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Integrative approaches to evaluate challenges faced by songbirds during migration and associated physiological trade-offs

by

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A thesis submitted in partial fulfillment of the requirements for the Degree of

Master of Science in Environmental Science

Thomas H. Gosnell School of Life Sciences

College of Science

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Rochester, New York

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Committee Approval

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Abstract

Migration is a demanding time for birds, and birds face numerous physiological, behavioral, and environmental challenges. As migratory birds balance the costs of numerous stressors during migration, this could result in detrimental physiological trade-offs. Understanding the trials and trade-offs that birds encounter during migration has important conservation implications. I utilized two component studies to better understand challenges encountered by migratory songbirds as well as to identify physiological metrics that may provide insight into these challenges. For the first study, I used nested PCR and DNA sequencing to determine parasite prevalence and diversity of avian haemosporidians in Canada Warblers (*Cardellina canadensis*) and Black-throated Blue Warblers (*Setophaga caerulescens*). I further evaluated if haemosporidian infections were related to immune condition, as assessed via total white blood cell (WBC) counts and heterophil/lymphocyte (H/L) ratios, or refueling patterns and/or migration timing. There was a high haemosporidian prevalence in sampled birds (51.5%). However, although haemosporidians appear to be a routine challenge faced by birds during migration, the analyses provided no evidence that Canada Warblers and Black-throated Blue Warblers experience physiological or behavioral trade-offs as a result of infections. In the second study, I used an enzyme-linked immunosorbent assay (ELISA) developed for migratory birds to measure heat shock protein 60 (HSP60) in three species of *Catharus* thrushes during spring and fall migration. HSP60 was significantly higher during spring migration relative to fall migration, and there was moderate variation in HSP60 across species. HSP60 was not strongly associated with white blood cell counts, scaled mass index, plasma uric acid, or migration arrival date. Results suggest that HSP60 could exhibit correlations with numerous stressors associated with the challenges of long-distance migratory flight and that HSP60 is a robust metric of physiological stress suitable for avian migration studies.

Overview

Migration is one of the most physiologically demanding periods of the songbird annual cycle (McWilliams et al. 2004). Birds are challenged to fly extreme distances, where cumulative annual flight lengths exceeding 20,000 km have been observed in individuals weighing as little as 12 g (DeLuca et al. 2019). Furthermore, migration is often characterized by extended continuous bouts of flight where suitable stopping locations are not present, and therefore birds must maximize energy reserves stored prior to flight (Klaassen 1996). Building fuel stores for flight is associated with its own challenges. Birds may accumulate fat stores equivalent to 50% of total body mass prior to long-distance flight (McWilliams et al. 2004), and significant physiological adjustments are necessary to sustain hyperphagic food intake associated with this storage (McWilliams and Karasov 2001). Birds must also devote substantial time and effort to foraging before flight to build fuel stores rapidly and adequately. As such, both the endurance exercise of migratory flight and preparation that precedes it impose tremendous energetic costs on a bird (Alerstam and Hedenstrom 1998, Wikelski et al. 2003). Migratory birds may also be challenged by severe weather en route (Klaassen et al. 2012), increased exposure to parasites and other non-parasitic agents (Altizer et al. 2011), or environmental pollutants (Seewagen 2020), all of which could act as additional stressors during an already physiologically demanding time.

Migratory birds must stop regularly at locations called stopover sites to replenish energy reserves and prepare for impending stretches of flight. Counterintuitively, most of the cumulative time and energy devoted to migration is spent at stopover sites (Alerstam and Hedenstrom 1998, Wikelski et al. 2003), and stopover periods provide birds with an opportunity to rest, avoid adverse flight conditions, and refuel prior to continuing migration. Although the majority of past research has focused on refueling behavior of birds at stopover sites, birds may also spend time during stopover to recover physiologically and restore oxidative balance and/or constitutive

immune function (Eikenaar et al. 2020b, Schmaljohann et al. 2022). Overall, because a large percentage of migration is spent at stopover sites, stopover duration is an important determinant of migration pace and can influence the rate at which birds can continue on to subsequent stages of the annual cycle. However, an increased risk of predation, competition, poor weather, and reductions in food availability are examples of the many challenges that birds may face at stopover sites (Klaassen et al. 2012). These challenges could serve as a roadblock in the demanding journey that is migration.

It is evident that birds face numerous challenges during both migratory flight and rest/recovery periods at stopover sites. As birds balance the physiological demands of managing these various challenges, this could prompt resource-related trade-offs to occur. Physiological systems (such as the immune system or endogenous antioxidant defenses) are often dependent on available energetic reserves (Owen and Moore 2008, Eikenaar et al. 2020a). Depletion of energy reserves—perhaps in response to the demands of long-distance flight or other associated stressors—can result in physiological trade-offs in migratory birds (Eikenaar et al. 2020a). In particular, trade-offs within the immune system itself are well documented in migratory birds. For example, birds with elevated immune defenses due to parasitic infections may experience trade-offs with refueling rates at stopover sites or migration pace as they balance elevated costs of the immune system (Klaassen et al. 2012, Hegemann et al. 2018). Another documented immune trade-off in migratory birds is that of immune function and antioxidant defenses. Eikenaar et al. (2018) showed that in migrating Eurasian Blackbirds, microbial killing capacity was negatively correlated with nonenzymatic antioxidant defenses, suggesting a physiological trade-off between innate immune function and antioxidant defenses to mitigate oxidative stress. Trade-offs such as those described can have a detrimental impact on migration pace and/or the

condition of a migratory bird when it reaches breeding or wintering grounds. This could negatively impact both subsequent migrations and long-term fitness and survival (Bearhop et al. 2004, Paxton and Moore 2015).

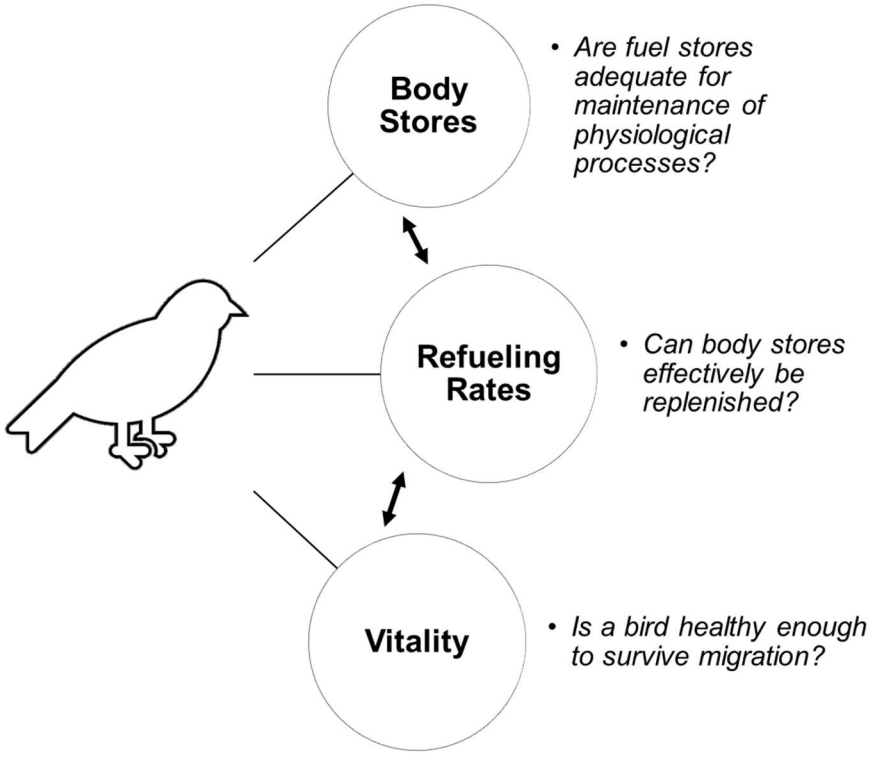

Figure I. Conceptual framework of physiological attributes important to migratory birds. Figure adapted from Klaassen et al. (2012).

Since a multitude of factors influence the ability of birds to survive migration, developing reliable methodology to assess the physiological condition of migratory songbirds is a conservation necessity. Klaassen et al. (2012) has proposed that researchers consider three core physiological attributes when evaluating the condition of birds during migration and for understanding potential physiological trade-offs: body stores, refueling rates, and vitality (Figure I). Body stores refer to the energy reserves immediately available to a bird—such as fat stores—and can be an important indicator of a bird's health and nutritional state (Klaassen et al. 2012,

Schmaljohann and Eikenaar 2017). Refueling rate comprises a bird's ability to forage and successfully replenish energy reserves, and it reflects both stopover site quality and food availability (Smith and McWilliams 2010) or a bird's energetic devotion to foraging. Vitality relates to a bird's health and ability to survive migration, and overall immune status and/or stress levels could provide insight into a migrant's chances of survival (Klaassen et al. 2012). Despite the importance of each of these attributes for migratory songbirds, evaluating the significance of interactions and relative trade-offs between these variables is an area that remains underexplored.

The objectives of this thesis are multifaceted with both basic and applied goals. Generally, I sought to measure various condition metrics (e.g. plasma metabolites, leukocyte counts, stress proteins) in birds during migration stopover on the south shore of Lake Ontario and evaluate the overall physiological condition of these birds. The study site for this work was Braddock Bay Bird Observatory, a well-known migration monitoring station that has set the stage for numerous stopover physiology studies. Additionally, I aimed to explore how potentially unfavorable scenarios—such as blood parasite infections or physiological stress—may force birds to make trade-offs as they balance immense energetic costs associated with these challenges. Questions pertaining to these goals were explored via two unique component studies.

Chapter 1 is a correlative study that explores the impact that avian malaria and related haemosporidian infections may have on the physiological condition of two species of Nearctic-Neotropical migratory songbirds—Canada Warblers (*Cardellina canadensis*) and Black-throated Blue Warblers (*Setophaga caerulescens*). I sought to investigate if harboring chronic haemosporidian infections may prompt migrating birds to make physiological or behavioral trade-offs as they maintain elevated costs of immune defenses.

Chapter 2 focuses on the utility of heat shock protein 60 (HSP60) as a stress biomarker for migratory birds. HSP60 is a highly conserved molecular chaperone protein used to mitigate physiological stress, yet few studies have investigated the importance of this protein to migratory birds. I developed an enzyme-linked immunosorbent assay (ELISA) to measure HSP60 in migratory bird samples, then I applied this method to quantify HSP60 in *Catharus* thrushes.

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Chapter 1

Haemosporidian parasites of Canada Warblers (*Cardellina canadensis*) and Black-throated Blue Warblers (*Setophaga caerulescens*): Prevalence, diversity, and associations with physiological condition during migration

Chapter 1 is formatted according to guidelines of Avian Conservation and Ecology and has been submitted to the journal for peer review.

ABSTRACT

Avian malaria (*Plasmodium* spp.) and related haemosporidian parasites (*Leucocytozoon* spp. and *Haemoproteus* spp.) are widespread in birds, yet studies investigating prevalence and diversity of haemosporidians are lacking for many Nearctic-Neotropical migrants. Furthermore, the impact that infections may have on the condition or behavior of birds during migration is still poorly understood. Here, we used nested PCR and DNA sequencing to determine parasite prevalence and identify genetic lineages of haemosporidians in Canada Warblers (*Cardellina canadensis*) and Black-throated Blue Warblers (*Setophaga caerulescens*) during their migration stopover on the south shore of Lake Ontario. We further evaluated if haemosporidian infections were related to immune condition, as assessed via total white blood cell (WBC) counts and heterophil/lymphocyte (H/L) ratios, or refueling patterns and/or migration timing. We found a haemosporidian prevalence of 51.5% in sampled birds (n = 66); however, this pattern differed by species. We detected 15 cytochrome *b* lineages of parasites, including 4 novel lineages. In Black-throated Blue Warblers, infected birds had significantly higher total WBC counts compared to uninfected birds. Across species, females had lower H/L ratios when infected, whereas the opposite trend was observed in males. Plasma metabolites (triglyceride, β-hydroxybutyrate, uric

acid) were not associated with infection status, and infection was not related to arrival date at the spring stopover site. Results provide insight into the diversity of haemosporidians that infect two species of migratory warblers and illustrate that blood parasite infections are a routine challenge that these birds face during migration. Although haemosporidian infections may be associated with a heightened immune response in some scenarios, the findings of this correlative study provide no evidence that Canada Warblers and Black-throated Blue Warblers experience physiological or behavioral trade-offs as a result of infections.

Key Words: eco-immunology; haemosporidian; leukocyte counts; migration; trade-off.

INTRODUCTION

Migration is a physiologically demanding period of a songbird's annual cycle. Prior to migration, birds deposit fat stores as great as 50% of total body mass and subsequently use both fat and endogenous protein to fuel nonstop, long-distance bouts of flight (McWilliams et al. 2004). Alongside supporting immense energetic costs of flight, migratory birds expend resources during intermittent refueling periods at stopover sites (Wikelski et al. 2003) while simultaneously balancing costly routine challenges including predation threats, sub-optimal environmental conditions, and maintenance of physiological traits including immunity and oxidative balance (Klaassen et al. 2012, Eikenaar et al. 2018, McWilliams et al. 2021). Additionally, parasites and other non-parasitic infections could serve as another cost of migration (Altizer et al. 2011). Migratory birds are exposed to diverse habitats and vectors, and they are often more vulnerable to acquiring infections due to immunosuppression during migration (Owen and Moore 2006, Altizer et al. 2011). Infections could reduce foraging efficiency or delay migration, which could result in harmful carry-over effects (Owen and Moore 2006, Risely et al.

2018). Considering that migratory birds are experiencing ongoing population declines (Bairlein 2016), understanding how parasites and non-parasitic diseases challenge birds during migration has important conservation implications.

