11-14-2015

RELATIONSHIPS OF HAEMOSPORIDIAN PARASITES TO POPULATIONS OF THEIR AVIAN HOSTS IN EASTERN NORTH AMERICA

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A Dissertation Submitted to The Graduate School at the University of Missouri-St. Louis
in partial fulfillment of the requirements for the degree
Doctor of Philosophy in Biology

May, 2015

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Dissertation Abstract

Avian Haemosporida are common, vector-transmitted blood parasites of birds throughout the world. During my dissertation research, I explored how multiple host species respond immunologically to natural infections in the wild (Chapter 1) and to experimental infections in the laboratory (Chapter 2). Despite their tractability as a model host-parasite system and a burgeoning literature on avian Haemosporida, little is known about how their populations interact across large areas (hereafter "regions"). I present data from parasite surveys of birds across eastern North America suggesting that continental parasite populations track host populations across the region, but also that the host breadth of a parasite can be variable across space and time (Chapter 3). Parasite lineages replace each other spatially within a host population, likely due to interspecific parasite competition mediated by host immune systems (Chapter 3). Parasite prevalence is positively related to host abundance within local assemblages (Chapter 4), but within host species across their ranges, prevalence does not vary with abundance (Chapters 3 and 4). Finally, a 12 year survey of parasites and their hosts in the Missouri Ozarks demonstrates that parasite populations vary through time, and that this variability is related to host breadth—specialist parasites (i.e., parasites infecting primarily one host) were more variable than generalist parasites (i.e., parasites infecting multiple hosts; Chapter 5). Overall my dissertation work contributes to the natural history and ecology of avian Haemosporidian parasites and their avian hosts, and to host-parasite ecological and evolutionary theory.

Acknowledgments

None of this work would have been possible without the support, guidance, encouragement, patience, and tireless editing of my advisor, Bob Ricklefs. The contributions of numerous collaborators were essential, particularly those of Michael Collins, Matt Medeiros, and Stéphane Cornet. My thesis committee provided useful insights and guidance throughout the course of the project. Funding agencies were generous in their support of my work, especially the Harris World Ecology Center, the Missouri and St. Louis Audubon Societies, the Curators of the Univeristy of Missouri, the NSF Malaria Research Coordination Network, and the Department of Biology (Raven Fellowship) and the Graduate School (Dissertation Fellowship) at UMSL.

I am lucky enough to be part of a family that has always supported me, and I thank them and my friends for their constant encouragement over the last few years. Finally, I thank my wife, Eloisa Sari, whose love and support have sustained me as long as I have known her.

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Chapter 1

The ecology of host immune responses to chronic avian haemosporidian infection

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Published as: Ellis VA, Kunkel MR, and RE Ricklefs. 2014. The ecology of host immune responses to chronic avian haemosporidian infection. Oecologia 176: 729-737.

Abstract
Host responses to parasitism in the wild are often studied in the context of single host-parasite systems, which provide little insight into the ecological dynamics of host-parasite interactions within a community. Here we characterized immune system responses to mostly low-intensity, chronic infection by haemosporidian parasites in a sample of 424 individuals of 22 avian host species from the same local assemblage in the Missouri Ozarks. Two types of white blood cells (heterophils and lymphocytes) were elevated in infected individuals across species, as was the acute-phase protein haptoglobin, which is associated with inflammatory immune responses. Linear discriminant analysis indicated that individuals infected by haemosporidians occupied a subset of the overall white blood cell multivariate space that was also occupied by uninfected individuals, suggesting that these latter individuals might have harbored other pathogens or that parasites more readily infect individuals with a specific white blood cell profile. DNA sequence-defined lineages of haemosporidian parasites were sparsely distributed across the assemblage of hosts. In one well-sampled host species, the red-eyed vireo (*Vireo olivaceus*), heterophils were significantly elevated in individuals infected with one but not another of two common parasite lineages. Another well sampled host, the yellow-breasted chat (*Icteria virens*), exhibited no differences in immune response to different haemosporidian lineages. Our results indicate that while immune responses to infection may be generalized across host species, parasite-specific immune responses may also occur.
Key words: *avian malaria, ecoimmunology, Haemosporida, haptoglobin, leukocytes*

Introduction

Parasites can adversely affect host individuals and populations (e.g., Day and Monk 1974; van Riper et al. 1986; Hudson et al. 1998; Lafferty and Kuris 2009), but relatively little is known about the responses of individual hosts to infection in natural populations. Several studies have documented the prevalence of haemosporidian parasites (*Plasmodium*, *Haemoproteus*, and *Leucocytozoon*) in local and regional avian assemblages (van Riper et al. 1986; Schall and Vogt 1993; Booth and Elliott 2003; Ricklefs et al. 2005; Szymanski and Lovette 2005), but few have examined variation in the immunological and physiological consequences of infection among host species in an ecological context (but see Apanius et al. 2000). That haemosporidian infection affects host physiology is not in question—experimental infections often have severe physiological consequences (Atkinson et al. 2000, 2001; Garvin et al. 2003; Palinauskas et al. 2008; Cellier-Holzem et al. 2010). Furthermore, comparisons of infected and

infection-free individuals, as well as applications of antimalarial drugs, have revealed fitness costs associated with haemosporidian infection in populations of wild birds (Merino et al. 2000; Marzal et al. 2005; Knowles et al. 2010).

Studies of the effects of haemosporidian parasites on hosts have mostly involved single host species and single parasite lineages (Atkinson et al. 2000, 2001; Garvin et al. 2003; Cellier-Holzem et al. 2010). However, in one experimental study, Palinauskas et al. (2008) infected five species of passerine birds with the same lineage of *Plasmodium relictum* and showed that infection dynamics and susceptibility to infection differed between the host species. This is not surprising, as species often differ in aspects of baseline immune function (Matson et al. 2006) and in immune responses to antigens (Horrocks et al. 2011). While much has been gained by placing immune function *per se* in an ecological context (Norris and Evans 2000), clearly there also is a need to place parasite-specific immune responses in an ecological context.

Parasites in the genus *Plasmodium* are thought to be more pathogenic than those in the related haemosporidian genus *Haemoproteus* because *Plasmodium* reproduces asexually in the blood stream of its vertebrate host while *Haemoproteus* does not (Valkiūnas 2005). Host individuals tend to maintain chronic infections of *Plasmodium* at a lower intensity in the blood than those of *Haemoproteus* (Fallon and Ricklefs 2008), presumably because the former is more dangerous to the host. Palinauskas et al. (2011) tackled the question of how hosts respond to different parasite lineages by infecting two species of birds with two lineages of parasites in the genus *Plasmodium*. They showed that the infection dynamics of the two parasite lineages differed across hosts, indicating potential differences in pathogenicity between lineages. White blood cell levels additionally have been shown to be elevated in haemosporidian-infected birds in some but not all populations of the same, or closely related, species (Ricklefs and Sheldon 2007).

To better understand the ecology of host parasite relationships, we sampled 424 individuals of 22 species from a local avian assemblage in the Ozarks of southern Missouri and investigated variation in host responses to haemosporidian infection. We looked for an effect of infection on circulating white blood cell levels, presumably indicators of host immune defense (Ots et al. 1998; Ricklefs and Sheldon 2007), and on the concentration of haptoglobin, an acute-phase protein of the immune system whose concentration increases during an inflammatory response (Matson et al. 2006). Cellier-Holzem (2010) documented elevated haptoglobin in canaries during experimental infection with *P. relictum* in a laboratory setting, but to date haptoglobin has not been examined in relation to haemosporidian infection in a natural setting, where parasitemias (i.e., the concentration of parasites in the peripheral circulation of an individual) are typically low (Valkiūnas 2005). We also tested for an effect of infection on individual mass and the proportional volume of red blood cells in the peripheral blood (hematocrit). For two well-sampled host species (*Vireo olivaceus* and *Icteria virens*), we assessed whether distinct molecular parasite lineages elicited different physiological responses from their common hosts.

Given the known fitness consequences of haemosporidian infection in wild birds (Merino et al. 2000; Marzal et al. 2005; Knowles et al. 2010), we predicted that haemosporidian infection would result in increased inflammation (as measured by haptoglobin) and a generally heightened immune response.

Materials and methods

From late May to early July 2011, we captured birds (n = 424) via mist-net in previously clear-cut areas of the Ozarks of southern Missouri (centroid geographic coordinates for sampling locations are 37°13′ N, 91°04′ W; for habitat description see Ricklefs et al. 2005). Upon capture, each bird was banded with a U.S. Fish and Wildlife issued metal leg band, sex and age were determined (if possible) using the criteria in Pyle (1997), morphometric measurements were taken, and a small blood sample (< 1% of the individual's body weight) was collected before releasing the bird. In this analysis, birds were aged as either hatch year (HY, i.e., born that summer) or after-hatch year (AHY). Sampling occurred only in the mornings from approximately 0600 to 1100. All sampling was conducted under federal permit #21688 and Missouri Department of Conservation permit #14967 and in accordance with University of Missouri – St. Louis IACUC (Protocol 309824-1).

Blood collection

We collected blood samples from the brachial vein of each bird using heparinized microcapillary tubes. From each blood sample we made two blood smears, and then added ca. 5-10 μL to a tube with 300 μL of lysis buffer (Longmire et al. 1997) for later DNA extraction. Remaining blood was kept on ice (<6 h) until centrifugation. After centrifugation, hematocrit was recorded, and plasma was placed in a microcentrifuge tube and frozen at -20°C. Blood smears were fixed on the day of collection for five minutes in methanol. Within two weeks of collection, blood smears were stained for 60 minutes in a modified Geimsa solution (Valkiūnas 2005). Due to logistical constraints in the field, we were able to collect plasma from only 151 individuals.

White blood cells, parasitemias, and haptoglobin

A subset (n = 235) of blood smears was chosen randomly for estimating white blood cell densities and haemosporidian parasitemias. Slides were read by a single person (MRK). White blood cell density was determined by counting the number of white blood cells per approximately 10^4 red blood cells at 400× magnification. We estimated the number of red blood cells by comparing each field with a series of standardized photographs with known numbers of red blood cells (Ricklefs and Sheldon 2007). The proportion of different types of white blood cells were also determined by counting and classifying 100 white blood cells at 1000× magnification as either heterophils, eosinophils, basophils, lymphocytes, or monocytes (Clark et al. 2009). Basophils were rare in our blood smears and so were excluded from our analyses. The presence or absence of blood parasites was also recorded, and if parasites were found, the parasitemia was estimated as the number of parasites per 10^4 red blood cells. We typically examined approximately 10-20 fields at 400× magnification in order to include 10^4 red blood cells.

Plasma samples were thawed and then analyzed for haptoglobin concentration using a commercially available kit (Tridelta Diagnostics, product code TP-801 Morris Plains, NJ), following the "manual method" provided by the manufacturer.

Molecular analysis

DNA was extracted from the blood stored in lysis buffer using a standard ammonium acetate-isopropanol protocol (Svensson and Ricklefs 2009). Polymerase chain reaction was used to screen the blood samples for haemosporidian infections, and a small region of the parasite cytb gene was sequenced in positive infections. Details of the protocol are

described in Fecchio et al. (2013). Molecular lineages were determined after sequencing positive infections and comparing those sequences to those in Ricklefs et al. (2005). Parasites with similar cytb sequences were distinguished as independent lineages when they occurred in different hosts in the same area, or were more divergent than such lineages. A subset of individuals had double peaks in the chromatogram of the sequenced infections potentially indicating the presence of multiple infections (i.e., different lineages infecting the same individual). All individuals with potential multiple infections were removed from the analysis. All unique sequences are available through GenBank (accession numbers listed in Supplementary Table 8). The sex of each individual was confirmed by molecular analysis following the protocol in Fridolfsson and Ellegren (1999).

Statistical analyses

All statistical analyses were performed using R version 2.15.1 (R Core Team 2012). We used log-likelihood G-tests to determine whether prevalence varied across host species, age, and sex. We determined potential predictors of the physiological variables using AICc to weight generalized linear models based on all possible combinations of the following variables: infection status, parasite genus, parasite lineage, host species, sex, age, and the interaction between infection status and host species. This analysis was performed using the *glmulti* package in R (Calcagno 2012).We report the best model (i.e., the model with the highest model weight) and its weight in the text. The model-averaged parameter estimates and unconditional variances (as calculated in Buckland et al. 1997) for all explanatory variables from the best models are reported in Supplementary Table 2.

To analyze variation in white blood cell response to infection we performed a linear discriminant analysis (LDA) to explore the white blood cell multivariate space. White blood cell frequencies (WBC type per 10^4 RBCs) were fourth-root transformed to normalize their distributions, and were checked for homogeneity of multivariate dispersions (Legendre and Legendre 2012) using the function 'betadisper' from the *vegan* package in R (Oksanen et al. 2012). We also repeated our analyses using higher taxonomic levels as nested random effects in our models (results not reported). Adding levels of taxonomy above species did not change the results of any of our analyses. We also analyzed the white blood cell response to infection by different parasite lineages in two hosts using linear regression models with Type III F-tests.

Results

Infection prevalence across host species, age, and sex

The most common infections were from the genera *Plasmodium* and *Haemoproteus* (subgenus *Parahaemoproteus*), however parasites belonging to the genus *Leucocytozoon* were also identified (Table 1). We used the RAxML BlackBox (Stamatakis et al. 2008; Figure 1) to produce a midpoint-rooted, maximum-likelihood phylogeny of the *Plasmodium* and *Haemoproteus* lineages in this analysis; only one *Leucocytozoon* lineage was found. We also compared our sequences with the MalAvi database (Bensch et al. 2009) and found that three lineages had associated morphospecies, which we listed on the phylogeny next to the lineage name (Figure 1). Overall infection prevalence determined by PCR varied significantly across host species ($G = 102$, $df = 21$, $P < 0.001$) ranging from 0 to 100 percent (Supplementary Table 1). Prevalence did not differ significantly between sexes ($G = 0.11$, $df = 1$, $P = 0.74$), but younger individuals (HY) were less often

infected than were older individuals (AHY; G = 12.7, df = 1, $P < 0.001$). However, the significant age effect could be due to heterogeneity of age distributions, infection intensities, and sample sizes across species (Ricklefs et al., 2005). To account for this potential source of bias, we ran a logistic regression with a quasibinomial error distribution with age class, species, and the interaction between the two as predictor variables weighted by the square root of the sample size of each species. Both species and the interaction between species and age class were not significant predictors of infection status, but age class alone was. Younger individuals (HY) were still less often infected than older individuals (AHY) in this analysis (estimate of coefficient for HY compared to AHY = -0.973, s.e. = 0.446, $P = 0.030$).

White blood cells and parasitemias

As is common in studies of haemosporidian infection in wild bird populations, most infections presented very low parasitemias (mean = 32, s.d. = 128.9 parasites in 10^4 red blood cells; Supplementary Figure 1). In our blood smear sample, 93.3% of infected individuals (70/75) had parasitemias under one percent (i.e., 100 parasites in 10^4 red blood cells). In 45.3% of samples that tested positive for infection using molecular methods, we failed to find a single parasite among 10^4 red blood cells. We therefore excluded parasitemia from our analyses and relied on PCR detection of infection, although including parasitemia as a separate variable did not change our results qualitatively. In a separate set of multiple regression analyses (results not shown), we found that parasitemia was not significantly related to any of the other physiological variables we measured. Furthermore, our main results did not change if we excluded birds with less than one or fewer than 100 parasites per 10^4 red blood cells, indicating that the physiological measurements we recorded were likely not related to parasitemia.

We explored the white blood cell multivariate space using linear discriminant analysis (LDA) to determine the position of infected and uninfected individuals within that space. The LDA correctly classified 91.7% of uninfected birds from white blood cell frequencies, while it classified only 22.7% of infected birds correctly. Thus, the multivariate space occupied by uninfected birds is larger than that occupied by infected birds and the latter is largely overlapped by the former, as shown by the distribution of uninfected and infected birds on the discriminant axis (Figure 2). The standardized discriminant function coefficients showed that heterophils (0.89), lymphocytes (1.63), and to a lesser extent eosinophils (0.25), were positively related to the discriminant axis while monocytes (-0.35) were negatively related. These results indicate that haemosporidian infected individuals have white blood cell profiles that occupy a particular part of the overall white blood cell multivariate space (elevated heterophils and lymphocytes), which is also occupied by some individuals that are not infected by haemosporidians.

We were also interested in which variables best predicted individual white blood cell responses. The AICc model with the highest model weight for heterophils (model weight = 0.25) included infection status, host species, and age as explanatory variables, as did the best model for lymphocytes (model weight = 0.44; Supplementary Tables 2, 3 and 4). Both models showed that heterophils and lymphocytes were elevated in haemosporidian infected individuals (estimates of coefficients 0.095 and 0.146, respectively). The best model for eosinophils included only host species (model weight =

0.26). The model with the highest weight for monocytes was a null model with no explanatory variables (model weight = 0.24, Supplementary Table 1).

Haptoglobin, hematocrit and body mass

Haptoglobin assays and hematocrit measurements were not equally distributed between infected and uninfected birds within most species, many of which were poorly sampled. Seven of the species in our analysis lacked data on haptoglobin for either uninfected or infected birds, and seven species had only one data point for one or both of those groups (Supplementary Tables 5 and 6). Accordingly, we analyzed data only from four better-sampled species (northern cardinal, *Cardinalis cardinalis*: uninfected = 3, infected = 3; red-eyed vireo, *Vireo olivaceus*: uninfected = 18, infected = 7; scarlet tanager, *Piranga olivacea*: uninfected = 6, infected = 6; yellow-breasted chat, *Icteria virens*: uninfected = 16, infected = 11). The model for haptoglobin concentration with the highest AICc weight in this analysis included infection status and host sex as explanatory variables (model weight = 0.17), and the estimated standardized coefficient for infection status (0.203) indicated that haptoglobin was elevated in infected individuals relative to uninfected individuals. The best models in our analysis of both hematocrit (model weight = 0.36) and mass (model weight = 0.36) included host species and age as explanatory variables and, interestingly, did not include infection status (Supplementary Tables 2, 5, 6 and 7).

Response to different parasite lineages by individuals of a single host species

In two well-sampled hosts, *Vireo olivaceus* (n = 56) and *Icteria virens* (n = 70), our data allowed us to test whether infected individuals responded differently to different parasite lineages. Many *V. olivaceus* individuals harbored one of two common lineages from the genus *Haemoproteus:* OZ10 (n = 13) and OZ12 (n = 14). From young individuals (HY) we recovered proportionally fewer infections of OZ12 relative to OZ10 ($G = 4.64$, $df = 1$, $P = 0.03$). Regardless of age, individuals infected with OZ10 also had higher levels of heterophils than did those infected with OZ12 ($F_{1,25} = 4.3$, $P = 0.05$), although they did not differ in terms of other types of white blood cells. *I. virens* harbored two lineages of *Plasmodium*: OZ01 (n = 5) and OZ08 (n = 14). Relative to OZ01, infections of OZ08 were found in fewer young individuals (HY) than in older individuals (AHY; $G = 4.75$, $df = 1$, $P = 0.03$). However, white blood cell levels did not differ significantly between individuals infected with one or the other lineage. We did not test for differences in haptoglobin in either of these cases because we lacked samples.

Discussion

Haemosporidian prevalence varied significantly across host species in our system, allowing us to investigate the ecological component of immune response to infection across hosts within a single community. We quantified circulating levels of white blood cells as a measure of immune function and response to infection, and found that two cell types (heterophils and lymphocytes) were elevated in infected individuals across species (Supplementary Table 2). Furthermore, we found no evidence for an interaction between host species and infection status in either heterophils or lymphocytes indicating that the response to infection was generalized across species.

We also quantified circulating levels of the acute-phase protein haptoglobin to assess the degree to which haemosporidian infection elicited an inflammatory immune response in the host. Haptoglobin has been shown to increase in experimentally infected

canaries in a laboratory setting (Cellier-Holzem et al. 2010) but it is unclear whether wild birds would respond similarly. We did, however, find that haptoglobin was elevated in infected individuals. Moreover, it did not vary across four of the better sampled hosts, indicating that wild birds do mount an inflammatory immune response to haemosporidian infection and that the response is consistent across species. In caged canaries, which are highly susceptible to infection, haptoglobin remained elevated up to twenty days post-infection, when parasitemias were higher than they were in the individuals in our study (Cellier-Holzem et al. 2010). Indeed, 45.3% of infected individuals in our study had parasitemias lower than 1 parasite in 10^4 host red blood cells, however those individuals had indistinguishable haptoglobin levels from individuals with higher parasitemias (analysis not shown).

We also applied a multivariate analysis to white blood cell responses to infection and found that infected individuals concentrate in a small portion of the overall white blood cell multivariate space characterized by elevated heterophil and lymphocyte counts (Figure 2). Moreover, haemosporidian infected birds share white blood cell profiles with some individuals that are uninfected by haemosporidians, but which might be infected by other parasites or pathogens that we did not sample. In chickens, for example, "infectious bronchitis virus" causes an increase in infiltration of heterophils and lymphocytes in various tissues (Raj and Jones 1997); lymphocytes in the peripheral blood also are known to interact with avian influenza viruses in chickens (Suarez and Shultz-Cherry 2000). Moreover, heterophils in the peripheral blood of chickens express a broad range of Toll-like receptor mRNAs, demonstrating their importance as the first line of defense against bacterial, viral, fungal, and other parasitic infections (Kogut et al. 2005). While further investigation is needed, these results indicate the potential for white blood cells as general indicators of infection. However, an alternative hypothesis is that haemosporidian parasites infect individuals that have a particular white blood cell profile (i.e., higher heterophils and lymphocytes) more readily than others. Ultimately experimental infections will need to be performed to resolve the mechanism behind the pattern we described.

Contrary to expectation, we found no differences between the effects of the two most common parasite genera (*Plasmodium* and *Haemoproteus*) on blood parameters. In one case, however, different parasite lineages of the genus *Haemoproteus* (OZ10 and OZ12) were associated with different levels of heterophils in infected individuals of *Vireo olivaceus*. We did not find a similar difference in any of the other variables measured. We also found no differences in host response to two well-sampled *Plasmodium* parasite lineages that infected *Icteria virens* emphasizing the idiosyncratic, complex nature of ecological interactions between hosts and parasites in this system. Haemosporidian lineage-specific responses by hosts have been documented in experimentally infected mouse populations (Jarra and Brown 1985), and Palinauskas et al. (2011) demonstrated that parasitemias of two different species of *Plasmodium* differed during the course of co-infection in two species of birds. While it is hard to quantify the level of parasite-specific immunity in our system without performing experimental infections, our results indicate the potential for variation in the specificity of immune responses to parasite lineages.

Parasite prevalence differed significantly among age classes in our sample, with younger individuals (HY) being less often infected than older individuals. Ricklefs et al.

(2005) found a similar, though non-significant, trend in birds from the same location sampled several years earlier. In a study of pied flycatchers (*Ficedula hypoleuca*), Dale et al. (1996) found that younger individuals were less often infected with *Haemoproteus* than were older individuals, but there was no significant effect of age on the prevalence of *Trypanosoma*, another blood parasite. When we analyzed infection data at the lineage level in two well sampled hosts, we found that some lineages infect a greater proportion of older individuals than expected while others do not, again hinting at the complexity of the ecological relationships between haemosporidian parasites and their hosts and perhaps suggesting differing parasite strategies. This overall pattern of younger individuals harboring fewer infections than older individuals might reflect a general accumulation of chronic infections in individuals with age, or could be the result of younger birds dying more often as a result of infection.

Measures of host condition can indicate the severity of health effect of parasite infection. On such measure is hematocrit, the proportion of red blood cells in the peripheral blood. We measured hematocrit in a subset of hosts, expecting from experimental studies that haemosporidian infection would depress hematocrit. This pattern has been shown in wild (Booth and Elliott 2003) and experimentally manipulated (Garvin et al. 2003; Palinauskas et al. 2008, 2009; Cellier-Holzem et al. 2010) avian populations. We did not detect such a pattern in our data. However, birds in our sample did not exhibit the high parasitemias of acute-stage infections observed by Cellier-Holzem et al. (2010), and while Booth and Elliott (2003) examined many of the same species that were present in our sample, their study was based on blood smears and therefore might have missed infected individuals with low parasitemias but nonetheless detected by PCR. In a study of sage-grouse (*Centrocercus urophasianus*), Dunbar et al. (2003) also failed to find a relationship between infection status and hematocrit in birds with low-intensity, chronic infections. Interestingly, we did not find a relationship between parasitemia and hematocrit in our study (analysis not shown). Booth and Elliott (2003) surveyed birds in Louisiana and Connecticut, however, and perhaps host responses to infection vary across the regional space.

Our study of the variation in host responses to haemosporidian infection across a single ecological assemblage of hosts illustrates the complexity of host-parasite interactions in this system. Our data show that while overall host immune responses to infection appear to be generalized, individual host species can respond differently to specific parasite DNA lineages. Future studies can contribute to our understanding of host-parasite interactions in this system most effectively through experimental infections. These types of studies could clarify the extent to which parasite lineage-specific immune responses exist across multiple host species.

Acknowledgements
Alicia Burke, Kristen Bird, Elyse Coffey, Linlin Meng, and William Burke assisted with field work and Eloisa Sari provided advice regarding lab procedures and insightful comments on an earlier version of the manuscript; Iris Levin, Matthew Medeiros, Eliot Miller, Michael Collins, Alix Matthews, Alison Hanson, and Jackson Roberts also provided helpful comments. The Whitney Harris World Ecology Center at UMSL funded this project through the Leo and Kay Drey Scholarship (VAE). The Missouri Department of Conservation provided housing and logistical support. Logistics were also facilitated

by John Faaborg. Laboratory funds were provided by the Curators of the University of Missouri (RER). All sampling occurred under appropriate federal and state permits and in accordance with the UMSL IACUC.

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Figure 1

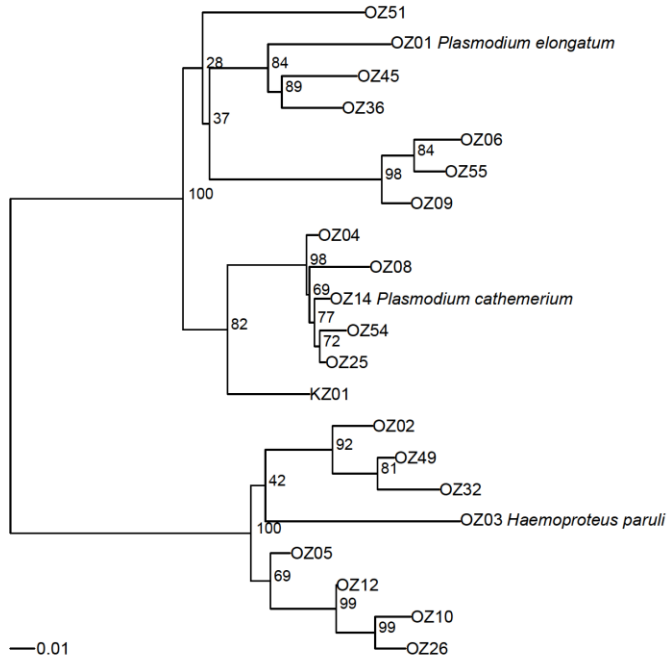


Fig. 1—Maximum-likelihood phylogeny of the *Plasmodium* and *Haemoproteus* lineages in this study. Three lineages had associated morphospecies, OZ01 is *Plasmodium elongatum* (GenBank Accession no. DQ368381), OZ03 is *Haemoproteus paruli* (AF465563), and OZ14 is *Plasmodium cathemerium* (AY377128).

