HOW PARASITES AFFECT, AND ARE AFFECTED BY, HOST PHYSIOLOGY, BEHAVIOR, AND BREEDING SYSTEM

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July 12, 2017

Samuel P. Slowinski

HOW PARASITES AFFECT, AND ARE AFFECTED BY, HOST PHYSIOLOGY, BEHAVIOR, AND BREEDING SYSTEM

Parasites comprise a striking diversity of lifeforms, and probably evolved from free-living organisms many times. It has even been argued that most species on earth are parasites. Parasites can have profound effects on their hosts, and the biology of hosts, in turn, affects the evolution and spread of parasites. My dissertation research examined the effects of parasites on the physiology, behavior, and breeding systems of their hosts, as well as the effects of host physiology and behavior on parasitism. In chapters one and two, I focused on the disease ecology of a natural system: haemosporidian (blood) parasites and an avian host, the dark-eyed junco. In chapter one, I measured the association between junco long-distance migration behavior and infection with haemosporidian parasites. I found that a migrant population of juncos maintains a significantly lower prevalence of haemosporidian parasite infections relative to a closely related and seasonally sympatric sedentary junco population, suggesting that long-distance host migration may be associated with reduced parasitism. In chapter two, I showed that experimental elevation of circulating testosterone levels in hosts does not affect the prevalence of haemosporidian parasite infections in a wild population of juncos, and that haemosporidian infections do not affect host telomere degradation. I also showed that the prevalence of haemosporidian parasite infections increases with host age. In chapters three and four, I used an experimental host-parasite system in the lab to assess how coevolving parasites affect the breeding system of their hosts. In chapter three, I showed that coevolving bacterial parasites (*Serratia marcescens*) can constrain the spread of self-fertilization into obligately outcrossing

populations of nematode (*Caenorhabditis elegans*) hosts. This result supports the Red Queen hypothesis and contributes to a large body of evidence that antagonistic coevolution between hosts and parasites can maintain biparental sex. Finally, in chapter four, I showed that the presence of parasites (*S. marcescens*) in the environment does not induce plastic changes in the propensity to outcross in hosts (*C. elegans*) capable of both outcrossing and self-fertilization, and that coevolutionary interactions with *S. marcescens* parasites does not cause *C. elegans* hermaphrodites to evolve a higher propensity to outcross.

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Parasites comprise a striking diversity of lifeforms, and parasites probably evolved from free-living organisms many times (Poulin 2007). It has even been argued that most of the species on earth are parasites (Windsor 1998). Parasites can have profound effects on their hosts, and the biology of hosts, in turn, affects the evolution and spread of parasites.

My dissertation research examined the effects of parasites on the physiology, behavior, and breeding systems of their hosts, as well as the effect of host physiology and behavior on parasitism. In chapters one and two of my dissertation, I focused on the disease ecology of a natural system: haemosporidian parasites and an avian host, the dark-eyed junco. The haemosporidians are a diverse group of obligate, heteroxenous, protist parasites that infect amphibians, reptiles, birds, and mammals, and use blood-sucking dipteran insects as vectors (Valkiūnas 2005). Haemosporidians include the malaria-causing genus *Plasmodium* which, infects hundreds of millions of people, and kills hundreds of thousands of people every year (Centers for Disease Control and Prevention Website 2015). For this reason, haemosporidian parasites have been the focus of intensive research in the biomedical community. Avian haemosporidians served a leading role as a model for human malaria in the first half of the 20th century, but interest in avian malaria faded in the latter half of the 20th century with the discovery of malaria parasites of rodents, which served as a more convenient lab model (Valkiūnas 2005). Still, the amazing diversity of avian haemosporidian parasites and of the hosts that they infect, makes them a great model for understanding the ecology and evolution of host-parasite interactions.

In chapter one of my dissertation, I measured the association between host long-distance migration behavior and haemosporidian parasite infections *within* a songbird host species, the dark-eyed junco. The relationship between host migration patterns and the prevalence, virulence,

and evolution of parasites has been an emerging area of interest in disease ecology. Long-distance host migrations can be predicted to *increase* or to *decrease* parasitism, depending on the assumptions that researchers make about the ecological context and the biology of the host and parasite (reviewed in Altizer et al. 2011). The importance of understanding the relationship between host migration and disease spread has taken on a new urgency in the face of global climate change and associated changes in animal migration patterns (reviewed in Altizer et al. 2011). To date, many studies on the relationship between host migration and parasitism have focused on invertebrate migratory hosts, with the monarch butterfly (*Danaus plexippus*) and its protozoan parasite *Ophryocystis elektroscirrha* serving as a popular model system (e.g. Altizer et al. 2000, Bradley and Altizer 2005, Bartel et al. 2011, Fritzsche McKay et al. 2016, Satterfield et al. 2016). In vertebrates, some research has found that long-distance migration is associated with reduced parasitism (e.g. Poulin et al. 2012) while other research suggests that migration can be associated with increased parasitism (e.g. Kelly et al. 2016), and very little research has focused on the relationship between parasitism and host migration *within* vertebrate host species. In the first chapter of my dissertation research I sought to fill in some of the gaps in our knowledge about the relationship between parasitism and migration within host species. In a correlative study, I compared haemosporidian parasite infections between a long-distance migrant population of a songbird host, and a closely related conspecific sedentary host population that shares wintering habitat.

In chapter two, I tested the prediction that experimental elevation of the circulating steroid hormone testosterone juncos would increase the prevalence of haemosporidian parasite infections. This prediction was based on a classic hypothesis, called the Immunocompetence Handicap Hypothesis (ICHH), which posits that testosterone increases investment of energy and

resources into reproduction at the expense of investment in immune function (Folstad and Karter 1992). The ICHH has received mixed support (Roberts et al. 2004). I also tested whether haemosporidian parasite infections were associated with an increased rate of telomere loss in junco hosts, which has recently been shown in other songbirds (Asghar et al. 2015, Asghar et al. 2016). The effect of chronic haemosporidian infections on telomere shortening has the important implication that long-term haemosporidian infections may be affecting the health and longevity of their hosts, even when infection intensities are low and even when there are no overt symptoms of infection. Finally, in chapter two, I also measured how haemosporidian parasite infection is associated with host age. Some previous studies have demonstrated that the prevalence of blood parasite increases with age in avian hosts (Garvin and Greiner 2003, Marzal et al. 2016), while other studies have demonstrated that infection prevalence decreases with age (van Oers et al. 2010, Hammers et al. 2016). Overall, the relationship between host age and infection prevalence is a fundamental natural history question that is largely unexplored in natural populations. In my research, I sought to help fill this gap using a rare resource: haemosporidian infection data from a long-term study of known-aged birds in a natural population.

In chapters three and four, I used an experimental host-parasite system in the lab to assess how coevolving parasites affect the breeding system of their hosts. How biparental sex is maintained as the dominant mode of reproduction in most plant and animal taxa is an important puzzle in evolutionary biology, given the enormous costs of biparental sexual reproduction (Maynard Smith 1978, Gibson et al. 2017). The Red Queen hypothesis (RQH) is a leading hypothesis to explain the maintenance of biparental sexual reproduction, and posits that coevolutionary interactions with parasites generates negative frequency dependent selection on

host populations, favoring biparental sex because it produces offspring with rare genotypes that are more likely to resist infection (Maynard Smith 1978, Hamilton 1980). The RQH has received broad empirical support across diverse study systems (reviewed in Lively and Morran 2014). In my dissertation research, I took advantage of a host-parasite experimental system (*Caenorhabditis elegans* hosts and *Serratia marcescens* parasites) that has emerged recently as a model for studying how antagonistic coevolutionary interactions can affect breeding system evolution.

In support of the RQH, previous research in this experimental system has already shown that coevolutionary interactions with parasites can favor increased rates of outcrossing (as opposed to self-fertilization) in host populations (Morran et al. 2011). In chapter three, I built on this previous research by testing the important and previously untested prediction of the RQH that coevolutionary interactions with parasites can constrain the spread of self-fertilization into outcrossing host populations.

Now that a large body of evidence has emerged showing that coevolutionary interactions with parasites can maintain high outcrossing rates in host populations, an important next step is to determine the mechanisms by which coevolving parasites can maintain high outcrossing rates in host populations. In chapter four, I addressed this question by testing whether exposure to parasites can induce plastic increases in the propensity of hosts to reproduce by outcrossing as opposed to self-fertilization, and whether long-term coevolutionary interactions with parasites can cause host populations to evolve higher outcrossing propensities.

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Chapter 1

Sedentary dark-eyed juncos (*Junco hyemalis*) maintain higher prevalence of haemosporidian infections than migratory juncos during seasonal sympatry

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Abstract

Several hypotheses relating migratory behavior to the likelihood of parasitism have entered the literature, some making similar predictions and others making conflicting predictions. To assess how migratory behavior of animal hosts is associated with parasitism, we compared haemosporidian parasite infections between two closely related populations of a common North American sparrow, the dark-eyed junco, that co-occur on shared wintering grounds during nearly half of their annual cycles. One population is sedentary and winters and breeds in the Appalachian Mountains. The other population is migratory and winters sympatrically with the sedentary population, then flies (≥ 900 km) northwards to breed in Canada, Alaska, and/or Northern New England. The populations were sampled in nature at the beginning of winter and again after confining them in a captive common environment until the spring. We found significantly higher prevalence of haemosporidian parasite infections in the sedentary

population, suggesting that long-distance migration may reduce the prevalence of parasite infections at the population level. Among infected juncos, we found no difference in parasite densities (parasitemias) between the sedentary and migrant populations. Our results are consistent with the Migratory Culling hypothesis, which posits that heavily infected animals are less likely to survive long-distance migration, and with the Migratory Escape hypothesis, which posits that long-distance migration allows host populations to seasonally escape areas of high levels of infestation. However, our results are inconsistent with the Migratory Exposure hypothesis. We found no evidence for differences in haemosporidian parasite phylogenetic diversity between populations, and no evidence for winter dormancy of the parasites. We found a positive co-variation between parasitemia and gonad mass, consistent with the immunocompetence handicap hypothesis. Overall, our results suggest that long-distance migration in host populations, and host breeding physiology, may markedly affect parasite infections.

Introduction

Long-distance migration is found in all major animal groups (Dingle and Drake 2007). Animals likely evolved long-distance migration to track seasonal resources (reviewed in Alerstam et al. 2003), avoid harsh environmental conditions, and/or reduce predation risk (McKinnon et al. 2010). Regardless of their evolutionary origins, long-distance migrations influence the physiology, behavior, and fitness of migratory animals throughout their annual cycles, and they fundamentally alter their interactions with pathogens and parasites. For example, as migratory animals move seasonally across the landscape, entirely new spatial and temporal dynamics emerge with respect to host-parasite interactions, adding complexity to our theoretical

frameworks and empirical characterizations of critically important topics in both disease ecology and host-parasite co-evolution. In the present study, we leveraged intra-specific variation in migratory strategies to examine how the migratory behavior of animal hosts relates to the prevalence, intensity, and diversity of their parasites, and the timing of parasite life cycles. Specifically, we compared haemosporidian parasite infections in a sedentary population of dark-eyed juncos (*Junco hyemalis carolinensis*) with infections in a closely related seasonally sympatric migrant junco population (*Junco hyemalis hyemalis*) in order to assess how host migratory strategies are associated with the prevalence and intensity (parasitemia) (objective 1), the phylogenetic diversity (objective 2), and the timing of seasonal dormancy (objective 3) of haemosporidian parasites. We also compared gonad mass, a metric of seasonal breeding physiology, with haemosporidian parasitemias in order to assess how host reproductive physiology may be linked to haemosporidian parasite infections (objective 4).

Several non-mutually exclusive hypotheses have been put forth to predict the association between long-distance host migratory behavior and the dynamics of parasite infections, including their prevalence, diversity, and virulence (reviewed in Altizer et al. 2011). The Migratory Culling hypothesis posits that heavily infected animals are less likely to survive the physiological stresses of long-distance migration (Bradley and Altizer 2005). Consequently, long-distance migration weeds out infected animals from host populations via natural selection. The Migratory Escape hypothesis posits that long-distance migration allows host populations to seasonally escape areas that have become heavily infested with parasites (Loehle 1995). Hence the Migratory Culling hypothesis and the Migratory Escape hypothesis both predict that parasite prevalence should be higher in sedentary host populations than in closely related migratory host

populations. This prediction has received some empirical support from studies on both migratory vertebrates and invertebrates (Altizer et al. 2000, Poulin et al. 2012, Satterfield et al. 2016).

On the other hand, the migratory exposure hypothesis (Hannon et al. 2016) posits that migrating through multiple different habitats may increase parasite exposure in migratory animals. Migratory animals may be exposed to different parasites at their breeding grounds, stopover sites, and wintering grounds, and consequently may accumulate a higher prevalence and diversity of parasites than sedentary animals (Moller and Erritzoe 1998). See Table 1 for a summary of hypotheses and predictions about how long-distance host migration should be associated with parasite prevalence.

Hypothesis	Prediction
Migratory Culling	**Sedentary prevalence > Migrant prevalence**
Migratory Escape	**Sedentary prevalence > Migrant prevalence**
Migratory Exposure	**Sedentary prevalence < Migrant prevalence**

Table 1: A summary of the primary hypotheses and predictions discussed in the present study, and the predictions they make about haemosporidian infection prevalence in the junco populations.

In support of the Migratory Exposure hypothesis, several studies have found that migratory species of birds harbor a higher diversity of parasites relative to non-migratory species (Jenkins et al. 2012, Koprivnikar and Leung 2015, Hannon et al. 2016). While such among-species comparisons suggest generally that host migration may increase exposure to parasites and infection risk, fewer studies have made comparisons within-species, where the interpretive challenges of phylogenetic history and host-specificity should be minimized. In the only published study that we are aware of that compared parasite prevalence with migratory propensity *within* a vertebrate host species, Kelly et al. (2016) found a higher prevalence of

haematozoan parasite infections in adult long-distance migrant song sparrows (*Melospiza melodia*) relative to conspecifics that migrated shorter distances. Given the conflicting associations between migration and parasitism in studies published so far, more research is needed to assess how migratory behavior is associated with parasite prevalence and diversity among closely related populations *within* vertebrate species.

Objective 1: Association between host migration and infection prevalence and parasitemia

In the present study, our first objective was to explore how variation in host migratory behavior is associated with the prevalence and parasitemia of a widespread and diverse class of parasites, the haemosporidia, that infect a songbird host, the dark-eyed junco (*Junco hyemalis*). Specifically, we compared haemosporidian parasite infections in the bloodstream, during seasonal sympatry, between a sedentary junco population and a closely related migrant junco population that share wintering grounds in Virginia, USA. Based on the Migratory Culling hypothesis and the Migratory Escape hypothesis, we predicted that the prevalence of haemosporidian parasite infections would be higher in the sedentary junco population than in the migrant population. Alternatively, based on the Migratory Exposure hypothesis, we predicted that the prevalence and diversity of haemosporidian parasites would be higher in the migrant population than the sedentary population (Table 1).

Objective 2: Association between host migration and parasite diversity

Our second objective was to test whether the diversity of haemosporidian parasites differed between parasites infecting juncos in the sedentary population versus the migrant population. Based on the Migratory Exposure hypothesis, we predicted that, if long-distance migration increases exposure of animals to diverse parasites, then the migratory population of

juncos should harbor a higher diversity of haemosporidian parasites relative to the sedentary junco population.

Objective 3: Measuring seasonality in the parasite lifecycle

In temperate climates, some avian haemosporidian parasites alternate between an active stage in the spring and summer, during which the parasites grow and replicate and produce transmissible life stages in the bloodstream of their host, and a dormant stage in the winter during which the parasites sequester in the host organs (reviewed in Valkiūnas 2005). Our third objective was to determine whether haemosporidian parasites in our study system exhibit this seasonal pattern. We predicted that prevalence and parasitemia of parasites detectable in the host blood stream would be lower during the winter (when parasites were dormant in the host organs) and would increase in the spring (when parasites re-emerged and started replicating in the blood stream). We tested these predictions by measuring haemosporidian prevalence and parasitemia in blood samples collected from juncos in both populations during the winter (December) and spring (early March and late March). Additionally, we hypothesized that, if haemosporidians are adapted to match the timing of their lifecycles with the availability of vectors in their environment, then the sedentary population (which breeds further South in a warmer climate where they likely encounter haemosporidian vectors earlier) would exhibit an earlier spring emergence of haemosporidians into the blood stream relative to the migrant population.

Objective 4: Association between host breeding physiology and haemosporidian parasitemias

Our fourth objective was to assess how haemosporidian parasite infections were associated with breeding physiology in late March *among-individuals* within the sedentary and migrant junco populations. We compared circulating parasitemias with previously published (Fudickar et al. 2016a) gonad mass data from the same juncos. In this analysis, gonad mass

serves as a metric of breeding condition. We predicted that, if investment in immune function trades off with investment in reproduction, as predicted by the immunocompetence handicap hypothesis (Folstad and Karter 1992), then parasitemias should be positively correlated with reproductive condition.

Methods

Study system

We compared haemosporidian parasite infections between two populations of dark-eyed junco hosts. Juncos in one of the populations (the sedentary population, *Junco hyemalis carolinensis*) are generally year-round residents, breeding during the spring and summer, with most individuals remaining near their territories during the non-breeding season. In contrast, the other population (the migrant population, *Junco hyemalis hyemalis*) co-occurs with *J. h. carolinensis* during non-breeding, but migrates northwards (≥ 900 km to Canada, Northern New England, and/or Alaska) to breed (Miller 1941, Nolan et al. 2002). The sedentary and migrant junco subspecies are thought to have diverged within the last 15,000 years, since the last major glaciation (Friis et al. 2016), and can be distinguished morphologically (Nolan et al. 2002). The sedentary and migrant junco subspecies do not hybridize at our field site in Virginia as far as we know. Haemosporidians are protozoan parasites that infect vertebrate red blood cells and are transmitted by dipteran insect vectors. Haemosporidians include the malaria-causing genus *Plasmodium*. Avian haemosporidian parasites can be pathogenic in wild populations (e.g. Valkiūnas 2005, Lachish et al. 2011, Asghar et al. 2015).

Bird capture, and sampling in the field

The details of the capture and housing methods used in this study have been described previously (Fudickar et al. 2016a). Briefly, sedentary (*Junco hyemalis carolinensis*) and migrant (*Junco hyemalis hyemalis*) juncos were captured from 4-12 December 2013, at the Mountain Lake Biological Station in Pembroke, VA (37.37°N, 80.52°W). Juncos were captured in mist-nets and potter traps baited with cracked corn and millet. Population status (sedentary or migrant) was determined based on bill coloration, plumage, and wing chord (Nolan et al. 2002). At the time of capture, a blood sample was collected by pricking the brachial vein with a sterile needle. For blood DNA samples collected in the field in December, about 150ul of blood was collected into a microcapillary tube, and was stored in a buffer preservative (Longmire's solution) at 4°C until the DNA was extracted.

Housing and sampling of birds during the captivity experiment

After capture, birds were housed briefly (1-10 days) in identical outdoor aviaries at the Mountain Lake Biological Station. On December 14, birds were transported to Bloomington, Indiana, where they were brought into an indoor aviary environment until they were resampled in early and late March. The purpose of resampling the juncos in captivity in the spring was to test the hypothesis that haemosporidian parasites go dormant in the host organs during the winter and re-emerge and start replicating in the host bloodstream during the spring. Because haemosporidian parasites are obligately vector-transmitted, and because there were no vectors in our indoor aviary environment, we assume that there was no transmission of haemosporidian parasites among our focal juncos while they were being held in captivity.

In the indoor aviary environment, birds were fed ad libitum, and maintained on a photoperiod that matched the natural photoperiod at their capture site (Mountain Lake Biological Station, VA, 37.37°N, 80.52°W). They were housed in mixed flocks with equal numbers of

migrants and sedentary birds until February 27, when they were individually housed in cages in seven replicate rooms. Each room housed 3 migrants and 3 sedentary birds. The temperature was maintained at 16º ± 2º C (Fudickar et al. 2016a). Blood DNA samples for measuring haemosporidian parasites were collected again from each bird in early March (March 4-5), and again in late March (March 25-27). After collection of blood from the birds in captivity, plasma was separated from the red blood cells by centrifugation, the plasma (supernatant) was removed from the tube with a sterile Hamilton syringe, and the red blood cells were stored at -20° C until the DNA was extracted.

Metyrapone implant treatments

For the purposes of another experiment, unrelated to the research goals of the present study, we implanted half the birds with metyrapone, a drug that blocks the production of glucocorticoids. We found no effect of metyrapone on any measures (haemosporidian parasitemia, circulating corticosterone and testosterone, morphology) so we pooled metyrapone implant treatment birds with control birds for all analyses presented in this study (see supplementary materials for details on metyrapone treatment).

Animal research ethics statement

All sampling procedures were approved by the Indiana University Institutional Animal Care and Use Committee and conducted under scientific collecting permits issued by the Virginia Department of Game and Inland Fisheries (permit 47553) and the US Fish and Wildlife Service (permit MB093279).

Measuring haemosporidian parasite infection

DNA Extractions

Genomic DNA (gDNA) was extracted from blood, using IBI Scientific MINI Genomic DNA kits (IB46701). For the December samples, about 100μL of avian blood/Longmire's solution from each sample was used for the DNA extractions. For the early March and late March samples, about 32.5 μl of red blood cells (after plasma had been removed) was used for the DNA extractions. gDNA was measured with a Nanodrop spectrophotometer (Take 3) and samples with insufficient quality or concentration of DNA were re-extracted prior to measuring haemosporidian infections.

Quantitative PCR to determine parasitemia and prevalence

We used a quantitative PCR (qPCR) to estimate the prevalence of haemosporidian parasite infections in the host populations, and the relative abundance (parasitemia) of haemosporidian parasites in the blood samples of infected birds (Knowles et al. 2010). Parasitemia levels may be predictive of the degree of pathology haemosporidian parasites are likely to cause in birds, as wild birds with higher parasitemias of some haemosporidian parasite lineages have been shown to experience greater reproductive and survival costs relative to birds with lower parasitemias (e.g. Asghar et al. 2011). We used the primers L9 5'-AAA-CAATTCCTAACAAAACAGC-3' and NewR 5'ACATCCAATCCATAATAAAGCA-3' which target a 188-bp region of the cytochrome B gene, previously published by (Knowles et al. 2010). qPCR estimates of parasitemia using these primers were previously shown to be strongly positively correlated with *Plasmodium* infection status determined by a restriction enzyme-based assay to distinguish among avian haemosporidians (Beadell and Fleischer 2005, Knowles et al. 2010).

Absolute quantification was done using qPCR. To create the PCR template for the standard curve, the full length haemosporidian *cytb* gene from a positive sample was amplified

using the primers DW2 5'-TAATGCCTAGACGTATTCCTGATTATCCAG-3' and DW4 5'-TGTTTGCTTGGGAGCTGTAATCATAATGTG-3' (Perkins and Schall, 2002). The amplicon (1356 bp) was purified, quantified, and the copy number was estimated. Serial dilutions over six orders of magnitude of this DNA then were used on each qPCR plate to create a standard curve. DNA for qPCR was quantified using Qubit® 2.0 Fluorometer and diluted to a working concentration of 2ng/μl (10 ng of DNA was used per reaction). The qPCR absolute quantification experiment was done on Applied Biosystems® 7500 Real-Time PCR system (Life Technologies, CA, USA) using Power SYBR® Green PCR Master Mix (Life Technologies, CA, USA).

Each reaction was run in triplicate and haemosporidian copy number (i.e. the number of haemosporidian cytochrome B gene copies per 10 ng of total DNA (host + parasite)) was estimated by calculating the mean value for the triplicate. Our qPCR estimates were not used to estimate the absolute number of parasites per bird, and may not be reliable for estimates of absolute copy number. However, we did use our qPCR estimates to compare the relative parasitemias of haemosporidian parasites among samples. All dissociation curves were examined for the presence of nonspecific amplification or primer dimer formation. None was detected.

For the purposes of determining infection prevalence, we scored each bird with a qPCR haemosporidian copy estimate of zero as uninfected, and we scored any bird with a positive (non-zero) haemosporidian copy estimate as infected. While infection prevalence was estimated using both the nested PCR results and the qPCR results, here we report only the infection prevalence results based on qPCR. Our prevalence estimates based on the nested PCR results were qualitatively similar. We only included birds for which we had parasitemia data at all three sampling time points in our analyses (i.e. we did not include birds with missing data). There

were 18 migrant juncos and 19 sedentary juncos included in our statistical analyses of prevalence and parasitemia.

Nested PCR and sequencing to determine parasite identities and evolutionary relationships

The concentration of DNA in the final extracted samples used for nested PCR ranged from 20.6-255.3 ng/µl. Haemosporidian infection was determined by amplifying the haemosporidian parasite cytochrome B (*cytb*) gene using published nested polymerase chain reaction (PCR) protocols (Hellgren et al. 2004). Each gDNA sample was screened for the presence of *Plasmodium* and *Haemoproteus* parasites using the external PCR primers HAEMNF and HAEMNR2 followed by the nested or internal primers HAEMF and HAEMR2 (Waldenström et al. 2004). To verify infection status of each sample, PCR was independently run twice per sample, followed by gel electrophoresis of the PCR products. A negative control (water and PCR reagents with no DNA template) was also run to confirm that there was no contamination of haemosporidian DNA in the PCR reagents. Gels were scored treatment blind by RDM and SPS to determine haemosporidian infection status. A sample was considered positive (infected) if there was a visible band (at 478bp) on at least one of the gel runs. Nested PCR products from positive samples were sequenced using Sanger sequencing at the Western South Dakota DNA Core Facility at Black Hills State University (BigDye v3.1 Cycle Sequencing; ABI3130 series Genetic Analyzer). Using Sequencher v5.4.6 software, the forward and reverse sequences were manually trimmed at 5' and 3' ends for poor quality base calls and assembled automatically into an alignment (contig). Any remaining ambiguous base calls were resolved manually. Ten sequences did not have good quality complimentary sequences. These were trimmed at the ends in Sequencher to include in the project with the assembled contigs.

