) persistence of blood-stage parasites below the assay detection threshold or (iii) parasite sequestration within fixed tissues, which is known to occur for both *P. relictum* and *P. circumflexum* in experimental infections (Valkiūnas 2005) and is strongly suggested by seasonal prevalence patterns in wild populations (Cosgrove *et al.* 2008). Long-term monitoring of experimental *Plasmodium* infections in canaries has shown that although parasitaemia often becomes microscopically undetectable for weeks or months, parasites are still present, as naïve hosts inoculated with blood from such individuals often develop infections (Manwell 1934). Regardless of how infections disappear from the blood, a consequence is that a significant undetectable reservoir of exposed and potentially infective individuals may be missed when infections are diagnosed solely using DNA- or microscopy-based detection of blood-stage parasites. The dynamic nature of infection status within individuals, coupled with the pronounced temporal and fine-scale spatial effects on prevalence reported here, suggest single time-point assessments of infection status may provide a poor indicator of host genetic resistance to avian malaria in wild birds. Measuring and accounting for variation in exposure will therefore be critical when attempting to measure resistance in natural settings. One promising approach would be to measure antibody levels (Atkinson *et al.* 2001; Jarvi *et al.* 2002) as well as parasitaemia, and thus permit a comparison of infection intensity among individuals known to have been exposed.

Although prevalence increased across age cohorts in this population (Table 1, Fig. 2a), the risk of becoming infected declined with host age: whilst infection risk in the first year of life was nearly 40%, infection risk in subsequent years was approximately halved (Fig. 4c). This reduced infection risk among older hosts could have a number of explanations. One possibility is that some individuals are resistant to infection and thus never develop chronic infections. A recent study has demonstrated that for *P. relictum* (cyt *b* lineage pSGS1), hosts show marked inter- and intraspecific variation in resistance to experimental infection (Palinauskas *et al.* 2008). The recovery of several Hawaiian honeycreeper species from the threat of extinction and re-establishment in areas of high malaria prevalence also highlights the potential for intraspecific variation in resistance to avian malaria (Foster *et al.* 2007). Consistent individual differences in behaviour, such that some individuals persistently avoid infection whilst others are quickly infected, could also play a role in this pattern. The potential for such processes in a population like that studied here is considerable, given that there is strong spatial variation in the risk of infection and that blue tits show high breeding site fidelity after postnatal dispersal, with around 25% breeding in the same nestbox in multiple years and over 90% in the same broad sector of the woodland (B.C. Sheldon, unpublished data). The stronger age-dependency in infection risk for *P. circumflexum* compared to *P. relictum* found here is consistent with such a behavioural explanation, as the spatial distribution of *P. circumflexum* prevalence is far more repeatable across years compared to *P. relictum* (S. Knowles, unpublished data). Experimental infection studies on a wider range of host species are needed to provide further insight into the extent of intraspecific variation in resistance to avian malaria. When combined, the two processes of infection loss and age-dependency of infection risk were able to account for the observed quadratic relationship between host age and prevalence found in cross-sectional analysis. This suggests that additional effects of infection-related mortality or dispersal are not necessary to explain this age-prevalence pattern, although dedicated survival analysis would be required to test definitively for such processes.

Conclusion

The findings here illustrate the ecological complexity of avian malaria epidemiology that can be observed within a single host population. Strong spatiotemporal effects as well as host factors influenced within-population heterogeneity in both infection status and parasitaemia. Thus, both these infection traits are underpinned by a combination of environmental and host factors, and simplistic assumptions about prevalence reflecting either exposure or intrinsic resistance among individuals or populations, are unrealistic. Moreover, infections within individuals were clearly dynamic, with a significant proportion of patent infections (over 20% for one *Plasmodium* species) disappearing from the blood on both short and long timescales, despite the use of sensitive qPCR diagnostics. These dynamics imply that population surveys using single time-point assessments of infection may often fail to recognize a reservoir of exposed, and potentially infective, individuals. The ability to distinguish between unexposed and exposed but immune individuals is likely to be critical for future studies of these parasites in wild populations, and efforts to further develop methods for achieving this should be a priority.

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Supporting information

Additional Supporting information may be found in the online version of this article.

Table S1 Comparison of *Plasmodium* detection by the nested PCR method of Waldenström *et al.* (2004), and a novel quantitative PCR assay, among samples tested by both methods

Table S2 Results from GLMM modelling of *Plasmodium* infection status (prevalence) variation across the Wytham blue tit population

Fig. S1 Increase in cumulative *Plasmodium* prevalence as more qPCR tests are performed. Means and 95% confidence intervals are shown.

Data S1 Supplementary information.

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