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

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21 Running head (48 characters): Seasonal variation in *Plasmodium* infection in blue tits
22 Summary 251 words, manuscript total 7072 words (including references), 4 figures, 2
23 tables.

24

25 **Summary**

26

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

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

52

53

54 **Introduction**

55

56 The prevalence of many infectious diseases varies markedly through time, from short-
57 term seasonal fluctuations to complex population dynamics (Altizer, Dobson, Hosseini *et*
58 *al.*, 2006; Dietz, 1976; Greenman, Kamo & Boots, 2004). The dynamics of vector-borne
59 diseases are particularly likely to vary with environmental conditions, as vectors are
60 sensitive to climatic conditions (Aron & May, 1982; Hess, Randolph, Arneberg *et al.*,
61 2001). For example, human malaria *Plasmodium* spp. shows marked seasonality in
62 transmission, largely due to the sensitivity of the mosquito vectors to climate (Childs,
63 Cattadori, Suwonkerd *et al.*, 2006; Hay, Myers, Burke *et al.*, 2000).

64

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

71 Grenfell, Hendry *et al.*, 1996). In addition, there may also be a reduction in herd
72 immunity that exposes older individuals to an increased risk of infection, resulting in the
73 epidemic spread of previously rare parasite genotypes (Altizer *et al.*, 2006; White *et al.*,
74 1996). Revealing the environmental and demographic drivers that contribute to seasonal
75 disease dynamics aids the understanding of disease epidemiology (Pascual & Dobson,
76 2005).

77

78 In tropical climates, avian malaria occurs year-round (Valkiūnas, 2005), whereas studies
79 in temperate regions report consistent seasonal variation: a peak in prevalence during
80 spring or the breeding season, followed by a decline during winter (Applegate, 1971;
81 Beaudoin, Applegate, David *et al.*, 1971; Kucera, 1981; Schrader, Walters, James *et al.*,
82 2003; Weatherhead & Bennett, 1991), although some studies have found higher
83 prevalence of some haematozoa in winter (Hatchwell, Wood, Anwar *et al.*, 2000).
84 Beaudoin *et al.* (1971) proposed a model to explain patterns of seasonal variation with
85 reference to the transmission requirements and life cycle of avian malaria parasites: a
86 peak in malaria prevalence is supposed to occur in late summer and autumn, when vector
87 populations (Cranston, Ramsdale, Snow *et al.*, 1987; Marshall, 1938) and the proportion
88 of immunologically naïve juveniles in the host population are high. Prevalence then drops
89 in winter as vector activity wanes and malaria parasites disappear from the blood, but not
90 necessarily body tissues, followed by a spring relapse of infection prior to the breeding
91 season.

92

93 The development of molecular tools for diagnosis of avian malaria infection based on
94 mitochondrial cytochrome-*b* lineage variation (Bensch, Stjernman, Hasselquist *et al.*,
95 2000; Fallon, Ricklefs, Swanson *et al.*, 2003; Hellgren, Waldenström & Bensch, 2004;
96 Waldenström, Bensch, Hasselquist *et al.*, 2004) allows avian malaria infections to be
97 examined in more detail than is possible using traditional light microscopy techniques
98 (Waldenström *et al.*, 2004). Estimates of diversity of around 200 species using
99 microscopy (Valkiūnas, 2005) may mask diversity to the order of 10,000 species as
100 revealed by molecular approaches (Bensch, Pérez-Tris, Waldenström *et al.*, 2004): most
101 ecological studies of malaria do not consider this diversity, a potentially important source
102 of variation in host-parasite interactions. Established parasitological techniques remain
103 important for identifying groups of lineages that are morphologically similar, a likely
104 indicator of similar parasite ecology (Valkiūnas, 2005). Here, we examine seasonal
105 variation in avian malaria infection in a woodland population of blue tits *Cyanistes*
106 *caeruleus* L., 1758, to test Beaudoin *et al.*'s (1971) model. We report marked seasonal
107 patterns of variation in infection that vary between parasite morphospecies and with host
108 age, based on screening more than 800 samples over three years.

