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1 **Abstract**

2
3 The development of molecular genetic screening techniques for avian blood parasites has
4 revealed many novel aspects of their ecology, including greatly elevated diversity, and
5 complex host-parasite relationships. Many previous studies of malaria in birds have treated
6 single study populations as spatially homogeneous with respect to the likelihood of
7 transmission of malaria to hosts, and we have very little idea whether any spatial
8 heterogeneity influences different malaria lineages similarly. Here, we report an analysis of
9 variation in the prevalence and lineage distribution of avian malaria infection with respect to
10 environmental and host factors, and their interactions, in a single blue tit *Cyanistes*
11 *caeruleus* population. Of nine *Plasmodium* *cyt-b* lineages found in 997 breeding individuals,
12 the three most numerous (pSGS1, pTURDUS1 and pBT7) were considered separately, in
13 addition to analyses of all malaria lineages pooled. Our analyses revealed marked spatial
14 differences in the prevalence and distribution of these lineages, with local prevalence of
15 malaria as a whole ranging from >60% to <10%. In addition, we found several more complex
16 patterns of prevalence with respect to local landscape features, host state, parasite genotype
17 and their interactions. We discuss the implications of such heterogeneity in parasite infection
18 at a local scale for the study of the ecology and evolution of infectious diseases in natural
19 populations. The increased resolution afforded by the combination of molecular genetic and
20 GIS (Geographical Information System) tools has the potential to provide many insights into
21 the epidemiology, evolution and ecology of these parasites in the future.

1 Introduction

2
3 The ecological context of host-parasite interactions can have marked effects on the
4 transmission and persistence of disease. Abiotic factors, such as microclimate and landscape,
5 can influence the transmission stages of parasites and therefore the prevalence of host
6 infection, in addition to biotic effects such as host age, sex and population density (Combes
7 2001; Wilson *et al.* 2001). An understanding of the influence of landscape ecology on host-
8 parasite interactions in wild populations is of particular relevance in a changing world of
9 climate change and habitat fragmentation. However, landscape ecology has only occasionally
10 been considered in ecological studies of disease: there may, therefore, be significant gaps in
11 our understanding of host-parasite ecology, as such effects may not be apparent in small scale
12 population studies (May 1999). In contrast, the importance of the landscape in which a host-
13 parasite interaction occurs has been increasingly studied in human malaria: the rise in drug
14 resistance has shifted the focus away from chemical interventions to risk management
15 (Dieckmann *et al.* 2002), revealing associations between malaria infection and factors such as
16 altitude and proximity to water in human populations (Foley *et al.* 2003; Balls *et al.* 2004;
17 Omumbo *et al.* 2005). Furthermore, the risk of mosquito-borne infection may be higher at the
18 edge of a host population, if infective vectors seek out an area of high host density (Ribeiro *et*
19 *al.* 1996; Smith *et al.* 2004).

20
21 Host factors such as age, sex and host population density may also influence host
22 parasite infection. Prevalence may increase with age as new infections accumulate, then
23 decrease as susceptible individuals die or resistant individuals become immune (Wilson *et al.*
24 2001). Male mammals and birds tend to have a higher prevalence of infection than females
25 (Poulin 1996; Schalk & Forbes 1997; McCurdy *et al.* 1998). Population density may also
26 influence the risk of infection, depending on how parasite transmission relates to host
27 population density (Keymer & Anderson 1979). Spatiotemporal variation in parasite infection
28 has often been supposed to contribute to the maintenance of genetic variation in host
29 resistance to parasites but rarely studied (Lively & Dybdahl 2000; Bensch & Åkesson 2003).
30 Ideally, the influence of these processes needs to be studied against the background of
31 environmental variation due to abiotic factors, since there may also be interactions between
32 biotic and abiotic factors.

33
34 Avian malaria, *Plasmodium* and *Haemoproteus* spp. (following the definition of
35 Pérez-Tris *et al.* 2005), is a vector-borne disease transmitted primarily by mosquitoes of the
36 genera *Culex*, *Aedes* and *Culiseta* (Valkiūnas 2005). These parasite taxa are globally
37 distributed (Valkiūnas 2005, Beadell *et al.* 2006), and our understanding of their diversity,
38 ecology, and relationships with their avian hosts has been increased by the application of
39 molecular genetic screening techniques to blood samples collected from wild hosts. For
40 example, estimates of global species diversity of the order of 200 species based on
41 microscopy, have been suggested to need revision to somewhere in the order of 10,000
42 species based on comparisons of nuclear and mitochondrial gene trees (Bensch *et al.* 2004).
43 Recent phylogeographical studies of host-parasite interactions involving avian malaria
44 suggest that this diversification is the result of frequent host-switching followed by local
45 diversification in new hosts, and rapid parasite dispersal (Fallon *et al.* 2005), although host
46 switching may be more common in *Plasmodium* than *Haemoproteus* (Beadell *et al.* 2004).
47 Avian malaria has long been a popular study system for research in behavioural and
48 evolutionary ecology, having been used to test ideas ranging from the relationship between
49 parasitism and sexual selection (Hamilton & Zuk 1982) to the role of immune system trade-

