Physiology & Behavior 102 (2011) 406–413

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

The effect of winter sex ratio on immune function and condition in a differential migrant

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ARTICLE INFO

Article history:
Received 9 June 2010
Received in revised form 25 October 2010
Accepted 26 November 2010
Available online 4 December 2010

Keywords:
Acquired immunity
Birds
Innate immunity
Non-breeding
Sexual segregation
Social interaction
Migratory behavior

ABSTRACT

The dark-eyed junco (*Junco h. hyemalis*) is a differential migrant in which females migrate farther south in winter than males. Hypotheses to explain this difference in migratory behavior have given limited consideration to physiological differences between the sexes, particularly with respect to immune function. We hypothesized that female migratory behavior could either be directly dependent on climate if migratory distance traveled is a product of interactions between climate and inherent sex differences in physiology, or indirectly dependent on climate owing to interactions between climate and aggressive encounters with males which are known to be behaviorally dominant to females and restrict their access to food. We tested the latter of these two hypotheses by measuring condition in females held in two sex ratio regimes in a common winter environment. Holding density constant in 28 replicate populations, we compared flocks composed of 100% female to mixed-sex flocks composed of 20% female. We assessed condition by measuring immune function via complement activity and response to a foreign antigen, keyhole limpet hemocyanin, as well as by indices of mass, fat, and pectoral muscle. Unexpectedly, females in mixed-sex flocks did not differ in condition from females housed only with females, despite increased aggressive interactions in mixed flocked cages. Combined, our results suggest that overwintering with males has no significant effect on female winter condition. Differential migration in the junco may be mediated by sex differences in ability to withstand harsh northern climate, as has been demonstrated in several mammalian systems, rather than dominance interactions.

1. Introduction

Many studies have considered the effects of sexual selection on the evolution of sex differences particularly with respect to the role of mating systems [reviewed in 1]. Less widely appreciated are the sex differences influenced by a species' ecology, in which case selection may favor adaptive responses/traits that will reduce competition between the sexes or that better fit each sex to its abiotic and biotic environment [2,3]. For example, differences between the sexes in their habitat preferences and dispersal behavior can lead males and females to settle in different locations and give rise to sexual segregation [4]. In many species of birds and mammals, the sexes differ in distance migrated (differential migration) leading males and females to settle at different non-breeding (hereafter winter) latitudes [4,5]. The distance an individual migrates into its winter range is influenced by selective forces (e.g., climate, disease prevalence, and food and refuge availability) that differ in their impact based on sex, age, and body size [reviewed in 2]. These selective forces result in tradeoffs involving the costs of migration, competition for winter resources, and the timing of return to the breeding range [2,5–11].

Birds are a classic model for examining migratory behavior, and the dark-eyed junco (*Junco hyemalis hyemalis*) is one such differential migrant in which females migrate further south during autumn than males [6,12,13]. Several hypotheses have been proposed to explain differential migration in this and other species including intrasexual competition for breeding resources, sex differences in the ability to withstand cold climate, and sex differences in the ability to compete effectively for food when it is in short supply, particularly when in the presence of the other sex [2,5,8,13–16]. These hypotheses tested singly, however, have received only moderate support in the junco and several other species and it is likely that multiple factors interact to give rise to differential migration [5,13,17–20].

In the junco, for example, sex differences in the ability to withstand cold climate may interact with sex differences in the ability to compete effectively for food or other resources in the face of dominance interactions. For females in particular, migratory behavior and overwinter survival could be explained by two competing hypotheses. First, differential migration could be directly dependent on climate if females are more likely than males to be negatively affected by extreme cold or unpredictable changes in weather. If so, differential migration could be a product of the interaction of winter

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doi:10.1016/j.physbeh.2010.11.034

climate and inherent sex differences in physiology such that females might be in poorer condition under harsh climates than mild climate, whether or not they wintered with males. Second, differential migration could be indirectly dependent on climate owing to interactions between climate and aggressive encounters or other consequences of living with males. That is, wintering with males, which are dominant to females [21,22], may have a negative impact on the physiology of females, reducing their winter condition and perhaps their relative survival, thus favoring females that make longer migrations.

To distinguish between these two explanations, we began by testing the latter of these two hypotheses. We investigated the effect of male presence on female winter condition by comparing females that did and did not overwinter with males. We controlled for density and stimulated dominance interactions by limiting the number of feeding sites, not the amount of food. This paradigm mimics natural conditions where winter weather such as snow can concentrate ground feeding birds at a small number of feedings sties. Studies in other species have also shown that relatively high-density environments such as these can have significant effects not only on the frequency of dominance interactions but also on physiological measures such as immune function [23].