109

110

111 **Methods**

112

113 *Host-parasite system*

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

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

124

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

134

135 *Sampling and molecular diagnosis of infection*

136 Blood samples of <20µL were taken, under licence, by brachial or jugular venepuncture
137 from blue tits in Wytham Woods, a ca. 380ha woodland in Oxfordshire, UK (51°47' N,
138 1°20'W) between May 2003 and June 2005. Birds were captured at nest boxes while

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

145

146 The presence/quality of extracted DNA was assessed by electrophoresing 2µl of the
147 extract on a 2% agarose gel containing ethidium bromide, and visualising under UV light.
148 Samples were then screened for the presence of *Plasmodium* and *Haemoproteus* using the
149 nested PCR method of Waldenström et al. (2004), amplifying a 478bp fragment of the
150 mitochondrial cytochrome-*b* gene. PCR reactions were performed in 25µl volumes, in
151 two separate rounds. First-round primers were HaemNF (5'-
152 CATATATTAAGAGAATTATGGAG-3') and HaemNR2 (5'-
153 AGAGGTGTAGCATATCTATCTAC-3'): each reaction contained contained 2µl of
154 genomic DNA, 0.125mM each dNTP, 0.2µM each primer, 3mM MgCl₂ and 0.25 units of
155 Platinum Taq polymerase (Invitrogen, CA, USA) with the accompanying PCR buffer at
156 1x final concentration. The thermal profile consisted of a 2 minute 94°C enzyme
157 activation step, followed by 20 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for
158 45 sec, ending with an elongation step of 72°C for 10 min. In the second PCR round,
159 primers HaemF (5'-ATGGTGCTTTTCGATATATGCATG-3') and HaemR2 were used
160 (5'-GCATTATCTGGATGTGATAATGGT-3'): the composition of the PCR reactions
161 was as above, except that 0.4µM of each primer and 0.5 units of Platinum Taq

162 Polymerase were used, and 2µl of the PCR product from the first round was used as
163 template instead of genomic DNA. The thermal profile for the second round PCR was the
164 same as for the first round, with the number of cycles increased from 20 to 35.
165
166 2-8µl of PCR products from the second round were run on 2% agarose gels stained with
167 ethidium bromide and visualised under UV light. Samples containing bands of 450-
168 600bp in size were prepared for sequencing using a Qiagen MinElute 96 UF PCR
169 purification kit and a QiaVac multiwell vacuum manifold. The purified PCR fragments
170 were then sequenced directly by dye terminator cycle sequencing (Big Dye v3.1), and
171 loaded on an ABI PRISM 310 automated sequencer (Applied Biosystems, CA, USA).
172 Sequences were edited in Sequencher v. 4.2 (GeneCodes Corp., MI, USA), and aligned in
173 ClustalX (Jeanmougin, Thompson, Gouy *et al.*, 1998). Sequences corresponding to
174 *Plasmodium* or *Haemoproteus* from known alignments were scored as positive for avian
175 malaria. Sequences corresponding to *Leucocytozoon* sequences were scored as negative
176 for the purposes of this study; while a study of the seasonal variation in *Leucocytozoon*
177 prevalence would certainly be of interest, the PCR test is not designed to amplify DNA
178 from these parasites, and is thus less efficient, particularly when either *Haemoproteus* or
179 *Plasmodium* are also present. Where possible, avian malaria sequences were further
180 characterised to the lineage level, with exact matches named as per existing lineages in
181 GenBank, whilst sequences differing by one or more base pairs from those in GenBank
182 were assigned new names. We report a new lineage, pBLUTI3 (now assigned GenBank
183 accession number DQ991069). Mixed infections were present at a low rate (ca. 2% in
184 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 (GLM) 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