1 offs with reproduction in structuring life histories (e.g. Norris et al. 1994; Richner et al. 1995;
2 Stjernman et al. 2004). However, the majority of ecological studies of malaria have not
3 considered either this diversity (a potentially important source of variation in host-parasite
4 interactions, since parasite virulence can vary among parasite lineages; Read & Taylor 2001),
5 or the possibility that prevalence and lineage distribution may vary with local landscape
6 features. The latter is an important consideration, because strong effects of the environment
7 (both biotic and abiotic) mean that the risk of exposure and infection may be very variable for
8 different individuals.

9
10 In this study, we examined variation in avian malaria infection with respect to
11 landscape and host factors on a local scale, in a single woodland population of blue tits
12 *Cyanistes caeruleus*. Using molecular diagnostic techniques, we considered avian malaria
13 infections at a high taxonomic resolution and a fine geographical scale, examining
14 associations between infection and a range of landscape and host features for separate
15 lineages, as well as for all lineages pooled. We report marked differences in the prevalence of
16 malaria with respect to lineage, landscape features, host characteristics, and the complex
17 interactions among these factors.

18 19 20 **Materials and Methods**

21 22 *Host and parasite*

23
24 Approximately 1160 nestboxes are monitored in Wytham Woods (51°46'N, 1°20'W), a
25 385ha woodland near Oxford, UK, where 250-450 pairs of blue tits breed annually (Perrins
26 1979). In this paper we report analysis of blood samples collected in 2001 and 2003-5, all of
27 which were collected from adult blue tits captured between day 6 and day 14 of the nestling
28 phase either within the nestbox by hand or using traps, or with mist nets in front of the nest
29 entrance. We thus analyse samples here that were all collected from hosts at the same point in
30 their annual cycle; as the study population is single-brooded, and breeds with a great degree
31 of synchrony, there is relatively little variation in the calendar date among samples. Host sex
32 was determined based on the presence (female) or absence (male) of a brood patch, age (first
33 year, or older) determined using plumage characteristics (Svensson 1992). A total of 997 blue
34 tits over four breeding seasons were included in the analyses of the associations between
35 breeding landscape, host factors and avian malaria infection. To avoid pseudoreplication in
36 cases where an individual bird was sampled in more than one year, one sample was randomly
37 chosen; therefore, each individual appears only once in the current analysis.

38 39 *Avian malaria diagnosis*

40
41 Blood samples were taken, under licence, by ulnar or jugular venipuncture. Samples were
42 stored in Queen's Lysis Buffer (Seutin *et al.* 1991), and DNA extracted using a DNeasy
43 Extraction Kit (Qiagen, Valencia, CA, USA). An assessment of the presence/quality of
44 extracted DNA was made by electrophoresing 2µl of the extract in 2% agarose containing
45 ethidium bromide and visualising it under UV light. The samples were screened for the
46 presence of *Plasmodium* and *Haemoproteus* using a nested PCR protocol (Waldenström *et al.*
47 *et al.* 2004), which amplifies a 478bp fragment of the mitochondrial cytochrome-b gene. The
48 PCR reactions were performed in 25µl volumes, in two separate rounds with positive and
49 negative controls. The first-round primers were HaemNF (5'-
50 CATATATTAAGAGAATTATGGAG-3') and HaemNR2 (5'-

1 AGAGGTGTAGCATATCTATCTAC-3'). Each reaction contained 2µl of genomic DNA,
2 0.125mM each dNTP, 0.2µM each primer, 3mM MgCl₂ and 0.25 units of Platinum Taq
3 Polymerase (Invitrogen, Carlsbad, California) with the accompanying PCR buffer at 1× final
4 concentration. The thermal profile consisted of a 2 minute 94°C enzyme activation step,
5 followed by 20 cycles of 94°C for 30sec, 50°C for 30sec, and 72°C for 45sec, ending with an
6 elongation step of 72°C for 10min. In the second round of PCR, primers HaemF (5'-
7 TGGTGCTTTTCGATATATGCATG-3') and HaemR2 were used (5'-
8 GCATTATCTGGATGTGATAATGGT-3'). The composition of the PCR reactions was as
9 above, except 0.4µM of each primer and 0.5 units of Platinum-Taq Polymerase were used,
10 and 2µl of the PCR product from the first round was used as template instead of genomic
11 DNA. The thermal profile for the second round PCR was the same as for the first, except the
12 number of cycles was increased from 20 to 35. 2-8µl of PCR products from the second round
13 were run on 2% agarose stained with ethidium bromide and visualised under UV. Samples
14 containing bands of 450-600bp in size were prepared for sequencing using Qiagen MinElute
15 96 UF PCR Purification Kits and QiaVac Multiwell vacuum manifolds. Purified PCR
16 fragments were sequenced directly by dye terminator cycle sequencing (Big Dye v3.1), and
17 loaded on a ABI PRISM 310 automated sequencer (Applied Biosystems, CA). Sequences
18 were edited in Sequencher v. 4.2 (GeneCodes Corp., MI), and aligned in ClustalX
19 (Thompson *et al.* 1997). Sequences corresponding to *Plasmodium* or *Haemoproteus* from
20 known alignments were scored as positive for avian malaria. Sequences corresponding to
21 *Leucocytozoon* sequences were scored as negative for the purposes of this study, and are not
22 considered further here. Based on the occurrence of double peaks in electropherograms,
23 mixed infections were present at a low rate (2.2% in 2004-5, S.C.L. Knowles *et al.* unpubl.)
24 and are not considered further here.