Avian malaria and related haemosporidians are common blood parasites in birds, and over 250 species of haemosporidians have been identified worldwide (Greiner et al. 1975, Valkiūnas 2005, Harl et al. 2020). Three genera of haemosporidians (*Plasmodium*, *Haemoproteus*, and *Leucocytozoon*) are most common in birds, and these parasites are transmitted to birds via insect vectors. MalAvi, a global database of haemosporidians, contains over 4,000 unique cytochrome *b* lineages of parasites that have been identified using molecular techniques (Bensch et al. 2009; http://130.235.244.92/Malavi/). Some parasite lineages are host-specific, and others are generalists found in a wide variety of avian families and are capable of host switching (Doussang et al. 2021). Potential pathogenicity of haemosporidian infections may vary by parasite lineage and host (Ellis et al. 2014). Genetic analyses of haemosporidian parasites of migratory birds provide a unique opportunity to gain insight into host-parasite interactions and parasite dispersal. Migratory birds are exposed to a diversity of parasites at breeding grounds, wintering grounds, or stopover habitats, and they could transmit parasites both among continents and host species (Pulgarín-R et al. 2019, De Angeli Dutra et al. 2021). However, despite their potential ecological and evolutionary significance, detailed studies on haemosporidian prevalence and diversity are still lacking for many migratory species, particularly Nearctic-Neotropical migrants.

Although haemosporidians may directly influence host reproduction and survival (Valkiūnas 2005, Asghar et al. 2011), the indirect effects that haemosporidian infections may have on the physiology and behavior of birds during migration are largely understudied. Birds

that are already physiologically depleted from migration may experience resource-related trade-offs as they balance physiological costs associated with chronically harboring haemosporidian infections. Specifically, haemosporidian-infected birds often exhibit elevated immune defenses relative to uninfected birds (Ricklefs and Sheldon 2007, Ellis et al. 2014, Emmenegger et al. 2018). Considering that the immune system incurs significant costs in terms of maintenance and activation (Martin et al. 2003), it could be hypothesized that infected birds with elevated immune defenses, which are often suppressed during migration (Owen and Moore 2006), could experience trade-offs as they balance the costs of migration and infection. In fact, migrating birds experience reduced antioxidant defenses and slower migration pace while managing elevated costs of the immune system (Eikenaar et al. 2018, Hegemann et al. 2018). Trade-offs of immunity with refueling rates or energetic condition may also be expected (Klaassen et al. 2012).

Despite generally severe physiological consequences for birds experimentally infected with haemosporidians (Atkinson et al. 2000, Garvin et al. 2003), prior studies that have investigated the effects of haemosporidian infections on free-living migratory birds have observed mixed results. Some infected birds may pause at stopover sites for increased periods of time or delay migration altogether, which could result in later migration timing (DeGroote and Rodewald 2010, Emmenegger et al. 2018, Hegemann et al. 2018). For instance, Emmenegger et al. (2018) showed that haemosporidian-infected birds appeared at a Mediterranean stopover site up to one month later compared to uninfected counterparts. Furthermore, radio-tracking of migrants during stopover on the Falsterbo Peninsula in southern Sweden provided fine-scale behavioral evidence that birds with blood parasite infections may show increased stopover duration or migrate later in the evening than uninfected birds (Hegemann et al. 2018). Conversely, experimentally infected Yellow-rumped Warblers (*Setophaga coronata*) showed no

difference in activity levels at a stopover site or stopover duration, suggesting that not all infected birds migrate at a slower pace (Howe 2022). Additionally, prior studies have observed conflicting results with regards to the impact of haemosporidians on metrics of host body condition during migration, and patterns may differ by age, sex, or host species (Garvin et al. 2006, DeGroote and Rodewald 2010, Cornelius et al. 2014). Additional work is needed to help elucidate the significance of haemosporidian infections to migratory songbirds.

Here, we studied Canada Warblers (*Cardellina canadensis*) and Black-throated Blue Warblers (*Setophaga caerulescens*) at a stopover site on the south shore of Lake Ontario to explore haemosporidian prevalence and diversity and investigate how infections may impact these birds during migration. The Canada Warbler and Black-throated Blue Warbler are long-distance Nearctic-Neotropical migrants that primarily overwinter in northern South America (Reitsma et al. 2020) and the Greater Antilles or Central America (Holmes et al. 2020), respectively, and both species breed in the northeastern United States or southern Canada. The Canada Warbler is a species of special conservation concern having experienced steady population declines the past 50 years and is classified as a threatened species in Canada (Reitsma et al. 2020). The first goal of this study was to evaluate parasite prevalence and identify genetic lineages of haemosporidians in these species. Despite some previous occurrence records of haemosporidians in Canada Warblers and Black-throated Blue Warblers (Greiner et al. 1975), relatively little is known regarding haemosporidian prevalences in these birds, and few prior studies have documented cytochrome *b* lineages of parasites in either species (Bensch et al. 2009; http://130.235.244.92/Malavi/). Second, we explored whether birds of these two species showed evidence of physiological or behavioral trade-offs when infected. We hypothesized that infected birds would display heightened immune defenses relative to uninfected birds. We

predicted that infected birds would simultaneously experience trade-offs with foraging success and migration pace, resulting in reduced refueling rates and later migration timing.

METHODS

Bird capture and sampling

Canada Warblers and Black-throated Blue Warblers were captured opportunistically during three spring migration seasons (2021-2023) at Braddock Bay Bird Observatory (BBBO), a migration-monitoring station on the south shore of Lake Ontario in the town of Hilton, New York, USA (43.3236°N, 77.7175°W). Shoreline habitats near BBBO have received attention as important stopover sites for migrating landbirds, in part due to their proximity to an ecological barrier and abundant food resources (Bonter et al. 2007, Smith 2013). The site primarily consists of early successional habitat and is surrounded by forests, agricultural lands, and water cover. Dominant shrub species include dogwoods (*Cornus* spp.), viburnums (*Viburnum* spp.), and honeysuckles (*Lonicera* spp.). Tree species include alder (*Alnus* spp.) and ash (*Fraxinus* spp.); however, the area has experienced significant changes in canopy cover in certain habitats due to loss of ash as a consequence of the spread of the Emerald Ash Borer (*Agrilus planipennis*) in this region. Bird capture occurred during normal operating hours (0-6 hours after sunrise) at the station and was conducted using mist-netting. Birds were extracted from mist nests and brought back to a central location where a blood sample was collected via brachial vein puncture using a 27.5 G needle, and approximately 70 ul of blood were collected in heparinized capillary tubes. Tubes were centrifuged within 5 hours to separate plasma from red blood cells, and blood fractions were separately frozen at -80 °C until laboratory analyses. Prior to release, birds were banded with a U.S. Geological Survey aluminum leg band, mass was determined using a

portable balance (± 0.1g), and wing chord was measured with a wing ruler (± 0.5 mm). Age and sex were determined when possible using Pyle (1997). Time of capture was recorded as the hour after sunrise of the net check (hereafter "capture hour"), and "bleed time" was noted as the number of minutes between net extraction and blood sampling.

Haemosporidian screening

Nested PCR

Birds were screened for haemosporidian parasites using a nested PCR approach (Hellgren et al. 2004). DNA was first extracted from red blood cell fractions of whole blood using the E.Z.N.A. blood DNA extraction kit (Omega Bio-Tek; Norcross, Georgia, USA). DNA concentration and purity were determined using a Nanodrop One spectrophotometer (Thermo Fisher Scientific; Waltham, Massachusetts, USA), and DNA extracts were diluted to a working concentration of 25 ng/ul. Extracted DNA (50-100 ng) was then used for PCR according to the protocol described by Hellgren et al. (2004). This protocol consists of an initial round of PCR to amplify a fragment of the cytochrome *b* gene common to *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* followed by a second round of PCR with two primer pairs that distinguishes between *Haemoproteus*/*Plasmodium* and *Leucocytozoon* infections. Infection status was assessed by running 5 ul of final PCR products on 2% agarose gels, and bands on the gel near 500 base pairs were interpreted as positive for parasites. All samples were screened twice to account for imperfect detection with the PCR (Hellgren et al. 2004), and multiple positive controls (samples known to be positive for *Plasmodium* and *Leucocytozoon* infections) and negative controls (prepared with sterile water in place of DNA) were implemented.

DNA sequencing

PCR products that screened positively for haemosporidians were sequenced twice in the forward and reverse directions. Sanger Sequencing was performed by GeneWiz (Azenta Life Sciences; South Plainfield, New Jersey, USA), and resulting sequences were assembled and edited using BioEdit (Hall 1999). Consensus sequences were identified to the genus and/or lineage level using NCBI BLAST or the MalAvi BLAST feature (Altschul et al. 1990, Bensch et al. 2009; MalAvi accessed January 2024). Sequences that differed by one or more nucleotides from previously published cytochrome *b* lineages in the MalAvi database were identified as novel lineages and were deposited in MalAvi and NCBI GenBank. We were unable to obtain both forward and reverse sequences for 8 samples; we only identified these samples to the genus level.

Physiological analyses

White blood cell counts

At the time of initial sampling, a blood smear for each bird was prepared from whole blood (Owen 2011). Smears were fixed using 100% methanol and stained with Hema-3 manual staining solution (Fisher Scientific; Waltham, Massachusetts, USA). Stained blood smears were analyzed using a compound light microscope at 1000x magnification. Counts were performed as in Owen and Moore (2006), where all leukocytes (heterophils, lymphocytes, monocytes, eosinophils, basophils) in the first 100 fields of view (FOV) were counted, and the total white blood cell (WBC) count was calculated as the number of white blood cells per 10,000 erythrocytes (with approximately 200 erythrocytes per field of view). The heterophil/lymphocyte (H/L) ratio was found by dividing the number of heterophils per 100 FOV by the number of

lymphocytes per 100 FOV. All leukocyte counts were performed by the same observer (G.L.O.). The total WBC count can reveal information about immunocompetence of an individual, whereas the H/L ratio can be used as an indicator of stress or a reallocation of resources within the immune system and is often elevated in birds experiencing physiological or environmental stressors (Owen and Moore 2006, Davis et al. 2008).

Plasma metabolite assays

Plasma metabolite assays were used to measure circulating plasma concentrations of triglyceride, uric acid, and β-hydroxybutyrate. Triglyceride and β-hydroxybutyrate are metrics of fat deposition and fat catabolism from fasting (Guglielmo et al. 2005), respectively; uric acid is an indicator of both exogenous and endogenous protein catabolism (Smith et al. 2007). Hence, all three metrics can provide information about refueling state and nutrient use. Plasma was diluted 1:3 with 0.9% NaCl prior to analyses. Triglyceride and uric acid concentrations were then measured using colorimetric endpoint assays modified for microwell plates, and β-hydroxybutyrate was quantified via a kinetic assay following the procedures described in Smith and McWilliams (2010). For each metabolite, all samples were measured in duplicate on 96-well plates using a Bio-Tek Synergy H1 microplate reader and accepted as valid when the coefficient of variation between replicates was <10%. Due to limited sample plasma volumes, plasma metabolite assays were not able to be performed for all samples; plasma triglyceride assays were prioritized in these instances.

Statistical analysis

Birds were pooled across all three years of this study to increase sample sizes for analyses. Differences in parasite prevalence by species were assessed with a Chi-square

goodness-of-fit test assuming equal probabilities between species. To investigate the influence of haemosporidian infections on migration timing, we fitted a general linear model (GLM) with capture date (ordinal day of year) as the dependent variable and infection status (infected vs. uninfected), species, and sex as predictors. Interactions between infection status and the other factors in the model were also included to ensure that potential differences in migration timing due to parasitism did not differ by species or sex. A similar modeling approach with GLMs was used to explore relationships between WBC counts and parasite infection status. We normalized the total WBC count using a $\log_{10}(x)+1$ transformation and the H/L ratio using a square root transformation. Separate models were created for the total WBC count and the H/L ratio; species, sex, and the interactions of these factors and infection status were included in the models. If an interaction was significant, we used a two-tailed Student's t-test assuming equal variances to further investigate the effect.

Relationships between plasma metabolites (triglyceride, uric acid, β-hydroxybutyrate) and parasite infection status were assessed using GLMs. Triglyceride and β-hydroxybutyrate were normalized with $\log_{10}(x)+1$ transformations prior to analyses; uric acid was normally distributed and was left untransformed. Because plasma metabolites may be sensitive to numerous covariates (Smith and McWilliams 2010), we used an Akaike's Information Criterion (AIC) model selection procedure to identify variables to include in models. All combinations of capture hour, bleed time, scaled mass index (body mass scaled for wing chord; Peig and Green 2009), sex, species, and day of year were included as predictors in candidate model sets for each metabolite separately. Covariates included in the top-ranked AIC models were then included in models with infection status as a categorial predictor. We originally included interactions between infection status and categorical covariates in the final models; however, no interactions

were significant and were ultimately removed. All analyses were performed with R 4.2.2 (R Core Team 2023) or JMP Pro 16 (SAS Institute 2021). AIC analyses were conducted using the R package *MuMIn* (Bartoń 2022). Means are presented with standard error (SE). Results were interpreted as statistically significant when $P < 0.05$.

RESULTS

We sampled 32 Canada Warblers (5 in 2021; 20 in 2022; 7 in 2023) and 34 Black-throated Blue Warblers (23 in 2022; 11 in 2023). For Canada Warblers, there were 12 males and 20 females. There were 17 male and 17 female Black-throated Blue Warblers. Median day of capture for Canada Warblers was May 23 (2021: May 27; 2022: May 22; 2023: May 24), while median day of capture for Black-throated Blue Warblers was May 19 (2022: May 19; 2023: May 18).

Haemosporidian prevalence

51.5% of warblers in this study (n = 66) were infected with haemosporidian parasites. *Plasmodium* infections were most common and were detected in 25.8% of birds. *Leucocytozoon* infections were found in 12.1% of birds, whereas *Haemoproteus* prevalence was 9.1%. Mixed infections of multiple parasite genera (*Leucocytozoon* and *Plasmodium*) were detected in 4.5% of birds. Overall infection prevalence was 67.6% in Black-throated Blue Warblers and 34.4% in Canada Warblers ($\chi^2 = 7.3$, df = 1, $P = 0.007$; Figure 1.1).

