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High rates of infection by blood parasites during the nestling phase in UK Columbids with notes on ecological associations

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RUNNING TITLE: Blood parasite infection in nestling Columbids

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SUMMARY

Studies of blood parasite infection in nestling birds rarely find a high prevalence of infection. This is likely due to a combination of short nestling periods (limiting the age at which nestlings can be sampled) and long parasite prepatent periods before gametocytes can be detected in peripheral blood. Here, we examine rates of blood parasite infection in nestlings from three Columbid species in the UK. We use this system to address two key hypotheses in the epidemiology of avian haemoparasites: first, that nestlings in open nests have a higher prevalence of infection; and second, that nestlings sampled at 14 days old have a higher apparent infection rate than those sampled at 7 days old. Open-nesting individuals had a 54% infection rate compared to 25% for box-nesters, probably due to an increased exposure of open-nesting species to dipteran vectors. Nestlings sampled at 14 days had a 68% infection rate compared to 32% in nestlings sampled at 7 days, suggesting that rates of infection in the nest are high. Further work should examine nestlings post-fledging to identify rates of successful parasite infection (as opposed to abortive development within a dead-end host) as well as impacts on host post-fledging survival and behaviour.

Key words: Haemoparasite, *Haemoproteus*, *Leucocytozoon*, nesting ecology, parasite, PCR

KEY FINDINGS

- We screened 70 nestlings from three Columbid species for blood parasite infection using PCR
- Nestlings in open nests had a higher prevalence of infection than nestlings in nestboxes
- Nestlings sampled at 14 days had a higher prevalence of infection than those sampled at 7 days
- Infection of nestlings appears widespread but detection may be limited by parasite biology
- Further research should investigate impacts of infection on post-fledging survival and behaviour

INTRODUCTION

The age of first infection is a key question in disease epidemiology. Previous studies of haemoparasite infection in nestling birds have failed to find evidence of widespread infection (Weatherhead and Bennett, 1991; Cosgrove *et al.* 2006; Zehtindjiev *et al.* 2011). These studies include those of open-nesting Red-winged Blackbirds *Agelaius phoeniceus* at 6-7 days old (Weatherhead and Bennett, 1991), Skylarks *Alauda arvensis* at 5-7 days old (Zehtindjiev *et al.* 2011), and box-nesting Blue Tits *Cyanistes caeruleus* at 11 days old (Cosgrove *et al.* 2006). Box-nesting species may be shielded from vector exposure due to their enclosed surroundings, but open-nesting species should be susceptible in areas of high vector activity due to their sessility and incomplete plumage. The lack of sensitivity to detect nestling infection in passerines is likely due to a combination of both the developmental time of the parasite, and the length of the nestling period during which sampling is possible. Following a bite from an infected vector, which injects parasite sporozoites into the blood stream, the parasites then enter a prepatent period where they retreat to the fixed tissues of the host. Here, they develop into gametocytes (the transmissible stage of the parasite), which are released into the peripheral blood stream and can be detected through serological sampling of the host. The majority of avian haemoparasites have a prepatent period of between 11 days and 3 weeks (Valkiūnas, 2005). However, the length of this prepatent period varies between parasite species: *Haemoproteus* has the longest prepatent period of generally between 14 and 38 days, *Leucocytozoon* usually between 4 and 15 days, and *Plasmodium* has the widest range, generally between 2 days and 3 months (Valkiūnas, 2005).

Sensitive PCR techniques, as used by Cosgrove *et al.* (2006) but not by Weatherhead and Bennett (1991) can amplify DNA from sporozoites during initial infection (Valkiūnas *et al.* 2009). A recent study of open-nesting Skylarks sampled at 5-7 days detected infection at rates of 9.9% by *Plasmodium* (Zehtindjiev *et al.* 2011). Any immune consequences of infection for rapidly-growing nestlings, or prevalence of dead-end infections at the nestling stage are currently unknown although infected adult birds often show altered immune parameters compared to uninfected individuals (e.g. Dunn *et al.* 2013).

