Research Article

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Leukocyte profiles vary with breeding latitude and infection status in a seasonally sympatric songbird

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Abstract: Billions of animals migrate annually in pursuit of food, safety, and reproduction. Long-distance migration can be energetically expensive, which can force trade-offs with investment in other physiological systems (e.g., suppressing immunity). Understanding the physiological impacts of migration is important to predict when and where such animals may be vulnerable to parasites and how changes to migration might affect infectious disease risks. To isolate relationships between migration and physiology from energetic tradeoffs and hormonal shifts associated with spring reproduction, we assessed differences in leukocyte profiles between seasonally sympatric resident and recently arrived fall migrant dark-eyed juncos (*Junco hyemalis*) in the Appalachian Mountains. When examining heterophil:lymphocyte (HL) ratios, which can elevate for long durations after even mild stressors, we found weak associations with migratory strategy (resident or migrant subspecies). In contrast, feather δ^2H values showed that HL ratios were highest for juncos breeding at more northern latitudes, and this relationship was strongest for birds that arrived at the overwintering site infected with haemosporidian parasites (*Plasmodium* and *Haemoproteus* spp.). These patterns were more pronounced and better indicated hematological responses to stressors when using multivariate analyses. Our findings suggest that short- and long-distance migration may be more energetically costly in the presence of infection and highlight how approximating breeding latitude and using multivariate analyses can help understand host physiology.

Keywords: white blood cells; ecoimmunology; hydrogen isotopes; migration; compositional data; *Junco hyemalis*; avian malaria; *Borrelia burgdorferi*

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1 Introduction

Each year, billions of animals migrate between their breeding and overwintering sites to track predictable changes in resources and habitats (e.g., foraging and breeding opportunities) [1, 2]. Migratory journeys can span thousands of kilometers and take several weeks to complete; for example, many birds undertake one-way migratory distances of over 10,000 kilometers [3], with arctic terns (*Sterna paradisaea*) migrating over 80,000 kilometers on an annual basis [4]. Long-distance migrations can require large energy expenditures [5, 6], with birds investing up to 50% of body mass in fat reserves for flight [7]. Migration and its carry-over effects can thus force energetic trade-offs and have negative effects on host immunity [8, 9], which could make migrants more susceptible to infection or relapse and shape the extent to which they transmit parasites [10, 11]. Understanding the physiological correlates of migratory activity is especially important in the context of predicting how environmental changes that alter migrations, such as urbanization and climate shifts, will alter wildlife health and pathogen spillover risks [12, 13].

Links between migration and physiology have been best assessed in birds [14, 15]. Many passerine species sampled in fall migration have low circulating plasma corticosterone (CORT, the main avian glucocorticoid) [16,

17]. In other cases, CORT is elevated in birds preparing to migrate, during the course of migration, or following migration. For example, bar-tailed godwits (*Limosa lapponica*) sampled when arriving at an initial stopover site during fall migration had higher plasma CORT than those sampled at a refueling site [18], and white-crowned sparrows (*Zonotrichia leucophrys*) with stimulated migratory restlessness also had elevated CORT [19]. However, CORT levels are typically only mildly elevated to facilitate energy mobilization, support development of migratory condition, and play a regulatory role during migration, rather than reflecting levels associated with highly stressful and life-threatening conditions [20–22].

In contrast, physiological studies have increasingly adopted leukocyte profiles to gain additional insights into how birds are affected by migratory activity [23–26]. Elevated glucocorticoids stimulate an influx of heterophils from bone marrow to blood while redistributing lymphocytes from blood to other tissue [27]; these shifts increase the ratio of heterophils to lymphocytes (i.e., HL ratios) in blood [28]. Whereas baseline CORT is altered within minutes by the acute stress of sampling [29], stress-induced changes in leukocytes occur more slowly (e.g., HL ratios can remain unchanged during a stressor and only begin to elevate hours after treatment) [30]. Leukocyte profiles can also shift in response to mild stressors, and these shifts can persist over long timescales [31]. For example, Eurasian kestrel (*Falco tinnunculus*) nestlings elevated HL ratios and CORT in response to starvation, but only HL ratios increased with less-severe stressors such as poor weather [32]. These HL ratios also remained elevated in CORT-implanted nestlings for a week after CORT returned to baseline [32], and long-term elevations in HL ratios have been supported by a comprehensive meta-analysis [33].

Accordingly, HL ratios could remain elevated following migration owing to the high energy expenditures, nutritional stress, and predation risks of these long-distance journeys [34]. However, associations between leukocyte profiles and bird migration have been inconsistent. Swainson's thrushes (*Catharus ustulatus*) sampled during fall and spring migration had higher HL ratios than those sampled inr their breeding season [35], and common eiders (*Somateria mollissima*) that migrated further to their breeding site had higher HL ratios [24]. However, skylarks (*Alauda arvensis*) sampled across their annual cycle showed no temporal variation in HL ratios [26], and migratory distance did not predict HL ratios in a community of shorebirds during their fall migration [25]. A limitation of such work may be the lack of comparison with sympatric non-migrants (i.e., residents). One such comparison of Neotropical flycatchers (*Elaenia chiriquensis* and *E. cristata*) found that the migratory species had lower HL ratios than the sympatric resident species during the breeding season [36]. However, comparisons between sympatric breeding residents and migrants may be confounded by energetic costs and hormonal shifts associated with reproduction [23, 37]. Studies of sympatric migratory and resident birds early in the non-breeding season could thus allow isolating the relationships between recent migration (i.e., carry-over effects) on leukocyte profiles independent of breeding physiology.

