Parasitology Research (2018) 117:919–928
https://doi.org/10.1007/s00436-018-5748-3

SHORT COMMUNICATION

Molecular characterization of avian malaria in the spotless starling (*Sturnus unicolor*)

Jaime Muriel[1] · Jeff A. Graves[2] · Diego Gil[1] · S. Magallanes[3] · Concepción Salaberria[4] · Miriam Casal-López[5] · Alfonso Marzal[3]

Received: 18 September 2017 / Accepted: 3 January 2018 / Published online: 11 January 2018

Abstract

We studied the prevalence and genetic diversity of malaria parasites in the poorly investigated spotless starling (*Sturnus unicolor*) breeding in central Spain, aiming to describe the phylogenetic relationships among them and with other haemosporidians infecting the genus *Sturnus*. A total of 180 nestlings and 180 adult individuals from four different breeding seasons were screened for haemosporidian parasites using a nested PCR approach for the genera *Plasmodium* and *Haemoproteus*. Although the malaria prevalence ranged between years, the overall prevalence was 6.94%. Adults had a higher prevalence than chicks: 12.77 vs. 1.11%, respectively. We molecularly characterized avian malaria isolated in peripheral blood samples taken from malaria-infected individuals. Sequence analyses revealed four unique *Plasmodium* lineages of avian malaria (STURUNI01, STURUNI02, SYAT05, SGS1) in our spotless starling population. The phylogenetic analysis showed a well-supported clade comprised by STURUNI01, STURUNI02, and SYAT05. The most common lineage (SYAT05) has been previously found in 26 other avian host species, including populations of spotless starling in Portugal. Because this sedentary species is widely distributed throughout the Iberian Peninsula, we suggest that the local transmission of these lineages might place migratory birds at infection risk.

Keywords Avian malaria · Haemoparasites · Host age · *Plasmodium* · *Sturnus unicolor*

Introduction

Over the last two decades, the role of host–parasite interactions has received great attention not only from the World Health Organization (Loy et al. 2016) but also from evolutionary ecologists and wildlife conservationists (Bensch et al. 2009; Atkinson et al. 2014). Malaria in mammals is a well-known example (especially in humans). However, birds and reptiles are also the targets of this globally widespread disease (Levine 1988). Avian malaria is an insect-borne disease induced by many different lineages of haemosporidians (Phylum Apicomplexa, order Haemosporida) of the genera *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* (Atkinson and van Riper 1991; Martinsen et al. 2008). The presence of these protozoan blood parasites in birds is very common, and it is estimated that around 68% of all bird species are susceptible to malaria (Atkinson et al. 2000; Valkiūnas et al. 2000). Haemosporidians affect most investigated bird species, both wild and domestic (Valkiūnas 2005). In those wild avian populations where malaria is common and the host parasites have co-evolved, it is difficult to assess the role of avian malaria in population dynamics. However, several studies have shown that avian malaria can have a negative impact on host fitness components (Atkinson and van Riper 1991; Sol et al. 2003; Marzal et al. 2005; Knowles et al. 2010; Palinauskas et al.

✉ Jaime Muriel
Jaime.muriel.redondo@gmail.com

[1] Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales (CSIC), José Gutiérrez Abascal 2, E-28006 Madrid, Spain

[2] School of Environmental and Evolutionary Biology, University of St. Andrews, St. Andrews, Fife KY16 9TH, UK

[3] Department of Anatomy, Cellular Biology and Zoology, University of Extremadura, Avda. de Elvas s/n, ES-06006 Badajoz, Spain

[4] Departamento de Etología, Fauna Silvestre y Animales de Laboratorio, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de Mexico, Av. Universidad #3000, Delegación Coyoacán, 04510 Ciudad de México, Mexico

[5] Departamento de Biodiversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales (CSIC), José Gutiérrez Abascal 2, E-28006 Madrid, Spain

2011; Asghar et al. 2015). Host parasite infection may be influenced by host population density (Keymer and Anderson 1979; Isaksson et al. 2013), as well as by other host demographic parameters such as sex (McCurdy et al. 1998; Asghar et al. 2011; Calero-Riestra and García 2016) and age (Wilson et al. 2001; Deviche et al. 2005; Marzal et al. 2016). These two factors could lead to differences in reproductive investment, behaviour and immune capacities between individuals (Sheldon and Verhulst 1996).

Historically, avian haemosporidian species have been classified on the basis of their host species and the morphological characteristics of their gametocytes observed trough an optical microscope. In this regard, a recent study has suggested that there are approximately 250 morphospecies of avian haemosporidians (Valkiūnas et al. 2014). However, thanks to the development of molecular tools, such as PCR protocols based on the amplification and sequencing of a fragment of the cytochrome b gene of parasites, molecular studies have identified and recorded over 2000 unique genetic haplotypes of avian malaria (Bensch et al. 2009; Outlaw and Ricklefs 2014; Clark et al. 2014). Furthermore, it has been found that different lineages could co-infect the same host individual (Pérez-Tris and Bensch 2005; Marzal et al. 2008) and that one single lineage can frequently infect a wide range of hosts (Waldenström et al. 2002; Pérez-Tris et al. 2007; Ricklefs et al. 2014). For example, *Plasmodium relictum* (SGS1) has been found in 32 different passerine families (Valkiūnas 2005; Bensch et al. 2009). Thus, the application of these state-of-the-art techniques has made possible the discovery of a wide diversity of parasites within the traditional morphospecies (Bensch et al. 2009). Currently, bird–haemosporidian interactions have become one of the best known host–parasite systems in the field of evolutionary ecology (Arriero and Møller 2008; Garamszegi and Møller 2012; Dodge et al. 2013). However, there are still significant unresolved taxonomic issues that limit the study of the complex multiple-parasite/multiple-host systems (Walther et al. 2016).

Numerous studies have focused on the study of avian haemosporidian diversity in passerines (Valkiūnas 2005), although not all species have received the same attention within each family or genus. For example, starlings and mynas of the genera *Sturnus* and *Acridotheres* (family Sturnidae; Zuccon et al. 2008) show a great difference in the variety of lineages described, where the Common Myna (*Sturnus tristis*, formerly *Acridotheres tristis*) have greater diversity of malaria haplotypes than starlings (MalAvi Database, Bensch et al. 2009). This may be related to a possible higher resistance to malaria in starlings, as shown by experimental studies (Palinauskas et al. 2011; Dimitrov et al. 2015) or, alternatively, by a research bias to mynas due to their popularity as pets in some regions. In fact, the Common Myna has been declared one of the world's 100 worst invasive alien species by the International Union for the Conservation of Nature (IUCN) (Lowe et al. 2004). Studies conducted in this species have identified up to 12 different lineages of avian haemosporidians (Clark et al. 2015; Beadell et al. 2006; Ishtiaq et al. 2007; Martinsen et al. 2008), while the MalAvi database (version 2.3.3 November 2017; Bensch et al. 2009) shows only 9 different lineages for 4 out of the 12 recognized species of starlings (Craig and Feare 2009).

