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Within-population variation in prevalence and lineage distribution of avian malaria in blue tits, *Cyanistes caeruleus*

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Abstract

The development of molecular genetic screening techniques for avian blood parasites has revealed many novel aspects of their ecology, including greatly elevated diversity and complex host–parasite relationships. Many previous studies of malaria in birds have treated single study populations as spatially homogeneous with respect to the likelihood of transmission of malaria to hosts, and we have very little idea whether any spatial heterogeneity influences different malaria lineages similarly. Here, we report an analysis of variation in the prevalence and cytochrome *b* lineage distribution of avian malaria infection with respect to environmental and host factors, and their interactions, in a single blue tit (*Cyanistes caeruleus*) population. Of 11 *Plasmodium* and *Haemoproteus* cytochrome *b* lineages found in 997 breeding individuals, the three most numerous (pSGS1, pTURDUS1 and pBT7) were considered separately, in addition to analyses of all avian malaria lineages pooled. Our analyses revealed marked spatial differences in the prevalence and distribution of these lineages, with local prevalence of malaria within the population ranging from over 60% to less than 10%. In addition, we found several more complex patterns of prevalence with respect to local landscape features, host state, parasite genotype, and their interactions. We discuss the implications of such heterogeneity in parasite infection at a local scale for the study of the ecology and evolution of infectious diseases in natural populations. The increased resolution afforded by the combination of molecular genetic and geographical information systems (GIS) tools has the potential to provide many insights into the epidemiology, evolution and ecology of these parasites in the future.

Keywords: avian malaria, *Haemoproteus*, host–parasite interactions, landscape ecology, *Plasmodium*, vector-borne disease

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Introduction

The ecological context of host–parasite interactions can have marked effects on the transmission and persistence of disease. Abiotic factors, such as microclimate and landscape, can influence the transmission stages of parasites and therefore the prevalence of host infection, in addition to biotic effects such as host age, sex and population density (Combes 2001; Wilson *et al*. 2001). An understanding of the influence of landscape ecology on host–parasite interactions in wild populations is of particular relevance in a changing world of climate change and habitat fragmentation. However, landscape ecology has only occasionally been considered in ecological studies of disease: there may therefore be significant gaps in our understanding of host–parasite ecology, as such effects may not be apparent in small-scale population studies (May 1999). In contrast, the importance of the landscape in which a host–parasite interaction occurs has been increasingly studied in human malaria, revealing associations between malaria infection and factors such as altitude and proximity to water in human populations (Foley *et al*. 2003; Balls *et al*. 2004; Omumbo *et al*. 2005). Furthermore, the risk of mosquito-borne infection may be higher at the edge of a host population, if infective vectors seek out an area of high host density (Ribeiro *et al*. 1996; Smith *et al*. 2004).

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Host factors such as age, sex and host population density may also influence host parasite infection. Prevalence may increase with age as new infections accumulate, then decrease as susceptible individuals die or resistant individuals become immune (Wilson *et al*. 2001). Male mammals and birds tend to have a higher prevalence of infection than females (Poulin 1996; Schalk & Forbes 1997; McCurdy *et al*. 1998). Population density may also influence the risk of infection, depending on how parasite transmission relates to host population density (Keymer & Anderson 1979). Spatiotemporal variation in parasite infection has often been supposed to contribute to the maintenance of genetic variation in host resistance to parasites, but only rarely has it been studied in wild populations (Lively & Dybdahl 2000; Bensch & Åkesson 2003). Ideally, the influence of these processes needs to be studied against the background of environmental variation due to abiotic factors, since there may also be interactions between biotic and abiotic factors.

Avian malaria, *Plasmodium* and *Haemoproteus* spp. (*sensu* Pérez-Tris *et al*. 2005; see also Valkiūnas *et al*. 2005), is a vector-borne disease, avian *Plasmodium* being transmitted primarily by mosquitoes (genera *Culex*, *Aedes* and *Culiseta*) and *Haemoproteus* by biting midges (Ceratopogonidae) and louse flies (Hippoboscidae) (Valkiūnas 2005). These parasite taxa are globally distributed (Valkiūnas 2005; Beadell *et al*. 2006), and our understanding of their diversity, ecology, and relationships with their avian hosts has been increased by the application of molecular genetic screening techniques to blood samples collected from wild hosts. For example, estimates of global species diversity of the order of 200 species based on microscopy, have been suggested to need revision to somewhere in the order of 10 000 species based on comparisons of nuclear and mitochondrial gene trees (Bensch *et al*. 2004). However, the majority of ecological studies of malaria have not considered either this diversity, a potentially important source of variation in host–parasite interactions since parasite virulence can vary among parasite lineages (Read & Taylor 2001), or the possibility that prevalence and lineage distribution may vary with local landscape features. The latter is an important consideration, because strong effects of the environment (both biotic and abiotic) mean that the risk of exposure and infection may be very variable for different individuals.

In this study, we examined variation in avian malaria infection with respect to landscape and host factors on a local scale, in a single woodland population of blue tits *Cyanistes caeruleus*. The development of sensitive and accurate molecular diagnostic techniques has allowed the study of avian malaria infections at a fine taxonomic resolution (Jarvi *et al*. 2002; Fallon *et al*. 2003; Waldenström *et al*. 2004), while the development of geographical information systems (GIS) allows ecological phenomena to be considered at a fine geographical scale. We integrated these techniques to examine associations between infection and a range of landscape and host features for separate lineages, as well as for all lineages pooled. We report marked differences in the prevalence of malaria with respect to lineage, landscape features, host characteristics, and the complex interactions among these factors.

