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This is a peer-reviewed, post-print (final draft post-refereeing) version of the following published document:

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). pp. 540-548.

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

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

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 $MgCl_2$ 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'-ATGGTGCTTTCGATATATGCATG-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

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

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

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:

χ^2=4.89, df=1, P=0.027) and significant variation between bimonthly periods (χ^2=5.89,

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 15th) also

retained species as a model factor (2-way analysis of deviance: χ^2=7.70, df=1, P=0.0055):

importantly, the season*species interaction was retained (χ^2=10.4, 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).

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*; good ness 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

Discussion

300

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

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

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

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	*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%)**

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 *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

5

Figure 1.

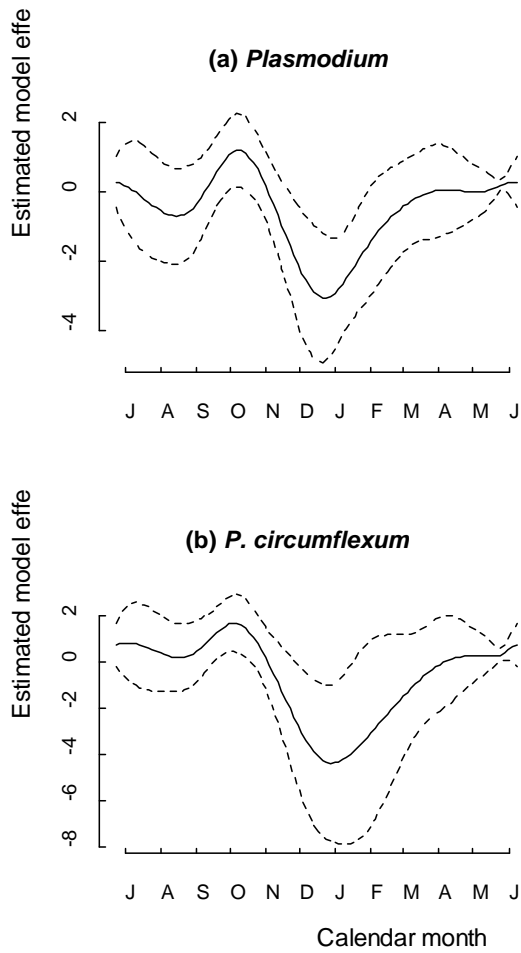Seasonal variation in the prevalence of *Plasmodium* infection in blue tits
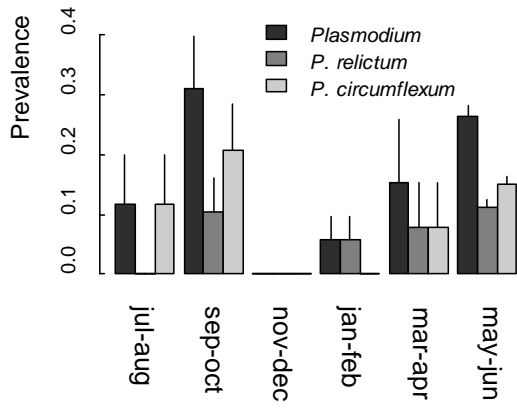
1 **Figure 2.**

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

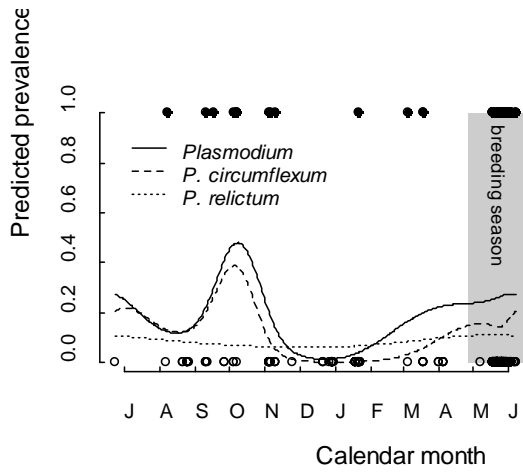3 *Plasmodium* and (b) *P. circumflexum* infection in blue tits