Figure 2

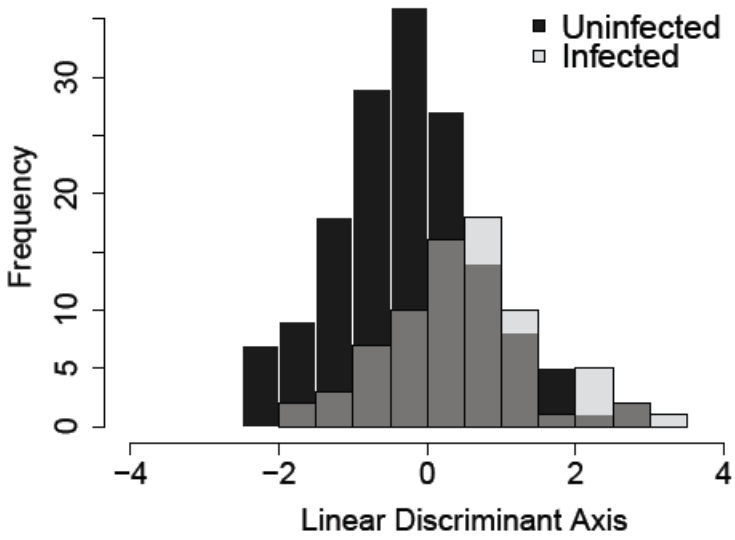


Fig. 2— Frequency distribution of individual scores separated into groups of haemosporidian-infected (light gray) and uninfected (black) individuals on the linear discriminant axis (LDA) based on the frequencies of four types of white blood cells. The

dark gray corresponds to areas of overlap in the frequency distributions of the two groups. Infected birds occupy a portion of the overall white blood cell multivariate space.

Table 1—Parasite lineages recovered from each host species in this sample from the Missouri Ozarks in 2011. Parasite lineages are listed in the first row of the table (with the prefix "OZ" omitted), and host species are listed in the first column with their respective sample sizes in parenthesis. Parasite lineages of the genus *Plasmodium* are highlighted in boldface font, *Leucocytozoon* in italics, and *Haemoproteus* in normal type. We did not find haemosporidian infection in four species (*Vermivora cyanoptera*, *Coccyzus americanus*, *Poecile carolinensis*, and *Setophaga discolor*).

	Lineage Name																						
Species	2	3	32	5	10	12	49	26	*59*	**1**	**4**	**6**	**8**	**9**	**55**	**54**	**51**	**KZ01**	**14**	**25**	**36**	**45**	Total
Icteria virens (70)	1					1			*1*	**5**			**14**				**1**	**1**	**1**				25
Vireo olivaceus (56)			1	3	13	14								**3**							**1**		35
Helmitheros vermivorus (42)																			**4**				4
Mniotilta varia (36)																			**1**			**1**	2
Vireo griseus (35)								5															5
Passerina cyanea (28)										**3**													3
Thryothorus ludovicianus (16)					1																	**1**	2
Piranga olivacea (15)	7	1																					8
Seiurus aurocapillus (14)												**1**											1
Parula americana (12)											**1**	**1**											2
Wilsonia citrina (11)												**1**											1
Cardinalis cardinalis (11)		1								**1**									**2**			**1**	5
Baeolophus bicolor (10)		1																	**1**	**1**			3
Oporornis formosus (7)															**1**	**1**			**1**				3
Icterus spurius (5)													**1**						**1**				2
Pirangra rubra (5)	2								*1*														3
Empidonax virescens (4)																			**1**				1
Spizella passerina (4)							4																4
Total	10	3	1	3	14	15	4	5	*2*	**9**	**1**	**3**	**15**	**3**	**1**	**1**	**1**	**1**	**12**	**1**	**1**	**3**	**109**

Supplementary Table 1

Supp. Table 1—Prevalence of haemosporidian infection separated by parasite genus, and including an overall prevalence with all parasite genera grouped.

Species	n	Total Prevalence	Plasmodium Prevalence	Haemoproteus Prevalence	Leucocytozoon Prevalence
Icteria virens	70	0.36	0.31	0.03	0.01
Vireo olivaceus	56	0.63	0.07	0.55	0
Helmitheros vermivorus	42	0.10	0.10	0	0
Mniotilta varia	36	0.06	0.06	0	0
Vireo griseus	35	0.14	0	0.14	0
Passerina cyanea	28	0.11	0.11	0	0
Vermivora cyanoptera	18	0	0	0	0
Thryothorus ludovicianus	16	0.13	0.06	0.06	0
Piranga olivacea	15	0.53	0	0.53	0
Seiurus aurocapillus	14	0.07	0.07	0	0
Parula americana	12	0.17	0.17	0	0
Wilsonia citrina	11	0.09	0.09	0	0
Cardinalis cardinalis	11	0.45	0.36	0.09	0
Coccyzus americanus	10	0	0	0	0
Poecile carolinensis	10	0	0	0	0
Baeolophus bicolor	10	0.3	0.2	0.1	0
Oporornis formosus	7	0.43	0.43	0	0
Icterus spurius	5	0.4	0.4	0	0
Setophaga discolor	5	0	0	0	0
Pirangra rubra	5	0.6	0	0.4	0.2
Empidonax virescens	4	0.25	0.25	0	0
Spizella passerina	4	1	0	1	0

Supplementary Table 2

Supp. Table 2—All the best models (i.e., highest-model weight according to AICc) found in our analysis. The estimate of the coefficient and the unconditional variance (estimated following Buckland et al. 1997) for each of the parameters in the best models are averaged across all models in the AICc analysis. Categorical explanatory variables all have two or more levels, one of which is not presented in the model tables as it is used as a baseline to which the estimate of the coefficients for the remaining levels should be compared. This is the standard way R estimates coefficients in linear models with categorical predictor variables. For example, the variable "Infection Status" has two levels, infected and uninfected. The estimate of the coefficient of the level infected in the

haptoglobin model is 0.203 ± 0.024 indicating that being infected increases haptoglobin levels about 0.2 times more than being uninfected.

Model: *Haptoglobin ~ Infection Status + Host Sex*			
Overall Model Weight	Parameter	Estimate of Coefficient	Variance
0.17	Intercept	0.703	0.015
	Infected	0.203	0.024
	Male	0.079	0.006
Model: *Heterophils ~ Infection Status + Host Species + Host Age*			
Overall Model Weight	Parameter	Estimate of Coefficient	Variance
0.25	Intercept	0.297	0.117
	Infected	0.095	0.011
	Icteria virens	0.778	0.147
	Vireo olivaceus	0.541	0.126
	Helmitheros vermivorus	0.447	0.118
	Mniotilta varia	0.394	0.119
	Vireo griseus	0.199	0.133
	Passerina cyanea	0.704	0.147
	Vermivora cyanoptera	0.384	0.124
	Thryothorus ludovicianus	0.695	0.148
	Piranga olivacea	0.629	0.140
	Seiurus aurocapillus	0.404	0.119
	Parula americana	0.476	0.133
	Wilsonia citrina	0.296	0.115
	Cardinalis cardinalis	0.554	0.135
	Coccyzus americanus	0.855	0.193
	Poecile carolinensis	0.282	0.118
	Baeolophus bicolor	0.478	0.133
	Oporornis formosus	0.495	0.157
	Setophaga discolor	0.272	0.258
	Hatch-year	0.079	0.006
Model: *Eosinophils ~ Host Species*			
Overall Model Weight	Parameter	Estimate of Coefficient	Variance
0.26	Intercept	1.154	0.004
	Icteria virens	0.320	0.124
	Vireo olivaceus	0.361	0.148
	Helmitheros vermivorus	0.194	0.067
	Mniotilta varia	0.083	0.036
	Vireo griseus	0.227	0.083
	Passerina cyanea	0.307	0.120
	Vermivora cyanoptera	0.253	0.095
	Thryothorus ludovicianus	0.228	0.082
	Piranga olivacea	0.330	0.132
	Seiurus aurocapillus	0.116	0.044
	Parula americana	0.130	0.050
	Wilsonia citrina	0.208	0.076
	Cardinalis cardinalis	0.238	0.087
	Coccyzus americanus	0.279	0.108

	Poecile carolinensis	0.242	0.090
	Baeolophus bicolor	0.266	0.101
	Oporornis formosus	0.260	0.106
	Setophaga discolor	0.150	0.099
Model: *Lymphocytes ~ Infection Status + Host Species + Host Age*			
Overall Model Weight	Parameter	Estimate of Coefficient	Variance
0.44	Intercept	1.478	0.082
	Infected	0.146	0.010
	Icteria virens	0.954	0.089
	Vireo olivaceus	0.552	0.088
	Helmitheros vermivorus	0.342	0.089
	Mniotilta varia	0.084	0.094
	Vireo griseus	0.948	0.098
	Passerina cyanea	0.470	0.093
	Vermivora cyanoptera	0.215	0.099
	Thryothorus ludovicianus	0.462	0.094
	Piranga olivacea	0.545	0.094
	Seiurus aurocapillus	0.299	0.094
	Parula americana	0.178	0.101
	Wilsonia citrina	0.330	0.099
	Cardinalis cardinalis	1.198	0.095
	Coccyzus americanus	0.432	0.10
	Poecile carolinensis	0.210	0.102
	Baeolophus bicolor	0.354	0.100
	Oporornis formosus	0.025	0.120
	Setophaga discolor	0.057	0.241
	Hatch-year	0.241	0.004
Model: *Monocytes ~ 1*			
Overall Model Weight	Parameter	Estimate of Coefficient	Variance
0.24	Intercept	0.976	0.003
Model: *Hematocrit ~ Host Species + Host Age*			
Overall Model Weight	Parameter	Estimate of Coefficient	Variance
0.36	Intercept	49.896	9.649
	Icteria virens	-6.207	10.493
	Vireo olivaceus	-0.427	11.220
	Helmitheros vermivorus	-2.522	11.121
	Mniotilta varia	-7.261	11.972
	Vireo griseus	-6.928	12.345
	Passerina cyanea	6.866	11.714
	Vermivora cyanoptera	-3.930	15.974
	Thryothorus ludovicianus	-2.683	11.155
	Piranga olivacea	-.813	16.107
	Seiurus aurocapillus	-5.328	15.865
	Parula americana	-0.419	19.515
	Cardinalis cardinalis	-1.541	12.279
	Coccyzus americanus	1.690	12.261
	Poecile carolinensis	-2.831	13.159
	Baeolophus bicolor	0.284	14.218
	Oporornis formosus	-1.900	28.454
	Setophaga discolor	-3.200	28.454

	Piranga rubrica	1.172	19.291
	Spizella passerina	-3.11	29.261
	Hatch-year	-1.046	1.484
Model: *Mass ~ Host Species + Host Age*			
Overall Model Weight	Parameter	Estimate of Coefficient	Variance
0.36	Intercept	12.535	1.839
	Icteria virens	11.731	1.948
	Vireo olivaceus	3.614	1.982
	Helmitheros vermivorus	0.176	2.037
	Mniotilta varia	-2.227	2.071
	Vireo griseus	-1.421	2.057
	Passerina cyanea	1.009	2.110
	Vermivora cyanoptera	-4.267	2.268
	Thryothorus ludovicianus	5.794	2.291
	Piranga olivacea	14.132	2.350
	Seiurus aurocapillus	5.743	2.370
	Parula americana	-5.459	2.456
	Icterus spurius	6.749	3.334
	Wilsonia citrina	-2.220	2.500
	Cardinalis cardinalis	29.445	2.528
	Coccyzus americanus	50.272	2.500
	Poecile carolinensis	-3.163	2.648
	Baeolophus bicolor	7.483	2.705
	Oporornis formosus	0.486	2.884
	Setophaga discolor	-5.485	3.299
	Piranga rubrica	15.409	3.310
	Spizella passerina	-1.289	3.701
	Hatch-year	-0.267	0.099

Supplementary Table 3

Supp. Table 3—White blood cell means and standard errors for all birds that tested PCR positive for haemosporidian infection.

Species	n	Het mean	Het SE	Eos mean	Eos SE	Lym mean	Lym SE	Mon mean	Mon SE
Icteria virens	19	1.29	0.07	1.20	0.06	2.69	0.11	1.00	0.13

Vireo olivaceus	29	1.01	0.1	1.40	0.08	2.28	0.07	0.79	0.12
Helmitheros vermivorus	4	0.98	0.11	1.02	0.1	2.16	0.11	0.92	0.14
Mniotilta varia	1	1.05	-	0.97	-	1.63	-	0.97	-
Vireo griseus	2	1.07	0.06	1.26	0.03	2.32	0.02	0.83	0.07
Passerina cyanea	3	1.01	0.19	1.22	0.06	2.10	0.26	1.24	0.43
Vermivora cyanoptera	0	-	-	-	-	-	-	-	-
Thryothorus ludovicianus	1	0.92	-	0.95	-	1.64	-	0.65	-
Piranga olivacea	8	1.09	0.08	1.34	0.11	2.24	0.12	1.21	0.11
Seiurus aurocapillus	1	1.16	-	1.43	-	2.28	-	2.28	-
Parula americana	1	0.78	-	0.78	-	1.22	-	0.50	-
Wilsonia citrina	1	0	-	1.52	-	2.12	-	1.49	-
Cardinalis cardinalis	5	1.08	0.35	1.11	0.31	2.81	0.27	1.10	0.29
Coccyzus americanus	0	-	-	-	-	-	-	-	-
Poecile carolinensis	0	-	-	-	-	-	-	-	-
Baeolophus bicolor	2	0.41	0.41	0.90	0.21	2.42	0.39	1.68	0.22
Oporornis formosus	1	0.74	-	1.07	-	1.69	-	0.67	-
Icterus spurius	0	-	-	-	-	-	-	-	-
Setophaga discolor	0	-	-	-	-	-	-	-	-
Pirangra rubra	0	-	-	-	-	-	-	-	-
Empidonax virescens	1	0	-	0.71	-	1.83	-	1.12	-
Spizella passerina	0	-	-	-	-	-	-	-	-

Supplementary Table 4

Supp. Table 4—White blood cell means and standard errors for all birds that tested PCR negative for haemosporidian infection.

Species	n	Het mean	Het SE	Eos mean	Eos SE	Lym mean	Lym SE	Mon mean	Mon SE
Icteria virens	12	1.05	0.16	1.39	0.07	2.45	0.12	1.01	0.16

Vireo olivaceus	8	0.77	0.18	1.28	0.09	1.99	0.17	0.89	0.27
Helmitheros vermivorus	15	0.74	0.1	1.11	0.05	1.92	0.12	1.04	0.08
Mniotilta varia	12	0.71	0.09	0.88	0.11	1.73	0.11	0.92	0.2
Vireo griseus	8	0.39	0.19	1.16	0.19	2.63	0.15	1.11	0.35
Passerina cyanea	10	1.1	0.09	1.33	0.08	2.08	0.12	1.04	0.16
Vermivora cyanoptera	9	0.71	0.15	1.21	0.11	1.83	0.17	0.85	0.08
Thryothorus ludovicianus	11	1.05	0.13	1.18	0.08	2.05	0.12	0.71	0.16
Piranga olivacea	0	-	-	-	-	-	-	-	-
Seiurus aurocapillus	12	0.69	0.1	0.91	0.06	1.87	0.09	0.82	0.13
Parula americana	7	0.81	0.14	1.00	0.08	1.85	0.2	0.92	0.11
Wilsonia citrina	8	0.65	0.15	1.07	0.11	1.79	0.15	0.84	0.15
Cardinalis cardinalis	6	0.84	0.29	1.23	0.34	2.95	0.16	1.43	0.17
Coccyzus americanus	9	1.22	0.09	1.25	0.1	1.9	0.15	1.04	0.16
Poecile carolinensis	9	0.62	0.13	1.19	0.18	1.90	0.17	1.27	0.12
Baeolophus bicolor	7	0.98	0.18	1.33	0.08	1.96	0.07	1.11	0.22
Oporornis formosus	3	0.87	0.21	1.27	0.27	1.58	0.36	0.97	0.15
Icterus spurius	0	-	-	-	-	-	-	-	-
Setophaga discolor	1	0.53	-	1.01	-	1.53	-	1.08	-
Pirangra rubra	7	1.01	0.08	1.35	0.09	2.19	0.08	1.22	0.24
Empidonax virescens	1	0.56	-	0.74	-	1.32	-	1.13	-
Spizella passerina	0	-	-	-	-	-	-	-	-

Supplementary Table 5

Supp. Table 5—Haptoglobin and hematocrit means, standard errors, and sample sizes for all birds that tested PCR positive for haemosporidian infection.

Species	n	Haptoglobin (mg/mL) mean	Haptoglobin SE	n	Hematocrit mean	Hematocrit SE
Icteria virens	11	1.03	0.15	1	44.23	0.85

				6		
Vireo olivaceus	18	0.99	0.1	9	50.12	1.2
Helmitheros vermivorus	2	0.59	0.36	0	-	-
Mniotilta varia	1	1.78	-	1	44.8	-
Vireo griseus	1	1.15	-	2	40.95	4.55
Passerina cyanea	1	1.79	-	2	65.8	6.2
Vermivora cyanoptera	0	-	-	0	-	-
Thryothorus ludovicianus	1	0.44	-	2	47.3	0.9
Piranga olivacea	6	0.68	0.12	2	50.8	2
Seiurus aurocapillus	1	1.89	-	1	42.9	-
Parula Americana	0	-	-	1	50.8	-
Wilsonia citrine	0	-	-	0	-	-
Cardinalis cardinalis	3	1.31	0.28	4	48.13	2.85
Coccyzus americanus	0	-	-	0	0	-
Poecile carolinensis	0	-	-	0	-	-
Baeolophus bicolor	1	1.08	-	2	52.8	2.3
Oporornis formosus	1	0.72	-	0	-	-
Icterus spurius	0	-	-	0	-	-
Setophaga discolor	0	-	-	0	-	-
Pirangra rubra	0	-	-	1	54.2	-
Empidonax virescens	0	-	-	0	-	-
Spizella passerina	2	1.27	0.17	1	47.1	-

Supplementary Table 6

Supp. Table 6— Haptoglobin and hematocrit means, standard errors, and sample sizes for all birds that tested PCR negative for haemosporidian infection.

Species	n	Haptoglobin (mg/mL) mean	Haptoglobin SE	n	Hematocrit mean	Hematocrit SE
Icteria virens	16	0.59	0.07	1	42.47	1.02

				7		
Vireo olivaceus	7	0.91	0.12	5	49.98	1.46
Helmitheros vermivorus	18	0.89	0.07	14	47.01	0.95
Mniotilta varia	9	0.81	0.05	10	43.64	1.52
Vireo griseus	6	1.16	0.19	9	43.68	2.2
Passerina cyanea	4	0.98	0.15	8	53.08	2.49
Vermivora cyanoptera	5	0.70	0.09	3	45.97	1.59
Thryothorus ludovicianus	4	0.93	0.28	11	47	1.29
Piranga olivacea	0	-	-	1	48.4	-
Seiurus aurocapillus	6	1.20	0.11	2	45.7	3.6
Parula Americana	1	1.16	-	1	47.6	-
Wilsonia citrine	0	-	-	0	-	-
Cardinalis cardinalis	3	0.52	0.05	4	48.2	2.59
Coccyzus americanus	7	0.52	0.12	6	51.95	1.26
Poecile carolinensis	1	0.43	-	8	46.15	0.91
Baeolophus bicolor	4	0.37	0.05	5	48.98	1.15
Oporornis formosus	0	-	-	1	48.1	-
Icterus spurius	2	0.653	0.17	0	-	-
Setophaga discolor	2	1.269	0.66	1	46.8	-
Pirangra rubra	6	0.766	0.15	1	46.5	-
Empidonax virescens	0	-	-	2	50	1.2
Spizella passerina	0	-	-	0	-	-

Supplementary Table 7

Supp. Table 7—Mean mass and standard error for host species summarized separately for infected and uninfected individuals.

Species	n	Mass (g) mean Infected	Mass SE Infected	n	Mass (g) mean Uninfected	Mass SE Uninfected
Icteria virens	25	24.45	0.30	45	24.04	0.19
Vireo olivaceus	35	16.23	0.17	21	15.87	0.30
Helmitheros vermivorus	4	12.18	0.34	38	12.61	0.12
Mniotilta varia	2	10.6	0.2	34	10.14	0.09
Vireo griseus	5	11.54	0.39	30	10.98	0.18
Passerina cyanea	3	14.3	0.61	25	13.35	0.19
Vermivora cyanoptera	0	-	-	18	8.11	0.14
Thryothorus ludovicianus	2	16.6	1.2	14	18.57	0.58
Piranga olivacea	8	26.69	0.49	7	26.44	0.56
Seiurus aurocapillus	1	19.2	-	13	18.09	0.36
Parula Americana	2	7.4	0.1	10	6.89	0.15
Wilsonia citrine	1	11.5	-	10	10.15	0.19
Cardinalis cardinalis	5	41.3	2.20	6	42.32	5.23
Coccyzus americanus	0	-	-	10	61.94	4.08
Poecile carolinensis	0	-	-	10	9.13	0.12
Baeolophus bicolor	2	20	0.3	7	19.81	0.57
Oporornis formosus	3	13.23	0.19	4	13.13	0.41
Icterus spurius	2	18.3	0.3	3	19.7	1.55
Setophaga discolor	0	-	-	5	7	0.14
Pirangra rubra	3	27.67	1.28	2	28.45	1.35
Empidonax virescens	1	11.8	-	3	12.8	0.26

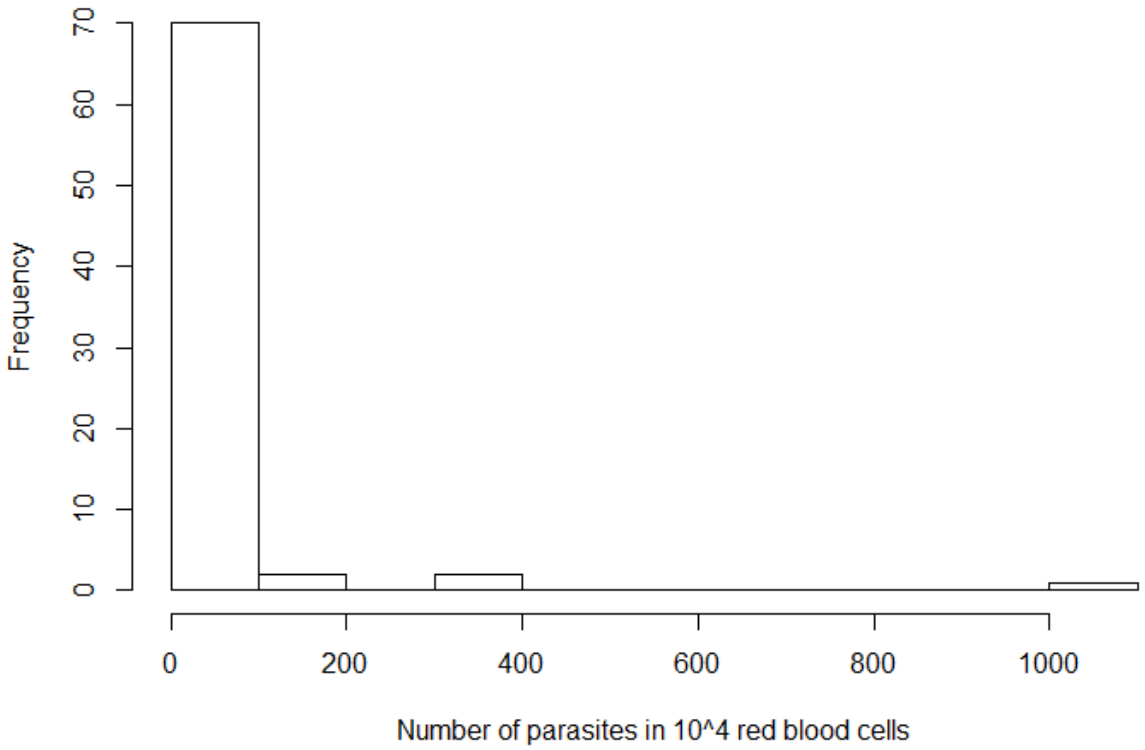
Spizella passerina	4	11.3	0.50	0	-	-

Supplementary Table 8

Supp. Table 8—Genbank accession numbers for all of the haemosporidian sequences identified in this study.

Lineage Name	Genbank Accession Number
OZ 01	GQ395654
OZ 02	AY455658
OZ 03	AF465563
OZ 04	AY540197
OZ 05	AY167241
OZ 06	AF465554
OZ 08	AF540207
OZ 09	AY540208
OZ 10	AF465576
OZ 12	AY817748
OZ 14	AY540210
OZ 25	AY540211
OZ 26	AY540212
OZ 32	AY540214
OZ 36	AY540220
OZ 45	HM222481
OZ 49	KF359931
OZ 51	KF359932
OZ 54	KF359933
OZ 55	KF359934
OZ 59	KF359935
KZ 01	KF359936

Supplementary Figure 1



Supplementary Figure 1—A frequency distribution of parasitemia, measured as the number of parasites in 10^4 red blood cells, for all the birds that both screened positive for infection using PCR and had their blood smears read. The distribution is highly skewed with many infected birds having very low level parasitemias.

References:

Buckland ST, Burnham KP, Augustin NH (1997) Model selection: an integral part of inference. Biometrics 53: 603-618

Chapter 2

Host immune responses to experimental infection of *Plasmodium relictum* (lineage SGS1) in domestic canaries (*Serinus canaria*)

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In revision at Parasitology Research

Abstract
Understanding the complexity of host immune responses to parasite infection requires controlled experiments which can in turn inform observational field studies. Birds and their malaria parasites provide a useful model for understanding host-parasite relationships, but lack a well described experimental context for how hosts respond immunologically to infection. Here we infected 10 canaries (*Serinus canaria*) with the avian malaria parasite *Plasmodium relictum* (lineage SGS1) in a controlled laboratory setting with 10 uninfected (control) birds. We repeatedly measured a suite of immunological blood parameters including the density of four white blood cell types, the concentration of the acute phase protein haptoglobin, and the bacteria-killing ability of blood plasma over a 25 day period covering the acute phase of the infection. Three infected and one control bird died during the course of the experiment. A multivariate statistical analysis of the immune indices revealed significant differences between infected and uninfected individuals between 5 and 14 days post infection (dpi). Group differences corresponded to reduced densities of lymphocytes (5 dpi), heterophils (8 dpi) and monocytes (11 and 14 dpi), and an increase in haptoglobin (14 dpi), in infected birds relative to uninfected controls, and no change in bacteria-killing. Upon re-running the analysis with only the surviving birds, immunological differences between infected and control birds shifted to between 11 and 18 dpi. However, we found no clear correlates between immune parameters and likelihood of surviving the infection. Our results demonstrate the dynamic and complex nature of avian immune function during the acute phase of malaria infection and provide a context for studies investigating immune function in wild birds.