To test how migratory behavior is associated with leukocyte profiles, we focus on the dark-eyed junco (*Junco hyemalis*), a temperate songbird with diverse migratory strategies across subspecies in North America [38]. Migratory slate-colored juncos (*J. h. hyemalis*) breed across Alaska, Canada, and the northern United States, migrating south in autumn and overwintering throughout the central and eastern United States (Fig. 1A). At the southern edge of this breeding range, in the Appalachian Mountains, resident slate-colored juncos (*J. h. carolinensis*) are sympatric with migrants overwintering from early fall through early spring, when *J. h. hyemalis* initiate northern migration and residents transition into breeding [39]. During seasonal sympatry, these subspecies forage in mixed flocks and are exposed to similar environmental conditions [40]. Sympatry following fall migration thus allows assessing whether recent migration is associated with differences in leukocyte profiles without confounding by tradeoffs with reproduction in residents. Following prior work in this system [39, 41], we refer to subspecies according to their migratory strategy (i.e., *J. h. hyemalis* as migrants and *J. h. carolinensis* as residents); however, migrants were only sampled after their fall migration.

We first tested whether sympatric migrants and residents differed in leukocyte profiles. Anticipating that stable isotopes of hydrogen in junco feathers could be a proxy for distance from the breeding grounds [42], we also asked whether variation in breeding latitude (coarsely approximating migratory distance) could explain individual differences in junco leukocyte profiles. We used not only HL ratios but also multivariate analyses to test these relationships, as shifts in the proportions of eosinophils and basophils can additionally shift in response to stressors [43, 44]. If fall migration carries mild or pronounced costs, we would predict that juncos breeding further from the wintering site show elevated HL ratios alongside fewer eosinophils. However, such patterns could be absent if southern migrations impose little to no energetic or nutritional costs. One alternative