Parasite lineages

We successfully obtained 29 complete DNA sequences from samples that tested positive for haemosporidians via nested PCR. We identified 15 distinct haemosporidian lineages (Table 1.1) homologous (99%-100%) to published cytochrome *b* lineages within the MalAvi database. Of these lineages, 11 had been previously identified in the MalAvi database, and 4 lineages had at least one fixed difference from any sequence in MalAvi and were identified as novel (Table 1.1). We detected 8 lineages in Canada Warblers, whereas 9 lineages were identified in Black-throated Blue Warblers. GEOTRI09 was the most common lineage (8 individuals) and was detected in both species. With the exception of GEOTRI09 and CNEORN1, all lineages were novel for these host species based on records in the MalAvi database. All lineages identified that were previously deposited in MalAvi had been detected in North America, except for the *Haemoproteus* lineage SCLCAU03, which had only ever been found in South America (Table A1; Appendix 1). Additionally, with the exception of SCLCAU03, all lineages that were 100% homologous to sequences in the MalAvi database had previously been documented in the family Parulidae (Table A1; Appendix 1).

Infection and migration timing

There was no significant association of infection status with arrival date, and this trend did not differ by species or sex (infection: $F_{1,60} = 1.2$, $P = 0.28$; species: $F_{1,60} = 3.7$, $P = 0.06$; sex: $F_{1,60} = 18.8$, $P < 0.001$; infection × species: $F_{1,60} = 2.3$, $P = 0.14$, infection × sex: $F_{1,60} = 0.02$, $P = 0.90$). In fact, mean day of capture was slightly but not significantly earlier for infected birds relative to uninfected birds in both Canada Warblers (Infected: May 22 ± 2 days; Uninfected:

May 23 ± 1 day) and Black-throated Blue Warblers (Infected: May 17 ± 2 days; Uninfected: May 21 ± 2 days).

Infection and physiological condition

We derived leukocyte counts [Canada Warbler: mean total WBC (per 10,000 erythrocytes) = 30.64 ± 3.16, mean H/L ratio = 0.22 ± 0.02; Black-throated Blue Warbler: mean total WBC (per 10,000 erythrocytes) = 27.59 ± 2.61, mean H/L ratio = 0.13 ± 0.02] and investigated their relationship with parasite infection status. There was a significant species × infection interaction (Table 1.2) in a model predicting total WBC counts. Infection did not relate to total WBC counts in Canada Warblers ($t_{30} = -1.3$, $P = 0.21$; Figure 1.2A). For Black-throated Blue Warblers, total WBC counts were significantly higher in infected birds relative to uninfected birds ($t_{32} = 3.0$, $P = 0.005$; Figure 1.2B). Infection status on its own was not associated with the H/L ratio; however, there was a significant sex × infection interaction (Table 1.2). Infected females had significantly lower H/L ratios than uninfected females ($t_{35} = -3.0$, $P = 0.004$; Figure 1.3A), whereas there was a non-significant trend toward higher H/L ratios in infected males compared to uninfected males ($t_{27} = 1.5$, $P = 0.13$; Figure 1.3B).

We measured plasma metabolites (Canada Warbler: mean triglyceride = 1.74 ± 0.12 mM, mean uric acid = 0.97 ± 0.07 mM, mean β-hydroxybutyrate = 2.30 ± 0.28 mM; Black-throated Blue Warbler: mean triglyceride = 1.71 ± 0.21 mM, mean uric acid = 1.24 ± 0.12 mM, mean β-hydroxybutyrate = 1.76 ± 0.30 mM) to explore refueling patterns in infected and uninfected birds. AIC model selection results showed that capture day, capture hour, and scaled mass index were the most important predictors of plasma triglyceride, species was the most important predictor of uric acid, and sex and scaled mass index were important predictors of β-

hydroxybutyrate (Table 1.3). Important covariates from the top-ranked models were included in respective GLMs for each of the three metabolites (Table 1.4). Haemosporidian infection status was not significantly related to any plasma metabolites (Table 1.4).

DISCUSSION

Migratory birds are confronted with numerous challenges en route, and parasites could serve as an additional constraint during migration (Klaassen et al. 2012). In this study, we explored haemosporidian parasite prevalence in two North American migratory warbler species and investigated physiological trade-offs that may occur in these birds as they balance costs of infection. Molecular parasite screening revealed that haemosporidian infections appear to be a routine challenge that migratory birds face, and warblers can be infected with a diversity of haemosporidian genera and genetic lineages. However, the results did not provide unequivocal evidence that these infections force birds to make detrimental physiological or behavioral trade-offs during migration. Infected Black-throated Blue Warblers did display elevated total WBC counts, but it did not appear that maintaining immune defenses was associated with compromised refueling or later migration timing. Although haemosporidians can at times be severely detrimental to their hosts (Atkinson et al. 2000), results agree with studies that have demonstrated that haemosporidian infections have a more subtle effect on the physiological condition and migration success of birds (Cornelius et al. 2014, Howe 2022). While the results of this study are largely correlative, we provide insight into the implications of this data and suggestions for future experimental work.

Haemosporidian infections were found in about half the birds in this study. This is not surprising considering that birds of the family Parulidae are innately susceptible to

haemosporidians (Greiner et al. 1975). Furthermore, results agree with prior studies that have found similar haemosporidian prevalences in birds during migration (Santiago-Alarcon et al. 2011, DeBrock et al. 2021, Emmenegger et al. 2023). However, a greater proportion of Black-throated Blue Warblers were infected relative to Canada Warblers. This trend is somewhat puzzling, as Black-throated Blue Warblers and Canada Warblers similarly breed in coniferous and deciduous forests with well-developed understory layers (Holmes et al. 2020, Reitsma et al. 2020). Sabo (1980) even proposed that these species may experience competition with one another due to their comparable habitat preferences and niche overlap in northern forests. Thus, differences in breeding habitat and nesting behavior are not likely to explain variation in exposure to insect vectors and associated haemosporidians, as has been proposed for other avian species (DeGroote and Rodewald 2010, Fecchio et al. 2022). Alternatively, Black-throated Blue Warblers often overwinter at or near sea level in the Greater Antilles (Holmes et al. 2020), whereas Canada Warblers can overwinter at elevations of up to 3,000 m in forests of Columbia, Ecuador, and Peru (Reitsma et al. 2020). Parasite vectors and associated haemosporidians are usually less abundant at high elevations (Zamora-Vilchis et al. 2012), which could be a factor in reduced parasite prevalence in Canada Warblers. For instance, *Culex* mosquitoes, which are common vectors for *Plasmodium* in Neotropical landscapes, are less abundant in locations in the vicinity of the Andes Mountains relative to low-elevation tropical areas (Rivero De Aguilar et al. 2018, Gorris et al. 2021). However, this explanation assumes that primary transmission of haemosporidians for these species occurs on the wintering grounds, which has been disputed for birds in the western hemisphere (Garvin et al. 2004, DeGroote and Rodewald 2010). Future studies that involve sampling these species at different locations throughout their annual cycle

could be useful for clarifying patterns in parasite transmission and for understanding why differences in prevalence may arise between seemingly similar species of the same family.

We detected 15 cytochrome *b* lineages of haemospordians (5 *Plasmodium*, 4 *Haemoproteus*, 6 *Leucocytozoon*), and 4 of these lineages were novel to the MalAvi database. This diversity of lineages across all three genera of haemosporidians is not surprising, as previous studies have reported high haemosporidian lineage richness in migratory birds (Jenkins et al. 2012, Pulgarín-R et al. 2019, DeBrock et al. 2021, De Angeli Dutra et al. 2021). For instance, De Angeli Dutra et al. (2021) showed that fully migratory species exhibit greater haemosporidian lineage richness compared to residents or partial migrants. Other studies have illustrated higher prevalence and diversity of parasites during spring and fall migration compared to overwintering periods, although haemosporidian prevalence is still highest during the breeding season (Pulgarín-R et al. 2019, Reinoso-Pérez et al. 2024). High richness of haemosporidians in migratory birds could be related to the extraordinary physiological costs of migration, increasing the susceptibility of birds to infections, alongside exposure to a wider variety of haemosporidians in different regions (Jenkins et al. 2012, De Angeli Dutra et al. 2021). Interestingly, one previously reported *Haemoproteus* lineage (SCLCAU03) that was detected in a Canada Warbler had only previously been reported in the MalAvi database in three species of birds that are endemic to South America: Gould's Jewelfront (*Heliodoxa aurescens)*, Silver-beaked Tanager (*Ramphocelus carbo)*, and Black-tailed Leaftosser (*Sclerurus caudacutus)*. The wintering range of the Canada Warber overlaps with the ranges of all three of these species in Colombia, Ecuador, and Peru (Billerman et al. 2022). We propose that the detection of SCLCAU03 in Canada Warblers in this study is suggestive of intercontinental transmission during the dispersal of this parasite lineage, although we recognize that haemosporidian lineages are not always

useful for determining migration routes (Pagenkopp et al. 2008, DeBrock et al. 2021). Additional information on vector preferences for host species would be useful for investigating this further.

We found that total WBC counts were elevated in infected birds, although this trend was only apparent in Black-throated Blue Warblers. Total WBC counts are often used as a metric of overall immunocompetence (Owen and Moore 2006), and numerous studies have illustrated via leukocyte counts that haemosporidian infections trigger increased immune activity in birds (Dunn et al. 2013, Cornelius et al. 2014, Ellis et al. 2014). Thus, elevated total WBC counts in infected Black-throated Blue Warblers could reflect heightened immune defenses to control haemosporidian infections. Differences in immune responses by Black-throated Blue Warblers compared to Canada Warblers could be related to the parasite genera that were most common in each species. The majority of infections (73.9%) in Black-throated Blue Warblers were either single or mixed infections including *Plasmodium*, whereas most Canada Warbler infections (72.7%) were with *Haemoproteus* or *Leucocytozoon*. *Plasmodium* parasites replicate asexually within peripheral blood; however, *Haemoproteus* and *Leucocytozoon* only replicate in tissues (Valkiūnas 2005). Thus, it is thought that *Plasmodium* infections may be more virulent than those caused by other haemosporidian genera, and birds may mount greater immune defenses to control *Plasmodium* infections at low levels of parasitemia (Fallon and Ricklefs 2008, Ellis et al. 2014). In fact, *Plasmodium* is often found at a lower intensity in the blood compared to *Haemoproteus* (Ricklefs and Sheldon 2007, Fallon and Ricklefs 2008). Elevated total WBC counts in infected Black-throated Blue Warblers could reflect heightened immune defenses to control *Plasmodium* infections. Perhaps Canada Warblers did not show this trend because most infections in this species were either with *Haemoproteus* or *Leucocytozoon*. We do caution, however, that other parasites and non-parasitic agents besides haemosporidians can influence

WBC counts (Reinoso-Pérez et al. 2020), and further work is necessary to confirm patterns observed here.

For female birds, the H/L ratio was significantly lower in infected birds, although an opposing trend, albeit not statistically significant, was observed in male birds. This pattern in the H/L ratio was consistent in both Canada Warblers and Black-throated Blue Warblers. Protandrous migration behavior, where males migrate earlier in the migration season and at a quicker pace compared to female birds (Morbey and Ydenberg 2001), could help explain these observed trends in the H/L ratio. Females may be more likely than males to mount a more specific and time-consuming immune response, which could be associated with elevated lymphocyte numbers and reduced H/L ratios (Dunn et al. 2013). Time-pressured male birds may not be able to afford costs associated with this type of immune defense. In fact, elevated H/L ratios in males could indicate that infections may actually function as a stressor for male birds as they simultaneously attempt to balance the costs of migrating at a rapid pace and managing infections (Davis et al. 2008).

Contrary to our expectations, we found no evidence that haemosporidian infections resulted in trade-offs with refueling/nutritional condition or migration timing in our study system. This result agrees with DeGroote and Rodewald (2010), who found a lack of an association between haemosporidian prevalence and refueling rates, but conflicts with prior studies that have demonstrated a relationship between infections and migration timing (DeGroote and Rodewald 2010, Emmenegger et al. 2018). Overall, if birds are making a trade-off to balance the costs of infection, it does not appear that they allow this trade-off to be with their migration stopover behavior or physiology. This could reflect coevolutionary relationships between avian hosts and blood parasites. Specifically, pathogens including avian haemosporidians can coevolve

low virulence in suitable hosts and will only display themselves as pathogenic in naïve populations (Ricklefs 2010). It is also possible that the costs of immune defenses are not great enough to trigger resource-related trade-offs, or trade-offs may only occur when birds are at their most physiologically depleted (such as after crossing an ecological barrier; as in Garvin et al. 2006) or are truly deprived of food (French et al. 2007, Dunn et al. 2013), which we do not believe to be the case with the birds in our study, as the majority of birds had visible subcutaneous fat stores upon arrival at the site.

Additionally, we acknowledge that a lack of an observed trade-off could be related to inherent sampling biases associated with studying haemosporidians in free-living birds. It is likely that birds with acute avian haemosporidian infections with high parasitemia may not be as likely to be captured via mist-netting or may not even survive to migrate (Valkiūnas 2005, Altizer et al. 2011, Mukhin et al. 2016). In fact, the majority of infections revealed by PCR in this dataset were not detectable by microscopy (G.L.O. pers. obs.), indicating low parasitemia. Thus, the birds sampled in our study likely represent individuals that have survived initial infections and are harboring chronic infections (Asghar et al. 2011). We caution that conclusions drawn from the results of this study can at most be generalized to birds with low-intensity infections. In fact, Asghar et al. (2011) found that migration arrival timing was later, and host reproductive success was reduced in birds with chronically elevated parasitemia as determined via qPCR and microscopy. Captive studies involving experimentally induced infections could be useful for further elucidating the effects that infections with differing levels of parasitemia may have on migratory birds. Additionally, responses to chronic parasitism may differ by host, so future work encompassing a broader range of species would be valuable. We also acknowledge

that sample size could have influenced non-significant results observed in this study, and sampling a greater number of birds in future work could help confirm patterns observed here.

