CHAPTER 5

Seasonal redistribution of immune function in a migrant shorebird: annual cycle effects override adjustments to thermal regime

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ABSTRACT

Throughout the annual cycle, demands on competing physiological systems change, and animals must allocate resources to maximize fitness. Immune function is one such system and is important for survival. Yet, detailed empirical data tracking immune function over the entire annual cycle are lacking for most wild animals. We measured constitutive immune indices once a month for a year on captive red knots (*Calidris canutus*). We also examined temperature as an environmental contributor to immune variation by manipulating ambient temperature to vary energy expenditure. To identify relationships among immune indices we performed principal component analysis (PCA). We found significant repeatability in immune indices over the annual cycle and co-variation of immune indices within- and among-individuals. This co-variation suggests "immune strategies" as individual traits among-individuals and the use of different immune strategies during different annual cycle stages within-individuals. Over the annual cycle, both higher cost phagocyte-based immunity and lower cost lymphocyte-based immunity were high during mass change, but there was a clear shift towards lower cost lymphocyte-based immunity during peak molt. Experimental manipulation of temperature had little effect on annual variation in immune function. This suggests that other environmental factors, such as food availability and disease, should also be examined in the future.

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INTRODUCTION

Throughout the annual cycle, demands on competing physiological systems change, and animals must allocate resources to maximize fitness (King 1974). Immune function represents one such physiological system and it contributes to host survival by limiting infection and by performing self maintenance duties such as clearing apoptotic cells. Given its importance, one might expect animals to maintain strong immune defenses throughout year. However, variability in immune defense is widespread (reviewed in Martin et al. 2008), and hypotheses about why this variation occurs include endogenous annual cycles, changes in disease threat and immuno-enhancement in anticipation of disease threat based on changes in environmental cues (Nelson et al. 2002). Variation may also occur because immune defense comes with costs as well as benefits. Building an immune system is developmentally costly and maintaining and using the system has energetic and immunopathology costs (Klasing 2004; Råberg et al. 1998; Schmid-Hempel and Ebert 2003). These costs have led to predictions about trade-offs between immune function and other activities (e.g. reproduction, migration, molt, thermoregulation; Lochmiller and Deerenberg 2000; Norris and Evans 2000; Sheldon and Verhulst 1996). However, evaluating these hypotheses is difficult without detailed empirical data (Hasselquist 2007).

Empirical data tracking immune variation over the annual cycle exist for humans and small mammals (Nelson et al. 2002), but are lacking for other animals. Avian studies, for example, have included comparisons between pairs of life cycle stages (e.g. breeding versus non-breeding or molt versus non-molt, see Martin et al. 2008 for a review), but have not detailed variation over the entire annual cycle. Furthermore, baseline data collected under controlled environmental, nutritional and pathogen pressure conditions are necessary to interpret data taken across seasons in the wild. In small mammals, such baseline data coupled with wild data show that complex physiology-environment interactions in the wild can mask underlying seasonal anticipatory patterns (Nelson et al. 2002).

In addition to the need for baseline data, controlled studies that manipulate environmental factors are needed to tease apart the relative importance of contributors to immune variation. Many environmental factors change over the annual cycle (i.e. temperature, pathogen pressure, photoperiod, food availability) and some, ambient temperature and photoperiod, have been linked to annual variation in immune function in mammals (Nelson et al. 2002). We focus on ambient temperature, which may also influence immune function in birds, especially species wintering in temperate regions where temperature greatly impacts energy expenditure (Wiersma and Piersma 1994). If immune function is traded-off when birds need to invest more on thermoregulation, then we predict that birds living in colder temperatures should show reduced immune function or a shift away from higher cost immunity (sensu Lee 2006, less phagocytosis/ inflammation).

Another environmental contributor to annual variation in immune function is pathogen pressure. Seasonal fluctuations in diseases have been detected in humans, mammals and birds (Nelson et al. 2002) and are likely to affect investment in immune

defense, but in the wild these patterns are extremely complex (Altizer et al. 2006). Even in captivity pathogen pressure is not easily manipulated; however, in some species it can be somewhat controlled by cleaning regimes (Buehler et al. 2008b). If pathogen pressure in captivity is relatively stable, then patterns of immune function observed in captivity may be interpreted in the context of anticipatory cycles, in species where annual patterns of pathogen pressure can be predicted.

