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The epidemiology underlying age-related avian malaria 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

individuals at birth, or because it may not be possible to infer age from morphological traits. Moreover, most studies of disease in wild bird populations have been conducted in study organisms that are short-lived relative to the scale of seasonal variation in transmission (Beadell et al. 2006, Ishtiaq et al. 2007, Ricklefs et al. 2005, Scheuerlein and Ricklefs 2004, Ventim et al. 2012). This highlights the need for more studies of parasite dynamics in longer-lived host species, which are poorly represented among avian studies (Bennett and Owens 2002)

In the absence of significant disease-induced mortality, the observed pattern of apparent prevalence is driven by rates of transmission and recovery. The direct measurement of transmission presents significant logistical challenges, requiring observation of contact rates and infection probabilities, both seldom directly observable outside the lab (for exceptions see Goeyvaerts et al. 2010, Kjaer et al. 2008). Insight into the transmission process is often gained indirectly, therefore, by estimating the force-of-infection (FOI), also known as the infection hazard, which is simply the per capita rate of infection of susceptible hosts. Though it does not reflect transmission per se (the density or frequency of infected individuals, and rates of transmission govern its magnitude), FOI is nonetheless an important metric for quantifying disease foothold in a population (Heisey et al. 2006, Long et al. 2010). In an endemic setting – one in which the disease is at, or near, dynamic equilibrium or at least changing slowly relative to the lifespan of the host – FOI can be estimated from age-prevalence data derived from cross-sectional sampling. Such data has long been used to estimate the force of infection in human populations using ‘catalytic’ models based on the proportional-hazards framework (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

infection models to estimate FOI in the face of different assumptions governing recovery and re-infection of individuals.

Methods

Host sampling

Mute swans (family Anatidae) are large (7-14kg) waterbirds found in lakes, rivers and coastal areas in temperate and oceanic climates in Europe & Asia, feeding in shallow water on aquatic vegetation. In Western Europe, mute swans are usually territorial and nest in isolated pairs close to the water's edge (Cramp and Simmons 1983), but uniquely in the UK a colony of mute swans is located on the south coast of England at Abbotsbury Swannery (50°35'N, 2°30'W) in The Fleet, a 14km tidal lagoon. The colony has been in existence since at least the 1300s and its population dynamics studied since the late 1960s (Perrins and Ogilvie 1981). The breeding population has increased steadily to become relatively stable at approximately 130 breeding pairs (1990-2012: McCleery et al. 2002); C.M. Perrins unpublished data). Most swans in the colony are ringed as cygnets shortly after hatching and are natally philopatric – only 5% of breeding females are immigrants (Charmantier et al. 2006a) – so the age of most adult birds is known. The majority of breeding pairs nest near brackish pools, ditches and streams or in nearby *Phragmites* reedbeds. Supplemental feeding and the protection of vulnerable breeding pairs in pens are part of the long-term management of the population, but this intervention does not

appear to cause significant differences in population ecology between the Abbotsbury colony and territorial swans in other parts of the UK (Perrins and Ogilvie 1981).

In August 2008, 115 birds of known ages between 1 and 19 years were blood sampled by ulnar or tarsal venepuncture under UK Home Office licence. All were recruits to the colony (i.e. hatched as cygnets at Abbotsbury) to avoid the potential confounding effects of immigration. 92 of these birds were of known sex, based on the consensus from observations of sexually dimorphic bill knob size (Horrocks et al. 2006), sexual behaviour and cloacal examination (Swan Study Group 2005) made during the 2008 capture and any preceding captures or resightings.

Parasite screening

Samples were stored in SET buffer (0.015 M NaCl, 0.05 M Tris, 0.001 MEDTA, pH 8.0), and DNA extracted using a standard ammonium acetate protocol with the final product eluted in Qiagen AE buffer (Qiagen, Valencia CA, USA). DNA was quantified in 100x dilutions using pico-green dye and diluted to a final concentration of 25ng/µl. Extraction products were screened for the presence of *Leucocytozoon, Plasmodium* and *Haemoproteus* infections following the protocol by (Hellgren et al. 2004). 3µl of PCR product was run on 2% agarose gel containing ethidium bromide with a 1Kbp DNA ladder for each sample, testing for *Leucocytozoon* or *Haemoproteus/Plasmodium* lineages. Standard positives were used from previous malaria work in blue tits *Cyanistes caeruleus* (Knowles et al. 2010, Wood et al. 2007). Clear, strong bands were taken as positive and the absence of a band as negative for infection. Negative samples were rescreened to verify parasite absence. Positive sample PCR products were cleaned using a