Keywords Avian malaria, bacteria-killing, ecoimmunology, haptoglobin, Haemosporida, *Plasmodium*

Introduction

Parasites adversely affect host individuals and populations and may exert strong selective pressures on the expression and evolution of host life history traits, including immune defenses. Studying the intimate relationships between hosts and parasites and how hosts fight infection and deal with the costs of parasitism is essential to understand variation in immune defenses in the wild. Ecoimmunology (Sheldon and Verhulst 1996; Demas and Nelson 2012) seeks explanations for this immune variation in wild animals, but ethical considerations often restrict researchers from conducting controlled experiments in the field. The inability to experiment in the field represents a barrier to disentangling the causes and the consequences of relationships between immune function and parasite infection. As such, laboratory studies relying on experimental infections represent an opportunity to study host-parasite interaction within a controlled framework and inform field studies.

Avian malaria parasites of the genus *Plasmodium* (order: Haemosporida) are globally distributed, common parasites of birds (Valkiūnas 2005; Beadell et al. 2006). The life-cycles of avian malaria parasites alternate between sexual reproduction in a dipteran host and asexual reproduction in an avian host (Cornet and Sorci *In Press*; Valkiūnas 2005). Upon parasite inoculation, and after the prepatent period which corresponds to the few days required to observe parasites in the blood, birds typically undergo an "acute phase" of infection leading to a surge of parasites in the peripheral blood (peak of parasitemia). Infection is usually rapidly controlled by the host immune system in birds (Atkinson et al. 2001), as well as in rodents (Taylor-Robinson et al. 1993; Oakley et al. 2013) and humans (Good et al. 2005). Following this immune activation, birds enter a "chronic phase" of infection characterized by a longer period of low parasitemia which may last the lifetime of the individual (Bishop et al. 1938; Valkiūnas 2005; Cornet et al. 2014, but see Latta and Ricklefs 2010 and Wood et al. 2013 for evidence that hosts can clear infections).

In the wild, captured birds rarely present acute phase infections (perhaps because of a sampling bias) and most studies comparing immune parameters and malaria infection in wild birds are restricted to the chronic phase of infection. Ricklefs and Sheldon (2007) looked at chronic haemosporidian infections (i.e., avian parasites in the genera *Haemoproteus* as well as *Plasmodium*) in relation to host white blood cell densities in the avian hosts *Turdus migratorius* (North America) and *Turdus grayi* (Panama). They found that *T. migratorius* had elevated lymphocytes and eosinophils in infected relative to uninfected birds in one but not another location (however, the locations were sampled in different seasons which may have also contributed to immune differences). Furthermore, they found that while *T. grayi* generally had lower white blood cell counts than *T. migratorius*, individuals appeared to maintain infections at lower parasitemias than their North American congeners. These results indicate that there may be variation in how avian immune systems have evolved to control malaria infections. In another study of haemosporidian parasites of birds, Ellis et al. (2014) found that heterophils and lymphocytes were elevated in chronically infected birds relative to uninfected birds across several host species within a single locality. The authors also found evidence of parasite-specific immune responses in one well-sampled host (*Vireo olivaceus*), but not

another (*Icteria virens*), again indicating some degree of variation in how hosts respond to chronic infections.

Whether or not a correlation is found between immune effectors and chronic parasitemia does not necessarily mean that the effector is involved in the response against the parasite. Such claims should be experimentally validated. To this end, the use of experimental infections is appropriate to study the relationship between the avian immune system and malaria infection. For example, Cellier-Holzem et al. (2010) infected canary hosts (*Serinus canaria*) with the malaria parasite *Plasmodium relictum* (lineage SGS1) and found an increase in the acute-phase immune system protein haptoglobin (Hp) in infected birds within 12 days of the initial infection. However, in a subset of birds re-infected with the same parasite, Hp again increased even though parasitemias were lower than in the initial infection, possibly suggesting that Hp alone may not control infection. In a similar experimental system, Bichet et al. (2012) demonstrated a role for nitric oxide in the blood in reducing parasitemia during the acute phase of infection. However, higher parasitemias in canaries with experimentally reduced nitric oxide concentrations did not result in higher physiological costs compared to control birds (Bichet et al. 2012), again suggesting the involvement of other parts of the immune system. In another experimental system, Atkinson et al. (2001) documented an increase in plasma antibodies in Hawaii amakihi (*Hemignathus virens*) only a week after infection with the parasite *P. relictum*. Antibodies remained in the host blood more than three years after initial infection and likely provided resistance to reinfection with the same malaria strain (Atkinson et al. 2001). These three experimental studies highlight that bird immune response to *Plasmodium* infection is complex and involves various effectors to control parasite infection.

Elucidating the relationship between avian immune defenses and malaria infection may best be accomplished by measuring a variety of immune parameters simultaneously to capture the complexity of the immune system (Matson et al. 2006a; Buehler et al. 2011). Here, we measured several immune parameters in canaries experimentally infected with the avian malaria parasite *P. relictum* (lineage SGS1). Immune parameters, including the densities of four white blood cell types, Hp, and the bacteria-killing ability (BKA) of the blood plasma, were continued for 25 days, thereby covering the entire acute phase of infection. BKA primarily reflects the activity of complement proteins (Matson et al. 2006b; Merrill, unpublished data). Complement proteins are able to opsonize or lyse invading cells (Esser 1994), as well as direct antigen to lymphoid organs thereby lowering the threshold for B-cell activation (Ochsenbein and Zinkernagel 2000). These proteins can be a critical component of host defenses against pathogens (Matson et al. 2006b). We took a multivariate statistical approach to better understand the immune system response to the acute phase of avian malaria infection, and sampled repeatedly during infection to capture fine-scale immune system dynamics. We were particularly interested in testing whether these commonly measured immune parameters were influenced by malaria infection in a controlled laboratory setting.

Materials and methods

Experimental design

Prior to the experiment, one year old canaries were screened for haemosporidian infections and sexed using the molecular protocols of Waldenström et al. (2004) and Griffiths et al. (1998), respectively. All birds were found to be negative for blood infections. We randomly assigned birds to the infected (n = 10) or control (i.e., uninfected; n = 10) treatment groups while maintaining a similar sex-ratio between groups (infected: 5 males, 5 females; control: 4 males, 6 females). We kept all birds in small indoor cages with *ad libitum* food and water.

We used a strain of *P. relictum* lineage SGS1 maintained in naïve canaries and *Culex pipiens* mosquitoes in captivity (Cornet et al. 2013; Cornet et al. 2014), initially isolated from house sparrows (*Passer domesticus*) in France. To infect birds we used the following protocol: ca. 200 µl of blood was collected from each of four parasite-infected donors and mixed with equal parts phosphate buffered saline solution (PBS) and 100 µl of the infected blood solution was injected intraperitoneally into each naïve canary assigned to the infected group. Similarly, control birds received a 100 µl injection of uninfected blood (sampled from 4 uninfected birds, prepared as previously described).

We monitored infected and control birds during a 25 day period. We took a blood sample (ca. 100 µl) from the brachial vein via a small puncture before the infection (0 days post-infection; hereafter, "dpi"), and then on 5, 8, 11, 14, 18, 21, and 25 dpi. At each sampling time point we weighed each bird and used the blood samples to quantify hematocrit (i.e., the volume of red blood cells per total volume of blood measured after centrifugation for 5 min at 10,000 rpm in a capillary tube) and to make a single blood smear. We centrifuged the remaining blood (4°C, 10 min, 10,000 rpm) to separate plasma from the packed red blood cells. Plasma was separated into different tubes for the Hp and BKA assays and immediately frozen at -80°C until the assays were run.

White blood cells and parasitemia

We stained all blood smears following a standard Geimsa based protocol (Valkiūnas 2005) and all smears were read by a single experienced person (MRK). White blood cell (WBC) densities were determined by first counting the number of WBCs per 10^4 red blood cells (RBC) at 400X magnification, providing a total WBC density estimate. We then determined the proportion of different WBC types by classifying 100 WBCs, read at 1000X magnification, as either heterophils, eosinophils, lymphocytes, or monocytes (Clark et al. 2009). Basophils were excluded from the analysis due to rarity in our samples. We calculated the density of each WBC type for a given sample by multiplying the proportion of cells (i.e., the count of 100 WBCs) by the total WBC density estimate. We estimated parasitemia by counting the number of parasitized RBCs present in approximately 10^4 RBC at 400X magnification.

Bacteria-killing assay (BKA) and haptoglobin (Hp)

The methods for the BKA assay we used were derived from Matson et al. (2006b), Millet et al. (2007) and Morrison et al. (2009). In brief, we added 5 µL of plasma to a combination of CO_2-independent media (Gibco, Invitrogen) plus 4mM L-glutamine (90 µL), and bacterial broth (10 µL), incubated the solution for 30 min at 40^{o}C, then pipetted out 50 µL in duplicate onto agar plates which were then incubated at 37^{o}C overnight. We counted the number of colonies the following day and compared them to control plates in which the bacterial broth and phosphate buffered saline (PBS) were incubated together without any plasma. BKA was determined by subtracting the mean number of colonies counted for a bird's two plates from the mean number of control colonies, and then dividing that by the control mean. We measured BKA at days 0, 11, 14, and 25 post-infection. We were unable to measure BKA on one individual at day 25 post-infection. We measured plasmatic haptoglobin concentrations for all individuals at all sampling time points using a commercially available kit (Tridelta Diagnostics, product code TP-801, Morris Plains, NJ) following the "manual method" provided by the manufacturer.

Statistical analyses

We took a multivariate approach to our immune system data. We fourth-root transformed the white blood cell densities to more closely approximate normal distributions. At each sampling point we performed a linear discriminant analysis (LDA) on the immune parameters (i.e., transformed WBC densities, BKA, and Hp; BKA and Hp were not normally distributed but several transformations did not change the results) and used experimental treatment (i.e., infected vs. control) as the grouping variable. The resulting standardized discriminant function coefficients indicate the relative importance of each immune variable in accounting for variation among experimental treatment at each sampling period (for a detailed description of LDA see Legendre and Legendre 1998). We inspected the standardized discriminant function coefficients and the distribution of the two experimental treatments across the linear discriminant axis and tested for a statistical difference between the experimental treatments in multivariate space using MANOVA with the Pillai-Bartlett trace. All MANOVAs were re-run using a randomization procedure to calculate *P*-values; all randomization-based *P*-values were similar to those reported here and are not reported separately. We also examined the relationship between immune variables (response variable) and natural log (plus one) transformed parasitemia (explanatory variable) across sampling points using linear mixed effects models in the R package lme4 (Bates et al. 2014); we calculated *P*-values for these models using the Kenward-Rogers approximation with the R packages pbkrtest (Halekoh and Højsgaard 2014) and lmerTest (Kuznetsova et al. 2014). We used non-parametric statistics for all other tests. We ran all of our statistical analyses in R v3.0.2 (R Core Team 2013), and all graphics were produced using the R package ggplot2 (Wickham 2009). The original data are available in Appendix A.

Results

Parasitemia dynamics

During the course of the experiment, three birds from the infected group and one control bird died (G = 1.3, df = 1, P = 0.25). Parasitemia in the infected group—excluding those birds that died—followed the typical rise and fall that characterizes the acute phase of infection: parasitemia peaked between 8 and 11 dpi, and subsequently decreased to low levels by 21 dpi (Figure 1). None of the control birds developed infections during the experiment. Two of the birds from the infected group died after 14 dpi with 91.45% and 50.99% of their RBCs parasitized; a third from the infected group that died after 21 dpi, had 69.14% of its RBCs parasitized at 14 dpi (Figure 1). All three of these individuals had significantly more parasitized RBCs (i.e., higher parasitemias) than the rest of the infected group at 14 dpi (Mann-Whitney U test, P = 0.02).

Comparison of infected and control groups

Linear discriminant analyses and MANOVAs revealed differences in immune parameters between experimental groups (i.e., infected and control) from 5 to 14 dpi. These differences generally corresponded to a decrease in lymphocytes (5 dpi), heterophils (8 dpi), and monocytes (11, 14 dpi), and to an increase in Hp (14 dpi) in infected relative to control birds (see Figures 2 and 3 for a graphical summary of the raw data, and Table 1 for the standardized discriminant function coefficients and MANOVA results).

We then re-ran the analyses without the four birds that died during the experiment (i.e., an analysis on "survivors" only). In this second set of analyses we found the differences in immune parameters between treatments to occur later, from 11 to 18 dpi (Table 2). These differences were related to an increase in BKA (11 dpi), a decrease in monocytes and an increase in Hp (14 dpi), and a decrease in heterophils and lymphocytes (18 dpi) in infected relative to control birds (Figures 2 and 3, and Table 2).

Hematocrit in infected birds dropped precipitously relative to controls between 8 and 11 dpi and recovered by about 18 dpi (Figure 2B). Interestingly, hematocrit in the infected group was only significantly related to parasitemia at 14 dpi (Spearman's rho = -0.74, P = 0.01). This negative relationship with hematocrit appears to be driven by two of the birds which later died from their infections (result not shown) although a comparison of hematocrit values of the three birds that died with the rest of the infected group at 14 dpi was not significant (Mann-Whitney U test, P = 0.17). We found no other relationships between hematocrit and parasitemia.

Correlates of immune function and survival

We tested for differences in immune parameters between infected birds that died ("non-survivors") and those that did not ("survivors"). At the start of the experiment (0 dpi), the seven survivors had significantly fewer monocytes in their peripheral blood (Mann-Whitney U test, P = 0.02) and higher hematocrit (P = 0.02) than the three non-survivors; the difference in hematocrit persisted to 5 dpi (P = 0.02), while the difference in monocytes did not (P = 0.38). While the multivariate analysis restricted to survivors (comparing infected and uninfected birds) revealed an increase in BKA at 11 dpi, we did

not find a significant difference between the BKA of survivors and non-survivors at 11 dpi ($P = 0.12$). The multivariate analysis restricted to survivors also revealed a decrease in heterophils and lymphocytes in infected birds relative to controls at 18 dpi, however we did not find significant differences between survivors and non-survivors at 18 dpi for either immune parameter ($P = 0.19$, $P = 0.25$, respectively).

We also tested for relationships between the immune parameters we measured and parasitemia in the infected group using survivors and non-survivors together. We first looked at all sampling periods together using linear mixed effects models with the individual bird identification set as a random effect. As expected we found a negative relationship between hematocrit and parasitemia (estimate of coefficient(β) = -0.02, standard error (se) = 0.003, $P < 0.001$). The results were mixed with regards to the relationship between immune parameters and parasitemia. Of the white blood cells (all fourth-root transformed prior to analysis), heterophils ($\beta = 0.04$, se = 0.02, $P = 0.01$) and eosinophils ($\beta = 0.06$, se = 0.02, $P = 0.02$) were positively related to parasitemia, while monocytes ($\beta = -0.06$, se = 0.02, $P = 0.002$) were negatively related to parasitemia, and lymphocytes ($\beta = -0.02$, se = 0.02, $P = 0.30$) were not related to parasitemia. Both Hp (log-transformed) and BKA were not related to parasitemia across sampling periods ($P = 0.17$ and 0.53, respectively). We also looked for relationships with parasitemia within sampling periods using non-parametric Spearman's correlation tests. We found positive relationships between Hp and parasitemia at 8 dpi (Spearman's rho = 0.66, $P = 0.04$), and between heterophils and parasitemia (rho = 0.66, $P = 0.04$) and eosinophils and parasitemia (rho = 0.71, $P = 0.03$) at 11 dpi. Finally, at 25 dpi we found a positive relationship between BKA and parasitemia (rho = 0.83, $P = 0.04$), however this was driven by the only two birds measured at this time point that had parasites detectable on their blood smears. Each of those two birds had one infected RBC in 10^4 RBCs, and had strong BKA values of 0.87 and 0.86. Of the remaining four birds measured for BKA, three had weak BKA values (less than 0.21) and one had a BKA value of 0.62.

Discussion

Immune responses to the initial, acute phase of *P. relictum* infection in canaries are clearly complex and dynamic over a short period of time. How well these immune responses in canaries mirror immune responses in other bird species is difficult to assess without further experimentation. Experimental infection of *P. relictum* in five passerine bird species revealed differences between hosts in susceptibility to infection and parasitemia during infection (Palinauskas et al. 2008). However, similar immune responses to haemosporidian infection have been documented across many host species in the wild (Ellis et al. 2014). Another potential caveat to our results is that we infected birds with blood, which may have itself caused an inflammatory immune response. While differences between treatments still reflect the effects of *P. relictum* it is difficult to know whether the immune profiles of birds infected via mosquito bite would be similar. We chose infect birds with a blood inoculum rather than with a mosquito bite so that we could standardize the number of parasites each bird was infected with. Nevertheless it is clear that future work should replicate this study using mosquitos to infect birds.

Within our experimental system, immune responses may become apparent as early as 5 dpi. Interestingly, the majority of the changes we document involve decreases in circulating white blood cells in infected relative to control birds. This result seems to contrast with previous studies. For example, in a study of blue jays (*Cyanocitta cristata*) experimentally infected with another haemosporidian parasite, *Haemoproteus danilewskyi*, Garvin et al. (2003) found an increase in circulating host lymphocytes and heterophils beginning one week post infection. There was some variation in the response as heterophils then dropped to control levels by the second week post infection before increasing again in the third week (see Figure 3 in Garvin et al. 2003). However, our results may not be contradictory since *Plasmodium* parasites reproduce asexually in the host blood stream while *Haemoproteus* parasites do not (Valkiūnas 2005), which may lead to different immune responses.

Ultimately, antibody production seems to be necessary for long-term control of malaria infections in birds (Atkinson et al. 2001) and other vertebrates (Good et al. 2005, but see caveats therein regarding the importance of cellular responses as well). Lymphocytes are involved in antibody production (Babcook et al. 1996), and have been demonstrated to produce anti-parasitic gamma interferons (IFN) in response to other parasites (Breed et al. 1997); gamma IFN are known to be involved in *Plasmodium* resistance (Schofield et al. 1987). However, we found no relationship between lymphocytes and parasitemia, and in fact found a decrease in lymphocytes in infected relative to control birds at multiple sampling points during our study. In a study of wild birds, Ellis et al. (2014) found elevated lymphocytes in birds infected with haemosporidian parasites. However, like most studies of wild birds and haemosporidian parasites Ellis et al. (2014) worked with chronically infected birds, whereas our study used birds with acute phase infections. It may be that lymphocytes only begin to play a role as infections move to the chronic phase; this is something that future experiments will need to confirm.

One of our most striking results was of survivors initiating immune responses to infection later than all the birds (i.e., survivors and non-survivors) grouped together. In rodent systems, control of the acute phase of *Plasmodium* infection is thought to be at least partially controlled by $CD4^+$ T cells (Taylor-Robinson et al. 1993), and ironically, the same cells may also contribute to pathology in the host (Oakley et al. 2013). It is interesting to note that survivors in our experiment maintained WBCs below or at the level of control birds throughout the experiment while non-survivors appeared to drive WBC densities up at certain times (Figure 3). The possibility that an early immune response to malaria may prove detrimental to the host cannot be assessed with the data from our experiment but certainly warrants further investigation.

Our experimental design does not allow us to determine whether differences in immune responses between survivors and non-survivors were directly related to parasite control, contributed to host pathology, or were simply consequences of an uncontrolled infection. However, it does appear that survivors were able to control their infections more effectively than non-survivors—two of the three non-survivors appeared unable to reduce their parasitemias at 8 and 11 dpi and parasitemia continued to increase until they

died, while the third non-survivor's parasitemia peaked later, at 18 dpi (Figure 1). Survivors started the experiment with fewer monocytes and higher hematocrit than non-survivors, and the difference in hematocrit continued through 5 dpi when parasites were first found on the blood smears of five of the ten infected birds (Figure 1). While high hematocrit may help buffer against the loss of RBCs during peak parasitemia, we found no statistical differences between the hematocrit of survivors and non-survivors at any sampling point after 5 dpi ($P > 0.17$ for all tests). Possibly, the initial differences in monocytes and hematocrit between survivors and non-survivors were spurious and unrelated to the subsequent infection dynamics, yet it is tempting to speculate. One possibility is that malaria infections are more effective in hosts with certain white blood cell profiles (in this case, elevated monocytes at the start of the experiment); a second is that non-survivors were suffering from an undetected illness (e.g., a virus) at the start of the experiment. Ellis et al. (2014) raised a similar issue when they showed that many uninfected birds had white blood cell profiles (characterized in that study primarily by elevated heterophils and lymphocytes) similar to individuals infected with haemosporidian parasites. This suggested that those uninfected individuals might have been suffering from a different type of infection, or that haemosporidian parasites were better able to establish in birds with that specific white blood cell profile. While it is unlikely that canaries in our experiment were fighting undetected infections, given that husbandry for all birds was identical before the start of the experiment, future experiments should consider co-infecting subjects with malaria and a non-malarial pathogen to determine possible consequences for infection dynamics. Such an approach has been employed frequently in rodent models, with one result being that malaria infections are more severe in the presence of other parasites (e.g., Graham et al. 2005; Knowles 2011).

Relationships between immune parameters and parasitemia might also shed light on how infections are successfully managed. At 8 dpi we found a positive relationship between Hp and parasitemia. Hp is a protein which often increases several-fold in vertebrates in response to infection or injury (Quaye 2008), and has been shown to be elevated across several avian species with chronic phase haemosporidian infections (Ellis et al. 2014) and in canaries with acute phase infection of *P. relictum* (Cellier-Holzem et al. 2010). In humans infected with the parasite *Plasmodium falciparum*, Hp may indeed play a role in controlling parasite infection (reviewed in Quaye 2008). However, in our data we found no evidence for a difference in Hp between survivors and non-survivors, and so we cannot guess at its true role in this system. We also found positive relationships between two WBCs (heterophils and eosinophils) and parasitemia. Heterophils phagocytize invading bacteria and increase in number during the inflammatory blood response in chickens and turkeys; their cellular defensins may also be lethal to a broad range of pathogens (reviewed in Harmon 1998). Eosinophils have also been implicated in parasite resistance (Kiesecker 2002). In birds suffering from chronic phase haemosporidian infections, various white blood cell types have been demonstrated to be elevated in relation to uninfected birds (Ots and Horak 1998; Apanius et al. 2000; Ellis et al. 2014), although this may vary by species and location (Ricklefs and Sheldon 2007). Understanding whether relationships between white blood cells and parasitemia (or parasite prevalence) are reflective of immunological control of infection,

or a by-product of infection, likely will require an experimental approach, such as experimentally elevating white blood cells in birds (Latimer et al. 1988), and then infecting birds with malaria and monitoring parasitemia and survival.

At 25 dpi we found a positive correlation between BKA and parasitemia. This relationship was based on two birds, each with only one infected RBC in 10^4 RBCs. BKA was also elevated in infected relative to control birds at 11 dpi in the multivariate analysis restricted to survivors, suggesting that it may play a role in the successful control of parasitemia. However, similar to the WBC and Hp responses, experiments where BKA levels can be manipulated are needed to fully elucidate its function during infection.

Overall our study provides an experimental context for how immune parameters commonly used in field studies (Boughton et al. 2011) respond to malaria infection in birds. Clearly more experiments are needed to fully elucidate the causes and consequences of these immune responses. Nevertheless our results are readily applicable to studies of ecoimmunology. For example, through fine-scale temporal sampling we show that immune responses may be highly dynamic over short time scales. Recent work has shown that immune profiles can vary across seasons and years (Buehler et al. 2008; Hegemann et al. 2012), but our study indicates that when birds are infected with malaria, immune profiles may even shift over a matter of days. We also confirm that multiple immune parameters should be measured to capture immunological variation in response to infection (Buehler et al. 2011). Future experimental studies should focus on manipulating the immune responses we have identified to elucidate their roles in controlling infection, and should attempt to integrate more avian species into this experimental framework.

Acknowledgments This work was supported in part by the NSF Malaria Research Coordination Network. The experiment was performed in the MIVEGEC lab in Montpellier, France. SC acknowledges support from a postdoctoral position funded by the ERC Starting Grant EVOLEPID 243054 to S. Gandon (CNRS, Montpellier). Thanks to J. Grindstaff (Oklahoma State University) for use of lab space for the BKA assay and to D. Fontenille (IRD, Montpellier) for supporting this project.

Ethical standards The experiment was approved by the Ethical Committee for Animal Experimentation (CNRS) and the French Ministry of Education and Research (permit number CEEA-LR-1051).

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

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Tables and Figures

Table 1— Standardized discriminant function coefficients resulting from the LDA performed on all birds (survivors and non-survivors) at each sampling period, with experimental treatment as the grouping variable. Note that the coefficients indicate relative importance of each measure in separating the treatments (see Methods). Also the Pillai-Bartlett trace from the MANOVA and associated *P*-value at each sampling period. A significant *P*-value indicates that the null hypothesis of no difference between treatments based on the immune variables can be rejected.

Standardized Discriminant Function Coefficients and MANOVA results (all birds)

Immune Variables	0dpi	5dpi	8dpi	11dpi	14dpi	18dpi	21dpi	25dpi
Heterophils	1.70	0.35	-5.43	1.39	-1.22	-4.23	-0.78	-6.19
Eosinophils	-1.16	1.20	1.68	1.32	-0.35	-0.58	0.25	1.33
Lymphocytes	-0.13	-3.60	-1.24	-2.09	0.89	-0.14	-1.07	11.35
Monocytes	1.40	1.17	-0.95	-1.91	-3.64	-1.12	0.86	-13.76
Hp	3.03	-3.29	-1.58	-0.21	8.75	-2.05	7.74	8.29
BKA	-0.12	NA	NA	1.93	-0.16	NA	NA	4.28
Pillai-Bartlett Trace	0.25	0.55	0.49	0.60	0.62	0.40	0.33	0.62
MANOVA *P*-value	0.63	0.03	0.08	0.03	0.03	0.23	0.38	0.16

Table 2— Standardized discriminant function coefficients resulting from the LDA restricted to survivors with experimental treatment as the grouping variable; for 25 dpi see Table 2 (only survivors are represented at 25 dpi). Also the Pillai-Bartlett trace from the MANOVA and associated *P*-value at each sampling period.

