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INDICES OF IMMUNE FUNCTION ARE LOWER IN RED KNOTS (*CALIDRIS CANUTUS*) RECOVERING PROTEIN THAN IN THOSE STORING FAT DURING STOPOVER IN DELAWARE BAY

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ABSTRACT.—Each year, hundreds of thousands of shorebirds use Delaware Bay, on the northeast coast of the United States, as a final stopover before migration to breeding areas. The bay provides them with abundant Horseshoe Crab (*Limulus polyphemus*) eggs, which they use to gain the fat stores necessary for continued migration and subsequent survival on the breeding grounds. However, abundant food attracts dense mixed-species flocks, which may facilitate pathogen transmission, and migration itself may suppress immune defense. Despite the potential importance of disease risk and immune function during migration, little is known about how immune function changes during stopover in migratory shorebirds. To examine this, we measured constitutive immune function in Red Knots (*Calidris canutus rufa*) during stopover in Delaware Bay. We found lower total leukocyte, lymphocyte, and monocyte concentrations, complement-mediated lysis, and haptoglobin activity in birds recovering protein after migration than in birds storing fat to fuel subsequent flight. We discuss two possible reasons for this result. First, fueling birds may have an increased rate of infection or may be bolstering immune defense in response to high antigen exposure. Second, recovering birds may be immuno-compromised because of the physical strain of migratory flight or as a result of adaptive tradeoffs between immune function and migration. Received 15 January 2009, accepted 26 August 2009.

Key words: *Calidris canutus*, Delaware Bay, immune function, migration, Red Knot, stopover.

Los Índices de Función Inmune son Menores en Individuos de *Calidris canutus* que se Encuentran Recuperando Proteínas que en los que Están Acumulando Grasas durante Paradas Migratorias en la Bahía Delaware

RESUMEN.—Cada año, cientos de miles de aves usan la Bahía Delaware, en la costa noreste de Estados Unidos, como última parada migratoria antes de migrar hacia las áreas de cría. Esta bahía provee abundantes recursos, como los huevos de *Limulus polyphemus*, que son usados para acumular grasas necesarias para continuar la migración y contribuyen a la supervivencia subsecuente en las áreas de cría. Sin embargo, la abundancia de alimento atrae bandadas mixtas con alta densidad de individuos, lo que puede facilitar la transmisión de patógenos, además de que la migración por sí sola también puede suprimir la defensa inmune. A pesar de la importancia potencial del riesgo de enfermedad y de la función inmune durante la migración, se sabe poco sobre cómo cambia la función inmune durante las paradas migratorias de las aves costeras. Para examinar esto, medimos la función inmune constitutiva de individuos de *Calidris canutus rufa* durante la parada migratoria en la Bahía Delaware. Encontramos lisis mediada por el complemento, actividad de haptoglobina y concentraciones totales de leucocitos, linfocitos y monocitos más bajas en aves que se encontraban recuperando los niveles proteicos después de la migración que en aves que se encontraban acumulando energía para el vuelo subsiguiente. Discutimos dos posibles razones para este resultado. Primero, las aves que se encuentran acumulando energía pueden tener una tasa más alta de infección o pueden estar reforzando su sistema inmune en respuesta a una alta exposición a antígenos. Segundo, las aves que se están recuperando pueden estar inmunológicamente comprometidas debido al esfuerzo físico durante el vuelo migratorio o como resultado de una solución de compromiso adaptativa entre la función inmune y la migración.

EACH YEAR, HUNDREDS OF THOUSANDS of shorebirds migrate through a small number of highly productive staging areas before the last leg of their journey to breeding areas in the Arctic (van Gils et al. 2005). These sites provide important food

resources that enable birds to store fat, which is essential for successful migration and important for survival on the breeding grounds if weather conditions become difficult (Morrison et al. 2005, 2007). However, despite abundant food resources, the risk

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of illness during stopover may be high. Ingestion of large amounts of food exposes the gastrointestinal tract to numerous novel antigens, large numbers of birds feeding in high-density flocks may facilitate disease transmission, and the strenuous exercise of migratory flight itself may compromise immune function (Buehler and Piersma 2008). Despite the potential importance of disease risk and immune function during migration, little is known about how immune function varies during stopover in long-distance migrants (but see Owen and Moore 2008a).

Here, we focus on Red Knots (*Calidris canutus rufa*) during spring migration in Delaware Bay, on the northeast coast of the United States, the last stopover site for shorebirds on the West Atlantic Flyway. Horseshoe Crabs (*Limulus polyphemus*) deposit their eggs on the bay's sandy beaches, providing abundant food for large numbers of shorebirds during spring migration (Castro and Myers 1993, Haramis et al. 2007). Red Knots stop in the bay area for 2–3 weeks (Harrington 1996, Gillings et al. 2009) and approximately double their body mass from 90–120 g to 180–240 g. Different wintering populations use the bay area, and stable-isotope analysis indicates that shorter-distance migrants eat mussels on the Atlantic coast, whereas longer-distance migrants feed on crab eggs within the bay (Atkinson et al. 2006). We captured birds on bay beaches, so our samples likely contained Red Knots that had just completed migrations of >8,000 km from stopover sites in South America (Piersma et al. 2005).