Materials and methods

Host and parasite

Approximately 1160 nestboxes are monitored in Wytham Woods (51°46′N, 1°20′W), a 385-ha woodland near Oxford, UK, where 250–450 pairs of blue tits breed annually (Perrins 1979). In this study, we report the analysis of blood samples collected in 2001 and 2003–2005, all of which were collected from adult blue tits captured between day 6 and day 14 of the nestling phase, either within the nestbox by hand or using traps, or with mist nets in front of the nest entrance. We thus analyse samples here that were all collected from hosts at the same point in their annual cycle; as the study population is single-brooded and breeds with a great degree of synchrony, there is little variation in the calendar date among samples. Sampling date was therefore not related to variation in the age of nestlings. Host sex was determined based on the presence (female) or absence (male) of a brood patch, age (1 year, or older) was determined using plumage characteristics (Svensson 1992). A total of 997 blue tits over four breeding seasons were included in the analyses of the associations between breeding landscape, host factors and avian malaria infection. To avoid pseudoreplication in cases where an individual bird was sampled in more than 1 year, one sample was randomly chosen; therefore, each individual appears only once in the current analysis.

Avian malaria diagnosis

Blood samples were taken, under licence, by ulnar or jugular venepuncture. Samples were stored in Queen's lysis buffer (Seutin *et al*. 1991), and DNA extracted using a DNeasy Extraction Kit (QIAGEN). An assessment of the presence/quality of extracted DNA was made by electrophoresing 2 μL of the extract in 2% agarose containing ethidium bromide, and visualizing it under UV light. The samples were screened for the presence of *Plasmodium* and *Haemoproteus* using a nested polymerase chain reactions (PCR) protocol (Waldenström *et al*. 2004), which amplifies a 478-bp fragment of the mitochondrial cytochrome *b* gene. The PCR tests were performed in 25 μL volumes, in two separate rounds with positive (*Plasmodium relictum* DNA) and negative (ddH20) controls. To avoid contamination, different pipettes and different sections of the laboratory were used for pre- and post-PCR work. Our subsequent screening protocols now use one negative control for every 15 samples (one every two rows of a PCR plate), and have

found no contamination in approximately 500 samples, indicating that contamination has not occurred during our screening procedures to any detectable extent. The first-round primers were HaemNF (5′-CATATATTAAGAGAATTATGGAG-3′) and HaemNR2 (5′-AGAGGTGTAGCATATCTATCTAC-3′). Each reaction contained 2 μL of genomic DNA, 0.125 mM each dNTP, 0.2 μM each primer, 3 mM $MgCl_2$ and 0.25 U of Platinum *Taq* Polymerase (Invitrogen) with the accompanying PCR buffer at 1× final concentration. The thermal profile consisted of a 2-min, 94 °C enzyme activation step, followed by 20 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s, ending with an elongation step of 72 °C for 10 min. In the second round of PCR, primers HaemF (5′-TGGTGCTTTCGATATATGCATG-3′) and HaemR2 were used (5′-GCATTATCTGGATGTGATAATGGT-3′). The composition of the PCRs was as above, except 0.4 μM of each primer and 0.5 U of Platinum-*Taq* Polymerase were used, and 2 μL of the PCR product from the first round was used as template instead of genomic DNA. The thermal profile for the second round PCR was the same as that for the first, except the number of cycles was increased from 20 to 35. 2–8 μL of PCR products from the second round were run on 2% agarose stained with ethidium bromide and visualized under UV light. Samples containing bands of 450–600 bp in size were prepared for sequencing using QIAGEN MinElute 96 UF PCR Purification Kits and QiaVac Multiwell vacuum manifolds. Purified PCR fragments were sequenced directly by dye terminator cycle sequencing (BigDye version 3.1), and loaded on an ABI PRISM 310 automated sequencer (Applied Biosystems). Sequences were edited in SEQUENCHER version 4.2 (GeneCodes), and aligned in CLUSTAL_X (Thompson *et al*. 1997). All lineage assignments included in our analyses showed a clean sequence across at least 450 bp of the 478 bp cytochrome *b* gene fragment examined in this protocol. Any unreadable sequences were not assigned to a particular lineage and excluded from analyses. Lineages pTURDUS1 and pBT7 differed by just 1 bp (0.25% of 478 bp): pBT7 infections showed a consistent C/T polymorphism from pTURDUS1 infections at exactly the same site, which was clear from the examination of sequences. Sequences corresponding to *Plasmodium* or *Haemoproteus* from known alignments were scored as positive for avian malaria. Sequences corresponding to *Leucocytozoon* sequences were scored as negative for the purposes of this study, and are not considered further here. Based on the occurrence of double peaks in electropherograms, mixed infections were present at a low rate (2.2% in 2004–2005; S. C. L. Knowles, C. L. Cosgrove, M. J. Wood, B. C. Sheldon, unpublished) and are not considered further here. The use of molecular diagnosis techniques without microscopic examination of blood smears would appear to be justified in this study, as cytochrome *b* lineage diversity has been revealed within avian malaria morphological species, and pilot data from this blue tit population suggest that the intensity of *Plasmodium* infection is usually below the limit of detection (one infected cell in 10 000) using microscopy (S. C. L. Knowles *et al*. unpublished). Phylogenetic relationships among parasite lineages were estimated using samples for which we had at least 450 bp of cytochrome *b* sequence. We used a mammalian malaria sequence (*Plasmodium reichenowi*, GenBank Accession no. AF069610), as an outgroup to root the tree. The neighbour-joining method was employed with a Kimura 2-parameter distance matrix in MEGA 2.1 (Kumar *et al*. 2001).