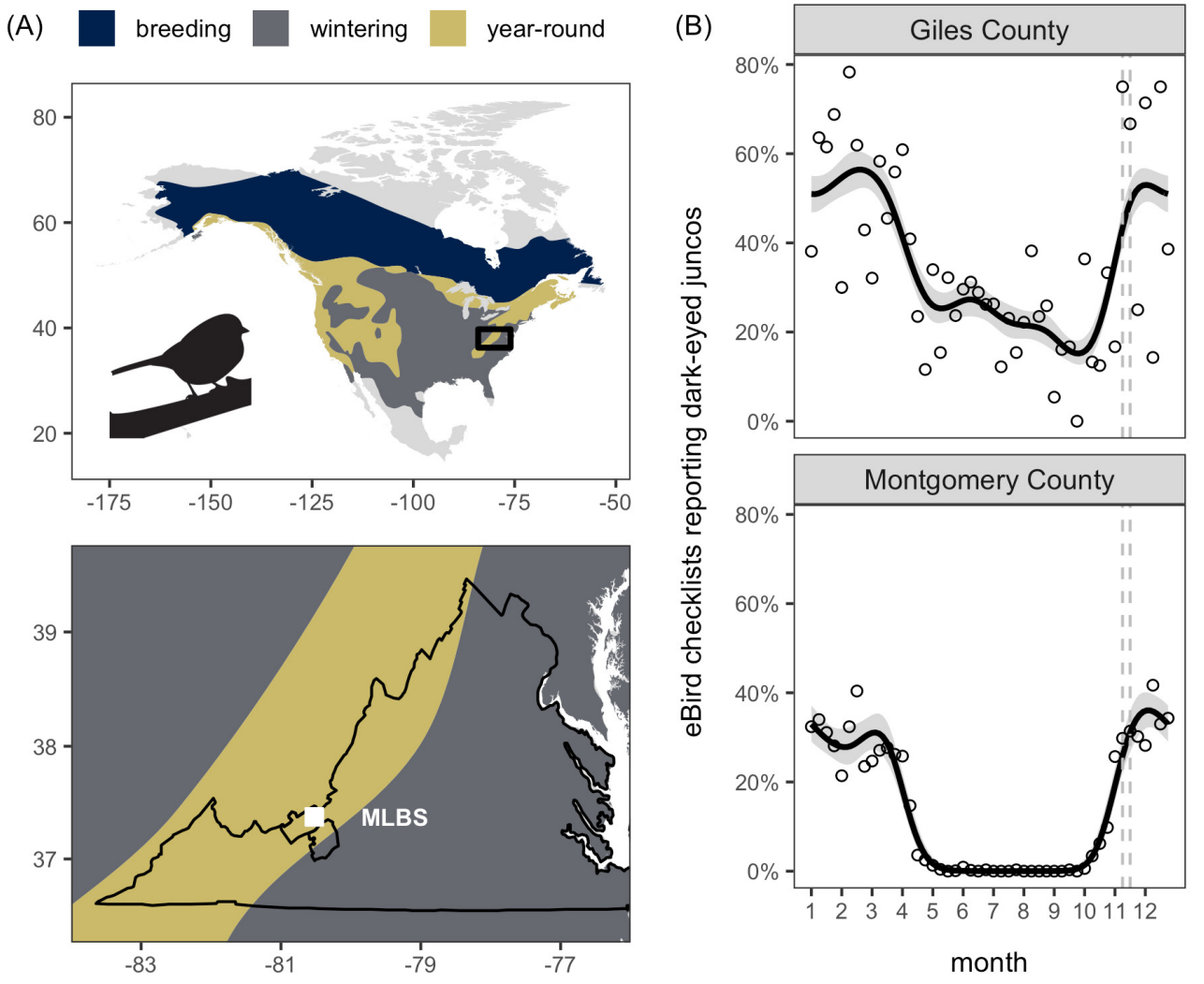

Fig. 1. (A) Geographic range of the dark-eyed junco stratified by breeding, wintering, and year-round distributions in relation to the study site at the Mountain Lake Biological Station (MLBS), Virginia, USA; Giles County and adjacent Montgomery County are also shown. Here, residents (*J. h. carolinensis*) mix with overwintering migrants (*J. h. hyemalis*) in the non-breeding season. The range shapefile is from BirdLife International and the International Union for Conservation of Nature [91]. The junco silhouette is from the Cornell Laboratory of Ornithology (https://www.allaboutbirds.org). (B) The proportion of eBird checklists reporting juncos was modeled across the annual cycle using a generalized additive model fit using the *mgcv* package in R, binomial errors, and a logit link. The model included Virginia county, a cyclic cubic spline for weeks, and their interaction [92]. Upper and lower bounds of the shaded regions represent 95% confidence intervals, with thick lines showing the mean predictions; points indicate raw eBird data. Results suggest that most migrant arrival occurs from mid-October to mid-November, overlapping with our sampling window (indicated by dashed lines).

explanation for this lack of difference could involve blood parasites. For example, past work showing similar HL ratios between passerines sampled during fall migration and in the breeding season could be explained by absent blood parasite infections [23], which have been associated with poorer condition, later timing of migration, and prolonged stopover durations [45–49]. In several songbird systems, including juncos, migrants have lower blood parasite prevalence than residents [41, 50, 51], supporting the idea that infection can be costly during migration [11]. We therefore assessed infection to control for blood parasitism in our analyses. However, we also predicted that leukocyte profiles could be especially indicative of recent stressors in the migratory subspecies when infections are present.

2 Methods

2.1 Study timing and junco sampling

From 7 to 11 November 2018, we sampled migrant and resident juncos using mist nets and seed bait at the University of Virginia Mountain Lake Biological Station in Pembroke, Virginia, United States (Fig. 1A). Although we cannot determine the arrival date of migrants without tracking technologies (e.g., light-level geolocators, large-scale radio-telemetry arrays) [52], general temporal trends from eBird checklists in Giles County and adjacent Montgomery County suggest most migrant juncos arrive from mid-October to mid-November (Fig. 1B) [53]. Similarly, prior work on migrant arrival at a similar latitude in Indiana suggests peak autumn arrivals occur within this timeframe [54]. Our sampling window, although narrow, therefore likely captured overwintering *J. h. hyemalis* with arrival times ranging from days to several weeks, for which hematological shifts stemming from migration could still be evident [31].

Each bird was fitted with an aluminum U.S. Fish & Wildlife Service leg band if not previously banded. We used plumage and bill color to identify migratory and resident subspecies as described previously [38]. Recaptured birds were confirmed as residents if banded in a prior breeding season. We were unable to classify subspecies for seven birds, which were excluded from analyses of migratory strategy. For all remaining juncos, we collected the first secondary feather on the right wing for stable isotopes of hydrogen (δ^2H values) and stored these in envelopes at room temperature. We determined age class from wing plumage and iris color and sex with wing chord length [38]. We determined mass to the nearest gram using a Pesola scale to estimate body condition through residuals of a linear model regressing wing length on mass [55]. We obtained 50–70 µL of blood by pricking the brachial vein with a sterile 26G needle, followed by collection with heparinized capillary tubes. Thin blood smears were prepared on glass slides and stained with Wright–Giemsa (Quick III, Astral Diagnostics). All birds were bled within at most two hours of capture to minimize impacts on differential leukocyte counts [43, 56], although 80% of birds were bled within one hour. Blood not used for smears was preserved in 1000 uL of Longmire's solution and stored at 4°C until DNA extraction for parasite diagnostics.

All avian sampling procedures were approved by the Indiana University Institutional Animal Care and Use Committee (18-030) and conducted under scientific collecting permits issued by the Virginia Department of Game and Inland Fisheries (064150) and the U.S. Fish and Wildlife Service (MB093279).

