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Historical Biogeography, Spatial Distribution, and Within-Host Interactions of Avian Haemosporidian Parasites (Apicomplexa, Haemosporida)

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by

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To my nieces, Estela and Bianca.

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ABSTRACT

This dissertation addresses several aspects of the biogeography and evolution of avian malarial parasites (Haemosporida: *Plasmodium* and *Haemoproteus*), and the interactions of these pathogens with their hosts and other avian blood parasites. In Chapter 1, I investigate change in haemosporidian assemblages on islands in the West Indies over millennial time scales, taking advantage of the historical isolation of islands by postglacial rising sea levels. I found that the prevalence of parasite lineages is highly dynamic over periods from decades to thousands of years. Millenial timescales are required for the turnover of lineages in insular assemblages, likely because of processes such as evolution of host resistance to individual parasite lineages. In Chapter 2, I report the occurrence of the highly invasive and virulent avian malaria parasite, *Plasmodium relictum*, in the endemic avifauna of Cuba. Using molecular markers that target a region of the parasite's merozoite surface protein gene, I determined that the *P. relictum* haplotype present in Cuba matches that of the malaria parasite that caused the population decline and extinction of several endemic Hawaiian birds. I suggested a time frame for the introduction of this parasite lineage on Cuba, and raised the possibility that avian malaria might be responsible for the remarkable absence from Cuba of several otherwise common and geographically widespread bird species in the West Indies, such as the bananaquit *Coereba flaveola*. In Chapter 3, I compare parasite prevalence in wintering shorebirds in two areas in Argentina: coastal, marine habitats of Patagonia generally lacking dipteran parasite vectors, and the shorelines of Mar Chiquita Lagoon, an inland freshwater basin, where landbirds exhibiting high parasite prevalence, as well as dipteran vectors, are also present. I found that haemosporidian infections are close to nil in both shorebird assemblages, even when these birds are exposed to parasite transmission. This study offered unprecedented evidence of the rarity of haemosporidian infections in shorebirds, regardless of suitable conditions for parasite transmission. In contrast to hypotheses based on the avoidance of parasite transmission areas, I propose that these birds are highly resistant to haemosporidian infections, presenting either a physiological barrier to the parasites, or quickly clearing infections as they appear. In Chapter 4, I investigate the occurrence of co-infections between *Plasmodium* spp. and *Trypanosoma* spp. parasites in a population of yellow-breasted chats (*Icteria virens*) in southern Missouri. I determine that individuals infected with avian malaria parasites are more likely also to host trypanosome parasites, when compared to individuals free of infections. This study also presents evidence that trypanosome infections may cause disease in birds. Overall, this dissertation supports hypotheses regarding the geographic and the host distribution of haemosporidian parasites, and how these distributions change over evolutionary periods of time.

INTELLECTUAL MERIT

This dissertation presents original research that advances the fields of ecology and evolution on several fronts related to the effects of haemosporidian parasites on populations and assemblages of avian host species. In the first chapter, I present a new method for quantifying changes in ecological assemblages over millennial timescales taking advantage of the joining and separating of islands on shallow banks in the West Indies archipelago, caused by glacial-cycle sea-level changes. I compare pairs of assemblages on islands that were connected during periods of low sea levels and subsequently isolated about 2.5 thousand years (Ka) ago by rising sea levels, to assemblages on islands that remained isolated, as well as assemblages sampled locally over up to two decades and assemblages sampled in the same year at distant localities on the same island. This approach could be applied to determine long-term variation in communities or assemblages of any taxonomic group, and is especially useful for the retrospective study of change in assemblages of organisms lacking fossil records. This method also could be applied to any set of assemblages composed of discrete units on a landscape that was affected by climate change during the Last Glacial Maximum (ca., 27 Ka), which include, but is not limited to, assemblages on other oceanic archipelagos and assemblages in regions with isolated Pleistocene refugia. Using this method, I demonstrate that avian haemosporidian assemblages on Caribbean islands are highly dynamic over a time frame of up to 2.5 Ka, during which prevalence and composition of lineages change significantly. This study not only offers an innovative approach to investigating host-parasite relationships over time-scales inaccessible through epidemiological and fossil records, but also presents unprecedented evidence of rapid dynamics of haemosporidian parasite assemblages over millennial time-scales. In the second chapter, I report the occurrence in Cuba of a highly pathogenic lineage of avian malaria, *Plasmodium relictum* GRW4 Pr9, which has been responsible for extinctions of native Hawaiian bird populations. I recommend, as high priority for the future assessment of the distribution of this lineage and other haemosporidian parasites in Cuba, as well as experimental infections to determine the effects of this parasite on resident and wintering avifauna of the West Indies. In the third chapter, I present evidence that one species of shorebird (Charadriiformes: Scolopacidae) is resistant to haemosporidian parasites. This finding supports the hypothesis that haemosporidian parasites reduce oxygen transport in the blood and impact performance during energy-demanding long-distance migration, setting up strong selection in shorebirds for resistance to parasite infection. Future studies should confirm that dipteran vectors take blood meals from Charadriiformes and whether, or not, infected shorebirds are competent hosts in which the haemosporidian life cycle is completed. Finally, in the fourth chapter, I demonstrate that haemosporidian parasites facilitate infection of individual hosts by another common avian blood parasite, *Trypanosoma* spp.. I also provide novel evidence that trypanosome parasites may cause disease in birds, and therefore may depress their host populations. These results indicate

the utility of future research on the effect of *Trypanosoma* spp. infections on avian hosts, as well as on the health consequences of parasitic co-infections for avian populations.

TABLE OF CONTENTS

CHAPTER 1.

THE DYNAMICS OF AVIAN HAEMOSPORIDIAN ASSEMBLAGES THROUGH MILLENNIAL TIMESCALES INFERRED FROM INSULAR BIOTAS OF THE WEST INDIES

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Abstract

Although introduced haemosporidian (malaria) parasites (Apicomplexa: Haemosporida) have hastened the extinction of endemic bird species in the Hawaiian Islands and perhaps elsewhere, little is known about the temporal dynamics of endemic malaria parasite populations. Haemosporidian parasites do not leave informative fossils and records of population change are lacking beyond a few decades. Here, we take advantage of the isolation of West Indian land-bridge islands by rising post-glacial sea levels to estimate rates of change in haemosporidian parasite assemblages over a millennial time frame. Several pairs of West Indian islands have been connected and separated by falling and rising sea levels associated with the advance and retreat of Pleistocene glaciers. We use island isolation following post-glacial sea-level rise, ca. 2.5 ka, to characterize long-term change in insular assemblages of haemosporidian parasites. We find that assemblages on formerly connected islands are as differentiated as assemblages on islands that have never been connected, and both are more differentiated than local assemblages sampled up to two decades apart. Differentiation of parasite assemblages on formerly connected islands reflects variation in the prevalence (i.e., frequency of infections) of shared haemosporidian lineages, whereas differentiation on islands isolated by millions of years reflects replacement of haemosporidian lineages infecting similar assemblages of avian host species.

Introduction

Insular biotas provide natural laboratories for characterizing change in host-parasite relationships over time(1). Haemosporidian parasites (genera *Plasmodium* and *Haemoproteus*, among others) are dipteran vector-transmitted protozoans that clonally reproduce in cells of vertebrates(2). The few fossils of ancient haemosporidians(3) are insufficient to analyze retrospectively the dynamics of these host-parasite interactions.

We have found that haemosporidian parasite assemblages of birds present remarkable geographic heterogeneity in the West Indies, even though common host species are widely distributed throughout the archipelago(4). Additionally, the frequencies of individual lineages in assemblages of avian haemosporidian parasites have been observed to vary over periods as short as one decade(5, 6). Here, we take advantage of the geographic history of islands in the West Indies to characterize changes in avian haemosporidian parasite assemblages over millennial time scales.

During the late Quaternary glaciations, which reached their maximum extent ca. 26 ka, increase in continental ice volume caused the global sea level to drop as much as 120 m, exposing land connections between islands lying on shallow banks(7). During periods of sea level lows, we presume that organisms could freely disperse between some pairs of present-day islands, tending to homogenize assemblages of birds and, presumably, their pathogens(7). Deglaciation started 7.5-10 ka, and ended ca. 2.5 ka when seas returned to currently observed levels and many pairs of islands were re-isolated (Figure 1)(8). We use island coalescence during glacial sea-level lows to define a natural experimental group in which host and parasite assemblages presumably were homogenized, then subsequently isolated, providing ca. 2.5 ka during which insular host-parasite assemblages could change independently and diverge.

We ask whether the period from the re-isolation of insular biotas by rising sea levels to the present has been sufficient for the homogenized parasite assemblages on the previously connected island pairs to become differentiated. We quantify differences in host and parasite assemblages by indices based on differences in parasite prevalence, as well as gains and losses of haemosporidian lineages on one island of a pair relative to the other. Finally, we ask whether differences in assemblage composition are due to replacement of lineages that are more or less closely related through evolution than one would expect of random replacement.

To establish relative points of comparison to the millennial timeframe of haemosporidian assemblage isolation, we compare parasite assemblages from islands that were formerly connected and assemblages from islands separated by deep-water channels for millions of years. These island pairs are relatively well matched in terms of size and ecology. We account for short-term variation by comparing parasite assemblages from the same island sampled between time intervals as short as two years and as long as two decades. Finally, we use pairwise comparisons between assemblages sampled at the same time, but at different locations within the same island, to assess parasite assemblage homogenization over distance and the assumption, implicit in our analysis, that assemblages were initially homogeneous across formerly connected islands. Assemblage comparisons, sample size, and year of collection are listed on Tables 1 and 2 of the Supplemental Material. We

analyze two types of parasite assemblages (Table 2, Supplemental Material). Island assemblages refer to all haemosporidian lineages sampled on an island. Host focal assemblages refer to haemosporidian lineages recovered from a suite of six common bird species across individual islands. We compare bird assemblages themselves to quantify the influence of variation in host composition on changes on haemosporidian assemblages over time.

Our analyses are based on blood samples obtained from 6,270 individuals of 143 avian host species across 17 islands of the West Indies archipelago, five pairs of which were connected until ca. 2.5 ka (Figure 1, Supplementary Tables 1 and 2). Parasite assemblage data come from 1,245 sequenced haemosporidian infections assigned to 71 phylogenetically distinct lineages, which are defined by nucleotide differences in a region of the parasites' mitochondrial cytochrome b gene(9). Beta (β) diversity is a between-sample component of variance used by community ecologists to quantify differences between two or more assemblages of organisms. Here, β diversity metrics are used to describe differences in the composition of haemosporidian parasite assemblages between pairs of islands, and over space and time within islands. We use these metrics to compare four groups of assemblage pairs associated with distinct points on a time-scale of island isolation: 1) islands never connected, and separated by millions of years; 2) islands connected until ca. 2.5 ka; 3) assemblages from the same island, but sampled over intervals of two years to two decades; 4) contemporary assemblages from the same island, isolated by distances comparable to the distances between islands, and testing the assumption of homogeneity between pairs of assemblages connected by land.

Results

Parasite assemblages on islands connected by land bridges until ca. 2.5 ka were as differentiated as pairs of assemblages on islands that have never been connected, and assemblages on both groups were considerably more differentiated than assemblages from the same island sampled at intervals of up to two decades, or between contemporaneous samples from the same island ($\beta_{Chao-Sørensen}$, island assemblages: χ^2=31.8, P<0.01; host focal assemblages: χ^2=6.0, P=0.04; Figure 2, Supplementary Table 4a and 4b). Avian host assemblages are not differentiated within these comparisons ($\beta_{Chao-Sørensen}$, avian assemblages: χ^2=3.9, P=0.2; Supplementary Table 4c); most of the common host species used in our analyses are widely distributed within the West Indies(10). Thus, the increasing dissimilarity between haemosporidian assemblages over time is not caused by changes in host assemblages.

Beta (β) diversity quantifies the variance between two assemblages and it can be partitioned into components that represent replacement of parasite lineages (i.e., coupled

gains and losses), differential gains or losses of lineages, and variation in lineage prevalence(10). The contribution of each partition might represent assembly processes that occur on distinct time-scales. Haemosporidian lineages often present short-term variation in prevalence(5, 6, 11), including cycles in the proportion of infected host individuals(5). Replacement of lineages might reflect sorting processes that constrain assemblage composition, including competition among parasite lineages, involving interactions through the host immune system and the evolution of host resistance.

Most parasite lineages on individual islands were replaced or lost over periods of 2.5 ka or more (host focal assemblages, $\beta_{Replacement}$ χ^2=9.3, P<0.01; $\beta_{Nestedness}$ χ^2=8.2, P<0.01; Figure 2, Supplementary Table 4a). Lineages within host focal assemblages were replaced almost twice as often between islands isolated for millions of years than between islands separated by ca. 2.5 ka. However, lineages were replaced between formerly connected assemblages more frequently than between local assemblages sampled over time intervals of up to two decades. Parasite lineage replacement in host focal assemblages was not related to replacement of host species, which do not vary across comparison groups ($\beta_{Replacement}$ avian assemblages: χ^2=5.4, P=0.07; Supplementary Table 4c). The overall lineage composition within an island appeared to be stable, as we observed no differences in the frequency with which lineages were replaced between island assemblages across all comparison groups ($\beta_{Replacement}$ χ^2=2.9, P=0.4; $\beta_{Nestedness}$ χ^2=2.3, P=0.5; Supplementary Table 4b), even though replacement in host focal assemblages was more frequent at longer time intervals. Hence, changes in lineage prevalence, not replacement or losses of lineages, are primarily responsible for the positive relationship between dissimilarity and isolation time in island assemblages. This suggests that although the composition of haemosporidian assemblages of an island remains stable through time, parasite lineages switch among different host species within an island. The observed temporal stability of insular haemosporidian assemblage composition is likely a result of limited parasite dispersal among islands within the archipelago, as differential gains and losses of lineages ($\beta_{Nestedness}$) did not explain differentiation of assemblages with increased isolation time (Supplementary Table 4b)(12).

Finally, we ask whether replacement of haemosporidian lineages involves closely or distantly related parasites. If closely related lineages replaced one another more often than expected by chance, parasite assemblages might be constrained by one or more traits that are shared by common descent through parasite evolutionary history. Secondly, if lineages are substituted at random, stochastic processes, such as ecological drift, are the major drivers of lineage composition on haemosporidian assemblages. Alternatively, if substituted lineages are distant relatives, competitive exclusion might constrain the

coexistence of closely related lineages within an island host fauna. We found that differential gain and loss of lineages over evolutionary time scales results in higher phylogenetic diversity of haemosporidian assemblages on islands isolated by deep marine channels ($\beta_{Phylo-Sor\ PD}$ χ^2=7.7, *P*=0.02; Supplementary Table 4a). However, replacement of haemosporidian lineages apparently is unrelated to phylogenetic relationships among parasites ($\beta_{Phylo-Sor\ Replacement}$, island assemblages: χ^2=4.8, *P*=0.2; host focal assemblages: χ^2=3.2, *P*=0.2; Supplementary Table 4a and 4b). This result mirrors the observation that the hosts of related parasites (i.e., sister lineages) are not more closely related than pairs of species drawn at random from the host taxonomic hierarchy(13). In the West Indies, we frequently observe locally prevalent and geographically widespread lineages replacing one another across islands of the archipelago (Figure 3)(12, 14).

Discussion

We have found that pairs of insular haemosporidian assemblages that were formerly homogenized, but isolated for the past ca. 2.5 ka, present decay in similarity comparable to pairs of assemblages that have never been connected (Figure 2). The most detailed retrospective record of endemic haemosporidian assemblages is from a breeding population of great reed warbler (*Acrocephalus arundinaceus*) from Scandinavia(5). Over the period of 17 years, *A. arundinaceus* hosts were mainly infected by three haemosporidian lineages that presented coupled periodicity in prevalence, with cycles every 3-4 years (5). On Puerto Rico and Saint Lucia, in the West Indies, changes on haemosporidian assemblages between intervals of approximately ten years are mostly due to variation in the prevalence of parasite lineages, but gains and losses of lineages were also observed (11). Similar patterns of temporal dynamics are observed for human malaria parasites. A 14-year cohort study of *Plasmodium falciparum* infections of humans in Brazil revealed temporal fluctuations in the frequency of allelic variants of the polymorphic merozoite surface protein gene (msp-1)(15). Mathematical models have suggested that parasite prevalence can present periodic and even deterministic chaotic behavior when host populations are exposed to pathogens with polymorphic antigens (16). Although we present strong evidence of temporal variation over evolutionary time-scales (Figure 2, Supplementary Table 4a and 4b, the mechanisms producing change in haemosporidian assemblages remain unknown.

Our results suggest long-term temporal variation on the regulatory effects of haemosporidian parasites on their host populations, and vice versa. In the initial stages of a host-parasite interaction, a population of parasites presents a strong selective pressure, and therefore a regulatory effect, over their host populations (i.e., periods of high parasite prevalence). Through time, host susceptibility wears off as hosts evolve immune

resistance, switching the direction of selective pressure towards parasite populations and reducing parasite prevalence(17). Haemosporidian parasites can depress avian populations on islands of the Lesser Antilles, where relative population sizes of two widespread and common species of bird were negatively related to the relative abundances of two common parasite lineages(14). Hence, the temporal variation in the prevalence of endemic parasite lineages in the West Indies (Figure 2) and elsewhere could reflect cycles of host-parasite evolution and co-evolution.

Islands in the West Indies present parasite assemblage stability, exhibited by the little variance on the frequency of lineage replacement through time (Figure 2). However, within an island, haemosporidian assemblages of specific host species present dynamic composition, in which replacement of lineages within subset assemblages within an island is more likely to happen over longer time scales of several thousands of years (Figure 2). This result suggests that differences in haemosporidian assemblages between islands are due to host switching of parasite lineages between host populations within an island, rather than replacement of lineages over the island as a whole; this observation is supported by the remarkable ability of host shifting among avian haemosporidian parasites (6, 13). Although we cannot assess the mechanisms that drive replacement and host shifting of lineages, we suggest that the observed "ecological drift" of lineage distributions across host species is consistent with the observed phylogenetic unpredictability of lineage replacement among host populations within an island (i.e., lineages are substituted at random across the parasite phylogeny). Insular assemblages of the West Indies represent a scenario of limited dispersion of parasite lineages (12), in whivh ecological drift would predict that the chance one lineage would replace another is directly proportional to its prevalence (i.e., probability of replacement drawn from the prevalence of the lineage in the island assemblage).

In conclusion, differentiation among haemosporidian assemblages that were presumed to be homogeneous on connected islands until 2.5 ka reflects variation in the prevalence of established parasite lineages, and not substitutions, gains, or losses of lineages. In contrast, turnover of lineages drives dissimilarity among haemosporidian assemblages between pairs of islands that have been isolated for longer periods, up to millions of years. Our results suggest limited dispersal of parasite lineages among insular host assemblages, and that parasite lineages of particular host species are replaced at random with respect to parasite phylogenetic relationship.

Methods

Study Sites. Field studies were conducted on 17 islands across the West Indies. Formerly connected pairs of islands included: 1) Grenada and Carriacou; 2) Antigua and Barbuda;

3) St. Kitts and Nevis; 4) Little Cayman and Cayman Brac; and 5) Eastern Puerto Rico and British Virgin Islands. Each assemblage from these five pairs of formerly connected islands was compared to assemblages on isolated islands, with the exception of Puerto Rico and British Virgin Islands for which we had no suitable comparison. The isolated islands in each comparison were, respectively: 1) St. Vincent; 2) Guadeloupe; 3) Montserrat; 4) Grand Cayman. Pair-wise comparisons between assemblages sampled on the same island, over intervals of up to two decades, were: Dominican Republic (2001, 2002, and 2014) and St. Lucia (1991 and 2000). In addition, we sampled haemosporidian assemblages geographically separated within the same island: 1) St. Eustatius; 2) St. Kitts; 3) Nevis; 4) St. Vincent. Detailed information about study sites and sample collection is provided in the Supplement.

Field methods. We captured birds with mist nets in representative habitats on each island, generally during the late spring and summer months. We took blood samples from captured individuals by brachial venipuncture and stored the samples in Puregene® (Germantown, MD, USA) or Longmire's lysis buffer. All samples were collected under IACUC protocols approved at the University of Pennsylvania (collections up to 1995), and at the University of Missouri-St Louis or National Aviary (after 1995), and under appropriate permits from the governments of the individual islands. Individual birds were released after blood sampling at the site of capture.

Laboratory methods. We extracted DNA from lysis buffer by isopropanol precipitation proceeded by removal of proteins by ammonium acetate precipitation. We used polymerase chain reaction (PCR) to detect the presence of haemosporidian infections by amplification of a highly conserved, 154-bp 16S rRNA-coding sequence of the parasite mitochondrial DNA18. Samples found to be infected in the first PCR step were further subjected to one or more nested PCR assays that amplify a phylogenetically informative region of the mitochondrial cytochrome b gene (cyt b) of haemosporidian parasites of the genera Plasmodium and Haemoproteus. For the first set of nested PCR assays, we used the outer primer pair 3932F and DW4R(18, 19)19,20 and the inner primer pair 413F and 926R(20)21. We also amplified and sequenced regions of the mitochondrial cyt b gene from positive samples using a variety of primer pairs and protocols(21-23)22-24. Our protocols often fail to recognize mixed infections, meaning that parasite lineage prevalence in host populations may be somewhat underestimated. We distinguished lineages of haemosporidian parasites based on pairwise nucleotide differences between sequences(9)11.

Phylogenetic tree reconstruction. We constructed a phylogenetic tree of 132 haemosporidian lineages from North America, Central America, and the Caribbean region. We used Geneious®

9.1.5 to generate an alignment of the 132 Plasmodium and Haemoproteus sequences together with 10 Leucocytozoon sequences used as outgroup(24). We used BEAST 2.4.2 and its applications(25) to obtain a Bayesian maximum clade credibility tree using an uncorrelated relaxed lognormal clock, a GTR+I+G substitution model, and a Yule speciation prior. Trees were sampled every 5,000th run throughout an MCMC chain of 10 million trees and 10% burn-in. Analyses were run using CIPRES cluster(26). The maximum clade credibility tree was summarized using TreeAnnotator 2.4.2 and visualized using FigTree 1.4.2 (Supplementary Data Figure 1).

Statistical analyses. For each haemosporidian lineage, we calculated the proportion of infected host individuals, which we refer as lineage prevalence. Two ratios were calculated using two populations of hosts: 1) captured individuals of six selected focal bird species (*island assemblages*); and 2) captured individuals of all haemosporidian-infected bird species on an island (*host focal assemblages)*. Focal host species were the most well-sampled and widespread avian species for which we had haemosporidian infections recorded. These were: 1) Bananaquit, *Coereba flaveola*; 2) Black-faced grassquit, *Tiaris bicolor*; 3) Common ground dove, *Columbina passerina*; 4) Lesser Antillean bullfinch, *Loxigilla noctis*; 5) Pearly-eyed thrasher, *Margarops fuscatus*; and 6) Scaly-breasted thrasher, *Margarops fuscus*. We used these lineage prevalence data to build island × parasite assemblage pair-wise comparison matrices. Due to limitations in sample size, we did not include comparisons of contemporary assemblages using host focal assemblage data. We then used these matrices to calculate metrics that describe and partition β diversity to characterize pair-wise assemblage comparisons.

We first calculated Chao-Sørensen dissimilarity, which estimates the difference between assemblages on two islands by taking into account lineage prevalence, the number of island-unique lineages, and the number of shared lineages between islands(*26*). Chao-Sørensen dissimilarity was calculated by

$$\beta_{\text{Chao-Sørensen}} = 1 - \frac{2UV}{U+V},$$

where *U* is the sum of the prevalence of all shared lineages (i.e., present on both islands) on one island, and *V* represents the same for the other island. When islands of a pair have no shared lineages, dissimilarity equals 1; when islands of a pair have identical composition and prevalence of haemosporidian lineages, the dissimilarity index equals 0.

We then used an additive partitioning method to decompose β diversity into two compartments with different biological meaning: turnover (i.e., replacement) and nestedness(*27*). This partitioning method is based on the Sørensen dissimilarity index, but also takes into account the proportion of shared lineages among assemblages.

$$\beta_{\text{Sørensen}} = \frac{b + c}{(2a) + b + c}$$

The parameter *a* represents the number of shared lineages, as above, and the parameters *b* and *c* represent, for each island, the number of lineages present on one island, but absent on the other. Therefore, $\beta_{\text{Sørensen}}$ combines differences between islands in both the total number of lineages and lineage identity. Following Baselga (2009), we calculated assemblage turnover as:

$$\beta_{Turnover} = \frac{min(b, c)}{a + min(b, c)}$$

Then, we calculated $\beta_{\text{Sørensen}} - \beta_{Turnover}$ as the nested component of β diversity ($\beta_{\text{Nestedness}}$). As an example of assemblage turnover, bananaquits (*Coereba flaveola*) from Grenada are infected by the lineages OZ21 and OZ04, whereas on Carriacou, an islet connected to Grenada by land bridges up until ca. 2.5 ka, OZ21 is replaced by lineage LA07. On Barbuda, bananaquit hosts are infected by two haemosporidian lineages, LA07 and OZ21. However, on Antigua, an island connected to Barbuda during the Last Glacial Maximum (LGM), bananaquits are infected solely by OZ21, which makes Antigua a nested subset of Barbuda's haemosporidian assemblage. We used the R package *betapart*(*28*) to calculate and partition $\beta_{\text{Sørensen}}$ dissimilarity.

