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1 The epidemiology underlying age-related avian malaria
2 infection in a long-lived host: the mute swan *Cygnus olor*

3

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17

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19 Disease dynamics, Anatidae, Haemosporida

20

21 **Abstract**

22 Quantifying the factors that predict parasite outbreak and persistence is a major challenge
23 for both applied and fundamental biology. Key to understanding parasite prevalence and
24 disease outbreaks is determining at what age individuals show signs of infection, and
25 whether or not they recover. Age-dependent patterns of the infection of a host population
26 by parasites can indicate among-individual heterogeneities in their susceptibility to, or
27 rate of recovery from, parasite infections. Here, we present a cross-sectional study of
28 avian malaria in a long-lived bird species, the mute swan *Cygnus olor*, examining age-
29 related patterns of parasite prevalence and modelling patterns of infection and recovery.
30 115 swans, ranging from one to nineteen years old, were screened for infection with
31 *Plasmodium*, *Haemoproteus* and *Leucocytozoon* parasites. Infections with three
32 cytochrome-b lineages of *Haemoproteus* were found (pooled prevalence 67%), namely
33 WW1 (26%), which is common in passerine birds, and two new lineages closely related
34 to WW1: MUTSW1 (25%) and MUTSW2 (16%). We found evidence for age-related
35 infection in one lineage, MUTSW1. Catalytic models examining patterns of infection and
36 recovery in the population suggested that infections in this population were not life-long
37 – recovery of individuals was included in the best fitting models. These findings support
38 the results of recent studies that suggest hosts can clear infections, although patterns of
39 infection-related mortality in older birds remain to be studied in more detail.

40

41

42 **Introduction**

43

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

60 A number of cross-sectional studies have reported differences in the degree of
61 parasitism in the wild, revealing variation in space or time (Altizer et al. 2006, Bensch
62 and Åkesson 2003, Cosgrove et al. 2008, Loiseau et al. 2010, Wood et al. 2007).

63 However, such studies do not always permit age-prevalence patterns to be examined in
64 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

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

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 a^{th} 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

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

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

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

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

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

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

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

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

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 $\mathbf{p}'_a = (p'_{a,0} \ p_{a,1} \ p_{a,2} \ p_{a,3})$, where the $p_{a,i}$ are the elements of \mathbf{p}_a and
 301 $p_{a,0} = 1 - p_{a,1} - p_{a,2} - p_{a,3}$. 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

330 **Results**

331

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

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

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

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

336 29/115: GenBank accession number GU319788) and MUTSW2 (15.7%, 18/115:

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

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

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

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

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

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

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

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

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

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

347 Age-specific variation in prevalence

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

349 infection appeared to rise steeply in older individuals, reaching a plateau of 80.6(±5.9)%

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

351 Analysis of deviance $\chi^2=14.6$, 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 ($\chi^2=12.4$, 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: $\chi^2=3.67$, 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 ($\chi^2=12.0$, 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: $\chi^2=8.18$, 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

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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