During migratory flight, Red Knots first use fat stores and then begin protein catabolism (van der Meer and Piersma 1994). At stopover sites, this process is reversed and birds recover protein before depositing fat. Atkinson et al. (2007) studied this relationship in Red Knots in Delaware Bay to determine the point at which birds have recovered protein lost during migratory flight and begin to deposit fat. They found that birds under a “breakpoint” of 133 g are recovering mainly protein and gain very little fat (~15%), whereas birds over 133 g gain mainly fat (~84%). In this way, body mass can indicate the progression of fueling during stopover.

We examined how immune function differed between newly arrived birds that were still recovering protein lost during migratory flight and fueling birds that were depositing fat. To examine immune function, we measured constitutive (non-induced) immune function because it represents the birds' first line of defense and is likely the most important defense during short stopovers when there is not enough time to mount an acquired response (Schmid-Hempel and Ebert 2003). Furthermore, this measure does not require keeping birds in captivity or recapturing birds during this very sensitive time in their migration. Specifically, we measured leukocyte concentrations (Campbell 1995), complement and natural antibody levels (Matson et al. 2005), and haptoglobin activity (Matson 2006).

METHODS

Birds and sampling.—Birds were captured using cannon nets between 16 and 28 May 2007 on the inner bay beaches of Slaughter Beach, Mispillion Harbor, and Port Mahon along the southwest coast of Delaware Bay. All sites were ≥ 20 km away from the Atlantic coast. At capture, biometrics were taken and birds were banded, weighed, and aged as adults on the basis of plumage characteristics (Prater et al. 1977). The sex of each bird was later determined

using molecular techniques (Baker et al. 1999). A total of 108 birds were caught (63% male, 37% female); however, 1 bird escaped before body mass was taken, and for 2 others we were unable to collect a sufficient volume of blood ($n = 105$).

Our data do not tell us exactly how long a given bird had been in Delaware Bay (see Gillings et al. 2009). However, we build upon previous work on Red Knot physiology, by van der Meer and Piersma (1994) and Atkinson et al. (2007), to categorize birds. Body mass can be broken down into structural (protein) and storage (fat) components, and fasting or migrating birds first draw on fat stores before catabolizing protein (van der Meer and Piersma 1994). Applying this model in reverse implies that when birds gain body mass they recover protein first and then fat. Atkinson et al. (2007) tested this idea using data from dissected Red Knots captured during migratory stopover in Delaware Bay. They found that the data fit a two-phase (broken stick) model with the dependent variable, proportion of body mass gained as fat, changing from 0.15 ± 0.12 to 0.84 ± 0.05 at a breakpoint of 133 ± 5 g (means $\pm$ SE; see Atkinson et al. 2007: fig. 2). In other words, the mean mass at which birds have recovered protein lost during migratory flight and begin to deposit fat as fuel for the next journey is ~133 g. Assuming that the Red Knots we measured followed the same pattern as those measured by Atkinson et al. (2007), we categorized birds into a protein-recovery group (body mass < 133 g, minimum = 95.9 g, maximum = 132.1 g, mean $\pm$ SD = 119.2 ± 10.8 g, $n = 24$) and a fuel-storage group (body mass > 133 g, minimum = 133.2 g, maximum = 201.6 g, mean $\pm$ SD = 163.8 ± 17.1 g, $n = 81$). We consider this a valid assumption because we worked with the same species in the same location at the same time of year and our sample is representative of the range of body mass examined by Atkinson et al. (2007, see fig. 2), approximately 90–133 g versus 133–230 g. We further assumed that birds still recovering protein had arrived in the bay more recently than those depositing fat stores.

We sterilized the area around the brachial vein with 70% ethanol and then collected 200–400 μ L of blood into heparinized capillary tubes (Fisher Emergo, Landsmeer, The Netherlands). Blood samples used to assay leukocyte concentrations were taken within 25 min (mean $\pm$ SD = 14.8 ± 5.5 min) of cannon net firing (first stress for the birds). Time-series experiments on Red Knots showed no effect of handling stress on leukocyte concentrations when blood was sampled within 30 min of first stress (Buehler et al. 2008a). Immediately after sampling, we made two blood smears and the remainder of the blood was stored on ice and transported to the laboratory. Blood samples not used for leukocyte analysis were taken within 2 h of capture (73.0 ± 51.8 min). Complement and natural antibody titers are insensitive to handling stress up to at least 2 h after first stress (Buehler et al. 2008a). Because the sensitivity of haptoglobin to handling stress has not been tested, we included time between capture and sampling as a covariate in statistics with haptoglobin as the response variable. Plasma was obtained by centrifuging blood samples for 10 min at $12,000 \times g$. The plasma was stored at -20°C in Delaware, transported frozen, and stored in The Netherlands at -80°C until processing.