We assessed winter condition in captive individuals via indices of mass, fat, pectoral muscle and measures of immune function. Mass, fat, and pectoral muscle are common estimates of physical condition in free-living birds and can give information about energy reserves and protein stores utilized in various metabolic (i.e. thermoregulation) and physiological functions (i.e. corticosterone released in response to stress) associated with breeding, migratory events, environmental perturbations, predation attempts, and social interactions [24–30]. Immune response is also an important tool for measuring the physical condition of vertebrates because immune response can have direct and indirect effects on survival and future reproduction [31–35]. Reduced immune function has been linked to poor energetic condition in migrating birds [36] and has also been associated with significant increases in basal metabolic rate in both birds and mammals [37,38]. Poor immune function can also negatively affect sexually selected characteristics, such as calling song [39] and plumage coloration [40]. Additionally, immune function is sensitive to variation in temperature, habitat (food and refuge availability), and social interactions [23,31,41–45].

In this study, we measured both acquired immune function (response to a foreign antigen keyhole limpet hemocyanin [KLH]) and innate immune function (levels of complement activity). Acquired (or adaptive) immunity is developed in response to specific antigens and deals with the direct killing of cells along with antibody production [46]. Innate immune function refers to nonspecific antigen defense mechanisms that are activated as a first response to eliminate microbes and prevent infection [46]. By measuring both acquired and innate immune responses, we were able to develop a more complete picture of an individual's immune function including potential tradeoffs between immune components and thus a better understanding of overall health and the ability to fight infection [34,47].

We predicted male presence and thus male dominance would result in reductions in not only acquired (i.e. fewer antibodies produced in response to KLH) and innate (i.e. lower levels of complement activity) immune responses, but also in indices of mass, fat, and pectoral muscle in females that overwintered with males, as compared to those that overwintered with only females. We based our prediction on previous studies that have shown male juncos are dominant to females and can restrict their access to food resources [22] and that subordinate status leads to reductions in non-breeding immune function [23,48]. To test our prediction, we captured female and male juncos from the wild during autumn migration and set them up in replicated flocks that differed in sex ratio. Some flocks consisted of females only; others consisted of females housed with males. All flocks were held in outdoor aviaries, and we made comparisons between females wintering with females (FWF) and females wintering with males (FWM). We also compared both categories of females to the males that were housed with females (MWF) to ascertain any differences between the sexes in winter condition.

2. Methods

2.1. Study species

The dark-eyed junco (*Junco hyemalis*) is a songbird species whose reproductive and migratory behavior has been extensively studied [49]. Data collected between 1950 and 1976 on migration schedules and population structure for the northern subspecies of junco (*J. h. hyemalis*) overwintering in the region of Bloomington IN, USA (39° 09′ 55″ N 86° 31′ 35″ W), as well as data from populations sampled at points throughout the winter range, have provided background on geographic variation in winter population structure and dynamics. Historic data from eastern North America have shown that female juncos winter farther from the breeding range and at more southerly latitudes than males [5,6]. Thus, percentages of females found across the range increases with decreasing latitude, approximately 20% at the northern portion of the range increasing to 100% in south [5]. In the winter, the junco's range extends from the northern United States and the extreme southeast of Canada to the southern US and portions of northern Mexico [49]. Settlement of the winter grounds occurs between mid-October and early December more or less simultaneously, as the sex and age classes that migrate farther also migrate earlier [50]. Junco males are generally larger than and dominant to females, reducing females' access to food resources [12,22].

2.2. Capture methods

We captured 280 juncos at baited mist nets and potter traps from October 15th through December 31st, 2006 during autumn migration [6,50] at Kent Farm Bird Observatory in Bloomington, IN and the Hoosier National Forest Paynetown State Recreation Area, IN. We captured migrants, many of which may have ultimately chosen different winter sites [50] in order to ensure that we 1) obtained enough females, as Indiana is in the northern portion of the female range where females are less abundant in winter, and 2) captured both males and females before the establishment of dominance hierarchies.