4

1 **Figure 3.**

2 Predictive models of seasonal variation in *Plasmodium* infection in blue tits
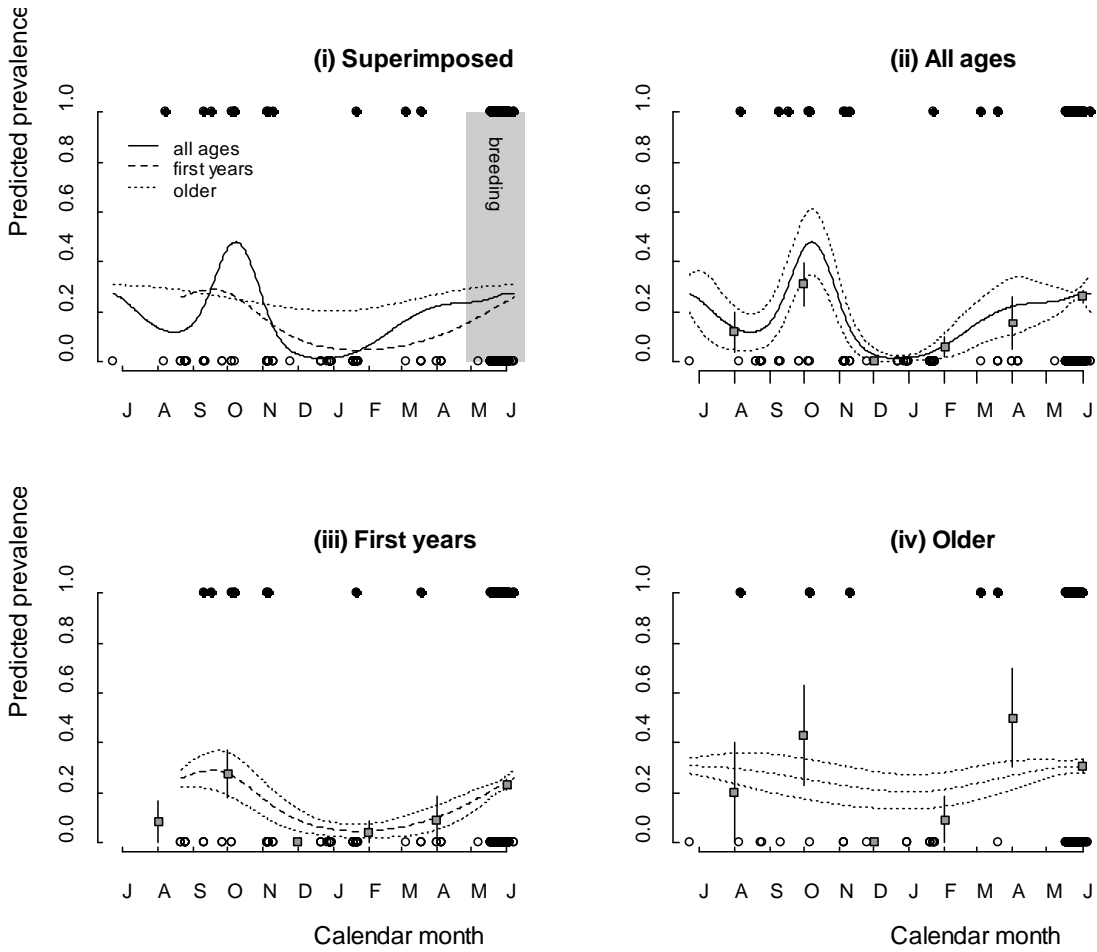
3