25 26 *Measurement of landscape using GIS techniques*

27
28 A GIS of the study site was constructed in 2005 (e.g. see Wilkin *et al.* 2006). This system
29 allowed us to plot accurately the location of each breeding blue tit's nest. Blue tits feed their
30 offspring on invertebrate prey, and forage in the immediate vicinity of their nest; hence using
31 the nestbox to represent location for breeding birds is justified. Other topographical features
32 from UK Ordnance Survey data were incorporated into the GIS: the shortest distance (m)
33 between each nestbox and i) the woodland edge and ii) the River Thames (Figure 1) was
34 calculated using GIS software (MapInfo Professional v7.8).

35
36 In order to test for spatial differences in prevalence, and the distribution of the
37 different malaria lineages, we categorised individual nestboxes based on the woodland
38 section to which they belonged. The population studies of tits in Wytham Woods have
39 traditionally been divided into nine separate areas (sections) for the purposes of delimiting
40 different parts of the study area (e.g. see Garant *et al.* 2005 for more detail). The sections are
41 arbitrary delineations of the study area with respect to malaria, and consequently they provide
42 a means to partition the population into sections in order to test for differences in prevalence
43 within the population.

44
45 In order to estimate territory size (and hence population density), tessellations
46 (Thiessen or Voronoi polygons) were formed around each breeding pair by placing boundary
47 lines equidistant between occupied nestboxes in each year. The area of these polygons is
48 necessarily inversely related to breeding density (Wilkin *et al.* 2006). As blue tits show a high
49 degree of sharing of avian malaria lineages with great tits *Parus major* (no significant
50 difference in lineage assemblages: C.L. Cosgrove *et al.* unpubl. data), boxes occupied by both

1 species were included so that tessellated territory size was a measure of interspecific density.
2 Blue tits and great tits are territorial during the breeding season (March to June), but more
3 loosely associated with the territory for the rest of the year (Perrins 1979). While tessellated
4 territory size is a geometric construct, it has been shown to be a useful measure of breeding
5 density in tits at Wytham, with strong relationships to many density-dependent life history
6 characters (Wilkin *et al.* 2006). Because it is calculated on an individual basis, is an
7 improvement on other methods such as distance to the nearest neighbour or the number of
8 pairs per unit area (e.g. Orell & Ojanen 1983; Both & Visser 2000).

9 10 *Statistical analysis*

11
12 Generalized linear modelling (GLM) was performed to assess associations between landscape
13 and host predictors on the presence or absence of infection in individual birds, either with
14 avian malaria as a whole or the three most numerous avian malaria lineages separately (see
15 below), both analyses using binomial errors and a logit link. Starting models were optimised
16 by backward stepwise elimination of non-significant terms, beginning with three-way
17 interactions and progressing to single order predictors. Terms were deleted from the model if
18 their removal caused a non-significant change in deviance ($P>0.05$). In landscape analyses,
19 potentially non-linear relationships between infection status and host and landscape/host
20 covariates were considered in statistical analyses using generalized additive modelling
21 (GAM), a generalized linear model (GLM) in which a smoothed function of a covariate can
22 be modelled alongside linear predictors (Wood & Augustin 2002). Linear covariates retained
23 in final models were substituted for GAM smoothed terms, the latter being retained if they
24 caused a significant reduction in deviance. Step functions were similarly considered.

25
26 Individual infection with pooled avian malaria *cyt-b* lineages, and infection with the
27 three most prevalent lineages (pSGS1, pTURDUS1 and pBT7: 36%, 34% and 16% of all
28 infections, respectively) were used as binary responses for analyses, as lineages at lower
29 prevalences (less than 4% of sampled hosts) prohibited the modelling of presence/absence
30 data because modelling algorithms failed to converge. pTURDUS1 and pBT7 are closely
31 related avian malaria lineages (<0.25% sequence divergence at *cyt-b*), being less related to
32 pSGS1 (>4% sequence divergence: Cosgrove C.L. *et al.* unpubl.). All statistical analyses
33 were conducted using R version 2.2.1. Means are presented ± 1 s.e.

