Blood parasites in Passeriformes in central Germany: Prevalence and lineage diversity of Haemosporida (*Haemoproteus*, *Plasmodium* and *Leucocytozoon*) in six common songbirds

Yvonne R. Schumm [Corresp., 1] , **Christine Wecker** [1] , **Carina Marek** [1] , **Mareike Wassmuth** [1] , **Anna Bentele** [1] , **Hermann Willems** [2] , **Gerald Reiner** [2] , **Petra Quillfeldt** [1]

[1] Department of Animal Ecology & Systematics, Justus Liebig University, Giessen, Germany

[2] Department of Clinical Veterinary Sciences, Justus Liebig University, Giessen, Germany

Corresponding Author: Yvonne R. Schumm
Email address: Yvonne.R.Schumm@bio.uni-giessen.de

Background: Avian Haemosporida are vector-borne parasites that commonly infect Passeriformes. Molecular analyses revealed a high number of different lineages and lineage specific traits like prevalence and host-specificity, but knowledge of parasite prevalence and lineage diversity in wild birds in Central Germany is still lacking.

Results: Blood samples from a total of 238 adult and 122 nestling songbirds belonging to six species were investigated for infections with avian haemosporidian genera and lineages (*Haemoproteus* spp., *Plasmodium* spp., *Leucocytozoon* spp.) and *Trypanosoma avium* using PCR, targeting the parasite mitochondrial cytochrome b gene and 18S ribosomal RNA. In total, the prevalence in adult birds was 31.3% infected with *Haemoproteus*, 12.5% with *Plasmodium* and 71.0% with *Leucocytozoon* (nestlings excluded). None of the tested birds was infected with *Trypanosoma avium*. Only in two nestling birds, aged 12-17 days, a *Leucocytozoon* spp. infection was proven. Among 225 successfully sequenced samples, we found four *Haemoproteus*, three *Plasmodium* and 19 *Leucocytozoon* lineages, including two new *Leucocytozoon* lineages. Furthermore, we report two new host-lineage associations.

Conclusions: As first study investigating avian haemosporidian parasites in Central Germany, we provide new information on genetic diversity of Haemosporida infecting Passeriformes. We show that even with a small sample size new lineages as well as previously unknown linkages between certain lineages and host species can be detected. This may help to elucidate the diversity of lineages as well as lineage-host-connections of avian Haemosporida.

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4   **Authors**: Yvonne R. Schumm[1*], Christine Wecker[1], Carina Marek[1], Mareike Wassmuth[1], Anna

5   Bentele[1], Hermann Willems[2], Gerald Reiner[2] & Petra Quillfeldt[1]

6   [1] Department of Animal Ecology & Systematics, Justus Liebig University, Heinrich-Buff-Ring 26-

7   32, 35392 Giessen, Germany.

8   [2] Department of Clinical Veterinary Sciences, Justus Liebig University, Frankfurter Strasse 112,

9   35392 Giessen, Germany.

10 * Corresponding author, Email address: Yvonne.R.Schumm@bio.uni-giessen.de

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13 Passeriformes. Molecular analyses revealed a high number of different lineages and lineage

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17 species were investigated for infections with avian haemosporidian genera and lineages

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20 prevalence in adult birds was 31.3% infected with *Haemoproteus*, 12.5% with *Plasmodium* and

21 71.0% with *Leucocytozoon* (nestlings excluded). None of the tested birds was infected with

22 *Trypanosoma avium*. Only in two nestling birds, aged 12-17 days, a *Leucocytozoon* spp.

23 infection was proven. Among 225 successfully sequenced samples, we found four

24 *Haemoproteus*, three *Plasmodium* and 19 *Leucocytozoon* lineages, including two new

25 *Leucocytozoon* lineages. Furthermore, we report two new host-lineage associations.

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30   diversity of lineages as well as lineage-host-connections of avian Haemosporida.

Introduction

Avian blood parasites (Hematozoa) infect both domestic and wild birds, and therefore they have been objects of intensive scientific research over a long period (Valkiūnas, 1996; Valkiūnas, 2005; Bensch et al., 2013). Among the most common blood parasite orders are the Haemosporida. These include among others the avian malaria-like genera *Haemoproteus* and *Leucocytozoon* as well as the pathogen of avian malaria *Plasmodium*. These avian Haemosporida belonging to the phylum Apicomplexa share a similar but complex life cycle (Schmid et al., 2017), including asexual stages of reproduction in a bird host and sexual stages within a vector (Valkiūnas, 2005; Santiago-Alarcon et al., 2012). Another common blood parasite is *Trypanosoma avium*, flagellate protozoans living in the bloodstream of birds (Hamilton et al., 2007). Hematophagous dipterans are the vectors of these parasites. *Leucocytozoon* parasites are vectored by blood-sucking dipterans of two families: black flies (Simuliidae) and biting midges (Ceratopogonidae) (Freund et al., 2016; Lotta et al., 2016). Ceratopogonid midges are also vectors for *Haemoproteus* spp*.* (Desser & Bennett, 1993). The main vectors for *Plasmodium* spp*.* are several species of mosquitos (Culicidae) (Medeiros et al., 2013). The transmission for *T. avium* remains still unclear with various blood-sucking insects mentioned as possible vectors (Votypka et al., 2002). Haemosporida are generally considered as pathogens with a low pathogenicity and harmless in bird populations (Wiersch et al., 2007; Ciloglu et al., 2016), but several studies demonstrated different costs on life-history traits associated with Haemosporida infections: Haemosporidian parasites can affect the body condition (Valkiūnas et al., 2006), reproductive success (e.g. Hunter et al., 1997; Merino et al., 2000; Marzal et al., 2005; Tomás et al., 2007a; Knowles et al., 2010) and the survival (e.g. Dawson & Bortolotti, 2000; Møller & Nielsen, 2007; Donovan et al., 2008; Bueno et al., 2010).

Haemosporida are abundant in many avian families and occur worldwide except in Antarctica (Valkiūnas, 2005; Bensch et al., 2009; Clark et al., 2014; Vanstreels et al., 2014). However, there are interspecific differences in the parasite prevalence (e.g. Bennett et al., 1993a, b; Valkiūnas, 2005) and in some orders, e.g. Passeriformes, the parasites are more abundant (Valkiūnas, 2005). On the basis of recent molecular studies, the diversity of Haemosporida species and lineages may be assessed more precisely. The species diversity

60   seems to be as high as the diversity of avian species (Bensch et al., 2004) or even higher

61   (Schmid et al., 2017) and thousands of lineages may exist (Szöllősi et al., 2011). Lineages can

62   differ from each other in only one single nucleotide (e.g. one substitution) of the mitochondrial

63   cytochrome b gene (Bensch et al., 2004; Hellgren et al., 2004; Bensch et al., 2009; Chagas et al.,

64   2017). For the order Passeriformes alone, 912 *Haemoproteus*, 852 *Plasmodium* and 600

65   *Leucocytozoon* lineages are deposited in the avian haemosporidian parasite database MalAvi

66   (Bensch et al. 2009, MalAvi database accessed August 2018). In addition, molecular surveys

67   vastly improved our understanding about the host specificity of avian haemosporidian

68   infections (Ciloglu et al., 2016). The Haemosporida occupy niches that vary from extreme host

69   generalization to extreme host specialization (Okanga et al., 2014). Generally, *Plasmodium* spp.

70   is known to be more host-generalized (e.g. Waldenström et al., 2002; Križanauskienė et al.,

71   2006; Dimitrov et al., 2010; Mata et al., 2015), whereas species of *Leucocytozoon* as well as

72   *Haemoproteus* are considered to be more host-specific (e.g. Waldenström et al., 2002; Beadell

73   et al., 2004; Forrester & Greiner, 2008; Dimitrov et al., 2010; Jenkins & Owens, 2011).

74   *Trypanosoma avium* seems to possess a relatively low host-specificity (Bennett et al., 1994;

75   Sehgal et al., 2001).

76   Furthermore, prevalence varies not merely interspecific but also with the age of the birds. A

77   common pattern observed in host-parasite assemblages is a higher abundance of parasites in

78   juvenile compared to adult birds (Sol et al. 2003). However, for blue tits nesting in nest boxes,

79   blood parasites are far less prevalent in nestlings than in adult birds (Cosgrove et al., 2006;

80   Martinez-de la Puente et al., 2013). Possibly because box-nesting species may be shielded from

81   vector exposure due to their enclosed surroundings (Dunn et al., 2017).

82   The aims of the present study were (i) to assess the prevalence of the Haemosporida:

83   *Plasmodium*, *Haemoproteus* and *Leucocytozoon* as well as *T. avium* in six species of wild

84   Passeriformes in Central Germany, (ii) to identify and compare the lineage diversity among the

85   birds and to record interspecific-shared lineages by means of mitochondrial cytochrome b

86   sequencing as well as (iii) to compare the prevalence in adult and nestling birds.

87   **Material & Methods**

88   Origin and preparation of the samples

89   Bird capture and sampling were carried out under license (Animal welfare officer of the

90   University of Giessen, no. 662_GP and 828_GP, and the Regierungspräsidium Giessen, no. 109-

91   2012 and 77-2016) in accordance with the German legislation. Blood samples from 360

92   Passeriformes of four families and six species (blue tit *Cyanistes caeruleus*, Paridae; great tit

93   *Parus major*, Paridae; coal tit *Periparus ater*, Paridae; eurasian tree sparrow *Passer montanus*,

94   Passeridae; european pied flycatcher *Ficedula hypoleuca*, Muscicapidae and eurasian nuthatch

95   *Sitta europaea*, Sittidae) were collected during April to June in the years 2015, 2017 and 2018

96   (Table 1).