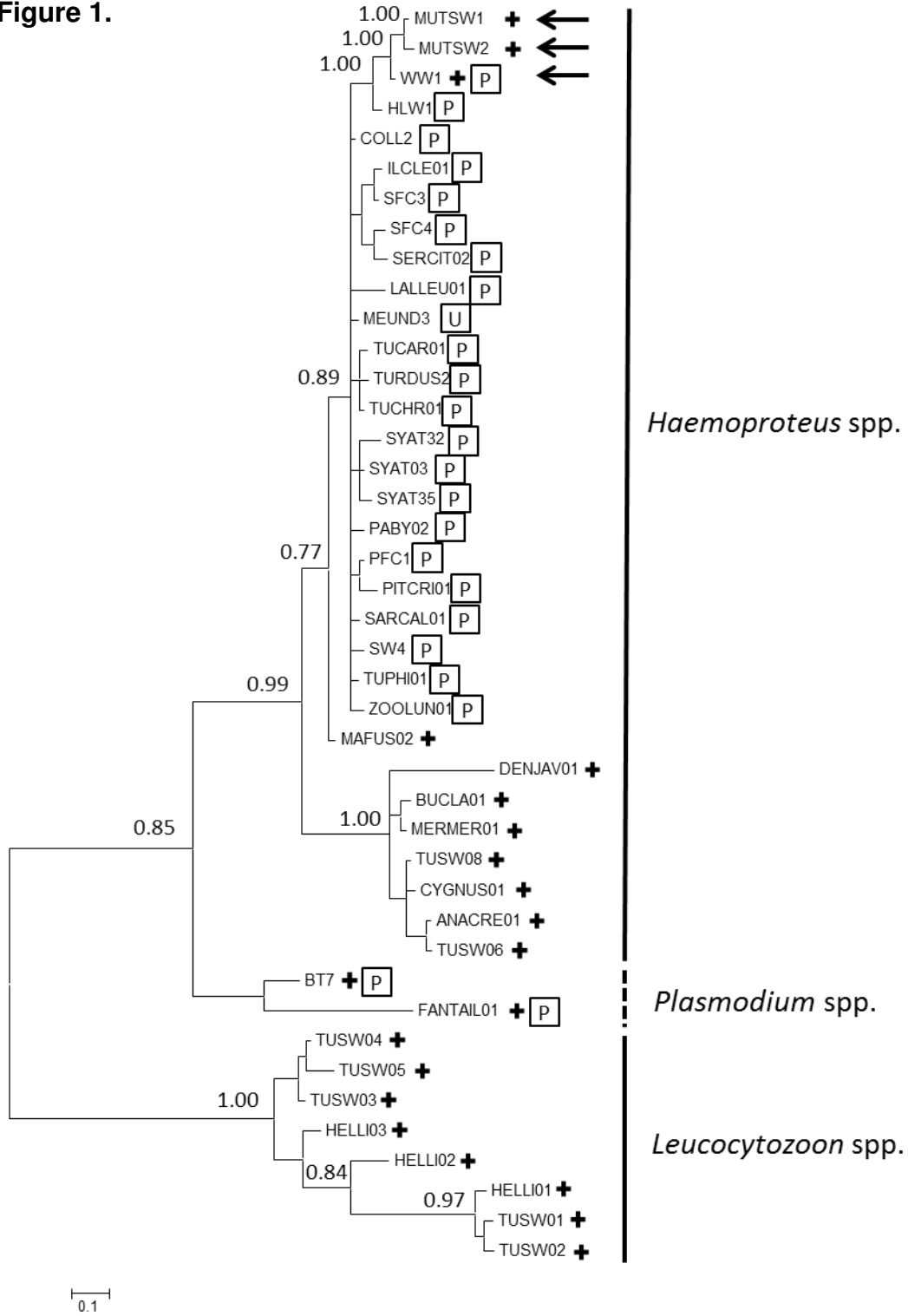
561 Figure 3.