Measurement of landscape using GIS techniques

A GIS of the study site was constructed in 2005 (Wilkin *et al*. 2007). This system allowed us to plot accurately the location of each breeding blue tit's nest. Blue tits feed their offspring on invertebrate prey, and forage in the immediate vicinity of their nest (Stauss *et al*. 2005); hence using the nestbox to represent location for breeding birds is justified. Other topographical features from UK Ordnance Survey data were incorporated into the GIS: the shortest distance (m) between each nestbox and (i) the woodland edge and (ii) the River Thames (Fig. 2a) was calculated using GIS software (MAPINFO Professional Version 7.8). As an initial test for broad spatial differences in prevalence, and the distribution of the different malaria lineages, we categorized individual nestboxes based on the woodland section to which they belonged. The population studies of tits in Wytham Woods have traditionally been divided into nine separate areas (sections, Fig. 2a) for the purposes of delimiting different parts of the study area (see Garant *et al*. 2005 for an example). The sections are arbitrary delineations of the study area with respect to malaria, and consequently they provide a means to partition the population into sections in order to test for differences in prevalence within the population as a whole. Sampling effort by woodland section and year is shown in Table 1.

To visualize spatial variation in avian malaria infection within the study population, malaria prevalence was interpolated between nestboxes (Fig. 2c–f) using inverse distance weighting (IDW). This technique uses a moving point average to interpolate pixel values and estimate local trends between spatially discontinuous and highly variable data, in this case infection status of breeding adult blue tits sampled at nest boxes. 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). In this case, the radii of the search and display areas were four times the average point density (400 m), as in Garant *et al*. (2005). While these maps do not generate statistic inferences, they remain a useful tool

Table 1 Sampling effort between woodland sections and sampling years

	Year				
Wood section	2001	2003	2004	2005	Total
B	23	19	50	58	150
C	37	13	22	30	102
CP	10	18	35	57	120
E	24	1	24	51	100
MP	19	12	32	45	108
O	22	9	73	76	180
P	0	7	16	18	41
SW	19	7	28	27	81
W	15	8	43	49	115
Total	169	94	323	411	997

Breeding blue tits were sampled in nine woodland areas in 4 years (2001, 2003–2005). The woodland sections are arbitrary divisions of the study site providing a means of partitioning the population into sections for an initial examination of spatial variation in avian malaria prevalence. Boundaries of woodland sections are shown in Fig. 2a.

for identifying potential spatial trends to inform subsequent statistical analyses. In order to estimate territory size (and hence population density), tessellations (Thiessen or Voronoi polygons) were formed around each breeding pair by placing boundary lines equidistant between occupied nestboxes in each year. The area of these polygons is necessarily inversely related to breeding density (Wilkin *et al*. 2006). As blue tits show a high degree of sharing of avian malaria lineages with great tits *Parus major* (no significant difference in lineage assemblages: C. L. Cosgrove, M. J. Wood, S. C. L. Knowles, K. P. Day, B. C. Sheldon, unpublished), boxes occupied by both species were included so that tessellated territory size was a measure of interspecific density. Blue tits and great tits are territorial during the breeding season (March to June), but more loosely associated with the territory for the rest of the year (Perrins 1979). While tessellated territory size is a geometric construct, it has been shown to be a useful measure of breeding density in tits at Wytham, with strong relationships to many density-dependent life-history characters (Wilkin *et al*. 2006). Because it is calculated on an individual basis, this measure is an improvement on other methods such as distance to the nearest neighbour or the number of pairs per unit area (Orell & Ojanen 1983; Both & Visser 2000). We estimated the parasite phylogenetic relationships among parasite lineages using samples for which we had at least 450 bp of cytochrome *b* sequence. We used a mammalian malaria sequence, *P. reichenowi* as an outgroup for rooting the tree (GenBank Accession no. AF069610). We used the neighbour-joining method with a Kimura 2-parameter distance matrix in MEGA 2.1 (Kumar *et al*. 2001).

Statistical analysis

Generalized linear modelling (GLZ) was performed to assess associations between landscape and host predictors on the presence or absence of infection in individual birds, with avian malaria as a whole (all 11 lineages pooled) and infection with the three most numerous avian malaria lineages separately (see below), using binomial errors and a logit link. Starting models were optimized by backward stepwise elimination of nonsignificant terms, beginning with three-way interactions and progressing to single order predictors. Terms were deleted from the model if their removal caused a nonsignificant change in deviance ($P > 0.05$). Nonsignificant factors were necessarily retained in final models where a higher–order interaction involving that factor was retained. In landscape analyses, potentially nonlinear relationships between infection status and host and landscape/host covariates were considered in statistical analyses using generalized additive modelling (GAM), a generalized linear model in which a smoothed function of a covariate can be modelled alongside linear predictors (Wood & Augustin 2002). Linear covariates retained in final models were substituted for GAM smoothed terms, the latter being retained if they caused a significant reduction in deviance. Step functions, such as a positive linear association followed by a plateau, were similarly considered as an alternative to smoothed function suggesting such a relationship (e.g. Fig. 3).