Reconstructing parasite phylogenies from parasite sequence data

Haemosporidian parasite sequences were aligned in Molecular Evolutionary Genetics Analysis Version 7.0 (MEGA7) (Kumar et al. 2016) using the ClustalW method. Following alignment, sequences were trimmed manually. The evolutionary history was inferred in MEGA using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). Initially, a phylogenetic tree was reconstructed using the parasite sequences from the all the infected juncos in the present study as well as all of the parasite sequences of all of the described haemosporidian morphospecies on the MalAvi database (Bensch et al. 2009). Subsequently, we removed the sequences of all the morphospecies in the MalAvi database, except for the morphospecies which most closely matched the parasite sequences from the juncos in our study, which we retained for phylogenetic reference. The tree with the highest log likelihood (-907.1152) is shown (Figure 1). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 31 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 320 positions in the final dataset.

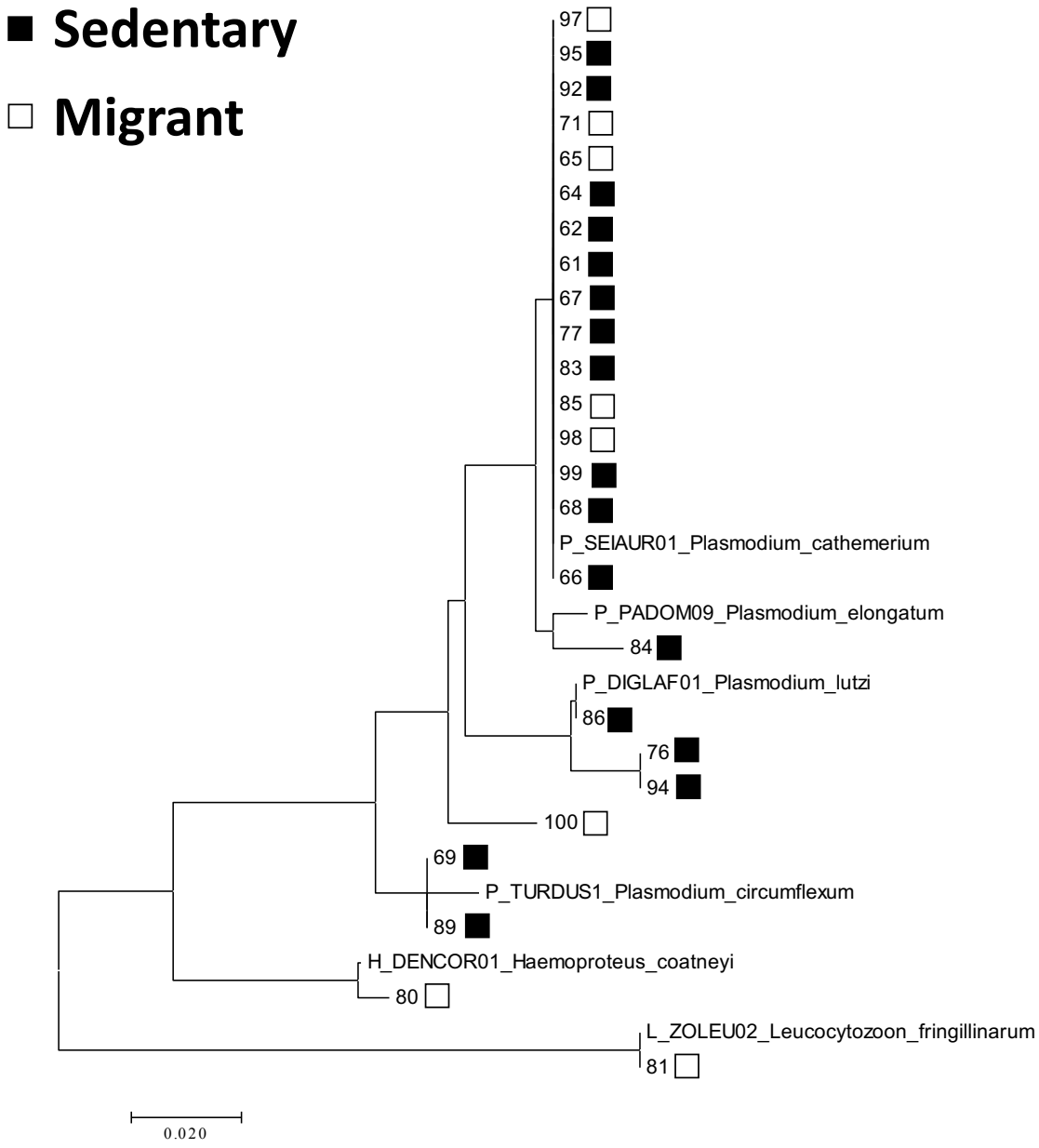

Figure 1: A maximum likelihood phylogeny of the haemosporidian parasites infecting the sedentary and migrant juncos in our study. Branch tips with filled boxes represent parasites sequenced from blood samples of birds in the sedentary population. Branch tips with unfilled boxes represent parasites sequenced from blood samples of birds in the migrant population. For reference, the parasite sequences from the described morphospecies on the MalAvi data base that most closely matched each parasite lineage in our phylogeny are included in the phylogenetic tree.

Measuring haemosporidian phylogenetic diversity

We used the mean genetic distance between all possible pairs of parasite sequences (including identical parasite sequences with pairwise distances of zero) within each population (sedentary and migrant) as a proxy for the parasite phylogenetic diversity (Vellend et al. 2011). We used the software program MEGA7 to calculate the phylogenetic distance between every possible pair of sequences within the sedentary population, and between every possible pair of sequences within the migrant population. We calculated the mean of the pairwise distances within the sedentary population and the mean of the pairwise distances in the migrant population using Microsoft Excel 2016. We subtracted the mean of the pairwise distances within the sedentary population from the mean of the pairwise distances within the migrant population in order to estimate the difference in phylogenetic diversity of the parasite communities infecting our focal host populations.

In order to determine whether the difference we observed in the phylogenetic diversity of the parasite communities infecting our sedentary vs. our migrant junco populations was statistically significant, we constructed a null model to predict the parasite phylogenetic diversities we would have expected to observe within each population assuming no association between host population and the structure and diversity of the haemosporidian parasite communities, and we compared our observed differences in phylogenetic diversity with the differences predicted by the null model. In order to construct our null model, we used Microsoft Excel 2016 to randomize the population assignments (sedentary or migrant) at the tips of the parasite phylogenetic tree, while preserving the topology and branch lengths of the tree. We randomized the population assignments at the tips of the tree assigning each tip a population status (either sedentary or migrant) with the probability of each population status equal to the frequencies of each population in our original (observed tree). After randomizing the population

assignments at the tips of the tree, we recalculated the mean pairwise distances for all possible pairwise comparisons in the sedentary and migrant populations. This process of randomizing population assignments at the tips of the trees and then recalculating population pairwise distances was reiterated 1,000 times. In our original (observed) tree there were 17 parasite sequences (including redundant sequences) sequenced from infected sedentary juncos, and 8 parasite sequences (including redundant sequences) sequenced from infected migrant juncos. In the 1,000 simulated phylogenies in our null model, the mean number of sequences from sedentary juncos was 17, with a standard deviation of 2.356, and the mean number of sequences from migrant juncos was eight with a standard deviation of 2.356.

In order to calculate the probability that we would have observed a difference as large or larger in the mean pairwise distances of parasites in the sedentary vs. the migrant populations, given no association between host population status and the parasite phylogeny (i.e. in order to calculate the significance of the difference in parasite phylogenetic diversities that we observed between the two populations), we ranked the absolute values of the distances between the mean pairwise distances in the sedentary and migrant populations across the 1,000 iterations. Then we compared the differences in the mean pairwise distances between the sedentary and migrant populations in our simulations (null model) with the difference between the mean pairwise distances we observed in our original (observed) parasite phylogenetic tree.

Measuring reproductive physiology

We use gonad mass as a proxy for reproductive condition. Details for measuring the gonad masses of the juncos in this study have been previously described in a previously published study comparing the seasonal timing of reproductive development in sedentary and

migrant juncos (Fudickar et al. 2016a). In the present study, we republish the gonad mass data, but with a larger sample size and with new analyses comparing the gonad masses with haemosporidian infection data. Briefly, at the end of the study (March 31 and April 1, 2014) birds were euthanized with isoflurane and the testes were immediately flash frozen and transferred on dry ice to a -80° freezer, where they were stored until weighed. Frozen testis mass of one testis from each bird was measured using a digital microbalance accurate to ± 0.001 g. We compared gonad masses (collected from birds euthanized March 31 and April 1) with haemosporidian parasitemias as measured at our last sampling time point (March 25-27).

Statistical methods

95% binomial confidence intervals around infection prevalence estimates were estimated using the online statistical calculator JavaStat http://statpages.info/confint.html. Phylogenetic analyses and calculations of pairwise distances between parasite sequences were performed in MEGA7. Calculations of the mean pairwise distances between all possible pairwise comparisons of parasite sequences within each population, and simulations to determine a null expectation for parasite phylogenetic diversity within each population, were conducted in Microsoft Excel 2016. All other statistical tests were run in SPSS version 24.

Comparing haemosporidian prevalence between populations (objective 1) and sampling time points (objective 3)

We used a generalized estimating equation (GEE) with band number (individual identity) as a subject variable, time point (December, early March, or late March) as a within-subject's variable, population (sedentary or migrant) as a between-subjects variable, and qPCR infection status (positive or negative) as our dependent variable. We used a robust estimator for the

covariance matrix with an exchangeable working correlation matrix structure, and we used a binary logistic regression for the type of model. Goodness of fit QIC = 138.1.

Post hoc tests

Because we found significant effects of population and of sampling time point on haemosporidian infection prevalence in the overall GEE model, we used post-hoc tests in the GEE model to assess whether there was a significant difference between infection prevalence in the sedentary vs. migrant populations within each of the three sampling time points, and to test for differences in haemosporidian infection prevalence across all time point pairwise comparisons.

Comparing haemosporidian parasitemias between populations (objective 1)

We used a Mann-Whitney U test to determine whether parasitemias differed between the sedentary and the migrant junco populations. For this analysis, we averaged the parasitemia score for each bird by summing the parasitemias from each of the three time points and then dividing the summed score by 3. Because we wanted to assess whether population was associated with parasitemias *within infected birds*, we excluded all the birds from both populations that were uninfected throughout the entire study from this analysis (i.e. we excluded all the birds with an average parasitemia of zero). By excluding uninfected birds, we reduced the sample size for this analysis from 19 sedentary juncos and 18 migrant juncos (all the juncos in the study) to 17 sedentary juncos and 9 migrant juncos (only the infected juncos in the study).

Repeatability of parasitemias across sampling time points

We ran a one-way ANOVA, including data from all three sampling time points, with band number (individual identity) as a factor and with parasitemia as the dependent variable. We calculated the repeatability of parasitemias across time points based on the ANOVA output, as

described in (Lessells and Boag 1987). We calculated the standard errors for our repeatability estimates as described in (Becker 1984).

Objective 4: Association between host breeding physiology and haemosporidian parasitemias

We used a generalized linear model to test whether population and gonad mass (our metric of breeding condition) were predictive of haemosporidian parasitemias. We used a linear type model with population as a factor, gonad mass as a covariate, and parasitemia as our response variable. We tested for an effect of population, an effect of gonad mass, and for a population*gonad mass interaction. We used a robust estimator for the covariance matrix. We initially ran the generalized linear model on all birds (infected and uninfected). However, in this analysis we detected significant heteroscedasticity in the data, in violation of the model's assumption of homoscedasticity. In order to confirm that our results were robust to violations of the assumption of homoscedasticity, we reran the generalized linear model on infected birds only (i.e. birds with non-zero parasitemias). The analysis of infected birds only did not violate the assumption of homoscedasticity (or any other assumptions of the generalized linear model). The results of the two analyses were not qualitatively different, so we conclude that the result of our generalized linear model is not an artifact of heteroscedasticity in the data set. We report the results of both analyses.

Results

Comparisons of parasite prevalence between populations (objective 1) and sampling time points (objective 3)

We found a significant effect of population on haemosporidian infection prevalence (Generalized Estimating Equation, Wald Chi-Square$_1$ = 8.666, P = 0.003, figure 2). We also found a

significant effect of time point on infection prevalence (Wald Chi-Square$_2$ = 7.562, P = 0.023, figure 2). We found no significant interactions between sampling time point and population (Wald Chi-Square$_2$ = 0.953, P = 0.621). Post hoc tests revealed that the sedentary population exhibited a significantly higher infection prevalence than the migrant population at each of the three sampling time points: December (P = 0.007), early March (P = 0.001), and late March (P = 0.007). Furthermore, post hoc tests revealed that infection prevalence did not significantly change from December to early March in the sedentary population (P = 0.135) or the migrant population (P = 0.560). There was a significant decrease in infection prevalence from early March to late March in the sedentary population (P = 0.024) and a marginally significant decrease in infection prevalence from early March to late March in the migrant population (P = 0.058). Overall, the infection prevalence did not change significantly from the beginning of the study (December) to the end of the study (late March) in the sedentary (P = 0.304) or the migrant (P = 0.303) population.

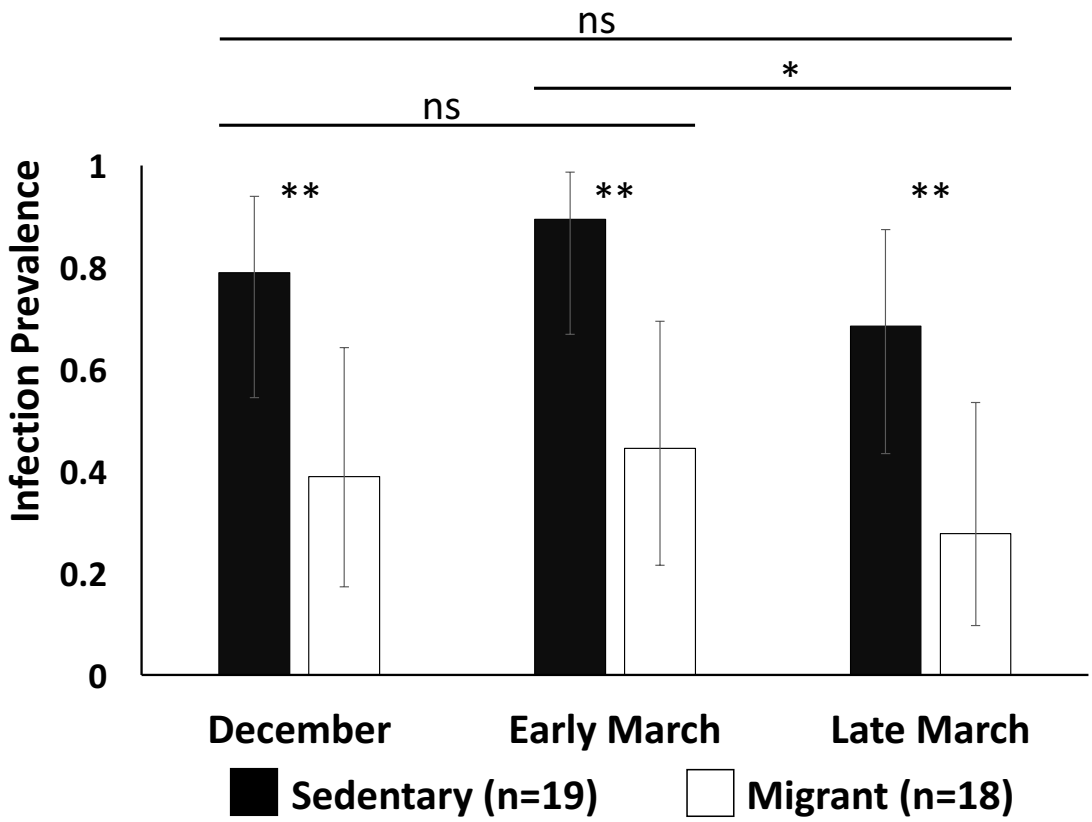

Figure 2: Haemosporidian infection prevalence in the blood, based on qPCR, in the sedentary population (filled bars) and the migrant population (unfilled bars). Error bars represent 95% binomial confidence intervals. Asterisks indicate significant differences between populations at each time point (1 asterisk $p < 0.05$, 2 asterisks $p < 0.01$). Horizontal bars indicate pairwise comparisons between infection prevalences across time points (n.s. indicates $p > 0.05$ for pairwise comparison).

Relationship between host population and parasitemias of infected birds

When considering only birds that were infected during at least one sampling time point during the study (average parasitemia > 0), we found no difference between the medians of the average parasitemia (averaged across the three sampling time points in the study) in the sedentary vs. the migrant population ($U = 77$, $z = 0.027$, $p = 1.0$, figure 3).

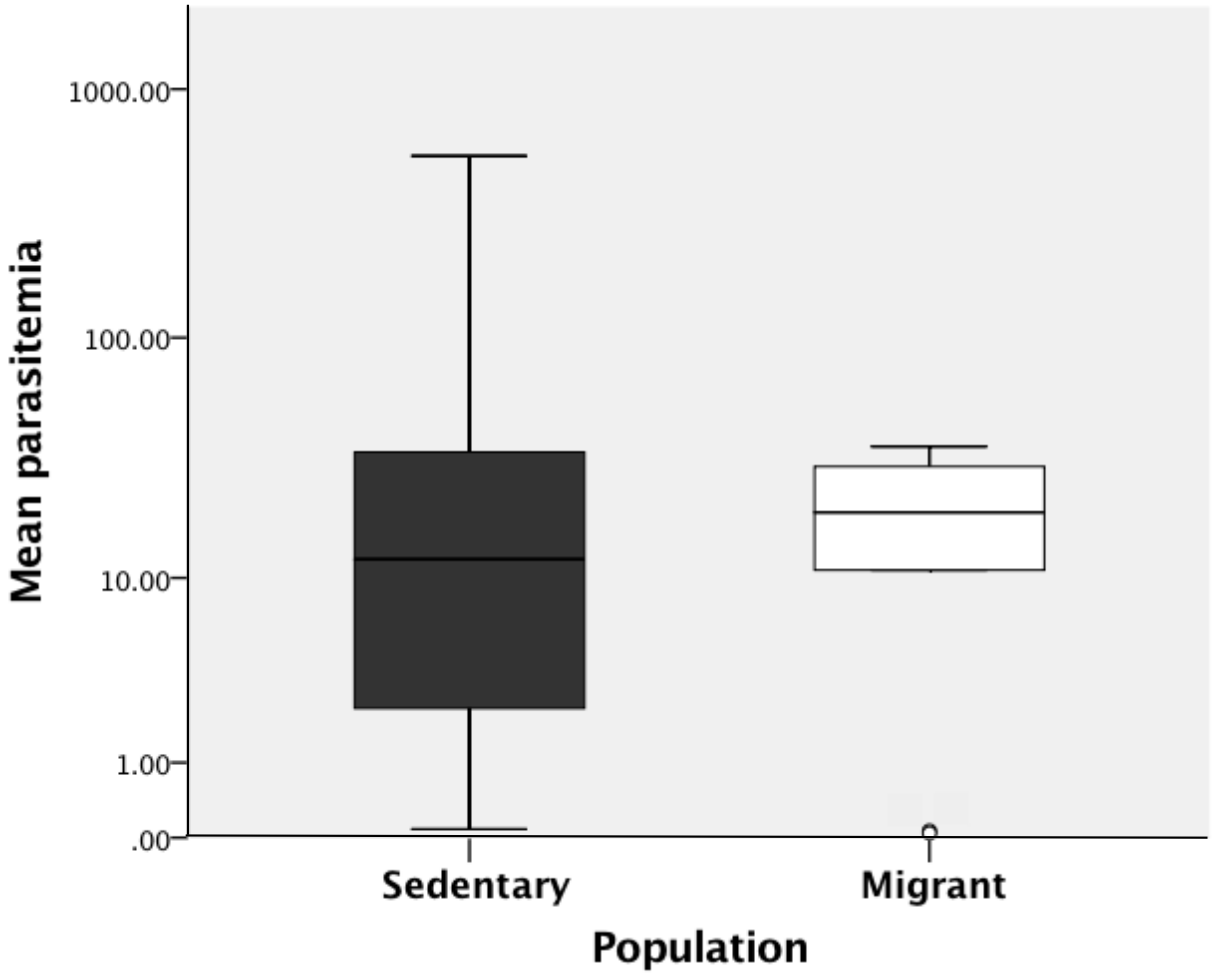

Figure 3: A boxplot showing the mean parasitemias (averaged across the three sampling time points: December, early March, and late March) of sedentary (filled) juncos and migrant (unfilled) juncos. Only infected juncos are included in this figure. Uninfected individuals (individuals with mean parasitemias of zero) were excluded. The horizontal lines inside the boxes represent the sample medians. The length of the box represents the interquartile range. Whiskers span all the data except for statistical outliers. Statistical outliers are points that are at least 1.5 box lengths away from the edge of the box.

Repeatability of parasitemias across time points

There were significant differences among individuals in parasitemias within the sedentary population (ANOVA, $F_{18, 38} = 2.046$, $P = 0.031$) and the migrant population ($F_{17, 36} = 2.406$, $P = 0.013$). The repeatability of parasitemias (also called the intraclass correlation coefficient) was 0.259 (s.e. = 0.1517) in the sedentary population and 0.3129 (s.e. 0.1547) in the migrant population. The repeatability of parasitemias can be visualized in figure 4.

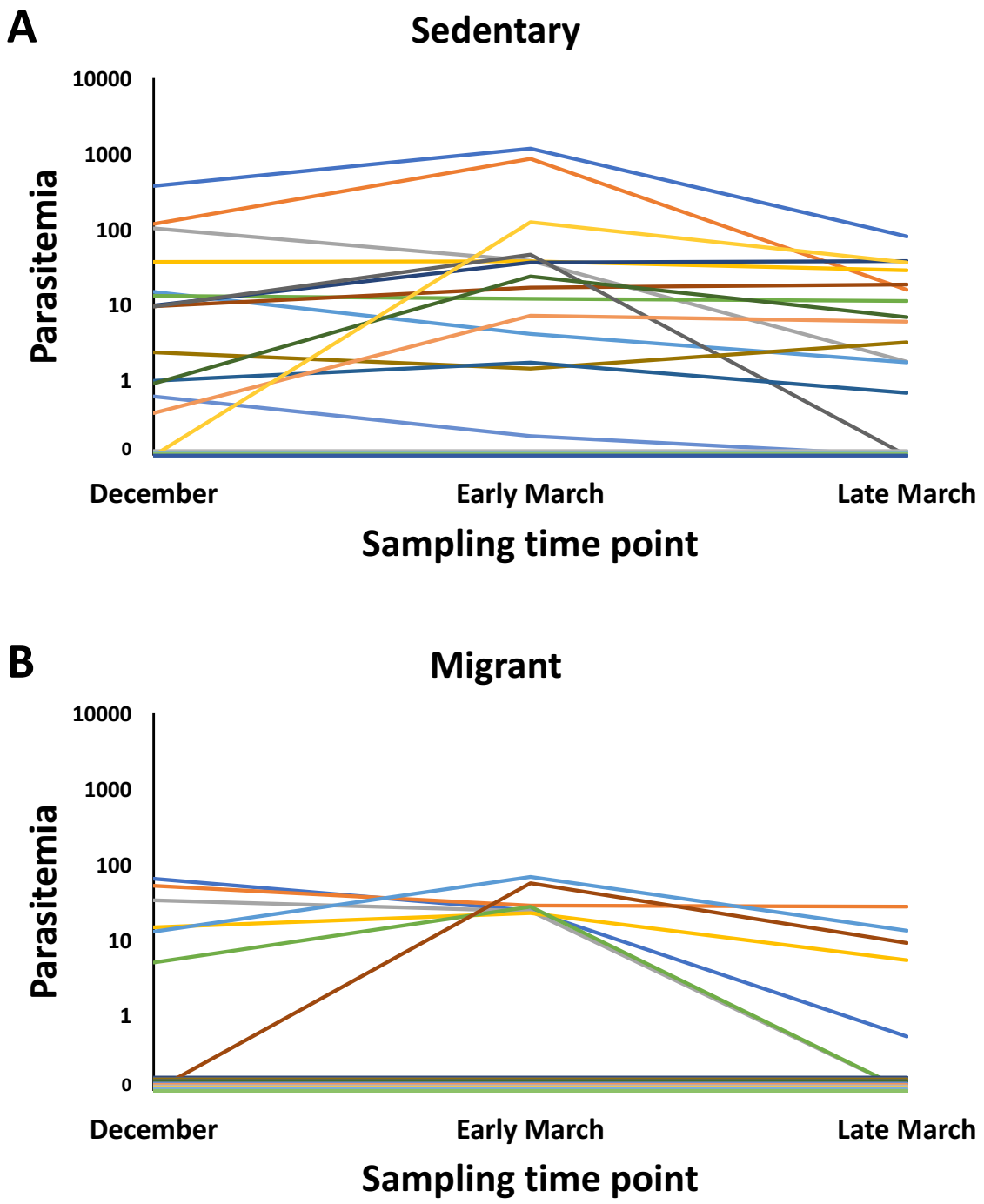

Figure 4: Haemosporidian parasitemias in the bloodstream (as determined by qPCR) of individual sedentary (panel A) and migrant (panel B) juncos across time points. Each colored line represents an individual junco. Lines representing uninfected birds stacked on top of each other at the bottom to visualize overlapping lines.

Objective 2: Association between host migration and parasite community composition and diversity

The mean pairwise distance between all possible pairs of parasite sequences in the sedentary population was 0.021237 substitutions per site (figure 5). The mean pairwise distance between all possible pairs of parasite sequences in the migrant population was 0.067396 substitutions per site (figure 5). The difference between the mean pairwise distances in the sedentary vs. the migrant populations was 0.46159. We found that our null model, with random population assignments on the tree, produced a difference in phylogenetic diversity between the two populations greater than or equal to the difference that we actually observed in 113 out of 1,000 runs (i.e. $P = 0.113$. Therefore, the difference in phylogenetic diversity that we observed between the sedentary and migrant populations was not statistically significant.