Immune assays: Leukocyte concentrations.—Total leukocyte concentrations provide information on circulating immune cells and current infection (Campbell 1995). Furthermore, the concentrations of different types of leukocytes (heterophils, lymphocytes, monocytes, and thrombocytes) may provide information

about other aspects of immune function. Heterophils are important phagocytes that respond immediately to novel pathogens during the initial immune response. Thrombocytes are also phagocytic and in addition play a role in blood clotting. Monocytes link constitutive, nonspecific (innate) defense to induced, specific (acquired) immune functions. Lymphocytes orchestrate the antibody and cell-mediated functions of the acquired immune system (Campbell 1995). Blood smears were stained (Giemsa Stain, Sigma-Aldrich, Germany), randomized, and counted blind to stopover progression by a single observer (D.M.B.) at 1,000 $\times$ magnification using the criteria in Campbell (1995). The first 100 leukocytes were counted and classified as heterophils, eosinophils, lymphocytes, or monocytes; however, eosinophils had a high proportion of zero values and were excluded from further analysis. While counting the first 100 leukocytes, we also recorded the number of thrombocytes seen as an estimate of the relative number of thrombocytes per leukocyte. Total leukocyte concentrations were obtained in combination with the blood smears using the indirect eosinophil Unopette method (Campbell 1995), following the manufacturer's instructions (no. 5877; BD, Franklin Lakes, New Jersey). The relative number of thrombocytes per leukocyte was also converted to a concentration using the total leukocyte count. Sample sizes for total leukocyte concentrations are smaller than for other assays because of the need to sample birds within 25 min of capture ($n = 38$). However, the sample still adequately covered a range of body masses below and above 133 g (body mass < 133 g, minimum = 107.1 g, maximum = 130.3 g, mean $\pm$ SD = 119.2 ± 8.8 g, $n = 7$; body mass > 133 g, minimum = 133.2 g, maximum = 195.9 g, mean $\pm$ SD = 162.4 ± 16.6 g, $n = 31$).

Immune assays: Hemolysis–hemagglutination.—The complement cascade and natural antibodies provide a first line of defense against spreading infections via cell lysis and link innate and acquired immunity (Ochsenbein and Zinkernagel 2000). We placed 25 μ L of plasma in the first and second rows of a 96-well plate and then from the second to the 11th rows we performed ten 1:2 dilutions using Dulbecco's PBS (Mauck et al. 2005). We then added 25 μ L of 1% of rabbit red blood cell suspension to each well and incubated the plates at 37°C for 90 min. After incubation, we tilted the plates 45° and then digitally scanned them (Epson Perfection 4990 scanner) for agglutination after 20 min and lysis after 90 min. The scans were randomized and were scored blindly by a single observer (D.M.B.) for lysis and agglutination using the criteria outlined in Matson et al. (2005). Complement activity was measured as the last titer at which the lysis of rabbit red blood cells occurred and natural antibody activity as the last titer at which the agglutination of rabbit red blood cells occurred (presented as the $-\log_2$ of the dilution titer).

Immune assay: Haptoglobin.—Haptoglobin is an acute phase protein that binds iron (heme) to keep it from providing nutrients to pathogens (Delers et al. 1988). Furthermore, heme is recognized as an important promoter of oxidative stress during exercise in humans (Cooper et al. 2002); thus, haptoglobin may also be important for binding heme during strenuous exercise. Haptoglobin was quantified from blood plasma following the "manual method" instructions provided with a commercially available assay kit (no. TP801; Tri-Delta Diagnostics, Morris Plains, New Jersey). Sample sizes for haptoglobin were smaller than for complement and

natural antibodies because we did not have enough plasma to conduct the assay for every individual ($n = 99$).

Statistical analyses.—We tested the residuals of parametric models for normality using one-sample Kolmogorov-Smirnov tests and visual examination of histograms. To achieve normality, haptoglobin activity was logarithmically (base 10) transformed and complement activity was transformed by raising 2.5 to the power of the lysis titer ($-\log_2$). Agglutination was not normally distributed, and transformation did not improve the situation. Levene's tests were used to examine homogeneity of variances. For leukocyte concentrations and lysis, P values for the Levene's test were between 0.05 and 0.10; thus, we show the results of both parametric and nonparametric models. In our general linear models or nonparametric tests, protein recovery and fuel storage were compared using the main effect "status". In the parametric models, we also included body mass ("mass") as a covariate and the mass*status interaction. Initially, sex was also included as a co-factor; however, the main effect of sex was never significant ($P > 0.75$, except haptoglobin $P = 0.08$) and the inclusion of sex did not affect the outcome of any model, so we present results from models excluding sex. Finally, because the sensitivity of haptoglobin activity to handling stress has not been tested, we included the covariate "tstress" (time in minutes between capture and blood sampling) in the model for haptoglobin. Because we performed multiple comparisons and Bonferroni corrections can be too conservative in ecological studies with small sample sizes (Nakagawa 2004), we present partial eta-squared values (η_p^2) as a measure of effect size. Statistical analyses were conducted using SPSS, version 14.0 (SPSS, Chicago, Illinois).