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

210

211

212 **Results**

213

214 Samples collected between autumn 2003 and summer 2005 from 816 individual blue tits
215 were screened for avian malaria infection. The prevalence of avian malaria infection
216 across the study period was 25.6%, comprising 24.4% *Plasmodium* and 0.8%
217 *Haemoproteus* (the latter genus is excluded from analyses due to low prevalence and the
218 potential for different seasonal patterns due to different vector ecologies: Valkiūnas,
219 2005). A total of 11 cytochrome-b lineages were identified: eight *Plasmodium* and three
220 *Haemoproteus* spp. (Table 1). Some *Plasmodium* lineages have been matched to
221 morphological species known from light microscopy (Hellgren, Križanauskiene,
222 Valkiūnas *et al.*, 2007; Palinauskas, Kosarev, Shapoval *et al.*, 2007; Valkiūnas,
223 Zehtindjiev, Hellgren *et al.*, 2007): we therefore analyse the seasonal pattern of
224 *Plasmodium* pooled across all lineages, in addition to the prevalence of the two most
225 common parasite morphospecies which together account for 93% of avian malaria
226 infections, namely *Plasmodium relictum* Grassi & Feletti, 1891 and *P. circumflexum*
227 Kikuth, 1931. As the prevalence of any single lineage never exceeded 10%, the available
228 sample sizes did not support the analysis of lineages within species. Two approximately
229 similar peaks of pooled *Plasmodium* prevalence were observed in May/June and
230 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 ($29.8 \pm 2.5\%$) compared to first-year birds ($20.5 \pm 1.9\%$). 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 ($17.1 \pm 2.1\%$) than first years ($11.5 \pm 1.5\%$). *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 $\chi^2=4.89$, $df=1$, $P=0.027$) and significant variation between bimonthly periods ($\chi^2=5.89$,
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: $\chi^2=7.70$, $df=1$, $P=0.0055$):
251 importantly, the season*species interaction was retained ($\chi^2=10.4$, $df=3$, $P=0.016$),
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).

254

255 We further examined the differences in seasonal variation in prevalence by constructing
256 predicted response models, which use final models (Table 2) to predict the variation in
257 prevalence over a hypothetical range of daily sampling dates, an approach that is useful to
258 visualise complex non-linear variation in prevalence (Fig. 4). The predicted response
259 models were judged to be a good reflection of observed prevalence data, because (i)
260 bimonthly prevalence (e.g. from Fig. 1) did not deviate significantly from the predicted
261 variation in prevalence shown in Fig. 4 (bimonthly observed vs. predicted prevalence for
262 pooled *Plasmodium*, *P. circumflexum*, *P. relictum*; goodness of fit χ^2 tests, df=5,
263 $P>0.90$), and (ii) observed and predicted bimonthly prevalence were significantly
264 correlated, with slopes close to unity, for pooled *Plasmodium* ($r=1.03$, $P=0.01$, $R^2=0.80$)
265 and *P. circumflexum* ($r=1.27$, $P=0.006$, $R^2=0.85$). These correlations reflect the retention
266 of smoothed date as a predictor of prevalence (Table 2), whereas no such correlation
267 existed between observed and predicted *P. relictum* prevalence ($r=0.36$, $P=0.22$,
268 $R^2=0.18$), for which smoothed date was not retained. Predicted response models for *P.*
269 *relictum* (Fig. 4c) are, therefore, presented merely for visual comparison with pooled
270 *Plasmodium* and *P. circumflexum*.

271

272 Comparing these plots between morphospecies reveals different seasonal patterns of
273 prevalence (Figs. 4a-c): both pooled *Plasmodium* and *P. circumflexum* showed a clear
274 pattern of seasonal variation including an autumn peak and an increase in prevalence
275 early in the year. *P. relictum* infection (the modelling of which retained a linear function
276 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

300 **Discussion**

301

302 Seasonal variation in *Plasmodium* prevalence in blue tits in our study population is
303 characterised by bimodal peaks in prevalence in autumn and spring, and a marked drop in
304 prevalence during winter. At first sight, this genus level pattern agrees with the model of
305 Beaudoin *et al.* (1971) for seasonal variation in avian malaria in temperate regions.

306 However, the two most prevalent avian *Plasmodium* morphospecies in our study
307 population showed different patterns of seasonal variation in prevalence: *P. circumflexum*
308 showed seasonal variation of a pattern similar to that for pooled *Plasmodium*, whereas *P.*
309 *relictum* prevalence was more stable. There was also clear age structure in the seasonality
310 of *Plasmodium* infection: first year birds showed a less marked spring relapse of
311 *Plasmodium* than older birds. The autumn peak in *Plasmodium* prevalence was largely
312 driven by *P. circumflexum*. As seasonal patterns vary between age classes and between
313 different *Plasmodium* morphospecies, we reject Beaudoin *et al.*'s model as it is not robust
314 to the underlying complexity of the blue tit-*Plasmodium* interaction in this population.