We applied a metric analogous to $\beta_{\text{Sørensen}}$ and its partitions to weight dissimilarity by the phylogenetic distance between lineages among assemblages(*29*). To calculate phylogenetic β diversity, we used the same parameters *a*, *b*, and *c* described above, but weighted by the total branch length of lineages from the haemosporidian phylogeny we generated. The phylogenetic diversity of an assemblage (PD) is the sum of the length of all branches from all lineages present in that assemblage. When comparing two assemblages, phylogenetic β diversity represents the variation in composition due to differences between the total PDs of each assemblage (i.e., phylogenetic nestedness), and due to the turnover of lineages that are not related to differences in PD among assemblages (phylogenetic turnover). PhyloSør and its partitions are calculated by

$$\beta_{Phylo\text{Sør}} = \frac{2PD_{total} + PD_k + PD_j}{PD_k + PD_j}$$

and

$$\beta_{Phylo\text{Sør}-\text{Turnover}} = \frac{min(PD_{total} - PD_k\ ,\ PD_{total} - PD_j)}{PD_k + PD_j - PD_{total} + min(PD_{total} - PD_k\ ,\ PD_{total} - PD_j)},$$

where PD_k and PD_j are the sum of branch lengths of lineages from assemblage *k* and *j*, respectively, and PD_{total} is the sum of branch lengths from all lineages present on

assemblages *k* and *j*.

$$\beta_{PhyloSør-PD} = \beta_{PhyloSør} - \beta_{PhyloSør-Turnover}$$

To determine whether assemblages are more evolutionarily related than expected by chance, which indicates environmental (including host) filtering, we compared phylogenetic and taxonomic β diversity among pairs of assemblages. Low phylogenetic and high taxonomic β diversity suggests that assemblages share closely related, but not identical lineages of parasites. In contrast, high phylogenetic and low taxonomic β diversity suggests exclusion of phylogenetically related lineages, potentially the result of parasite competition. We also calculated the standardized effect size (*SES*) for $\beta_{PhyloSør}$ and its partitions, using the formula:

$$SES = \frac{\beta_{obs} - mean(\beta_{null})}{sd(\beta_{null})}$$

The parameter β_{obs} represents the value of the phylogenetic β diversity index derived from the data, whereas mean(β_{null}) and sd(β_{null}) represent the average and the standard deviation of a null distribution of phylogenetic β diversity indices calculated by shuffling labels of lineages in the phylogeny 999 times. Results are displayed in the Supplementary Data Figure 2.

Finally, we used linear mixed models to test the null hypothesis that assemblage dissimilarity does not change across the four comparison groups: between islands that have never been connected; between islands connected during the Pleistocene LGM and isolated for 2.5 ka; between samples separated by short periods of time (i.e., years or decades); and between contemporary samples from the same island. In one set of models, we combined all pair-wise comparisons from assemblages of host focal species and treated avian host species identity as a random intercept in the models. Note that comparisons using pooled host focal assemblages did not include contemporary samples from the same island due to sampling limitations. In a second set of models, we used data from the overall island haemosporidian assemblages and treated groups of island comparisons as a random intercept in the models. The first set of models describes changes on haemosporidian assemblages of determined host species, occurring within the same island, whereas the second set of models provides an overview of changes on island assemblages in their entirety. In both sets of models, by adding a random intercept we not only acknowledge that there is variation in dissimilarity within each set of comparisons, but also address the unbalanced design across groups of pair-wise comparisons (Supplemental Table 2). We ran one model per β diversity metric, requiring ten models for pooled host focal assemblages and ten models for island assemblages. All models were weighted by sample size, calculated as the sum of the total number of host

individuals captured on each island. Linear mixed models were run using the R package lme4(*30*).

References

1. R. Ricklefs, E. Bermingham, The West Indies as a laboratory of biogeography and evolution. Philos. Trans. R. Soc. Lond., B, Biol. Sci. 363, 2393–2413 (2008).

2. G. Valkiūnas, Avian malarial parasites and other haemosporidia (Taylor and Francis Publishing, London, 2004).

3. G. Poinar Jr, *Plasmodium dominicana* n. sp. (Plasmodiidae: Haemospororida) from Tertiary Dominican amber. Syst Parasitol. 61, 47–52 (2005).

4. S. Fallon, E. Bermingham, R. E. Ricklefs, Host specialization and geographic localization of avian malaria parasites: a regional analysis in the Lesser Antilles. American Naturalist. 165, 466–480 (2005).

5. S. Bensch et al., Temporal dynamics and diversity of avian malaria parasites in a single host species. J Anim Ecol. 76, 112–122 (2007).

6. V. A. Ellis et al., Local host specialization, host-switching, and dispersal shape the regional distributions of avian haemosporidian parasites. Proceedings of the National Academy of Sciences. 112, 11294–11299 (2015).

7. W. R. Peltier, R. G. Fairbanks, Global glacial ice volume and Last Glacial Maximum duration from an extended Barbados sea level record. Quaternary Science Reviews. 25, 3322–3337 (2006).

8. G. F. Camoin, L. F. Montaggioni, C. J. R. Braithwaite, Late glacial to post glacial sea levels in the Western Indian Ocean. Marine Geology. 206, 119–146 (2004).

9. M. Svensson-Coelho et al., Diversity, prevalence, and host specificity of avian *Plasmodium* and *Haemoproteus* in a Western Amazon Assemblage. Ornithological Monographs. 76, 1–47 (2013).

10. H. A. Raffaele, J. W. Wiley, O. H. Garrido, J. I. Raffaele, Birds of the West Indies (Princeton University Press, Princeton, 2003).

11. S. M. Fallon, R. E. Ricklefs, S. C. Latta, E. Bermingham, Temporal stability of insular avian malarial parasite communities. Proceedings of the Royal Society B: Biological Sciences. 271, 493–500 (2004).

12. R. E. Ricklefs, J. Dodge Gray, S. C. Latta, M. Svensson Coelho, Distribution anomalies in avian haemosporidian parasites in the southern Lesser Antilles. Journal of Avian Biology. 42, 570–584 (2011).

13. R. E. Ricklefs et al., Species formation by host shifting in avian malaria parasites. Proceedings of the National Academy of Sciences. 111, 14816–14821 (2014).

14. R. E. Ricklefs, L. Soares, V. A. Ellis, S. C. Latta, Haemosporidian parasites and avian host population abundance in the Lesser Antilles. Journal of Biogeography. 43, 1277–1286 (2016).

15. N. S. Silva, L. A. Silveira, R. L. Machado, M. M. Póvoa, M. U. Ferreira, Temporal and spatial distribution of the variants of merozoite surface protein-1 (MSP-1) in *Plasmodium falciparum* populations in Brazil. Annals of Tropical Medicine and Parasitology. 94, 675–688 (2000).

16. S. Gupta, Chaos, persistence, and evolution of strain structure in antigenically diverse infectious agents. Science. 280, 912–915 (1998).

17. R. M. May, R. M. Anderson, Population biology of infectious diseases: Part II. Nature. 280, 455–461 (1979).

18. S. L. Perkins, J. J. Schall, A molecular phylogeny of malarial parasites recovered from cytochrome b gene sequences. The Journal of Parasitology. 88, 972–978 (2002).

19. K. J. Olival, E. O. Stiner, S. L. Perkins, Detection of *Hepatocystis* sp. in southeast asian flying foxes (Pteropodidae) using microscopic and molecular methods. Journal of Parasitology. 93, 1538–1540 (2007).

20. R. E. Ricklefs et al., Community relationships of avian malaria parasites in southern Missouri. Ecological Monographs. 75, 543–559 (2005).

21. S. Bensch et al., Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proceedings of the Royal Society B: Biological Sciences. 267, 1583–1589 (2000).

22. O. Hellgren, J. Waldenström, S. Bensch, A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. Journal of Parasitology. 90, 797–802 (2004).

23. D. C. Outlaw, R. E. Ricklefs, Rerooting the evolutionary tree of malaria parasites. Proceedings of the National Academy of Sciences. 108, 13183–13187 (2011).

24. A. J. Drummond, M. A. Suchard, D. Xie, A. Rambaut, Bayesian Phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. 29, 1969–1973 (2012).

25. M. A. Miller, W. Pfeiffer, T. Schwartz, Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Proceedings of the GCE Workshop (2010).

26. A. Chao, R. L. Chazdon, R. K. Colwell, T.-J. Shen, A new statistical approach for assessing similarity of species composition with incidence and abundance data. Ecology Letters. 8, 148–159 (2004).

27. A. Baselga, Partitioning the turnover and nestedness components of beta diversity. Global Ecol Biogeography. 19, 134–143 (2009).

28. A. Baselga, C. D. L. Orme, betapart: an R package for the study of beta diversity. Methods Ecol Evol. 3, 808–812 (2012).

29. F. Leprieur et al., Quantifying phylogenetic beta diversity: distinguishing between "true" turnover of lineages and phylogenetic diversity gradients. PLoS ONE. 7, e42760 (2012).

30. D. Bates, M. Mächler, B. Bolker, S. Walker, Fitting linear mixed-effects models using lme4. Journal of Statistical Software. 67, 1–48 (2015).

Figures

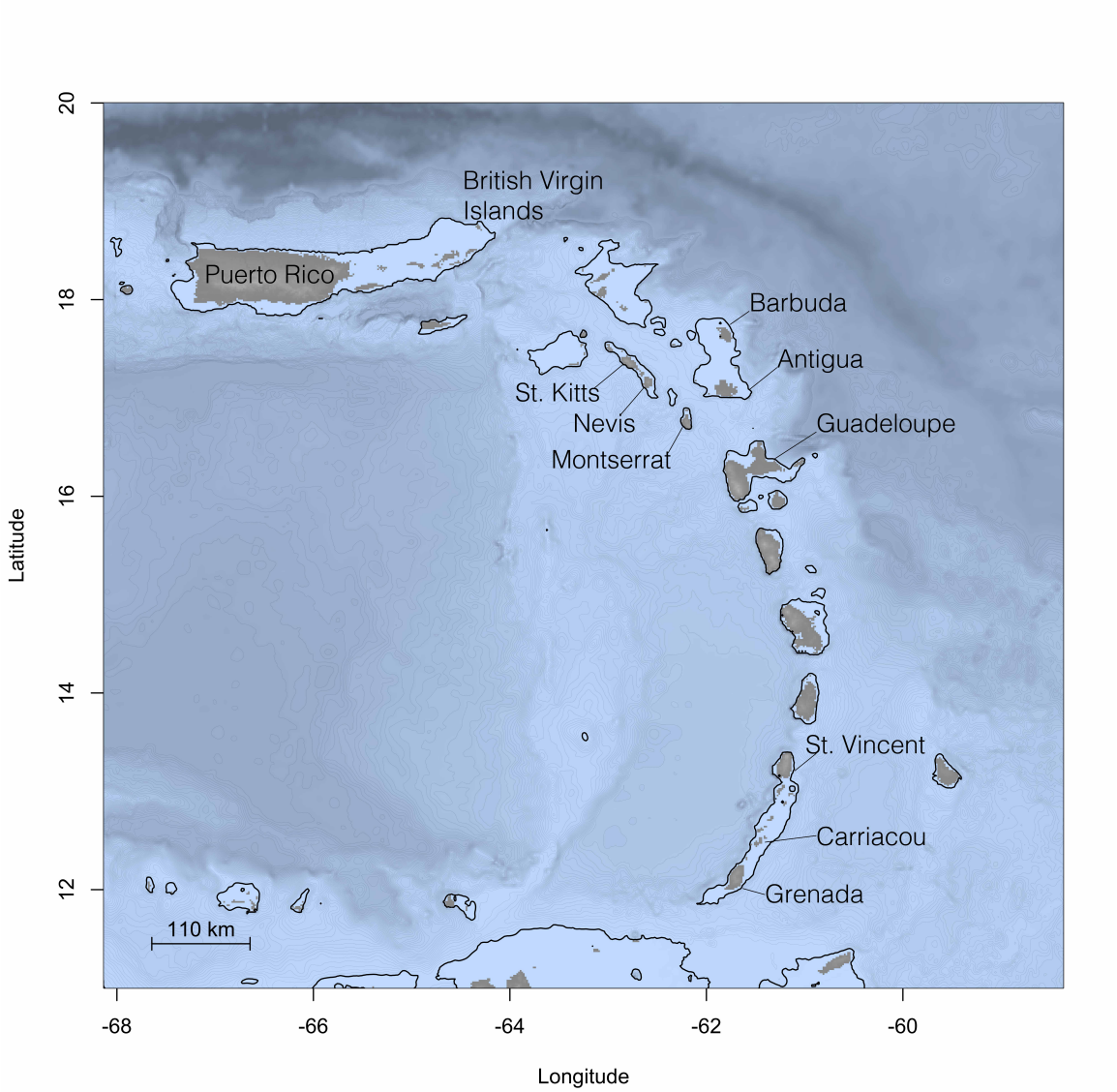


Figure 1: Bathymetric map representing part of the West Indies archipelago. Puerto Rico and islands of the Lesser Antilles are represented. Black contours delineate the 100 m isobath (i.e., depth contour). This isobath approximates shorelines at the most recent sea level minimum, ca. 26 ka. Islands that were formerly connected, as well as their isolated counterparts, are labeled; the Cayman Islands are located to the west in the Greater Antilles, outside the map area. Bathymetry data were obtained from the National Oceanic and Atmospheric Administration (NOAA).

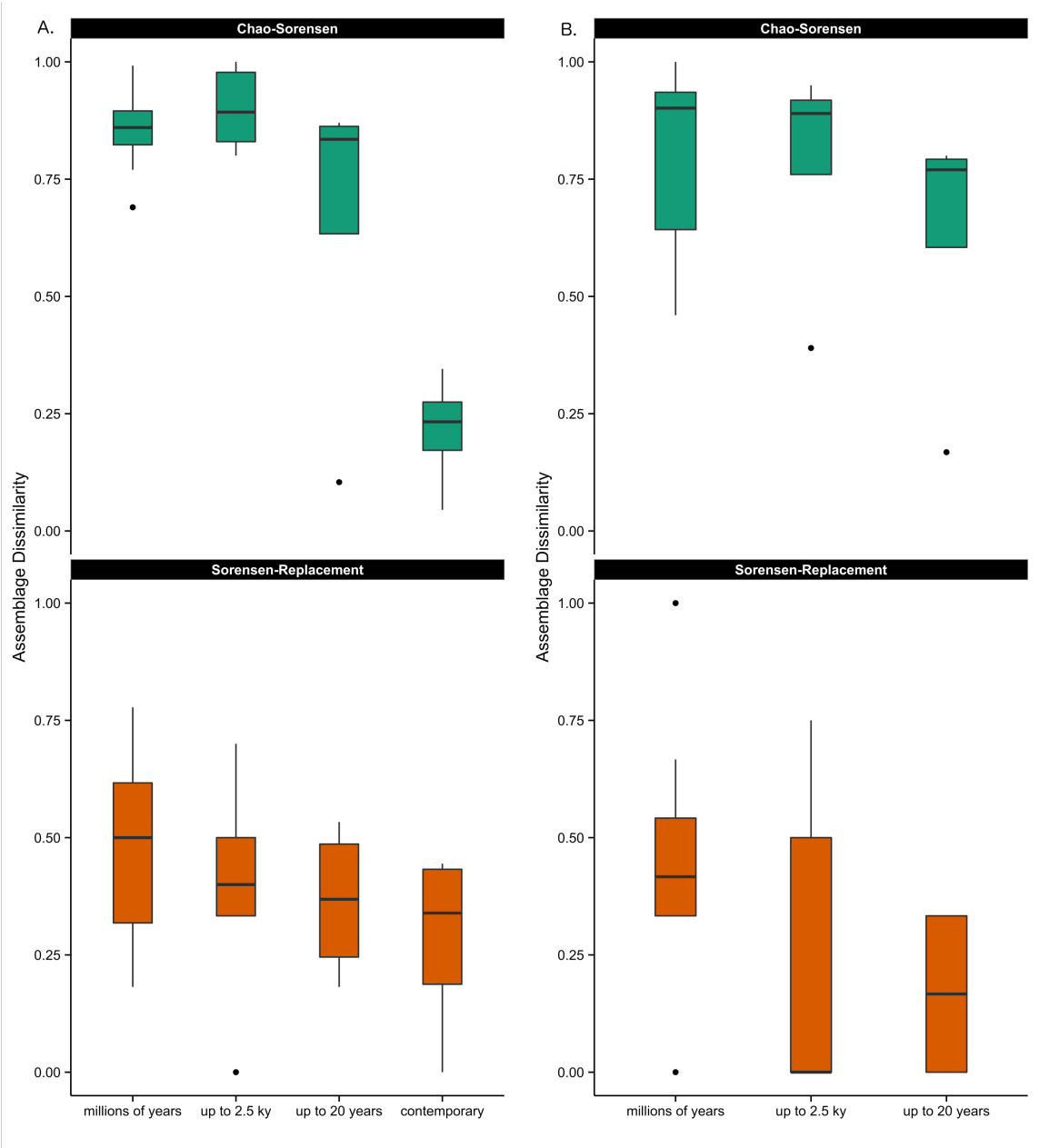


Figure 2: Haemosporidian assemblage dissimilarity across groups of island pair-wise comparisons. Dissimilarity is represented by two metrics: Chao-Sørensen (i.e., overall dissimilarity describing differences in lineage composition and prevalence) and Sørensen-Replacement (i.e., dissimilarity describing differences in lineage composition only), which are summarized for each group of pair-wise assemblage comparison. Chao-Sørensen estimates dissimilarity between two assemblages based on (i) the prevalence of parasite lineages, (ii) the number of lineages shared between two assemblages, and (iii) the number of unique lineages in each assemblage. Chao-Sørensen values vary from 0 for identical assemblages, to 1 for assemblages that differ completely with respect to one, or a combination, of the metric components. Sørensen-Replacement is a compositional dissimilarity metric that measures only the sharing, or not, of lineages and attributes zero

weight to differences in prevalence. When two assemblages of hosts support the same lineages of parasites, Sørensen-Replacement = 0; when no lineages are shared, Sørensen-Replacement = 1. The left panel (A) summarizes dissimilarity metrics for pair-wise comparisons based the overall haemosporidian assemblage within each sample. On island haemosporidian assemblages, dissimilarity measured by Chao-Sørensen (top left) increases with isolation time (P_{χ^2} <0.01; χ^2 = 31.8), but replacement of lineages (bottom left) is unrelated to assemblage isolation (P_{χ^2} = 0.4; χ^2 = 2.9). The right panel (B) summarizes dissimilarity metrics for pair-wise comparisons based on haemosporidian assemblages of bananaquits (*Coereba flaveola*), which similarly to island assemblages, show a patter of decay in assemblage similarity with isolation time based on the Chao-Sørensen dissimilarity metric (P_{χ^2} = 0.04; χ^2 = 6.0). However, replacement of haemosporidian lineages on bananaquit assemblages increase with isolation time (P_{χ^2} < 0.01; χ^2 = 9.3). Contemporary comparisons of haemosporidian assemblages from banaquits were not included in the analyses due to sampling limitation.

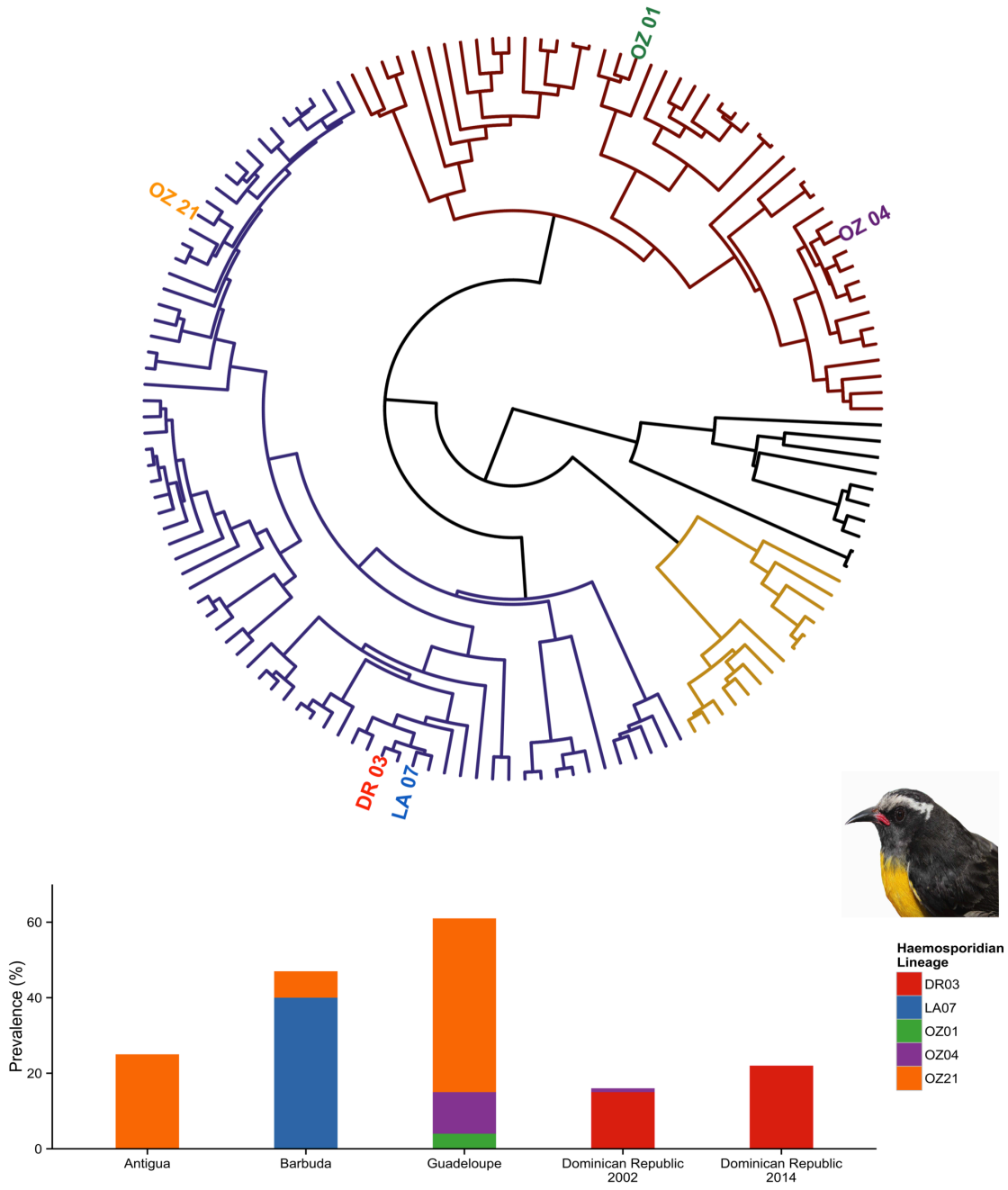


Figure 3: Haemosporidian assemblages from selected populations of Coereba flaveola (bananaquit). Lineage OZ21 (Haemoproteus coatneyi, a host generalist) occurs in three out of five samples; its prevalence is inversely related to that of the bananaquit specialist lineage, LA07 (Haemoproteus sp.). Antigua and Barbuda were connected by a broad land until 2.5 ka, but both islands have always been separated from Guadeloupe by deep marine channels. The parasite lineages on Antigua and Barbuda differ from those on Guadeloupe in prevalence, composition, and number of parasite lineages; parasite assemblages in the Dominican Republic that are separated by a short time interval have similar composition, with variation in the prevalence of the dominant lineage DR03,

which is closely related to the bananaquit specialist LA07. The phylogeny represents 132 lineages of Haemosporidian parasites from North America, Central America, and the Caribbean. Lineages found infecting the five populations of C. flaveola are labeled in the phylogeny (maximum clade credibility tree), showing that lineage composition and replacement on parasite assemblages are phylogenetically uncorrelated. Branch colors correspond to the four major haemosporidian genera: black for *Leucocytozoon* sp., the outgroup; brown for *Plasmodium* sp. lineages; blue for *Haemoproteus (Haemoproteus)* lineages; and gold for *Haemoproteus (Parahaemoproteus)* lineages.

Supplementary Tables

Supplementary Table 1: Sampling summary across 17 islands of the archipelago of the West Indies, displaying the year of sampling on each island, and the number of blood samples collected on each year (in between parenthesis). Islands are ordered on the table based on their geographic position, with northernmost islands on the top; geographic coordinates for each island are given.

Island	Year (Number of Samples)	Coordinates
Grand Cayman	2002 (281)	19.31, 81.23
Cayman Brac	2002 (186)	19.72, 79.79
Little Cayman	2002 (89)	19.69, 80.04
Dominican Republic	1994 (18); 1995 (89); 2001 (612); 2002 (642); 2014 (600)	18.78, 70.35
Eastern Puerto Rico	1993 (149)	18.29, 65.70
British Virgin Islands	2001 (150)	18.47, 64.56
St. Eustatius	2015 (375)	17.49, 62.97
St. Kitts	2012 (321)	17.34, 62.77
Nevis	2012 (353)	17.15, 62.58
Barbuda	1993 (95)	17.62, 61.77
Antigua	1993 (88)	17.10, 61.81
Montserrat	1993 (150)	16.73, 62.18
Guadeloupe	1993 (171)	16.11, 61.66
St. Lucia	1991(226); 2000 (200)	13.86, 60.97
St. Vincent	2008 (334)	13.21, 61.20
Carriacou	2013 (241)	12.47, 61.44
Grenada	2002 (477)	12.12, 61.68

Supplementary Table 2: Pair-wise comparisons of haemosporidian parasite assemblages. Pairs of islands are represented by rows, and the total number of pair-wise comparisons is shown in between parenthesis. Columns represent the type of host haemosporidian assemblages were derived from. Island assemblage represents all haemosporidian infections retrieved within an island, and the remaining columns represent assemblages of individual host species, which we refer as focal assemblages. Species codes represent the following species: CFA, *Coereba flaveola* (bananaquit); LNO, *Loxigilla noctis* (Lesser Antillean bullfinch); CPA, *Columbina passerina* (common ground dove); *Tiaris bicolor* (black-faced grassquit); *Margarops fuscus* (pearly-eyed thrasher); and *Margarops fuscatus* (scaly breasted thrasher).