Here, we screen nestling columbids from three species: European Turtle Doves *Streptopelia turtur* (hereafter referred to as Turtle Doves), Stock Doves *Columba oenas* and Woodpigeons *Columba palumbus*; for infection by *Haemoproteus*, *Plasmodium* and *Leucocytozoon* parasites using PCR. These three species all nest within farmland in the UK, with Turtle Doves and Woodpigeons making open nest platforms in scrubby habitats or hedgerows in farmland, and Stock Doves nesting in tree holes and artificial boxes. Turtle Dove nestlings remain in the nest for up to 14 days, and Stock Doves and Woodpigeons for up to 30 days (Robinson, 2016). Turtle Dove nestlings can be handled and samples taken at up to 7 days, and Woodpigeon and Stock Doves nestlings at up to 14 days. We use sensitive PCR techniques to amplify parasite DNA from avian blood to infer the frequency and potential importance of haemoparasite infection during the nestling phase for disease epidemiology and test the following hypotheses:

1) Nestlings in open nests have higher parasite prevalence than nestlings in nestboxes

2) Nestlings with a longer exposure period (i.e. those sampled at a later age) have higher parasite prevalence than those with a shorter exposure period

MATERIALS AND METHODS

Study sites and nest location

Turtle Dove, Woodpigeon and Stock Dove nestlings were sampled at sites in Cambridgeshire, Essex, Norfolk and Suffolk during June – September in 2011 - 2013. All sites were predominantly arable farmland and are those detailed in Dunn *et al.* (2015), with the addition of 3 new sites in Essex, Norfolk and Bedfordshire in 2013 (nearest towns Great Wigborough: 51°47'N, 0°51'E; March: 52°32'N, 0°5'E; and Sandy: 52°7'N, 0°17'W). Nests were located by cold searching of suitable habitat for Woodpigeon and Turtle Doves, by tracking radiotagged Turtle Doves back to their nests (these were tagged as part of a wider autecological study), and by liaising with landowners with nestboxes containing Stock Doves present on their land. Once located, nests were monitored regularly until hatching; if hatch day was unknown, nestlings were aged by comparison of feather growth to nestlings of known ages.

Blood sampling and parasite detection

Blood was taken through venipuncture of the brachial vein and stored frozen until subsequent analysis. Two blood smears were created for each nestling and fixed with methanol in the field. Slides were subsequently stained with RAPI-DIFF stain (Biostain Ready Reagents, Manchester, UK) and examined using a AmScope B120C-E1 microscope (AmScope, Irvine, CA). To determine whether infection in nestlings was associated with immune activity, we examined white blood cells (WBCs) under oil immersion at x100 magnification in order to calculate the proportions of heterophils and lymphocytes in 100 WBCs. The ratio of heterophils to lymphocytes (H:L ratio) indicates an increased stress response,

139 which can be caused by parasite infection (e.g. Figuerola *et al.* 1999; Davis *et al.*
140 2008). To determine whether infection was patent at this age, or whether we
141 were likely to be detecting sporozoites only in PCR positive birds (Valkiūnas *et*
142 *al*., 2009), we examined slides from PCR positive birds only under x40
143 magnification to confirm presence or absence of intracellular gametocytes in at
144 least 10,000 erythrocytes. Where we subsequently refer to 'infected' birds, we
145 are referring to those that tested positive through PCR, rather than through
146 microscopy.
147
148 DNA was extracted from 10 - 30μl of whole blood using a DNeasy blood and
149 tissue kit (Qiagen, Manchester, UK) according to the manufacturer's instructions.
150 Successful DNA extraction was confirmed by using a Nanodrop ND-1000
151 Spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE) and DNA was
152 diluted to a working concentration of 25 – 100 ng/ μl.
153
154 Blood parasite presence or absence was determined through PCR using four
155 primer sets targeting the cytochrome b gene region (Table 1). Primer sets were
156 chosen as part of a wider study aiming to detect co-infection in Columbids
157 (Dunn *et al.* unpubl). All PCR reactions were carried out in a 10ul reaction
158 volume containing 1 X QIAGEN Multiplex PCR buffer (containing 3mM $MgCl_2$,
159 dNTP mix and HotStarTaq DNA Polymerase; Qiagen, Manchester, UK), 0.2 μM of
160 each primer and 1 μl template DNA. A positive control of DNA from an adult bird
161 with known infection and a negative control containing deionised water in place
162 of DNA were included with each PCR reaction to ensure successful amplification
163 and lack of contamination respectively. As multiple PCR runs can produce