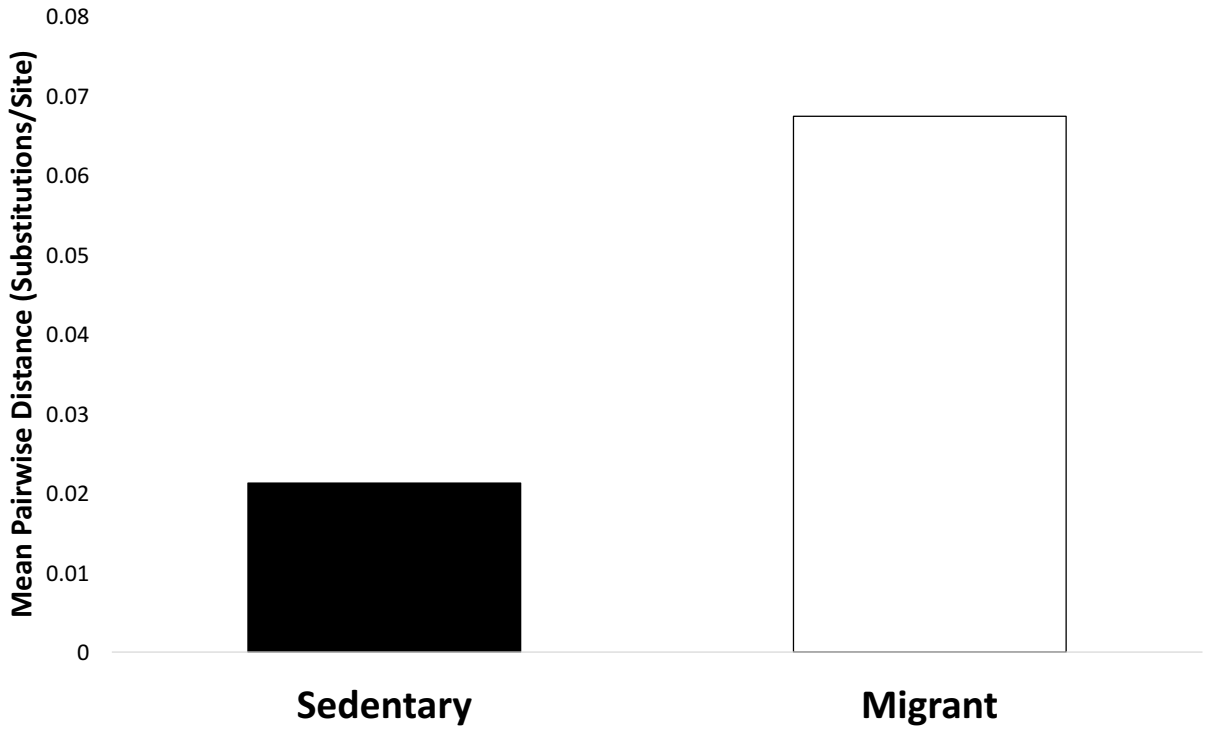

Figure 5: Mean pairwise distance (substitutions/site) across all possible pairwise comparisons of parasite sequences from infected juncos in the sedentary population (black bar, n = 17) vs the migrant population (white bar, n = 8).

Objective 4: Association between host breeding physiology and haemosporidian parasitemias

When all birds (infected and uninfected) were included in our analysis, we found a significant effect of gonad mass (Generalized Linear Model, Wald Chi-Square$_1$ = 5.145, P = 0.023) on haemosporidian parasitemias. However, we found no significant effect of population (Wald Chi-Square$_1$ = 1.964, P = .136) and no significant gonad mass*population interaction on haemosporidian parasitemias (Wald Chi-Square$_1$ = 1.399, P = 0.237, figure 6).

Similarly, when only infected birds (parasitemia ≠ 0) were included in our analysis, we found a significant effect of gonad mass (Generalized Linear Model, Wald Chi-Square$_1$ = 9.850, P = 0.002) on haemosporidian parasitemias, and we found no significant effect of population (Wald Chi-Square$_1$ = 1.450, P = 0.229) and no significant gonad mass*population interaction on haemosporidian parasitemias (Wald Chi-Square$_1$ = 0.164, P = 0.685, figure 6).

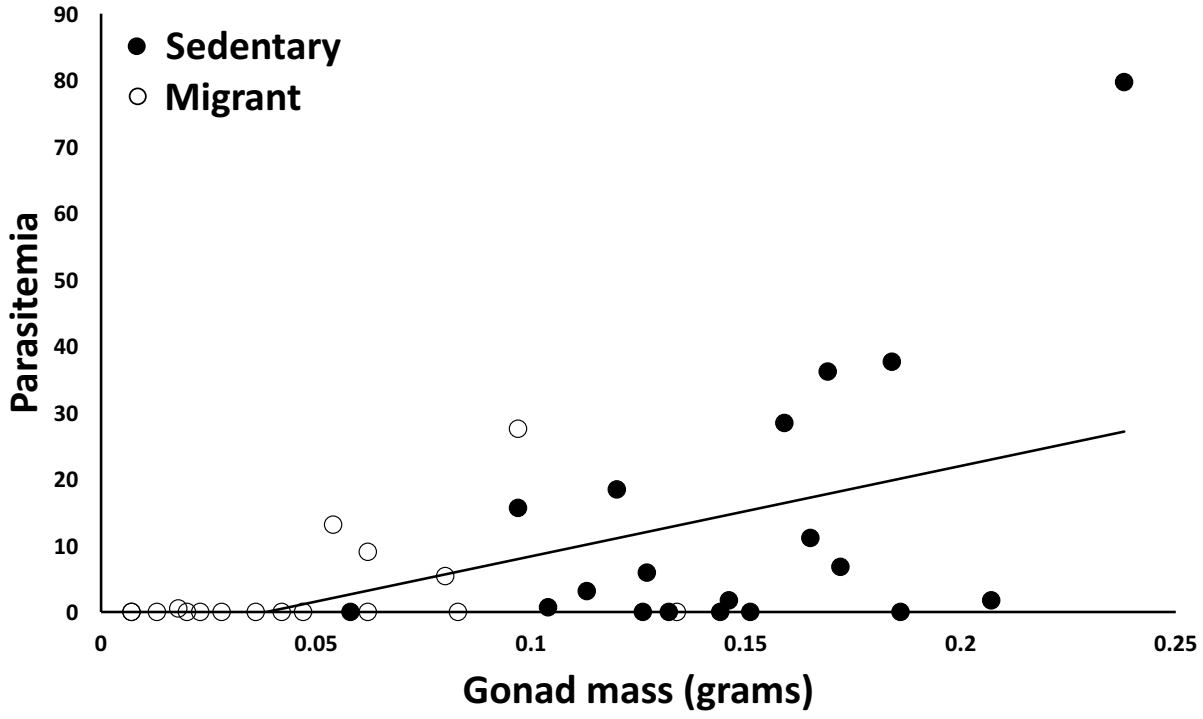

Figure 6: A bivariate plot showing gonad mass (in grams, measured in late March) vs. haemosporidian

parasitemia in the blood (as determined by qPCR, also measured in late March) for sedentary juncos (filled circles) and migrant juncos (unfilled circles). The trend line indicates the line of best fit for the populations pooled.

Discussion

We found that a sedentary population of dark-eyed juncos exhibited a higher prevalence of haemosporidian parasite infections throughout the winter and early spring relative to a closely related and seasonally sympatric migrant junco population. This result suggests that long-distance migration may be associated with reduced parasitism in juncos, a pattern which is consistent with the Migratory Culling hypothesis and the Migratory Escape hypothesis, but not with the Migratory Exposure hypothesis. Among infected birds, we found no difference in parasitemia between the sedentary and migrant populations. Parasite phylogenetic diversity did not differ between the sedentary and migrant junco populations, suggesting that long-distance migration does result in the accumulation of diverse parasite lineages in migrant juncos. We found no evidence that the haemosporidian parasites in this study exhibit seasonal dormancy. Finally, we found that haemosporidian parasitemia was positively correlated with gonad mass, suggesting that investment in reproductive physiology may trade off with investment in immune function.

Objective 1: Association between host migration and infection prevalence and parasitemia

We found that a sedentary population of dark-eyed juncos maintains a higher prevalence of haemosporidian parasite infections, relative to a closely related migrant population of juncos, during seasonal sympatry in the winter and early spring. Among infected juncos, we found no difference between the parasitemias circulating in the bloodstream of sedentary vs. migrant juncos. These results are consistent with the Migratory Culling hypothesis and the Migratory Escape hypothesis, and suggest that long-distance migration may reduce the prevalence of

haemosporidian parasites in birds. The higher prevalence of parasites that we observed in sedentary hosts is also consistent with previous empirical research showing that migration is associated with reduced parasitism in monarch butterflies (Altizer et al. 2000, Satterfield et al. 2016) and galaxiid fishes (Poulin et al. 2012).

On the other hand, previous research in another avian blood-parasite system demonstrated a very different relationship between host migration and parasitism relative to what we observed in juncos. Kelly et al. (2016) found that longer-distance adult song sparrow migrants were *more* likely, on average, to be infected with blood-borne parasites than sparrows that migrated shorter distances. We don't know why we found that long-distance migration is associated with reduced parasitism in juncos while Kelly et al. (2016) found the opposite pattern in a closely related host. Perhaps the relationship between parasitism and host migration is highly dependent on the biology of the host and/or parasite, or on the ecological context. Future research should investigate why the association between long-distance migration and parasitism varies across study systems.

Migratory culling and migratory escape are non-mutually exclusive mechanisms that each could explain the reduced prevalence of haemosporidian parasites that we observed in migrant juncos. With our data set, it is not possible to eliminate either of these potential mechanisms. Future research should assess the contribution of each mechanism to the population difference that we observed. For example, testing whether parasites reduce flight performance or increase the costs of long-distance flight in birds, as has been demonstrated in invertebrates (Bradley and Altizer 2005), could provide a more direct test of the migratory culling hypothesis in our study system. A comparison of the density of infected vectors at the breeding site for our sedentary juncos (Mountain Lake Biological Station in Virginia) versus at breeding sites for our

migrant juncos (in Alaska, Canada, and/or Northern New England) could provide a more direct test for the Migratory Escape hypothesis, as the Migratory Escape hypothesis predicts that migrant birds should experience reduced haemosporidian parasite exposure at their breeding grounds relative to sedentary birds.

We reject the Migratory Exposure hypothesis as a primary driver of variation in infection prevalence between our study populations. The Migratory Exposure hypothesis predicts that sedentary host populations should exhibit *lower* parasite infection prevalence relative to closely related migrant populations. Therefore, our observation that a sedentary host population exhibited a *higher* prevalence of haemosporidian parasite infections directly contradicts a key prediction of the Migratory Exposure hypothesis.

Objective 2: Association between host migration and parasite diversity

The migratory exposure hypothesis posits that long-distance migration exposes host populations to diverse parasites, and predicts that migrant host populations should be infected by a higher diversity of parasites relative to closely related sedentary host populations. Our results do not support this prediction. We found no significant difference between the diversity of haemosporidian parasites infecting juncos in the sedentary population versus the migrant population. Therefore, it appears that long-distance migration does not expose the juncos in our focal populations to a higher diversity of parasites relative to not migrating. That said, we did find a trend (not statistically significant) toward a higher diversity of parasites in our sedentary population relative to our migrant population. It's possible that long-distance migration does result in the exposure to, and accumulation of, more diverse parasites relative to not migrating, but that this pattern was not detectable given our small sample size. There were only 17 sedentary parasite sequences and eight migrant parasite sequences in our haemosporidian

phylogeny. Future research should compare the diversity of haemosporidian parasites infecting sedentary versus migrant juncos in a larger sample of infected juncos.

Objective 3: Measuring seasonality in the parasite lifecycle

Contrary to our expectations, we found no evidence for winter dormancy of the haemosporidian parasites. A high prevalence of haemosporidian parasite infections was detectable in the blood stream in midwinter (December) in both host populations, suggesting that haemosporidian parasites do not sequester and go dormant in the host organs in our study system. Similarly, we found no evidence for a spring reemergence of haemosporidian parasites from the host organs into the host bloodstream, as neither the prevalence nor parasitemia of haemosporidian infections increased over the course of the study in either population.

We propose three possible explanations for why we did not observe a detectable increase in the prevalence or parasitemia of haemosporidian parasites in the bloodstream from December to late March. First, it's possible that the pattern of dormancy in the organs during the winter, followed by replication in the bloodstream during the spring and summer, may not be universal for avian haemosporidian parasites in temperate regions, and may not occur in our study populations. Some previous studies have found a seasonal pattern in wild bird populations in which haemosporidian parasites are detectable at high prevalence in the bloodstream during the spring and summer but not detectable in the bloodstream, or only detectable at low prevalence in the bloodstream, during the winter (Cosgrove et al. 2008, Deviche et al. 2010, Cornelius et al. 2014), suggesting a pattern of parasite dormancy in the winter. On the other hand, another study found that a high prevalence of haemosporidian infections was detectable in the bloodstream of rusty blackbirds (*Euphagus carolinus*) during the winter in Mississippi and Arkansas, suggesting that winter dormancy of haemosporidian parasites may not occur in those populations (Barnard

et al. 2010). Our observation that a high prevalence of haemosporidian parasites was detectable in the host bloodstream in the middle of the winter (December) is consistent with the (Barnard et al. 2010) study, and could suggest that seasonal cycling between winter dormancy in the organs and spring replication in the bloodstream does not occur in some temperate avian haemosporidian populations.

Alternatively, while the high prevalence of infections that we measured in December clearly demonstrates that haemosporidians are still circulating in the blood stream during the winter, and are not completely sequestered in the host organs, it is still possible that haemosporidians in our study system exhibit partial dormancy and sequestration in the organs, and could be induced by the right seasonal cues to increase the abundance and activity in the host blood stream. Therefore, we propose that a second possible explanation for why we did not observe a seasonal increase in parasite prevalence or parasitemia over the course of our study is that not all of the seasonal environmental cues required to induce spring reemergence of haemosporidians into the host bloodstream were present in the indoor aviary environment where the juncos were housed in our study. The juncos in our study experienced a lengthening photoperiod, set to match the natural photoperiod at their capture site in Virginia. This lengthening photoperiod was previously shown to be a sufficient cue of seasonal change to induce reproductive development in the sedentary population in the aviary environment and preparation for migration in the migrant population (Fudickar et al. 2016a, Fudickar et al. 2016b). However, perhaps the lengthening photoperiod in the indoor aviary environment was not a sufficient cue, on its own, to induce parasites to reemerge from dormancy in the host organs and to start replicating in the bloodstream. It has been suggested that, in addition to photoperiodic cues, the activity and replication of avian haemosporidian parasites may respond

plastically to supplemental cues such as changes in temperature, food availability, or exposure to vectors (Cornet et al. 2014). In support of this explanation, Cornet et al. (2014) demonstrated that exposure of birds chronically infected with the haemosporidian parasite *Plasmodium relictum* to uninfected *Culex pipiens* mosquito vectors induces an increase in the *Plasmodium* parasitemia in the bloodstream, suggesting that direct cues of vector presence may induce *Plasmodium* parasites to emerge into the bloodstream and/or to start replicating in the bloodstream. Our indoor aviary was vector-free, and our study birds were maintained at a constant temperature and fed ad libitum, so the parasites in our study may not have been exposed to the supplemental cues that could be necessary to induce reemergence and replication in the host bloodstream. To our knowledge, no previous studies have measured seasonal activity of avian haemosporidian parasites in a controlled indoor aviary environment. Future research should manipulate photoperiod in combination with manipulations in supplemental cues in a controlled indoor aviary environment to assess whether lengthening photoperiod, supplemental cues, or a combination of lengthening photoperiod and supplemental cues can induce the reemergence and replication of dormant haemosporidian parasites.

A third possible explanation for why we did not observe a spring reemergence of haemosporidian parasites into the host bloodstream in our study is that the photoperiod at the end of our study may not have been sufficiently long to induce the emergence and to stimulate the reproduction of haemosporidian parasites. The study ended when the birds were sacrificed in the beginning of April. Perhaps the seasonal reemergence and reproductive activation of haemosporidian parasites into the bloodstream of the juncos in our study populations does not occur until mid-April or early May, and thus a photoperiod at least the length of the photoperiod

that juncos would experience in April or May would be required to induce their reemergence and replication.

Overall, we found no evidence that haemosporidian parasites exhibit winter dormancy and sequestration in the host organs, or that haemosporidians exhibit a reemergence into the host bloodstream in the spring. Our failure to detect winter dormancy followed by spring reemergence could suggest that haemosporidian parasites don't exhibit winter dormancy in our study system, that supplemental cues in addition to photoperiodic cues are required to induce spring reemergence of parasites into the bloodstream, or that a photoperiod longer than the photoperiod which our juncos experienced in late March would be required to induce spring reemergence of the parasites. Given that we did not detect evidence for seasonal cycling of haemosporidian parasite activity, we were not able to test our prediction that haemosporidian parasites should exhibit an earlier reemergence into the host bloodstream in sedentary juncos which breed further South relative to migrant juncos which breed further North.

Objective 4: Association between host breeding physiology and haemosporidian parasitemias

In spring, when the juncos in our study were coming into reproductive condition, we found a positive correlation between circulating haemosporidian parasitemias and gonad mass. This association is consistent with the Immunocompetence Handicap Hypothesis, and suggest that testosterone may induce allocation of resources into reproductive traits at the expense of allocation in immune function, which could result in increased haemosporidian parasitemias in juncos with large gonads.

Conclusions

In the present study, we have demonstrated that sedentary juncos maintain a higher prevalence (but not parasitemia) of haemosporidian parasite infections throughout the winter and

early spring relative to migrant juncos in a seasonally sympatric population. Our results suggest that long-distance migration may reduce the prevalence of avian haemosporidian parasites. Migratory culling and migratory escape are non-mutually exclusive alternative mechanisms that could potentially explain why long-distance migration was associated with reduced infection prevalence. With our data set we are unable to eliminate either of these mechanisms. We reject migratory exposure as a primary mechanism explaining differences in haemosporidian infection prevalence between our study populations. Also contrary to the migratory exposure hypothesis, we found no significant difference in phylogenetic diversity between the parasite communities infecting juncos in the sedentary versus the migrant junco populations. We found no evidence that haemosporidian parasites in our study system exhibit dormancy and sequestration in the host organs during the winter. It's possible that haemosporidians do not exhibit winter dormancy in our study system, or that haemosporidians exhibit partial dormancy but supplemental cues, or a longer photoperiod than that experienced in our study, would be required to induce the reemergence and activity of haemosporidians in the host bloodstream. We found, that haemosporidian parasitemia was positively correlated with gonad mass, suggesting that testosterone may increase susceptibility of juncos to haemosporidian parasites. Future research should expand on this work by comparing haemosporidian infection prevalence, parasitemia, community composition and diversity, and seasonality across more sedentary and migrant populations within a vertebrate host species, to assess whether migrant populations consistently exhibit a lower prevalence of infections, as we observed in this study.

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Chapter 2

Haemosporidian parasite infections increase with host age, but do not explain testosterone's effect on telomere loss in a songbird population

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Abstract

Many traits likely interact to influence the longevity of organisms. One trait that is believed to have a strong influence on longevity is telomere length. Telomeres shorten with each round of cell replication, and telomere length has been shown to predict the longevity of animals. Recent research has demonstrated that experimental elevation in circulating levels of testosterone can increase the rate of telomere degradation in a wild passerine, the dark-eyed junco. We proposed and tested the hypothesis that the prevalence and parasitemia of haemosporidian parasites increase in juncos with experimentally elevated testosterone, and that haemosporidian parasites *cause* the increased rate of telomere degradation observed in testosterone-elevated juncos. We measured haemosporidian infections in a wild population of juncos before and at least one year after each junco was treated with either a testosterone treatment implant (to

elevate circulating testosterone levels) or a control (empty) implant. We found no effect of testosterone implants on haemosporidian parasite prevalence. Furthermore, we found no effect of haemosporidian infections on telomere degradation. Hence we reject the hypothesis that haemosporidian parasites cause the accelerated telomere loss observed in testosterone-elevated birds. We also found that, irrespective of implant treatments, haemosporidian infection prevalence increased with increasing host age. This latter result suggests that, following the acute stage of infection, haemosporidian-infected juncos likely sustain long-term chronic infections which they rarely clear completely. As a result, haemosporidian infections accumulate over the host life-span.

Introduction

Many traits likely interact to influence the longevity of organisms. One trait that is believed to have a strong influence on longevity is telomere length. Telomeres are repetitive DNA sequences that, along with shelterin proteins, cap the ends of chromosomes in eukaryotes (Blackburn 2005). Telomeres shorten with each round of cell replication, and telomere length has been shown to predict longevity in captive and wild animals (Heidinger et al. 2012, Asghar et al. 2015). Telomere length can be influenced by genetics (Slagboom et al. 1994), maternal effects (Asghar et al. 2015), psychological stress (Epel et al. 2004), reproductive effort (Heidinger et al. 2012), and parasite infections (Asghar et al. 2015, Asghar et al. 2016). Recent research has demonstrated that experimental elevation in circulating levels of the steroid hormone testosterone can also increase the rate of telomere degradation in a wild passerine (Heidinger et al. unpublished manuscript).

In the present study, we propose and test a mechanism by which testosterone could increase the rate of telomere degradation. Previous research provides correlative (Cornelius et al. 2014) and experimental (Deviche and Parris 2006) evidence that testosterone can increase the intensity (parasitemia) of haemosporidian parasite infections in wild passerines. Furthermore, haemosporidian parasite infections have been shown to increase the rate of telomere degradation in wild passerines (Asghar et al. 2015, Asghar et al. 2016). Hence, we propose that haemosporidian parasites mediate the relationship between testosterone and telomere degradation. We propose that haemosporidian parasite prevalence and parasitemia increases in birds with experimentally elevated testosterone, and that haemosporidian parasites *cause* the increased rate of telomere degradation that Heidinger et al. (unpublished manuscript) observed in birds with experimentally elevated testosterone.

We tested whether haemosporidian parasites mediate the link between testosterone and telomere degradation by measuring haemosporidian parasite infections in blood samples collected from wild dark-eyed juncos (*Junco hyemalis*) before and after a long-term experimental manipulation of circulating testosterone levels. We predicted that, relative to control juncos, the prevalence of haemosporidian parasites circulating in the blood stream would increase in juncos with experimentally elevated circulating testosterone levels, and that haemosporidian-infected juncos would experience more rapid telomere degradation relative to uninfected juncos. The sample of juncos in our study is the same sample of birds that were observed to experience an increased rate of telomere degradation by (Heidinger et al. unpublished manuscript). Hence our study is a direct test of the hypothesis that haemosporidian parasites mediate the effects of

testosterone on telomere degradation observed by (Heidinger et al. unpublished manuscript).

We also investigated the relationship between host age and haemosporidian parasite infection prevalence. Avian haemosporidian infections are characterized by a brief acute infection stage in which parasitemia may reach high levels in the host blood stream, followed by a long chronic infection stage during which low parasitemias may persist for years in the host blood stream if the host is unable to completely clear the infection (reviewed in Valkiūnas 2005). We predicted that, if after surviving the initial, acute, stage of parasite infection, infected hosts clear their infections and develop immunity against future infections, then infection prevalence should decrease with host age as most older hosts become immune. This pattern has been observed in a free-ranging island population of Seychelles warblers (*Acrocephalus sechellensis*) infected by the haemosporidian parasite lineage GRW1 (*Haemoproteus nucleocondensus*) (van Oers et al. 2010, Hammers et al. 2016). Alternatively, if after surviving the initial acute stage of infection, infected individuals sustain long-term chronic infections, which they fail to clear completely, then haemosporidian infections may accumulate as hosts age and infection prevalence should increase with age. This pattern has been observed in a free-ranging population of blue jays (*Cyanocitta cristata*) infected with the haemosporidian parasite species *Haemoproteus danilewskyi* (Garvin and Greiner 2003), and in a population of house martins (*Delichon urbica*) infected with diverse blood parasites (*Trypanosoma,* microfilariae, and haemosporidians) (Marzal et al. 2016).

Materials and Methods

Study system and study site

Dark-eyed juncos (the hosts)

The dark-eyed junco (*Junco hyemalis*) is a small, common, North American passerine (Nolan et al. 2002). In the present study, we focused on a sedentary population of slate-colored dark-eyed juncos (*Junco hyemalis carolinensis*) that winter and breed at the Mountain Lake Biological Station in the Appalachian Mountains in Giles County, Virginia (37.37°N, 80.52°W). The demography, breeding behavior, and breeding physiology of this junco population has been intensively studied for more than three decades (reviewed in Ketterson et al. 2001).

Haemosporidians (the parasites)

The haemosporidians are a diverse group of obligate, heteroxenous, protist parasites that infect amphibians, reptiles, birds, and mammals, and use blood-sucking dipteran insects as vectors (Valkiūnas 2005). Haemosporidians include the malaria-causing genus *Plasmodium*. Avian haemosporidian parasites can negatively affect the fitness of their hosts (e.g. Knowles et al. 2010, Martinez de la Puente et al. 2010, Asghar et al. 2011, Asghar et al. 2015).

Testosterone implant treatments

Our protocol for experimentally manipulating circulating testosterone levels in free-living juncos has been previously described (e.g. Ketterson et al. 2001, Gerlach and Ketterson 2016). Briefly, male juncos were treated with two subcutaneous 10-mm Silastic implants filled with crystalline testosterone (treatment group) or empty (control group) and sealed at both ends. Silastic implants are porous, allowing the hormone to slowly diffuse across the tubing and enter systemic circulation. Testosterone implant

treatments maintained testosterone at sex-typical peak levels throughout the breeding season (Ketterson et al. 1991, Ketterson et al. 1992). After blocking by capture site within the study area, treatment group was determined by coin flip. Implants were typically given during the early breeding season (mid-April to mid-May), and removed at the end of the breeding season (late July to August) if the bird was recaptured. Birds recaptured the following year were given new implant treatments, and only birds that received the same treatment every year were selected for inclusion in this study. Only adults ≤ 1 year old were treated with implants. The implant treatments to manipulate circulating testosterone levels were given to breeding male juncos from between the years 1993 to 2000.