RESULTS

Birds recovering protein had significantly lower total leukocyte concentrations, complement-mediated lysis, and haptoglobin activity than fueling birds (Fig. 1A–C and Table 1). All leukocyte components (heterophils, lymphocytes, monocytes, and thrombocytes) showed the same pattern of lower concentrations during protein recovery, but the trends reached statistical significance only in lymphocytes and monocytes (Fig. 2 and Table 1). For measures of total leukocytes, lymphocytes, monocytes, and haptoglobin the covariate "mass" had a marginally negative effect (Table 1), which was most pronounced in birds storing fuel. In other words, within birds categorized as storing fuel, heavier birds had lower scores than lighter birds for these indices of immune function. Natural antibody-mediated agglutination did not differ between protein recovery and fuel storage (Fig. 1D and Table 1). In addition to a main effect of "status" for lysis, "mass" and "status" interacted such that lysis decreased slightly with mass in birds gaining protein and increased with mass in birds gaining fat ($F = 8.29$, $df = 1$ and 101, $P = 0.005$, $\eta_p^2 = 0.08$). However, the data only just met the assumptions of homogeneity of variance, and the nonparametric test for "status" effect, which could not take the interaction into account, did not reach statistical significance (Table 1). Nevertheless, lysis increased significantly with body mass, as indicated by a nonparametric correlation (Spearman's $\rho = 0.26$, $P = 0.007$).

Handling time had a significant effect in the model for haptoglobin activity ($F = 19.6$, $df = 1$ and 95, $P < 0.001$, $\eta_p^2 = 0.17$). Birds experiencing the longer handling times had lower haptoglobin

FIG. 1. Indices of constitutive immune function in Red Knots on stopover at Delaware Bay in May 2007 in either the protein-recovery or the fat-storage phase. Data on (A) total leukocyte concentrations, (B) lysis, (C) haptoglobin, and (D) agglutination are shown as box plots in which the median (thick line), interquartile range (boxes), range (whiskers), and outliers (circles) are depicted. See Table 1 for full statistics.

TABLE 1. Statistical tests for indices of immune function in Red Knots during stopover in Delaware Bay in 2007. Parametric general linear models compared birds of different status (recovering protein vs. storing fuel) and included mass as a covariate. Models for lysis also included a status * mass interaction term, and models for haptoglobin included a handling-time covariate (see text). Because of potential problems with small sample sizes and non-homogeneity of variance, we also present nonparametric tests of status (Mann-Whitney tests) to confirm patterns found in parametric models. Effect sizes are partial eta-squared (η_p^2). Significance at the $P < 0.05$ level is shown in bold, and trends where $0.1 > P > 0.05$ are shown in italics.

Immune function	Status				Mass				Nonparametric tests			
	df	F	P	η_p^2	df	F	P	η_p^2	U	W	z	P
Total leukocytes (μL^{-1})	1 and 35	9.40	0.004	0.21	1 and 35	3.93	0.05	0.10	41.5	69.5	-2.52	0.01
Heterophils (μL^{-1})	1 and 35	2.33	0.13	0.06	1 and 35	0.34	0.56	0.01	68.0	96.0	-1.53	0.13
Lymphocytes (μL^{-1})	1 and 35	8.26	0.007	0.19	1 and 35	4.02	0.05	0.10	45.0	73.0	-2.39	0.02
Monocytes (μL^{-1})	1 and 35	2.37	0.02	0.15	1 and 35	3.88	0.06	0.10	69.0	97.0	-1.49	0.14
Thrombocytes (μL^{-1})	1 and 35	0.44	0.51	0.01	1 and 35	0.08	0.78	<0.01	85.0	113.0	-0.88	0.39
Lysis (-log2)	1 and 101	8.35	0.005	0.08	1 and 101	3.14	0.08	0.03	756.0	1,056.5	-1.67	0.09
Agglutination (-log2) ^a									827.0	4,148.0	-1.22	0.22
Haptoglobin (mg μL^{-1}) ^b	1 and 95	12.3	0.001	0.12	1 and 95	3.86	0.05	0.04				

^aParametric tests were not performed because Kolmogorov-Smirnov and Levene's tests indicated a non-normal distribution.