This research suggests that migratory birds can be infected with a diversity of haemosporidian parasites, but these infections are not necessarily associated with the physiological condition and migration success of birds. Although we did not observe any physiological or behavioral trade-offs as a result of infection in this study, our results have conservation and management implications when considered alongside the importance of adequate stopover habitat for migrating birds. Some infected birds in this study displayed elevated immune defenses, and resource-related trade-offs due to immune costs may be more likely if birds do not have access to high-quality stopover sites with abundant food resources. We recommend further research to explore the ways by which stopover site quality may relate to the physiological condition and habitat needs of migratory birds infected with haemosporidians. Migratory birds—particularly those faced with infections—manage numerous physiological challenges. Disruptions (e.g. loss of adequate stopover habitat) to this delicate balancing act could prove detrimental to the overall vitality and associated survival of migratory birds (Klaassen et al. 2012).

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FIGURES

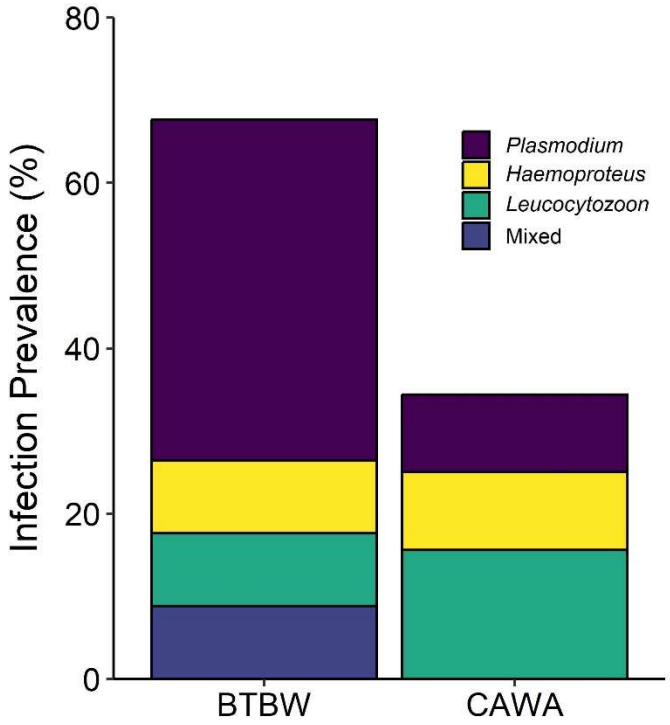

Figure 1.1. Infection prevalence (%) for three genera of haemosporidians (*Plasmodium*, *Haemoproteus*, and *Leucocytozoon*) in Canada Warblers (n = 32) and Black-throated Blue Warblers (n = 34). 'Mixed' denotes a single bird was infected with multiple parasite genera. BTBW = Black-throated Blue Warbler; CAWA = Canada Warbler.

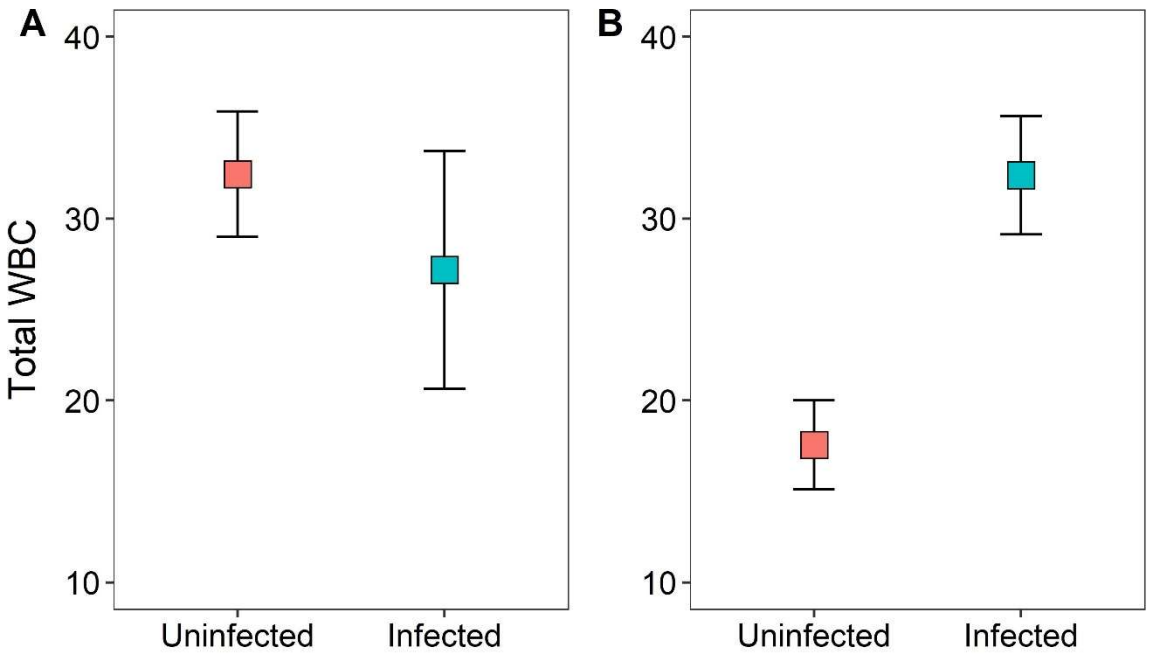

Figure 1.2. Total white blood cell (WBC) counts (mean ± SE) by haemosporidian infection status for Canada Warblers (**A**) and Black-throated Blue Warblers (**B**). Untransformed total WBC counts are presented though transformed values were used in all analyses. In Black-throated Blue Warblers, total WBC counts significantly differed ($P < 0.05$) by infection status, whereas there was no difference in Canada Warblers (see Results).

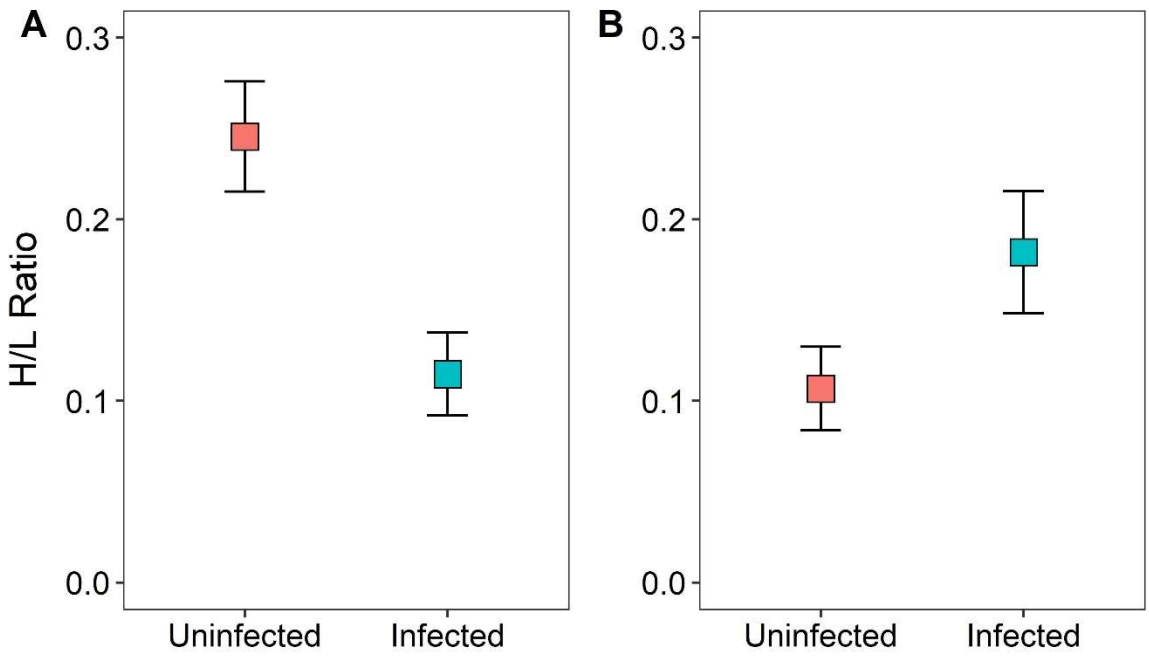

Figure 1.3. Heterophil/lymphocyte (H/L) ratios (mean ± SE) by haemosporidian infection status for female birds (**A**) and male birds (**B**). Data represent both Canada Warblers and Black-throated Blue Warblers. Untransformed H/L ratios are presented though transformed values were used in all analyses. In female birds, the H/L ratio significantly differed ($P < 0.05$) by infection status, whereas there was no difference in males (see Results).

TABLES

Table 1.1. Haemosporidian cytochrome *b* lineages detected in Canada Warblers and Black-throated Blue Warblers. Lineages were identified via comparison to previously identified parasite lineages in the MalAvi database.

	MalAvi Lineage	Genus	N
Canada Warbler	CARCAN02†	*Haemoproteus*	1
	CARCAN03†	*Haemoproteus*	1
	CARCAN04†	*Leucocytozoon*	1
	CNEORN01	*Leucocytozoon*	1
	COLBF21	*Leucocytozoon*	1
	DENCOR05	*Leucocytozoon*	1
	GEOTRI09	*Plasmodium*	1
	SCLCAU03	*Haemoproteus*	1
Black-throated Blue Warbler	CNEORN01	*Leucocytozoon*	1
	DUMCAR01	*Leucocytozoon*	1
	GEOTRI01	*Plasmodium*	6
	GEOTRI09	*Plasmodium*	7
	GEOTRI13	*Leucocytozoon*	1
	PASILI01	*Haemoproteus*	2
	PIPERY02	*Plasmodium*	1
	RAMCAR01	*Plasmodium*	1
	SETCAE01†	*Plasmodium*	1

†Novel parasite lineage in MalAvi.

Table 1.2. Parameter estimates from general linear models predicting the total white blood cell (WBC) count and the heterophil/lymphocyte (H/L) ratio in Canada Warblers and Black-throated Blue Warblers. Species (Canada Warbler vs. Black-throated Blue Warbler), sex (male vs. female), and infection (infected vs. uninfected) were coded in models as dichotomous factors Parameter estimates for two-level factors are for the factor stated in parentheses compared to the other factor.

Model	Parameter	β ± SE	t	*P*
Total WBC (n = 66)	Intercept	2.21 ± 0.09	25.1	<0.001
	Species (Canada Warbler)	0.25 ± 0.10	2.7	0.01
	Sex (Male)	-0.05 ± 0.10	-0.6	0.58
	Infection (Infected)	0.28 ± 0.11	2.5	0.02
	Species × Infection	-0.38 ± 0.13	-2.9	0.006
	Sex × Infection	-0.02 ± 0.13	-0.1	0.90
H/L Ratio (n = 66)	Intercept	0.36 ± 0.05	6.5	<0.001
	Species (Canada Warbler)	0.16 ± 0.06	2.6	0.01
	Sex (Male)	-0.17 ± 0.06	-2.8	0.008
	Infection (Infected)	-0.07 ± 0.07	-1.1	0.29
	Species × Infection	-0.05 ± 0.08	-0.7	0.51
	Sex × Infection	0.24 ± 0.08	2.9	0.005

Table 1.3. Model selection results for top-ranked models that explain variation in plasma metabolites (triglyceride, uric acid, β-hydroxybutyrate) measured in Canada Warblers and Black-Throated Blue Warblers. Models with ΔAIC < 2 are provided. Abbreviations: K, number of estimable regression parameters including intercept and variance; AIC_c, small-sample Akaike information criterion; ΔAIC_c, difference between AIC_c value of the model of interest and the minimum AIC_c value; w_i, Akaike weight.

	Model[†]	**K**	**AIC_c**	**ΔAIC_c**	w_i
Triglyceride (n = 41)	Y=DOY+HAS+SMI	5	-28.00	0.00	0.15
	Y=DOY+HAS	4	-27.60	0.40	0.12
	Y=DOY+SMI	4	-27.24	0.77	0.10
Uric acid (n = 34)	Y=Species	3	30.99	0.00	0.11
	Y=BT+Species	4	31.34	0.35	0.10
	Y=1	2	32.63	1.65	0.05
	Y=DOY+Species	4	32.76	1.77	0.05
	Y=BT	3	32.76	1.78	0.05
β-hydroxybutyrate (n = 27)	Y=Sex+SMI	4	-7.35	0.00	0.12
	Y=SMI	3	-6.39	0.96	0.08
	Y=Sex	3	-5.84	1.50	0.06
	Y=SMI+Species	4	-5.57	1.78	0.05
	Y=DOY+Sex+SMI	5	-5.51	1.83	0.05

[†]Parameters: Y = physiological variable, HAS = capture hour after sunrise, DOY = ordinal day of year, SMI = scaled mass index, BT = bleed time.

Table 1.4. Parameter estimates from general linear models to determine whether haemosporidian infection is associated with plasma triglyceride, uric acid, or β-hydroxybutyrate. Species (Canada Warbler vs. Black-throated Blue Warbler), sex (male vs. female), and infection (infected vs. uninfected) were coded in models as dichotomous factors. Parameter estimates for two-level factors are for the factor stated in parentheses compared to the other factor.