Determining host age

The juncos in the present study were part of an intensive long-term demographic study. As part of the demographic study the entire junco population was censused every spring (mid-April to mid-May) and at the end of every summer (late July to August). During each census, nearly every bird in the population was banded for individual identification. During each census the age of each bird was estimated based on plumage and morphology (juveniles can be distinguished from adults based on plumage, and one-year-old adults can be distinguished from ≤ two-year-old adults based on plumage color and eye color (Nolan et al. 2002)). Additionally, throughout the breeding season, a team of researchers intensively searched for nests, and nests were monitored until fledging. All nestlings were banded for individual identification at age day 6. Therefore, the exact age, in years, was known for every bird that was first banded as a nestling, juvenile, or a one-year-old, which was the case for every bird in the present study.

Blood sample collection and DNA extraction

Blood DNA samples, for assays to measure haemosporidian infections and to measure telomere lengths, were collected by pricking the brachial vein with a sterile needle and collecting about 150 μl into a microcapillary tube. Immediately after collection, blood samples were transferred into an Eppendorf tube where they were suspended in a DNA buffer solution (Longmire's solution) in which they were stored (at 4° C or at -20° C) until DNA was extracted. Blood samples were collected when the first implant treatment was given (i.e. pre-treatment) and when each subsequent implant treatment was given (i.e. post-treatment). Blood samples in Longmire's solution (preservative buffer) were shipped (frozen) from the Ketterson lab at Indiana University to the Heidinger lab at North Dakota State University. Genomic DNA (gDNA) was extracted by the Heidinger lab group at North Dakota State University NDSU. Our DNA extraction of the samples in this study has been previously described (Heidinger et al. unpublished manuscript). Briefly, 100 μl of blood (in Longmire's buffer solution) was mixed with 100 μl phosphate buffer solution. Then DNA was extracted using Macherey-Nagel Nucleospon Blood Kits (Macherey-Nagel, Bethlehem, PA, USA), following the manufacturer's instructions (Bauer et al. 2016). A Nanodrop 8000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA) was used to determine DNA purity and concentration. DNA integrity was verified by electrophoresis on a 2% agarose gel and blood samples remained in excellent condition. Telomere length was measured in the Heidinger lab at NDSU and extracted gDNA was then shipped (frozen) back to the Ketterson lab at Indiana University for measuring haemosporidian parasite infections.

Measuring telomere length

Our methods for measuring telomeres in the blood DNA samples in this study have been previously described (Heidinger et al. unpublished manuscript). Briefly, telomere lengths were measured using quantitative PCR (qPCR) using an Mx3000P (Stratagene, San Diego, CA, USA) using methods described by (Cawthon 2002) and adapted for dark-eyed juncos. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a single copy control gene. We verified the suitability of GAPDH using a melt curve analysis, which indicated that the dissociation curve had a single peak at the expected melting temperature (T_m) of 82.0°C. In addition, the PCR product was run on a 2% agarose gel to confirm the amplification of a single product at the expected 98 bp.

We ran GAPDH and telomere reactions in triplicate on separate plates and we measured the number of PCR cycles necessary to accumulate sufficient fluorescent signal to cross a threshold (*Ct*). Each 25 µl reaction contained 20 ng of DNA and either telomere or GAPDH primers at a 200/200 nM concentration mixed in 12.5 µl of perfeCTa SYBR green supermix Low ROX (Stratagene). The following primers to amplify the reactions were used: telomeres - forward tel1b (5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and reverse tel2b (5'- GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3') and zebra finch GAPDH - forward (5'-AACCAGCCAAGTACGATGACAT-3') and reverse GAPDH (5'-CCATCAGCAGCAGCCTTCA-3'). The qPCR reaction conditions were as follows: telomeres - 10 min at 95 °C, followed by 27 cycles of 15 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C, finishing with 1 min at 95 °C, 30 s at 58 °C, and 30 s at 95 °C and GAPDH - 10 min at 95 °C, followed by 40 cycles of 30 s at 95 °C and 30 s at 60 °C, finishing with 1 min at 95 °C, 30 s at 55 °C, and 30 s at 95 °C.

Each plate also contained a dark-eyed junco reference sample that was used to create a 5-point standard curve (40, 20, 10, 5, 2.5 ng) and to measure reaction efficiencies and inter-plate variation in relative telomere length. Average *Ct* values were used to calculate the relative telomere length (T/S ratio) according to the following formula: $2^{\Delta\Delta Ct}$,where $\Delta\Delta Ct = (Ct^{telomere} - Ct^{GAPDH})$ reference sample – $(Ct^{telomere} - Ct^{GAPDH})$ focal sample (Stratagene 2007). The samples were all within the range of the standard curve. The average reaction efficiencies for the GAPDH (mean ± 1 SEM: 91.9 ± 0.81) and telomere (mean ± 1 SEM: 89.3 ± 0.58) plates were similar and in all cases were between 85-115%. The inter-plate variation for the T/S ratio was 4.7%.

Measuring haemosporidian infections

Nested PCR and sequencing

Haemosporidian parasite infections in the junco blood samples were measured in the Center for the Integrative Study of Animal Behavior Laboratory at Indiana University, Bloomington. To determine haemosporidian infection status, the haemosporidian parasite cytochrome B (*cytb*) gene was amplified using published nested polymerase chain reaction (PCR) protocols (Hellgren et al. 2004). Each gDNA sample was amplified using the external PCR primers HAEMNF and HAEMNR2. Following the first round of amplification, PCR product from the first round of amplification was amplified again using the nested or internal primers HAEMF and HAEMR2, which have been shown to very efficiently amplify *Plasmodium* and *Haemoproteus cytb* sequences.

Scoring gels

Gels were scored treatment blind by SPS and AG. Because our nested PCR primers amplify haemosporidian DNA (if present) to produce a 478bp DNA sequence

(Hellgren et al. 2004), a sample was considered positive if it produced a visible band (at 478bp). Samples that produced no visible band, and samples that produced only a very faint visible band at 478bp, or that produced visible bands elsewhere on the gel, were rerun (up to four times). If a sample produced no visible band after two runs it was considered negative. If the infection status of a sample was still ambiguous after the sample had been rerun multiple times, the sample was excluded from our analysis.

Selecting samples for analyses in this study

We restricted our analysis to male juncos that were implanted and sampled at least twice and that were sampled in different years (i.e. male juncos for which we had a pre-treatment sample and at least one post-treatment sample in a subsequent year). We excluded birds for which we were not able to determine infection status for at least two time points (i.e. pretreatment and at least one post-treatment time point). We only included juncos that received the same treatment (testosterone or control) every time they were implanted.

Statistical methods

95% binomial confidence intervals around infection prevalence estimates were estimated using the online statistical calculator JavaStat http://statpages.info/confint.html. All other statistical tests were run in SPSS version 24.

Determining whether infection status and sampling time point were associated with telomere lengths

For this analysis, we restricted the data set to birds that were either infected every time that they were sampled (we call this category "Stayed infected") or uninfected every time they were sampled (we call this category "Stayed uninfected). We excluded birds

that were measured as infected at least once *and also* measured as uninfected at least once. We only included the first and last sampling time points for each bird (i.e. we excluded middle sampling time points for birds that were sampled more than twice). Telomere lengths were significantly non-normally distributed. Natural log transformation of the telomere lengths improved normality, so we analyzed the ln transformed telomere lengths. We ran a 2-way mixed ANOVA in SPSS with ring number as the subject variable, sampling time point as a within-subjects factor, and infection status as a between-subjects factor.

Determining whether testosterone implant treatment and sampling time point were associated with haemosporidian infection prevalence

We used a Generalized Estimating Equation (GEE) to test whether testosterone implant treatment (T vs. C) and sampling time point affected the probability that a bird would be infected. For this analysis, we only included data from the first and the last sampling time point for each bird (i.e. we excluded middle samples from birds that were sampled more than twice). We treated band number (individual identity) as a subject variable and sampling time point (first or last) as a within-subjects variable. We used a robust estimator for the covariance matrix with an exchangeable working correlation matrix structure, and we used a binary logistic regression for the type of model. We treated infection status (positive or negative) as the response variable. We included main effects of time point and implant treatment in the binomial model, and we also tested for a time point*implant treatment interaction. QLC = 155.632.

Results

Relationship between infection status, sampling time point, and telomere length

The telomere data from the samples in this study has already been described in another study (Heidinger et al. unpublished manuscript) in the context of measuring how testosterone implants affect the rate of telomere degradation. Here, we republish the telomere data with new analyses to measure the association between haemosporidian infections and telomere degradation.

There was no significant sample point*infection status interaction on telomere lengths ($F_{1, 43} = 0.014$, $P = 0.907$, figure 1). There was no significant main effect of sampling time point on telomere lengths ($F_{1, 43} = 0.031$, $P = 0.860$, figure 1). There was no significant main effect of infection status on telomere lengths ($F_{1, 43} = 1.900$, $P = 0.175$, figure 1).

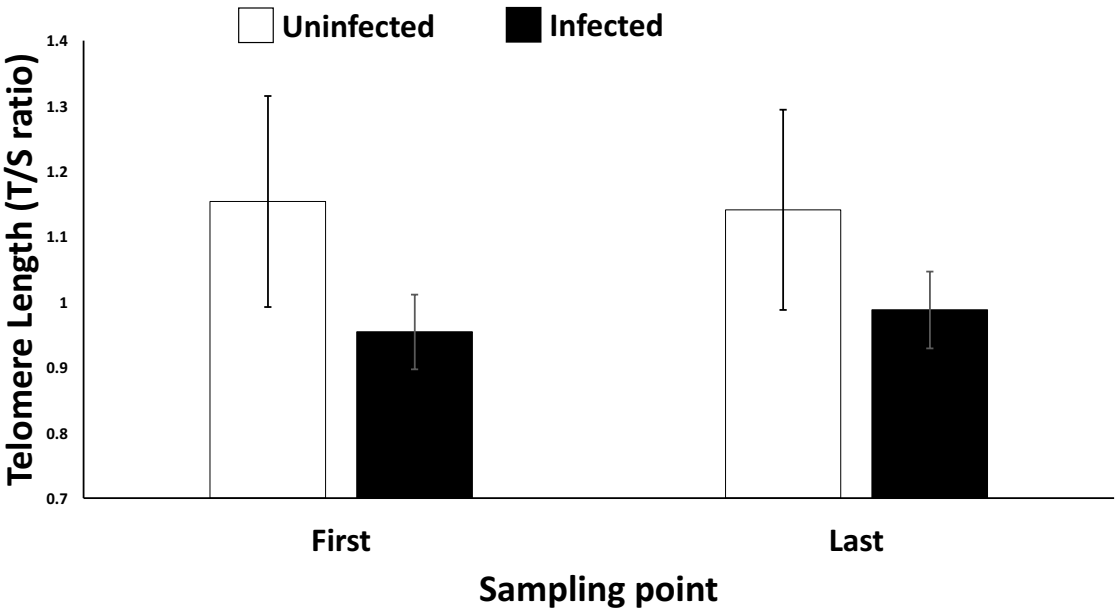

Figure 1: Telomere lengths at the first and last sampling time points in the study. Telomere lengths are expressed as a T/S ratio, the ratio of telomere repeat copy number to single gene (GAPDH) repeat copy number. Unfilled bars represent juncos that were uninfected every time they were sampled (n = 6). Filled bars represent juncos that were infected every time they were

sampled (n = 39). Data from juncos that gained or lost infections during the study are not included in this figure. Error bars represent ± one standard error of the mean.

Relationship between implant treatment and sampling time point and infection status

There was no significant main effect of treatment on infection status (Wald Chi-Square$_1$ = 0.128, P = 0.721, figure 2). There was no significant time-point-by-treatment interaction (Wald Chi-Square$_1$ = 0.312, P = 0.576, figure 2). There was a significant main effect of sampling time point on haemosporidian infections. Infection prevalence was significantly higher at the last sampling time point than the first sampling time point (Wald Chi-Square$_1$ = 15.023, $P < 0.001$, figure 2), suggesting that infection prevalence increases with host age. The relationship between host age and infection prevalence can also be visualized in figure 3.

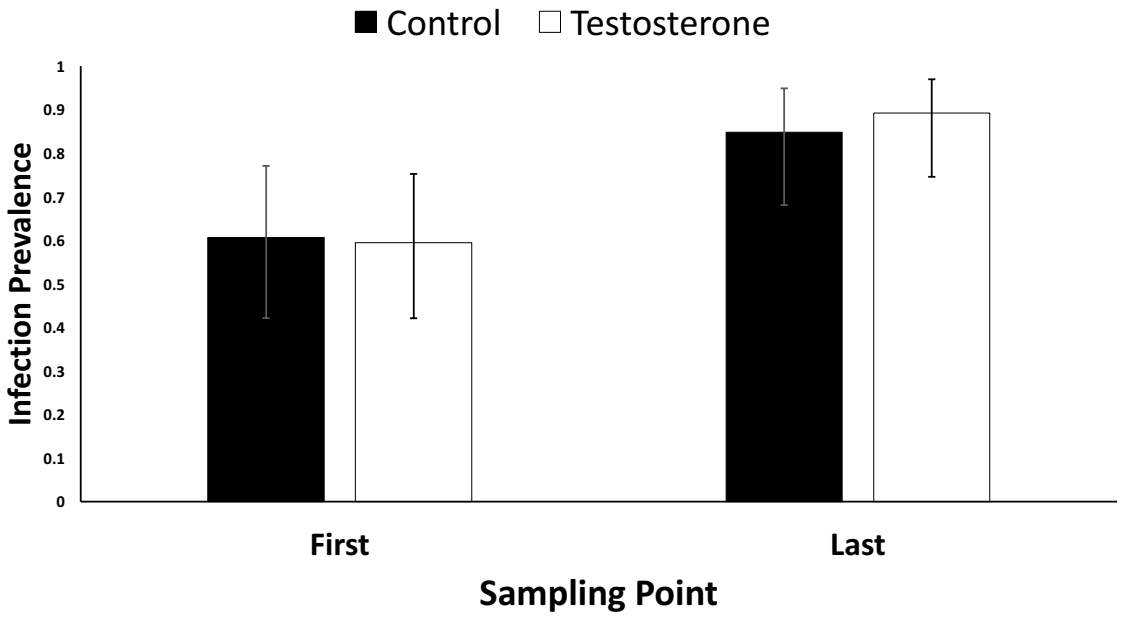

Figure 2: Infection prevalence (i.e. proportion of individuals infected) at the first and last sampling time points in the study. Filled bars represent control (empty) implant male juncos (n = 33). Unfilled bars represent testosterone implant male juncos (n = 37). Error bars represent 95% binomial confidence intervals.

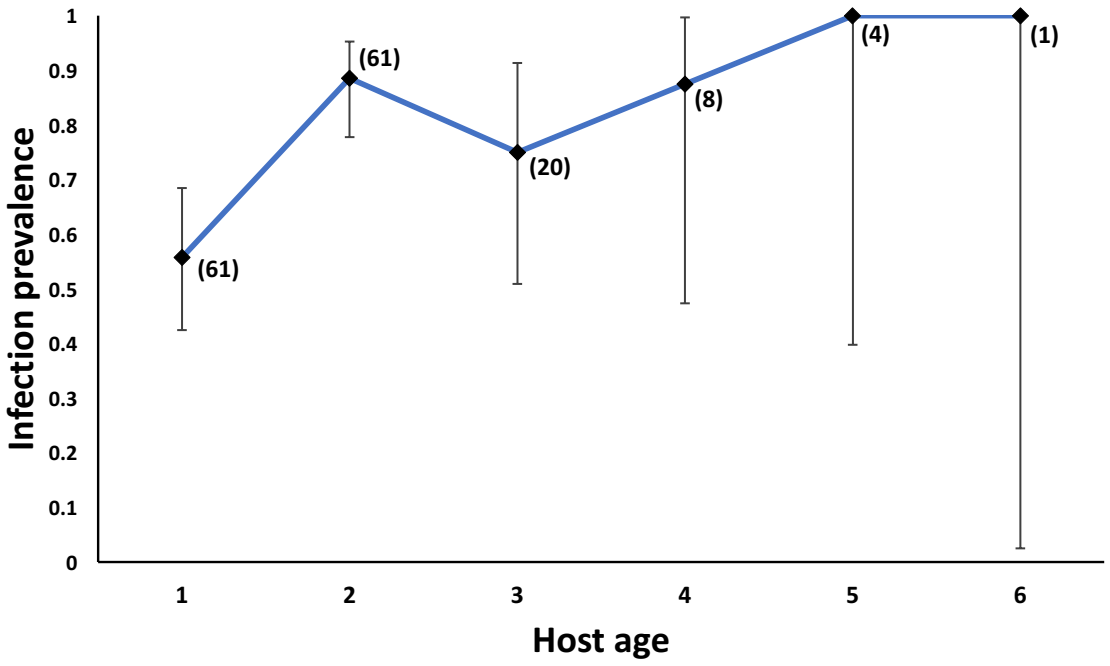

Figure 3: Haemosporidian infection prevalence (i.e. proportion of individuals infected) charted as a function of host age (in years). Error bars represent 95% binomial confidence intervals. Numbers in parentheses represent sample sizes for each age.

Discussion

We found no effect of experimental elevation of circulating testosterone on haemosporidian infection prevalence in a free-living population of dark-eyed juncos (*Junco hyemalis*). Furthermore, we found no significant effect of haemosporidian parasite infections on telomere degradation. Taken together these results suggest that haemosporidians are unlikely to explain how experimental elevation of testosterone increases the rate of telomere loss in wild juncos. We found a significant positive relationship between host age and haemosporidian parasite infection prevalence. This result suggests that, following the acute stage of infection, haemosporidian-infected juncos likely sustain long-term chronic infections, which they rarely clear completely.

Haemosporidian infections appear to accumulate over the host life-span, resulting in a higher prevalence of infection in older birds relative to younger birds.

The immunocompetence handicap hypothesis (ICHC) posits that the steroid hormone testosterone increases investment of energy and resources into reproduction at the expense of investment in immune function (Folstad and Karter 1992). Therefore, the ICHC predicts that testosterone reduces immunocompetence, making organisms more susceptible to infection by parasites (Folstad and Karter 1992). Based on the ICHC, and based on past research providing correlative (Cornelius et al. 2014) and experimental (Deviche and Parris 2006) evidence that testosterone is positively associated with the intensity of haemosporidian parasite infections, we predicted that, relative to control juncos, the prevalence of haemosporidian parasites circulating in the blood stream would increase in juncos with experimentally elevated circulating testosterone levels. We found no support for this prediction. While haemosporidian infection prevalence did increase in testosterone-implanted birds over the course of the study, prevalence also increased in control-treatment birds, and there was no significant interaction between implant treatment and sampling time point on infection status. This result could indicate that testosterone does not increase the susceptibility of hosts to haemosporidian parasite infections, at least in our study system. Alternatively, it's possible that testosterone does increase the susceptibility of juncos to haemosporidian parasite infections, but we were not able to detect this effect with our sample size. Given the high prevalence (prevalence = 0.85, 95% confidence interval: 0.68-0.95) of haemosporidian parasite infections that we measured in the control population at the final sampling time point, a testosterone-induced increase in haemosporidian parasite infection prevalence would be difficult to

detect. It's also possible that very high levels of testosterone would increase susceptibility to haemosporidian parasite infections, but that the dose in the testosterone implants used in this study was not sufficient to induce changes in susceptibility. It's worth noting, however, that the testosterone implants used in this study were sufficient to induce significant increases in singing and courtship behaviors, along with significant reductions in defensive behaviors at the nest and offspring feeding behavior in the males in this study (reviewed in Gerlach and Ketterson 2016).

We found no effect of haemosporidian parasite infections on the rate of telomere degradation in wild juncos. This result contradicts recent research showing that haemosporidian parasite infections accelerated telomere degradation in songbirds: the great reed warbler (*Acrocephalus arundinaceus*) (Asghar et al. 2015) and the Eurasian siskins (*Spinus spinus*) (Asghar et al. 2016). We do not know why Asghar et al. (2015) and Asghar et al. (2016) found that haemosporidian infections accelerate telomere loss in songbird hosts while we found no such effect. Perhaps the effect of haemosporidian infections on telomere loss is highly system specific, with only some haemosporidian species or lineages causing telomere degradation in hosts, or with only some host species susceptible to the effects of haemosporidians on telomeres. Alternatively, it's possible that haemosporidian parasites do cause telomere degradation in juncos, but that we were not able to detect this effect because our sample size was insufficient, or because the parasitemias (parasite densities or intensities) in the infected juncos in our study were too low to affect telomeres. We did not measure parasitemias, and it is possible that the juncos in our study were much more lightly infected with haemosporidians than the warblers in the (Asghar et al. 2015) study or the siskins in the (Asghar et al. 2016) study.

Based on our observation that testosterone implants did not increase haemosporidian parasite infection prevalence, and our observation that haemosporidian parasite infections were not associated with telomere degradation, we reject the hypothesis that haemosporidians mediate the relationship between testosterone implants and telomere loss in our study population of juncos. Experimental elevation of testosterone must have caused accelerated telomere shortening via a different mechanism. Perhaps testosterone-induced increases in investment in reproductive behaviors led to an acceleration of telomere loss. Previous research has demonstrated that reproduction can increase telomere loss in birds (Heidinger et al. 2012). Alternatively, elevated testosterone may have increased oxidative stress, which can accelerate telomere loss (von Zglinicki 2002). Future research should test these alternative mechanisms that could explain how experimental elevation of testosterone accelerates telomere loss.

We found a positive relationship between host age and haemosporidian parasite infection prevalence. This pattern suggests that infected individuals may sustain long-term chronic haemosporidian infections, which they fail to clear completely, and that infections accumulate in hosts as they age. Our result is consistent with another study showing that infection prevalence with the haemosporidian parasite *Haemoproteus danilewskyi* is positively associated with host age in a population of blue jays in South Central Florida (Garvin and Greiner 2003). Our result is also consistent with a study showing that the prevalence of blood parasite infections is positively associated with age in a population of host martins. However, the opposite pattern has been observed in a population of Seychelles warblers infected by the haemosporidian parasite lineage GRW1

(*Haemoproteus nucleocondensus*), which shows a significantly reduced prevalence in older birds (van Oers et al. 2010, Hammers et al. 2016).

Why do some species/populations of birds exhibit an increase in blood parasite infection prevalence as hosts age while prevalence decreases with host age in other species/populations of birds? The relationship between parasite prevalence and host age may be highly dependent on the biology of the host and parasite, or on the ecological context. Perhaps some host species can more effectively clear blood parasites following the acute stage of infection, while other host species are unable to completely clear blood parasites, allowing chronic infections to persist and accumulate over time. Or perhaps the diversity of parasites in an environment affects the relationship between host age and prevalence. The Seychelles warbler population that exhibited a decline in haemosporidian prevalence with host age was an isolated island population. After sampling thousands of birds over many years, researchers have only detected one lineage of haemosporidian parasites GRW1 (*Haemoproteus* nucleocondensus) infecting this population (van Oers et al. 2010, Hammers et al. 2016). Since this host population is likely exposed to a very low diversity of haemosporidian parasites, hosts that clear their infections and develop immunity may remain immune to all future parasite exposures that they are likely to experience for the remainder of their lives, because they likely will only be exposed to the same strain to which they are immune (Hammers et al. 2016). Therefore, in this island warbler population, older individuals are likely to be immune and therefore to stay uninfected, which could drive the observed negative relationship between infection prevalence and host age. On the other hand, in host populations that are exposed to a high diversity of blood parasites, prior exposure to a haemosporidian parasite may not confer

immunity to all the haemosporidian parasite lineages that a host is likely to encounter in the future. Therefore, in host populations exposed to diverse parasites, host immunity may be less likely to prevent parasite infections from accumulating over the course of the host life span (Hammers et al. 2016). Future research should explore the causes and consequences of variation in the relationship between host age and parasite prevalence among host populations.

Conclusions

Here we have shown that experimental elevation of testosterone did not affect haemosporidian parasite infection prevalence in a wild population of dark-eyed juncos. Furthermore, we have shown that haemosporidian parasite infections were not associated with the rate of telomere degradation. Based on these results, we reject the hypothesis that haemosporidian parasites caused the accelerated telomere loss in juncos with elevated testosterone, observed by (Heidinger et al. unpublished manuscript). Future research should test testosterone-induced changes in reproductive behavior, and oxidative stress, as potential mechanisms mediating the effects of testosterone implants on telomere degradation. We also found a positive relationship between haemosporidian parasite infections and host age, suggesting that juncos rarely, if ever, completely clear chronic haemosporidian parasite infections, and suggesting that haemosporidian parasite infections accumulate in hosts as they age.

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Chapter 3

Coevolutionary interactions with parasites constrain the spread of self-fertilization into outcrossing host populations

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Abstract

Given the cost of sex, outcrossing populations should be susceptible to invasion and replacement by self-fertilization or parthenogenesis. However, biparental sex is common in nature, suggesting that cross-fertilization has substantial short-term benefits. The Red Queen hypothesis (RQH) suggests that coevolution with parasites can generate persistent selection favoring both recombination and outcrossing in host populations. We tested the prediction that coevolving parasites can constrain the spread of self-fertilization relative to outcrossing. We introduced wild-type *Caenorhabditis elegans* hermaphrodites, capable of both self-fertilization and outcrossing, into *C. elegans* populations that were fixed for a mutant allele conferring obligate outcrossing. Replicate *C. elegans* populations were exposed to the parasite *Serratia marcescens* for 33 generations under three treatments: a control (avirulent) parasite treatment, a fixed (non-evolving) parasite treatment, and a copassaged (potentially coevolving) parasite treatment. Self-fertilization rapidly invaded *C. elegans* host populations in the control and the fixed-parasite treatments, but remained rare throughout the entire experiment in the copassaged treatment. Further, the frequency of the wild-type allele (which permits selfing) was strongly positively correlated with the frequency of self-fertilization across host populations at the end of the experiment. Hence, consistent with the RQH, coevolving parasites can limit the spread of self-fertilization in outcrossing populations.