2.2 Feather hydrogen isotopes

Because adult juncos complete their annual molt cycle before leaving breeding habitats and juveniles retain wing feathers during their first annual cycle [38], feather δ^2H values can not only reflect migratory strategy but also reveal additional variation in approximate breeding latitude based on isotopic variation in precipitation [42, 57]. Following typical feather cleaning procedures (e.g., [39]), feathers from 59 juncos were washed of oils using a 2:1 chloroform:methanol solution and allowed to air dry in a fume hood [39]. Two replicates of ~0.2 mg tissue were clipped from the distal end of each feather and loaded into silver capsules for hydrogen analysis. Because a fraction of hydrogen atoms in feather tissue can readily exchange with atmospheric water vapor of unknown composition [58], we controlled for this effect by forcing isotopic equilibration with a water vapor of known isotopic composition in a flow-through chamber system at 115 °C [59]. Equilibrated feather samples were then analyzed for hydrogen isotopic composition using a thermal conversion elemental analyzer coupled with a ThermoFinnigan Delta Plus XP isotope ratio mass spectrometer at the Indiana University Stable Isotope Research Facility. δ^2H values are reported in standard per mil notation (‰) relative to Vienna Standard Mean Ocean Water using two reference materials: USGS77 (polyethylene powder) and hexatriacontane 2 (C_{36} n-alkane 2). Analytical precision was ±3 ‰ for δ^2H values. We then calculated the isotopic composition of each feather's non-exchangeable hydrogen [60]; means of these corrected values per two replicates were used in further analyses. We used a linear model in R to test how feather δ^2H values varied with migratory strategy (n=53) [61].

2.3 Blood parasites

We extracted genomic DNA from 100 µL blood and Longmire's solution mixture using RSC Whole Blood DNA Kits (Maxwell) run on a Maxwell RSC at Indiana University; DNA was eluted into 70 µL of elution buffer. We used nested PCR to assess infection with two groups of blood parasites previously detected in juncos: *Borrelia burgdorferi* [62] and haemosporidians (*Plasmodium* and *Haemoproteus* spp.) [41, 63]. Both are transmitted in spring and summer by arthropod vectors present at MLBS (Table S1). Whereas haemosporidians can be transmitted in winter in some

contexts (e.g., [64]), their vectors were likely not active during our sampling (Table S1). Nested PCR is highly sensitive, and PCR positivity for both parasite groups in November could indicate either chronic or relapsed infections [65, 66]. Using previously published primers and protocols (2–3 µL of template per primary reaction), we targeted the *flaB* gene for *B. burgdorferi* sensu lato [67] and the cytochrome *b* gene for haemosporidians [68, 69].

For haemosporidians, acute infections display high parasitemia, whereas chronic or relapsed infections show very low intensity [70]. To differentiate chronic from acute infections, we complemented PCR with analyzing blood smears for evidence of infection in peripheral blood. One observer (DJB) screened approximately 100 fields of view at 1000X magnification (oil immersion) for *Plasmodium* spp. and *Haemoproteus* spp.; we also noted infection with larger parasites previously found in juncos (*Leucocytozoon* spp. and *Trypanosoma* spp. [63, 71]). PCR-positive birds without visible infection in peripheral blood can indicate chronic infection [66].

We used a Chi-squared test with the *p* value generated via a Monte Carlo procedure with 1000 simulations to assess whether PCR positivity for haemosporidians was associated with blood smear positivity. We then used the *prevalence* package to derive infection prevalences and 95% confidence intervals. We first used two separate GLMs with binomial errors and a logit link to assess whether the odds of infection varied by migratory strategy and breeding latitude. We then repeated these analyses using GLMs with Poisson errors and a log link for intensity data.

2.4 Leukocyte profiles

We recorded the percentage of heterophils, lymphocytes, monocytes, eosinophils, and basophils by counting 100 leukocytes at 1000X magnification (oil immersion) from blood smears [72]. One observer (DJB) performed all differential white blood cell counts.

Differential white blood cell data are compositional: changes in the proportion of one leukocyte type correspond to changes in each of the other four leukocyte types [73]. As analyses of compositional data deal with logratio transformations [74], we treated zeros as values below the detection limit and replaced them with 50% of the bounds for detection (i.e., 0.5%) [75].

We first derived HL ratios and used a Grubbs test to assess outliers [76]. We next used two linear models with $\log_{10}$-transformed HL ratios as the response variable and either migratory strategy or breeding latitude (δ^2H values) as the primary predictor variable. For each model, we statistically adjusted for sex, age, and body condition and considered the interaction between migratory phenotype and blood parasite infection. Owing to missing values for some predictors, these models were fit to reduced datasets (n=53 and n=49 for migratory strategy and δ^2H values).

As the relative abundances of leukocytes such as eosinophils and basophils can also shift in response to stressors [30, 43, 44], we next used a multivariate approach to holistically analyze our compositional leukocyte data. We used two separate permutational multivariate analyses of variance (PERMANOVAs) with the *vegan* package to quantify the proportion of variance in leukocyte composition explained by the same predictor variables as in our analyses of HL ratios [77, 78]. Both PERMANOVAs used Euclidean distances on the isometric logratio (ILR)–transformed relative leukocyte abundances [79]. We visualized differences using a robust principal components analysis (PCA) with the *robCompositions* package [80, 81].