Model	Parameter[†]	β ± SE	t	*P*
Triglyceride (n = 41)	Intercept	2.22 ± 0.62	3.6	<0.001
	Infection (Infected)	0.00 ± 0.05	-0.02	0.99
	DOY	-0.01 ± 0.00	-2.9	0.007
	HAS	0.03 ± 0.02	1.7	0.09
	SMI	0.07 ± 0.04	1.6	0.11
Uric Acid (n = 34)	Intercept	1.28 ± 0.15	8.8	<0.001
	Infection (Infected)	-0.05 ± 0.13	-0.4	0.69
	Species (Canada Warbler)	-0.29 ± 0.14	-2.0	0.05
β-hydroxybutyrate (n = 27)	Intercept	2.46 ± 0.69	3.5	0.002
	Infection (Infected)	0.03 ± 0.08	0.3	0.75
	Sex (Male)	0.14 ± 0.08	1.9	0.07
	SMI	-0.13 ± 0.07	-1.9	0.07

[†]Parameters: Y = physiological variable, HAS = capture hour after sunrise, DOY = ordinal day of year, SMI = scaled mass index.

Appendix 1

Table A1. Haemosporidian cytochrome *b* lineages previously documented in the MalAvi database that were detected in Canada Warblers or Black-throated Blue Warblers during stopover on the south shore of Lake Ontario.

Genus	Lineage	Accession Number	Host(s)[†]	N	Known host families[‡]	Region
Plasmodium	GEOTRI01	EF011170	BTBW	6	Fringillidae, Parulidae, Turdidae, Vireonidae	North & South America
	GEOTRI09	EU328173	CAWA, BTBW	8	Certhiidae, Fringillidae, Hirundinidae, Mimidae, Paridae, Parulidae, Strigidae, Turdidae	North, Central & South America
	PIPERY02	OR063105	BTBW	1	Corvidae, Fringillidae, Parulidae	North America
	RAMCAR01	KF482346	BTBW	1	Fringillidae, Icteridae, Mimidae, Parulidae	North & South America
Leucocytozoon	CNEORN01	DQ355976	CAWA, BTBW	2	Aegithalidae, Certhiidae, Fringilidae, Icteridae, Mimidae, Paridae, Parulidae	North, Central & South America
	COLBF21	KR052953	CAWA	1	Certhiidae, Fringillidae, Icteridae, Paridae, Parulidae, Picidae, Turdidae, Tyrannidae, Vireonidae	North America
	DENCOR05	KF314797	CAWA	1	Fringillidae, Parulidae	North America
	DUMCAR01	MF817811	BTBW	1	Fringillidae, Icteridae, Mimidae, Parulidae, Turdidae	North America
	GEOTRI13	OR063135	BTBW	1	Fringillidae, Parulidae	North America
Haemoproteus	PASILI01	KJ584597	BTBW	2	Corvidae, Fringillidae, Paridae, Parulidae, Turdidae	North America
	SCLCAU03	MN458625	CAWA	1	Fringillidae, Furnariidae, Trochilidae	South America

[†]Host alpha codes: CAWA = Canada Warbler; BTBW = Black-throated Blue Warbler.
[‡]Families reflect previous host family names as published in the MalAvi database and may not represent the most recently updated host taxonomic classification.

Chapter 2

Techniques for quantification of heat shock protein 60 in songbirds and applications in migration physiology research

ABSTRACT

Migration is a challenging time for birds, and birds use numerous physiological mechanisms to survive this demanding period of the annual cycle. Heat shock protein 60 (HSP60) is a highly conserved molecular chaperone protein, and past work has illustrated that this protein could be important to migratory songbirds, particularly while they are mitigating physiological stress. However, only a handful of studies have investigated patterns of HSP60 in free-living migratory birds, and laboratory techniques to quantify HSP60 are still limited. Here, we optimized an enzyme-linked immunosorbent assay (ELISA) to measure HSP60 in migratory bird red blood cell samples. We then used ELISA to quantify HSP60 in three species of *Catharus* thrushes (Swainson's Thrush, Gray-cheeked Thrush, Hermit Thrush) during spring and fall migration and performed a preliminary analysis of factors that may influence HSP60. Western blotting results showed that the primary antibody used for ELISA demonstrated high specificity for HSP60. Our ELISA had a consistent pattern of linearity from 6.25-50 ng/mL, and it displayed high precision with minimal inter-assay variability. Exploratory analyses suggested moderate variability in HSP60 across species, where there was a non-significant trend toward elevated HSP60 in Hermit Thrushes relative to Swainson's Thrushes or Gray-cheeked Thrushes. HSP60 was significantly higher during spring migration when compared to fall migration. There was no significant relationship of HSP60 with plasma uric acid, white blood cell counts, scaled mass index, or migration arrival date. Our results suggest that HSP60 could have associations with some

stressors related to the challenges of long-distance migratory flight. This study illustrates that ELISA is an effective method for quantification of HSP60 in migratory birds, and we propose that HSP60 is a robust metric of physiological stress suitable for avian migration studies.

INTRODUCTION

Migration is a time of immense physiological exertion for songbirds. Migratory birds challenge the limits of endurance, where small songbirds are capable of long-distance, non-stop flights with limited opportunities for rest and recovery. Transoceanic flights of nearly 3,000 km and lasting as long as 3 days have been documented in small songbirds weighing just 12 g (DeLuca et al. 2019). To fuel this long-distance endurance flight, migratory birds nearly double their body weight in fat (McWilliams et al. 2004). Additionally, migratory birds catabolize lean mass, primarily endogenous protein from their own internal muscles and organs, as both fuel for flight and as a water source (McWilliams et al. 2004, Gerson and Guglielmo 2011). This strenuous activity imposes numerous physiological stressors on a bird. Long-distance flight is associated with high metabolic activity, and this results in an increase of harmful pro-oxidants produced by the mitochondria, constituting significant oxidative stress (Swanson 2010, Skrip et al. 2015). Furthermore, migratory birds are confronted with hypoxic conditions as they ascend to high altitudes during flight (Laguë 2017). In addition to flight, rest periods at stopover sites are associated with significant energy expenditure (Wikelski et al. 2003). Migratory birds also face numerous environmental challenges, threats from parasites and disease, and predation, all of which can serve as additional stressors during an already energetically demanding time (Wikelski et al. 2003, Klaassen et al. 2012). Although many studies have focused on developing a greater understanding of avian migration, there is still a limited understanding of the physiological

mechanisms that birds use balance the costs of stressors faced during migration, particularly at the cellular and molecular level.

Heat shock proteins (HSPs) may serve as a molecular mechanism that birds use to mitigate physiological stress, and they have been proposed as novel biomarker of stress for birds during migration (Klaassen et al. 2012). HSPs constitute a family of highly conserved molecular chaperone proteins, and they are produced as an immediate response to protein damage from a variety of environmental stressors (Feder and Hofmann 1999, Sørensen et al. 2003). Cellular functions of HSPs include prevention of protein aggregation, degradation of damaged proteins, and assistance with protein folding (Sørensen et al. 2003). Although HSPs are well known for their ability to help organisms adapt to hyperthermic conditions (Lindquist and Craig 1988, Feder and Hofmann 1999), a variety of nonthermal stressors—including pesticides, toxins, parasites and disease, hypoxia, oxidative stress, and intense physical activity—can also induce HSP production (Lewis et al. 1999, Sørensen et al. 2003). Considering that HSPs have critical roles in many diverse ecological systems, it is likely that HSPs are also an important physiological trait in birds. For instance, HSPs could act as physiological buffer in a variety of stressful situations for migratory birds.

Despite the numerous ecological and evolutionary roles of HSPs, limited studies have examined the importance of HSPs in the context of the challenges of avian migration. The few that have investigated HSPs in migratory birds have provided compelling evidence that HSPs could be an important physiological trait of migration. For example, Lobato et al. (2010) found that both corticosterone and HSP60 were elevated in migratory birds that had just arrived on the breeding grounds. This trend was especially apparent in early arriving individuals, which may experience increased allostatic load due to harsher conditions experienced during migration in

early spring (Smith and Moore 2005). It was speculated that this relationship could reflect HSP synthesis during a time of increased energy demands and need for O_2 supply (Lobato et al. 2010). Additionally, it was found that some heat shock genes were upregulated in migratory birds that had just crossed the Sahara Desert-Mediterranean Sea ecological barrier (Bounas et al. 2024). This result suggested that HSPs may be an important mechanism that migratory birds use to facilitate muscle fatigue, nutritional deprivation, and oxidative stress during long-distance flight (Bounas et al. 2024). These prior studies suggest noteworthy associations between HSPs and physiological stress experienced by migratory birds, but the role of HSPs in bird migration warrants additional investigation.

Understanding the physiological mechanisms that birds use to respond to stressful life periods such as migration is an important objective of avian conservation physiology. Stress—which can be defined as the mechanisms used by an animal when confronted with a scenario that elicits a defense response—is not without costs (Siegel 1980), and balancing the costs of physiological stressors could result in trade-offs with other physiological or behavioral processes that could impact fitness and survival. As an example, trade-offs between physiologically demanding activities and immunocompetence are well documented in avian eco-immunology (Siegel 1980, Owen and Moore 2006, Hasselquist and Nilsson 2012, Eikenaar et al. 2018, 2020). These trade-offs with immunity could lead to a migratory bird being more susceptible to detrimental diseases or parasite infections (Owen and Moore 2006). Other stress-related trade-offs pertinent to migratory birds include refueling trade-offs or those related to migration pace, which could result in delayed migration or poor body condition and have harmful carry-over effects to other periods of the annual cycle (Lobato et al. 2010, Bounas et al. 2024).

Several methodological challenges have impeded initiatives to use HSPs as a routine stress metric in avian migration research. Western blots have served as the primary laboratory technique for HSP analyses in ecological studies (Lewis et al. 1999, Moreno et al. 2002, Tomás et al. 2004). However, this technique is only semi-quantitative, as it is based on the quantification of resolved western blot images with densitometry, and these measurements of immunoreactivity should ideally be scaled with total protein or a housekeeping protein. Furthermore, western blots are inefficient for analyzing a large number of samples, as often necessary in studies of free-living birds. More recently, researchers have turned to examining gene expression patterns as a proxy of HSP production (Bounas et al. 2024). However, quantifying gene expression is reliant on assumptions—most notably that transcribed mRNA is actually translated to a final protein product, which is not always the case (Griffin et al. 2002). Enzyme-linked immunosorbent assays (ELISAs) could be the most effective and direct measurement technique for quantifying HSPs in ecological studies (Lewis et al. 1999). ELISAs are more efficient than western blotting for analyzing a large number of samples, and they offer advantages over gene expression measurement in that they involve direct protein quantification. Although some past works have utilized commercially available kits—most often adapted for human serum or cell lysates—to measure HSPs in free-living birds (Herring et al. 2011, O'Dell et al. 2014), the ELISA technique has been largely under-utilized in ornithological studies.

Here we measured HSP60 in three species of *Catharus* thrushes during their migration stopover on the south shore of Lake Ontario. Although there are many types of HSPs—with HSP60, HSP70, and HSP90 being the most commonly measured—we chose to measure HSP60 due to its numerous associations with a variety of environmental stressors (see Table 2.1 for a summary of past work on HSP60) outside of thermal stress (Hill et al. 2013). HSP70 and HSP90

are more often associated with hyperthermic conditions, and HSP60 has been shown to reflect a wider range of stressors experienced by wild birds (Lindquist and Craig 1988, Moreno et al. 2002). An objective of this study was to adapt a standard western blotting procedure for HSP60 (Tomás et al. 2004) into an ELISA tailored to measure HSP60 in migratory bird red blood cell samples. We sought to improve the quantification method for HSP60 in birds to enhance the ability to use HSP60 as a standard stress metric in migration research. HSPs resolve logistical challenges associated with other commonly used metrics of stress (e.g. corticosterone) in that they do not fluctuate quickly in response to handling and capture stress, and they could be a robust metric of physiological stress (Vleck et al. 2000, Tomás et al. 2004). We also performed a preliminary analysis of factors that may influence HSP60 in migratory birds. We investigated correlations of HSP60 with physiological metrics including fuel stores, uric acid (as a measure of protein catabolism), and total and differential white blood cell counts. We additionally explored the relationships of HSP60 with migration timing and season. We hypothesized that HSP60 would exhibit a negative relationship with body condition and immune defenses, and there would be a positive relationship with uric acid. We predicted that early arriving birds would show the highest HSP60 levels, and HSP60 would be elevated in spring migrants compared to birds in fall.

METHODS

Study site, bird capture, and blood sampling

Bird sampling was conducted at Braddock Bay Bird Observatory (BBBO) on the south shore of Lake Ontario in the town of Hilton, New York, USA (43.3236°N, 77.7175°W). This location is an important stopover site for migratory songbirds and provides adequate refueling habitat for birds during both spring and fall migration (Smith 2013). Study site characteristics

have been previously documented by Bonter et al. (2007), and the site mainly includes early successional habitat surrounded by forests, agricultural lands, and water cover. Although once dominated by alder (*Alnus* spp.) and ash (*Fraxinus* spp.), the area more recently experienced significant changes in canopy cover in certain habitats due to loss of ash as a consequence of the spread of the Emerald Ash Borer (*Agrilus planipennis*) in this region.

The focal species in this study were the Swainson's Thrush (*Catharus ustulatus*), Gray-cheeked Thrush (*Catharus minimus*), and Hermit Thrush (*Catharus guttatus*). We selected these species because these are migratory birds that use the south shore of Lake Ontario as stopover habitat during migration and are not known to breed at the site. Additionally, the Swainson's Thrush and Gray-cheeked Thrush exhibit a long-distance migration strategy, while the Hermit Thrush is considered a short-distance migrant, allowing for a comparison of migration strategy. Thrushes were sampled using mist-netting during normal operating hours (0-6 h after sunrise) at BBBO during spring 2022 and 2023 (April 15 – May 31) and fall 2022 (August 15– October 31). Birds were removed from mist nets and brought back to a central location for banding and blood sampling. Birds were banded with a U.S. Geological Survey aluminum leg band, morphometric data was recorded, and birds were aged according to Pyle (1997). Sex was later determined molecularly because Swainson's Thrushes cannot be sexed reliably in the field (Pyle 1997). DNA was extracted from red blood cells using the E.Z.N.A. blood DNA extraction kit (Omega Bio-Tek; Norcross, Georgia, USA), and PCR-based methods were used to determine the sex of each bird (Fridolfsson and Ellegren 1999). Prior to banding and measurement, a blood sample was collected from each bird via brachial vein puncture using a 27.5 G needle, and approximately 70 ul of blood were collected in heparinized capillary tubes. Tubes were centrifuged at 10,000 rpm for 10 min to separate plasma from red blood cells, and plasma and cellular fractions were

separately frozen at -80 °C until subsequent laboratory analyses. Time of capture was recorded as the hour after sunrise of the net check (hereafter "capture hour"), and "bleed time" was noted as the number of minutes between net extraction and blood sampling. A blood smear slide was prepared from whole blood at the time of blood collection, where it was then fixed using 100% methanol and stained within 2 weeks (Owen 2011). Blood smear slides were stained with Wright-Giemsa solution (Hema-3 manual staining system; Fisher Scientific, Waltham, Massachusetts, USA).