	Island Assemblage	CFA	LNO	CPA	TBI	MFU	MFT
Pairs of assemblages on islands isolated for millions of years (Never connected), *N*=34							
Grand Cayman *vs*. Little Cayman (2)							
Grand Cayman *vs.* Cayman Brac (2)							
Guadeloupe *vs.* Antigua (5)							
Guadeloupe *vs.* Barbuda (3)							
Montserrat *vs.* St. Kitts (5)							
Montserrat *vs.* Nevis (7)							
St. Vincent *vs*. Carriacou (6)							
St. Vincent *vs*. Grenada (4)							
Pairs of assemblages on islands connected up until 2.5 ka (LGM connected), *N*=15							
Little Cayman *vs*. Cayman Brac (2)							
East Puerto Rico *vs*. British Virgin Is. (2)							
Antigua *vs*. Barbuda (3)							
St. Kitts *vs*. Nevis (5)							
Carriacou *vs*. Grenada (3)							
Pairs of assemblages sampled between short periods of time (Short-term isolation), *N*=15							
Dominican Republic 2001 vs. 2002 (3)							
Dominican Republic 2001 vs. 2014 (3)							
Dominican Republic 2002 vs. 2014 (3)							
St. Lucia 1991 vs. 2000 (6)							
Pairs of assemblages within an island (Contemporary assemblage), *N*=4							
St. Eustatius (1)							
St. Kitts (1)							
Nevis (1)							
St. Vincent (1)							

Supplementary Table 3: Mean and standard deviation for seven metrics of β diversity comparing pairs of haemosporidian assemblages on islands of the West Indies archipelago. $\beta_{\text{Chao-Sørensen}}$ estimates dissimilarity between two assemblages based on the prevalence of parasite lineages, the number of lineages shared between two assemblages, and the number of unique lineages in each assemblage. $\beta_{\text{Chao-Sørensen}}$ values vary from 0 for identical assemblages, to 1, for assemblages that differ completely with respect to one, or a combination, of the three metric components. $\beta_{\text{Sørensen}}$ is a compositional dissimilarity metric that measures only the sharing, or not, of lineages and attributes zero weight to differences in prevalence, and can be partitioned into a measure of dissimilarity due to substitutions of lineages among islands ($\beta_{\text{Replacement}}$), and differences in gains and losses of lineages, estimating the extent at which one assemblage is a subset of the other ($\beta_{\text{Nestednedness}}$). $\beta_{\text{Phylo-Sør}}$ is the phylogenetic correspondent of $\beta_{\text{Sørensen}}$, in which dissimilarity is weighted by branch lengths taken from a phylogeny of haemosporidian lineages. The replacement component of $\beta_{\text{Phylo-Sør}}$ corresponds to differences in parasite assemblages that are attributed to replacement of lineages that do not change the phylogenetic diversity of assemblages, whereas the PD (i.e., phylogenetic diversity, the sum of branch lengths of all lineages within an assemblage) estimates the amount of dissimilarity between assemblages due to differences in the total branch length of assemblages. *SES*, or standard effect size, is a metric calculated for each compartment of phylogenetic β diversity (PhyloSor and its partition components, Replacement and PD), and represents how much the observed dissimilarity deviates from a null expectation. Assemblages with phylogenetic diversity higher than expected by chance (i.e., overdispersion, *SES*>1.96) represent assembly processes related to competitive exclusion and evolution of host resistance. Pairs of assemblages more similar than expected by chance (i.e., phylogenetic clustering, *SES*>1.96) suggest that replacement takes place among closely related lineages, a process correspondent to environmental filtering.

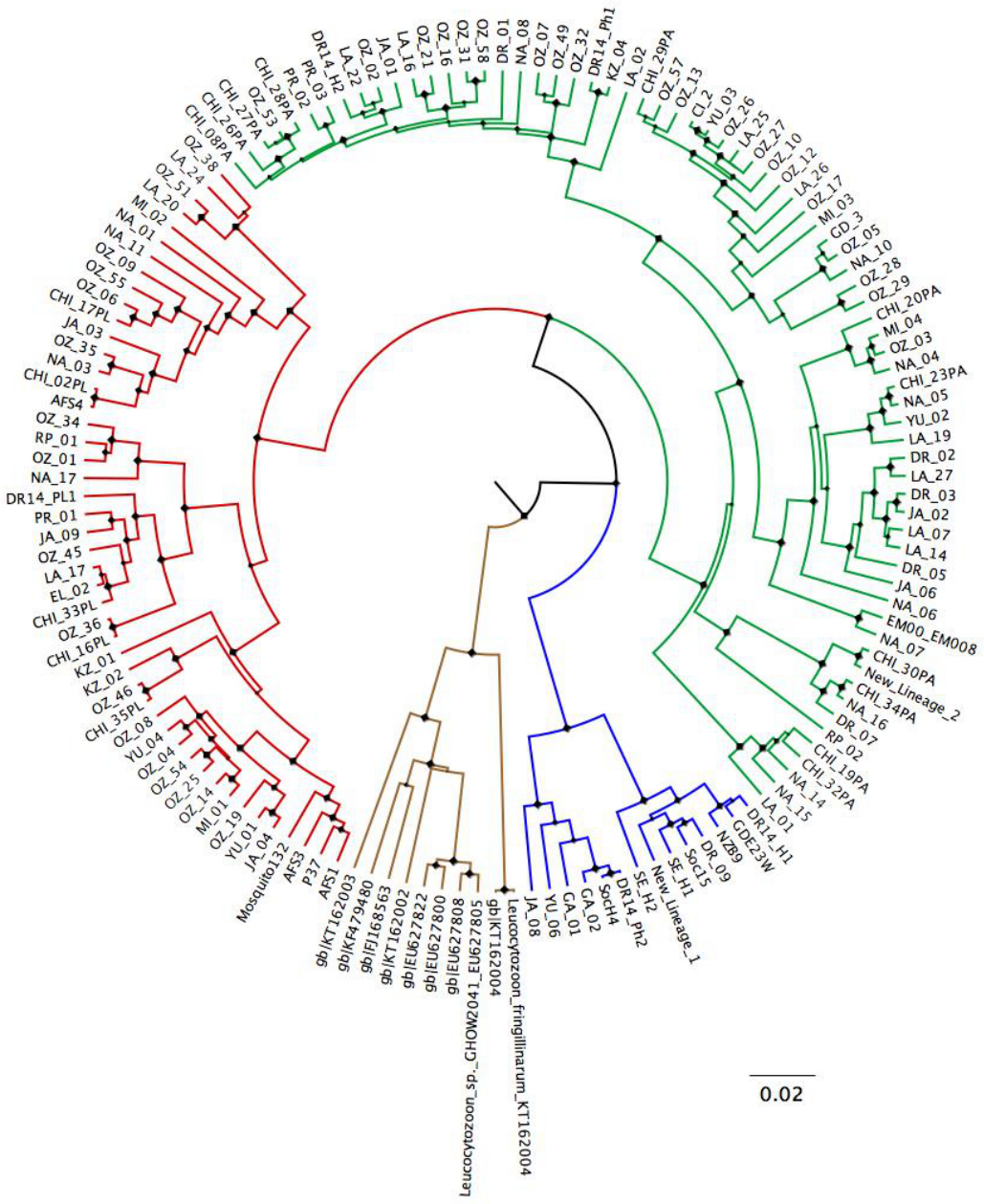
Island comparison	$\beta_{\text{Chao-Sørensen}}$	$\beta_{\text{Sørensen}}$	$\beta_{\text{Replacement}}$	$\beta_{\text{Nestedness}}$	$\beta_{\text{Phylo-Sør}}$	$\beta_{\text{Phylo-Sør Replacement}}$	$\beta_{\text{Phylo-Sør PD}}$	$SES\ \beta_{\text{Phylo-Sør}}$	$SES\ \beta_{\text{Phylo-Sør Replacement}}$	$SES\ \beta_{\text{Phylo-Sør PD}}$
Combined focal host assemblages, *Coreba flaveola*, *Tiaris bicolor*, *Loxigilla noctis*, *Columbina passerina*, *Margarops fuscus*, and *Margarops fuscatus*.										
Never connected	0.8 (0.2)	0.6 (0.3)	0.5 (0.4)	0.1 (0.2)	0.3 (0.2)	0.2 (0.2)	0.1 (0.1)	-0.4 (1.2)	-0.7 (1.0)	0.2 (1.0)
LGM connected	0.7 (0.2)	0.5 (0.3)	0.3 (0.4)	0.2 (0.2)	0.3 (0.2)	0.1 (0.2)	0.2 (0.1)	0.3 (0.7)	0.5 (0.6)	-0.1 (0.8)
Short-term isolation	0.5 (0.2)	0.4 (0.1)	0.2 (0.2)	0.2 (0.1)	0.3 (0.1)	0.1 (0.1)	0.2 (0.1)	-0.1 (1.2)	-1.1 (0.9)	1.0 (1.9)
Island assemblages										
Never connected	0.9 (0.1)	0.6 (0.2)	0.5 (0.2)	0.1 (0.1)	0.4 (0.2)	0.2 (0.2)	0.1 (0.2)	0.3 (0.9)	0.1 (1.1)	0.2 (1.4)
LGM connected	0.9 (0.1)	0.6 (0.2)	0.4 (0.3)	0.2 (0.2)	0.4 (0.1)	0.2 (0.1)	0.2 (0.2)	0.7 (1.0)	-0.4 (0.6)	1.3 (1.5)
Short-term isolation	0.7 (0.4)	0.4 (0.2)	0.4 (0.2)	0.0 (0.0)	0.2 (0.1)	0.2 (0.1)	0.1 (0.1)	0.3 (1.0)	0.8 (1.0)	-0.5 (0.5)
Contemporary	0.2 (0.1)	0.4 (0.1)	0.3 (0.2)	0.1 (0.2)	0.2 (0.1)	0.1 (0.1)	0.1 (0.1)	-0.8 (0.6)	-0.9 (0.6)	-0.2 (0.6)
Host assemblages										
Never connected	0.2 (0.1)	0.4 (0.1)	0.2 (0.1)	0.2 (0.1)						
LGM connected	0.3 (0.2)	0.4 (0.1)	0.2 (0.1)	0.2 (0.2)						
Short-term isolation	0.1 (0.0)	0.2 (0.1)	0.1 (0.1)	0.1 (0.1)						

Supplementary Table 4: Parameter estimates from the linear mixed effect models testing the null hypothesis that haemosporidian assemblages isolated for millions of years are as similar as assemblages isolated for short periods of time, or even assemblages that have never been separated. We run one model per dissimilarity metric for two types of assemblages: combined dissimilarity metrics from comparisons between parasite assemblages retrieved from focal host species, and dissimilarity metrics from comparisons between overall island haemosporidian assemblage.

Dissimilarity Metric (x)	Predictor (y)	Effec	S.E.	t-value	Pz	χ2	Pχ2
A. Combined pair-wise comparisons between haemosporidian assemblages of six avian host species							
$\beta_{\text{Chao-Sørensen}}$	**Never connected**	**0.8**	**0.1**	**12.7**	**0.0**	**6.0**	**0.04**
	LGM connected	-0.1	0.1	-0.7	0.5		
	Short-term isolation	**-0.2**	**0.1**	**-2.4**	**0.0**		
□Sørensen	Never connected	0.4	0.1	3.6	0.0	4.7	0.09
	LGM connected	0.1	0.1	1.4	0.2		
	Short-term isolation	0.2	0.1	2.0	0.0		
$\beta_{\text{Replacement}}$	**Never connected**	**0.5**	**0.1**	**4.2**	**0.0**	**9.3**	**0.009**
	LGM connected	**-0.2**	**0.1**	**-2.2**	**0.0**		
	Short-term isolation	**-0.3**	**0.1**	**-2.8**	**0.0**		
$\beta_{\text{Nestedness}}$	**Never connected**	**0.9**	**0.0**	**33.2**	**0.0**	**8.2**	**0.01**
	LGM connected	**-0.1**	**0.0**	**-2.0**	**0.0**		
	Short-term isolation	**-0.1**	**0.1**	**-2.6**	**0.0**		
$\beta_{\text{Phylo-Sør}}$	Never connected	0.3	0.0	5.7	0.0	0.9	0.6
	LGM connected	0.0	0.1	0.8	0.4		
	Short-term isolation	0.0	0.1	-0.3	0.8		
$\beta_{\text{Phylo-Sør Replacement}}$	Never connected	0.2	0.1	3.0	0.0	3.2	0.2
	LGM connected	0.0	0.1	-0.4	0.7		
	Short-term isolation	-0.1	0.1	-1.8	0.1		
$\beta_{\text{Phylo-Sør PD}}$	**Never connected**	**0.1**	**0.0**	**4.0**	**0.0**	**7.7**	**0.02**
	LGM connected	0.1	0.0	1.8	0.1		
	Short-term isolation	**0.1**	**0.0**	**2.6**	**0.0**		
Dissimilarity Metric (x)	**Predictor (y)**	**Effec**	**S.E.**	**t-value**	**Pz**	**χ2**	**Pχ2**
B. Pair-wise comparisons between overall island haemosporidian assemblages							
$\beta_{\text{Chao-Sørensen}}$	**Never connected**	**0.9**	**0.1**	**8.5**	**0.0**	**31.8**	**0.00**
	LGM connected	0.0	0.0	0.2	0.8		
	Short-term isolation	**-0.4**	**0.2**	**-2.1**	**0.0**		
	Contemporary isolated	**-0.6**	**0.1**	**-8.5**	**0.0**		
$\beta_{\text{Sørensen}}$	Never connected	0.4	0.1	4.8	0.0	4.5	0.2
	LGM connected	0.0	0.1	0.4	0.7		

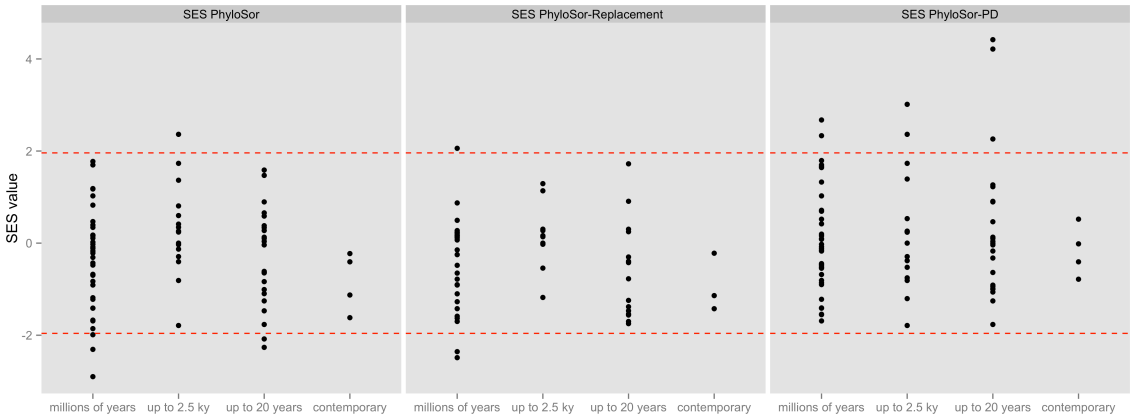
	Short-term isolation	0.3	0.2	1.9	0.1		
	Contemporary isolated	0.1	0.1	0.9	0.4		
$\beta_{\text{Replacement}}$	Never connected	0.5	0.1	5.8	0.0	2.9	0.4
	LGM connected	0.1	0.1	0.8	0.4		
	Short-term isolation	0.2	0.2	1.1	0.3		
	Contemporary isolated	0.1	0.1	1.2	0.2		
$\beta_{\text{Nestedness}}$	Never connected	0.9	0.1	15.1	0.0	2.3	0.5
	LGM connected	0.0	0.1	-0.7	0.5		
	Short-term isolation	0.1	0.1	0.9	0.4		
	Contemporary isolated	-0.1	0.1	-0.6	0.5		
$\beta_{\text{Phylo}-\text{Sør}}$	Never connected	0.4	0.1	6.8	0.0	5.0	0.2
	LGM connected	0.0	0.1	0.2	0.9		
	Short-term isolation	-0.2	0.1	-1.7	0.1		
	Contemporary isolated	-0.1	0.1	-1.2	0.2		
$\beta_{\text{Phylo}-\text{Sør Replacement}}$	Never connected	0.3	0.1	4.7	0.0	4.8	0.2
	LGM connected	-0.1	0.1	-1.2	0.2		
	Short-term isolation	-0.1	0.1	-1.2	0.2		
	Contemporary isolated	-0.1	0.1	-1.7	0.1		
$\beta_{\text{Phylo}-\text{Sør PD}}$	Never connected	0.1	0.1	2.2	0.0	2.4	0.5
	LGM connected	0.1	0.1	1.2	0.2		
	Short-term isolation	-0.1	0.1	-0.5	0.6		
	Contemporary isolated	0.0	0.1	0.4	0.7		
Dissimilarity Metric (x)	**Predictor (y)**	**Effec**	**S.E.**	**t-value**	**Pz**	**χ2**	**Pχ2**
C. Pair-wise comparisons between host assemblages							
$\beta_{\text{Chao}-\text{Sørensen}}$	Never connected	0.3	0.1	3.9	0.0	3.0	0.2
	LGM connected	0.0	0.0	-0.4	0.7		
	Short-term isolation	-0.2	0.1	-1.6	0.1		
$\beta_{\text{Sørensen}}$	Never connected	0.4	0.0	8.0	0.0	2.8	0.2
	LGM connected	0.0	0.1	-0.5	0.6		
	Short-term isolation	-0.2	0.1	-1.6	0.1		
$\beta_{\text{Replacement}}$	Never connected	0.2	0.0	7.0	0.0	5.4	0.07
	LGM connected	-0.1	0.0	-2.2	0.0		
	Short-term isolation	-0.1	0.1	-1.0	0.3		
$\beta_{\text{Nestedness}}$	Never connected	0.2	0.1	2.9	0.0	3.0	0.2
	LGM connected	0.1	0.1	1.2	0.2		
	Short-term isolation	-0.1	0.1	-0.8	0.4		

Supplementary Figures



Supplementary Data Figure 1: Bayesian maximum clade credibility tree of 132 haemosporidian lineages from North America, Central America, and the Caribbean region. Phylogenetic relationships were reconstructed using 10 *Leucocytozoon* sequences as outgroup (highlighted in brown), and applying an uncorrelated relaxed lognormal clock, a GTR+I+G substitution model, and a Yule speciation prior. *Plasmodium* sp. lineages were highlighted in red, *Haemoproteus (Parahaemoproteus)* sp. in green, and *Haemoproteus (Haemoproteus)* sp. in blue. Diamonds represent nodes with posterior

probability higher than 80%.



Supplementary Data Figure 2: Standardized effect size (*SES*) values for phylogenetic beta diversity index PhyloSør and its partitioned components, PhyloSør-Replacement and PhyløSor-PD, among haemosporidian assemblage pair-wise comparison groups. Each point represents a pair-wise comparison, and each pane represents the *SES* values for a phylogenetic beta diversity metric. The dashed red line represents the lower (-1.96) and upper (1.96) cut-off limits for *SES* values. Pairs of assemblages with phylogenetic beta diversity higher than expected by chance (i.e., overdispersion, *SES*>1.96) represent assembly processes related to competitive exclusion and evolution of host resistance. Pairs of assemblages more similar than expected by chance (i.e., phylogenetic clustering, *SES*>1.96) suggest that replacement takes place among closely related lineages, a process correspondent to environmental filtering. None of the pair-wise comparison groups showed

CHAPTER 2.

THE MALARIA PARASITE *PLASMODIUM RELICTUM* IN THE ENDEMIC AVIFAUNA OF EASTERN CUBA

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Abstract

Island populations are vulnerable to introduced pathogens, as evidenced by extinction of several endemic Hawaiian birds caused by the malaria parasite, *Plasmodium relictum*. Among avian malaria parasites (order Haemosporida; genera *Plasmodium* and *Haemoproteus*) surveyed in the vicinity of Guantánamo Bay, Cuba, nine of 363 individuals were infected with the *Plasmodium relictum* lineage GRW4. Five of the infected individuals were endemic Cuban grassquits (*Tiaris canorus*). A sequence of the merozoite surface protein gene from *T. canorus* infected with GRW4 matched that of the Hawaiian haplotype Pr9. Cuba lacks several bird species, including the bananaquit *Coereba flaveola*, that are ubiquitous elsewhere in the West Indies. Thus, from the standpoint of avian conservation in the Caribbean Basin, it will be important to determine the distribution of *P. relictum* in Cuba and its pathogenicity in local birds, including species absent from the island.

Introduction

In 1826, the Royal Navy warship HMS Wellington sailed from the west coast of Mexico to the island of Maui, making history as the vessel that introduced a mosquito vector of avian pox and avian malaria, *Culex quinquefasciatus*, to the Hawaiian archipelago (Warner 1968). Beginning at the end of the 19th century, mortality attributed to the pox virus and, somewhat later, an introduced avian malaria pathogen caused the extinction, or dramatic population decline, of several endemic Hawaiian bird species, especially among the honeycreepers of the endemic subfamily Drepanidinae (Atkinson & LaPointe 2009a). *Plasmodium relictum* (Apicomplexa: Haemosporida), a causative agent of avian malaria, has been listed among the 100 most invasive species on Earth (Lowe et al. 2000). In such remote areas as the Hawaiian Islands, where the endemic avifauna has evolved resistance to few pathogens (Matson 2006; Beadell et al. 2007), infections by newly invasive pathogens can have severe health consequences (Diamond 1999). For example, experimental infections from single bites of the vector *C. quinquefasciatus* bearing sporozoites (i.e., the parasite's infective stage to vertebrate hosts) of *P. relictum* caused 65%

mortality in Hawaii amakihis (*Hemignathus virens*) (Atkinson et al. 2000).

Species limits are not well defined for haemosporidian parasites (Outlaw & Ricklefs 2014; Perkins 2014). Several avian malaria lineages distinguished on the basis of sequences of the parasite's mitochondrial cytochrome *b* (cyt *b*) gene have been attributed to the morphological species *P. relictum* (Beadell et al. 2006). The lineage GRW4, which has been identified in 39 bird species belonging to 13 families, is among the most widespread of the *P. relictum* lineages, and in Hawaii it is the only avian malaria lineage, as defined by nucleotide variation in the cytochrome *b* gene (Beadell et al. 2006). This lineage is rare in New World birds (Figure 1), having been found primarily in introduced European species, such as the house sparrow *Passer domesticus* (Marzal et al. 2011; Ewen et al. 2012). Phylogeographic analyses suggest that GRW4, together with its dipteran mosquito vector, *C. quinquefasciatus*, originated in the Old World, likely in tropical Africa (Fonseca et al. 2000; Beadell et al. 2006; Valkiünas et al. 2007).

Here we report the presence of *P. relictum* GRW4 in the endemic avifauna of eastern Cuba. We discuss the origins of GRW4 in the New World and speculate on the possible role of *P. relictum* in the near absence of several common and widespread West Indian bird species from the avifauna of Cuba.

Methods

A field team under the supervision of P. Marra captured birds with mist nets in the surroundings of Guantanamo Bay, on the southeast coast of Cuba (19° 55′ N, 75° 07′ W), between January 10 and February 4, 2004. Blood samples were obtained from captured individuals by brachial venipuncture. Following centrifugation, the red blood cells were frozen in microcapillary tubes and later extracted using Puregene Cell Lysis Buffer (Gentra Systems, Minneapolis, MN, USA; currently QIAGEN, Hilden, Germany). All samples were collected under IACUC protocols approved at the Smithsonian Institution (03-355) and analyzed in the senior author's laboratory at the University of Missouri-St. Louis.

We isolated DNA from extracted blood samples using standard isopropanol precipitation preceded by enzymatic protein digestion and precipitation with ammonium acetate. We tested the quantity and quality of the extracted DNA using a Nanodrop Epoch™ Take3™ multivolume plate reader spectrophotometer (ThermoFisher Scientific, Waltham, MA) and by running 6 µL of each extracted sample for 60 min in a 2% TAE agarose gel stained with ethidium bromide. We repeated DNA extractions for samples that produced no band on the gel, and used spin-columns to purify samples that presented low DNA-to-contaminant ratios (DNeasy Kit, QIAGEN, Valencia, CA).

We screened all samples for presence of either of the haemosporidian genera *Plasmodium* or *Haemoproteus* by a PCR that targets a highly conserved 154-bp 16S rRNA-coding sequence of the parasite mitochondrial DNA, using the primers 343F and 496R (Fallon et al. 2003). All samples that tested negative were screened a second time to reduce false negative results. Samples found to be infected in the first PCR step were further subjected to one or more nested PCR assays that amplify a phylogenetically informative region of the parasite's cytochrome *b*

(cyt *b*) gene. For the first set of nested PCR assays, we used the outer primer pair 3932F and DW4R (Perkins & Schall 2002; Olival et al. 2007) and the inner primer pair 413F and 926R (Ricklefs et al. 2005). When a sample was positive at the initial PCR screening, but failed to amplify in the first nested cyt *b* assay, we ran a second nested PCR using the HAEM primers (Waldenstrom et al. 2002). Two positive and one negative control were included in all PCR reactions. Forward sequencing of cyt *b* products was conducted at Eurofins MWG Operon (Louisville, KY) following manufacturer's protocols. We matched cyt *b* sequences to known haemosporidian lineages using BLAST® (Basic Local Alignment Search Tool) against lineages deposited in GenBank®, MalAvi (Bensch et al. 2009), and our local lineage database. We distinguished as new haemosporidian lineages sequences that differed by one non-silent nucleotide substitution or at least three silent nucleotide substitutions from known sequences. Sequences for new lineages were deposited in GenBank® [KX958175, KX958176, and KX958177].