While sampling within 1–2 hours of capture should minimize effects of acute stress on differential leukocyte counts [43, 56], differences in time between capture and blood collection (i.e., handling time) could introduce additional variation. We thus assessed the sensitivity of our results by including handling time as a covariate in separate linear models and PERMANOVAs.

3 Results

3.1 Migratory strategy and hydrogen isotopes

We sampled 36 residents, 27 migrants, and seven unclassified juncos. Residents had higher feather δ^2H values than migrants ($F_{1,51}$=105, $R2$=0.67, p<0.001; Fig. S1). Resident feather δ^2H values ranged from –71 to –35‰ ($\bar{x}$=–49, SD=8) while migrant feather δ^2H values ranged from –108 to –55‰ ($\bar{x}$=–83, SD=15). Feather δ^2H values in migrants were approximately twice as variable than those in residents, suggesting correspondingly broad variation in breeding latitude.

3.2 Blood parasite prevalence and intensity

PCR targeting the haemosporidian cytochrome *b* gene showed that 50% of juncos had haemosporidian parasites in blood (32/64, 95% CI: 38–62%). Residents and overwintering migrants did not differ in their odds of haemosporidian infection (OR=1.75, p=0.29; Fig. S2A),

and infection status did not vary with breeding latitude (feather δ^2H values; OR=1.00, p=0.76; Fig. S2B). No juncos displayed PCR evidence of *B. burgdorferi* infection (i.e., the *flaB* gene).

In contrast to the PCR results, analysis of blood smears showed low prevalence of visible haemosporidian infection (9/70, 95% CI: 7–23%), for which we only detected *Plasmodium* spp. and *Haemoproteus* spp. However, PCR results and blood smear results were strongly associated ($\chi 2$=5.77, p=0.02); microscopy-positive infections were only detected in PCR-positive birds, and no PCR-negative birds had visible haemosporidian infection (Fig. S3). Intensity was greater in residents ($\chi 2$=181, df=1, p<0.001; Fig. S4A), but removing a high-intensity outlier (G=8.24, U<0.01, p<0.001) minimized this relationship ($\chi 2$=1.83, df=1, p=0.18). Intensity did not vary with feather δ^2H values ($\chi 2$=1.66, df=1, p=0.20; Fig. S4B). Most microscopy-visible infections were of low intensity; excluding microscopy-negative birds resulted in a median intensity of 2 infected erythrocytes in 100 fields of view (ranging from 1–180 infected erythrocytes). This suggests that most PCR-positive juncos had chronic infections [66]. Owing to the high zero inflation of our haemosporidian intensity data, we only used PCR data in our subsequent analyses of leukocyte profiles (with infection here referring to haemosporidian parasites).

3.3 Analysis of HL ratios

Junco HL ratios varied from 0.005 to 2.12 ($\bar{x}$=0.14, SD=0.28), and a Grubbs test on the $\log_{10}$-transformed values detected no outliers (G=2.82, U=0.88, p=0.27). In our first model, HL ratios were only weakly related to migratory strategy after accounting for sex, age, and body condition (F1,46=2.94, p=0.09), and we observed no interaction with infection status (Table 1). Migrants had weakly higher HL ratios than residents (β=0.19), irrespective of infection status (Fig. 2A).

In our second model with feather δ^2H values replacing migratory strategy, HL ratios were strongly related to breeding latitude (F1,42=5.28, p=0.03), the effect of which was marginally dependent upon infection status after accounting for sex, age, and body condition (F1,42=3.13, p=0.08; Table 1). Uninfected juncos showed no relationship between feather δ^2H values and HL ratios (β=−0.005, 95% CI: −0.01 to 0.005). However, HL ratios were negatively associated with feather δ^2H values for PCR-positive juncos (β=−0.02, 95% CI: −0.03 to −0.005), suggesting that HL ratios were highest for birds breeding further from the overwintering site (Fig. 2B). These patterns were more pronounced when handling time was included in the model (Table S2).

Table 1. ANOVA table for linear models predicting $\log_{10}$-transformed HL ratios as a function of either migratory strategy or feather δ^2H values, infection status, and the interaction with migratory phenotype, alongside adjustment for sex, age, and body condition. See Table S2 for equivalent models that also account for the time between capture and blood collection.

Model 1 (*n*=53): *R2*=0.04	*df*	*F*	*p*
Migratory strategy	1	2.94	0.09
Haemosporidian infection	1	1.17	0.29
Migratory strategy * haemosporidian infection	1	1.82	0.18
Sex	1	0.30	0.59
Age	1	0.63	0.44
Body condition	1	0.17	0.68
Residuals	46		
Model 2 (*n*=49): *R2*=0.11	*df*	*F*	*p*
Feather δ^2H values	1	5.28	0.03
Haemosporidian infection	1	3.08	0.09
Feather δ^2H values * haemosporidian infection	1	3.13	0.08
Sex	1	1.50	0.23
Age	1	<0.01	0.97
Body condition	1	1.96	0.17
Residuals	42		