Introduction

Substantial costs are associated with biparental sexual reproduction relative to uniparental forms of reproduction such as self-fertilization and parthenogenesis (Maynard Smith 1978). For example, the inability of males to directly produce their own offspring reduces the per-capita

growth rate of biparental sexual populations by as much as one half (Maynard Smith 1978). Given this cost, populations that reproduce via biparental outcrossing should be susceptible to invasion and replacement by mutants capable of uniparental reproduction (Fisher 1941; Maynard Smith 1978; Charlesworth 1980; Agrawal and Lively 2001). However, asexual reproduction and obligate self-fertilization are rare in nature, relative to outcrossing. For example, obligate asexuality occurs in less than 1% of animal species (Bell 1982; Judson and Normark 1996), and very few plant or animal species exhibit obligate self-fertilization (Goodwillie et al. 2005; Jarne and Auld 2006). Although these patterns suggest that the costs of cross-fertilization are outweighed by compensatory short-term advantages, the nature of these advantages has been debated for at least several decades.

The Red Queen hypothesis (RQH) proposes that coevolving parasites can impose selection against common host genotypes, resulting in an advantage to producing outcrossed, genetically variable progeny (Jaenike 1978; Hamilton 1980). This idea has received support from studies of natural host populations of both plants and animals (Lively 1987; King et al. 2011; Verhoeven and Biere 2013; Vergara et al. 2014). In addition, recent laboratory experiments have shown that coevolving parasites can favor increased rates of outcrossing in mixed-mating (outcrossing and self-fertilizing) host populations. For example, Morran et al. (2011) found that androdioecious mixed-mating populations of the nematode host *Caenorhabditis elegans* evolved and maintained higher outcrossing rates when experimentally exposed to the coevolving bacterial parasite, *Serratia marcescens*. Morran et al. (2011) also found found that obligately selfing populations of *C. elegans* went extinct within 20 generations of coevolution with *S. marcescens*, while mixed-mating and obligately outcrossing populations coevolving with *S. marcescens* parasites persisted for the entire experiment (30 generations).

While there is now evidence that coevolutionary interactions with parasites can provide a selective benefit to outcrossing, it is unknown whether parasite-mediated selection is, by itself, sufficient to explain the long-term maintenance of obligate outcrossing, which depends on the ability of host populations to resist invasion by mutants that reproduce via self-fertilization. A critical question thus remains open: can coevolving parasites constrain the spread of self-fertilization into obligately outcrossing host populations? Here we provide a direct test of this question by introducing an allele that confers the ability for self-fertilization into replicate outcrossing host populations. We then measure the effects of coevolutionary and non-coevolutionary interactions with a parasite on the spread of self-fertilization in the experimental host populations.

The nematode host *Caenorhabditis elegans* is an ideal model organism for studying the effect of host-parasite interactions on breeding-system evolution, because *C. elegans* utilizes several modes of reproduction, and it is a genetically and experimentally tractable system. The nematode has two sexes: hermaphrodites (XX) and males (XØ) (Brenner 1974). Hermaphrodites reproduce either by self-fertilization (selfing) or by cross-fertilization with males (outcrossing); hermaphrodites cannot mate with each other. Outcrossing produces a 1:1 ratio of males to hermaphrodites, while almost 100% of selfed offspring are hermaphrodites (Brenner 1974). Because of this relationship between reproductive mode and male frequency in the offspring, the frequency of outcrossing and the frequency of selfing in *C. elegans* populations can be accurately estimated based on male frequencies (Stewart and Phillips 2002). Additionally, *C. elegans* has been shown to coevolve with the virulent bacterial parasite *Serratia marcescens* (Morran et al. 2011; Morran et al. 2013; Morran et al. 2014; Gibson et al. 2015).

The mutant allele *fog-2(q71)* can transform the *C. elegans* breeding system from mixed mating to obligately outcrossing. The *fog-2(q71)* allele (hereafter called the "obligate-outcrossing allele") is an autosomal recessive allele with a loss-of-function mutation that prevents sperm production in hermaphrodites, thereby functionally transforming hermaphrodites into females (Schedl and Kimble 1988). The obligate-outcrossing allele has no known effects on males (Schedl and Kimble 1988). In contrast, hermaphrodites harboring the *fog-2(wt)* allele (which we will refer to as the "mixed-mating allele") are capable of both self-fertilization and of outcrossing with any males, regardless of the males' genotypes at the *fog-2* locus. Hence the mixed-mating breeding system and the obligately outcrossing breeding system can freely intermix whenever they occur together in the same population. Previous work in this study system has shown that the mixed-mating allele rapidly outcompetes the obligate-outcrossing allele in *C. elegans* populations under standard laboratory conditions (i.e. in the absence of a parasite) (Stewart and Phillips 2002; Katju et al. 2008). This result is consistent with the prediction that the 2-fold cost of sex should make outcrossing populations susceptible to invasion and replacement by self-fertilizing mutants (Fisher 1941; Charlesworth 1980; Agrawal and Lively 2001).

In the present study, we tested the prediction that coevolutionary interactions with parasites can constrain the spread of self-fertilization, and prevent self-fertilization from rising to fixation in outcrossing host populations. We introduced homozygous mixed-mating (i.e. *fog-2(wt/wt)*) *C. elegans* nematodes into obligately outcrossing *C. elegans* populations that were fixed for the obligate-outcrossing allele. Following introduction of the mixed-mating individuals, we tracked the spread of self-fertilization in the *C. elegans* host populations under three parasite treatment regimes. In the control treatment (1) hosts were exposed to avirulent, heat-killed *S.*

marcescens bacteria. In the fixed-parasite treatment (2) hosts were exposed to the same isogenic stock population of the highly virulent *S. marcescens* parasite every generation. In the copassaged treatment (3) *C. elegans* hosts were exposed every generation to potentially coevolving *S. marcescens*. We predicted that in the control treatment self-fertilization would rapidly spread into host populations, due to the 2-fold cost of outcrossing. In the fixed-parasite treatment we predicted that self-fertilization would initially remain rare while the host populations adapted to the novel parasite environment, but that self-fertilization would eventually spread once the hosts had adapted to the fixed-parasite population. In the copassaged treatment, we predicted that negative frequency-dependent selection induced by the coevolving parasite would constrain the spread of self-fertilization into host populations. Finally, we predicted that, following experimental evolution, the frequency of the mixed-mating allele (which restores hermaphroditism and permits self-fertilization) should be tightly positively correlated with the frequency of self-fertilization across experimental populations.

Materials and methods

Establishment of the starting populations

Caenorhabditis elegans strains are derived from a single individual nematode isolated from a natural population and maintained as stock populations. Our laboratory group previously obtained the CB4856 strain (originally from Hawaii, USA) from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN) (Morran et al. 2011). Prior to our experiment the obligate-outcrossing allele, *fog-2(q71)*, was backcrossed into a systematically inbred strain of CB4856 (PX382) to generate the obligate-outcrossing strain PX386 (fixed for the obligate-outcrossing allele) (Morran et al. 2009b). The obligate-outcrossing allele contains a

nonsense mutation G → A, which results in a non-functional gene relative to the wild-type, preventing the production of viable sperm in hermaphrodites (Katju et al. 2008).

Prior to our experiment, during three consecutive generations, four near-isogenic replicate populations of (obligately outcrossing) PX386 were independently mutagenized at 40mM of EMS for 4 hours (Morran et al. 2011). While being maintained separately, the populations received random and independent mutation loads (Morran et al. 2011). This procedure induced approximately 1,000 point mutations per lineage in each population (Epstein and Shakes 1995; Morran et al. 2011). Following EMS mutagenesis, as part of another experiment, (Morran et al. 2011), each population was then divided into two different treatments, a control treatment and a fixed-parasite treatment and underwent 30 generations of experimental evolution prior to our use of the experimental lines in the present experiment. The resulting experimental populations, following 30 generations of selection in the prior experiment, as well as the mutagenized ancestral population (pre-experimental evolution) were used as the starting populations in the current study. See our supplementary material for the details of the experimental evolution our nematode populations underwent prior to the start of our experiment. Experimental treatment in the prior study did not significantly affect the evolution of selfing rates in our experiment.

Prior to our experiment, we established mixed-mating strains by introgressing the mixed-mating allele into each of our 12 replicate obligately outcrossing genetic backgrounds (described above) through a series of 7 backcrosses. In this way, we established 12 mixed-mating strains, each associated with an obligately outcrossing population with the same genetic background. This introgression approach made it unlikely that any differences in the fitness of the mixed-mating allele and the obligate-outcrossing allele in our experimental populations would be

confounded by differences in the genetic backgrounds of the mixed-maters and the obligate outcrossers. See supplementary material for details of our establishment of the mixed-mating strains.

Introduction of the mixed-mating invaders into obligately outcrossing populations

At the initiation of our experiment, approximately 38 wild-type (mixed-mating) nematodes from each of 12 replicate mixed-mating lines were liquid transferred onto the first generation Serratia selection plates (SSPs) with approximately 713 nematodes from the obligate-outcrossing populations from which the mixed-maters were derived to produce a mixed (obligate outcrossers and mixed-maters) population with a starting frequency of about 5% mixed-maters. See figure 1 for a depiction of the sexes, *fog-2* genotypes, and modes of reproduction available to hosts at the start of our experiment. Following introduction of the mixed-maters into our replicate obligately outcrossing populations, each replicate was passaged separately on SSPs for 33 host generations.

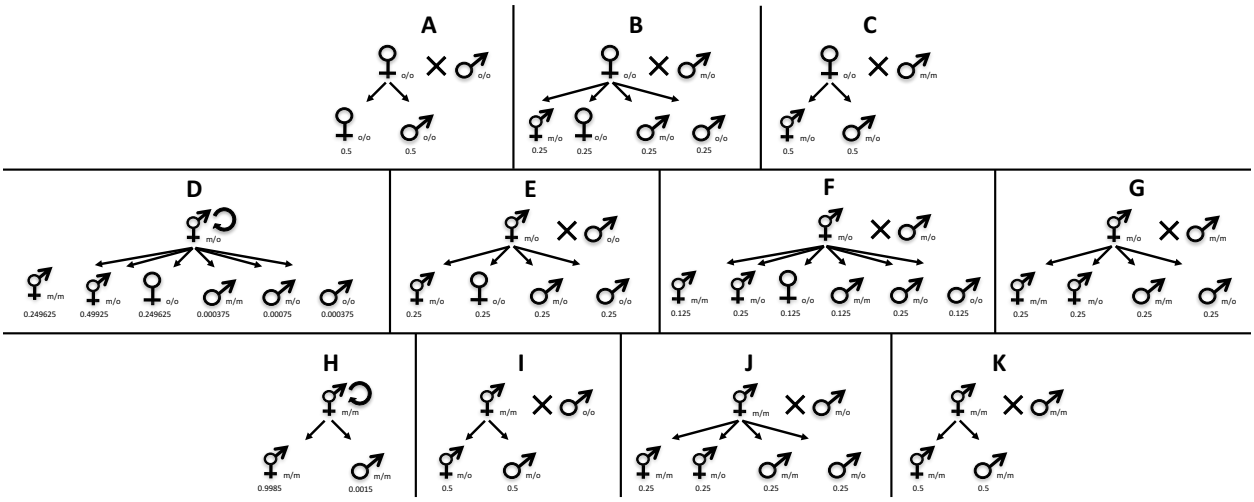

Figure 1) A conceptual diagram depicting all of the possible reproductive combinations that can occur between different genotypes of males, females, and hermaphrodites in our experiment, and the expected frequencies of sexes and genotypes in the offspring that would result from each of these possible crosses. The expected frequencies of sexes and genotypes in the offspring of selfing hermaphrodites include males which are produced by nondisjunction of the X chromosome during meiosis (Brenner 1974). Our figure depicts males produced at a nondisjunction rate of 0.0015 (panels D and H). 95% of the individuals in our generation zero host populations were obligately outcrossing males and females which were homozygous for the obligate-outcrossing allele, designated as "o" in the figure. 5% of the individuals in our generation zero host populations were homozygous for the mixed-mating allele (designated as 'm' in the figure). Almost all of the mixed-mating homozygotes at the start of our experiment were hermaphrodites, although there may also have been rare males homozygous for the mixed-mating allele. Because mixed-mating hermaphrodites can mate with males carrying the obligate-outcrossing allele (panels E, F, I, and J), and because obligately outcrossing females can mate with males carrying the mixed-mating allele (panels B and C), the mixed-mating and obligately outcrossing breeding systems could freely mix in our experimental populations. The mixed-mating allele is dominant, so hermaphrodites heterozygous at the *fog-2* locus (i.e. hermaphrodites with the 'm/o' genotype) were capable of both self-fertilization (panel D) and outcrossing (panels E, F, and G).

Experimental evolution on Serratia Selection Plates (SSPs)

The details of our experimental evolution protocol on SSPs have been previously described in (Morran et al. 2011). Briefly, we constructed SSPs by pouring 24 mL of autoclaved NGM Lite (US Biological, Swampscott, MA) into a 10 cm Petri dish. One side of the plate was seeded with 50 µl of *E. coli* (the food source) while the other side of the plate was seeded with 50 µl of live *S. marcescens* (for the fixed-parasite and copassaged populations) or with 50 µl of heat-killed *S. marcescens* (for the control populations). The bacteria solutions used to seed our

SSPs were grown in 5 mL test tubes of Lysogeny broth (LB) for 24 hours at 28 degrees C. The *E. coli* and *S. marcescens* inocula were both spread across their respective sides of the plate using a sterile spreader. The plate was then incubated for 24 hours at 28 degrees to allow the bacteria to grow. Following incubation, 20 µl of ampicillin (100 mg/mL) was streaked across the plate between the lawns of *E. coli* and *S. marcescens.* Approximately 750 nematodes were liquid transferred in M9 buffer onto the *S. marcescens* side of the plate. Relatively constant nematode population sizes were maintained across all three treatments by counting the number of individual nematodes in random 10 µl samples of our nematodes/buffer liquid transfer solutions prior to transfers to estimate the density of nematodes in the transfer solutions, and by adjusting the volume of solution transferred to the fresh SSP every generation to ensure that approximately 750 individual nematodes were transferred. When necessary, to avoid transferring large volumes of nematode/buffer solution to fresh SSPs, we would concentrate the nematodes in our transfer buffer solution by leaving the solution in an Eppendorf tube for a couple of minutes to allow the nematodes to settle to the bottom of the tube, and then discarding the buffer solution off the top of the tube, leaving a concentrated solution of nematodes at the bottom of the tube for transfer. We maintained populations on SSPs at 20°C.

Our experimental setup required *C. elegans* to crawl through the *S. marcescens* parasite (live or heat-killed) to reach their food source of *Escherichia coli* (Morran et al. 2009b). Four days after the nematodes were introduced onto a SSP, a random sample of approximately seven-hundred and fifty individual nematodes were selected from the *E. coli* side of the plate and transferred to repeat the process. Most of the transferred nematodes were L4 larva, however, our protocol did not explicitly synchronize our populations during experimental evolution. In the copassaged treatment, bacterial cells were harvested every generation from the bodies of 20 dead

nematodes (per line) for passage to the next SSP. Bacterial cells were harvested from the carcasses of nematodes that died on the *S. marcescens* lawn by picking the carcasses into 1mL of M9 buffer, and centrifuging the solution at 3,000 RPM for 3 minutes, discarding the supernatant, and rinsing 5 times with 1mL of M9 (Morran et al. 2011). Harvested bacterial cells were streaked on NGM lite agar which was incubated for 24 hours at 28°C. A random sample of 10 bacterial colonies which grew on the agar plate were used to inoculate 5mL of Lysogeny broth (LB). Bacterial communities were grown in the LB solution for 24 hours at 28 degrees. Following bacterial growth 50 µl of bacterial culture was then used to seed the next SSP onto which the copassaged nematode population would be plated. A thick lawn of *S. marcescens* grew up on the SSPs prior to nematode exposure. The whole bacterial community (*S. marcescens* parasites as well as contaminating *E. coli*) harvested from the bodies of dead nematodes was used to grow the next generation of parasites. However, when the recycled parasite lineages became visibly contaminated with *E. coli*, each line was purified by streaking the bacterial communities on an agar plate, and by selecting a random sample of 20 *S. marcescens* colonies (as determined by their red color) for passage to the next generation. In this way our copassaged treatment protocol selected for resistance in the host and infectivity and virulence in the coevolving parasite (Morran et al. 2011). Fixed-parasite treatment SSPs were always seeded with the same ancestral stock population of *S. marcescens* (SM2170), while control SSPs were always seeded with heat-killed *S. marcescens*. The same stock population of *S. marcescens* (SM2170) that served as the non-evolving parasite in our fixed treatment, was the starting parasite population in all of our copassaged lines.

Male frequency was measured at generations 10, 15, 21, and 33 following the protocol described in Stewart and Phillips (2002). Specifically, we measured male frequencies by

following a transect on the plates and scoring the sex of each nematode in a random sample of 200 adult and L4 larva individuals (Stewart and Phillips 2002). Outcrossing rates were calculated from male frequency data by multiplying the male frequency by 2 and adjusting for males produced by nondisjunction of the X chromosome during meiosis (Stewart and Phillips 2002). Selfing rates were calculated by subtracting the outcrossing rate from 1 (Stewart and Phillips 2002). In order to estimate the selfing rates of our combined (mixed-maters + obligate outcrossers) populations at generation zero we calculated the selfing rates of our mixed-mating lines (prior to combining the mixed-mating lines with the obligately-outcrossing lines) based on male frequencies, and then we multiplied the selfing rate of each mixed-mating line by 0.05 (the starting frequency of mixed-maters in our combined populations) to determine the selfing rate in the overall (mixed-mating and obligately-outcrossing) populations. Samples from all replicate populations were cryogenically frozen at generations 0, 10, 15, 21, and 33.

Measuring the mixed-mating allele frequencies

In order to determine whether the frequency of the mixed-mating allele was correlated with the frequency of selfing across our experimental populations, we thawed and reanimated a subsample of 12 host populations that had been frozen at host generation 33 (the experimental endpoint). We calculated the post-thaw selfing rate based on male frequencies (using the same selfing-rate estimation methods described above). We used molecular techniques to measure the frequency of the mixed-mating allele in our reanimated host populations. See supplementary material for our detailed protocol for measuring *fog-2* genotype frequencies.

Statistical methods

We used an ANOVA to test whether the generation 10 selfing rates differed by treatment. Because our starting populations were distributed evenly among our three treatments, the selfing

rate was the same across all three treatments at generation zero. Therefore, any significant differences observed in the selfing rates across treatments at generation 10 would reflect differences in the evolution of selfing rates during the first 10 generations of evolution. The assumption of homogeneity of variances was violated in the generation 10 selfing rate data, as assessed by Levene's test for equality of variances ($P = 0.015$), so we report the results of a Welch's ANOVA on the generation 10 selfing rates. We used Games-Howell post hoc tests on all pairwise treatment comparisons to determine which treatments differed significantly in their mean selfing rates.

Similarly, we used an ANOVA to test whether treatment significantly affected the selfing rates at generation 33 (our experimental endpoint). We used Tukey post hoc tests across all treatment pairwise comparisons to determine which treatments means differed significantly. Finally, we used a one sample t-test to assess whether the mean selfing rate in the copassaged treatment populations at generation 33 (our experimental endpoint) was significantly greater than zero.

We used a linear regression analysis to test whether the frequency of individuals with mixed-mating genotypes was associated with the self-fertilization frequency (as measured post-thaw) across our reanimated nematode populations at generation 33 (our experimental endpoint). All p-values reported are 2-tailed unless otherwise specified. All statistical tests were run using SPSS version 23.

Results

The selfing rates were not equal across all treatments at generation 10 of the experiment (*Welch's* $F_{2,\ 18.331} = 24.497$, $P < 0.001$), indicating that treatment significantly affected the

evolution of selfing rates during the first 10 generations of experimental evolution. Specifically, post hoc Games-Howell tests indicated that the mean selfing rate in the control treatment was significantly greater than the fixed-parasite treatment ($P = 0.005$), and the copassaged treatment ($P < 0.001$). This indicates that selfing spread more quickly in the controls than in either parasite treatment during the first 10 generations of evolution. There was no significant difference between the copassaged and fixed-parasite treatment selfing rates at generation 10 ($P = 0.645$) (figure 2).

The selfing rates were not equal across all treatments at generation 33, the experimental endpoint ($F_{2, 33} = 54.864$, $P < 0.001$). The selfing rate was significantly lower in the copassaged treatment than in the fixed-parasite treatment (Tukey HSD $P < 0.001$), and the selfing rate was significantly lower in the fixed-parasite treatment than in the control treatment (Tukey HSD $P = 0.015$) (figure 2).

The copassaged treatment selfing rate at our experimental endpoint was significantly greater than zero, suggesting that some selfing persisted throughout the entire experiment in at least some of the copassaged treatment host populations (mean selfing rate = 0.054, stdv = 0.086, $t_{11} = 2.185$, P (1-tailed) = 0.026) (figure 2).

Overall, the frequency of self-fertilization increased rapidly throughout the entire experiment in our control treatment populations. In our fixed-parasite treatment populations, selfing remained low initially, and then increased rapidly. In our copassaged populations, the selfing rate remained low throughout the entire experiment, although some selfing persisted in at least some of our copassaged populations (figure 2).

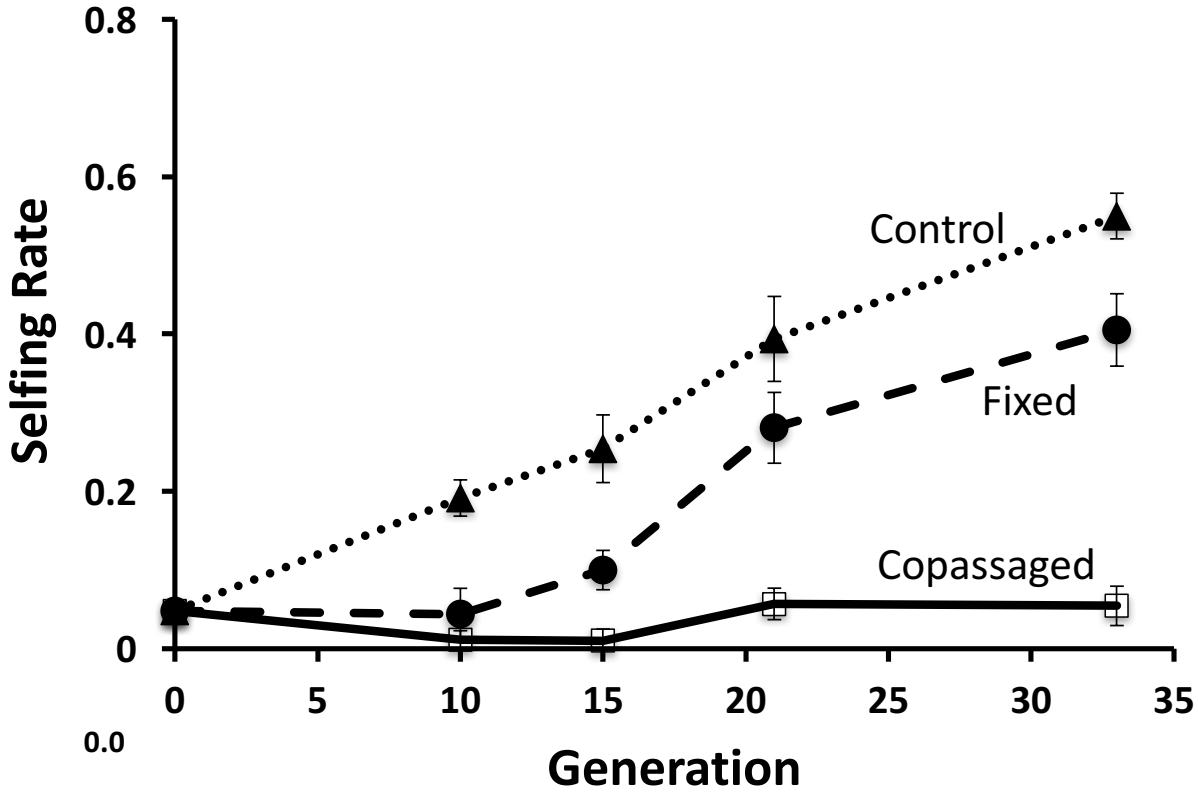

Figure 2) Mean selfing rates (± one standard error) over the course of the experiment. Host populations were exposed to three different treatments: control (heat-killed *S. marcescens*; dotted line and triangular markers), fixed-parasite treatment (fixed strain of *S. marcescens*; dashed line and circle markers), copassaged (copassaged *S. marcescens*; solid line and square markers) for 33 generations.

Evolution of the mixed-mating allele frequency

At generation 33 (our experimental endpoint), we found a highly significant positive relationship between the frequency of individuals with mixed-mating genotypes and the frequency of selfing across experimental populations ($r^2 = 0.911$, $F_{1,11} = 101.949$, $P < 0.001$) (figure 3).

