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Bottlenecks, budgets and immunity

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CHAPTER 10

Increasing immune indices in Red Knots suggest high pathogen pressure during stopover in Delaware Bay

Deborah M. Buehler, B. Irene Tieleman and Theunis Piersma

ABSTRACT

Each year thousands of shorebirds use Delaware Bay, USA, as a final stopover before migration to breeding areas. The bay provides them with an abundant food source, horseshoe crab (*Limulus polyphemus*) eggs, which they use to gain mass very rapidly. However, because the birds feed in dense mixed-species flocks, which may facilitate pathogen transmission, pathogen pressure in Delaware Bay may be high. We therefore predicted increased immune indices in birds over the course of stopover. To test this, we measured constitutive immune function in Red Knots (*Calidris canutus*) during stopover in Delaware Bay. We found lower total leukocytes, complement mediated lysis and haptoglobin in new arrivals (<133 grams) than in fuelling birds (≥ 133 grams). This result supports the idea of high pathogen pressure in Delaware Bay and suggests that fuelling birds have an increased rate of infection or are up-regulating of immune indices in response to pathogen pressure.

Submitted

INTRODUCTION

Each year millions of shorebirds migrate through a small number of highly productive staging areas before the last leg of their journey to breeding areas (van Gils et al. 2005a). These sites provide important food resources, but may also have high pathogen pressures. Delaware Bay, USA is one such stopover, where birds rapidly gain mass by feeding on an abundance of horseshoe crab (*Limulus polyphemus*) eggs (Castro and Myers 1993, Haramis et al. 2007). However, from a pathogen pressure perspective, Delaware Bay may be high risk for shorebirds. In the bay, birds feed in dense mixed-species flocks of Red Knots (*Calidris canutus*), Sanderlings (*Caldiris alba*), Semipalmated Sandpipers (*Calidris pusilla*), Ruddy Turnstones (*Arenaria interpres*) and Laughing Gulls (*Larus atricilla*; Botton et al. 1994). On bay beaches, flocks are so dense that it is common for birds to be feeding on substrate covered with the feces of other birds (D. M. Buehler pers. obs.). Thus, the spread of diseases, especially those with fecal-oral transmission, may be high (Altizer et al. 2006). As an example, avian influenza has been detected in both shorebirds and gulls in Delaware Bay (Krauss et al. 2007).

To capitalize on their well described stopover ecology, we focused on Red Knots in this research. Knots from different wintering populations use the Delaware 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, thus our samples likely contain knots from stopover sites in South America. There, in contrast to dense mixed-species flocks in Delaware Bay, knots feed in single-species flocks dispersed over large areas of restinga (González et al. 1996). Red Knots arrive in Delaware Bay exhausted after migrations of over 8000 km (Piersma et al. 2005), and must achieve weights of at least 180 grams by late May or early June in order to reach the Arctic on time and with sufficient stores to breed successfully (Baker et al. 2004, Morrison et al. 2005). During migration, knots first use fat stores and then cross a “breakpoint” and begin protein catabolism (van der Meer and Piersma 1994). At stopover sites, this process is reversed and birds first recover protein before depositing fat. Atkinson et al. (2007) model the relationship between the total mass of an individual and whether it is depositing protein or fat. They find that newly arrived birds under 133 grams are recovering protein and gain very little fat (15%), whereas birds over 133 grams gain approximately 84% fat. In this way, body mass indicates a Red Knot’s progression during stopover.

Given the high disease potential in Delaware Bay we predict increasing immune function over the course of the stopover, such that newly arrived birds, which are still recovering protein, should have lower immune indices than birds that have been in the bay longer and are depositing fat. Increased immune indices in wild birds, with unknown health status, can mean that birds are fighting a current infection or that they have up-regulated immune function to avoid infection in an environment with high pathogen pressure. We measure constitutive (non-induced) immune function because it represents the birds’ first line of defense and is likely the most important defense during short stopovers where there is not enough time to mount an acquired response (Schmid-Hempel and Ebert 2003). Furthermore, it does not require keeping birds in

captivity or recapturing birds during this very sensitive time in their migration. Specifically we measured complement and natural antibody levels (Matson et al. 2005), haptoglobin concentrations (Matson 2006) and leukocyte concentrations (Campbell 1995).