Standardized Discriminant Function Coefficients and MANOVA results (surviving birds only)

Immune Variables	0dpi	5dpi	8dpi	11dpi	14dpi	18dpi	21dpi
Heterophils	-1.83	1.83	-5.11	0.39	-0.78	-3.34	0.02
Eosinophils	2.87	0.70	1.78	-0.18	-0.67	-1.31	0.13
Lymphocytes	-1.90	-4.14	-1.13	-2.93	1.54	-3.90	-1.13
Monocytes	1.32	1.02	-0.78	-1.94	-7.26	-0.29	1.21
Hp	3.39	-1.65	-0.88	-1.06	7.10	-0.22	9.69
BKA	0.08	NA	NA	2.44	-0.80	NA	NA
Pillai-Bartlett Trace	0.18	0.51	0.39	0.73	0.61	0.72	0.48
MANOVA *P*-value	0.91	0.16	0.34	0.03	0.12	0.01	0.20

Figure 1

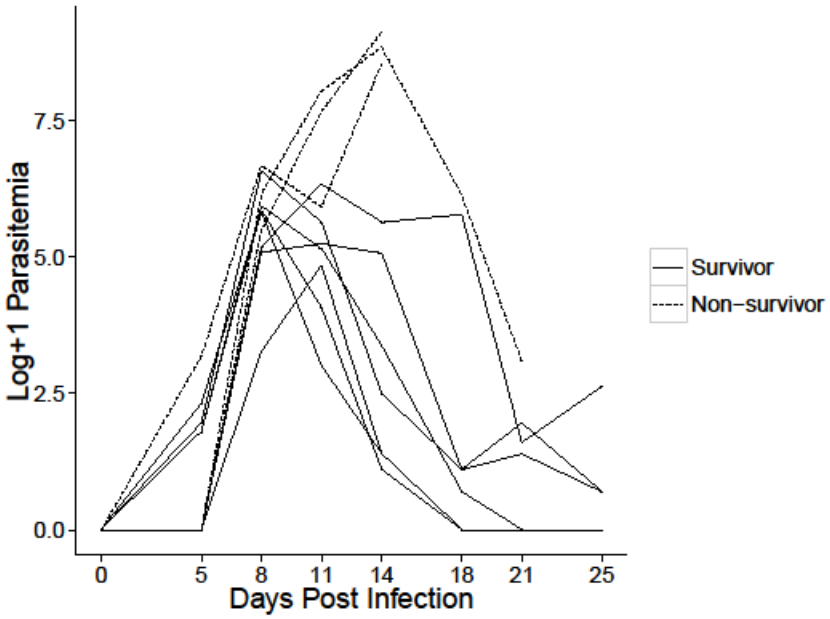

Figure 1—The natural logarithm of parasitemia plus one in the infected group measured as the number of infected red blood cells (RBCs) per 10^4 RBCs. Each line represents an individual in the infected group, solid lines represent those individuals which survived the experiment (survivors), and dashed lines are individuals which died at some point during the experiment (non-survivors).

Figure 2

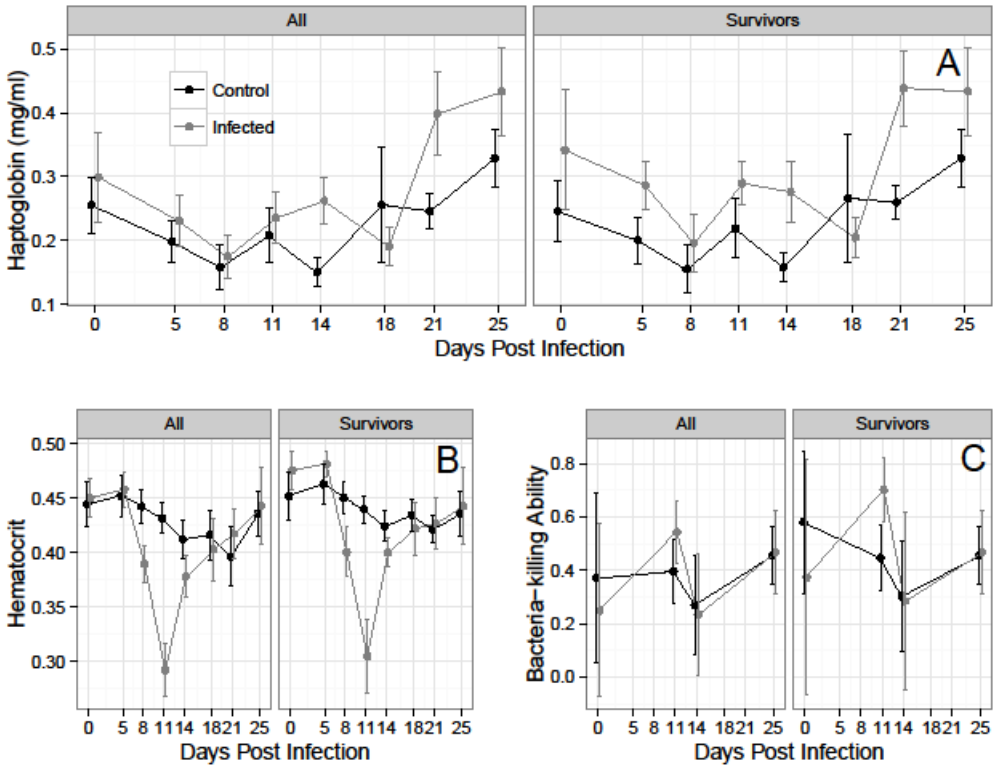

Figure 2—The means and standard errors of haptoglobin (**A**), hematocrit (i.e., the proportion of red blood cells in the blood; **B**), and bacteria-killing ability (i.e., the proportion of bacteria killed by blood plasma *in vitro*; **C**). All three graphics are separated into categories "All" and "Survivors", the first represents the data with all birds grouped together, the second only uses data from those birds that survived until the end of the experiment. Treatments are separated by color with control birds represented by black lines and infected birds by gray lines.

Figure 3

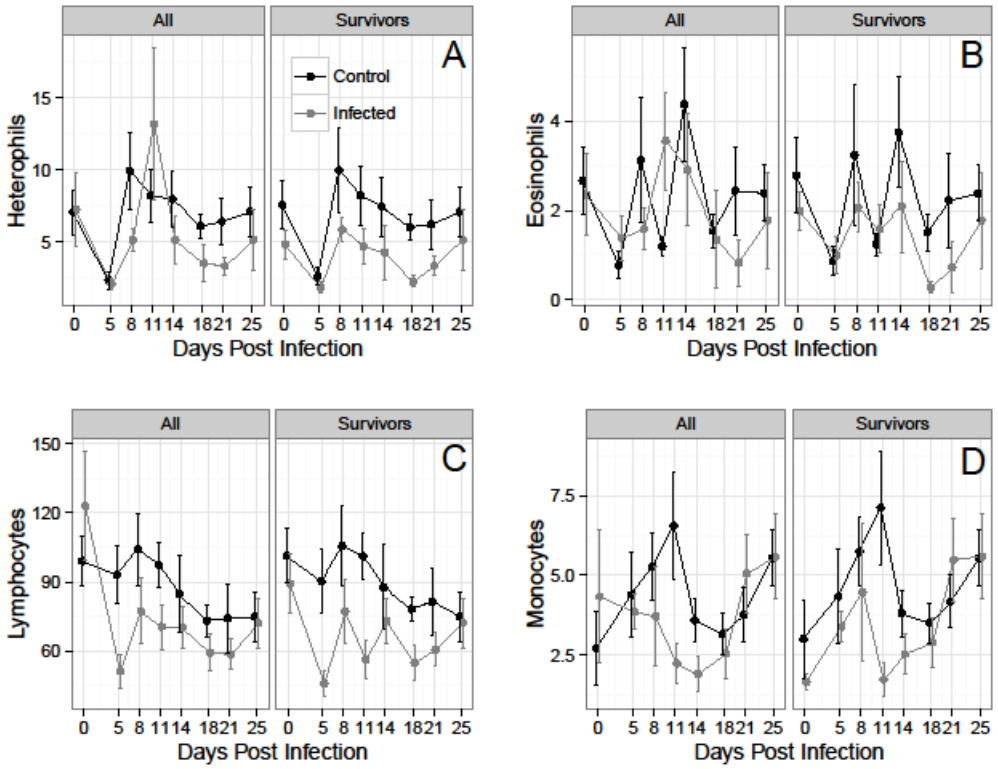

Figure 3—The means and standard errors of untransformed numbers of heterophils (**A**), eosinophils (**B**), lymphocytes (**C**), and monocytes (**D**). The units for all four of these white blood cell types is number of cells per 10^4 red blood cells. As in Figure 2, graphics are separated into categories of all birds together ("All") and only birds that survived until the end of the experiment ("Survivors"). Treatments are separated by color with control birds represented by black lines and infected birds by gray lines.

Appendix A

Bird.ID	Sex	Treatment	Days_Post_Infection	Mass	Hematocrit	Heterophil	Eosinophil	Lymphocyte	Monocyte	Parasitemia	Hp	BKA
Rouge11	M	Control	0	21.8	0.413793	16.8	8.4	142.8	0	0	0.239244	0.8121
Noir11	F	Control	0	24	0.428571	6.3	3.15	88.2	7.35	0	0.483703	-1.54235
Rouge18	M	Infected	0	20	0.428571	6.27	2.85	46.74	0.57	0	0.314211	0.836299
Noir15	M	Control	0	24.3	0.4	8.47	2.42	110.11	0	0	0.363103	0.840569
Noir12	M	Infected	0	18.5	0.421053	3.45	1.15	108.1	2.3	0	0.85854	0.785053
Rouge13	F	Control	0	24.8	0.375	2.49	1.66	78.85	0	0	0.343546	-1.51957
Argent0105	M	Infected	0	23.9	0.363636	5.2	0	249.6	5.2	0	0.128422	0.419217
Noir16	F	Control	0	22.9	0.333333	10.2	2.72	55.08	0	0	0.310952	0.765125
Rouge12	F	Infected	0	23.5	0.409091	29.16	9.72	262.44	22.68	0	0.118644	0.264057
Noir17	F	Infected	0	22.2	0.4	4	0	92	4	0	0.353325	-0.80356
Rouge7	F	Control	0	24	0.55	0	0	162.68	3.32	0	0.337027	0.874733
Noir3	F	Control	0	23	0.5	5.52	4.6	80.96	0.92	0	0.193611	0.767972
Rouge4	F	Infected	0	23.2	0.5	4.12	1.03	95.79	2.06	0	0.350065	0.423488
Noir8	M	Control	0	23.7	0.481481	5.52	1.38	60.72	1.38	0	0.183833	0.916014
Rouge10	F	Infected	0	24.5	0.478261	2.24	2.24	105.28	2.24	0	0.095828	-2.24555
Noir7	F	Control	0	21.4	0.5	3.3	1.1	102.3	3.3	0	0.059974	0.883274
Noir2	M	Infected	0	23.3	0.5	4.44	3.33	101.01	1.11	0	0.353325	0.994306
Jaune14	F	Infected	0	23.1	0.55	3.15	0.45	39.6	1.8	0	0.281617	0.874733
Noir10	M	Infected	0	26.4	0.451613	10.01	2.86	128.7	1.43	0	0.14146	0.945907
Rouge14	M	Control	0	25.2	0.461538	11.97	1.33	109.06	10.64	0	0.043677	0.904626
Rouge11	M	Control	5	21.9	0.444444	6.8	1.36	127.84	0	0	0.161017	
Noir11	F	Control	5	24	0.441176	0.72	0.72	67.68	2.88	0	0.095828	
Rouge18	M	Infected	5	20.3	0.5	2.22	0.74	31.08	2.96	0	0.411995	
Noir15	M	Control	5	23.3	0.391304	2.73	2.73	85.54	0	0	0.193611	
Noir12	M	Infected	5	19.4	0.473684	0.51	0.51	45.9	4.08	6	0.249022	
Rouge13	F	Control	5	24.6	0.357143	0	0	117.12	4.88	0	0.183833	
Argent0105	M	Infected	5	23.8	0.35	2.94	4.9	86.24	3.92	0	0.102347	
Noir16	F	Control	5	22.8	0.393939	2.84	1.42	124.96	12.78	0	0.115385	
Rouge12	F	Infected	5	22.5	0.416667	3.96	1.98	85.14	7.92	0	0.017601	

Noir17	F	Infected	5	22.7	0.444444	0.92	0	19.09	2.99	23	0.183833	
Rouge7	F	Control	5	22.9	0.555556	3.6	0	167.4	9	0	0.154498	
Noir3	F	Control	5	22.4	0.45	2.96	1.48	64.38	5.18	0	0.232725	
Rouge4	F	Infected	5	23.5	0.533333	0.36	0.36	32.4	2.88	0	0.3794	
Noir8	M	Control	5	23.5	0.5	0.76	0	36.48	0.76	0	0.454368	
Rouge10	F	Infected	5	25.2	0.461538	1.96	1.96	43.12	1.96	5	0.305054	
Noir7	F	Control	5	21.4	0.515152	1.42	0	67.45	2.13	0	0.133574	
Noir2	M	Infected	5	24	0.5	3.2	0.4	34.4	2	0	0.223827	
Jaune14	F	Infected	5	24.7	0.454545	1.54	0	70.84	4.62	9	0.12154	
Noir10	M	Infected	5	27.8	0.448276	2.96	2.96	62.9	5.18	5	0.314079	
Rouge14	M	Control	5	25.2	0.47619	1.58	0	71.1	6.32	0	0.256919	
Rouge11	M	Control	8	22.6	0.409091	5.65	0	101.7	5.65	0	0.320096	
Noir11	F	Control	8	23.8	0.391304	4.05	0	66.42	10.53	0	0.145608	
Rouge18	M	Infected	8	19.6	0.5	4.32	1.08	19.98	1.62	176	0.106498	
Noir15	M	Control	8	23.4	0.47619	22.7	9.08	195.22	0	0	0.193742	
Noir12	M	Infected	8	19.5	0.407407	6.3	1.89	52.29	2.52	708	0.193742	
Rouge13	F	Control	8	23.9	0.37037	9.36	2.08	90.48	1.04	0	0.187726	
Argent0105	M	Infected	8	22.5	0.35	2.76	0.69	62.1	3.45	243	0.025271	
Noir16	F	Control	8	21	0.4	27.3	13	87.1	2.6	0	0.028279	
Rouge12	F	Infected	8	22.7	0.363636	1.57	0	155.43	0	471	0.1787	
Noir17	F	Infected	8	23	0.375	6	0.75	16	2.25	800	0.169675	
Rouge7	F	Control	8	23.4	0.526316	1.93	0	185.28	5.79	0	0.335138	
Noir3	F	Control	8	22.2	0.448276	5.7	1.9	81.7	5.7	0	0.124549	
Rouge4	F	Infected	8	22.8	0.421053	10.08	2.24	95.2	4.48	25	0.06438	
Noir8	M	Control	8	23.2	0.47619	7.5	1.5	56.25	9.75	0	0.091456	
Rouge10	F	Infected	8	25.3	0.318182	4.26	4.26	132.06	0	373	0.413357	
Noir7	F	Control	8	21.6	0.444444	9.62	3.7	55.5	5.18	0	0.145608	
Noir2	M	Infected	8	24.4	0.416667	6.5	1.3	105.3	16.9	161	0.157641	
Jaune14	F	Infected	8	25	0.409091	3.15	0	58.59	1.26	332	0.148616	
Noir10	M	Infected	8	28.2	0.333333	6.37	3.64	76.44	4.55	344	0.287004	
Rouge14	M	Control	8	25.1	0.481481	5.28	0	120.12	6.6	0	0.00722	
Rouge11	M	Control	11	22.8	0.441176	2.5	1.25	116.25	5	0	0.148616	0.229147
Noir11	F	Control	11	24.3	0.384615	8.01	1.78	75.65	3.56	0	0.070397	-0.03164
Rouge18	M	Infected	11	18.1	0.421053	2.7	0.6	23.4	3.3	559	0.326113	0.6721

Noir15	M	Control	11	23.7	0.40625	8.72	1.09	97.01	2.18	0	0.21781	0.24640
Noir12	M	Infected	11	18.2	0.2	9.38	4.69	50.92	1.34	277	0.45246	0.69127
Rouge13	F	Control	11	21.7	0.36111	8.25	0.75	63.75	1.5	0	0.10349	-0.06424
Argent0105	M	Infected	11	22.4	0.27227	16.24	8.12	84.68	6.96	2128	0.01925	0.36375
Noir16	F	Control	11	23	0.42105	12.4	1.24	102.92	6.2	0	0.02879	0.01438
Rouge12	F	Infected	11	20.6	0.27778	54.88	9.8	129.36	1.96	3108	0.05535	0.08341
Noir17	F	Infected	11	22	0.23594	27.72	6.6	96.36	1.32	369	0.24789	0.08149
Rouge7	F	Control	11	23.4	0.46875	3.36	0	156.24	8.4	0	0.33814	0.61647
Noir3	F	Control	11	21.9	0.41176	1.22	0.61	56.73	2.44	0	0.46750	0.38063
Rouge4	F	Infected	11	24.3	0.33333	4.4	1.76	78.32	3.52	125	0.31708	0.87152
Noir8	M	Control	11	23.9	0.48	6.23	0.89	75.65	6.23	0	0.31407	0.95379
Rouge10	F	Infected	11	24.5	0.16667	2.12	1.06	49.82	0	170	0.29602	0.02396
Noir7	F	Control	11	21.2	0.44444	21.6	2.7	99.9	10.8	0	0.20641	0.83700
Noir2	M	Infected	11	23.2	0.36111	4.2	1.68	78.12	0	188	0.18753	0.95206
Jaune14	F	Infected	11	23.1	0.30769	1.26	0.42	39.06	1.26	19	0.20012	0.83317
Noir10	M	Infected	11	27.1	0.34375	8.7	0.87	74.82	2.61	57	0.25047	0.86193
Rouge14	M	Control	11	24.8	0.5	9.66	1.61	130.41	19.32	0	0.18124	0.77564
Rouge11	M	Control	14	21.5	0.41667	19.44	8.1	131.22	3.24	0	0.15607	0.68204
Noir11	F	Control	14	23.3	0.34483	0.61	1.22	53.68	5.49	0	0.14348	-0.77431
Rouge18	M	Infected	14	17	0.35294	13.44	5.76	74.88	1.92	279	0.20012	0.47906
Noir15	M	Control	14	23.5	0.40909	12.15	10.8	109.35	2.7	0	0.29137	0.24627
Noir12	M	Infected	14	19.2	0.42105	1.56	0	35.1	2.34	11	0.45815	0.53726
Rouge13	F	Control	14	21.1	0.30435	12.45	9.96	58.93	1.66	0	0.08055	-0.00923
Argent0105	M	Infected	14	20.3	0.25	5.25	1.05	96.6	1.05	9145	0.25991	-0.00923
Noir16	F	Control	14	21.8	0.40909	4.2	1.05	27.65	1.75	0	0.03964	-0.05891
Rouge12	F	Infected	14	20.3	0.31818	14.28	12.24	74.46	0	6914	0.12146	0.28318
Noir17	F	Infected	14	17.4	0.41176	1.84	1.15	19.78	0.23	5099	0.31025	0.07452
Rouge7	F	Control	14	23.3	0.43478	2.16	0	205.2	8.64	0	0.20272	0.63177
Noir3	F	Control	14	22.9	0.4	9.46	2.58	70.52	3.44	0	0.16866	-0.47622
Rouge4	F	Infected	14	24.3	0.41667	8.8	3.3	93.5	3.3	3	0.30396	-0.01916
Noir8	M	Control	14	23.3	0.44828	4	2	32.4	1.6	0	0.17453	0.92335

Rouge10	F	Infected	14	25	0.3888	0.65	0	58.5	5.85	28	0.366	-1.552
Noir7	F	Control	14	21.2	0.48	11.66	6.36	84.8	3.18	0	0.156	0.649
Noir2	M	Infected	14	23.4	0.3888	0.98	0	96.04	0.98	158	0.303	0.967
Jaune14	F	Infected	14	24	0.375	3.24	0	48.6	2.16	3	0.225	0.638
Noir10	M	Infected	14	26.3	0.4583	1.11	5.55	103.23	1.11	2	0.074	0.941
Rouge14	M	Control	14	25	0.4761	3.36	1.68	74.76	4.2	0	0.086	0.903
Rouge11	M	Control	18	21.5	0.4	4.85	0.97	88.27	2.91	0	0.067	
Noir11	F	Control	18	23.2	0.3888	4.74	3.95	67.15	3.16	0	0.134	
Rouge18	M	Infected	18	16.8	0.3	3.6	0.45	37.8	3.15	322	0.203	
Noir15	M	Control	18	23.2	0.3888	5.76	2.88	84.48	2.88	0	0.134	
Noir12	M	Infected	18	18.7	0.3846	3.2	0	59.52	1.28	2	0.222	
Rouge13	F	Control	18	19.5	0.25	6.48	1.8	27.72	0	0	0.162	
Noir16	F	Control	18	21.2	0.44	10.32	0.86	71.38	3.44	0	0.105	
Rouge12	F	Infected	18	19.3	0.2666	12.32	8.96	90.72	0	464	0.096	
Rouge7	F	Control	18	23.2	0.4705	2.08	0	94.64	7.28	0	0.341	
Noir3	F	Control	18	22.5	0.4444	7.4	2.22	64.38	0	0	0.181	
Rouge4	F	Infected	18	23.5	0.4444	2.22	0.74	68.82	2.22	0	0.250	
Noir8	M	Control	18	23.6	0.4166	9.68	0.88	73.04	4.4	0	0.322	
Rouge10	F	Infected	18	24.8	0.5	0.49	0	45.08	3.43	1	0.219	
Noir7	F	Control	18	20.8	0.5238	6.12	0.68	57.12	4.08	0	0.086	
Noir2	M	Infected	18	23.4	0.4444	1.98	0	90.09	6.93	2	0.140	
Jaune14	F	Infected	18	23.9	0.4545	1.98	0.66	27.72	2.64	0	0.071	
Noir10	M	Infected	18	25.1	0.4285	2.32	0	55.1	0.58	0	0.325	
Rouge14	M	Control	18	24.5	0.44	3.33	1.11	103.23	3.33	0	1.021	
Rouge11	M	Control	21	21.7	0.3684	5.64	2.82	126.9	5.64	0	0.293	
Noir11	F	Control	21	22.9	0.4285	4.83	2.07	60.03	2.07	0	0.152	
Rouge18	M	Infected	21	17.3	0.3571	5.88	4.2	69.72	4.2	4	0.385	
Noir15	M	Control	21	23	0.4117	19.14	10.44	135.72	8.7	0	0.244	
Noir12	M	Infected	21	18.1	0.4	1.53	0	41.82	7.65	6	0.405	
Rouge13	F	Control	21	17.2	0.1739	8.36	4.4	9.24	0	0	0.124	
Noir16	F	Control	21	20.5	0.4117	3.33	1.11	32.19	0.37	0	0.168	
Rouge12	F	Infected	21	19.1	0.3529	3	1.5	43.5	2	21	0.116	
Rouge7	F	Control	21	23.4	0.4	4.16	0	94.64	5.2	0	0.341	
Noir3	F	Control	21	22.4	0.3913	0.24	0	20.88	2.88	0	0.353	

Rouge4	F	Infected	21	22.8	0.368421	5.4	0	84.6	0	0	0.645316	
Noir8	M	Control	21	23.2	0.444444	6.49	1.18	48.97	2.36	0	0.357086	
Rouge10	F	Infected	21	24.4	0.5	1.44	0	42.72	3.84	0	0.533227	
Noir7	F	Control	21	20.2	0.5	4.45	0.89	79.21	4.45	0	0.200961	
Noir2	M	Infected	21	23	0.5	4.3	0	75.68	6.02	3	0.589271	
Jaune14	F	Infected	21	23.6	0.470588	2.25	0	37.35	5.4	0	0.293034	
Noir10	M	Infected	21	24.6	0.391304	2.61	0.87	72.21	11.31	0	0.220977	
Rouge14	M	Control	21	23.7	0.434783	7.45	1.49	134.1	5.96	0	0.224098	
Rouge11	M	Control	25	22.1	0.416667	2.52	0.63	55.44	4.41	0	0.156926	0.533662
Noir11	F	Control	25	23.3	0.4	3.48	2.61	73.95	6.96	0	0.525022	-0.02627
Rouge18	M	Infected	25	16.8	0.363636	4.5	1.35	38.25	0.9	13	0.357086	
Noir15	M	Control	25	23.3	0.388889	14.84	5.3	81.62	4.24	0	0.361089	0.395731
Noir12	M	Infected	25	17.6	0.388889	17.29	7.98	101.08	6.65	1	0.361089	0.871921
Noir16	F	Control	25	20.1	0.4	12.87	3.96	78.21	3.96	0	0.172938	0.244663
Rouge7	F	Control	25	23.2	0.5	2.66	0	121.03	9.31	0	0.349079	0.295567
Noir3	F	Control	25	21.9	0.428571	3.44	1.29	36.55	1.72	0	0.22498	0.17734
Rouge4	F	Infected	25	21.4	0.315789	3.36	1.92	41.76	0.96	0	0.369095	0.065681
Noir8	M	Control	25	23.2	0.4	3.78	1.62	42.66	5.94	0	0.461169	0.906404
Rouge10	F	Infected	25	24.1	0.526316	0	0	52.92	10.08	0	0.621297	0.09688
Noir7	F	Control	25	20.2	0.578947	5.76	1.28	53.12	3.84	0	0.461169	0.896552
Noir2	M	Infected	25	22.9	0.555556	4.2	0	71.4	8.4	1	0.73739	0.860427
Jaune14	F	Infected	25	23.4	0.521739	2	0	93	5	0	0.385108	0.210181
Noir10	M	Infected	25	24.5	0.428571	4.8	1.2	106.8	7.2	0	0.204964	0.692939
Rouge14	M	Control	25	23.7	0.409091	14.31	4.77	130.38	9.54	0	0.253002	0.665025

Chapter 3

Regional community structure of avian haemosporidian parasites in eastern North America

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Abstract

We surveyed the avifauna of eastern North America for haemosporidian blood parasites (*Plasmodium* and *Haemoproteus*) to characterize a regional parasite community and understand the forces that determine parasite distributions. Distributions of parasite populations generally reflected those of their hosts across the region. However, controlling statistically for the interdependence between hosts and parasites revealed that while local host assemblages were related to regional climatic gradients, parasite assemblages were not. Furthermore, parasite assemblage similarity did not decrease with distance when controlling for host assemblages and climate, suggesting that parasites disperse readily within the distributions of their hosts. Host specialization varied in some parasite lineages over short time periods and small geographic distances independently of the diversity of available hosts and the diversity of parasite lineages. Nonrandom spatial turnover was apparent in parasite lineages infecting one well-sampled host within a single year suggesting competition between parasite lineages within hosts. Overall, populations of avian hosts generally determine haemosporidian parasite geographic distributions. However, parasites are not dispersal-limited within their host distributions, and they may switch hosts relatively frequently.

