Cosgrove, Catherine L. and Wood, Matthew J. and Day, Karen P. and Sheldon, Ben C. (2008). Seasonal variation in Plasmodium prevalence in a population of blue tits Cyanistes caeruleus. Journal of Animal Ecology, 77 (3) 540-548.

Seasonal variation in *Plasmodium* prevalence in a population of blue tits *Cyanistes caeruleus*

Catherine L. Cosgrove[1†‡], Matthew J. Wood[1*†], Karen P. Day[2] & Ben C. Sheldon[1]

[1] Edward Grey Institute, Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK

[2] Department of Medical Parasitology, New York University, 341 East 25th Street, New York, NY 10010, USA

[*] Corresponding author: matt.wood@zoo.ox.ac.uk

Telephone +44 1865 281999

Fax +44 1865 271168

[†] Joint first authors

[‡] Current address: The Wellcome Centre for Human Genetics, Roosevelt Drive, Oxford OX3 7BN, UK

Email addresses: matt.wood@zoo.ox.ac.uk, catherine.cosgrove@well.ox.ac.uk, karen.day@med.nyu.edu, ben.sheldon@zoo.ox.ac.uk

Running head (48 characters): Seasonal variation in *Plasmodium* infection in blue tits

Summary 251 words, manuscript total 7072 words (including references), 4 figures, 2 tables.

Summary

1. Seasonal variation in environmental conditions is ubiquitous and can affect the spread of infectious diseases. Understanding seasonal patterns of disease incidence can help to identify mechanisms, such as the demography of hosts and vectors, which influence parasite transmission dynamics.
2. We examined seasonal variation in *Plasmodium* infection in a blue tit *Cyanistes caeruleus* population over three years using sensitive molecular diagnostic techniques, in light of Beaudoin *et al.*'s (1971) model of seasonal variation in avian malaria prevalence in temperate areas. This model predicts a within-year bimodal pattern of spring and autumn peaks with a winter absence of infection
3. Avian malaria infections were mostly *Plasmodium* (24.4%) with occasional *Haemoproteus* infections (0.8%). Statistical non-linear smoothing techniques applied to longitudinal presence/absence data revealed marked temporal variation in *Plasmodium* prevalence, which apparently showed a within-year bimodal pattern similar to Beaudoin *et al.*'s model. However, of the two *Plasmodium* morphospecies accounting for most infections, in only (*Plasmodium circumflexum*) did seasonal patterns support Beaudoin *et al.*'s model. On closer examination there was also considerable age structure in infection: Beaudoin *et al.*'s seasonal pattern was observed only in first year and not older birds. *Plasmodium relictum* prevalence was less seasonally variable.
4. For these two *Plasmodium* morphospecies, we reject Beaudoin *et al.'s* model as it does not survive closer scrutiny of the complexities of seasonal variation among

Plasmodium morphospecies and host age classes. Studies of host-parasite interactions should consider seasonal variation whenever possible. We discuss the ecological and evolutionary implications of seasonal variation in disease prevalence.

Introduction

The prevalence of many infectious diseases varies markedly through time, from short-term seasonal fluctuations to complex population dynamics (Altizer, Dobson, Hosseini *et al.*, 2006; Dietz, 1976; Greenman, Kamo & Boots, 2004). The dynamics of vector-borne diseases are particularly likely to vary with environmental conditions, as vectors are sensitive to climatic conditions (Aron & May, 1982; Hess, Randolph, Arneberg *et al.*, 2001). For example, human malaria *Plasmodium* spp. shows marked seasonality in transmission, largely due to the sensitivity of the mosquito vectors to climate (Childs, Cattadori, Suwonkerd *et al.*, 2006; Hay, Myers, Burke *et al.*, 2000).

Host demography might play a greater role in the transmission dynamics of avian as compared to human malaria, as the temporally discrete breeding and migratory periods of avian hosts give rise to seasonally regular fluctuations in host abundance and the proportion of susceptible individuals in the host population, due to the relatively synchronous recruitment of immunologically naïve juveniles to the host population and the arrival of migrant birds (and their parasites) to the wider bird community (White,

Grenfell, Hendry *et al.*, 1996). In addition, there may also be a reduction in herd immunity that exposes older individuals to an increased risk of infection, resulting in the epidemic spread of previously rare parasite genotypes (Altizer *et al.*, 2006; White *et al.*, 1996). Revealing the environmental and demographic drivers that contribute to seasonal disease dynamics aids the understanding of disease epidemiology (Pascual & Dobson, 2005).

In tropical climates, avian malaria occurs year-round (Valkiūnas, 2005), whereas studies in temperate regions report consistent seasonal variation: a peak in prevalence during spring or the breeding season, followed by a decline during winter (Applegate, 1971; Beaudoin, Applegate, David *et al.*, 1971; Kucera, 1981; Schrader, Walters, James *et al.*, 2003; Weatherhead & Bennett, 1991), although some studies have found higher prevalence of some haematozoa in winter (Hatchwell, Wood, Anwar *et al.*, 2000). Beaudoin *et al.* (1971) proposed a model to explain patterns of seasonal variation with reference to the transmission requirements and life cycle of avian malaria parasites: a peak in malaria prevalence is supposed to occur in late summer and autumn, when vector populations (Cranston, Ramsdale, Snow *et al.*, 1987; Marshall, 1938) and the proportion of immunologically naïve juveniles in the host population are high. Prevalence then drops in winter as vector activity wanes and malaria parasites disappear from the blood, but not necessarily body tissues, followed by a spring relapse of infection prior to the breeding season.

The development of molecular tools for diagnosis of avian malaria infection based on mitochondrial cytochrome-*b* lineage variation (Bensch, Stjernman, Hasselquist *et al.*, 2000; Fallon, Ricklefs, Swanson *et al.*, 2003; Hellgren, Waldenström & Bensch, 2004; Waldenström, Bensch, Hasselquist *et al.*, 2004) allows avian malaria infections to be examined in more detail than is possible using traditional light microscopy techniques (Waldenström *et al.*, 2004). Estimates of diversity of around 200 species using microscopy (Valkiūnas, 2005) may mask diversity to the order of 10,000 species as revealed by molecular approaches (Bensch, Pérez-Tris, Waldenström *et al.*, 2004): most ecological studies of malaria do not consider this diversity, a potentially important source of variation in host-parasite interactions. Established parasitological techniques remain important for identifying groups of lineages that are morphologically similar, a likely indicator of similar parasite ecology (Valkiūnas, 2005). Here, we examine seasonal variation in avian malaria infection in a woodland population of blue tits *Cyanistes caeruleus* L., 1758, to test Beaudoin *et al.*'s (1971) model. We report marked seasonal patterns of variation in infection that vary between parasite morphospecies and with host age, based on screening more than 800 samples over three years.

Methods

Host-parasite system

Avian malaria, caused by *Plasmodium* and *Haemoproteus* spp. (*sensu* Pérez-Tris, Hasselquist, Hellgren *et al.*, 2005; see Valkiūnas, Anwar, Atkinson *et al.*, 2005 for an

alternative view), is a globally distributed vector-borne disease (Beadell, Ishtiaq, Covas *et al.*, 2006; Valkiūnas, 2005). *Plasmodium* is transmitted primarily by mosquitoes (Culicidae), and *Haemoproteus* by biting midges (Ceratopogonidae) and louse flies (Hippoboscidae); parasite transmission is therefore dependent on vector activity, between spring and autumn in temperate areas (Valkiūnas, 2005). Blue tits (Paridae) are small passerine birds that take readily to nestboxes, laying eggs in spring with the peak of broods hatching (in the south of England) in late April-early May. Chicks fledge 16-18 days later, with the last chicks fledging in early June (Perrins, 1979).