315

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

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

328

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

342

343 Parasite prevalence has been reported to increase prior to the breeding season in
344 temperate wild bird populations, known as the ‘spring relapse’ (Applegate, 1971; Box,
345 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

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

372

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

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

395

396 This study is reliant upon sensitive molecular diagnostic techniques, (Waldenström *et al.*,
397 2004), knowledge of the taxonomy of avian *Plasmodium* in relation to molecular data
398 (Hellgren *et al.*, 2007; Valkiūnas *et al.*, 2007) and categorisation of hosts into first year
399 and older birds. Without these factors, the ‘two peaks and a trough’ model of seasonal
400 variation in avian malaria prevalence (Beaudoin *et al.*, 1971) would have been accepted
401 by our study, when in fact the seasonal pattern of *Plasmodium* variation in blue tits in our
402 study is a complex combination of different patterns, both between *Plasmodium*
403 morphospecies and (in the case of *P. circumflexum*) between age classes. An additional
404 factor not considered here is that there may be marked spatial differences in the
405 prevalence and distribution of different parasite species. Indeed, we know this to be the
406 case for the present study population, which shows spatial variation in both the overall
407 prevalence of malaria and in the distribution of morphospecies (Wood, Cosgrove, Wilkin
408 *et al.*, 2007). There are some intriguing parallels between the temporal patterns revealed
409 here and the spatial ones described elsewhere (Wood *et al.*, 2007): in both cases, *P.*
410 *relictum* shows a broader distribution, while *P. circumflexum* shows a more clustered
411 distribution.

412

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

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

424

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

438 diseases might respond to climate change (Kovats, Campbell-Lendrum, McMichael *et al.*,
439 2001; Rogers & Randolph, 2000). Any study that aims to understand individual
440 heterogeneity in infection in avian malaria should consider both temporal (this study) and
441 spatial variation (Wood *et al.*, 2007) as contributory factors. Continued research promises
442 increasing understanding of the ecology of avian malaria, and the epidemiology of
443 vector-borne disease in general.

444

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450 valuable comments on the manuscript.

451

452

453 **Table and Figure legends**

454

455 **Table 1.**

456 A total of 816 individual blue tits, sampled between autumn 2003 and summer 2005 were
457 screened for avian malaria infection. Mitochondrial cytochrome-*b* lineages were assigned
458 using molecular techniques (see Methods), shown in the ‘Lineage’ column; the prefix “p”
459 denotes *Plasmodium*, and “h” denotes *Haemoproteus*. The frequency of infection of each
460 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

484 **Figure 2.**

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

492

493 **Figure 3.**

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

503

504 **Figure 4.**

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

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

517

518 **Table 1.**

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

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

520

1 **Table 2.**

2 Generalized additive models (GAM) examining seasonal variation in the prevalence of

3 *Plasmodium* infection in blue tits

4

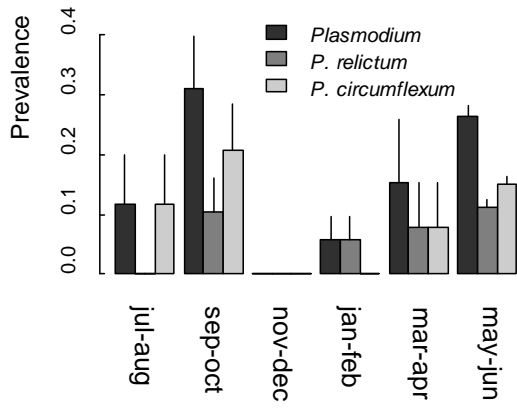
Factor	parameter estimate	Z	P
(a) Pooled <i>Plasmodium</i>			
Age	0.45±0.17	2.66	0.0078
Smoothed sample date: estimated df = 5.56, $\chi^2 = 19.3$, $P < 0.013$			
(b) <i>P. circumflexum</i>			
Age	0.42±0.21	2.04	0.042
Smoothed sample date: estimated df = 4.91, $\chi^2 = 16.6$, $P = 0.034$			
(c) <i>P.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