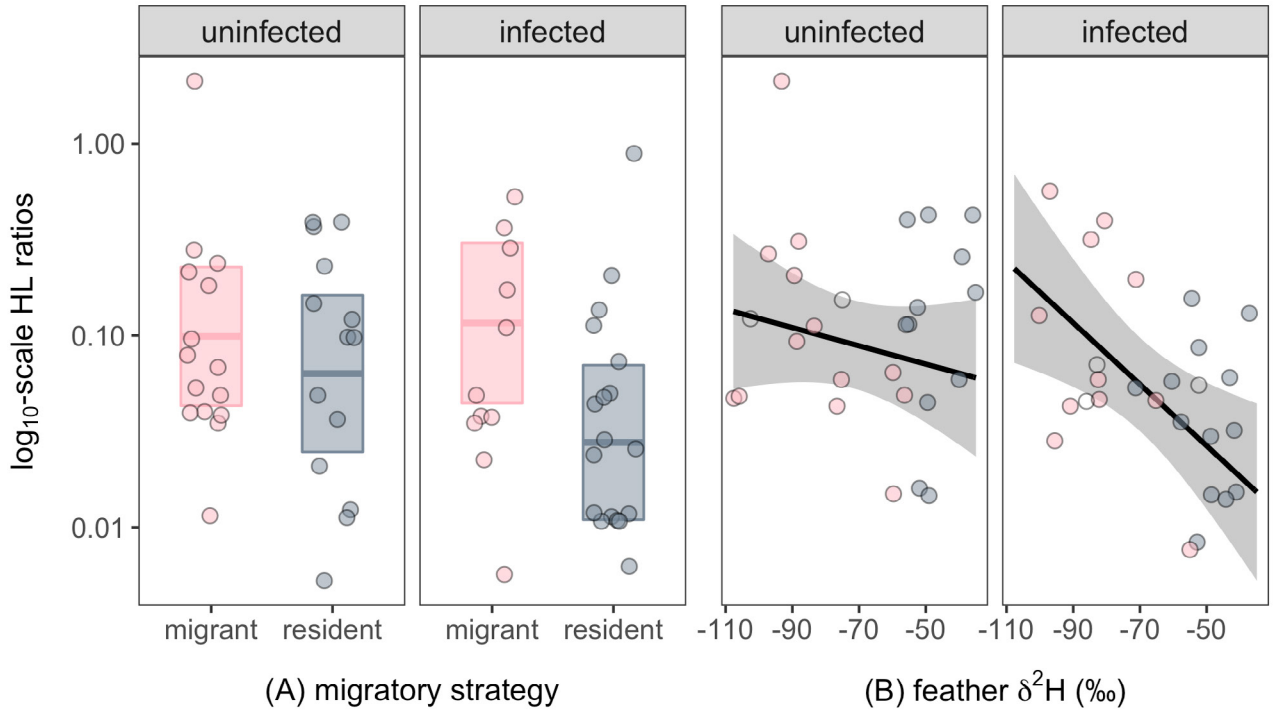

Fig. 2. Modeled relationships between $\log_{10}$-transformed HL ratios and (A) migratory strategy or (B) feather δ^2H values. All models included an interaction with haemosporidian infection status and adjusted for sex, age, and body condition. Upper and lower bounds represent 95% confidence intervals, with thick lines showing the mean predictions. Points are colored by migratory strategy, with empty circles showing juncos with equivocal subspecies assignments.

3.4 Compositional leukocyte profiles

The first two principal components (PC1 and PC2) explained 75% of the variation in junco leukocyte composition. PC1 loaded positively by eosinophils (0.74) and heterophils (0.29) and negatively by lymphocytes (−0.38), monocytes (−0.35), and basophils (−0.31). PC2 loaded positively primarily by heterophils (0.83) and weakly by basophils (<0.01) and negatively by eosinophils (−0.50), lymphocytes (−0.19), and monocytes (−0.15). Movement from the southwestern to the northeast quadrant of PC space largely indicates hematological shifts in relation to stressors (e.g., heterophilia, lymphopenia, and eosinopenia) [30, 43, 44].

PERMANOVAs including migratory strategy or feather δ^2H values showed that the interaction between migratory phenotype and haemosporidian infection status were the primary correlate of leukocyte composition (Table 2). In the first PERMANOVA, the interaction between migratory strategy and infection explained 7% of the variance in leukocyte profiles ($F_{1,46}$=4.44, p<0.01), with body condition explaining a similar degree of variance (Table 2). The robust PCA showed that uninfected migrants and residents overlapped in leukocyte composition. Infected juncos showed more separation with migratory strategy, with migrants having more heterophils and eosinophils and fewer lymphocytes, monocytes, and basophils than residents (Fig. 3A).

In the second PERMANOVA, the interaction between feather δ^2H values and infection explained 8% of the variance in leukocyte composition ($F_{1,42}$=4.45, p<0.01), whereas body condition explained 6% of this variance (Table 2). We used a *k*-means clustering algorithm to visualize differences in feather δ^2H groups, which showed high overlap in leukocyte composition for all breeding latitude groups for uninfected juncos (Fig. 3B). However, infected birds showed clear separation with δ^2H groupings, for which likely low-latitude breeders (−60 to −35‰) had fewer heterophils and eosinophils but more monocytes and lymphocytes. In contrast, juncos that likely breed at northern latitudes (−86 to −65‰, −108 to −88‰) grouped separately as having more heterophils but fewer eosinophils, lymphocytes, monocytes, and basophils (Fig. 3B).

