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1 # The epidemiology underlying age-related avian malaria

2 # infection in a long-lived host: the mute swan *Cygnus olor*

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Abstract

Quantifying the factors that predict parasite outbreak and persistence is a major challenge for both applied and fundamental biology. Key to understanding parasite prevalence and disease outbreaks is determining at what age individuals show signs of infection, and whether or not they recover. Age-dependent patterns of the infection of a host population by parasites can indicate among-individual heterogeneities in their susceptibility to, or rate of recovery from, parasite infections. Here, we present a cross-sectional study of avian malaria in a long-lived bird species, the mute swan *Cygnus olor*, examining age-related patterns of parasite prevalence and modelling patterns of infection and recovery. 115 swans, ranging from one to nineteen years old, were screened for infection with *Plasmodium*, *Haemoproteus* and *Leucocytozoon* parasites. Infections with three cytochrome-*b* lineages of *Haemoproteus* were found (pooled prevalence 67%), namely WW1 (26%), which is common in passerine birds, and two new lineages closely related to WW1: MUTSW1 (25%) and MUTSW2 (16%). We found evidence for age-related infection in one lineage, MUTSW1. Catalytic models examining patterns of infection and recovery in the population suggested that infections in this population were not life-long – recovery of individuals was included in the best fitting models. These findings support the results of recent studies that suggest hosts can clear infections, although patterns of infection-related mortality in older birds remain to be studied in more detail.

Introduction

Parasite dynamics are the result of a complex interplay between exposure, transmission, disease-induced morbidity or death, immune-mediated recovery, and host life history (Hudson et al. 2002), each of which may be influenced by environmental or among-individual heterogeneities (Wilson 2002). Quantifying these basic processes allows us to understand the factors that govern parasite outbreaks and long-term persistence (Dobson and Foufopoulos 2001), and can reveal potentially important associations between biotic and abiotic components of the environment and parasite performance (Patz et al. 2000). Such studies are an essential prerequisite for the effective design of control and eradication programs, and provide a basis for delineating the fundamental drivers of parasite dynamics in space and time. In the absence of more detailed longitudinal study, the most commonly available data for inferring key epidemiological parameters is apparent prevalence, the proportion of animals in a population that test positive for an infection (Heisey et al. 2006). Such data are relatively easy to collect compared to logistically challenging longer term studies, and when coupled with measurements of the age of individuals provide a relatively straightforward means of comparing key epidemiological parameters among host populations or parasite lineages.

A number of cross-sectional studies have reported differences in the degree of parasitism in the wild, revealing variation in space or time (Altizer et al. 2006, Bensch and Åkesson 2003, Cosgrove et al. 2008, Loiseau et al. 2010, Wood et al. 2007). However, such studies do not always permit age-prevalence patterns to be examined in detail, either because age may not be known due to constraints in the marking of

65   individuals at birth, or because it may not be possible to infer age from morphological

66   traits. Moreover, most studies of disease in wild bird populations have been conducted in

67   study organisms that are short-lived relative to the scale of seasonal variation in

68   transmission (Beadell et al. 2006, Ishtiaq et al. 2007, Ricklefs et al. 2005, Scheuerlein and

69   Ricklefs 2004, Ventim et al. 2012). This highlights the need for more studies of parasite

70   dynamics in longer-lived host species, which are poorly represented among avian studies

71   (Bennett and Owens 2002)

72   In the absence of significant disease-induced mortality, the observed pattern of

73   apparent prevalence is driven by rates of transmission and recovery. The direct

74   measurement of transmission presents significant logistical challenges, requiring

75   observation of contact rates and infection probabilities, both seldom directly observable

76   outside the lab (for exceptions see Goeyvaerts et al. 2010, Kjaer et al. 2008). Insight into

77   the transmission process is often gained indirectly, therefore, by estimating the force-of-

78   infection (FOI), also known as the infection hazard, which is simply the per capita rate of

79   infection of susceptible hosts. Though it does not reflect transmission per se (the density

80   or frequency of infected individuals, and rates of transmission govern its magnitude), FOI

81   is nonetheless an important metric for quantifying disease foothold in a population

82   (Heisey et al. 2006, Long et al. 2010). In an endemic setting – one in which the disease is

83   at, or near, dynamic equilibrium or at least changing slowly relative to the lifespan of the

84   host – FOI can be estimated from age-prevalence data derived from cross-sectional

85   sampling. Such data has long been used to estimate the force of infection in human

86   populations using 'catalytic' models based on the proportional-hazards framework

87   (Anderson and May 1985, Bundy et al. 1987, Farrington et al. 2001, Grenfell and

88   Anderson 1985, Keiding 1991, Keiding et al. 1996), and occasionally in a wildlife disease

89   setting (Caley and Hone 2002, Heisey et al. 2006, Hudson and Dobson 1997, Woolhouse

90   and Chandiwana 1992).

91   Avian malaria in long term study populations offers a useful model system for the

92   study of ecological drivers of disease in wild populations. Avian malaria, *Plasmodium*

93   and *Haemoproteus* spp. (*sensu* Pérez-Tris et al. 2005; see Valkiūnas et al. 2005 for a

94   contrasting view), is a vector-borne disease transmitted by haematophagous Diptera.

95   Avian *Plasmodium* is transmitted primarily by mosquitoes (Culicidae) and *Haemoproteus*

96   by biting midges (Ceratopogonidae) and louse flies (Hippoboscidae) (Valkiūnas 2005).

97   The development of sensitive and accurate molecular diagnosis techniques for avian

98   malaria (Hellgren et al. 2004) has revealed a substantial and unexpected diversity of

99   malaria lineages (Bensch et al. 2004), many showing marked variation between lineages

100   in associations with biotic and abiotic factors (Cosgrove et al. 2008, Knowles et al. 2011,

101   Wood et al. 2007).

102   In this paper we report the results of a cross-sectional survey of the prevalence of

103   avian malaria parasites in a resident, colonial population of mute swans *Cygnus olor* in

104   which the majority of individuals are of known age. Mute swans are relatively long-lived

105   birds, with some individuals living beyond 20 years (Charmantier et al. 2006b, McCleery

106   et al. 2002). There are relatively few reports of avian malaria in other swan species

107   (Bennett et al. 1984, Ramey et al. 2012, Ricklefs and Fallon 2002, Valkiūnas 2005). We

108   examine age-specific variation in the apparent prevalence of infection with specific

109   lineages of avian malaria using non-parametric regression, and then construct catalytic

110   infection models to estimate FOI in the face of different assumptions governing recovery

111   and re-infection of individuals.

