Avian haemosporidian infections in rufous-collared sparrows in an Andean dry forest: diversity and factors related to prevalence and parasitaemia

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Abstract

Despite intensive research during the last few decades, understanding of ecological and physiological factors related to haemosporidian infections in birds is still fragmentary. Since more model organisms are needed in order to understand these infections in the wild, we analysed avian haemosporidian infections in the rufous-collared sparrow (*Zonotrichia capensis*, Emberizidae) in a dry forest of the Ecuadorian Andes. Parasite diversity was screened using molecular and morphological approaches. By molecular diagnosis, we identified three lineages that were phylogenetically placed in the context of molecular haemosporidian diversity and associated with a morphospecies. By microscopy, we identified five described morphospecies and one additional undescribed morphospecies. We found that avian haemosporidian prevalence on the study site was 76.3%. Additionally, we used a series of generalized linear models to explore the potential relationship of parasite prevalence and parasitaemia with a set of variables related to physiological and environmental conditions. Although our results revealed associations of haemosporidian infections with precipitation, age and sampling site, the models only explained a small fraction of the variation.

Introduction

Avian haemosporidian infections are caused by *Plasmodium* malaria parasites, but also by evolutionary closely related 'malaria-like' parasites *Haemoproteus* belonging to Apicomplexa protozoans, order Haemosporida (Pérez-Tris *et al.*, 2005). These parasites infect the organs and blood of avian hosts (Valkiūnas, 2005), and are transmitted by dipteran vectors (Santiago-Alarcón *et al.*, 2012). Despite intensive research during the last few decades, understanding of ecological and physiological factors related to the acquisition of infection, as well as its consequences in both hosts and vectors, is still fragmentary. For example, parasitaemia may increase with vector abundance, but not necessarily because of novel parasite transmissions, but as a plastic response of parasites to increased probability of transmission to other hosts (e.g. Cornet *et al.*, 2014). In the same way, effects of infection on host fitness need detailed studies, especially in wild populations (e.g. Knowles *et al.*, 2011; Asghar *et al.*, 2015; Coon *et al.*, 2016). Thus, it is important to keep identifying 'model organisms' that may be easily studied in the wild, to reach a deeper understanding of avian haemosporidian infections, especially in the tropics.

The rufous-collared sparrow, *Zonotrichia capensis*, has a broad geographic distribution from southern Mexico to Patagonia, Argentina, including non-Amazonian Brazil, the Guianas and Venezuela, occupying open spaces, from sea-level to high elevation (Rising and Jaramillo, 2017). In Ecuador, it is common along the Andes, at 1500–3500 m a.s.l. (Ridgely and Greenfield, 2001). Prevalence of haemosporidian infections in this host has been reported to reach up to 42% of local populations at mid altitude (2000–3300 m a.s.l.) in Andean forests of western Peru (Jones *et al.*, 2013) and up to 73% in the foothills of the Ecuadorian Amazon (1500 m a.s.l.; Escallón *et al.*, 2016). Such high prevalence has been associated with higher major histocompatibility complex diversity, suggesting important parasite-mediated selection on these genes central in the immune response (Jones *et al.*, 2014).

Given the high prevalence of haemosporidian infections reported for rufous-collared sparrows and the implications that it may have for understanding infection dynamics and host resilience, we focused our study on a population of this species in an underrepresented and relatively simple ecosystem: the Andean dry forest. To obtain a first approximation to the system we: (1) identified molecular lineages and morphospecies of Haemosporida parasites infecting this host, as well as their prevalence and parasitaemia; (2) assessed differences in prevalence and parasitaemia caused by different parasites and (3) analysed biotic and abiotic

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factors associated with infection status and parasitaemia. Based on the relationship between water availability and vector abundance, we hypothesize that both parasitaemia and prevalence of infection would be highly dependent on precipitation in this dry forest ecosystem. Additionally, low diversity of parasite lineages would be expected given a relative low diversity of potential hosts found previously in the study area (Cadena-Ortiz et al., 2015), and infection status (infected vs non-infected) by different parasite lineages should have different effects on host body condition.

Materials and methods

Study area and field methods

Fieldwork was conducted at Bosque Protector Jerusalem (BPJ), a 1110-ha protected tropical dry forest, ~10 km north of Quito (00° 00′05″N, 78°21′18″W), in the Andean valley of Guayllabamba, Ecuador (Fig. 1). The dry season takes place from May to August (125 mm rainfall average), while the wet season occurs from September to April (360 mm rainfall average); average annual temperature under shade is 19 °C (data provided by Instituto Nacional de Meteorología e Hidrología, INAMHI). The study area is mostly covered by 'semi-deciduous forest and scrub', characteristic of the northern portion of the inter-Andean valleys of Ecuador (MAE, 2013). This ecosystem is dominated by algarrobo trees (Acacia macracantha), upon which abundant epiphytic vegetation develops, as well as cacti of various genera (e.g. Opuntia sp. and Cleistocactus sp.).

Mist-netting was carried out between December 2012 and June 2013, at four sampling sites that aimed to account for the environmental heterogeneity of the reserve (Fig. 1). Sites 1 and 3 were located over a trail within the 'core' of the dry forest, and were separated by 320 m. These two sites are bordered on both sides by dense, native vegetation. There, the canopy is dominated by Acacia sp. (<4 m), harbouring a great variety of epiphytes, and Cactaceae are abundant in the understory. Sites 2 and 4 were established on a more disturbed area of the reserve and separated by 200 m. Site 2 was located within a plantation of Citrus sp. and Persea americana (<3 m); along the borders of this plantation, remnant patches of native vegetation still exist, alternated by introduced trees of Eucalyptus sp. (<10 m). Site 4 was located on a path within the dry forest, where native vegetation exists but in lower density than on sites 1 and 3. Sites 1 and 3 were separated from sites 2 and 4 by more than 600 m. Each site was visited in sequential order (1–4), conducting a total of six visits per site (1.5-day visit per month, per site). At each site, we placed seven mist-nets (four shelves, 12 m × 2.5 m; 36 mm mesh). Nets were opened from 6:00 to 18:00 h the first day, and from 6:00 to 12:00 the second day. All birds captured were weighed to the nearest 0.1 g, and measured to the nearest 0.01 mm (bill, tarsus) or the nearest millimetre (wing). We obtained blood samples from the jugular or brachial vein using disposable needles; 5–20 μl were used to prepare a blood smear (for microscopic analysis) and 20 μl were preserved in 99% ethanol solution (for molecular analysis). At the end of this process, birds were banded and released. After air drying, smears where fixed with methanol for 3 min in the field and then stained with Giemsa for 60 min in the laboratory (Waldenström et al., 2004).

Molecular lineage identification and phylogenetic relationships

To determine the identity of haemosporidian lineages, as well as their evolutionary relationships, we performed phylogenetic analyses based on partial sequences of the mitochondrial gene cytochrome b (cytb). Total genomic DNA was extracted from blood using a lysis protocol with proteinase K and protein precipitation with guanidine thiocyanate (Chomczynski, 1993). DNA amplification was conducted based on a non-nested polymerase chain reaction (PCR) protocol, using primers HaemF and HaemR2 (Waldenström et al., 2004). PCR reactions were prepared in 25 μl volumes with 5 μl of genomic DNA, 3 mM MgCl$_2$, PCR buffer (Invitrogen Inc., Carlsbad, USA), 0.4 mM dNTPs, 0.6 μM of each primer and 1.25 U Platinum Taq polymerase (Invitrogen), using the PCR profile described in Waldenström et al. (2004). Final PCR products were stained with SYBR Safe (Invitrogen) and visualized on a 1% agarose gel. When samples tested negative, we used 1 μl of the PCR product, along with the same reagent proportions and thermocycler profile (using 20 cycles) in a second PCR, to try to rule out false negatives. PCR amplicons from positively infected individuals were purified using ExoSAP-IT (Affymetrix Inc., Santa Clara, USA) and sequenced in an ABI 3730XL sequence analyzer (Applied Biosystems Inc., Foster City, USA), with the same primers used for PCR. Chromatograms of parasite cytb were assembled and edited with Geneious 5.1.7 (Kearse et al., 2012).