Keywords: Avian malaria | community assembly | emerging infectious disease | Haemosporida | macroecology | parasite communities

Introduction

A regional community can be thought of as a set of species whose distributions partially overlap within a large geographic area (Gleason 1926; Ricklefs 2008). The structure of

the regional community (i.e., the relative abundances of species across space and the degree to which populations overlap) is governed by local (e.g., interspecific competition) and regional (e.g., species diversification) processes (Ricklefs 1987). Although regional communities include all species, parasites and pathogens are rarely considered integral community members (Poulin 1999). Indeed, the impact of parasites on community structure is frequently associated with epidemics—often following introductions to non-native regions—that have driven naïve hosts to extinction or near extinction (Day & Monk 1974; van Riper III *et al.* 1986; Sinclair *et al.* 2007). However, parasites likely play a critical role in driving regional community structure. They can represent a large proportion of the community biomass (Kuris *et al.* 2008) and can be involved in the majority of links in a community food web (Lafferty, Dobson & Kuris 2006), and they may influence regional diversity by variously driving (Page 2003) or slowing (Ricklefs 2010) host diversification.

Parasite populations are difficult to integrate into community studies, partly because they are distributed across multiple dimensions—space, host species, and host individuals (Combes 2001)—and also because parasites are difficult to sample. Parasites tend to specialize on one or a few host species, but host-breadth may vary across a parasite's range (Poulin, Krasnov & Mouillot 2011). Compounding this complexity, most community studies of hosts and parasites are local and ignore regional patterns and processes.

Regional studies of birds and their dipteran-vectored haemosporidian blood parasites (Apanius *et al.* 2000; Fallon, Bermingham & Ricklefs 2005; Hellgren, Perez-Tris & Bensch 2009; Ishtiaq *et al.* 2010; Levin *et al.* 2011, 2013; Ricklefs *et al.* 2011; Loiseau *et al.* 2012) have shown that many haemosporidian parasites are heterogeneously distributed across space despite the availability of suitable hosts. Host-parasite associations evidently are not shaped by vector-host encounter dynamics (Medeiros, Hamer & Ricklefs 2013), and local coevolutionary relationships between parasites and their hosts likely influence geographic distributions of both host and parasite populations (Apanius *et al.* 2000; Fallon *et al.* 2005; Ricklefs 2010). However, regional studies of these parasites have been mostly restricted to individual host species (Ishtiaq *et al.* 2006; Fallon, Fleischer & Graves 2006; Durrant *et al.* 2008; Sehgal *et al.* 2011; Marzal *et al.* 2011; Swanson, Lyons & Bouzat 2014; Scordato & Kardish 2014).

We surveyed local assemblages of avian haemosporidian parasites across eastern North America and related the distributions of individual parasite lineages to regional climate variation and to the distribution and abundance of their hosts. Axes derived from ordinations of hosts and parasites by their distributions across sample locations were correlated, suggesting interdependence of host and parasite population distributions. However, when controlling statistically for that interdependence, local host assemblages responded strongly to environmental gradients and became more distinct with increasing geographic separation, but parasite assemblages did not. This finding suggests that haemosporidian parasites disperse readily across the distributions of their host populations in eastern North America, independent of regional climate and geographic distance. The degree to which some parasite lineages specialized on particular hosts varied across years and locations. Despite evidence of pathogenicity of haemosporidian parasites in birds (Asghar *et al.* 2015), correlation coefficients between host abundances and parasite relative abundances across the region were statistically indistinguishable

from random. Taken together, these results suggest that the distributions of parasite populations largely follow the distributions of their hosts, but that parasites readily switch hosts across their ranges.

Results

Parasite populations track populations of their hosts. We screened 5867 individuals of 99 bird species from 13 locations in eastern North America (Fig. 1), mostly from the order Passeriformes, and found 1720 (29.3%) infected with haemosporidian parasites of the genera *Plasmodium* or *Haemoproteus*. Overall we recovered 87 distinct parasite lineages (see Table S1 for parasite lineage details; see Materials and Methods for lineage determination). We used ordinations to place sampling locations on axes ecologically meaningful to birds and to parasites (*sensu* (James 1971; Ricklefs 2011). We used Non-metric Multidimensional Scaling (NMDS) with three axes to ordinate "community" sampling locations (i.e., sites where sampling was not restricted to focal species; Fig. 1, Table S2) separately by bird species abundances and by parasite relative abundances. We restricted this analysis to 33 parasite lineages sampled 10 or more times across the community samples and to 64 host species infected at least once by any of the 87 parasite lineages (indicating suitability) within the region (Table S3). We compared location scores for birds and parasites on the three NMDS axes with canonical correlation. Pillai's trace (the sum of the squared canonical correlations) differed significantly from a random expectation ($P = 0.012$) indicating interdependence between host and parasite populations across the region.

Differences between regional populations of hosts and parasites might reflect dispersal limitation (i.e., geographic distance), environment (e.g., climate or habitat variables), and interactions among hosts and parasites (Fig. 2). To evaluate these relationships, we calculated partial Mantel coefficients for the hypothesized connections in Fig. 2 (Table 1). Partial Mantel coefficients represent the strength of correlation between two distance matrices while controlling for the effect of a third (Legendre & Legendre 1998). For example, the effect of the environment on hosts may not be independent of space (distance between localities). Therefore, the correlation between hosts and environment controlling for the effect of geographic distance can be computed as a partial Mantel coefficient. Because space and climate are independent of birds and parasites, we tested their relationship with a standard Mantel test, which involves no control for a third variable. Here we used distance matrices representing geographic distances between sampling locations (space), and Euclidean distances between sampling locations based on climate (environment). The latter matrix consisted of Euclidean distances based on the first five principal components (PCA) scores for 19 climatic variables (BioClim variables, http://www.worldclim.org/) downloaded for each location, weighted by the proportion of variation explained by each PCA axis (Fig. S1, Table S4). We also determined dissimilarities between sites based on their parasite lineages or host assemblages. These were calculated as Bray-Curtis dissimilarities (Odum 1950; Bray & Curtis 1957; Legendre & Legendre 1998) which are appropriate for comparing species abundance data (see Materials and Methods). Partial Mantel tests between these distance matrices revealed that while host populations are related to variation in climate across eastern North America, parasite populations, when controlling for the effect of hosts, are not (Table 1). Furthermore, parasite community similarity does not decline with distance

(i.e., parasite distributions were not spatially restricted ((Nekola & White 1999) when controlling for hosts, suggesting that parasites disperse readily across the region within their host populations.

Host specialization. The host-breadth of a parasite may vary geographically or temporally, and it may be limited by the phylogenetic relatedness of potential host species (Poulin *et al.* 2011). Indeed, in our Chicago sample, each *Plasmodium* parasite lineage is associated with a single host taxon at the superfamily level (Medeiros *et al.* 2013). To elucidate the importance of host phylogeny on parasite distributions across the region, we created a phylogenetic distance matrix for all hosts infected at least once by any of the 33 parasite lineages sampled 10 or more times (60 host species). We then calculated a second matrix by computing Bray-Curtis dissimilarities between those hosts based on the number of times each host species was infected with each of the 33 parasite lineages. A Mantel test comparing these two matrices showed a weak, but significant correlation ($r = 0.23$, $P < 0.001$) indicating that parasite host distribution is constrained to more closely related hosts than expected by chance.

To quantify the host-breadth of each parasite, we used the Gini-Simpson index (Jost 2006), which accounts for the number of infections on each host species (Poulin *et al.* 2011). We weighted the index by the phylogenetic distance between hosts using the formula for Rao's Quadratic Entropy (Rao's QE, (Rao 1982; Medeiros, Ellis & Ricklefs 2014); see Materials and Methods for formula). Although ecologists often distinguish generalist and specialist parasites, host-breadth in the 33 parasite lineages sampled 10 or more times was continuously distributed (Fig. S2) and did not differ statistically from a unimodal distribution (Hartigans' dip test, $D_{33} = 0.047$, $P = 0.87$, (Hartigan & Hartigan 1985).

When all years were pooled, parasite lineages recovered at least four times from each of at least four community sampling locations exhibited variation in local host-breadth across the region (Fig. 3). A linear mixed effects model with parasite lineage as a random effect showed no effect of local phylogenetically-weighted bird diversity (Rao's QE using host species infected at least once in the region) on parasite host-breadth ($F_{1,21.4} = 1.26$, $P = 0.27$), suggesting that variation in host-breadth is not simply due to the number of available hosts. Furthermore there was no effect of local parasite diversity on parasite host-breadth ($F_{1,21.2} = 2.41$, $P = 0.14$). For example, parasite lineage LA01 was recovered exclusively from *Dumetella carolinensis* (DCA) in Chicago, Illinois (23/157 DCA hosts infected; years sampled 2006, 2007), Connecticut (4/45; 2002 and 2003), and Michigan (11/94; 2012). However, in the 2013 Tennessee sample, LA01 was recovered from the hosts *Mimus polyglottos* (2/9 infected), *Cardinalis cardinalis* (1/36), and *Spinus tristis* (1/19), while the two DCA hosts sampled in Tennessee were both uninfected. LA01 also infected DCA exclusively in the western Chicago location (6/7) in 2014, while it infected DCA (2/6) and *Toxostoma rufum* (1/7) in Champaign, Illinois, in the same year (although those were not community samples). To determine whether local host-breadth differed from a random expectation, we restricted our dataset to those five host species infected by LA01. We then shuffled all parasite lineages infecting those hosts within sampling locations and recalculated a randomized host-breadth for LA01 (9999 randomizations) and compared it with observed host-breadth. In Chicago the host-breadth of LA01 was lower than expected by chance ($P < 0.001$), while in Tennessee it was

higher than expected by chance (P = 0.019). The host-breadth of LA01 did not differ from random in Connecticut and Michigan because there were no infected alternate hosts in either location.OZ06 also exhibited great variability in its host-breadth (Fig. 4). The host-breadth of OZ06 was lower than expected based on a random distribution in Michigan (P = 0.003), Indiana (P < 0.001), and Tennessee (P = 0.030), but was no different than random in Chicago (P = 0.758) and the Ozarks (P = 0.943).

Such variation may reflect temporal change in the host-breadth of parasites. Within individual years, parasite lineages sampled more than three times at multiple locations mostly showed little variation in host-breadth. However, in 2013, OZ14 infected the host *M. melodia* almost exclusively in Pennsylvania (6/12 *M. melodia* infected, also 1/3 *Pipilo erythrophthalmus*, and 1/1 *Pheucticus ludovicianus*), but infected a variety of species in Tennessee (6/50 *Passerina cyanea* individuals infected, and 12 more infections in nine other species) and Indiana (six infections recovered across five species). Host-breadth of OZ14 was higher than expected based on a random distribution in Indiana (P = 0.050), no different than expected in Tennessee (P = 0.127), and, while low, still within the random expectation in Pennsylvania (P = 0.082). Overall, these results demonstrate that parasite host-breadth can indeed exhibit geographic variation independent of temporal effects, and that these differences do not merely reflect the array of potential host species available or the local diversity of parasites.

Competition among parasites. Tests for competition between parasite lineages across the region are likely confounded by idiosyncratic host-parasite associations. Nevertheless, we did find evidence of parasite lineage replacement across locations within our best-sampled host in 2013, *C. cardinalis* (CCA, Fig. 4). We restricted our analysis to the four locations in which CCA was well-sampled in 2013 and to parasite lineages recovered at least 9 times from CCA across those locations. Prevalences of all parasite lineages of CCA except for OZ14 were significantly heterogeneous across sampling locations (Table 2). Furthermore, at each location CCA harbored a single dominant parasite lineage, making each location's parasite assemblage distinct. To test whether these parasite communities differed more from each other than one would expect by chance, we calculated the mean Bray-Curtis dissimilarity between the four locations based on parasite lineage prevalence and compared it to a distribution of randomized average dissimilarities. We created a randomized parasite-by-location matrix by shuffling parasite lineages among infected birds, recalculating prevalence for each lineage at each site, and then calculating the randomized mean Bray-Curtis dissimilarity 9999 times. Mean observed dissimilarity between sites based on parasite prevalence greatly exceeded the randomized average dissimilarities (P < 0.001, Fig. S3), confirming that location-parasite combinations were more distinct than expected by chance.

Parasites and host abundance. We tested for a relationship between host abundance and parasite relative abundance by calculating pairwise Spearman correlation coefficients (ρ) between all host abundances and parasite relative abundances across the region. We hypothesized that pathogenicity of these parasites (Asghar *et al.* 2015) would result in more negative correlations than expected by chance. We restricted our analysis to "community" sampling locations and parasite lineages sampled at least 10 times and hosts infected at least once (results did not qualitatively change using the full dataset).

Our analysis included the abundances of 64 host species and the relative abundances of 33 parasite lineages at each sampling location, resulting in 2112 pairwise correlations. Mean observed ρ was -0.012 $\pm$ 0.008 SE, which did not differ significantly from the distribution of mean ρ values obtained by randomizing the parasite frequency matrix (by row shuffling 9999 times) and recalculating the mean ρ each time ($P = 0.628$). Furthermore, the observed SE was no different than the distribution of randomized SEs ($P = 0.147$).

Discussion

Our survey and analysis of haemosporidian blood parasites of birds in eastern North America demonstrate that the distributions of parasite populations strongly mirror those of their hosts, with broad-scale climatic gradients and barriers to dispersal playing minimal structuring roles even though the distributions of populations of avian hosts were related to environmental gradients when controlling for the effects of parasites (Table 1). Because parasite transmission takes place primarily during the warm summer months (as evidenced by infections in hatch-year birds in late summer; (Ricklefs *et al.* 2005; Ellis, Kunkel & Ricklefs 2014; Medeiros *et al.* 2014), haemosporidian parasites probably are largely buffered against variation in climate (average summer temperature varied between 19.9 and 26.9 ºC across our sampling locations). While we do not know the extent to which the populations of the parasites' dipteran vectors track hosts, in at least one location in the region (Chicago, IL) parasite-host associations appeared unrelated to vector-host encounter rates (Medeiros *et al.* 2013). Interestingly, regional studies of small mammals and their flea ecto-parasites have shown that flea assemblages can respond to distance between sampling locations, and local habitat and climate characteristics even on the same host species (Krasnov *et al.* 1997, 2005), perhaps because they are more exposed to the environment than haemosporidian parasites.

Distributions of parasite populations across the region seemed to also be influenced by parasite competition within hosts and by localized host-switching. In our best sampled host, *C. cardinalis*, we found evidence of nonrandom, distinct parasite assemblages at each of four sampling locations within a single year (Fig. 4). For example, parasites LA22 and NA04 seem to replace each other as the most common parasites of *C. cardinalis* in Louisiana and Mississippi, although both lineages infect *C. cardinalis* in both locations. This suggests that besides being limited to the distribution of certain host populations, parasite distributions may also be determined by competition within host populations across the region. However, we do not know the extent to which such competition is mediated by the host immune system through localized lineage-specific immunity (Bonneaud *et al.* 2006). Furthermore, parasite host-breadth can vary across time and space (Fig. 3) even when controlling for the diversity of available hosts and the local diversity of parasites, indicating the importance of host-switching in determining parasite distributions across the region.

Finally, while theoretical (Anderson & May 1978) and empirical (Hudson, Dobson & Newborn 1998) studies suggest that parasites may often limit host population size, correlations between host and parasite populations across the region did not differ from random suggesting that haemosporidian parasites do not limit the population densities of their hosts in this region. Taken together our data reveal that populations of haemosporidian parasites are largely structured by populations of their hosts, while host

populations seem to be structured to some extent by environmental and geographic gradients and not by haemosporidian parasites. However, parasites appear to interact within host species and to shift between hosts over short time periods and across short distances.

Materials and Methods

Field methods. We captured birds with mist-nets at 13 locations across eastern North America (Fig. 1) during summer months (primarily late May – August with minimal sampling in April and September; removal of April and September samples did not qualitatively change results) from 1999 to 2014 (Table S2). We took a small (ca. 10 μL) blood sample from the brachial vein of each bird and stored the blood in Puregene® (Germantown, Maryland) or Longmire's (Longmire, Maltbie & Baker 1997) lysis buffer. We collected all samples under appropriate state and federal permits and IACUC protocols.

Laboratory methods. We extracted DNA from blood samples using an ammonium acetate-isopropanol precipitation protocol (Svensson & Ricklefs 2009). We screened DNA samples for haemosporidian parasites using a PCR protocol designed to amplify a small section of mitochondrial parasite DNA (Fallon *et al.* 2003). We then amplified a portion of the cytochrome *b* gene in positive samples using several primer pairs and protocols (Waldenström *et al.* 2004; Fallon *et al.* 2005; Ricklefs *et al.* 2005; Fecchio *et al.* 2013). We identified unique parasite lineages based on their cytochrome *b* sequences, and on their host and geographic distributions (Svensson-Coelho *et al.* 2013; Ricklefs *et al.* 2014). Multiple infections were separated by phasing (Browning & Browning 2011) where possible. Genbank Accession numbers for all lineages can be found in Table S1.

Statistical analysis. All analyses were performed in R v3.1.2 (R Core Team 2014), all graphics were created with the ggplot2 package (Wickham 2009) in R, and we report two-tailed P values for all tests. We used the "metaMDS" function in the vegan package (Oksanen *et al.* 2015) in R for Non-Metric Multidimensional Scaling (NMDS) and set the number of axes in the NMDS ordinations to three. We compared the ordinations with Canonical Correlation Analysis (CCorA) using the function "CCorA" in the vegan package (significance was tested by permutation with 9999 trials). We calculated Bray-Curtis dissimilarities between locations with the "vegdist" function in the vegan package. Bray-Curtis dissimilarity between two sampling locations (1 and 2) is calculated by

$$\frac{\sum_{j=1}^{p}|y_{1j}-y_{2j}|}{\sum_{j=1}^{p}(y_{1j}+y_{2j})}$$

where *y* represents the number (or frequency) of individuals sampled of species *j*, and *p* represents the total number of species sampled over both locations (Legendre & Legendre 1998).

We created a geographic distance matrix between locations with the "rdist.earth" function in the fields package (Nychka, Furrer & Sain 2014) in R. We compared distance matrices with Mantel and partial Mantel tests using functions "mantel" and "mantel.partial" (method = "spearman") in the vegan package. Mantel statistics were tested for significance by permutation (9999 trials) following (Legendre & Legendre

1998). We tested for a departure from unimodality in the frequency of host breadth values using Hartigans' dip test (Hartigan & Hartigan 1985) with the function "dip.test" in the diptest package (Maechler 2014) in R. Linear mixed effects models were run with the lme4 R package (Bates *et al.* 2014), and denominator degrees of freedom for F tests were calculated using the "Kenward-Roger" approach (Kenward & Roger 1997) implemented in the lmerTest (Kuznetsova, Brockhoff & Christensen 2014) and pbkrtest (Halekoh & Højsgaard 2014) packages in R.

Host abundance. Our mist-net effort varied across locations and years and therefore provided unreliable estimates of avian abundance. To estimate avian abundance we downloaded route data from the North American Breeding Bird Survey (https://www.pwrc.usgs.gov/bbs/). We selected routes deemed acceptable by the survey organizers (i.e., routes that met all survey requirements in a particular year) located within 80km of our sampling locations, and we used route data corresponding to the year each location was sampled, plus one year before and one year after our sample was taken. For example, Chicago, IL was sampled in 2006 and 2007, so we used route data from 2005 to 2008 within the 80-km buffer (for the locations sampled in 2014 we used route data from years 2013 and 2014). We then averaged bird species abundances across routes and across years for each sampling location. We spatial and temporal buffers to account for potential variability in abundance estimates due to environmental heterogeneity within routes (Bart, Hopschen & Peterjohn 1995) and observer error (Sauer, Peterjohn & Link 1994), but our results did not change qualitatively with the size of these buffers.

Parasite host-breadth. We calculated host-breadth for each parasite lineage using Rao's QE (Rao 1982; Pavoine, Ollier & Pontier 2005; Chao, Chiu & Jost 2010) defined by the formula

$$\frac{1}{2}\sum_{i,j}^{S} t_{ij} p_i p_j$$

where t_{ij} is a matrix of phylogenetic distances between host species *i* and *j* observed to be infected by a given parasite lineage (divided by two to obtain average phylogenetic distance), p_i is the proportion of infections by the parasite in host species *i* (i.e., the number of individuals of host species *i* infected by the parasite divided by the total number of individuals infected by that parasite), p_j is the proportion of infections by the parasite in host species *j*, and *S* is the total number of host species. Our parasite host-breadth score varies from zero (complete host specialization) to

$$\frac{1}{2}\sum_{i,j}^{S} t_{ij} S^{-2}$$

which represents a maximally generalized parasite (i.e., a parasite that infects all hosts in the community equally; however, an alternative might be that a perfect generalist would infect all hosts in direct proportion to host abundance), and is equivalent to a phylogenetically-weighted Gini-Simpson diversity index. We calculated Rao's QE using the "raoD" function in the picante package (Kembel *et al.* 2010) in R and report the

"Dkk" value the function produces. We used the phylogeny of Jetz et al. (Jetz *et al.* 2012) to estimate phylogenetic relationships between bird species. Based on parasites sampled at least 10 times over the community sampling locations, we showed a strong relationship between Rao's QE and the Gini-Simpson index applied to parasite host-breadth (Fig. S4). Because of the apparent effect of host phylogeny, we used Rao's QE as a metric of parasite host-breadth for all analyses.

Acknowledgments
We thank Brandt Ryder, Pete Marra, Keegan Tranquillo, Peter Pyle, and the Indiana MAPS teams for assistance collecting samples from Indiana; Alicia Burke for assistance collecting samples in the Ozarks; Field Biologist Brenda Keith and the Kalamazoo Valley Bird Observatory/Kalamazoo Nature Center for collecting samples in Michigan; the Connecticut Audubon Society for providing samples from their banding station; Steve Latta for providing parasite sequences from Alabama; Woody Walstrom and Diana Outlaw for providing samples from Mississippi; Emma Levenson and Kathleen Riley for assistance with data collection in LA; most of our work would not have been possible without the help of numerous local government and conservation agencies. We also thank the many volunteers and organizers of the Breeding Bird Survey. V.A.E. thanks Fabrício R. Santos and LBEM for providing support and a place to write. Field and laboratory work was supported by the Harris World Ecology Center, the St. Louis Audubon Society, the Missouri Audubon Society, the Curators of the University of Missouri, the NSF Malaria Research Coordination Network, and a University of Missouri-St. Louis Dissertation Fellowship awarded to V.A.E.

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Figures and Tables

Table 1—Results of partial Mantel tests comparing hypothesized relationships between space (i.e., geographic distance between sites), the environment (i.e., climatic differences between sites), birds (i.e., host community dissimilarity between sites), and parasites (i.e., parasite community dissimilarity between sites) identified in Fig. 2. We report the partial Mantel correlation coefficient (r_p) and associated P value. The relationship between space and environment was tested with a standard Mantel test.

Relationship between	Controlling for	$\mathbf{r_p}$	**P**

Space	Environment	None	**0.410**	0.036
Birds	Environment	Parasites	**0.694**	< 0.001
Birds	Space	Parasites	**0.504**	0.012
Birds	Environment	Space	**0.682**	0.001
Birds	Space	Environment	**0.438**	0.022
Parasites	Environment	Birds	0.092	0.333
Parasites	Space	Birds	0.097	0.302
Parasites	Environment	Space	0.294	0.081
Parasites	Space	Environment	0.199	0.178
Birds	Parasites	Environment	0.256	0.076
Birds	Parasites	Space	**0.335**	0.027

Table 2—Results of G-tests comparing the prevalence of each well-sampled parasite of the host *Cardinalis cardinalis* in 2013 across sampling locations. Prevalence data are shown graphically in Fig. 4.

	LA22	**NA04**	**OZ01**	**OZ03**	**OZ14**
$G_{(df = 3)}$	53.73	58.72	8.46	67.1	6.82
P	< 0.001	< 0.001	0.037	< 0.001	0.078

Figure 1

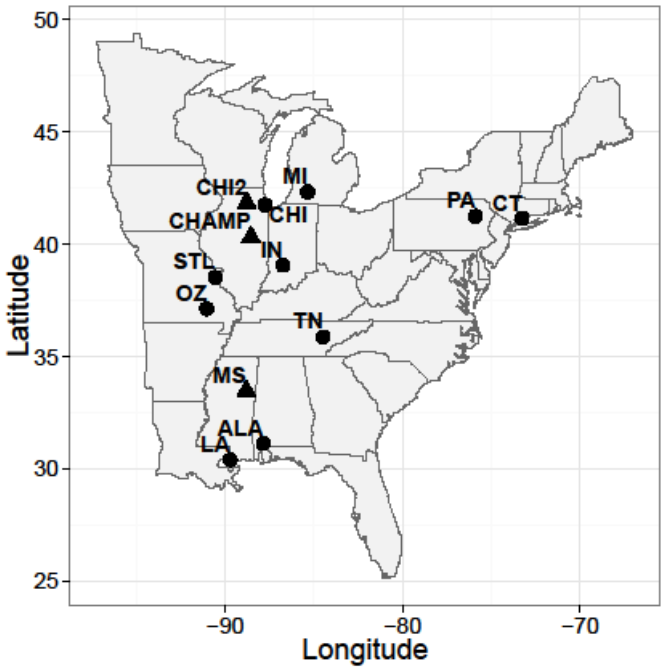

Fig. 1—Sampling locations. Circles are "community" samples (i.e., sampling was not restricted to focal bird species), triangles are samples of one or a few bird species only (Table S2). Location codes are as follows: **ALA** is Alabama**, CHAMP** is Champaign (Illinois), **CHI** is Chicago (Illinois), **CHI2** is western Chicago (Illinois), **CT** is Connecticut, **IN** is Indiana, **LA** is Louisiana**, MI** is Michigan, **MS** is Mississippi, **OZ** is Ozarks (Missouri), **PA** is Pennsylvania, **STL** is St. Louis (Missouri), and **TN** is Tennessee.

