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Immune profiles vary seasonally, but are not significantly related to migration distance or natal dispersal, in a migratory songbird

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A central tenet of ecoimmunology is that an organism's environment shapes its optimal investment in immunity. For example, the benefits of acquired (relatively pathogen-specific) versus innate (non-specific) immune defences are thought to vary with the risk of encountering familiar versus unfamiliar pathogens. Because pathogen communities vary geographically, individuals that travel farther during seasonal migration or natal dispersal are predicted to have higher exposure to novel pathogens, and lower exposure to familiar pathogens, potentially favoring investment in innate immunity. During the breeding season, migratory animals' exposure to familiar pathogens should increase, potentially favoring investment in acquired immunity. We hypothesized that song sparrows *Melospiza melodia* adjust their constitutive immune profiles in response to risk of encountering novel versus familiar pathogens. We predicted that individuals migrating longer distances (inferred from stable hydrogen isotope analysis of claws) and less philopatric individuals (inferred from microsatellite assignment testing) would rely more heavily on acquired than innate defences. We also predicted that reliance on acquired defences would increase throughout the early breeding season. Consistent with trade-offs between acquired and innate defences, levels of immunoglobulin Y (acquired) varied negatively with macrophage phagocytosis activity (innate). Levels of acquired relative to innate immunity did not vary significantly with migration distance or philopatry, but increased throughout the early breeding season. Macrophage phagocytosis was not significantly repeatable between years. Song sparrows appear to shift from innate defences immediately after migration, to acquired defences with increasing time at the breeding grounds. These patterns highlight the plasticity of constitutive immune defences in migratory animals.

Keywords: constitutive immunity, deuterium, dispersal, migration, stable isotopes, trade-offs

Parasites are taxonomically widespread and geographically ubiquitous (Schmid-Hempel 2011). Because few organisms can successfully avoid exposure to parasites, being able to resist, eliminate or tolerate infection is a key component of host fitness. As a result, multicellular organisms have evolved diverse anti-parasitic defences. However, immune defences are costly to develop, maintain and deploy (Klasing 2004, Lee 2006). Thus, trade-offs between immunity and other life history traits, and even trade-offs within the immune system itself, are inevitable. The idea that infectious disease risk shapes the optimal immune profiles of host species and individuals is a central principle of ecoimmunology (Sheldon and Verhulst 1996, Møller and Erritzøe 1998, Nelson et al. 2002).

In vertebrate animals, immune defences can be broadly categorized as constitutive or induced and, independently, as innate or acquired (Schmid-Hempel and Ebert 2003, Buehler et al. 2008a). Along the constitutive-induced axis, constitutive defences provide a baseline level of expression of protective cells and molecules, and additional induced defences can be activated in response to infection or other immune challenges. Along the innate-acquired axis, innate defences provide nonspecific protection against a broad array of pathogens, whereas acquired defences use immune memory to generate a recognition system of antigen-specific responses (Janeway et al. 2005; Buehler et al. 2008a; Demas et al. 2011). The initial development of acquired defences is relatively costly and slow, but once in place, such defences act rapidly and efficiently upon repeat exposure to a specific pathogen. Thus, innate and acquired defences differ in their relative effectiveness against novel versus familiar pathogens: innate defences comprise a first line of protection against novel pathogens, whereas acquired defences are most effective when re-encountering familiar pathogens (Lee 2006). The relative risk of encountering new versus familiar

pathogens should thus affect the optimal balance between innate versus acquired immune defences. For example, species and populations with longer development, lifespans and generation time invest more in acquired immune defences, relative to those with shorter development and generation time (Lee 2006; Martin et al. 2006, 2007).

Even within a population, the relative risk of encountering novel vs familiar pathogens is likely to vary both across individuals and throughout the annual cycle. Pathogen communities vary geographically (Pagenkopp et al. 2008), suggesting that individuals engaging in long-distance movements such as seasonal migration and natal dispersal are likely to encounter new pathogens (Møller and Erritzøe 1998, Møller et al. 2004, Fincher and Thornhill 2008). Individual variation in migration distance and natal dispersal could thus influence the optimal balance between innate and acquired immune defence. Similarly, for migratory animals, the relative risk of encountering unfamiliar pathogens versus re-encountering familiar pathogens is likely to decrease with increasing time spent in a particular area (for example, on the breeding grounds). This may promote a seasonal transition from innate to acquired defences throughout the early breeding season.