Sequences depicting single infections were aligned automatically in Clustal X2 (Larkin et al., 2007) to identify samples infected with identical lineages. Sequences showing double peaks in chromatograms were treated as coinfections and resolved through computational phasing in the program PHASE (Stephens and Donnelly, 2003) as implemented in DNAsp 5 (Librado and Rozas, 2009). In the PHASE analysis, we included nine sequences with coinfections and ten additional sequences – three identified during molecular diagnosis (see Results) and seven additional sequences that correspond to the molecular lineages associated with morphospecies found during morphological identification (five Haemoproteus (Parahaemoproteus) coatneyi, one H. (P.) erythrogravidus and one Plasmodium (Novyella) nucleophilum; see Results). The analysis was run with default parameters (except for 1000 iterations, thinning interval = 10, burn-in = 200, and no recombination).

Unique sequences were compared to those archived in MalAvi (Bensch et al., 2009) and GenBank (https://www.ncbi.nlm.nih.gov/genbank/); sequences with a match lower than 100% were considered new lineages. To compare our sequences with published ones, we assembled an alignment that contained unique sequences that were 98–99% identical in MalAvi and GenBank (but restricted to hosts in the Neotropics), as well as lineages reported to infect Z. capensis. Alignment of compiled sequences was performed in Clustal X2 separately for both genera, using Leucocytozoon fringillinarum (TFUS04; GenBank: JQ815435) as outgroup.

Before estimating phylogenetic trees, we used PartitionFinder 2 (Lanfear et al., 2016) to choose the best model of nucleotide substitution for each dataset (run with branch lengths linked, all models, Akaike Information Criterion (AIC), and greedy algorithm). The best partition scheme for Plasmodium (by codon position) was TrN (Tamura-Nei) + I (invariable sites) + G (gamma distribution) (first), F81 (Felsenstein 1981) (second) and GTR (general time reversible) + G (third); for Haemoproteus, it was GTR + I + G (first), HKY (Hasegawa-Kishino-Yano) + I + G (second) and GTR + G (third). Phylogenetic analyses were performed separately by genus in MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003) with 2 million generations, sampling each 1000 trees and burning 250, retaining 1750 trees used to obtain a 50% majority-rule consensus tree.

Morphological identification

Blood smears were examined using a Leica DM750 microscope (Leica Microsystems, Heerbrugg, Switzerland) at 400× for 10 min and then at high magnification 1000× for 20 min to detect haemoparasites. From positive slides, we obtained images representing
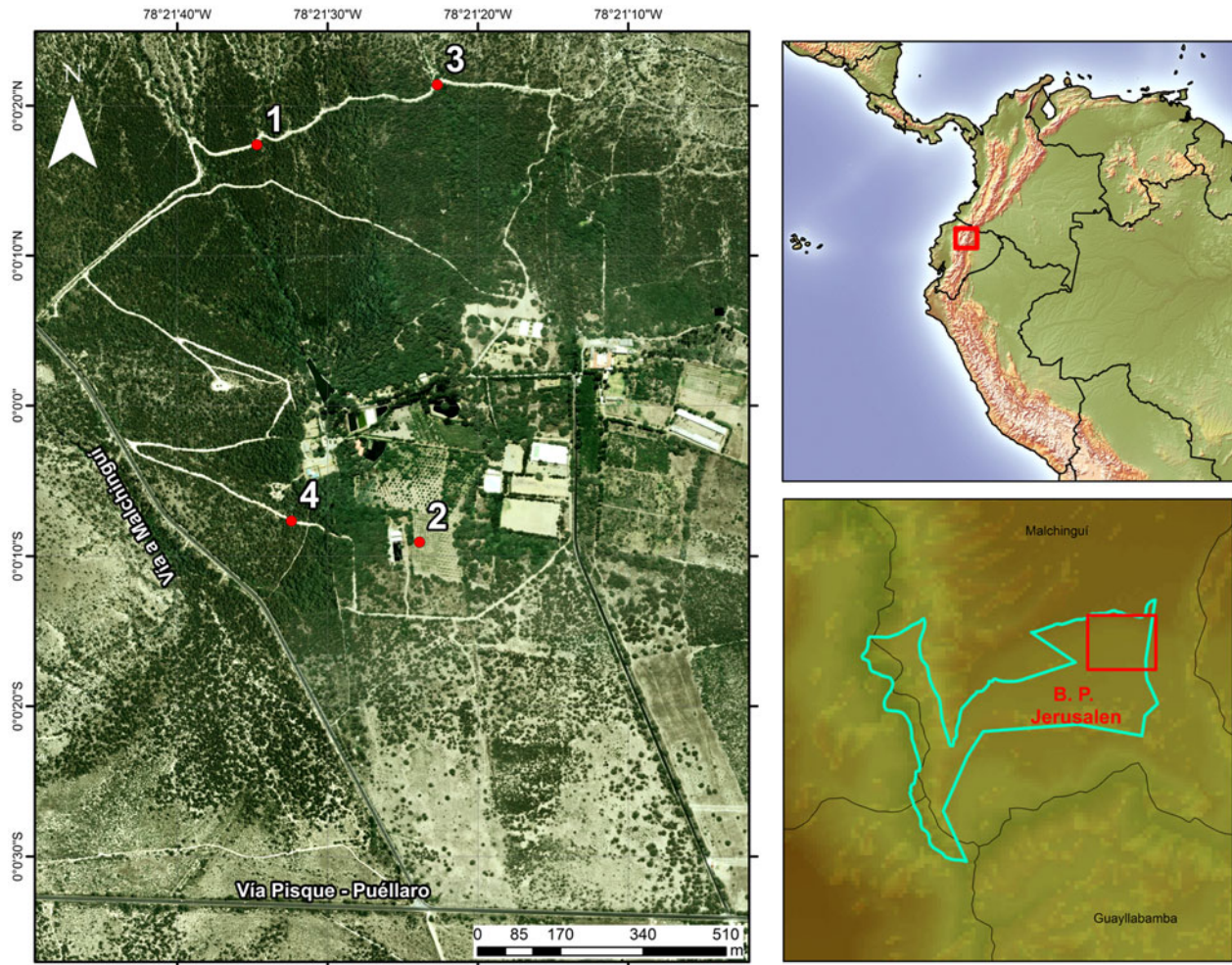
Fig. 1. Study area: sampling sites within Bosque Protector Jerusalem and location of Bosque Protector Jerusalem.

different parasite blood stages using a Leica EC3 camera and processed with LAS EZ software (Leica Microsystems Ltd., Switzerland, 2012). Taxonomic determination of parasites was made using Valkiūnas (2005) and Valkiūnas and Iezhova (2018) keys as well as recent descriptions of new species (Walther *et al.*, 2014; Mantilla *et al.*, 2016).