For each bird, we recorded mass (nearest 0.5 g), as well as indices of pectoral muscle [26], and fat [27]. Briefly, the scale for pectoral muscle was: 1, breast muscle concave to the keel, and keel prominently displayed; 2, breast muscle even with the keel; and 3, breast muscle bulging on either side of the keel, keel not prominent. Fat deposition was scaled as: 0, no fat; 1, trace lining either the fossa or abdomen; 2, fat filling the fossa; 3, fat filling the fossa and the abdomen; 4, fat bulging from either the fossa and the abdomen; 5 fat bulging from both the fossa and the abdomen. Following collection of blood samples, we determined sex and age using wing chord measurements (to nearest 0.5 mm) and plumage characteristics as described by Ketterson and Nolan [6]. Individuals were then separated by sex and held outdoors in same-sex flight cages (2.44 m × 34.16 m × 7.32 m) at Kent Farm Bird Observatory (KFBO) in Bloomington, Indiana (39.16°N, 86.49°W) until flock formation, detailed below.

Upon capture, we collected a 10 μl blood sample from the alar vein to confirm sex using the sex-linked CHD gene on the W chromosome [51]. Samples were stored at 4 °C in Longmire's [52] solution until analysis. DNA was extracted using standard phenol-chloroform protocols, and the CHD loci were amplified with fluorescently labeled primers P8 (5′-CTCCCAAG-GATGAGRAAYTG-3′) and P2 (5′-TCTGCATCGCTAAATCCTTT-3′) (Operon) in 10 μl PCR reactions. The resulting product was then diluted (1:20) and mixed with a molecular size standard (GeneScan-500 LIZ, Applied

Biosystems), and fragment size was analyzed with the ABI 3730 DNA Analyzer and the GeneMapper® 4.0 software. Females were identified genetically by the presence of 2 bands (346 bp and 387 bp) and males by one band (346 bp).

2.3. Flock formation

On January 5th, 2007, day 0, birds were transferred from flight cages into 28 flocks of 10 birds each (Fig. 1). In a preliminary study, we found this density was sufficient to induce dominance interactions (O'Neal et al. unpublished data), and it falls within the natural range of flock size [53,54]. Flocks were balanced in 28 identical outdoor compartments (2.44 m × 2.44 m × 2.44 m). Every two days, birds received 450 g of a mixture of white millet and crushed sunflower seeds. This amount of food can be considered abundant, but it was distributed in one food dish (37 cm × 18 cm × 9 cm) per cage to promote ongoing dominance interactions. We also provided each cage with one water dish of the same size containing ~1 l of water. Density and age class were kept constant within compartments, with 22 cages containing only birds in their first year (AY) and 6 cages consisting of only birds older than one year (AO). The sex ratio in each cage was either 100% female (females with females, FWF), simulating conditions in the far south, or 20% female (females with males, FWM), simulating conditions in the far north of the junco winter range. All 6 of the cages containing AO birds were in the mixed-sex treatment. To account for the reduction in statistical power associated with the small number of females in mixed-sex cages (two females, eight males) we increased the number of replicates for the 20% female treatment to 18 cages, resulting in 10 cages with 100% female.

FWM & MWF 20%	FWM & MWF 20%
FWF 100%	FWM & MWF 20%
FWM & MWF 20%	FWF 100%
FWF 100%	FWM & MWF 20%
FWM & MWF 20%	FWF 100%
FWM & MWF 20%	FWF 100%
FWF 100%	FWM & MWF 20%
FWF 100%	FWF 100%
FWM & MWF 20%	FWF 100%
FWF 100%	FWM & MWF 20%
FWM & MWF 20%	FWM & MWF 20%
FWF 100%	FWM & MWF 20%
FWF 100%	FWM & MWF 20%
FWM & MWF 20%	FWF 100%

Fig. 1. Schematic diagram of flock set up. Individuals were divided between 28 compartments in flocks of 10 birds each. Density and age class were kept constant within compartments and sex ratio in each cage was either 100% female (FWF) or 20% female (FWM and MWF). To account for the reduction in statistical power associated with the small number of females in mixed-sex cages we increased the number of replicates for the 20% female treatment to 18 cages, resulting in 10 cages with 100% female.

All procedures used in this study were approved by the Indiana University Bloomington Institutional Animal Care and Use Committee (Study# 06-242).