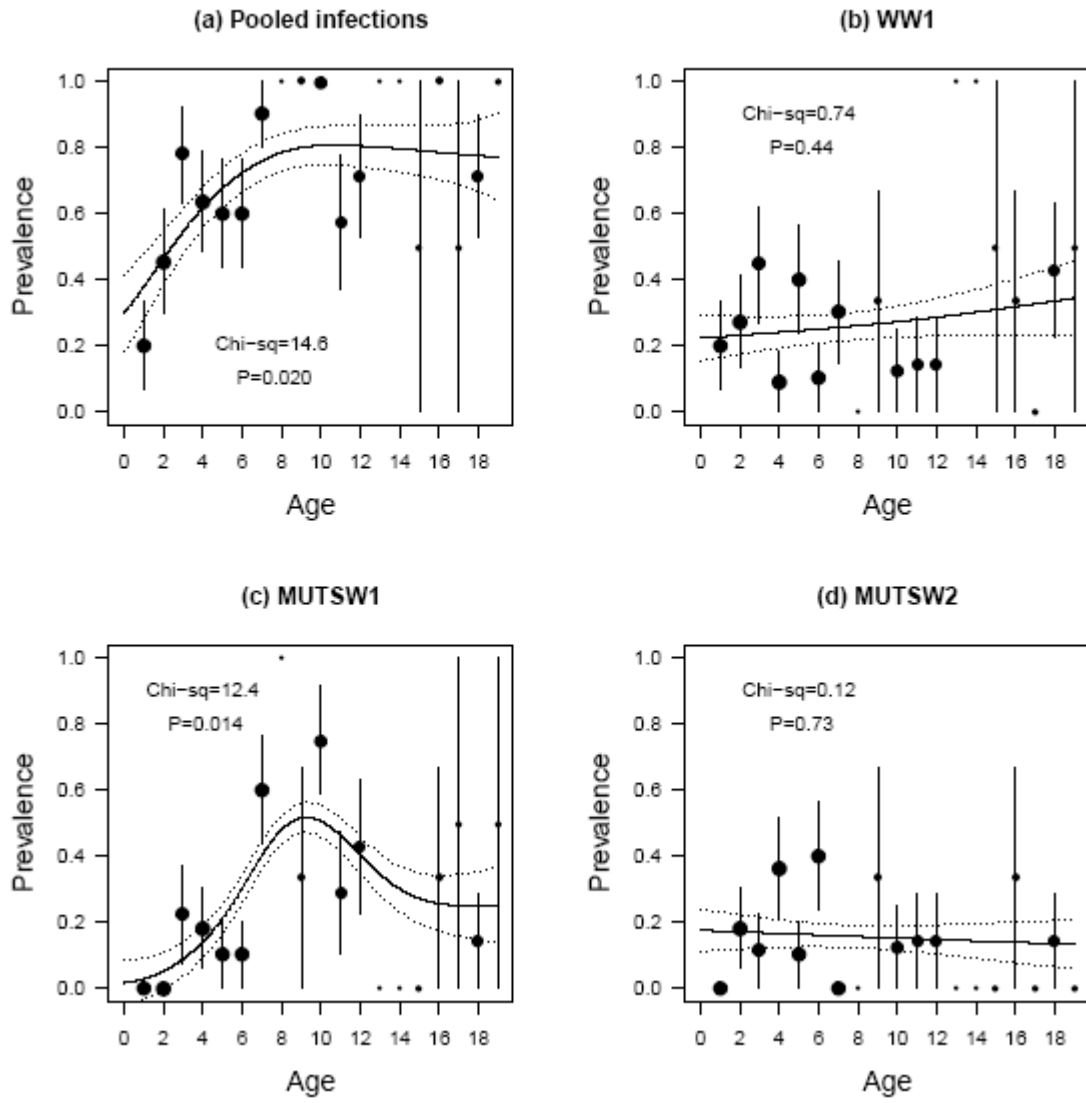
562 Predicted age-prevalence patterns of mute swan malaria from catalytic models. Results of
563 models for (a) pooled Haemoproteus infections and (b-d) infection with three
564 cytochrome-b Haemoproteus lineages. Solid lines indicate predicted age-prevalence
565 curves for the fitted SIR-case without 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.