^bNonparametric tests were not performed because transformation achieved normality and a handling-time covariate was required.

FIG. 2. Differential leukocyte concentrations in Red Knots on stopover at Delaware Bay in May 2007 in either the protein-recovery or the fat-storage phase. Data for (A) heterophils, (B) lymphocytes, (C) monocytes, and (D) thrombocytes are presented as box plots in which the median (thick line), interquartile range (boxes), range (whiskers), outliers (circles), and extremes (stars) are shown. See Table 1 for full statistics.

(Pearson's correlation $r = -0.40$, $P < 0.001$). However, this effect was not significant within the first 25 min of capture ($r = 0.13$, $P = 0.48$).

DISCUSSION

We found lower total leukocyte, lymphocyte, and monocyte concentrations, along with lower complement-mediated lysis and haptoglobin activity, in birds that were recovering protein after migratory flight than in birds that were depositing fat during stopover in Delaware Bay. Heterophil and thrombocyte concentrations showed similar patterns that were not statistically significant. Only natural antibody titers did not differ, a result that is not surprising. Natural antibodies are unique among the immune indices measured in that they are not flexible over the annual cycle (Buehler et al. 2008b). Therefore, it is unlikely that natural antibody activity would be flexible over the shorter period of migratory stopover. However, our data indicate that other aspects of

constitutive immune function can change quickly in response to environmental conditions, even within the 2- to 3-week window that migratory birds spend on stopover. The differences that we report may stem from a variety of causes, and here we discuss two possibilities: (1) fueling birds may have an increased rate of infection or be bolstering immune defense in response to high antigen exposure at the feeding grounds, and (2) recovering birds may be immuno-compromised because of the physical strain of migratory flight or as a result of adaptive tradeoffs between immune function and migration.

Assuming that the birds recovering protein arrived in the bay more recently than those depositing fat stores, immune indices may be higher in fueling birds because they have spent more time exposed to antigens in the bay. From a disease-risk perspective, avian influenza has been detected in both shorebirds and gulls in Delaware Bay (Krauss et al. 2007, Hanson et al. 2008), and Red Knots are known to feed in mixed-species flocks (Botton et al. 1994, Vahl et al. 2007) so dense that birds often ingest substrate covered with

the feces of other birds (D. M. Buehler pers. obs.). Such conditions may increase the spread of diseases (Altizer et al. 2006), especially those with fecal-oral transmission. Studies examining immune function and infection status in wild birds are rare. However, we know that experimental injection with lipopolysaccharide (which mimics bacterial infection) increases mean haptoglobin activity to 3 $\times$ that of control birds (Buehler et al. 2009). We report mean haptoglobin activity 1.4 $\times$ higher in fueling than in recovering birds. This indicates that some fueling birds may harbor bacterial or other infections but that it is unlikely that all fueling birds in our sample were infected. Even if pathogenic disease is not present, birds consume copious amounts of food during stopover, thereby exposing the gastrointestinal tract to numerous antigens. Because a major function of the immune system is protection against opportunistic microbes (Janeway et al. 2004, Clark 2008), these circumstances alone may trigger elevated immune function.

Alternatively, recovering birds may have lower immune indices than fueling birds as a result of nonadaptive or adaptive tradeoffs between immune function and migration (*sensu* Owen and Moore 2008a, b). Migratory flight represents strenuous activity, with energy expenditure reaching up to 7 or 8 times the basal metabolic rate in some species (e.g., Gill et al. 2005, Videler 2005). In humans, strenuous activity has been associated with changes in immune function consistent with the patterns we describe: lowered lymphocyte concentrations (Nieman and Pedersen 1999) and reduced complement-mediated lysis (Smith et al. 1990, Wolach et al. 1998). Furthermore, haptoglobin reserves may be depleted during recovery from strenuous activity if haptoglobin is used to bind oxidative iron released during exercise (Cooper et al. 2002). In addition to potentially unavoidable changes in immunity, immune function may be adaptively down-regulated. Energetically costly aspects of immune function may be down-regulated to reallocate energy to migratory flight (Norris and Evans 2000, Klasning 2004). Furthermore, potentially damaging aspects of immune function such as inflammatory or sickness responses may also be down-regulated during migratory flight to avoid immunopathology (Råberg et al. 1998).

To test the idea that immune function is compromised or down-regulated in birds recovering protein, data on immune indices over the entire annual cycle are needed. It is known that the indices that we measured vary over the annual cycle in Red Knots living in captivity (up to 4 $\times$ mean minimum values for total leukocytes and 3 $\times$ for lymphocytes and lysis; Buehler et al. 2008b). However, we cannot directly compare immune index values, because conditions experienced by captive and free-living birds differ considerably and because the birds studied by Buehler et al. (2008b) did not originate from the West Atlantic Flyway. Here, we report smaller differences (1.8 $\times$ for mean total leukocytes and lymphocytes, and 1.1 $\times$ for lysis), which may be understandable given that we are measuring within an annual cycle stage (migration, or more specifically migratory stopover) rather than between them.