Qiavac multiwell vacuum manifold.. To identify cytochrome-*b* lineages, BigDye (Applied Biosystems, Foster City, CA, USA) sequencing reactions were run with the forward primer for each lineage (F or FL). Sequences were edited and aligned in Sequencher 4.2 (GeneCodes Corp., Ann Arbor, MI, USA) using known, widespread malarial lineages as an alignment reference obtained from the MalAvi database (Bensch et al. 2009, accessed 18th February 2013). Novel lineages were identified by BLAST search against Genbank and then by comparison with all Haemosporidian lineages in the MalAvi database. Novel lineages were then named to indicate the bird host, using the five-letter species codes (as used by the British Trust for Ornithology), i.e. MUTSW for mute swan, suffixed by a number for each new lineage. Data on novel lineages were submitted to GenBank and MalAvi databases.

Phylogenetic analysis

To place the lineages found in this study in a phylogenetic context, we used the MalAvi database (Bensch et al. 2009) to identify (i) all lineages of *Haemoproteus*, *Plasmodium* and *Leucocytozoon* previously found in Anatidae, and (ii) the closest known relatives of the lineages found in this study. The latter were selected by constructing a neighbour-joining phylogeny – a Jukes-Cantor model as implemented in Geneious ver. R6 (Biomatters Ltd., Auckland, New Zealand: http://www.geneious.com) – including all available lineages to identify a well-defined clade of lineages in which the lineages found in this study occurred (for selection of clusters see Appendix 2). A Bayesian phylogeny was then constructed from the selected lineages using MrBayes (Huelsenbeck and 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

age-prevalence variation between parasite lineages, we used generalized additive mixed modelling (GAMM, Wood 2006). Each host individual was represented by three data per individual reflecting infection with each of three lineages, with individual identity fitted as a random effect and varying coefficient smoothing with respect to infection with each lineage. Once GAMM model selection was completed using maximum likelihood, the model was refitted using REML for extraction of parameter estimates. These analyses were conducted using the packages mgcv 1.7-13 and gamm4 0.1-5 in R 2.15.0 (R Core Team 2012). Means are presented ±1 standard error.

2. Catalytic model

The observed age-prevalence data is multinomial, with four possible outcomes corresponding to individual infection status: uninfected ($Y = 0$), infected with lineage WW1 (see results) ($Y = 1$), infected with MUTSW1 ($Y = 2$), or infected with MUTSW2 ($Y = 3$). Two assumptions are made:

(i) That infections are avirulent, such that infection-induced mortality is negligible and can be ignored. This assumption was traditionally accepted by early studies of avian blood parasites (Bennett et al. 1988, Valkiūnas 2005). While a growing number of correlative studies have been equivocal on the virulence of avian malaria in stable host populations (Asghar et al. 2011, Atkinson et al. 2008, Lachish et al. 2011a), recent experimental studies have detected the detrimental effects of avian malaria in wild passerine bird populations (Knowles et al. 2010, Martinez-de la Puente et al. 2010, Marzal et al. 2005, Merino et al. 2000). In view of the equivocal evidence from avian 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.

In order to construct the likelihood under a particular model we need to calculate the probability that an individual has a given infection status (0, 1, 2, or 3) at each age, up to the maximum age observed. Because the FOI and recovery rates (if present) are constant with respect to age, the required likelihood can be calculated by iterating matrix projection model describing the transitions among infection states in successive ages. In the SI-case with strain varying FOI, the matrix projection model has the form

$$\mathbf{p}_a = \Psi^a \mathbf{p}_0 , \tag{1}$$

where $\mathbf{p}_a$ denotes the distribution vector of states at age a, $\mathbf{p}_0$ is the initial distribution vector, and Ψ^a is the transition matrix for infection raised to the a^{th} power. Since individuals are uninfected at birth the initial distribution vector is simply $\mathbf{p}_0 = \begin{pmatrix} 1 & 0 & 0 & 0 \end{pmatrix}^T$. The infection matrix Ψ has the form

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

where π is the probability an individual avoids infection over the course of a year and θ_i is the probability that an individual is infected by strain *i*. These probabilities are expressed in terms of the FOI for each strain, λ_i, such that $\pi = e^{-\lambda_1 - \lambda_2 - \lambda_3}$ and $\theta_i = \frac{\lambda_i}{\lambda_1 + \lambda_2 + \lambda_3}$. The likelihood is then

$$y_i \sim Multinom\left(1, \mathbf{p}_a\right), \tag{3}$$

where y_i denotes the status of individual i. Readers familiar with survival analysis will recognise that the FOI can also be estimated with a competing risks survival model,

where the "hazard" associated with each infection process is λ_i. However, we use the above formulation because it is easily extended to incorporate recovery.