For all infections genotyped as *P. relictum* GRW4, we ran a nested PCR assay that targets a highly variable region of the *P. relictum* nuclear DNA, the merozoite surface protein (msp1) gene (Hellgren et al. 2013; 2014). The msp1 gene accumulates nucleotide variation 3.2–4.8 times faster than the cyt *b* gene, and is therefore more useful for resolving fine-scale genetic variation within *Plasmodium relictum* lineages (Hellgren et al. 2014). Outer PCR reactions were conducted in a volume of 10 µL composed by 2 µL of DNA template, 0.2 µL of each 3F and 3R primers at 10 µM (Hellgren et al. 2013), 5 µL of ImmoMix^{TM} Red (Bioline, Tauntom, MA), and 2.6 µL of ddH_2O. Inner PCR reactions had final volumes of 25 µL, consisting of 2 µL of DNA amplicon from the outer reaction, 0.6 µL of each 3FN and 3RN primers at 10 µM (Hellgren et al. 2014), 14.4 µL of ImmoMix^{TM} Red (Bioline, Tauntom, MA), and 7.5 µL of ddH_2O. Thermocycling conditions for both outer and inner assays were those described by Hellgren et al. (2014). We ran a 6 µL sample from the inner assay for 20 min in a 2% TAE agarose gel stained with ethidium bromide, and positive samples were submitted to Eurofins MWG Operon (Louisville, KY) for forward sequencing.

Results

We obtained 363 blood samples from 23 species in 11 families of birds (Table 1). Initial screening detected haemosporidian parasites in 59 (16%) samples. The concentrations of final DNA extractions were low, and we were able to amplify and sequence a region of the parasites' mitochondrial cyt *b* gene from only 20 (34%) of the individuals that initially tested positive. Among these sequences, we identified seven phylogenetically distinct haemosporidian lineages. The lineage representing *P. relictum* GRW4 (AY099041) was the most common parasite, having been detected in nine individuals of five host species distributed across four avian families (Table 1). The other identified haemosporidian lineages are common on several host species across the Greater Antilles (KZ04 gb KX958176, OZ02 gb KX958177 and YU02 gb HM222483), or widely distributed in the West Indies (GA01 gb HM222486, *H. multipigmentatus*) (Fallon et al. 2005; Ricklefs et al. 2011). The lineage KZ04 is common on North American warblers wintering in the West Indies and in the Yucatán Peninsula. The lineage OZ02 is widely distributed across bird species, occurring on host populations sampled on North and Central America, as well as on the Caribbean region. We identified a new *Haemoproteus* sp. lineage, designated CUH01

(KX958175) in four individuals of three resident species: one common ground dove (*Columbina passerina*), two Oriente warblers (*Teretistris fornsi*), and one Cuban grassquit (*Tiaris canorus*). The common ground dove and the two Oriente warblers carrying lineage CUH01 were also co-infected with the *Haemoproteus* lineage OZ02.

Five of the nine individuals infected with GRW4 were Cuban grassquits, which belong to the West Indian radiation of Coerebinae (Thraupidae) (Burns et al. 2010; Barker et al. 2015); the grassquit is endemic to the island of Cuba (Raffaele et al. 2003). GRW4 was also detected in three other resident species: a single common ground dove of 19 sampled, one of seven red-legged thrushes (*Turdus plumbeus*), and one of five yellow warblers (*Setophaga petechia gundlachi*). These three species are common resident birds on Cuba; the dove occurs throughout the West Indies archipelago, while the thrush is restricted to the Greater Antilles (Raffaele et al. 2003). Yellow warblers have breeding populations across many islands in the West Indies, and on Cuba residents overlap with fall transient migrants from North America (Klein & Brown 1994; Raffaele et al. 2003). One Cape May warbler, a migratory species, also was infected with GRW4. Cape May warblers winter in the Greater Antilles and Central America, with breeding populations distributed in the northeastern United States and broadly across southern Canada.

We amplified and sequenced a fragment of the merozoite surface protein (msp1) gene from one Cuban grassquit infected with GRW4. The sequence was identical to allele Pr 9 (gb KJ850280), identified as the most common allele out of the five unique msp1 alleles that have been described worldwide for GRW4 and it is the allele found in the Hawaiian *P. relictum* (Hellgren et al. 2014).

Discussion

We report the presence of the highly invasive and virulent avian malaria parasite *P. relictum* in the endemic avifauna of eastern Cuba. The *P. relictum* haplotype (GRW4 Pr9) that infects some Cuban birds is the same haplotype that has caused extinction and population decline in the Hawaiian endemic avifauna (Atkinson et al. 1995; Hellgren et al. 2014). GRW4 Pr9 is also the most prevalent *P. relictum* lineage in Hawaii, where it has been identified in four endemic species of honeycreepers on four of the islands in the Hawaiian archipelago (Hellgren et al. 2014). The only other GRW4 msp1 allele present in Hawaii (Pr 8) was found only in Kauai amakihis (*Hemignathus kauaiensis*). Prior to this study, the only known infections of Pr 9 outside Hawaii have been in introduced house sparrows (*P. domesticus*) in Tampa, Florida, and St. Louis, Missouri, in the continental United States, and in central Brazil and on the island of Bermuda in the North Atlantic Ocean (Hellgren et al. 2014).

The rarity of *P. relictum* GRW4 in the New World might be due to an incompatibility with novel hosts. In Europe, GRW4 is most commonly detected in Afrotropical migrants upon their arrival on their breeding grounds (Bensch et al. 2007; Hellgren et al. 2007), and infections in the resident avifauna are rare, even though experimental infections have demonstrated that GRW4 is able to develop sporozoites in *Culex pipiens pipiens*, which are common dipteran vectors in Northern Europe (Valkiünas et al. 2015). Additionally, like many pathogens, GRW4 exhibits host specialization. When nineteen individuals from six European bird species were injected with blood from a great reed warbler (*Acrocephalus arundinaceus)* infected with GRW4, only five

individuals developed infections (i.e., exhibited parasitemias), four of which were from the same species, *A. arundinaceus*, as the donor individual (Dimitrov et al. 2015). Except for Hawaii, the effect of GRW4 infections on New World birds has not been determined, and we cannot exclude the possibility that infections are infrequently detected because they can be lethal. Differently from what has been reported in Europe and North America, and because in our study GRW4 was the most common haemosporidian lineage infecting Cuban birds in our sample, this lineage apparently can infect a wide range of host species. Because we lacked blood smears for the Cuban samples, we cannot confirm directly that these parasites complete their life cycles within these hosts, which would only be supported by the presence on blood smears of fully developed gametocytes (i.e., the parasite's infective stage to invertebrate hosts). However, the presence of GRW4 in several endemic Cuban species indicates local transmission and implies local completion of the parasite life cycle.

Avian malaria is of special concern when introduced parasites are detected in naïve host populations that lack immunity against the parasites and are highly susceptible to infections (Ricklefs 2010). When Hawaiian native iiwis (*Vestiaria coccinea*) were experimentally infected with *P. relictum*, all birds developed symptoms of the disease and subsequently died. In contrast, nutmeg mannikins (*Lonchura punctulata*), which are introduced in Hawaii, were refractory to infection by *P. relictum* (Atkinson et al. 1995). Prior to the present study, the haplotype GRW4 Pr9 had been found in only five host species, four of which are endemic Hawaiian birds and the fifth being the cosmopolitan house sparrow *Passer domesticus* (Hellgren et al. 2014). In North and South America, GRW4 is the most common lineage found in introduced house sparrows, and it has been suggested that the lineage switched to native hosts as *P. domesticus* expanded its distribution (Marzal et al. 2011). The presence of GRW4 P9 on house sparrows in the USA (Hellgren et al. 2014) and on resident Cuban birds (this study) suggest that infections detected in the migratory Cape May warbler wintering in Cuba could have been acquired on either breeding or wintering grounds.

In Hawaii, *P. relictum* was likely introduced by the mid-19th century (Atkinson & LaPointe 2009b), and the introduction of the mosquito vector *Cx. quinquefasciatus* in 1826 constrains the date of earliest potential transmission of avian malaria. When *Cx. quinquefasciatus* first arrived in Cuba also is not known, but the history of European colonization of islands on the Caribbean suggests that these mosquito vectors could have been introduced much earlier to Cuba than they were to Hawaii. Sugar plantations were established in Cuba in the 16th century, which coincided with the beginning of the slave trade from West Africa (Knight 1997); both GRW4 and *Cx. quinquefasciatus* have evolutionary origins in West Africa (Fonseca et al. 2000; Hellgren et al. 2014). If vector and parasite hitchhiked with ships coming from West Africa, *P. relictum* might have become established on other islands in the West Indies. Avian populations on the West Indies have been extensively surveyed for haemosporidian parasites (Fallon et al. 2004; 2005; Ricklefs et al. 2011; Svensson-Coelho & Ricklefs 2011; Latta 2012) and, with the exception of Cuba, we have only two records of *P. relictum* GRW4 in the archipelago: one Greater Antillean bullfinch (*Loxigilla violacea*) in the Dominican Republic in 2001, and one Tropical mockingbird (*Mimus gilvus*) in Grenada in 2002.

An early introduction of *P. relictum* in Cuba raises the possibility that avian malaria might be responsible for the remarkable absence, or near absence, from Cuba of several otherwise common and geographically widespread bird species in the West Indies, including the bananaquit *C. flaveola*, the black-faced grassquit (*Tiaris bicolor*), and the Greater Antillean bullfinch (*Loxigilla violacea*). Competition with island endemics has been suggested as preventing the establishment of these species on Cuba (Lack 1976; Ricklefs 2010), however the three mentioned species are abundant on other islands of the West Indies, including close-by Jamaica and Hispaniola. Additionally, bananaquits present one of the rare cases of "reverse colonization", with ancestral populations that originated in the Greater Antilles between 1.7 and 4 mya and spread widely throughout the West Indies, but also colonized the tropical American mainland (Bellemain et al. 2008). The phylogeographic history of *C. flaveola* excludes dispersal limitation as an explanation for the absence of the species on Cuba, particularly inasmuch as bananaquits in Quintana Roo on the Yucatan Peninsula of Mexico are most closely related to the population in the Bahamas Islands (Bellemain et al. 2008), with Cuba in between. The most likely cause of the absence of several species of widespread West Indian birds from the island of Cuba remains one or more pathogens, among which *P. relictum* is a likely candidate.

We report the presence of the avian malaria haplotype *P. relictum* GRW4 Pr9 in the endemic avifauna of Cuba. This parasite is rare in the New World, and haplotype Pr9 has been associated with extinction and population decline in the native Hawaiian avifauna. In view of the drastic impacts of this avian malaria lineage in Hawaii, assessing the broader effects and potential threat of avian malaria in the Cuban avifauna is a high priority. The coincidental presence in Cuba of GRW4 and absence of several common West Indian bird species from the island suggest that experimental infection by GRW4 of individuals of these missing species from other island populations would be highly informative. Moreover, given the high host-switching potential of avian malaria parasites, and the presence of wintering North American migratory birds on the island, local transmission of *P. relictum* on Cuba raises the potential for spread of the parasite more widely. At this point, determining the distribution of *P. relictum* GRW4 Pr9 and other haemosporidian parasites on Cuba, and the effects of these parasitic infections on resident and wintering avifauna, should be high priorities.

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References

Atkinson, C.T. & LaPointe, D.A. (2009a). Introduced avian diseases, climate change, and the future of Hawaiian honeycreepers. J. Avian Med. & Surg., 23, 53–63.

Atkinson, C.T. & LaPointe, D.A. (2009b). Ecology and pathogenicity of avian malaria and pox. In: Conservation biology of Hawaiian forest birds (eds. Pratt, T.K., Atkinson, C.T., Banko, P.C., Jacobi, J. & Woodworth, B.L.). Yale University Press, New Haven.

Atkinson, C.T., Dusek, R.J., Woods, K.L. & Iko, W.M. (2000). Pathogenicity of avian malaria in

experimentally-infected Hawaii Amakihi. J. Wildl. Dis., 36, 197–204.

Atkinson, C.T., Woods, K.L., Dusek, R.J., Sileo, L.S. & Iko, W.M. (1995). Wildlife disease and conservation in Hawaii: Pathogenicity of avian malaria (*Plasmodium relictum*) in experimentally infected Iiwi (*Vestiaria coccinea*). Parasitol., 111, S59–S69.

Barker, F.K., Burns, K.J., Klicka, J., Lanyon, S.M. & Lovette, I.J. (2015). New insights into New World biogeography: An integrated view from the phylogeny of blackbirds, cardinals, sparrows, tanagers, warblers, and allies. The Auk, 132, 333–348.

Beadell, J., Atkins, C., Cashion, E. & Jonker, M. (2007). Immunological change in a parasite-impoverished environment: divergent signals from four island taxa. PLoS One, 2, e896.

Beadell, J.S., Ishtiaq, F., Covas, R., Melo, M., Warren, B.H., Atkinson, C.T., Bensch, S., Graves, G.R., Jhala, Y.V., Peirce, M.A., Rahmani, A.R., Fonseca, D.M. & Fleischer, R.C. (2006). Global phylogeographic limits of Hawaii's avian malaria. Proc. Biol. Sci., 273, 2935–2944.

Bellemain, E., Bermingham, E. & Ricklefs, R.E. (2008). The dynamic evolutionary history of the bananaquit (*Coereba flaveola*) in the Caribbean revealed by a multigene analysis. BMC Evol. Biol., 8, 240.

Bensch, S., Hellgren, O. & Pérez-Tris, J. (2009). MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome blineages. Mol. Ecol., 9, 1353–1358.

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, 112–122.

Burns, K.C., Berg, J., Bialynicka-Birula, A., Kratchmer, S. & Shortt, K. (2010). Tree diversity on islands: assembly rules, passive sampling and the theory of island biogeography. J. Biogeo., 37, 1876–1883.

Diamond, J.M. (1999). Guns, germs, and steel: the fates of human society. W. W. Norton & Company, New York.

Dimitrov, D., Palinauskas, V., Iezhova, T.A., Bernotienė, R., Ilgūnas, M., Bukauskaitė, D., Zehtindjiev, P., Ilieva, M., Shapoval, A.P., Bolshakov, C.V., Markovets, M.Y., Bensch, S. & Valkiūnas, G. (2015). *Plasmodium* spp.: An experimental study on vertebrate host susceptibility to avian malaria. Exp. Parasitol., 148, 1–16.

Ewen, J.G., Bensch, S., Blackburn, T.M., Bonneaud, C., Brown, R., Cassey, P., Clarke, R.H. & Pérez-Tris, J. (2012). Establishment of exotic parasites: the origins and characteristics of an avian malaria community in an isolated island avifauna. Ecol. Lett., 15, 1112–1119.

Fallon, S., Bermingham, E. & Ricklefs, R.E. (2005). Host specialization and geographic localization of avian malaria parasites: a regional analysis in the Lesser Antilles. Am. Nat., 165, 466–480.

Fallon, S.M., Ricklefs, R.E., Latta, S.C. & Bermingham, E. (2004). Temporal stability of insular avian malarial parasite communities. Proc. Royal Soc. B, 271, 493–500.

Fallon, S.M., Ricklefs, R.E., Swanson, B.L. & Bermingham, E. (2003). Detecting avian malaria: an improved polymerase chain reaction diagnostic. J. Parasitol., 89, 1044–1047.

Fonseca, D.M., LaPointe, D.A. & Fleischer, R.C. (2000). Bottlenecks and multiple introductions: population genetics of the vector of avian malaria in Hawaii. Mol. Ecol., 9, 1803–1814.

Hellgren, O., Atkinson, C.T., Bensch, S., Albayrak, T., Dimitrov, D., Ewen, J.G., Kim, K.S., Lima, M.R., Martin, L., Palinauskas, V., Ricklefs, R., Sehgal, R.N.M., Valkiūnas, G., Tsuda,

Y. & Marzal, A. (2014). Global phylogeography of the avian malaria pathogen *Plasmodium relictum* based on MSP1 allelic diversity. Ecography, 38, 842–850.

Hellgren, O., Kutzer, M., Bensch, S., Valkiünas, G. & Palinauskas, V. (2013). Identification and characterization of the merozoite surface protein 1 (msp1) gene in a host-generalist avian malaria parasite, *Plasmodium relictum* (lineages SGS1 and GRW4) with the use of blood transcriptome. Malaria J., 12, 381.

Hellgren, O., Waldenström, J., Pérez-Tris, J., Szöllősi, E., SI, Ö., Hasselquist, D., Krizanauskiene, A., Ottosson, U. & Bensch, S. (2007). Detecting shifts of transmission areas in avian blood parasites — a phylogenetic approach. Mol. Ecol., 16, 1281–1290.

Klein, N.K. & Brown, W.M. (1994). Intraspecific molecular phylogeny in the yellow warbler (*Dendroica petechia*), and implications for avian biogeography in the West Indies. Evol., 48, 1914.

Knight, F. (1997). The slave societies of the Caribbean. UNESCO, London.

Lack, D. (1976). Island biology, illustrated by the land birds of Jamaica. University of California Press., Berkeley.

Latta, S.C. (2012). Avian research in the Caribbean: past contributions and current priorities. J. Field Ornit., 83, 107–121.

Lowe, S., Browne, M., Boudjelas, S. & De Poorter M. (2000). 100 of the world's worst invasive alien species: a selection from the global invasive species database. The Invasive Species Specialist Group (ISSG) of the World Conservation Union (IUCN), Auckland.

Marzal, A., Ricklefs, R.E., Valkiūnas, G., Albayrak, T., Arriero, E., Bonneaud, C., Czirják, G.A., Ewen, J., Hellgren, O., Hořáková, D., Iezhova, T.A., Jensen, H., Krizanauskiene, A., Lima, M.R., de Lope, F., Magnussen, E., Martin, L.B., Møller, A.P., Palinauskas, V., Pap, P.L., Pérez-Tris, J., Sehgal, R.N.M., 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, e21905.

Matson, K.D. (2006). Are there differences in immune function between continental and insular birds? Proc. Biol. Sci., 273, 2267–2274.

Olival, K.J., Stiner, E.O. & Perkins, S.L. (2007). Detection of *Hepatocystis* sp. in southeast asian flying foxes (*Pteropodidae*) using microscopic and molecular methods. *Journal of* Parasitol., 93, 1538–1540.

Outlaw, D.C. & Ricklefs, R.E. (2014). Species limits in avian malaria parasites (Haemosporida): how to move forward in the molecular era. Parasitol., 141, 1223–1232.

Perkins, S.L. (2014). Malaria's many mates: past, present, and future of the systematics of the order Haemosporida. J. Parasitol., 100, 11–25.

Perkins, S.L. & Schall, J.J. (2002). A molecular phylogeny of malarial parasites recovered from cytochrome b gene sequences. J. Parasitol., 88, 972–978.

Raffaele, H.A., Wiley, J.W., Garrido, O.H. & Raffaele, J.I. (2003). Birds of the West Indies. Princeton University Press, Princeton.

Ricklefs, R.E. (2010). Host–pathogen coevolution, secondary sympatry and species diversification. Philos. Trans. R. Soc. Lond., B, Biol. Sci., 365, 1139–1147.

Ricklefs, R.E., Dodge Gray, J., Latta, S.C. & Svensson Coelho, M. (2011). Distribution anomalies in avian haemosporidian parasites in the southern Lesser Antilles. J. Avian Biol., 42, 570–584.

Ricklefs, R.E., Swanson, B.L., Fallon, S.M., MartÍnez-AbraÍn, A., Scheuerlein, A., Gray, J. & Latta, S.C. (2005). Community relationships of avian malaria parasites in southern Missouri. Ecol. Monog., 75, 543–559.

Svensson-Coelho, M. & Ricklefs, R.E. (2011). Host phylogeography and beta diversity in avian haemosporidian (Plasmodiidae) assemblages of the Lesser Antilles. J. Anim. Ecol., 80, 938–946.

Valkiūnas, G., Zehtindjiev, P., Hellgren, O., Ilieva, M., Iezhova, T.A. & Bensch, S. (2007). Linkage between mitochondrial cytochrome b lineages and morphospecies of two avian malaria parasites, with a description of *Plasmodium (Novyella) ashfordi* sp. Parasitol. Res., 100, 1311–1322.

Valkiūnas, G., Žiegytė, R., Palinauskas, V., Bernotienė, R., Bukauskaitė, D., Ilgūnas, M., Dimitrov, D. & Iezhova, T.A. (2015). Complete sporogony of *Plasmodium relictum* (lineage pGRW4) in mosquitoes *Culex pipiens pipiens*, with implications on avian malaria epidemiology. Parasitol. Res., 114, 3075–3085.

Waldenstrom, J., Bensch, S., Kiboi, S., Hasselquist, D. & Ottosson, U. (2002). Cross-species infection of blood parasites between resident and migratory songbirds in Africa. Mol. Ecol., 11, 1545–1554.

Warner, R.E. (1968). The role of introduced diseases in the extinction of the endemic Hawaiian avifauna. The Condor, 70, 101–120.

Table

Table 1: Samples of birds in Guantánamo Bay, Cuba. The column labeled "Infected" represents positive samples identified through PCR amplification of a coding region for the 16S small subunit of ribosomal RNA. The number of infections sequenced represents the positives amplified and sequenced using primers that target the parasite's cytochrome *b* coding region. Migratory species are represented by M, resident species by R, and resident species that are also endemic to Cuba by E. GRW4 is *Plasmodium relictum*; GA01 is *Haemoproteus (Haemoproteus) multipigmentatus* (HM222486); CUH01 (KX958175), OZ02 (KX958177), KZ04 (KX958176), YU02 (HM222483), and LA01 (GQ395656) are *Haemoproteus* spp.

Species	Migrant (M) or Resident (R) (E = endemic)	Infected	Sequenced	Total	Haemosporidian Lineages
Ardeidae					
Green Heron (*Butorides virescens*)	R			6	
Columbidae					
Common Ground-dove (*Columbina passerina*)	R	7	4	19	GRW4, CUH01, GA01, OZ02
Strigidae					
Cuban Pygmy Owl (*Glaucidium siju*)	R,E			5	
Picidae					

Cuban Green Woodpecker (*Xiphidiopicus percussus*)	R,E			1	
Tyrannidae					
Cuban Pewee (*Contopus caribaeus*)	R	1		1	
LaSagra's Flycatcher (*Myiarchus sagrae*)	R			10	
Parulidae					
Black and White Warbler (*Mniotilta varia*)	M			2	
Cape May Warbler (*Setophaga tigrina*)	M	2	1	26	GRW4
Northern Parula (*Setophaga americana*)	M	6	1	17	KZ04
Northern Waterthrush (*Parkesia noveboracensis*)	M			14	
Oriente Warbler (*Teretistris fornsi*)	R,E	6	2	8	CUH01, OZ02
Ovenbird (*Seiurus aurocapilla*)	M			3	
Prairie Warbler (*Setophaga discolor*)	M			7	
Western Palm Warbler (*Stetophaga palmarum*)	M	6	1	25	GA01
Yellow Warbler (*Setophaga petechia*)	M	1	1	5	GRW4
Mimidae					
Northern Mockingbird (*Mimus polyglottos*)	R	6	2	15	YU02
Turdidae					
Red-legged Thrush (*Turdus plumbeus*)	R	5	1	7	GRW4
Thraupidae					
Cuban Grassquit (*Tiaris canorus*)	R,E	17	7	66	GRW4, GA01, LA01
Icteridae					
Black-cowled Oriole (*Icterus prosthemelas*)	M			16	
Cuban blackbird (*Dives atroviolaceus*)	R,E	1		73	
Shiny Cowbird (*Molothrus bonariensis*)	R			7	
Tawny-shouldered Blackbird (*Agelaius humeralis*)	R			27	
Emberizidae					
Yellow-faced Grassquit (*Tiaris olivaceus*)	R	1		3	
Total		**59**	**20**	**363**	

Figure.

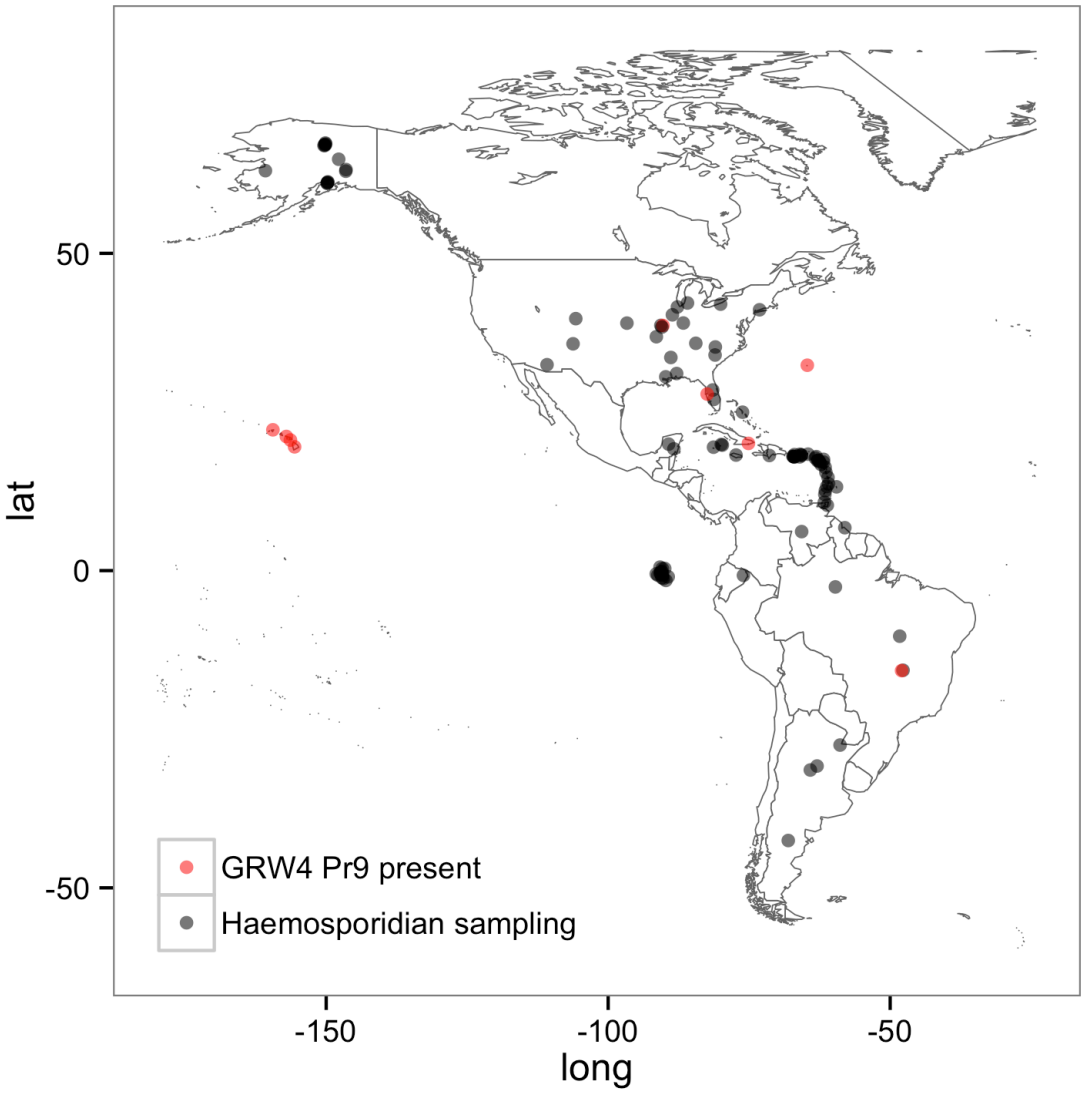

Figure 1: Map of showing locations where haemosporidian parasites were surveyed in bird populations (black). Red points represent areas where the *Plasmodium relictum* haplotype GRW4 Pr9 was detected.

