Differential prevalence and diversity of haemosporidian parasites in two sympatric closely related non-migratory passerines

ANNA DUBIEC[1]*, EDYTA PODMOKŁA[2], MAGDALENA ZAGALSKA-NEUBAUER[3], SZYMON M. DROBNIAK[2], ANETA ARCT[2], LARS GUSTAFSSON[4] *and* MARIUSZ CICHOŃ[2]

[1] *Museum and Institute of Zoology, Polish Academy of Sciences, Wilcza 64, 00-679 Warszawa, Poland*
[2] *Institute of Environmental Sciences, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland*
[3] *Ornithological Station, Museum and Institute of Zoology, Polish Academy of Sciences, Nadwiślańska 108, 80-680 Gdańsk, Poland*
[4] *Department of Ecology and Genetics, Evolutionary Biology Centre, Uppsala University, Norbyvägen 18 D, SE-752 36 Uppsala, Sweden*

(*Received 17 August 2015; revised 24 March 2016; accepted 30 March 2016; first published online 13 May 2016*)

SUMMARY

Haemosporidian parasites infecting birds show distinct heterogeneity in their distribution among host species. However, despite numerous studies on the prevalence and diversity of parasite communities across species, very little is known on patterns of differences between them. Such data is lacking because up to date the majority of studies explored the patterns of variation in infections in different years, different time of sampling within a year or a breeding cycle, different study sites or was based on a small sample size, all of which may affect the estimates of prevalence and parasite diversity. Here, the prevalence, richness and diversity of haemosporidian parasites from the genera *Plasmodium* and *Haemoproteus* were studied in two closely related non-migratory hole-nesting passerines: Great Tits and Blue Tits. Birds were sampled in sympatrically breeding populations during two seasons at the same stage of their breeding cycle – late nestling care. Great Tits were more prevalently infected with *Plasmodium* and *Haemoproteus* parasites (97·1 *vs* 71·2%), harboured a higher proportion of multiple infections (26·2 *vs* 3·2%) and had a more diverse parasite community (11 *vs* 5 parasite lineages) than Blue Tits. Observed differences between two host species are discussed with reference to their breeding densities and immunological and behavioural characteristics.

Key words: *Cyanistes caeruleus*, haemosporidian parasites, parasite diversity, prevalence, parasite richness, *Parus major*.

INTRODUCTION

Parasite distribution shows distinct heterogeneity in wild animal populations (Poulin, 2007). In different species, which are hosts for a given type of parasites (e.g. helminths, mites, ticks), the proportion of infected individuals (i.e. prevalence), richness and diversity of the parasite community may range from very similar up to very dissimilar (Clayton and Moore, 1997; Schmid-Hempel, 2011). Such pattern of variation in parasitic infections may be primarily associated with host life-history traits, host ecology and the parasite characteristics. Since parasites may constitute the important selection drivers in animal populations (Schmid-Hempel, 2011), exploring the patterns of variation in parasitic infections among host species is important for understanding many of the biological processes, e.g. mate choice (Hamilton and Zuk, 1982).

Birds act as hosts for a highly diverse and geographically widely distributed vector-transmitted haematozoan parasites from the genera *Plasmodium* and *Haemoproteus* (phylum: Apicomplexa, order: Haemosporida) (Valkiūnas, 2005). The majority of bird species have been confirmed to harbour these parasites (Atkinson and Van Riper, 1991), although the prevalence and their diversity substantially differ among the host species (Scheuerlein and Ricklefs, 2004). In some species, none or only a small fraction of individuals in the population are being infected (Yohannes *et al*. 2009; Krams *et al*. 2012), while in the others all or nearly all birds in the population carry an infection (Van Rooyen *et al*. 2013). Despite the great number of studies focusing on the prevalence and diversity of haemosporidian parasites, surprisingly little is known about patterns of differences in these two parameters among bird species. This is because in most studies data originate either from different years, different time of sampling within a year or a breeding cycle, different study sites or is based on a small sample size, all of which may affect the estimates of prevalence and parasite diversity (Jovani and Tella,

* Corresponding author: Museum and Institute of Zoology, Polish Academy of Sciences, Wilcza 64, 00-679 Warszawa, Poland. Tel.: 0048 22 629 32 21. Fax: 0048 22 629 63 02. E-mail: adubiec@miiz.waw.pl

doi:10.1017/S0031182016000779

2006; Arriero and Møller, 2008; Svoboda *et al*. 2009; Zamora-Vilchis *et al*. 2012). Among- and within-year variability in the prevalence and the diversity of haemosporidian communities is common in avian hosts (Garvin and Greiner, 2003; Bensch *et al*. 2007; Cosgrove *et al*. 2008). In temperate regions the prevalence peaks in the spring, which coincides with the relapse of chronic infections (Valkiūnas, 2005; Cosgrove *et al*. 2008). Commonly observed differences in the prevalence and parasite diversity among geographically distant sites (Pagenkopp *et al*. 2008; Szöllősi *et al*. 2011) are probably primarily driven by different local communities of vectors and parasites as well as environmental conditions which may affect the activity of vectors and the development of parasites (Martínez-de la Puente *et al*. 2009). The number of screened host individuals and the number of infected individuals affect the estimates of the prevalence and parasite richness respectively, as at low sample sizes the accuracy of the prevalence estimates is low and richness maybe underestimated (Jovani and Tella, 2006; Jenkins and Owens, 2011). Because of these factors, the most reliable estimates of the prevalence and diversity of the parasite community in different species should be derived from large sample sizes collected at the same location and the same year. There are only few studies presenting data on the prevalence and diversity of haemosporidian parasites in closely related bird species, which fulfil these requirements (Shurulinkov and Chakarov, 2006; Wiersch *et al*. 2007; Kulma *et al*. 2013; Scordato and Kardish, 2014). However, they mostly consider migratory species, in which differential prevalence and parasite diversity at breeding grounds may at least partly result from differences in vector and parasite exposure at stopover and wintering sites. For example, long distance migrants – Collared (*Ficedula albicollis*) and Pied Flycatchers (*Ficedula hypoleuca*) – sampled at the breeding site on the island of Öland (Sweden) – have been shown to differ in the prevalence of infection with haemosporidian parasites by 50% (Kulma *et al*. 2013). Interestingly, the Pied Flycatcher – the more prevalently infected species – had a less diverse parasite community.