Some lineages of *Plasmodium* (such as GRW4 and OZ14) or *Haemoproteus* (hLAMPUR01) have been recorded in the common starling *Sturnus vulgaris* (Beadell et al. 2006; R.E. Ricklefs, personal observation in Matthews et al. 2016; Valkiūnas et al. 2014; respectively). Here, we report the results of a molecular characterization of avian malaria parasites in its sister species (Zuccon et al. 2008), the spotless starling (*S. unicolor*), a passerine widely distributed throughout one of the most important passages of migratory birds between Europe and Africa. Previous information regarding haemosporidians in this species is scarce and relatively recent (Drovetski et al. 2014; Mata et al. 2015; Muriel et al. 2017). The aim of this descriptive and PCR-based study is twofold. On the one hand, we aim to document the molecular identity of blood parasites infecting a Spanish colony of spotless starlings throughout different years, thereby broadening our knowledge of the diversity of haemosporidian lineages and their phylogenetic relationships in this globally distributed genus. On the other hand, we present an analysis of prevalence of these parasites, examining differences in relation to sex and age that may provide information about patterns of transmission and host resistance.

Materials and methods

Study area and species

This study was conducted in four different breeding seasons (2007, 2011, 2012, 2013) in a nest box population of spotless starlings (*Sturnus unicolor*) located in central Spain (Soto del Real, Madrid, ca. 40° 45′ N, 3° 48′ W, 920–940 m above sea level). The study area is covered by a deciduous woodland of oak (*Quercus pyrenaica*) and ash (*Fraxinus angustifolius*) with abundant open areas used by grazing cattle. It exhibits a continental Mediterranean climate [Köppen–Geiger climate classification: Csb category (reviewed in Peel et al. 2007)] with hot, dry summers. The spotless starling is a relatively long-lived and colonial passerine species that exhibits a facultatively polygynous breeding system (Moreno et al. 1999; Veiga 2002). This species shows sexually dimorphic characters (Cordero et al. 2001; Aparicio et al. 2001), and is closely related to the common starling (*Sturnus vulgaris*) (Zuccon et al. 2008). The spotless starling is mainly sedentary and restricted to Iberia, south-eastern France, the islands of Sicily, Sardinia and Corsica and north-west Africa (Craig

and Feare 2009). Females can lay up to two clutches per season in our study area; the first is started in early April, and the second around the end of May (Salaberria et al. 2014; Muriel et al. 2015). Modal clutch size is five eggs (López-Rull et al. 2007), and fledglings leave the nest around 22 days of age (Cramp 1998).

Field sampling

We caught male and female starlings by traps placed inside nest boxes during the pre-laying period in the 4 years of study (from early March until the first egg of the colony was laid, usually in early April). Those new recruits that had not been marked in previous years were individually marked with a metal ring. From every individual captured, a blood sample was collected from the brachial vein and stored in absolute ethanol at ambient temperature during fieldwork, and then at − 20 °C until molecular analyses. In 2007, we randomly selected 60 adult individuals (30 males and 30 females) to test for the presence of haemosporidian parasites in the circulating blood. From 30 nest boxes, we also took blood samples from two random nestlings (14 days old) per brood, which were processed in the same way in order to determine the prevalence of these parasites in the early stages of development ($n = 60$). During 2007, sampling was evenly distributed between the two breeding attempts, in order to cover the entire breeding season. In addition, and following the same sampling protocol, we randomly selected 40 nestlings and 40 adult individuals (20 males and 20 females) from 2011, 2012 and 2013 to achieve the figure of 240 individuals, although adults were only sampled during the pre-laying period. All sampled birds (total sample size = 360 individuals) were captured and handled with the corresponding permissions and in compliance with the requirements of both regional and Spanish authorities.

Molecular parasite screening

Parasite infections were detected in blood samples using molecular methods (Hellgren et al. 2004; Waldenström et al. 2004). Genomic DNA from avian blood samples was extracted in the laboratory using a standard ammonium acetate protocol (Green et al. 2012). The quality and suitability for PCR analysis of extracted DNA was examined by 1% agarose gel electrophoresis and ethidium bromide staining, and the concentrations were evaluated by optical density at 260 nm (NanoDrop 1000 Spectrophotometers, Thermo Scientific Inc., Waltham, MA). After quantification, extracted DNA was diluted to a standard working concentration of 25 ng/μl. To detect haemosporidian parasites, we used a set of complementary PCR methods targeted to amplify a 479-bp-long fragment of the mitochondrial cytochrome b gene of avian *Haemoproteus* and *Plasmodium* following a modification of the nested PCR protocol developed by Waldenström et al. (2004). We used the nested PCR approach of Hellgren et al. (2004), with an initial amplification of a 617-bp-long fragment common for both genera, with a subsequent nested PCR using combinations of primers from Bensch et al. (2000) and Hellgren et al. (2004). The amplification was evaluated by running 2.5 μl of the final PCR on a 2% agarose gel stained with ethidium bromide under UV light, looking for bands of the appropriate size. We used negative controls (using ddH_2O instead of genomic DNA as template) and positive controls (using DNA from an individual with known malarial infections) to ascertain that the outcome of each PCR run was not affected by contamination.

Thirteen out of the 17 positive samples from 2007 could not be considered for molecular sequencing analysis because of inadequate storage conditions after PCR amplification, and only 4 samples had DNA of enough quality after storage to be sequenced (2 male and 2 female individuals). However, all positive samples from 2011 to 2013 were considered in the sequencing analyses. PCR products were purified with the MSB® Spin PCRapace Clean-Up Kit (Stratec, Birkenfeld, Germany). After purification, fragments of samples from 2007 were bidirectionally sequenced on an ABI 3730 genetic analyser (provided by the Gene Pool Sequencing facility, University of Edinburgh), while those fragments from 2011 to 2013 were sequenced with the forward primers and resolved on an ABI 3130 genetic analyser (provided by the Bioscience Applied Techniques facility, SAIUEx). We edited and aligned sequences using the program BioEdit (Hall 1999), and we used a sequence divergence of at least one nucleotide to define lineages (Waldenström et al. 2002). Mixed infections were recognized by the presence of double peaks on the electropherograms (Pérez-Tris and Bensch 2005).