Prevalence and mean parasitaemia

Prevalence was assessed based on molecular lineage identification. Values of parasitaemia (intensity of infection) were obtained from 79 blood smears of infected samples (18–20 smears randomly selected per month). Blood smears were screened under an Olympus CX31 microscope (Olympus Optical Co. Ltd., Tokyo, Japan) at 1000× magnification, counting the number of infected, uninfected and immature erythrocytes (polychromatophils) until a total of 10 000 cells per smear were examined (Waldenström *et al.*, 2004). Prevalence and mean parasitaemia were analysed using software Quantitative Parasitology 3.0 (Rózsa *et al.*, 2000; Reiczigel and Rózsa, 2005). Estimation of confidence intervals (CIs) for prevalence was performed with the Sterne method (Reiczigel, 2003). Mean parasitaemia and 95% CIs were estimated with the BCa method, using 2000 bootstrap replicates (Rózsa *et al.*, 2000). Significant differences in prevalence between lineages were estimated using the unconditional exact test, whereas significant differences in mean parasitaemia between lineages were estimated using a bootstrap-*t* test with 2000 replicates (Rózsa *et al.*, 2000).

Factors associated with haemosporidian infections

To determine how biotic or abiotic factors are related to infection status (presence/absence of infection) and parasitaemia, and to explore the associations between infection and body condition, we performed a series of generalized linear model (GLM) analyses in R (R Development Core Team, 2008) using the MASS package (Venables and Ripley, 2002). Factors related to infection status were assessed using a logistic regression with binomial distribution and logit link function. Infection status was selected as dependent variable, and the following variables were selected as factors: age (adult, subadult) and body condition, as biotic factors; total monthly precipitation (mm), sampling site (S1, S2, S3, S4) and capture date, as abiotic factors. Body condition was calculated as the residuals from an ordinary least squares linear regression of body mass (dependent variable) against tarsus length (independent variable) (Green, 2001). Once the general model was obtained by including all factors, a backward AIC-based stepwise model selection was performed to find the minimal adequate model. Differences among factor levels were evaluated by examining the estimates of the model.

Factors related to parasitaemia were explored by means of a GLM assuming a negative binomial distribution to account for overdispersion of the data. Parasitaemia was selected as dependent variable and the factors were the same selected previously for analysing infection status (AIC-based model selection performed as previously). Finally, we investigated the associations among parasite lineages, coinfections and body condition. Body condition was selected as dependent variable and parasite lineage, presence

of coinfections (detected by either morphology or genetic sequencing), age, date of measurement, monthly precipitation and sampling site as factors (AIC-based model selection performed as previously). In all models, multicollinearity among factors was inspected by the variance inflation factor. Goodness of fit was investigated by inspecting the difference between null and residual deviance of the models.

Results

Relative abundance and movement of individuals

We captured 871 individuals from 36 species of birds. Of this total, 226 were *Z. capensis*, which represented a relative abundance of 26% in mist net captures, the highest in this study. We recorded 20 recaptures, 17 within the same sampling sites, and three of birds banded at site 2 and recaptured at site 4 or *vice versa*.

Molecular lineage identification and phylogenetic relationships

We analysed a total of 177 samples of *Z. capensis* by PCR and sequencing, 135 of which were positive for Haemosporida infection (Table 1). A total of 86 samples showed single infections for Haemoproteus ZC1 (GenBank: KC480265; Jones *et al.*, 2013); 34 for Plasmodium BAEBIC02 (GenBank: KF537291; Walther *et al.*, 2014; González *et al.*, 2015) and four for a new lineage of *Plasmodium*, Plasmodium ZOCAP15 (GenBank: MK077679). Phasing of sequences showing coinfections identified five fully resolved haplotypes (no phase uncertainties and phase probabilities = 1.0); three were coinfections by Plasmodium BAEBIC02 and Haemoproteus ZC1, and two by Haemoproteus ZC1 and Plasmodium ZOCAP15. Among the four sequences that were not resolved, three were regarded as combinations of BAEBIC02 and themselves, and one was regarded as a combination of lineage ZC1 and another mixed infection in the sample, both with high uncertainty (phase probabilities > 0.51). Thus, in further analyses we only included the five, fully resolved coinfections.

Phylogenetic analyses based on 34 unique sequences of related avian *Plasmodium* lineages placed the new lineage, ZOCAP15, in a clade with other *P. cathemerium* lineages (Fig. 2). Phylogenetic analyses based on 45 unique sequences of related avian *Haemoproteus* placed ZC1 as a distinctive lineage, together with lineages that infect *Z. capensis* and other Passeriformes, which have not been associated with any morphospecies (Fig. 3).

Morphological identification and its association with molecular identification

Morphological identification was performed by microscopy on a fraction of the molecularly diagnosed samples (Table 1). This analysis allowed us to identify six morphospecies infecting *Z. capensis*: *Plasmodium (Novyella) homopolare*, *P. (Haemamoeba) cathemerium*, *P. (N.) nucleophilum*, *Haemoproteus (Parahaemoproteus) coatneyi*, *H. (P.) erythrogravidus*, and an undescribed morphospecies that we call *Haemoproteus (P.)* sp. 1 (Fig. 4).

From 74 screened samples with single infections for molecular linage *Haemoproteus (P.)* ZC1, 64 showed single microscopic infections by *Haemoproteus (P.)* sp. 1 (Table 1); thus, hereafter, we refer to this molecular lineage as *Haemoproteus (P.)* sp1 (ZC1). Out of 25 screened samples with single infections by molecular linage Plasmodium BAEBIC02, 20 samples were only infected by morphospecies *P. (N.) homopolare* (Table 1); thus, hereafter, we refer to the molecular lineage as *P. (N) homopolare* (BAEBIC02). One sample infected with lineage ZOCAP15 was

heavily infected by *Plasmodium (H.) cathemerium* and *H. (P.) erythrogravidus* (Table 1). Given that phylogenetic analyses placed the new lineage, ZOCAP15, in a clade with other *P. (H.) cathemerium* lineages and the screened sample was infected with *Plasmodium (H.) cathemerium*, hereafter, we refer to this molecular lineage as *P. (H.) cathemerium* (ZOCAP15). Nine samples with molecular coinfections showed single infections by microscopy, whereas 16 samples with molecular single infections showed coinfections by microscopy (Table 1).

Prevalence and mean parasitaemia

Prevalence of haemosporidian parasites in *Z. capensis* was 76.3% based on molecular diagnosis (Table 2). Prevalence by lineage considered samples bearing single infections or fully resolved coinfections. Prevalence for *Haemoproteus (P.)* sp. 1 (ZC1) was significantly higher than for *P. (N.) homopolare* (BAEBIC02) (exact *P* value [two-tailed] < 0.0001). Mean parasitaemia for avian haemosporidians was 84.1 infected erythrocytes/10 000 erythrocytes counted (Table 2), whereas mean parasitaemia was also significantly higher for *Haemoproteus (P.)* sp. 1 (ZC1) than for *P. (N.) homopolare* (*t*-statistic = −2.789; bootstrap *P* value [two-tailed] = 0.011).

Factors associated with haemosporidian infections

When analysing the predictors of infection status, AIC-based model selection resulted in a model that included total monthly precipitation, age and site as predictors. Model estimates indicated that more individuals were infected on months with higher precipitation (estimate = 0.01, *z*-value = 2.25, *P* < 0.02); more adults than subadults were infected (estimate = 2.18, *z*-value = 4.25, *P* < 0.0001); and there were less individuals infected on site 2 compared to individuals in sites 1 and 4 (estimate = −1.51, *z*-value = −2.01, *P* = 0.04) but not from site 3 (Fig. 5A). The model explained 13.3% of the variation in infection status.