Individual infection with pooled avian malaria cytochrome *b* lineages, and infection with the three most prevalent lineages (pSGS1, pTURDUS1 and pBT7: 36%, 34% and 16% of all infections, respectively) were used as binary responses for analyses; lineages at lower prevalence (less than 4% of sampled hosts) prohibited the modelling of presence/absence data because modelling algorithms failed to converge. The avian malaria lineages pTURDUS1 and pBT7 are closely related (< 0.25% sequence divergence at cytochrome *b*), being less related to pSGS1 (> 4% sequence divergence: C. L. Cosgrove *et al*. unpublished). All statistical analyses were conducted using R version 2.2.1. Means are presented ± 1 standard error.

Results

The overall prevalence of avian malaria (i.e. *Plasmodium* and *Haemoproteus*) within this sample was 28.4% ($n = 997$), comprising 11 different cytochrome *b* lineages (Table 2). The three most common were pSGS1 (prevalence 10.2%), pTURDUS1 (9.7%), and pBT7 (4.6%); the overwhelming majority of infections were with *Plasmodium*, with only nine individuals (0.9%) infected with *Haemoproteus* (Table 2).

We first visualized the distribution of infection within the study site as a means of informing the statistical analysis, by generating interpolated maps of malaria prevalence

Table 2 Diversity of avian malaria lineages in the Wytham blue tit population

Parasite taxon	Lineage	GenBank Accession no.	N positive	Prevalence (%)
Plasmodium relictum	pSGS1*	AF495571	102	10.2
Plasmodium sp.	pTURDUS1*	AF495576	97	9.7
Plasmodium sp.	pBT7*	AY393793	46	4.6
Plasmodium relictum	pGRW11	AY831748	13	1.3
Plasmodium sp.	pBLUTI1	DQ991068	6	0.6
Plasmodium sp.	pBLUTI2	DQ991072	1	0.1
Plasmodium sp.	pBLUTI4	DQ991070	1	0.1
Plasmodium sp.	pBLUTI5	DQ991071	1	0.1
Pooled *Plasmodium*†	—	—	277	27.8
Haemoproteus sp.	hWW1	AF254971	3	0.3
Haemoproteus minutus	hTURDUS2	DQ060772	2	0.2
Haemoproteus sp.	hBLUTI1	DQ991068	1	0.1
Pooled *Haemoproteus*†	—	—	9	0.9
Plasmodium + *Haemoproteus*	—	—	283	28.4

Based on sequence data from a 478-bp fragment of the mitochondrial cytochrome *b* gene, 11 lineages of avian malaria were detected in a total of 997 blue tit malaria diagnoses. Infections of all 11 avian malaria lineages were pooled for analysis, in addition to infections with lineages with higher than 4% prevalence (*), infection with each of which was analysed separately. The prevalences of pooled lineages of *Plasmodium* and *Haemoproteus* are shown (†), including infections that could not be assigned to a particular lineage and accounting for mixed infections, which were present at a low rate (2.2% in 2004–2005, S. C. L. Knowles, C. L. Cosgrove, M. J. Wood, B. C. Sheldon, unpublished). ‡Cytochrome *b* lineages matched to morphological species (Valkiūnas *et al*. 2007; Hellgren *et al*. 2007). A neighbour-joining tree of these lineages is shown in Fig. 1.

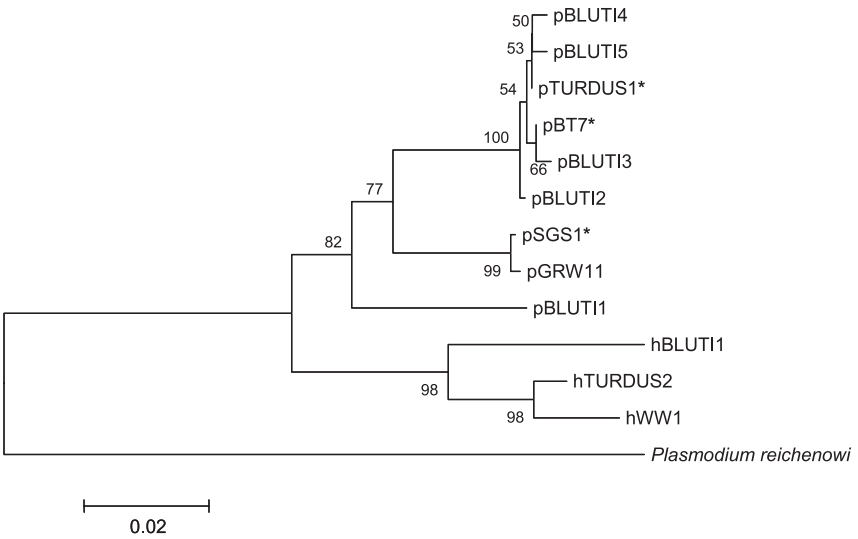

Fig. 1 Neighbour-joining tree of avian malaria lineages in blue tits in Wytham Woods. The phylogenetic relationships among parasite lineages were estimated using samples for which we had at least 450 bp of cytochrome *b* sequence. We used a mammalian malaria sequence, *Plasmodium reichenowi* as an outgroup to root the tree. The neighbour-joining method was used, with a Kimura 2-parameter distance matrix in MEGA 2.1 (Kumar *et al*. 2001).

using GIS software (Fig. 2c–f). These maps indicated that the distribution of avian malaria infection, when all infections were pooled, was concentrated mainly in the northwest of the study area (Fig. 2c). This prevalence mapping procedure also suggested that the three most common lineages showed different distributions: pSGS1 (Fig. 2d) was widely, but patchily distributed, whereas pBT7 (Fig. 2e) and pTURDUS1 (Fig. 2f) were much more restricted to the northern edge of the study site. We tested statistically for broad spatial differences in the prevalence of malaria, and in the prevalence of these three commonest lineages, by testing the effect of woodland section (Fig. 2a) on prevalence. The prevalence of pooled malaria lineages varied markedly between woodland sections (analysis of deviance: $\chi^2 = 71.2$, $P < 0.001$). Considering the three most numerous lineages, prevalence varied both by lineage ($\chi^2 = 28.6$, $P < 0.001$) and woodland section ($\chi^2 = 68.1$, $P < 0.001$). In addition, we tested the lineage*woodland section interaction with respect to prevalence ($\chi^2 = 54.2$, $P < 0.001$), which confirmed that the lineages are differently distributed in space, as suggested by the visual inspection of the interpolated maps.