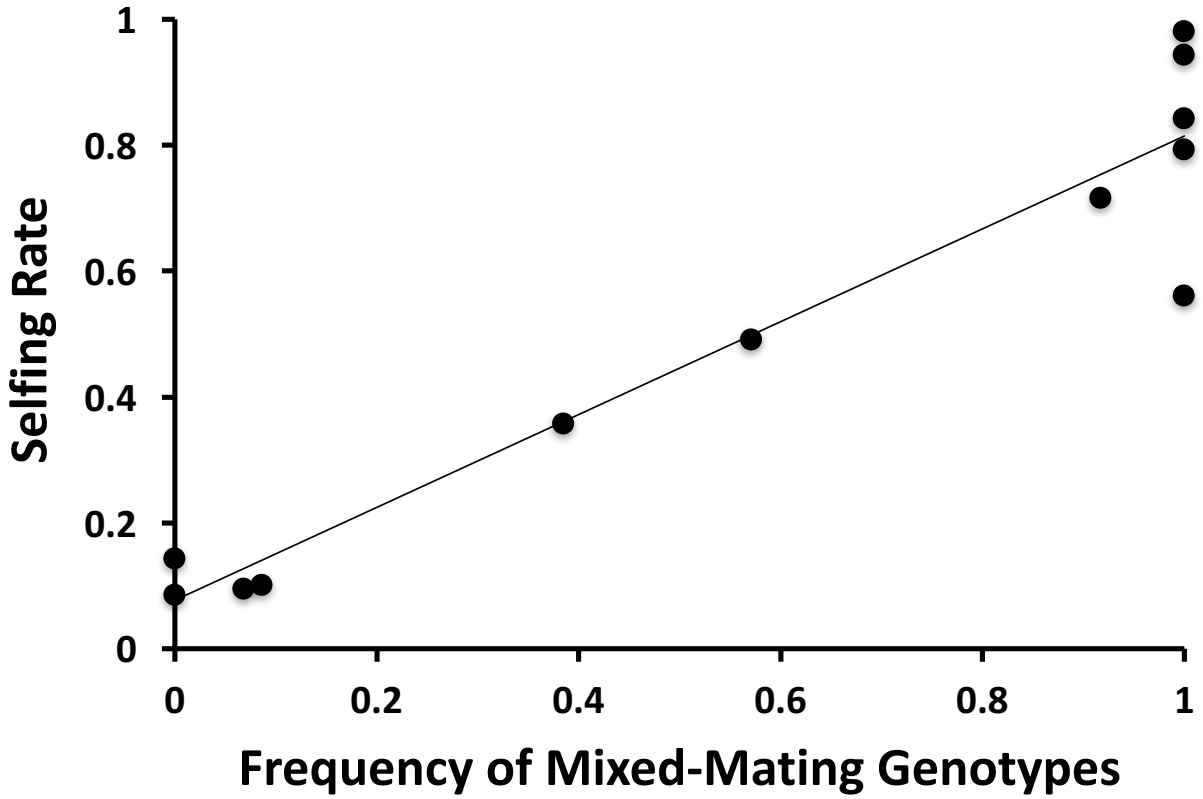

Figure 3) A bivariate plot showing the frequency of individuals with mixed-mating genotypes (i.e. the frequency of individuals with genotypes that permit both outcrossing and selfing) plotted against selfing rates for 12 host populations at generation 33 (our experimental endpoint).

Discussion

We tested a key prediction of the Red Queen hypothesis: coevolutionary interactions with parasites can constrain the spread of self-fertilization within outcrossing host populations. In our experimental laboratory system, we found that, in the absence of a coevolving parasite, self-fertilization rapidly invaded outcrossing populations of the nematode host *C. elegans*. Repeated exposure to a fixed (non-evolving) strain of the virulent bacterial parasite *S. marcescens* delayed, but ultimately did not prevent the spread of self-fertilization into host populations. However, the spread of self-fertilization into host populations was constrained throughout our entire 33-

generation experiment in our copassaged parasite treatment (Fig. 1), suggesting that antagonistic coevolutionary interactions with a virulent parasite can render obligate outcrossing resistant to invasion and replacement by self-fertilization. We also found that the spread of self-fertilization into outcrossing host populations was tightly linked with the spread of an allele that restored hermaphroditism and permitted self-fertilization (i.e. the mixed-mating allele *fog-2(wt)*).

Our observation that self-fertilization spread rapidly in control (no parasite) conditions is consistent with theoretical models which predict that biparental sex is costly (Fisher 1941; Maynard Smith 1978; Charlesworth 1980; Agrawal and Lively 2001). Furthermore, our results are consistent with previous empirical work in this study system which has demonstrated that self-fertilization rapidly invades outcrossing *C. elegans* populations under standard laboratory conditions (Stewart and Phillips 2002; Cutter 2005; Katju et al. 2008).

Our results also indicate that exposure to a novel environment may temporarily impede the invasion of self-fertilization. In our fixed-parasite treatment lines, the selfing rate remained low for the first 10 generations of our experiment, and then increased rapidly. We believe that the most likely explanation for this result is that outcrossing may have been favored in the fixed-parasite treatment populations at the beginning of the experiment, because it facilitated host adaptation to their novel parasite environment. However, after the fixed-parasite treatment host populations adapted to their static parasite environment, selection may have shifted. Selection could have favored selfing following adaptation because, as the host populations adapted to the fixed parasite, resistant host genotypes may have become common in the host populations. Once resistant host genotypes became common, outcrossing may have been selected against because it breaks up resistant host gene combinations. Additionally, selection may have favored selfing

following adaptation to the novel environment because of the numerical cost of outcrossing (Maynard Smith 1978).

Our fixed-parasite treatment results are consistent with previous work in androdioecious (wild-type) *C. elegans* populations which showed that exposure to non-evolving *Serratia marcescens* parasites initially results in increases in *C. elegans* host outcrossing rates, but that outcrossing is only temporarily favored over selfing in a static parasite environment (Morran et al. 2009b; Morran et al. 2011). Additionally, our results are consistent with experimental work in the facultatively sexual rotifer *Brachionus calyciflorus*, which showed that, when rotifers were initially exposed to a novel environment selection favored sexual reproduction, however, following adaptation to the novel environment selection favored asexual reproduction (Becks and Agrawal 2012).

While adaptation to a novel environment temporarily impeded the spread of self-fertilization in the fixed-parasite treatment, the spread of self-fertilization was constrained throughout the entire experiment in our copassaged treatment in which both the host and the parasite could evolve, and potentially coevolve. This result suggests that a coevolving parasite may provide the persistent selection necessary to maintain outcrossing in host populations in the long term. However, while the spread of selfing was constrained in the copassaged populations, a low frequency of self-fertilization did persist over the course of the entire experiment in at least some of our copassaged host populations. This persistence of some selfing, even in an environment with a coevolving parasite, suggests that parasite-mediated selection may favor some degree of self-fertilization in hosts. This result is consistent with theoretical models predicting that, when parasite virulence is high, parasites can select for mixtures of selfed and outcrossed host progeny (Agrawal and Lively 2001).

The rate at which self-fertilization spread in our experimental populations was probably highly dependent on the frequency with which mixed-mating hermaphrodites within our experimental populations self-fertilized their own offspring rather than reproduced by outcrossing with males. The selfing rate of hermaphrodites within *C. elegans* populations composed of both obligate outcrossers and mixed-maters is unknown. However, (Morran et al. 2009a) manipulated male frequencies in the *C. elegans* strain CB4856 (the strain from which the experimental populations in the present study were derived), and found that the selfing rates of hermaphrodites increases as the male frequency in the population decreases. Furthermore, parasite treatment may have affected the selfing rate of hermaphrodites within our experimental populations. Perhaps differences in the selfing rates of hermaphrodites could help explain treatment differences in the rate at which self-fertilization spread into experimental host populations.

Overall, our results provide strong evidence that coevolutionary interactions with parasites can constrain the spread of self-fertilization and prevent selfing from rising to fixation in outcrossing host populations in a controlled laboratory setting. Furthermore, the spread of the mixed-mating allele, which restores hermaphroditism and permits selfing, was tightly linked with the spread of selfing in our experimental populations. Taken together with other laboratory and field studies, our results strongly support the Red Queen hypothesis.

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Supplementary Material

Establishment of the obligately outcrossing populations

Prior to our experiment, following EMS mutagenesis of the nematode populations used in our experiment, each experimental population was divided into two different treatments, a control treatment and a fixed-parasite treatment. Each treatment underwent 30 generations of experimental evolution as part of another study (Morran et al. 2011). In the control treatment of the Morran et al. (2011) study, the populations were serially passaged on *Serratia* selection plates (SSPs) containing heat-killed *Serratia marcescens* (strain SM2170) for 30 generations. In the fixed-parasite treatment the populations were exposed to the same stock population of SM2170 for 30 generations, as described in (Morran et al. 2011). Samples of each population were frozen at generation 0 and generation 30 of the Morran et al. (2011) study. 4 replicate populations at generation 0, generation 30 of the fixed *S. marcescens* treatment, and generation 30 of the control treatment (for a total of 12 replicate starting populations) were reanimated and used as the starting obligately outcrossing populations in the current experiment. We used the experimental lines described above, with different histories of evolution with the *S. marcescens* parasite, as our starting populations so that we could assess the effect of previous adaptation to the parasite on the evolution of selfing rates in host populations in our experiment. However, we found no significant effects of previous evolutionary history on selfing rates. Furthermore, there were no significant interactions between previous evolutionary history and treatment in our experiment on selfing rates. Because we found no effects of previous evolutionary history on the evolution of selfing rates in our experiment, we did not include the previous evolutionary history of our source populations as a factor in our analyses.

Establishment of the mixed-mating invader strains

The invading wild-type (mixed-mating) individuals that we introduced into each replicate obligately outcrossing *C. elegans* population at the start of our experiment were established by independently introgressing the mixed-mating allele into each of our 12 replicate obligately outcrossing genetic backgrounds through a series of 7 backcrosses. The source of the wild-type allele was PX382, a systematically inbred wild-type (mixed-mating) strain of CB4856. After the 7th backcross a single homozygous *fog-2(wt)* hermaphrodite was isolated from each line and permitted to self on a small *E. coli*-seeded petri dish. This created a mixed-mating line that was derived from a single genotype. Each mixed-mating invader line (one genotype) shared its genetic background with the obligately outcrossing population into which the introgression had occurred. All populations of wild-type lines and of their associated obligately outcrossing lines were frozen at generation zero and then samples of each replicate were reanimated at the initiation of our experiment. At the initiation of our experiment, each mixed-mating strain was introduced into the obligately outcrossing population that shared its same genetic background.

Our approach of introgressing the wild-type allele into each obligately outcrossing genetic background prior to our experiment minimized the genetic differences between the mixed-maters and the obligate outcrossers in each of our replicate experimental populations (apart from any alleles tightly linked to the *fog-2(wt)* allele, which may have remained associated with the *fog-2(wt)* allele during our introgression). Therefore, our introgression approach made it unlikely that any differences in the fitness of the mixed-mating allele and the obligate-outcrossing allele in our experimental populations would be confounded by differences in the genetic backgrounds of the mixed-maters and the obligate outcrossers.

Measuring genotype frequencies at the fog-2 locus

We thawed a subsample of 12 nematode host populations that had been cryogenically frozen at generation 33, our experimental endpoint. The subsample of reanimated nematode populations was balanced across our 3 experimental treatments. In each reanimated nematode population, we isolated and extracted the DNA from a random sample of 38 larval nematodes. We used a polymerase chain reaction (PCR) to amplify the *fog-2* locus in the extracted DNA from each nematode, using the published primers F1RFLP, FogR4short, and FogR3 (Theologidis et al. 2014). We validated our results by running a negative control (reagents with no template DNA), and positive controls (2 nematodes with known homozygous *fog-2(wt, wt)* genotypes, and 2 nematodes with known homozygous *fog-2(q71, q71)* genotypes), on every gel. Consistent with previous results, (Theologidis et al. 2014), we found that *fog-2(wt/wt)* (mixed-mating) homozygotes yielded a 295 bp DNA band that can be visualized on agarose gels, while *fog-2(q71/q71)* (obligately outcrossing) homozygotes yielded a 264 bp band. Additionally, we ran a known heterozygote *fog-2(wt, q71)* control on a subset of our gels. Contrary to previous results, (Theologidis et al. 2014), we found that our heterozygote *fog-2(wt, q71)* controls yielded only one visible DNA band (295 bp). We don't know why the 264 bp band was not visible for heterozygotes, perhaps the 295 bp band was preferentially amplified under our PCR conditions. Therefore, heterozygotes could not be distinguished from *fog-2(wt, wt)* individuals in our assay.

Gels were scored by a treatment-blind observer (AB) to determine the frequency of *fog-2* genotypes in the sample of nematodes assayed from each experimental population. Because the *fog-2(wt)* allele is dominant, hermaphrodites that carry either one or two copies of the *fog-2(wt)* allele are capable of both selfing and outcrossing. Therefore, we refer to genotypes with at least one copy of the *fog-2(wt)* allele as "mixed-mating genotypes", since such genotypes permit both selfing and outcrossing when expressed in an individual with two X chromosomes. Because both

heterozygotes and *fog-2(wt, wt)* homozygotes produced a single 295 bp DNA band in our assays, any nematode that produced a 295 bp band was scored as a "mixed-mating genotype". Individuals that produced a 264 bp band were scored as "obligate-outcrossing genotypes". The frequency of individuals with mixed-mating genotypes in each experimental population was estimated by dividing the number of individuals that produced a 295 bp band by the total number of individuals that produced bands (either 295 bp or 264 bp) in the population sample.

Chapter 4

Does outcrossing propensity in an androdioecious host respond plastically to exposure to parasites in the environment or evolve in response to coevolution with parasites?

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Abstract

Despite substantial costs, biparental sex is the dominant mode of reproduction across most plant and animal taxa. The Red Queen hypothesis (RQH) posits that coevolutionary interactions with parasites can favor biparental sex in hosts, despite the costs. This hypothesis has been supported by recent studies showing that coevolutionary interactions with the virulent bacterial parasite *Serratia marcescens* can maintain high outcrossing rates in the androdioecious nematode host *Caenorhabditis elegans*. Here we propose and test two non-mutually exclusive mechanisms that could explain how coevolving parasites maintain high outcrossing rates in *C. elegans* host populations: 1) short-term exposure to parasites induces plastic increases in the propensity for hosts to reproduce by outcrossing (as opposed to self-fertilization), and 2) parasites cause coevolving parasites to evolve increased outcrossing propensities. We find no evidence that parasites cause plastic or evolved changes in host outcrossing propensity. We recommend future research to investigate alternative mechanisms that could explain how coevolving parasites maintain high outcrossing rates in *C. elegans* host populations. In particular we propose that

continuous culling of selfed offspring from host populations could maintain high outcrossing rates, even in the absence of parasite-induced changes in host outcrossing propensity.

Introduction

Despite substantial costs (Maynard Smith 1978, Gibson et al. 2017), biparental sex is the dominant mode of reproduction across most plant and animal taxa (Bell 1982, Vrijenhoek 1998). The Red Queen hypothesis (RQH) posits that coevolutionary interactions with parasites can produce negative-frequency dependent selection on hosts, perhaps favoring biparental sexual reproduction in host populations despite the costs (Jaenike 1978, Hamilton 1980). The RQH has received substantial empirical support, including several recent experimental studies that have demonstrated that coevolutionary interactions with the virulent bacterial parasite *Serratia marcescens* can maintain high outcrossing rates in the androdioecious nematode host *Caenorhabditis elegans* (Morran et al. 2011, Slowinski et al. 2016, Cho et al. unpublished manuscript). Specifically, Morran et al. (2011) showed that wildtype androdioecious *C. elegans* populations maintained high outcrossing rates when copassaged with the virulent parasite, while control populations of the nematode, which did not coevolve with a parasite, maintained low outcrossing rates. On the other hand, Slowinski et al. (2016) and Cho et al. (unpublished manuscript) manipulated the host breeding system using a mutant allele called *fog-2(q71)*, or the obligate outcrossing allele, which blocks the production of viable sperm in hermaphrodites, functionally transforming hermaphrodites into females. Slowinski et al. (2016) and Cho et al. (unpublished manuscript) showed that self-fertilization was unable to spread into obligately outcrossing populations of the nematode host *Caenorhabditis elegans* when host populations

were copassaged with the virulent bacterial parasite *Serratia marcescens,* while self-fertilization spread rapidly into control host populations that were not exposed to a coevolving parasite.

In the present study, we investigate the mechanisms by which coevolving parasites can maintain high outcrossing rates and prevent the spread of selfing in host populations. It has already been established that coevolving parasites can select against alleles that permit self-fertilization in *C. elegans* hosts. For example, Slowinski et al. (2016) demonstrated that the spread of self-fertilization was strongly positively correlated with the spread of *fog-2(wt),* the mixed-mating allele which permits self-fertilization, and Cho et al. (unpublished manuscript) showed that the *fog-2(wt)* allele spread rapidly into control host populations, but did not spread into host populations that were coevolving with the parasite. Taken together, these results demonstrate that coevolutionary interactions with the parasite prevented self-fertilization from spreading into host populations *by preventing the spread of a host allele which permits self-fertilization.* Hence parasite-mediated selection altered the evolution of the host breeding system by selecting on genetic variation at the *fog-2* locus that determines whether or not hosts are capable of reproducing by self-fertilization.

While parasite-mediated selection on genetic variation that determines whether or not hosts are capable of self-fertilization may be the primary mechanism by which parasites can prevent self-fertilization from spreading into outcrossing host populations, as observed by (Slowinski et al. 2016) and (Cho et al. unpublished manuscript), parasites may affect host population breeding systems in other ways as well. In the present study we propose and test an additional mechanism which could help explain how parasites maintain high outcrossing rates host populations. We propose that parasites cause hermaphroditic hosts to increase their propensity to reproduce by outcrossing as opposed to self-fertilization. Parasites could cause

increased outcrossing propensity in hermaphroditic hosts in two ways. First, hermaphrodites may plastically increase their outcrossing propensity in order to diversify their offspring in response to exposure to parasites in their environment. We will refer to this as the "plasticity of outcrossing propensity hypothesis". Here we define outcrossing propensity as the frequency with which hermaphrodites will outcross, given a standardized opportunity for outcrossing with a male. Previous studies have demonstrated that, in some species, hosts can plastically change their reproductive strategy to diversify their offspring in response to parasite exposure. For example, Singh et al. (2015) demonstrated that *Drosophila melanogaster* plastically increases the production of recombinant offspring after infection with parasites. Soper et al. (2014) showed that both males and females in the freshwater snail host *Potamopyrgus antipodarum* increased their number of different mating partners when exposed to a sterilizing trematode parasite. Here we test whether *C. elegans* also diversify their offspring in response to parasite exposure, by increasing their propensity to reproduce by outcrossing as opposed to self-fertilization when a parasite is detected in the environment. If outcrossing propensity increases in response to parasite exposure, then parasite-induced plastic changes in the outcrossing propensity of hermaphroditic hosts could help prevent the spread of self-fertilization, and maintain high outcrossing rates in host populations repeatedly exposed to a virulent parasite. The plasticity of outcrossing hypothesis predicts that hermaphrodites that experience a short-term parasite exposure should exhibit a higher propensity to outcross relative to hermaphrodites that have not experienced parasite exposure. We test this prediction by comparing the outcrossing propensity of *C. elegans* that have experienced a brief exposure to a virulent parasite during development (parasite-exposure treatment) versus the outcrossing propensity of *C. elegans* that have not been exposed

to a parasite (control treatment), with both treatments assayed in a standard, parasite-free environment.

The second way in which parasites could increase the propensity of hermaphroditic hosts to reproduce by outcrossing is that coevolutionary interactions with parasites could select for high outcrossing propensity in *C. elegans* hermaphrodites, and, consequently, hermaphrodites in *C. elegans* populations that coevolve with a virulent parasite could evolve a higher propensity to reproduce by outcrossing. The evolution of higher outcrossing propensity in *C. elegans* hermaphrodites coevolving with parasites could help maintain high outcrossing rates in *C. elegans* populations. We will refer to this as the "evolution of outcrossing propensity hypothesis". The evolution of outcrossing propensity hypothesis predicts that *C. elegans* hermaphrodites sampled from populations with a history of coevolution with a parasite should exhibit a higher propensity to outcross relative to hermaphrodites sampled from control populations with no history of coevolution. We tested this prediction by assaying the outcrossing propensity, in a controlled, parasite-free environment, of *C. elegans* hermaphrodites sampled from populations that were passaged under three different parasite treatments for 24 host generations in another study (Cho et al. unpublished manuscript) prior to our assays. In one treatment (heat-killed parasite), *C. elegans* hosts were passaged in an environment with heat-killed (avirulent) *Serratia marcescens* bacterial parasites. In the second treatment (static parasite), *C. elegans* hosts were exposed every generation to a fixed, non-evolving virulent strain of the *Serratia marcescens* parasite. In the third treatment (copassaged parasite), *C. elegans* hosts were copassaged with a virulent strain of *Serratia marcescens.* In the copassaged treatment both host and parasite were permitted to evolve and potentially to coevolve. We predicted that hermaphrodites sampled from the copassaged parasite treatment should exhibit a higher

propensity to outcross relative to hermaphrodites sampled from the heat-killed parasite and static parasite treatment populations.

Materials and Methods

Outcrossing propensity assays

Rationale for the outcrossing propensity assays

The purpose of our outcrossing propensity assays was to standardize the opportunity for *C. elegans* hermaphrodites to outcross, and then to test how experimental manipulation of parasite exposure, and experimental manipulation of coevolutionary history with a parasite, affects the frequency with which *C. elegans* hermaphrodites reproduce by outcrossing. We define "outcrossing propensity" as the frequency with which a *C. elegans* hermaphrodite will reproduce by outcrossing, given a standardized opportunity to reproduce by outcrossing or by self-fertilization. In our "plasticity of outcrossing propensity" experiment, we measured *C. elegans* outcrossing rates in a standard environment after manipulating *C. elegans* parasite exposure during development, to determine how brief parasite exposure during development affects hermaphrodite outcrossing propensity. In our "evolution of outcrossing propensity" experiment we sampled hermaphrodites from populations that had previously evolved in different parasite environments, and we assayed them in a standard (parasite-free) environment to assess how their history of evolution/coevolution with a parasite affected their propensity to outcross.

Synchronizing lines for outcrossing propensity assays

Prior to outcrossing propensity assays, the life-stages of the nematodes in assay populations were synchronized using a "hatch off" protocol (Stiernagle 2006). Nematode populations in liquid M9 solution were treated with 120 µl of 60% bleach solution mixed into

1,000 µl of M9/nematodes solution to kill all the nematode life stages except the eggs (Stiernagle 2006). Following exposure to the bleach solution, nematodes were washed with sterile deionized water and then continuously mixed in an M9 solution for 24 hours in a tube rotator, during which period the eggs hatched into L1 larvae. L1 larvae were transferred onto plates that had been constructed by pouring 24 mL of autoclaved nematode growth medium (NGM) Lite (US Biological, Swampscott, MA) onto a 10 cm Petri dish. The NGM had been seeded with a lawn of 60 µl of OP50 *E. coli* which had grown overnight in a 28° C incubator before the nematodes were transferred onto the plate. After the L1 nematodes were transferred onto the seeded *E. coli* plate they grew at 20° C for 48 hours until they matured into L4 larvae.

General protocol for assaying outcrossing propensity

Our outcrossing propensity assay protocol, and the source of our experimental lines, differed slightly between our "plasticity of outcrossing propensity" experiment and our "evolution of outcrossing propensity" experiment (see sections below for experiment-specific details). The general outcrossing propensity protocol was loosely based on (Bahrami and Zhang 2013), as follows:

After our nematode populations underwent the hatch-off procedure and matured to the L4 larval stage, we randomly selected and picked one L4 hermaphrodite onto a "mating plate". The L4 hermaphrodite on each mating plate was paired with a single randomly selected L4 male. Mating plates are small agar plates that serve as arenas in which replicate hermaphrodites can experience a standardized environment with a standardized opportunity to reproduce by outcrossing with a male or by self-fertilization. The proportion of offspring produced by outcrossing can be easily estimated based on male frequencies (Bahrami and Zhang 2013). Mating plates were constructed by pouring 4 mL of autoclaved NGM Lite (US Biological,

Swampscott, MA) into a 4 cm Petri dish. For the assays in the plasticity of outcrossing propensity experiment, the mating plates were seeded with 20 μl of *E. coli*, which was spread to cover the entire surface of the agar in the 4 cm Petri dish. For the assays in the evolution of outcrossing propensity experiment, the mating plates were seeded with 50 μl of *E. coli*, which was dropped on the surface of the agar in the middle of the Petri dish and not spread. Mating plates were incubated at 28° C for 24 hours to allow the *E. coli* to grow prior to transferring nematodes onto the mating plates. This process was repeated to create replicate mating plates for each experimental nematode line (and treatment) that we assayed (see below for details). We will refer to the L4 individuals that we picked onto the mating plates as the "parents" on the mating plates, because they are the reproductive individuals whose outcrossing propensity we assayed. The parents were left together on each mating plate for 48 hours, during which time the hermaphrodite on each mating plate had the opportunity to mate with the male and reproduce by outcrossing, or, alternatively, to reproduce by self-fertilization. After 48 hours, the parents, which had matured into adults, were removed from the mating plates, and their offspring, which were mostly eggs or early stage larvae, and which were easily distinguishable from the parents, were left behind to mature on the mating plates. Mating plates in which we could not find and remove both parents, or in which one of the parents had died during the 48-hour window of mating opportunity, were excluded from our analyses. Forty-eight hours after the parents were removed, we counted the male frequency of the offspring (which had matured into adults) on each mating plate by following a transect along the plate and scoring the sex of the first 200 nematodes that we saw. On mating plates with less than 200 nematodes, we scored the sex of all the nematodes on the plate. If we were unable to score the sex of at least 20 offspring the mating plate was excluded from our analysis, because estimating male frequencies based on samples

with a small number of individual worms is associated with a high level of sampling error. Outcrossing rates of the parents on our mating plates were estimated by doubling the male frequency of the offspring and adjusting for rare non-disjunction males, as described in (Stewart and Phillips 2002).