White blood cell counts

White blood cell counts were performed as a measure of immunological condition. Prepared blood smears were read under a compound light microscope at 1000x magnification. All leukocytes (lymphocytes, heterophils, monocytes, eosinophils, basophils) within 100 monolayer fields of view (approx. 200 erythrocytes per field of view) were counted. The total white blood cell count was calculated as the number of white blood cells per 10,000 erythrocytes, while the heterophil/lymphocyte ratio was calculated as the number of heterophils per 100 fields of view divided by the number of lymphocytes per 100 fields of view (Owen and Moore 2006).

Uric acid assays

Uric acid is a measure of protein catabolism, and elevated plasma uric acid concentrations can be reflective of endogenous protein catabolism in migratory birds; however, protein consumed in the diet can also affect uric acid (Smith et al. 2007, Gutierrez Ramirez et al. 2022). Plasma uric acid concentrations were measured spectrophotometrically by endpoint assay

(TECO Diagnostics, Anaheim, California, USA) using procedures described by Smith and McWilliams (2010). Plasma was diluted by a factor of 4 with 0.9% NaCl prior to analyses, and all samples were measured in duplicate wells of 96-well microplates. Measurements were repeated between replicates until the coefficient of variation between wells was <10%.

Analyzing HSP60

ELISA

HSP60 concentrations were determined using ELISA adapted from a western blotting protocol described by Tomás et al. (2004). Cell lysates were obtained from red blood cell fractions (Tomás et al. 2004), and total protein in cell lysates was quantified using a Bradford assay (Bio-Rad, Hercules, California, USA) with bovine serum albumin as the standard. To perform the ELISA, 100 ul of samples (diluted 1:5 in PBS) and standards (6.25-50 ng/mL recombinant human HSP60) were loaded in duplicate wells in high-binding 96-well microplates (Greiner BioOne, Kremsmünster, Austria) and incubated overnight (4 °C). A blank (PBS) was also included on the plates in duplicate wells. Attention was given to figures of merit for the assay method (linear range, limit of quantification, inter- and intra-assay variation). A series of standards of varying concentration of recombinant human HSP60 were preliminarily analyzed to determine the linear range for the assay. Additionally, we evaluated replicate measurements of blank samples to ensure our lowest standard and samples were above the limit of quantification (10x the standard deviation of the blank absorbance replicates) for the method. The stated concentration range followed Beer's Law with strong R^2 values of the linear fit of calibration curves. Plates were blocked with 200 ul 1X PBS and 3% powdered milk for 1 hour at room temperature then washed. Plates were then incubated for 2 h at 37°C with 100 ul anti-HSP60

monoclonal antibody (H3524, Clone LK2; Sigma-Aldrich, St. Louis, Missouri, USA) diluted 1:1,000, followed by a 2 h incubation at room temperature with 100 ul of 1:5,000 goat anti-mouse IgG (A4416; Sigma-Aldrich, St. Louis, Missouri, USA). Finally, 100 ul of TMB substrate (Fisher Scientific, Waltham, Massachusetts, USA) was added to all wells and incubated for 30 min in the dark at room temperature. The reaction was stopped with 50 ul phosphoric acid, and the absorbance of the plate was read at 450 nm with a Bio-Tek Synergy H1 microplate reader (Bio-Tek, Winooski, Vermont, USA). HSP60 concentrations were determined using a standard curve, and final HSP60 values were calculated as the concentration of HSP60 divided by total protein. An inter-assay control (a pooled cell lysate from migratory passerine red blood cells) was included on each plate to assess validity of the ELISA results. A general linear model including concentration as the independent variable and absorbance as the dependent variable with ELISA plate × concentration as an interaction term was used to evaluate whether slopes of standard curves varied among all assays.

Western blots

A subset of samples was analyzed via western blotting to verify specificity of the primary antibody for ELISA. HSP60 was detected via western blotting using methods previously described by Tomás et al. (2004) with some modifications. As in Tomás et al. (2004), total protein was extracted from red blood cell fractions and quantified using a Bradford assay (Bio-Rad, Hercules, California, USA). Protein extracts (70 ug) were then loaded onto Bolt 10% Bis-tris Plus polyacrylamide gels (Fisher Scientific, Waltham, Massachusetts, USA) and separated via SDS-PAGE at constant voltage (200 V). Electroblot transfer from polyacrylamide gels to nitrocellulose membranes was then completed using the iBlot2 instrument. Western blotting of the transfer membrane was performed using the instructions and reagents from the Pierce Fast

Western Blot Kit (Fisher Scientific, Waltham, Massachusetts, USA) and a primary antibody for HSP60 (H3524, Clone LK2; Sigma-Aldrich, St. Louis, Missouri, USA) diluted 1:1,000. Western blots were imaged using chemiluminescent detection (Azure 300; Azure Biosystems, Dublin, California, USA). A protein marker was included on western blots to evaluate the size of resolved protein bands.

Statistical analyses

HSP60 concentrations of thrushes were not normally distributed, so we transformed HSP60 using a $\log_{10}(x)+1$ transformation prior to all analyses involving HSP60 as a dependent variable. Wing chord length was significantly related to body mass ($F_{1,103} = 30.04$, $P < 0.001$), so mass was scaled based on wing chord length using an equation established by Peig and Green (2009) to create a scaled mass index (SMI). We performed a preliminary analysis of potential covariates that may influence HSP60 levels. Covariates included year, capture hour after sunrise, bleed time, and sex. For categorical variables, we independently assessed the importance of each variable using one-way analysis of variance (ANOVA). We assessed the significance of continuous covariates using Pearson's correlation coefficients.

We then explored relationships between HSP60 and main effects of season and species using one-way ANOVA after confirming equal variances. The effects of SMI, capture day of year (analyzed separately by spring and fall migration seasons), plasma uric acid concentrations, total WBC, and the H/L ratio were evaluated univariately using simple linear regression. All analyses were performed with R 4.2.2 (R Core Team 2023) or JMP Pro 16 (SAS Institute 2021). Results were accepted as significant when $P < 0.05$. Summary statistics are reported as mean ± SE.

RESULTS

Performance of HSP60 analyses

Western blotting results demonstrated specificity of the primary antibody for HSP60, as all samples showed a band detected by chemiluminescent detection at the 60 kDa marker (Figure 2.1). An analysis of a series of standards of varying concentration of recombinant human HSP60 suggested a linear range for the assay from 6.25-50 ng/mL (Figure 2.2). Across all assays, the slope of the standard curve ranged from 0.0057-0.0064, and the linear fit of the standard curves had an average R^2 value of 0.990 ± 0.004 (Figure 2.2). There was no evidence that the slopes of the standard curves differed based on ELISA plate (ELISA plate × concentration: $F_{4,14} = 0.55$, $P = 0.70$; Figure 2.2). In this study, the inter-assay coefficient of variation based on the calculated assay control well concentration was 9.1%, and the average intra-assay coefficient of variation based on duplicate sample wells was 2.8%. For all bird samples measured using the ELISA method (n = 105), HSP60 concentrations corrected for total protein concentration ranged from 3.46-86.38 ng/mg with a mean of 18.30 ± 1.35 ng/mg.

Bird sampling

We sampled 105 thrushes across spring 2022 (n = 14), fall 2022 (n = 55), and spring 2023 (n = 36). Sampling efforts for each species by season can be found in Table 2.2. Median capture dates in spring were May 24 for Swainson's Thrush, May 24 for Gray-cheeked Thrush, and May 3 for Hermit Thrush. In fall, median capture dates were September 8 for Swainson's Thrush, September 23 for Gray-cheeked Thrush, and October 15 for Hermit Thrush. The majority of sampled birds (84/105) were juvenile ('hatch-year' in fall or 'second-year' in spring). Thus, ages were pooled for all analyses. There were 59 female birds (Swainson's Thrush: n = 40; Gray-

cheeked Thrush: n = 5; Hermit Thrush: n = 14), and 45 birds were male (Swainson's Thrush: n = 26; Gray-cheeked Thrush: n = 13; Hermit Thrush: n = 6).

Assessing covariates associated with HSP60

Prior to physiological analyses to assess main effects of interest, we performed a preliminary analysis of covariates that may be expected to influence HSP60 concentrations in this study. One-way ANOVA results showed that capture year and sex did not affect HSP60 concentrations (Year: $F_{1,103} = 1.06$, $P = 0.30$; Sex: $F_{1,102} = 1.67$, $P = 0.20$). Temporal continuous covariates were unimportant in influencing HSP60, as we found no significant correlation of HSP60 with capture hour after sunrise (r = -0.01, $P = 0.90$) or bleed time (r = 0.05, $P = 0.60$). Thus, no covariates were included in subsequent physiological analyses.

Associations of HSP60 with species, migration timing, and physiological variables

A summary of physiological variables measured in this study can be found in Table 2.2. There was no significant effect of species on HSP60 ($F_{2,102} = 2.48$, $P = 0.09$; Figure 2.3), although there was a trend toward higher HSP60 concentrations in Hermit Thrushes relative to Swainson's Thrushes or Gray-cheeked Thrushes. There was a significant relationship between HSP60 concentration and migration season ($F_{1,103} = 5.46$, $P = 0.02$; Figure 2.4), where HSP60 concentrations were elevated in spring relative to fall (Figure 2.4). We found that uric acid, SMI, the total WBC count, and the H/L ratio were not significantly related to HSP60 (uric acid: $F_{1,99} = 2.63$, $P = 0.11$; SMI: $F_{1,103} = 3.34$, $P = 0.07$; total WBC: $F_{1,102} = 0.46$, $P = 0.50$; H/L ratio: $F_{1,102} = 1.48$, $P = 0.23$; Figure 2.5). Capture day of year did not influence HSP60 concentrations in spring ($F_{1,48} = 0.54$, $P = 0.47$) nor in fall ($F_{1,53} = 0.46$, $P = 0.50$).

DISCUSSION

HSPs are molecular chaperone proteins with a diversity of conserved functions across organisms (Lindquist and Craig 1988). HSPs could be an important cellular mechanism used by birds to mitigate physiological stress (Bounas et al. 2024), yet their applications in studies involving migratory birds are little explored. The goal of this study was to improve the quantification method for HSP60 and then apply this technique in a field study on migratory birds, which have experienced dramatic population declines and are a group of conservation importance (Bairlein 2016). We provide several insights into the logistics of using HSP60 as a stress metric, variables that HSP60 may be associated with in migratory birds, and the performance of our ELISA technique to measure HSP60.

ELISA was an effective technique for analyzing HSP60 in a large number of samples from migratory birds. The ELISA in our study was adapted from standard western blotting procedures for HSP60 previously used for songbirds (Tomás et al. 2004), making the results from this study more clearly comparable to past work in contrast to studies that have utilized commercial ELISA kits. Additionally, the measurements from this study were quantitative, as they involved measurement of HSP60 concentrations in cell lysates using a standard curve, along with the standardization of these measurements based on total protein in centrifuged packed red blood cell volumes. Our ELISA demonstrated high precision, low inter-assay variability, and a repeatable linear relationship of the standard across assays. ELISA performance overall was consistent, and we believe the ELISA protocol described here could be useful for future avian migration studies. We advise further research to evaluate variables (e.g. temperature, freeze-thawing of antibodies) that may contribute to slight inter-assay variation in ELISA results among plates to fully ensure that the ELISA is repeatable in all scenarios. As another consideration, we

only measured HSP60 in red blood cell lysates in this study. Future work encompassing a wider range of tissue types and associated cell lysates could be valuable, as protein quality and content may vary considerably among animal tissues (Lewis et al. 1999).

We tested the relationship between HSP60 and covariates that may influence patterns in this protein. We found no evidence of variation between years in our study, suggesting that HSP60 is a robust metric that is unlikely to drastically fluctuate in response to routine variation in environmental conditions from year to year. Sex was unimportant in influencing HSP60 in this study, suggesting that male and female *Catharus* thrushes do not differ in HSP60 levels during migration. However, protandry is a well-documented phenomenon in birds (Morbey and Ydenberg 2001), particularly during spring migration; thus, it may be expected that birds of different sexes may experience different stressors during migration. Thus, we recognize that sex could be an important variable in other species and should be considered as a potential confounding variable that could influence HSP60 concentrations. Temporal covariates such as capture hour after sunrise and bleed time were also unimportant in influencing HSP60 levels in this study. This agrees with O'Dell et al. (2014), where it was found that HSP60 was not influenced by handling time of birds. Thus, we provide additional evidence that HSP60 does not change in short time spans, such as in between capture in a mist net and blood sampling. Therefore, HSP60 would not be expected to fluctuate in response to capture stress, a typical logistical challenge associated with stress metrics such as corticosterone (O'Dell et al. 2014).