In order to construct the likelihood for the SIS- and SIR-case we assume that the infection and recovery processes occur independently and sequentially each year. This approximation, which simplifies the modelling, is justified by the observation that insect vectors are absent over the winter, so that transmission necessarily occurs in late spring and summer. We also assume that within-year recovery during the spring/summer transmission period is negligible, and can be ignored; an assumption that may be justified on two counts: Firstly, previous studies of avian haemosporidia indicate that if a bird survives the initial acute phase of infection, usually occurring on being first exposed to infection as a juvenile, there follows a chronic phase of infection that persists for an extended period of time at low parasitaemia (Valkiūnas 2005, Zehtindjiev et al. 2008). Most individuals in this study were sampled as adults with no noticeable symptoms of infection, so we assume that these infections are in the chronic, stable, low intensity phase of infection and therefore unlikely to change infection status during the transmission period. Secondly, between-year repeatability of individual avian malaria infection is lower than within-year repeatability (Knowles et al. 2011). In the SIS-case with strain varying FOI, the matrix projection model used to construct the likelihood has the form

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

where $\mathbf{p}_a$, $\mathbf{p}_0$ and Ψ are defined as above and Λ denotes the transition matrix for recovery to the susceptible state. The recovery matrix Λ has the form

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

where ϕ denotes the probability that an individual remains infected over autumn and winter. This probability is expressed in terms of the recovery rate, ρ, such that $\phi = e^{-\rho}$. The likelihood is then given by equation 3 above. In the SIR-case with strain varying FOI the matrix projection model used to construct the likelihood has the form

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

where $\mathbf{p}_a$, $\mathbf{p}_0$ and Ψ are defined as above and Ω denotes the transition matrix for recovery to the immune state. The recovery matrix Ω has the form

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

where η denotes the probability that an individual remains infected over autumn and winter, which is expressed in terms of the recovery rate, γ, such that $\eta = e^{-\gamma}$. The likelihood is again given by equation 3 above, but with age specific distribution vector $\mathbf{p}_a$ 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 $p_{a,0} = 1 - p_{a,1} - p_{a,3} - p_{a,3}$. This is because we do not (explicitly) track the immune class, yet the observed uninfected class includes both susceptible and recovered individuals.

The strain-independent FOI version of each model is obtained by simply setting $\lambda_1 = \lambda_2 = \lambda_3$ in infection matrix Ψ. Calculation of the likelihood and likelihood

maximisations were carried out with the R statistical programming language (R Core Team 2012). Univariate confidence intervals for model parameters were estimated from profile likelihoods. Models were compared using the small sample AIC_c, since the ratio of the number of observations to parameters in the highest dimensional models is small (Burnham and Anderson 2002).

3. Avian malaria infection and survival

To address one of the assumptions of catalytic modelling, that infection was avirulent, we scrutinised resighting and recapture data of swans sampled and screened for avian malaria infection in 2008. Every two years, approximately 99% of the population is captured during a 'round-up' of the swans at Abbotsbury; the identities of birds captured at round-ups in 2009 and 2011 was supplemented by data from individually colour-marked individuals resighted breeding in the colony in these years – typically around 50 breeding adults are not captured at each round-up (C.M. Perrins, unpublished data). Therefore, we examine survival until 2009 and 2011 as two measures of mortality that may have been influenced by infection status at sampling in 2008. Included as factors in this analysis were infection status and individual age, the latter included both as age in years and categorised as young (0-9) or old (10-19 years) to examine the potential effects of infection-related mortality in later life. These data were analysed using a generalized additive model (Wood 2006) to examine the effect of parasite infection and age on survival until 2009 or 2011, with binomial errors and a logit link. Models were optimised by backward stepwise deletion: a predictor was deleted if its removal from the model made a non-significant change in model deviance (Analysis of deviance, $P>0.05$).