In the present study, we take 15th June as a biologically meaningful start to the sampling year, because of (i) the addition to the population of many newly fledged young by this time (all nestling tits had fledged by 15th June), (ii) the age transition from first year (previous year's nestlings) to older adults that occurs at this time, and (iii) the timing of feather moult in blue tits, in mid to late summer. It is also difficult to catch blue tits at our study site during late June and early July using mist-nets at artificial food stations, resulting in a natural break in sampling at the beginning of our sampling year on 15th June. Therefore, figures in this paper show the year's sampling beginning in summer, with date shown by calendar month for clarity.

Sampling and molecular diagnosis of infection

Blood samples of <20µL were taken, under licence, by brachial or jugular venepuncture from blue tits in Wytham Woods, a ca. 380ha woodland in Oxfordshire, UK (51°47' N, 1°20'W) between May 2003 and June 2005. Birds were captured at nest boxes while

feeding nestlings, and using mist nets at feeding stations approximately weekly at other times of the year. Sex was determined by plumage characteristics or, during the breeding season, on the presence/absence of a brood patch (Svensson, 1992). Blood samples were stored in Queen's lysis buffer (Seutin, White & Boag, 1991), and DNA extracted using a DNeasy extraction kit (Qiagen, CA, USA). One sample from each individual is analysed here, giving a total of 816 sampled individuals.

The presence/quality of extracted DNA was assessed by electrophoresing 2µl of the extract on a 2% agarose gel containing ethidium bromide, and visualising under UV light. Samples were then screened for the presence of *Plasmodium* and *Haemoproteus* using the nested PCR method of Waldenström et al. (2004), amplifying a 478bp fragment of the mitochondrial cytochrome-*b* gene. PCR reactions were performed in 25µl volumes, in two separate rounds. First-round primers were HaemNF (5´-CATATATTAAGAGAATTATGGAG-3´) and HaemNR2 (5´-AGAGGTGTAGCATATCTATCTAC-3´): each reaction contained contained 2µl of genomic DNA, 0.125mM each dNTP, 0.2µM each primer, 3mM $MgCl_2$ and 0.25 units of Platinum Taq polymerase (Invitrogen, CA, USA) with the accompanying PCR buffer at 1x final concentration. The thermal profile consisted of a 2 minute 94°C enzyme activation step, followed by 20 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 45 sec, ending with an elongation step of 72°C for 10 min. In the second PCR round, primers HaemF (5'-ATGGTGCTTTCGATATATGCATG-3') and HaemR2 were used (5'-GCATTATCTGGATGTGATAATGGT-3'): the composition of the PCR reactions was as above, except that 0.4µM of each primer and 0.5 units of Platinum Taq

Polymerase were used, and 2µl 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 for the first round, with the number of cycles increased from 20 to 35.

2-8µl of PCR products from the second round were run on 2% agarose gels stained with ethidium bromide and visualised under UV light. Samples containing bands of 450-600bp in size were prepared for sequencing using a Qiagen MinElute 96 UF PCR purification kit and a QiaVac multiwell vacuum manifold. The purified PCR fragments were then sequenced directly by dye terminator cycle sequencing (Big Dye v3.1), and loaded on an ABI PRISM 310 automated sequencer (Applied Biosystems, CA, USA). Sequences were edited in Sequencher v. 4.2 (GeneCodes Corp., MI, USA), and aligned in ClustalX (Jeanmougin, Thompson, Gouy *et al.*, 1998). 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; while a study of the seasonal variation in *Leucocytozoon* prevalence would certainly be of interest, the PCR test is not designed to amplify DNA from these parasites, and is thus less efficient, particularly when either *Haemoproteus* or *Plasmodium* are also present. Where possible, avian malaria sequences were further characterised to the lineage level, with exact matches named as per existing lineages in GenBank, whilst sequences differing by one or more base pairs from those in GenBank were assigned new names. We report a new lineage, pBLUTI3 (now assigned GenBank accession number DQ991069). Mixed infections were present at a low rate (ca. 2% in 2004-5, S.C.L. Knowles *et al*. unpubl.) and are not considered here.

185

186 *Statistical analysis*

187 Examining only linear changes of parasite prevalence through time can mask complex
188 oscillations in disease prevalence (Pascual & Dobson, 2005), so we employed a statistical
189 approach that seeks the best linear or non-linear fit to prevalence data. Seasonal variation
190 in the prevalence of malaria infection was examined using generalized additive modelling
191 (GAM), essentially a generalized linear model (GLZ) in which a smoothed function of a
192 covariate (sample date) can be considered alongside conventional linear predictors and
193 their interactions (Hastie, 1990). The smoothed term uses a cyclic spline for continuity
194 between the end and beginning of each year. More complex functions are penalised such
195 that a linear function would be retained if more parsimonious, with smoothing parameters
196 selected by penalized likelihood maximization via generalized cross validation (Wood,
197 2004). We incorporated a smoothed function of sampling date as a model term while
198 examining associations between malaria infection and linear functions of sampling date,
199 year, host age, and sex (and their interactions), using binomial errors and a logit link. This
200 starting model was optimised by the backward stepwise elimination of non-significant
201 terms, beginning with higher-order interactions. Interactions between conventional
202 factors were considered, but as those involving smoothed date cannot be incorporated
203 into GAMs, potential interactions between the smoothed date term and any retained linear
204 terms were examined by constructing GAMs subsetted by the retained term (e.g. age, see
205 Results). In order to compare seasonal patterns of prevalence between *Plasmodium*
206 morphospecies, we tested the factorial interaction between season (four three-month
207 periods beginning 15th June) and parasite species. In all models, terms were retained if

their removal caused a significant change ($P<0.05$) in model deviance. Means are presented ±1 standard error.

Results

Samples collected between autumn 2003 and summer 2005 from 816 individual blue tits were screened for avian malaria infection. The prevalence of avian malaria infection across the study period was 25.6%, comprising 24.4% *Plasmodium* and 0.8% *Haemoproteus* (the latter genus is excluded from analyses due to low prevalence and the potential for different seasonal patterns due to different vector ecologies: Valkiūnas, 2005). A total of 11 cytochrome-b lineages were identified: eight *Plasmodium* and three *Haemoproteus* spp. (Table 1). Some *Plasmodium* lineages have been matched to morphological species known from light microscopy (Hellgren, Križanauskiene, Valkiūnas *et al.*, 2007; Palinauskas, Kosarev, Shapoval *et al.*, 2007; Valkiūnas, Zehtindjiev, Hellgren *et al.*, 2007): we therefore analyse the seasonal pattern of *Plasmodium* pooled across all lineages, in addition to the prevalence of the two most common parasite morphospecies which together account for 93% of avian malaria infections, namely *Plasmodium relictum* Grassi & Feletti, 1891 and *P. circumflexum* Kikuth, 1931. As the prevalence of any single lineage never exceeded 10%, the available sample sizes did not support the analysis of lineages within species. Two approximately similar peaks of pooled *Plasmodium* prevalence were observed in May/June and September/October, with a steep decline in infection in winter (Fig. 1).