MATERIALS AND METHODS

Birds

As part of an ongoing monitoring program in Delaware Bay, birds were captured using cannon nets between 16 and 28 May 2007. At capture biometrics were taken and birds were banded, weighed and aged as adults based on plumage characteristics (Prater et al. 1977). Sexes were later determined using molecular techniques (Baker et al. 1999). A total of 108 birds were caught (63% male, 37% female); however, one bird escaped before body mass was taken and for two others we were unable to collect a sufficient volume of blood (n = 105).

Stopover progression

Our data do not tell us exactly how long a given bird has been in Delaware Bay. However, the dynamics of fueling can be used as an indicator of a bird's stopover progression. Newly arrived individuals weighing less than 133 grams are recovering protein, whereas individuals weighing more than 133 grams are gaining mostly fat (Atkinson et al. 2007). This difference in the physiology of fuelling gives an indication of how long a bird has been in the bay. Thus, we used body mass to categorize birds into a protein recovery group (< 133 grams) and a fuel storage group (≥ 133 grams).

Blood sampling

We collected 200 to 400 μl of blood into heparinized capillary tubes (Fisher Emergo) after sterilizing the area around the brachial vein with 70% ethanol. To obtain baseline values for leukocyte concentrations, blood samples used for this assay were always taken within 25 min (mean ± SD = 14.8 ± 5.5 min.) of cannon net firing (first stress for the birds). Time-series experiments show no change in leukocyte counts within 30 min of capture (Buehler et al. 2008c). 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 two hours of capture (73.0 min ± 51.8 min.). Complement and natural antibody titers are insensitive to capture and handling times up to two hours (Buehler et al. 2008c). Because the sensitivity of haptoglobin has not been tested, we included time between capture and sampling as a covariate in all statistics with haptoglobin as the response variable. Plasma was obtained by centrifuging blood samples for 10 min at 12000 x 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

Leukocyte concentrations provide information on circulating immune cells and current infection (Campbell 1995). After staining (Giemsa Stain, Sigma-Aldrich, Germany) blood smears were examined at 1000X magnification under oil immersion and the first 100 leukocytes were counted and classified as heterophils, eosinophils, lymphocytes or monocytes. Basophils were extremely rare (< 0.5%) and were not included in the counts. Eosinophils were included in the counts, but because they had a high proportion of zero values were excluded from further analysis. While counting the first 100 leukocytes, thrombocytes were also recorded as an estimate of the relative number of thrombocytes per leukocyte. Blood smears were randomized and counted blind to stopover progression by a single observer (DMB) using the criteria in Campbell (1995) Total leukocyte concentrations were obtained in combination with the blood smears using the indirect eosinophil Unopette method (Campbell 1995) following the manufacturers instructions (No. 5877; Becton Dickinson). Sample sizes for total leukocyte concentrations are smaller than for other assays due to the need to sample birds within 25 min of capture (n = 38).

Hemolysis-hemagglutination assay

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). The amount of haemoglobin released from lysed rabbit red blood cells (hemolysis) indicates complement action and the agglutination of rabbit red blood cells indicates natural antibody activity. Following the procedure outlined in Matson et al. (2005), we placed 25 μl of plasma in the first and second rows of a 96-well plate and then from the second to the eleventh rows we performed ten 1:2 dilutions using Dulbecco's PBS (Mauck et al. 2005). We then added 25 μl 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 (Epson Perfection 4990 scanner) them for agglutination after 20 min and lysis after 90 min. The scans were randomized and were scored blindly by a single observer (DMB) for lysis and agglutination using the criteria outlined in Matson et al. (2005).

Haptoglobin assay

Haptoglobin is an acute phase protein that binds iron (haem) to keep it from providing nutrients to pathogens (Delers et al. 1988). Haptoglobin was quantified from blood plasma following the 'manual method' instructions provided with a commercially available assay kit (#TP801; Tri-Delta Diagnostics, Inc., Morris Plains, NJ). Sample sizes for haptoglobin are smaller than for complement and natural antibodies because we did not have enough plasma to conduct the assay for five birds (n = 100).