For the analysis of parasitaemia, a global model was fitted by assuming a Poisson distribution; however, the analysis of the residual deviance and the degrees of freedom detected overdispersion. Thus, to correct for overdispersion we applied a negative binomial distribution. AIC-based stepwise model selection identified a final model that only retained site as factor. Here, our results indicated that individuals from site 2 presented the lowest parasitaemia (estimate = −1.65, *z*-value = −3.11, *P* < 0.0001) (Fig. 5B). The model explained 11.1% of the variation in parasitaemia.

In the analysis of associations of parasite lineages and coinfections with body condition, stepwise AIC-based model selection resulted in a model where the presence of coinfections and site were selected as factors (no relationship between parasite lineage and body condition was observed). Estimates from the minimal adequate model showed individuals from sites 2 and 4 (site 2: estimate = 1.19, *t*-value = 3.02, *P* < 0.01, site 4: estimate = 1.33, *t*-value = 3.26, *P* < 0.01), and individuals with presence of coinfections (estimate = 0.65, *t*-value = 1.97, *P* = 0.05) as having better body condition (Fig. 5C). The model explained 14.8% of the variation in body condition. No multicollinearity was observed among factors in all models.

Discussion

Diversity of haemosporidians

We identified three molecular lineages of haemosporidian parasites: *Plasmodium (N.) homopolare* (BAEBIC02), *Haemoproteus (P.)* sp1 (ZC1) and *Plasmodium (H.) cathemerium* (ZOCAP15). Lineage *Plasmodium (N.) homopolare* (BAEBIC02) was first

Table 1. Association of molecular lineage (amplified by PCR) and morphospecies (identified by microscopy) found in *Z. capensis* sampled at Bosque Protector Jerusalem Quito-Ecuador. The first morphospecies denotes the morphospecies responsible for the main infection, whereas the other represents a coinfection

Molecular lineage	d/s[1]	Morphospecies by microscopy	d/s[2]
ZC1 (GenBank: KC480265)	86/177	*Haemoproteus* (*Parahaemoproteus*) sp. 1.	64/74
		Haemoproteus (*P.*) sp. 1., *H.* (*P.*) *coatneyi*	6/74
		P. (*Haemamoeba*) *cathemerium*, *Haemoproteus* (*P.*) sp. 1	1/74
		Haemoproteus (*P.*) sp. 1., *H.* (*P.*) *erythrogravidus*	3/74
BAEBIC02 (GenBank: KF537291)	34/177	*P.* (*Novyella*) *homopolare*	20/25
		P. (*N.*) *homopolare*, *H.* (*P.*) *coatneyi*	1/25
		P. (*N.*) *nucleophilum*, *P.* (*N.*) *homopolare*	3/25
		Haemoproteus (*P.*) sp1, *P.* (*H.*) *cathemerium*	1/25
ZOCAP15 (GenBank: MK077679)	4/177	*P.* (*H.*) *cathemerium*, *H.* (*P.*) *erythrogravidus*	1/1
Coinfection BAEBIC02/ZC1	3/177	*P.* (*N.*) *homopolare*	2/2
Coinfection ZOCAP15/ZC1	2/177	*Haemoproteus* (*P.*) sp. 1	1/1
Coinfection undetermined	4/177	*Haemoproteus* (*P.*) sp. 1	3/3
Lineage undetermined	2/177	Not screened	
Total[a]	135/177		106/106

[a]Totals are different because not all samples diagnosed by PCR were diagnosed by morphology. Out of the 135 samples that were positive by PCR, four were undetermined coinfections and two were not identified to lineage.
(d/s[1]) number of samples detected positive for molecular lineage/number of samples screened by PCR-sequencing.
(d/s[2]) number of samples detected positive for morphospecies/number of samples screened by microscopy once molecular lineage was determined.
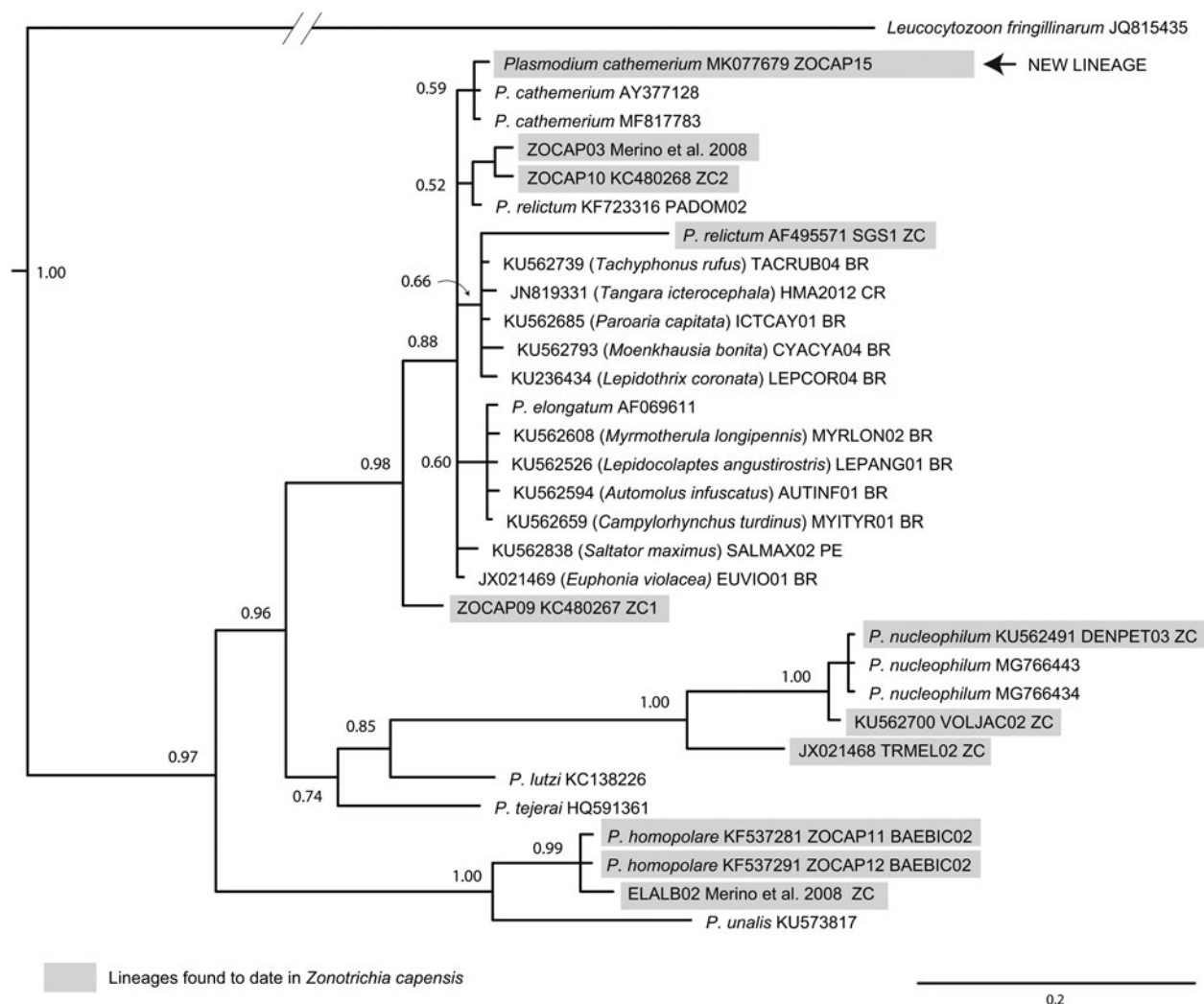