Chapter 3.

LOW PREVALENCE OF HAEMOSPORIDIAN PARASITES IN SHOREBIRDS

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Abstract
Although shorebirds (Charadriiformes: suborders Scolopaci and Charadrii) face physiological stress during migration and experience a wide range of vector-suitable habitats across their distributions, haemosporidian parasites (order Haemosporida) have rarely been detected in these species worldwide. We ask whether shorebirds remain infection-free when wintering in areas where parasite transmission occurs among landbird species. Blood samples were collected at three locations in Argentina: Patagonia, with an avian assemblage dominated by shorebird species; Mar Chiquita Lagoon, a high-salinity water basin where shorebird and landbird species co-occur; and inland sites in Chaco and Cordoba provinces, where host assemblages included mostly landbird species. We screened 650 samples for the presence of haemosporidian infections and sequenced a part of the parasite mitochondrial cytochrome *b* gene to determine parasite lineages. Of 342 landbirds, 91 were infected with haemosporidian parasites, compared to only three of 318 shorebirds. Wintering location had no effect on the prevalence of haemosporidian parasites in shorebirds. Haemosporidian infections are rare in shorebirds even when these co-occur with landbird species in inland areas suitable for parasite transmission. The few infections detected in shorebirds might represent spillover infections from landbirds, as the parasite lineages were also found in common landbird species. We suggest that shorebirds are resistant to haemosporidian parasites, and either do not become infected or quickly clear occasional infections.

Introduction
Haemosporidian parasites (Apicomplexa: order Haemosporida; genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*) are vector-transmitted protozoans that replicate in the tissues of their vertebrate hosts (Valkiūnas 2004), and that are known to reduce host

reproductive success and survival (Atkinson *et al*. 2000, LaPointe *et al*. 2005, Asghar *et al*. 2011, Asghar *et al*. 2015). These parasites infect most species of birds that have been sampled, with a conspicuous exception of many shorebirds (Charadriiformes: primarily Charadriidae and Scolopacidae) (Greiner *et al*. 1975, Mendes *et al*. 2005, Yohannes *et al*. 2008, D'Amico and Baker 2010). Greiner *et al*. (1975) screened 84 blood films from five Charadriidae species from the Western Hemisphere and found only a single individual (1.1%) black-bellied plover (*Pluvialis squatarola*) infected with *Leucocytozoon* parasites. They also examined 1156 blood films of 25 Scolopacidae species, and found only eight infections (0.7% of potential host individuals), five of which (0.4%) carried *Plasmodium* infections. Using PCR to identify infections, Mendes *et al*. (2005) found only six individuals of marine shorebirds (0.7%) with circulating haemosporidian parasites out of 816 individuals screened across the East-Atlantic (Europe-Africa) flyway. Yohannes *et al*. (2008) also used PCR to search for haemosporidian parasites in 114 pectoral sandpipers (*Calidris melanotus*) and 84 semipalmated sandpipers (*C. pusilla*), finding only three *Plasmodium* infections (2.6%) in *C. melanotus*. D'Amico and Baker (2010) used PCR to screen 341 blood samples from red knots (*Calidris canutus*) caught in the Delaware Bay, New Jersey (USA), and found only one individual (0.3%) infected with *Plasmodium* parasites. Clark *et al*. (2016) discovered 40 haemosporidian infections (3. 6%) in 1125 screened Australian wader samples. The scarcity of infections in migratory shorebirds is unexpected because haemosporidian parasites have been detected in most well-sampled bird species across all biogeographic regions of the world, except Antarctica (Bensch *et al*. 2009, Perkins 2014).

Three complementary hypotheses might explain why haemosporidian infections are rarely detected in shorebirds. First, the 'parasite avoidance' hypothesis proposes that species travel longer distances to avoid pathogen transmission, breeding and wintering in relatively parasite-free environments (Curtis 2014). Indeed, some authors have suggested that parasite avoidance might determine whether individuals migrate or not, how far they travel, and even where they stop (Lokki and Saurola 2004, Poulin *et al*. 2012, Gohli *et al*. 2015). Piersma (1997) suggested that shorebirds which use coastal marine wetlands rather than inland freshwater habitats tend to be less parasitized because marine environments do not support abundant dipteran vector populations. Testing the parasite avoidance hypothesis is challenging because the absence of detectable infections in shorebirds may indicate absence of suitable parasite vectors, resistance to or rapid clearing of infections, or absence of parasites from the peripheral blood while present in other tissues (Svensson-Coelho *et al.* 2016). Moreover, this hypothesis might have little relevance for birds in general, because migratory passerine species, some of which travel as far as shorebird species, are commonly infected by haemosporidian parasites (Hellgren *et al*. 2007), often with higher prevalence and parasite diversity than nonmigratory species (Pérez-Tris *et al*. 2004, Jenkins *et al*. 2012, Hellgren *et al*. 2013). Second, the 'migration culling' hypothesis states that the physiological stress of migration (Nebel *et*

al. 2013) removes infected individuals from host populations, reducing prevalence and transmission of parasites in subsequent host generations (Bradley and Altizer 2005). Over time, through the elimination of susceptible hosts, migration culling could reduce pathogen prevalence, leading to reduced transmission or even to parasite extirpation. Migration culling can be tested only by monitoring parasite prevalence during host migration; the hypothesis would be supported if prevalence declined as a host population progresses through its migratory route (Bartel *et al*. 2011). Third, the 'parasite resistance' hypothesis suggests that shorebirds might have strong intrinsic immunity to parasite infection, which either prevents infection or clears infections rapidly. Accordingly, such species would be expected to exhibit low parasite prevalence throughout the annual cycle, even where parasite transmission is indicated locally by infections in other, resident species. This hypothesis is consistent with the general absence of infections by blood-borne pathogens in shorebirds (Greiner *et al*. 1975), but experimental infections have not been conducted in shorebirds to determine whether they are resistant to haemosporidian infections.

In this study, to test the parasite resistance hypothesis, we screened wintering shorebirds for haemosporidian parasites in an area that we hypothesize to have a high infection risk for resident landbirds. We compare parasite prevalence between populations of shorebird species wintering at an inland site in Argentina, in association with haemosporidian-infected individuals of many resident passeriform bird species, and at coastal sites in Patagonia, with few other potential host species and, presumably, few vectors. Specifically, we compare infections from four locations: two subtropical inland areas with abundant landbird species but lacking shorebirds (Cordoba city and Monte Alto, in Chaco province), one inland saline lakeside area that supports both landbird and shorebird species (Mar Chiquita Lagoon), and several arid, temperate coastal sites in southern Patagonia, where host assemblages are exclusively shorebirds. Infections in resident landbirds at Mar Chiquita Lagoon indicate local parasite transmission.

The comparative framework of our study allows us to make predictions related to the parasite avoidance and the parasite resistance hypotheses. Specifically, if both landbirds and post-breeding migratory shorebirds at Mar Chiquita Lagoon were infected by parasites, we would conclude that the general absence of parasites in shorebirds elsewhere is a result of their reduced exposure to parasite transmission (supporting the parasite avoidance hypothesis). If landbirds are infected at Mar Chiquita Lagoon, but shorebirds are not, provided that shorebirds are present during the parasite transmission season we would conclude that shorebirds are inherently resistant to infection by haemosporidians (supporting the parasite resistance hypothesis).

Two characteristics determine the suitability of an area for parasite transmission: competent invertebrate vectors and vertebrate host reservoirs. Because we did not assess

vectors in this study, we judge the suitability of an area for parasite transmission by the presence of infections in resident species. If Mar Chiquita Lagoon is a stopover or wintering site having low transmission risk, the diversity and prevalence of haemosporidian infections in shorebirds would be comparable to that in coastal environments of Patagonia, and landbirds would exhibit lower parasite diversity and prevalence there than their counterparts inland at Cordoba and Chaco. Alternatively, if shorebirds were generally resistant to haemosporidian parasites, the prevalence of infections should be low both in Patagonia and at Mar Chiquita Lagoon, and we would expect landbirds at Mar Chiquita Lagoon to be infected at frequencies comparable to those of landbirds at the sites in Cordoba and Chaco. Our analyses support the second hypothesis of well-developed parasite resistance in shorebirds.

Materials and methods

Field methods. Blood samples were collected in four regions of Argentina (Fig 1), principally during the southern hemisphere summer: Monte Alto in Chaco province (January through December, 2004), Cordoba city (January and February, 2005) and Mar Chiquita Lagoon (December 2004, October through November 2006) in Cordoba province, and fifteen localities in Patagonia (October through November 2004, January, April and December 2005, as well as November 2006). Numbers of landbirds and shorebirds captured at each location are presented in Tables S1 and S2 of the Supplemental Material. Lowland semi-arid vegetation interspersed with urban and farming areas characterizes Monte Alto and Cordoba city. Mar Chiquita Lagoon is a post-Pliocene water basin that experiences variation in its water levels and salinity (Reati *et al*. 1996). Water levels remained stable from 1980 through 2004, when a drought caused water levels decrease, leading to an increase in salinity and the exposure of coastal areas by 2006. Variation in the hydrologic regime of the lagoon impacts shorebird populations in the area, with numbers of individuals increasing as water levels decline and salinity increases (Reati *et al*. 1996, Nores 2011). Our samples from the Patagonian region of Argentina, which included only charadriiform species, were obtained in Chubut and Santa Cruz provinces from lakeside or coastal areas (Fig 1). For each individual captured, a small blood sample, generally <10 mL, was taken, after which the bird was released. Blood samples were stored in 70% ethanol at room temperature until laboratory analysis.

Molecular detection, genotyping, and phylogenetic reconstruction. We removed approximately 1 mm^3 of clotted blood from each sample and transferred it to a new microcentrifuge tube. Samples were then placed in a heated water bath at 50 °C for five days, or until all the ethanol had evaporated. The tubes with the dried blood were then filled with 1000 µL of Longmire's lysis buffer (Longmire *et al*. 1997), and placed in a heated bath at 50 °C for five days or until all the blood had been incorporated into the solution. We extracted DNA from dissolved blood samples using standard isopropanol

precipitation preceded by enzymatic protein digestion and precipitation with ammonium acetate. We tested the quantity and quality of the extracted DNA using a Nanodrop Epoch™ Take3™ multivolume plate reader spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and by running 6 µL of each extracted sample for 60 min in a 2% TAE agarose gel stained with ethidium bromide. We repeated DNA extraction for samples that presented low DNA-to-contaminant ratios and samples that produced no band on the gel.

We genotyped haemosporidian parasites in two steps. First, we screened all samples for infection with either of the haemosporidian genera *Plasmodium* or *Haemoproteus* by a PCR that targets a highly conserved, 154-bp 16S rRNA-coding sequence of the parasite mitochondrial DNA, using the primers 343F and 496R (Fallon *et al*. 2003). All samples that tested negative were screened twice to reduce false negative results.

Samples found to be infected in the first PCR step were further subjected to one or more nested PCR assays that amplify a phylogenetically informative region of the mitochondrial cytochrome *b* gene (cyt *b*) of haemosporidian parasites of the genera *Plasmodium* and *Haemoproteus*. For the first set of nested PCR assays, we used the outer primer pair 3932F and DW4R (Perkins and Schall 2002, Olival *et al*. 2007) and the inner primer pair 413F and 926R (Ricklefs *et al*. 2005). When a sample was positive at the initial PCR screening, but failed to amplify in the first nested cyt *b* assay, we ran a second nested PCR using the HAEM primers (Waldenström *et al*. 2002). Two positive and one negative control were used on all PCR reactions. Forward sequencing of cyt *b* products was conducted at Beckman Coulter Genomics (Danvers, MA, USA), following the manufacturer's protocols. Because our screening primers can amplify infections by other apicomplexan parasites, we sequenced the 154-bp amplicons from shorebird samples that failed to amplify on all cyt *b* assays.

We matched cyt *b* sequences to known haemosporidian lineages using BLAST® (Basic Local Alignment Search Tool) against lineages deposited in GenBank®, MalAvi (Bensch *et al*. 2009), and our local lineage database. We distinguished as new haemosporidian lineages, sequences that differed by one non-silent nucleotide substitution or at least three silent nucleotide substitutions from known sequences. Sequences for new lineages were deposited in GenBank® (numbers KU258504-KU258521). Phylogenetic relationships among the parasite lineages were determined by maximum likelihood phylogeny reconstruction using a GTR + gamma model of nucleotide substitution and 100 bootstrap replicates. Sequence editing and phylogenetic analyses were performed using CLC Main Workbench version 7.0 (QIAGEN, http://www.clcbio.com).

Statistical analyses. We used generalized linear mixed models with a binomial error distribution to test: 1) the null hypothesis that the prevalence of haemosporidian parasites

is comparable between shorebirds and landbirds in Mar Chiquita Lagoon; and 2) whether haemosporidian prevalence differed between Chaco province, Cordoba city, and Mar Chiquita Lagoon. One the first model, we used the classification landbird vs. shorebird as a two level-fixed effect, and on the second model we used the sampling locality as a four-level fixed effect. To control for variation on prevalence attributed to differences on avian species composition between locations, we added a random intercept that corresponds the taxonomic family of each individual bird. The dependent variable on both models was the prevalence of haemosporidian infections (binary, infected *vs.* non-infected). We considered a sample to be infected with haemosporidian parasites only when positive PCR result was confirmed by sequencing of the parasite's 16S rRNA or cyt *b* regions. Analyses were run using the package lme4 in R (Bates *et al*. 2015).

Results

Three of the 318 shorebirds sampled were positive for haemosporidian infections, revealing that these parasites are rare in shorebird species even when these birds are found in habitats where infections are known to occur in landbirds (Fig 2, Table 1). Below we divide our results in three sections that describe host assemblage composition, parasite assemblage composition, and finally the distribution of haemosporidian infections on shorebirds and landbirds across Monte Alto, Cordoba city, Mar Chiquita Lagoon, and Patagonia.

Host assemblage composition. We screened 650 blood samples, of which 332 were from 53 species of landbirds and 318 were from 16 species of shorebirds (Table 1 and Table S1 of the Supplemental Material). Ten of the shorebird species are long-distance migrants that breed at high latitudes on the arctic tundra of the northern hemisphere; the other six species migrate within the Southern hemisphere, wintering in sub-tropical regions and breeding primarily in cold, south-temperate areas. Eleven of the landbird species migrate within the Southern Hemisphere, and the remaining 42 are resident locally in the sample areas. Landbirds were sampled during winter months in Cordoba city and Monte Alto, which skewed our sampling of migratory landbirds by sampling more species that breed in temperate regions of the Southern hemisphere, and winter in subtropical areas.

Host samples from Monte Alto and Cordoba were dominated by landbird species; no shorebird was caught in Monte Alto, and individuals of only four charadriiform species were caught in Cordoba: one southern lapwing (*Vanellus chilensis*), three collared plovers (*Charadrius collaris*), one white-rumped sandpiper (*Calidris fuscicolis*), and four pectoral sandpipers (*Calidris melanotus*). The Mar Chiquita Lagoon sample had a mixed host composition, including 18 landbird species, eight of which were also caught in Cordoba and Monte Alto, and 11 shorebird species, eight of which also were caught in Patagonia. No landbird species was sampled in Patagonia, but we did sample two least seedsnipes (*Thinocorus rumicivorus*), which is a charadriiform wader of temperate and

sub-tropical grasslands. *T. rumicivorus* individuals, like those of *V. chilensis* caught in Cordoba, often occur far from water bodies, even though they are more closely related to shorebirds than to landbirds. The differences in sample composition between Patagonia and the inland areas of Cordoba and Monte Alto reflected the dominance of shorebird species in the first location.

Haemosporidian phylogenetic diversity. We were able to sequence the cyt *b* gene and assign lineages to 68% (91/134) of the infections of landbird species, and to two of the 20 infections of shorebird species (Table 1). We confirmed the haemosporidian infection from one *Charadrius falklandicus* caught in Mar Chiquita by sequencing the parasite's 16S SSU rRNA, but we were unable to amplify the parasite's cyt *b* of these shorebird infections. When looking at populations of shorebirds, landbirds, or both groups across locations, we found no difference in parasite incidence between the sampling years. Parasites were assigned to 33 haemosporidian lineages, including 13 *Haemoproteus* spp. and 20 *Plasmodium* spp. (Fig. 3), 10 and 8 of which, respectively, were new to us. The *Plasmodium* lineages ArgP1, ArgP3, ArgP7, ArgP8, and ArgP9 each occurred only once, and these were from host species represented by single captures (Table S1). Among the *Haemoproteus* lineages, ArgH1 and ArgH9 were each found only once, in individuals of the ground dove *Columbina picui*. ArgH1 is a close match to *Haemoproteus sacharovi* (Bensch *et al*. 2009); ArgH9 falls within a clade that includes lineages GA02 and SocH4, which are dove specialist parasites (Fallon *et al*. 2005, Carlson *et al*. 2013). Lineages ArgH2 and ArgH3 likely are generalist parasites, as they were recovered from three and seven host species, respectively.

Site and host distribution of haemosporidian lineages. After controlling for differences in taxonomic composition between avian assemblages, the prevalence of haemosporidian parasites was comparable in Monte Alto (30%) and Cordoba (27%), marginally lower (10%) at Mar Chiquita Lagoon (Table 2), and close to nil in Patagonia. The most common lineage in Monte Alto, Cordoba, and Mar Chiquita was *Haemoproteus* sp. SocH4, first described in the Socorro ground dove (*Zenaida graysoni*), a resident endemic on Socorro Island, off the west coast of Mexico (Carlson *et al*. 2013). The second most prevalent lineage was *Haemoproteus* (*Parahaemoproteus*) ArgH2, which clusters with haplotype ArgH3 and haplotype CEhapH, the latter also described from the Brazilian Cerrado, a region with similar host composition and comparable climate to the Chaco province and to Cordoba, where ArgH2 was found.
Compared to landbirds, shorebirds in Mar Chiquita Lagoon had lower, indeed close to nil, haemosporidian prevalence (Table 2). Only three shorebird individuals out of 318 captured were infected with haemosporidian parasites: one white-rumped sandpiper (*Calidris fuscicollis*) at Mar Chiquita, one southern lapwing (*Vanellus chilensis*) at Cordoba, and a two-banded plover (*Charadrius falklandicus*) in Patagonia. The infection of *C. fuscicollis* was genotyped as a *Haemoproteus* lineage (ArgH2), which was also

found in a tyrant flycatcher (*Elaenia* sp.), a rufous hornero (*Furnarius rufus*), and a great kiskadee (*Pitangus sulphuratus*), which are common suboscine passerine landbirds in the region, and which could serve as reservoirs for this lineage. In Cordoba, we found another *Haemoproteus* lineage (ArgH10) infecting *V. chilensis*, as well as both sampled individuals of the bay-winged cowbird (*Molothrus badius*).

We were unable to amplify the cyt *b* gene of the parasites infecting two individuals of *C. falklandicus* in Patagonia, but we confirmed that at least one of these was a haemosporidian infection by sequencing the 154 bp 16S rRNA coding region, which matched haemosporidian sequences in GenBank. In 15 cases, however, the screening primers based on the 16S rRNA coding region (the 343F/496R primer pair) amplified 144 to 240 bp of DNA from *C. fuscicollis*. These 15 cases also correspond to samples that showed negative on the first PCR assay using the 16S rRNA screening primers, but appeared positive on the second 16S rRNA assay that attempted to identify false negatives. Apparently, this primer pair sometimes anneals to a region of *C. fuscicollis* genome, which could be misinterpreted as a haemosporidian infection without sequencing additional parasite DNA markers.

Discussion

We found that haemosporidian infections were rare in shorebird species compared to landbirds within the same environment (Mar Chiquita Lagoon) providing support for the hypothesis that shorebird species are resistant to haemosporidian infections. A shorebird in Mar Chiquita Lagoon, where we detected haemosporidian parasites in 26% of the landbirds, is as unlikely to be infected as a shorebird in Patagonia, where infection prevalence is close to nil. Infections are rarely detected in shorebirds generally, and when parasites are present, they usually exhibit low prevalence (Greiner *et al*. 1975, Mendes *et al*. 2005, Yohannes *et al*. 2008, Clark *et al*. 2016, this study). Haemosporidian parasites have been found in populations of migratory shorebirds of the east Atlantic flyway between Europe and Africa, though most of these infections were detected at inland, freshwater wintering sites in Africa, which presumably are suitable for parasite transmission owing to both competent host reservoirs and vectors (Mendes *et al*. 2005). However, even at inland, freshwater sites in Africa, Mendes *et al*. (2005) found haemosporidian infections in only 1 of 37 individuals of shorebird species that typically winter along saline shorelines, contrasting with 18% (48/261) of individuals of shorebird species that typically winter in freshwater habitats ($G_{adj} = 7.8$, d,f, = 1, $P < 0.01$). It is noteworthy that 40 of the 48 infections detected in shorebirds by Mendes *et al*. (2005) were found in ruffs (*Philomachus pugnax*), known to occur in freshwater wetlands. The results from Mendes *et al*. (2005), combined with evidence from our study that shorebird species rarely harbour haemosporidian infections, even when they occur in inland areas with infected resident landbird species, suggest that shorebird species that typically winter along saline shorelines effectively resist or suppress haemosporidian infection.

Why shorebirds generally have so few haemosporidian infections is not understood (Greiner *et al*. 1975, Piersma 2002). Proposed mechanisms have included limited vector exposure (Mendes *et al*. 2005), and even the size of the areas of the geographic distributions during breeding and wintering (Piersma 1997, Piersma 2003, Yohannes *et al*. 2008). Greiner *et al*. (1975) highlighted the scarcity of haemosporidian parasites in blood smears of shorebird species, suggesting a physiological barrier of parasitism in these species, as shorebirds occur in areas known to have vectors able to transmit the parasites. Our results also support the hypothesis that these birds are either incompatible hosts for haemosporidian parasites, in the sense of preventing parasite development, or are highly resistant, controlling or eliminating infections even when parasites are able to complete their life-cycle within the host.

Most shorebirds that breed at high latitudes in the northern hemisphere undertake long-distance migrations to wintering areas. Infections, particularly by blood parasites, likely increase the stress of migration (Jenni-Eiermann *et al*. 2014) and would therefore favour individuals with strong immune responses that reduce or eliminate haemosporidian and other blood parasites (Nebel *et al.* 2013). Strong immune resistance to haemosporidian parasites might be associated with the relatively long development periods and lifespans of shorebird species. Longer incubation periods allow more time for diversification of B-cell lines that will express functionally different immunoglobulins (Killpack and Karasov 2012). Studies in precocial galliforms have shown that B-cell lines differentiate mostly during embryonic development (Ratcliffe 2006). In altricial landbirds, incubation time is inversely related to the prevalence of hemoparasites (Ricklefs 1992), but this relationship has yet to be investigated in precocial birds. Little is known about the susceptibility of Charadriidae birds to infection or their ability to mount immune responses against parasites (except for Piersma 1997, Mendes 2006).

Failure to detect haemosporidian infections should also be taken into account with respect to differences in prevalence between shorebird and landbird species. Given the high survival rates of migratory shorebirds (Sandercock 2003), we believe it is unlikely that parasites are highly pathogenic to these hosts, to the point that parasite-caused mortality would lead to under-detection of infections. However, under-detection might occur because haemosporidian parasites initially develop in tissues other than the blood, and infected hosts that do not present gametogeny or schizogony in red blood cells might test negative if the parasite survey is solely based on blood samples. In ruffs, for instance, haemosporidian lineages have been detected by PCR in tissues other than blood, including pectoral muscle, brain, and liver (Mendes *et al*. 2013).

The marginally lower parasite prevalence in Mar Chiquita compared to Monte Alto and Cordoba suggests that the high salinity in the coastal areas of the lagoon might limit

vector reproduction. Although the abundance of dipteran vectors might be lower in the shoreline of the lagoon, ornithophilic vectors must be present in the area because resident land birds exhibit normal prevalence of haemosporidian parasites. Amiron and Brewer (1995) determined the feeding preferences of mosquitoes in Mar Chiquita by looking at the frequency of dipterans captured in traps baited with turtles, rabbits, or chickens. The traps baited with chickens collected 69% of the mosquitos, all of which belonged to four species of *Culex* sp. (Almirón and Brewer 1995), including *C. quinquefasciatus*, a known vector of avian malaria (Fonseca *et al*. 1998). As far as we are aware, ornithophilic biting midges (*Culicoides*, Diptera), which are the vectors of *Haemoproteus* sp. parasites, have not been surveyed in the area. We also acknowledge that untested variables could explain the scarcity of infections on shorebird species; these include differences in microhabitat usage among host species, which could lead differential vector exposure. Future studies on vector ecology should focus on surveying avian parasite vectors as well as determining vector-feeding preferences, particularly on landbirds compared to shorebirds.