Cloning of multiple infections

We examined sequences showing double peaks for multiple infections by cloning and sequencing (Pérez-Tris and Bensch 2005). Cloning was performed using the pGEM®-T Easy Vector System (Promega) according to the manufacturer's protocol. Ligation reactions were set up using purified PCR products, and cultures of transformed high-efficiency competent cells were plated onto LB/ampicillin/IPTG/X-Gal plates. Bacterial transformations were carried out using *Escherichia coli* DH5α. Plasmid DNA was isolated and purified using QIAprep® Spin Miniprep kit (Qiagen) according to the manufacturer's instructions. We sequenced using the universal primers M13F and M13R three clones from each sample for which we observed co-infections. The identities of the parasites involved were assessed by comparing the double peak patterns with previously known sequences of avian parasite haplotypes obtained from MalAvi database (Bensch et al. 2009) and GenBank. The new lineages were coded following

the nomenclature of the MalAvi database (Bensch et al. 2009) and deposited in GenBank.

Phylogenetic analyses

For the molecular approach, we analysed lineages of *Plasmodium* sp. and *Haemoproteus* sp. known to infect the genus *Sturnus*. We used Malavi database for obtaining the sequences of the lineages of malaria parasites that are known to infect the species of the genus *Sturnus* (Bensch et al. 2009). A cytochrome b sequence from *Theileria annulata* (KF732030.1) was used as outgroup. Phylogenetic relationships were evaluated using samples for which we had at least 481 bp of cytochrome b gene. Phylogenetic analyses were performed using Bayesian inference (BI) implemented in MrBayes v. 3.2 (Ronquist et al. 2012). The Akaike information criterion (Akaike 1973) implemented in jModeltest (Posada 2008) was used to determine the evolutionary model that best fit the data. In this case, GTR+I+G model was selected (R (a) [AC] = 1.0000, R(b) [AG] = 367.325, R(c) [AT] = 1221.5, R(d) [CG] = 332.73, R(e) [CT] = 7235.9, R(f) [GT] = 1.0000, p-inv = 0.3780). Bayesian inference was performed using two independent runs of four Markov Monte Carlo coupled chains (MCMC) of 5 × 106 generations each to estimate the posterior probability distribution. Topologies were sampled every 100 generations, and a majority rule consensus tree was estimated after discarding the first 10% of generations as burn-in. The robustness of the clades was assessed using Bayesian posterior probabilities.

Results and discussion

Parasite prevalence

The spotless starling is a passerine with a more restricted distribution than the common starling (Craig and Feare 2009), so it has received comparatively less attention in parasitological studies. Some previous information suggests that common starlings may be infected by avian malaria (Rothschild and Clay 1953; Janovy 1966; Martinsen et al. 2007; Valkiunas et al. 2014). However, this information is scarce and relatively recent for the non-migratory spotless starling (Drovetski et al. 2014; Mata et al. 2015; Muriel et al. 2017). Although the colonial lifestyle of spotless starlings may be taken to suggest a higher prevalence derived from a greater attraction of ornitophilic mosquitoes (Ventim et al. 2012), our population showed a relatively low malaria prevalence (*Plasmodium* and *Haemoproteus* combined), with an overall prevalence of 6.94% ($N = 360$). This low prevalence is likely associated with a reduction in transmission rates due to the scarcity of suitable vectors (Tella et al. 1999; Sol et al. 2000; Ortego and Espada 2007) or to variation in mosquito-feeding preferences (Martínez-de la Puente et al. 2015a). For example, it has been shown that some vectors, such as the mosquito *Culex pipiens*, have a high preference for blackbirds and magpies but significantly avoid rock doves and common starlings (Rizzoli et al. 2015). Since the spotless starling is a sedentary species, the infection most likely takes place locally or within a restricted regional range. Additionally, although we do not know the degree of virulence of lineages that we found, weaker, infected individuals could increase their mortality rate during the acute phase of the disease, leading to an underestimation of their prevalence during the breeding season (Bensch et al. 2007). Because the statistical power was reduced due to this low prevalence (the statistical design contained too many zero counts), we explored parasite distribution between breading seasons, breeding attempts, age classes and sexes using chi-squared tests for associations between each factor and infection status. In line with previous evidence showing that the prevalence of vector-borne diseases (i.e. malaria) is particularly responsive to yearly changes in environmental factors (Githeko et al. 2000), we found a strong inter-annual variation in haemosporidian infection in common starlings ($\chi^2 = 18.13$, $P < 0.001$; see Table 1 for prevalence data). These differences could be explained by annual variations in environmental factors such as temperature or rainfall that can affect the abundance of insect vectors and therefore the spread of the infectious diseases (Higgs and Beaty 2005). Similarly, previous studies have also shown between year variations in avian

Table 1 Prevalence of *Plasmodium/Haemoproteus* sp. infection in adult and nestling spotless starlings in four different breeding seasons (2007, 2011, 2012 and 2013) in Soto del Real, Madrid

Breeding season	Infection status	Adults		Nestlings	Total
		Male	Female		
2007	Infected	5	10	2	17
	Uninfected	25	20	58	103
	Total (*n*)	30	30	60	120
	Prevalence (%)	16.67	33.33	3.33	14.17
2011	Infected	0	2	0	2
	Uninfected	20	18	40	78
	Total (*n*)	20	20	40	80
	Prevalence (%)	0	10	0	1.66
2012	Infected	3	3	0	6
	Uninfected	17	17	40	74
	Total (*n*)	20	20	40	80
	Prevalence (%)	15	15	0	5
2013	Infected	0	0	0	0
	Uninfected	20	20	40	80
	Total (*n*)	20	20	40	80
	Prevalence (%)	0	0	0	0

haemosporidian prevalence, suggesting that extrinsic parameters such as environmental conditions affecting vector distribution may explain such fluctuations (Bensch et al. 2007; Ganser et al. 2016; Wilkinson et al. 2016).

Malaria prevalence in the spotless starling population did not show significant differences between first and second broods in 2007 ($\chi^2 = 1.28$, $P = 0.256$). Thus, in the remainder analyses, we pooled all data without considering brood order. We found that the overall malaria prevalence in adults was higher than in nestlings ($\chi^2 = 18.95$, $P < 0.001$). In fact, only two out of a total of 180 chicks showed malarial infection (1.11%), whereas in the case of adults, the proportion was much larger (23 out of 180, 12.77%). We propose several alternative hypotheses to explain these differences. First, once infected with haemosporidians, birds can maintain the infection for years (Valkiūnas 2005). Thus, adult usually have greater prevalence of infections than younger individuals because of their longer period of contact with vectors and hence greater chances to be infected. Second, vectors may not be able to infect nestlings during their stay in the nest, and active transmission often occurs after they leave the nest (Valkiūnas 2005). Third, although malaria infection has been detected in chicks from different bird species (Calero-Riestra and García 2016), the prepatent period (the period between infection with a parasite and the parasite detection in the blood stream) is usually longer than 2 weeks for *Haemoproteus* and *Plasmodium* species (Valkiūnas 2005). Therefore, although nestlings could already be infected in their nests, we would often fail to detect the malaria infection in nestlings of 14 days old because the disease has not yet reached the bloodstream at the time of sampling (Cosgrove et al. 2006, but see Van Oers et al. 2010). In the case of adults, there were no significant differences in the extent of parasitism between males and females ($\chi^2 = 2.27$, $P = 0.131$; see Table 1 for prevalence), although females tended to have a higher parasitemia than males. Similar results have been reported in other species (i.e. Bentz et al. 2006; Asghar et al. 2011), and it has been suggested that females may be the more parasitized gender due to the higher physiological costs of reproduction (but see Calero-Riestra and García 2016). We did not sex nestlings given their low malaria prevalence.