Here, we test the hypothesis that the immune phenotype of migratory song sparrows (*Melospiza melodia*) reflects individual and seasonal variation in the risk of encountering familiar versus unfamiliar pathogens. Individuals in the study population vary markedly in latitudinal migration distance, as inferred from stable hydrogen isotope analysis (Kelly et al. 2016, Lymburner et al. 2016), and in natal philopatry, as inferred from genetic assignment tests (Kelly et al. 2016). Migration distance varies consistently among individuals across years in this study system (repeatability = 0.41), and is associated with natal dispersal such that individuals that migrate longer

distances are also more likely to have immigrated from outside the local breeding population (Kelly et al. 2016). We predicted that longer-distance migrants and dispersing individuals would rely more heavily on innate immune defences, reflecting their increased exposure to novel pathogens. Conversely, shorter-distance migrants and philopatric individuals should rely more heavily on acquired defences, reflecting their increased exposure to familiar pathogens. We also predicted that the balance between innate and acquired immune function would vary seasonally. Specifically, we predicted a shift from innate defences to acquired defences as the early breeding season progressed, reflecting seasonal changes in the relative risk of encountering new versus familiar pathogens.

Seasonal variation in immune profiles could reflect factors other than an increasing familiarity of pathogens as the breeding season progresses. First, associated with maternal transfer of immunity to offspring, levels of circulating immunoglobulins increase in female birds prior to egg-laying (Saino et al. 2001). Because individual lay date was not known for most females in our study, we could not control statistically for variation in breeding phenology relative to sampling date. Instead, we tested for sex-specific effects of date on immune profiles, to assess whether seasonal shifts in immunity might reflect maternal antibody allocation. Second, tradeoffs between migratory flight and immune defence (e.g. Nebel et al. 2012) could generate seasonal shifts in immunity, as recovering body reserves after migration allows more energy to be invested into adaptive or innate immune defence. To assess this possibility, we included size-corrected mass (a proxy for energy reserves) as a covariate in models predicting immunity.

Methods

a) Study site and field methods

Study subjects were 100 song sparrows breeding on land owned by the Queen's University Biological Station, at a site near Newboro, Ontario, Canada (44.633°N, 76.330°W). We captured subjects in seed-baited Potter traps between 15 April – 14 May, 2013 (N = 54) and 14 April – 9 May, 2014 (N = 60, including 14 individuals captured in both years). Capture dates corresponded to shortly after spring migration (first return to the breeding grounds: 3 April and 1 April in 2013 and 2014 respectively) through early nesting (first egg date: 14 May and 8 May in 2013 and 2014 respectively). We ran traps between 07:00 – 10:30 each day, checking each trap at least once per hour.

We collected up to 200 µL of blood for immune and genetic analyses, by brachial venepuncture the first time each bird was captured. We collected blood samples using sterile techniques (Millet et al. 2007), and to minimize effects of handling stress on immune response (Buehler et al. 2008b), we sampled within 8 min of researchers approaching the trap. We measured mass to the nearest 0.1 g using a spring-loaded scale, measured tarsus length and unflattened wing length to the nearest 0.1 mm using dial calipers, and determined sex based on the presence (male) or absence (female) of a cloacal protuberance. We used previous years' banding records to categorize birds to age class (i.e. second-year, hereafter SY, or after-second-year, hereafter ASY; see Kelly et al. 2016 for details). We clipped a sample of claw tissue for stable-isotope analysis of overwinter latitude (details below), outfitted the bird with a numbered USFWS aluminum leg band and a unique combination of three colored plastic leg bands if not already banded, and released the bird at the site of capture. All subjects were re-captured or re-sighted later in the season, suggesting that

all were resident breeders. Animal procedures were approved by the Animal Use Subcommittee at the University of Western Ontario (protocol 2008-054).