Fig. 2. Phylogenetic position of *P.* (*N.*) *homopolare* (BAEBIC02) and the new lineage, *Plasmodium* (*H.*) *cathemerium* (ZOCAP15), among closely related lineages of *Plasmodium*, based on a Bayesian phylogenetic analysis of 480 base pairs; Bayesian posterior probabilities are showed over nodes. Scientific names in parentheses are hosts were lineages have been detected.
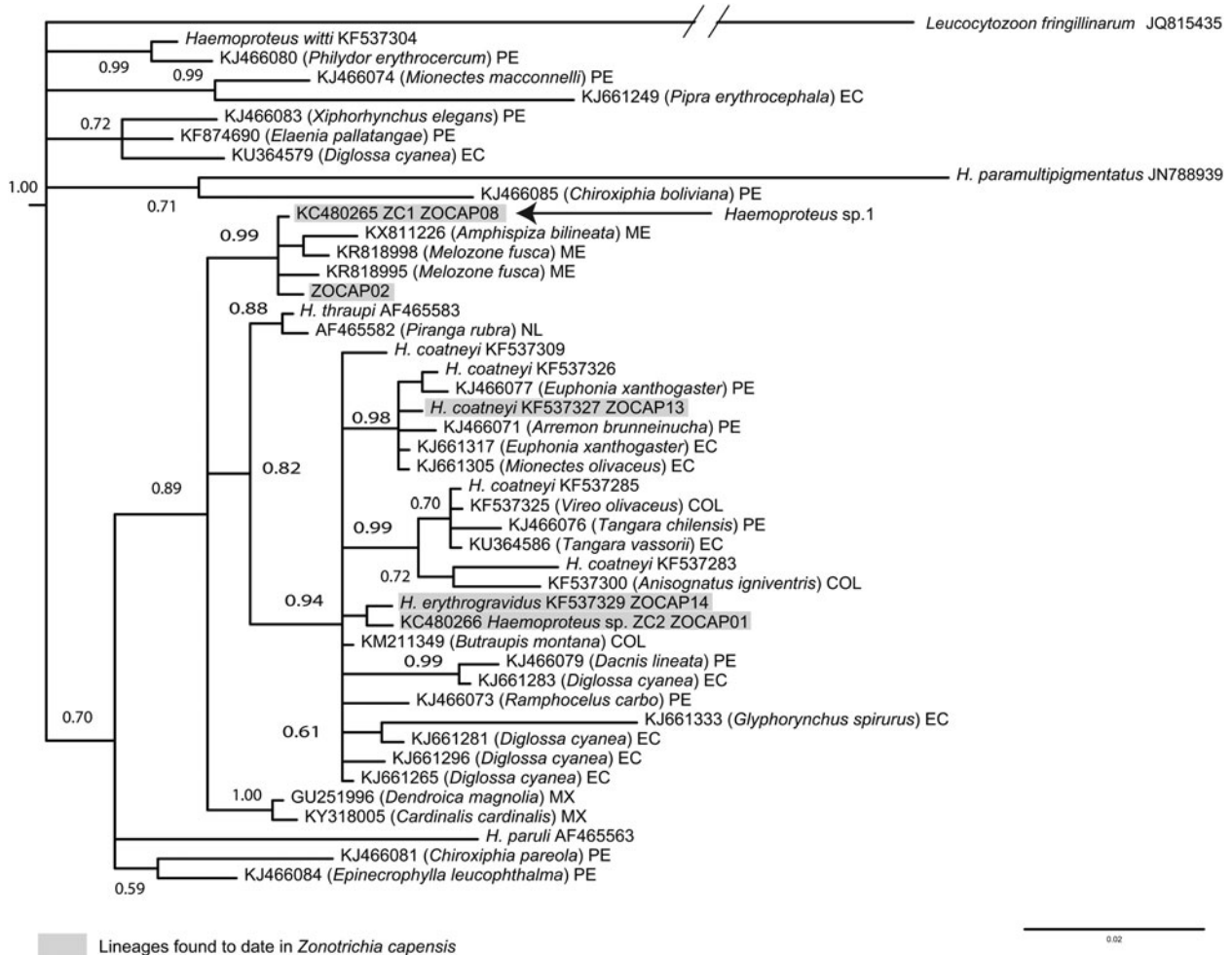
Fig. 3. Phylogenetic position of *Haemoproteus* (*P.*) sp. 1 (ZC1) among closely related lineages of *Haemoproteus*, based on a Bayesian phylogenetic analysis of 480 base pairs; Bayesian posterior probabilities are showed over nodes. Scientific names in parentheses are hosts were lineages have been detected.
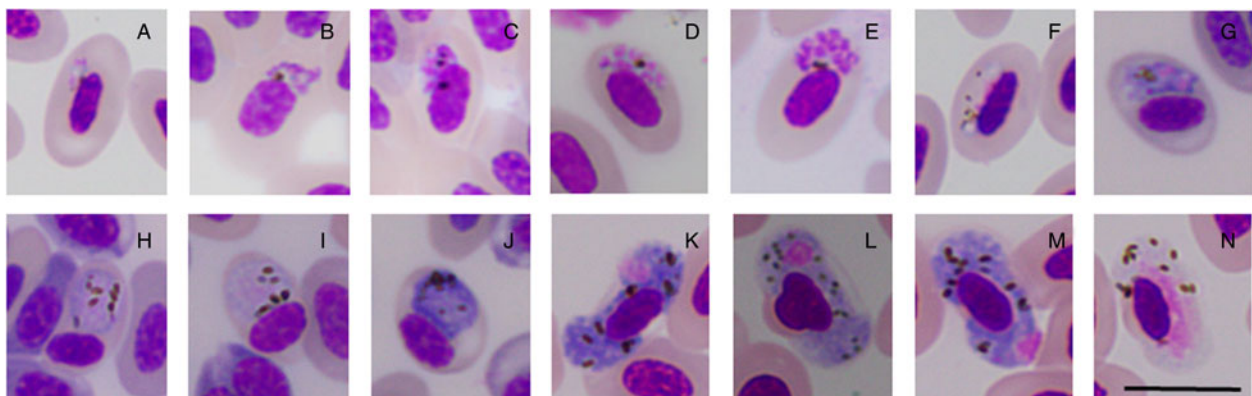

Fig. 4. Morphological features of some haemosporidian parasites from the blood of *Zonotrichia capensis*. *Plasmodium* (*N.*) *nucleophilum*: (A) trophozoites, (B–E) erythrocytic meronts, (F) young microgametocytes. *Plasmodium* (*H.*) *cathemerium*: (G) erythrocytic meront, (H, I) microgametocytes, (J) macrogametocyte. *Haemoproteus* (*P.*) sp. 1: (K–M) macrogametocytes, (N) microgametocyte. Giemsa-stained blood films. Scale bar = 10 *μ*m.

isolated from *Baeolophus bicolor* (Paridae) in the United States (Martinsen *et al.*, 2007) and it has been found infecting *Z. capensis* in Peru (Marzal *et al.*, 2015) and Colombia (Walther *et al.*, 2014; González *et al.*, 2015), as well as other 14 species of passerines from Alaska to Peru (Galen and Witt, 2014; Oakgrove *et al.*, 2014; Walther *et al.*, 2014; González *et al.*, 2015). *Haemoproteus* sp. ZC1 infects *Z. capensis* in Peru (Jones *et al.*, 2013), other Emberizidae in México (Reinoso-Pérez *et al.*, 2016; Ham-Dueñas *et al.*, 2017), and *Troglodytes aedon* in Peru (Galen and Witt,

2014). This lineage shows considerable genetic distance from those of other *Haemoproteus* morphospecies (Fig. 3), making the associated morphospecies a good candidate for morphological description.