231

232 A non-linear smoothed function of sampling date was retained as the most suitable
233 temporal predictor of pooled *Plasmodium* prevalence (Table 2a). Host age was also
234 retained in the model: over the year as a whole, prevalence was 45% higher in older birds
235 (29.8±2.5%) compared to first-year birds (20.5±1.9%). Year, host sex and a linear date
236 function were not retained (Table 2a). A residual plot of the final model describing
237 seasonal variation in prevalence (Fig. 2a) shows two prevalence peaks, one in autumn and
238 one in the breeding season in spring, with a marked drop in prevalence in winter. Similar
239 analyses, treating the morphospecies separately, produced contrasting results: the *P.*
240 *circumflexum* model retained a smoothed date function similar to that for pooled
241 *Plasmodium* (Fig. 2b and Fig. 3), and an age effect (Table 2b); prevalence was again
242 higher in older birds (17.1±2.1%) than first years (11.5±1.5%). *P. relictum* retained a
243 weak linear date function in preference to non-linear smoothed functions, increasing
244 gradually over the year, but with no age effect (Table 2c). Analysis of morphospecies
245 prevalence by bimonthly periods (as in Fig. 1) retained parasite species as a model factor,
246 reflecting a difference in overall prevalence across the year (2-way analysis of deviance:
247 χ^2=4.89, df=1, P=0.027) and significant variation between bimonthly periods (χ^2=5.89,
248 df=1, P=0.015), but no interaction term. Analysing prevalence variation by of the
249 sampling year (seasons being four, three-month periods beginning on June 15th) also
250 retained species as a model factor (2-way analysis of deviance: χ^2=7.70, df=1, P=0.0055):
251 importantly, the season*species interaction was retained (χ^2=10.4, df=3, P=0.016),
252 indicating different patterns of seasonal variation in prevalence, at the level of three-
253 month seasons, shown by the two *Plasmodium* morphospecies (Fig. 3).

We further examined the differences in seasonal variation in prevalence by constructing predicted response models, which use final models (Table 2) to predict the variation in prevalence over a hypothetical range of daily sampling dates, an approach that is useful to visualise complex non-linear variation in prevalence (Fig. 4). The predicted response models were judged to be a good reflection of observed prevalence data, because (i) bimonthly prevalence (e.g. from Fig. 1) did not deviate significantly from the predicted variation in prevalence shown in Fig. 4 (bimonthly observed vs. predicted prevalence for pooled *Plasmodium*, *P. circumflexum*, *P. relictum*; good ness of fit χ^2 tests, df=5, P>0.90), and (ii) observed and predicted bimonthly prevalence were significantly correlated, with slopes close to unity, for pooled *Plasmodium* (r=1.03, P=0.01, R^2=0.80) and *P. circumflexum* (r=1.27, P=0.006, R^2=0.85). These correlations reflect the retention of smoothed date as a predictor of prevalence (Table 2), whereas no such correlation existed between observed and predicted *P. relictum* prevalence (r=0.36, P=0.22, R^2=0.18), for which smoothed date was not retained. Predicted response models for *P. relictum* (Fig. 4c) are, therefore, presented merely for visual comparison with pooled *Plasmodium* and *P. circumflexum*.

Comparing these plots between morphospecies reveals different seasonal patterns of prevalence (Figs. 4a-c): both pooled *Plasmodium* and *P. circumflexum* showed a clear pattern of seasonal variation including an autumn peak and an increase in prevalence early in the year. *P. relictum* infection (the modelling of which retained a linear function in preference to a smoothed date function, Table 2c) showed a relatively stable seasonal

277 pattern of prevalence, if somewhat lower in winter. This strongly suggests that seasonal
278 variation in *P. circumflexum* prevalence is largely responsible for the observed seasonal
279 variation in pooled *Plasmodium* prevalence.
280
281 Considering subsets of these predicted prevalence models by age class showed that the
282 seasonal pattern of pooled *Plasmodium* infection differs markedly by host age (Fig 4a).
283 All age classes show evidence of a post-breeding peak in *Plasmodium* in autumn, but
284 older birds show a more marked increase in prevalence in early spring. This indicates that
285 the age structure in seasonal variation in pooled *Plasmodium* prevalence between age
286 classes (Table 2a) lies in the putative 'spring relapse' period. *P. circumflexum* showed
287 evidence for an autumn peak in prevalence, which was most apparent in first year blue
288 tits; notably an obvious spring relapse was absent regardless of age (Fig. 4b). As
289 modelling of *P. relictum* prevalence retained a linear function in preference to a
290 smoothed date function (Table 2c), and a poor fit was found between observed and
291 predicted *P. relictum* prevalence, examining predictive models subsetted by age is not
292 justified statistically for this morphospecies, so we may not draw conclusions from the
293 age-subsetted model of predicted *P. relictum* prevalence (Fig. 4c). Only a linear date
294 function, and not age, was not retained in the modelling of *P. relictum* prevalence. This
295 linear date function, suggesting a slight increase in prevalence over the year (Table 2c),
296 indicates that the prevalence of *P. relictum* is less seasonally variable than *P.*
297 *circumflexum*.
298
299

Discussion

Seasonal variation in *Plasmodium* prevalence in blue tits in our study population is characterised by bimodal peaks in prevalence in autumn and spring, and a marked drop in prevalence during winter. At first sight, this genus level pattern agrees with the model of Beaudoin *et al.* (1971) for seasonal variation in avian malaria in temperate regions. However, the two most prevalent avian *Plasmodium* morphospecies in our study population showed different patterns of seasonal variation in prevalence: *P. circumflexum* showed seasonal variation of a pattern similar to that for pooled *Plasmodium*, whereas *P. relictum* prevalence was more stable. There was also clear age structure in the seasonality of *Plasmodium* infection: first year birds showed a less marked spring relapse of *Plasmodium* than older birds. The autumn peak in *Plasmodium* prevalence was largely driven by *P. circumflexum.* As seasonal patterns vary between age classes and between different *Plasmodium* morphospecies, we reject Beaudoin *et al.*'s model as it is not robust to the underlying complexity of the blue tit-*Plasmodium* interaction in this population.

Following the post-breeding/fledging phase in June, blue tits showed a peak in prevalence of pooled *Plasmodium* (and *P. circumflexum*) in autumn (Figs. 2, 4a&b). This October peak might result from new transmission to previously uninfected birds, rather than a relapse of previously infected birds, which could result either from a reduction in herd immunity or the addition of immunologically naïve juveniles into the population during the breeding season (Altizer *et al.*, 2006). The October *Plasmodium*/*P. circumflexum* prevalence peak seen in first-year birds (Fig. 4b) necessarily represents new transmission,

since these birds are new recruits to the population and so cannot have been previously infected. This post-fledging period is considerable a gap in our knowledge of the ecology of tits: after fledging, they are not easily trapped, so causes of the high rates of post-fledging mortality are poorly understood (Perrins, 1979). Assessing the impact of avian malaria on the survival of juveniles presents an important challenge.

In winter, the prevalence of pooled *Plasmodium* infections and the *P. circumflexum* morphospecies declined dramatically in both first year and adult birds, most likely due to a cessation of transmission and decline of existing malaria parasites from the blood, with negligible blood stages surviving the winter. *P. relictum* was also absent in winter, but present at a stable prevalence for the rest of the year (Fig. 4c). Avian *Plasmodium* spp. survive the lack of transmission during the winter by remaining in host tissues (Valkiūnas, 2005); our use of sensitive PCR-based screening methods in this study suggests that *Plasmodium* infections were indeed absent from the blood during in November and December (Fig. 1), as these techniques can detect approximately one malaria parasite per 10^5 erythrocytes (Waldenström *et al.*, 2004). It is possible that some malaria parasites are better adapted to surviving the winter than others, an idea supported by the markedly different seasonal patterns shown by *P. relictum* and *P. circumflexum* (Fig. 3).