Generalized linear modelling of infection status of breeding blue tits revealed several complex associations

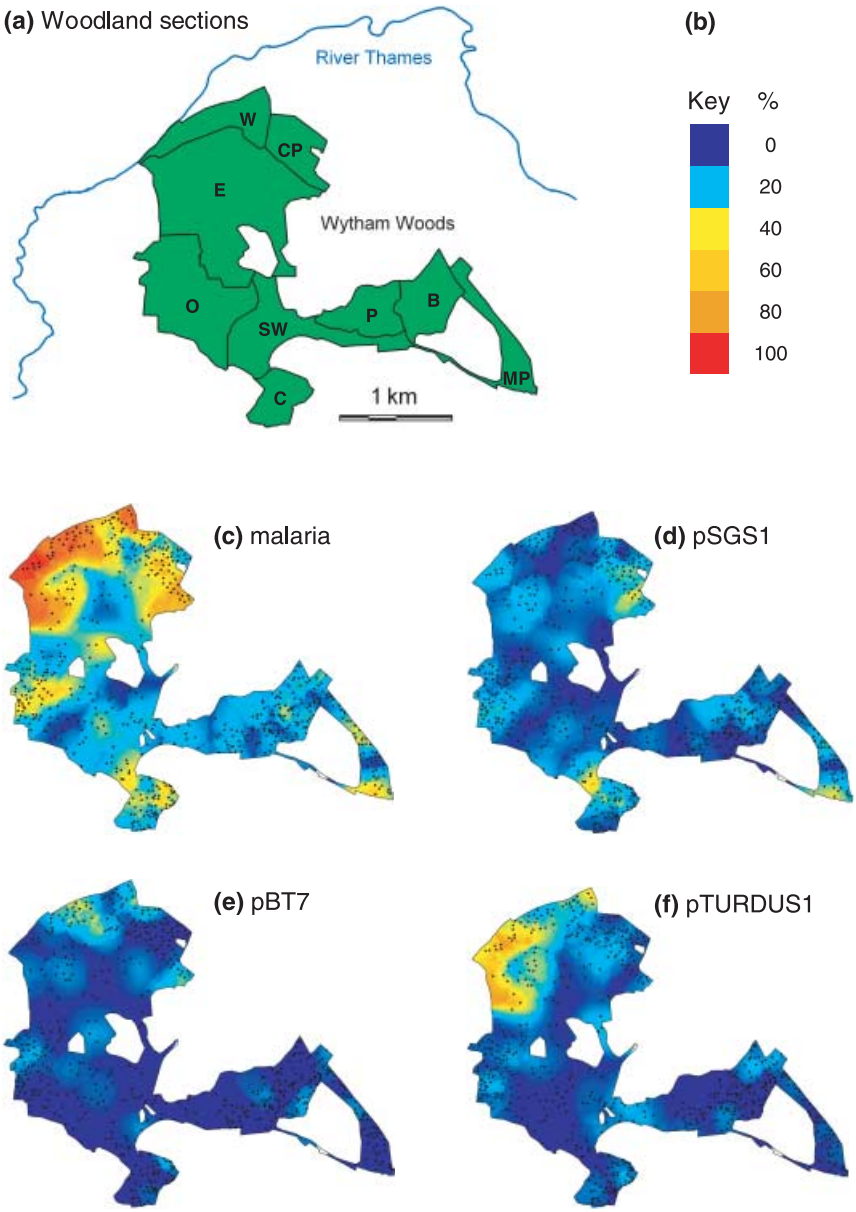

Fig. 2 Interpolated prevalence maps describing within-population variation in avian malaria infection in blue tits. (a) Boundaries of woodland sections used in the initial analysis of spatial variation in prevalence. Interpolated avian prevalence maps are shown with reference to the key (b): (c) all malaria lineages pooled, and the three most common *Plasmodium* lineages; (d) pSGS1, (e) pTURDUS1 and (f) pBT7, as identified by cytochrome *b* lineages. Interpolations maps were generated using inverse distance weightings of infection data from nest box sampling locations, to visualize spatial variation in the prevalence of avian malaria in blue tits (see Methods for details). Data were pooled for the years 2001, 2003–5. Filled circles indicate nest box locations at which sampling was conducted.

between avian malaria infection and both landscape and host factors (Table 3). Infection with two lineages (pTURDUS1 and pBT7), and for all avian malaria lineages pooled, decreased farther away from the nearby River Thames, which runs along the edge of the study site. Smoothed functions (Fig. 3) provided a significantly better fit than a linear or step function in all cases (changes in model residual deviance, $P < 0.05$). Curvilinear relationships with distance from the river (Fig. 3) indicate that the smoothing function is detecting an increase in prevalence in the extreme south and east of the study site (most apparent in the interpolated map of pooled malaria prevalence; Fig. 2c), which are farthest from the river. In contrast, there was no influence of this landscape feature on the prevalence of SGS1 (Fig. 2d).