2.4. Measuring immune function

We assessed acquired immune function as antibody production in response to injection with keyhole limpet hemocyanin (KLH, EMD Biosciences Inc.), a novel non-pathogenic protein antigen that is highly immunogenic but does not elicit sickness behaviors, following procedures adapted from Hasselquist et al. [55] and successfully employed with juncos (Casto et al. unpublished data). We began sampling 14 days after the formation of flocks because previous studies have indicated this is adequate time for the establishment of stable dominance hierarchies [56]. On day 14, we collected a 100 μl blood sample and gave a single intramuscular injection of 100 μl of 1 mg ml^{-1} KLH in 1 mL of Freund's incomplete adjuvant (Sigma F5506). On day 25 we collected a second 100 μl blood sample (primary immune response). On day 30 a second KLH injection was given and on day 38 (the peak of the secondary immune response in juncos, Casto et al. unpublished data) another 100 μl blood sample was drawn (secondary immune response).

Following blood collection, plasma was drawn off and kept frozen until assayed by enzyme-linked immunosorbent assay (ELISA) for measures of response to KLH.

To assess innate immune function, a portion of the plasma samples (20 μl) drawn on days 14, 25, and 38 were also used to assay for hemolytic complement activity, which is an estimate of the ability of complement and natural antibodies to lyse foreign blood cells and thus an indicator of an individual's ability to mount an innate immune response [33]. Before birds were released back into their respective flocks, we also measured mass, pectoral muscle, and fat.

2.5. KLH assay

We measured anti-KLH IgG concentrations [55,57] using microtiter plates coated with antigen incubated overnight at 4 °C with 0.5 mg/ml KLH in sodium bicarbonate buffer (pH 9.6). The following day, plates were washed with PBS-Tween (PBS, Sigma, 08057) in an automatic plate washer, coated with milk blocking buffer (5% non-fat dry milk in PBS) overnight at 4 °C, and then washed again with PBS. Thawed plasma samples were diluted 1:20 in PBS and added in duplicate to randomly assigned wells of the antigen coated plates. Plates were sealed and incubated at 37 °C for 3 h, then washed with PBS. Positive control samples (pooled plasma from juncos diluted in PBS) and negative control samples (pooled sera from KLH-naïve juncos, diluted in PBS) were also added in duplicate. We then added 100 μl of a 1:500 dilution of rabbit anti-starling IgG (courtesy of G.F. Ball and R.J. Nelson) to the wells as a secondary antibody, and plates were sealed and incubated for 1 h at 37 °C. This antibody has previously been shown to possess sufficient cross-reactivity to junco plasma [58]. Plates were then washed with PBS, and 150 μl of alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Aldrich) diluted 1:500 in PBS was added to each well. Plates were again incubated at 37 °C for 1 h and subsequently washed with PBS. 150 μl of the enzyme substrate p-nitrophenyl phosphate (1 mg/ml in diethanolamine substrate buffer, Sigma Aldrich) was added to each well and plates were incubated for 20 min. We measured the optical density (OD) of each well at 405 nm with a microplate reader (Bio-Rad) and calculated the mean OD for each set of duplicate wells. Sample concentrations of IgG are expressed as a percentage OD of the plate

positive control. High percentages are indicative of higher antibody production.

2.6. Hemolytic complement assay

For precise titrations of hemolytic complement, the dilution of plasma that will lyse 50% of the indicator red blood cells is determined as the CH_{50}. Using methods described in Greives et al. (2006), 25 μl of a 0.6% suspension of washed sheep red blood cells (SRBC, MP Biomedicals 55876) and 25 μl of 1:40 dilution of rabbit anti-SRBC (Sigma-Aldrich) were added to a microtiter plate containing (in duplicate) 5 μl of plasma, diluted 1:40 in veronal buffer (Cambrex). Positive control (0% lysis, veronal bufffer in 65 μl SRBC) and negative control (100% lysis, water in 65 μl SRBC) were also added in duplicate. The plate was then sealed and shaken for 5 min before being incubated at 37 °C for 90 min. Next, plates were centrifuged for 5 min at 500 rpm. We then transferred 60 μl of supernatant to a new plate, and absorbance was measured at 405 nm to determine optical density (OD). To calculate CH_{50}, a log-linear function was created and used to extrapolate the point at which 50% lysis occurred [59]. We then calculated percent lysis for each well using the percent OD of the sample compared to the OD for the positive control (100% lysis). Higher percentage lysis indicated higher concentrations of complement protein.