b) Immune assays

Overview and timing:

We tested multiple measures of constitutive (baseline) immunity, including both cellular and complement-mediated innate defences and an acquired defence. All agents were approved by the Biosafety Committee at the University of Western Ontario (protocol BIO-UWO-0133). Immune assays included whole-blood- as well as plasma-based measures. Whole-blood-based assays (macrophage phagocytosis; details below) were conducted in the field, and initiated within 40 minutes of sampling. To ensure sterility under field conditions, we conducted these assays in a Plexiglass dead-air box equipped with a HEPA filtration system (Kubli and MacDougall-Shackleton 2014). We assayed macrophage phagocytosis activity against the gram-negative bacteria *Escherichia coli* in 2013 and 2014. For plasma-based assays (hemagglutination and immunoglobulin Y assays; see below), we kept the remainder of the blood sample cool on ice for several hours, then isolated plasma by centrifugation and stored it at -20°C until laboratory analysis. Plasma-based analyses were run in 2013 only.

Macrophage phagocytosis assay:

As an indicator of *cellular innate* constitutive immunity, we measured *in vitro* phagocytic activity of macrophages (Millet et al. 2007). We reconstituted Bioparticles® of *E. coli* (E-2864) fluorescently labeled with BODIPY FL (Molecular Probes), in sterile, tissue-grade PBS plus 2 mM sodium azide according to the

manufacturer's protocol. In the field, following Kubli and MacDougall-Shackleton (2014) we diluted 10 µL of whole blood in CO_2-independent media plus 4 mM L-glutamine, 1% fetal bovine serum (FBS), and 1% penicillin-streptomycin (Invitrogen) to a final volume of 200 µL. A fresh dilution of Bioparticles® was prepared each morning in cold, sterile media to a working concentration of 15,000 particles/ µL.

We added 20 µL of diluted blood and 76 µL of bacterial suspension to each well of an eight-chamber slide (Nunc Lab-Tek), preparing one slide of eight wells per individual. Slides were incubated at 40.5°C for 15 min, placed on ice to end phagocytosis, washed twice with 95 µL cold sterile media, and then fixed with 100% methanol. Slides were stored in a lightproof container and later examined under a fluorescent microscope with an excitation/absorption spectrum of 505/513 nm. We examined 400 adherent cells per slide (approximately 50 cells per well), and scored phagocytic capacity as the proportion of macrophages containing at least one fluorescent particle.

Hemagglutination assay:

As an indicator of *complement-mediated innate* constitutive immunity, we estimated titers of natural antibodies by scoring the ability of plasma to agglutinate rabbit red blood cells (RRBC; Matson et al. 2005). We prepared twofold serial dilutions of freshly thawed plasma into PBS across a 96-well plate, using one column of 12 wells and a total of 50 µL plasma per bird. Two columns of each plate contained chicken plasma (Sigma-Aldrich) as a positive control, and the remaining six columns used song sparrow plasma. Each well contained a total volume of 25 µL, with plasma dilutions ranging from 1 (i.e., undiluted plasma, row 1) to 1:1024 in PBS (row 11).

Row 12 of each column contained 25 µL PBS and no plasma, and served as a negative control.

We added 25 µL of 1% RRBC suspension (Rockland Immunochemicals) to each well, covered plates and incubated them at 37°C for 90 min, and tilted plates at a 45° angle for 20 min. Plates were scanned at 300 dpi with a top-lit flatbed scanner (Epson V600), and two independent observers each noted the weakest dilution of plasma that was sufficient to induce agglutination. Thus, lower values reflect greater agglutination ability. We then incubated plates at 20°C for 70 min and scanned again to assess lysis of RRBC by plasma (Matson et al. 2005). However, similar to previous findings in this species (Kubli and MacDougall-Shackleton 2014), we observed very few instances of lysis, thus we report only the hemagglutination results below.