Results

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(±5.9)% prevalence at ten years of age: overall a significant age-prevalence relationship (GAM:

Analysis of deviance χ^2=14.6, est.df=2.18, P=0.020; Figure 2a). Prevalence of infection with the three comprising lineages revealed varying patterns. WW1 and MUTSW2 showed no significant age-related variation (P>0.05; Figures 2b,d), however MUTSW1 infection showed significant age-related variation (χ^2=12.4, est.df=3.21, P=0.014), increasing to a peak of 51.6(±9.7)% prevalence at approximately nine years of age before declining in older individuals (Figure 2c). In a direct test of age-dependent variation in infection, lineage identity did not have an overall significant effect on age-prevalence variation (GAMM: χ^2=3.67, df=2, P=0.16), although a significant age:lineage interaction was detected for MUTSW1: the age-related pattern of infection for MUTSW1 was found to be significantly different to that of pooled infection with other *Haemoproteus* lineages (χ^2=12.0, est.df=2.5, P=0.0045). Sex was not retained as a significant predictor of infection (GAM: P>0.05).

Epidemiological modelling

The relative performance of the six catalytic models is summarised in Table 1. The best model was the strain-independent force-of-infection (FOI) version of the susceptible-immune-recovered (SIR) model; this model predicts that the FOI does not vary among strains, and that individuals recover into a fully immune class. However, the AIC differences associated with the three remaining models that also include recovery processes were all less than 1, revealing very similar weights of evidence for these alternatives. The AIC difference of both models excluding the recovery were greater than 10, indicating that models excluding a recovery process were (relatively) very poor approximating models for the age-prevalence data. Taken together, these results provide

strong evidence against the possibility that *Haemoproteus* infections are life-long in the Abbotsbury mute swan population. However, our analysis was unable to resolve the nature of the recovery process (i.e. no significant preference between susceptible-immune-susceptible (SIS) and SI-recovered (SIR) models), and was not able to establish unequivocally whether the FOI varies among strains.

The maximum likelihood estimate of the per-strain FOI under the best model (SIR) is 0.10 (0.07-0.15, 95% CI), which implies that the annual probability of infection by any strain is 0.26 (0.18-0.36, 95% CI), whereas the estimated per-strain FOI under the strain-independent FOI model (capturing recovery back into a susceptible class: SIS) was very similar to that of the best model (0.12, 0.07-0.25 95% CI), implying a similar annual probability of infection by any strain (0.30, 0.20-0.53 95% CI). Although the predicted recovery rate under this alternative model was relatively higher than that of the best model, this rate is still very low (0.076, 0.023-0.19 95% CI) and corresponds to an annual recovery probability of only 0.08 (0.02-0.18 95% CI).

The predicted age-prevalence curves under each of the fitted models are summarised in Figure 3. Figure 3a shows the predicted relationship under the models assuming strain-independent FOI. It is clear that both the SIR and SIS models yield very similar relationships, which explains why we were unable to resolve differences among the two types of model. The remaining three figures (3b-d) summarise the predictions derived from the models allowing between-strain variation in the FOI. Though among strain differences in the predicted age-prevalence relationships can be detected ‘by eye’, this variation is very low (Table 1).

3. Avian malaria infection and survival

Infection with avian malaria at capture in 2008, either as pooled or as individual *Haemoproteus* lineages, was not associated with subsequent survival until August 2009, and age was not a contributory factor whether incorporated as a potentially non-linear effect or categorised as 'young' (0-9 years) or 'old' (10-19 years) (GAM: P>0.3) . Age-dependent mortality was detected in data on survival until 2011 (as expected, older birds were less likely to survive: χ^2=8.18, est.df=1.61, P=0.017), but this effect was not related to infection, either as pooled or lineage-specific *Haemoproteus* infections (GAM: age:infection interactions, P>0.5). Given the few old birds in our sample (40/115, 34.8%), we interpret the results of these basic analyses of survival with caution.

Discussion

We found that 67% of the mute swan population at Abbotsbury was infected with avian malaria, with one of three cytochrome-*b* lineages of *Haemoproteus*: namely WW1 (prevalence 26%) and two novel lineages not previously reported in previous studies, named MUTSW1 (25%) and MUTSW2 (15%). We found evidence for different age-prevalence patterns between lineages, with only MUTSW1 showing significant variation in prevalence with age, although this was not reflected in varying epidemiological parameters between lineages. Catalytic modelling found most support for models of age-dependent prevalence models including recovery from infection, rejecting models of life-long infection by a considerable margin.