It is important to note that factors that differ among individuals (e.g., arrival time and condition) may affect the way different immune indices respond to antigen exposure or physiological stress during stopover. In Delaware Bay, arrival may be asynchronous (Gillings et al. 2009) and differences in condition may be linked to arrival time, with birds in good condition arriving early and birds in poor condition arriving late (Baker et al.

2004, Atkinson et al. 2007). Because Red Knots must achieve a body mass of ≥ 180 g to reach the Arctic on time and with sufficient stores to breed successfully (Baker et al. 2004, Morrison et al. 2005), late-arriving birds may trade immune function for fuel storage. This idea may be of importance for the conservation of shorebirds that use Delaware Bay, because the consequences of such tradeoffs are likely greater in years with insufficient crab eggs. Indeed, population declines in Red Knots have been linked to reduced food abundance in Delaware Bay, and survival was negatively correlated with reduced intestinal and liver masses (organs potentially important for gastrointestinal immune defense), especially in late-arriving birds (Baker et al. 2004). Unfortunately, we do not have data on arrival times, and we recommend future studies to test these ideas.

In summary, our data showed a clear difference in immune function between birds recovering protein and birds fueling for the next leg of migration by storing fat. Although we cannot determine the cause of this variation, this study opens the door for future research to examine the contributions of antigen exposure and physiological stress to variation in immune function during migratory stopover. Such research should include measures of immune function throughout the annual cycle to determine whether immune function in recovering birds is low or immune function in fueling birds is high. Furthermore, to examine the possibility that antigen exposure underlies the patterns we observed, studies that concurrently assess immune function, disease prevalence, and pathogen pressure will be needed in free-living birds throughout the flyway. Finally, experimental studies that, for example, measure immune indices before and after flight in a wind tunnel will help to elucidate the effects of migratory flight on immune function while controlling for pathogen pressure and current infection. Whatever the cause of the patterns we report, they suggest that lower levels of immune function may be part of the flight stage of the migratory phenotype (*sensu* Piersma 2002), whereas higher levels of immune function may characterize the fueling stage of migration. If this is the case, we predict a pattern of increasing (from protein recovery to fuel storage) and then decreasing (from fuel storage to departure) immune function as stopover progresses. This idea is supported by the finding that within birds storing fuel, heavier birds had lower leukocyte concentrations and haptoglobin than lighter birds. In the future, more detailed studies of migratory stopover that compare newly arrived and fueling birds with birds that have reached departure mass will be important for testing this idea.