Red knots (*Calidris canutus islandica*) are medium-sized (100-200 g) long distance migrant shorebirds with well studied annual cycles (Piersma 2007). In this subspecies, pathogen pressures are predicted to be highest during migration (Buehler and Piersma 2008) when the birds pass through a variety of environments where they might encounter novel pathogens (Møller and Erritzøe 1998). During breeding in the Arctic knots are widely dispersed decreasing the risk of disease transmission. Little is known about pathogen pressure during fall migration, molt and wintering; however it is likely lower than during spring migration (Buehler and Piersma 2008). Fall migration is less synchronous than spring migration and birds are aggregated in smaller flocks (Battley et al. 2004). Given appropriate photoperiod cues, captive red knots exhibit pre-migratory fattening, mass loss and molt cycles comparable to those of free-living birds (Jenni-Eiermann et al. 2002; Piersma et al. 1995). Furthermore, because captive knots do not migrate, anticipatory cycles corresponding to pathogen pressure, which might be masked by extreme physical activity in migrating birds, may also be detected.

The immune system can be divided along an innate (non-specific) and acquired (specific) axis and along a constitutive (non-induced) and induced axis (Schmid-Hempel and Ebert 2003). Immune defense may shift between or even within these axes to provide "optimal immune strategies" in different situations (Lee 2006; Martin et al. 2007b; Schmid-Hempel and Ebert 2003). We chose constitutive immunity to examine resource allocation over the annual cycle because it is effective at controlling multiple pathogen types and responds immediately to threats, making it an evolutionarily relevant first line of defense. Furthermore, mediators of constitutive immunity must be maintained even when not in use, generating costs that may be important in physiological trade-offs during the annual cycle (Martin et al. 2008; Schmid-Hempel and Ebert 2003). Finally, because a response is not induced and immunological memory is not stimulated, repeated measures of individuals throughout the annual cycle can be made. We quantified three categories of constitutive immune indices covering a range of protective functions. First, microbial killing ability which measures the functional capacity of blood to limit microbial infection (Millet et al. 2007; Tieleman et al. 2005). Second, leukocyte concentrations which provide information on circulating immune cells, and differential leukocyte counts which are useful in multivariate analysis in terms of their relationship to functional measures of immunity such as microbial killing. Heterophils and eosinophils mediate innate immunity against novel pathogens and are important phagocytes, monocytes link innate and acquired defence, and lymphocytes mediate pathogen specific antibody and cell-mediated responses of the acquired immune system (Campbell 1995). Third, levels of complement and natural antibodies (Matson et al. 2005) which provide a first line of defense against spreading infections via cell lysis, and link innate and acquired immunity (Ochsenbein and Zinkernagel 2000).

In this study we describe how immune function changes over the annual cycle of red knots and how ambient temperature influences this pattern. To do so, we sampled captive birds once a month over a complete annual cycle. We manipulated thermoregulatory costs using treatments of cold, warm and variable temperatures (the latter tracking outdoor conditions). We first establish the repeatability of immune indices, then examine how different indices co-vary, and finally analyze changes over the annual cycle and the effects of thermoregulatory costs.

MATERIALS AND METHODS

Birds, aviaries and experimental treatments

In August and September 2004 (n = 26) and February 2005 (n = 4) red knots, *C. c. islandica*, were captured in the Dutch Wadden Sea (53°31'N 6°23'E) and brought into captivity at the Royal Netherlands Institute for Sea Research (NIOZ). At capture the birds were ringed, weighed, and aged (all older than two years; Prater et al. 1977) and we later determined sexes using molecular techniques (Baker et al. 1999). We randomly assigned the birds (total n = 30, 21 females, 9 males) to one of three treatment groups in identical indoor aviaries (4.5m x 1.5m x 2.3m high). The birds had free access to food (mudsnails *Hydrobia ulvae*), freshwater for drinking, and before the start of measurements all birds had at least one month to acclimate to captivity. Photoperiod was set to track the seasonal changes in day length in the northern Netherlands for all cages. To verify that all study birds were healthy, we performed weekly assessments of body mass, feather condition and the skin of the feet, elbows (carpals) and keel for signs of local inflammation (e.g. bumblefoot).