Pooled avian malaria lineages and pBT7 both showed significant annual variation in prevalence of infection (Tables 3 and 4). Sex was retained as a significant factor only in analyses of pooled avian malaria infection, with higher prevalence for males than for females (Table 3a: male 29.8 ± 1.4%, female 27.0 ± 1.4%). The prevalence of infection was higher in older birds, both for pooled avian malaria and all three analysed lineages (Table 3: pSGS1 first years 8.15 ± 0.87%, older 13.1 ± 1.1%; pTURDUS1 first years 7.97 ± 0.86%, older 12.1 ± 1.0%; pBT7 first years 3.1 ± 0.72%, older 6.7 ± 1.2%). These analyses thus show that the prevalence of malaria is, to some extent, dependent on the location of the sampled individual, its age and sex, and that there may be annual fluctuations in the prevalence of some lineages.

Table 3 Statistical modelling of variation in avian malaria infection in blue tits

Factor	Parameter estimate	*Z*	*P*
(a) Pooled malaria model			
Year	0.37 ± 0.094	3.90	< 0.0001
Age (older)	0.36 ± 0.21	1.30	0.086
Sex (male)	729 ± 240	3.04	0.0024
Year*sex	0.37 ± 0.12	–3.06	0.0025
Age*sex	0.69 ± 0.30	2.32	0.021
Smoothed distance to river: estimated d.f. = 2.72, $\chi^2 = 54.0$, $P < 0.0001$			
Model residual deviance = 1080.4			
(b) pSGS1 model			
Age (older)	2.42 ± 0.15	15.9	< 0.0001
Model residual deviance = 651.9			
(c) pTURDUS1 model			
Age (older)	2.47 ± 0.21	–11.9	< 0.0001
Sex (male)	–0.51 ± 0.33	–1.54	0.12
Age*sex	1.42 ± 0.47	2.97	0.0028
Smoothed distance to river: estimated d.f. = 3.43, $\chi^2 = 74.2$, $P < 0.0001$			
Model residual deviance = 548.6			
(d) pBT7 model			
Year	–5.41 ± 0.82	–6.61	< 0.0001
Age (older)	1.55 ± 0.53	2.97	0.003
Tesselated territory size	0.00085 ± 0.00005	1.57	0.11
Age*tessellated territory size	–0.00016 ± 0.00007	–2.16	0.031
Smoothed distance to river: estimated d.f. = 3.82, $\chi^2 = 33.1$, $P < 0.0001$			
Model residual deviance = 323.7			

The results of generalized linear modelling of infection with pooled avian malaria cytochrome *b* lineages and the three most common lineages (pSGS1, pTURDUS1 and pBT7) are shown; predicted by year, landscape and host factors; using binomial errors and a logit link. Those predictors remaining after model optimization are shown with statistics describing their contribution to the final model.

A number of interaction terms were retained in the final models of avian malaria infection prevalence. In the case of pooled avian malaria lineages, female infection increased over the study period while male infection was more variable, causing a significant year*sex interaction (Fig. 4a, Table 3a); infection probability also increased with age more markedly in males than in females (Fig. 4b, Table 3a). At the level of individual lineages, pTURDUS1 infection also showed an age*sex interaction: increasing prevalence with age in males was not apparent in females (Fig. 4c, Table 3c). In the case of pBT7, infection increased with territory size in first year birds whereas it decreased in older birds (Fig. 4d). Hence, the effect of individual state differences on infection may also be environmentally dependent.

Discussion

In an analysis of a single blue tit population, we found marked and sometimes complex associations between infection with avian malaria and both landscape and host predictors at a local scale. At a simple spatial level, the prevalence of avian malaria as a whole and prevalence of infection with the three most numerous lineages varied between woodland sections. Variation in prevalence between woodland sections itself varied between lineages, indicating that different lineages had different spatial distributions (Fig. 2d–f). At a finer spatial scale, infection with avian malaria as a whole, and infection with two very closely related *Plasmodium* lineages (pTURDUS1 and pBT7, based on cytochrome *b* similarity), increased strongly with increasing proximity to a large body of water, the River Thames, but this was not true for the most abundant lineage in the population, pSGS1, which had a more scattered distribution. In lineage-specific models, infection increased with host age but age was not a significant factor in the model of pooled malaria lineages. Strong temporal variation in infection was also detected: pooled malaria infection and pBT7 infection varied significantly with year of sampling, with a steady increase in malaria infection in females, but not males, during the study period. Distance to the woodland edge, and breeding site altitude were not retained as significant predictors of infection, while tessellated territory size was only retained a predictor of infection with one malaria lineage, pBT7, as an interaction with host age.