Immunoglobulin Y (IgY):

As a measure of *acquired* constitutive immunity, we assessed circulating concentration of IgY, the functional equivalent of mammalian IgG and the major constitutive antibody in birds (Warr et al. 1995). Following Bourgeon and Raclot (2006) we measured IgY using an enzyme-linked immunosorbent assay (ELISA). First, to identify the appropriate plasma dilution for use in song sparrows, for each of 12 song sparrows we added 2 µL plasma to 998 µL of dilution solution, consisting of 0.1 M sodium carbonate and 0.1 M sodium bicarbonate solutions mixed to a pH of 9.6, in a flat bottom 96-well plate (Corning #3596). We then performed twofold serial dilutions into dilution solution, for final dilutions ranging from 1:500 to 1:64 000.

The plate was covered and incubated for 1 h at 37°C, then for 24 h at 4°C. We then washed the plate twice with 200 µL of PBS-Tween solution (0.05% Tween

dissolved in PBS), added 100 µL of 5% powdered milk dissolved in PBS-Tween solution to each well, and incubated again for 1 h at 37°C. We again washed the plate twice with PBS-Tween, then added 100 µL of anti-chicken IgY (Sigma A9046) diluted 1:250 in PBS-Tween solution to each well. We incubated the plate for 2 h at 37°C, washed each well again with PBS-Tween, and added 100 µL of reveal solution (0.0031% hydrogen peroxide in ABTS [2-2' azino-bis (3-ethyl-benzthiazoline-6-sulphonic acid)]) to each well. The plate was incubated for a final time for 1 h at 37°C, then absorbance at 405 nm read immediately using a microplate reader (BIO-RAD iMark).

We calculated the average absorbance (i.e. IgY level) for each dilution across all 12 samples in the pilot procedure, and plotted average absorbance units as a function of plasma concentration. Slope was maximal at a plasma dilution of 1:4000, so the final analysis used plasma at this dilution rather than across a range of dilutions. Final assays were conducted in duplicate for each individual (two wells of 1:4000 plasma in dilution solution) with two wells per plate serving as a negative control (no plasma; dilution solution only). Conditions for the final assays were otherwise as described above, and for each sample we estimated IgY concentration based on average absorbance for the two duplicate wells.

c) Stable isotope analysis of overwinter latitude

We estimated the latitude at which each individual had overwintered using stable isotope analysis of deuterium (∂^2H) content of winter-grown claw tissue. Details of this analysis are provided elsewhere (Kelly et al. 2016, Lymburner et al. 2016), but in brief, we took advantage of a latitudinal gradient in the ∂^2H content of growing-season precipitation across North America. Amount-weighted values of ∂^2H increase

248 with decreasing latitude, and because these isotopic patterns are transferred up the
249 food web to the consumer, the latitude at which metabolically inert tissues were
250 grown can be estimated (Wassenaar and Hobson 2000; Hobson et al. 2014).
251 In the field, we clipped the distal 2.5 mm from each bird's back claw. Rates of
252 claw growth in white-throated sparrows (*Zonotrichia albicollis*), which are closely
253 related and ecologically similar to song sparrows, indicate that this sample should
254 correspond to tissue grown during the winter months (Kelly et al. 2016). Claw
255 samples were stored, cleaned of surface oils, and prepared as described elsewhere
256 (Kelly et al. 2016), then analyzed for nonexchangeable hydrogen at Environment
257 Canada's Stable Isotope Laboratory (Saskatoon, Canada), using online continuous-
258 flow isotope ratio mass spectrometry (CF-IRMS) on a Micromass Isoprime mass
259 spectrometer (Micro-mass UK, Manchester, UK) interfaced with a Eurovector
260 elemental analyzer (Milan, Italy). Isotopic measurements were performed on H_2 gas
261 derived from high-temperature (1350°C) flash pyrolysis of claw samples and keratin
262 standards.
263 To correct for the effects of H exchange with ambient water vapour, we
264 analyzed three Environment Canada keratin standards (caribou hoof standard, CBS: -
265 197‰; spectrum keratin, SPK: -121.6‰; kudu horn standard, KHS: -54.1‰) using
266 the comparative equilibrium method (Wassenaar and Hobson 2003). Based on within-
267 run replicate analyses of five of each keratin standard, the analytical precision was
268 estimated to be ± 2‰. We report non-exchangeable ∂^2H values expressed in delta
269 notation of units per mil (‰) and normalized on the Vienna Standard Mean Ocean
270 Water- Standard Light Antarctic Precipitation (VSMOW-SLAP) scale.
271
272