The finding of the new lineage, ZOCAP15, couples with the first report of the *Plasmodium* (*H.*) *cathemerium* morphospecies in *Z. capensis*. Its low prevalence may indicate that it is either rare in this population or highly virulent. One individual carrying this parasite was analysed by microscopy and showed one of the

Table 2. Prevalence and parasitaemia by haemosporidian parasites in *Z. capensis*

Molecular lineage	Morphospecies	Prevalence[a] (*n* = 177)	Prevalence 95% CI	Mean parasitaemia (*n*)[b]	Mean parasitaemia 95% CI
ZC1	*Haemoproteus (Parahaemoproteus)* sp1	51.40%	44.06–58.77%	104.63 (38)	82.24–174.61
BAEBIC02	*P. (Novyella) homopolare*	20.9%	15.48–27.63%	45.89 (18)	30.78–74.61
ZOCAP15	*Plasmodium (Haemamoeba) cathemerium*	3.4%	1.49–7.25%	–	–
All lineages		76.3%	69.26–82.04%	84.1 (79)	63.19–114.25

[a]Prevalence of infection was calculated based on molecular results and includes single infections and unambiguous coinfections by molecular lineage.
[b]Parasitaemia (infected erythrocytes in 10 000 cells counted) was calculated based on single infections by lineage and morphospecies; total parasitaemia (all lineages) includes individuals with single and mixed infections from any lineage or morphospecies. *n*: number of samples for which parasitaemia was analysed by microscopy; –: not calculated because of low sampling size.
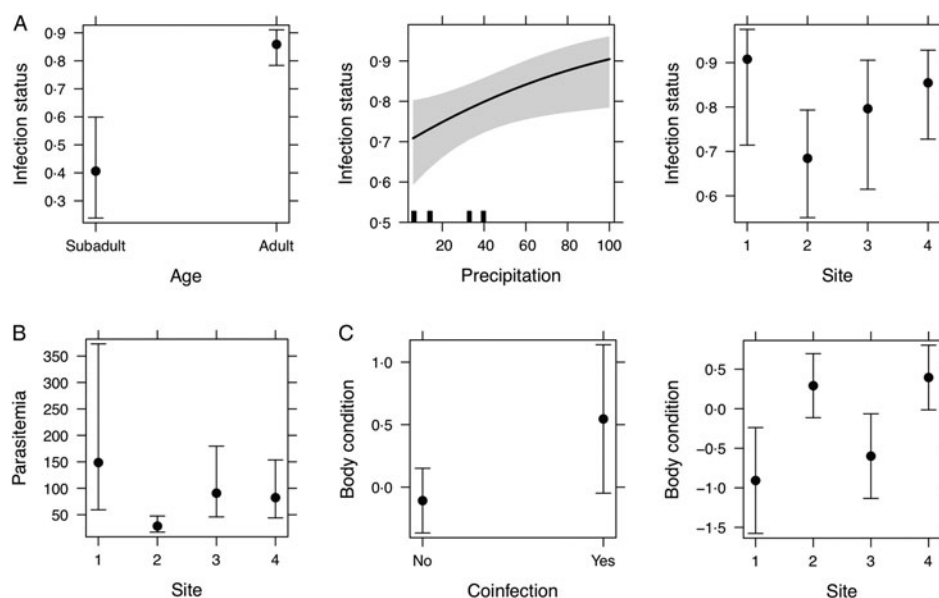

Fig. 5. Effect plots from models investigating infection status (A), intensity of infection (B) and body condition (C). Grey shaded area and the error bars indicate standard error (s.e.).

highest parasitaemias our study (481 infected cells/10 000 cells counted). *Plasmodium (H.) cathemerium* Hartman, 1927 is a generalist parasite with a wide range of hosts (Bennett *et al.*, 1993; Valkiūnas, 2005) that may cause severe forms of malaria (Gabaldón *et al.*, 1988; Valkiūnas, 2005; Vanstreels *et al.*, 2015). Thus, it would be important to investigate the prevalence of *Plasmodium (H.) cathemerium* in this community, not only in *Z. capensis* – which is definitively a competent host, as gametocytes were observed in the blood smear – but also in other species.

Together with previous studies (González *et al.*, 2015; Mantilla *et al.*, 2016), our results confirm at least six morphospecies of haemosporidians infecting *Z. capensis* and add to the current knowledge by associating morphospecies *Haemoproteus (P.)* sp1 to molecular lineage ZC1. Interestingly, three morphospecies not detected by molecular diagnosis – *Plasmodium (N.) nucleophilum*, *H. (P.) coatneyi* and *H. (P.) erythrogravidus* – were detected by microscopy in coinfection with *P. (N.) homopolare* or *Haemoproteus (P.)* sp1. This result may be caused by differences in parasitaemia or by the differential ability of the primers to amplify parasite DNA in mixed infections, especially when both *Haemoproteus* and *Plasmodium* are present (Bernotienė *et al.*, 2016).

Given that our sampling effort for a single locality has been considerably higher than in previous studies of *Z. capensis* (e.g. Jones *et al.*, 2013; González *et al.*, 2015; Marzal *et al.*, 2015), our results may be reflecting the actual diversity of haemosporidians in this population. Rather than the low diversity initially expected, we found the highest diversity of haemosporidian parasites infecting *Z. capensis* on a single site. Additionally, coinfections were

relatively frequent compared to previous studies (e.g. Cosgrove *et al.*, 2008; Levin *et al.*, 2013; Ferraguti *et al.*, 2017; but see Marzal *et al.*, 2008). This condition was detected in 7% of molecular diagnoses and 15% of microscopic diagnoses (Table 1), stressing the need for both kinds of diagnoses in the survey of haemoparasites (see Carlson *et al.*, 2018; Pacheco *et al.*, 2018).

Prevalence and parasitaemia

Prevalence of haemosporidians in *Z. capensis* in this Andean dry forest (76%) is higher than that reported by Jones *et al.* (2013) in Andean Peru (42%) and similar to that found by Escallón *et al.* (2016) in the Andean foothills of the Amazon (73%). Prevalence of lineage *P. (N.) homopolare* (BAEBICO2) (19.2%) is higher than that reported by Marzal *et al.* (2015) for *Z. capensis* in the eastern slope of the Andes of Peru (15.15%), whereas prevalence of *Haemoproteus* sp1 (ZC1) (48.6%) was also higher than that reported by Jones *et al.* (2013) in Peru (25%). Overall, high prevalence of haemosporidians in our study could be related to habitat, latitude, regional or community-level assemble of parasites (e.g. Fecchio *et al.*, 2017), or high abundance of *Z. capensis*, the highest in the avian community of the study site (see Ellis *et al.*, 2017). Higher prevalence and parasitaemia of *Haemoproteus* sp1 (ZC1) over *P. (N.) homopolare* (BAEBICO2) might be related to the relatively mild or sublethal effects previously reported for *Haemoproteus* (Garvin *et al.*, 2003), but further studies are necessary to confirm the pathology of this lineage infecting *Z. capensis*.