2.7. Behavioral observations

Beginning March 2nd, day 56, following immune challenges, each of the 28 flocks was videotaped for 1 h in order to determine the rate of aggressive interactions for the flock. We chose to measure aggressive interactions near the end of the study to avoid the effects of handling stress and observer disturbance on behavior. Additionally, we wanted to ensure that observations were being made of established dominance hierarchies, the outcome of initial competition for dominance rank which is a better indicator of an individual's social status during immune sampling. Following standard protocols, one hour prior to taping food dishes were removed to increase activity and replaced for taping [23,45,56]. We began taping between 1500 and 1700 h to capture the pre-sunset feeding time. Individuals fed one at a time at the food source and generally, females were the last to feed in mixed-sexed flocks (O'Neal pers. observations). The rate of aggressive interactions was measured as the number of displacements that occurred over the food dish independent of sex. Due to damaged video recordings, 9 flocks (5 100% female flocks and 4 20% female flocks) were not included in the analyses.

2.8. Statistical analysis

We analyzed the impact of sex ratio on mass, pectoral muscle, complement activity and response to KLH using a linear mixed model with a Satterthwaite correction to account for unequal sample sizes across groups (SPSS version 15.0). KLH and complement activity were log transformed for normality. Group [females that lived with males (FWM), females that lived exclusively with females (FWF), and males that lived with females (MWF)] and time point of sampling (14, 25, and 38 days) were included as fixed factors, and cage as a random factor for all analyses. Time-point was also included as a repeated measure in the complete model. Additionally, we made pair-wise comparisons of groups at each time point to determine which treatment was responsible for any observed main effects. We also used Pearson's correlation to determine if any tradeoffs existed between immune measures at the individual level after primary and secondary challenges (25 and 38 days). In all immune comparisons, covariates (age and indices of mass, pectoral muscle, and fat), included as fixed effects, were removed from the final model if they fell above the 0.05 significance level.

We analyzed the effect of treatment (mixed sex cages vs. same sex cages) on aggressive interactions using an ANOVA with the dependent variable of interaction rate square root transformed for normality. In addition, we used linear regression to analyze interaction rates with respect to averaged (across the cage) secondary KLH response and average complement activity measured at 38 days to determine whether competition had any effect on immune response. We chose the secondary KLH measure and the final complement measure since these were closest to the time of behavioral observation and would give a more accurate interpretation of immune profiles during behavioral trials. For all analysis, tests were two-tailed and significance was calculated at the $p = 0.05$ significance level. Means reported are estimated marginal means (EMM).

3. Results

3.1. Group affected mass but not indices of fat and pectoral muscle

Group and sampling time significantly affected mass (Group: $F(2,73.01) = 13.33$, $p<0.001$; Time: $F(2,488.10) = 428.65$, $p<0.001$; Table 1), but there was no significant interaction between time and group ($F(4,487.71) = 0.093$, $p = 0.985$). A Bonferroni post hoc analysis revealed that the group effect was driven by males, with males weighing approximately 1 g more than females of both groups but that females did not differ significantly from each other (estimated marginal means (EMM) mass by group: FWM = 19.92 ± 0.261; FWF = 19.84 ± 0.195; MWF = 20.91 ± 0.155; $F(2,74.97) = 13.33$, $p<0.001$). A Bonferroni post-hoc analysis also revealed that mass declined significantly between each time point (EMM mass by time: 14 days = 21.45 ± 0.141; 25 days = 20.36 ± 0.137; 38 days = 18.87 ± 0.138; $F(2,488.09) = 428.65$, $p<0.001$). Fat scores did not differ significantly by group but did decline over time (EMM mass by time: 14 days = 2.52 ± 0.057; 25 days = 2.21 ± 0.054; 38 days = 1.85 ± 0.055; $F(2,493.92) = 68.21$; $p<0.001$; Table 1). Age and pectoral muscle did not vary significantly with group or time.

3.2. Acquired immune function varied with group and time

Analysis of group and sampling time together indicated a significant main effect of time ($F(1,220.51) = 267.41$; $p<0.001$) and a trend towards

Table 1
Estimated marginal means (±SE) for mass, fat, complement activity and response to KLH by time and treatment group. Letters (a, b, and c) denote significant differences across group (FWM, FWF, and MWF) while numbers (i, ii, and iii) denote significant differences across time points (14, 25, and 38 days). Both mass and fat declined significantly with time and mass was higher in males compared to females. KLH response increased significantly between primary and secondary responses but only differed significantly among groups with respect to FWF and MWF. Complement activity declined significantly between 14 days and 25 and 38 days.