97   Sample sites were located in and closely around the city Giessen (50° 35' 2.584" N

98   8° 40' 42.251" E, Hesse, central Germany). All birds were captured at their nest boxes by hand

99   and blood-sampled by brachial venipuncture. The blood was stored on Whatman FTA classic

100   cards (Whatman®, UK). For DNA isolation a 3 x 3 mm piece of each sample was cut out of the

101   FTA card with a sterile scalpel blade. Subsequently the DNA was extracted according to the

102   ammonium-acetate protocol by Martinez et al. (2009) and purified with NZYspintech-columns

103   (NZYTech, Lda.-Genes & Enzymes, Portugal) or Zymo-Spin™ IIC columns (Zymo Research, USA).

104   The presence and concentration of DNA were confirmed and determined with NanoDrop2000c

105   UV-Vis Spectrophotometer (NanoDrop Technologies, USA). If the DNA concentration was higher

106   than 80 ng/µl, samples were diluted to 30 ng/µl.

107   Parasite screening

108   A partial amplification of the mitochondrial cytochrome b gene of the different Haemosporida

109   was accomplished by polymerase chain reaction (PCR) with the respective primers (Table 2).

110   For the detection of *Haemoproteus*/*Plasmodium* species the primer pair HaemF (5'-

111   ATGGTGCTTTCGATATATGCATG -3') and HaemR2 (5'- GCATTATCTGGATGTGATAATGGT -3')

112   (Bensch et al., 2000) was used. For *Leucocytozoon* detection we applied primer pair HaemFL (5'-

113   ATGGTGTTTTAGATACTTACATT -3') and HaemNR3 (5'- ATAGAAAGATAAGAAATACCATTC -3')

114   (Hellgren et al., 2004). To check *T. avium* infections the accomplished primer pair was TryF (5'-

115   ATGCACTAGGCACCGTCG -3') and TryR (5'- GGAGAGGGAGCCTGAGAAATA -3') (Martinez-de la

116   Puente et al., 2013) targeting the 18S ribosomal RNA (Table 2).

117   Nestlings were only checked for possible *Leucocytozoon* spp. and *T. avium* infections.

118   The PCR reactions with HaemF and HaemR2 consisted of 20 µl reaction volumes containing 2 µl

119   DNA template (14.2 – 80 ng/µl), 1.2 µl of each primer (10 µM), 10 µl of InnuMix PCR Master Mix

120   (2x, Analytik Jena AG, Germany) and 5.6 µl nuclease-free water. The PCR reaction for the

121   detection of *Leucocytozoon* spp. contained 2.5 µl DNA template (8.0 - 80 ng/µl), 0.6 µl of each

122   primer (20 µM), 10.6 µl InnuMix PCR Master Mix and 5.7 µl nuclease-free water. For *T. avium*

123   evidence the PCR reaction volume was 20 µl consisting of 2.5 µl DNA template, 0.6 µl of each

124   primer (20 µM), 10.6 µl DreamTaq PCR Master Mix (Thermo Fisher Scientific, Germany) and

125   5.7 µl nuclease-free water. The PCR reaction parameters using thermal cyclers peqSTAR 96Q

126   (Peqlab, Germany) and Tone (Biometra, Germany) are given in Table 2. A positive as well as a

127   negative control were included in each run to ensure PCR was working properly. PCR amplicons

128   were visualized using QIAxcel Advanced (Qiagen, Switzerland) high-resolution capillary gel

129   electrophoresis. Samples rendering a clear peak during gel electrophoresis were Sanger

130   sequenced bi-directional by Microsynth-Seqlab (Sequence Laboratories Goettingen GmbH,

131   Germany).

132   Phylogenetic and statistical analyses

133   The forward and reverse sequences were assembled and trimmed in CLC Main Workbench

134   7.6.4 (CLC Bio, Qiagen, Denmark). PCR and sequencing were repeated, if not all nucleotides of a

135   sequence could be determined unambiguously. Sequences were excluded from network

136   construction when repetitions did not improve sequence quality.

137   The consensus sequences were assigned to a parasite lineage by BLAST (BLASTN

138   2.3.0+, Zhang, 2000) using the database MalAvi (Bensch et al., 2009). Constructions of lineage

139   networks for each Haemosporida genus, using the median-joining network method, were

140   performed with PopART 1.7 (Bandelt et al., 1999; Leigh & Bryant, 2015) after aligning the

141   sequences in BioEdit v7.2.5 (Hall, 1999). For these alignments, we used 123 *Leucocytozoon*

142   (478 bp), 39 *Haemoproteus* (463 bp) and 14 *Plasmodium* consensus sequences (440 bp).

143   Statistical evaluation of comparing prevalences and number of interspecific shared

144   lineages per genus was performed with R (R Development Core Team 2013) using the R

145   Package R commander. To compare the equality of proportions of parameters mentioned

146   above the frequency distribution test Pearson`s Chi^2-test was applied. A significance level of

147    $p < 0.05$ was used.

148   **Results**

149   Blood parasite prevalence

150   We detected three Haemosporida parasite genera in the local bird population in and around

151   Giessen. The overall prevalence was 31.3% for *Haemoproteus*, 12.5% for *Plasmodium* and

152   71.0% for *Leucocytozoon* (nestlings excluded) (Table 3). Only blue tits and great tits were

153   infected with all three genera. Coal tits were infected with *Plasmodium* and *Leucocytozoon* and

154   tree sparrows with *Plasmodium* and *Haemoproteus*. Nuthatches showed infections with

155   *Haemoproteus* and *Leucocytozoon*. For pied flycatchers we only found evidence of

156   *Haemoproteus* infections (Table 3).

157   Prevalence rates of *Leucocytozoon* spp. were highest in blue tits (94.8%). The highest

158   prevalence of *Plasmodium* infections occurred in coal tits (25.0%). *Haemoproteus* was almost

159   equally common in blue tits (36.2%) and great tits (40.6%) (Table 3). The prevalence for the

160   three haemosporidian genera in adult birds was different between the genera (Pearson`s Chi²-

161   test: $\chi^2 = 129.1$, df = 2, $p < 0.001$). Comparing the prevalence of *Haemoproteus* and

162   *Plasmodium*, *Haemoproteus* spp. was more prevalent significant (Pearson`s Chi²-test: $\chi^2 = 13.2$,

163   df = 1, $p < 0.001$), and *Leucocytozoon* was significantly more prevalent than the other two

164   genera (Pearson`s Chi²-test: *Haemoproteus*/*Leucocytozoon*: $\chi^2 = 53.7$, df = 1, $p < 0.001$;

165   *Plasmodium*/*Leucocytozoon*: $\chi^2 = 114.0$, df = 1, $p < 0.001$). For none of the tested birds a *T.*

166   *avium* infection was detected. In blue tit nestlings a haemosporidian infection could not be

167   detected by PCR. In great tit nestlings we proved an infection with *Leucocytozoon*, although the

168   prevalence with 3.5% tits infected (2 out of 57 nestlings) was low.

169   Lineage diversity

170   In total, 26 haemosporidian lineages could be identified in the MalAvi database. We found four

171   *Haemoproteus* spp., three *Plasmodium* spp. and 19 *Leucocytozoon* spp*.* lineages (Table 4). Great

172   and blue tits showed similar high lineage diversities (n = 15 lineages: 2 *Haemoproteus*, 1

173   *Plasmodium* and 12 *Leucocytozoon* lineages; n = 17: 1 *Haemoproteus*, 3 *Plasmodium* and 13

174   *Leucocytozoon* lineages, respectively). We identified two new *Leucocytozoon* lineages

175   (CYACAE02, GenBank accession number MH758695 and CYACAE03, MH758696) infecting blue

176   tits which differ each in 0.21% (1 nucleotide) from their closest MalAvi match. For new lineages

177   PCR and sequencing were performed twice to verify the results.

178   The lineage diversity of the other four species was less pronounced probably due to

179   smaller sample sizes. For pied flycatchers we detected one *Haemoproteus* lineage (PFC1). For

180   tree sparrows we found three lineages (1 *Haemoproteus*: PADOM03 and 2 *Plasmodium*: GRW11

181   and SGS1), for coal tits one lineage each for *Plasmodium* (TURDUS1) and *Leucocytozoon*

182   (PARUS19) and for nuthatches one *Haemoproteus* (PARUS1*)* and two *Leucocytozoon* lineages

183   (PARUS7 and PARUS20).

184   Several sequences (n= 11) could not be assigned to a single reference sequence due to

185   an insufficient sequence length or quality. In these cases, the sequences were determined to

186   the closest related reference lineages (Table S1).

187   Host specificity

188   Maximally three out of six Passeriformes species were found infected with the same lineage

189   (Table 4). We recorded the highest number of lineages occurring in several host species for

190   *Plasmodium*. According to the BLAST results, all identified *Plasmodium* lineages occurred in

191   more than one species (Table 4). In contrast, smaller percentages of lineages infecting more

192   than one host species, were found for *Leucocytozoon* (36.8% of the lineages infected two host

193   species and only 5.3% (one lineage: PARUS19) infected three host species) and *Haemoproteus*

194   with 25% (only one lineage (PARUS1) infected more than one host species) (Table 4). The

195   difference in the percentage of interspecific shared lineages was not significant between the

196   three Haemosporida genera (Pearson`s Chi²-test: χ^2 = 6.82, df = 4, p = 0.15).