Here, we compare the prevalence and diversity of haemosporidian parasites from the genera *Plasmodium* and *Haemoproteus* in two closely related non-migratory passerines – the Great Tit (*Parus major*) and the Blue Tit (*Cyanistes caeruleus*) based on molecular screening of blood samples. While data on these two parameters of haemosporidian infections are available from several populations of each species (e.g. Wood *et al*. 2007; Stjernman *et al*. 2008; Szöllősi *et al*. 2011; Ferrer *et al*. 2012; Van Rooyen *et al*. 2013), only a single study looked into interspecific infection patterns with these parasites in sympatrically breeding populations (Lachish *et al*. 2012). However, this study did not examine the composition of the parasite communities. Here, we compare sympatric populations of Great and Blue Tits, sampled in the same years and the same point of the annual cycle, for the prevalence of infection with *Plasmodium* and *Haemoproteus* parasites and richness and the diversity of parasite community at location characterized by a high infection frequency. Great and Blue Tits share an array of characteristics in their breeding biology and ecology. Both species are small and short-living insectivorous hole-nesters. Only females build the nest and incubate the eggs, while both parents feed the young. However, Blue Tits invest more in a single breeding attempt per unit of body mass since they lay larger clutches than Great Tits (Cramp, 1985). We predicted that Great and Blue Tits either (i) do not differ in the prevalence given their similar biology and ecology and close genetic relatedness, (ii) Blue Tits are more prevalently infected than Great Tits because they may exhibit stronger reproductive effort-mediated immunosuppression (Knowles *et al*. 2009) as a consequence of their higher investment in reproduction, or (iii) Blue Tits are less prevalently infected than Great Tits because of their predicted lower exposure to vectors resulting from the presence in their nests of plants with insect repelling properties (Petit *et al*. 2002). We had no clear prediction about the diversity of the parasite community, although given close genetic relatedness of the two host species we expected that they would harbour similar parasite community (Ricklefs and Fallon, 2002; Davies and Pedersen, 2008).

MATERIALS AND METHODS

Data were collected as part of two projects focusing on fitness consequences of infection with malaria parasites in Great and Blue Tits (Podmokła *et al*. 2014*a*; unpublished results). Birds were sampled during two breeding seasons (2011–2012) in nest-box breeding populations in Southern Gotland, Sweden (57°03′N, 18°17′E). The study site consists of over ten large and several small wood plots (primarily deciduous) separated by arable areas (Fig. 1).

From the middle of April nest-boxes were regularly inspected to determine the first egg-laying date, hatching date (day = 0) and the parameters of reproductive success. Adult Blue Tits were sampled for blood once over the nesting period, during the nestling stage (modal nestlings' age at catching of adult birds – 14 days), while Great Tits – twice: during the nest-building stage and the nestling stage (modal nestling age at catching of adult birds – 14 days). Birds were caught with traps installed inside the box or with mist nets located in the vicinity of the nest box. The subset of breeding birds of both species was subject to an additional treatment. In the case of Blue Tits, some pairs had their brood size increased by three

https://doi.org/10.1017/S0031182016000779 Published online by Cambridge University Press

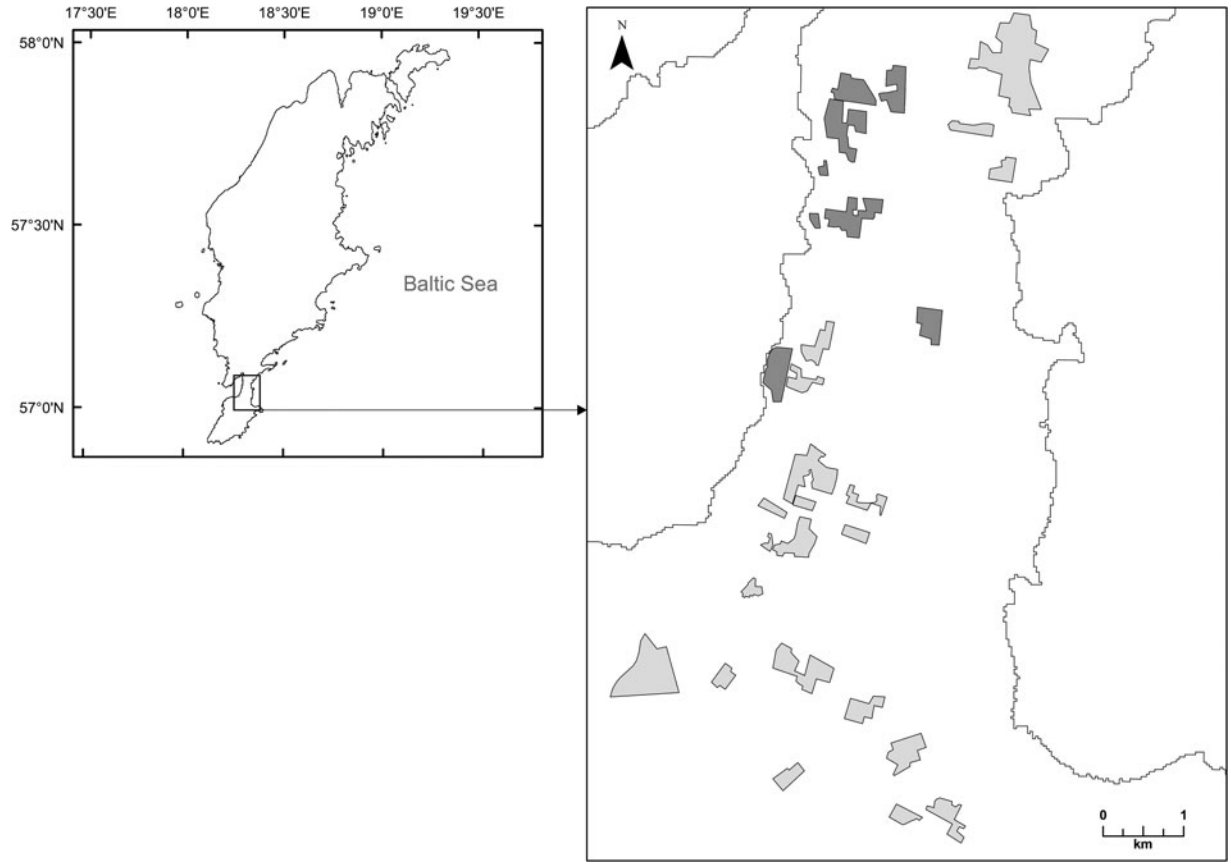

Fig. 1. The map of study plots monitored for box-breeding Great and Blue Tits in 2011 and 2012 on the island of Gotland (Sweden). Plots with individuals used for inter-species comparisons of the prevalence and the diversity of haemosporidian infections are depicted in dark grey.

nestlings on day 2 post-hatching (for a detailed description of the treatment see Podmokła *et al*. 2014*a*), while in Great Tits some females were injected during the nest-building stage with either a physiological salt or an anti-malarial drug – primaquine. Because the experimental increase of brood size is expected to elevate reproductive effort, which in turn may induce immunosuppression and increase blood parasitaemia (Knowles *et al*. 2009), and the primaquine may potentially eradicate malaria parasites and influence the probability of developing a new infection (Marzal *et al*. 2005), birds subject to these treatments (21 Blue Tits and 50 Great Tits) were excluded from the analyses. Moreover, because the probability of acquiring infection with malaria parasites may differ at a local spatial scale (e.g. Wood *et al*. 2007), only birds breeding in plots where individuals of both species were sampled (seven wood plots in 2011 and eight wood plots in 2012) were considered in the analyses (Fig. 1). The size of these plots ranges from ca. 0·7 to 19·8 ha and the mean yearly breeding density in these plots in 2011–2012 was 1·8 and 0·6 pairs ha^{-1} for Great and Blue Tits, respectively.