112

113

114   **Methods**

115

116   *Host sampling*

117   Mute swans (family Anatidae) are large (7-14kg) waterbirds found in lakes, rivers and

118   coastal areas in temperate and oceanic climates in Europe & Asia, feeding in shallow

119   water on aquatic vegetation. In Western Europe, mute swans are usually territorial and

120   nest in isolated pairs close to the water's edge (Cramp and Simmons 1983), but uniquely

121   in the UK a colony of mute swans is located on the south coast of England at Abbotsbury

122   Swannery (50°35'N, 2°30'W) in The Fleet, a 14km tidal lagoon. The colony has been in

123   existence since at least the 1300s and its population dynamics studied since the late 1960s

124   (Perrins and Ogilvie 1981). The breeding population has increased steadily to become

125   relatively stable at approximately 130 breeding pairs (1990-2012: McCleery et al. 2002);

126   C.M. Perrins unpublished data). Most swans in the colony are ringed as cygnets shortly

127   after hatching and are natally philopatric – only 5% of breeding females are immigrants

128   (Charmantier et al. 2006a) – so the age of most adult birds is known. The majority of

129   breeding pairs nest near brackish pools, ditches and streams or in nearby *Phragmites*

130   reedbeds. Supplemental feeding and the protection of vulnerable breeding pairs in pens

131   are part of the long-term management of the population, but this intervention does not

132   appear to cause significant differences in population ecology between the Abbotsbury

133   colony and territorial swans in other parts of the UK (Perrins and Ogilvie 1981).

134   In August 2008, 115 birds of known ages between 1 and 19 years were blood

135   sampled by ulnar or tarsal venepuncture under UK Home Office licence. All were

136   recruits to the colony (i.e. hatched as cygnets at Abbotsbury) to avoid the potential

137   confounding effects of immigration. 92 of these birds were of known sex, based on the

138   consensus from observations of sexually dimorphic bill knob size (Horrocks et al. 2006),

139   sexual behaviour and cloacal examination (Swan Study Group 2005) made during the

140   2008 capture and any preceding captures or resightings.

141

142   *Parasite screening*

143   Samples were stored in SET buffer (0.015 M NaCl, 0.05 M Tris, 0.001 MEDTA, pH 8.0),

144   and DNA extracted using a standard ammonium acetate protocol with the final product

145   eluted in Qiagen AE buffer (Qiagen, Valencia CA, USA). DNA was quantified in 100x

146   dilutions using pico-green dye and diluted to a final concentration of 25ng/μl. Extraction

147   products were screened for the presence of *Leucocytozoon, Plasmodium* and

148   *Haemoproteus* infections following the protocol by (Hellgren et al. 2004). 3μl of PCR

149   product was run on 2% agarose gel containing ethidium bromide with a 1Kbp DNA

150   ladder for each sample, testing for *Leucocytozoon* or *Haemoproteus/Plasmodium*

151   lineages. Standard positives were used from previous malaria work in blue tits *Cyanistes*

152   *caeruleus* (Knowles et al. 2010, Wood et al. 2007). Clear, strong bands were taken as

153   positive and the absence of a band as negative for infection. Negative samples were

154   rescreened to verify parasite absence. Positive sample PCR products were cleaned using a

155   Qiavac multiwell vacuum manifold.. To identify cytochrome-*b* lineages, BigDye

156   (Applied Biosystems, Foster City, CA, USA) sequencing reactions were run with the

157   forward primer for each lineage (F or FL). Sequences were edited and aligned in

158   Sequencher 4.2 (GeneCodes Corp., Ann Arbor, MI, USA) using known, widespread

159   malarial lineages as an alignment reference obtained from the MalAvi database (Bensch

160   et al. 2009, accessed 18th February 2013). Novel lineages were identified by BLAST

161   search against Genbank and then by comparison with all Haemosporidian lineages in the

162   MalAvi database. Novel lineages were then named to indicate the bird host, using the

163   five-letter species codes (as used by the British Trust for Ornithology), i.e. MUTSW for

164   mute swan, suffixed by a number for each new lineage. Data on novel lineages were

165   submitted to GenBank and MalAvi databases.

166

167   *Phylogenetic analysis*

168   To place the lineages found in this study in a phylogenetic context, we used the MalAvi

169   database (Bensch et al. 2009) to identify (i) all lineages of *Haemoproteus*, *Plasmodium*

170   and *Leucocytozoon* previously found in Anatidae, and (ii) the closest known relatives of

171   the lineages found in this study. The latter were selected by constructing a neighbour-

172   joining phylogeny – a Jukes-Cantor model as implemented in Geneious ver. R6

173   (Biomatters Ltd., Auckland, New Zealand: http://www.geneious.com) – including all

174   available lineages to identify a well-defined clade of lineages in which the lineages found

175   in this study occurred (for selection of clusters see Appendix 2). A Bayesian phylogeny

176   was then constructed from the selected lineages using MrBayes (Huelsenbeck and

177   Ronquist 2001) with a GTR-inv.gamma model allowing for 6 different gamma

178   categories. The total run length was 1000000 with trees sampled every 200th step. After

179   discarding the first 10000 trees, the remaining trees were used to construct a consensus

180   tree. The phylogeny was visualised using MEGA 5.0 (Kumar et al. 2008)

181   *Statistical analysis*

182   1. Age-related variation in prevalence

183   We used generalized additive models (GAM) to accommodate potentially non-

184   linear relationships between parasite prevalence and age (Hudson et al. 2002, Wilson

185   2002). GAMs allow the expected value of the response to vary as a smooth function of a

186   predictor (host age in this case) alongside conventional linear or categorical predictors

187   and their interactions (Wood 2006). First, we analysed infection with each parasite

188   lineage separately: starting models incorporated a smoothed function of age and host sex

189   as model predictors, using binomial errors and a logit link. Beginning with the interaction

190   term, which was introduced as separate smoothed age functions for each sex, predictors

191   were eliminated from the model if removal resulted in a non-significant change in model

192   deviance ($P > 0.05$), using likelihood ratio tests with penalised likelihoods and a backward

193   stepwise procedure. Patterns of prevalence were visualized by calculating the predicted

194   fitted response of each GAM of sample date on parasite infection: this approach applies

195   the estimated model effects to a hypothetical range of sampling ages to calculate the

196   fitted response and associated confidence estimates. GAMs were not forced through the

197   origin (as would be appropriate for a disease without vertical transmission, i.e. zero

198   prevalence at age zero), because birds in their first year of life (zero years) would, in fact,

199   have been weeks or months old at the time of sampling (late summer) and therefore

200   cannot be assumed to be free of avian malaria infection. To test directly for differences in

201   age-prevalence variation between parasite lineages, we used generalized additive mixed

202   modelling (GAMM, Wood 2006). Each host individual was represented by three data per