Parasite prevalence has been reported to increase prior to the breeding season in temperate wild bird populations, known as the 'spring relapse' (Applegate, 1971; Box, 1966; Schrader *et al.*, 2003; Valkiūnas, 2005). Experimental studies have implicated day

346 length and hormone levels in inducing relapse (Applegate, 1970; Valkiūnas, Bairlein,
347 Iezhova *et al.*, 2004). Pooled *Plasmodium* infection shows, and *P. relictum* infection
348 suggests, a spring peak in prevalence, prior to the onset of the breeding season in mid-
349 May (Fig. 3). This may be due to relapse, or if infected birds die during the winter the
350 spring peak may result from re-infection with newly transmitted parasites. Contrary to
351 this latter interpretation is that vector populations are unlikely to have reached their peak
352 until later in the year (Cranston *et al.*, 1987; Marshall, 1938). Therefore, it is reasonable
353 to suggest that the spring 'relapse' in prevalence among older birds is indeed due to a
354 relapse of old infections rather than to new transmission.
355
356 Previous studies report marked differences in the prevalence of avian malaria between
357 first year and older birds, but the direction of this effect is not consistent in previous
358 studies (Dale, Kruszewicz & Slagsvold, 1996; Kucera, 1979; Merilä & Andersson, 1999;
359 Sol, Jovani & Torres, 2000, 2003; Valkiūnas, 2005). Predicted models of seasonal
360 variation in *Plasmodium* prevalence between age classes in our blue tit population (Fig.
361 4) suggest that the age structure lies in the spring relapse: pooled age classes showed an
362 autumn peak in prevalence, but older birds had a more marked spring peak than first-
363 years (Fig. 4a). From February to the breeding season, prevalence increased steadily in
364 first-years, but more rapidly in older birds. Although young birds breed later than older,
365 more experienced, birds, the difference in breeding time is small (2-3 days) so is unlikely
366 to account for the large discrepancy in relapse between age groups. Examining the age
367 structure of infection by morphospecies revealed that the pattern seen in pooled
368 *Plasmodium* prevalence was due to seasonal variation between both morphospecies and

age class: the autumn peak in pooled *Plasmodium* can be attributed to *P. circumflexum* in first years (Fig. 4b), and our data hint that the spring relapse in pooled *Plasmodium* may be attributable to *P. relictum* in older birds (Fig. 4c).

The different seasonal patterns of prevalence between these two *Plasmodium* morphospecies suggest that *P. circumflexum* transmission may benefit from the post-fledging peak in numbers of immunologically naïve individuals or a reduction in herd immunity. Potential spring relapses of *P. relictum* in older birds may represent lineages transmitted only before the eggs hatch, and so not transmitted to first years after fledging. Given that *P. relictum* is the most ubiquitous and least host-restricted of the avian Plasmodia, one may speculate that it has a more successful transmission strategy than *P. circumflexum*. This hypothesis would be supported if spring relapse in *P. relictum* but not *P. circumflexum* was confirmed by further study, as *P. relictum* gametocytes are more infective to vectors in spring than in autumn (Valkiūnas, 2005). The higher infectivity of *P. relictum* in spring coincides with the arrival of migratory bird species and precedes the increase in the host population, potentially facilitating the parasite's spread and persistence. Such speculation requires improved knowledge of the ecology of avian malaria in resident and migrant birds at Wytham. The autumn peak in *Plasmodium* prevalence, particularly in *P. circumflexum*, coincides with a peak in the post-fledging dispersal of first year birds, presenting an opportunity for malaria parasites to disperse with their hosts; older birds, having already bred and held a territory, disperse less far than first years (Perrins, 1979). The epidemiological consequences of age-structure, both in the seasonal variation of prevalence between *Plasmodium* morphospecies and in

dispersal distance, are intriguing. Clearly, our understanding of the epidemiology of host-parasite interactions involving avian Plasmodia would be enhanced by the study of vector specificities and the seasonal availability of compatible vectors.

This study is reliant upon sensitive molecular diagnostic techniques, (Waldenström *et al.*, 2004), knowledge of the taxonomy of avian *Plasmodium* in relation to molecular data (Hellgren *et al.*, 2007; Valkiūnas *et al.*, 2007) and categorisation of hosts into first year and older birds. Without these factors, the 'two peaks and a trough' model of seasonal variation in avian malaria prevalence (Beaudoin *et al.*, 1971) would have been accepted by our study, when in fact the seasonal pattern of *Plasmodium* variation in blue tits in our study is a complex combination of different patterns, both between *Plasmodium* morphospecies and (in the case of *P. circumflexum*) between age classes. An additional factor not considered here is that there may be marked spatial differences in the prevalence and distribution of different parasite species. Indeed, we know this to be the case for the present study population, which shows spatial variation in both the overall prevalence of malaria and in the distribution of morphospecies (Wood, Cosgrove, Wilkin *et al.*, 2007). There are some intriguing parallels between the temporal patterns revealed here and the spatial ones described elsewhere (Wood *et al.*, 2007): in both cases, *P. relictum* shows a broader distribution, while *P. circumflexum* shows a more clustered distribution.

We found no evidence that the seasonal pattern of infection differed between years (Table 2), although the possibility of annual variation in seasonal patterns is suggested by

variation in the prevalence of some avian malaria lineages between breeding seasons (Wood *et al.*, 2007). Between-year fluctuations in parasite prevalence are commonly reported for vector-borne and other diseases, suggesting that more long-term data is required to examine between-year variation in avian malaria in our study population (e.g. see (Bensch, Waldenström, Jonzen *et al.*, 2007). There was no significant difference between the malaria prevalence of males and females throughout the year, in contrast to several field studies showing differences in parasite prevalence between the sexes of breeding wild birds (Applegate, 1971; Merilä & Andersson, 1999; Richner, Christe & Oppliger, 1995).

Our data demonstrate that studies of the ecology of parasites in wild populations should take account of temporal variation within years (i.e. seasonal variation) in at least three contexts. First, overall prevalence varies both with date and with host activity, meaning that both factors must be known to make sense of any variation in prevalence, unless sampling is restricted to specific temporal and activity classes. Second, prevalence varies with host demographic factors, and the seasonal pattern differs among different host age groups. Third, the seasonal pattern of prevalence differs among malaria parasite morphospecies. Identifying the transmission periods when hosts and infective vectors meet is crucial here: the study of vector ecology would greatly enhance our understanding of the seasonality of avian malaria in our study system. Host-vector and vector-parasite associations are poorly understood at present (Boete & Paul, 2006). In a broader context, understanding the causes of seasonal variation in transmission might be attempted at a wider geographic scale (Pérez-Tris & Bensch, 2005), or in the context of how these

diseases might respond to climate change (Kovats, Campbell-Lendrum, McMichael *et al.*, 2001; Rogers & Randolph, 2000). Any study that aims to understand individual heterogeneity in infection in avian malaria should consider both temporal (this study) and spatial variation (Wood *et al.*, 2007) as contributory factors. Continued research promises increasing understanding of the ecology of avian malaria, and the epidemiology of vector-borne disease in general.

Acknowledgments

The first two authors made an equal contribution to this paper. We thank Simon Griffith, Iain Barr, Louise Rowe, Joanne Chapman and numerous Wytham fieldworkers for their invaluable assistance in the field. CLC and MJW were supported by a NERC grant to KPD and BCS. Sarah Knowles, Freya Fowkes and two anonymous reviewers made valuable comments on the manuscript.

Table and Figure legends

Table 1.

A total of 816 individual blue tits, sampled between autumn 2003 and summer 2005 were screened for avian malaria infection. Mitochondrial cytochrome-*b* lineages were assigned using molecular techniques (see Methods), shown in the 'Lineage' column; the prefix "p" denotes *Plasmodium*, and "h" denotes *Haemoproteus*. The frequency of infection of each avian malaria lineage is shown, categorised by host species.