34 35 36 **Results**

37
38 The overall prevalence of avian malaria (i.e. *Plasmodium* and *Haemoproteus*) within this
39 sample was 28.4% (n=997), comprising 12 different *cyt-b* lineages (Table 1). The three most
40 common were pSGS1 (prevalence 10.2%), pTURDUS1 (9.7%), and pBT7 (4.6%); the
41 overwhelming majority of infections were with *Plasmodium*, with only six individuals (0.6%)
42 infected with three separate *Haemoproteus* lineages.

43
44 We first visualised the distribution of infection within the study site as a means of
45 informing the statistical analysis, by generating interpolated maps of malaria prevalence
46 using GIS software (Figures 1a-d). These maps indicated that the distribution of avian
47 malaria infection, when all infections were pooled, was concentrated mainly in the north-west
48 of the study area (Figure 1a). This mapping procedure also suggested that the three most
49 common lineages showed different distributions: pSGS1 (Figure 1b) was widely, but
50 patchily distributed, whereas pTURDUS1 (Figure 1c) and pBT7 (Figure 1d) were much more

1 restricted to the northern edge of the study site. We tested for spatial differences in the overall
2 prevalence of malaria, and in the prevalence of these three commonest lineages, by testing the
3 effect of woodland section (see Materials and Methods) on prevalence. The prevalence of
4 pooled malaria lineages varied markedly between woodland sections (analysis of deviance:
5 $\chi^2=71.2$, $P<0.001$). Considering the three most numerous lineages, prevalence varied both by
6 lineage ($\chi^2=28.6$, $P<0.001$) and woodland section ($\chi^2=68.1$, $P<0.001$). In addition, we tested
7 the lineage*woodland section interaction with respect to prevalence ($\chi^2=54.2$, $P<0.001$),
8 which confirmed that the lineages are differently distributed in space, as suggested by the
9 visual inspection of the interpolated maps.

10
11 Generalized linear modelling of infection status of breeding blue tits revealed several
12 complex associations between avian malaria infection and both landscape and host factors
13 (Table 2). Infection with two lineages (pTURDUS1 and pBT7), and for all avian malaria
14 lineages pooled, decreased further away from the nearby River Thames, which runs along the
15 edge of the study site. Smoothed functions (Figure 2) provided a significantly better fit than a
16 linear or step function in all cases (changes in model residual deviance, $P<0.05$). In contrast,
17 there was no influence of this landscape feature on prevalence of SGS1 (Figure 1b).

18
19 Pooled avian malaria lineages and pBT7 both showed significant annual variation in
20 prevalence of infection, (Figure 3, Table 3), in both cases being highest in the final two years
21 of the study. Sex was retained as a significant factor only in analyses of pooled avian malaria
22 infection, with marginally higher prevalence for males than for females (Table 2a: male
23 $29.8\pm 1.4\%$, female $27.0\pm 1.4\%$). The prevalence of infection was higher in older birds, both
24 for pooled avian malaria and all three analysed lineages (Table 2: pSGS1 first years
25 $8.15\pm 0.87\%$, older $13.1\pm 1.1\%$; pTURDUS1 first years $7.97\pm 0.86\%$, older $12.1\pm 1.0\%$; pBT7
26 first years $3.1\pm 0.72\%$, older $6.7\pm 1.2\%$). These analyses thus show that the prevalence of
27 malaria is, to some extent, dependent on the location of the sampled individual, its age and
28 sex, and that there may be annual fluctuations in the prevalence of some lineages.

29
30 A number of interaction terms were retained in the final models of avian malaria
31 infection prevalence. In the case of pooled avian malaria lineages, female infection increased
32 over the study period while male infection was more variable, causing a significant year*sex
33 interaction (Figure 4a, Table 2a); infection probability also increased with age more markedly
34 in males than in females (Figure 4b, Table 2a). At the level of individual lineages,
35 pTURDUS1 infection also showed an age*sex interaction: increasing prevalence with age in
36 males was not apparent in females (Figure 4c, Table 2c). In the case of pBT7, infection
37 increased with territory size in first year birds whereas it decreased in older birds (Figure 4d).
38 Hence, the effect of individual state differences on infection may also be environmentally-
39 dependent.