Despite these low overall prevalence results, the spotless starling could be a key local reservoir of avian malaria infections because this species is considered a common target for haemosporidian vectors as suggested by the presence of the P_SYAT05 lineage in mosquito's salivary glands in the Iberian Peninsula (Ventim et al. 2012), and hence could spill over the infection to other species. Nonetheless, to be considered as a reservoir host species, haemosporidian gametocytes (parasite forms that are capable of transmission from bird hosts to mosquito vectors, Valkiūnas 2005) should be present in the blood of spotless starlings, as it was the case in hummingbirds as key reservoir host species for *Haemoproteus witti* (Moens et al. 2016). Unfortunately, in the present study, we did not screen blood smears, and thus, we could not assess the presence of haemosporidian gametocytes in the blood stream of starlings. However, a pilot unpublished study (Muriel, unpublished data) has microscopically identified microgametocytes and macrogametocytes in spotless starlings, suggesting that this bird species may indeed act as a potential reservoir host.

Parasite diversity

In the present study, 4 out of the 17 malaria-positive samples from 2007 and all those from 2011 to 2013 were used to sequence blood parasites. We compared the sequences obtained from our samples with homologous sequences of other avian parasite haplotypes obtained from MalAvi database (version 2.3.3 November 2017; Bensch et al. 2009) and GenBank. Four different parasite haplotypes of *Plasmodium* were found (Table 2, Fig. 1). Interestingly, two of them had not been previously described (P_STURUNI01 and P_STURUNI02). Nevertheless, P_SYAT05 and P_SGS1 had already been found infecting 18 and 71 different genera of passerines, respectively (Bensch et al. 2009). Both lineages were also associated to the same area in another study carried out in Portugal (Ventim et al. 2012). Two out of four sequenced samples from 2007 had mixed infections, in which P_SYAT05 was always present. Even though the closest phylogenetic relation of the spotless starling is the common starling (Zuccon et al. 2006), available data shows that they do not

Table 2 Results of parasite screening, showing the number of infections of each parasite cytochrome b haplotype in each breeding season for males and females in adult spotless starlings

Parasite genus	Haplotype	GenBank accession no.	2007		2011		2012		2013	
			Male	Female	Male	Female	Male	Female	Male	Female
Plasmodium	P_SYAT05	DQ847271	2	2	–	2	1	3	–	–
Plasmodium	P_SGS1	AF495571	–	–	–	–	1	–	–	–
Plasmodium	P_STURUNI01	JQ671031	–	1	–	–	–	–	–	–
Plasmodium	P_STURUNI02	JQ671032	1	–	–	–	1	–	–	–

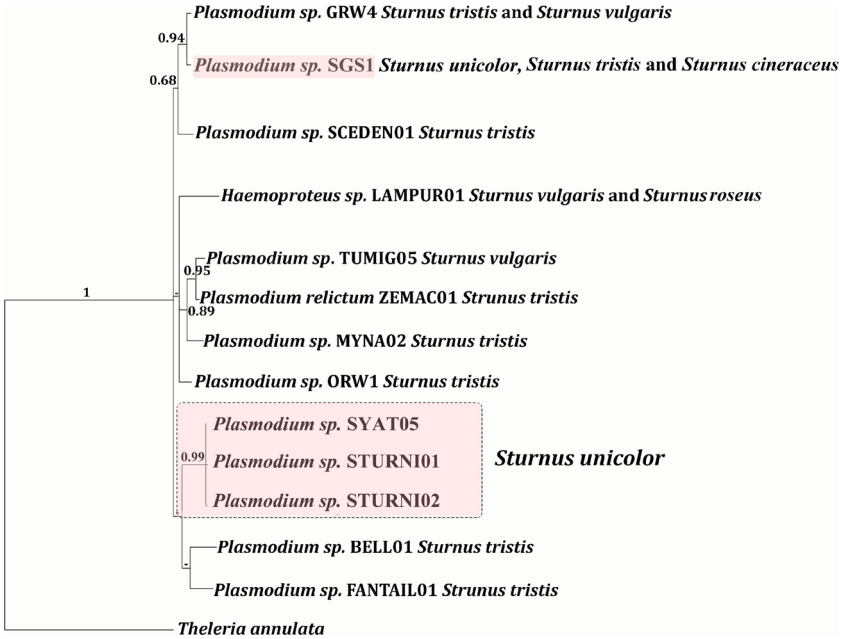

Fig. 1 Phylogenetic tree of lineages obtained in this study (coloured boxes) combined with lineages from MalAvi database (version 2.3.3 November 2017; Bensch et al. 2009). Phylogenetic tree rendered by Bayesian inference of the cytochrome b gene. Numbers on branches indicate posterior probability values (values below 0.60 are indicated as −)

share malaria lineages (Fig. 1). Lineages found in the common starling such as GRW04 (Beadell et al. 2006), TUMIG05 (Martinsen et al. 2007), and LAMPUR01 (Valkiūnas et al. 2014) do not match those found in the spotless starling (Drovetski et al. 2014; Mata et al. 2015; this study). The host generalist P_SYAT05 lineage has also been detected in the blood meal of *Culex theileri*, which had been taken from spotless starling donors (Ventim et al. 2012). Subsequent studies described the presence of P_SYAT05 in wild adult spotless starlings from Portugal (Drovetski et al. 2014; Mata et al. 2015). On the other hand, P_SGS1 is shared with other species within the genus *Sturnus*, such as *Sturnus tristis* and *Sturnus cineraceus* (Beadell et al. 2006; Inumarua et al. 2017). It is known that the *P. relictum* lineage SGS1 is widespread and actively transmitted on every continent except Antarctica (Marzal et al. 2011; Howe et al. 2012; Marzal et al. 2015), although the common starling seems to be resistant to this lineage (Palinauskas et al. 2008). As far as we know, this parasite species had not been previously reported infecting *S. unicolor* (MalAvi database version 2.3.3 November 2017; Bensch et al. 2009). We did not verify the presence of parasites in vectors, but the lineages described in this study might have been potentially transmitted by the *Aedes* mosquitoes, since we have documented blood sucking by this species on day 6 posthatch on spotless starling nestlings (see Fig. 2). In fact, a study has shown that P_SYAT05 is transmitted by *Aedes albopictus* (Martínez-de la Puente et al. 2015b). However, further studies are required to analyse the competence of *Aedes* species as vectors in the transmission of these malaria lineages. From the point of view of the vector, the prevalence of the different lineages could be conditioned by the differences in distribution patterns of mosquitoes among sites and its specificity. In addition, the overall prevalence could be conditioned not only by the virulence of each lineage (Read and Taylor 2001) but also by the presence of multiple infections (Frank 1996; Bensch et al. 2004; Marzal et al. 2008).