203   individual reflecting infection with each of three lineages, with individual identity fitted

204   as a random effect and varying coefficient smoothing with respect to infection with each

205   lineage. Once GAMM model selection was completed using maximum likelihood, the

206   model was refitted using REML for extraction of parameter estimates. These analyses

207   were conducted using the packages mgcv 1.7-13 and gamm4 0.1-5 in R 2.15.0 (R Core

208   Team 2012). Means are presented ±1 standard error.

209

210   2. Catalytic model

211   The observed age-prevalence data is multinomial, with four possible outcomes

212   corresponding to individual infection status: uninfected ($Y = 0$), infected with lineage

213   WW1 (see results) ($Y = 1$), infected with MUTSW1 ($Y = 2$), or infected with MUTSW2

214   ($Y = 3$). Two assumptions are made:

215   (i) That infections are avirulent, such that infection-induced mortality is negligible

216   and can be ignored. This assumption was traditionally accepted by early studies of avian

217   blood parasites (Bennett et al. 1988, Valkiūnas 2005). While a growing number of

218   correlative studies have been equivocal on the virulence of avian malaria in stable host

219   populations (Asghar et al. 2011, Atkinson et al. 2008, Lachish et al. 2011a), recent

220   experimental studies have detected the detrimental effects of avian malaria in wild

221   passerine bird populations (Knowles et al. 2010, Martinez-de la Puente et al. 2010,

222   Marzal et al. 2005, Merino et al. 2000). In view of the equivocal evidence from avian

223   malaria studies and the lack of evidence from long-lived birds in general (and our study

224   population in particular), we take the lack of virulence as a starting assumption for our

225   catalytic models. We re-examine this assumption in the Discussion.

226   (ii) That the disease is endemic, such that disease incidence remains constant

227   within the transmission period. Long-term studies of avian malaria in host populations are

228   infrequent (Lachish et al. 2011b, Westerdahl et al. 2005), but generally do not support the

229   existence of epidemic outbreaks of infection in stable host populations (Atkinson et al.

230   2008). Under these assumptions the age-prevalence data can be used to estimate the FOI,

231   that is, the rate at which susceptible individuals acquire infection (Heisey et al. 2006). We

232   can also estimate parameters describing what happens once individuals become infected

233   for the first time: (i) infections are life-long and there is no recovery; (ii) individuals

234   recover to become fully susceptible again; (iii) individuals recover and to possess lifelong

235   immunity. Adopting the conventional language of epidemiology, we refer to these as the

236   SI-, SIS-, and SIR-case, respectively. Since the FOI will differ among strains when either

237   the prevalence or the transmission rates (or both) vary by strain, we consider two further

238   possibilities for each scenario: the FOI is constant with respect to the strain; or the FOI

239   varies by strain. Thus, we fit a total of six models to our age-prevalence data. In each, we

240   assume that co-infection does not occur, an assumption justified as all sequence

241   electropherograms were carefully examined for mixed infections and none was observed

242   (Pérez-Tris and Bensch 2005). While we accept that these modelling assumptions place

243   restrictions on the conclusions that may be drawn from our analyses, we believe that this

244   modelling approach is justified by the utility of estimating FOI, which is often difficult to

245   estimate in wild populations (McCallum et al. 2001), for example to enable comparison

246   of FOI within and between studies.

247   In order to construct the likelihood under a particular model we need to calculate

248   the probability that an individual has a given infection status (0, 1, 2, or 3) at each age, up

249   to the maximum age observed. Because the FOI and recovery rates (if present) are

250   constant with respect to age, the required likelihood can be calculated by iterating matrix

251   projection model describing the transitions among infection states in successive ages. In

252   the SI-case with strain varying FOI, the matrix projection model has the form

253          $\mathbf{p}_a = \Psi^a \mathbf{p}_0$,   (1)

254   where $\mathbf{p}_a$ denotes the distribution vector of states at age a , $\mathbf{p}_0$ is the initial distribution

255   vector, and Ψ^a is the transition matrix for infection raised to the ath power. Since

256   individuals are uninfected at birth the initial distribution vector is simply $\mathbf{p}_0 = (1\ 0\ 0\ 0)^T$.

257   The infection matrix Ψ has the form

258          $$\Psi = \begin{pmatrix} \pi & 0 & 0 & 0 \\ (1-\pi)\theta_1 & 1 & 0 & 0 \\ (1-\pi)\theta_2 & 0 & 1 & 0 \\ (1-\pi)\theta_3 & 0 & 0 & 1 \end{pmatrix},$$ (2)

259   where π is the probability an individual avoids infection over the course of a year and θ_i

260   is the probability that an individual is infected by strain i. These probabilities are

261   expressed in terms of the FOI for each strain, λ_i , such that $\pi = e^{-\lambda_1 - \lambda_2 - \lambda_3}$ and

262     $\theta_i = \frac{\lambda_i}{\lambda_1 + \lambda_2 + \lambda_3}$. The likelihood is then

263          $y_i \sim Multinom(1,\ \mathbf{p}_a),$ (3)

264   where y_i denotes the status of individual i . Readers familiar with survival analysis will

265   recognise that the FOI can also be estimated with a competing risks survival model,

266   where the "hazard" associated with each infection process is λ_i. However, we use the

267   above formulation because it is easily extended to incorporate recovery.

268   In order to construct the likelihood for the SIS- and SIR-case we assume that the

269   infection and recovery processes occur independently and sequentially each year. This

270   approximation, which simplifies the modelling, is justified by the observation that insect

271   vectors are absent over the winter, so that transmission necessarily occurs in late spring

272   and summer. We also assume that within-year recovery during the spring/summer

273   transmission period is negligible, and can be ignored; an assumption that may be justified

274   on two counts: Firstly, previous studies of avian haemosporidia indicate that if a bird

275   survives the initial acute phase of infection, usually occurring on being first exposed to

276   infection as a juvenile, there follows a chronic phase of infection that persists for an

277   extended period of time at low parasitaemia (Valkiūnas 2005, Zehtindjiev et al. 2008).

278   Most individuals in this study were sampled as adults with no noticeable symptoms of

279   infection, so we assume that these infections are in the chronic, stable, low intensity

280   phase of infection and therefore unlikely to change infection status during the

281   transmission period. Secondly, between-year repeatability of individual avian malaria

282   infection is lower than within-year repeatability (Knowles et al. 2011). In the SIS-case

283   with strain varying FOI, the matrix projection model used to construct the likelihood has

284   the form

285          $$\mathbf{p}_a = \left(\Lambda \Psi \right)^a \mathbf{p}_0 , \qquad\qquad (4)$$

286   where $\mathbf{p}_a$, $\mathbf{p}_0$ and Ψ are defined as above and Λ denotes the transition matrix for

287   recovery to the susceptible state. The recovery matrix Λ has the form

288
$$\Lambda = \begin{pmatrix} 1 & 1-\phi & 1-\phi & 1-\phi \\ 0 & \phi & 0 & 0 \\ 0 & 0 & \phi & 0 \\ 0 & 0 & 0 & \phi \end{pmatrix},$$
(5)

289 where ϕ denotes the probability that an individual remains infected over autumn and

290 winter. This probability is expressed in terms of the recovery rate, ρ, such that $\phi = e^{-\rho}$.