Finally, the identified *Haemoproteus* sp. lineages from shorebirds were also present in passerine hosts in the study areas, suggesting that the occasional infections in shorebird species represent spillover infections from common landbirds. Similarly, Mendes *et al.* (2013) found that most lineages detected in tissue samples of ruffs were also found in several common passerine species in both Africa and Europe. *Haemoproteus* and *Plasmodium* parasites can have broad host breadth, being able to infect host species of different genera and even different families (Bensch *et al*. 2000). However, because we lacked blood smears for the Argentine samples, we could not determine whether the few infections in shorebirds produced gametocytes and, therefore, could be transmitted to other birds (Valkiūnas 2004). Serological tests of immune responses could reveal whether shorebird individuals had been infected, but had not developed the blood stages of the infection, and therefore were under-detected in PCR screenings (Jarvi *et al*. 2002). Future studies should determine the frequency of parasite exposure through immune assays, and look for evidence of haemosporidian gametocytes in blood smears, testing the hypothesis that parasites often fail to complete their life cycles within shorebirds once infection takes place. Overall, our study indicates that haemosporidian infections are rare in shorebird species that winter along high salinity shorelines, even in areas of high transmission risk, implying that shorebirds resist haemosporidian infection.

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Data Accessibility

Sequences for new lineages were deposited in GenBank® (numbers KU258504-KU258521).

References

Almirón W.R. & Brewer M.M. 1995. Preferencia de hospedadores de Culicidae (Diptera) recolectados en el centro de la Argentina. Rev. Saúde Pública 29:108–114.

Asghar M., Hasselquist D. & Bensch S. 2011. Are chronic avian haemosporidian infections costly in wild birds? J. Avian Biol. 42:530–537.

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 347:436–438.

Atkinson C.T., Dusek R.J., Woods K.L. & Iko W.M. 2000. Pathogenicity of avian malaria in experimentally-infected Hawaii Amakihi. J. Wildl. Dis. 36:197–204.

Bartel R.A., Oberhauser K.S., de Roode J.C. & Altizer S.M. 2011. Monarch butterfly migration and parasite transmission in eastern North America. Ecology 92:342–352.

Bates, D., Mächler, M., Bolker, B. & Walker, S. 2015. Fitting linear mixed-effects models using lme4. J. Stat. Soft. 67:1–48.

Bensch, S., Stjernman, M., Hasselquist, D., Örjan, Ö., Hannson, B., Westerdahl, H. & Pinheiro, R.T. 2000. Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proc. B., 267:1583–1589.

Bensch S., Hellgren O. & Pérez-Tris J. 2009. MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome blineages. Mol. Ecol. 9:1353–1358.

Bradley C.A. & Altizer S. 2005. Parasites hinder monarch butterfly flight: implications for disease spread in migratory hosts. Ecol. Let. 8:290–300.

Carlson J.S., Martínez-Gómez J.E., Valkiūnas G., Loiseau C., Bell D.A. & Sehgal R.N. 2013. Diversity and phylogenetic relationships of hemosporidian parasites in birds of socorro island, México, and their role in the re-introduction of the Socorro dove (*Zenaida graysoni*). J. Parasitol. 99:270–276.

Clark N.J., Clegg S. & Klaassen M. 2016. Migration strategy and pathogen risk: non-breeding distribution drives malaria prevalence in migratory waders. Oikos (*in press*).

Curtis, V.A. 2014. Infection-avoidance behaviour in humans and other animals. Tren. Immun. 35:457–464.

D'Amico V.L. & Baker A.J. 2010. A rare case of *Plasmodium (Haemamoeba) relictum* infection in a free-living Red Knot (*Calidris canutus rufa*, Scolopacidae). J. Ornithol. 151: 951–954.

Fallon S., Bermingham E. & Ricklefs R.E. 2005. Host specialization and geographic localization of avian malaria parasites: a regional analysis in the Lesser Antilles. Am. Nat. 165:466–480.

Fallon S.M., Ricklefs R.E., Swanson B.L. & Bermingham E. 2003. Detecting avian malaria: an improved polymerase chain reaction diagnostic. J. Parasitol. 89:1044–1047.

Fonseca D.M., Atkinson C.T. & Fleischer R.C. 1998. Microsatellite primers for *Culex pipiens quinquefasciatus*, the vector of avian malaria in Hawaii. Mol. Ecol. 7:1617–1619.

Gohli J., Lifjeld J.T. & Albrecht T. 2015. Migration distance is positively associated with sex-linked genetic diversity in passerine birds. Ethol. Ecol. & Evol. 28: 42–52.

Greiner E.C., Bennett G.F., White E.M. & Coombs R.F. 1975. Distribution of the avian hematozoa of North America. Can. J. Zool. 53:1762–1787.

Hellgren O., Waldenström J., Pérez-Tris J., Szöll E., Si Ö., Hasselquist D., Krizanauskiene A., Ottosson U. & Bensch S. 2007. Detecting shifts of transmission areas in avian blood parasites – a phylogenetic approach. Mol. Ecol. 16:1281–1290.

Hellgren O., Wood M.J., Waldenström J. Hasselquist D., Ottosson U., Stervander M. & Bensch S. 2013. Circannual variation in blood parasitism in a sub-Saharan migrant passerine bird, the garden warbler. J. Evol. Biol. 26:1047–1059.

Jarvi S.I., Schultz J.J. & Atkinson C.T. 2002. PCR diagnostics underestimate the prevalence of avian malaria (*Plasmodium relictum*) in experimentally-infected passerines. J. Parasitol. 88:153–158.

Jenkins K.T., Thomas G.H., Hellgren O. & Owens I.P.F. 2012. Migratory behavior of birds affects their coevolutionary relationship with blood parasites. Evolution 66:740–751.

Jenni-Eiermann S., Jenni L., Smith S. & Costantini D. 2014. Oxidative stress in endurance flight: an unconsidered factor in bird migration. PLoS ONE 9:e97650.

Killpack T.L. & Karasov W.H. 2012. Ontogeny of adaptive antibody response to a model antigen in captive altricial zebra finches. PLoS ONE 7:e47294.

LaPointe D.A., Goff M.L. & Atkinson C.T. 2005. Comparative susceptibility of introduced forest-dwelling mosquitoes in Hawai'i to avian malaria, *Plasmodium relictum*. J. Parasitol. 91:843–849.

Lokki H. & Saurola P. 2004. Comparing timing and routes of migration based on ring encounters and randomization methods. Anim. Biodiv. Cons. 27: 163–174.

Longmire J.L., Maltbie M. & Baker R.J. 1997. Use of "lysis buffer" in DNA isolation and its implications for museum collections. Occ. Pap. Mus. Texas Tech U. 163:1–3.

Mendes L. 2006. Variation in the innate and acquired arms of the immune system among five shorebird species. J. Exp. Biol. 209:284–291.

Mendes L., Pardal S., Morais J., Antunes S., Ramos J.A., Pérez-Tris J. & Piersma T. 2013. Hidden haemosporidian infections in Ruffs (*Philomachus pugnax*) staging in Northwest Europe en route from Africa to Arctic Europe. Parasitol. Res. 112:2037–2043.

Mendes L., Piersma T., Lecoq M., Spaans, B. & Ricklefs R. 2005. Disease-limited distributions? Contrasts in the prevalence of avian malaria in shorebird species using marine and freshwater habitats. Oikos 109:396–404.

Nebel, S., Buehler, D.M., MacMillan, A. & Guglielmo, C.G. 2013. Flight performance of western sandpipers, *Calidris mauri*, remains uncompromised when mounting an acute phase immune response. J. Exp. Biol. 216: 2752–2759.

Nores M. 2011. Long-term waterbird fluctuations in Mar Chiquita Lake, Central Argentina. Waterbirds 34:381–388.

Olival K.J., Stiner E.O. & Perkins S.L. 2007. Detection of *Hepatocystis* sp. in southeast asian flying foxes (*Pteropodidae*) using microscopic and molecular methods. J. Parasitol. 93:1538–1540.

Perkins S.L. 2014. Malaria's many mates: past, present, and future of the systematics of the order Haemosporida. J. Parasitol. 100:11–25.

Perkins S.L. & Schall J.J. 2002. A molecular phylogeny of malarial parasites recovered from cytochrome b gene sequences. J. Parasitol. 88:972–978.

Pérez-Tris J., Bensch S., Carbonell R., Helbig A.J. & Telería J.L. 2004. Historical diversification of migration patterns in a passerine bird. Evolution 58:1819–1832.

Piersma T. 1997. Do global patterns of habitat use and migration strategies co-evolve with relative investments in immunocompetence due to spatial variation in parasite pressure? Oikos 80:623–631.

Piersma T. 2002. Energetic bottlenecks and other design constraints in avian annual cycles. Integr. Comp. Biol. 42:51–67.

Piersma T. 2003. "Coastal" versus" inland" shorebird species: interlinked fundamental dichotomies between their life-and demographic histories? Bull. Wader Study Group 100:5–9.

Poulin R., Closs G.P., Lill A.W.T., Hick A.S., Herman K.K. & Kelly D.W. 2012.

Migration as an escape from parasitism in New Zealand Galaxiid fishes. Oecologia 169:955–963.

Ratcliffe M.J.H. 2006. Antibodies, immunoglobulin genes and the bursa of Fabricius in chicken B cell development. Dev. Comp. Immunol. 30:101–118.

Reati G.J., Florín M., Fernández G.J. & Montes C. 1996. The Laguna de Mar Chiquita (Córdoba, Argentina): A little known, secularly fluctuating, saline lake. Int. J. Salt Lake Resear. 5:187–219.

Ricklefs R.E. 1992. Embryonic development period and the prevalence of avian blood parasites. Proc. Biol. Sci. 89:4722.

Ricklefs R.E., Swanson B.L., Fallon S.M., Martínez-Abraín, A., Scheuerlein A., Gray J. & Latta S.C. 2005. Community relationships of avian malaria parasites in southern Missouri. Ecol. Mon. 75:543–559.

Svensson-Coelho, M., Silva, G.T., Santos, S.S., Miranda, L.S., Araújo-Silva, L.E., Ricklefs, R.E., Miyaki, C.Y. & Maldonado-Coelho, M. 2016. Lower detection probability of avian *Plasmodium* in blood compared to other tissues. J. Parasitol. in press.

Sandercock B.K. 2003. Estimation of survival rates for wader populations: a review of mark-recapture methods. Bull. Wader Study Group 100:163–175.

Valkiūnas G. 2004. Avian malarial parasites and other haemosporidia. Taylor and Francis Publishing, London.

Waldenström J, Bensch S, Kiboi S, Hasselquist D. & Ottosson U. 2002. Cross-species infection of blood parasites between resident and migratory songbirds in Africa. Mol. Ecol. 11:1545–1554.

Yohannes E., Križanauskienė A., Valcu, M., Bensch S. & Kempenaers B. 2008. Prevalence of malaria and related haemosporidian parasites in two shorebird species with different winter habitat distribution. J. Ornithol. 150:287–291.

Tables

Table 1: Numbers of landbird and shorebird samples analysed per location. Numbers within the parentheses represent the number of infected individuals detected using conservative screening primers that amplify a region of the 16S SSU rRNA of haemosporidian parasites and, in bold type, individuals that tested positive and were successfully sequenced and genotyped using primers that amplify a region of the parasite's mitochondrial cytochrome *b* gene.

Area	**Landbirds**	**Shorebirds**
Monte Alto (Chaco province)	87 (40/**26**)	0
Cordoba city (Cordoba province)	176 (68/**49**)	7 (2/**1**)
Laguna Mar Chiquita (Cordoba province)	61 (18/**16**)	119 (16/**1**)
Patagonia	0	197 (2/**0**)

Table 2: Parameter estimate, standard error, *P* value for the two generalized linear mixed effect models testing the differences in prevalence between landbirds and shorebirds on Mar Chiquita lagoon, and the differences in prevalence between sampling locations. In both models, the taxonomic family of each individual bird was used as a random intercept to account for differences in species composition between comparison groups.

Model 1 H_0: Prevalence of haemosporidian parasites is comparable between shorebirds and landbirds in Mar Chiquita Lagoon.			
Parameter	**Estimate**	**Std. Error**	***P***
Landbird (intercept)	-1.5	0.6	0.01
Shorebird	-2.9	1.4	0.04
Parameter (Random Effect)	**Variance**	**Std. Deviation**	
Avian Family	0.2	0.4	
Model 2 H_0: Prevalence of haemosporidian parasites is comparable between sampling locations.			
Parameter (Fixed Effect)	**Estimate**	**Std. Error**	***P***
Cordoba (intercept)	-1.4	0.3	<0.01
Monte Alto	0.3	0.3	0.4
Mar Chiquita Lagoon	-0.6	0.4	0.09
Patagonia	-21.8	142.0	0.9
Parameter (Random Effect)	**Variance**	**Std. Deviation**	
Avian Family	0.5	0.7	

Figures

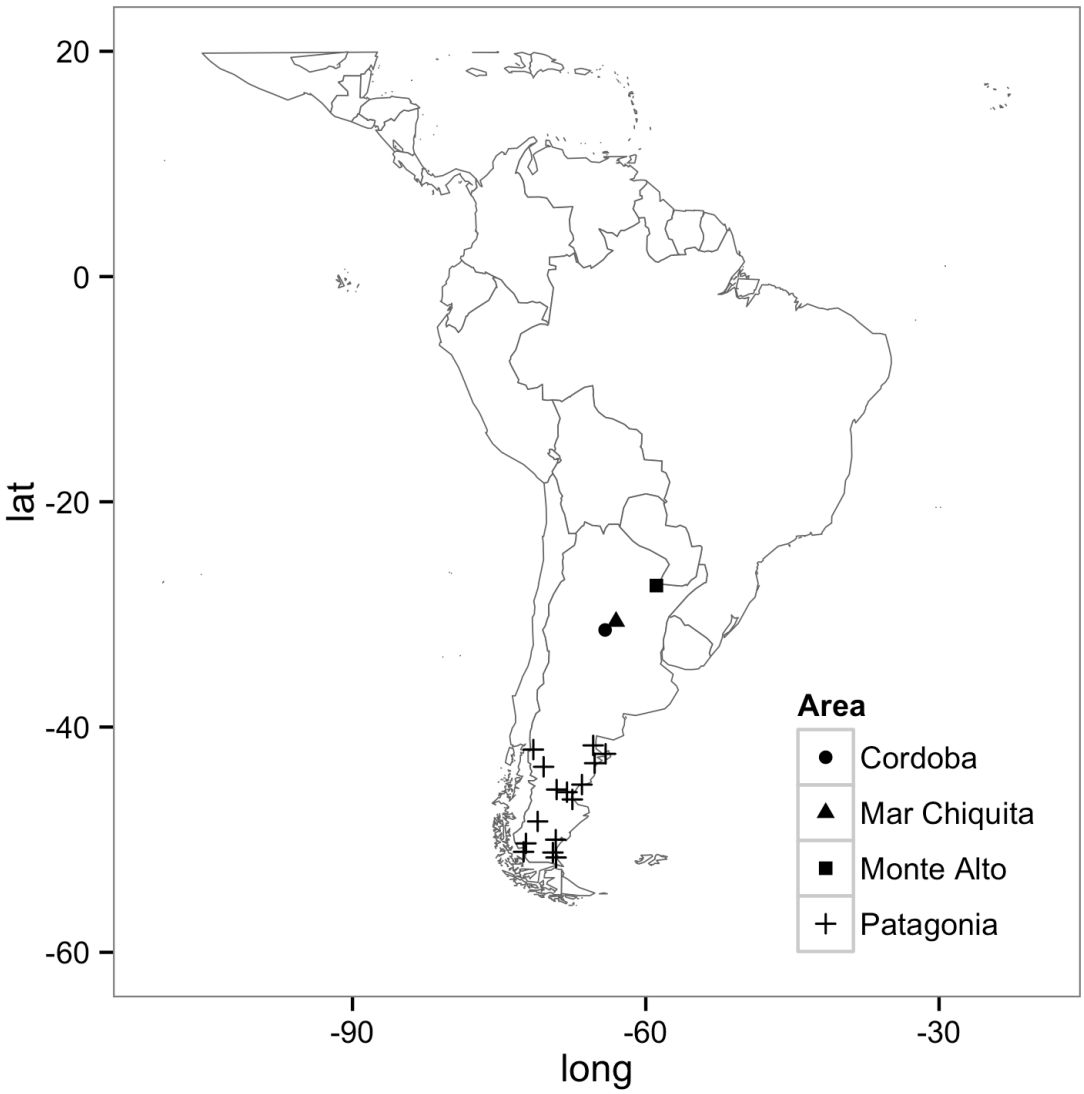

Figure 1. Map showing the sample locations: Monte Alto (Chaco province), Cordoba province (Mar Chiquita Lagoon and Cordoba city), and fifteen localities in Patagonia, which were either coastal or bordered bodies of fresh water.

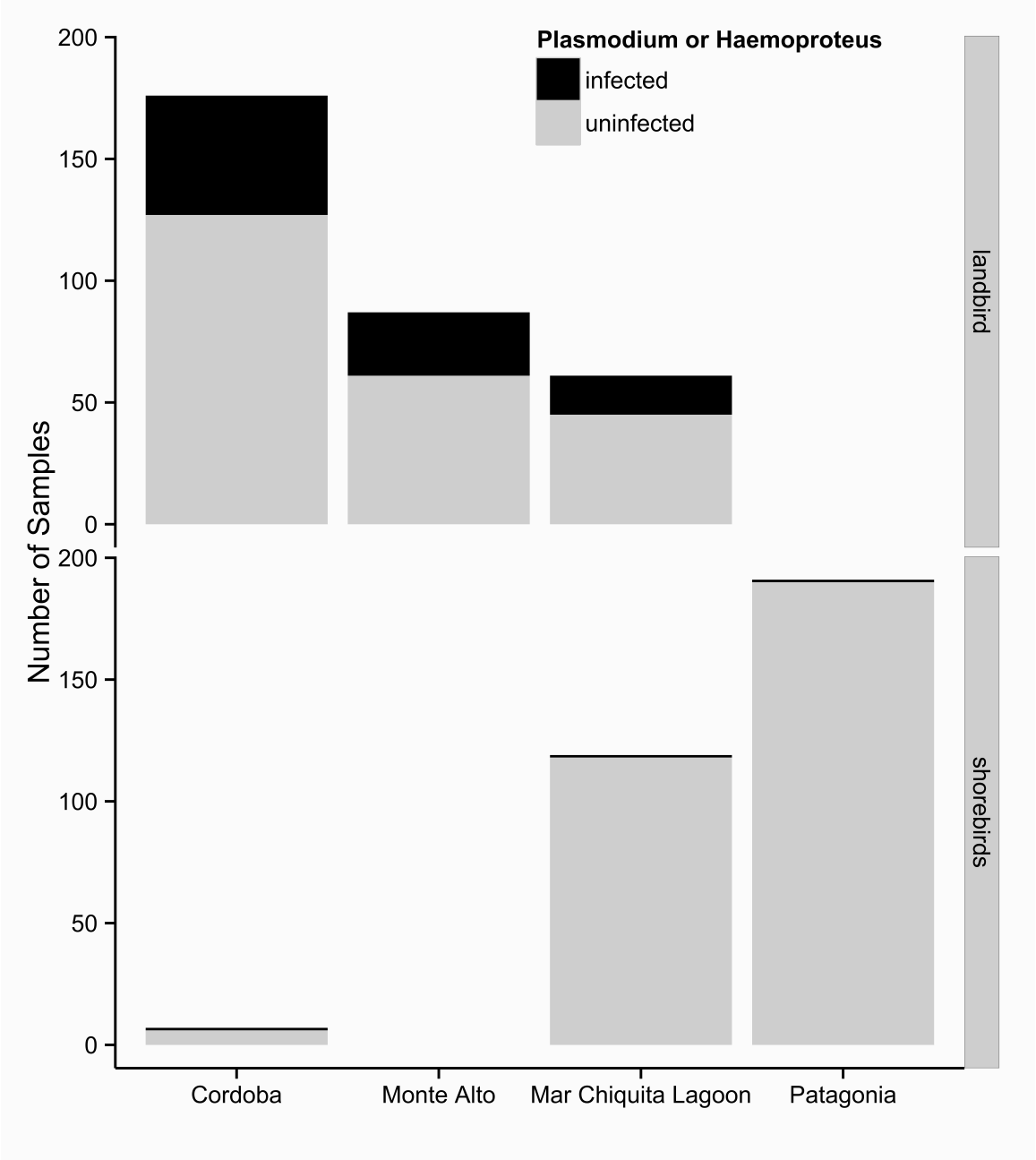

Figure 2. Graph representing the number of landbird and shorebird individuals infected with haemosporidian parasites in Cordoba, Monte Alto (Chaco Province), Mar Chiquita Lagoon, and Patagonia.

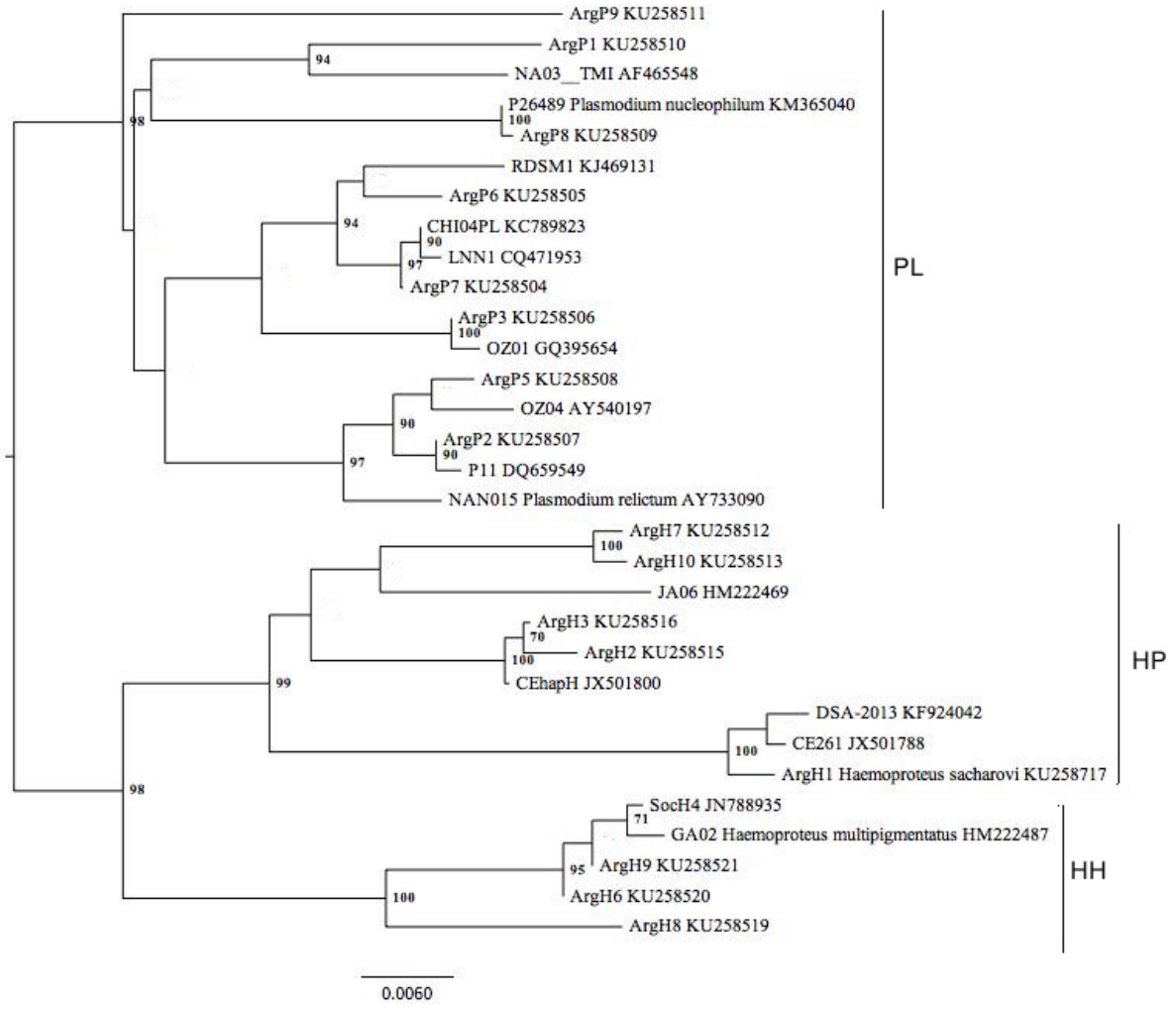

Figure 3. Maximum likelihood phylogeny of haemosporidian lineages found infecting avian hosts in Argentina constructed using a GTR + gamma model of nucleotide substitution. New lineages are labelled with the code Arg. PL, HP and HH correspond to the clades grouping haemosporidian lineages of the genera *Plasmodium*, *Haemoproteus* (subgenus *Parahaemoproteus*) and *Haemoproteus* (subgenus *Haemoproteus*), respectively. Numbers at the nodes represent bootstrap support values above 70%, based on 100 replicates.

Table S1: Samples of birds in four locations in Argentina. The numbers within parentheses represent positive samples identified through PCR amplification of a coding region for the 16S small subunit of ribosomal RNA. The number of infections sequenced represents the positives amplified and sequenced using primers that target the parasite's cytochrome *b* coding region. *Plasmodium* lineages in italics; *Haemoproteus* (*Haemoproteus*) lineages in bold type; *Haemoproteus* (*Parahaemoproteus*) lineages in normal type. Shorebird species are shaded in grey.