461 * Mitochondrial cytochrome-*b* lineages previously matched to morphological species
462 (Hellgren *et al.*, 2007; Palinauskas *et al.*, 2007; Valkiūnas *et al.*, 2007).
463 † Some sequences could not be resolved to a particular malaria lineage, but in some cases
464 could be resolved to either *Plasmodium* or *Haemoproteus.*
465 ‡ Percentages in parentheses indicate the overall population prevalence, which do not sum
466 to pooled prevalence due to low frequency (ca. 2%) mixed infections (S.C.L. Knowles et
467 al. unpublished).
468
469 **Table 2.**
470 Final Generalized Additive Models (GAMs) are shown, examining seasonal variation in
471 (a) pooled *Plasmodium* infections, (b) *P. circumflexum* and (c) *P. relictum*. In each
472 model, a smoothed function of sample date was modelled alongside linear predictors and
473 their interactions (linear date, host age, host sex and sampling year) using binomial errors
474 and a logit link. Each model was optimised by the backward stepwise elimination of non-
475 significant terms, beginning with higher order interactions. Model terms were retained if
476 their removal caused a significant change ($P<0.05$) in model deviance. No interactions
477 were retained in final models.
478
479 **Figure 1.**
480 A total of 816 blue tits sampled between autumn 2003 and summer 2005 are analysed
481 here. Avian malaria infection was diagnosed using molecular techniques (see Methods).
482 Error bars represent ±1 s.e.
483

Figure 2.

The estimated effect of the smoothed function of date on prevalence is shown, controlling for other model effects (e.g. host age, see Table 2). Generalized additive modelling (GAM) was used to incorporate potential non-linear variation in prevalence (see Methods). Note the marked peak in prevalence in October-November, a reduced prevalence in mid-winter (December-January), another peak in prevalence in early spring (March) before the breeding season (May-June). Dotted lines about plotted functions show the Bayesian credible intervals of the model.

Figure 3.

Predictive models were constructed to visualise variation in prevalence with sampling date and age, for *Plasmodium* infection, *P. circumflexum* and *P. relictum*, each using the best non-linear smoothed function of sampling date (Table 2; *P. relictum* retained a linear function in modelling, but a smoothed function is used here for comparison). Their respective predicted prevalences through the year were then extrapolated from the model fitted to prevalence data (e.g. Fig. 2). Points on each graph show the pooled *Plasmodium* infection status of birds used in generating the predictive model, i.e. those positive (black circles) and negative (open circles) for infection. Multiple samples on a particular day are overlaid, so these points under-represent the extent of sampling.

Figure 4.

These plots follow the rationale in Fig. 3; predicted prevalence is shown for (a) *Plasmodium* infection, (b) *P. circumflexum* and (c) *P. relictum*, by age category to

illustrate the age structure in infection (Table 2): (i) age classes superimposed, (ii) all ages, (iii) first years and (iv) older birds. Smoothed date function and host age were not retained in the modelling of *P. relictum* prevalence, and therefore is shown here (Fig. 3c) merely for comparison. Circles on each graph show the infection status of birds used in generating the predictive model, multiple samples on a particular day are overlaid and so under-represent the extent of sampling. Grey squares show observed mean bimonthly prevalence: predicted prevalence showed a good fit with observed prevalence data for *Plasmodium* ($r=1.03$, $P=0.01$, $R^2=0.80$) and *P. circumflexum* ($r=1.27$, $P=0.006$, $R^2=0.85$), but not for *P. relictum* ($r=0.36$, $P=0.22$, $R^2=0.18$). Predicted prevalence is plotted only within the range of observed data.

Table 1.

Diversity and abundance of avian malaria in blue tits from Wytham Woods

Lineage	GenBank no.	Morphospecies	N infected
pSGS1	AF495571	*Plasmodium relictum**	72 (8.8%)
pGRW11	AY831748	*Plasmodium relictum**	12 (1.5%)
pBLUTI3	DQ991069	*Plasmodium relictum**	1 (0.1%)
		***Plasmodium relictum**‡**	**84 (10.3%)**
pTURDUS1	AF495576	*Plasmodium circumflexum**	74 (9.1%)
pBT7	AY393793	*Plasmodium circumflexum**	38 (4.7%)
pBLUTI4	DQ991070	*Plasmodium circumflexum**	1 (0.1%)
pBLUTI5	DQ991071	*Plasmodium circumflexum**	1 (0.1%)
		***Plasmodium circumflexum**‡**	**113 (13.8%)**
pBLUTI1	DQ991068	*Plasmodium* spp. unknown	4 (0.5%)
		Unresolved *Plasmodium* lineages†	17 (2.1%)
		Pooled *Plasmodium* spp.‡	**199 (24.4%)**
hTURDUS2	DQ060772	*Haemoproteus minutus**	3 (0.4%)
hWW1	AF254971	*Haemoproteus* spp. unknown	1 (0.1%)
hBLUTI1	DQ991077	*Haemoproteus* spp. unknown	1 (0.1%)
		Unresolved *Haemoproteus* lineages†	2 (0.2%)
		Pooled *Haemoproteus* spp.‡	**7 (0.8%)**
		Unresolved avian malaria†	5 (0.6%)
		Pooled avian malaria‡	**209 (25.6%)**

Table 2.

Generalized additive models (GAM) examining seasonal variation in the prevalence of *Plasmodium* infection in blue tits

Factor	parameter estimate	*Z*	*P*
(a) Pooled *Plasmodium*			
Age	0.45±0.17	2.66	0.0078
Smoothed sample date: estimated df = 5.56, χ^2 = 19.3, *P* < 0.013			
(b) *P. circumflexum*			
Age	0.42±0.21	2.04	0.042
Smoothed sample date: estimated df = 4.91, χ^2 = 16.6, *P* = 0.034			
(c) *P. relictum*			
Linear date	0.0052±0.0027	1.96	0.050

Figure 1.

Seasonal variation in the prevalence of *Plasmodium* infection in blue tits

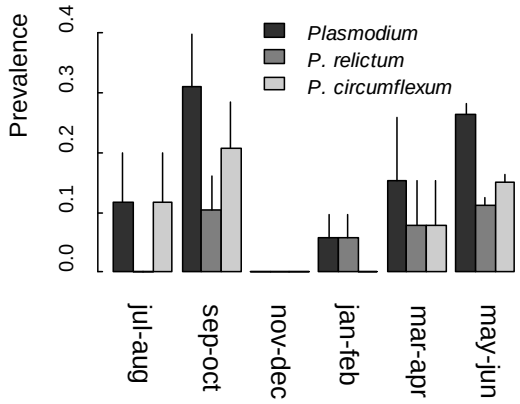


Figure 2.

Smoothed residual models of the seasonal variation in prevalence of (a) pooled *Plasmodium* and (b) *P. circumflexum* infection in blue tits

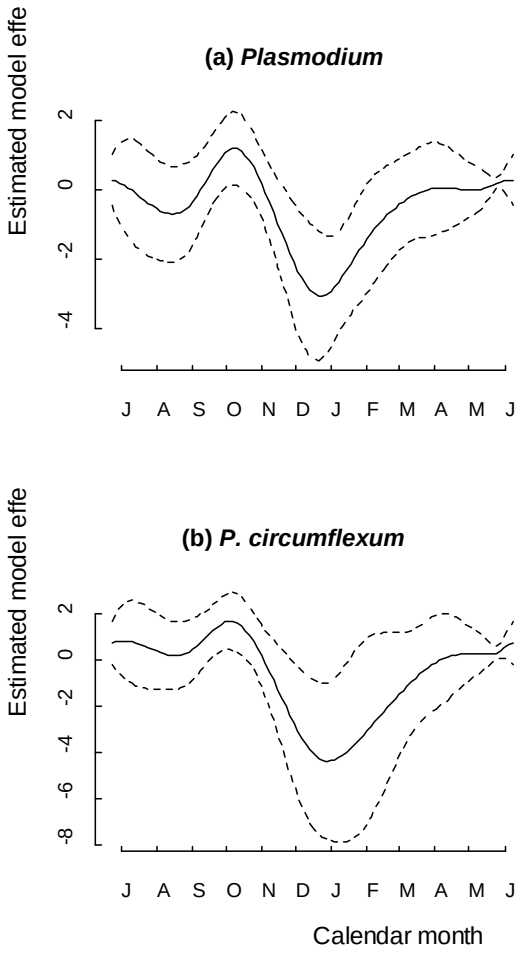


Figure 3.