197   Lineage Networks

198   The networks for each haemosporidian genus revealed the occurrence of four lineages for

199   *Haemoproteus* (Fig. 1), three for *Plasmodium* (Fig. 2) and 18 for *Leucocytozoon* (Fig. 3). In the

200   *Leucocytozoon* network sequences from the samples NK17_P01 (*C. caeruleus*) and NK17_Y40

201 (*C. caeruleus*) occurred each separately from all the other sequences and had a BLAST of

202 maximum 99% match with lineages deposit in MalAvi, indicating a different, new lineage

203 (named CYACAE02 and CYACAE03). The genetic divergence to the closest MalAvi match of

204 CYACAE02 (sample NK17_P01) is 0.21% (1 nucleotide difference to PARUS22, HM234031). The

205 difference of CYACAE03 (sample NK17_Y40) to its closest related match PARUS19 (99% match

206 in 477 bp) is 0.21%. The genetic divergence for the *Leucocytozoon* lineages in the network was

207 0.21 – 7.95%. All found lineages of the dataset are separated at least by one mutation

208 (equivalent to one hatch mark in the network). With the exception of PARUS19 and PARUS74

209 (Fig. 3). The mutation differentiating these lineages is located in the cytochrome b gene before

210 the fragment we used for network construction.

211 The *Haemoproteus* network (Fig. 1) shows that the lineage PFC1 (*H. pallidus*), occurring in all

212 three positively tested pied flycatchers, and the lineage PADOM03, found in one tree sparrow,

213 are clearly separated from the other *Haemoproteus* spp. lineages. *Haemoproteus* lineages had a

214 genetic divergence of 1.08 – 5.18%. The *Plasmodium* network (Fig. 2) illustrates the three

215 lineages (SGS1, GRW11 and TURDUS1) found in this study. The results of this network lead to

216 the assumption that TURDUS1 exclusively infects coal tits. However, it must be noted that also

217 a blue tit was infected with TURDUS1, but the sample sequence was too short to be used for

218 the alignment. *Plasmodium* lineages in the network ranged from 0.23 – 4.32% in their genetic

219 divergence.

220 **Discussion**

221 Prevalence in nestling birds

222 Contrary to a high percentage of adult blue and great tits infected with at least one

223 haemosporidian parasite we did not detect a single haemosporidian or *T. avium* infection in

224 nestling blue tits and a very low infection rate of *Leucocytozoon* spp. in great tit nestlings.

225 Generally, nestlings should be highly susceptible to vector-borne infection diseases due to their

226 confinement to the nest, nakedness and immunological naivety during nestling period (Baker,

227 1975). Possibly, more nestlings might have been infected in our study, but our methodology did

228 not accomplish detection if the disease was still at the prepatent stage. Infections cannot be

229   detected immediately after transmission because of the prepatency period (i.e. the period

230   between initial infection and the release of gametocytes into the peripheral blood). We can

231   distinguish between parasites with shorter prepatency periods like *Leucocytozoon* (5 to 6 days)

232   (Desser & Bennett, 1993) or *T. avium* (24 to 48 hours) (Bennett, 1970), and with longer

233   prepatency periods like *Haemoproteus* or *Plasmodium*. The period for *Haemoproteus* spp.

234   varies from 11 to 21 days, for *Plasmodium* it can last from few days up to more than one month

235   until an infection is detectable in the peripheral blood, depending on host and parasite species

236   (Valkiūnas, 2005; Cosgrove et al., 2006). Alternatively, it could simply mean that the nestlings

237   had not been infected yet.

238   To test if an infection was transmitted already, it would have been necessary to remove

239   nestlings from their nests, raising them in vector-free cages and checking regularly for

240   subsequent development of patent infections. Valkiūnas (2005) applied this approach in

241   chaffinch (*Fringilla coelebs*) nestlings. In his study, only two out of 67 chicks (3%), removed from

242   the nest at 6 to 12 days of age, subsequently developed infections. Contrary, the infection rate

243   of 25 to 50 day old wild fledglings was 36.2%, suggesting that most infections occurred after the

244   nestlings had left the nest (Valkiūnas, 2005). The very low rate of infections in nestling tits in

245   our study might also result from a lack of vector activity during the nestling period as in

246   northern temperature climes dipteran vector populations reach their peak not until late

247   summer (Beaudoin et al., 1971).

248   Prevalence in adult birds

249   Within Germany, only a few studies deal with the prevalence and distribution of infections with

250   avian haemosporidian parasites (microscopic examination: Haberkorn, 1984; Krone et al., 2001;

251   PCR-based methods: Wiersch et al., 2007; Jenkins & Owens, 2011; Santiago-Alarcon et al.,

252   2016) (see Table 5 for an overview of prevalences in great and blue tits sampled in Germany).

253   Wiersch et al. (2007) sampled birds in the northern part of Germany. Infection prevalences in

254   great tits were 30.4% for *Haemoproteus* and 46.4% for *Plasmodium*. In line with the present

255   study, coal tits had no infections with *Haemoproteus* spp. and a similar infection rate with

256   *Plasmodium* spp. (18.7%). The infection rate for pied flycatchers with *Haemoproteus* spp. (0.9%)

257 was less than in the present study (21.4%). In contrast to our findings, Wiersch et al. (2007)

258 reported *Plasmodium* infections in pied flycatchers (5.9%). Santiago-Alarcon et al. (2016) found

259 no haemosporidian infection in eurasian nuthatches in Germany (20.0% *Haemoproteus* spp.

260 and 30.0% *Leucocytozoon* spp. in the present study) and Haberkorn (1984) found no

261 haemosporidian infections in coal tits (25.0% *Plasmodium* spp. and 25.0% *Leucocytozoon* spp. in

262 this study). As far as we know, no other data are available for european tree sparrow infection

263 rates in Germany.

264 In contrast to our results, low infection rates in blood smears from great tits were reported by

265 Haberkorn (1984) (*Haemoproteus* spp.: 7.3%, *Leucocytozoon* spp.: 1.2%). These variations in

266 prevalences might be based on methodological differences.

267 Jenkins & Owens (2011) recorded 33% *Leucocytozoon* prevalence in south Germany for great

268 tits and 50% for blue tits, which is much lower than the prevalences reported here for the two

269 species (77.3% and 94.8%, respectively). However, some studies report even higher prevalences

270 especially for the avian malaria pathogen *Plasmodium* (e.g. 91% for blue tits from Switzerland,

271 Glaizot et al., 2012; 100% in a blue tits population in England, Szöllősi et al., 2011) and for *T.*

272 *avium* (e.g. 49% of infected blue tits in Spain, Fargallo & Merino, 2004; 40% in female blue tits

273 in Spain, Tomás et al., 2007b).

274 However, it should be considered that sensitive PCR-based diagnostics are able to detect

275 sporozoites of *Leucocytozoon* in the peripheral blood (Valkiūnas et al., 2009). Sporozoites are

276 transmitted to the bird during the blood meal of the vector fly. As it is unclear whether all of

277 these sporozoites result in an actual infection of the host, the evidence of haemosporidian

278 lineages by PCR based method does not necessarily allow the conclusion that the parasites

279 complete their entire life cycle in the host (Valkiūnas et al., 2009).

280 The high prevalence of *Leucocytozoon* spp. in blue and great tits in this study compared

281 to the parasite genera *Plasmodium* and *Haemoproteus* may be associated with the vector

282 abundance and behavior. Dipteran vectors of some Haemosporida genera may be more strictly

283 ornithophilic than Culicidae vectors of *Plasmodium* spp., which feed on a broader range of

284 vertebrates, reducing their potential for transmitting diseases to birds (Savage et al., 2009). The

285 reasons for the high *Leucocytozoon* prevalence in our study area Hesse compared to other parts

286   of Germany are speculative. One reason might be that Hesse is the most richly forested of all

287   German states (42% of the state area are forests) (Forest report Hesse, 2015). Moreover, these

288   forest sites are mostly near-natural and with a lot of woodland-running-waters, that are mostly

289   (70%) in a good ecological condition (Forest report Hesse, 2015). As Simuliidae, the vectors of

290   all *Leucocytozoon* species (except *L. caulleryi*, that is vectored by a biting midge; Lotta et al.,

291   2016), need running waters for reproduction (Lacey & Merrit, 2003) the conditions for their

292   reproduction in Hesse are good according to the habitat parameters mentioned above.

293   However, we did not record any habitat parameters during our study and similarly no

294   nationwide data throughout Germany on black fly distribution is available at the moment.

295   Therefore, it is not possible to test regional prevalence of *Leucocytozoon* depending on the

296   distribution and density of black flies so far.

297   Unfortunately, many studies do not consider all three avian haemosporidian genera and

298   especially *Leucocytozoon* is underrepresented in the literature (van Rooyen et al., 2013a).

299   Hence, a detailed comparison of prevalences in different avian hosts is difficult to assess.

300   However, comparison of our results with studies from Germany and Europe show that the

301   prevalences of avian haemosporidian infections differ among local bird populations (e.g.