Birds were ringed, sexed based on the presence of a brood patch and aged as yearlings or at least 2-year-old based on ringing records and plumage characteristics (Svensson, 1992). Blood samples were obtained by venipuncture from the wing vein using non-heparinized capillaries and stored in 96% ethanol in ambient temperature. Genomic DNA was extracted from the blood using either Chelex (Bio-Rad, Munich, Germany) in the case of Blue Tits (Walsh *et al*. 1991) or an ammonium acetate method in the case of Great Tits. The repeatability of PCR results based on chelex and ammonium acetate DNA isolation methods was 97·8% ($n = 45$ Blue Tit samples from 2015) with a slightly lower detection of infection with the chelex method (40 positive samples *vs* 41 positive samples based on ammonium acetate method). The presence of haemosporidian parasites (genus *Haemoproteus* and *Plasmodium*) was assessed by amplifying 478 bp long fragment of the mitochondrial cytochrome *b* gene using nested polymerase chain reaction (Waldenström *et al*. 2004). This method is very sensitive and allows one to detect the infection with sensitivity of one infected cell per 10 000 erythrocytes. PCR conditions followed the protocol of Cosgrove *et al*. (2008) and PCR products were processed as described in Podmokła *et al*. (2014*a*, *b*). In short, PCR products were run on 2% agarose gel and PCR products of all positive samples were purified and then sequenced uni-directionally (except for novel lineages, which were sequenced bi-directionally) from the 5′ end with

https://doi.org/10.1017/S0031182016000779 Published online by Cambridge University Press

the primer HaemF with an automated ABI 3130 DNA analyser (Applied Biosystems). Sequences were edited, aligned and compared with the MalAvi database (Bensch *et al*. 2009) using BioEdit software (Hall, 1999). When individuals harboured multiple infections, i.e. infections caused by two or more lineages simultaneously (indicated by double peaks in the chromatogram), parasite lineages were in most cases assigned following the visual comparison of sequences with the pool of lineages known to occur at the study site. The reliability of this method was confirmed by cloning of PCR products in ten individuals with multiple infections (see Podmokła *et al*. 2014*b* for more details). When unambiguous identification of the lineages was not possible, PCR products were cloned. In the case of unique, not previously described lineages (the lineage was considered unique when a difference of at least one nucleotide was present, Bensch *et al*. 2009), the PCR and sequencing of the sample yielding such sequence were repeated to exclude the possibility of an error. Novel lineages were named following recommendation of Bensch *et al*. (2009) and deposited in GenBank (accession no. KU695262- KU695264).

Statistical analyses

To avoid potential differences between host species associated with timing of sampling within the breeding cycle, only samples collected during the nestling period were considered in this study. In total, 124 blood samples from Blue Tits and 363 samples from Great Tits were considered. In the case of six Blue Tits and 38 Great Tits, the samples were collected in both breeding seasons. One positive Blue Tit sample was excluded from the statistical analyses because the amplified product was shorter than the targeted fragment of the parasite gene. Moreover, some samples were excluded from the analyses of the infection type (single *vs* multiple infections) and the parasite diversity because parasite lineages could not be identified either because of the poor quality of the PCR product or when cloning of the PCR product of multiple infections did not yield all unique lineages. The final data set for the analyses of infection prevalence included 64 female/59 male and 84 yearling/39 older Blue Tit samples and 160 female/203 male and 187 yearling/176 older Great Tit samples. Mean estimates of prevalence were calculated using the infection rates recorded in each season and were based on the dataset which included only one record (selected randomly) per individual to omit the issue of non-independence of data in individuals sampled twice.

Data on infection prevalence were analysed using restricted maximum-likelihood method (REML) implemented in the R package ASReml-R (Butler 2009). Since the data was expressed as binary contrasts (presence/absence of infection) we have used generalized linear mixed models with a logit link function. All models were run for 50 optimizing iterations, but in all cases convergence to the maximum-likelihood estimate was achieved within no more than ten iterations.

We have analysed four types of models. In the first three models the response variable described the prevalence of parasitic infection in each individual (three different response variables: presence/absence of haemosporidian infection, presence/absence of *Plasmodium* infection and presence/absence of *Haemoproteus* infection). Prevalence at the parasite genus level was based on individuals harbouring single and multiple infections. Consequently, birds which were co-infected with *Plasmodium* and *Haemoproteus* were scored as positive for both parasite types. In the fourth model, run on the dataset containing only infected individuals, the response variable described the presence/absence of the multiple infections. Each model included a random categorical effect of individual identity (to account for repeated inclusion of the same individuals in the dataset) and a set of fixed effects. The first three models included: host species, year of sampling, individual age and sex, and all second-order interactions, which contained the term species with the remaining fixed terms. The fourth model (the prevalence of multiple infections) included only species, year of sampling and their interaction because of the very low frequency of multiple infections in Blue Tits (see the Results). Non-significant interactions were removed sequentially, starting with those with the highest P-values. Fixed effects were tested using conditional Wald tests (equivalent to conventional F test) with Satterthwaite method of approximating the number of degrees of freedom.

Richness is expressed as the total number of detected lineages and the lineage diversity as the Shannon index, which combines information on species richness and relative abundance (Shannon and Weaver, 1962). The index and its 95% confidence intervals (CIs) were calculated using the software EstimateS v. 9.1.0 (Colwell, 2013). CIs were estimated based on 1000 randomizations. The diversity was considered to differ between compared sets of samples, if CIs did not overlap the mean of the other group. Richness and lineage diversity were calculated based on the dataset containing only one record per individual sampled twice.

RESULTS

Prevalence of infection with haemosporidian parasites

Both host species were frequently infected with haemosporidian parasites with, on average, over 70% of individuals in each species infected during the breeding period with either one or two of the surveyed parasite genera. In general, Great Tits were more frequently infected than Blue Tits in terms

https://doi.org/10.1017/S0031182016000779 Published online by Cambridge University Press

Table 1. The association between the probability of infection with haemosporidian parasites in Blue and Great Tits on Gotland (Sweden) with species, year, age (yearlings or older birds) and sex as fixed effects

Fixed effect	DF	denDF	*F*	*P*
Overall infection				
Species	1	424·4	44·60	<0·001
Year	1	381·5	11·69	<0·001
Age	1	467·3	11·33	<0·001
Sex	1	213·9	0·60	0·441
Plasmodium infection				
Species	1	402·3	2·75	0·098
Year	1	480·0	32·76	<0·001
Age	1	480·0	16·68	<0·001
Sex	1	413·3	0·03	0·872
Haemoproteus infection				
Species	1	479·0	21·53	<0·001
Year	1	479·0	12·12	<0·001
Age	1	479·0	1·48	0·224
Sex	1	398·9	0·22	0·636
Species*age	1	479·0	7·32	0·007

Data for overall hamosporidian infections and infections with either *Plasmodium* or *Haemoproteus* parasites are presented separately. Full models contained all fixed effects and two-way interactions which included the factor 'species'. Non-significant interactions were removed sequentially, starting with those with the highest *P*-values. Each model included a random categorical effect of individual identity to account for repeated inclusion of individuals sampled in both breeding seasons.

of the overall prevalence as well as the prevalence of *Haemoproteus* infections, while there was no difference between the host species in the *Plasmodium* prevalence (mean overall prevalence: Great Tits – 97·1%, Blue Tits – 71·2%; mean *Plasmodium* prevalence: Great Tits – 71·4%, Blue Tits – 62·7%; mean *Haemoproteus* prevalence: Great Tits – 38·4%, Blue Tits – 9·8%; Table 1, Fig. 2). The prevalence of infections (overall as well as the prevalence at the parasite genus level) differed between the seasons (Table 1). Overall and *Plasmodium* prevalence decreased from 2011 to 2012, while *Haemoproteus* prevalence increased during this period (Fig. 2).