Predictive models of seasonal variation in *Plasmodium* infection in blue tits

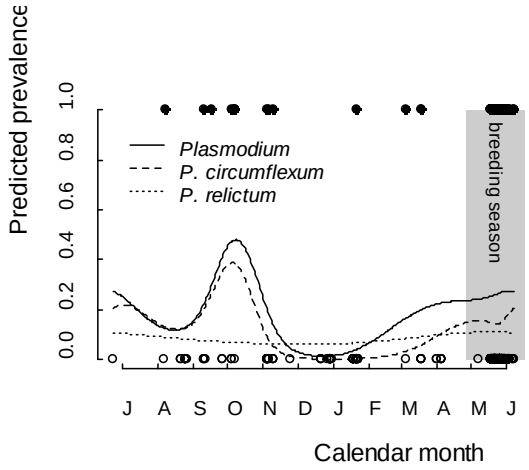


Figure 4a-c

Predicted prevalence of *Plasmodium* in blue tits

(a) Pooled *Plasmodium*

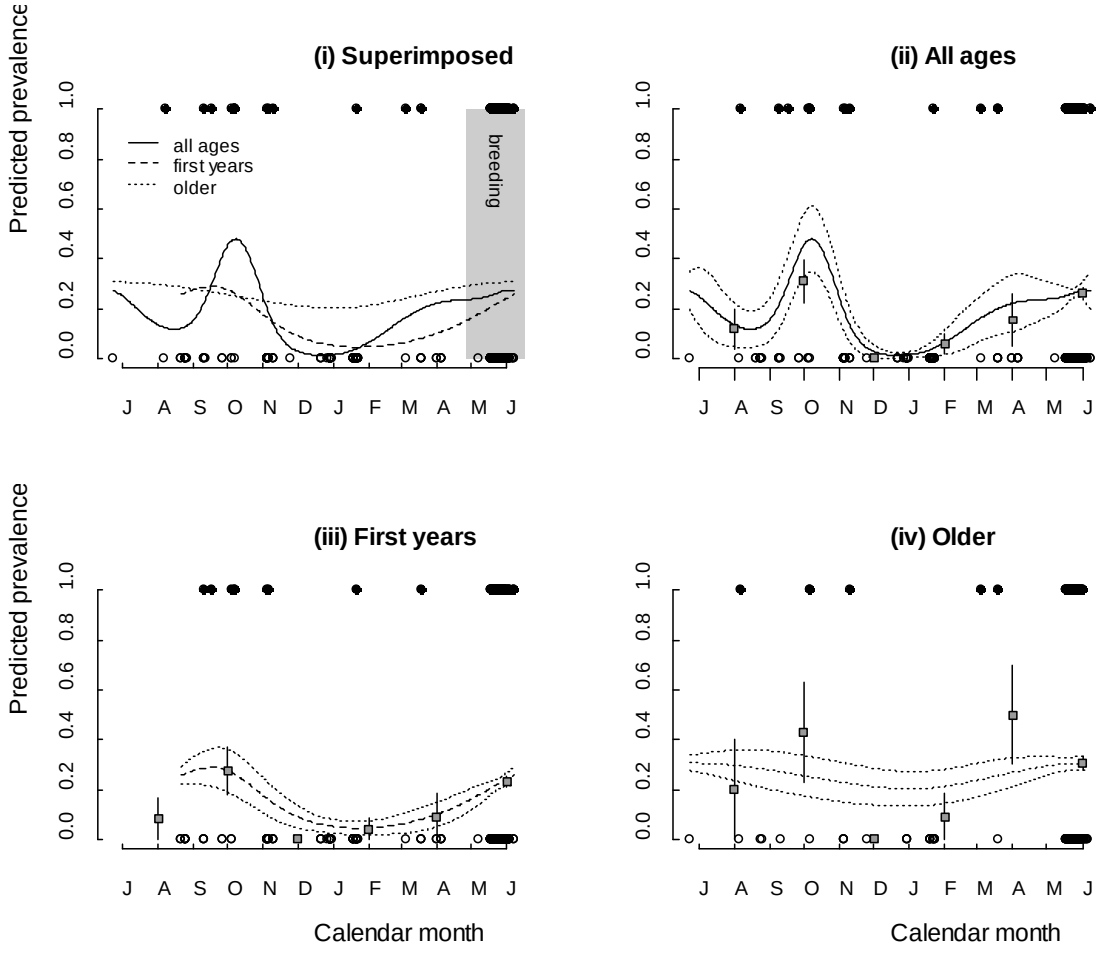


Figure 4a-c

Predicted prevalence of *Plasmodium* in blue tits

(b) *P. circumflexum*

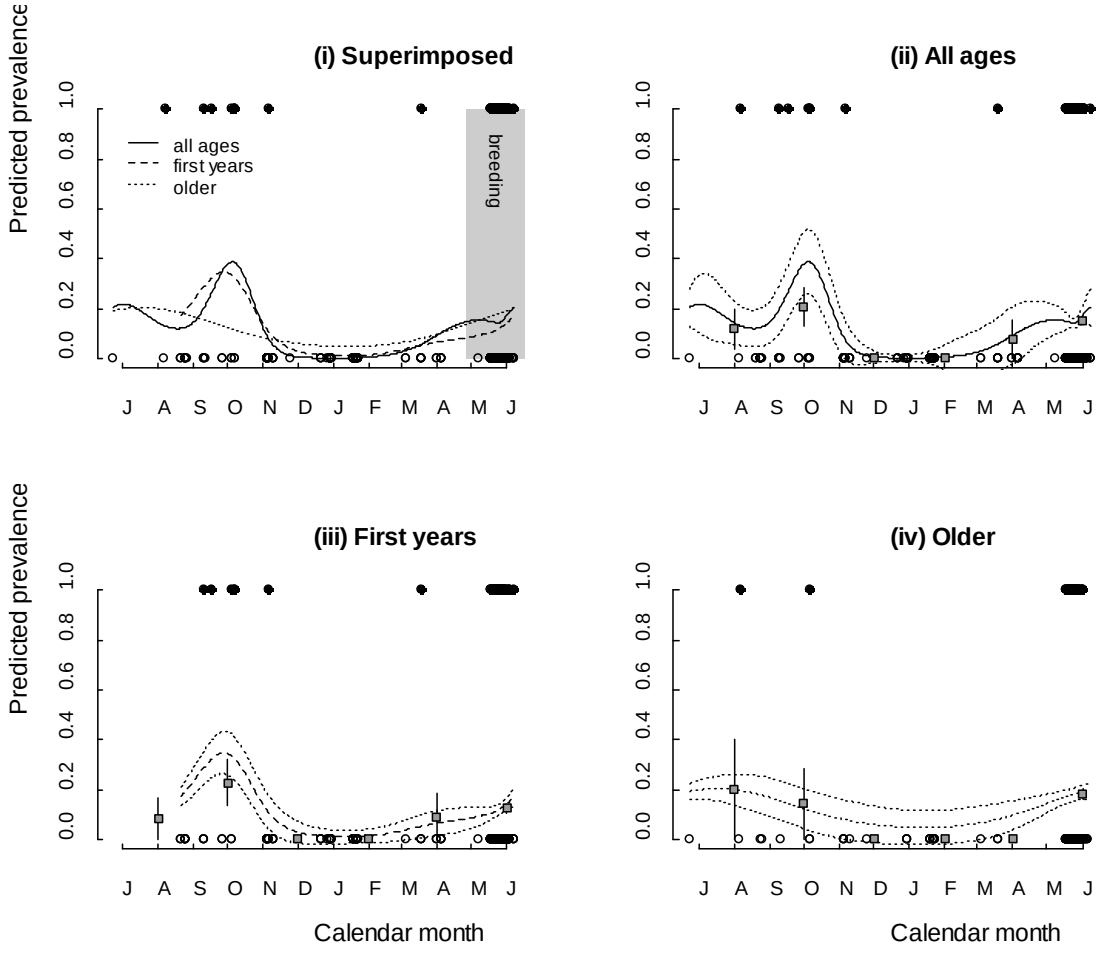
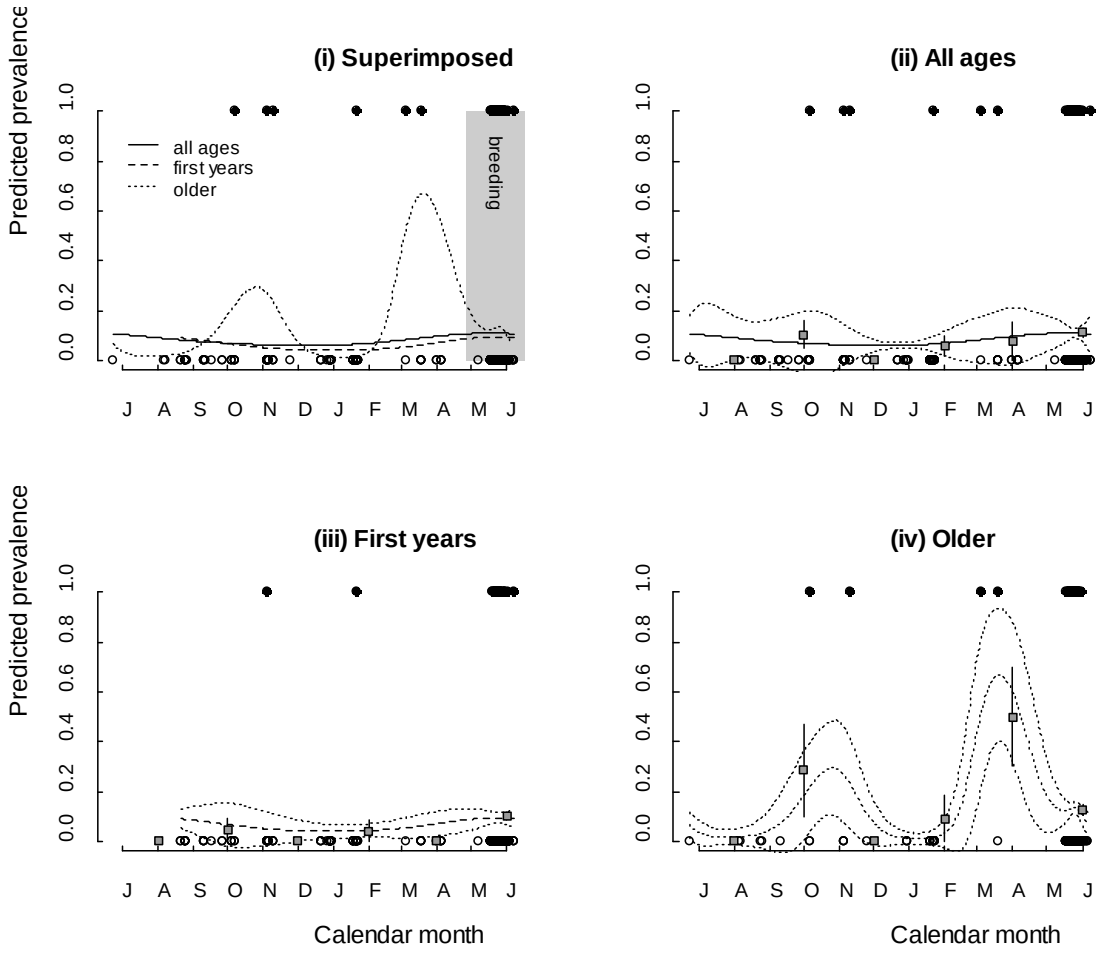


Figure 4a-c

Predicted prevalence of *Plasmodium* in blue tits by host age and parasite morphospecies

(c) *P. relictum*



References

Altizer, S., Dobson, A., Hosseini, P., Hudson, P., Pascual, M. & Rohani, P. (2006) Seasonality and the dynamics of infectious diseases. *Ecology Letters*, 9, 467-484.

Applegate, J.E. (1970) Population changes in latent avian malaria infections associated with season and corticosterone treatment. *Journal of Parasitology*, 56, 439-&.

Applegate, J.E. (1971) Spring relapse of *Plasmodium relictum* infections in an experimental field population of English sparrows (*Passer domesticus*). *Journal of Wildlife Diseases*, 7, 37-42.

Aron, J.L. & May, R.M. (1982). The population dynamics of malaria. In *Population Dynamics and Infectious Disease* (ed R.M. Anderson), pp. 139-179. Chapman and Hall, London, UK.

Beadell, J.S., Ishtiaq, F., Covas, R., Melo, M., Warren, B.H., Atkinson, C.T., Bensch, S., Graves, G.R., Jhala, Y.V., Peirce, M.A., Rahmani, A.R., Fonseca, D.M. & Fleischer, R.C. (2006) Global phylogeographic limits of Hawaii's avian malaria. *Proceedings of the Royal Society B-Biological Sciences*, 273, 2935-2944.