Classification	Migratory /Resident	Monte Alto	Cordoba	Mar Chiquita	Patagonia	Total	Infections Sequenced	Lineages
SCOLOPACIDAE								
Calidris fuscicollis	M		1 (1)	57 (15)	62	120	1	ArgH2
Calidris bairdii	M				29	29		
Micropalama himantopus	M			27 (1)		27		
Calidris melanotos	M		4	11	2	17		
Tringa flavipes	M			11	6	17		
Limosa haemastica	M				7	7		
Calidris alba	M				3	3		
Steganopus tricolor	M			2		2		
Calidris canutus	M				1	1		
CHARADRIIDAE								
Charadrius falklandicus	M/R*			4	58 (2)	62	1	**
Vanellus chilensis	R		1 (1)	2	3	6	1	ArgH10
Charadrius collaris	R		3	3		6		
Oreopholus ruficollis	M			1		1		
Pluvialis dominica	M			1		1		
HAEMATOPODIDAE								
Haematopus leucopodus	M				17	17		
THINOCORIDAE								
Thinocorus rumicivorus	R				2	2		
COLUMBIDAE								
Columbina picui	R	8 (6)	28 (13)	39 (12)		75	23	ArgH1, **ArgH9**, *ArgP5*, CEhapH, **GA02**, *OZ04*, **SocH4**
Zenaida auriculata	R		5 (2)			5	2	ArgH5, DSA-2013
Leptotila verreauxi	R	2 (1)				2	1	**ArgH8**
Columba maculosa	R		1			1		
TYRANNIDAE								
Pitangus sulphuratus	R	1 (1)	46 (13)	2 (1)		49	15	ArgH2, ArgH3, *ArgP7*, *NA03*
Elaenia sp.	R	7 (4)	3			10	3	ArgH2, ArgH3, *CHI04PL*

Xolmis irupero	R			3		3		
Pyrocephalus rubinus	M		2			2		
Griseotyrannus aurantioatrocristatus	M			1 (1)		1	1	*ArgP1*
Tyrannus melancholicus	R	1 (1)				1	1	ArgH2
Tyrannus savana	M		1 (1)			1	1	ArgH3
Stigmatura budytoides	R	1 (1)				1	1	ArgH3
FURNARIIDAE								
Furnarius rufus	R	6 (2)	31 (13)	3 (2)		40	17	ArgH2, ArgH3, *ArgP2*, *CHI04PL*, *LINN1, OZ01*, *P11*
Coryphistera alaudina	R	6 (2)		1		7	1	ArgH3
Pseudoseisura lophotes	R		5 (3)			5		
Lepidocolaptes angustirostris	R	3				3		
Xiphocolaptes major	R	1				1		
Campylorhamphus falcularius	R	1				1		
Anumbius annumbi	R	1 (1)				1		
TURDIDAE								
Turdus amaurochalinus	M	12 (5)		2 (1)		14	4	*ArgP6*, *ArgP8*, *P11*, **SocH4**
Turdus rufiventris	R	6 (4)				6	1	*ArgP6*
THRAUPIDAE								
Thraupis sayaca	M	11 (3)				11	2	*P26879*, **SocH4**
Sicalis flaveola	R		9 (2)			9	2	ArgH4, *ArgP2*
Sporophila caerulescens	M		6 (3)			6	1	*ArgP2*
Poospiza melanoleuca	R	4 (2)				4	1	*OZ04*
Coryphospingus cucullatus	R	3	1			4		
Poospiza nigrorufa	R		1 (1)			1		
Poospiza ornata	M		1			1		
Embernagra platensis	R			1		1		
EMBERIZIDAE								
Saltator aurantiirostris	R	4 (3)	2	1		7		
Zonotrichia capensis	R		5 (3)	1 (1)		6	1	**ArgH6**
Lophospingus pusillus	R		1 (1)			1	1	JA06
Ammodramus humeralis	R		1 (1)			1		
PASSERIDAE								
Passer domesticus	R		10 (3)			10	2	ArgH5, *NAN015*
CUCULIDAE								
Coccyzus melacoryphus	R	3 (2)	4 (1)			7	1	*ArgP9*

COTINGIDAE								
Phytotoma rutila	M		5 (2)			5	2	*ArgP2, RDSM1*
MIMIDAE								
Mimus triurus	M		5 (3)			5	2	*ArgP2, P11*
ICTERIDAE								
Molothrus bonariensis	R		2	1		3		
Molothrus badius	R	1 (1)	1 (1)			2	2	ArgH10
Cacicus solitarius	R	2 (1)				2	1	ArgH7
Molothrus rufoaxillaris	R			1		1		
TROGLODYTIDAE								
Troglodytes aedon	M		2 (2)			2	1	*ArgP2*
TITYRIDAE								
Pachyramphus polychopterus	R		2			2		
PSITTACIDAE								
Myiopsitta monachus	R		2			2		
CERYLIIDAE								
Chloroceryle americana	R	1		1		2		
HIRUNDINIDAE								
Tachycineta meyeni	R			1		1		
PICIDAE								
Picoides mixtus	M			1		1		
BUCCONIDAE								
Nystalus maculatus	R			1		1		
FALCONIDAE								
Milvago chimango	R		1 (1)			1	1	*ArgP3*
CAPRIMULGIDAE								
Caprimulgus longirostris	R	1				1		
Hydropsalis brasiliana	R			1		1		
CARDINALIDAE								
Cyanocompsa brissonii	R	1				1		
FRINGILLIDAE								
Carduelis magellanica	R		1 (1)			1		
TOTAL		87 (40)	183 (78)	180 (34)	190 (2)	650 (154)	93	

***** Mar Chiquita Lagoon has a breeding population of *Charadrius falklandicus.*

****** Haemosporidian infection confirmed by sequencing a 154-bp fragment of the parasite's mitochondrial genome encoding for the 16S subunit of rRNA.

Notes: CEhapH has been found in passerine species occurring in the arid regions of Cerrado and Caatinga in Brazil; *CHI04PL* is very common in North American thrushes, especially *Turdus migratorius*; **DSA-2013** and **CE261** are closely related to the lineage MODO1, which has been previously recorded only in mourning doves of

North America (*Zenaida macrorura*); **GA02** is common in common ground doves (*Columbina passerina*) in the West Indies and Central America; JA06, recovered from two bananaquits (*Coereba flaveola*) in Jamaica; *NA03*, has been found in several migratory passerine species that winter in Chile and Argentina, and also in the austral residents Falkland thrush (*Turdus falklandii*) and Juan Fernandez tit-tyrant (*Anairetes fernandezianus*); *LINN1* is a lineage that frequently infects Passeriformes, previously detected only in hosts caught in Europe and Oceania. *NAN015* (or GRW03) is a cosmopolitan lineage, found in the Americas, Europe, Africa, Asia and Oceania; *OZ01*, widespread and abundant in many North American hosts, common in the West Indies and Mexico, and occasional in South America; *OZ04* has been recovered from many hosts in the West Indies, and also infrequently in Trinidad, Tiputini (Amazonian Ecuador), and migrant passerines in Misssouri; *P11*, *P26489* and *RDSM* are common generalist lineages of passerine species from South America.

Table S2: Number of samples from landbirds (L) and shorebirds (S) sampled at each location, between 2004 and 2006.

	2004	2005	2006
Cordoba		L=176; S=7	
Monte Alto	L=87		
Patagonia	S=31	S=159	
Mar Chiquita	L=61; S=79		S=40

CHAPTER 4.

CO-INFECTIONS OF HAEMOSPORIDIAN AND TRYPANOSOME PARASITES IN A NORTH AMERICAN SONGBIRD

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Summary

Hosts frequently harbor multiple parasite infections, yet patterns of parasite co-occurrence are poorly documented in nature. In this study, we asked whether two common avian blood parasites, one haemosporidian and one trypanosome, affect each other's occurrence in individuals of a single host species. We used molecular genotyping to survey protozoan parasites in the peripheral blood of yellow-breasted chats (Aves: Passeriformes [Parulidae]: *Icteria virens*) from the Ozarks of southern Missouri. We also determined whether single and co-infections differently influence white blood cell and polychromatic erythrocyte counts, the latter being a measure of regenerative anemia. We found a positive association between the haemosporidian and trypanosome parasites, such that infection by one increases the probability that an individual host is infected by the other. Adult individuals were more likely than juveniles to exhibit haemosporidian infection, but co-infections and single trypanosome infections were not age-related. We found evidence of pathogenicity of trypanosomes in that infected individuals exhibited similar levels of regenerative anemia as birds infected with haemosporidian parasites of the genus *Plasmodium*. Counts of white blood cells did not differ with respect to infection status.

Introduction

Multiple infections by different parasites are commonly observed within single host individuals (Cox, 2001; Alizon *et al.*, 2013), and such coexisting parasites may interact to either enhance or reduce the pathologic effects of infection (Alizon *et al.*, 2013;

Budischak *et al.*, 2015). Co-infection also can influence host survival (Klemme *et al.*, 2016). For example, survival of laboratory mice infected with *Trypanosoma brucei* increased when the mice were experimentally infected with a second, less virulent, genotype (Balmer *et al.*, 2009). In another set of experimental infections of mice, two helminth species and one bacterial strain affected each other's virulence in co-infection, a result of direct competition for host resources and of interaction with the host immune system (Budischak *et al.*, 2015). While within-host parasite interactions may influence parasite virulence, and therefore, the consequences of parasitism, co-infections in wild populations remain under-studied.

In one-host-one-parasite systems, the evolution of virulence shifts the trade-off between parasite replication and host mortality; high host mortality can outweigh the benefits to the parasite of increased parasite transmission (de Roode *et al.*, 2008; Alizon *et al.*, 2013). However, co-infections can make such trade-offs irrelevant by causing parasite virulence to increase beyond the transmission-host mortality threshold (de Roode *et al.*, 2005). For example, red crossbills (*Loxia curvirostra*) co-infected with two species of malaria parasites, *Plasmodium relictum* and *P. ashfordi*, develop higher parasitemias of both infections than birds infected with either one of these parasite species alone (Palinauskas *et al.*, 2011; Dimitrov *et al.*, 2015). Increased virulence is not the only possible outcome of co-infection. Apparent competition is often detected in co-infections, as one parasite infection can interact through the host immune system to influence the probability of acquiring a subsequent infection (Mideo, 2009). For instance, field voles (*Microtus agrestis*) are more or less likely to acquire new infections over time depending on pre-existing parasite infections, regardless of their body condition (Telfer *et al.*, 2010).

Here, we ask how two common protozoan parasites influence each other's occurrence in a host population of yellow-breasted chats (Parulidae, *Icteria virens*) in North America. Avian haemosporidian parasites (order Haemosporida, genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon*) are protozoans transmitted by blood-feeding dipterans, replicating in the tissues of their vertebrate hosts, and undergoing sexual reproduction and sporulation in the midgut of the their invertebrate dipteran vectors (Valkiūnas *et al.*, 2005). Experimental infections indicate that haemosporidian parasites reduce host activity, reproductive success, and longevity (Atkinson *et al.*, 2000; Palinauskas *et al.*, 2008; Knowles *et al.*, 2009; Palinauskas *et al.*, 2009; Knowles *et al.*, 2010; Asghar *et al.*, 2015). The principal vectors for *Plasmodium* parasites are Culicidae mosquitos; *Haemoproteus* parasites are transmitted by biting midges (Ceratopogonidae: *Culicoides*) and hippoboscid flies (Hippoboscidae); *Leucocytozoon* parasites are vectored by Simuliidae black-flies (Valkiūnas, 2004).

Trypanosomes (class Kinetoplastida: order Trypanosomatida) are transmitted by a variety

of vectors, including mosquitoes, mites, black flies, hippoboscids, and midges (Votýpka *et al.*, 2004; Van Dyken *et al.*, 2006; Černý *et al.*, 2011; Ivkovic *et al.*, 2013). In contrast to haemosporidian parasites, which are transmitted to the vertebrate host by inoculation of sporozoites along with the vector's saliva, trypanosomes actively reach the host's blood stream by crossing mucosal membranes (Dresser *et al.*, 1975). Metacyclic trypomastigotes, the trypanosome infective stage from invertebrate to vertebrate host, may develop in the insect's hindgut, although *Trypanosoma culicavium* infecting *Culex* spp. mosquitos develop first in the midgut, and later migrate to the stomodeal valve of the insect host (Votýpka *et al.*, 2012), showing that development strategies in the invertebrate vector are variable. Transmission to the vertebrate host can happen via abraded skin, with parasites entering the host through the epidermis, or via an oral route, whereby birds feed on infected vectors and the parasites enter the bloodstream by crossing the mucosa of the bird's oral cavity, esophagus, or crop (Baker, 1956a; Votýpka and Svobodová, 2004). Although little is known about the pathogenicity of trypanosome infections in birds, these parasites are reported to cause subclinical infections of low parasitemia in avian hosts (Baker, 1956b; Valkiūnas *et al.*, 2004; Zídková *et al.*, 2012), with extremely variable prevalence across host species, ranging from zero to as high as 100% (Greiner *et al.*, 1975a; Kirkpatrick and Lauer, 1985; Kirkpatrick and Suthers, 1988; Sehgal *et al.*, 2001).

Yellow-breasted chats are the largest of the North American warblers, although their taxonomic placement is not fully resolved (Lovette *et al.*, 2010). They typically inhabit dense shrublands and second-growth forests (Morris *et al.*, 2013; Reidy *et al.*, 2014). Individuals breed across most of the United States, southern British Columbia and Alberta, as well as northern Mexico; the winter range extends from Mexico to Panama. Yellow-breasted chats that we have sampled across Missouri, Tennessee, and Mexico are mainly parasitized by one specialist lineage of haemosporidian parasite, designated in our laboratory as *Plasmodium* sp. OZ08 (GenBank accession HM222485). Surveys of blood parasites in North America have detected trypanosomes in blood films of *I. virens* (Stabler *et al.*, 1966; Greiner *et al.*, 1975b; Kirkpatrick and Suthers, 1988). During a recent investigation of white blood cell frequencies in birds infected with haemosporidian parasites (Ellis *et al.*, 2014), we detected trypanosome parasites in the blood films of yellow-breasted chats sampled in the Ozarks plateau of southern Missouri. Accordingly, in this study, we sought to determine whether: 1) occurrences of haemosporidian and trypanosome parasites are associated in this host population; 2) co-infections exhibit an age or sex bias; and 3) co-infections affect host condition, using, as proxies, regenerative anemia and white blood cell counts.

Materials and Methods

Blood sampling

Yellow-breasted chats were caught using standard mist-net protocols. Sampling was

originally conducted to survey haemosporidian parasites of avian assemblages in the Ozarks plateau of southern Missouri (91.03°W, 37.12°N), during the summer of the years 1999-2002 and again in 2011 (Ricklefs *et al.*, 2005; Svensson-Coelho *et al.*, 2013; Ellis *et al.*, 2015). All sampling was covered under state and federal permits, with field protocols approved by an Institutional Animal Care and Use Committee (IACUC) at the University of Missouri-St. Louis. For each individual captured, a small blood sample was taken through brachial venipuncture and stored in Longmire's lysis buffer (Longmire *et al.*, 1997) and a thin blood film was prepared in the field. We fixed all blood smears with methanol on the same day, and stained them with Giemsa within 30 days, following a standard staining protocol (Valkiūnas, 2004). Individuals were identified as after-hatch year (AHY), and hatch-year (HY) birds, which we refer to as adults and juveniles, respectively. All birds were released at the site of capture.

Molecular detection and genotyping of parasites

We extracted DNA from blood samples using a standard isopropanol precipitation protocol, preceded by enzymatic protein digestion and precipitation with ammonium acetate. Detection and genotyping of haemosporidian infections have been described elsewhere (Ricklefs *et al.*, 2005; Svensson-Coelho *et al.*, 2013; Ellis *et al.*, 2015). Detection and genotyping of trypanosome parasites were based on a nested PCR assay that amplifies a 770 bp fragment of the 18S SSU rRNA gene sequence, adapted from Valkiūnas et al. (2011). The outer PCR assay used the primers Tryp763 and Tryp1016, with 10 μL of master mix containing 0.6 μM of each primer, 200 μM of each dNTP, 1.2 mM of $MgCl_2$, 8 mM of Tris-HCl, 40 mM of KCl, 0.1 μL Taq DNA polymerase and 2 μL of DNA template. The inner PCR assay used 0.5 μL of DNA template from the outer reaction, in a 20 μL reaction volume with the primers Tryp99 and Tryp957. The PCR master mix for the inner reaction contained 0.6 μM of each primer, 200 μM of each dNTP, 1.2 mM of $MgCl_2$, 8 mM of Tris-HCl, 40 mM of KCl, 0.5 μL of Taq DNA polymerase. Thermocycler conditions for the outer essay were: initial denaturation at 95°C for 5 min, 5 cycles of 95°C for 1 min, 45°C for 30 sec, and 65°C for 1 min, followed by 35 cycles of 95°C for 1 min, 50°C for 30 sec, 72°C for 1 min, and a final extension at 65°C for 10 min. On the inner PCR assay, thermocycler conditions were: initial denaturation of 96°C for 3 min, 25 cycles of 96°C for 30 sec, 58°C for 1 min, and 72°C for 30 sec, and a final extension at 72°C for 7 min. We measured the DNA concentration and purity from all samples that tested negative, and confirmed that negative results were not due to extraction failures. We used the product of the inner PCR assay for bidirectional sequencing with BigDye Terminator v3.1 using ABI PRISM® 3100 DNA Analyzer (Applied Bio Systems®, USA) at the University of Missouri-St. Louis. DNA sequences were edited and analyzed on CLC Main Workbench version 7.0 (QIAGEN, http://www.clcbio.com).

Molecular sexing of hosts

For all individuals whose sex we could not determine based on plumage differences observed in the field (N=100), we used molecular sexing based on the PCR amplification of CDH1 genes found in avian sex chromosomes (Fridolfsson and Ellegren, 1999). Primers were 2550F and 2718R, in a PCR master mix with a final volume of 10 µL and 1 µL of genomic DNA. The master mix contained 0.3125 units of Taq DNA polymerase, 1.5 µL of 10X buffer, 2.5mM of each dNTP, 1.625 mM of $MgCl_2$, and 0.5 µM of each primer. Thermal cycling conditions were as follows: 2 minutes at 94 ºC, followed by 31 cycles of 45 seconds at 94 ºC, 45 seconds at 48 ºC, 45 seconds at 72 ºC, and final elongation for 10 minutes at 72 ºC. Sex was determined by the number of bands after running 8 µL of amplicon on a 2% agarose gel for 40 min.

Microscopy

We selected slides for microscopic viewing based on the PCR detection of parasites. We analyzed five slides for each of the following groups of individuals: 1) negative for both haemosporidian and trypanosome parasites; 2) positive for both haemosporidian and trypanosome parasites; 3) positive only for haemosporidian parasites; and 4) positive only for trypanosome parasites (four slides analyzed). We examined blood slides using an Olympus 224 CX31 light microscope, equipped with a Leica ICC50 HD digital camera and Leica Application Suite, version 4.4.0, imaging software. We took 100 pictures, corresponding to 100 fields at high magnification (x 1000), and used ImageJ to count the number of cell nuclei on each picture (Girish and Vijayalakshmi, 2004; Gering and Atkinson, 2004). We manually recorded the numbers of heterophils, eosinophils, basophils, lymphocytes, thrombocytes, as well as the number of polychromatic erythrocytes (PE) and the number of mature erythrocytes infected with haemosporidian parasites. Infections by haemosporidian parasites can lead to elevated counts of heterophils (Ellis *et al.*, 2014), which could be costly to the host due to tissue damage related to inflammatory responses (Ots *et al.*, 1998). Decreased numbers of lymphocytes could signal immunosuppression, as these cells are responsible for the production of antibodies against infective agents (Ots *et al.*, 1998). PE are erythrocytes prematurely released, or just released, from the bone marrow into the blood stream. PE have basophilic cytoplasm and cell nuclei showing clumps of condensed chromatin. Elevated levels of PE may signify regenerative anemia, which is a condition caused by either blood loss or hemolysis (Atkinson *et al*. 2014; Samour, 2006). The proportion of each white blood cell type was calculated as the number of cells per 10^4 non-polychromatic erythrocytes.

Statistical analyses

We used Fisher's exact tests and log linear tests for two-way and three-way contingency

analysis, respectively. We applied generalized linear models with a binomial error distribution to determine the effects of individual parasite lineages on the presence/absence of a second parasite. The first of these models tested the effects of haemosporidian lineages on the occurrence of trypanosome parasites. In this model, presence/absence of trypanosome parasites in each individual host was the response variable, and the presence/absence of each lineage of haemosporidian parasite was a multi-level categorical predictor. The second model followed the same reasoning, but with trypanosome lineages as the predictor variable and presence/absence of haemosporidian parasites as the response variable. Blood cell count data were represented as proportions of cells relative to 10^4 non-polychromatic erythrocytes. We used ANOVA and the post-hoc Tukey's HSD to compare hematological parameters determined through microscopy between groups of birds with single and co-infections. All analyses were performed in R (R Core Team, 2015).

Results

We screened 153 individual yellow-breasted chats, of which 52 were infected with haemosporidian parasites, 40 were infected with trypanosomes, and 23 harbored co-infections with lineages of both parasites. Genotyping identified ten haemosporidian lineages, with 33 (63%) of the infections being *Plasmodium* lineage OZ08, and 7 (13%) *Plasmodium* lineage OZ01 (Fig. 1). We observed five trypanosome lineages, 29 (72.5%) of which were assigned to the lineage Tryp01, which closely matches (nucleotide pairwise difference of 1.6%) a SSU rRNA sequence of *Trypanosoma bennetti* (Genbank accession AJ223562), first isolated from an American kestrel (*Falco sparverius*) (Haag, 1998). The lineage Tryp04 also matches *T. bennetti* AJ223562 (nucleotide pairwise difference of 2.2%). Tryp02 and Tryp03 do not match any identifiable morphotype sequence available in Genbank. The trypanosome lineages Tryp01-Tryp03 have an average nucleotide pairwise difference of 2%; Tryp04 differs by an average of 6.5% from Tryp01-Tryp03. Sequences for new trypanosome lineages were deposited in Genbank: accession numbers KX179915 – KX179918.

We determined the sex of 149 individuals, of which 81 were males and 68 were females. Sexes did not differ in the prevalence of single or co-infections (Table 1). Overall, 102 individuals were identified adults, and 42 as juveniles. Adults were more likely than juveniles to be infected with haemosporidian parasites; however, we found no effect of age on the occurrence of trypanosome infections (Table 2). We had determined in the field the age of 30 out of the 33 individuals infected with the specialist *Plasmodium* lineage OZ 08, and 28 of these were adults. Additionally, only ten adults harbored infections by lineages other than OZ 08. Co-infections were more common in adults than in juveniles, though the difference was only marginally significant (Table 2).

An individual *I. virens* carrying one parasite infection was almost four times more likely

to be carrying a second type of parasite (Fisher's exact test, $P = 0.0004$, odds ratio = 3.9, *CI* = 1.7–8.9). The *Plasmodium* lineage OZ08, the most common haemosporidian lineage infecting yellow-breasted chats (Fig. 1), was positively associated with the occurrence of trypanosome infections in individual hosts. That is, if an individual was infected with OZ08, it would more likely be infected with any given lineage of trypanosome parasite (Table 3). The *Plasmodium* lineage OZ04, recovered from only three individuals, showed a positive, marginally significant relationship with trypanosome infections; two of the three hosts infected with OZ04 also were infected with trypanosome parasites (Table 3). When we looked at the effects of trypanosome lineages on the probability of harboring haemosporidian infections, we found that Tryp01, the most frequent trypanosome lineage, increased the probability of an individual host also being infected with a haemosporidian parasite (Table 3).

Counts of polychromatic erythrocytes (PE), white blood cells, and erythrocytes infected with haemosporidian parasites are summarized in Table S1 of the Supplemental Material. Yellow-breasted chats that tested negative for both haemosporidian and trypanosome infections had lower levels of PE compared to individuals with either single or co-infections ($F = 11.9$; $P < 0.001$, Tables S1 and S2 of the Supplemental Material). Individuals infected with both *Plasmodium* spp. and *Trypanosoma* spp. had levels of haemosporidian-infected erythrocytes comparable to individuals infected only with *Plasmodium* spp. parasites (*t*-test = -0.4366, *d.f.* = 4, $P = 0.7$). The proportion of heterophils, eosinophils, basophils, lymphocytes, monocytes, and thrombocytes did not differ among individuals that were uninfected, co-infected, or singly infected.

Discussion

We found a positive association between haemosporidian and trypanosome infections, meaning that individuals infected by one parasite are more likely to carry a second protozoan infection. Specifically, the *Plasmodium* lineage OZ08 was positively associated with the trypanosome lineage Tryp01. A relationship between haemosporidian and trypanosome parasites was similarly observed in Alaskan passerine birds, where *Trypanosoma* infections were positively associated with *Leucocytozoon* and *Haemoproteus* infections, but not with *Plasmodium* infections (Oakgrove *et al.*, 2014). These associations are unlikely to result solely from these parasites being co-vectored, as the transmission routes of avian trypanosomes and some avian haemosporidian parasites may differ (Baker, 1956a; c; Molyneux and Robertson, 1974; Votýpka and Svobodová, 2004). We also found that trypanosome infections cause comparable levels of regenerative anemia as *Plasmodium* infections, which provides evidence of disease related to *Trypanosoma*.

An association between trypanosome and haemosporidian parasites also has been reported in Eurasian sparrowhawks (*Accipeter nisus*) and common buzzards (*Buteo buteo*) (Svobodová *et al.*, 2015). For individuals of these raptor species, co-infections involving *Trypanosoma* and *Leucocytozoon* parasites were more frequent than co-infections involving *Trypanosoma* and the other two haemosporidian parasite genera (Svobodová *et al.*, 2015). Blackflies can vector the *Trypanosoma* and *Leucocytozoon* parasites of sparrowhawks and buzzards, but *Haemoproteus* and *Plasmodium* parasites require other vectors (Svobodová *et al.*, 2015). The co-infections by *Trypanosoma* and *Leucocytozoon* in sparrowhawks and buzzards could be due to co-transmission by blackflies. However, we find it unlikely that co-transmission is responsible for the observed association between *Trypanosoma* and *Plasmodium* in yellow-breasted chats. First, although we have not identified the vectors of trypanosomes for yellow-breasted chats, *Trypanosoma culicavium* isolated from *Culex* mosquitoes is the only known case of avian trypanosome parasites potentially transmitted by mosquitoes (Votýpka *et al.*, 2012). Second, *Trypanosoma-Plasmodium* co-infection might not be survived by mosquito vectors. Experimental infections of avian haemosporidians in Culicidae vectors show that infections with a single parasite species reduce mosquito survival rates, even in naturally occurring (i.e., co-evolved) parasite-vector associations (see Ferguson and Read, 2002; Valkiūnas *et al*., 2014).