This study suggests that the convex age-prevalence curve of *Haemoproteus* lineage MUTSW1 in mute swans at Abbotsbury (Figure 2c) may be due to hosts recovering from infection. These results contrast to some extent with previous studies, mainly based on longitudinal studies of short-lived captive birds (Atkinson et al. 2008, Palinauskas et al. 2008, Valkiūnas 2005), which observed an initial critical phase of infection that the host may or may not survive, followed by a decrease in the number of parasites in the bloodstream to a low, stable level that continues for an extended period of time, perhaps the remaining life time of the host (Valkiūnas 2005). Chronic infections are typically of low parasitaemia, but above the detection threshold of the molecular diagnosis techniques applied in this study (Knowles et al. 2010, Palinauskas et al. 2008), so it is unlikely that the loss of infection we report here is a result of a reduction of parasitaemia to undetectable levels. Mute swans live much longer than bird hosts examined in previous studies examining age-related patterns of malaria infection, approximately 10 years on average (McCleery et al. 2002), so host age-related parasite dynamics may be different in short and long lived birds: the fall in MUTSW1 prevalence in older mute swans may be a result of mechanisms that are not apparent in relatively short lived hosts. Acquired immunity (Anderson and May 1985, Crombie and Anderson 1985, Dobson et al. 1990, Woolhouse et al. 1991), and age-related behavioural variation in exposure to infection (Altizer et al. 2003, Halvorsen 1986) may contribute to the convex age-prevalence curve of MUTSW1, but further study would be required to identify the mechanisms involved. Parasite-mediated viability selection may also result in a convex age-infection pattern: higher mortality of infected young individuals will remove them from the population to result in a lower prevalence of disease in older

individuals (Sol et al. 2003, van Oers et al. 2010). Age-specific patterns of infection may simply remain as echoes of past epidemics, with some age cohorts retaining chronic disease infection acquired in previous outbreaks of a disease no longer transmitted in a population (Long et al. 2010). Furthermore, if senescence results in higher parasite-induced mortality in older individuals, perhaps mediated by a deteriorating immune system in older individuals (Vleck et al. 2007, Lavoie 2005), then older infected individuals will be removed from the population with a subsequent decline in the prevalence and intensity of infection. Although we found no evidence for an interaction between infection and mortality in this study, this may have been masked by our cross-sectional 'snapshot' sampling – examining this potential mechanism to explain declining prevalence with age would be an important goal for future studies, particularly to scrutinise infection dynamics and mortality in older individuals. Further work would be necessary to resolve the potential mechanisms underlying age-specific variation in avian malaria infection in this population, and in wild populations more generally, involving detailed longitudinal studies of marked individuals combined with extensive infection screening (Lachish et al. 2011b, Westerdahl et al. 2005, Atkinson and Samuel 2010, van Oers et al. 2010), which would enable (i) the estimation of transitions between infection states (Atkinson and Samuel 2010, Faustino et al. 2004, Jennelle et al. 2007, Lachish et al. 2011a, Senar and Conroy 2004), (ii) a more detailed monitoring of infection-dependent mortality that may contribute to age-prevalence patterns, and (iii) the examination of the widely indicated importance of transmission in early life (Cosgrove et al. 2008, Valkiūnas 2005, Hasselquist et al. 2007). The benefits of molecular diagnosis in 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

common in wildfowl, being a confirmed cause of mortality in young Anatidae (ducks, geese and swans) including mute swans (Mörner and Wahlström 1983, Valkiūnas 2005), yet no such infections were found in this mute swan colony, perhaps due to the inability of blackfly vectors to tolerate the tidally variable salinity of The Fleet lagoon at the Abbotsbury Swannery (Williams and Williams 1998). The lack of malaria screening in water birds making it difficult to draw comparisons with other, more intensively surveyed taxa (mostly Passeriformes), generally smaller birds that can be conveniently sampled by mist-netting or while breeding in artificial nest boxes.

In conclusion, this study found a high prevalence of avian malaria infection in a mute swan colony comprised of three lineages of *Haemoproteus*, two of which were novel. Age-dependent variation in infection was found for just one of these lineages. Catalytic modelling provided strong evidence for recovery from *Haemoproteus* infections. The Abbotsbury mute swan colony would be a useful model system for the further study of pathogen outbreaks and persistence: the long-term study of individually-marked populations has brought considerable benefits to the fundamental understanding of ecology and evolution (Clutton-Brock and Sheldon 2010). The integration of systematic diagnosis of disease in wild populations has the potential to stimulate advances in the ecology and evolution of infectious disease.