Factors associated with haemosporidian infections

Our analyses produced both expected and puzzling results. Infection status was predicted by monthly precipitation, age and sampling site. First, a positive effect of precipitation on infection status is likely to be related to better conditions for vector reproduction, which would increase the acquisition of new infections or trigger higher parasitaemia within an already infected host (Cornet *et al.*, 2014). Although higher prevalence has been tied to reproduction of the host (e.g. Knowles *et al.*, 2011; Ham-Dueñas *et al.*, 2017), this scenario is not plausible given the apparently continuous reproduction of *Z. capensis* at the study site (Cadena-Ortiz, personal observation), which contrasts with reports of reproduction seasonality in two other nearby localities, but at higher elevation (Papallacta at 3300 m a.s.l. and Pintag at 2900 m a.s.l.; Moore *et al.*, 2005). Future studies should aim at monitoring reproductive activities of the host, prevalence and parasitaemia, as well as abundance and diversity of vectors in this locality, simultaneously. Second, we found more infections among adults than among subadults, which has been found in previous studies (e.g. Wood *et al.*, 2007). This difference is usually attributed to the increased probability of acquiring and maintaining chronic infections with age or, alternatively, higher mortality of subadults (Davidar and Morton, 1993). Third, we found that fewer individuals were infected on site 2. This result agrees with the lower parasitaemia found on site 2. Lower prevalence and parasitaemia on site 2 could result from lower abundance of vectors within the *Citrus* sp.—*P. americana* plantation. In fact, habitats with more natural vegetation may favour higher richness and abundance of vector species (*Culicoides* and Cuilicidae) through the availability of breeding places (Mullen, 2002; Eldridge, 2005). Thus, areas with less natural vegetation, like site 2, could have lower abundance and diversity of vectors, explaining lower prevalence and parasitaemia on this site.

When exploring the predictors of body condition, we found that individuals bearing coinfections, and those from sites 2 and 4, had better body condition. Recaptures in the study area reflect that there is only limited movement of *Z. capensis* among sites, explaining why sites seem to be acting as independent 'islands', with a few exceptions of movement between sites 2 and 4 (which are relatively closer). Given that individuals from site 2 had less prevalence and parasitaemia, it is not surprising that they have better body condition (although body condition could not predict levels of prevalence and parasitaemia); however, we may not explain why individuals from site 4 had better body condition. It is also difficult to explain why individuals with coinfections would show better body condition than those with single infections (although results are marginally significant). This is specially puzzling since mixed infections are expected to have more detrimental effects on body condition because of higher compromise of the immune system (Marzal *et al.*, 2008).

Concluding remarks

The low percentages of variation explained by our models suggest that variables not measured in this study may affect infection status, parasitaemia and body condition in the rufous-collared sparrow. Still, our results support the idea that *Z. capensis* could be a good model organism for studying the dynamics of avian haemosporidian infections given its high abundance, apparently high resilience to infections, and relatively high diversity of parasites (and coinfections). Future studies should analyse a more comprehensive list of variables and interactions, as well as a greater sample, in order to contribute to a more integrative understanding of avian haemosporidian infections in this host.

Conflict of interest. None.

Ethical standards. Bird manipulation performed during this study followed both ethical and legal procedures, and tried to minimize suffering and stress.

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References

Asghar M, Hasselquist D, Hansson B, Zehtindjiev P, Westerdahl H and Bensch S (2015) Hidden cost of infection: chronic malaria accelerates telomere degradation and senescence in wild birds. *Science* **347**, 436–438.

Bennett GF, Bishop MA and Peirce MA (1993) Checklist of the avian species of *Plasmodium* marchiafava & Celli, 1885 (Apicomplexa) and their distribution by avian family and Wallacean life zones. *Systematic Parasitology* **26**, 171–179.

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 I, 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.

Cadena-Ortiz H, Varela S, Bahamonde-Vinueza D, Freile JF and Bonaccorso E (2015) Birds of Bosque Protector Jerusalem, Guayllabamba Valley, Ecuador. *Check List* (*Luis Felipe Toledo*) **11**, 1770.

Carlson JS, Nelms B, Barker CM, Reisen WK, Sehgal RNM and Cornel AJ (2018) Avian malaria coinfections confound infectivity and vector competence assays of *Plasmodium homopolare*. *Parasitology Research* **117**, 2385–2394.

Chomczynski P (1993) A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* **15**, 532–537.

Coon CCA, García-Longoria L, Martin LB, Magallanes S, de Lope F and Marzal A (2016) Malaria infection negatively affects feather growth rate in the housesparrow (*Passer domesticus*). *Journal of Avian Biology* **47**, 779–787.

Cornet S, Nicot A, Rivero A and Gandon S (2014) Evolution of plastic transmission strategies. *PLoS Pathogens* **10**, e1004308.

Cosgrove CL, Wood MJ, Day KP and Sheldon BC (2008) Seasonal variation in *Plasmodium* prevalence in a population of blue tits *Cyanistes caeruleus*. *Journal of Animal Ecology* **77**, 540–548.

Davidar P and Morton ES (1993) Living with parasites: prevalence of a blood parasite and its effect on survivorship in the purple martin. *The Auk* **110**, 109–116.

Eldridge BF (2005) Mosquitoes, the Culicidae. In Marquardt WC (ed.), *Biology of Disease Vectors 2ª Ed.* New York, USA: Academic Press, pp. 95–111.

Ellis VA, Medeiros MCI, Collins MD, Sari EHR, Coffey ED, Dickerson RC, Lugarini C, Stratford JA, Henry DR, Merrill L, Matthews AE, Hanson AA, Roberts JR, Joyce M, Kunkel MR and Ricklefts RE (2017) Prevalence of avian haemosporidian parasites is positively related to the abundance of host species at multiple sites within a region. *Parasitology Research* **116**, 73–80.

Escallón C, Weinstein NM, Tallant JA, Wojtenek W, Rodríguez-Saltos CA, Bonaccorso E and Moore IT (2016) Testosterone and Haemosporidian parasites along a Tropical elevational gradient in Rufous-collared sparrows, (*Zonotrichia capensis*). *Journal of Experimental Zoology* 00A, 1–10.

Fecchio A, Svensson-Coelho M, Bell J, Ellis VA, Medeiros MC, Trisos CH, Blake JG, Loiselle BA, Tobias JA, Fanti R, Coffey ED, de Faria IP, Pinho JB, Felix G, Braga EM, Anciaes M, Tkach V, Bates J, Witt C, Weckstein JD, Ricklefs RE and Farias IP (2017) Host associations and turnover of haemosporidian parasites in manakins (Aves: Pipridae). *Parasitology* 144, 984–993.

Ferraguti M, Martínez-de la Puente J, Bensch S, Roiz D, Ruiz S, Viana DS, Soriguer RC and Figuerola J (2017) Ecological determinants of avian malaria infections: an integrative analysis at landscape, mosquito and vertebrate community levels. *Journal of Animal Ecology* 87, 1–14.

Gabaldón A, Ulloa G and Zerpa N (1988) *Plasmodium cathemerium*, cepa de Icteridae inoculable a palomas, patos y pavos; sus vectores y utilidad en enseñanza e investigación. *Boletín de la Dirección de Malariología y Saneamiento Ambiental* 28, 53–68.

Galen SC and Witt CC (2014) Diverse avian malaria and other haemosporidian parasites in Andean house wrens: evidence for regional co-diversification by host-switching. *Journal of Avian Biology* 45, 374–386.

Garvin MC, Homer BL and Greiner EC (2003) Pathogenicity of *Haemoproteus danilewskyi*, Kruse, 1890, in blue jays (*Cyanocitta cristata*). *Journal of Wildlife Diseases* 39, 161–169.

González AD, Lotta IA, García LF, Moncada LI and Matta NE (2015) Avian haemosporidians from Neotropical highlands: evidence from morphological and molecular data. *Parasitology International* 64, 48–59.