In both host species sex of the individual did not explain the variation in the prevalence (both overall and at the parasite genus level); however, the probability of being infected was associated with host age (Table 1). Specifically, the overall prevalence and the prevalence of infection with *Plasmodium* were in both species more common among older birds (mean overall prevalence: Great Tits: 95·3% in yearlings *vs* 99·0% in older birds, Blue Tits: 65·6% in yearlings *vs* 83·3% in older birds; mean *Plasmodium* prevalence: Great Tits: 64·2% in yearlings *vs* 79·0% in older birds, Blue Tits: 59·8% in yearlings *vs* 68·8% in older birds), while in the case of infection with *Haemoproteus* age-related changes in the prevalence differed between the host species. In Blue Tits mean prevalence was over twice higher in older birds than in yearlings, while in Great Tits older birds were slightly less prevalently infected than yearlings (Great Tits: 41·6% in yearlings *vs* 35·5% in older birds, Blue Tits: 6·8% in yearlings *vs* 16·6% in older birds).

Great and Blue Tits differed in the frequency of multiple infections, however, the proportion of individuals which harboured such infections did not differ between years (species: $F_{1,\ 432\cdot0} = 13\cdot95$, $P < 0\cdot001$, year: $F_{1,\ 432\cdot0} = 1\cdot821$, $P = 0\cdot178$). In the subset of infected individuals, multiple infections were found on average in 26·2% of Great Tits and 3·2% of Blue Tits. The most common were double infections – 83·4% (mean prevalence) in Great Tits and all multiple infections in Blue Tits. Great Tits also harboured triple (15·3%) and quadruple infections (1·4%).

Richness and diversity of the parasite community

In total, 11 parasite lineages were detected in the set of Great and Blue Tit samples used in the present study (Fig. 3, Appendix 1). Among eight lineages, which have been already described, five belong to the *Plasmodium* genus (BT7 and TURDUS1 representing morphospecies *P. circumflexum*, GRW11 and SGS1 representing *P. relictum* and SW2 representing *P. homonucleophilus*) and three to the *Haemoproteus* genus (PARUS1, PHSIB1 and WW2 representing *H. majoris*). Out of 11 detected lineages Great and Blue Tits shared five lineages (BT7, PARUS1, PHSIB1, SGS1 and TURDUS1), while six lineages, including three novel ones (GRW11, SW2, WW2, PARUS65, PARUS66 and PARUS67) were found only in Great Tits. Based on the comparison of novel lineages with the pool of lineages deposited in MalAvi database, PARUS65 most closely matches lineages from the *Haemoproteus* genus (the closest genetic similarity with PARUS1), while PARUS66 and PARUS67 most closely match lineages from *Plasmodium* genus (the closest genetic similarity with SW2 and TURDUS1, respectively).

The diversity of the parasite community was higher in Great Tits than Blue Tits (Shannon index and 95% CI, 2011: Great Tits 1·64, 1·56–1·72; Blue Tits 0·61, 0·37–0·84; 2012: Great Tits 1·90, 1·82–1·98; Blue Tits: 1·12, 0·94–1·30). Although both Tit species were most commonly infected with *Plasmodium* lineage TURDUS1, the distribution of infections with other lineages co-occurring in both species differed (Fig. 3, Appendix 1). For example, *Haemoproteus* lineage PHSIB1 showed a moderate infection rate in Great Tits, while in Blue Tits it was the least prevalent lineage among all detected in this species. In both host species, some lineages were found only sporadically with the mean annual prevalence <2% (Great Tits: GRW11, PARUS65, PARUS66, PARUS 67, Blue Tits: PHSIB1).

https://doi.org/10.1017/S0031182016000779 Published online by Cambridge University Press

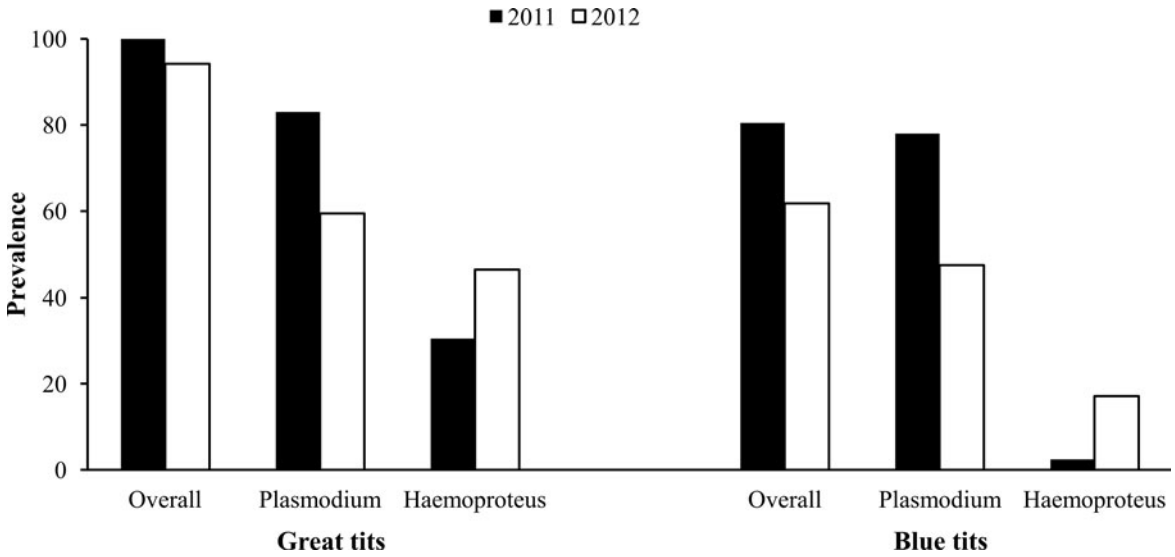

Fig. 2. The overall prevalence and the prevalence of infections with *Plasmodium* and *Haemoproteus* in Great and Blue Tits sampled during the late nestling period on Gotland (Sweden) in two breeding seasons. Data is presented separately for each season. Individuals co-infected with *Plasmodium* and *Haemoproteus* were scored as positive for both parasite genera. The number of screened individuals: Great Tits – 118 in 2011 and 207 in 2012, Blue Tits – 41 in 2011 and 76 in 2012.