4 Discussion

Understanding the physiological correlates of migration is important to predict when and where migrants may be most vulnerable to infectious disease and the extent

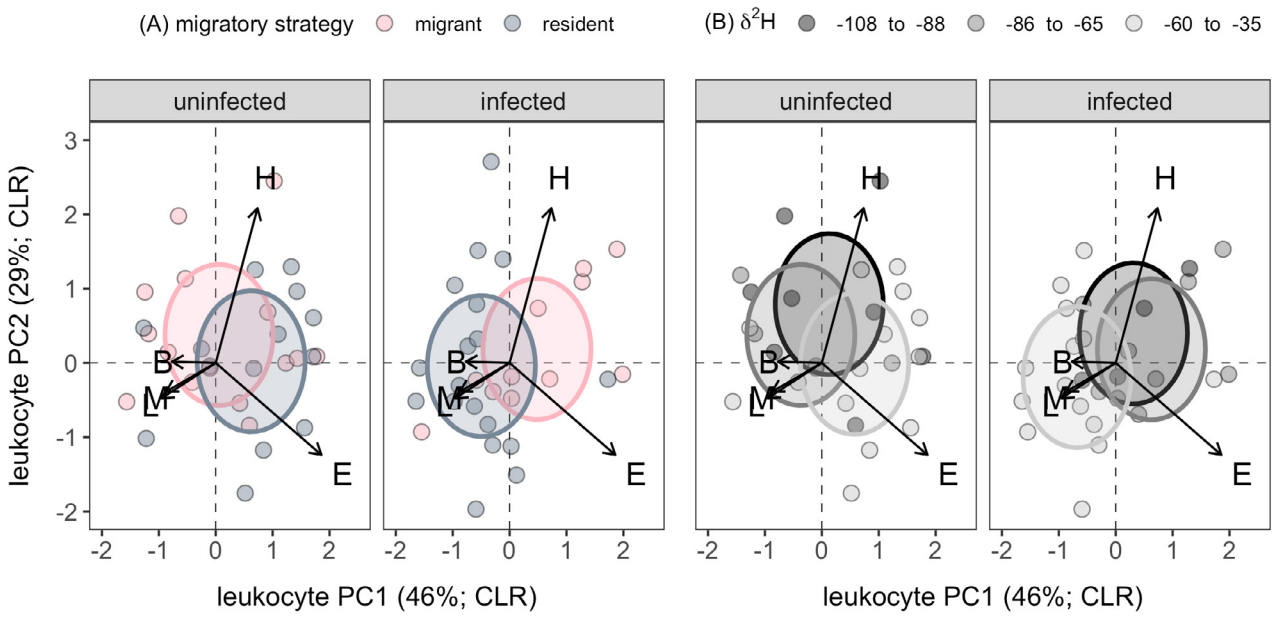

Fig. 3. Multivariate relationships between (A) migratory strategy or (B) feather δ²H values and junco leukocyte composition. Biplots show the first two back-transformed centered logratio (CLR) coordinates from a robust PCA of ILR-transformed leukocyte proportions (H=heterophils, L=lymphocytes, M=monocytes, E=eosinophils, B=basophils). Movement from the southwestern to the northeast quadrant of PC space largely indicates hematological shifts in relation to stressors (e.g., heterophilia, lymphopenia, and eosinopenia). Points are colored by migratory strategy or k-means clustering of feather δ²H values with 95% data ellipses. Results are stratified by haemosporidian infection status owing to significant interaction terms.

Table 2. Results of PERMANOVA analyses explaining variation in junco leukocyte composition (ILR-transformed proportions) as a function of either migratory strategy or feather δ²H values alongside sex, age, body condition, infection status, and interaction terms.

PERMANOVA 1 (*n*=53)	*df*	*F*	*R2*	*p*
Migratory strategy	1	2.36	0.04	0.06
Haemosporidian infection	1	0.10	0.02	0.41
Migratory strategy * haemosporidian infection	1	4.44	0.07	<0.01
Sex	1	1.00	0.02	0.40
Age	1	2.13	0.04	0.08
Body condition	1	4.06	0.07	<0.01
Residuals	46		0.75	
PERMANOVA 2 (*n*=49)	*df*	*F*	*R2*	*p*
Feather δ²H values	1	1.37	0.03	0.24
Haemosporidian infection	1	1.37	0.03	0.24
Feather δ²H values * haemosporidian infection	1	4.45	0.08	<0.01
Sex	1	0.47	<0.01	0.74
Age	1	1.46	0.03	0.22
Body condition	1	3.30	0.06	0.02
Residuals	42		0.77	

to which they facilitate cross-species transmission [10, 11]. Using leukocyte profiles of a seasonally sympatric songbird (*Junco hyemalis*), we demonstrate that birds likely traveling further from their breeding site during fall migration had leukocyte profiles consistent with hematological shifts in relation to stressors (e.g., high HL ratios, heterophilia, lymphopenia, and eosinopenia). However, these patterns were primarily observed for birds that tested positive for haemosporidian parasites, suggesting that short- and long-distance migrations may carry greater energetic costs when coupled with infections. These patterns were most pronounced when using feather δ^2H values and multivariate analyses, highlighting the benefits of approximating breeding latitude and of compositional data analysis for understanding physiology in the context of animal migration.