Green AJ (2001) Mass/length residuals: measures of body condition or generators of spurious results? *Ecology* 82, 1473–1483.

Ham-Dueñas JG, Chapa-Vargas L, Stracey CM and Huber-Sannwald E (2017) Haemosporidian prevalence and parasitaemia in the Black-throated sparrow (*Amphispiza bilineata*) in central-Mexican dryland habitats. *Parasitology Research* 116, 2527–2537.

Jones MR, Cheviron ZA and Carling MD (2013) Spatial patterns of avian malaria prevalence in *Zonotrichia capensis* on the western slope of the Peruvian Andes. *Journal of Parasitology* 99, 903–905.

Jones MR, Cheviron ZA and Carling MD (2014) Variation in positively selected major histocompatibility complex class I loci in rufous-collared sparrows (*Zonotrichia capensis*). *Immunogenetics* 66, 693–704.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Mentjies P and Drummond A (2012) Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* (*Oxford, England*) 28, 1647–1649.

Knowles SCL, Wood MJ, Alves R, Wilkin TA, Bensch S and Sheldon BC (2011) Molecular epidemiology of malaria prevalence and parasitaemia in a wild bird population. *Molecular Ecology* 20, 1062–1076.

Lanfear R, Frandsen PB, Wright AM, Senfeld T and Calcott B (2016) Partitionfinder 2: new methods for selecting partitioned models of evolution for molecular and phylogenetic analyses. *Molecular Biology and Evolution* 34, 772–773.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ and Higgins DG (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* (*Oxford, England*) 23, 2947–2948.

Levin II, Zwiers P, Deem SL, Geest EA, Higashiguchi JM, Iezhova TA, Jimenez-Uzcategui GD, Kim H, Morton JP, Perlut NG, Renfrew RB, Sari EHR, Valkiūnas G and Parker PG (2013) Multiple lineages of avian malaria parasites (*Plasmodium*) in the Galapagos Islands and evidence for arrival *via* migratory birds. *Conservation Biology* 27, 1366–1377.

Librado P and Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* (*Oxford, England*) 25, 1451–1452.

MAE (Ministerio del Ambiente del Ecuador) (2013) *Sistema de Clasificación de los Ecosistemas del Ecuador Continental*. Quito, Ecuador: Subsecretaría de Patrimonio Natural.

Mantilla S, González AD, Lotta IA, Moens M, Pacheco A, Escalante AA, Valkiūnas G, Moncada LI, Pérez-Tris J and Matta NE (2016) *Haemoproteus erythrogravidus* n. sp. (Haemosporida, Haemoproteidae): description and molecular characterization of a widespread blood parasite of birds in South America. *Acta Tropica* 159, 86–94.

Martinsen ES, Sidor IF, Flint S, Cooley J and Pokras MA (2007) Morphologically defined subgenera of *Plasmodium* from avian hosts: test of monophyly by phylogenetic analysis of two mitochondrial genes. *Parasitology* 134, 483–490.

Marzal A, Bensch S, Reviriego M, Balbontin J and de Lope F (2008) Effects of malaria double infection in birds: one plus one is not two. *Journal of Evolutionary Biology* 21, 979–987.

Marzal A, García-Longoria L, Cárdenas Calligros JM and Sehgal RNM (2015) Invasive avian malaria as an emerging parasitic disease in native birds of Peru. *Biological Invasions* 17, 39–45.

Moore IT, Bonier and Wingfield FJC (2005) Reproductive asynchrony and population divergence between two tropical bird populations. *Behavioral Ecology* 16, 755–762.

Mullen GR (2002) Biting midges (Ceratopogonidae). In Mullen G and Durden L (eds), *Medical and Veterinary Entomology*. San Diego, USA: Academic Press, pp. 163–183.

Oakgrove KS, Harrigan RJ, Loiseau C, Giers S, Seppi B and Sehgal RNM (2014) Distribution, diversity and drivers of blood-borne parasite coinfections in Alaskan bird populations. *International Journal for Parasitology* 44, 717–727.

Pacheco MA, Matta NE, Valkiunas G, Parker PG, Mello B, Stanley Jr CE, Lentino M, Garcia-Amado MA, Cranfield M, Pond SL and Escalante AA (2018) Mode and rate of evolution of haemosporidian mitochondrial genomes: timing the radiation of avian parasites. *Molecular Biology Evolution* 35, 383–403.

Pérez-Tris J, Hasselquist D, Hellgren O, Krizanauskiene A, Waldenström J and Bensch S (2005) What are malaria parasites? *Trends in Parasitology* 21, 209–211.

R Development Core Team (2008) A language and environment for statistical computing. *R Foundation for Statistical Computing* 1, 2673.

Reiczigel J (2003) Confidence intervals for the binomial parameter: some new considerations. *Statistics in Medicine* 22, 611–621.

Reiczigel J and Rózsa L (2005) Quantitative Parasitology 3.0. Budapest.

Reinoso-Pérez MT, Canales-Delgadillo JC, Chapa-Vargas L and Riego-Ruiz L (2016) Haemosporidian parasite prevalence, parasitemia, and diversity in three resident bird species at a shrubland dominated landscape of the Mexican highland plateau. *Parasites and Vectors* 9, 307.

Ridgely RS and Greenfield PJ (2001) *The Birds of Ecuador*. Ithaca, USA: Cornell University Press.

Rising J and Jaramillo A (2017). Rufous-collared sparrow (*Zonotrichia capensis*). In: del Hoyo J, Elliott A, Sargatal J, Christie DA and de Juana E. (eds), *Handbook of the Birds of the World Alive*. Barcelona: Lynx Edicions. Available at http://www.hbw.com/node/61910 (accessed 23 August 2017).

Ronquist F and Huelsenbeck JP (2003) Mrbayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* (*Oxford, England*) 19, 1572–1574.

Rózsa L, Reiczigel J and Majoros G (2000) Quantifying parasites in samples of hosts. *Journal of Parasitology* 86, 228–232.

Santiago-Alarcón D, Palinauskas V and Schaefer HM (2012) Diptera vectors of avian Haemosporidian parasites: untangling parasite life cycles and their taxonomy. *Biological Reviews* 87, 928–964.

Stephens M and Donnelly P (2003) A comparison of Bayesian methods for haplotype reconstruction from population genotype data. *American Journal of Human Genetics* 73, 1162–1169.

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

Valkiūnas G and Iezhova T (2018) Keys to the avian malaria parasites. *Malaria Journal* 17, 212.

Vanstreels RET, da Silva-Filho RP, Kolesnikovas CKM, Bhering RCC, Ruoppolo V, Epiphanio S, Amaku M, Junior FCF and Martins Braga EM (2015) Epidemiology and pathology of avian malaria in penguins undergoing rehabilitation in Brazil. *Veterinary Research* 46, 30.

Venables WN and Ripley BD (2002) MASS: modern applied statistics with S. *Issues of Accuracy and Scale* 868.

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

Walther EL, Valkiūnas G, González AD, Matta NE, Ricklefs RE, Cornel A and Sehgal RNM (2014) Description, molecular characterization, and patterns of distribution of a widespread New World avian malaria parasite (Haemosporida: Plasmodiidae), *Plasmodium* (*novyella*) *homopolare* sp. nov. *Parasitology Research* 113, 3319–3332.

Wood MJ, Cosgrove CL, Wilkin TA, Knowles SCL, Day KP and Sheldon BC (2007) Within-population variation in prevalence and lineage distribution of avian malaria in blue tits, *Cyanistes caeruleus*. *Molecular Ecology* 16, 3263–3273.