	FWM	FWF	MWF
Mass (g)			
14 days	21.2 (0.295)$^{b,\ i}$	21.1 (0.209)$^{b,\ i}$	22.1 (0.166)$^{a,\ i}$
25 days	20.1 (0.281)$^{b,\ ii}$	20.0 (0.205)$^{b,\ ii}$	21.0 (0.164)$^{a,\ ii}$
38 days	18.5 (0.286)$^{b,\ iii}$	18.5 (0.207)$^{b,\ iii}$	19.6 (0.165)$^{a,\ iii}$
Fat			
14 days	2.50 (0.127)^{i}	2.49 (0.082)^{i}	2.58 (0.066)^{i}
25 days	2.22 (0.117)^{ii}	2.21 (0.079)^{ii}	2.21 (0.064)^{ii}
38 days	1.95 (0.120)^{iii}	1.81 (0.081)^{iii}	1.81 (0.066)^{iii}
KLH response			
Primary (25 days)	0.463 (0.024)^{i}	0.431 (0.016)^{a,i}	0.509 (0.013)$^{b,\ i}$
Secondary (38 days)	0.647 (0.026)^{ii}	0.653 (0.017)^{ii}	0.659 (0.014)^{ii}
Complement			
14 days	4.07 (0.148)^{i}	4.00 (0.094)^{i}	4.20 (0.076)^{i}
25 days	3.31 (0.131)^{ii}	3.45 (0.092)^{ii}	3.48 (0.078)^{ii}
38 days	3.25 (0.195)^{ii}	3.20 (0.145)^{ii}	3.24 (0.111)^{ii}

significance with respect to group ($F(2,55.61) = 2.91$; $p = 0.063$). There was also a significant interaction between group and time ($F(2,219.80) = 6.16$; $p = 0.003$), and pair-wise post-hoc comparisons revealed an increase in antibody production in all groups between the primary and secondary injections (Table 1).

Additionally, we analyzed the effect of group at each injection time point because the secondary response, as a measure of an individual's immunological memory, is highly influenced by the primary response. Primary antibody production was significantly affected by group ($F(2,61.68) = 4.76$; $p = 0.012$), and post-hoc comparisons of primary antibody production indicated a significant difference between FWF and MWF ($F(2, 69.84) = 4.76$; $p = 0.027$): FWF had lower antibody production than MWF in response to the priming injection (EEM: FWF = 0.431 ± 0.021; MWF = 0.510 ± 0.017; Fig. 2). There were, however, no significant pair-wise comparisons between FWM and FWF or FWM and MWF. Additionally, group had no significant effect on secondary antibody response ($F(2,49.3) = 0.066$, $p = 0.936$), even when primary antibody response was included in the analysis as a covariate to control for variation in first exposure ($F(2,69.29) = 0.461$, $p = 0.633$).

3.3. Innate immune function varied with time but not group

We found no significant differences between groups in complement activity ($F(2,97.47) = 0.787$, $p = 0.458$; Table 1), but complement activity did decrease significantly with time ($F(2,392.8) = 45.63$, $p < 0.001$; Table 1). Post-hoc Bonferroni indicated a significantly higher complement activity at 14 days as compared to response at 25 and 38 days, which did not differ from each other (EMM: 14 days = 4.09 ± 0.066; 25 days = 3.41 ± 0.062; 38 days = 3.23 ± 0.091). Comparing the three groups at each time point indicated no significant effects of group on complement activity at any time point. At the individual level, there were no significant relationships between complement activity and KLH responses at any time point (25 days (primary): $r = 0.062$, $p = 0.372$; 38 days (secondary): $r = -0.021$, $p = 0.849$).

3.4. Dominance interactions were more frequent in mixed flocks

Dominance interaction rate in mixed-sex cages (20% female), independent of sex, was significantly higher than that in same-sex cages (EMM: 20% female = 2.79 ± 0.257; 100% female = 1.72 ± 0.429; $F(1,4.22) = 4.58$, $p = 0.047$; Fig. 3). Linear regression of aggressive interaction rate on secondary KLH response and complement activity at 38 days revealed no significant relationships (KLH: $t = 1.69$, $p = 0.109$, adjusted $r^2 = 0.094$; Complement: $t = 1.19$, $p = 0.249$, adjusted $r^2 = 0.023$).