164 additional positives (e.g. Lachish *et al.* 2011), each negative PCR reaction was
165 repeated twice to confirm the absence of parasites; a single positive PCR was
166 interpreted as an infected bird.
167
168 The PCR protocol consisted of a denaturation step of 95°C for 15 minutes
169 followed by 35 cycles of primer-specific timings and annealing temperatures
170 (Table 1), with a terminal extension step of 72°C for 10 minutes. PCR protocols
171 were carried out on a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster
172 City, CA). PCR products were visualised on a 1% agarose gel stained with
173 SYBR® Safe (ThermoFisher Scientific, Paisley, UK). Positive samples were sent
174 for sequencing by Eurofins Genomics (Wolverhampton, UK) to confirm the
175 identity of parasites and identify lineages.
176
177 *Statistical analyses*
178 Analyses were carried out in R version 3.3.0 "Supposedly Educational" (R Core
179 Team, 2016). To test for year or species differences in parasite prevalence, we
180 constructed a binomial generalised linear mixed-effects model (GLMM) with a
181 logit link function using the *lme4* package. Fixed factors were year and host
182 species (both as categorical variables) and we designated nest ID as a random
183 effect to control for non-independence of nestmates.
184
185 To test our hypotheses, we grouped species according to nest-type (open-nesting
186 or box-nesting) and sampling age (14 days or 7 days; detailed in Table 2), testing
187 both of these as fixed predictor variables within a binomial GLMM with parasite

188 presence or absence as the response variable and random effects as described
189 above.
190
191 To determine whether infection in nestlings was associated with immune
192 activity, as represented by the ratio of heterophils to lymphocytes, we
193 constructed a general linear model (GLM) using the *stats* package (R Core Team,
194 2016), with the proportion of heterophils in 100 WBCs as the response variable
195 and assumed a quasibinomial error distribution. Predictor variables were the
196 proportion of lymphocytes in 100 WBCs, host species and parasite infection
197 status as determined by PCR.
198

199 RESULTS

200 We screened blood samples from 70 nestlings from 42 nests. These comprised
201 33 Turtle Dove nestlings from 19 nests, 29 Woodpigeon nestlings from 18 nests
202 and 8 Stock Dove nestlings from 5 nests. Parasite prevalence differed between
203 species (GLMM, χ_2^2=6.48, p=0.04), being higher in Woodpigeons at 79% than in
204 Stock Doves at 25% (z=2.36, p=0.02); Turtle Dove prevalence was 30% and did
205 not differ significantly from either of the other two species (Stock Dove: z=1.22,
206 p=0.22; Woodpigeon z=1.00, p=0.32). Parasite prevalence also differed between
207 years (GLMM, χ_2^2=6.42, p=0.04), with model predictions (to control for variation
208 in sampling effort between species across years) being highest in 2011 (74%;
209 n=29), followed by 2012 (42%; n=17) and lowest in 2013 (17%; n=24).
210
211 *Ecological predictors of prevalence*
212 Nestlings in open nests had a higher blood parasite prevalence than those in
213 boxes (GLMM, χ^2_1=7.93, p=0.005; Open-nesting: 54% infected; Box-nesting: 25%
214 infected). Nestlings sampled at 14 days old had a higher parasite prevalence than
215 those sampled at 7 days old (GLMM, χ^2_1=14.01, p<0.001; long exposure: 68%;
216 short exposure: 32%).
217
218 *Parasite infection and Immune response*
219 We examined 62 blood slides to determine WBC differentials (8 slides were
220 excluded due to poor quality smears). Intracellular gametocytes, both early stage
221 and mature, were observed in 44.1% of blood smears from PCR positive birds:
222 50% of Turtle Dove blood smears (n = 5), 50% of Stock Dove blood smears (n =
223 1) and 41% of Woodpigeon blood smears (n = 9). We found no evidence for an

224 association between infection status, as determined by PCR, and immune status
225 (GLM, F=0.62, p=0.43; infected: 0.52 ± 0.02; uninfected: 0.52 ± 0.02).
226
227 *Sequence identity*
228 We obtained 27 sequences with good quality reads, corresponding to both
229 *Haemoproteus* and *Leucocytozoon*. *Leucocytozoon* sequences were obtained from
230 14 individuals (two Turtle Doves, one Stock Dove and 11 Woodpigeons) and
231 *Haemoproteus* infections were obtained from nine individuals (two Turtle Doves,
232 two Stock Doves and five Woodpigeons). Six individuals (five Woodpigeons and
233 one Stock Dove) were infected by multiple strains. Three Woodpigeons were
234 each infected by two *Leucocytozoon* strains, one Woodpigeon and one Stock Dove
235 with both *Leucocytozoon* and *Haemoproteus*, and one Woodpigeon with two
236 *Haemoproteus* strains. We found no evidence for infection by *Plasmodium* spp.
237
238 We found 17 distinct parasite lineages within our population (Table 3). These
239 had their closest matches to 10 different strains identified through BLAST
240 searches; 6 *Haemoproteus* and 4 *Leucocytozoon.* No strain had complete coverage
241 of the partial region of cytochrome b covered by the Malavi database (Bensch *et*
242 *al.* 2009). Eleven sequences from five different lineages were a 99% match to the
243 *Leucocytozoon* strain KT779209, first detected in a Red Turtle Dove *Streptopelia*
244 *tranquebarica* from Taiwan (Huang *et al.* unpubl.). Three sequences from two
245 lineages were a closest match to the *Haemoproteus* strain AB741490 (first
246 detected in an Oriental Turtle Dove, *Streptopelia orientalis* from Japan;
247 Yashimura *et al.* unpubl). The *Leucocytozoon* strain EU627792 (initially detected
248 in a Barn Owl *Tyto alba*, from Northern California; Ishak *et al.* 2008) was a 100%