The variable treatment birds (n = 12) were divided equally between two aviaries ventilated with outdoor air and maintained at outdoor temperature. During the experiment, variable treatment birds experienced a mean temperature of 15.4 ± 4.8°C (maximum of 25.5°C, 24 June 2005; minimum of 4.6°C, 6 March 2006). This treatment approximates temperatures experienced by free-living *C. c. islandica*, which spend ten months a year in the temperate climate of western Europe (Piersma 2007). Warm treatment birds (n = 12) were also divided between two aviaries and were maintained at 24.7 ± 1.8°C (i.e. within the zone of thermoneutrality; Piersma et al. 1995; Wiersma and Piersma 1994). Cold treatment birds (n = 6) were kept in a single aviary maintained at 4.9 ± 1.2°C. These temperature differences led to a 14% higher mass specific BMR in the cold than in the warm birds in February and March 2005 (Vézina et al. 2006). All groups were similar in terms of sex ratio and morphometrics (Vézina et al. 2006).

During the experiment, three birds died (all female). Two cold treatment birds that died in April and August 2005 were replaced sequentially in June (with a female captured in February 2005) and September (with a male captured in August 2005). Both were given at least a month to acclimate to captivity before entering the experiment. One warm treatment bird that died in February 2006 was not replaced because there was insufficient time to capture and acclimate a new bird before the end of the experiment.

Blood Sampling

Birds were sampled monthly with sampling order randomized by cage and individual. We always collected blood in the morning between 10:30 and 11:00 am (mean ± SD = 10:44 ± 5 min). Each day we sampled two birds within 20 min of entering an aviary (mean ± SD = 6.89 min ± 4.48). We thoroughly sterilized the area around the brachial vein with 70% ethanol and then collected about 600 _l of blood into pre-sterilized, heparinized capillary tubes. Immediately after sampling, we made blood smears and transported the remaining blood in sterile boxes to the laboratory for further processing within an hour of sampling.

Body mass and molt

Throughout the experiment, all birds were weighed and scored for molt and plumage color once a week. We scored wing molt by ranking the left primary feathers on a scale of 0 to 5 (0 for old feathers, 5 for fully grown new feathers, 1 to 4 for growing feathers depending on completeness; Ginn and Melville 1983). We scored body molt by examining the breast area and using the breast molt index (BMI; 0 = no growing feathers, 1 < 25% growing feathers, 2 = 25 to 50% growing feathers, 3 > 50% growing feathers; Piersma and Jukema 1993). Because we were interested in molt as a whole, we calculated total molt (TM) as the sum of BMI and the number of growing primaries (feathers in wing molt categories 1 through 4).

Measuring immune function

Microbial killing abilities

We followed the basic procedure of Millet et al. (2007) and Tieleman et al. (2005) and performed the assay in a sterile working environment (CleanAir CA/R3 Flow Cabinet, CleanAir Techniek B.V., Woerden, The Netherlands). To minimize the effects of different antigen-exposure histories, we used three ubiquitous microbial strains *Escherichia coli* (ATCC 8739), *Candida albicans* (ATCC 10231), and *Staphylococcus aureus* (ATCC 6538; see Appendix A from further details). First, we reconstituted lyophilized microbial pellets following the manufacturers instructions (MicroBioLogics, St Cloud, MN). Then, we diluted freshly-collected blood in CO_2-independent media (#18045-054, Invitrogen, The Netherlands). After subdividing the diluted blood samples for different incubation times (*E. coli* dilution 1:10, incubation 10 and 20 min; *C. albicans* dilution 1:10, incubation 30 and 60 min; *S. aureus* dilution 1:4, incubation 60 and 120 min) we added 20 μl of each microbial suspension to each subsample. These mixtures (totaling 220 μl) were incubated at 41°C and 75 μl was spread evenly onto agar plates in duplicate. Inverted plates were incubated at 36°C overnight; colonies were counted the following day.