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LITERATURE CITED

- ALTIZER, S., A. DOBSON, P. HOSSEINI, P. HUDSON, M. PASCUAL, AND P. ROHANI. 2006. Seasonality and the dynamics of infectious diseases. *Ecology Letters* 9:467–484.
- ATKINSON, P. W., A. J. BAKER, K. A. BENNETT, N. A. CLARK, J. A. CLARK, K. B. COLE, A. DEKINGA, A. DEY, S. GILLINGS, P. M. GONZÁLEZ, AND OTHERS. 2007. Rates of mass gain and energy deposition in Red Knot on their final spring staging site is both time- and condition-dependent. *Journal of Applied Ecology* 44:885–895.
- ATKINSON, P. W., A. J. BAKER, K. A. BENNETT, N. A. CLARK, J. A. CLARK, K. B. COLE, A. DEY, A. G. DUIVEN, S. GILLINGS, P. M. GONZÁLEZ, AND OTHERS. 2006. Using stable isotope ratios to unravel shorebird migration and population mixing: A case study with Red Knot *Calidris canutus*. Pages 535–540 in *Waterbirds around the World* (G. C. Boere, C. A. Galbraith, and D. A. Stroud, Eds.). Stationery Office, London.
- BAKER, A. J., P. M. GONZÁLEZ, T. PERSMA, L. J. NILES, I. DE LIMA SERRANO DO NASCIMENTO, P. W. ATKINSON, N. A. CLARK, C. D. T. MINTON, M. K. PECK, AND G. AARTS. 2004. Rapid population decline in Red Knots: Fitness consequences of decreased refuelling rates and late arrival in Delaware Bay. *Proceedings of the Royal Society of London, Series B* 271:875–882.
- BAKER, A. J., T. PERSMA, AND A. D. GREENSLADE. 1999. Molecular vs. phenotypic sexing in Red Knots. *Condor* 101:887–893.
- BOTTON, M. L., R. E. LOVELAND, AND T. R. JACOBSEN. 1994. Site selection by migratory shorebirds in Delaware Bay, and its relationship to beach characteristics and abundance of horseshoe crab (*Limulus polyphemus*) eggs. *Auk* 111:605–616.
- BUEHLER, D. M., N. BHOLA, D. BARJAKTAROV, W. GOYMANN, I. SCHWABL, B. I. TIELEMAN, AND T. PERSMA. 2008a. Constitutive immune function responds more slowly to handling stress than corticosterone in a shorebird. *Physiological and Biochemical Zoology* 81:673–681.
- BUEHLER, D. M., F. ENCINAS-VISO, M. PETIT, F. VÉZINA, B. I. TIELEMAN, AND T. PERSMA. 2009. Limited access to food and physiological trade-offs in a long distance migrant shorebird. II. Constitutive immune function and the acute-phase response. *Physiological and Biochemical Zoology* 82:561–571.
- BUEHLER, D. M., AND T. PERSMA. 2008. Travelling on a budget: Predictions and ecological evidence for bottlenecks in the annual cycle of long-distance migrants. *Philosophical Transactions of the Royal Society of London, Series B* 363:247–266.
- BUEHLER, D. M., T. PERSMA, K. MATSON, AND B. I. TIELEMAN. 2008b. Seasonal redistribution of immune function in a shorebird: Annual-cycle effects override adjustments to thermal regime. *American Naturalist* 172:783–796.
- CAMPBELL, T. W. 1995. *Avian Hematology and Cytology*, 2nd ed. Iowa State University Press, Ames.
- CASTRO, G., AND J. P. MYERS. 1993. Shorebird predation on eggs of the horseshoe crabs during spring stopover on Delaware Bay. *Auk* 110:927–930.
- CLARK, W. R. 2008. *In Defense of Self: How the Immune System Really Works*. Oxford University Press, New York.
- COOPER, C. E., N. B. J. VOLLAARD, T. CHOUÉIRI, AND M. T. WILSON. 2002. Exercise, free radicals and oxidative stress. *Biochemical Society Transactions* 30:280–285.
- DELERS, F., G. STRECKER, AND R. ENGLER. 1988. Glycosylation of chicken haptoglobin: Isolation and characterization of three molecular variants and studies of their distribution in hen plasma before and after turpentine-induced inflammation. *Biochemistry and Cell Biology* 66:208–217.
- GILL, R. E., JR., T. PERSMA, G. HUFFORD, R. SERVIRANCKX, AND A. RIEGEN. 2005. Crossing the ultimate ecological barrier: Evidence for an 11 000-km-long nonstop flight from Alaska to New Zealand and eastern Australia by Bar-tailed Godwits. *Condor* 107:1–20.
- GILLINGS, S., P. W. ATKINSON, A. J. BAKER, K. A. BENNETT, N. A. CLARK, K. B. COLE, P. M. GONZÁLEZ, K. S. KALASZ, C. D. T. MINTON, L. J. NILES, AND OTHERS. 2009. Staging behavior in Red Knot (*Calidris canutus*) in Delaware Bay: Implications for monitoring mass and population size. *Auk* 126:54–63.
- HANSON, B. A., M. P. LUTTRELL, V. H. GOEKJIAN, L. NILES, D. E. SWAYNE, D. A. SENNE, AND D. E. STALLKNECHT. 2008. Is the occurrence of avian influenza virus in Charadriiformes species and location dependent? *Journal of Wildlife Diseases* 44:351–361.
- HARAMIS, G. M., W. A. LINK, P. C. OSENTON, D. B. CARTER, R. G. WEBER, N. A. CLARK, M. A. TEECE, AND D. S. MIZRAHI. 2007. Stable isotope and pen feeding trial studies confirm the value of horseshoe crab *Limulus polyphemus* eggs to spring migrant shorebirds in Delaware Bay. *Journal of Avian Biology* 38:367–376.
- HARRINGTON, B. 1996. *The Flight of the Red Knot: A Natural History Account of a Small Bird's Annual Migration from the Arctic Circle to the Tip of South America and Back*. W.W. Norton, New York.
- JANEWAY, C. A., P. TRAVERS, M. WALPORT, AND M. SHLOMCHIK. 2004. *Immunobiology: The Immune System in Health and Disease*, 6th ed. Garland, New York.
- KLASING, K. C. 2004. The costs of immunity. *Acta Zoologica Sinica* 50:961–969.
- KRAUSS, S., C. A. OBER, J. FRANKS, D. WALKER, K. JONES, P. SEILER, L. NILES, S. P. PRYOR, J. C. OBENAUER, C. W. NAEVE, AND OTHERS. 2007. Influenza in migratory birds and evidence of limited intercontinental virus exchange. *PLoS Pathogens* 3:e167.
- MATSON, K. D. 2006. Are there differences in immune function between continental and insular birds? *Proceedings of the Royal Society of London, Series B* 273:2267–2274.
- MATSON, K. D., R. E. RICKLEFS, AND K. C. KLASING. 2005. A hemolysis–hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. *Developmental & Comparative Immunology* 29:275–286.
- MAUCK, R. A., K. D. MATSON, J. PHILIPSBORN, AND R. E. RICKLEFS. 2005. Increase in the constitutive innate humoral immune system in Leach's Storm-Petrel (*Oceanodroma leucorhoa*) chicks is negatively correlated with growth rate. *Functional Ecology* 19:1001–1007.
- MORRISON, R. I. G., N. C. DAVIDSON, AND T. PERSMA. 2005. Transformations at high latitudes: Why do Red Knots bring body stores to the breeding grounds? *Condor* 107:449–457.
- MORRISON, R. I. G., N. C. DAVIDSON, AND J. R. WILSON. 2007. Survival of the fittest: Body stores on migration and survival in Red Knots *Calidris canutus islandica*. *Journal of Avian Biology* 38:479–487.
- NAKAGAWA, S. 2004. A farewell to Bonferroni: The problems of low statistical power and publication bias. *Behavioral Ecology* 15:1044–1045.