d) Genetic analysis of philopatry

As part of a previous study, we inferred natal philopatry for all study subjects using genetic assignment tests based on microsatellite genotypes (Kelly et al. 2016). We collected blood for genetic analysis, extracted DNA, and genotyped birds at 12 microsatellite loci [Mme 1 and 12 (Jeffrey et al. 2001); Pdoμ 5 (Griffith et al. 1999); and Sosp 1, 2, 3, 4, 5, 7, 9, 13 and 14 (Sardell et al. 2010)] as detailed elsewhere (Kelly et al. 2016).

As reported elsewhere (Kelly et al. 2016), we entered each bird's microsatellite genotype into GeneClass2 (Piry et al. 2004) together with the genotypes of an additional 308 song sparrows previously captured at our study site or at one of ten other sites within 50 km. We inferred natal philopatry using the Lhome option, which does not require that all potential source populations have been sampled. Lhome ranges between 0 and 1, and represents the probability of an individual's genotype occurring in the site from which it was sampled. Thus, higher values of Lhome indicate relatively philopatric individuals, while lower values indicate individuals more likely to have immigrated (dispersed) from outside the capture site. Values of Lhome were arcsine-transformed for use in linear models predicting immune response, to improve normality of model residuals (details below).

e) Data analysis

Scaled mass

As an estimate of relative energy reserves, we corrected mass for structural size (wing length) using a scaled mass index (equation [2] in Peig and Green (2009)), based on measurements from birds captured in April 2013 and 2014. We tested for sex differences in scaled mass using *aov* in base R, version 3.3.2 (R Core Team 2016).

298 We tested for seasonal changes in scaled mass using a linear model regression (*lm* in
299 base R; scaled mass ~ capture date, coded as days since 1 April).
300
301 *Correlations between immune measures, repeatability, and PCA*
302 We used Pearson's correlation to investigate relationships between immune
303 measures. For the 14 individuals sampled in both years of the study, we also
304 calculated the repeatability of macrophage phagocytosis of *E. coli* based on among-
305 and within-individual components of variance (Lessells and Boag 1987) derived from
306 a one-way ANOVA. To reduce dimensionality within the 2013 immune dataset, we
307 zero-centered and scaled the three immune variables studied that year (macrophage
308 phagocytosis of *E. coli*; hemagglutination; IgY). We conducted a principal
309 components analysis (PCA) on the correlation matrix, and saved unrotated factor
310 scores, using *prcomp* in base R.
311
312 *Predictors of immune function*
313 We used *lm* in base R to construct linear models predicting immune PC scores
314 for the 2013 dataset. We first constructed a fully-saturated model including claw δ^2H
315 (interpreted as latitudinal migration distance), arcsine-transformed Lhome (interpreted
316 as natal philopatry), capture date (days since 1 April) and scaled mass (calculated as
317 described above; interpreted as energy reserves) as predictors of interest, along with
318 the potential nuisance variables of age class and sex. We confirmed normality of
319 residuals and fit to other model assumptions by visually inspecting residuals and Q-Q
320 plots, then used the *anova* command in base R to compare this fully-saturated model
321 to a nested model without age class and sex as predictors.

Some predictors of interest were correlated with one another (i.e., claw ∂^2H and arcsine-transformed Lhome, Pearson's $r_{1,47}$ = -0.22; date and scaled mass, Pearson's $r_{1,47}$ = 0.36). To assess the risk of bias due to collinear model terms, we calculated variance inflation factors (VIFs) from a linear model with the predictors claw ∂^2H, arcsine Lhome, date, and scaled mass, using *vif* in the R package car (Fox and Weisberg 2011). VIFs for these variables ranged from 1.14 to 1.21, well below the threshold of 5 that would suggest biasing of parameter estimates, so all four variables were retained for analysis.