40 41 42 **Discussion**

43
44 In an analysis of a single blue tit population, we found marked, and sometimes complex,
45 associations between infection with avian malaria and both landscape and host predictors at a
46 local scale. At a simple spatial level, the prevalence of avian malaria as a whole and
47 prevalence of infection with the three most numerous lineages varied between woodland
48 sections. Variation in prevalence between woodland sections itself varied between lineages,
49 indicating that different lineages had different spatial distributions (Figure 1). At a finer
50 spatial scale, infection with avian malaria as a whole, and infection with two very closely

1 related *Plasmodium* lineages (pTURDUS1 and pBT7, based on cytochrome-*b* similarity),
2 increased strongly with increasing proximity to a large waterbody, the River Thames, but this
3 was not true for the most abundant lineage in the population, pSGS1, which had a more
4 scattered distribution. In lineage-specific models, infection increased with host age but was
5 not a significant factor in the model of pooled malaria lineages. Strong temporal variation in
6 infection was also detected: pooled malaria infection and pBT7 infection varied significantly
7 with year of sampling, with a steady increase in malaria infection in females, but not males,
8 during the study period. Distance to the woodland edge, and breeding site altitude were not
9 retained as significant predictors of infection, while tessellated territory size was only
10 retained a predictor of infection with one malaria lineage, pBT7, as an interaction with age.
11

12 Such striking patterns of spatial heterogeneity at a local scale demonstrate that
13 environmental heterogeneity should be considered in studies of host-parasite interactions;
14 local estimates of prevalence range from >60% to <10% in this study population over as little
15 as 1 km. Hence, for a given individual, the likelihood of infection by malaria may depend to a
16 great extent on factors such as natal site (if infection occurs early in life), the degree of post-
17 natal dispersal, and the choice of breeding site, some of which may be under the control of
18 individuals, but others which are unlikely to be. Such environmental factors might easily
19 overwhelm individual differences in reproductive effort-parasite defence allocation, or
20 individual differences in parasite resistance, and suggest that host populations should not be
21 assumed to be homogeneous with respect to infection risk. The integration of parasite data at
22 high taxonomic resolution acquired using molecular techniques with landscape data at a high
23 geographical resolution has revealed complex and subtle ecological relationships that would
24 remain undetected using microscopy.
25

26 If such patterns of parasite distribution are consistent between years, as was the case
27 for two of the three lineages of avian malaria in this study, then is it reasonable to suggest
28 that spatially-dependent host-parasite co-evolution might also occur within scales similar to
29 our study site? While levels of immigration and host dispersal are quite substantial in this
30 population (and in blue tits in general: see Tufto et al. 2005), the potential exists for host local
31 adaptation to avian malaria infection to occur on a local scale in species that show reduced
32 dispersal. In addition, if dispersal is non-random with respect to resistance phenotype, then
33 local adaptation might occur even in the face of marked dispersal (see Garant et al. 2005 for
34 an example of this process in a different context). Further exploration of this idea would
35 necessitate studying the virulence of avian malaria lineages in this study population and its
36 variation within the study site, as modelling approaches suggest optimal virulence varies in
37 relation to habitat quality (Hochberg & Holt 2002). Longitudinal studies of spatiotemporal
38 patterns of host-parasite interactions could make an important contribution in this context, but
39 are uncommon at present.
40

41 The marked association between infection (as pooled malaria lineages and two
42 further, closely related, *Plasmodium* lineages, pTURDUS1 and pBT7) and proximity to a
43 nearby river suggests that vector larval habitat may be of considerable importance in
44 determining the patterns observed here: vector ecology is likely to be a crucial link. The
45 distribution of infective stages often predicts distribution of infected hosts (Wilson *et al.*
46 2001) and an increased risk of malaria in humans has been reported in proximity to water
47 bodies, i.e. supposed mosquito breeding sites (van der Hoek *et al.* 2003; Balls *et al.* 2004;
48 Munyekenye *et al.* 2005; Omumbo *et al.* 2005, though see Clarke et al. 2002 for a contrasting
49 finding). Investigation of the life cycle and behaviour of the mosquito species in our study
50 system and their vector competency with respect to the different avian malaria lineages is

1 clearly needed. Preliminary investigations of mosquito ecology at our study site have found
2 seven species of mosquito from the genera *Culex*, *Aedes* and *Culiseta* (M.J.Wood et al.
3 unpubl.). Revealing the vector-parasite competence relationships would prove particularly
4 useful in explaining our observed patterns of heterogeneity in avian malaria infection in terms
5 of vector abundance.

6
7 Age was retained as a significant predictor in the models of pSGS1, pTURDUS1 and
8 pBT7 infection, being higher in older birds, but was not retained for the model of pooled
9 malaria lineages. This age effect was more evident in males, with interaction between age and
10 sex for pooled malaria and pTURDUS1. Whether this age structure in infection results from
11 an accumulation of infection with age, or a loss of susceptible birds that become infected
12 requires detailed analysis of repeated samples from individuals. In addition, while we found
13 no evidence that population density was directly related to the probability of infection, we did
14 find an age-specific effect of density for one of the lineages. Whether avian malaria infection
15 is subject to host-density dependent effects needs to be subjected to experimental analysis, as
16 relying on natural variation in density is potentially influenced by non-random settlement of
17 individuals.

