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Research Paper

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

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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 66 birds; however, there was a greater haemosporidian prevalence in Black-throated Blue Warblers relative to Canada Warblers. 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.

Hémosporidies chez la Paruline du Canada (*Cardellina canadensis*) et la Paruline bleue (*Setophaga caerulescens*) : prévalence, diversité et liens avec l'état physiologique pendant la migration

RÉSUMÉ. Le paludisme aviaire (*Plasmodium* sp.) et les hémosporidies apparentées (*Leucocytozoon* sp. et *Haemoproteus* sp.) sont largement répandus chez les oiseaux, mais les études portant sur la prévalence et la diversité des hémosporidies font défaut pour de nombreux migrateurs néotropicaux et néarctiques. De plus, l'impact que les infections peuvent avoir sur l'état ou le comportement des oiseaux pendant la migration est encore mal compris. Dans la présente étude, nous avons utilisé la PCR nichée et le séquençage d'ADN pour déterminer la prévalence des parasites et les lignées génétiques d'hémosporidies chez la Paruline du Canada (*Cardellina canadensis*) et la Paruline bleue (*Setophaga caerulescens*) pendant leur halte migratoire sur la rive sud du lac Ontario. Nous avons également évalué si les infections aux hémosporidies étaient liées à l'état immunitaire – évalué au nombre total de globules blancs (GB) et au ratio hétérophiles/lymphocytes (H/L) – ou aux habitudes de ravitaillement et/ou au moment de la migration. Nous avons trouvé une prévalence d'hémosporidies de 51,5 % chez 66 oiseaux; toutefois, la prévalence d'hémosporidies était plus élevée chez la Paruline bleue que chez la Paruline du Canada. Nous avons détecté 15 lignées de cytochrome *b* de parasites, dont 4 nouvelles lignées. Chez les Parulines bleues, les oiseaux infectés avaient un nombre total de GB significativement plus élevé que les oiseaux non infectés. Toutes espèces confondues, les femelles avaient des ratios H/L plus faibles lorsqu'elles étaient infectées, alors que la tendance inverse était observée chez les mâles. Les métabolites plasmatiques (triglycérides, β-hydroxybutyrate, acide urique) n'étaient pas associés à l'état d'infection, et l'infection n'était pas liée à la date d'arrivée à la halte migratoire printanière. Nos résultats offrent un aperçu de la diversité des hémosporidies qui infectent deux espèces de parulines migratrices et illustrent le fait que les infections par des parasites sanguins représentent un défi récurrent auquel ces oiseaux sont confrontés pendant la migration. Bien que les infections par les hémosporidies puissent être liées à une réponse immunitaire accrue dans certains scénarios, les résultats de cette étude corrélative ne fournissent aucune preuve que des compromis physiologiques ou comportementaux opèrent à la suite d'infections chez les Parulines du Canada et les Parulines bleues.

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

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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 because of 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 4000 unique cytochrome *b* lineages of parasites that have been identified using molecular techniques (Bensch et al. 2009). 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 and 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). 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 because of 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 because of 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 polymerase chain reaction (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 eight 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 the health and immunocompetence of an individual, and it could be associated with ongoing disease processes (Owen and Moore 2006). 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%. Because of 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 association of haemosporidian infections with 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 23 May (2021: 27 May; 2022: 22 May; 2023: 24 May), while median day of capture for Black-throated Blue Warblers was 19 May (2022: 19 May; 2023: 18 May).

Haemosporidian prevalence

Haemosporidian parasites were detected in 51.5% of 66 warblers in this study. *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, $P = 0.007$; Fig. 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) 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 four lineages had at least one fixed difference from any sequence in MalAvi and were identified as novel (Table 1). We detected eight lineages in

Fig. 1. Infection prevalence (%) for three genera of haemosporidians (*Plasmodium*, *Haemoproteus*, and *Leucocytozoon*) in Canada Warblers (*Cardellina canadensis*; n = 32) and Black-throated Blue Warblers (*Setophaga caerulescens*; n = 34). 'Mixed' denotes a single bird was infected with multiple parasite genera. BTBW = Black-throated Blue Warbler; CAWA = Canada Warbler.

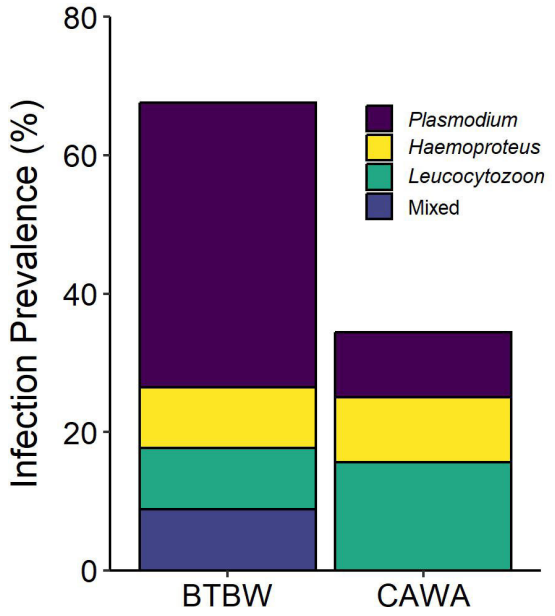


Table 1. Haemosporidian cytochrome *b* lineages detected in Canada Warblers (*Cardellina canadensis*) and Black-throated Blue Warblers (*Setophaga caerulescens*). Lineages were identified via comparison to previously identified parasite lineages in the MalAvi database.