match to one lineage and a 99% match to two more. Two *Haemoproteus* strains representing three lineages and one *Leucocytozoon* strain were closest match to strains previously detected in unspecified species in Africa (KJ488710, KJ488802 and KJ488907; Drovetski *et al.* 2014) and one *Haemoproteus* strain representing two lineages and one *Leucocytozoon* strain had their closest GenBank match to a strain previously detected in an Oriental Turtle Dove in Japan (AB741491 and AB741508; Yashimura *et al.* unpubl). The remaining *Haemoproteus* sequence, representing one lineage, had its closest match to a strains isolated from a Rock Pigeon *Columba livia* from a Brazilian zoo (KU131585; Chagas *et al.* 2016).

DISCUSSION

Our results indicate high rates of haemoparasite infection in free-living Columbid nestlings. These data were used to test two hypotheses addressing key questions in avian parasite epidemiology. We found support for both of our hypotheses, suggesting that rates of haemoparasite infection at the nestling stage are high, especially for open-nesting species, and that detection of infection is more likely for species with longer nestling periods.

We found a relatively high rate of infection by haemoparasites within nestlings in our population, with an overall prevalence of 50% (62% of nests contained at least one infected nestling). Studies of nestling passerines have tended to find extremely low rates of infection: Cosgrove *et al.* (2006) found no evidence of infection by either *Plasmodium* or *Haemoproteus* in 195 fourteen-day-old nestling Blue Tits using sensitive PCR techniques, although they did find one nestling to be infected by *Leucocytozoon*. Weatherhead and Bennett (1991) found infection in only one (out of 119 examined) 10 day old Red-Winged Blackbird nestlings, although this study was prior to the use of PCR for parasite detection. More recently, Zehtindjiev *et al.* (2011) detected *Plasmodium* infection in 9.9% of 71, 5-7 day old, Skylark nestlings and Calero-Riestra and Garcia (2016) detected *Plasmodium* and *Haemoproteus* at 45% prevalence in 7-11 day old Tawny Pipits *Anthus campestris* using PCR. We found no evidence of an association between infection and an immune response, suggesting either that we were detecting infections before birds had time to elicit an immune response, or that growing nestlings may not prioritise resource allocation to immunity over growth (e.g. Hasselquist and Nilsson, 2012).

We found open-nesting Columbids to have higher rates of infection than those nesting in boxes, although our sample size for box-nesting birds was small. This is not surprising as the dipteran vectors of haemoparasites may be more likely to locate nestlings in open nests, than those in nestboxes and this may also explain the discrepancy between infection rates in box-nesting Blue Tits (Cosgrove *et al.* 2006) compared to open-nesting Skylarks and Tawny Pipits (Zehtindjiev *et al*. 2011; Calero-Riestra and García, 2016). A notable exception to this occurs in the Eurasian Roller *Coracias garrulus*, where the ectoparasitic vector *Carnus hemapterus* inhabits nest cavities and repeatedly feeds on both adult and nestling birds within a cavity, parasitising nestlings with infected parents soon after hatching (Václav *et al.* 2016).