291 The likelihood is then given by equation 3 above. In the SIR-case with strain varying FOI

292 the matrix projection model used to construct the likelihood has the form

293
$$\mathbf{p}_a = \left(\Omega\Psi\right)^a \mathbf{p}_0,$$
(6)

294 where $\mathbf{p}_a$, $\mathbf{p}_0$ and Ψ are defined as above and Ω denotes the transition matrix for

295 recovery to the immune state. The recovery matrix Ω has the form

296
$$\Omega = \begin{pmatrix} 1 & 0 & 0 & 0 \\ 0 & \eta & 0 & 0 \\ 0 & 0 & \eta & 0 \\ 0 & 0 & 0 & \eta \end{pmatrix},$$
(7)

297 where η denotes the probability that an individual remains infected over autumn and

298 winter, which is expressed in terms of the recovery rate, γ, such that $\eta = e^{-\gamma}$. The

299 likelihood is again given by equation 3 above, but with age specific distribution vector

300 $\mathbf{p}_a$ replaced with $\mathbf{p}'_a = \left(p'_{a,0}\ p_{a,1}\ p_{a,2}\ p_{a,3}\right)$, where the $p_{a,i}$ are the elements of $\mathbf{p}_a$ and

301 $p_{a,0} = 1 - p_{a,1} - p_{a,3} - p_{a,3}$. This is because we do not (explicitly) track the immune class,

302 yet the observed uninfected class includes both susceptible and recovered individuals.

303   The strain-independent FOI version of each model is obtained by simply setting

304 $\lambda_1 = \lambda_2 = \lambda_3$ in infection matrix Ψ. Calculation of the likelihood and likelihood

305   maximisations were carried out with the R statistical programming language (R Core

306   Team 2012). Univariate confidence intervals for model parameters were estimated from

307   profile likelihoods. Models were compared using the small sample AIC_c, since the ratio

308   of the number of observations to parameters in the highest dimensional models is small

309   (Burnham and Anderson 2002).

310

311   3. Avian malaria infection and survival

312   To address one of the assumptions of catalytic modelling, that infection was avirulent, we

313   scrutinised resighting and recapture data of swans sampled and screened for avian

314   malaria infection in 2008. Every two years, approximately 99% of the population is

315   captured during a 'round-up' of the swans at Abbotsbury; the identities of birds captured

316   at round-ups in 2009 and 2011 was supplemented by data from individually colour-

317   marked individuals resighted breeding in the colony in these years – typically around 50

318   breeding adults are not captured at each round-up (C.M. Perrins, unpublished data).

319   Therefore, we examine survival until 2009 and 2011 as two measures of mortality that

320   may have been influenced by infection status at sampling in 2008. Included as factors in

321   this analysis were infection status and individual age, the latter included both as age in

322   years and categorised as young (0-9) or old (10-19 years) to examine the potential effects

323   of infection-related mortality in later life. These data were analysed using a generalized

324   additive model (Wood 2006) to examine the effect of parasite infection and age on

325   survival until 2009 or 2011, with binomial errors and a logit link. Models were optimised

326   by backward stepwise deletion: a predictor was deleted if its removal from the model

327   made a non-significant change in model deviance (Analysis of deviance, P>0.05).

328

329

Results

331

Avian malaria was diagnosed in 67.0% (77/115) of the mute swans sampled in this study.

All infections belonged to the genus *Haemoproteus*, comprising the cytochrome-*b* lineage

WW1 (prevalence 26.1%, 30/115) and two previously unreported lineages differing by

one base pair difference in a 433bp cytochrome-*b* sequence, namely MUTSW1 (25.2%,

29/115: GenBank accession number GU319788) and MUTSW2 (15.7%, 18/115:

GenBank accession number GU319789). All three lineages were very closely related to

each other: MUTSW1 & 2 differed by just one nucleotide, and they in turn differed to

WW1 by 2 and 3 substitutions respectively. The phylogenetic relationships between these

lineages do not indicate a close connection with those previously found in Anatidae

(Figure 1): these three lineages sit instead in a phylogenetic cluster containing

Haemoproteus lineages previously found exclusively in passerine bird species (with the

exception of lineage MEUND3, found in the budgerigar *Melopsittacus undulates*). For a

full list of the hosts species in which the lineages shown in Figure 1 have been found, see

Appendix 1. All lineages of *Plasmodium*, *Leucocytozoon* and *Haemoproteus* previously

found in Anatidae are phylogenetically distant to the lineages found in this study.

Age-specific variation in prevalence

On examining the age-dependent pattern of infection of pooled *Haemoproteus* infections,

infection appeared to rise steeply in older individuals, reaching a plateau of 80.6($\pm$5.9)%

prevalence at ten years of age: overall a significant age-prevalence relationship (GAM:

351   Analysis of deviance χ^2=14.6, est.df=2.18, P=0.020; Figure 2a). Prevalence of infection

352   with the three comprising lineages revealed varying patterns. WW1 and MUTSW2

353   showed no significant age-related variation (P>0.05; Figures 2b,d), however MUTSW1

354   infection showed significant age-related variation (χ^2=12.4, est.df=3.21, P=0.014),

355   increasing to a peak of 51.6($\pm$9.7)% prevalence at approximately nine years of age before

356   declining in older individuals (Figure 2c). In a direct test of age-dependent variation in

357   infection, lineage identity did not have an overall significant effect on age-prevalence

358   variation (GAMM: χ^2=3.67, df=2, P=0.16), although a significant age:lineage interaction

359   was detected for MUTSW1: the age-related pattern of infection for MUTSW1 was found

360   to be significantly different to that of pooled infection with other *Haemoproteus* lineages

361   (χ^2=12.0, est.df=2.5, P=0.0045). Sex was not retained as a significant predictor of

362   infection (GAM: P>0.05).