HSP60 exhibited moderate variation across species, where it appeared that Hermit Thrushes may have slightly elevated HSP60. Hermit Thrushes are short-distance migrants and migrate earlier than Swainson's Thrushes and Gray-cheeked Thrushes during spring migration and later in the season during fall migration in the Northeast. In fact, Hermit Thrushes in this

study arrived on average 21 days earlier than Swainson's and Gray-cheeked Thrushes in spring and 23 days later than either of these species in fall. Thus, Hermit Thrushes may be exposed to different environmental stressors than other *Catharus* thrush species during migration. Past work has shown that birds that arrive the earliest in spring encounter poor environmental conditions and low food availability, which has been shown to result in increased stress levels in these individuals (Smith and Moore 2005, Oberkircher and Pagano 2018). This pattern would be expected to be similar in late fall—in fact, previous work on *Catharus* thrushes has shown that Hermit Thrushes have elevated H/L ratios compared to Swainson's or Gray-Cheeked Thrushes during fall migration (Pagano et al. 2023). This is likely due to reduced food availability and poorer environmental conditions—such as lower temperatures and reduced vegetation cover—in late fall (Pagano et al. 2023). Therefore, the relationship of species and HSP60 in this study could be reflective of HSP60 being responsive to a diversity of stressors associated with adverse conditions during migration. Interestingly, unlike our initial hypothesis, arrival date itself was not an important predictor of HSP60. Thus, species variation could be attributable to more than just differences in passage timing. For instance, perhaps migration strategy could be important, as Hermit Thrushes exhibit a short-distance migration strategy, while Swainson's and Gray-cheeked Thrushes are long-distance migrants. Additional work is necessary to clarify factors that may contribute to variation in HSP60 across migratory species.

HSP60 was elevated during spring migration relative to fall migration. This pattern agrees with numerous studies that have illustrated that spring migration is a more physiologically stressful time for birds in comparison to fall migration (Owen and Moore 2006, Oberkircher and Pagano 2018). Birds are more time-pressured during spring migration compared to fall, and they may encounter more headwinds and less food, which may act as stressors during spring

migration (Nilsson et al. 2013). As an additional physiological explanation, birds arriving at this site in spring may simply be more physiologically depleted than birds arriving at the site in fall due to migration distance covered upon arrival at the stopover site. Braddock Bay is located on the south shore of Lake Ontario, and it is much nearer to the northern breeding grounds of many species compared to the overwintering locations of these species in the southern U.S. or Central and South America. Thus, in spring, birds will have traveled thousands of kilometers upon arrival at the stopover site, whereas in fall, many birds will have just begun their migration. A similar explanation was posed to explain seasonal variation in the H/L ratio in migrating thrushes, where birds that had just crossed the Gulf of Mexico in spring had higher H/L ratios than fall birds that had not yet underwent this long-distance flight (Owen and Moore 2006). HSP60 may not become elevated in migratory birds until they have undergone severe physiological disruption from long-distance flight, and this could explain why we observed higher HSP60 in spring migration compared to fall migration in birds in this study. Wind-tunnel studies involving experimental flights of varying distances alongside subsequent measurements of HSP60 would be useful for further investigating this relationship.

HSP60 was not significantly related to plasma uric acid in this study, although there was a weak positive relationship. Uric acid is an indicator of protein catabolism, and plasma uric acid concentrations are elevated after lean mass catabolism (Gutierrez Ramirez et al. 2022). Although fat is the primary fuel source of flight, migratory birds routinely burn lean mass during migration, both as an additional fuel source and as a water supply (McWilliams et al. 2004, Gerson and Guglielmo 2011). Perhaps HSP60 could be produced in response to migration-induced physiological stressors, including lean mass catabolism, oxidative stress, muscle fatigue, and nutritional deprivation, as has been suggested for other families of HSPs (Bounas et al.

2024). Heat shock proteins are important for maintaining cellular machinery in situations of internal stress (Sørensen et al. 2003), such as when lean mass is being catabolized. Perhaps a lack of obvious correlation between HSP60 and uric acid could be because uric acid is associated with dietary protein catabolism as well as endogenous protein catabolism (Smith et al. 2007), which complicates the interpretation of the relationship between HSP60 and uric acid in this study of birds that are presumably refueling during stopover. Future work with more direct measurement of lean mass catabolism (e.g. a study involving quantitative magnetic resonance) could help clarify whether or not there is a relationship between HSP60 and endogenous protein catabolism. Additionally, unlike our initial prediction, there was no relationship between HSP60 and a body condition index, which also warrants further investigation with regards to how HSP60 is related to fuel utilization during migratory flight.

The total WBC count has been interpreted as an overall metric of immunocompetence in past work on migratory birds (Owen and Moore 2006). The lack of a correlation between HSP60 and the total WBC count in this study suggests that stressors that induce HSP60 production do not necessarily result in trade-offs with immune function, as may be expected based on theories of stress-induced immunosuppression (Owen and Moore 2006). It is possible that the costs associated with producing HSPs and responding to stressors are simply not great enough to elicit a trade-off with immune function. We also recognize that the total WBC count can vary unpredictably in response to stressors including disease, and there is some inconsistency in the ecological literature with how immunocompetency can be defined in response to total WBC counts (Davis et al. 2008). Measurement of additional immune metrics would be helpful for clarifying the relationship between HSP60 and immune function. HSP60 was also not strongly correlated with the H/L ratio, although there was a weak positive relationship between these

variables. The H/L ratio is moderately correlated with corticosterone, yet it is a more reliable indicator of chronic stress (Vleck et al. 2000, Davis et al. 2008). This trend does agree with past work suggesting that the H/L ratio and HSP60 are both reliable metrics of stress, yet they may respond to subtly different stressors in birds (Moreno et al. 2002).

In conclusion, this study illustrated that HSP60 could have important roles for migratory birds, and we provide details on an ELISA method for measurement of this protein. We believe the results of this work provide evidence that HSPs are a meritorious stress metric in bird migration research. As suggested by others (Klaassen et al. 2012), we propose that HSPs should be considered as an additional condition metric in physiological studies of migratory birds. Migratory birds are in decline, and these population decreases are only likely to become more dramatic with increasing threats of climate change, habitat loss, and invasive species (Bairlein 2016). Physiological studies of free-living birds and the challenges they face are vital for informing conservation and management efforts. Migration is a significant period of mortality for songbirds, and habitat quality encountered during migration (e.g. at stopover sites) could limit migratory performance and survivorship (Newton 2006). As an example, HSPs could be utilized as a physiological indicator to understand how the condition of birds varies in response to habitat quality (Albano 2012).

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FIGURES

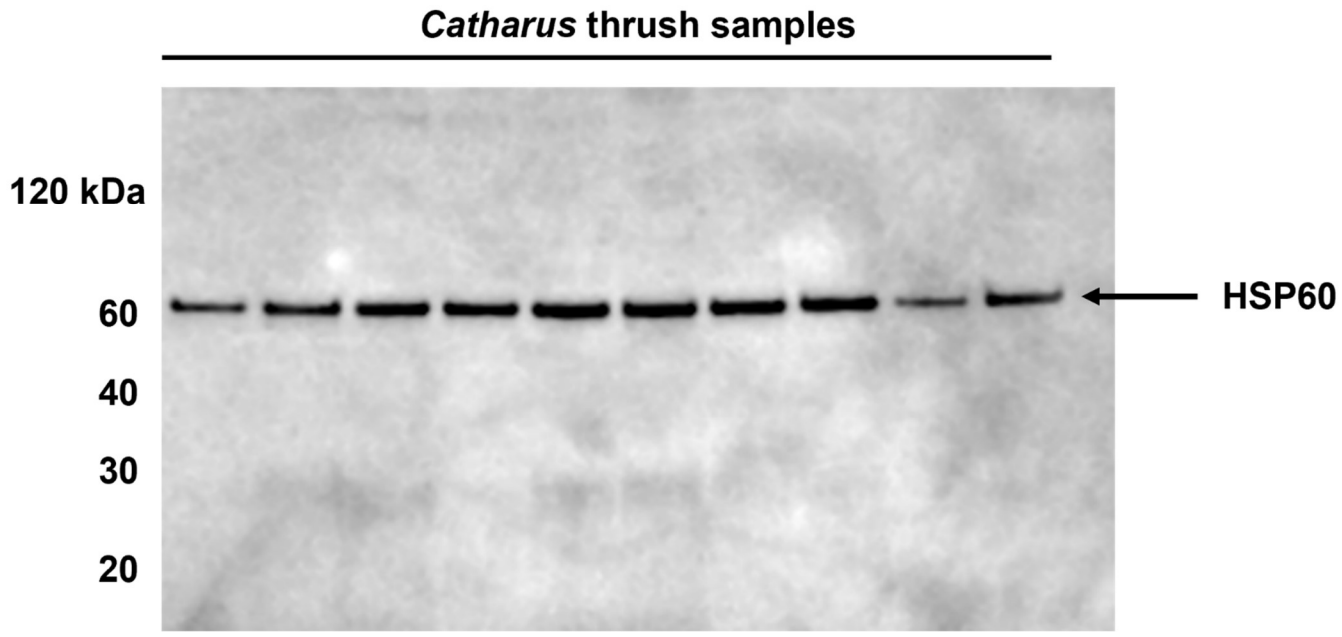

Figure 2.1. Western blot to detect heat shock protein 60 (HSP60) for samples from 10 individual *Catharus* thrushes.

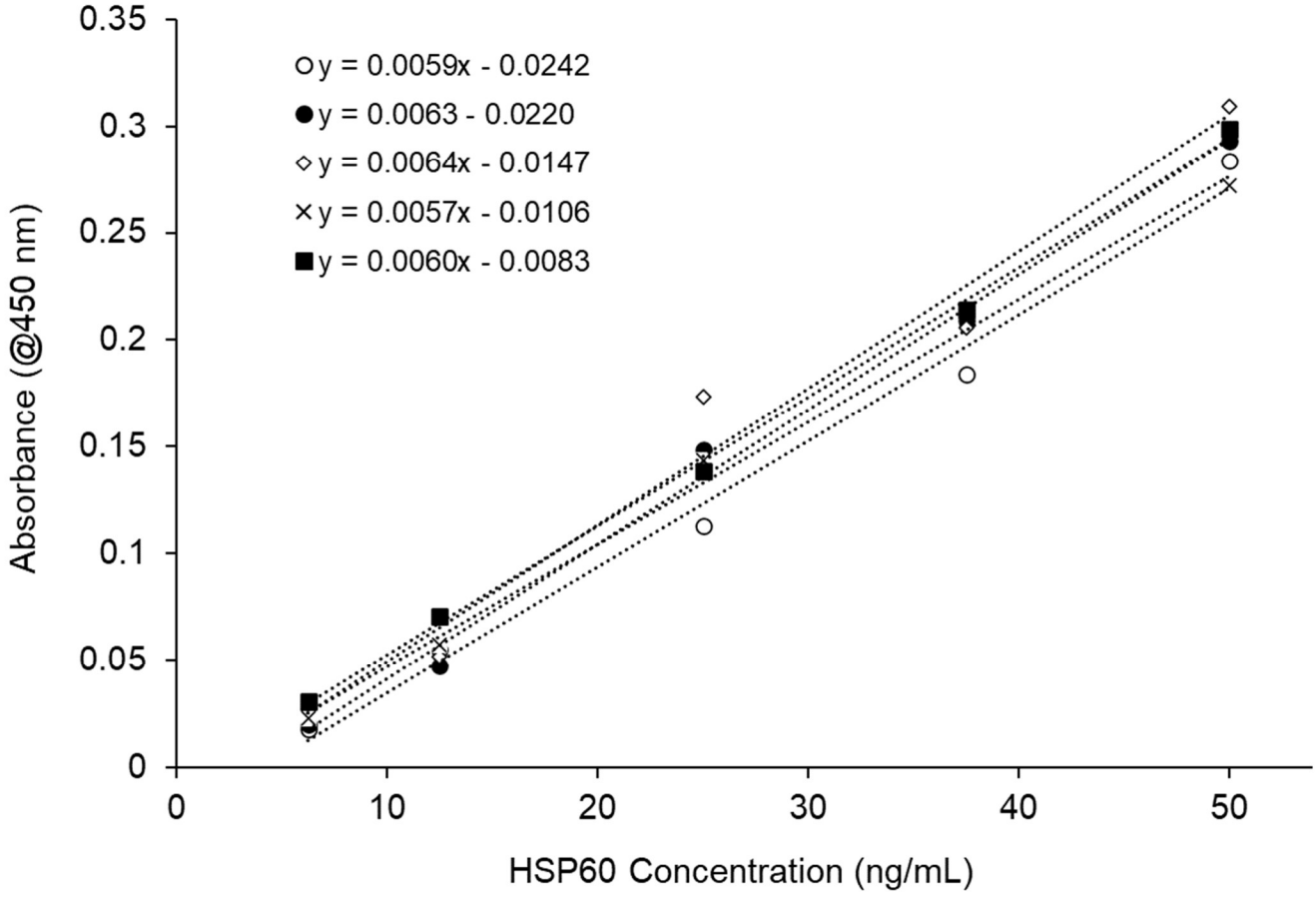

Figure 2.2. Heat shock protein 60 (HSP60) ELISA standard curves. Dilutions of the standard (recombinant human HSP60) were prepared from 6.25-50 ng/mL. Standard curves were fitted using the average of replicate wells.