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Figure Legends

Figure 1.

A Bayesian phylogram of lineages found in this study (indicated by arrows), with lineages previously found in hosts belonging to the Anatidae (+) and a selection of closely-related lineages (see Appendix 1). 'P' marks lineages previously found in passerine birds, 'U' those found only in the budgerigar *Melopsittacus undulatus*. All lineages belonging to the genus *Haemoproteus* marked with a P have been found previously only in passerine birds (for full list of hosts see Appendix 1). Numbers on branches represent posterior probabilities.

Figure 2.

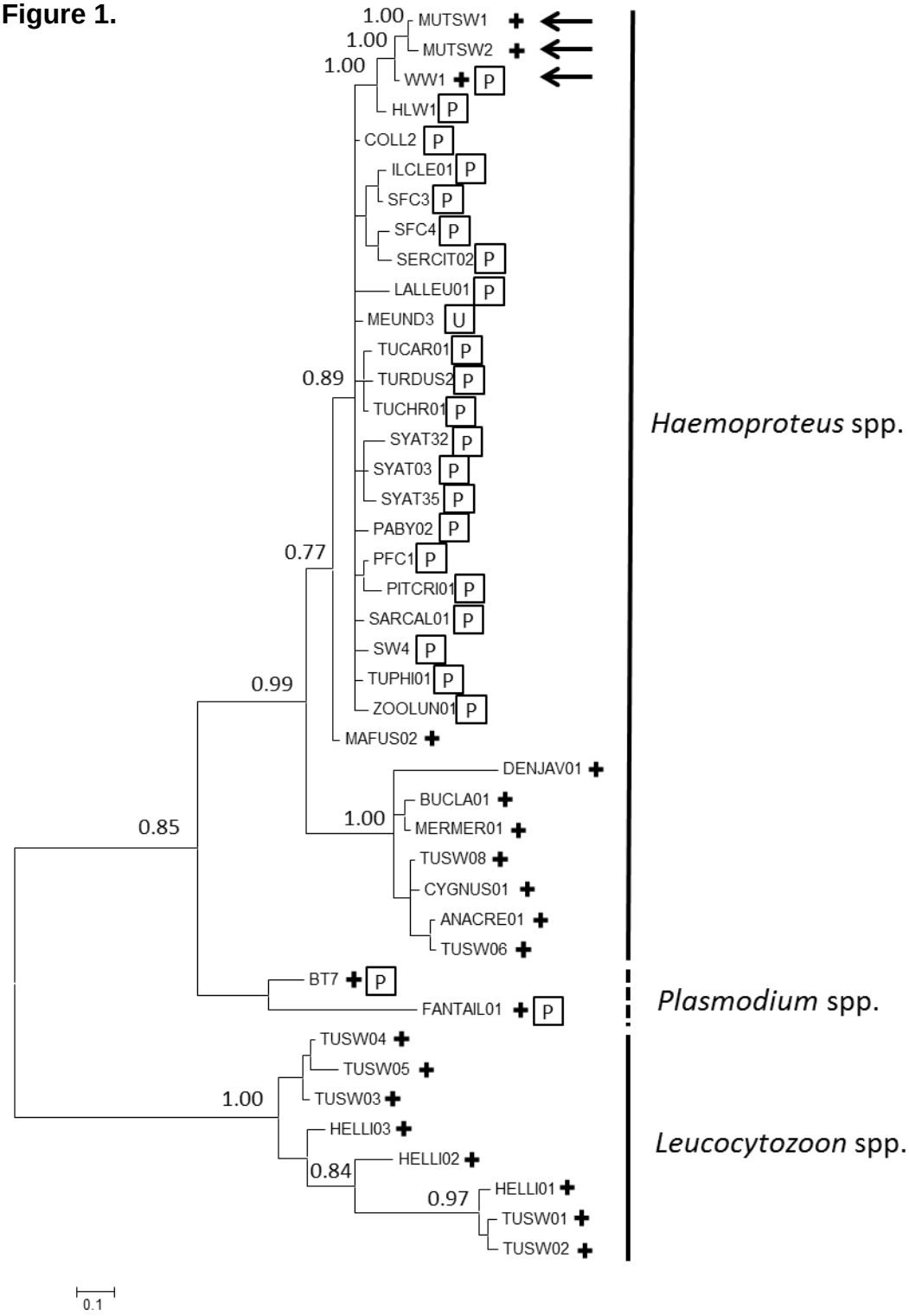
Age-specific variation in avian malaria infection. (a) Pooled infections, all of which were *Haemoproteus* spp., and (b-d) showing variation in three cytochrome-*b* lineages of *Haemoproteus.* The size of points reflects the sample size, bars indicate standard error.