We used an information theoretic approach (Burnham and Anderson 2002) to compare support for sixteen alternative models predicting immunity (i.e. one model set for each retained immune PC). Each model comprised a different combination of claw ∂^2H, Lhome, date, and scaled mass, and each model set included a null model (e.g. PC1 ~ 1). We compiled model-averaged parameter estimates from the full set of AICc-ranked candidate models using the natural averaging method (Burnham and Anderson 2002) implemented in *model.avg* in the R package MuMIn (Bartoń 2016). As a complementary analysis to determine whether maternal antibody allocation might underlie seasonal changes in immunity, we split the dataset by sex and conducted model ranking and averaging as above, but separately for each sex.

Results

Scaled mass

Following the recommendations of Peig and Green (2009), we used wing rather than tarsus as the length (structural size) variable, because wing length had the stronger correlation with mass on a log scale for our sample (r_{wing} = 0.42, r_{tarsus} = 0.32). We calculated the scaling coefficient b_{SMA} to be 1.90; and set L_0 as the sample average

wing length (i.e., 64.2 mm). Scaled mass was greater in females (mean ± SD = 22.91 g ± 2.30) than males (21.37 g ± 1.84; $F_{1,47} = 6.58$, $p = 0.014$), and increased with date ($R^2 = 0.11$, $F_{1,47} = 7.13$, $p = 0.010$).

Correlations between immune measures, repeatability, and PCA loadings

For the three immune variables measured in 2013, macrophage phagocytosis (a cellular innate defence) was negatively correlated to levels of IgY (an acquired defence; $r_{52} = -0.29$, $p = 0.03$). Plasma agglutination, a complement-mediated innate defence reflecting levels of natural antibody, was not significantly correlated with macrophage phagocytosis ($r_{52} = 0.13$, $p = 0.36$) or with IgY ($r_{52} = -0.004$, $p = 0.98$). Among the 14 individuals sampled in both 2013 and 2014, macrophage phagocytosis was not significantly repeatable between years (ANOVA, $F_{1,13} = 0.43$, $s^2 = 0.0084$, $s^2_A = -0.0003$, repeatability = -0.04, $p = 0.93$).

Principal component analysis of the 2013 dataset identified two components with eigenvalues ≥ 1 (Table 1), which we retained for further analysis. Positive values of immune PC1 were associated primarily with low macrophage activity against E. coli, but high levels of IgY. Thus, we interpreted PC1 as investment in acquired, as opposed to innate, constitutive defence. Positive values of immune PC2 were associated primarily with low concentrations of plasma required to induce agglutination, that is, with high levels of natural antibody. Neither PC1 nor PC2 differed between age classes (PC1: SY 0.28 ± 1.19, ASY -0.38 ± 1.08, $F_{1,47} = 3.70$, $p = 0.06$; PC2: SY -0.10 ± 0.98, ASY 0.09 ± 1.08, $F_{1,47} = 0.41$, $p = 0.52$) or sexes (PC1: females -0.08 ± 1.30, males 0.18 ± 1.04, $F_{1,47} = 0.59$, $p = 0.45$; PC2: females -0.15 ± 1.23, males 0.10 ± 0.69, $F_{1,47} = 0.74$, $p = 0.39$); values are reported as means ± SD. Comparing fully-fitted models predicting immune PC1 and PC2 with and without

the nuisance variables of age class and sex confirmed that including these variables did not improve model fit (ANOVA, PC1: $F = 0.32$, $p = 0.73$; PC2: $F = 0.39$, $p = 0.68$).

Predictors of immune function

Of the sixteen candidate models predicting immune PC1, models containing date as a covariate were substantially better-ranked than models not containing date (Table 2). Averaging across the full set of models predicting PC1 determined that this immune component increased with date (Figure 1), but did not vary significantly with claw ∂^2H (interpreted as seasonal migration distance), Lhome (interpreted as natal philopatry), or scaled mass (interpreted as energy reserves; Table 3a; Figure 1). Sex-specific analyses yielded qualitatively similar results; PC1 increased with date for both males and females but did not vary significantly with claw ∂^2H, Lhome, or scaled mass (Figure 1; Supplementary Table 1).