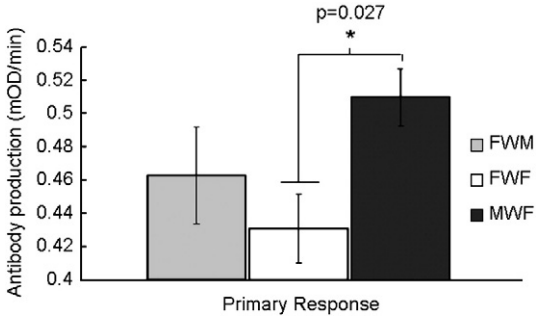

Fig. 2. Estimated marginal means (±SE) of antibody response to injection with KLH by injection time and group. Antibody response to KLH increased significantly with injection time. Males that overwinter with females (MWF) displayed a significantly higher primary response than females that wintered with females (FWF).

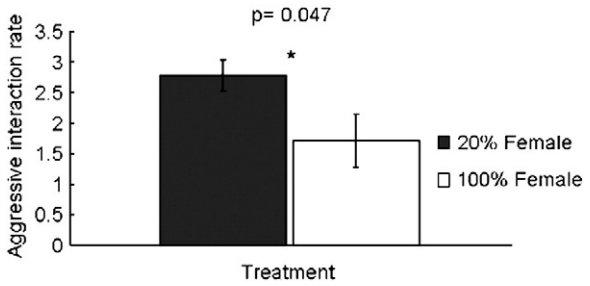

Fig. 3. Estimated marginal means (±SE) of rate of aggressive interactions per hour by treatment. Interaction rates over food sources were significantly higher in mixed-sex treatments (20% female) than same-sex treatments (100% female).

4. Discussion

We asked whether female migratory behavior in the junco might be indirectly dependent on climate as mediated by social dominance interactions over food resources. Specifically, we investigated the effects on females of cohabiting with males which are dominant to females and can restrict their access to food possibly impairing immune function. We predicted that females that overwintered with males (FWM) would have lower immune function than those that overwintered only with females (FWF). Despite evidence of increased incidence of aggressive interactions in mixed-sex cages, we found no significant differences in immune measures between FWM and FWF. Contrary to what we predicted, FWF had the lowest primary response to KLH, and this difference was significant when compared to males wintering with females (MWF). These differences, however, did not hold for their secondary response to KLH or for measures of complement activity at any time point. Combined, our results suggest that overwintering with males has no significant effects on female winter immune function and suggest that female migratory behaviors in the junco may be mediated by other factors including sex differences in the impact of harsh northern climate that are not mediated directly by dominance interactions.

4.1. Variation in immune response with treatment

Group significantly mediated immune function during initial measures of response to KLH (primary response, 25 days), but had no significant effect thereafter or on measures of complement activity. The reduced humoral response in FWF compared to MWF, although not FWF compared to FWM, suggests some initial immunological cost for FWF. Such costs might arise out of the establishment of social hierarchies (establishing dominance or accommodating to subordinance), which have been reported to affect immune function [23,44,48,60,61], from direct competition for food resources [62–64], or both. It is unclear, however, why individuals in the mixed-sexed treatment would not face these same costs or undergo similar interactions, unless social status in these cages was somehow pre-established (e.g. females are always subordinate to males) so fewer challenges over status took place. It is possible that this pre-establishment of status, if it exists, may have freed FWM from having to allocate energy resources towards dominance and competitive interactions and allowed them to direct energy towards maintaining their immune function at a level comparable to males.

Overall, the apparent lack of differences in immune function between the sexes is somewhat surprising, though these results do follow recent data collected on variation in non-specific immune function in free-living juncos (O'Neal et al. in prep). Generally, immune function is thought to be mediated by physiological and ecological/social mechanisms which can vary with the life history of each sex, but it should be noted that the majority of these studies have been conducted in breeding individuals [65–67]. Sex differences in immune function are often attributed to the immunosuppressive effects of testosterone, with

the result that males, the sex that generally displays higher levels of testosterone, show a lower immune response compared to females [68,69 but see 55]. Testosterone levels, however, are low in both sexes during the non-breeding season and are unlikely to affect immune response. Thus, we may not have observed sex differences in immune response because testosterone profiles are similar between the sexes at this time, as has been suggested by Hasselquist et al. [55]. Winter immune responses could also be the product of seasonal interactions, such that winter immune function is an extension of immune status on the breeding grounds or during migration. For example, the more robust immune response observed in breeding females compared to males [65,66,70,71] may act to buffer winter immunosupression if the cost of maintaining of immune cells, proteins, and immunoglobulins developed during breeding is low [72,73]. Junco males may exhibit a degree of immunoenhancement in preparation for non-breeding events, as seen in many other species, [31,74].