Figure 2

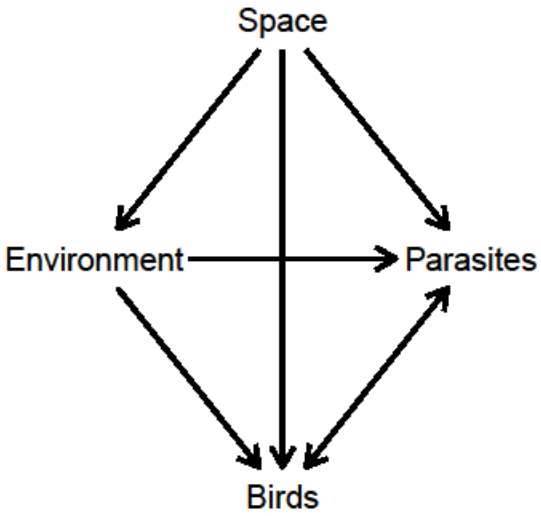

Fig. 2—Path diagram of hypothesized interactions between space (i.e., geographic distance between sampling locations), environment (i.e., climatic differences between sampling locations), bird and parasite communities (i.e., differences in species richness and abundances of birds and of parasite lineages respectively, between sampling locations), which are all represented as distance matrices. We test these hypotheses with partial Mantel tests which allow for the calculation of correlations between two distance matrices while controlling for the effect of a third. For example, birds and parasites are positively correlated (r = 0.335, P = 0.027) even when controlling for the effect of space (Table 1).

Figure 3

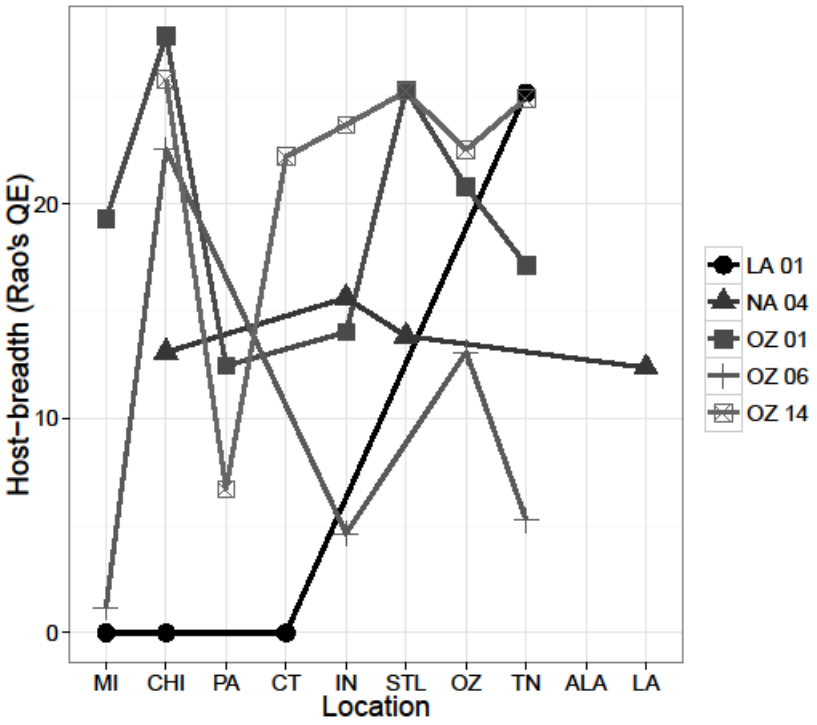

Fig. 3—Parasite host-breadth (calculated as Rao's Quadratic Entropy) for parasite lineages sampled at least four times at each of at least four sampling locations, pooling data from all years, showing clear variation in host-breadth across the region. Locations are organized from North to South.

Figure 4

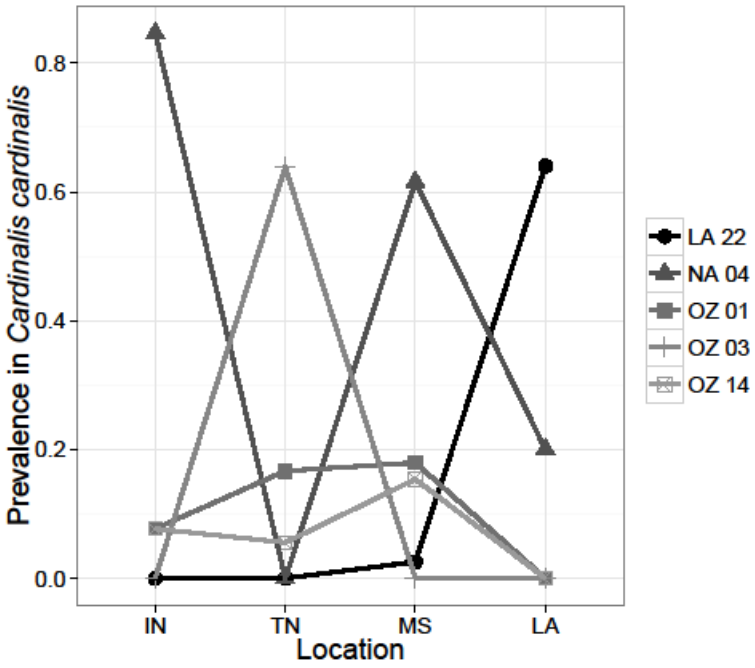

Fig. 4—Prevalences of well-sampled parasite lineages on the host *Cardinalis cardinalis* at four locations in 2013. Prevalence of four of five parasites was significantly heterogeneous across space (Table 2) and parasite assemblages within this host exhibited significant spatial turnover (mean Bray-Curtis dissimilarity between sites was significantly greater than random, $P < 0.001$; Fig. S3).

Supporting Information, Figures

Figure S1

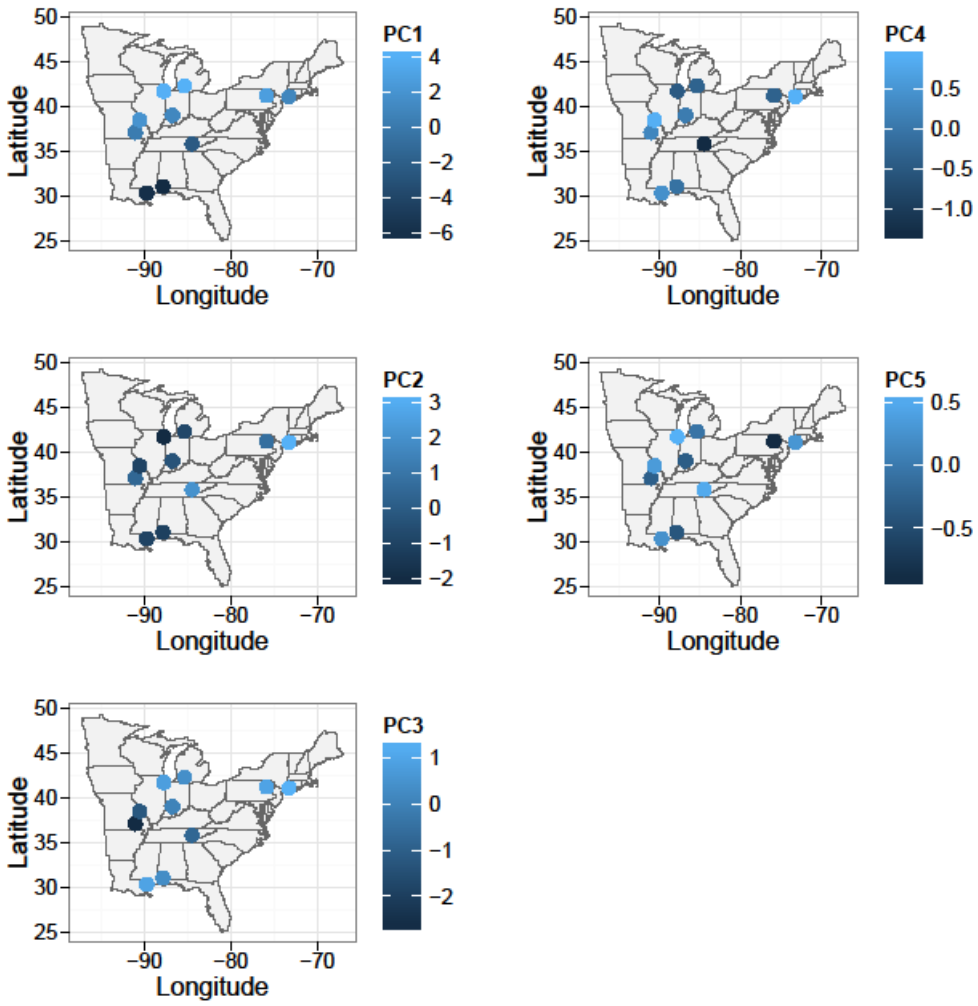

Fig. S1—The first five axes of a principal components analysis (PCA) ordinating 19 bioclim variables downloaded from http://www.worldclim.org/ for each sampling location (Table S4).

Figure S2

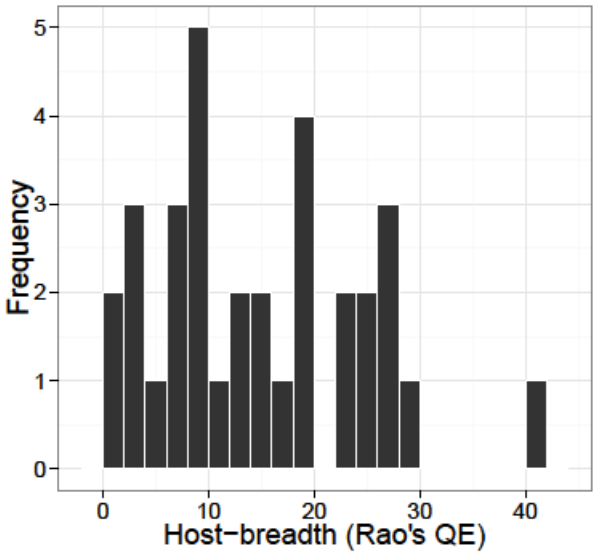

Fig. S2—Frequency distribution of parasite host-breadth (Rao's Quadratic Entropy) from parasite lineages sampled at least 10 times across all locations. It does not differ from a unimodal distribution (i.e., it is not bimodal; Hartigans' dip test, $D_{33} = 0.047$, $P = 0.87$).

Figure S3

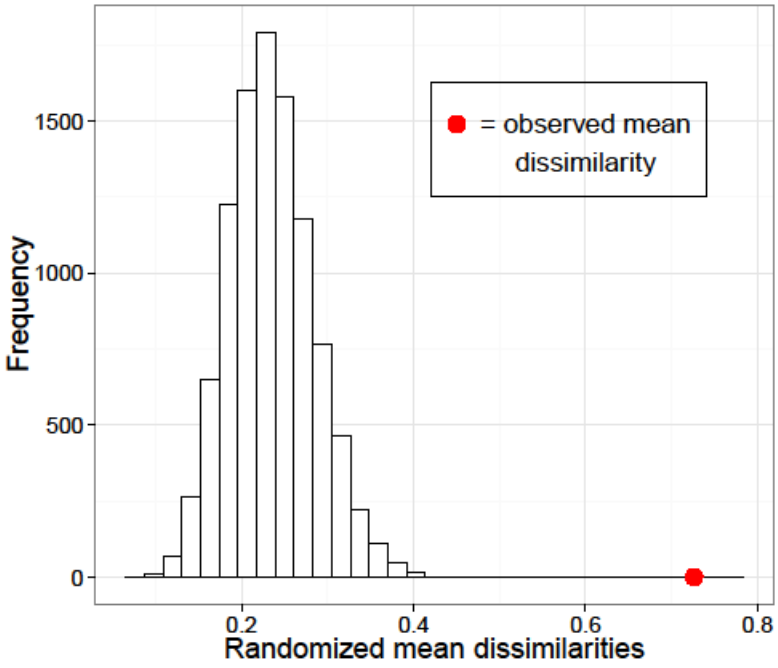

Fig. S3—The distribution of randomized mean dissimilarities between four locations where *Cardinalis cardinalis* was well-sampled in 2013 based on the prevalence of five

parasite lineages at each location. Observed dissimilarity between locations was greater than the random distribution indicating that parasite by location combinations were more unique than expected by chance alone ($P < 0.001$).

Figure S4

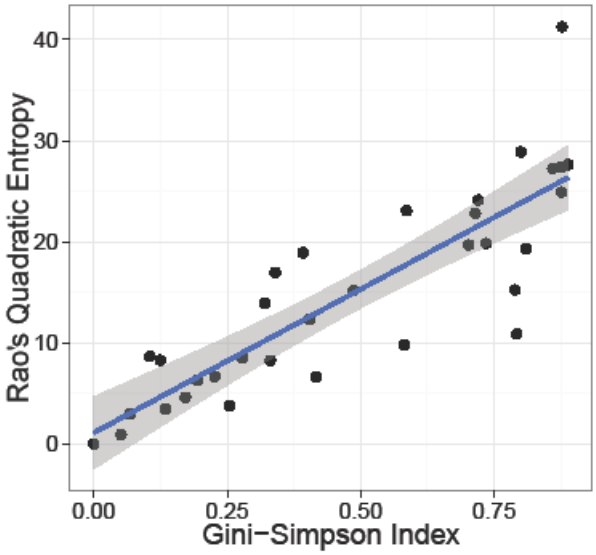

Fig. S4—The relationship between parasite host-breadth, calculated by Rao's Quadratic Entropy, and the Gini-Simpson Index for parasites sampled at least 10 times across community sampling locations ($R^2 = 0.724$, $P < 0.001$). The blue line is the predicted linear relationship and is surrounded by 95% confidence intervals.

Supporting Information, Tables

Table S1—All parasite lineages recovered in our study and the number of times each was sampled overall (N), its genus and Genbank number where the sequence can be recovered.

Lineage	N	Genus	Genbank Number
OZ 14	257	*Plasmodium*	AY540210, HM222474
OZ 01	253	*Plasmodium*	GQ395654, HQ287549, GQ395654, GQ141574
OZ 35	194	*Plasmodium*	HM222474-HM222480
NA 04	115	*Haemoproteus*	AF465562
OZ 10	80	*Haemoproteus*	AF465576
OZ 08	66	*Plasmodium*	AF540207, HM222485
LA 01	57	*Haemoproteus*	AF465572
OZ 03	55	*Haemoproteus*	AF465563

OZ 05	55	*Haemoproteus*	AY167241
OZ 06	52	*Plasmodium*	AF465554
KZ 01	50	*Plasmodium*	AY455663, KF359936
CHI 02PL	44	*Plasmodium*	KC789821
NA 15	35	*Haemoproteus*	GQ395665, GQ141584
CHI 08PA	28	*Haemoproteus*	KM280616, KM065800
OZ 02	28	*Haemoproteus*	AY455658
OZ 12	28	*Haemoproteus*	AY817748, GQ395673
OZ 45	27	*Plasmodium*	HM222481, HM222482
CHI 35PL	26	*Plasmodium*	KM280608
TI P25L	23	*Plasmodium*	KC680673, KC680702, KC680706
LA 22	22	*Haemoproteus*	HQ287537
CHI 05PL	21	*Plasmodium*	KC789824
CHI 20PA	20	*Haemoproteus*	KM065797, KM280614
CHI 09PL	18	*Plasmodium*	KC789828
CHI 30PA	18	*Haemoproteus*	KM280621, KM280624, KM280635
OZ 49	18	*Haemoproteus*	KJ910306, KF359931
EL 02	16	*Plasmodium*	KM598210
OZ 07	16	*Haemoproteus*	AF465580
OZ 04	15	*Plasmodium*	AY540197, GQ395669, GQ395657, KC680705, KC680692, KC680679
OZ 26	15	*Haemoproteus*	AY540212
OZ 16	14	*Haemoproteus*	AY817750
JA 01	12	*Plasmodium*	KM598212
OZ 17	11	*Haemoproteus*	GQ395632, AY817751, AY167244
CHI 23PA	10	*Haemoproteus*	KM280611
NA 01	9	*Plasmodium*	AF465547
OZ 13	9	*Haemoproteus*	AY540209, AY540209, GQ395674, GU252006
OZ 09	8	*Plasmodium*	AY540208, KC680672
OZ 25	8	*Plasmodium*	AY540211, GQ395679, GQ141593
IN 01	7	*Haemoproteus*	KM598226
NA 05	6	*Haemoproteus*	AF465564
OZ 38	6	*Plasmodium*	AF465559, HQ287545
CHI 16PL	5	*Plasmodium*	KC680714

MI 02	5	*Plasmodium*	KM598221
NA 14	5	*Haemoproteus*	AY540205
OZ 54	5	*Plasmodium*	KF359933
OZ 58	5	*Haemoproteus*	KM598223
PR 03	5	*Haemoproteus*	AY455659
OZ 36	4	*Plasmodium*	AY540220, AY167248
YU 01	4	*Plasmodium*	DQ838997, DQ659549, HQ287539, GQ395688, GQ141598
GAM 06	3	*Plasmodium*	KP771715
KZ 02	3	*Plasmodium*	AY540195
NA 16	3	*Haemoproteus*	HM222472, GQ395666, GU252003
OZ 19	3	*Plasmodium*	AY455661
OZ 21	3	*Haemoproteus*	AY167242, HQ287540
OZ 28	3	*Haemoproteus*	AY817753
OZ 53	3	*Haemoproteus*	KJ91307, KM065799
OZ 55	3	*Plasmodium*	KF359934
TI P10	3	*Plasmodium*	KC680707
CHI 26PA	2	*Haemoproteus*	KM280632
IN 04	2	*Haemoproteus*	KP771716
MI 01	2	*Plasmodium*	KM598218
NA 11	2	*Plasmodium*	AF465549
OZ 27	2	*Haemoproteus*	AY817752
CE hapH	1	*Haemoproteus*	JX501902
CHI 17PL	1	*Plasmodium*	JN792148, EU627845
CHI 19PA	1	*Haemoproteus*	KM280617
CHI 27PA	1	*Haemoproteus*	KM280631
CHI 28PA	1	*Haemoproteus*	KM280633
DR 07	1	*Haemoproteus*	HM222464
IN 02	1	*Plasmodium*	KM598227
IN 05	1	*Plasmodium*	KP771717
JA 04	1	*Plasmodium*	KM598213
LA 07	1	*Haemoproteus*	GQ395658, GU252000
LSW 01	1	*Plasmodium*	KP771718
MI 03	1	*Haemoproteus*	KM598220
MI 04	1	*Haemoproteus*	KM598219
MI 05	1	*Plasmodium*	KM598228
NA 10	1	*Haemoproteus*	AY540202

OZ 29	1	*Haemoproteus*	AY817754
OZ 31	1	*Haemoproteus*	AF465582
OZ 32	1	*Haemoproteus*	AY540214
OZ 34	1	*Haemoproteus*	AY540216, GQ395682, GQ141596
OZ 43	1	*Haemoproteus*	AY817755
OZ 51	1	*Plasmodium*	KJ910311, KF359932
OZ 57	1	*Haemoproteus*	GU252022
OZ 60	1	*Plasmodium*	KP771719
TN 24	1	*Haemoproteus*	KM065796
YU 03	1	*Haemoproteus*	GQ395690, GU252009

Table S2—Information on each sampling location, including the total number of samples collected (N).

Location	Abbrev.	Longitude	Latitude	Sample Years	N	Sample Type
Alabama	ALA	-87.84833	31.13	2001	108	Community-level
Champaign	CHAMP	-88.54445	40.3126	2014	37	Restricted to several species
Chicago	CHI	-87.7607	41.74647	2006, 2007	2023	Community-level
western Chicago	CHI2	-88.78047	41.83926	2014	13	Restricted to several species
Connecticut	CT	-73.25889	41.145	2002, 2003	301	Community-level
Indiana	IN	-86.75176	39.06639	2012, 2013	500	Community-level
Louisiana	LA	-89.71083	30.4025	2013	157	Community-level
Michigan	MI	-85.34944	42.32667	2012	381	Community-level
Mississippi	MS	-88.812	33.474	2013	39	Restricted to one species, *Cardinalis cardinalis*
Ozarks	OZ	-91.0374	37.12555	1999, 2000, 2001, 2002, 2005, 2007, 2011	1438	Community-level
Pennsylvania	PA	-75.88861	41.24472	2012, 2013	204	the 2012 sample was restricted to several species, the 2013 sample was community-level

St. Louis	STL	-90.5625	38.52472	2008, 2009	338	Community-level
Tennessee	TN	-84.48111	35.87361	2013	328	Community-level

Table S3—Host and parasite associations at each of our sampling locations. Three letter codes under "Species" column refer to host species names which can be found in Table S5. See .xls file, available upon request (vincenzoaellis@gmail.com).

Table S4—Results of a principal components analysis (PCA) of 19 bioclim variables downloaded from http://www.worldclim.org/ for each of our community sampling locations. We report the proportion of variance and cumulative variance explained by each axis up to eight axes, although we only used five in our analysis.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Proportion of variance	0.7435	0.1312	0.08014	0.02375	0.01187	0.00695	0.00211	0.00038
Cumulative variance	0.7435	0.8747	0.95481	0.97856	0.99043	0.99738	0.99949	0.99988

Table S5—Host species codes corresponding to three letter host species codes in Table S3. "N" refers to sample size, "AOU species code" is a four letter code for host species commonly used by bird banders. The "Genus" and "Species" columns reflect the taxonomy used at http://birdtree.org/. The "Current" column represents current taxonomy (when different) based on the American Ornithological Union's Checklist of North American Birds (http://checklist.aou.org/).

Host species code	**N**	**AOU species code**	**Genus**	**Species**	**Current**	**Common Name**
PDO	565	HOSP	*Passer*	*domesticus*		House Sparrow
TMI	491	AMRO	*Turdus*	*migratorius*		American Robin
CCA	353	NOCA	*Cardinalis*	*cardinalis*		Northern Cardinal
DCA	348	GRCA	*Dumetella*	*carolinensis*		Gray Catbird
VOL	284	REVI	*Vireo*	*olivaceus*		Red-eyed Vireo
PCY	231	INBU	*Passerina*	*cyanea*		Indigo Bunting
VGR	220	WEVI	*Vireo*	*griseus*		White-eyed Vireo

MME	219	SOSP	*Melospiza*	*melodia*		Song Sparrow
IVI	210	YBCH	*Icteria*	*virens*		Yellow-breasted Chat
CTR	209	AMGO	*Carduelis*	*tristis*	*Spinus tristis*	American Goldfinch
WCI	142	HOWA	*Wilsonia*	*citrina*	*Setophaga citrina*	Hooded Warbler
HMU	136	WOTH	*Hylocichla*	*mustelina*		Wood Thrush
OFO	130	KEWA	*Oporornis*	*formosus*	*Geothlypis formosa*	Kentucky Warbler
MVA	128	BAWW	*Mniotilta*	*varia*		Black-and-White Warbler
EVI	127	ACFL	*Empidonax*	*virescens*		Acadian Flycatcher
GTR	110	COYE	*Geothlypis*	*trichas*		Common Yellowthroat
HVE	108	WEWA	*Helmitheros*	*vermivorum*		Worm-eating Warbler
SAU	106	OVEN	*Seiurus*	*aurocapilla*		Ovenbird
CME	101	HOFI	*Carpodacus*	*mexicanus*	*Haemorhous mexicanus*	House Finch
TLU	91	CAWR	*Thryothorus*	*ludovicianus*		Carolina Wren
PPU	82	DOWO	*Picoides*	*pubescens*		Downy Woodpecker
BBI	71	TUTI	*Baeolophus*	*bicolor*		Tufted Titmouse
SVU	69	EUST	*Sturnus*	*vulgaris*		European Starling
QQU	68	COGR	*Quiscalus*	*quiscula*		Common Grackle
APH	60	RWBL	*Agelaius*	*phoeniceus*		Red-winged Blackbird
PAM	59	NOPA	*Parula*	*americana*		Northern Parula
SRU	57	AMRE	*Setophaga*	*ruticilla*		American Redstart
VPI	50	BWWA	*Vermivora*	*pinus*	*Vermivora cyanoptera*	Blue-winged Warbler
SNO	46	NOWA	*Seiurus*	*noveboracensis*	*Parkesia noveboracensis*	Northern Waterthrush

SPU	45	FISP	*Spizella*	*pusilla*		Field Sparrow
PCA	43	CACH	*Parus*	*carolinensis*	*Poecile carolinensis*	Carolina Chickadee
ZAL	43	WTSP	*Zonotrichia*	*albicollis*		White-throated Sparrow
POL	41	SCTA	*Piranga*	*olivacea*		Scarlet Tanager
DPE	38	YEWA	*Dendroica*	*petechia*	*Setophaga petechia*	Yellow Warbler
MAT	37	BHCO	*Molothrus*	*ater*		Brown-headed Cowbird
ZMA	36	MODO	*Zenaida*	*macroura*		Mourning Dove
CFU	30	VEER	*Catharus*	*fuscescens*		Veery
DMA	30	MAWA	*Dendroica*	*magnolia*	*Setophaga magnolia*	Magnolia Warbler
BCE	29	CEDW	*Bombycilla*	*cedrorum*		Cedar Waxwing
PCE	29	BGGN	*Polioptila*	*caerulea*		Blue-gray Gnatcatcher
PER	29	EATO	*Pipilo*	*erythrophthalmus*		Eastern Towhee
SPA	29	CHSP	*Spizella*	*passerina*		Chipping Sparrow
DDI	26	PRAW	*Dendroica*	*discolor*	*Setophaga discolor*	Prairie Warbler
LSW	26	SWWA	*Limnothlypis*	*swainsonii*		Swainson's Warbler
CVI	25	EAWP	*Contopus*	*virens*		Eastern Wood-Pewee
TRU	25	BRTH	*Toxostoma*	*rufum*		Brown Thrasher
ETR	23	WIFL	*Empidonax*	*traillii*		Willow Flycatcher
SCR	23	WBNU	*Sitta*	*carolinensis*		White-breasted Nuthatch
DCN	21	CAWA	*Wilsonia*	*canadensis*	*Cardellina canadensis*	Canada Warbler
IGA	19	BAOR	*Icterus*	*galbula*		Baltimore Oriole
PAT	19	BCCH	*Parus*	*atricapillus*	*Poecile atricapillus*	Black-capped

						Chickadee
SMO	19	LOWA	*Seiurus*	*motacilla*	*Parkesia motacilla*	Louisiana Waterthrush
SPH	18	EAPH	*Sayornis*	*phoebe*		Eastern Phoebe
TAE	17	HOWR	*Troglodytes*	*aedon*		House Wren
CCR	16	BLJA	*Cyanocitta*	*cristata*		Blue Jay
DCO	16	YRWA	*Dendroica*	*coronata*	*Setophaga coronata*	Yellow-rumped/Myrtle Warbler
DPN	16	CSWA	*Dendroica*	*pensylvanica*	*Setophaga pensylvanica*	Chestnut-sided Warbler
VGI	16	WAVI	*Vireo*	*gilvus*		Warbling Vireo
TBC	14	TRSW	*Tachycineta*	*bicolor*		Tree Swallow
MGR	13	SWSP	*Melospiza*	*georgiana*		Swamp Sparrow
CAM	12	YBCU	*Coccyzus*	*americanus*		Yellow-billed Cuckoo
PRU	12	SUTA	*Piranga*	*rubra*		Summer Tanager
VRU	11	NAWA	*Vermivora*	*ruficapilla*	*Leiothlypis ruficapilla*	Nashville Warbler
DCR	10	BTBW	*Dendroica*	*caerulescens*	*Setophaga caerulescens*	Black-throated Blue Warbler
DDO	10	YTWA	*Dendroica*	*dominica*	*Setophaga dominicana*	Yellow-throated Warbler
PCI	10	PROW	*Protonotaria*	*citrea*		Prothonotary Warbler
SSI	10	EABL	*Sialia*	*sialis*		Eastern Bluebird
MPO	9	NOMO	*Mimus*	*polyglottos*		Northern Mockingbird
ISP	8	OROR	*Icterus*	*spurius*		Orchard Oriole
MCA	8	RBWO	*Melanerpes*	*carolinus*		Red-bellied Woodpecker
RCA	8	RCKI	*Regulus*	*calendula*		Ruby-crowned