In the present study, we analysed differences in parasite prevalence between breeding seasons, ages and sexes infecting spotless starlings, as well as the molecular identity of its avian haemosporidians (*Plasmodium* and *Haemoproteus* genera). The presence of parasites in the bloodstream was not conditioned by the breeding attempt or the gender of adult hosts, but we found differences between years and age classes. Thus, malaria prevalence had strong inter-annual variability, and it was much lower in nestlings than adults. Despite the overall low prevalence found in this population, we observed that the parasite diversity was relatively high after sequencing up to four different lineages of malaria parasites from 12

Fig. 2 *Aedes* mosquito blood sucking on spotless starling nestling on day 6 posthatch. The arrow tip points to the location of the vector. Picture credit: Jaime Muriel

infected starlings. The phylogenetic analysis using a Bayesian inference revealed a well-supported monophyletic group formed by STURUNI01, STURUNI02 and SYAT05 (Fig. 1). In addition, we report for the first time the presence of *P. relictum* (lineage SGS1) in the spotless starling, despite reported resistance shown by its sister species, the common starling. This work highlights the importance of this species as a reservoir of different malaria lineages, which could have consequences in the transmission of the disease to other avian species. Although the prevalence is low in the spotless starling, this species is very abundant and widely distributed in the Iberian Peninsula, so it could be considered a local reservoir of avian malaria. Numerous migratory birds that use this route in their way to or from Africa could be at infection risk, especially for host-generalized lineages of avian Haemosporidia (Mata et al. 2015), such as SYAT05 and SGS1.

Acknowledgments This study was financed by the projects CGL2008-03501 (Ministerio de Ciencia e Innovación) to DG and CGL2015-6465P (Ministerio de Economía y Competitividad) and IB16121 (Junta de Extremadura) to AM. JM was supported by a FPI grant (BES-2009-021383) from the Ministerio de Ciencia e Innovación (MICINN). We performed all the laboratory work at the School of Biology at the University of St Andrews and the Department of Zoology at the University of Extremadura. Sequencing was performed by the Gene Pool Sequencing facility, University of Edinburgh. The authors appreciate the technical and human support provided by Facility of Bioscience Applied Techniques of SAIUEx (financed by UEx, Junta de Extremadura, MICINN, FEDER, and FSE). The authors would like to thank sincerely Dr. A. Pérez-Rodríguez for his useful guidelines in interpreting sequences and identifying new lineages. We also thank two anonymous referees for their constructive suggestions that helped to improve our study.

Compliance with ethical standards All applicable international, national and/or institutional guidelines for the care and use of animals were followed. Capture and manipulation of birds were authorized by the Consejería de Medio Ambiente (Comunidad de Madrid, Spain) under licence from the Spanish institutional authorities (Consejería de Medio Ambiente 10/0245533.09/13; and 11009 ringing licence to DG from Centro de Migración de Aves de SEO/BirdLife). Permission to work in the study area was granted by the Ayuntamiento de Soto del Real, Spain.

Conflict of interest The authors declare that they have no conflict of interest.

References

Akaike H (1973) Maximum likelihood identification of Gaussian autoregressive moving average models. Biometrika 60(2):255–265.https://doi.org/10.1093/biomet/60.2.255

Aparicio JM, Cordero PJ, Veiga JP (2001) A test of the hypothesis of mate choice bassed on heterozygosity in the spotless starling. Anim Behav 61:1001–1006

Arriero E, Møller AP (2008) Host ecology and life-history traits associated with blood parasite species richness in birds. J Evol Biol 21(6):1504–1513. https://doi.org/10.1111/j.1420-9101.2008.01613.x

Asghar M, Hasselquist D, Bensch S (2011) Are chronic avian haemosporidian infections costly in wild birds? J Avian Biol 42(6):530–537. https://doi.org/10.1111/j.1600-048X.2011.05281.x

Asghar M, Hasselquist D, Hansson B, Zehtindjiev P, Westerdahl H, Bensch S (2015) Hidden costs of infection: chronic malaria accelerates telomere degradation and senescence in wild birds. Science 347(6220):436–438. https://doi.org/10.1126/science.1261121

Atkinson CT & van Riper C III (1991) Pathogenicity and epizootiology of avian haematozoa: *Plasmodium, Leucocytozoon*, and *Haemoproteus.* In Loye JE, Zuk M, editors. Bird–parasite interactions. Ecology, evolution, and behavior. New York: Oxford University Press p 19–48

Atkinson CT, Dusek RJ, Woods KL, Iko WM (2000) Pathogenicity of avian malaria inexperimentally-infected Hawaii Amakihi. J Wildl Dis 36(2):197–204. https://doi.org/10.7589/0090-3558-36.2.197

Atkinson CT, Utzurrum RB, Lapointe DA, Camp RJ, Crampton LH, Foster JT, Giambelluca TW (2014) Changing climate and the altitudinal range of avian malaria in the Hawaiian Islands—an ongoing conservation crisis on the island of Kaua'i. Glob Change Biol 20(8): 2426–2436. https://doi.org/10.1111/gcb.12535

Beadell JS, Ishtiaq F, Covas R, Melo M, Warren BH, Atkinson CT, Bensch S, Graves GR, Jhala YV, Peirce MA (2006) Global phylogeographic limits of Hawaii's avian malaria. Proc R Soc Lond B 273(1604):2935–2944

Bensch S, Stjernman M, Hasselquist D, Östman Ö, Hansson B, Westerdahl H, Torres-Pinheiro R (2000) Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proc R Soc Lond B Biol Sci 267(1452):1583–1589. https://doi.org/10.1098/rspb.2000.1181

Bensch S, Pérez-Tris J, Waldenström J, Hellgren O (2004) Linkage between nuclear and mitochondrial DNA sequences in avian malaria parasites: multiple cases of cryptic speciation? Evolution 58(7): 1617–1621. https://doi.org/10.1111/j.0014-3820.2004.tb01742.x

Bensch S, Waldenström J, Jozén N, Westerdahl H, Hansson B, Sejberg D, Hasselquist D (2007) Temporal dynamics and diversity of avian malaria parasites in a single host species. J Anim Ecol 76(1):112–122. https://doi.org/10.1111/j.1365-2656.2006.01176.x

Bensch S, Hellgren O, Peréz-Tris J (2009) MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. Mol Ecol Resour 9(5): 1353–1358. https://doi.org/10.1111/j.1755-0998.2009.02692.x