5

1 **Figure 1.**

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

3

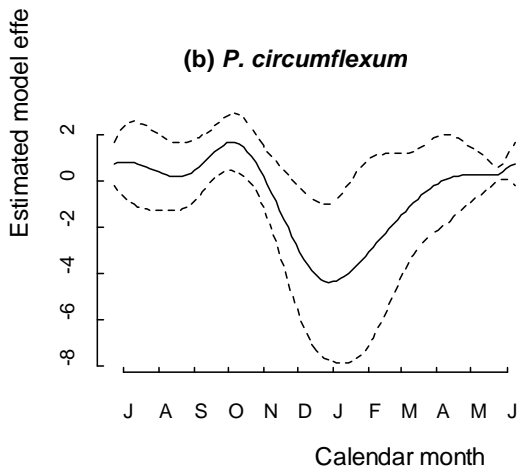
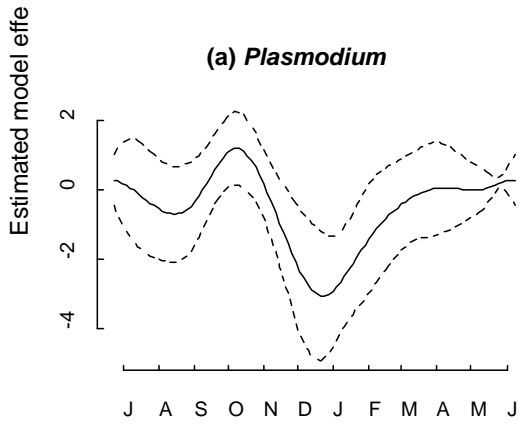