Such striking patterns of spatial heterogeneity at a local scale demonstrate that environmental heterogeneity should be considered in studies of host–parasite interactions; local

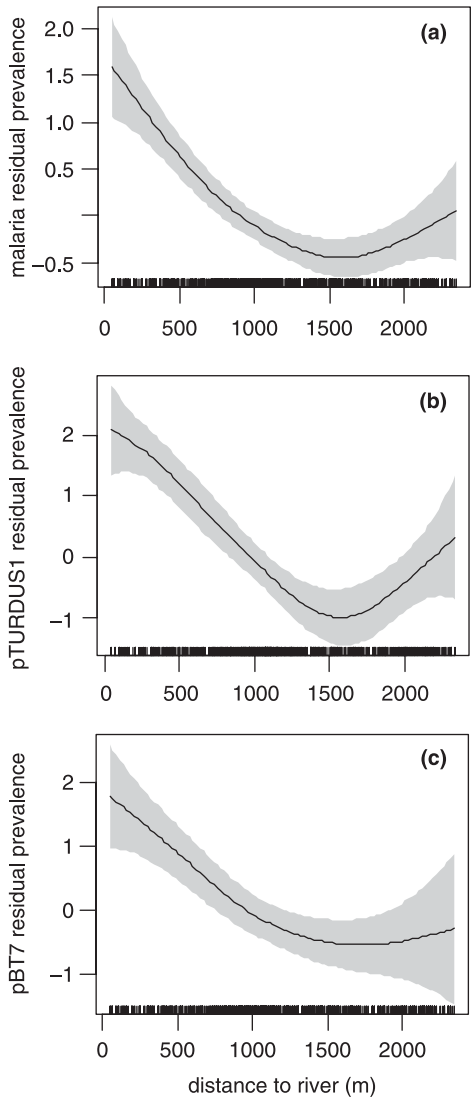

Fig. 3 Variation in avian malaria infection with proximity to the River Thames. Distance from the nearby River Thames, as a smoothed function, was retained as a significant predictor of the infection of blue tits with (a) pooled avian malaria lineages ($\chi^2 = 54.0$, $P < 0.0001$), (b) pTURDUS1 ($\chi^2 = 74.2$, $P < 0.0001$) and (c) pBT7 ($\chi^2 = 33.1$, $P < 0.0001$; see Table 3). The *y*-axis represents the residual prevalence from the smoothed model, shaded areas accompanying smoothed lines represent standard errors, and *x*-axis tick marks indicate the location of sample points relative to the river.

Table 4 Yearly contrasts in avian malaria prevalence in blue tits

	2001	2003	2004
(a) Pooled malaria			
2001	—	—	—
2003	0.041*	—	—
2004	< 0.0001***	< 0.0001***	—
2005	< 0.0001***	< 0.0001***	0.73
(b) pBT7			
2001	—	—	—
2003	0.99	—	—
2004	0.051	0.053	—
2005	0.028*	0.029*	0.80

Year was retained as a significant factor in the models of pooled malaria infection (Table 3a) and pBT7 infection (Table 3d). The significance values associated with generalized linear modelling treatment contrasts between years are shown.

estimates of prevalence range from over 60% to less than 10% in this study population over as little as a kilometre. Hence, for a given individual, the likelihood of infection by malaria may depend to a great extent on factors such as natal site (if infection occurs early in life), the degree of postnatal dispersal, and the choice of breeding site, some of which may be under the control of individuals, but others which are unlikely to be so. Such environmental factors might easily overwhelm individual differences in reproductive effort-parasite defence allocation, or individual differences in parasite resistance, and suggest that host populations should not be assumed to be homogeneous with respect to infection risk. The integration of parasite data at high taxonomic resolution acquired using molecular techniques with landscape data at a high geographical resolution has revealed complex and subtle ecological relationships.

If such patterns of parasite distribution are consistent between years, as was the case for two of the three lineages of avian malaria in this study, then is it reasonable to suggest that spatially dependent host–parasite co-evolution might also occur within scales similar to our study site? While levels of immigration and host dispersal are quite substantial in this population, and in blue tits in general (Tufto *et al*. 2005), the potential exists for host local adaptation to avian malaria infection to occur on a local scale in species that show reduced dispersal. In addition, if dispersal is nonrandom with respect to resistance phenotype, then local adaptation might occur even in the face of marked dispersal (see Garant *et al*. 2005 for an example of this process in a different context). Further exploration of this idea would necessitate studying the virulence of avian malaria lineages in this study population and its variation within the study site: modelling approaches suggest optimal virulence varies in relation to habitat quality (Hochberg & Holt 2002). The influence of host dispersal on spatiotemporal variation in parasite infections (Boulinier *et al*. 2000) also warrants further investigation. The timing of transmission of avian malaria lineages may vary in relation to the seasonal activity of vectors: some parasites may be transmitted during the nestling or post-fledging period, suggesting that natal environment may also be a