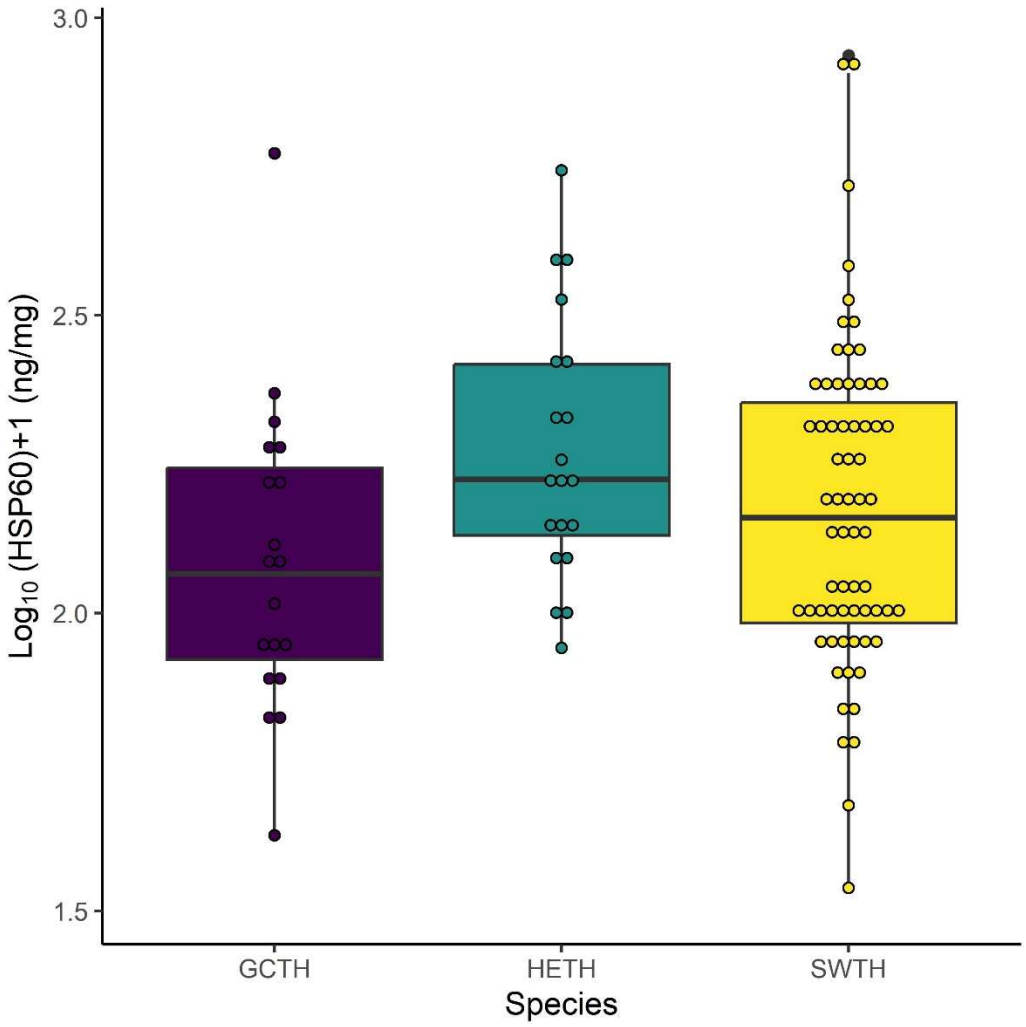

Figure 2.3. Heat shock protein 60 (HSP60) concentrations in Gray-cheeked Thrushes (GCTH; n = 19), Hermit Thrushes (HETH; n = 20), and Swainson's Thrushes (SWTH; n = 66). Log-transformed HSP60 was used in analyses. Each data point represents the HSP60 concentration of a sample. Lower and upper fences of box plots are the 25th and 75th percentiles, and the median is in between. Vertical extending lines represent the most extreme values within 1.5 interquartile range of the 25th and 75th percentiles.

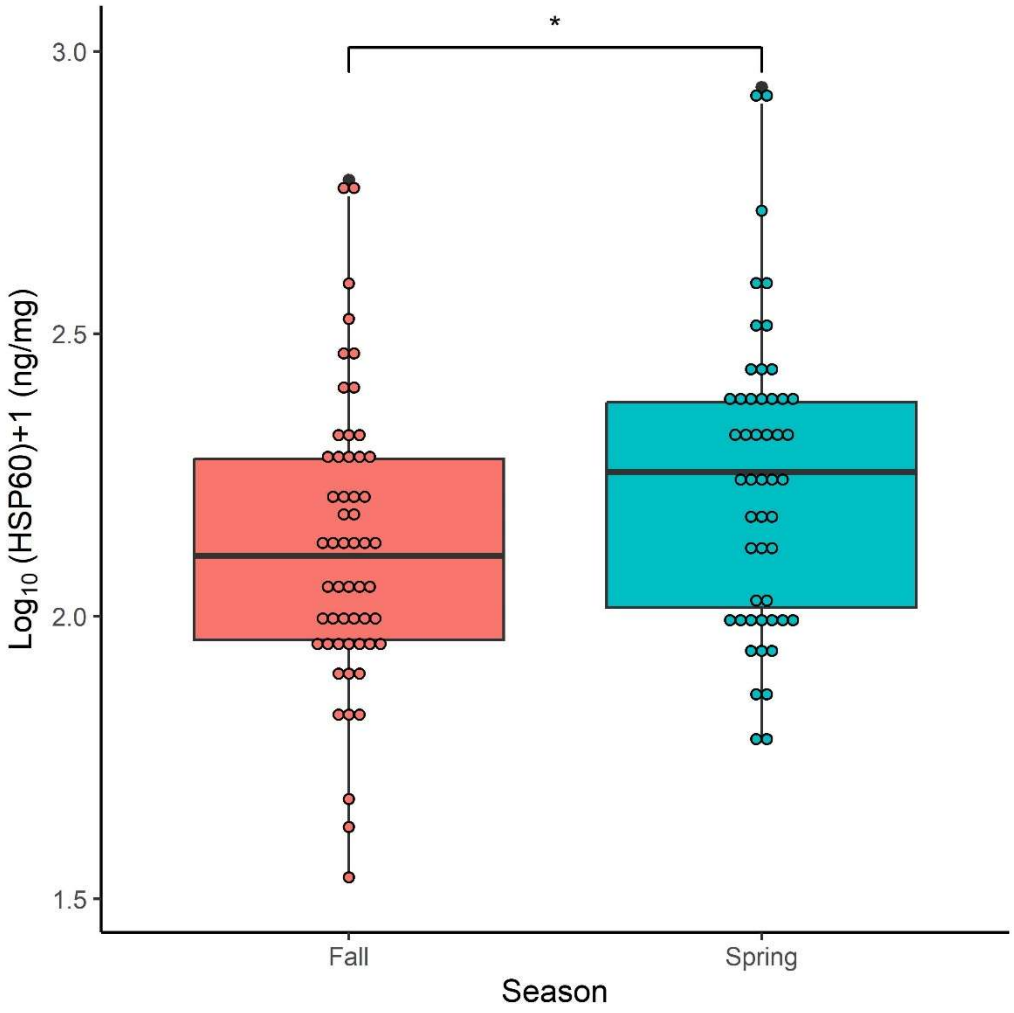

Figure 2.4. $Log_{10}(x) + 1$ transformed heat shock protein 60 (HSP60) concentrations in thrushes sampled in fall (n = 55) vs. spring (n = 50) migration. Each data point represents the HSP60 concentration of a sample. Lower and upper fences of box plots are the 25th and 75th percentiles, and the median is in between. Vertical extending lines represent the most extreme values within 1.5 interquartile range of the 25th and 75th percentiles. * indicates that there was a statistically significant difference in HSP60 ($P < 0.05$) between groups.

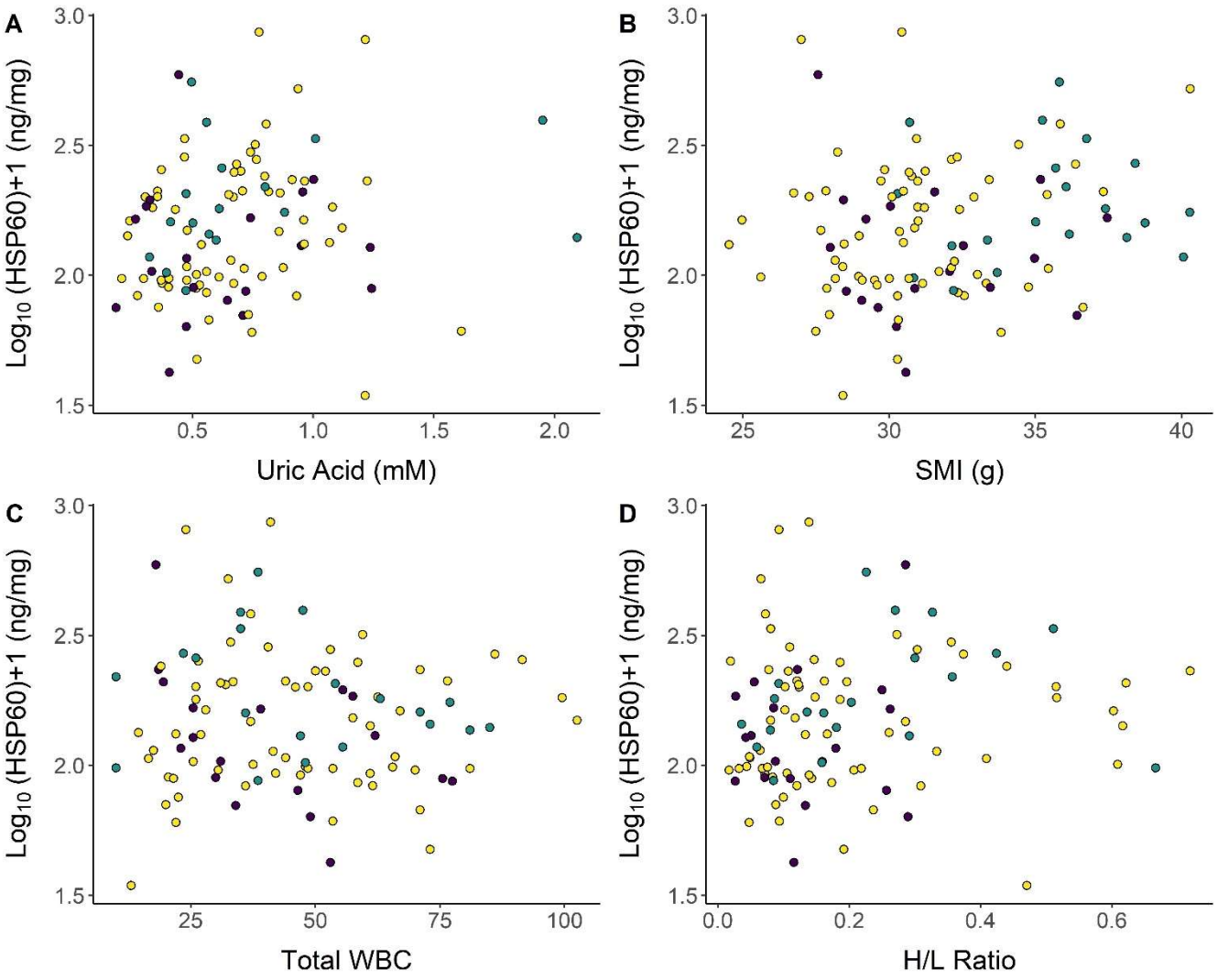

Figure 2.5. Relationships between $\log_{10}(x) + 1$ transformed heat shock protein 60 (HSP60) concentration and plasma uric acid concentrations (A), scaled mass index (SMI; B), the total white blood cell (WBC) count (C), and the H/L ratio (D). Species represented include Gray-cheeked Thrush (purple), Hermit Thrush (blue), and Swainson's Thrush (yellow).

TABLES

Table 2.1. Key findings from studies that measured heat shock protein 60 (HSP60) in free-living birds.

Reference	Species	Life Stage	Significant Finding(s)/Conclusion
(Moreno et al. 2002)	Pied Flycatcher	Nestlings	HSP60 was associated with food restriction and sibling competition.
(Martinez-Padilla et al. 2004)	Eurasian Kestrel	Nestlings	Smaller nestlings had higher HSP60 levels than larger nestlings suggesting HSP60 could indicate competition-related stress.
(Morales et al. 2006)	Pied Flycatcher	Breeding	Higher HSP60 levels were associated with lower humoral immunity.
(Lobato et al. 2010)	Pied Flycatcher	**Migration**	HSP60 was positively correlated with corticosterone; migratory flight may promote increased HSP production.
(Herring et al. 2011)	White Ibis	Nestlings	HSP60 was elevated in a year of reduced food availability.

Table 2.2. Average (mean ± SE) seasonal values for physiological variables in three species of *Catharus* thrushes (n = 105) sampled during spring migration (2022 and 2023) and fall migration (2022).

Species	Spring Migration (n)	Fall Migration (n)
Swainson's Thrush		
Body Condition (g)	32.02 ± 0.53 (37)	29.30 ± 0.35 (29)
Uric Acid (mM)	0.74 ± 0.05 (37)	0.57 ± 0.05 (28)
H/L Ratio[a]	0.18 ± 0.03 (37)	0.24 ± 0.03 (29)
Total WBC (/10,000 RBCs)[b]	44.19 ± 3.53 (37)	47.78 ± 4.04 (29)
HSP60 (ng/mg)	22.17 ± 2.94 (37)	13.52 ± 1.28 (29)
Gray-cheeked Thrush		
Body Condition (g)	33.70 ± 1.35 (6)	30.28 ± 0.58 (13)
Uric Acid (mM)	0.74 ± 0.10 (6)	0.57 ± 0.10 (13)
H/L Ratio	0.08 ± 0.02 (6)	0.16 ± 0.03 (12)
Total WBC (/10,000 RBCs)	41.42 ± 9.43 (6)	41.00 ± 5.22 (12)
HSP60 (ng/mg)	13.20 ± 2.47 (6)	15.62 ± 3.92 (13)
Hermit Thrush		
Body Condition (g)	35.50 ± 1.42 (7)	35.27 ± 0.77 (13)
Uric Acid (mM)	0.94 ± 0.26 (5)	0.67 ± 0.14 (12)
H/L Ratio	0.30 ± 0.08 (7)	0.20 ± 0.04 (13)
Total WBC (/10,000 RBCs)	40.71 ± 10.02 (7)	51.50 ± 5.46 (13)
HSP60 (ng/mg)	22.06 ± 3.51 (7)	20.90 ± 3.84 (13)

[a]Ratio of heterophils to lymphocytes
[b]White blood cells per 10,000 red blood cells