18
19 The use of molecular techniques to examine parasite infections at high taxonomic
20 resolution is uncovering high parasite species diversity in avian malaria (Bensch *et al.* 2004).
21 In this study, two closely related lineages (pTURDUS1 and pBT7, <0.25% sequence
22 divergence,) showed much closer similarity in their associations with landscape and host
23 factors than a third lineage (pSGS1; >4% sequence divergence from both pTURDUS1 and
24 pBT7); this may reflect similarities in vector ecology or transmission requirements and
25 suggests that there may be considerable scope for comparative studies of the transmission
26 requirements of avian malaria lineages (see Pérez-Tris & Bensch 2005; Wood & Cosgrove
27 2006). Our results of the analysis of pooled malaria lineages should therefore be approached
28 with a degree of caution, as it may not be meaningful to analyse pooled malaria *cyt-b* lineage
29 data if different lineages have different vector-parasite relationships or transmission
30 requirements, adding redundant complexity to statistical analyses.

31
32 We detected annual variation in the prevalence of one lineage, pBT7, and pooled
33 malaria infection, but no such pattern in infection with the two other lineages. Numerous
34 reports exist of such temporal variation in parasite infection (e.g. Schall & Marghoob 1995;
35 Bensch & Åkesson 2003; Altizer *et al.* 2004), but few studies are able to suggest a
36 mechanism. Whether the annual variation in infection in this study is a result of fluctuations
37 in environmentally driven variation in vector transmission, parasite-mediated population
38 cycles (Hudson *et al.* 1998) or patterns of selection with respect to parasite resistance (Little
39 & Ebert 2001; Westerdahl *et al.* 2004) remains to be seen: longer time-series may help to
40 resolve these possibilities in the present case.

41
42 There is a clear need for more studies to disentangle relationship between landscape
43 heterogeneity, vector abundance and host effects on host infection, which will require the
44 continued cross-fertilization of the approaches of spatial and landscape ecology,
45 epidemiology and parasitology and the further development of the statistical tools to analyze
46 wildlife disease systems. The use of molecular diagnostic techniques and GIS techniques to
47 approach these questions should prove extremely valuable in the future, since they greatly
48 expand the resolution with which such questions can be addressed.

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28
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35 **Figure legends**

37 **Figure 1. Maps of variation in avian malaria infection in a woodland population of blue** 38 **tits**

39 Interpolated maps of spatial variation in the prevalence of avian malaria in blue tits at
 40 Wytham, near Oxford, are shown for (a) all malaria lineages pooled, and the three most
 41 common *Plasmodium* lineages; (b) pSGS1, (c) pTURDUS1 and (d) pBT7, as identified by
 42 common cytochrome-*b* lineages. Data were pooled for the years 2001, 2003-5. Maps were
 43 generated using MapInfo Professional v7.8.

45 **Figure 2. Variation in avian malaria infection with proximity to the River Thames**

46 Smoothed distance from the nearby River Thames was retained as a significant predictor of
 47 the infection of blue tits with (a) pooled avian malaria lineages (smoothing function $\chi^2 = 54.0$,
 48 $P < 0.0001$), (b) pTURDUS1 ($\chi^2 = 74.2$, $P < 0.0001$) and (c) pBT7 ($\chi^2 = 33.1$, $P < 0.0001$;
 49 Table 2). The y-axis represents the residual prevalence from the smoothed model, shaded

1 areas about smoothed lines represent standard errors and x-axis tick marks indicate sample
2 points.

3

4 **Figure 3. Annual variation in avian malaria infection**

5 Year was retained as a significant predictor of (a) infection with pooled malaria lineages ($Z =$
6 $3.20, P < 0.0001$) and (b) pBT7 infection ($Z = -6.61, P < 0.0001$) showed significant variation
7 between years (see also Tables 2&3).

8

9 **Figure 4. Interactions between factors predicting avian malaria infection**

10 (a) Annual variation in pooled malaria infection varied with sex; with an apparent increase in
11 infection with year in females, but not in males (year*sex interaction: $Z = -3.06, P = 0.0025$).

12 (b) Pooled malaria infection increased with age in both sexes, but more sharply in males
13 (age*sex interaction: $Z = 2.31, P = 0.021$).

14 (c) pTURDUS1 infection increased with age in
15 males, but not in females (age*sex interaction: $Z = 2.97, P = 0.0028$). (d) pBT7 infection
16 increased with territory size in first years, but decreased with territory size in older birds
(age*territory size interaction: $Z = -2.16, P = 0.031$).

Table 1. Diversity of avian malaria lineages in the Wytham blue tit population

Based on sequence data from a 478bp fragment of the mitochondrial cytochrome-*b* gene, 12 lineages of avian malaria were detected in a total of 997 blue tit malaria diagnoses. Infections of all 12 avian malaria lineages were pooled for analysis, in addition to infections with higher than 4% prevalence (asterisked*), infection with each of which was analysed separately.

