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

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

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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
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 \textit{et al.}, 2000; Fallon, Ricklefs, Swanson \textit{et al.}, 2003; Hellgren, Waldenström & Bensch, 2004; Waldenström, Bensch, Hasselquist \textit{et al.}, 2004) allows avian malaria infections to be examined in more detail than is possible using traditional light microscopy techniques (Waldenström \textit{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-Trís, Waldenström \textit{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 \textit{Cyanistes caeruleus} L., 1758, to test Beaudoin \textit{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.

\textbf{Methods}

\textit{Host-parasite system}

Avian malaria, caused by \textit{Plasmodium} and \textit{Haemoproteus} spp. (\textit{sensu} Pérez-Trís, Hasselquist, Hellgren \textit{et al.}, 2005; see Valkiūnas, Anwar, Atkinson \textit{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 <20 µL 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´-ATGGTGCTTTCCATATGATG-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.
Statistical analysis

Examining only linear changes of parasite prevalence through time can mask complex oscillations in disease prevalence (Pascual & Dobson, 2005), so we employed a statistical approach that seeks the best linear or non-linear fit to prevalence data. Seasonal variation in the prevalence of malaria infection was examined using generalized additive modelling (GAM), essentially a generalized linear model (GLZ) in which a smoothed function of a covariate (sample date) can be considered alongside conventional linear predictors and their interactions (Hastie, 1990). The smoothed term uses a cyclic spline for continuity between the end and beginning of each year. More complex functions are penalised such that a linear function would be retained if more parsimonious, with smoothing parameters selected by penalized likelihood maximization via generalized cross validation (Wood, 2004). We incorporated a smoothed function of sampling date as a model term while examining associations between malaria infection and linear functions of sampling date, year, host age, and sex (and their interactions), using binomial errors and a logit link. This starting model was optimised by the backward stepwise elimination of non-significant terms, beginning with higher-order interactions. Interactions between conventional factors were considered, but as those involving smoothed date cannot be incorporated into GAMs, potential interactions between the smoothed date term and any retained linear terms were examined by constructing GAMs subsetted by the retained term (e.g. age, see Results). In order to compare seasonal patterns of prevalence between Plasmodium morphospecies, we tested the factorial interaction between season (four three-month 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, Zehtindjie, 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).
A non-linear smoothed function of sampling date was retained as the most suitable temporal predictor of pooled *Plasmodium* prevalence (Table 2a). Host age was also retained in the model: over the year as a whole, prevalence was 45% higher in older birds (29.8±2.5%) compared to first-year birds (20.5±1.9%). Year, host sex and a linear date function were not retained (Table 2a). A residual plot of the final model describing seasonal variation in prevalence (Fig. 2a) shows two prevalence peaks, one in autumn and one in the breeding season in spring, with a marked drop in prevalence in winter. Similar analyses, treating the morphospecies separately, produced contrasting results: the *P. circumflexum* model retained a smoothed date function similar to that for pooled *Plasmodium* (Fig. 2b and Fig. 3), and an age effect (Table 2b); prevalence was again higher in older birds (17.1±2.1%) than first years (11.5±1.5%). *P. relictum* retained a weak linear date function in preference to non-linear smoothed functions, increasing gradually over the year, but with no age effect (Table 2c). Analysis of morphospecies prevalence by bimonthly periods (as in Fig. 1) retained parasite species as a model factor, reflecting a difference in overall prevalence across the year (2-way analysis of deviance: \(\chi^2=4.89, \text{df}=1, P=0.027\)) and significant variation between bimonthly periods (\(\chi^2=5.89, \text{df}=1, P=0.015\)), but no interaction term. Analysing prevalence variation by of the sampling year (seasons being four, three-month periods beginning on June 15\(^{th}\)) also retained species as a model factor (2-way analysis of deviance: \(\chi^2=7.70, \text{df}=1, P=0.0055\)): importantly, the season*species interaction was retained (\(\chi^2=10.4, \text{df}=3, P=0.016\)), indicating different patterns of seasonal variation in prevalence, at the level of three-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*; goodness of fit $\chi^2$ 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
pattern of prevalence, if somewhat lower in winter. This strongly suggests that seasonal variation in *P. circumflexum* prevalence is largely responsible for the observed seasonal variation in pooled *Plasmodium* prevalence.

Considering subsets of these predicted prevalence models by age class showed that the seasonal pattern of pooled *Plasmodium* infection differs markedly by host age (Fig 4a). All age classes show evidence of a post-breeding peak in *Plasmodium* in autumn, but older birds show a more marked increase in prevalence in early spring. This indicates that the age structure in seasonal variation in pooled *Plasmodium* prevalence between age classes (Table 2a) lies in the putative ‘spring relapse’ period. *P. circumflexum* showed evidence for an autumn peak in prevalence, which was most apparent in first year blue tits; notably an obvious spring relapse was absent regardless of age (Fig. 4b). As modelling of *P. relictum* prevalence retained a linear function in preference to a smoothed date function (Table 2c), and a poor fit was found between observed and predicted *P. relictum* prevalence, examining predictive models subsetted by age is not justified statistically for this morphospecies, so we may not draw conclusions from the age-subsetted model of predicted *P. relictum* prevalence (Fig. 4c). Only a linear date function, and not age, was not retained in the modelling of *P. relictum* prevalence. This linear date function, suggesting a slight increase in prevalence over the year (Table 2c), indicates that the prevalence of *P. relictum* is less seasonally variable than *P. circumflexum*. 