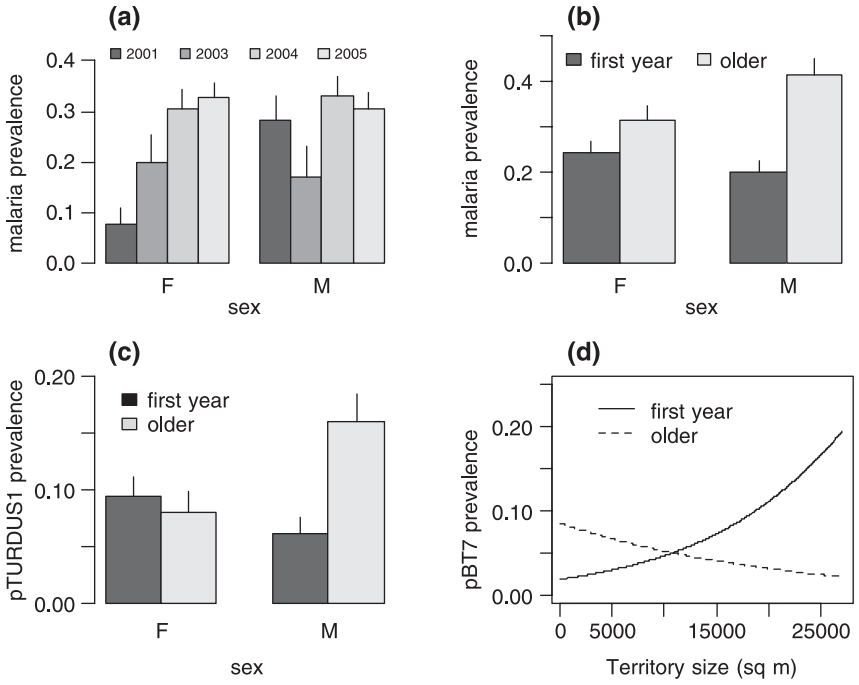

Fig. 4 Interactions between factors predicting avian malaria infection. (a) Annual variation in pooled malaria infection varied between females (F) and males (M); with an apparent increase in infection with year in females, but not in males (year*sex interaction: $Z = -3.06$, $P = 0.0025$). (b) Pooled malaria infection increased with age in both sexes, but more sharply in males (age*sex interaction: $Z = 2.31$, $P = 0.021$). (c) pTURDUS1 infection increased with age in males, but not in females (age*sex interaction: $Z = 2.97$, $P = 0.0028$). (d) the relationship between pBT7 infection and territory size differed between first years and older birds (age*territory size interaction: $Z = -2.16$, $P = 0.031$).

contributing factor to infection status as an adult. In temperate areas, avian malaria infections early in the year tend to be relapses of the previous year's infections (Beaudoin *et al*. 1971); such relapses may be less likely to reflect environmental conditions, such as distance to a river, than new infections acquired on breeding territories. Longitudinal studies of spatiotemporal patterns of host–parasite interactions could make an important contribution in this context, but are uncommon at present.

The marked association between infection (as pooled malaria lineages and as two further and closely related *Plasmodium* lineages, pTURDUS1 and pBT7) and proximity to a nearby river suggests that the wetness of potential vector larval habitat may be of considerable importance in determining the patterns observed here: vector ecology is likely to be a crucial link. The distribution of infective stages often predicts distribution of infected hosts (Wilson *et al*. 2001) and an increased risk of malaria in humans has been reported in proximity to bodies of water, i.e. supposed mosquito breeding sites (van der Hoek *et al*. 2003; Balls *et al*. 2004; Munyekenye *et al*. 2005; Omumbo *et al*. 2005). Investigation of the life cycles and behaviour of the mosquito species in our study system and their vector competency with respect to the different avian malaria lineages is clearly needed. Preliminary investigations of mosquito ecology at our study site have found seven species of mosquito from the genera *Culex*, *Aedes* and *Culiseta* (M. J. Wood, D. A. Cullen, R. M. Mallis, B. C. Sheldon, unpublished). Revealing the vector–parasite competence relationships would prove particularly useful in explaining our observed patterns of heterogeneity in avian malaria infection in terms of vector abundance.

Age was retained as a significant predictor in the models of pSGS1, pTURDUS1 and pBT7 infection prevalence, being higher in older birds, but was not retained for the model of pooled malaria lineages. This age effect was more evident in males, with interaction between age and sex for pooled malaria and pTURDUS1. Whether this age structure in infection results from an accumulation of infection with age, or a loss of susceptible birds that become infected requires detailed analysis of repeated samples from individuals; further longitudinal data from our study population should allow us to examine this question. In addition, while we found no evidence that population density was directly related to the probability of infection, we did find an age-specific effect of density for one of the lineages (pBT7). Whether avian malaria infection is subject to host-density dependent effects needs to be subjected to experimental analysis, as relying on natural variation in density is potentially influenced by nonrandom settlement of individuals.

The use of molecular techniques to examine parasite infections at high taxonomic resolution is uncovering high parasite species diversity in avian malaria (Bensch *et al*. 2004). In this study, two closely related lineages (pTURDUS1 and pBT7, < 0.25% sequence divergence) showed much closer similarity in their associations with landscape and host factors than a third lineage (pSGS1; > 4% sequence divergence from both pTURDUS1 and pBT7); this may reflect similarities in vector ecology or transmission requirements and suggests that there may be considerable scope for comparative studies of the transmission requirements of avian malaria lineages (Pérez-Tris & Bensch 2005; Wood & Cosgrove 2006). Our results of the analysis of pooled malaria lineages

should therefore be approached with a degree of caution, as it may not be meaningful to analyse pooled malaria cytochrome *b* lineage data if different lineages have different vector–parasite relationships or transmission requirements, adding redundant complexity to statistical analyses.

We detected annual variation in the prevalence of one lineage, pBT7, and pooled malaria infection, but no such pattern in infection with the two other lineages. Numerous reports exist of such temporal variation in parasite infection (Schall & Marghoob 1995; Bensch, Åkesson 2003; Altizer *et al*. 2004), but few studies are able to suggest a mechanism. Whether the annual variation in infection in this study is a result of fluctuations in environmentally driven variation in vector transmission, parasite-mediated population cycles (Hudson *et al*. 1998) or patterns of selection with respect to parasite resistance (Little & Ebert 2001; Westerdahl *et al*. 2004) remains to be seen; longer time series may help to resolve these possibilities in the present case.

There is a clear need for more studies to disentangle relationship between landscape heterogeneity, vector abundance and host effects on host infection, which will require the continued cross-fertilization of the approaches of spatial and landscape ecology, epidemiology and parasitology and the further development of the statistical tools to analyse wildlife disease systems. The use of molecular diagnostic techniques and GIS techniques to approach these questions should prove extremely valuable in the future, since they greatly expand the resolution with which such questions can be addressed.

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