Bentz S, Rigaud T, Barroca M, Martin-Laurent F, Bru D, Moreau J, Faivre B (2006) Sensitive measure of prevalence and parasitaemia of haemosporidia from European blackbird (*Turdus merula*) populations: value of PCR-RFLP and quantitative PCR. Parasitology 133(06):685–692. https://doi.org/10.1017/S0031182006001090

Calero-Riestra M, García JT (2016) Sex-dependent differences in avian malaria prevalence and consequences of infections on nestling growth and adult condition in the Tawny pipit, *Anthus campestris*. Malar J 15(1):178. https://doi.org/10.1186/s12936-016-1220-y

Clark NJ, Clegg SM, Lima MR (2014) A review of global diversity in avian haemosporidians (*Plasmodium* and *Haemoproteus*: Haemosporidia): new insights from molecular data. Int J Parasitol 44(5):329–338. https://doi.org/10.1016/j.ijpara.2014.01.004

Clark NJ, Olsson-Pons S, Ishtiaq F, Clegg SM (2015) Specialist enemies, generalist weapons and the potential spread of exotic pathogens: malaria parasites in a highly invasive bird. Int J Parasitol 45(14): 891–899. https://doi.org/10.1016/j.ijpara.2015.08.008

Cordero PJ, Vinuela J, Aparicio JM, Veiga JP (2001) Seasonal variation in sex ratio and sexual egg dimorphism favouring daughters in first clutches of the spotless starling. J Evol Biol 14(5):829–834. https://doi.org/10.1046/j.1420-9101.2001.00320.x

Cosgrove CL, Knowles SCL, Day KP, Sheldon BC (2006) No evidence for avian malaria infection during the nestling phase in a passerine bird. J Parasitol 92(6):1302–1304. https://doi.org/10.1645/GE-878R.1

Craig AJFK & Feare CJ (2009) Family Sturnidae (Starlings) in *Handbook of the Birds of the World*, Vol.14, eds J. del Hoyo, A Elliott, and D Christie (Barcelona: Lynx Edicions), 654-758

Cramp S (1998) The complete birds of the western Palaearctic. University Press, OptiMedia, CD-ROM, Oxford

Deviche P, McGraw K, Greiner E (2005) Interspecific differences in hematozoan infection in Sonoran Desert Aimophila sparrows. J Wildl Dis 41(3):532–541. https://doi.org/10.7589/0090-3558-41.3.532

Dimitrov D, Palinauskas V, Iezhova TA, Bernotienė R, Ilgūnas M, Bukauskaitė D, Zehtindjievb P, Anatoly M, Casimir S, Mikhail B, Markovetsd Y, Benschc S, Valkiūnasa G (2015) *Plasmodium* spp.: an experimental study on vertebrate host susceptibility to avian malaria. Exp Parasitol 148:1–16. https://doi.org/10.1016/j.exppara.2014.11.005

Dodge M, Guers SL, Sekercioğlu ÇH, Sehgal RN (2013) North American transmission of Hemosporidian parasites in the Swainson's thrush (*Catharus ustulatus*), a migratory songbird. J Parasitol 99(3):548–553. https://doi.org/10.1645/GE-3134.1

Drovetski SV, Aghayan SA, Mata VA, Lopes RJ, Mode NA, Harvey JA, Voelker G (2014) Does the niche breadth or trade-off hypothesis explain the abundance–occupancy relationship in avian Haemosporidia? Mol Ecol 23(13):3322–3329. https://doi.org/10.1111/mec.12744

Frank SA (1996) Models of parasite virulence. Q Rev Biol 71(1):37–78. https://doi.org/10.1086/419267

Ganser C, Gregory AJ, McNew LB, Hunt LA, Sandercock BK, Wisely SM (2016) Fine-scale distribution modeling of avian malaria vectors in north-central Kansas. J Vector Ecol 41(1):114–122. https://doi.org/10.1111/jvec.12202

Garamszegi LZ, Møller AP (2012) The interspecific relationship between prevalence of blood parasites and sexual traits in birds when considering recent methodological advancements. Behav Ecol Sociobiol 66(1):107–119. https://doi.org/10.1007/s00265-011-1259-2

Githeko AK, Lindsay SW, Confalonieri UE, Patz JA (2000) Climate change and vector-borne diseases: a regional analysis. Bull World Health Organ 78(9):1136–1147

Green M, Hughes H, Sambrook J, MacCallum P (2012) Molecular cloning: a laboratory manual, fourth edn. CSH Press, UK

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Hellgren O, Waldenstrom J, Bensch S (2004) A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. J Parasitol 90(4):797–802. https://doi.org/10.1645/GE-184R1

Higgs S & Beaty BJ (2005) Natural cycles of vector-borne pathogens. Biology of disease vectors, In Biology of disease vectors 2nd edn (eds Marquardt, W. C. Et al.) Elsevier Academic Press (New York)

Howe L, Castro IC, Schoener ER, Hunter S, Barraclough RK, Alley MR (2012) Malaria parasites (*Plasmodium* spp.) infecting introduced, native and endemic New Zealand birds. Parasitol Res 110(2):913–923. https://doi.org/10.1007/s00436-011-2577-z

Janovy J Jr (1966) Epidemiology of *Plasmodium hexamerium* Huff, 1935, in meadowlarks and starlings of the Cheyenne Bottoms, Barton County, Kansas. J Parasitol 52(3):573–578. https://doi.org/10.2307/3276329

Inumarua M, Muratab K, Sato Y (2017) Prevalence of avian Haemosporidia among injured wild birds in Tokyo and environs, Japan. Int J Parasitol Parasites Wildl 6(3):299–309. https://doi.org/10.1016/j.ijppaw.2017.09.007

Isaksson C, Sepil I, Baramidze V, Sheldon BC (2013) Explaining variance of avian malaria infection in the wild: the importance of host density, habitat, individual life-history and oxidative stress. BMC Ecol 13(1):15. https://doi.org/10.1186/1472-6785-13-15

Ishtiaq F, Gering E, Rappole JH, Rahmani AR, Jhala YV, Dove CJ, Milensky C, Olson SL, Peirce MA & and Fleischer RC (2007). Prevalence and diversity of avian hematozoan parasites in Asia: a regional survey. J Wildlife Diseases 43(3):382–398, DOI: https://doi.org/10.7589/0090-3558-43.3.382

Keymer AE, Anderson RM (1979) The dynamics of infection of *Tribolium confusum* by *Hymenolepis diminuta*: the influence of infective-stage density and spatial distribution. Parasitology 79(02):195–207. https://doi.org/10.1017/S0031182000053282

Knowles SCL, Palinauskas V, Sheldon BC (2010) Chronic malaria infections increase family inequalities and reduce parental fitness: experimental evidence from wild bird population. J Evol Biol 23(3):557–569. https://doi.org/10.1111/j.1420-9101.2009.01920.x