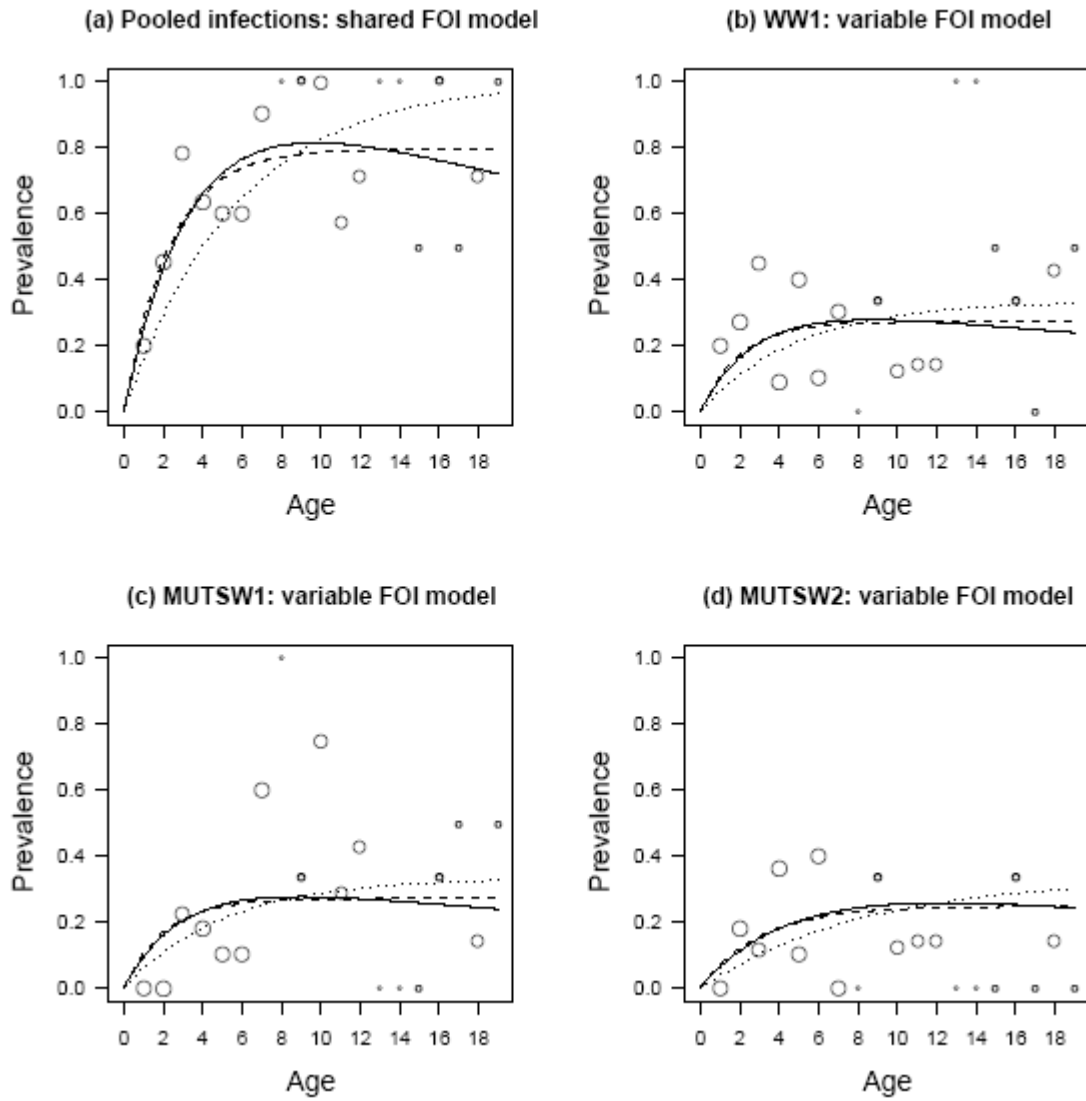
568

569 **Figure 1.**

570







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)
<i>SI</i>	-155.3	316.8	3	11.7
<i>SI</i> _{$\lambda_1 = \lambda_2 = \lambda_3$}	-157.1	316.3	1	11.2
<i>SIS</i>	-148.8	305.9	4	0.8
<i>SIS</i> _{$\lambda_1 = \lambda_2 = \lambda_3$}	-150.6	305.3	2	0.2
<i>SIR</i>	-148.7	305.7	4	0.6
<i>SIR</i> _{$\lambda_1 = \lambda_2 = \lambda_3$}	-150.5	305.1	2	0.0

580

581

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