Beaudoin, R.L., Applegate, J.E., David, D.E. & McLean, R.G. (1971) A model for the ecology of avian malaria. *Journal of Wildlife Diseases*, 7, 5-13.

Bensch, S., Pérez-Tris, J., Waldenström, J. & Hellgren, O. (2004) Linkage between nuclear and mitochondrial DNA sequences in avian malaria parasites: Multiple cases of cryptic speciation? *Evolution*, 58, 1617-1621.

Bensch, S., Stjernman, M., Hasselquist, D., Ostman, O., Hansson, B., Westerdahl, H. & Pinheiro, R.T. (2000) Host specificity in avian blood parasites: a study of

Plasmodium and *Haemoproteus* mitochondrial DNA amplified from birds. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 267, 1583-1589.

Bensch, S., Waldenström, J., Jonzen, N., Westerdahl, H., Hansson, B., Sejberg, D. & Hasselquist, D. (2007) Temporal dynamics and diversity of avian malaria parasites in a single host species. *Journal of Animal Ecology*, 76, 112-122.

Boete, C. & Paul, R.E.L. (2006) Can mosquitoes help to unravel the community structure of *Plasmodium* species? *Trends in Parasitology*, 22, 21-25.

Box, E.D. (1966) Blood and tissue protozoa of the English Sparrow (*Passer domesticus*) in Galveston, Texas. *Journal of Protozoology*, 13, 204-208.

Childs, D.Z., Cattadori, I.M., Suwonkerd, W., Prajakwong, S. & Boots, M. (2006) Spatiotemporal patterns of malaria incidence in northern Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 100, 623-631.