Levine ND (1988) The protozoan phylum Apicomplexa. CRC Press, Baca Raton

López-Rull I, Celis P, Gil D (2007) Egg colour covaries with female expression of a male ornament in the spotless starling (*Sturnus unicolor*). Ethology 113(10):926–933. https://doi.org/10.1111/j.1439-0310.2007.01400.x

Lowe S, Browne M, Boudjelas S & De Poorter M (2004) 100 of the World's worst invasive alien species—a selection from the global invasive species database. Invasive Species Specialist Group (Species Survival Commission) of the World Conservation Union (IUCN). Downloaded from http://www.iucngisd.org/gisd/100_worst.php on 20-12-2017

Loy DE, Liu W, Li Y, Learn GH, Plenderleith LJ, Sundararaman SA, Sharp PM, Hahn BH (2016) Out of Africa: origins and evolution of the human malaria parasites *Plasmodium falciparum* and *Plasmodium vivax*. Int J Parasitol 47:87–97

Martínez-de la Puente J, Figuerola J, Soriguer R (2015a) Fur or feather? Feeding preferences of species of Culicoides biting midges in Europe. Trends Parasitol 31(1):16–22. https://doi.org/10.1016/j.pt.2014.11.002

Martínez-de la Puente J, Muñoz J, Capelli G, Montarsi F, Soriguer R, Arnoldi D, Rizzoli A, Figuerola J (2015b) Avian malaria parasites in the last supper: identifying encounters between parasites and the invasive Asian mosquito tiger and native mosquito species in Italy. Malar J 14(1):32. https://doi.org/10.1186/s12936-015-0571-0

Martinsen ES, Waite JL, Schall JJ (2007) Morphologically defined subgenera of Plasmodium from avian hosts: test of monophyly by phylogenetic analysis of two mitochondrial genes. Parasitology 134(04):483–490. https://doi.org/10.1017/S0031182006001922

Martinsen ES, Perkins SL, Schall JJ (2008) A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): evolution of life-history traits and host switches. Mol Phylogenet Evol 47(1):261–273. https://doi.org/10.1016/j.ympev.2007.11.012

Marzal A, De Lope F, Navarro C, Møller AP (2005) Malarial parasites decrease reproductive success: an experimental study in a passerine bird. Oecologia 142(4):541–545. https://doi.org/10.1007/s00442-004-1757-2

Marzal A, Bensch S, Reviriego M, Balbontin J, De Lope F (2008) Effects of malaria double infection in birds: one plus one is not two. J Evol Biol 21:979–987.

Marzal A, Ricklefs RE, Valkiūnas G, Albayrak T, Arriero E, Bonneaud C, Czirják GA, Ewen J, Hellgren O, Hořáková D, Iezhova TA, Jensen H, Križanauskienė A, Lima MR, de Lope F, Magnussen E, Martin LB, Møller AP, Palinauskas V, Pap PL, Pérez-Tris J, Sehgal RNM, Soler M, Szöllősi E, Westerdahl H, Zetindjiev P, Bensch S (2011) Diversity, loss, and gain of malaria parasites in a globally invasive bird. PLoS One 6(7):e21905. https://doi.org/10.1371/journal.pone.0021905

Marzal A, García-Longoria L, Cárdenas Callirgos JM, Sehgal RN (2015) Invasive avian malaria as an emerging parasitic disease in native birds of Peru. Biol Invasion 17(1):39–45. https://doi.org/10.1007/s10530-014-0718-x

Marzal A, Balbontín J, Reviriego M, García-Longoria L, Relinque C, Hermosell IG, Magallanes S, López-Calderón C, de Lope F, Møller AP (2016) A longitudinal study of age-related changes in *Haemoproteus* infection in a passerine bird. Oikos 125(8):1092–1099. https://doi.org/10.1111/oik.02778

Mata VA, da Silva LP, Lopes RJ, Drovetski SV (2015) The Strait of Gibraltar poses an effective barrier to host-specialised but not to host-generalised lineages of avian Haemosporidia. Int J Parasitol 45(11):711–719. https://doi.org/10.1016/j.ijpara.2015.04.006

Matthews AE, Ellis, VA, Hanson AA, Roberts JR, Ricklefs RE, Collins MD (2016) Avian haemosporidian prevalence and its relationship to host life histories in eastern Tennessee. J Ornithol 157(2):533–548

McCurdy DG, Shutler D, Mullie A, Forbes MR (1998) Sex-biased parasitism of avian hosts: relations to blood parasite taxon and mating system. Oikos 82(2):303–312. https://doi.org/10.2307/3546970

Moens MAJ, Valkiūnas G, Paca A, Bonaccorso E, Aguirre N, Pérez-Tris J (2016) Parasite specialization in a unique habitat: hummingbirds as reservoirs of generalist blood parasites of Andean birds. J Anim Ecol 85(5):1234–1245. https://doi.org/10.1111/1365-2656.12550

Moreno J, Veiga JP, Cordero PJ, Minguez E (1999) Effects of paternal care on reproductive success in the polygynous spotless starling *Sturnus unicolor*. Behav Ecol Sociobiol 47(1-2):47–53. https://doi.org/10.1007/s002650050648

Muriel J, Salmón P, Nunez-Buiza A, Salas FD, Pérez-Rodríguez L, Puerta M, Gil D (2015) Context-dependent effects of yolk androgens on nestling growth and immune function in a multibrooded passerine. J Evol Biol 28(8):1476–1488. https://doi.org/10.1111/jeb.12668

Muriel J, Pérez-Rodríguez L, Ortiz-Santaliestra ME, Puerta M, Gil D (2017) Sex-specific effects of high yolk androgen levels on constitutive and cell-mediated immune responses in nestlings of an altricial passerine. Physiol Biochem Zool 90(1):106–117. https://doi.org/10.1086/688445

Ortego J, Espada F (2007) Ecological factors influencing disease risk in eagle owls *Bubo bubo*. Ibis 149(2):386–395. https://doi.org/10.1111/j.1474-919X.2007.00656.x

Outlaw DC, Ricklefs RE (2014) Species limits in avian malaria parasites (Haemosporida): how to move forward in the molecular era. Parasitology 141(10):1223–1232. https://doi.org/10.1017/S0031182014000560

Palinauskas V, Valkiūnas G, Bolshakov VC, Bensch S (2008) Effects of *Plasmodium relictum* (lineage P-SGS1) on experimentally infected passerine birds. Exp Parasitol 120(4):372–380. https://doi.org/10.1016/j.exppara.2008.09.001

Palinauskas V, Valkiūnas G, Bolshakov CV, Bensch S (2011) *Plasmodium relictum* (lineage SGS1) and *Plasmodium ashfordi* (lineage GRW2): the effects of the co-infection on experimentally infected passerine birds. Exp Parasitol 127(2):527–533. https://doi.org/10.1016/j.exppara.2010.10.007