363

364   *Epidemiological modelling*

365   The relative performance of the six catalytic models is summarised in Table 1. The best

366   model was the strain-independent force-of-infection (FOI) version of the susceptible-

367   immune-recovered (SIR) model; this model predicts that the FOI does not vary among

368   strains, and that individuals recover into a fully immune class. However, the AIC

369   differences associated with the three remaining models that also include recovery

370   processes were all less than 1, revealing very similar weights of evidence for these

371   alternatives. The AIC difference of both models excluding the recovery were greater than

372   10, indicating that models excluding a recovery process were (relatively) very poor

373   approximating models for the age-prevalence data. Taken together, these results provide

374   strong evidence against the possibility that *Haemoproteus* infections are life-long in the

375   Abbotsbury mute swan population. However, our analysis was unable to resolve the

376   nature of the recovery process (i.e. no significant preference between susceptible-

377   immune-susceptible (SIS) and SI-recovered (SIR) models), and was not able to establish

378   unequivocally whether the FOI varies among strains.

379   The maximum likelihood estimate of the per-strain FOI under the best model

380   (SIR) is 0.10 (0.07-0.15, 95% CI), which implies that the annual probability of infection

381   by any strain is 0.26 (0.18-0.36, 95% CI), whereas the estimated per-strain FOI under the

382   strain-independent FOI model (capturing recovery back into a susceptible class: SIS) was

383   very similar to that of the best model (0.12, 0.07-0.25 95% CI), implying a similar annual

384   probability of infection by any strain (0.30, 0.20-0.53 95% CI). Although the predicted

385   recovery rate under this alternative model was relatively higher than that of the best

386   model, this rate is still very low (0.076, 0.023-0.19 95% CI) and corresponds to an annual

387   recovery probability of only 0.08 (0.02-0.18 95% CI).

388   The predicted age-prevalence curves under each of the fitted models are

389   summarised in Figure 3. Figure 3a shows the predicted relationship under the models

390   assuming strain-independent FOI. It is clear that both the SIR and SIS models yield very

391   similar relationships, which explains why we were unable to resolve differences among

392   the two types of model. The remaining three figures (3b-d) summarise the predictions

393   derived from the models allowing between-strain variation in the FOI. Though among

394   strain differences in the predicted age-prevalence relationships can be detected 'by eye',

395   this variation is very low (Table 1).

396

397   *3. Avian malaria infection and survival*

398   Infection with avian malaria at capture in 2008, either as pooled or as individual

399   *Haemoproteus* lineages, was not associated with subsequent survival until August 2009,

400   and age was not a contributory factor whether incorporated as a potentially non-linear

401   effect or categorised as 'young' (0-9 years) or 'old' (10-19 years) (GAM: P>0.3) . Age-

402   dependent mortality was detected in data on survival until 2011 (as expected, older birds

403   were less likely to survive: χ^2=8.18, est.df=1.61, P=0.017), but this effect was not related

404   to infection, either as pooled or lineage-specific *Haemoproteus* infections (GAM:

405   age:infection interactions, P>0.5). Given the few old birds in our sample (40/115, 34.8%),

406   we interpret the results of these basic analyses of survival with caution.

407

408

409   **Discussion**

410

411   We found that 67% of the mute swan population at Abbotsbury was infected with avian

412   malaria, with one of three cytochrome-*b* lineages of *Haemoproteus*: namely WW1

413   (prevalence 26%) and two novel lineages not previously reported in previous studies,

414   named MUTSW1 (25%) and MUTSW2 (15%). We found evidence for different age-

415   prevalence patterns between lineages, with only MUTSW1 showing significant variation

416   in prevalence with age, although this was not reflected in varying epidemiological

417   parameters between lineages. Catalytic modelling found most support for models of age-

418   dependent prevalence models including recovery from infection, rejecting models of life-

419   long infection by a considerable margin.

420   This study suggests that the convex age-prevalence curve of *Haemoproteus*

421   lineage MUTSW1 in mute swans at Abbotsbury (Figure 2c) may be due to hosts

422   recovering from infection. These results contrast to some extent with previous studies,

423   mainly based on longitudinal studies of short-lived captive birds (Atkinson et al. 2008,

424   Palinauskas et al. 2008, Valkiūnas 2005), which observed an initial critical phase of

425   infection that the host may or may not survive, followed by a decrease in the number of

426   parasites in the bloodstream to a low, stable level that continues for an extended period of

427   time, perhaps the remaining life time of the host (Valkiūnas 2005). Chronic infections are

428   typically of low parasitaemia, but above the detection threshold of the molecular

429   diagnosis techniques applied in this study (Knowles et al. 2010, Palinauskas et al. 2008),

430   so it is unlikely that the loss of infection we report here is a result of a reduction of

431   parasitaemia to undetectable levels. Mute swans live much longer than bird hosts

432   examined in previous studies examining age-related patterns of malaria infection,

433   approximately 10 years on average (McCleery et al. 2002), so host age-related parasite

434   dynamics may be different in short and long lived birds: the fall in MUTSW1 prevalence

435   in older mute swans may be a result of mechanisms that are not apparent in relatively

436   short lived hosts. Acquired immunity (Anderson and May 1985, Crombie and Anderson

437   1985, Dobson et al. 1990, Woolhouse et al. 1991), and age-related behavioural variation

438   in exposure to infection (Altizer et al. 2003, Halvorsen 1986) may contribute to the

439   convex age-prevalence curve of MUTSW1, but further study would be required to

440   identify the mechanisms involved. Parasite-mediated viability selection may also result in

441   a convex age-infection pattern: higher mortality of infected young individuals will

442   remove them from the population to result in a lower prevalence of disease in older

443   individuals (Sol et al. 2003, van Oers et al. 2010). Age-specific patterns of infection may

444   simply remain as echoes of past epidemics, with some age cohorts retaining chronic

445   disease infection acquired in previous outbreaks of a disease no longer transmitted in a

446   population (Long et al. 2010). Furthermore, if senescence results in higher parasite-

447   induced mortality in older individuals, perhaps mediated by a deteriorating immune

448   system in older individuals (Vleck et al. 2007, Lavoie 2005), then older infected

449   individuals will be removed from the population with a subsequent decline in the

450   prevalence and intensity of infection. Although we found no evidence for an interaction

451   between infection and mortality in this study, this may have been masked by our cross-

452   sectional 'snapshot' sampling – examining this potential mechanism to explain declining

453   prevalence with age would be an important goal for future studies, particularly to

454   scrutinise infection dynamics and mortality in older individuals. Further work would be

455   necessary to resolve the potential mechanisms underlying age-specific variation in avian

456   malaria infection in this population, and in wild populations more generally, involving

457   detailed longitudinal studies of marked individuals combined with extensive infection

458   screening (Lachish et al. 2011b, Westerdahl et al. 2005, Atkinson and Samuel 2010, van

459   Oers et al. 2010), which would enable (i) the estimation of transitions between infection

460   states (Atkinson and Samuel 2010, Faustino et al. 2004, Jennelle et al. 2007, Lachish et