Parasite taxon	Lineage	GenBank accession number	N positive	Prevalence (%)
<i>Plasmodium</i> sp.	SGS1*	AF495571	102	10.2
<i>Plasmodium</i> sp.	TURDUS1*	AF495576	97	9.7
<i>Plasmodium</i> sp.	BT7*	AY393793	46	4.6
<i>Plasmodium</i> sp.	GRW11	AY831748	13	1.3
<i>Plasmodium</i> sp.	BLUTI1	DQ991068	6	0.6
<i>Plasmodium</i> sp.	BLUTI2	DQ991072	1	0.1
<i>Plasmodium</i> sp.	BLUTI4	DQ991070	1	0.1
<i>Plasmodium</i> sp.	BLUTI5	DQ991071	1	0.1
<i>Plasmodium</i> sp.	SW2	AF495572	1	0.1
<i>Haemoproteus</i> sp.	WW1	AF254971	3	0.3
<i>Haemoproteus</i> sp.	TURDUS2	DQ060772	2	0.2
<i>Haemoproteus</i> sp.	BLUTI1	DQ991068	1	0.1

9

Table 2. Statistical modelling of variation in avian malaria infection in blue tits

The results of generalized linear modelling of infection with pooled avian malaria *cyt-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 optimisation are shown, with statistics describing their contribution to the final model.

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 df = 2.72, $\chi^2 = 54.0$, $P < 0.0001$			
<i>model residual deviance = 1080.4</i>			
(b) pSGS1 model			
age (older)	2.42±0.15	15.9	<0.0001
<i>model residual deviance = 651.9</i>			
(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 df = 3.43, $\chi^2 = 74.2$, $P < 0.0001$			
<i>model residual deviance = 548.6</i>			
(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*tesselated territory size	-0.00016±0.00007	-2.16	0.031
smoothed distance to river: estimated df = 3.82, $\chi^2 = 33.1$, $P < 0.0001$			
<i>model residual deviance = 323.7</i>			

1 **Table 3. Year by year contrasts in avian malaria prevalence in blue tits**

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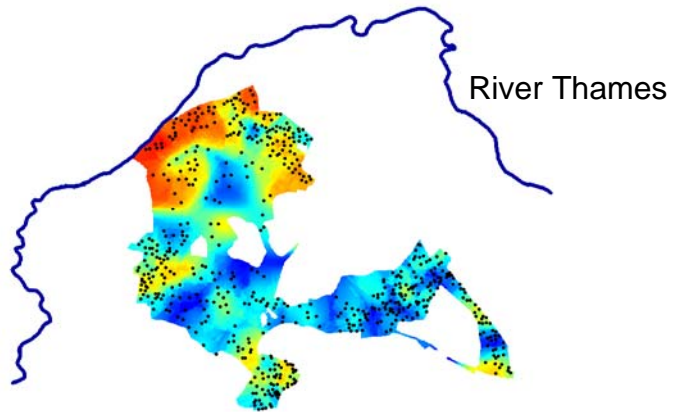
Year was retained as a significant factor in the models of pooled malaria infection (Table 2a, Figure 3b) and pBT7 infection (Table 2d, Figure 3b). The significance values associated with GLM treatment contrasts between years are shown.

(a) Pooled malaria	2001	2003	2004
2001	-	-	-
2003	0.041*	-	-
2004	<0.0001***	<0.0001***	-
2005	<0.0001***	<0.0001***	0.73

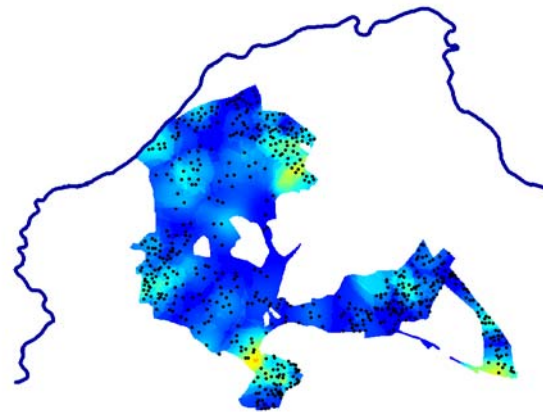
(b) pBT7	2001	2003	2004
2001	-	-	-
2003	0.99	-	-
2004	0.051	0.053	-
2005	0.028*	0.029*	0.80

1 **Figure 1. Maps of variation in avian malaria infection in a woodland population of blue tits**