Source of the nematode lines used in our outcrossing propensity assays

The lab strains used in the present study were derived from the nematode strain CB4856, originally from Hawaii, which was purchased by Patrick Phillips' laboratory group from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN). Prior to our experiment, a line called PX382 was derived by systematically inbreeding the strain CB4856 (Morran et al. 2009). An obligate outcrossing strain called PX386 was derived from the inbred strain by systematically backcrossing the mutant allele *fog-2(q71)* into the genetic background of PX382 (Morran et al. 2009). Prior to our experiment, during three consecutive generations, 5 near-isogenic populations of PX386 and 5 near-isogenic populations of PX382 were mutagenized at 40mM of EMS for four hours to create 5 genetically diverse mixed-mating strains (fixed for the mixed-mating allele (*fog-2(wt)*) and 5 genetically diverse obligately outcrossing strains (fixed for the obligate outcrossing allele (*fog-2(q71)*) (Morran et al. 2011). This procedure induced approximately 1,000 point mutations per lineage in each population (Epstein and Shakes 1995, Morran et al. 2011). The genetically diverse mixed-mating and obligately outcrossing nematode populations were passaged for 30 generations under 3 experimental treatments (Morran et al. 2011). One of the genetically diverse mixed-mating populations (called CW1) that was passaged for 30 generations under the control (no parasite) treatment in the (Morran et al. 2011) study was used as the source of hermaphrodite and male parents on the mating plates in one of our plasticity of outcrossing propensity assays. One of the

genetically diverse obligately outcrossing strains (called F5) that was frozen prior to the (Morran et al. 2011) study (i.e. an obligately outcrossing strain that was an ancestor to the populations in the (Morran et al. 2011)) was used as the source of male parents on the mating plates in our plasticity of outcrossing propensity assays. The lab strain N2 (which we had never previously exposed to *S. marcescens* parasites, and which had not undergone EMS mutagenesis) was the source of hermaphrodite parents (paired with F5 males) in the second plasticity of outcrossing propensity assay.

Prior to the present study, the mixed-mating allele from the PX382 strain was introgressed through a series of seven backcrosses into the genetic background of 12 obligately outcrossing populations from the (Morran et al. 2011) study to create 12 mixed-mating populations each representing a genetic background derived from one of the obligately outcrossing populations (Slowinski et al. 2016). Cho et al. (unpublished manuscript) combined nematodes from one of those obligately outcrossing populations (which had evolved under the control treatment in the (Morran et al. 2011) study), with nematodes from its associated mixed-mating strain (i.e. with nematodes bearing the mixed mating allele and the same genetic background), derived in the (Slowinski et al. 2016) study, at a starting frequency of 10% mixed-maters, to create a "trioecious" experimental population (composed of males, females, and hermaphrodites). The trioecious population was divided into four replicate populations, and each of the four replicate populations was divided into three experimental treatments, which were passaged on *Serratia* selection plates (SSPs) for 24 host *C. elegans* generations (Cho et al. unpublished manuscript). Briefly, nematode populations in the heat-killed parasite treatment were passaged on SSPs seeded with heat-killed (avirulent) SM2170 *Serratia* strain. Nematode populations in the static parasite treatment were passaged on SSPs seeded with a stock, virulent,

but non-evolving population of SM2170. Nematode populations in the copassaged treatment were copassaged with an evolving (and potentially coevolving) strain of SM2170, which was originally seeded from the same stock population used in the fixed parasite treatment, but which was permitted to evolve over the course of the study. Nematode populations were passaged in all three treatments for 24 host generations in the Morran lab at Emory University. After 24 generations of experimental evolution, the evolved nematode strains (from each of the three experimental treatments) were shipped (on agar plates, unfrozen) to SPS in the Lively lab at Indiana University, where the outcrossing propensity assays for the evolution of outcrossing propensity study were conducted (see below for outcrossing propensity assay details).

Plasticity of outcrossing propensity experiment

To assess whether *C. elegans* outcrossing propensity changes plastically in response to brief exposure to parasites, we assayed the outcrossing propensity of a random sample of *C. elegans* that had been briefly exposed during the fourth larval stage of their development to SM2170, a virulent strain of the bacterial parasite *Serratia marcescens*. We compared the outcrossing propensity of *S. marcescens*-exposed *C. elegans* with the outcrossing propensity of control *C. elegans*, which were sampled randomly from the same population, but which had not been exposed to a parasite. Prior to each assay we synchronized a nematode population, in a hatch-off procedure as described above, by exposure to a bleach solution to kill all the life-stages except the eggs. Following the hatch off, the synchronized population of L1 larvae nematodes were deposited onto a 10-cm agar plate seeded with the *E. coli* strain OP50, which functioned as a food source for the nematodes. The larvae were permitted to grow and develop on the *E. coli* for 48 hours. After 48 hours, half of the population of nematodes, which had matured into L4 larvae, were randomly selected for a parasite-exposure treatment, and were liquid transferred

onto a 10-cm agar plate that had been seeded with live SM2170 strain of *Serratia marcescens* and grown overnight in a 28° C incubator prior to the transfer of nematodes. The remaining half of the population (i.e. the control group) was liquid transferred onto a 10-cm agar plate that had been seeded with OP50 (an avirulent strain of *E. coli*) and grown overnight in a 28° C incubator prior to the transfer of nematodes. After approximately two hours of exposure to SM2170 (treatment group) or OP50 (control group) the worms were transferred onto sterile filter paper set in the middle of 10 cm seeded *E. coli* plates. After the worms crawled off the filter paper and onto the *E. coli* lawn, L4 hermaphrodites and males were randomly selected and individually picked in pairs (one male and one hermaphrodite per plate) onto mating plates. The purpose of allowing the worms to crawl over filter paper before transferring them onto mating plates was to allow the worms to shed *S. marcescens* cells that may have attached to their cuticles during the brief parasite exposure treatment, so that the worms would not carry parasite cells with them onto the mating plates. The outcrossing propensity of the worms on the mating plates was assayed as described above in the section called *General Protocol for Assaying Outcrossing Propensity*. The plasticity of outcrossing propensity assay was conducted twice. In the first assay, parasite-exposed and control hermaphrodites from the genetically diverse strain CW1-30, which had been previously passaged in the control treatment in the (Morran et al. 2011) study for 30 generations, were each paired on mating plates with males from their same population and exposure treatment. In the second plasticity of outcrossing propensity assay, parasite-exposed and control hermaphrodites from the inbred lab strain N2 were each paired with unexposed (control) males from the genetically diverse, obligately outcrossing ancestral strain F5 from the (Morran et al. 2011) experiment.

Evolution of outcrossing propensity experiment

Maintenance of the experimental nematode populations prior to outcrossing propensity assays

Experimental nematode lines were established by Cho et al. (unpublished manuscript) at Emory University as described above and were shipped to SPS at Indiana University. Experimental lines were maintained at 15° C at Indiana University and transferred once per week onto fresh agar plates seeded with OP50, until their outcrossing propensity was assayed. At 15° C the *C. elegans* life cycle is completed in about one generation per week. Outcrossing propensity was assayed between four weeks (first experimental line that was assayed) and nine weeks (final experimental line that was assayed) after the experimental lines were removed from selection in the (Cho et al. unpublished manuscript) experiment. Therefore, we estimate that the experimental populations were maintained out of selection (in a standard laboratory environment with no parasite) for between four and nine generations after they were removed from their respective parasite treatments and before we assayed their outcrossing propensity. We assayed all the treatments of one replicate before moving onto assaying the next replicate, so the amount of time that experimental populations were maintained out of selection prior to being assayed did not differ across treatments. We measured the male frequencies in each of the experimental lines that served as the source populations for our outcrossing propensity assays, and confirmed that our experimental populations had maintained treatment differences in male frequencies during the period in which they were maintained out of selection prior to our outcrossing propensity assays (i.e. the copassaged experimental populations had maintained significantly higher male frequencies than the control and the fixed treatment experimental populations).

Evolution of outcrossing propensity assays

Outcrossing propensity was assayed on 10 replicate mating plates from each of the four replicate control treatment populations and on 10 replicate mating plates from each of the four

replicate fixed treatment host populations. Outcrossing propensity was assayed on 20 replicate mating plates from each of the four replicate copassaged host populations. The populations assayed in this experiment evolved from trioecious starting populations (i.e. populations composed of hermaphrodites, females, and males) which segregated both the mixed-mating allele *fog-2(wt)* and the obligate outcrossing allele *fog-2(q71*). Most of these populations were still composed of all three sexes (hermaphrodites, females, and males) and still segregated both the mixed-mating allele and the obligate outcrossing allele when we assayed them, although some of the populations might have lost one or the other allele over the course of experimental evolution (Cho et al. unpublished manuscript). Hermaphrodites (i.e. individuals with two X chromosomes that express the mixed-mating allele) cannot be distinguished morphologically from females (i.e. individuals with two X chromosomes that express the obligate outcrossing allele) (Schedl and Kimble 1988). When picking mating plates for these assays we paired each L4 male with a partner who we determined based on morphology to be either a hermaphrodite or a female. Because females must outcross with males to reproduce, whereas hermaphrodites can reproduce by either outcrossing or by self-fertilization, it was critical for us to distinguish between hermaphrodites and females in the analysis of our outcrossing propensity assay results. To determine which mating plates had female mothers and which had hermaphrodite mothers, we used molecular methods to genotype the mothers at the *fog-2* locus, after the outcrossing propensity assays. When we removed the parents from the mating plates after 48 hours of mating opportunity, we picked the mother into a proteinase k solution and her DNA was extracted for *fog-2* genotyping assays (see below). See figure 1 for a conceptual visualization of our experimental design.

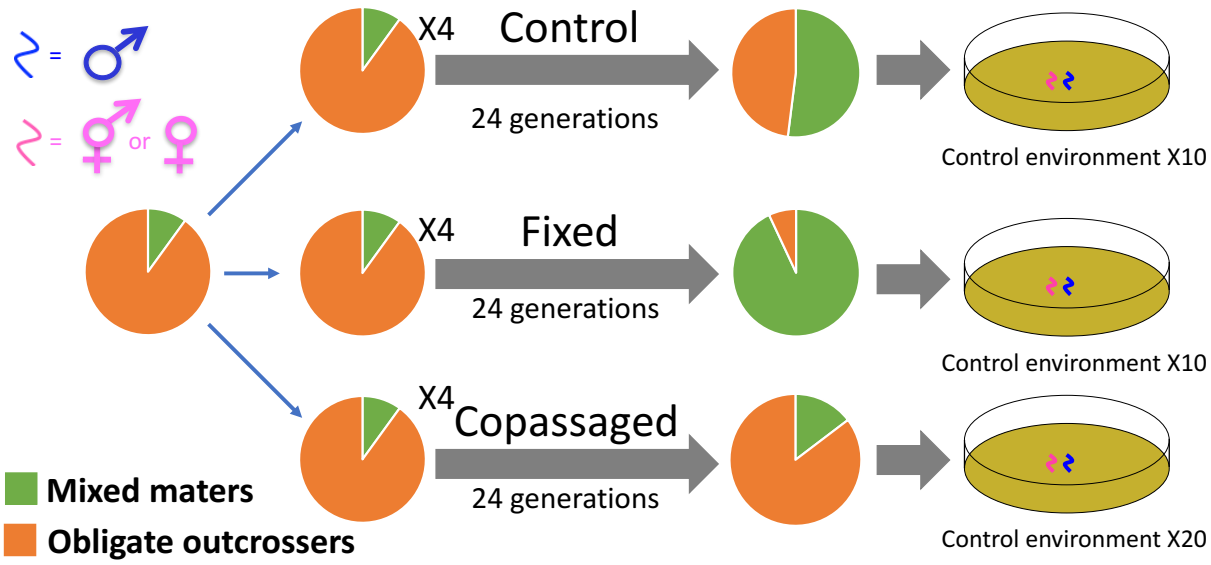

Figure 1: A visual representation of the experimental design we used to test whether outcrossing propensity in host populations evolved in response to coevolutionary interactions with parasites. An ancestral nematode population composed of 10% mixed-mating (wildtype) *C. elegans,* represented in green, and 90% obligate outcrossing (*fog-2(q71)*) *C. elegans*, represented in orange was divided into three experimental treatments. Within each treatment, populations were then divided into 4 experimental replicates. Replicate populations evolved for 24 generations under the three experimental treatments. Mixed mating spread in the control and fixed populations but did not spread (much) in the copassaged populations (Cho et al. unpublished manuscript). After 24 generations, hermaphrodite/females (represented in pink) and males (represented in blue) were paired on replicate mating plates. The sex of the mother (hermaphrodite or female) was determined after the assay based on molecular genotyping. The outcrossing rate on each mating plate was determined based on offspring male frequencies.

Genotyping assays to determine the sex of the mother on each mating plate

Because we were unable to distinguish hermaphrodites from females morphologically, we extracted DNA from the mothers (i.e. the hermaphrodite or female parents on our mating plates) and used polymerase chain reactions (PCRs) to amplify the *fog-2* locus to determine the *fog-2* genotype of the mother from each of our mating plates. Each individual mother had her DNA extracted and amplified by PCR in a separate reaction. DNA was extracted when the parents were removed from the mating plates (48 hours after they were picked as L4 larvae onto the mating plates) by picking the nematodes into a proteinase K solution and thermal-cycling the

solution. Extracted DNA was stored frozen until it was used in the PCRs. We used the published PCR primers F1RFLP, FogR4short, and FogR3 (Theologidis et al. 2014). We validated our results by running positive controls (i.e. a nematode with known homozygous *fog-2(wt, wt)* genotype and a nematode with known homozygous *fog-2(q71, q71)* genotype) on every gel. Nematodes with a *fog-2(wt, wt)* genotype yield a 295 bp DNA band that can be visualized on agarose gels, while *fog-(q71, q71)* genotypes yield a 264 bp band (Theologidis et al. 2014, Slowinski et al. 2016). Previous studies have reported conflicting results for genotyping heterozygous *fog-2(wt, q71)* individuals using these primers. Theologidis et al. (2014) reported that *fog-2(wt, q71)* heterozygotes exhibited a double band pattern, while Slowinski et al. (2016) reported that *fog-2(wt, q71)* heterozygotes yielded only one visible DNA band (295 bp) and therefore were indistinguishable from *fog-2(wt, wt)* individuals in the assays. Because the goal of our PCR assays was to determine the sex (hermaphrodite or female) of the mother on each mating plate, it was not important for us to distinguish between *fog-2(wt, q71)* heterozygous genotypes and *fog-2(wt, wt)* homozygous genotypes because both genotypes will express a hermaphrodite phenotype. Therefore, in our assays, we scored individuals as hermaphrodites whenever we observed at least one DNA band at 295 bp, and we scored individuals as females when we observed only one band at 264 bp. Gels were scored by SPS. SPS was blind regarding the offspring sex ratio when he scored the *fog-2* genotype of each mother on the gels. As additional controls, we ran the PCRs and gels on known females *(fog-2(q71, q71))* that had mated with mixed-mating males *(fog-2(wt, wt))* and on known hermaphrodites *(fog-2(wt, wt))* that had mated with obligately outcrossing males *(fog-2(q71, q71))* to confirm that the male's sperm inside mated mothers does not amplify and alter the apparent genotype of the mother in our genotyping assays.

Statistical methods

Excluded data

We excluded mating plates (two plates excluded from the plasticity of outcrossing propensity study, zero plates excluded from the evolution of outcrossing propensity study) from our data set if we were unable to find the male or the hermaphrodite/female when we went back to pick the parents off of the mating plates 48 hours after they had originally been picked onto the mating plates. We also excluded mating plates (six plates excluded from the plasticity of outcrossing propensity study, four plates excluded from evolution of outcrossing propensity study) on which there were fewer than 20 offspring which we were able to score for our offspring male frequency counts, because, due to sampling error, the uncertainty in our estimates of outcrossing rates is much higher on plates with few offspring. In the evolution of outcrossing propensity project we also excluded plates (n = 36) for which we were unable to conclusively determine the sex (hermaphrodite or female) of the mother. In the evolution of outcrossing propensity project, data from mating plates with hermaphrodite mothers, and data from mating plates with female mothers, were analyzed separately (see below).

Estimating outcrossing rates from male frequencies

Because of the relationship between parent outcrossing rates and the sex ratio of their offspring, the outcrossing rates in *C. elegans* populations can be accurately estimated based on population male frequencies (Stewart and Phillips 2002). We used the following equation to estimate population outcrossing rates (Stewart and Phillips 2002):

$$Outcrossing\ rate = (Male\ frequency - 0.0015) \times 2$$

in which 0.0015 is subtracted from the male frequency in the equation as a correction for selfed males produced by non-disjunction of the X chromosome during meiosis.

Plasticity of mating propensity in response to parasite exposure

We used a 2-way ANOVA to test whether nematode strain (CW1-30 or N2) and parasite exposure treatment (parasite-exposed or control) affected outcrossing rates on our mating plates. We treated experimental line and parasite exposure treatment as fixed factors, and outcrossing rate as our dependent variable.

Evolution of outcrossing propensity

Hermaphrodite outcrossing rates

We used a one-way ANOVA to test whether the outcrossing rates of hermaphrodites differed among treatments. For this analysis, within each experimental treatment, we pooled the mating plates from all of the replicate host populations.

Female offspring sex ratios

We used a one-sample t-test to assess whether the offspring sex ratio of nematodes that were determined to be females (based on our molecular sexing assays) differed significantly from an even sex ratio of 50% males and 50% hermaphrodites/females. For this analysis, we considered all mating plates with female mothers (mating plates pooled across treatments and across population replicates).

Results

Plasticity of outcrossing propensity

In a 2-way ANOVA we found no significant nematode strain by treatment interaction on outcrossing rates ($F_{1,48} = 0.003$, $p = 0.959$). We found no significant main effect of parasite

exposure treatment on outcrossing rates ($F_{1,48} < 0.312$, $p = 0.579$). However, there was a statistically significant main effect of nematode strain on outcrossing rates ($F_{1,48} = 117.913$, $p < 0.001$, figure 2).

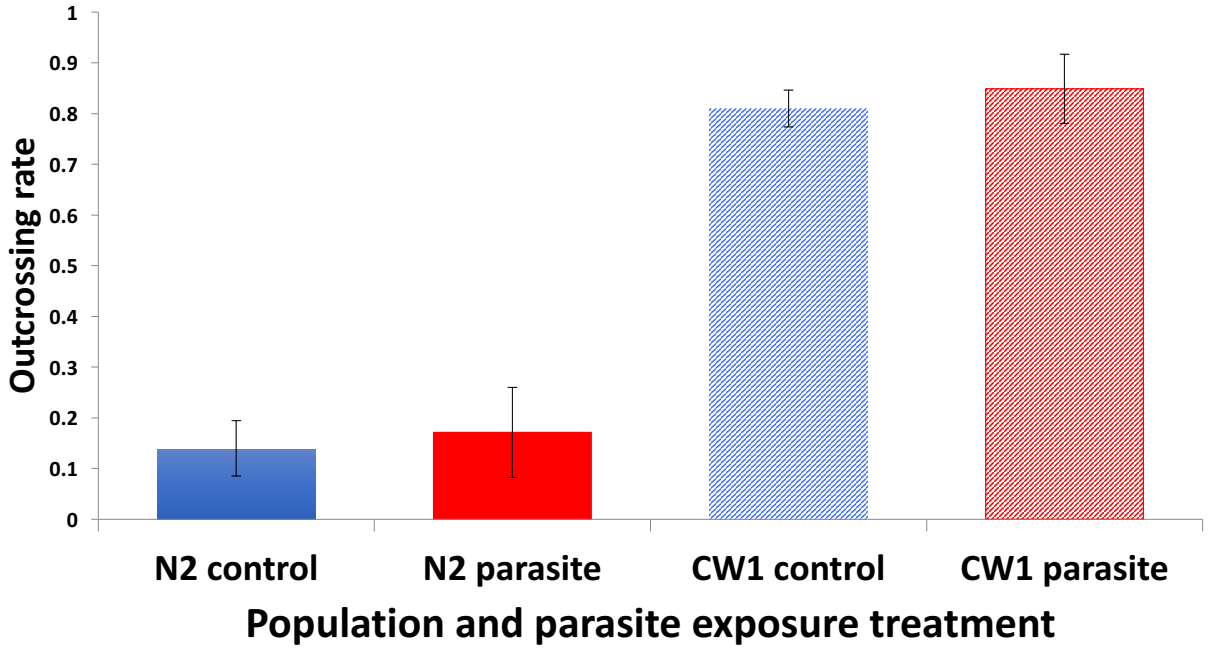

Figure 2: Outcrossing rates of hermaphrodites on mating plates following exposure to avirulent *E. coli* in the control treatment (blue) or to *S. marcescens* strain SM2170 in the parasite exposure treatment (red). N2 lab strain represented by solid bars. CW1 strain (derived from CB4856) represented by dotted bars. Error bars represent ± one standard error of the mean.

Evolution of outcrossing propensity

Comparing hermaphrodite outcrossing rates among treatments

We found no effect of experimental treatment on the outcrossing rates on mating plates with hermaphrodite mothers, as determined by molecular genotyping assays (one-way ANOVA, $F_{2, 60} = 0.043$, $P = 0.958$, figure 3).

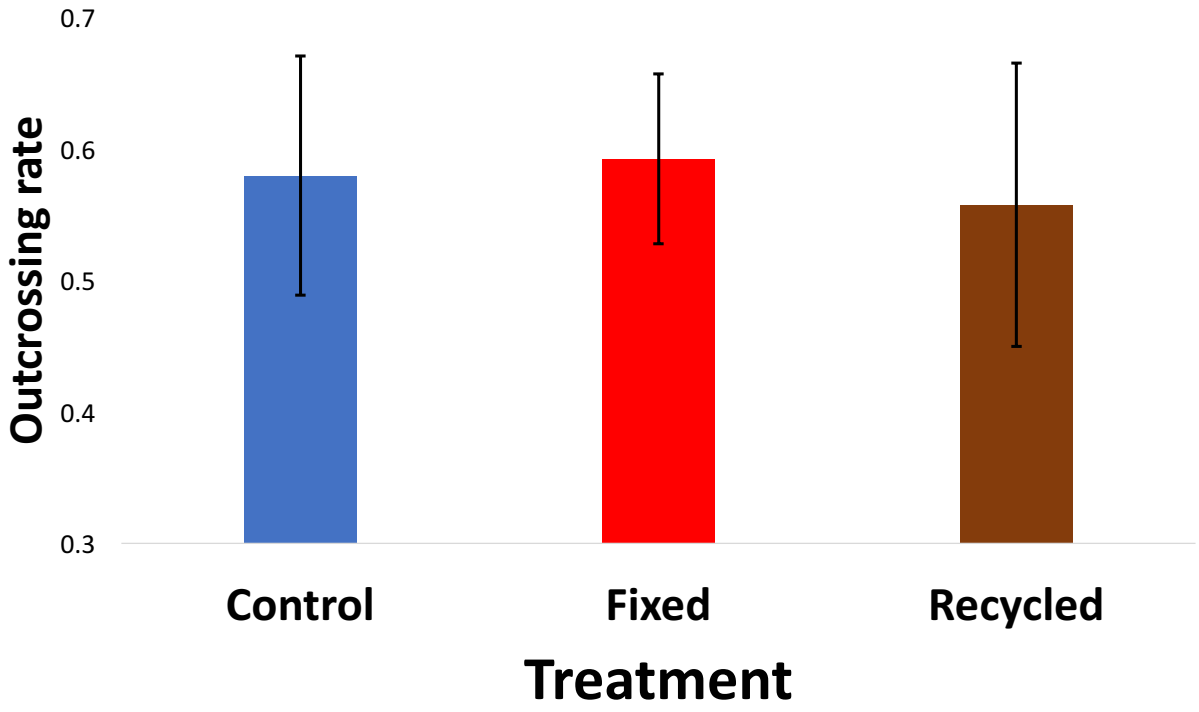

Figure 3: Outcrossing rates on mating plates with hermaphrodite mothers from the control treatment (blue), the fixed-parasite treatment (red), and the recycled parasite treatment (brown). Error bars represent ± one standard error of the mean.

Offspring sex ratio on mating plates with female mothers

We found that the male frequency of the offspring on mating plates with female mothers, as determined by our molecular genotyping assays, did not differ significantly from 0.5 (mean male frequency = 0.508349, 95% CI: 0.484-0.533, $t_{30} = 0.704$, $p = 0.487$).

Discussion

We found no effect of brief parasite exposure on outcrossing propensity in *C. elegans*. This result suggests that *C. elegans* do not plastically increase their propensity to outcross in response to exposure to parasites in their environment. Furthermore, we found no effect of evolution/coevolution with a parasite on outcrossing propensity in *C. elegans*. This result

suggests that *C. elegans* outcrossing propensity does not evolve in response to selective pressure from a coevolving parasite, at least not on the evolutionary timescale of this experiment (24 generations). Previous research has demonstrated that coevolutionary interactions with parasites can maintain high outcrossing rates in *C. elegans* host populations (Morran et al. 2011, Slowinski et al. 2016, Cho et al. unpublished manuscript). Based on the results of the present study, we reject the hypothesis that parasite-induced plastic or evolved changes in host outcrossing propensity explain how coevolutionary interactions with parasites can maintain high outcrossing rates in *C. elegans* host populations. Future research should test alternative mechanisms that could explain how parasites maintain high outcrossing rates in *C. elegans* host populations. In particular, ongoing and future work by our research group will test the hypothesis that outcrossed offspring have higher survival when exposed to a coevolving parasite, and that selective culling of selfed hosts by the parasite can explain how the coevolving parasite maintains high outcrossing rates in host populations.

Plasticity of outcrossing propensity

We found no effect of brief exposure to the bacterial parasite *Serratia marcescens* on outcrossing propensity in *C. elegans*. This result contrasts with recent research in other host-parasite study systems showing that parasites can induce plastic changes in host reproductive behaviors, allowing hosts to diversify their offspring in response to exposure to parasites in their environment. In particular, Singh et al. (2015) demonstrated that the fruit fly host *Drosophila melanogaster* can plastically increase their production of recombinant offspring in response to infection with diverse parasites, and Soper et al. (2014) demonstrated that the snail host *Potamopyrgus antipodarum* increases its number of mating partners in response to exposure to the sterilizing trematode parasite *Microphallus sp.* 'livelyi'. We do not know why studies in

other host-parasite systems have found strong evidence that hosts plastically diversify their offspring in response to parasite exposure, while we found no such evidence in our *C. elegans/S. marcescens* host/parasite system. The ability for hosts to diversify their offspring in response to parasite exposure may be system specific, with *D. melanogaster* and *P. antipodarum* exhibiting this response to parasites while *C. elegans* does not. Alternatively, it is possible that parasites can induce plastic changes in *C. elegans* outcrossing propensity, but that the parasite treatment that *C. elegans* was exposed to in our study was not sufficient to induce this effect. We exposed *C. elegans* to *S. marcescens* for about two hours during the 4th larval stage of development. Perhaps a longer exposure, or exposure to the parasite during a different developmental stage, would be necessary to induce plastic changes in outcrossing propensity. However, it is noteworthy that in another study, brief exposure to the *S. marcescens* strain Db11 (less than six hours) was sufficient for intact *S. marcescens* bacteria to get into the host intestinal lumen and start proliferating, and also to induce upregulation of host genes associated with immune defense (Mallo et al. 2002).