Of the sixteen candidate models predicting immune PC2, the null model was the top-ranked (Table 4) although several models had similar AICc values. Averaging across the full set of models predicting PC2 confirmed that this immune component did not vary significantly with claw ∂^2H, Lhome, date or scaled mass (Table 3b).

Discussion

The pace of life hypothesis, when applied to ecoimmunology, posits that variation among species and populations in the balance between different types of immunity reflects position along a slow- versus fast-living axis. In particular, slow- versus fast-living taxa differ in the extent to which individuals re-encounter pathogens to which they have previously been exposed, and thus, the benefits of investing in acquired

immune defence (Lee 2006, Martin et al. 2006, 2007). We extended this logic to the individual level, reasoning that stable individual variation in long-distance movements (seasonal migration and natal dispersal), and in the case of migration, the recency of such movements, should also influence exposure to novel versus familiar pathogens and thus the optimal balance between innate and acquired defences. Consistent with trade-offs within the immune system, measures of an innate defence (macrophage phagocytosis) were negatively associated with a measure of acquired immunity (IgY). However, individual variation in the balance between innate and acquired defence did not vary significantly with migration distance or natal dispersal tendency. The strongest predictor of this innate/acquired balance was date, consistent with individuals shifting from primarily innate to primarily acquired defence throughout the early breeding season (i.e., with increasing time at the breeding grounds and time since spring migration).

Correlations between, and repeatability of, immune measures

The vertebrate immune system is complex, with multiple inter-related components. Similar to previous findings from this study system (Kubli and MacDougall-Shackleton 2014), not all the immune defences examined here were positively correlated. Macrophage phagocytosis of *E. coli*, a cellular innate defence, was negatively related to IgY level, a non-cellular acquired defence. This pattern is consistent with trade-offs or compensatory relationships between different branches of immunity (e.g. Martin et al. 2007; Wegner et al. 2007; Forsman et al. 2008). However, inferring trade-offs is problematic without experimental manipulation, for example as accomplished by Keil and colleagues (2001). Moreover, we measured only one, non-cellular, component of acquired immunity (IgY). Thus, while we

interpret the negative relationship between IgY and macrophage phagocytosis as reflecting a trade-off between acquired and innate defence, an alternative explanation is the trade-off is instead between cellular and non-cellular defences. Undermining this alternative, however, we found no relationship between macrophage phagocytosis and the hemagglutination ability of plasma (a non-cellular innate defence). The diversity of relationships we observed among even a handful of assays highlights the risks of drawing far-reaching conclusions about 'immunocompetence' from studies -- including the current one-- that examine a limited number of immune parameters.

Our finding that macrophage phagocytosis was not significantly repeatable among years highlights another danger in interpreting immune parameters as stable indicators of 'quality'. That is, we found no evidence for consistent individual differences, at least in this measure of immunity. Power to detect among-individual variation was likely constrained somewhat by sample size (N = 14 individuals with measurements repeated in both years), and substantial within-individual variation was likely enhanced by the long time interval (one year) between measurements. However, even in captive experiments where environmental differences among individuals are likely minimal and the interval between measurements relatively short, immune measures have low repeatability (e.g. swelling response to phytohemagglutinin in European starlings *Sturnus vulgaris* and zebra finches *Taenopygia guttata*; Granbom et al. 2005, Love et al. 2008). Such results, combined with variation in immune responses among and within seasons (e.g. this study; Eikenaar and Hegemann 2016) highlight the remarkable plasticity of vertebrate immunity, and suggest that immune measures should be interpreted as snapshots of dynamic systems, not as stable traits.

Predictors of immune function

We hypothesized that song sparrows adaptively allocate resources to innate versus acquired defences in response to their relative risk of encountering unfamiliar versus familiar pathogens. We reasoned that this relative risk should vary among individuals, reflecting variation in their overwinter latitude (migration distance) and natal dispersal tendency. However, neither migration distance (inferred from claw ∂^2H) nor natal dispersal (inferred from genetic assignment testing) reliably predicted the balance between our measures of innate and acquired defence. At least three factors, not mutually exclusive, may help to explain this lack of relationship.