Our finding of a higher infection rate in birds sampled at 14 days old compared to 7 days old supports the suggestion that haemoparasite infection occurs at high rates in the nest, but that the time taken for infections to reach patency combined with the limited nestling period of many species may limit detection in hosts during this life stage. In support of this for two species of open-cup ground-nesting birds with similar ecologies, Zehtindjiev *et al.* (2011) detected a relatively low prevalence (9.9%) of *Plasmodium* infection in 5-7 day old Skylark nestlings, but and Calero-Riestra and Garcia (2016) detected *Plasmodium* and *Haemoproteus* at 45% prevalence in 7-11 day old Tawny Pipits *Anthus campestris*. Studies of raptor nestlings, which can be sampled later in the developmental period than passerines, tend to find higher rates of nestling

310 infection. For example, a 100% *Leucocytozoon* infection rate was found in 23-34
311 day old Northern Goshawk *Accipiter gentilis* nestlings (Jeffries *et al.* 2015).
312
313 Examination of blood smears found that only 44% of PCR positive birds in our
314 study showed evidence of circulating intracellular gametocytes. We did not
315 sequence *Plasmodium* within our population so this result suggests that some
316 *Haemoproteus* lineages are able to reach patency in very young birds (e.g. Jeffries
317 *et al.* 2015; Václav *et al.* 2016). The presence of multiple co-infections in some
318 birds and the lack of good quality sequence for all PCR-positive birds means that
319 we cannot reliably examine genus-specific prevalence within our population.
320 However, the presence of multiple strains within some nestlings leads to the
321 question of whether some dipterans can successfully vector multiple parasite
322 strains simultaneously. In many cases we may have been detecting circulating
323 sporozoites following initial infection (Valkiūnas *et al.* 2009). Whilst there are
324 likely to be differences in the length of the prepatent period between the
325 multiple parasite lineages found in our population (e.g. Valkiūnas, 2005),
326 differences in prepatent period are unlikely to alter either our ability to detect
327 sporozoites through PCR, or our conclusions. This then leads to the question of
328 whether sporozoites from these parasite strains are able to reach patency in
329 Columbid hosts. All 7 *Haemoproteus* strains found in this study for which host
330 data was provided in GenBank (n=5 lineages; 3 GenBank strains) had previously
331 been isolated from Columbids (Chagas *et al.* 2016; Yoshimura *et al.* unpubl;) and
332 5 of these strains were also found infecting adults within our population (Dunn
333 *et al.* unpubl.). From the 10 *Leucocytozoon* lineages identified in this study for
334 which host data was provided in GenBank (n=9 lineages; 3 GenBank strains), two

had previously been isolated from Columbids (Huang *et al.* unpubl. Yoshimura *et al.* unpubl.); 5 lineages were also isolated from adult Columbids at our study sites lending support to the suggestion that these infections were likely to reach patency within nestlings in our population.

In summary, we found a high prevalence of haemoparasite infection in three species of Columbid nestling sampled at 7-14 days old. The box-nesting species (Stock Dove) had a lower parasite prevalence than open-nesting species (Turtle Dove and Woodpigeon), and within the open-nesting species we were more likely to detect parasites in 14 day old Woodpigeon nestlings compared to 7 day old Turtle Dove nestlings. We identified 17 lineages of *Haemoproteus* and *Leucocytozoon* parasites, 10 of which were also isolated from adult Columbids in our population (Dunn *et al.* unpubl.), suggesting that a high proportion of nestling infections are likely to reach patency, as opposed to being dead-end infections. Further work should focus on examining the stage of infection in a wider range of species, as well as assessing the behaviour and survival of nestlings post-fledging to determine any long-term impacts of infection in the nest.

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460 Table 1. Primer sets used in this study to screen nestling Columbids for haemoparasites. For each cycle, the primer-specific annealing

461 and extension times and temperatures are shown. HMRf is the reverse complement of HMRr from Merino *et al.* (2008)

Primer set	Primer sequence (5' – 3')	Annealing	Extension
L15368 (Fallon *et al.* 2003)	AAAAATACCCTTCTATCCAAATCT	50°C/ 60 s	72°C/ 90 s
H15730 (Fallon *et al.* 2003)	CATCCAATCCATAATAAAGCAT		
HMRf	GGTAGCTCTAATCCTTTAGG	52°C/ 60 s	72°C/ 90 s
H15730 (Fallon *et al.* 2003)	CATCCAATCCATAATAAAGCAT		
Leunew1F (Quillfeldt *et al.* 2014)	GGWCAAATGAGTTTCTGGG	56°C/ 30 s	72°C/ 60 s
LDRd (Merino *et al.* 2008)	CTGGATGWGATAATGGWGCA		
3760f (Beadell *et al.* 2004)	GAGTGGATGGTGTTTTAGAT	59°C/ 90 s	72°C/ 90 s
HMRr (Merino *et al.* 2008)	CCTAAAGGATTAGAGCTACC		

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Table 2. Number of samples analysed, split by species and year.