Evolution of outcrossing propensity

We found no difference in the outcrossing propensity of hermaphrodites across experimental evolution treatments. This result suggests that *C. elegans* outcrossing propensity does not evolve in response to evolutionary, or coevolutionary, interactions with parasites, at least on the evolutionary time scale of our experiment (24 host generations). One possible explanation for why outcrossing propensity did not evolve in response to coevolutionary interactions with the parasite is that coevolution with the parasite did not produce strong selection on outcrossing propensity. However, we consider this explanation to be unlikely. Morran et al. (2011), Slowinski et al. (2016), and Cho et al. (unpublished manuscript) all found

that *C. elegans* host populations coevolving with the *S. marcescens* parasite maintained high outcrossing rates relative to host populations not coevolving with the parasite, which strongly suggests that the coevolving parasite would select for hosts that preferentially reproduce by outcrossing as opposed to self-fertilization. An alternative possible explanation for why were unable to detect evolution of outcrossing propensity in *C. elegans* hosts in response to coevolution with the *S. marcescens* parasite is that perhaps there was little or no additive genetic variance for outcrossing propensity in the host populations in our experiment. In this scenario, the evolution of outcrossing propensity would be constrained even if coevolution with the parasite strongly selected for increased outcrossing propensity. We consider this possibility to be more likely. While Bahrami and Zhang (2013) measured major differences in outcrossing propensity among diverse *C. elegans* lab strains, suggesting high genetic variance for outcrossing propensity *among* lab strains, less is known about the genetic variance for outcrossing propensity *within* lab strains. The *C. elegans* lines used in our experiment, which were derived from the CB4856 lab strain originally from Hawaii, were mutagenized by exposure to ethyl methan-sulfonate (EMS) prior to our experiment (Morran et al. 2011). Mutagenesis infused novel genetic variation into the experimental populations, but we don't know whether or not mutagenesis introduced genetic variation at loci that might affect outcrossing propensity. Future research should assess whether the experimental populations in our study exhibit heritable genetic variation for outcrossing propensity, and whether a lack of genetic variation for outcrossing propensity could explain why outcrossing propensity did not evolve in response to coevolution with the parasite in our study.

In spite of our observation that parasites do not induce plastic changes in *C. elegans* outcrossing propensity, and that outcrossing propensity does not evolve quickly in *C. elegans*

populations in response to coevolutionary interactions with parasites, three recent studies have all demonstrated that coevolutionary interactions with parasites can maintain high outcrossing rates in *C. elegans* host populations (Morran et al. 2011, Slowinski et al. 2016, Cho et al. unpublished manuscript). How can parasites maintain high outcrossing rates if they do not affect host outcrossing propensity?

In the Slowinski et al. (2016) study and in the Cho et al. (unpublished manuscript) study, host populations segregated different alleles at the *fog-2* locus, which affect breeding system. The *fog-2(wt)* allele, which we call the mixed-mating allele, permits hermaphrodites to reproduce by either outcrossing or self-fertilization. The mutant *fog-2(q71)* allele, which we call the obligate outcrossing allele, blocks the production of viable sperm in hermaphrodites, functionally transforming hermaphrodites into females by forcing them to outcross with males in order to reproduce. Because of this heritable variation for breeding system at the *fog-2* locus, parasite-mediated selection in these studies could have maintained high outcrossing rates by favoring males and "females" (who have to reproduce by outcrossing) over hermaphrodites (which can reproduce by self-fertilization). In this way, parasites could have selected for obligate outcrossing genotypes without requiring any changes in the outcrossing propensity or outcrossing behavior of hermaphrodites. In fact, Cho et al. (unpublished manuscript) found that, after 24 generations of experimental evolution, *C. elegans* populations that were copassaged with the parasite had maintained much higher frequencies of the obligate outcrossing allele relative to control populations (passaged in the absence of the parasite) and fixed-treatment populations (passaged every generation on the same stock non-evolving parasite population). This suggests that coevolving parasites maintained high host outcrossing rates, at least in part, by selecting for obligately outcrossing host genotypes.

On the other hand, Morran et al. (2011) demonstrated that coevolution with parasites can maintain high levels of outcrossing in *C. elegans* populations composed entirely of hermaphrodites and males (i.e. in populations fixed for the mixed-mating allele). In such populations, parasites cannot select for obligately outcrossing genotypes, because all hermaphrodites are capable of self-fertilization. We propose that, in the Morran et al. (2011) study, parasites maintained high rates of host outcrossing by selectively culling selfed individuals from the host populations. Relative to outcrossing, self-fertilization produces more offspring with common genotypes, and the RQH predicts that selfed offspring with common genotypes are, on average, more susceptible to coevolving parasites. In wildtype (mixed-mating) *C. elegans* populations, outcrossing produces a 50/50 ratio of male to hermaphrodite offspring, while almost 100% of the offspring produced by self-fertilization are hermaphrodites. If selfed offspring are more susceptible to infection by coevolving parasites, as predicted by the RQH, then selfed individuals will be selectively removed from populations coevolving with a parasite. Because almost all selfed individuals are hermaphrodites, this would result in reducing the frequency of hermaphrodites (and increasing the frequency of males) which could result in increased outcrossing opportunity for the hermaphrodites remaining in the population. This mechanism, of selective culling of selfed hosts by the coevolving parasite, could maintain high outcrossing rates in host populations, even in the absence of any parasite induced changes in hermaphrodite outcrossing propensity. Current and future work by our research group will test the prediction that, relative to outcrossed offspring, selfed offspring experience greater mortality when exposed to a coevolving parasite.

Conclusions

Several recent studies have demonstrated that coevolutionary interactions with the virulent bacterial parasite *Serratia marcescens* can maintain high outcrossing rates in the nematode host *Caenorhabditis elegans* (Morran et al. 2011, Slowinski et al. 2016, Cho et al. unpublished manuscript). In the present study, we tested whether parasite-induced changes in host outcrossing propensity could explain how coevolving parasites maintain high outcrossing rates in host populations. Specifically, we tested whether short term exposure to parasites can induce plastic increases in host outcrossing propensity, and whether long term experimental evolution with a coevolving parasite can lead to the evolution of increased outcrossing propensity in host populations. We found no evidence that parasites induce plastic or evolved changes in host outcrossing propensity. This result suggests that parasite induced changes in host outcrossing propensity are unlikely to explain how coevolutionary interactions with parasites maintain high outcrossing rates in host populations, at least in our experimental study system. Ongoing and future research will test alternative mechanisms that could explain how coevolving parasites maintain high host outcrossing rates, especially the hypothesis that parasites selectively cull selfed hermaphrodites from host populations.

Acknowledgements

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Diversity program for a Predoctoral Fellowship to SPS, as well as for providing funding for reagents and materials for the experiments (grant number 2 T32 HD049336-11A1).

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Slowinski, S. P., L. T. Morran, R. C. Parrish, E. R. Cui, A. Bhattacharya, C. M. Lively, and P. C. Phillips. 2016. Coevolutionary interactions with parasites constrain the spread of self-fertilization into outcrossing host populations. Evolution **70**:2632-2639.

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Conclusions

In chapter one, I found that a long-distance migrant population of juncos maintains a significantly lower prevalence of haemosporidian parasite infections relative to a closely related and seasonally sympatric sedentary junco population, suggesting that long-distance host migration may be associated with reduced parasitism in juncos. My result is consistent with some previous research showing that long-distance host migration is associated with reduced parasitism (e.g. Altizer et al. 2000, Poulin et al. 2012, Satterfield et al. 2016). However, my result contrasts with other research showing that long-distance host migration can be associated with increased parasitism (Kelly et al. 2016). Overall, my results point to the need for more research to determine the circumstances under which host migration increases parasitism, versus the circumstances under which host migration should be expected to reduce parasitism.

In chapter two, contrary to the predictions of the Immunocompetence Handicap Hypothesis (ICHH), I found no effect of testosterone elevation on haemosporidian parasite infection prevalence. However, my results should not be taken as a strong rejection of the ICHH because the post-treatment prevalence of haemosporidian infections was very high in the control (unmanipulated) juncos in my study. Given the high prevalence of infection in control juncos, it would have been very hard to detect a testosterone-induced increase in haemosporidian parasitism, even if testosterone was immunosuppressive. I found no relationship between haemosporidian infections and host telomere degradation, contrary to two recent studies showing that haemosporidian parasite infections can accelerate telomere loss in avian hosts (Asghar et al. 2015, Asghar et al. 2016). I believe my results highlight the need for research to determine why haemosporidian parasites accelerate telomere loss in some contexts but not others. Finally, in chapter two, I showed that the prevalence of haemosporidian parasite infections increases with

host age. This result raises important questions for future research. For example, what mechanisms cause haemosporidian parasite prevalence to increase with host age in juncos? How does host immune function change with age? Why did I find a positive relationship between host age and haemosporidian infections in juncos while previous work (van Oers et al. 2010, Hammers et al. 2016) has found a negative relationship between host age and infection prevalence in another songbird, the Seychelles warbler?

In chapter three, I showed that coevolving bacterial parasites (*Serratia marcescens*) can constrain the spread of self-fertilization into obligately outcrossing populations of nematode (*Caenorhabditis elegans*) hosts. This result supports the Red Queen hypothesis and contributes to a large body of evidence that antagonistic coevolution between hosts and parasites can maintain biparental sex. While self-fertilization was unable to spread in host populations coevolving with the virulent parasite, self-fertilization was also not eliminated. This result raises important questions about how much outcrossing is necessary to evade infection from coevolving parasites, and about the conditions under which both outcrossing and self-fertilization can be maintained simultaneously. Furthermore, my results highlighted the need for more research to investigate the mechanisms by which coevolving parasites can maintain high outcrossing rates in host populations, an area that I investigated in chapter four.

In chapter four, I showed that the presence of parasites (*S. marcescens*) in the environment does not induce plastic changes in the propensity to outcross in hosts (*C. elegans*) capable of both outcrossing and self-fertilization, and that coevolutionary interactions with *S. marcescens* parasites does not cause *C. elegans* hosts to evolve a higher propensity to outcross. My results suggest that parasites do not maintain high outcrossing rates in host populations by causing increases in the propensity of hosts to reproduce by outcrossing. This raises the

important question: If coevolving parasites do not increase host outcrossing propensity, then how do coevolving parasites maintain high outcrossing rates in host populations? I recommend that future research address this question and I propose that differential susceptibility of selfed versus outcrossed host offspring may offer a possible solution.

Summary of conclusions

Overall, in a natural avian host/haemosporidian parasite system, I have demonstrated that long-distance host migration is associated with reduced parasite infection prevalence, that hosts in breeding condition are more intensely infected by parasites than hosts in non-breeding condition, that experimental elevation of testosterone does not affect parasite infection prevalence, that parasite infection does not affect the rate of host telomere degradation, and that infection prevalence is positively associated with host age. In a laboratory experimental system, I have shown that coevolutionary interactions with parasites can constrain the spread of self-fertilization into outcrossing host populations, but that parasites do not induce plastic or evolved changes in host outcrossing propensity.

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Bauer, C. M., B. J. Heidinger, E. D. Ketterson, and T. J. Greives. 2016. A migratory lifestyle is associated with shorter telomeres in a songbird (Junco hyemalis). Auk **133**:649-653.

Hammers, M., J. Komdeur, S. A. Kingma, K. Hutchings, E. A. Fairfield, D. L. Gilroy, and D. S. Richardson. 2016. Age-specific haemosporidian infection dynamics and survival in Seychelles warblers. Scientific reports **6**.

Kelly, T. R., H. L. MacGillivray, Y. Sarquis-Adamson, M. J. Watson, K. A. Hobson, and E. A. MacDougall-Shackleton. 2016. Seasonal migration distance varies with natal dispersal and predicts parasitic infection in song sparrows. Behavioral Ecology and Sociobiology **70**:1857-1866.

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Satterfield, D. A., F. X. Villablanca, J. C. Maerz, and S. Altizer. 2016. Migratory monarchs wintering in California experience low infection risk compared to monarchs breeding year-round on non-native milkweed. Integrative and Comparative Biology **56**:343-352.

van Oers, K., D. S. Richardson, S. A. Saether, and J. Komdeur. 2010. Reduced blood parasite prevalence with age in the Seychelles Warbler: selective mortality or suppression of infection? Journal of Ornithology **151**:69-77.

Sam Slowinski

EDUCATION

Ph.D.	Indiana University, Evolution, Ecology, and Behavior, GPA: 4.0, Advisors: Ellen Ketterson and Curt Lively, August, 2017
BA	Oberlin College, Biology, GPA: 3.55, Advisor: Mary Garvin, June 2010

RESEARCH APPOINTMENTS

Upcoming	Postdoctoral researcher in the Integrative Biology Program, University of California, Berkeley, advisor Michael Shapira
2011-present	Ph.D. student in Evolution, Ecology, and Behavior, Indiana University
2011	Research intern, Archbold Biological Station
2010	Research assistant supervised by Mary Garvin, Oberlin College
2010	Research intern supervised by Randall Hughes and David Kimbro, Florida State University Coastal and Marine Laboratory
2009	Research intern supervised by Anne Royer and Jeffrey Conner, Kellogg Biological Station, Michigan State University
2009	Research intern supervised by Mark Frey, The Presidio Trust of San Francisco
2008	Research intern supervised by Walter Tschinkel, Florida State University

PUBLICATIONS (* indicates undergraduate co-author)

Published

2 **Slowinski, S.[1],** Morran, L.[1], Parrish, R.*, Cui, E.*, Bhattacharya, A., Lively, C., and Phillips, P. 2016. Coevolutionary interactions with parasites constrain the spread of self-fertilization into outcrossing host populations. Evolution **70**:2632-2639.
[1]Equal author contribution

1 Whittaker, D., Gerlach, N., **Slowinski, S.**, Corcoran, K., Winters, A., Soini, H., Novotny, M., Ketterson, E., and Theis, K. 2016. Social Environment Has a Primary Influence on the Microbial and Odor Profiles of a Chemically Signaling Songbird. Frontiers in Ecology and Evolution 4. DOI: 10.3389/fevo.2016.00090

In Review

1 Whittaker, D., Rosvall, K., Slowinski, S., Soini, H., Novotny, M., Ketterson, E. Chemical signals reflect uropygial gland androgen sensitivity and predict aggression: implications for the role of the periphery in chemosignaling. In review at Journal of Comparative Physiology A.

In preparation

3 **Slowinski**, **S. P.**, Fudickar, A. M., Hughes, A.*, Mettler, R. D., Gorbatenko, O., Spellman, G. M., Ketterson, E. D., Atwell, J. W. Sedentary dark-eyed juncos (*Junco hyemalis*) maintain higher prevalence of haemosporidian infections than migratory juncos during seasonal sympatry.

2 **Slowinski, S.**, Geissler, A., Bergeon Burns, C., Gerlach, N., Heidinger, B., Ketterson, E. Haemosporidian parasite infections increase with host age, but do not explain testosterone's effect on telomere loss in a songbird population.

1 **Slowinski, S.,** Penley, M., Lively, C., Morran, T. Does outcrossing propensity in an androdioecious host respond plastically to exposure to parasites in the environment or evolve in response to coevolution with parasites?

RESEARCH GRANTS

2016 American Ornithologists Union Research Award ($2,344)
2016 Indiana Academy of Sciences Senior Research Grant ($2,344)
2016 American Society of Naturalists Student Research Award ($2,000)
2016 Animal Behavior Society Student Research Grant ($1,000)
2015 Society for Integrative and Comparative Biology Grant in Aid of Research ($1,000)
2015 Indiana University Graduate and Professional Student Organization Research Award ($1,000)
2013 Sigma Xi, Grant in Aid of Research Award ($1,000)
2013 Society for the Study of Evolution, Rosemary Grant Award ($2,250)

FELLOWSHIPS AND TRAVEL AWARDS

Fellowships

2016 National Institute of Health Common Themes in Reproductive Diversity Predoctoral Fellowship
2015 Margaret Walton Scholarship for Mountain Lake
2015 Center for the Integrative Study of Animal Behavior Predoctoral Fellowship
2014 NIH Common Themes in Reproductive Diversity Predoctoral Fellowship
2014 Margaret Walton Scholarship for Mountain Lake
2013 Oberlin College Alumni Fellowship

Travel Awards

2016 Finalist for the W. D. Hamilton Award for Outstanding Student Presentation, Society for the Student of Evolution, Austin, TX
2016 Indiana University Biology Departmental Travel Award
2016 Indiana University Center for Integrative Study of Animal Behavior, Travel Award
2016 North American Ornithological Conference, Student Travel Award
2015 Indiana University Biology Departmental Travel Award
2014 Indiana University Biology Departmental Travel Award
2014 Indiana University Center for Integrative Study of Animal Behavior, Travel Award
2013 Indiana University Center for Integrative Study of Animal Behavior, Travel Award

TEACHING

Teaching appointments

2016 Associate Instructor for A502, Research and Professional Ethics for the Bio-behavioral Sciences, Indiana University

2015	Associate Instructor for P451, Human Physiology, Indiana University
2014	Associate Instructor for L376, Biology of Birds, Indiana University
2013	Associate Instructor for Z375, Invertebrate Biology Laboratory, Indiana University
2013	Biology Instructor for Indiana University Foundations in Science and Mathematics Summer Program for Local High School Students
2011-2012	Associate Instructor for L113, Introductory Biology Laboratory, Indiana University

Guest lectures

2017	Guest lecture on the evolution of sex for A379 (History of the Evolution Controversy), Indiana University
2016	Guest lecture on olfaction in birds for L376 (Biology of Birds), Indiana University
2013	Guest lecture on the mechanisms of evolution for L113 (Introductory Biology Laboratory), Indiana University

UNDERGRADUATES MENTORED

NSF-REU interns	*Name of student and project*
2016	Aidan Geissler: How testosterone implants affect haemosporidian parasite infections and telomere degradation in songbirds
2014	Virnaliz Cruz: The transmission of microbes from mother to nestlings in songbirds
2013	Domonique Jackson: How coevolution with parasites affects outcrossing propensity in *C. elegans* hermaphrodites
2012	Kaitlin Alford: The role of chemical signals in courtship behavior of songbirds

Indiana University	*Name of student and project*
2014-2017	Eric Cui, (L490 Research Credit): The effect of coevolutionary interactions with parasites on the evolution of an allele that permits self-fertilization in hosts
2013-2014	Raymond Parrish II, (L490 Research Credit): Coevolutionary interactions with parasites constrain the spread of self-fertilization into outcrossing host populations

OUTREACH

2015 and 2016	Leader of hands-on interactive birding activities and live-bird handling for Wondercamp, a summer science enrichment program for elementary students in Bloomington, IN
2015 and 2016	Volunteer at Indiana University's "Science Fest", an annual program for kids and parents to come learn about science at Indiana University. I led an activity in which kids could try capturing food with bird beak models to see how beak shape matches diet.
2012-2016	Mentor for the Groups Scholars Program STEM Initiative to promote and enhance the experiences of first-generation, underrepresented students in STEM, Indiana University
2012-2013	Biology tutor for a student at IV Tech Community College
2012	Coach for the *'Designer Genes'* Science Olympiad team, Bloomington High School South

2012 Wonderlab (children's science museum) volunteer

PROFFESIONAL DEVELOPMENT

2014 Third International Malaria Research Coordination Network Workshop on Malaria and Related Haemosporidian Parasites of Wildlife, Shepherdstown, WV

PRESENTATIONS AND POSTERS (* indicates undergraduate co-author)

Contributed presentations

2017 **Samuel P. Slowinski**, Adam M. Fudickar, Alex Hughes*, Raeann D. Mettler, Oxana Gorbatenko, Garth M. Spellman, Ellen D. Ketterson, Jonathan W. Atwell. Parasite prevalence in sympatry is associated with seasonal migration. Center for the Integrative Study of Animal Behavior annual meeting, Indiana University, Bloomington, IN.

2016 **Samuel P. Slowinski**, Adam M. Fudickar, Alex Hughes*, Raeann D. Mettler, Oxana Gorbatenko, Garth M. Spellman, Ellen D. Ketterson, Jonathan W. Atwell. Sedentary dark-eyed juncos (*Junco hyemalis*) maintain a higher prevalence of haemosporidian infections than migratory juncos in a seasonally sympatric population. Indiana University, Evolution, Ecology, and Behavior Brown Bag Seminar Series.

2016 **Slowinski, S.[1],** Morran, L.[1], Parrish, R.*, Cui, E. *, Bhattacharya, A., Lively, C., and Phillips, P. Coevolutionary interactions with parasites constrain the spread of self-fertilization into outcrossing host populations. Evolution annual meeting, Austin, TX.
[1]Equal author contribution

2016 **Slowinski, S.[1],** Morran, L.[1], Parrish, R.*, Cui, E. *, Bhattacharya, A., Lively, C., and Phillips, P. Coevolving parasites constrain the spread of self-fertilization into outcrossing host populations, but do not induce plastic changes in host mating propensity. Evolution Discussion Group. Indiana University, Bloomington.
[1]Equal author contribution

2014 **Slowinski, S.,** Whittaker, D., Spellman, G., Mettler, R., Soini, H., Novotny, M. Ketterson, E. The mechanism of *Plasmodium*-induced alterations in host attractiveness to vectors. Third International Malaria Research Coordination Network Workshop on Malaria and Related Haemosporidian Parasites of Wildlife, Shepherdstown, WV.

2014 **Slowinski, S.[1],** Morran, L.[1], R. Parrish*, E. Cui.*, C. Lively, and P. Phillips. The effect of parasites on the ability of self-fertilization to invade outcrossing host populations. Evolution annual meeting, Raleigh, NC.
[1]Equal author contribution

2014 **Slowinski, S.[1],** Morran, L.[1], R. Parrish*, E. Cui.*, C. Lively, and P. Phillips. The effect of parasites on the ability of self-fertilization to invade outcrossing host populations. Common Themes in Reproductive Diversity breakfast seminar series, Indiana University.
[1]Equal author contribution

2013 **Slowinski, S.[1],** Morran, L.[1], R. Parrish*, E. Cui.*, C. Lively, and P. Phillips. The effect of parasites on the ability of self-fertilization to invade outcrossing host populations. Indiana University, Evolution, Ecology, and Behavior Brown Bag Seminar Series.
[1]Equal author contribution

Posters

2016 **Samuel P. Slowinski**, Adam M. Fudickar, Alex Hughes*, Raeann D. Mettler, Oxana Gorbatenko, Garth M. Spellman, Ellen D. Ketterson, Jonathan W. Atwell. Sedentary songbirds (*Junco hyemalis*) exhibit higher prevalence of *Plasmodium* infections relative to coexisting conspecifics throughout winter and early spring. North American Ornithological Conference. Washington, D.C.

2016 **Samuel P. Slowinski**, Adam M. Fudickar, Alex Hughes*, Raeann D. Mettler, Oxana Gorbatenko, Garth M. Spellman, Ellen D. Ketterson, Jonathan W. Atwell. Sedentary songbirds (*Junco hyemalis*) exhibit higher prevalence of *Plasmodium* infections relative to coexisting conspecifics throughout winter and early spring. Ecology and Evolution of Infectious Diseases Annual Meeting, Ithaca, NY.

2015 **Slowinski, S.[1],** Morran, L.[1], R. Parrish*, E. Cui.*, A. Bhattacharya, C. Lively, and P. Phillips. The effect of parasites on the ability of self-fertilization to invade outcrossing host populations. Ecology and Evolution of Infectious Diseases Annual Meeting, Athens, GA.
[1]Equal author contribution

2015 **Slowinski, S. P.,** D. J. Whittaker, H. A. Soini, M. V. Novotny, R. D. Mettler, O. Gorbatenko, G. M. Spellman, E. D. Ketterson. *Plasmodium* Infections May Alter the Production of Volatile Compounds in the Avian Preen Gland. CISAB Animal Behavior Conference, Indiana University, Bloomington, IN.

2015 **Slowinski, S. P.[1],** L. Morran[1], R. Parrish*, E. Cui*, A. Bhattacharya, C. Lively, P. Phillips. The effect of parasites on the ability of self-fertilization to invade obligately outcrossing host populations. Midwest Ecology and Evolution Conference, Indiana University, Bloomington, IN.
[1]Equal author contribution
Presented by Eric Cui

2014 **Slowinski, S.**, D. Whittaker, H. Soini, M. Novotny, R. Mettler, G. Spellman, E. Ketterson. The effect of haemosporidian infections on the production of volatile compounds in the avian preen gland. The American Ornithologists Union Annual Meeting, Estes Park Colorado.

2013 **Slowinski, S.,** D. Whittaker, E. Ketterson. Odor sharing among kin in birds: assessing whether female songbirds transfer preen oil to their nestlings during

brooding. The Society for Integrative and Comparative Biology Annual Meeting. San Francisco, CA.

2013 **Slowinski, S.,** D. Whittaker, E. Ketterson. Odor sharing among kin in birds: assessing whether female songbirds transfer preen oil to their nestlings during brooding. CISAB Animal Behavior Conference, Indiana University, Bloomington, IN.

PROFESSIONAL SERVICE

Peer review for scientific journals

2015 Ethology
2014 Chemical Signals in Vertebrates
2013 Nature Communications

Peer review for grant and fellowship committees

2017 National Fellowships Committee for Graduate Women in Science

Organizing conferences, judging presentations, panel discussions

2017 IU CISAB Animal Behavior Conference, Poster Committee
2016 IU Center for the Integrative Study of Animal Behavior (CISAB) Conference, Undergraduate Poster Judge
2016 IU CISAB Animal Behavior Conference, Poster Committee
2015 IU CISAB REU program panelist for a session about grad student life
2015 IU CISAB Animal Behavior Conference, Poster Committee
2014 IU CISAB Animal Behavior Conference, Program Committee
2013 IU CISAB Animal Behavior Conference, Poster Committee