302   Haberkorn, 1984; Wiersch et al., 2007; Santiago-Alarcon et al., 2016). The factors causing these

303   local differences are still poorly understood. Szöllősi et al. (2011) showed that the distribution

304   and prevalence of avian malarial parasite species are influenced by multiple factors, such as

305   host and dipteran vector density, habitat characteristics or climatic conditions (see also Wood

306   et al., 2007; Merino et al., 2008). Moreover, prevalence seems to be a lineage-specific trait

307   (Szöllősi et al., 2011). That shows the importance to investigate not only prevalence for the

308   parasite genera, but rather identify the different parasite lineages infecting regional bird

309   populations.

310   Lineage diversity and host specificity

311   By using molecular phylogeny, we detected different lineages for each parasite genus. Few

312   lineages differed by only one nucleotide, resulting in low genetic divergences. Other authors

313   (e.g. Bensch et al., 2000; Chagas et al., 2017) also found low sequence divergences in

314   Haemosporida. In this study, the two new *Leucocytozoon* lineages (CYACAE02 and CYACAE03)

315   differ in one nucleotide each from their closest matching lineage, indicating they may have

316   diverged only recently (Bensch et al., 2000).

317   The lineage PFC1 (*H. pallidus*), infecting only pied flycatchers in this study, is clearly

318   separated in the phylogenetic tree as well as in the network. It is possible that the infection

319   with this lineage was transmitted outside Germany, as pied flycatchers, wintering in Africa, are

320   the only long-distance migratory bird species in our study and infection with the PFC1 lineage

321   might be vectored from dipteran vectors in Africa. However, the distribution of vectors on

322   wintering and breeding grounds, especially for *Haemoproteus*, is poorly understood (Dubiec et

323   al., 2017) and transmission of PFC1 lineage possibly also occurs in Europe (Jones, 2017).

324   Generally, host specificity of haemosporidian lineages differs among the genera with

325   host specialists being predominant among *Haemoproteus* and *Leucocytozoon* lineages but

326   being absent among *Plasmodium* lineages (Mata et al., 2015). This general pattern of host

327   specificity, with *Haemoproteus* being the most host specialized and *Plasmodium* being more

328   host generalized, is supported by several studies (e.g. Ricklefs & Fallon, 2002; Waldenström et

329   al., 2002; Križanauskienė et al., 2006; Dimitrov et al., 2010; Jenkins & Owens, 2011; Drovetski et

330   al., 2014; Okanga et al., 2014; Mata et al., 2015; Ciloglu et al., 2016). Less studies regarding host

331   specificity of *Leucocytozoon* lineages exist, therefore it is necessary to do further investigations

332   to confirm *Leucocytozoon* lineages to be, as assumed, host-specific mostly at avian order level

333   and in some cases even on species level (Forrester & Greiner, 2008; Ciloglu et al., 2016). In the

334   present study, we found no significant difference in the host specificity of the three

335   haemosporidian genera. But due to small sample sizes and closely related host species (all

336   within the order Passeriformes) general patterns should be proven with a higher number of

337   samples and an increased range of species.

338   Our data complements existing knowledge about host specificity and distribution of

339   some individual lineages as we obtained first records for lineages infecting a specific host

340   species. The two *Leucocytozoon* lineages PARUS20 (isolated from sample NK17_C05,

341   MH758693) and PARUS7 (isolated from samples NK17_C04, MH758692 and NK17_C14,

342   MH758694) were not detected in eurasian nuthatches (*S. europaea*) prior to this study (Bensch

343 et al. 2009, MalAvi database accessed August 2018). This is also the first record of the PARUS7

344 lineage in the family Sittidae. Host specificity seems not to be determined by parasite genera

345 but by the single lineages comparable with the lineage-specific prevalences. For example,

346 several studies (e.g. Ricklefs & Fallon, 2002; Beadell et al., 2009; Loiseau et al., 2012) suggest

347 that few lineages of avian malaria pathogen *Plasmodium* exhibit extreme generalization,

348 whereas other lineages seem to be constrained to certain host families or even host species.

349 Host shifts are often associated with a change in pathogen virulence (Toft & Karter, 1990).

350 Therefore, invading a new host may increase or decrease parasite virulence (Bull, 1994). This

351 might be the case for the two *Leucocytozoon* lineages (PARUS20 and PARUS7) infecting

352 nuthatches.

353 **Conclusion**

354 In summary, we found avian malaria and avian malaria-like pathogens of three genera

355 (*Plasmodium, Haemoproteus* and *Leucocytozoon*) infecting common Passeriformes in Central

356 Germany. The findings presented here provide knowledge about the distribution and

357 prevalence of avian haemosporidian parasites in a geographic region, which has not yet been

358 subject to studies investigating this kind of parasites. On the basis of a relatively small sample

359 size we found numerous lineages and detected several first records of lineage infections as well

360 as two new *Leucocytozoon* lineages. Comparison with studies from other parts of Germany

361 pointed out regional differences in Haemosporida prevalence, in particular for *Leucocytozoon*.

362 Understanding these patterns resulting in regional differences could be important in future to

363 understand the epidemiology of blood parasites in wild bird populations.

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370   **References**

371   Baker JR. 1975. Epizootiology of some haematozoic protozoa of English birds. Journal of Natural
372   History 9:601-609.
373
374   Bandelt H, Forster P, Röhl A. 1999. Median-joining networks for inferring intraspecific
375   phylogenies. Molecular Biology and Evolution 16:37-48.
376
377   Beadell JS, Gering E, Austin J, Dumbacher JP, Peirce MA, Pratt TK, Atkinson CT, Fleischer RC.
378   2004. Prevalence and differential host-specificity of two avian blood parasite genera in the
379   Australo-Papuan region. Molecular Ecology 13:3829-3844.
380
381   Beadell JS, Covas R, Gebhard C, Ishtiaq F, Melo M, Schmidt BK, Perkins S, Graves GR, Fleischer R.
382   2009. Host associations and evolutionary relationships of avian blood parasites from West
383   Africa. International Journal for Parasitology 39:257-266.
384
385   Beaudoin RL, Applegate JE, David DE, Mclean RG. 1971. A model for the ecology of avian
386   malaria. Journal of Wildlife Diseases 7:5-13.
387
388   Bennett GF. 1970. *Trypanosoma avium Danilewsky* in the avian host. Canadian Journal of
389   Zoology 48:803-807.
390
391   Bennett GF, Bishop MA, Peirce MA. 1993a. Checklist of the avian species of *Plasmodium*
392   *Marchiafava* and *Celli*, 1885 (Apicomplexa) and their distribution by avian family and Wallacean
393   life zones. Systematic Parasitology 26:171-179.
394
395   Bennett GF, Peirce MA, Ashford RW 1993b. Avian haematozoa: mortality and pathogenicity.
396   Journal of Natural History 27:993-1001.
397
398   Bennett GF, Earle RA, Squires-Parsons D. 1994. Trypanosomes of some sub-Saharan birds.
399   Onderstepoort Journal of Veterinary Research 61:263-271.
400
401   Bensch S, Stjernman M, Hasselquist H, Ostman O, Hansson B, Westerdahl H, Torres Pinheiro R.
402   2000. Host specificity in avian blood parasites: A study of *Plasmodium* and *Haemoproteus*
403   mitochondrial DNA amplified from birds. Proceedings of the Royal Society of London Series B:
404   Biological Sciences 267:1583-1589.
405
406   Bensch S, Perez-Tris J, Waldstrom J, Hellgren O. 2004. Linkage between nuclear and
407   mitochondrial DNA sequences in avian malaria parasites: multiple cases of cryptic speciation?
408   Evolution 58:1617-1621.
409
410   Bensch S, Hellgren O, Perez-Tris J. 2009. MalAvi: a public database of malaria parasites and
411   related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages.
412   Molecular ecology resources 9:1353-1358.

413

414   Bensch S, Hellgren O, Križanauskienė A, Palinauskas V, Valkiūnas G, Outlaw D, Ricklefs RE. 2013.
415   How can we determine the molecular clock of malaria parasites? Trends in Parasitology 29:363-
416   369.

417

418   Bueno MG, Lopez RP, de Menezes RM, Costa-Nascimento MdeJ, Lima GF, Araujo RA, Guida FJ,
419   Kirchgatter K. 2010. Identification of *Plasmodium relictum* causing mortality in penguins
420   (*Spheniscus magellanicus*) from Sao Paulo Zoo, Brazil. Veterinary Parasitology 173:123-127.

421

422   Bull JJ. 1994. Perspective-virulence. Evolution 48:1423-1437.

423

424   Chagas CRF, Valkiūnas G, Guimarães LdO, Monteiro EF, Guida FJV, Simões RF, Rodrigues PT,
425   Luna EJdA, Kirchgatter K. 2017. Diversity and distribution of avian malaria and related
426   haemosporidian parasites in captive birds from a Brazilian megalopolis. Malaria Journal 16:83
427   DOI: 10.1186/s12936-017-1729-8.

428

429   Ciloglu A, Yildirim A, Duzlu O, Onder Z, Dogan Z, Inci A. 2016. Investigation of avian
430   haemosporidian parasites from raptor birds in Turkey, with molecular characterisation and
431   microscopic confirmation. Folia Parasitologica 63 DOI: 10.14411/fp.2016.023.

432

433   Clark NJ, Clegg SM, Lima MR. 2014. A review of global diversity in avian haemosporidians
434   (*Plasmodium* and *Haemoproteus*: Haemosporida): new insights from molecular data.
435   International Journal for Parasitology 44:329-338.