First, as noted above, the vertebrate immune system is complex and we measured only a small number of immune parameters. This undermines our ability to draw definitive conclusions about individual variation in the overall balance between general and pathogen-specific defences. In particular, cellular mechanisms of acquired immunity developed during the first year of life may effectively 'vaccinate' individuals against pathogens encountered on the wintering grounds or on stopover (Møller et al. 2004). Second, our assumption that long-distance movements (seasonal migration, natal dispersal) increase the relative risk of encountering novel as opposed to familiar pathogens, may be incorrect. However, at least one important class of pathogens affecting migratory birds (haematozoan parasites) shows pronounced geographic variation (Pagenkopp et al. 2008). Furthermore, migrant species harbour a greater diversity of parasites than do resident species (Figuerola and Green 2000), although comparable information at the individual level is lacking. Third, limitations to phenotypic plasticity may constrain the ability of individuals to allocate resources optimally to innate versus acquired pathways.

Whereas the balance between innate and acquired defences did not vary predictably with individual variation in long-distance movement, we did observe a shift towards increasing levels of acquired immunity (IgY) relative to innate immunity (macrophage phagocytosis) as the breeding season progressed. Because we observed this pattern in males as well as females, we think it unlikely that this seasonal trend is driven exclusively by sex-specific changes in immunoglobulin levels as females approach laying. Because models including size-corrected mass did not perform better than models without this covariate, we also think it unlikely that our findings result from a seasonal improvement in energetic condition. Instead, our findings are consistent with adaptive allocation based on the relative risk of encountering new versus familiar pathogens and thus, the relative benefits of general versus specific immune defences. Novel pathogen encounters are likely to be highest during migration then decrease with increasing time spent in a single location (in this case, on the breeding grounds).

Similar to our findings, immunoglobulin concentrations increase throughout the breeding season in great tits *Parus major* (Pap et al. 2010), and phagocytosis activity decreases in captive red knots *Calidris canutus* (Buehler et al. 2008). Mechanistically, such variation may be mediated by seasonal variation in sex hormones and corticosterone (e.g. Evans et al. 2000, Casto et al. 2001, O'Neal and Ketterson 2012). Indeed, previous work in this study population found that plasma testosterone levels in males vary negatively with macrophage phagocytosis and also increase between early April and early May (Kubli 2011). Baseline levels of plasma corticosterone did not vary seasonally or predict measures of constitutive innate immunity in that study (Kubli 2011). However, in house sparrows *Passer domesticus*, sensitivity to corticosterone as assessed by glucocorticoid receptor binding in the

spleen peaks during the pre-laying period (Lattin et al. 2013). In light of the immunoenhancing effects of short-term increases in glucocorticoids (Dhabhar and McEwen 1999), this seasonal variation in sensitivity may represent an adaptation to increased risk of parasitism and wounding during early breeding (Lattin et al. 2013). Finally, we note that similar to our findings, Buehler et al. (2008a) detected no seasonal change in plasma agglutination, a non-cellular component of innate immunity. Thus, different components of immunity may respond differently to specific immune risks associated with stages in the annual cycle.

In conclusion, we found evidence for seasonal modulation, but not movement-related variation, in the immune profiles of wild migratory birds. Our findings provide partial, but not complete, support for the hypothesis that individuals adjust the balance between specific and nonspecific immune defences based on the relative risk of encountering novel versus familiar pathogens. Determining whether pathogen communities show greater variation temporally (e.g., over the course of the breeding season) or geographically (e.g., at scale of tens or hundreds of kilometres, corresponding to typical distances for natal dispersal and seasonal migration respectively) seems likely to cast light on this issue. Most conclusively, our findings add to a growing body of research demonstrating the complexity of vertebrate immunity and the risk of inferring "immunocompetence" either from a single measure or at a single point in time.

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659

Figure Legends

Figure 1: Reliance on acquired over innate constitutive innate immune defence (immune PC1) increased with date throughout the early breeding season in song sparrows. Filled and open symbols denote males and females, respectively.