461   al. 2011a, Senar and Conroy 2004), (ii) a more detailed monitoring of infection-

462   dependent mortality that may contribute to age-prevalence patterns, and (iii) the

463   examination of the widely indicated importance of transmission in early life (Cosgrove et

464   al. 2008, Valkiūnas 2005, Hasselquist et al. 2007). The benefits of molecular diagnosis in

465   such studies are clear, but it would be important to include the preparation of blood films

466   to complement molecular diagnoses: Haemosporidian parasites might sometimes infect

467   non-natural hosts without completing their lifecycle to the infective gametocyte stage,

468   perhaps allowing degraded parasite DNA to be detected in the blood and thus the

469   erroneous conclusion that the presence of a DNA lineage is evidence of a competent host

470   (see Olias et al 2011 for an example of abortive development). Although it is unlikely

471   that the three mute swan lineages detected in this study are examples of abortive

472   development (high prevalence of infection, close relationship between lineages), the

473   collection of data for microscopy remains important in an age of molecular diagnostics.

474   Assumptions of the epidemiology of avian malaria used in the construction of

475   catalytic models should be realistic for their results to be reliable. Avian malaria parasites

476   were assumed to be avirulent; traditionally accepted from reports of mortality confined to

477   outbreaks in poultry (Atkinson et al. 2008, Valkiūnas 2005) but contradicted by recent

478   experimental studies of wild bird populations that detect more subtle negative effects of

479   infection on reproductive effort and success (Knowles et al. 2010, Marzal et al. 2005,

480   Merino et al. 2000) and survival (Martinez-de la Puente et al. 2010). While a general

481   pattern exists for avian malaria infection to be negatively correlated with reproductive

482   effort (Knowles et al. 2009), detecting the consequences of infection as host mortality

483   have proved more elusive. All the infections detected in the current study were

484   *Haemoproteus*, generally accepted to be less virulent than *Plasmodium* or *Leucocytozoon*

485   haemosporidian parasites in stable host populations, in contrast with studies of the

486   devastating effects of avian *Plasmodia* introduced to naïve host populations (Atkinson

487   and Samuel 2010). Recapture/resighting of the swans in this study since sampling in 2008

488   revealed no significant effect of infection on the probability of survival. Although an

489   experimental approach would be preferable, we do not detect any obvious consequences

490   of *Haemoproteus* infection for survival in this mute swan colony, and suggest that our

491   assumption of avirulent *Haemoproteus* infections is a reasonable working hypothesis

492   with which to estimate the force-of-infection (FOI), a valuable epidemiological

493   parameter.

494   Few studies have examined water birds for avian malaria infection using modern

495   molecular diagnostic techniques, although two studies report avian malaria in the Tundra

496   swan *Cygnus columbianus* in North America (Ricklefs and Fallon 2002, Ramey et al.

497   2012). Considering the distribution of the avian malaria lineages previously reported in

498   swans in this and other studies (Bensch et al. 2009) reveals that the *Haemoproteus*

499   lineage WW1 has previously been found in ten bird species, all of which are passerine

500   birds from the Western Palaearctic or (with the exception of the paddyfield warbler

501   *Acrocephalus agricola*) from the Palaearctic-African migratory flyway (Bensch and

502   Åkesson 2003, Hellgren et al. 2007, Krizanaskiene et al. 2006, Ventim et al. 2012, Wood

503   et al. 2007). WW1 is the closest known relative to the other two lineages found in this

504   study, so if the lineages MUTSW1/2 are exclusive to mute swans it is possible that a host

505   shift has occurred whereby WW1 shifted into Anatidae and subsequently diversified into

506   MUTSW1 & 2 (Figure 1). Avian malaria lineages of the genus *Haemoproteus* are known

507   to be more host-specific than *Plasmodium* (Beadell et al. 2004), but WW1's appearance

508   in mute swans would appear to be an exception to this reported pattern. It is clear from

509   the phylogenetic relationship of *Haemoproteus* lineages in this study that infection is not

510   always related to the evolutionary history of that host: hosts of relatively distant shared

511   ancestry may share highly similar blood parasites (Figure 1). *Leucocytozoon* infection is

512   common in wildfowl, being a confirmed cause of mortality in young Anatidae (ducks,

513   geese and swans) including mute swans (Mörner and Wahlström 1983, Valkiūnas 2005),

514   yet no such infections were found in this mute swan colony, perhaps due to the inability

515   of blackfly vectors to tolerate the tidally variable salinity of The Fleet lagoon at the

516   Abbotsbury Swannery (Williams and Williams 1998). The lack of malaria screening in

517   water birds making it difficult to draw comparisons with other, more intensively surveyed

518   taxa (mostly Passeriformes), generally smaller birds that can be conveniently sampled by

519   mist-netting or while breeding in artificial nest boxes.

520   In conclusion, this study found a high prevalence of avian malaria infection in a

521   mute swan colony comprised of three lineages of *Haemoproteus*, two of which were

522   novel. Age-dependent variation in infection was found for just one of these lineages.

523   Catalytic modelling provided strong evidence for recovery from *Haemoproteus*

524   infections. The Abbotsbury mute swan colony would be a useful model system for the

525   further study of pathogen outbreaks and persistence: the long-term study of individually-

526   marked populations has brought considerable benefits to the fundamental understanding

527   of ecology and evolution (Clutton-Brock and Sheldon 2010). The integration of

528   systematic diagnosis of disease in wild populations has the potential to stimulate

529   advances in the ecology and evolution of infectious disease.

530

531

543

544

545   **Figure Legends**

546

547   *Figure 1.*

548   A Bayesian phylogram of lineages found in this study (indicated by arrows), with

549   lineages previously found in hosts belonging to the Anatidae (+) and a selection of

550   closely-related lineages (see Appendix 1). 'P' marks lineages previously found in

551   passerine birds, 'U' those found only in the budgerigar *Melopsittacus undulatus*. All

552   lineages belonging to the genus *Haemoproteus* marked with a P have been found

553   previously only in passerine birds (for full list of hosts see Appendix 1). Numbers on

554   branches represent posterior probabilities.