Figure 3.

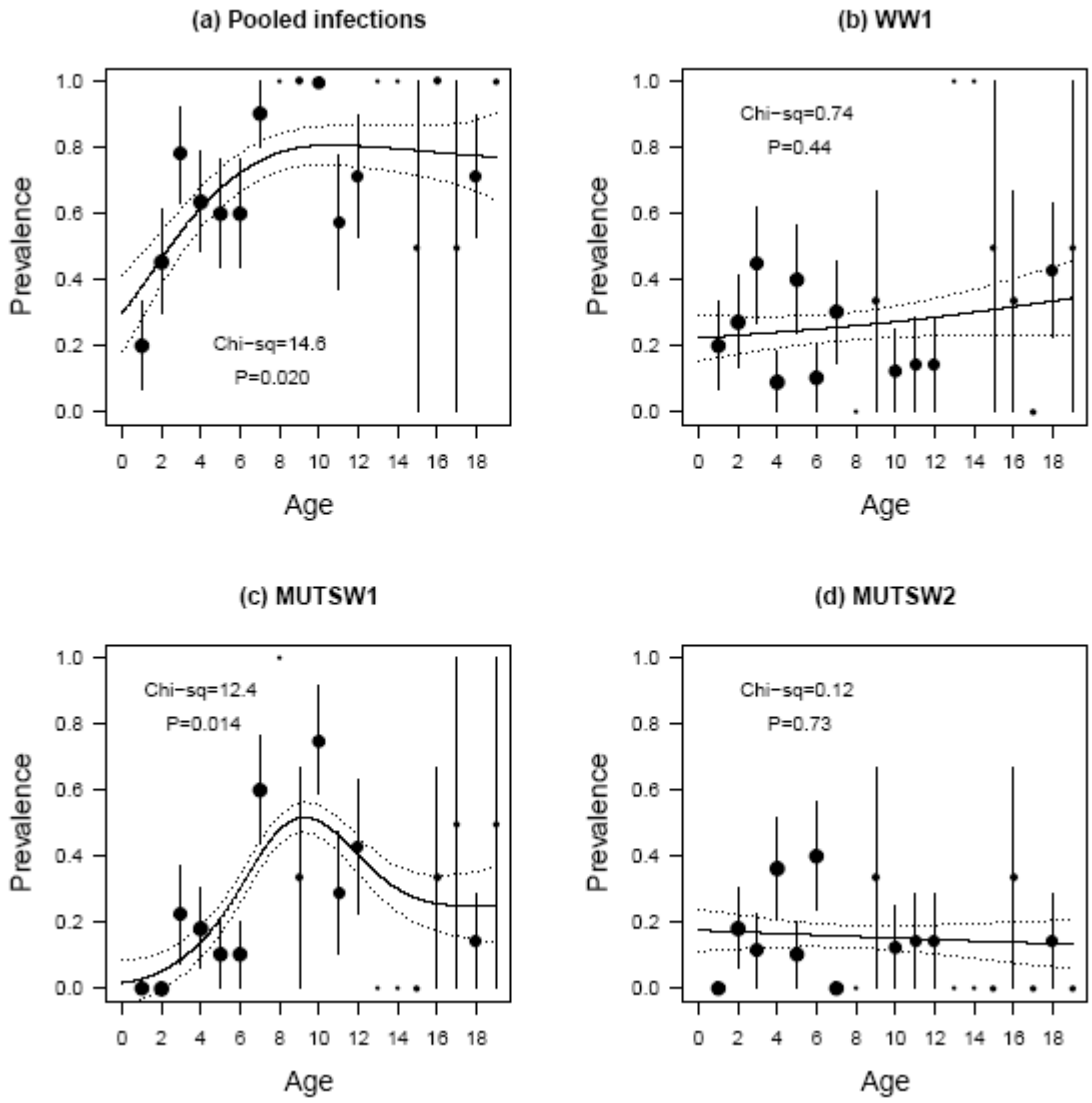
Predicted age-prevalence patterns of mute swan malaria from catalytic models. Results of models for (a) pooled *Haemoproteus* infections and (b-d) infection with three cytochrome-*b Haemoproteus* lineages. Solid lines indicate predicted age-prevalence curves for the fitted SIR-case without strain-varying force of infection; dashed lines show the fitted SIS-case without strain-varying force of infection; and dotted lines show the fitted SI-case without strain-varying force of infection. Point size reflects sample size.