436

437   Cosgrove CL, Knowles SCL, Day KP, Sheldon BC. 2006. No evidence for avian malaria infection
438   during the nestling phase in a passerine bird. The Journal of Parasitology 92:1302-1304.

439

440   Dawson RD, Bortolotti GR. 2000. Effects of hematozoan parasites on condition and return rates
441   of American Kestrels. The Auk: Ornithological Advances 117:373-380.

442

443   Desser SS, Bennett GF. 1993. The genera *Leucocytozoon*, *Haemoproteus* and *Hepatocystis*. In:
444   Kreier JP, ed. Parasitic Protozoa. San Diego, California: Academic Press, 273-307.

445

446   Dimitrov D, Zehtindjiev P, Bensch S. 2010. Genetic diversity of avian blood parasites in SE
447   Europe: cytochrome b lineages of the genera *Plasmodium* and *Haemoproteus* (Haemosporida)
448   from Bulgaria. Acta Parasitologica 55:201-209.

449

450   Donovan TA, Schrenzel M, Tucker TA, Pessier AP, Stalls IH. 2008. Hepatic hemorrhage,
451   hemocoelom, and sudden death due to *Haemoproteus* infection in passerine birds: eleven
452   cases. Journal of Veterinary Diagnostic Investigation 20:304-313.

453

454   Drovetski SV, Aghayan SA, Mata VA, Lopes RJ, Mode NA, Harvey JA, Voelker G. 2014. Does the
455   niche breadth or trade-off hypothesis explain the abundance-occupancy relationship in avian
456   Haemosporidia? Molecular ecology 23:3322-3329.

457

458 Dubiec A, Podmokła E, Harnist I, Mazgajski TD. 2017. Haemoparasites of the pied flycatcher:
459 inter-population variation in the prevalence and community composition. Parasitology 145:912-
460 919 DOI: 10.1017/S0031182017001913.
461

462 Dunn JC, Stockdale JE, Bradford EL, McCubbin A, Morris AJ, Grice PV, Goodman SJ, Hamer KC.
463 2017. High rates of infection by blood parasites during the nestling phase in UK Columbids with
464 notes on ecological associations. Parasitology 144:622-628.
465

466 Ejiri H, Sato Y, Kim KS, Hara T, Tsuda Y, Imura T, Murata K, Yukawa M. 2011. Entomological
467 study on transmission of avian malaria parasites in a zoological garden in Japan: bloodmeal
468 identification and detection of avian malaria parasite DNA from blood-fed mosquitoes. Journal
469 of Medical Entomology 48:600-607.
470

471 Fargallo JA, Merino S. 2004. Clutch size and haemoparasite species richness in adult and
472 nestling blue tits. Écoscience 11:168-174.
473

474 Forest report Hesse. 2015. Regionaler Waldbericht Hessen 2015. Regionale PEFC-Arbeitsgruppe
475 Hessen e.V. (eds.). https://pefc.de/media/filer_public/c2/2d/c22d6ce7-3bcb-4580-aee1-
476 c465b85dd094/hessen_waldbericht_2015.pdf
477

478 Forrester DJ, Greiner EC. 2008. Leucocytozoonosis. In: Atkinson CT, Thomas NJ, Hunter DB, eds.
479 Parasitic Diseases of Wild Birds. Ames, Iowa: Blackwell Publishing, 54-107.
480

481 Freund D, Wheeler SS, Townsend AK, Boyce WM, Ernest HB, Cicero C, Sehgal RNM. 2016.
482 Genetic sequence data reveals widespread sharing of *Leucocytozoon* lineages in corvids.
483 Parasitology Research 115:3557-3565.
484

485 Glaizot O, Fumagalli L, Iritano K, Lalubin F, Van Rooyen J, Christe P. 2012. High Prevalence and
486 Lineage Diversity of Avian Malaria in Wild Populations of Great Tits (*Parus major*) and
487 Mosquitoes (*Culex pipiens*). PLoS One 7(4):e34964 DOI: 10.1371/journal.pone.0034964.
488

489 Haberkorn A. 1984. Observations on malaria in European perching birds (Passeriformes).
490 Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene Series A 256:288-295.
491

492 Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis
493 program for Windows 95/98/NT. Nucleic Acids Symposium 41:95-98.
494

495 Hamilton PB, Gibson WC, Stevens JR. 2007. Patterns of co-evolution between trypanosomes
496 and their hosts deduced from ribosomal RNA and protein-coding gene phylogenies. Molecular
497 Phylogenetics and Evolution 44:15-25.
498

499   Hellgren O, Waldenström J, Bensch S 2004. A new PCR assay for simultaneous studies of
500   *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. Journal of Parasitology
501   90:797-802.
502
503   Hunter DB, Rohner C, Currie DC. 1997. Mortality in fledgling great horned owls from black fly
504   hematophaga and leucocytozoonosis. Journal of Wildlife Diseases 33:486-491.
505
506   Jenkins T, Owens IP. 2011. Biogeography of avian blood parasites (*Leucocytozoon* spp.) in two
507   resident hosts across Europe: phylogeographic structuring or the abundance-occupancy
508   relationship? Molecular Ecology 20:3910-3920.
509
510   Jones W. 2017. Parasitism and speciation in a changing world. Introductory Research Essay No.
511   104, Uppsala University.
512
513   Knowles SCL, Palinauskas V, Sheldon BC. 2010. Chronic malaria infections increase family
514   inequalities and reduce parental fitness: experimental evidence from a wild bird population.
515   Journal of Evolutionary Biology 23:557-569.
516
517   Križanauskienė A, Hellgren O, Kosarev V, Sokolov L, Bensch S, Valkiūnas G. 2006. Variation in
518   host specificity between species of avian hemosporidian parasites: evidence from parasite
519   morphology and cytochrome B gene sequences. Journal of Parasitology 92:1319-1324.
520
521   Krone O, Priemer J, Streich J, Soemmer J, Langgemach T, Lessow O. 2001. Haemosporida of
522   birds of prey and owls from Germany. Acta Protozoologica 40:281-289.
523
524   Lacey LA, Merritt RW. 2003. The Safety of Bacterial Microbial Agents Used for Black Fly and
525   Mosquito Control in Aquatic Environments. In Hokkanen HMT, Hajek AE (eds.) Enviromental
526   impacts of Microbial Insecticides. Progress in Biological Control, Volumne 1, Springer,
527   Dordrecht.
528
529   Leigh JW, Bryant D. 2015. PopART: Full-feature software for haplotype network construction.
530   Methods in Ecology and Evolution 6:1110-1116.
531
532   Loiseau C, Harrigan RJ, Robert A, Bowie RCK, Thomassen HA, Smith TB, Sehgal RNM. 2012. Host
533   and habitat specialization of avian malaria in Africa. Molecular Ecology 21:431-441.
534
535   Lotta AL, Pacheco MA, Escalante AA, Gonzalez AD, Mantilla JS, Moncada LI, Adler PH, Matta NE.
536   2016. *Leucocytozoon* Diversity and Possible Vectors in the Neotropical highlands of Colombia.
537   Protist 167:185-204.
538
539   MalAvi. A Database for avian haemosporidian parasites. Table: Host And Sites. Available at
540   http://mbio-serv2.mbioekol.lu.se/Malavi/ (downloaded 09 August 2018, 3.25 PM).
541

542 Martinez J, Martinez-de La Puente J, Herrero J, Del Cerro S, Lobato E, Aguilar JR, Vasquez RA,
543 Merino S. 2009. A restriction site to differentiate *Plasmodium* and *Haemoproteus* infections in
544 birds: on the inefficiency of general primers for detection of mixed infections. Parasitology
545 136:713-722.

547 Martinez-de la Puente J, Martinez J, Rivero-de Aguilar J, Del Cerro S, Merino S. 2013. Vector
548 abundance determines *Trypanosoma* prevalence in nestling blue tits. Parasitology 140:1009-
549 1015.

551 Marzal A, de Lope F, Navarro C, Moller AP. 2005. Malarial parasites decrease reproductive
552 success: an experimental study in a passerine bird. Oecologia 142:541-545.

554 Mata VA, da Silva LP, Lopes RJ, Drovetski SV. 2015. The Strait of Gibraltar poses an effective
555 barrier to host-specialised but not to host-generalised lineages of avian Haemosporidia.
556 International Journal for Parasitology 45:711-719.

558 Medeiros MCI, Hamer GL, Ricklefs RE. 2013. Host compatibility rather than vector–host-
559 encounter rate determines the host range of avian *Plasmodium* parasites. Proceedings of the
560 royal society B 280:20122947 DOI: 10.1098/rspb.2012.2947.

562 Merino S, Moreno J, Sanz JJ, Arriero E. 2000. Are avian blood parasites pathogenic in the wild?
563 A medication experiment in blue tits (*Parus caeruleus*). Proceedings of the Royal Society B
564 267:2507-2510.

566 Merino S, Moreno J, Vásquez RA, Martínez J, Sánchez-Monsálvez I, Estades CF, Ippi S, Sabat P,
567 Rozzi R, McGehee S. 2008. Haematozoa in forest birds from southern Chile: latitudinal gradients
568 in prevalence and parasite lineage richness. Austral Ecology 33:329-340.

570 Møller AP, Nielsen JT. 2007. Malaria and risk of predation: A comparative study of birds.
571 Ecology: 88:871-881.

573 Okanga SM, Cumming GS, Hockey PAR, Nupen L, Peters JL. 2014. Host speciation and co-
574 speciation in avian hemosporidia in the Western Cape, South Africa. PLoS One 9(2):e86382 DOI:
575 10.1371/journal.pone.0086382.