						Kinglet
VFL	7	YTVI	*Vireo*	*flavifrons*		Yellow-throated Vireo
CAU	6	NOFL	*Colaptes*	*auratus*		Northern Flicker
CMN	6	GCTH	*Catharus*	*minimus*		Gray-cheeked Thrush
MCR	6	GCFL	*Myiarchus*	*crinitus*		Great Crested Flycatcher
VPE	6	TEWA	*Vermivora*	*peregrina*	*Oreothlypis peregrina*	Tennessee Warbler
PVI	5	HAWO	*Picoides*	*villosus*		Hairy Woodpecker
CVR	4	BOBW	*Colinus*	*virginianus*		Northern Bobwhite
EFV	4	YBFL	*Empidonax*	*flaviventris*		Yellow-bellied Flycatcher
DFU	3	BLBW	*Dendroica*	*fusca*	*Setophaga fusca*	Blackburnian Warbler
DVI	3	BTNW	*Dendroica*	*virens*	*Setophaga virens*	Black-throated Green Warbler
GCA	3	BLGR	*Passerina*	*caerulea*		Blue Grosbeak
OPH	3	MOWA	*Oporornis*	*philadelphia*	*Geothlypis philadelphia*	Mourning Warbler
CER	2	BBCU	*Coccyzus*	*erythropthalmus*		Black-billed Cuckoo
CGU	2	HETH	*Catharus*	*guttatus*		Hermit Thrush
EAL	2	ALFL	*Empidonax*	*alnorum*		Alder Flycatcher
HRS	2	BASW	*Hirundo*	*rustica*		Barn Swallow
PMN	2	ETSP	*Passer*	*montanus*		Eurasian Tree Sparrow
CCN	1	NOHA	*Circus*	*cyaneus*		Northern Harrier
CFM	1	BRCR	*Certhia*	*familiaris*		Brown Creeper

DCE	1	CERW	*Dendroica*	*cerulea*	*Setophaga cerulea*	Cerulean Warbler
DPA	1	PAWA	*Dendroica*	*palmarum*	*Setophaga palmarum*	Palm Warbler
DPI	1	PIWA	*Dendroica*	*pinus*	*Setophaga pinus*	Pine Warbler
DST	1	BLPW	*Dendroica*	*striata*	*Setophaga striata*	Blackpoll Warbler
EMI	1	LEFL	*Empidonax*	*minimus*		Least Flycatcher
PLD	1	RBGR	*Pheucticus*	*ludovicianus*		Rose-breasted Grosbeak
SDE	1	ECDO	*Streptopelia*	*decaocto*		Eurasian Collared-Dove
VBE	1	BEVI	*Vireo*	*bellii*		Bell's Vireo
VPH	1	PHVI	*Vireo*	*philadelphicus*		Philadelphia Vireo

Chapter 4

Low haemosporidian parasite prevalence in locally rare avian hosts at multiple sites within a regional community

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Abstract

Little is known regarding the relationship between the probability of infection by parasites (prevalence) and host abundance. Here we survey a regional community of birds and their haemosporidian parasites (genera *Plasmodium* and *Haemoproteus*) in eastern North America and show that parasite prevalence is positively related to the abundance of host species within local assemblages, although the strength of the relationship varies between assemblages within the region. Accordingly, at the local scale, rare hosts are less frequently parasitized than abundant hosts. Densities of white blood cells (potential general indicators of infection) were also positively related to host species abundance at one location in the region, suggesting that the abundance-prevalence relationship might extend to other parasites. Relationships between parasite prevalence and the abundance of a host species at multiple locations across its range were insignificant for all host species except *Turdus migratorius*, the abundance of which was weakly negatively related to the prevalence of *Haemoproteus* parasites. Interestingly, three nonnative host species had lower prevalence than native species in the location where they were best sampled despite being relatively abundant, offering evidence in favor of the Enemy Release Hypothesis.

Key words: avian malaria, Enemy Release Hypothesis, *Haemoproteus*, host abundance, *Plasmodium*

Introduction

Local species assemblages are typically composed of many rare and only a few common species (McGill *et al.* 2007). However, locally rare species may be common in other parts

of their ranges (Murray & Lepschi 2004). Furthermore, phylogenetically informed analyses show that abundance is an evolutionarily labile trait (McGill 2008; Ricklefs 2011, 2012). Such observations suggest that localized, species-specific forces act independently across regions to determine abundance. One hypothesis proposes that local coevolution between parasites and their hosts may generate such abundance patterns (Ricklefs 2011, 2012). Indeed, several studies have shown that specialized soil pathogens influence the local abundance of temperate (Packer & Clay 2000, 2003) and tropical tree species (Mangan *et al.* 2010), and various parasites (including viruses) may depress population densities of their vertebrate hosts (e.g., Hudson, Dobson & Newborn 1998; LaDeau, Kilpatrick & Marra 2007). Moreover, nonnative species are often more common in their introduced ranges than in their native ranges, potentially as a result of having left their parasites behind (the "Enemy Release Hypothesis", Torchin *et al.* 2003; Marzal *et al.* 2011).

In the case of trees, individuals of rare species have a lower probability of survival in the presence of conspecifics than do individuals of abundant species (Comita *et al.* 2010), in part a result of increased sensitivity to host-specialized soil microbes (Mangan *et al.* 2010), and potentially other natural enemies (Janzen 1970). However, relationships between host abundance and parasite prevalence (i.e., the proportion of hosts infected) in vertebrates remain poorly explored both across and within host species. Several studies have shown that parasite prevalence increases with host population density (Arneberg *et al.* 1998; Brown *et al.* 2001; Krasnov, Khokhlova & Shenbrot 2002; O'Brien & Brown 2011), but such relationships are both parasite- and host-specific (Isaksson *et al.* 2013, Stanko, Krasnov & Morand 2006). Finally, few studies have examined the relationship between parasite prevalence and the relative abundance of different host species in a particular area. Ricklefs *et al.* (2005) found a U-shaped relationship between the prevalence of haemosporidian parasites and the relative abundance of hosts in a sample of birds in southern Missouri, where the rarest and commonest bird species had the highest prevalence. Importantly, Ricklefs *et al.* (2005) used host sample size (from mist-net captures, summed across years) as a proxy for local abundance, which may misrepresent the relative abundance of some species (Ralph *et al.* 2004).

Here, we attempt to clarify the relationship between avian host abundance and parasite prevalence in a regional community of birds and their dipteran-vectored, haemosporidian blood parasites (genera *Plasmodium* and *Haemoproteus*). According to one hypothesis, parasite control of host abundance might result in negative relationships between parasite prevalence both across and within host species abundance. Such patterns would suggest that abundant hosts are better able to resist infection than rare hosts. Alternatively, prevalence might increase with host abundance as a result of a higher probability of host-parasite encounters (mediated through host-vector encounters) in more common hosts, resulting in rare species being relatively free from infection. In a previous study from one sampling location in the region, we showed that increased white blood cell (WBC) densities in avian hosts may be general indicators of infection (Ellis, Kunkel & Ricklefs 2014). We therefore tested the relationship between WBC densities and host species abundance in that location to see if infection in general varied with abundance. Finally, the Enemy Release Hypothesis predicts that nonnative hosts reach high relative abundance as a result of losing their parasites, and that generalized parasites

should impact native more than nonnative hosts (Keane & Crawley 2002). We test the latter prediction by comparing the prevalence of native and nonnative hosts within a single assemblage in the region where three nonnative host species were particularly well-sampled.

Methods

We collected small blood samples (< 1% of an individual's body weight) from 5867 birds from 99 species (mostly in the order Passeriformes) across 13 locations in eastern North America (Fig. 1) from 1999 to 2014. Most of our sampling locations were "community samples," meaning that we did not restrict sampling to focal bird species; however, samples in three locations (CHAMP, CHI2, and MS) targeted one or a few species (see Appendix). The majority of samples were taken between late May and August, but minimal sampling also occurred in April and September (removal of April and September samples did not qualitatively change our results). All birds were released after capture, and all sampling took place under appropriate federal and state permits and IACUC protocols. We stored blood samples in Puregene® (Germantown, Maryland) or Longmire's (Longmire, Maltbie & Baker 1997) lysis buffer, and made blood smears for later quantification of white blood cells (Ellis *et al.* 2014).

Molecular analyses

We extracted DNA from samples stored in lysis buffer using a standard ammonium-acetate, isopropanol precipitation protocol (Svensson & Ricklefs 2009), and screened DNA samples using a PCR protocol designed to amplify a small fragment of haemosporidian mitochondrial DNA (Fallon *et al.* 2003). For samples that were positive for haemosporidian parasite DNA, we amplified a portion of the parasite cytochrome *b* gene using one of several protocols (Waldenström *et al.* 2004; Fallon, Bermingham & Ricklefs 2005; Ricklefs *et al.* 2005; Fecchio *et al.* 2013). We defined molecular lineages based on their cytochrome *b* sequences and their host and geographic distributions (Svensson-Coelho *et al.* 2013; Ricklefs *et al.* 2014). We separated mixed infections by phasing when possible (Browning & Browning 2011). Lineage names and GenBank Accession numbers are provided in the Appendix.

Host abundance

Mist-net capture effort was not standardized across locations or across years within locations and therefore did not provide reliable estimates of host abundance. We therefore estimated host abundance from the North American Breeding Bird Survey (https://www.pwrc.usgs.gov/bbs/). We selected routes deemed acceptable by the survey organizers (i.e., routes that met all survey requirements in a particular year) located within 80km of our sampling locations, and we used route data corresponding to the year each location was sampled, plus one year before and one year after our sample was taken. For example, Chicago, IL was sampled in 2006 and 2007, so we used route data from 2005 to 2008 within the 80-km buffer (for the locations sampled in 2014 we used route data from years 2013 and 2014). We then averaged bird species abundances across routes and across years for each sampling location. We used spatial and temporal buffers to account for potential bias due to observer error (Sauer, Peterjohn & Link 1994), but our results did not change qualitatively with the size of these buffers.

White blood cells (WBCs)

We quantified the densities of four types of WBCs on a subset of 235 blood smears from the Ozarks sampling location (Fig. 1) as previously described in Ellis *et al.* (2014). WBC densities are reported as the number of WBCs per 10^4 red blood cells.

Statistical analyses

We used R v.3.1.2 (R Core Team 2014) for all analyses. To test for a general relationship between abundance and parasite prevalence within locations we created a logistic mixed-effects model in the lme4 package in R (Bates *et al.* 2014), with individual infection status as the response variable, host species abundance as the explanatory variable, and random effects (intercepts) of both location and host species.

To better explore the particular relationships of parasite prevalence to both across-host and within-host species abundances, we used generalized linear models (sample size weighted) with binomial error distributions, or quasibinomial error distributions when overdispersion was detected (i.e., more variance in the data than predicted by the model, Bolker *et al.* 2009). For tests of prevalence and across-host-species abundance, we fit one model for each of our best sampled locations (N = 7 locations, Table 1), where each replicate in the model was a host species, the response variable was prevalence, and the explanatory variable was abundance. For tests of prevalence and within-host species abundance we fit one model for each of the host species sampled in at least eight locations throughout the region (N = 5 host species, Table 2). In these within-host-species models, each replicate was a location within the range of the host species in question, and the response and explanatory variables were prevalence and abundance of that host species, respectively.

For all models we used only those host species within a particular location that had at least two individuals sampled at that location so as not to bias against rare species. However, our results did not change qualitatively using different sample size cut-offs as high as eight individuals sampled per species in a particular location. All models were repeated with prevalence based on *Plasmodium* and *Haemoproteus* parasites together (total prevalence) as well as separately. We excluded the three nonnative species we sampled (*Passer domesticus*, *Sturnus vulgarus*, *Haemorhous mexicanus*) from our dataset for all analyses except the generalized linear model comparing prevalence and across-host-species abundance in Chicago, IL where they were best sampled. In the Chicago model, a categorical variable coding for native and nonnative host species was used as a covariate (no significant interaction was detected with abundance) allowing us to control for the effect of nonnatives on the prevalence abundance relationship and also allowing us to compare prevalence between natives and nonnatives. *H. mexicanus* is arguably less of a nonnative than the other two nonnatives since its native distribution extends across western North America. However, some of the haemosporidian parasites of *H. mexicanus* show geographic structuring between their western (native) and eastern (nonnative) ranges (Kimura, Dhondt & Lovette 2006) so we considered them nonnatives for this study.

Finally we related WBCs to host abundance at one location—Ozarks, MO—using negative binomial mixed effects models in the glmmADMB package in R (Fournier *et al.* 2012; Skaug *et al.* 2014). Each replicate in these models was an individual, and we used

host species as a random effect (intercept). We ran models with WBC density as the response variable, and with combinations of host abundance, infection status, and their interaction as explanatory variables. We compared model fits using AIC*c* (Akaike information criterion weighted for small sample size) with the bbmle package in R (Bolker & R Core Team 2014). We used the natural logarithm of host abundance plus one (to account for zeros in host abundance) in all analyses and we created all graphics with the ggplot2 package in R (Wickham 2009).

Results

Across- and within-host-species abundance and parasite prevalence

We found 1720 individuals (29.3%) infected by 87 parasite lineages across the region (Appendix). We created a logistic mixed-effects model relating the infection status of each host individual to the log-transformed abundance of its species at the location it was sampled, incorporating host species and sampling location as random effects (only using "community" sampling locations, see Appendix). The estimate of the coefficient for abundance in the model was positive ($\beta = 0.34$, 95% CI = 0.17, 0.50), and it fit the data better than a null model (i.e., one with no predictor variable but the same random effects structure; likelihood ratio test, $P < 0.001$). Separate models using prevalence of *Plasmodium* ($\beta = 0.25$, 95% CI = 0.05, 0.45, $P < 0.001$ compared with a null model) and *Haemoproteus* ($\beta = 0.55$, 95% CI = 0.31, 0.79, $P < 0.001$) only were generally similar.

We further explored the relationship between parasite prevalence and across-host-species abundance at separate locations using generalized linear models. In each of the seven best-sampled locations, these models generally show a positive relationship between parasite prevalence and across-host-species abundance, as suggested by the logistic mixed effects model (Table 1). Furthermore, in the case of Chicago, nonnative hosts were less often infected (total prevalence) than natives ($\beta = -1.34 \pm 0.24$ SE, $P < 0.001$, Fig. 2). This relationship also held for the prevalence of *Plasmodium* parasites ($\beta = -0.88 \pm 0.27$ SE, $P = 0.002$), and none of the nonnative hosts were infected with *Haemoproteus* parasites in Chicago. We also compared parasite prevalence to within-host-species abundance for several of our well-sampled hosts and generally found non-significant relationships (Table 2; we also found non-significant relationships using less well-sampled host species—results not shown). The one exception was the host *Turdus migratorius*, the abundance of which was weakly negatively related to the prevalence of *Haemoproteus* parasites across its sampled range (Fig. 3).

White blood cells and interspecific host abundance

In the Ozarks, MO location, we quantified WBC densities from 235 individuals of 17 host species as previously described in Ellis *et al.* (2014). We set up several negative binomial mixed effects models with WBC density per individual as the response variable, and combinations of log-transformed host species abundance at that location, infection status, and their interaction as response variables with host species included as a random effect. The best model (based on AIC*c*) included host abundance and infection status as explanatory variables but not their interaction (model weight = 0.54, Fig. 4). The model showed a positive effect of host species abundance on total WBC density ($\beta = 0.33$, 95% CI = 0.11, 0.64), and slightly higher WBCs in infected as compared to uninfected individuals ($\beta = 0.2$, 95% CI = -0.04, 0.41; while the 95% CI overlaps zero for all WBC

types combined, this effect varies in strength with the WBC type analyzed, Ellis *et al.* 2014), and fit the data better than a null model (P = 0.002). The results of separate models for each WBC type were similar to the result of modeling total WBC only and so are not reported separately. WBC densities may be general indicators of infection (Ellis et al. 2014), suggesting that the positive relationship between across-host-species abundance and haemosporidian prevalence may extend to other parasites.

Discussion

Our data demonstrate that within the local assemblages we surveyed, haemosporidian parasites generally infect a higher proportion of abundant hosts than rare hosts. However, within the ranges of individual hosts, parasite prevalence does not generally vary with abundance. These results suggest that variation in local parasite prevalence across host species is related to host-parasite encounter rates (mediated through vector-host encounter rates). We did find one weak negative relationship between within-host-species abundance and parasite prevalence in one host in our dataset (Fig. 3, Table 2). However, without experiments, it is difficult to know whether this negative relationship indicates that parasites limit the population size of the host. Nevertheless, it is worth noting that avian haemosporidian parasites can be highly pathogenic and lead to reduced host survival in some cases (Asghar *et al.* 2015).

In a previous study of birds and their haemosporidian parasites from the Ozarks (Ellis *et al.* 2014), birds infected with haemosporidian parasites generally had higher WBC densities than those that were not infected, but many uninfected birds also had high WBC densities. This finding lead the authors to hypothesize that elevated WBC densities might be general indicators of infection in that system. Here we show that WBC densities are also positively related to across-host-species abundance in the Ozarks even when controlling for the effect of infection status. If WBCs are indeed general indicators of infection, this would suggest that the positive relationship between parasite prevalence and across-host-species abundance could be generalized to other types of parasites. In a study of cliff swallows (*Petrochelidon pyrrhonota*) in Nebraska, Brown & Brown (2002) showed experimentally that increased spleen size was a general indicator of infection by ectoparasites and perhaps other types of parasites. Furthermore, spleen size increased with colony size (i.e., host population density) in these birds suggesting that the transmission of multiple parasites increased with host density.

One of the predictions of the Enemy Release Hypothesis is that nonnative hosts are less impacted by generalized parasites than native hosts (Keane & Crawley 2002). However, many major tests of the hypothesis have focused on the prediction than overall parasite prevalence is lower in the nonnative range of the host than in its native range, rather than comparing prevalence between nonnatives and natives in the same assemblage (e.g., Torchin *et al.* 2003; Marzal *et al.* 2011). Here we show that nonnative hosts had lower parasite prevalence than native hosts in one local assemblage, despite reaching high abundances (Fig. 2). This suggests that nonnative hosts are likely less impacted by the parasites they encounter in their new ranges than the native hosts they coexist with.

Overall our results are consistent with the idea that parasite infection rates are positively related to location-dependent parasite-host encounter rates. This is supported by the haemosporidian parasite data and by the WBC data from the Ozarks, which

suggest that the relationship might be extended to other parasites. We also found that nonnative host species can be less often parasitized than native hosts despite reaching high abundances, offer support to the Enemy Release Hypothesis. While vectors-host encounter rates likely do not determine host-parasite associations (Medeiros, Hamer & Ricklefs 2013), future work should investigate the potential effect of vector feeding rates in possibly driving parasite prevalence within local assemblages.

Acknowledgments
We thank Brandt Ryder, Pete Marra, Keegan Tranquillo, Peter Pyle, and the Indiana MAPS teams for assistance collecting samples from Indiana; Alicia Burke for assistance collecting samples in the Ozarks; Field Biologist Brenda Keith and the Kalamazoo Valley Bird Observatory/Kalamazoo Nature Center for collecting samples in Michigan; the Connecticut Audubon Society for providing samples from their banding station; Steve Latta for providing parasite sequences from Alabama; Woody Walstrom and Diana Outlaw for providing samples from Mississippi; Emma Levenson and Kathleen Riley for assistance with data collection in LA. Our work would not have been possible without the help of numerous government and conservation agencies. We also thank the many volunteers and organizers of the Breeding Bird Survey. Field and laboratory work was supported by the Harris World Ecology Center, the St. Louis Audubon Society, the Missouri Audubon Society, the Curators of the University of Missouri, the NSF Malaria Research Coordination Network, and a University of Missouri-St. Louis Dissertation Fellowship awarded to V.A.E.

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Tables and Figures

Table 1—Results of generalized linear models of the effect of log-transformed host abundance on parasite prevalence (Total prevalence, *Plasmodium* prevalence only, and *Haemoproteus* prevalence only) within the best sampled locations within the region. We report the estimate of the coefficient of log-transformed abundance (β), its 95% confidence interval, and P value. Introduced species were removed from the dataset prior to running these models, except in the case of the Chicago models where a categorical variable coding for native and nonnative host species was included as a covariate.

			Confidence interval		
Location	**Prevalence**	**β**	**2.5%**	**97.5%**	**P value**
Chicago	Total	0.36	0.19	0.55	< **0.001**
	Plasmodium	0.55	0.31	0.81	< **0.001**
	Haemoproteus	-0.12	-0.38	0.15	0.383
Connecticut	Total	0.37	0.07	0.7	**0.02**
	Plasmodium	0.78	0.15	1.7	**0.04**
	Haemoproteus	0.23	-0.12	0.59	0.204
Indiana	Total	0.86	0.39	1.38	**0.002**
	Plasmodium	0.65	0.18	1.13	**0.013**
	Haemoproteus	0.76	0.09	1.44	**0.035**
Michigan	Total	0.47	-0.04	1.04	0.099
	Plasmodium	0.41	-0.19	1.09	0.219
	Haemoproteus	0.45	-0.47	1.57	0.397
Ozarks	Total	0.71	0.37	1.08	< **0.001**
	Plasmodium	0.37	0.04	0.71	**0.038**
	Haemoproteus	0.85	0.28	1.51	**0.01**
St. Louis	Total	0.66	0.25	1.11	**0.005**
	Plasmodium	0.47	0.07	0.91	**0.033**
	Haemoproteus	0.56	0.05	1.13	**0.048**
Tennessee	Total	0.72	0.19	1.3	**0.017**
	Plasmodium	0.4	-0.12	0.94	0.155
	Haemoproteus	0.8	-0.06	1.81	0.1

Table 2—Results of generalized linear models of the effect of log-transformed host abundance on parasite prevalence (Total prevalence, *Plasmodium* prevalence only, and *Haemoproteus* prevalence only) for the five best sampled host species in the region. **N** refers to the number of locations each host species was sampled (at least two individuals sampled per location). We report the estimate of the coefficient of log-transformed abundance (β), its 95% confidence interval, and P value. Models for all other host species were non-significant (P > 0.05).

Host	N	Prevalence	β	Confidence interval 2.5%	97.5%	P value
Cardinalis cardinalis	12	Total	0.21	-1.79	2.48	0.846
		Plasmodium	-0.95	-2.47	0.35	0.21
		Haemoproteus	0.84	-1.57	3.6	0.51
Turdus migratorius	9	Total	-0.56	-1.73	0.42	0.327
		Plasmodium	0.63	-0.02	1.35	0.112
		Haemoproteus	-2.07	-3.63	-0.66	**0.023**
Dumetella carolinensis	9	Total	-0.15	-0.63	0.32	0.55
		Plasmodium	0.01	-0.6	0.63	0.976
		Haemoproteus	-0.23	-0.94	0.47	0.538
Vireo olivaceus	8	Total	0.25	-0.24	0.75	0.352
		Plasmodium	-0.09	-0.75	0.76	0.827
		Haemoproteus	0.28	-0.14	0.71	0.241
Passerina cyanea	8	Total	-0.28	-2.07	1.62	0.765
		Plasmodium	-0.31	-2.2	1.71	0.755
		Haemoproteus	0.27	-0.99	1.89	0.727

Fig. 1

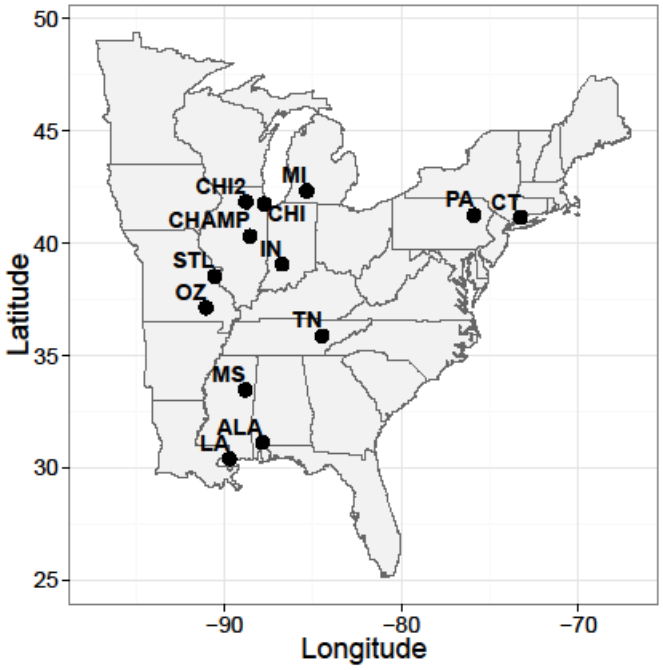

Figure 1—Sampling locations. Detailed information on each location can be found in the Appendix. Location codes are as follows: **ALA** is Alabama, **CHAMP** is Champaign (Illinois), **CHI** is Chicago (Illinois), **CHI2** is western Chicago (Illinois), **CT** is Connecticut, **IN** is Indiana, **LA** is Louisiana, **MI** is Michigan, **MS** is Mississippi, **OZ** is

Ozarks (Missouri), **PA** is Pennsylvania, **STL** is St. Louis (Missouri), and **TN** is Tennessee.