Cranston, P.S., Ramsdale, C.D., Snow, K.P. & White, G.B. (1987) *Adults, larvae and pupae of British mosquitoes (Culicidae)*. Freshwater Biological Association, Ambleside, UK.

Dale, S., Kruszewicz, A. & Slagsvold, T. (1996) Effects of blood parasites on sexual and natural selection in the pied flycatcher. *Journal of Zoology*, 238, 373-393.

Dietz, K. (1976) The incidence of infectious diseases under the influence of seasonal fluctuations. *Lecture Notes in Biomathematics*, 11, 1-15.

Fallon, S.M., Ricklefs, R.E., Swanson, B.L. & Bermingham, E. (2003) Detecting avian malaria: An improved polymerase chain reaction diagnostic. *Journal of Parasitology*, 89, 1044-1047.

Greenman, J., Kamo, M. & Boots, M. (2004) External forcing of ecological and epidemiological systems: a resonance approach. *Physica D-Nonlinear Phenomena*, 190, 136-151.

Hastie, T.T., R. (1990) *Generalized Additive Models*. Chapman and Hall, London, UK.

Hatchwell, B.J., Wood, M.J., Anwar, M. & Perrins, C.M. (2000) The prevalence and ecology of the haematozoan parasites of European blackbirds, *Turdus merula*. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, 78, 684-687.

Hay, S.I., Myers, M.F., Burke, D.S., Vaughn, D.W., Endy, T., Ananda, N., Shanks, G.D., Snow, R.W. & Rogers, D.J. (2000) Etiology of interepidemic periods of mosquito-borne disease. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 9335-9339.

Hellgren, O., Križanauskiene, A., Valkiūnas, G. & Bensch, S. (2007) Diversity and phylogeny of mitochondrial cytochrome *b* lineages from six morphospecies of avian *Haemoproteus* (Haemosporida, Haemoproteidae). *Journal of Parasitology*, 93, 889–896

Hellgren, O., Waldenström, J. & Bensch, S. (2004) A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. *Journal of Parasitology*, 90, 797-802.

Hess, G.R., Randolph, S.E., Arneberg, P., Chemini, C., Furlanello, C., Harwood, J., Roberts, M.G. & Swinton, J. (2001). Spatial aspects of disease dynamics. In *The Ecology of Wildlife Diseases* (eds P.J. Hudson, A. Rizzoli, B.T. Grenfell, H. Heesterbeek & A.P. Dobson), pp. 102-118. Oxford University Press, Oxford, UK.

Jeanmougin, F., Thompson, J.D., Gouy, M., Higgins, D.G. & Gibson, T.J. (1998) Multiple sequence alignment with Clustal X. *Trends in Biochemical Sciences*, 23, 403-405.

Kovats, R.S., Campbell-Lendrum, D.H., McMichael, A.J., Woodward, A. & Cox, J.S. (2001) Early effects of climate change: do they include changes in vector-borne disease? *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, 356, 1057-1068.

Kucera, J. (1979) *Leucocytozoon* in Central European birds: II. Ecological studies. *Journal of Protozoology*, 26, suppl. 43a.

Kucera, J. (1981) Blood parasites of birds in Central Europe: III. *Plasmodium* and Central Europe. *Folia Parasitologica*, 28, 303-312.

Marshall, J.F. (1938) The British Mosquitoes. British Musuem, London, UK.

Merilä, J. & Andersson, M. (1999) Reproductive effort and success are related to haematozoan infections in blue tits. *Ecoscience*, 6, 421-428.

Palinauskas, V., Kosarev, V.V., Shapoval, A.P., Bensch, S. & Valkiūnas, G. (2007) Comparison of mitochondrial cytochrome b lineages and morphospecies of two avian malaria parasites of the subgenera *Haemamoeba* and *Giovannolaia* (Haemosporida: Plasmodiidae). *Zootaxa*, 1626, 39-50

Pascual, M. & Dobson, A. (2005) Seasonal patterns of infectious diseases. *Plos Medicine*, 2, 18-20.

Pérez-Tris, J. & Bensch, S. (2005) Dispersal increases local transmission of avian malarial parasites. *Ecology Letters*, 8, 838-845.

Pérez-Tris, J., Hasselquist, D., Hellgren, O., Križanauskiene, A., Waldenström, J. & Bensch, S. (2005) What are malaria parasites? *Trends in Parasitology*, 21, 209-211.

Perrins, C.M. (1979) *British Tits*. Collins, Glasgow.

Richner, H., Christe, P. & Oppliger, A. (1995) Paternal investment affects prevalence of malaria. *Proceedings of the National Academy of Sciences, USA*, 92, 1192-1194.

Rogers, D.J. & Randolph, S.E. (2000) The global spread of malaria in a future, warmer world. *Science*, 289, 1763-1766.

Schrader, M.S., Walters, E.L., James, F.C. & Greiner, E.C. (2003) Seasonal prevalence of a haematozoan parasite of red-bellied woodpeckers (*Melanerpes carolinus*) and its association with host condition and overwinter survival. *Auk*, 120, 130-137.

Seutin, G., White, B.N. & Boag, P.T. (1991) Preservation of avian blood and tissue samples for DNA analyses. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, 69, 82-90.

Sol, D., Jovani, R. & Torres, J. (2000) Geographical variation in blood parasites in feral pigeons: the role of vectors. *Ecography*, 23, 307-314.

Sol, D., Jovani, R. & Torres, J. (2003) Parasite mediated mortality and host immune response explain age-related differences in blood parasitism in birds. *Oecologia*, 135, 542-547.

Svensson, L. (1992) *Identification guide to European passerines*. 4th edn. Natural History Museum, Stockholm.

Valkiūnas, G. (2005) *Avian Malaria Parasites and other Haemosporidia*. CRC Press, Boca Raton, FL, USA.

Valkiūnas, G., Anwar, A.M., Atkinson, C.T., Greiner, E.C., Paperna, I. & Peirce, M.A. (2005) What distinguishes malaria parasites from other pigmented haemosporidians? *Trends in Parasitology*, 21, 357-358.

Valkiūnas, G., Bairlein, F., Iezhova, T.A. & Dolnik, O.V. (2004) Factors affecting the relapse of *Haemoproteus belopolskyi* infections and the parasitaemia of *Trypanosoma* spp. in a naturally infected European songbird, the blackcap, *Sylvia atricapilla*. *Parasitology Research*, 93, 218-222.

Valkiūnas, G., Zehtindjiev, P., Hellgren, O., Ilieva, M., Iehzova, T.A. & Bensch, S. (2007) Linkage between mitochondrial cytochrome b lineages and morphospecies of two avian malaria parasites, with a description of *Plasmodium (Novyella) ashfordi* sp. nov. *Parasitology Research*, 100, 1311-1322.

Waldenström, J., Bensch, S., Hasselquist, D. & Ostman, O. (2004) A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. *Journal of Parasitology*, 90, 191-194.

Weatherhead, P.J. & Bennett, G.F. (1991) Ecology of red-winged blackbird parasitism by hematozoa. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, 69, 2352-2359.

White, K.A.J., Grenfell, B.T., Hendry, R.J., Lejeune, O. & Murray, J.D. (1996) Effect of seasonal host reproduction on host-macroparasite dynamics. *Mathematical Biosciences*, 137, 79-99.

Wood, M.J., Cosgrove, C.L., Wilkin, T.A., Knowles, S.C.L., Day, K.P. & Sheldon, B.C. (2007) Within-population variation in prevalence and lineage distribution of avian malaria in blue tits *Cyanistes caeruleus*. *Molecular Ecology*, 16, 3263–3273.

Wood, S.N. (2004) Stable and efficient multiple smoothing parameter estimation for generalized additive models. *Journal of the American Statistical Association*, 99, 673-686.