577 Perez-Tris J, Bensch S. 2005. Dispersal increases local transmission of avian malarial parasites.
578 Ecology Letters 8:838-845.

580 Ricklefs RE, Fallon SM. 2002. Diversification and host switching in avian malaria parasites.
581 Proceedings of the Royal Society B 269:885-892.

583 Santiago-Alarcon D, Palinauskas V, Schaefer HM. 2012. Diptera vectors of avian haemosporidian
584 parasites: untangling parasite life cycles and their taxonomy. Biological Reviews of the
585 Cambridge Philosophical Society 87:928-964.

586

587 Santiago-Alarcon D, MacGregor-Fors I, Kühnert K, Segelbacher G, Schaefer HM. 2016. Avian
588 haemosporidian parasites in an urban forest and their relationship to bird size and abundance.
589 Urban Ecosystems 19:331-346.

590

591 Savage AF, Robert V, Goodman SM, Raharimanga V, Raherilalao MJ, Andrianarimisa A, Ariey F,
592 Greiner EC. 2009. Blood parasites in birds from Madagascar. Journal of Wildlife Diseases
593 45:907-920.

594

595 Schmid S, Fachet K, Dinkel A, Mackenstedt U, Woog F. 2017. Carrion crows (*Corvus corone*) of
596 southwest Germany: important hosts for haemosporidian parasites. Malaria Journal 16:369
597 DOI: 10.1186/s12936-017-2023-5.

598

599 Scordato ESC, Kardish MR. 2014. Prevalence and beta diversity in avian malaria communities:
600 host species is a better predictor than geography. Journal of Animal Ecology 83:1387-1397.

601

602 Sehgal RNM, Jones HI, Smith TB. 2001. Host specificity and incidence of *Trypanosoma* in some
603 African rainforest birds: a molecular approach. Molecular Ecology 10:2319-2327.

604

605 Sol D, Jovani R, Torres J. 2003. Parasite mediated mortality and host immune response explain
606 age-related differences in blood parasitism in birds. Oecologia 135: 542-547.

607

608 Szöllősi E, Cichoń M, Eens M, Hasselquist D, Kempenaers B, Merino S, Nilsson JÅ, Rosivall B,
609 Rytkönen S, Török J, Wood MJ, Garamszegi LZ. 2011. Determinants of distribution and
610 prevalence of avian malaria in blue tit populations across Europe: separating host and parasite
611 effects. Journal of Evolutionary Biology 24:2014-2024.

612

613 Toft CA, Krater AJ. 1990. Parasite-host coevolution. Trends in Ecology & Evolution 5:326-329.

614

615 Tomás G, Merino S, Moreno J, Morales J, Martìnez-de la Puente J. 2007a. Impact of blood
616 parasites on immunoglobulin level and parental effort: a medication field experiment on a wild
617 passerine. Functional Ecology 21:125-133.

618

619 Tomás G, Merino S, Moreno J, Morales M. 2007b. Consequences of nest reuse for parasite
620 burden and female health and condition in blue tits, *Cyanistes caeruleus*. Animal Behaviour
621 73:805-814.

622

623 Valkiūnas GA. 1996. Ecological implications of Hematozoa in birds. Bulletin for the Scandinavian
624 Society for Parasitology 6:103-113.

625

626 Valkiūnas G. 2005. Avian Malaria Parasites and Other Haemosporidia. Boca Raton, Florida: CRC
627 Press.

628

629   Valkiūnas G, Zickus T, Shapoval AP, Iezhova TA. 2006. Effect of *Haemoproteus belopolskyi*
630   (Haemosporida: Haemoproteidae) on body mass of the blackcap *Sylvia atricapilla*. Journal of
631   Parasitology 92:1123-1125.
632
633   Valkiūnas G, Iezhova TA, Loisseau C, Sehgal RNM. 2009. Nested cythochrome b polymerase
634   chain reaction diagnostics detect sporozoites of haemosporidian parasites in peripheral blood
635   of naturally infected birds. Journal of Parasitology 95:1512-1515.
636
637   Valkiūnas G, Palinauskas V, Križanauskienė A, Bernotienė R, Kazlauskienė R, Iezhova TA. 2013.
638   Further observations on in vitro hybridization of hemosporidian parasites: patterns of ookinete
639   development in *Haemoproteus spp*. Journal of Parasitology 99:124-136.
640
641   Vanstreels RET, Miranda FR, Ruoppolo V, Almeida Reis AO de, Costa ES, Lira Pessôa AR de,
642   Torres JPM, Cunha LST da, Cruz Piuco R da, Valiati VH, González-Acuña D, Labruna MB, Petry
643   MV, Epiphanio S, Catão-Dias JL. 2014. Investigation of blood parasites of pygoscelid penguins at
644   the King George and Elephant Islands, South Shetlands Archipelago, Antarctica. Polar Biology
645   37:135-139.
646
647   Van Rooyen J, Lalubin F, Glaizot O, Christe P. 2013a. Altitudinal variation in haemosporidian
648   parasite distribution in great tit populations. Parasites & Vectors 6:139 DOI: 10.1186/1756-
649   3305-6-139.
650
651   Van Rooyen J, Lalubin F, Glaizot O, Christe P. 2013b. Avian haemosporidian persistence and co-
652   infection in great tits at the individual level. Malaria Journal 12:40 DOI: 10.1186/1475-2875-12-
653   40.
654
655   Votypka J, Obornik M, Volf P, Svobodova M. 2002. *Trypanosoma avium* of raptors
656   (Falconiformes): phylogeny and identification of vectors. Parasitology 125:253-263.
657
658   Waldenström J, Bensch S, Kiboi S, Hasselquist D, Ottosson U. 2002. Cross-species infection of
659   blood parasites between resident and migratory songbirds in Africa. Molecular Ecology
660   11:1545-1554.
661
662   Wiersch SC, Lubjuhn T, Maier WA, Kampen H. 2007. Haemosporidian infection in passerine
663   birds from Lower Saxony. Journal of Ornithology 148:17-24.
664
665   Wood MJ, Cosgrove CL, Wilkin TA, Knowles SC, Day KP, Sheldon BC. 2007. Within-population
666   variation in prevalence and lineage distribution of avian malaria in blue tits, *Cyanistes*
667   *caeruleus*. Journal of Animal Ecology 16:3263-3273.
668
669   Zhang Z, Schwartz S, Wagner L, Miller W. 2000. A greedy algorithm for aligning DNA sequences.
670   Journal of Computational Biology 7:203-214.
671

Figure 1

Median-joining network of mitochondrial cytochrome b gene lineages of *Haemoproteus* spp. (n=39, 463bp fragment)

Circles represent lineages, and the circle sizes are proportional to the lineage frequencies in the population. One hatch mark represents one mutation. Sampled host species are represented by different colors. Lineage names are noted at the associated circles.