4.2. Dominance interactions had no significant relationship with immune function

Several studies have indicated that social interactions, which might arise out of competition for resources, can also affect immune function [48,75,76]. We found that despite a significant difference in aggressive interaction rates between mixed- and same-sex treatments, aggressive interactions seemed to have no effect on immune measures. Individuals from mixed-sex treatments with high aggressive interactions did not have lower immune function than individuals from same-sex treatments with a lower interaction rate. Social interactions are thought to impact immune function because these interactions interfere with meeting metabolic requirements needed to mount an immune response [44,45,48]. In this instance, we may not have observed differences in immune function between treatments or the sexes because there may have been no differences in energetic requirements or in short-term resource allocation to competing demands such as thermoregulation or immune function. It remains possible that if we had restricted food quantity as opposed to offering food ad lib (which allowed individuals to obtain required nutrients even though they may have had restricted access to food), we might have observed differences in immune function between groups and possibly the sexes.

Across all groups we also found that while humoral immune function increased over time, mass declined over time despite abundant food. Declines in mass and fat are generally indicative of a reduction in energy reserves for possible use in thermoregulation [77], mounting an immune response [37,78, but see 79], or both, although in this instance we cannot rule out the possibility that individuals may have perceived captivity as a high quality, stable environment lessening the need to put on energy reserves. In sum, while abundant food may have prevented detection of significant differences between the sexes in winter immune function, not all our observations are consistent with this default explanation and further experiments would be required to determine how food availability and competition might interact to influence immune function.

4.3. Immune function varied with time

While increases in secondary immune responses to KLH were unexpected considering the large declines in mass despite ad lib food [but see 79], immunoenhancement in non-breeding individuals has been documented in several species [80–83, but see 84]. These increases are thought to be a mechanism for keeping parasite activities to a minimum during winter months when resource availability and environment are variable [31,85] and could also act to ensure good condition and assist in early arrival to breeding grounds [86]. Additionally, enhancement could be a response to increases in social interactions associated with winter flocking or novel environments, both of which could introduce individuals to diseases or parasites [but see 48,87,88]. The innate immune response, however, followed a somewhat opposite pattern with complement activity declining with time. This decline in complement activity is surprising considering that innate responses are generally thought to have lower development and maintenance costs compared to adaptive responses such as antibody production which have higher development but low maintenance costs [reviewed in 73]. An increase in KLH antibody production with time suggests that the costs of antibody maintenance may indeed be low since secondary responses or immunological memory was shown to be high across treatment groups. The decline in complement activity, however, suggests that despite the low development and maintenance costs associated with such nonspecific immune defense mechanisms, these mechanisms may be abated when the immune system is faced with challenges from previously encountered antigens. That is, individuals may favor specific immune defenses over nonspecific defenses when dealing with re-infections. Indeed, there was a negative relationship at the individual level between complement activity and KLH responses measured after secondary challenge (38 days) but this relationship was not statistically significant. Thus, we cannot rule out that declines in complement activity were mediated by associated declines in mass and fat, particularly because complement was measured in association with the simultaneous measurement of response to KLH, but it is also possible that other physiological processes varied over time and were responsible or even that the birds were fighting infections at the time of measurement.

4.4. Summary

We hypothesized that the interaction of winter physiology and male dominance could affect winter condition in females and act as a possible mediator of differential migration in the junco. Though we cannot rule out completely the role of cohabiting with males as the mediator of differential migration in the junco because food was present at all times, our results suggest that differential migration in the junco may be mediated instead by some other factor. Possibilities are numerous, but primary candidates include two hypotheses that have been successful in explaining sexual segregation in ungulate systems: sex differences in the effects of winter climate on lifetime reproductive success that are independent of interactions with males (e.g., sex differences in metabolic rate) and the suitability of habitat that favors over winter survival in males and females respectively and varies geographically in its availability [4,20]. Additionally, recent hypotheses considering seasonal interactions, in particular carry-over effects from the breeding grounds that may favor longer migrations by females, or a combination of some other selective factors [9,13,20] remain promising in this system.

Acknowledgments

We thank Dr. M. Scotti, E. Snajdr, K. Cain, and the rest of the United Junco Workers for their assistance in capturing birds, setting up flocks and taking immune measures. We also thank S. Dickinson from Indiana University's Statistical Consulting Center for statistical assistance and Dr. D. Whittaker, Dr. S. French, and Dr. G. Demas for helpful discussions and suggestions on the manuscript. This research was funded in part by the Indiana Academy of Sciences and the Office for the Vice-Provost for Research Indiana University.

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