555

556   *Figure 2.*

557 Age-specific variation in avian malaria infection. (a) Pooled infections, all of which were

558 *Haemoproteus* spp., and (b-d) showing variation in three cytochrome-*b* lineages of

559 *Haemoproteus.* The size of points reflects the sample size, bars indicate standard error.
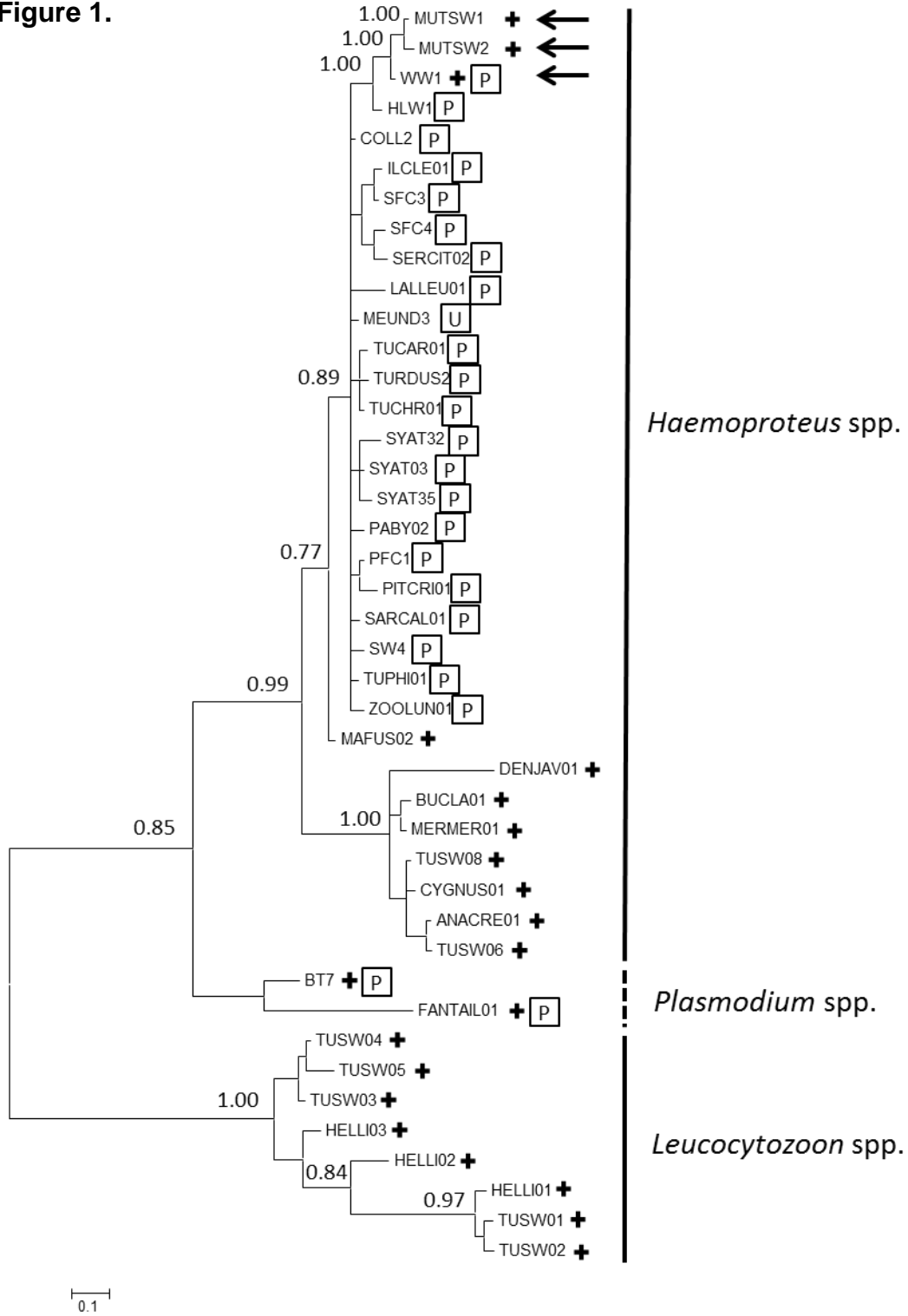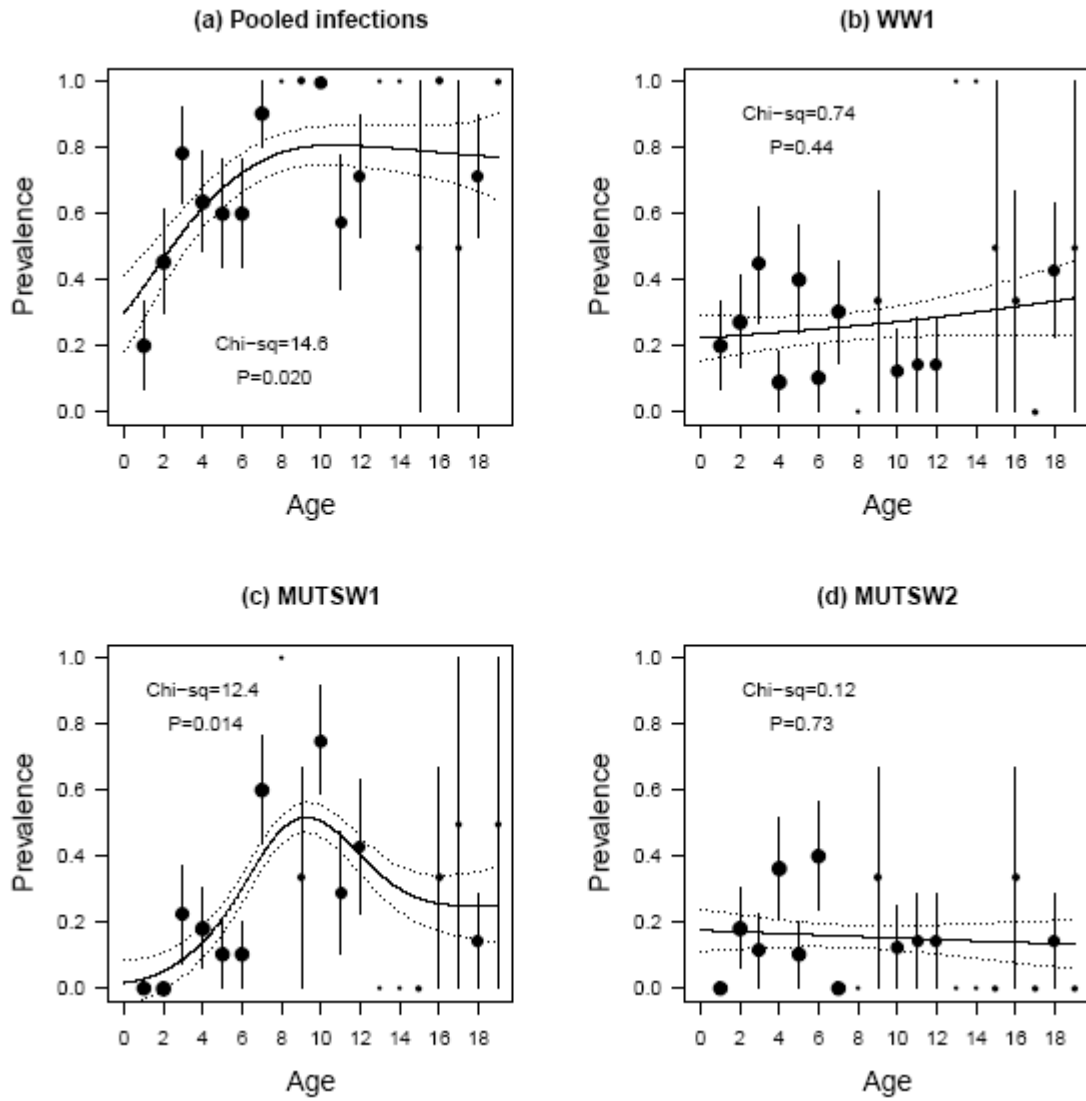
560