PHSIB1

PARUS1

PADOM03

10 samples

1 sample

- C. caeruleus
- P. major
- P. montanus
- F. hypoleuca
- S. europaea

PFC1

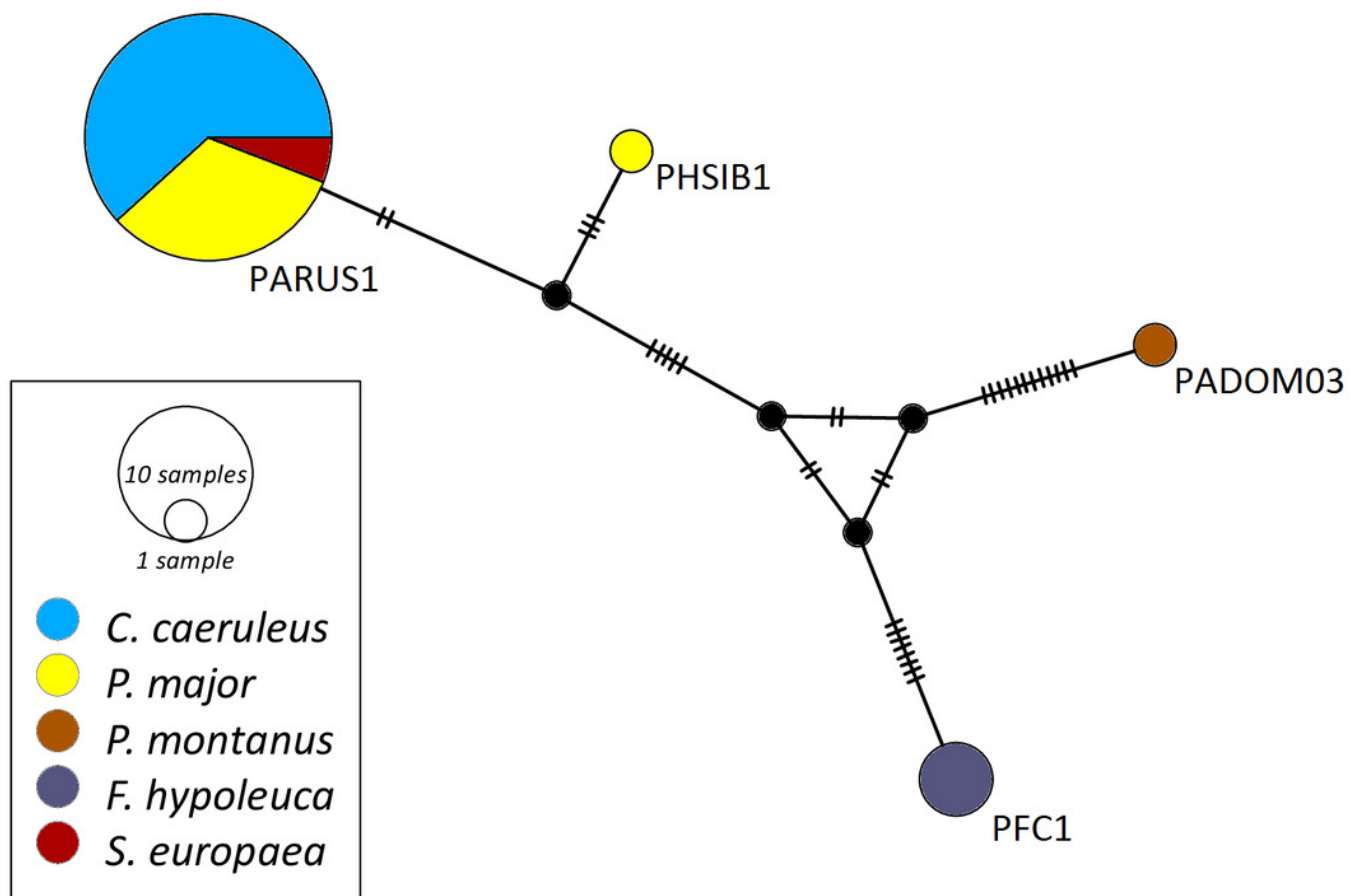



Figure 2

Median-joining network of mitochondrial cytochrome b gene lineages of *Plasmodium* spp. (n=14, 440bp fragment).

Circles represent lineages, and the circle sizes are proportional to the lineage frequencies in the population. One hatch mark represents one mutation. Sampled host species are represented by different colors. Lineage names are noted at the associated circles.

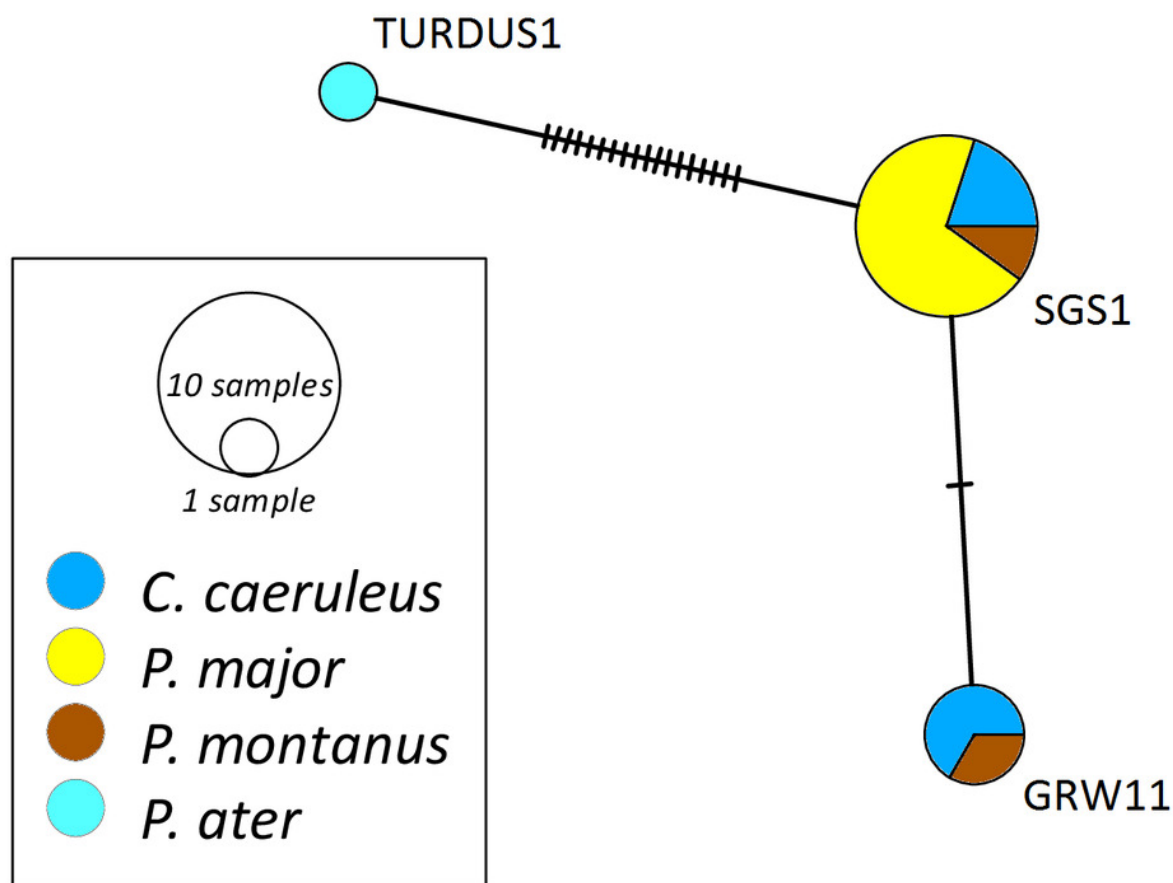
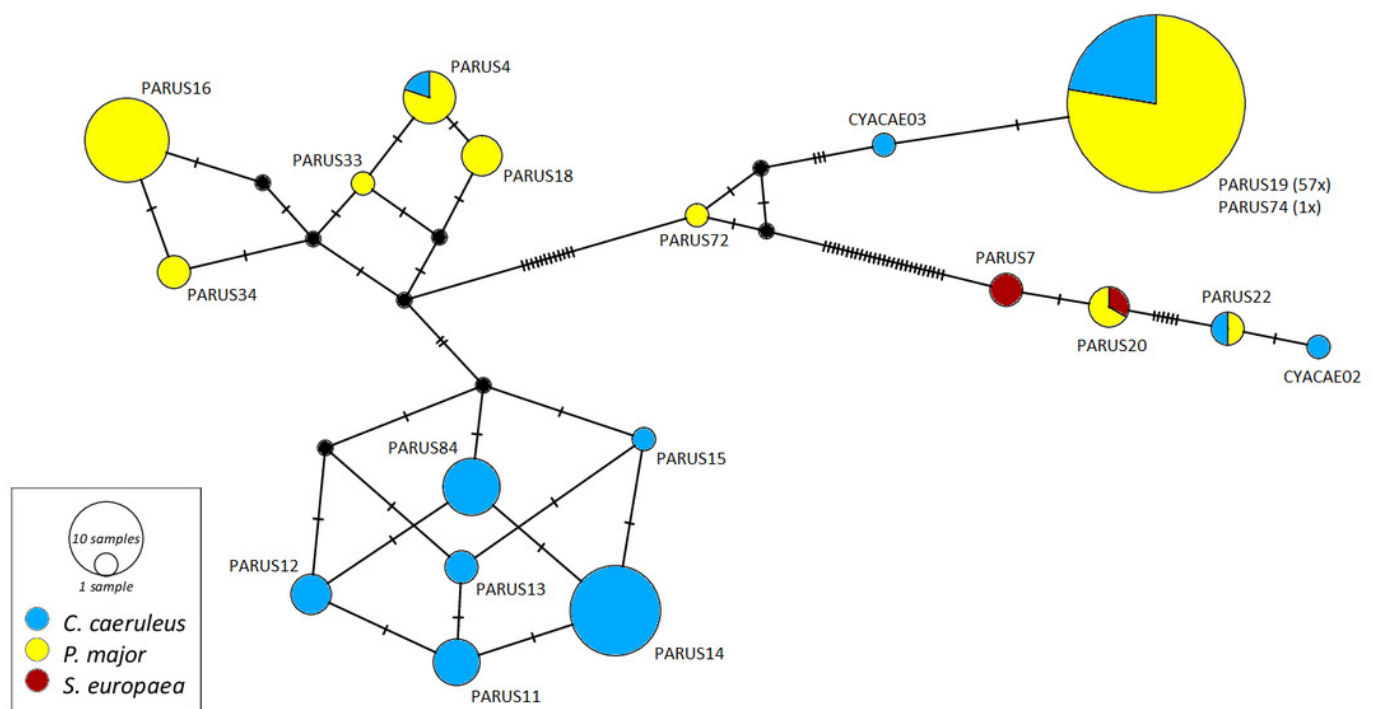



Figure 3

Median-joining network of mitochondrial cytochrome b gene lineages of *Leucocytozoon* spp. (n=123, 478bp fragment).

Circles represent lineages, and the circle sizes are proportional to the lineage frequencies in the population. One hatch mark represents one mutation. Sampled host species are represented by different colors. Lineage names are noted at the associated circles.

Numbers of sampled adult and nestling songbirds.

Sample sizes per sex and parasite genus are given (P = *Plasmodium* spp., H = *Haemoproteus* spp., L = *Leucocytozoon* spp. and T = *Trypanosoma avium*).