Fig. 2

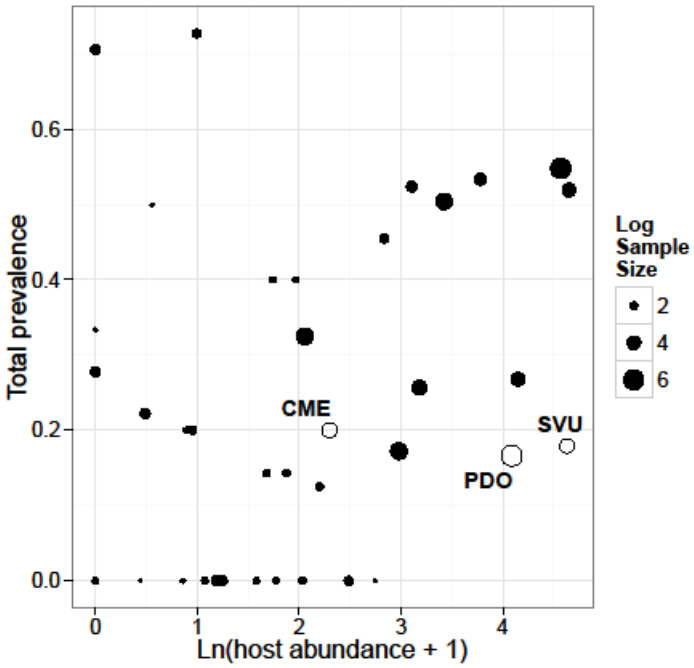

Figure 2—Total prevalence is significantly related to across-host-species abundance in Chicago ($P < 0.001$) when controlling for the effect of the three nonnative host species (hollow circles); CME = *Haemorhous mexicanus*, PDO = *Passer domesticus*, SVU = *Sturnus vulgarus*. Nonnative hosts had lower total prevalence than native hosts ($P < 0.001$). The size of points are scaled to the natural logarithm of sample size.

Fig. 3

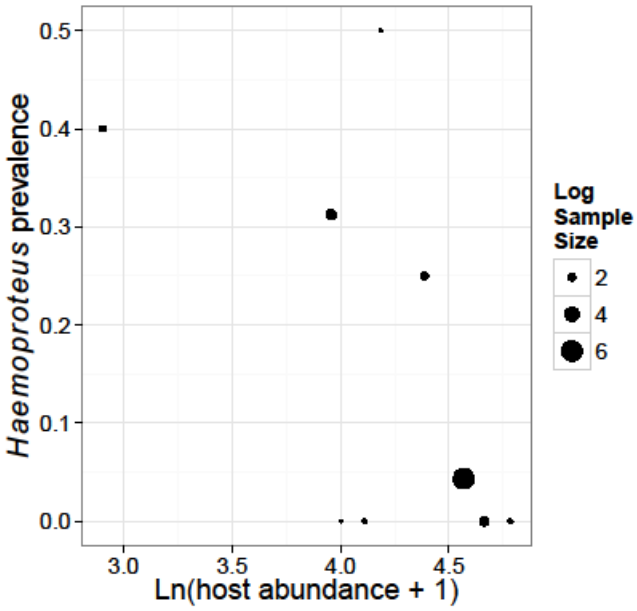

Figure 3—Prevalence of *Haemoproteus* parasites is weakly negatively related to the abundance of the host *Turdus migratorius* (P = 0.023) across the host's sampled range. The smallest points represent a sample size of two individuals per location (Connecticut and Pennsylvania), and the largest point represents a sample size of 438 individuals (Chicago); the size of the points are scaled to the natural logarithm of sample size.

Fig. 4

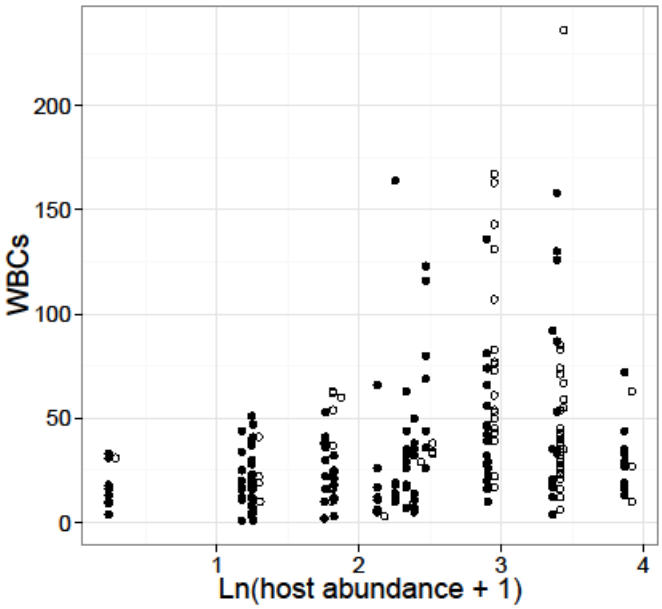

Figure 4—White blood cells (WBCs) per 10^4 red blood cells in relation to across-host-species abundance (N = 17 host species) in the Ozarks, MO. Empty circles (slightly offset for clarity) are individuals infected by a haemosporidian parasite lineage, and filled circles are individuals uninfected by haemosporidian parasites. The best fitting model showed a positive relationship between WBCs and host abundance and a slight increase in WBCs in infected birds (model weight = 0.54).

Appendix
(available upon request, vincenzoaellis@gmail.com)

Chapter 5

Temporal variation in avian haemosporidian parasite prevalence in relation to host-breadth

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Abstract

We surveyed an assemblage of birds and their haemosporidian blood parasites (*Plasmodium* spp. and *Haemoproteus* spp.) over a 12 year period to investigate temporal variation in parasite prevalence (proportion of infected hosts). Overall we identified 11 well-sampled parasite lineages, seven of which exhibited significant variation from year-to-year. Unlike in similar host-parasite assemblages, parasite lineages were not restricted to closely related host species. The distribution of host-breadths (the diversity of host species parasites infect) among those 11 lineages was bimodal, and suggested that parasites were either specialized on a single host species (specialists, N = 4) or infected a broad range of hosts (generalists, N = 7). The standard deviation of prevalence across years (a measure of temporal variability in local parasite population size) was higher in specialist parasite lineages than in generalists, revealing a potential cost to specialization. Finally, average pairwise correlations across years between host abundances and the prevalence of each parasite lineage were more negative than expected by chance, possibly a consequence of parasites limiting host abundance locally.

Introduction

Understanding the emergence and persistence of infectious diseases has important implications for protecting populations of both humans and wildlife (Schrag & Wiener 1995; Daszak, Cunningham & Hyatt 2000; Anderson *et al.* 2004). Mathematical models exploring these themes in human parasites have a long history (see review in Mandal, Sarkar & Sinha 2011). However, parasites that infect multiple host species have only recently begun to receive attention from theorists. One proposal has been that the host-breadth of a parasite (i.e., the diversity of hosts a parasite infects; Poulin, Krasnov & Mouillot 2011) may influence its ability to persist (i.e., not go extinct) in a location (Dobson 2004).

Several studies of parasites infecting wildlife offer glimpses into the short-term temporal dynamics of host parasite associations. For instance, in a 17-year study of the great reed warbler (*Acrocephalus arundinaceus*), Bensch *et al.* (2007) found that the prevalence (i.e., the proportion of host individuals infected) of each of the three most common haemosporidian blood parasites were coupled and cycled regularly with a periodicity of three to four years. Similarly, in a 13-year study of the malaria parasite *Plasmodium mexicanum* infecting a lizard host *Sceloporus occidentalis*, parasite prevalence appeared to cycle at a ten-year interval (Schall & Marghoob 1995). In one of the most extensive temporal parasite-host studies to date, Hudson, Dobson & Newborn

(1998) demonstrated that populations of Scottish red grouse (*Lagopus lagopus scoticus*) and a nematode parasite cycled inversely through time.

Studies of multiple parasites infecting multiple host species over time are less common, but may capture greater temporal variation in parasite populations. For instance, Fallon *et al.* (2004) sampled haemosporidian parasites in bird assemblages on two West Indian islands at two time points almost 10 years apart and found loss and gain of several parasite lineages. Their results hint at the potential for great variation in parasite populations over time in that system, including frequent local extinction and recolonization. Similarly, Svensson-Coelho *et al.* (2013) found significant variation in some haemosporidian parasites recovered from birds over a nine year survey at one locality in Amazonian Ecuador.

Here we report on a survey of avian haemosporidian parasites (*Plasmodium* and *Haemoproteus*) in a single local assemblage of birds over 12 years. We document temporal variation in parasite prevalence and explicitly test whether this variation is related to parasite host-breadth, and whether it is related to temporal changes in host abundance.

Methods

We obtained blood samples (< 1% body weight) from small landbirds (mostly in the order Passeriformes) captured via mist-net from late May to July in the Ozarks of southern Missouri (37°13′ N, 91°04′ W) during 1999-2002, 2005, 2007, and 2011 (for detailed location information and collection procedures see Ricklefs *et al.* 2005 and Ellis, Kunkel & Ricklefs 2014). Sampling effort varied between years (Table 1). We stored blood samples in Puregene® (Germantown, Maryland) or Longmire's (Longmire, Maltbie & Baker 1997) lysis buffer for later DNA extraction. All sampling was performed under appropriate state and federal permits and with the approval of the University of Missouri-St. Louis IACUC.

Molecular analyses

We extracted DNA from the blood samples in lysis buffer using an ammonium acetate, isopropanol precipitation procedure (Svensson & Ricklefs 2009). We screened all samples for haemosporidian DNA using a PCR protocol designed to amplify a small fragment of the parasite mitochondrial genome (Fallon *et al.* 2003). For positive samples we then amplified a section of the parasite cytochrome *b* gene using a combination of protocols (Waldenström *et al.* 2004; Fallon, Bermingham & Ricklefs 2005; Ricklefs *et al.* 2005; Fecchio *et al.* 2013). We designated unique parasite lineages based on cytochrome *b* sequences and host and geographic distributions (Ricklefs *et al.* 2014). We separated mixed infection by phasing when possible (Browning & Browning 2011). Individuals with ambiguous infections due to sequencing error or the inability to phase mixed infections were removed from the analysis. All recovered lineages and their GenBank Accession numbers can be found in the Appendix A (available upon request, vincenzoaellis@gmail.com).

Host abundance

We estimated host abundance from the North American Breeding Bird Survey (https://www.pwrc.usgs.gov/bbs/). We downloaded data from routes within 200 km the

study site for each year of sampling (results were generally robust to changes in this distance). We chose a relatively large distance to increase the number of routes included and therefore reduce the variance of abundance estimates (Sauer, Peterjohn & Link 1994) and we selected only routes that were classified as acceptable by the survey organizers. We then calculated the average abundance of each species across routes within each year.

Statistical analyses

All statistical tests were run in R v.3.1.2 (R Core Team 2014). We calculated annual prevalence of parasite lineages in two ways. The first is an assemblage-level estimate of prevalence, calculated as the number of infections of a parasite in a particular year, divided by the total number of individual birds sampled in that year. This metric assumes that parasites can infect any host in the assemblage, which may be a reasonable assumption given that avian haemosporidian parasites appear to switch hosts nearly at random on an evolutionary timescale (Ricklefs *et al.* 2014). The second prevalence calculation is based on host species from which a particular parasite was recovered at least once during the survey (i.e., suitable hosts). This metric is calculated as the number of infections of a given parasite in a given year divided by the total number of individuals of suitable host species sampled during that year, and assumes fixity in the host species parasites infect. We compared prevalence between years using likelihood ratio G-tests with the function "likelihood.test" in the R package Deducer (Fellows 2012). We calculated non-parametric Spearman rank-order correlations to compare parasite prevalence and host abundance across years. We performed several randomization tests, described in the Results section, and we report two-tailed P values for all of those tests.

We calculated the host-breadth of each parasite lineage using the Gini-Simpson index (Jost 2006) of all the host individuals each parasite infected across all the years of the survey. We used the "raoD" function, and returned its "Dkk" value in the R package picante (setting the "phy" argument to "NULL", Kembel *et al.* 2010) to implement the Gini-Simpson index. We also compared host phylogenetic distances, estimated using the phylogeny of Jetz *et al.* (2012), to a distance matrix representing the diversity of parasite lineages hosts share, using a mantel test run with the function "mantel" in the vegan package in R (Oksanen *et al.* 2015). The latter distance matrix was calculated as Bray-Curtis dissimilarities (Odum 1950; Bray & Curtis 1957; Legendre & Legendre 1998) between hosts based on the parasites they were infected by. All data analyzed for this study can be found in Appendix B (available upon request, vincenzoaellis@gmail.com).

Results

Parasite host-breadth and temporal variation in prevalence

We screened 1438 individuals of 49 species and found 329 infected with haemosporidian parasites (22.9%). Overall total parasite prevalence did not vary significantly across years ($G = 7.05$, $df = 6$, $P = 0.316$; Table 1). We recovered 42 distinct parasite lineages (Appendix A), of which, 11 were recovered more than eight times (Table 2). Of those 11 lineages, the prevalence of seven varied significantly through time (Table 2, Fig. 1).

To determine if parasite distributions across hosts were constrained by host phylogenetic relatedness (e.g., Medeiros, Hamer & Ricklefs 2013) we compared a matrix of host phylogenetic distances to a matrix of host distances based on the parasites they shared (see Methods) with a Mantel test. We restricted this analysis to the 11 parasite

lineages recovered more than eight times and to host species they infected at least once in the sample (N = 25 host species). The Mantel test was not significant (r = 0.04, P = 0.335) suggesting that parasite distributions are not constrained by host relatedness in this assemblage.

We therefore calculated parasite host-breadth using a Gini-Simpson index, which does not account for phylogenetic relationships between hosts. The distribution of host-breadth was markedly bimodal (Fig. 2), suggesting the existence of specialist and generalist parasites. Indeed, parasite lineages with host-breadths less than 0.5 seemed to mostly specialize on two common hosts in the assemblage, *Vireo olivaceus* (primarily infected by lineages OZ 10, OZ 12, and OZ 05) and *Icteria virens* (primarily infected by OZ 08).

To capture temporal variation in prevalence with a single metric, we calculated the standard deviation of arcsin, square root transformed prevalence across years for each parasite. We calculated this metric using prevalence based on all hosts in the assemblage, and separately based on hosts the parasites were observed infecting. We ran two ANOVAs (restricted to parasite lineages sampled more than eight times) with the standard deviation of parasite prevalence across years (as previously described either using assemblage-wide prevalence or prevalence based on the parasite's observed hosts) as the response variable, and a categorical variable coding for specialists (host-breadth < 0.5) and generalists (host-breadth > 0.5), and another coding for parasite genus (*Plasmodium*/*Haemoproteus*) as explanatory variables. Parasite genus was not significant in either model (P > 0.3 in both models), and so was dropped. The standard deviation of parasite prevalence across years was greater in specialists than in generalists in the final models, although the effect was more pronounced and statistically significant when prevalence was calculated using the observed hosts of the parasites ($F_{1,9}$ = 9.18, P = 0.014), rather than all the hosts in the assemblage ($F_{1,9}$ = 4.21, P = 0.071; Fig. 3).

Prevalence and host abundance

To clarify the relationship between host abundance and parasite prevalence across years, we calculated pairwise Spearman correlations (ρ) between the abundances of all hosts and the prevalences of all parasite lineages across years. We then compared the mean observed correlation coefficient to a randomized distribution of mean correlations obtained by row-shuffling the matrix of host abundances and re-calculating the correlations (9999 times). We restricted this analysis again to the 11 parasite lineages sampled more than eight times, and to all hosts sampled (whether infected or not). However, three host species that we sampled were not observed on Breeding Bird Survey routes during the sampling years (*Setophaga magnolia*, *Oreothlypis peregrina*, and *Cardellina canadensis*), and so were dropped from this analysis, leaving 46 host species, and 506 pairwise correlations between hosts and parasites. Regardless of whether prevalence was calculated using the entire host assemblage (mean ρ = -0.16 $\pm$ 0.02 SE) or only the observed hosts of each parasite (mean ρ = -0.14 $\pm$ 0.02), mean correlation coefficients were more negative than expected by chance (P = 0.007 and P = 0.038, respectively). Interestingly, the most negative correlations we observed were between hosts and parasites that were not associated in our survey. For example, the *Plasmodium* parasite OZ 01 was negatively related (ρ = -0.96, using both prevalence measures) to the abundance of the host *Baeolophus bicolor*, which was never recorded as infected with OZ

01. Similarly, the *Plasmodium* parasite OZ 08, primarily found infecting the host *Icteria virens*, was negatively related to the abundances of *Mniotilta varia* (ρ = -0.95) and *Seiurus aurocapilla* (ρ = -0.95), while never having been recorded infecting either species. In both cases, the hosts involved were well-sampled (Appendix B).

Discussion

We found substantial variation in the prevalence of multiple haemosporidian parasite lineages in a local assemblage of birds over a 12-year period, and this variation was related to host-breadth of the parasite lineages. Specialized parasites seemed to vary more through time than generalist parasites (Fig. 1 and 3). This result suggests that the ability to utilize more host species might buffer parasites against temporal variation and potentially lead to greater parasite persistence over time (Dobson 2004).

We suspect that specialized parasites are more variable through time than generalists because of temporal changes in the proportion of immune individuals of their primary host species. In a three year study of *Plasmodium falciparum* in a human population in Sudan, Babiker, Satti & Walliker (1995) found significant temporal antigenic variability in the parasite, potentially a result of changes in the proportion of resistant hosts, among other possibilities. However, we do not have data to address this hypothesis and can only speculate. Perhaps some of the variation we observed can be explained by temporal changes in the local assemblage of vectors and the parasites those vectors harbored (Kim & Tsuda 2012; Lalubin *et al.* 2013). Kim & Tsuda (2012) recorded densities of mosquitos and the diversity of haemosporidian parasites they were infected by in a single locality in Japan over a four year period. The authors found that the lineage composition of parasites within at least one mosquito vector (*Culex inatomii*) appeared to change between years. Such changes in the associations of vectors and parasites might lead to prevalence changes in the vertebrate host assemblage as well.

We also found no evidence of parasite-host associations being constrained by host phylogenetic relatedness. This is in contrast with a study of a similar host-parasite assemblage in Chicago, IL (Medeiros *et al.* 2013). This result indicates that the importance of the evolutionary relatedness of hosts may change across the region, highlighting the geographic idiosyncrasies of host-parasite relationships. Finally, we found that parasite prevalence is generally negatively related to host abundance across years in this location. Interestingly, the most negative correlations we found were between hosts and parasites that were not associated in our study. While these correlations could be spurious (in the sense that hosts and parasites are not responding to one another, but to other unmeasured variables), it is tempting to speculate that parasites may be limiting host population size within this local assemblage. Furthermore, high mortality rates in birds infected by particularly pathogenic parasites might bias capture rates and prevent us from recording all host parasite associations (Valkiūnas 2005).

Overall our results are indicative of how little we know about variation in assemblages of multiple hosts and multiple parasites through time. Ultimately studies should attempt to incorporate both geographic and temporal scales into studies of host-parasite associations, as many temporal trends may depend on geography. For example, in a 30 year study of monarch butterflies (*Danaus plexippus*) and a protozoan parasite *Ophryocystis elektroscirrha* at various locations across North America, Altizer, Oberhauser & Brower (2000) showed that temporal variation in parasite prevalence did

not vary predictably across years and was location dependent. Ecological assemblages of multiple interacting hosts and parasites across a region are likely to be no less complicated, but deserve attention in the effort to understand the dynamics of emerging infectious disease.

Acknowledgments
We thank Alicia Burke, John Faaborg, and the entire Missouri Ozark Forest Ecosystem Project for providing support and access to banding stations in the Ozarks; the Missouri Department of Conservation for providing housing and logistical support; Melanie Kunkel for providing invaluable help collecting samples in 2011; many students in the Ricklefs Lab at UMSL for laboratory assistance; and Matt Medeiros and Eloisa Sari for helpful discussions. We also thank the thousands of volunteers and organizers of the North American Breeding Bird Survey for all their work putting together a wonderful dataset. The study was funded by the Curators of the University of Missouri, the Whitney Harris World Ecology Center, and the Audubon Society of Missouri. VAE was supported by a University of Missouri-St. Louis Dissertation Fellowship.

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Figures and Tables

Table 1—Number of host individuals sampled (N) and the number of infected individuals in each year of the survey. The probability of infection did not differ by year (G = 7.05, df = 6, P = 0.316).

Year	N	Infected
1999	204	56
2000	136	37
2001	209	42
2002	190	35
2005	105	25
2007	111	24
2011	483	110

Table 2—The 11 best sampled parasite lineages, including the genus they belong to, the number of infections recorded across all years (N), and host-breadth (HB). We report two G statistics and associated P values from tests comparing whether the probability of infection of each lineage varied by year, calculated by either assuming parasites could infect all hosts in the assemblage (Assemblage), or could only infect hosts they were observed infecting at least once (Host Range); see Methods for further details.

				Assemblage		Host Range	
Lineage	Genus	N	HB	$G_{df=6}$	P value	$G_{df=6}$	P value
OZ 01	*Plasmodium*	30	0.802	8.00	0.238	5.34	0.501
OZ 02	*Haemoproteus*	20	0.770	11.98	0.063	11.49	0.074
OZ 03	*Haemoproteus*	14	0.786	8.89	0.180	8.26	0.220
OZ 04	*Plasmodium*	14	0.776	24.01	**0.001**	24.25	< **0.001**
OZ 05	*Haemoproteus*	26	0.269	16.16	**0.013**	14.34	**0.026**
OZ 06	*Plasmodium*	12	0.861	5.40	0.493	5.95	0.428
OZ 08	*Plasmodium*	43	0.174	27.76	< **0.001**	30.39	< **0.001**
OZ 10	*Haemoproteus*	64	0.091	24.78	< **0.001**	31.22	< **0.001**
OZ 12	*Haemoproteus*	22	0.169	18.38	**0.005**	15.93	**0.014**
OZ 14	*Plasmodium*	19	0.848	15.35	**0.018**	12.95	**0.044**
OZ 16	*Haemoproteus*	9	0.840	31.11	< **0.001**	30.37	< **0.001**

Fig. 1

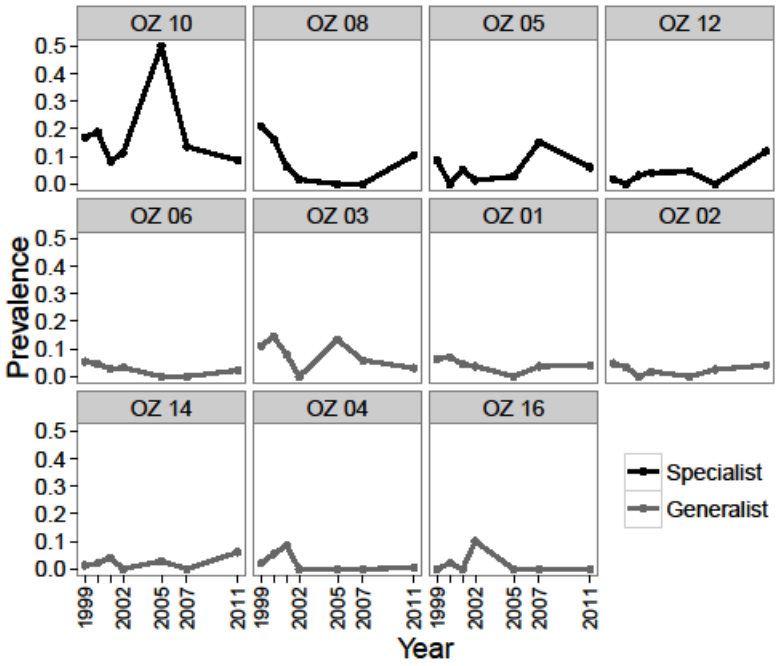

Figure 1—Annual prevalences of the 11 parasite lineages recovered more than eight times. Here prevalence was calculated using only the hosts parasites were observed infecting (see Methods). The shade of the lines corresponds to the host-breadth of the parasites with black lines representing host specialists and gray lines representing host generalists.

Fig. 2

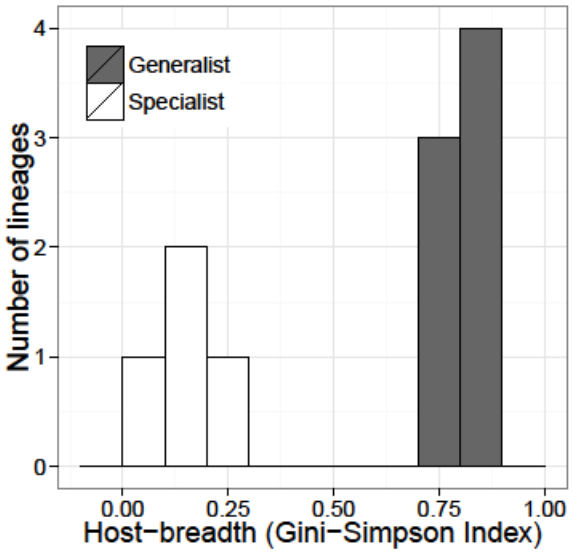

Figure 2—A histogram of host-breadths (calculated as a Gini-Simpson index of the hosts parasites were found infecting) of the 11 parasite lineages recovered more than eight times appears bimodal, suggesting that that some parasites (those with host-breadths lower than 0.5) are host specialists, and others (those with host-breadths greater than 0.5) are host generalists.

Fig. 3

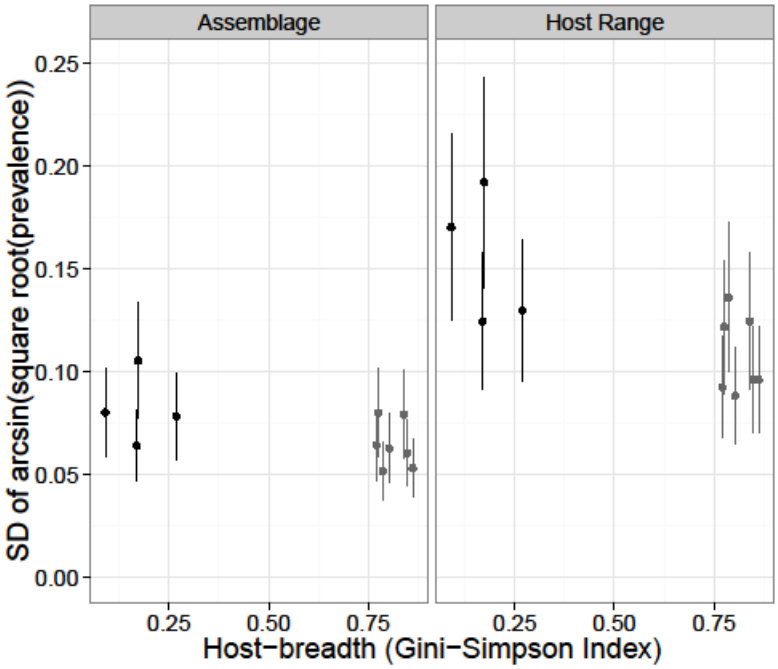

Figure 3—Points represent the standard deviations of arcsin, square root transformed prevalences across years and parasite host-breadths for each parasite lineage. Error bars are standard errors of the standard deviations. The two panels represent the two ways of calculating prevalence—either using the whole host assemblage (Assemblage), or using only the hosts that parasites were found infecting (Host Range); see Methods for details. Specialist parasites are colored in black and generalists in gray. Standard deviation of the prevalences of specialist parasites were higher than those of generalist parasites although the effect was weaker in the assemblage-based calculation of prevalence compared with the calculating prevalence based on hosts within a parasite's host range ($P = 0.071$ and $P = 0.014$, respectively).