561 *Figure 3.*

562 Predicted age-prevalence patterns of mute swan malaria from catalytic models. Results of

563 models for (a) pooled *Haemoproteus* infections and (b-d) infection with three

564 cytochrome-*b Haemoproteus* lineages. Solid lines indicate predicted age-prevalence

565 curves for the fitted SIR-case without strain-varying force of infection; dashed lines show

566 the fitted SIS-case without strain-varying force of infection; and dotted lines show the

567 fitted SI-case without strain-varying force of infection. Point size reflects sample size.

568

Figure 1.

571 **Figure 2**

(a) Pooled infections

Chi-sq=14.6
P=0.020

(b) WW1

Chi-sq=0.74
P=0.44

(c) MUTSW1

Chi-sq=12.4
P=0.014

(d) MUTSW2

Chi-sq=0.12
P=0.73

572

Figure 3

(a) Pooled infections: shared FOI model

(b) WW1: variable FOI model

(c) MUTSW1: variable FOI model

(d) MUTSW2: variable FOI model
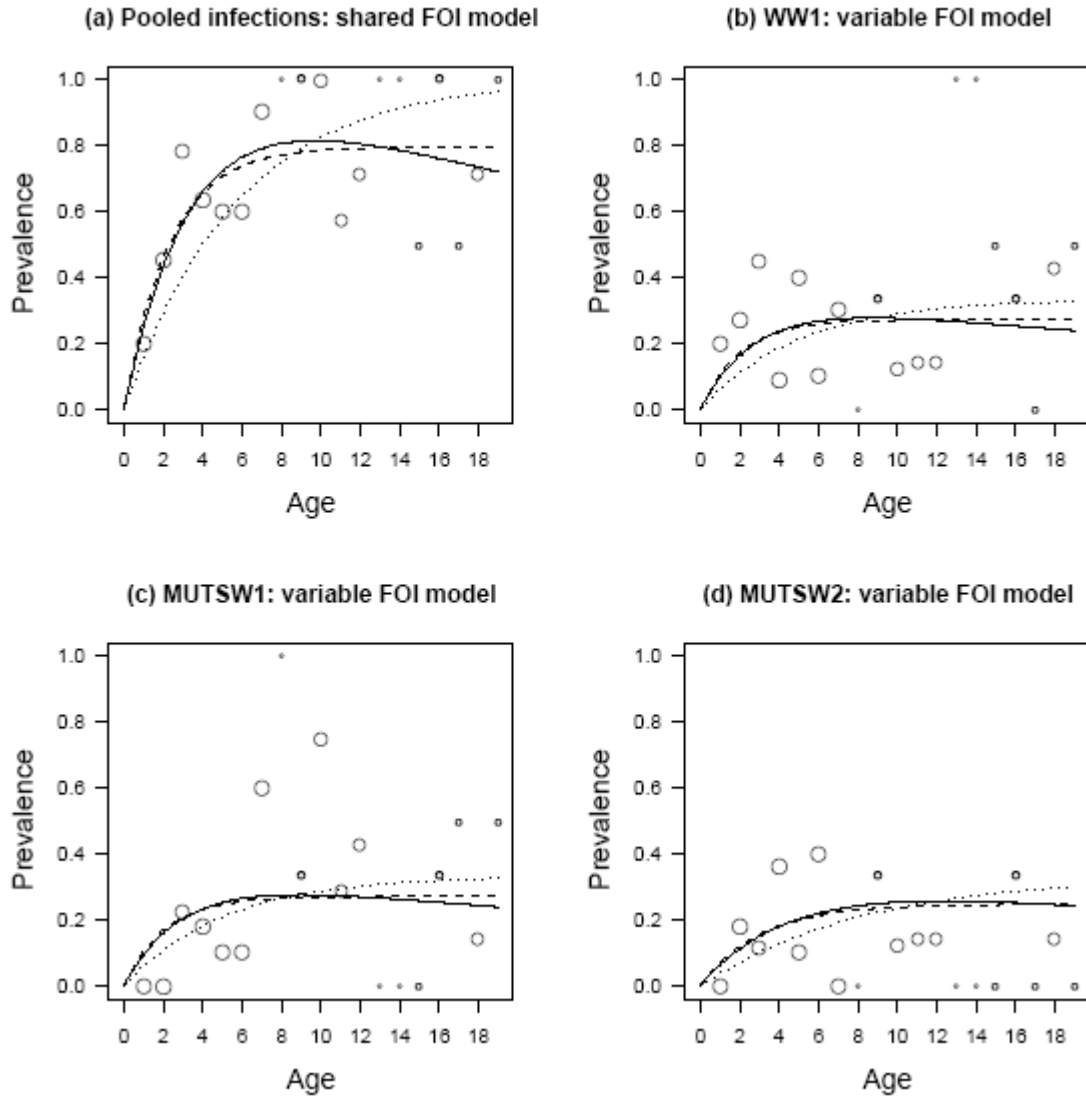
575 **Table 1.**

576 Performance of the six catalytic models fitted to age-prevalence data of *Haemoproteus*

577 infection in swans at Abbotsbury. Δ_i values in bold type indicate the models that were

578 within 2 AIC units of the best model.

579

Model	Log Likelihood	Small sample AIC (AIC_c)	Number of parameters	AIC differences (Δ_i)
SI	-155.3	316.8	3	11.7
$SI_{\lambda_1 = \lambda_2 = \lambda_3}$	-157.1	316.3	1	11.2
SIS	-148.8	305.9	4	**0.8**
$SIS_{\lambda_1 = \lambda_2 = \lambda_3}$	-150.6	305.3	2	**0.2**
SIR	-148.7	305.7	4	**0.6**
$SIR_{\lambda_1 = \lambda_2 = \lambda_3}$	-150.5	305.1	2	**0.0**

580

581

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