Peel MC, Finlayson BL, McMahon TA (2007) Updated world map of the Köppen–Geiger climate classification. Hydrol Earth Syst Sci 11(5):1633–1644. https://doi.org/10.5194/hess-11-1633-2007

Posada D (2008) jModelTest: phylogenetic model averaging. Mol Biol Evol 25(7):1253–1256. https://doi.org/10.1093/molbev/msn083

Pérez-Tris J, Bensch S (2005) Diagnosing genetically diverse avian malarial infections using mixed-sequence analysis and TA-cloning. Parasitology 131(1):15–23. https://doi.org/10.1017/S003118200500733X

Pérez-Tris J, Hellgren O, Križanauskienė A, Waldenström J, Secondi J, Bonneaud C, Fjeldså J, Hasselquist D, Bensch S (2007) Within-host speciation of malaria parasites. PLoS One 2(2):e235. https://doi.org/10.1371/journal.pone.0000235

Read AF, Taylor LH (2001) The ecology of genetically diverse infections. Science 292(5519):1099–1102. https://doi.org/10.1126/science.1059410

Ricklefs RE, Outlaw DC, Svensson-Coelho M, Medeiros MC, Ellis VA, Latta S (2014) Species formation by host shifting in avian malaria parasites. Proc Natl Acad Sci U S A 111(41):14816–14821. https://doi.org/10.1073/pnas.1416356111

Rizzoli A, Bolzoni L, Chadwick EA, Capelli G, Montarsi F, Grisenti M, Martínez de la Puente J, Muñoz J, Figuerola J, Soriguer R, Anfora G, Di Luca M, Rosà R (2015) Understanding West Nile virus ecology in Europe: *Culex pipiens* host feeding preference in a hotspot of virus emergence. Parasit Vectors 8(1):213. https://doi.org/10.1186/s13071-015-0831-4

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across large model space. Syst Biol 61(3):539–542. https://doi.org/10.1093/sysbio/sys029

Rothschild M, Clay T (1953) Fleas, flukes and cuckoos. Readers Union, Collins, London, pp 235–237

Salaberria C, Celis P, Lopez-Rull I, Gil D (2014) Effects of temperature and nest heat exposure on nestling growth, dehydration and survival in a Mediterranean hole-nesting passerine. Ibis 156(2):265–275. https://doi.org/10.1111/ibi.12121

Sheldon BC, Verhulst S (1996) Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. Trends Ecol Evol 11(8):317–321. https://doi.org/10.1016/0169-5347(96)10039-2

Sol D, Jovani D, Torres J (2000) Geographical variation in blood parasites in feral pigeons: the role of vectors. Ecography 23(3):307–314. https://doi.org/10.1111/j.1600-0587.2000.tb00286.x

Sol D, Jovani R, Torres J (2003) Parasite mediated mortality and host immune response explain age-related differences in blood parasitism in birds. Oecologia 135(4):542–547. https://doi.org/10.1007/s00442-003-1223-6

Tella JL, Blanco G, Forero MG, Gajón A, Donázar JA, Hiraldo F (1999) Habitat, world geographic range, and embryonic development of hosts explain the prevalence of avian hematozoa at small spatial and phylogenetic scales. Proc Natl Acad Sci U S A 96(4):1785–1789. https://doi.org/10.1073/pnas.96.4.1785

Valkiūnas G, Olsen H, Iezhova T (2000) Haemoparasites in the hooded crow, *Corvuscorone cornix L.*, in the vicinity of Copenhagen. Bull Scand Soc Parasitol 10:1–4

Valkiūnas G (2005) Avian malaria parasites and other Haemosporidia. CRC Press, Boca Raton

Valkiunas G, Palinauskas V, Ilgunas M, Bukauskaite D, Dimitrov D, Bernotiene R, Zehtindjiev P, Ilieva M, Iezhova T (2014) Molecular characterization of five widespread avian haemosporidian parasites (Haemosporida), with perspectives on the PCR-based detection of haemosporidians in wildlife. Parasitol Res 113:2551–2263

Van Oers K, Richardson D, Sæther S, Komdeur J (2010) Reduced blood parasite prevalence with age in the Seychelles warbler: selective mortality or suppression of infection? J Ornithol 151(1):69–77. https://doi.org/10.1007/s10336-009-0427-x

Veiga, J.P. 2002. Estornino Negro—*Sturnus unicolor*. In: Carrascal LM, Salvador A (eds) Enciclopedia Virtual de los Vertebrados Españoles Madrid: Museo Nacional de Ciencias Naturales. http://www.vertebradosibericos.org/

Ventim R, Ramos JA, Osório H, Lopes RJ, Pérez-Tris J, Mendes L (2012) Avian malaria infections in western European mosquitoes. Parasitol Res 111(2):637–645. https://doi.org/10.1007/s00436-012-2880-3

Waldenström J, Bensch S, Kiboi S, Hasselquist D, Ottosson U (2002) Crossspecies infection of blood parasites between resident and migratory songbirds in Africa. Mol Ecol 11(8):1545–1554. https://doi.org/10.1046/j.1365-294X.2002.01523.x

Waldenström J, Bensch S, Hasselquist D, Östman Ö (2004) A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. J Parasitol 90(1):191–194. https://doi.org/10.1645/GE-3221RN

Walther EL, Carlson JS, Cornel A, Morris BK, Sehgal RN (2016) First molecular study of prevalence and diversity of avian Haemosporidia

in a Central California songbird community. J Ornithol 157(2):549–564. https://doi.org/10.1007/s10336-015-1301-7

Wilkinson LC, Handel CM, Van Hemert C, Loiseau C, Sehgal RN (2016) Avian malaria in a boreal resident species: long-term temporal variability, and increased prevalence in birds with avian keratin disorder. Int J Parasitol 46(4):281–290. https://doi.org/10.1016/j.ijpara.2015.12.008

Wilson K, Bjørnstad ON, Dobson AP, Merler S, Poglayen G, Randolph S, Read AF, Skorping A (2001) Heterogeneities in macroparasite infections: patterns and processes. In: Hudson PJ, Rizzoli A, Grenfell BT, Heesterbeek H, Dobson AP (eds) The Ecology of Wildlife Diseases. Oxford University Press, Oxford, pp 6–44

Zuccon D, Cibois A, Pasquet E, Ericson PGP (2006) Nuclear and mitochondrial sequence data reveal the major lineages of starlings, mynas and related taxa. Mol Phylogenet Evol 41(2):333–344. https://doi.org/10.1016/j.ympev.2006.05.007

Zuccon D, Pasquet E, Ericson PGP (2008) Phylogenetic relationships among PalearcticOriental starlings and mynas (genera *Sturnus* and *Acridotheres*: Sturnidae). Zool Scr 37(5):469–481. https://doi.org/10.1111/j.1463-6409.2008.00339.x