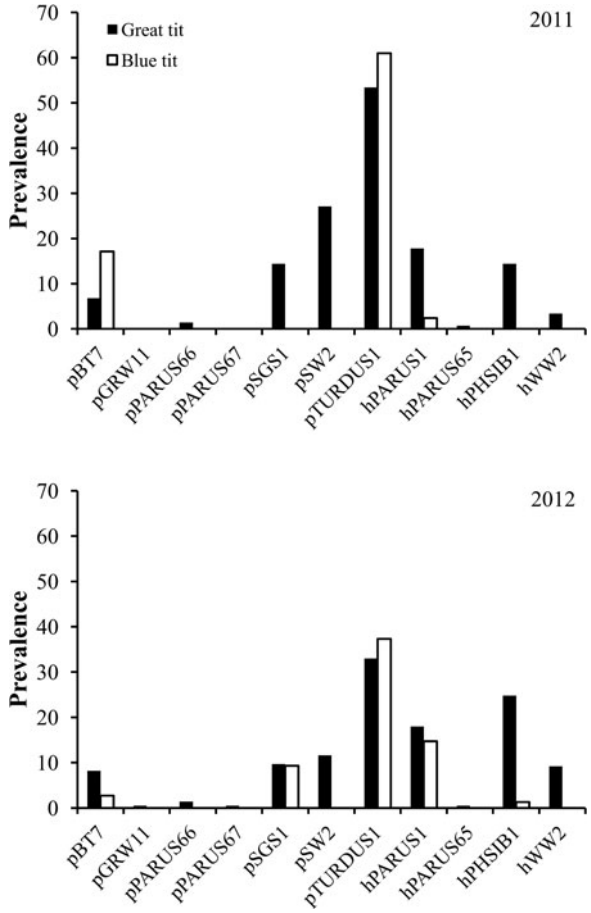

Fig. 3. The composition and the prevalence of *Plasmodium* and *Haemoproteus* lineages detected in sympatric populations of Great and Blue Tits on Gotland (Sweden) in two breeding seasons. Data is presented separately for 2011 and 2012.

DISCUSSION

Based on screening a large sample of individuals from sympatric populations we show that Blue Tits and Great Tits – two closely related passerines – differ in the prevalence and diversity of infections with haemosporidian parasites. Great Tits not only are more prevalently infected with these vector-transmitted parasites, but also harbour a higher proportion of multiple infections and have a more diverse parasite community than Blue Tits. The same pattern of interspecific difference in haemosporidian prevalence in sympatric populations of these two host species has been found in Southern UK (Lachish *et al*. 2012). Since both study sites differ in an array of parasite- and host-associated characteristics (e.g. prevalence, composition of the parasite assemblage, the frequency of multiple infections, ratio of Blue Tit/Great Tit population density), a higher frequency of infections in Great Tits seems to be a consistent pattern.

In general, closely related host species from sympatric populations are expected to show similarity in the susceptibility to parasitic infections and the parasite community because of the common evolutionary background (Ricklefs and Fallon, 2002; Davies and Pedersen, 2008) and exposure to the same vectors in the habitat they occupy. However, currently available data, including this study, seems to challenge this prediction. Not only closely related migratory species breeding sympatrically differ in the prevalence and composition of the haemosporidian community (Shurulinkov and Chakarov, 2006; Kulma *et al*. 2013; Scordato and Kardish, 2014), which may be largely attributed to contracting

https://doi.org/10.1017/S0031182016000779 Published online by Cambridge University Press

infections at stopover and wintering sites, but importantly such differences occur in closely related non-migratory species (Lee *et al*. 2006; Wiersch *et al*. 2007, Lachish *et al*. 2012, but see Jenkins and Owens, 2011 for no difference in infection rates of *Leucocytozoon*, the sister genus to *Plasmodium* and *Haemoproteus*, in Great and Blue Tits).

Several factors, including immunological and behavioural characteristics, may contribute to differences between Great and Blue Tits in the prevalence and diversity of the haemosporidian parasite community. Direct comparison of immune function activity of two study species is currently lacking, so it is not possible to make any association between the two parameters reflecting the susceptibility to haemosporidian parasites and immunocompetence. However, in two other closely related passerines – House (*Passer domesticus*) and Tree Sparrows (*Passer montanus*) – immune defences have been shown to differ and the species with higher antibody responsiveness – the House Sparrow – has been also found to be less prevalently infected with haemosporidians from the genera *Plasmodium* and *Haemoproteus* (Lee *et al*. 2006). In general, based on studies in mammals, it may be expected that species with stronger antibody responsiveness should be better able to control and clear chronic haemosporidian infections (Taylor-Robinson, 1995). Regardless of the potential differences in the constitutive and adaptive immunity, immune reaction to infection may differ between these two species if immune function is differently modulated by reproductive effort. Blue Tits invest more in a single breeding event than Great Tits, because they lay more eggs and consequently rear more nestlings per unit of their body mass. As a consequence more pronounced suppression of immune function resulting from a trade-off between immunity and reproductive effort (Knowles *et al*. 2009) should be expected in this species. Moreover, in the study area adult Great Tits seem to be more prone than Blue Tits to infestation/infection with other parasitic and pathogenic agents including ticks and pox viruses (own observation). Infestation/infection with other parasites and pathogens may suppress the immune function (e.g. ticks' saliva contains molecules which activate an anti-inflammatory TH2 response; Andrade *et al*. 2005) resulting in a higher probability of developing haemosporidian infection if the parasite gets transmitted by the vector.

The majority of 1-year old females of both host species are infected already at the nest building stage (Great Tits) or late incubation stage (Blue Tits, unpublished results). Since a prepatent period in *Plasmodium* and *Haemoproteus* parasites lasts from a few days to several weeks (Valkiūnas, 2005) and some vectors (e.g. mosquitoes) become active at the earliest at the incubation stage of both Tit species, such infections are most probably contracted by juveniles during the previous year. Such pattern of acquiring infection may indicate that other factors, not associated with reproduction-mediated reallocation of resources, play a role in this process. One factor, which may substantially contribute to differences between species, is the exposure to vectors transmitting haemosporidian parasites. Such differences may be especially present during the nestling stage. Blue Tits rear larger broods, which should attract more blood-sucking dipterans, because more nestlings should produce more cues, which are used by ornithophilic arthropods to locate the host (Russell and Hunter, 2005; Allan *et al*. 2006). However, on the other hand, Blue Tits incorporate in their nests plants producing volatile compounds (Petit *et al*. 2002; review in Dubiec *et al*. 2013), which may act as repellent against parasite-transmitting insects (Lafuma *et al*. 2001; Krams *et al*. 2013, but see Tomás *et al*. 2012). Krams *et al*. (2013) showed that 1-month old Great Tit fledglings from nest boxes treated with the insect repellent (citronella oil) had much lower prevalence and intensity of infection with haemosporidian parasites than fledglings from untreated nest boxes. In the study area some Blue Tit nests contain green plant material (own observation), which may indicate that nestlings of this species are exposed to fewer parasite-transmitting dipterans than Great Tit nestlings. Other potential behaviourally mediated mechanism of different exposure to vectors includes exploitation of foraging microhabitats which vary in vector abundance.