Species	2011	2012	2013	Nest type	Age of sampling
Stock dove	3	5	0	Box	14 days
Turtle dove	7	3	24	Open	7 days
Woodpigeon	19	10	0	Open	14 days

Table 3. Summary table of lineages identified in this study along with host species (TD: Turtle Dove; WP: Woodpigeon; SD: Stock Dove), the closest matching strain on GenBank, % coverage, % identity and the number of nestlings within which the lineage was found. * indicates a lineage also found in adults within our study area. Assignment of lineage names within our study is non-consecutive as our overall study includes adults, data for which will be reported elsewhere (Dunn *et al.* unpubl.).

Lineage (this study)	**Parasite species**	**Host species**	**Sequence length (bp)**	**GenBank Match**	**% overlap**	**% identity**	**Number of nestlings**	**Citation**	**GenBank Accession Number**
A*	Leucocytozoon	WP	506	EU627792	99	99	1	Ishak *et al.* 2008	KX832555
B*	Leucocytozoon	SD, WP	340	KT779209	100	99	1	Huang *et al.* unpubl.	KX832556
C*	Leucocytozoon	TD, WP	352	KT779209	100	99	5	Huang *et al.* unpubl.	KX832557
D*	Leucocytozoon	WP	549	KT779209	100	99	3	Huang *et al.* unpubl.	KX832558
E*	Leucocytozoon	WP	618	KT779209	100	99	2	Huang *et al.* unpubl.	KX832559
K	Leucocytozoon	WP	395	KT779209	100	97	1	Huang *et al.* unpubl.	KX832565
L	Leucocytozoon	WP	506	EU627792	99	99	1	Ishak *et al.* 2008	KX832566

M*	Haemoproteus	SD	807	KJ488802	99	100	1	Drovetski *et al.* 2014	KX832567
S	Leucocytozoon	TD	383	EU627792	100	100	1	Ishak *et al.* 2008	KX832573
W*	Haemoproteus	WP	794	KU131585	98	97	2	Chagas *et al.* 2016	KX832577
AA*	Haemoproteus	WP	666	KJ488710	100	99	1	Drovetski *et al.* 2014	KX832581
AH	Leucocytozoon	WP	395	KJ488907	99	97	1	Drovetski *et al.* 2014	KX832588
AI	Haemoproteus	WP	395	AB741491	100	98	1	Yashimura *et al.* unpubl	KX832589
AM	Haemoproteus	SD	395	AB741491	100	94	1	Yashimura *et al.* unpubl	KX832593
AR	Leucocytozoon	SD	339	AB741508	99	92	1	Yashimura *et al.* unpubl	KX832598
BB*	Haemoproteus	TD, WP	419	AB741490	100	99	2	Yashimura *et al.* unpubl	KX832608
BH*	Haemoproteus	TD, WP	384	AB741490	100	99	2	Yashimura *et al.* unpubl	KX832614

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Appendix 1

a) Full model results from a GLMM testing whether nest type or age of sampling influence the likelihood of infection by blood parasites. Results presented for each term are Estimate, standard error (SE), degrees of freedom (df), χ^2 statistic and p value. F statistics and p values are calculated for each variable (excluding the intercept) by comparing models with and without each term. For factors, Estimates are presented for the level in brackets in the Variable column, relative to the reference level. Nest ID is designated as a random effect (Variance: 0.282, Standard deviation: 0.53)

Variable	Estimate	SE	df	χ^2	p
Intercept	-1.192	0.093			
Nest type (open)	2.630	1.164	1	7.930	0.005
Age of sampling (7 days)	-2.333	0.800	1	14.010	<0.001

b) Full results from a GLM testing whether the presence of blood parasites in nestling columbids is associated with immune performance (heterophil: lymphocyte ratio). Results presented for each term are Estimate, standard error (SE), degrees of freedom (df), F statistic and p value. F statistics and p values are calculated for each variable (excluding the intercept) by comparing models with and without each term. For factors, Estimates are presented for the level in brackets in the Variable column, relative to the reference level.

Variable	Estimate	SE	df	F	p
Intercept	1.99	0.07			
Lymphocytes	-4.28	0.14	1	960.14	<0.001
Species (Turtle Dove)	-0.02	0.05	2	0.47	0.63
Infection status (positive)	0.03	0.03	1	0.62	0.434