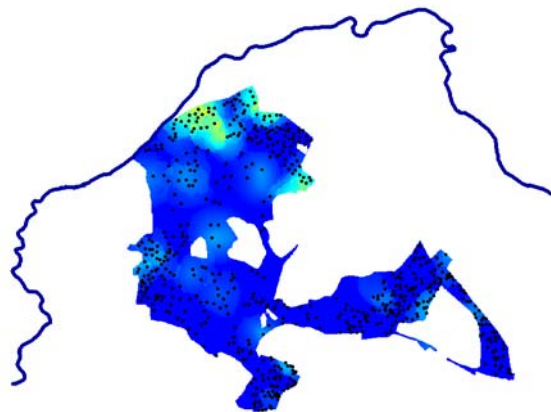
(a) Pooled avian malaria lineages



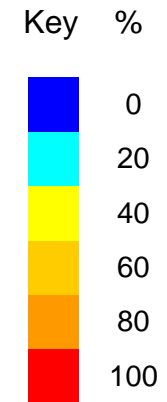
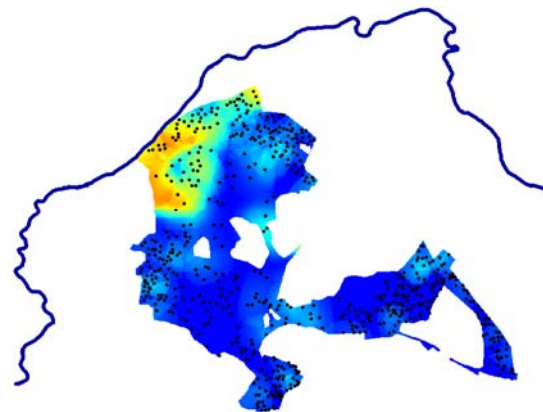
(b) pSGS1



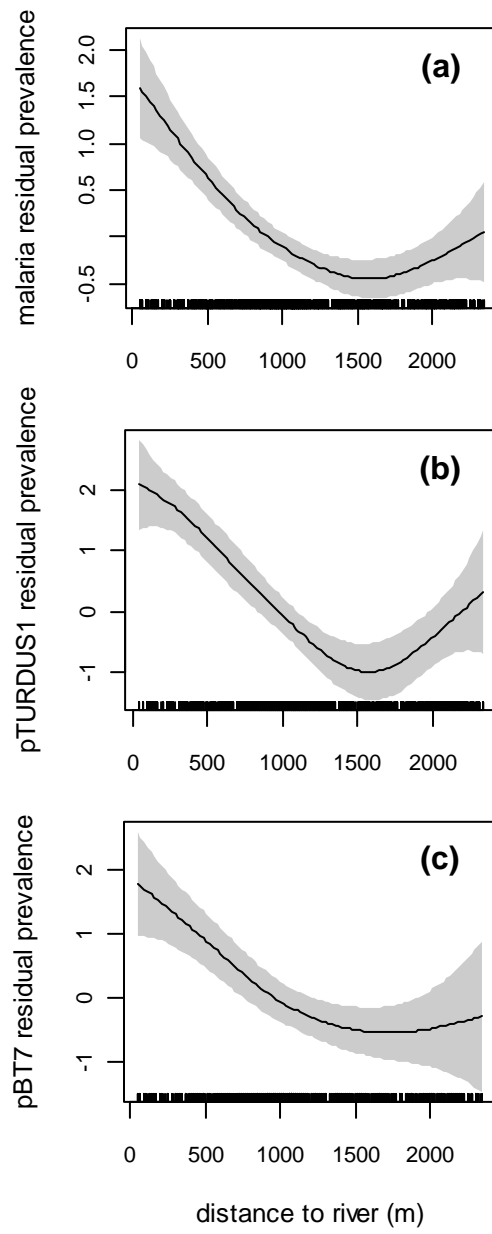
(c) pBT7



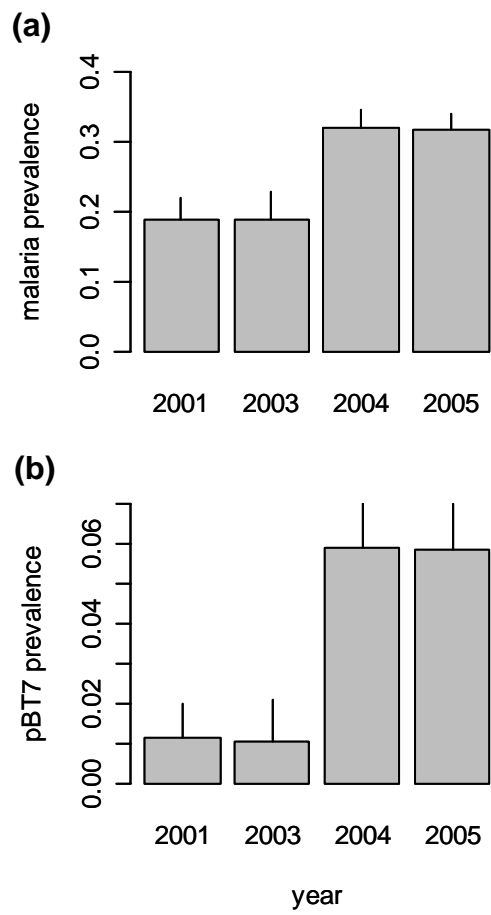
(d) pTURDUS1



1 **Figure 2. Variation in avian malaria infection with proximity to the River Thames**
2



1 **Figure 3. Annual variation in avian malaria infection**
2



1 **Figure 4. Interactions between factors predicting avian malaria infection**
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