1 **Figure 2.**

2 Smoothed residual models of the seasonal variation in prevalence of (a) pooled

3 *Plasmodium* and (b) *P. circumflexum* infection in blue tits

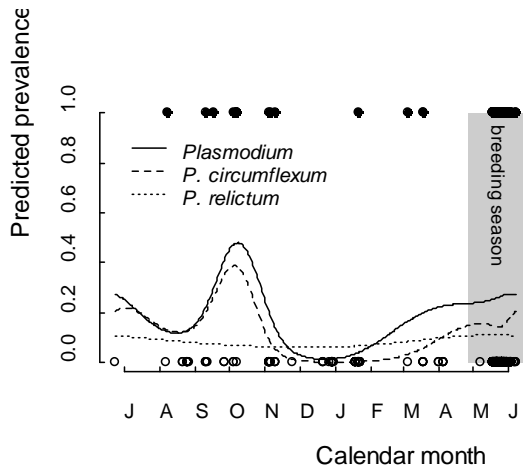
4



1 **Figure 3.**

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

3



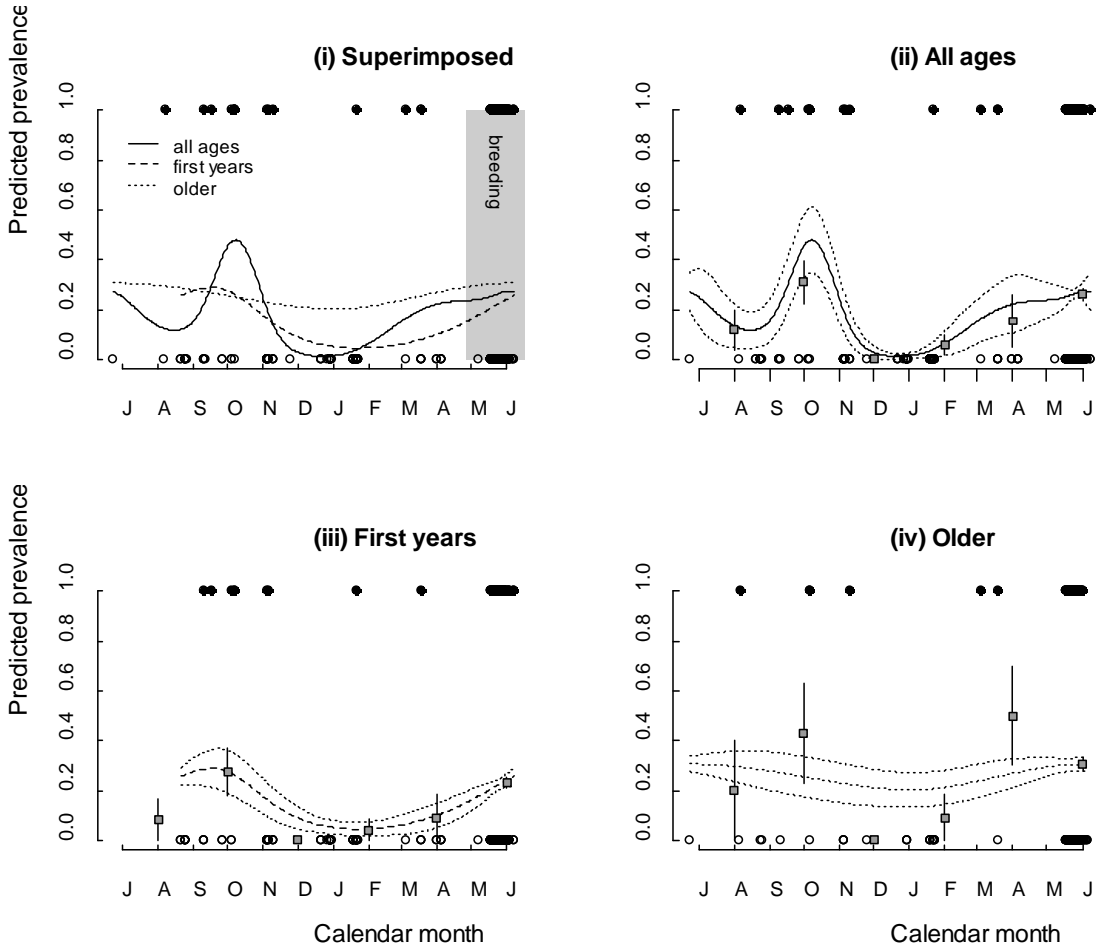
1 **Figure 4a-c**

2 Predicted prevalence of *Plasmodium* in blue tits