Statistical analyses

We used general linear models or non-parametric tests to examine the effect of stopover progression (protein recovery or fuel storage) on immune function. We

included sex in our models as a co-factor (sex was never statistically significant and models including and excluding sex produced the same result) and time between capture and blood sampling as a covariate for haptoglobin concentrations. Covariates were removed from models where $P > 0.05$. Haptoglobin concentrations were logarithmically (base 10) transformed to achieve normality of the data and model residuals. Agglutination and lysis were not normally distributed and transformation did not improve the situation so we show the results of both parametric and nonparametric models. Leukocyte data and model residuals were normally distributed; however, we show the results of both parametric and nonparametric models as count data are often skewed. We used SPSS 14.0 for statistical tests and report mean ± SD in the text.

RESULTS

We found lower total leukocytes, complement mediated lysis and haptoglobin in birds recovering protein than in fuelling birds (Fig. 10.1A to C, Table 10.1). Natural antibody mediated agglutination did not differ between protein recovery and fuel storage (Fig. 10.1D, Table 10.1). Like total leukocytes, heterophils, lymphocytes, monocytes and thrombocytes all showed the same pattern of lower concentrations during protein recovery, but these trends did not reach statistical significance (Fig. 10.2, Table 10.1).

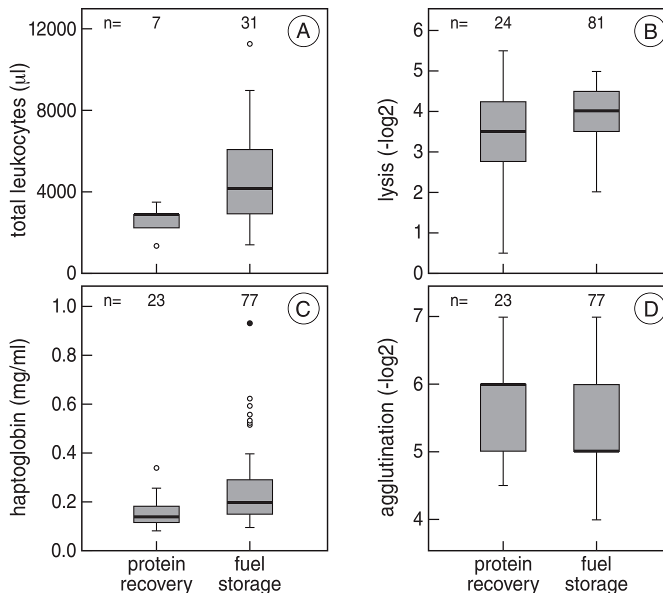

Figure 10.1. Total leukocyte concentrations (A), lysis (B) and haptoglobin (C) were lower during protein recovery than during fuel storage. Agglutination (D) did not differ during the two phases of stopover progression. Box plots show the median (thick line), interquartile range (boxes), range (whiskers), outliers (open dots) and extremes (black dots). See Table 10.1 for statistics.

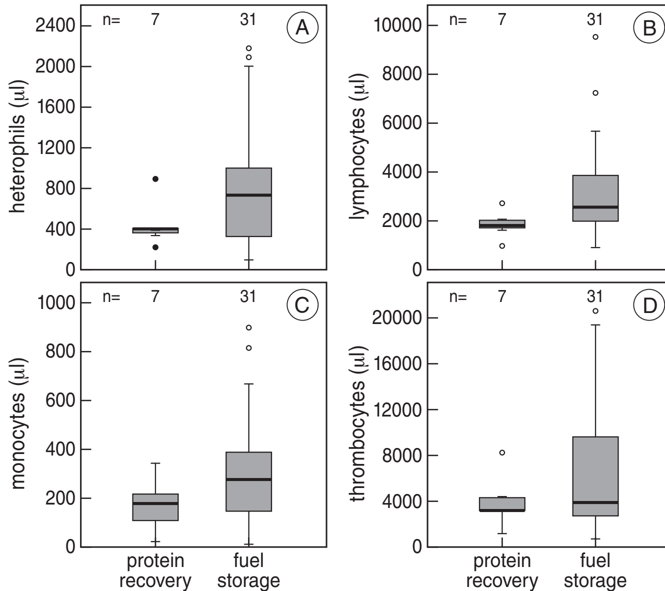

Figure 10.2. Heterophils (A), lymphocytes (B), monocytes (C) and thrombocytes (D) also show a trend for lower concentrations during protein recovery than during fuel storage. Box plots show the median (thick line), interquartile range (boxes), range (whiskers), outliers (open dots) and extremes (black dots). See Table 10.1 for statistics.