The probability of infection with haemosporidian parasites may also be associated with density of either conspecific or heterospecific hosts. Generally, some theoretical models suggest that the probability of infection with vector-transmitted parasites follows the dynamics along the continuum between pure density-dependent to pure frequency-dependent transmission (density and frequency refer in the models to infected individuals, Antonovics *et al*. 1995). However, in birds, the probability of vector-transmitted haemosporidians in some host-parasite systems has been shown to be well explained by the overall local host density (Ortego and Cordero, 2010; Lachish *et al*. 2012; Isaksson *et al*. 2013). Lachish *et al*. (2012) showed that in sympatric populations of Blue and Great Tits, infected primarily with two *Plasmodium* morphospecies – *P. circumflexum* and *P. relictum*, the probability of belonging to *P. circumflexum* cluster for nest-boxes occupied by Great Tits increased with local density of Great Tits and Blue Tits, while for boxes occupied by Blue Tits – with density of Great Tits, but not conspecifics. We may not exclude that at our study site certain density-dependent processes of acquiring and/or developing of infection also occur contributing to a higher infection frequency in Great Tits.

In both species age but not sex of the host was associated with variation in infection prevalence. Higher

https://doi.org/10.1017/S0031182016000779 Published online by Cambridge University Press

frequency of infection with haemosporidian parasites in birds at least 2-year-old than in yearlings observed in the current study (overall and *Plasmodium* infections in Great and Blue Tits and *Haemoproteus* infections in Blue Tits) is commonly found in birds (Kulma *et al*. 2014; Marzal *et al*. 2016, but see Zylberberg *et al*. 2015 for the lack of age-related patterns) and is being attributed to the increased probability of the vector encounter with age. Interestingly, in the case of *Haemoproteus* in Great Tits older birds were slightly less prevalently infected than yearlings. Possible mechanisms behind such a pattern include a competitive exclusion of *Haemoproteus* lineages by some *Plasmodium* lineages (Beadell *et al*. 2004) and differential mortality rates in *Haemoproteus*-infected Great and Blue Tits.

We found a much higher frequency of multiple infections in Great than Blue Tits. Similarly to the general prevalence of infections, the interspecific differences in the frequency of multiple infections in these two host species may be explained by aforementioned factors including immunological and behavioural characteristics and difference in the density. It has to be noted, that the actual rates of multiple infections in both species are possibly markedly higher than reported because PCR protocols underestimate this parameter (Bernotienė *et al*. 2016). Limited detection of parasite lineages simultaneously infecting the host may be associated with preferential amplification of lineages with higher parasitaemia (Pérez-Tris and Bensch, 2005; Valkiūnas *et al*. 2006).

Prevalence differed between years, which is a pattern commonly found in studies of haemosporidian infections in birds (Bensch *et al*. 2007). Changes in the prevalence between years are most probably associated with fluctuations in the population size and the activity of vectors, which are known to be affected by temperature and rainfall (Martínez-de la Puente *et al*. 2009). Apart from its effect on vectors, temperature may also affect rates of parasite development within vectors (Hoshen and Morse, 2004). All these factors may in turn affect the transmission frequency of haemosporidian parasites. Since at the parasite genus level only *Plasmodium* prevalence decreased, while *Haemoproteus* prevalence increased, most probably weather conditions negatively affected only the population of mosquitoes, but not dipterans transmitting *Haemoproteus*.

Based on the subset of samples used in this study, Great Tits harbour richer and more diverse parasite community than Blue Tits. This pattern holds even after taking into account four lineages (GRW11, PADOM02, SW2 and WW2, all occurring at very low frequencies), which have been found in the study population of Blue Tits in a set of nearly 1400 samples collected in years 2008–2014. It has to be noted, however, that Great and Blue tits are not necessarily the competent hosts for all detected lineages, especially those occurring sporadically, because haemosporidians may sometimes replicate in non-competent hosts without forming the infective stages, the gametocytes (Valkiūnas *et al*. 2009). To rule out such a possibility, assessing the presence of gametocytes in blood smears is necessary.

In order to better understand the patterns of haemosporidian infection rates and the variation in the parasite community composition between Blue and Great Tits as well as in other avian hosts, studies at different sites within hosts' distribution range and in different habitats are required.

ACKNOWLEDGEMENTS

We thank two anonymous reviewers for valuable comments on an earlier version of the manuscript. We also thank Giulia Casasole, Ewa Pośliński, Joanna Sudyka and Javier Lázaro Tapia for assistance with the fieldwork, Adam Krupski for assistance with molecular analyses and Kevin Fletcher for improving the manuscript's English. The study conforms to the legal requirements of Sweden.

FINANCIAL SUPPORT

Financial support was provided by the Polish National Science Centre (A.D., grant number N N303 818340), (E.P., grant number 2011/03/N/NZ8/02106); and the Polish Ministry of Science and Higher Education (M.C., grant number N N304 409838), (S.M.D., grant number N N304 061140). The long-term nest box study was supported by The Swedish Research Council (to L.G.).

REFERENCES

Allan, S. A., Bernier, U. R. and Kline, D. L. (2006). Laboratory evaluation of avian odors for mosquito (Diptera: Culicidae) attraction. *Journal of Medical Entomology* **43**, 225–231.

Andrade, B. B., Texeira, C. R., Barral, A. and Barral-Netto, M. (2005). Haematophagous arthropod saliva and host defense system: a tale of tear and blood. *Anais da Academia Brasileira de Ciências* **77**, 665–693.

Antonovics, J., Iwasa, Y. and Hassell, M. P. (1995). A generalized model of parasitoid, veneral and vector-based transmission processes. *American Naturalist* **145**, 661–675.

Arriero, E. and Møller, A. P. (2008). Host ecology and life-history traits associated with blood parasite species richness in birds. *Journal of Evolutionary Biology* **21**, 1504–1513.

Atkinson, C. T. and Van Riper, C., III. (1991). Pathogenecity and epizootiology of avian haematozoa: *Plasmodium*, *Leucocytozoon*, and *Haemoproteus*. In *Bird-parasite Interactions* (ed. Loye, J. E. and Zuk, M.), pp. 19–48. Oxford University Press, Oxford, UK.

Beadell, J. S., Gering, E., Austin, J., Dumbacher, J. P., Peirce, M. A., Pratt, T. K., Atkinson, C. T. and Fleischer, R. C. (2004). Prevalence and differential host-specificity of two avian blood parasite genera in the Australo-Papuan region. *Molecular Ecology* **13**, 3829–3844.

Bensch, S., Waldenström, J., Jonzén, N., Westerdahl, H., Hansson, B., Sejberg, D. and Hasselquist, D. (2007). Temporal dynamics and diversity of avian malaria parasites in a single host species. *Journal of Animal Ecology* **76**, 112–122.

Bensch, S., Hellgren, O. and Pérez-Tris, J. (2009). MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. *Molecular Ecology Resources* **9**, 1353–1358.

Bernotienė, R., Palinauskas, V., Iezhova, T., Murauskaitė, D. and Valkiūnas, G. (2016). Avian haemosporidian parasites (Haemosporida): a comparative analysis of different polymerase chain reaction assays in detection of mixed infections. *Experimental Parasitology* **163**, 31–37.

Butler, D. (2009). asreml: asreml() fits the linear mixed model. R package version 3.0. http://www.vsni.co.uk

Clayton, D. H. and Moore, J. (1997). *Host-Parasite Evolution. General Pricinples and Avian Models*. Oxford University Press, Oxford, UK.

https://doi.org/10.1017/S0031182016000779 Published online by Cambridge University Press

Colwell, R. K. (2013). EstimateS: Statistical estimation of species richness and shared species from samples. Version 9. User's Guide and application published at: http://purl.oclc.org/estimates

Cosgrove, C. L., Wood, M. J., Day, K. P. and Sheldon, B. C. (2008). Seasonal variation in *Plasmodium* prevalence in a population of blue tits *Cyanistes caeruleus*. *Journal of Animal Ecology* **77**, 540–548.