1 **Figure 4a-c**

2 Predicted prevalence of *Plasmodium* in blue tits

3

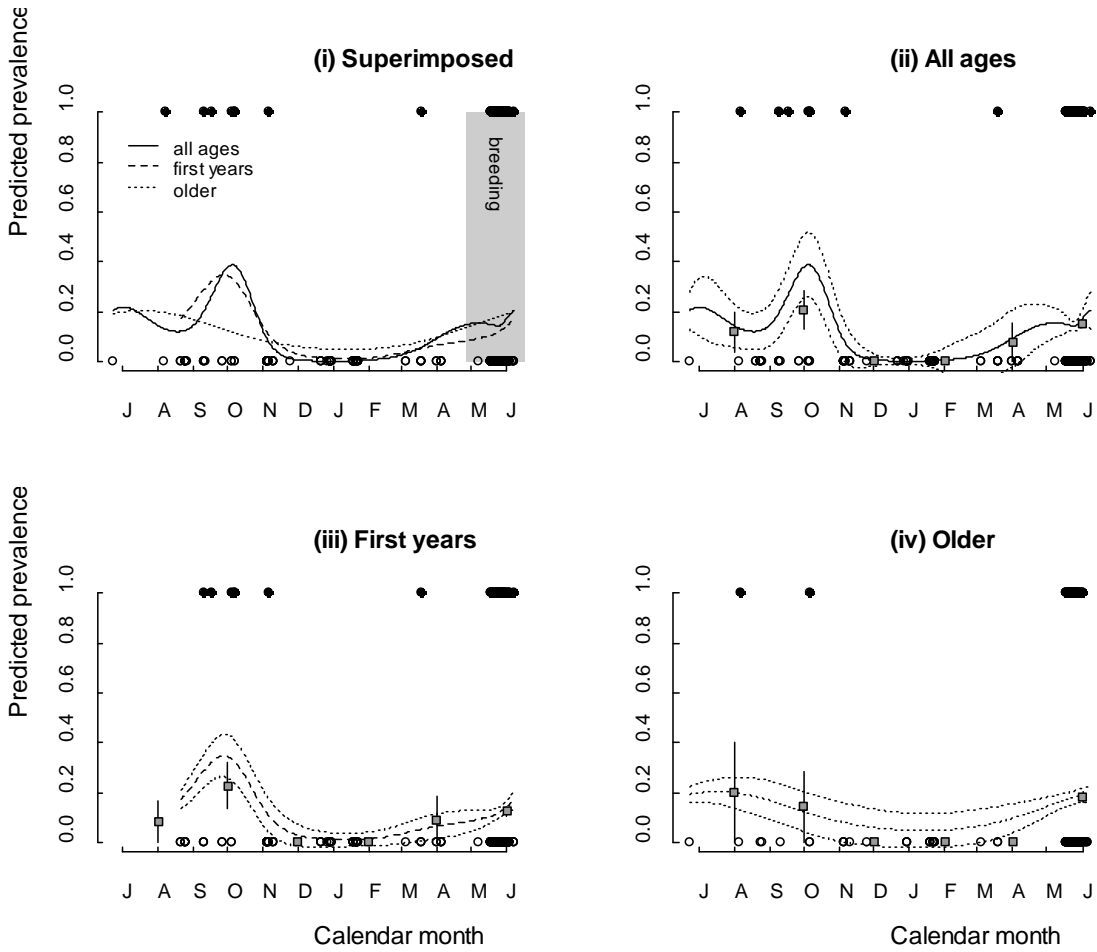4 **(a) Pooled *Plasmodium***

5

1 **Figure 4a-c**

2 Predicted prevalence of *Plasmodium* in blue tits
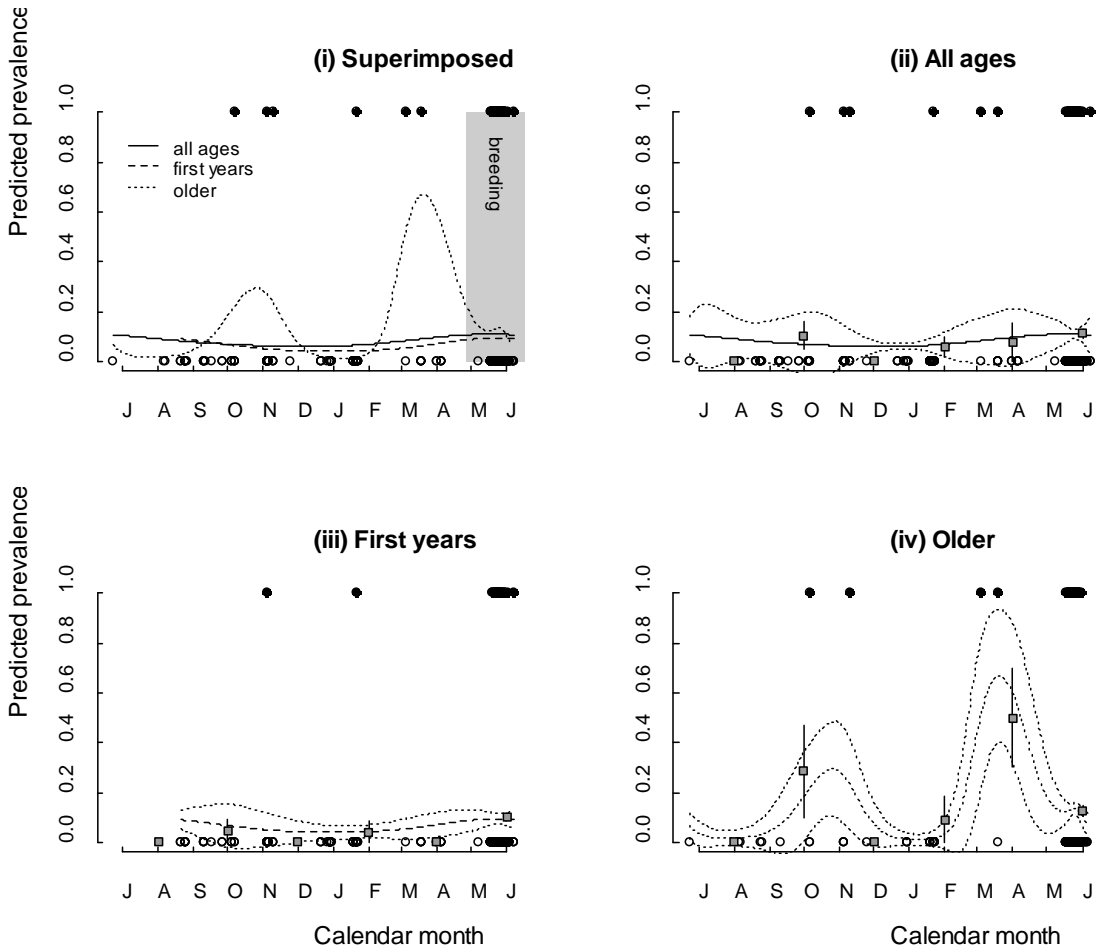
3

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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