Empirical evidence suggests that the association of haemosporidian and trypanosome infections could be even stronger than what we observed, since trypanosome parasites are difficult to detect in the peripheral blood of birds (Kirkpatrick and Suthers, 1988; Apanius, 1991). Experimental infections of *Trypanosoma avium* in canaries demonstrated that, upon ingestion of infected louse-flies, metacyclic trypomastigotes first migrated to the host lymph nodes (Baker, 1956c). Within a few days post infection, and after growing in size in the lymph nodes, individual trypanosomes could be found in the peripheral blood, where they remained for two to three months before migrating to the bone marrow (Baker, 1956c; Molyneux and Robertson, 1974; Valkiūnas *et al.*, 2004; Votýpka and Svobodová, 2004). Thus, the narrow window of detection of the parasite in the peripheral blood suggests that we likely underestimated the prevalence of trypanosome parasites in our study population. However, our samples were collected during the summer months, when trypanosomes are known to reappear in the peripheral blood of infected birds, and when haemosporidian infections also relapse (Valkiūnas *et al.*, 2004). Additionally, detection bias in our molecular methods might have resulted in underestimating the strength of the association between haemosporidian and trypanosome parasites; in the presence of co-infections of either type of parasite (i.e., multiple haemosporidians or multiple trypanosomes infecting the same individual host), a PCR reaction may amplify one parasite over the other (Pérez-Tris and Bensch, 2005). However, this bias does not affect the probability of detecting one of two or more infections of either type of parasite.

We detected five lineages of *Trypanosoma* parasites infecting a single host population. In samples from 71 bird species caught in African rainforests, Sehgal *et al*. (2001) found eight trypanosome lineages, four of which were found infecting olive sunbirds (*Cyanomitra olivacea)*. In an assemblage of 49 bird species in Alaska, only *Trypanosoma avium* was detected (Oakgrove *et al.*, 2014). As trypanosomes are known to have low host specificity (Bennett *et al.*, 1994; Sehgal *et al.*, 2001), each of the lineages identified in this study might infect multiple bird species in the Ozarks region. Our findings also suggest high genetic diversity of *Trypanosoma* lineages, which might correspond to species other than *T. bennetti* depending on analyses of morphological characters and further genetic markers.

The lack of support for sex-biased incidence of infection is consistent with the findings of previous studies. For example, Kirkpatrick and Suthers (1988) analyzed blood films of 697 individuals from 59 bird species and found no differences between males and females in the prevalence of *Plasmodium*, *Haemoproteus*, and *Trypanosoma*, or co-infections amongst the three genera of parasites. In our study, haemosporidian infections were found more often in adults than juveniles, which is consistent with at least three hypotheses: (1) mortality due to haemosporidian infections is higher among juveniles, leading to detection bias favoring infections in older individuals; (2) infections accumulate with age, meaning that older birds have been exposed more to infected vectors than juveniles; (3) most infections in adults correspond to relapses of chronic infections acquired in previous years. Hypothesis (1) seems unlikely, given the high local abundance of yellow-breasted chats. Some combination of (2) and (3) more likely explains the higher prevalence of infections in adults. Previous studies have demonstrated that older birds are not only more likely to be infected than juveniles (Svobodová *et al.*, 2015), but also that they are more likely to be harbor specialist rather than generalist parasites, possibly reflecting differences in the ability of specialist versus generalist parasites to infect hosts that they share (Medeiros *et al.*, 2014).

Haemosporidian parasite relapse in spring is an adaptation of the parasite to seasonal host susceptibility, availability of naïve hosts, and vector availability (Cornet *et al.*, 2014; Pérez-Rodríguez *et al.*, 2015). Additionally, in chronically infected birds in temperate regions, trypanosomes spend the winter in the bone marrow, and only migrate to the peripheral blood during spring and summer months (Baker, 1956a; c). Kirkpatrick and Suthers (1988) found hatch year (HY) North American birds to have half the haemosporidian and trypanosome infections as after hatch year (AHY) individuals. The difference between our findings and those of Kirkpatrick and Suthers (1988) may be due to the different methods used to detect trypanosomes; they surveyed trypanosomes using blood cultures, which boosts the detectability of these parasites when they are uncommon

in the peripheral blood (Stabler *et al.*, 1966; Kirkpatrick and Lauer, 1985). Thus, although PCR methods have high sensitivity when it comes to detection of blood parasites, it is possible that we failed to detect some developing trypanosome infections in juvenile birds.

While the pathogenicity of some avian haemosporidian lineages is fairly well understood (Valkiūnas 2004), little is known about the consequences of *Trypanosoma* infections for birds. The similar white blood cell levels of infected and uninfected birds is consistent with previous studies, in which white-blood cell responses to blood parasite infections occurred in some populations of hosts, but not others (Ricklefs and Sheldon, 2007). Ellis *et al*. (2014) found that haemosporidian-infected birds of the Ozarks presented higher levels of heterophils and lymphocytes than non-infected individuals. The difference in results between this study and Ellis *et al*. (2014) could be due to the latter being a broader analysis of the effects of any haemosporidian infection on the white-blood cell profiles of several host species, whereas this study focused on *Plasmodium*-infected individuals from one population of a single host species.

We found evidence for regenerative anemia associated with trypanosome parasites on the same level as that caused by haemosporidian infections. Elevated numbers of polychromatic erythrocytes (PE) in trypanosome-infected individuals is evidence of disease. Although we found a statistically significant difference in PE between trypanosome-infected and uninfected individuals, the number of slides available for analysis was limited, and we interpret these results as evidence that trypanosome infections have the potential to cause disease. More importantly, we recommend that future studies investigating the effect of avian trypanosome infections include in their design some measure of anemia. Although reports are scarce, ours is not the first to associate avian *Trypanosoma* parasites with disease-like symptoms in birds. Experimental infections of *Trypanosoma bouffardi* in canaries revealed myocarditis, nephritis, congestion of the spleen, and myofibril degeneration in infected subjects (Molyneux *et al.*, 1983). However, experimental studies of infection of *Trypanosoma* in canaries have not detected multiplication of the parasite (Baker, 1956c; Votýpka and Svobodová, 2004), which suggests that canaries are not natural hosts for the parasites (Chatterjee and Ray, 1971). When *Trypanosoma avium* was experimentally inoculated in red-whiskered bulbuls (*Otocompsa jocosa*), Chatterjee and Ray (1971) observed trypomastigotes multiplying by binary fission in the blood and bone marrow. Yet, the evidence of multiplication by binary fission does not make the pathogenicity of avian trypanosomes straightforward to understand, as parasites are not multiplying within the erythrocytes, as are *Plasmodium* schizonts. Moreover, comparable numbers of polychromatic erythrocytes between single and co-infected individuals suggest that either haemosporidian or trypanosome parasites have suppressed virulence in co-infections.

In conclusion, we have demonstrated a positive association between *Plasmodium* and *Trypanosoma* infections in yellow-breasted chats, with evidence of pathogenicity of trypanosomes in these hosts. The association between these two protozoans could arise from a negative effect of one parasite on the host's immune response to the other. Future studies might focus on determining whether such an association remains during host migration and wintering. The basic life cycle of avian trypanosome parasites and their pathogenicity in vertebrate hosts remains poorly understood. However, the evidence presented here sheds some light on the mechanisms of within-host interactions between trypanosomes and haemosporidians, two common parasites of birds.

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References

Alizon, S., de Roode, J. C. and Michalakis, Y. (2013). Multiple infections and the evolution of virulence. *Ecology letters* 16, 556–567.

Apanius, V. (1991). Avian trypanosomes as models of hemoflagellate evolution. *Parasitology Today* 7, 87–90.

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

Atkinson, C.T., Saili, K.S., Utzurrum, R.B. & Jarvi, S.I. (2014) Experimental evidence for evolved tolerance to avian malaria in a wild population of low elevation Hawai'i 'Amakihi (*Hemignathus virens*). *EcoHealth* 10, 366–375.

Atkinson, C. T., Dusek, R. J., Woods, K. L. and Iko, W. M. (2000). Pathogenicity of avian malaria in experimentally-infected Hawaii Amakihi. *Journal of Wildlife Diseases* 36, 197–204.

Baker, J. R. (1956a). Studies on *Trypanosoma avium* Danilewsky 1885. II. Transmission by *Ornithomyia avicularia*. *Parasitology* 46, 321–334.

Baker, J. R. (1956b). Studies on T*rypanosoma avium* Danilewsky 1885. I. Incidence in some birds of Hertfordshire. *Parasitology* 46, 308–320.

Baker, J. R. (1956c). Studies on *Trypanosoma avium* Danilewsky 1885. III. Life cycle in vertebrate and invertebrate hosts. *Parasitology* 46, 335–352.

Balmer, O., Stearns, S. C., Schötzau, A. and Brun, R. (2009). Intraspecific competition between co-infecting parasite strains enhances host survival in African trypanosomes. *Ecology* 90, 3367–3378.

Bennett, G. F., Earle, R. A. and Squires-Parsons, D. (1994). Trypanosomes of some sub-Saharan birds. *The Onderstepoort journal of veterinary research* 61, 263–271.

Budischak, S. A., Sakamoto, K., Megow, L. C., Cummings, K. R., Urban, J. F., Jr. and Ezenwa, V. O. (2015). Resource limitation alters the consequences of co-infection for both hosts and parasites. *International Journal for Parasitology* 45, 455–463.

Chatterjee, D. K. and Ray, H. N. (1971). Some observations on the morphology and developmental stages of *Trypanosoma avium bakeri* ssp.nov. from the red-whiskered bulbul (*Otocompsa jocosa* Linn.). *Parasitology* 62, 331–338.

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

Cox, F. E. (2001). Concomitant infections, parasites and immune responses. *Parasitology* 122 Suppl, S23–38.

Černý, O., Votýpka, J. and Svobodová, M. (2011). Spatial feeding preferences of ornithophilic mosquitoes, blackflies and biting midges. *Medical and Veterinary Entomology* 25, 104–108.

de Roode, J. C., Helinski, M. E. H., Anwar, M. A. and Read, A. F. (2005). Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. *The American Naturalist* 166, 531–542.

de Roode, J. C., Yates, A. J. and Altizer, S. (2008). Virulence-transmission trade-offs and population divergence in virulence in a naturally occurring butterfly parasite. *Proceedings of the National Academy of Sciences* 105, 7489–7494.

Desser, S. S., McIver, S. B. and Jez, D. (1975). Observations on the role of simuliids and culicids in the transmission of avian and anuran trypanosomes. *International Journal for Parasitology* 5, 507–509.

Dimitrov, D., Palinauskas, V., Iezhova, T. A., Bernotienė, R., Ilgūnas, M., Bukauskaitė, D., Zehtindjiev, P., Ilieva, M., Shapoval, A. P., Bolshakov, C. V., Markovets, M. Y., Bensch, S. and Valkiūnas, G. (2015). Plasmodium spp.: An experimental study on vertebrate host susceptibility to avian malaria. *Experimental Parasitology* 148, 1–16.

Ellis, V. A., Collins, M. D., Medeiros, M. C. I., Sari, E. H. R., Coffey, E. D., Dickerson, R. C., Lugarini, C., Stratford, J. A., Henry, D. R., Merrill, L., Matthews, A. E., Hanson, A. A., Roberts, J. R., Joyce, M., Kunkel, M. R. and Ricklefs, R. E. (2015). Local host specialization, host-switching, and dispersal shape the regional distributions of avian haemosporidian parasites. *Proceedings of the National Academy of Sciences* 112, 11294–

11299.

Ellis, V. A., Kunkel, M. R. and Ricklefs, R. E. (2014). The ecology of host immune responses to chronic avian haemosporidian infection. *Oecologia* 176, 729–737.

Ferguson, H.M. and Read, A.F. (2002) Why is the effect of malaria parasites on mosquito survival still unresolved? *Trends in parasitology*, 18, 256–261.

Fridolfsson, A. K. and Ellegren, H. (1999). A simple and universal method for molecular sexing of non-ratite birds. *Journal of Avian Biology* 116–121.

Gering, E. and Atkinson, C. T. (2004). A Rapid Method for Counting Nucleated Erythrocytes on Stained Blood Smears by Digital Image Analysis. *Journal of Parasitology* 90, 879–881.

Girish, V. and Vijayalakshmi, A. (2004). Affordable image analysis using NIH Image/ImageJ. *Indian journal of cancer* 41, 47–47.

Greiner, E. C., Bennett, G. F., White, E. M. and Coombs, R. F. (1975a). Distribution of the avian hematozoa of North America. *Canadian journal of zoology* 53, 1762–1787.

Greiner, E. C., Bennett, G. F., White, E. M. and Coombs, R. F. (1975b). Distribution of the avian hematozoa of North America. *Canadian journal of zoology* 53, 1762–1787.

Haag, J. (1998). The molecular phylogeny of trypanosomes: evidence for an early divergence of the Salivaria. *Molecular and Biochemical Parasitology* 91, 37–49.

Ivkovic, M., Kesic, M., Mihaljevic, Z. and Kudela, M. (2013). Emergence patterns and ecological associations of some haematophagous blackfly species along an oligotrophic hydrosystem. *Medical and Veterinary Entomology* 28, 94–102.

Kirkpatrick, C. E. and Lauer, D. M. (1985). Hematozoa of raptors from southern New Jersey and adjacent areas. *Journal of wildlife diseases* 21, 1–6.

Kirkpatrick, C. E. and Suthers, H. B. (1988). Epizootiology of blood parasite infections in passerine birds from central New Jersey. *Canadian journal of zoology* 66, 2374–2382.

Klemme, I., Louhi, K.-R. and Karvonen, A. (2016). Host infection history modifies co-infection success of multiple parasite genotypes. *The Journal of animal ecology* 85, 591–597.

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

Knowles, S. C. L., Wood, M. J. and Sheldon, B. C. (2010). Context-dependent effects of parental effort on malaria infection in a wild bird population, and their role in reproductive trade-offs. *Oecologia* 164, 87–97.

Longmire, J. L., Maltbie, M. and Baker, R. J. (1997). Use of “lysis buffer” in DNA

isolation and its implications for museum collections. *Occasional Papers of the Museum of Texas Tech University* 163, 1–3.

Lovette, I. J., Pérez-Emán, J. L., Sullivan, J. P., Banks, R. C., Fiorentino, I., Córdoba-Córdoba, S., Echeverry-Galvis, M., Barker, F. K., Burns, K. J., Klicka, J., Lanyon, S. M. and Bermingham, E. (2010). A comprehensive multilocus phylogeny for the wood-warblers and a revised classification of the Parulidae (Aves). *Molecular Phylogenetics and Evolution* 57, 753–770.

Medeiros, M. C. I., Ellis, V. A. and Ricklefs, R. E. (2014). Specialized avian Haemosporida trade reduced host breadth for increased prevalence. *Journal of Evolutionary Biology* 27, 2520–2528.

Mideo, N. (2009). Parasite adaptations to within-host competition. *Trends in Parasitology* 25, 261–268.

Molyneux, D. H. and Robertson, E. (1974). Ultrastructure of the bloodstream forms of an avian trypanosome *Trypanosoma bouffardi. Annals of Tropical Medicine and Parasitology* 68, 369–77.

Molyneux, D. H., Cooper, J. E. and Smith, W. J. (1983). Studies on the pathology of an avian trypanosome (T. *bouffardi*) infection in experimentally infected canaries. *Parasitology* 87 (Pt 1), 49–54.

Morris, D. L., A Porneluzi, P., Haslerig, J., Clawson, R. L. and Faaborg, J. (2013). Results of 20 years of experimental forest management on breeding birds in Ozark forests of Missouri, USA. *Forest Ecology and Management* 310, 747–760.

Oakgrove, K. S., Harrigan, R. J., Loiseau, C., Guers, S., Seppi, B. and Sehgal, R. N. M. (2014). Distribution, diversity and drivers of blood-borne parasite co-infections in Alaskan bird populations. *International Journal for Parasitology* 44, 717–727.

Ots, I., MurumAgi, A. and HOrak, P. (1998) Haematological health state indices of reproducing Great Tits: methodology and sources of natural variation. *Functional Ecology*, 12, 700–707.

Palinauskas, V., Valkiūnas, G., Bolshakov, C. V. and Bensch, S. (2008). *Plasmodium relictum* (lineage P-SGS1): Effects on experimentally infected passerine birds. *Experimental Parasitology* 120, 372–380.

Palinauskas, V., Valkiūnas, G., Bolshakov, C. V. and Bensch, S. (2011). *Plasmodium relictum* (lineage SGS1) and Plasmodium ashfordi (lineage GRW2): The effects of the co-infection on experimentally infected passerine birds. *Experimental Parasitology* 127, 527–533.

Palinauskas, V., Valkiūnas, G., Kriħanauskienė, A., Bensch, S. and Bolshakov, C. V. (2009). *Plasmodium relictum* (lineage P-SGS1): Further observation of effects on

experimentally infected passeriform birds, with remarks on treatment with Malarone™. *Experimental Parasitology* 123, 134–139.

Pérez-Rodríguez, A., la Hera, de, I., Bensch, S. and Pérez-Tris, J. (2015). Evolution of seasonal transmission patterns in avian blood-borne parasites. *International Journal for Parasitology* 45, 605–611.

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

R Core Team (2015). R: A language and environment for statistical computing. *R Foundation for Statistical Computing*, Vienna, Austria. URL http://www.R-project.org/.

Reidy, J. L., Thompson, F. R., III and Kendrick, S. W. (2014). Breeding bird response to habitat and landscape factors across a gradient of savanna, woodland, and forest in the Missouri Ozarks. *Forest Ecology and Management* 313, 34–46.

Ricklefs, R. E. and Sheldon, K. S. (2007). Malaria prevalence and white-blood-cell response to infection in a tropical and in a temperate thrush. *The Auk* 124, 1254-1266.

Ricklefs, R. E., Swanson, B. L., Fallon, S. M., MartÍnez-AbraÍn, A., Scheuerlein, A., Gray, J. and Latta, S. C. (2005). Community relationships of avian malaria parasites in southern Missouri. *Ecological Monographs* 75, 543–559.

Samour, J. (2006). *Diagnostic value of hematology*. (eds. Harrison, G. and Lightfoot, T. Clinical Avian Medicine.

Sehgal, R. N. M., Jones, H. I. and Smith, T. B. (2001). Host specificity and incidence of Trypanosoma in some African rainforest birds: a molecular approach. *Molecular Ecology* 10, 2319–2327.

Stabler, R. M., Holt, P. A. and Kitzmiller, N. J. (1966). *Trypanosoma avium* in the blood and bone marrow from 677 Colorado birds. *Journal of Parasitology* 52, 1141–1144.

Svensson-Coelho, M., Blake, J. G., Loiselle, B. A., Penrose, A. S., Parker, P. G. and Ricklefs, R. E. (2013). Diversity, prevalence, and host specificity of avian *Plasmodium* and *Haemoproteus* in a western Amazon assemblage. *Ornithological Monographs* 76, 1–47.

Svobodová, M., Weidinger, K., Peške, L., Volf, P., Votýpka, J. and Voříšek P. (2015). Trypanosomes and haemosporidia in the buzzard (*Buteo buteo*) and sparrowhawk (*Accipiter nisus*): factors affecting the prevalence of parasites. *Parasitology Research*, 114, 551–560.

Telfer, S., Lambin, X., Birtles, R., Beldomenico, P., Burthe, S., Paterson, S. and Begon, M. (2010). Species interactions in a parasite community drive infection risk in a wildlife population. *Science* 330, 243–246.

Valkiūnas, G. (2004). *Avian malarial parasites and other haemosporidia*. Taylor and

Francis Publishing, London.

Valkiūnas, G., Anwar, A. M., Atkinson, C. T., Greiner, E. C., Paperna, I. and Peirce, M. A. (2005). What distinguishes malaria parasites from other pigmented haemosporidians? *Trends in parasitology* 21, 357–358.

Valkiūnas, G., Bairlein, F., Iezhova, T. and Dolnik, O. V. (2004). Factors affecting the relapse of haemoproteus belopolskyi infections and the parasitaemia of *Trypanosoma* spp. in a naturally infected European songbird, the blackcap, *Sylvia atricapilla*. *Parasitology Research* 93, 218–222.

Valkiūnas, G., Kazlauskienė, R., Bernotienė, R., Bukauskaitė, D., Palinauskas, V. and Iezhova, T.A. (2014) *Haemoproteus* infections (Haemosporida, Haemoproteidae) kill bird-biting mosquitoes. *Parasitology Research* 113, 1011–1018.

Van Dyken, M., Bolling, B., Moore, J. L., Blair, C., Beaty, B., Black, W. C. and Foy, B. (2006). Molecular evidence for trypanosomatids in *Culex* mosquitoes collected during a West Nile virus survey. *International Journal for Parasitology* 36, 1015–1023.

Votýpka, J. and Svobodová, M. (2004). *Trypanosoma avium*: experimental transmission from black flies to canaries. *Parasitology Research* 92, 147–151.

Votýpka, J., Lukeš, J. and Oborník, M. (2004). Phylogenetic relationship of T*rypanosoma corvi* with other avian trypanosomes. *Acta Protozoologica* 43, 225–231.

Votýpka, J., Szabova, J., Radrova, J., Zidkova, L. and Svobodová, M. 2012. *Trypanosoma culicavium* sp. nov., an avian trypanosome transmitted by *Culex* mosquitoes. International Journal of Systematic and Evolutionary Microbiology 62, 745–754.

Zídková, L., Cepicka, I., Szabová, J. and Svobodová, M. (2012). Biodiversity of avian trypanosomes. *Infection, Genetics and Evolution* 12, 102–112.

Figure

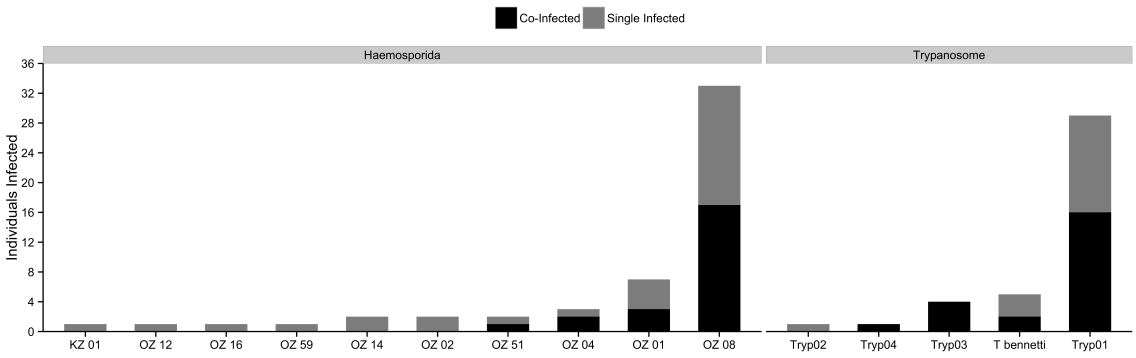

Figure 1. Prevalence of lineages of and haemosporidian (left) and trypanosome (right) parasites retrieved from yellow-breasted chats sampled in the Ozarks (MO).

Supplemental Material

Table S1. Proportion of polychromatic erythrocytes (PE), and white blood cell differentials of yellow-breasted chats (*Icteria virens*). Each row is a group of individual hosts, and each cell represents the mean and standard deviation (in parenthesis) for all individuals within the groups. Proportions were calculated based on cell counts over 10^4 non-polychromatic erythrocytes.

	PE	**Eosinophils**	**Lymphocytes**	**Thrombocytes**	**Haemosporidian**
Uninfected	0.02 (0.00)	0.001 (0)	0.005 (0.002)	0.003 (0.004)	
Co-infected	0.065 (0.02)	0.0002 (0.0004)	0.004 (0.002)	0.001 (0.001)	0.001 (0.001)
Haemosporidian Infected	0.059 (0.01)	0.0002 (0.0004)	0.005 (0.002)	0.002 (0.0007)	0.0007 (0.0009)
Trypanosome Infected	0.059 (0.007)	0.001 (0.001)	0.002 (0.0006)	0.005 (0.005)	

The average proportion of heterophils for uninfected individuals was 0.0004 (0.0005); monocytes were 0.0005 (0.0001) for trypanosome-infected birds. Basophil counts were zero as well as otherwise noted for heterophils and eosinophils. Only one trypanosome parasite was observed on a slide of an individual that tested negative for haemosporidian parasites.

Table S2. Post-hoc Tukey HSD test of comparisons of the proportion of polychromatic erythrocytes (PE) between the four groups of *Icteria virens* blood films analyzed. Represented on the table are the average differences between the groups, the lower and upper confidence intervals (CI), as well as the P-values. Significant comparisons were highlighted in bold.

	Average Difference	**Lower CI**	**Upper CI**	**P**
Co-infected *vs.* Uninfected	0.04	0.02	0.07	**0.0005**
Plasmodium infected *vs.* Uninfected	0.04	0.02	0.06	**0.002**
*Trypanosom*a infected *vs.* Uninfected	0.04	0.01	0.07	**0.003**
Plasmodium infected *vs.* Co-infected	-0.005	-0.03	0.02	0.92
*Trypanosom*a infected *vs*. Co-infected	-0.005	-0.03	0.02	0.93
Plasmodium infected *vs. Trypanosoma* infected	-0.0001	-0.03	0.03	1.0