Cramp, S. (ed). (1985). *Handbook of the Birds of Europe, the Middle East and North Africa: the Birds of the Western Palearctic*, Vol. **VIII**. Oxford University Press, Oxford, UK.

Davies, T. J. and Pedersen, A. B. (2008). Phylogeny and geography predict pathogen community similarity in wild primates and humans. *Proceedings of the Royal Society of London B* **275**, 1695–1701.

Dubiec, A., Góźdź, I. and Mazgajski, T. D. (2013). Green plant material in avian nests. *Avian Biology Research* **6**, 133–146.

Ferrer, E. S., García-Navas, V., Sanz, J. J. and Ortego, J. (2012). Molecular characterization of avian malaria parasites in three Mediterranean blue tit (*Cyanistes caeruleus*) populations. *Parasitology Research* **111**, 2137–2142.

Garvin, M. C. and Greiner, E. C. (2003). Epizootiology of *Haemoproteus danilewskyi* (Haemosporina: Haemoproteidae) in blue jays (*Cyanocitta cristata*) in Southcentral Florida. *Journal of Wildlife Diseases* **39**, 1–9.

Hall, T. (1999). *BioEdit. Biological Sequence Alignment Editor for Windows*. North Carolina State University, NC, USA, http://www.mbio.ncsu.edu/BioEdit/bioedit.html

Hamilton, W. D. and Zuk, M. (1982). Heritable true fitness and bright birds: a role for parasites? *Science* **218**, 384–387.

Hoshen, M. and Morse, A. (2004). A weather-driven model of malaria transmission. *Malaria Journal* **3**, 32–46.

Isaksson, C., Sepil, I., Baramidze, V. and Sheldon, B. C. (2013). Explaining variance of avian malaria infection in the wild: the importance of host density, habitat, individual life-history and oxidative stress. *BMC Ecology* **13**, 15.

Jenkins, T. and Owens, I. P. F. (2011). Biogeography of avian blood parasites (*Leucocytozoon* spp.) in two resident hosts across Europe: phylogeographic structuring or the abundance-occupancy relationship? *Molecular Ecology* **20**, 3910–3920.

Jovani, R. and Tella, J. L. (2006). Parasite prevalence and sample size: misconceptions and solutions. *Trends in Parasitology* **22**, 214–218.

Knowles, S. C. L., Nakagawa, S. and Sheldon, B. C. (2009). Elevated reproductive effort increases blood parasitaemia and decreases immune function in birds: a meta-regression approach. *Functional Ecology* **23**, 405–415.

Krams, I., Suraka, V., Rattiste, K., Āboliņš-Ābols, M., Krama, T., Rantala, M. J., Mierauskas, P., Cīrule, D. and Saks, L. (2012). Comparative analysis reveals a possible immunity-related absence of blood parasites in Common Gulls (*Larus canus*) and Black-headed Gulls (*Chroicocephalus ridibundus*). *Journal of Ornithology* **153**, 1245–1252.

Krams, I. A., Suraka, V., Rantala, M. J., Sepp, T., Mierauskas, P., Vrublevska, J. and Krama, T. (2013). Acute infection of avian malaria impairs concentration of haemoglobin and survival in juvenile altricial birds. *Journal of Zoology* **291**, 34–41.

Kulma, K., Low, M., Bensch, S. and Qvarnström, A. (2013). Malaria infections reinforce competitive asymmetry between two *Ficedula* flycatchers in a recent contact zone. *Molecular Ecology* **22**, 4591–4601.

Kulma, K., Low, M., Bensch, S. and Qvarnström, A. (2014). Malaria-infected female collared flycatchers (*Ficedula albicollis*) do not pay the cost of late breeding. *PLoS ONE* **9**, e85822.

Lachish, S., Knowles, S. C. L., Alves, R., Sepil, I., Davies, A., Lee, S., Wood, M. J. and Sheldon, B. C. (2012). Spatial determinants of infection risk in a multi-species avian malaria system. *Ecography* **35**, 1–12.

Lafuma, L., Lambrechts, M. M. and Raymond, M. (2001). Aromatic plants in bird nests as a protection against bloodsucking flying insects? *Behavioural Processes* **56**, 113–120.

Lee, K. A., Martin, L. B., II, Hasselquist, D., Ricklefs, R. E. and Wikelski, M. (2006). Contrasting adaptive immune defenses and blood parasite prevalence in closely related *Passer* sparrows. *Oecologia* **150**, 383–392.

Martínez-de la Puente, J., Merino, S., Lobato, E., Rivero-de Aguilar, J., del Cerro, S., Ruiz-de-Castañeda, R. and Moreno, J. (2009). Does weather affect biting fly abundance in avian nests? *Journal of Avian Biology* **40**, 653–657.

Marzal, A., de Lope, F., Navarro, C. and Møller, A. P. (2005). Malarial parasites decrease reproductive success: an experimental study in a passerine bird. *Oecologia* **142**, 541–545.

Marzal, A., Balbontín, J., Reviriego, M., García-Longoria, L., Relinque, C., Hermosell, I. G., Magallanes, S., López-Calderón, C., de Lope, F. and Møller, A. P. (2016). A longitudinal study of age-related changes in *Haemoproteus* infection in a passerine bird. *Oikos*, in press. doi: 10.1111/oik.02778.

Ortego, J. and Cordero, P. J. (2010). Factors associated with the geographic distribution of leucocytozoa parasitizing nestling eagle owls (*Bubo bubo*): a local spatial-scale analysis. *Conservation Genetics* **11**, 1479–1487.

Pagenkopp, K. M., Klicka, J., Durrant, K. L., Garvin, J. C. and Fleischer, R. C. (2008). Geographic variation in malarial parasite lineages in the common yellowthroat (*Geothlypis trichas*). *Conservation Genetics* **9**, 1577–1588.

Petit, C., Hossaert-McKey, M., Perret, P., Blondel, J. and Lambrechts, M. M. (2002). Blue tits use selected plants and olfaction to maintain an aromatic environment for nestlings. *Ecology Letters* **5**, 585–589.

Podmokła, E., Dubiec, A., Drobniak, S. M., Arct, A., Gustafsson, L. and Cichoń, M. (2014*a*). Avian malaria is associated with increased reproductive investment in the blue tit. *Journal of Avian Biology* **45**, 219–224.

Podmokła, E., Dubiec, A., Drobniak, S. M., Arct, A., Gustafsson, L. and Cichoń, M. (2014*b*). Determinants of prevalence and intensity of infection with malaria parasites in the Blue Tit. *Journal of Ornithology* **155**, 721–727.

Poulin, R. (2007). *Evolutionary Ecology of Parasites*, 2nd Edn. Princeton University Press, Princeton, NJ.

Pérez-Tris, J. and Bensch, S. (2005). Diagnosing genetically diverse avian malarial infections using mixed-sequence analysis and TA-cloning. *Parasitology* **131**, 15–23.