	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.

Canada Warblers, whereas nine lineages were identified in Black-throated Blue Warblers. GEOTRI09 was the most common lineage (eight 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 relationship between infection and migration timing was not influenced 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: $F_{1,60} < 0.1$, $P = 0.90$). In fact, mean day of capture was slightly but not significantly earlier for infected birds relative to uninfected birds in both Canada Warblers (Infected: 22 May ± 2 days; Uninfected: 23 May ± 1 day) and Black-throated Blue Warblers (Infected: 17 May ± 2 days; Uninfected: 21 May ± 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 2) in a model predicting total WBC counts. Infection did not relate to total WBC counts in Canada Warblers ($t_{30} = -1.3$, $P = 0.21$; Fig. 2A). For Black-throated Blue Warblers, total WBC counts were significantly higher in infected birds relative to uninfected birds ($t_{32} = 3.0$, $P = 0.005$; Fig. 2B). Infection status on its own was not associated with the H/L ratio; however, there was a significant sex × infection interaction (Table 2). Infected females had significantly lower H/L ratios than uninfected females ($t_{35} = -3.0$, $P = 0.004$; Fig. 3A), whereas there was a non-significant trend toward higher H/L ratios in infected males compared to uninfected males ($t_{27} = 1.5$, $P = 0.13$; Fig. 3B).

Table 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 (*Cardellina canadensis*) and Black-throated Blue Warblers (*Setophaga caerulescens*). 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

Fig. 2. Total white blood cell (WBC) counts (mean ± SE) by haemosporidian infection status for Canada Warblers (*Cardellina canadensis*; A) and Black-throated Blue Warblers (*Setophaga caerulescens*; 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).

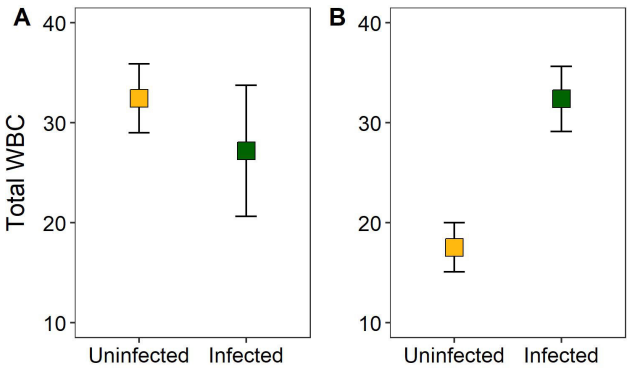
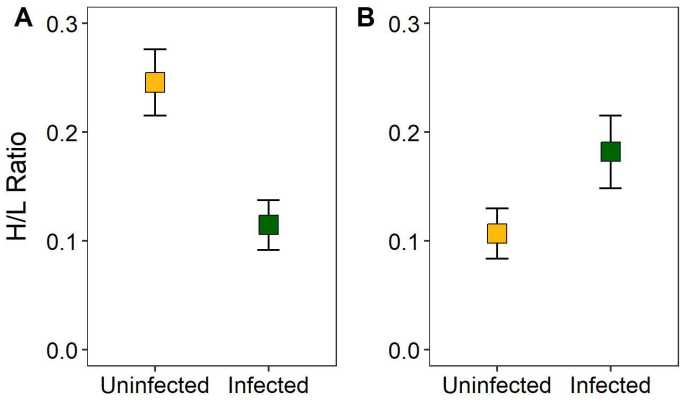


Fig. 3. Heterophil/lymphocyte (H/L) ratios (mean ± SE) by haemosporidian infection status for female birds (A) and male birds (B). Data represent both Canada Warblers (*Cardellina canadensis*) and Black-throated Blue Warblers (*Setophaga caerulescens*). Untransformed H/L ratios are presented though transformed 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).



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. 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 3). Important covariates from the top-ranked models were included in respective GLMs for each of the three metabolites (Table 4). Haemosporidian infection status was not significantly related to any plasma metabolites (Table 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). Although the results of this study are largely correlative, we provide insight into the implications of the 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 routinely carry haemosporidians (Greiner et al. 1975). Furthermore, results agree with prior studies that have

Table 3. Model selection results for top-ranked models that explain variation in plasma metabolites (triglyceride, uric acid, β-hydroxybutyrate) measured in Canada Warblers (*Cardellina canadensis*) and Black-throated Blue Warblers (*Setophaga caerulescens*). Models with $\Delta AIC < 2$ 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 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 [*Cardellina canadensis*] vs. Black-throated Blue Warbler [*Setophaga caerulescens*]), 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.

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 because of 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 3000 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 haemosporidians (five *Plasmodium*, four *Haemoproteus*, six *Leucocytozoon*), and four 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, De Angeli Dutra et al. 2021, DeBrock 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 (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 because 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., *personal observation*), 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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Data Availability:

Sequencing data for novel haemosporidian lineages are available in MalAvi and NCBI GenBank (accession numbers: OR817924, OR817925, OR817927, OR817928). All other data used in this manuscript are available upon reasonable request.

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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.