DISCUSSION

Constitutive immune function increased from protein recovery to fuel storage in Red Knots during stopover in Delaware Bay. This general increase in leukocytes, haptoglobin and complement suggests an increased rate of actual infection or an up-regulation of immune indices to protect against infection in an environment with high pathogen pressure. It may also suggest that birds arrive with depleted constitutive immunity, which they need to rebuild while fueling. Although we do not have the data to tease these possibilities apart, it is clear that birds that have progressed further in their fueling, and have likely been exposed to pathogen pressure in Delaware Bay longer, have higher constitutive immunity. High levels of constitutive immune function during the period of spring migration have also been found in captive Red Knots that do not actually migrate and have *ad libitum* access to food (Buehler et al. 2008a). Our data suggest that this increase in captivity might indicate that captive birds bolster immune function in anticipation of high pathogen pressure in the wild.

The lack of difference in natural antibody titers between protein recovery and fuel storage is not surprising. Natural antibodies are unique among the immune indices measured in this study in that they are not plastic over the annual cycle (Buehler et al. 2008a). Furthermore, natural antibody levels do not seem to be affected by current

Table 10.1. Statistical tests for differences in immune indices between protein recovery and fuel storage. Statistics were performed on log 10 transformed haptoglobin values. For all other variables the results of both parametric and non-parametric analyses are shown. Significance at the $P < 0.05$ level is shown in bold and trends where $0.1 > P > 0.05$ are shown in italics.

Response	Parametric GLM			Non-parametric tests			
	df	*F*	*P*	*U*	*W*	*z*	*P*
Total leukocytes (per μl)	1,37	5.24	**0.03**	41.5	69.5	-2.52	**0.01**
Heterophils (per μl)	1,37	2.70	0.11	68.0	96.0	-1.53	0.13
Lymphocytes (per μl)	1,37	3.94	*0.06*	45.0	73.0	-2.39	**0.02**
Monocytes (per μl)	1,37	2.37	0.13	69.0	97.0	-1.49	0.14
Thrombocytes (per μl)	1,37	1.73	0.20	85.0	113.0	-0.88	0.39
Lysis (-log2)	1,104	4.95	**0.03**	756.0	1056.5	-1.67	*0.09*
Agglutination (-log2)	1,104	1.73	0.20	827.0	4148.0	-1.22	0.22
Haptoglobin (mg/ml)	1,99	6.20	**0.01**	transformed and covariate			

infection (Matson et al. 2005). Therefore they are not likely to increase as a result of actual infection or in response to pathogen pressure.

An intriguing detail of our data is a marked increase in the variability of leukocyte counts and haptoglobin between birds under 133 grams and those above 133 grams. This variability might indicate differences in individual quality in birds using Delaware Bay. High quality birds may be able to up-regulate immune function to higher levels or, conversely, low quality birds may have higher scores because they are sicker. Either way, this increased variability during fueling suggests individual differences in the ability of birds to resist infection. These quality differences may be linked to arrival time and the need for late arriving individuals to "catch up" (Baker et al. 2004, Atkinson et al. 2007). Late arriving birds may trade-off immune function for fuel storage since there are negative fitness consequences associated with leaving Delaware Bay below 180 grams (Baker et al. 2004). This trade-off is likely to be even more extreme in years with insufficient crab eggs such as 2000, 2003 and 2005 (Atkinson et al. 2007) when birds are crowded into even denser flocks because fewer of the bay's beaches contain eggs (N. Clark, pers. comm.). Larger datasets with data from early and late arriving birds will allow closer examination of this variability.

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