Species	Year of sampling	Number of specimens (n)					
		Adult (male/female)				Nestling	
		P	H	L	T	L	T
Blue tit (*C. caeruleus*)	2017	**58** (30/28)	**58** (30/28)	**58** (30/28)	**58** (30/28)	**65**[a]	**65**[a]
Great tit (*P. major*)	2015/2018	**32** (14/18)	**32** (14/18)	**142** (49/93)	**68** (18/50)	**57**[b]	**57**[b]
Coal tits (*P. ater*)	2017	**4** (2/2)	**4** (2/2)	**4** (2/2)	**4** (2/2)	0	0
Eurasian tree sparrow (*P. montanus*)	2015/2017	**10** (1/9)	**10** (1/9)	**10** (1/9)	**10** (1/9)	0	0
European pied flycatcher (*F. hypoleuca*)	2015/2017	**14** (2/12)	**14** (2/12)	**14** (2/12)	**14** (2/12)	0	0
Eurasian nuthatch (*S. europaea*)	2015/2017	**10** (3/7)	**10** (3/7)	**10** (3/7)	**10** (3/7)	0	0

1 [a]Age: 16 days

2 [b]Age: 12 – 17 days

Primer pairs and their PCR conditions used for blood parasite screening.

Primer pair	Fragment size (bp)	Initial denaturation	Denaturation Annealing Extension	Cycles	Final extension	Target gene
HaemF HaemR2	480	3 min / 94 °C	30 sec / 94 °C 30 sec / 55 °C 45 sec / 72 °C	35	10 min / 72 °C	Cytochrome b
HaemFL HaemNR3	600	3 min / 94 °C	30 sec / 94 °C 30 sec / 51 °C 45 sec / 72 °C	35	10 min / 72 °C	Cytochrome b
TryF TryR	122	10 min / 95 °C	15 sec / 95 °C 30 sec / 56 °C 60 sec / 72 °C	40	10 min / 72 °C	18S rRNA

Haemosporida and *Trypanosoma avium* prevalence in six songbird species.

Species (No. of samples)	*Haemoproteus* spp.		*Plasmodium* spp.		*Leucocytozoon* spp.		*Trypanosoma avium*	
	positive / negative	Prevalence [%]	positive / negative	Prevalence [%]	positive / negative	Prevalence [%]	positive / negative	Prevalence [%]
C. caeruleus								
Adult (58)	21 / 37	**36.2**	6 / 52	**10.3**	55 / 3	**94.8**	0 / 58	**0.0**
Nestling (65)					0 / 65	**0.0**	0 / 65	**0.0**
P. major								
Adult (142) [a]	13 / 19	**40.6**	7 / 25	**21.9**	110 / 32	**77.5**	0 / 68	**0.0**
Nestling (57)					2 / 55	**3.5**	0 / 57	**0.0**
P. ater								
Adult (4)	0 / 4	**0.0**	1 / 3	**25.0**	1 / 3	**25.0**	0 / 4	**0.0**
P. montanus								
Adult (10)	1 / 9	**10.0**	2 / 8	**20.0**	0 / 10	**0.0**	0 / 10	**0.0**
F. hypoleuca								
Adult (14)	3 / 11	**21.4**	0 / 14	**0.0**	0 / 14	**0.0**	0 / 14	**0.0**
S. europaea								
Adult (10)	2 / 8	**20.0**	0 / 10	**0.0**	3 / 7	**30.0**	0 / 10	**0.0**
Total	40 / 88	**31.3**	16 / 112	**12.5**	171 / 189	**47.5**	0 / 286	**0.0**
Nestlings excluded					169 / 69	**71.0**	0 / 164	**0.0**

1   [a] For *Haemoproteus* spp. and *Plasmodium* spp. the sample size was 32 and for *Trypanosoma avium* 68 adult great tits.

Parasite lineages found in the six songbird species with their closest MalAvi match, the respective accession number and query cover in %.

Parasite	Lineage (MalAvi)	Accession Number (GenBank)	n	Match [%]	Host species (Number of individuals)	Lineage prevalence [%][†]	Reference Accession Number
Haemoproteus [a]	PARUS1	JQ778282	34	100	*C. caeruleus* (21) *P. major* (11) *S. europaea* (2)	15.1	Glaizot et al. 2012
Haemoproteus	PADOM03	KJ488647	1	100	*P. montanus* (1)	0.4	Drovetski et al. 2014
Haemoproteus	PHSIB1	KJ396634	1	100	*P. major* (1)	0.4	Scordato & Kardish 2014
H. pallidus	PFC1	JX026899	3	100	*F. hypoleuca* (3)	1.3	Valkiūnas et al. 2013
Plasmodium	GRW11	AY831748	3	100	*C. caeruleus* (2) *P. montanus* (1)	1.3	Perez-Tris & Bensch 2005
Plasmodium	SGS1	AB542064	11	99 - 100	*C. caeruleus* (3) *P. major* (7) *P. montanus* (1)	4.9	Ejiri et al. 2011
P. circumflexum	TURDUS1	KP000842	2	100	*C. caeruleus* (1) *P. ater* (1)	0.9	Ciloglu et al. 2016
Leucocytozoon [b]	PARUS4	KJ488615	11	99 - 100	*P. major* (10) C. caeruleus (1)	4.9	Mata et al. 2015
Leucocytozoon	PARUS7	KJ488817	3	97 - 100	*P. major (1) S. europaea* (2)	1.3	Mata et al. 2015
Leucocytozoon	PARUS11	HM234019	5	99 - 100	*C. caeruleus* (5)	2.2	Jenkins & Owens 2011
Leucocytozoon	PARUS12	HM234020	3	100	*C. caeruleus* (3)	1.3	Jenkins & Owens 2011
Leucocytozoon	PARUS13	HM234021	2	100	*C. caeruleus* (2)	0.8	Jenkins & Owens 2011
Leucocytozoon	PARUS14	HM234022	15	100	*C. caeruleus* (15)	6.7	Jenkins & Owens 2011
Leucocytozoon	PARUS15	HM234023	1	100	*C. caeruleus* (1)	0.4	Jenkins & Owens 2011
Leucocytozoon	PARUS16	HM234024	14	100	*P. major* (14)	6.2	Jenkins & Owens 2011
Leucocytozoon	PARUS18	HM234026	6	99 - 100	*C. caeruleus* (2) *P. major* (4)	2.7	Jenkins & Owens 2011
Leucocytozoon	PARUS19	HM234027	67	99 - 100	*C. caeruleus* (13) *P. major* (53) *P. ater* (1)	29.8	Jenkins & Owens 2011
Leucocytozoon	PARUS20	KJ488629	4	99 - 100	*P. major* (3) *S. europaea* (1)	1.8	Mata et al. 2015
Leucocytozoon	PARUS22	HM234031	2	100	*P. major* (1) *C. caeruleus* (1)	0.9	Jenkins & Owens 2011
Leucocytozoon	PARUS33	JX867108	2	100	*P. major* (2)	0.9	van Rooyen et al. 2013b
Leucocytozoon	PARUS34	JX855049	2	100	*P. major* (2)	0.9	van Rooyen et al. 2013b
Leucocytozoon	PARUS72	KJ488759	3	99	*P. major* (3)	1.3	Mata et al. 2015
Leucocytozoon	PARUS74	KJ488766	9	100	*P. major* (6) *C. caeruleus* (3)	4.0	Mata et al. 2015
Leucocytozoon	PARUS84	KJ488911	6	100	*C. caeruleus* (6)	2.7	Mata et al. 2015
Leucocytozoon	CYACAE02	MH758695	1	100	*C. caeruleus* (1)	0.4	New lineage
Leucocytozoon	CYACAE03	MH758696	3	98 - 100	*P. major* (1) *C. caeruleus* (2)	1.3	New lineage

1   † Percentage of each lineage among all infected birds (n= 225).
2   ᵃ 1 sample could be determined as *Haemoproteus* spp., but could not be assigned to one certain lineage by BLAST against the MalAvi database
3   due to an insufficient sequence quality.
4   ᵇ 10 samples could be determined as *Leucocytozoon* spp., but could not be assigned to one certain lineage by BLAST against the MalAvi database
5   due to an insufficient sequence length and quality.

Overview of publications dealing with Haemosporida and *Trypanosoma avium* prevalences in great and blue tits sampled in Germany.

Research method, sample sizes, study region and prevalences of the different blood parasites are given (P = *Plasmodium* spp., H = *Haemoproteus* spp., L = *Leucocytozoon* spp. and T = *Trypanosoma avium*). NT = Not tested in the listed study.

Reference	Study region	Method	Species (sample size)	Prevalence P	Prevalence H	Prevalence L	Prevalence T
Haberkorn 1984	Western and northern Germany	Microscopic examination	*P. major* (82)	2.4%	8.5%	1.2%	0.0%
			C. caeruleus (66)	0.0%	7.6%	1.5%	0.0%
Wiersch et al. 2007	Northern Germany	PCR-based	*P. major* (56)	46.4%	30.4%	NT	NT
Jenkins & Owens 2011	Southern Germany	PCR-based	*P. major* (33)	NT	NT	33.3%	NT
			C. caeruleus (24)	NT	NT	41.6%	NT
Santiago-Alarcon et al. 2016	Southwestern Germany	PCR-based	*P. major* (43)		46.5%[a]		NT
			C. caeruleus (6)		0.0%[a]		NT
Present study	Central Germany	PCR-based	*P. major* (142) [b]	21.9%	40.6%	94.8%	0.0%
			C. caeruleus (58)	10.3%	36.2%	77.5%	0.0%

2 [a] Only overall prevalence for the three Haemosprida was given in the publication (20 from 43 great tits were infected); no prevalences for the

3 single genera were mentioned.

4 [b] For *Haemoproteus* spp. and *Plasmodium* spp. the sample size was 32 and for *Trypanosoma avium* 68 adult great tits.