3

4 **(a) Pooled *Plasmodium***

5



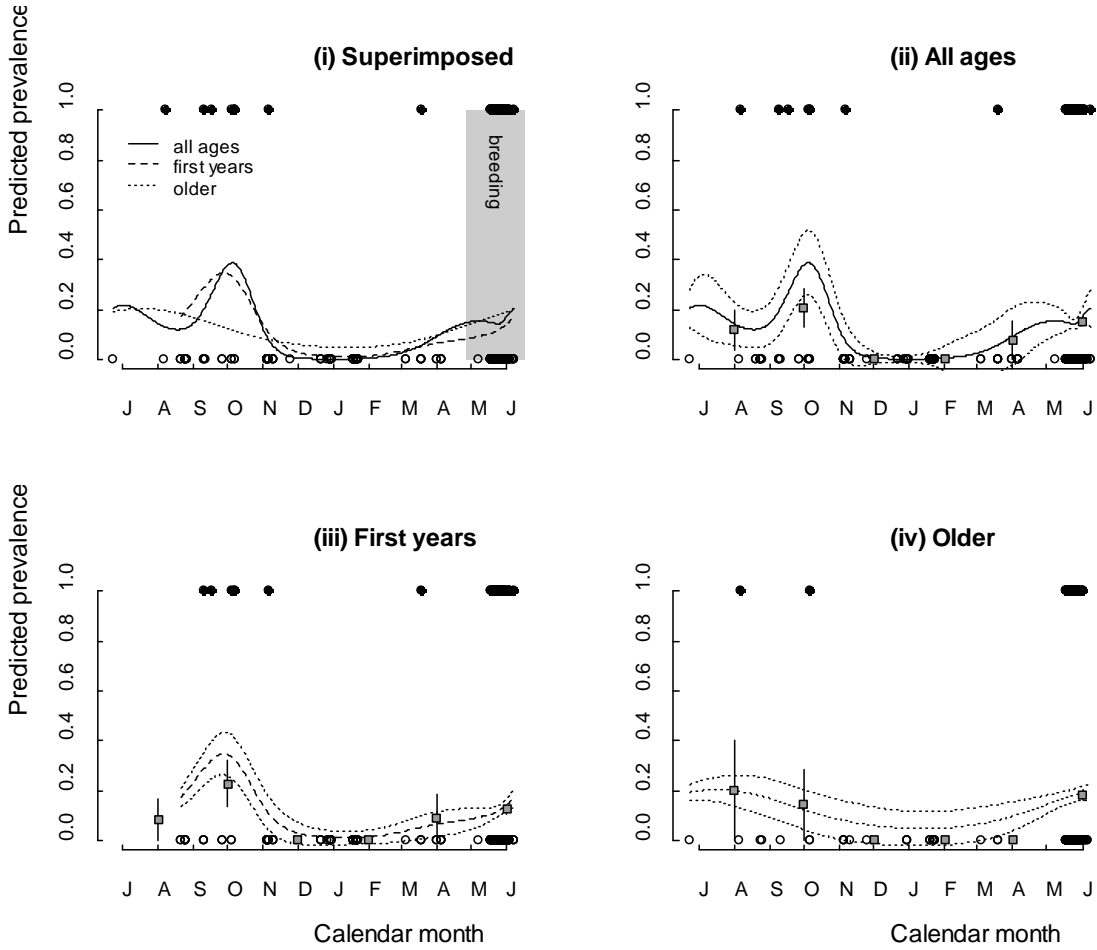
1 **Figure 4a-c**

2 Predicted prevalence of *Plasmodium* in blue tits

3

4 **(b) *P. circumflexum***

5



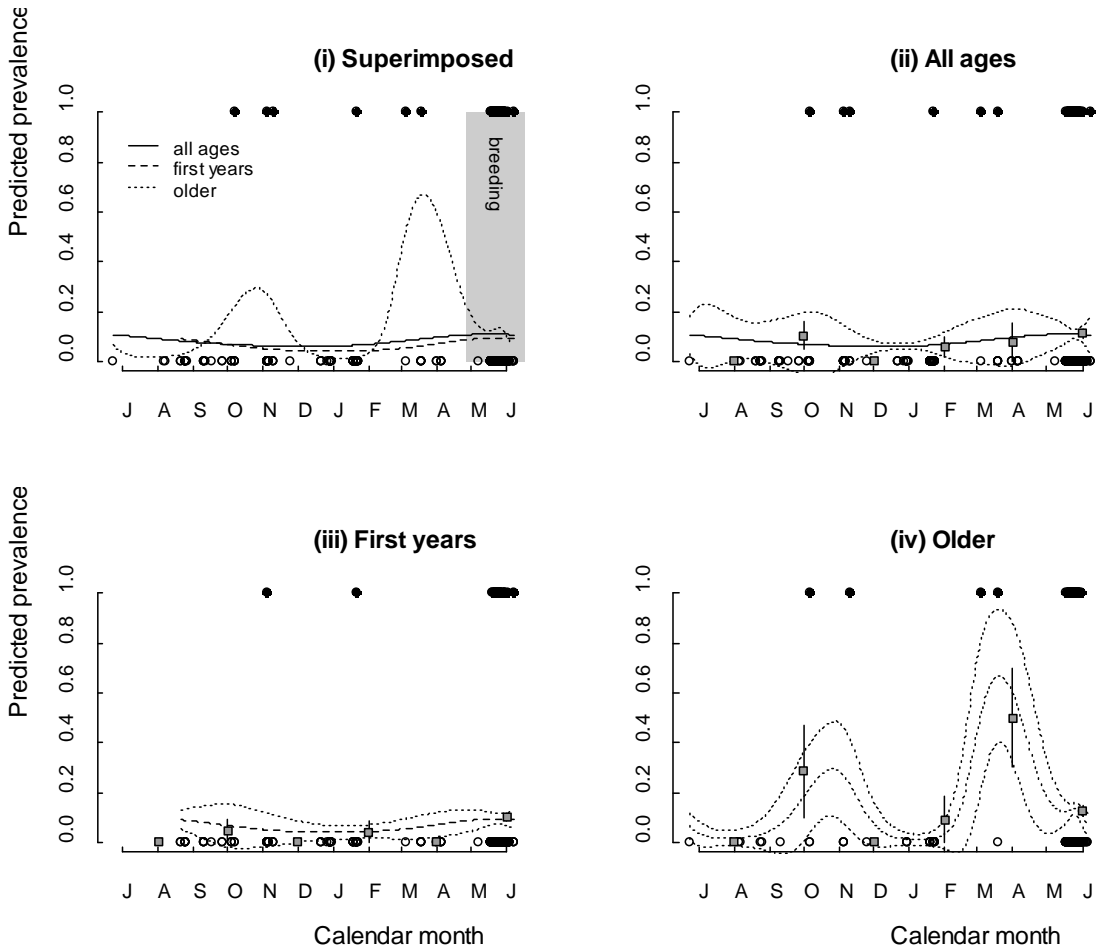
1 **Figure 4a-c**

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

3

4 **(c) *P. relictum***

5



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