- NIEMAN, D. C., AND B. K. PEDERSEN. 1999. Exercise and immune function: Recent developments. *Sports Medicine* 27:73–80.
- NORRIS, K., AND M. R. EVANS. 2000. Ecological immunology: Life history trade-offs and immune defense in birds. *Behavioral Ecology* 11:19–26.
- OCHSENBEIN, A. F., AND R. M. ZINKERNAGEL. 2000. Natural antibodies and complement link innate and acquired immunity. *Immunology Today* 21:624–630.
- OWEN, J. C., AND F. R. MOORE. 2008a. Relationship between energetic condition and indicators of immune function in thrushes during spring migration. *Canadian Journal of Zoology* 86:638–647.
- OWEN, J. C., AND F. R. MOORE. 2008b. Swainson's Thrushes in migratory disposition exhibit reduced immune function. *Journal of Ethology* 26:383–388.
- PIERSMA, T. 2002. Energetic bottlenecks and other design constraints in avian annual cycles. *Integrative and Comparative Biology* 42:51–67.
- PIERSMA, T., D. I. ROGERS, P. M. GONZÁLEZ, L. ZWARTS, L. J. NILES, I. DE LIMA SERRANO DO NASCIMENTO, C. D. T. MINTON, AND A. J. BAKER. 2005. Fuel storage rates before northward flights in Red Knots worldwide: Facing the severest constraint in tropical intertidal environments? Pages 262–273 *in* *Birds of Two Worlds: The Ecology and Evolution of Migration* (R. Greenberg and P. P. Marra, Eds.). Johns Hopkins University Press, Baltimore, Maryland.
- PRATER, A. J., J. H. MARCHANT, AND J. VUORINEN. 1977. Guide to the Identification and Aging of Holarctic Waders. British Trust for Ornithology, Tring, United Kingdom.
- RÅBERG, L., M. GRAHN, D. HASSELQUIST, AND E. SVENSSON. 1998. On the adaptive significance of stress-induced immunosuppression. *Proceedings of the Royal Society of London, Series B* 265:1637–1641.
- SCHMID-HEMPEL, P., AND D. EBERT. 2003. On the evolutionary ecology of specific immune defence. *Trends in Ecology and Evolution* 18:27–32.
- SMITH, J. K., D. S. CHI, G. KRISH, S. REYNOLDS, AND G. CAMBRON. 1990. Effect of exercise on complement activity. *Annals of Allergy* 65:304–310.
- VAHL, W. K., J. VAN DER MEER, K. MEIJER, T. PIERSMA, AND F. J. WEISSING. 2007. Interference competition, the spatial distribution of food and free-living foragers. *Animal Behaviour* 74:1493–1503.
- VAN DER MEER, J., AND T. PIERSMA. 1994. Physiologically inspired regression models for estimating and predicting nutrient stores and their composition in birds. *Physiological Zoology* 67:305–329.
- VAN GILS, J. A., P. F. BATTLE, T. PIERSMA, AND R. DRENT. 2005. Reinterpretation of gizzard sizes of Red Knots world-wide emphasises overriding importance of prey quality at migratory stop-over sites. *Proceedings of the Royal Society of London, Series B* 272:2609–2618.
- VIDELER, J. J. 2005. *Avian Flight*. Oxford University Press, Oxford, United Kingdom.
- WOLACH, B., A. ELIAKIM, R. GAVRIELI, E. KODESH, Y. AROM, M. SCHLESINGER, AND B. FALK. 1998. Aspects of leukocyte function and the complement system following aerobic exercise in young female gymnasts. *Scandinavian Journal of Medicine & Science in Sports* 8:91–97.

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