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
length and hormone levels in inducing relapse (Applegate, 1970; Valkiūnas, Bairlein, Iezhova et al., 2004). Pooled *Plasmodium* infection shows, and *P. relictum* infection suggests, a spring peak in prevalence, prior to the onset of the breeding season in mid-May (Fig. 3). This may be due to relapse, or if infected birds die during the winter the spring peak may result from re-infection with newly transmitted parasites. Contrary to this latter interpretation is that vector populations are unlikely to have reached their peak until later in the year (Cranston et al., 1987; Marshall, 1938). Therefore, it is reasonable to suggest that the spring ‘relapse’ in prevalence among older birds is indeed due to a relapse of old infections rather than to new transmission.

Previous studies report marked differences in the prevalence of avian malaria between first year and older birds, but the direction of this effect is not consistent in previous studies (Dale, Kruszewicz & Slagsvold, 1996; Kucera, 1979; Merilä & Andersson, 1999; Sol, Jovani & Torres, 2000, 2003; Valkiūnas, 2005). Predicted models of seasonal variation in *Plasmodium* prevalence between age classes in our blue tit population (Fig. 4) suggest that the age structure lies in the spring relapse: pooled age classes showed an autumn peak in prevalence, but older birds had a more marked spring peak than first-years (Fig. 4a). From February to the breeding season, prevalence increased steadily in first-years, but more rapidly in older birds. Although young birds breed later than older, more experienced, birds, the difference in breeding time is small (2-3 days) so is unlikely to account for the large discrepancy in relapse between age groups. Examining the age structure of infection by morphospecies revealed that the pattern seen in pooled *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.

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**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.
Mitochondrial cytochrome-\(b\) lineages previously matched to morphological species (Hellgren et al., 2007; Palinauskas et al., 2007; Valkiūnas et al., 2007).

† Some sequences could not be resolved to a particular malaria lineage, but in some cases could be resolved to either \textit{Plasmodium} or \textit{Haemoproteus}.

‡ Percentages in parentheses indicate the overall population prevalence, which do not sum to pooled prevalence due to low frequency (ca. 2\%) mixed infections (S.C.L. Knowles et al. unpublished).

\textbf{Table 2.}

Final Generalized Additive Models (GAMs) are shown, examining seasonal variation in (a) pooled \textit{Plasmodium} infections, (b) \textit{P. circumflexum} and (c) \textit{P. relictum}. In each model, a smoothed function of sample date was modelled alongside linear predictors and their interactions (linear date, host age, host sex and sampling year) using binomial errors and a logit link. Each model was optimised by the backward stepwise elimination of non-significant terms, beginning with higher order interactions. Model terms were retained if their removal caused a significant change (P<0.05) in model deviance. No interactions were retained in final models.

\textbf{Figure 1.}

A total of 816 blue tits sampled between autumn 2003 and summer 2005 are analysed here. Avian malaria infection was diagnosed using molecular techniques (see Methods). Error bars represent ±1 s.e.
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

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

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

<table>
<thead>
<tr>
<th>Factor</th>
<th>parameter estimate</th>
<th>Z</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Pooled <em>Plasmodium</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.45±0.17</td>
<td>2.66</td>
<td>0.0078</td>
</tr>
<tr>
<td>Smoothed sample date: estimated df = 5.56, $\chi^2 = 19.3$, $P &lt; 0.013$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) <em>P. circumflexum</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.42±0.21</td>
<td>2.04</td>
<td>0.042</td>
</tr>
<tr>
<td>Smoothed sample date: estimated df = 4.91, $\chi^2 = 16.6$, $P = 0.034$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(c) <em>P. relictum</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linear date</td>
<td>0.0052±0.0027</td>
<td>1.96</td>
<td>0.050</td>
</tr>
</tbody>
</table>
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

![Graph showing predicted prevalence of Plasmodium, P. circumflexum, and P. relictum over the calendar month]

**Legend:**
- **Plasmodium**
- **P. circumflexum**
- **P. relictum**
Figure 4a-c

Predicted prevalence of *Plasmodium* in blue tits

(a) Pooled *Plasmodium*

(i) Superimposed

(ii) All ages

(iii) First years

(iv) Older

Calendar month
Figure 4a-c

Predicted prevalence of *Plasmodium* in blue tits

(b) *P. circumflexum*

![Graphs showing predicted prevalence of *P. circumflexum* in different age groups (superimposed, all ages, first years, older) across calendar months.](image-url)
Figure 4a-c

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

(c) *P. relictum*

(i) Superimposed

(ii) All ages

(iii) First years

(iv) Older
References


Plasmodium and Haemoproteus mitochondrial DNA amplified from birds.

Proceedings of the Royal Society of London Series B-Biological Sciences, 267, 1583-1589.