569 **Figure 1.**

570



571 **Figure 2**



572

573 **Figure 3**

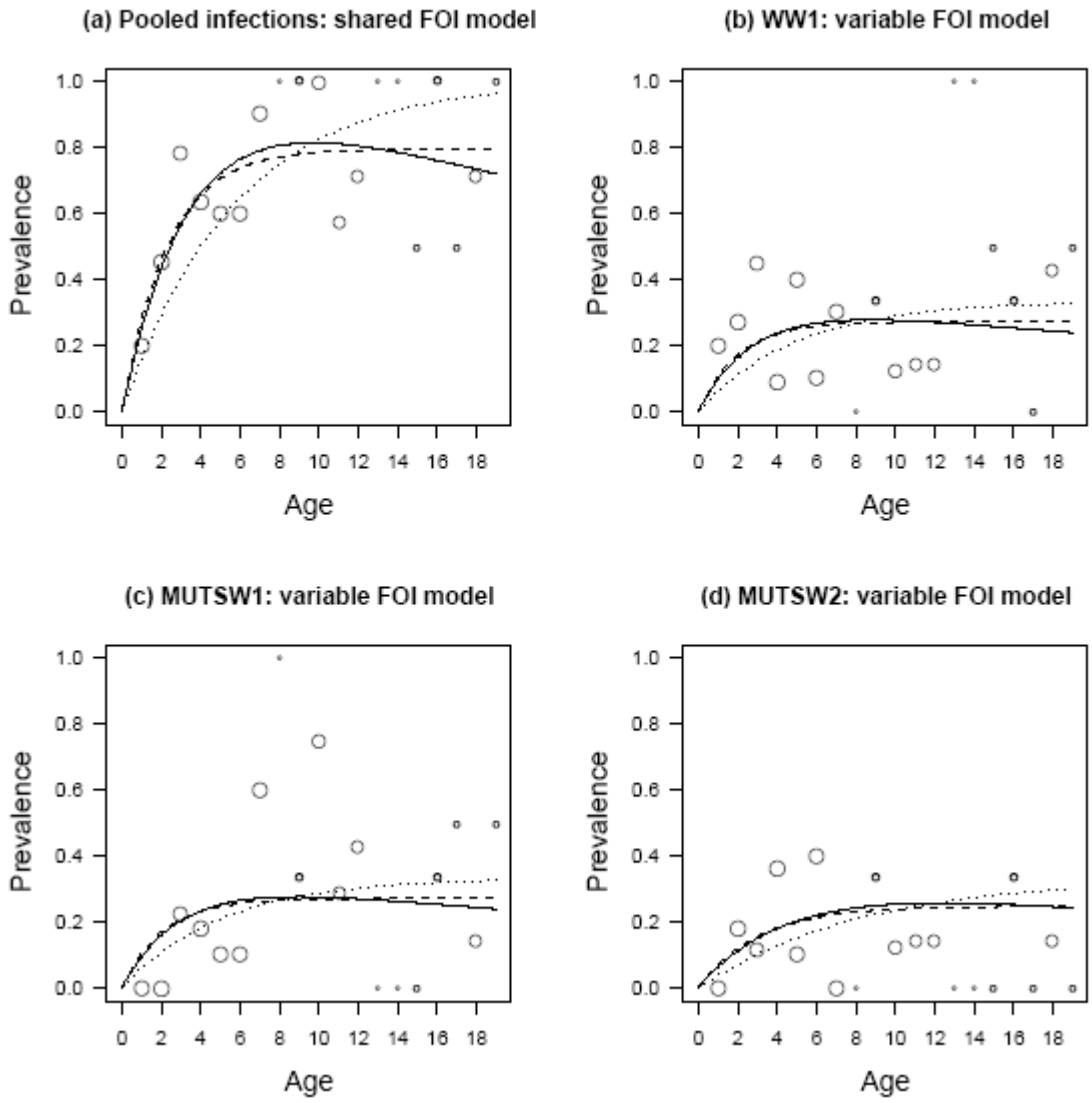


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Table 1.

Performance of the six catalytic models fitted to age-prevalence data of *Haemoproteus* infection in swans at Abbotsbury. Δ_i values in bold type indicate the models that were within 2 AIC units of the best model.

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**

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