To isolate effects of recent migration as a possible stressor independently of the energetic tradeoffs and hormonal shifts associated with spring reproduction, we studied seasonally sympatric resident and migrant juncos in early November. Following eBird data (Fig. 1B), this period coincides with the start of seasonal sympatry in the weeks to days following pulses of migrant arrival [38]. However, migratory strategy had weak associations with leukocyte profiles, especially when examining HL ratios. Classifying juncos by subspecies alone likely obscured relevant variation in breeding latitude, for which our proxy (feather δ^2H values) displayed twice as much variation in migrants than residents. This greater variation of δ^2H values in *J. h. hyemalis* implies a broad range of possible breeding origins, in agreement with past work on this overwintering population [39]. Given the known geographic variation in hydrogen isotope composition of meteoric waters [82], these feather δ^2H values suggest likely breeding origins along a north and northwestern gradient across North America. In contrast, less variation in feather δ^2H values of residents (*J. h. carolinensis*) could stem from altitudinal migrations in the Appalachian Mountains [83]. Importantly, such variation in breeding latitude across individuals better explained variation in leukocyte profiles during seasonal sympatry. We thus emphasize using feather δ^2H values to approximate breeding origins, or direct quantification of migratory distance with tracking technologies, could provide further insights into links between migration and physiology [52]. Additional isotopic systems could help resolve migratory origins, based on geographic variation in vegetation type (carbon) or bedrock geology (strontium) [84].

The effects of recent migration on leukocyte profiles were also clearest for birds that tested positive for haemosporidian parasites (*Plasmodium* and *Haemoproteus* spp.). Both are transmitted by biting fly (Diptera) vectors mostly in spring and summer, and parasites may remain dormant in peripheral host tissue after acute primary infection [70]. Because visible infections in blood smears were rarer than those detected through PCR, and because intensities were generally low, PCR positivity of overwintering birds likely indicates chronic or relapsed infection [66]. This suggests chronic haemosporidian infections could have significant effects on bird physiology, complementing other studies showing that such infections can be associated with poorer condition, later migration timing, and prolonged stopover durations [45–49]. Coupling experimental infections with photoperiod manipulations to simulate the annual cycle (e.g., [85]) could help establish causality between haemosporidians and migratory ability.

Although we found an interactive effect of haemosporidian infection and migration on junco leukocyte profiles, we did not detect our other target parasite, *B. burgdorferi* sensu lato, in either residents or overwintering migrants. The *Ixodes* tick vectors mostly parasitize birds from spring through summer [86], limiting exposure in late fall (Table S1). However, as migration can reactivate *Borrelia* infection in birds [65], we expected to detect bacteria in blood from recently arrived migrants. Zero prevalence of *Borrelia* in these populations could suggest minimal vector exposure in the breeding season or that reactivation is more likely to occur prior to spring migration, as experimental manipulation of photoperiod has triggered reactivation of latent infection in redwings (*Turdus iliacus*) [65]. Alternatively, detection ability may have been limited by small blood volumes and possible low concentrations of bacteria. Future work across the duration of the non-breeding season could accordingly better capture spring relapse of both *Borrelia* and haemosporidian infections and their associations with avian physiology [87].

Alongside suggesting that future work assess physiology of seasonally sympatric migrants and residents throughout the non-breeding season (i.e., following, independent of, and prior to migration), our study highlights several other areas of needed research. Importantly, our sample size was limited, potentially underpowering our analyses. Greater temporal replication (e.g., assessing leukocyte profiles of sympatric migrants and residents not only within but also across multiple non-breeding seasons) could increase power and improve inference. When considering leukocyte profiles, our use of multivariate analyses also better partitioned leukocyte variation than HL ratios and provided additional insights into how cells such as eosinophils simultaneously varied

in response to infection and recent migration. We thus encourage future use of such statistical methods when analyzing leukocyte profiles [30, 31]. We also suggest that the patterns identified here (i.e., hematology indicative of recent stressors in infected short- and long-distance migrants) be confirmed with long-term, integrated measures of hypothalamic–pituitary–adrenal activity such as feather corticosterone [88] or biomarkers such as expression of heat shock proteins [89]. To explicitly link hematological differences with immunosuppression, future work could also quantify functional measures of defense (e.g., microbicidal assays) and organismal traits (e.g., host tolerance and competence) in relation to migratory phenotypes [90]. Such studies will be important to understand and predict how changing animal migrations, including those driven by anthropogenic factors such as climate change and supplemental food in urban and agricultural habitats, affect wildlife health and infectious disease risks [10, 12, 13].

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