Ricklefs, R. E. and Fallon, S. M. (2002). Diversification and host switching in avian malaria parasites. *Proceedings of the Royal Society of London B* **269**, 885–892.

Russell, C. B. and Hunter, F. F. (2005). Attraction of *Culex pipiens/restuans* (Diptera: Culicidae) mosquitoes to bird uropygial gland odors at two elevations in the Niagara region of Ontario. *Journal of Medical Entomology* **42**, 301–305.

Scheuerlein, A. and Ricklefs, R. E. (2004). Prevalence of blood parasites in European passeriform birds. *Proceedings of the Royal Society of London B* **271**, 1363–1370.

Schmid-Hempel, P. (2011). *Evolutionary Parasitology*. Oxford University Press, Oxford, UK.

Scordato, E. S. C. and Kardish, M. R. (2014). Prevalence and beta diversity in avian malaria communities: host species is a better predictor than geography. *Journal of Animal Ecology* **83**, 1387–1397.

Shannon, C. E. and Weaver, W. (1962). *The Mathematical Theory of Information*. University of Illinois Press, Urbana.

Shurulinkov, P. and Chakarov, N. (2006). Prevalence of blood parasites in different local populations of reed warbler (*Acrocephalus scirpaceus*) and great reed warbler (*Acrocephalus arundinaceus*). *Parasitology Research* **99**, 588–592.

Stjernman, M., Råberg, L. and Nilsson, J.-Å. (2008). Maximum host survival at intermediate parasite infection intensities. *PLoS ONE* **3**, e2463.

Svensson, L. (1992). *Identification Guide to European Passerines*, 4th Edn. BTO, Stockholm, Sweden.

Svoboda, A., Marthinsen, G., Turčoková, L., Lifjeld, J. T. and Johnsen, A. (2009). Identification of blood parasites in old world warbler species from the Danube River Delta. *Avian Diseases* **53**, 634–636.

Szöllősi, E., Cichoń, M., Eens, M., Hasselquist, D., Kempenaers, B., Merino, S., Nilsson, J.-Å., Rosivall, B., Rytkönen, S., Török, J., Wood, M. J. and Garamszegi, L. Z. (2011). Determinants of distribution and prevalence of avian malaria in blue tit populations across Europe: separating host and parasite effects. *Journal of Evolutionary Biology* **24**, 2014–2024.

Taylor-Robinson, A. W. (1995). Regulation of immunity to malaria – valuable lessons learned from murine models. *Parasitology Today* **11**, 334–342.

Tomás, G., Merino, S., Martínez-de la Puente, J., Moreno, J., Morales, J., Lobato, E., Rivero-de Aguilar, J. and del Cerro, S. (2012). Interacting effects of aromatic plants and female age on nest-dwelling ectoparasites and blood-sucking flies in avian nests. *Behavioural Process* **90**, 246–253.

Valkiūnas, G. (2005). *Avian Malaria Parasites and Other Haemosporidia*. CRC Press, Boca Raton, FL.

Valkiūnas, G., Bensch, S., Iezhova, T. A., Križanauskienė, A., Hellgren, O. and Bolshakov, C. V. (2006). Nested cytochrome *b* polymerase chain reaction diagnostics underestimate mixed infections of avian blood haemosporidian parasites: microscopy is still essential. *Journal of Parasitology* **92**, 418–422.

Valkiūnas, G., Iezhova, T. A., Loiseau, C. and Sehgal, R. N. M. (2009). Nested cytochrome b polymerase chain reaction diagnostics detect sporozoites of hemosporidian parasites in peripheral blood of naturally infected birds. *Journal of Parasitology* **95**, 1512–1515.

Van Rooyen, J., Lalubin, F., Glaizot, O. and Christe, P. (2013). Altitudinal variation in haemosporidian parasite distribution in great tit populations. *Parasites & Vectors* **6**, 139.

Waldenström, J., Bensch, S., Hasselquist, D. and Östman, Ö. (2004). A new nested PCR method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. *Journal of Parasitology* **90**, 191–194.

Walsh, P. S., Metzger, D. A. and Higuchi, R. (1991). Chelex® 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* **10**, 506–513.

Wiersch, S. C., Lubjuhn, T., Maier, W. A. and Kampen, H. (2007). Haemosporidian infection in passerine birds from Lower Saxony. *Journal of Ornithology* **148**, 17–24.

Wood, M. J., Cosgrove, C. L., Wilkin, T. A., Knowles, S. C. L., Day, K. P. and Sheldon, B. C. (2007). Within-population variation in prevalence and lineage distribution of avian malaria in blue tits, *Cyanistes caeruleus*. *Molecular Ecology* **16**, 3263–3273.

Yohannes, E., Križanauskienė, A., Valcu, M., Bensch, S. and Kempenaers, B. (2009). Prevalence of malaria and related haemosporidian parasites in two shorebird species with different winter habitat distribution. *Journal of Ornithology* **150**, 287–291.

Zamora-Vilchis, I., Williams, S. E. and Johnson, C. N. (2012). Environmental temperature affects prevalence of blood parasites of birds on an elevation gradient: implications for disease in a warming climate. *PLoS ONE* **7**, e39208.

Zylberberg, M., Derryberry, E. P., Breuner, C. W., MacDougall-Shackleton, E. A., Cornelius, J. M. and Hahn, T. P. (2015). Haemoproteus infected birds have increased lifetime reproductive success. *Parasitology* **142**, 1033–1043.

APPENDIX 1

Table A1. The parasite assemblage and the number of birds infected with *Plasmodium* and *Haemoproteus* lineages in Great and Blue Tits on Gotland (Sweden) in years 2011–2012. Dataset includes only one record (selected randomly) per individual for birds which were sampled in both years (six Blue Tits and 38 Great Tits). Number of individuals harbouring a given lineage was calculated based on the set of birds with single and multiple infections. *N* in brackets denotes the number of individuals screened molecularly for the presence of haemosporidian infections.

			No. of individuals harbouring the lineage			
			Great Tit		Blue Tit	
Parasite lineage	Parasite taxon	GenBank accession no	2011 ($n = 118$)	2012 ($n = 206$)	2011 ($n = 41$)	2012 ($n = 75$)
BT7	*Plasmodium circumflexum*	AY393793	8	17	7	2
GRW11	*Plasmodium relictum*	AY831748	0	1	0	0
PARUS66	*Plasmodium* sp.	KU695263	0	3	0	0
PARUS67	*Plasmodium* sp.	KU695264	0	1	0	0
SGS1	*Plasmodium relictum*	AF495571	17	20	0	7
SW2	*Plasmodium homonucleophilum*	AF495572	32	24	0	0
TURDUS1	*Plasmodium circumflexum*	AF495576	63	68	25	28
PARUS1	*Haemoproteus majoris*	AF254977	21	37	1	11
PARUS65	*Haemoproteus* sp.	KU695262	0	1	0	0
PHSIB1	*Haemoproteus majoris*	AF495565	17	51	0	1
WW2	*Haemoproteus majoris*	AY831755	4	19	0	0

https://doi.org/10.1017/S0031182016000779 Published online by Cambridge University Press