For all strains we calculated the proportion of microorganisms killed as one minus the number of colonies on blood plates (described above) relative to the number of colonies on inoculate control plates (200 μl of media mixed with 20 μl of microorganism without incubation). These inoculate plates reflect the initial situation when the blood starts to act on the microorganism, thus best mimicking the situation of a bird responding to a pathogen.

Incubation times for *C. albicans* and *S. aureus* were long enough to allow microbial growth in some cases (i.e. negative killing – see Appendix 5.1). To take this into account, we prepared extra inoculate plates which were incubated the same amount of time as plates containing microorganism and blood. We included counts from these plates in our statistical models as "incubated controls" (see Statistics).

Circulating cellular immunity

At the end of each month blood smears were randomized and counted blind to treatment by a single observer (DMB). After staining (Giemsa Stain, Sigma-Aldrich, Germany), the smears were examined at 1000x magnification with oil immersion and the first 100 leukocytes were counted and classified as heterophils, eosinophils, lymphocytes or monocytes. We were unable to distinguish B and T cells on our blood smears, thus both are included in the lymphocyte count. Basophils were extremely rare (< 0.5%) and were therefore not included in the counts. The number of thrombocytes seen while counting the first 100 leukocytes was also recorded as an estimate of the relative number of thrombocytes per leukocyte. In combination with the blood smears, we obtained leukocyte concentrations using the indirect eosinophil Unopette method (Campbell 1995) following the manufacturers instructions (No. 5877; Becton Dickinson).

Complement and natural antibodies

We performed the assay as described by Matson et al. (2005). We pipetted 25 μl of plasma into the first and second rows of columns B to G in 96-well plates. Chicken serum (Gibco, ref: 16110-082) was used as a positive standard in columns A and H. Using Dulbecco's PBS (Mauck et al. 2005), we serially diluted the plasma and controls from row two to row 11 and left the 12th row as a negative control (PBS only). We then added 25 μl of 1% of rabbit red blood cell suspension to all wells and incubated the plates at 37°C for 90·min. After incubation, plates were tilted at a 45° angle and were scanned (Epson Perfection 4990 scanner) for agglutination after 20 minutes and lysis after 90 minutes. The scans were randomized with respect to sample origin, plate, and location within the plate and were scored blindly for lysis and agglutination by a single observer (DMB). Hemolysis, indicating complement and other lytic proteins, was identified by the absence of intact cells and the presence of free hemoglobin. Hemagglutination, indicating natural antibody activity, was identified by clumped red blood cells. Scores reflect the last plasma dilution in the dilution series (i.e. rows 2 to 11) exhibiting lysis or agglutination (see Matson et al. 2005 Fig 1, for an example).

Statistics

We used 1-sample Kolmogorov–Smirnov tests and visual examination of histograms to examine response variables and model residuals for normality. Although we calculated microbial killing as the proportion of colonies killed, the data were better described by normal than by binomial distributions (Appendix 5.1). Leukocyte data were right skewed and were log10 transformed. After transformation, all variables and the residuals of the models were normally distributed. Thus, we performed univariate analyses

using a linear mixed model with treatment and month as fixed factors and cage and bird as random factors. This approach allowed us to incorporate the nested and repeated measures design of our experiment (bird within cage within treatment, and individual birds sampled monthly) and to include all individuals, even those with missing values (Quinn and Keough 2002). In all comparisons, we ran the models including and excluding an effect of sex. Sex was never significant and never changed the outcome of the model, thus the statistics presented are from models excluding sex. We also included mass and time from cage entry to blood sampling as covariates in all models. For microbial killing capacities, we included the number of colonies inoculated, the ratio of incubated control and inoculation, and the microorganism suspension age (in days) as covariates. Covariates were sequentially removed from the models when not statistically significant at the 0.05 level. Effect sizes were calculated as generalized eta-squared (η_G^2; Olejnik and Algina 2003) and repeatability was calculated following Lessells and Boag (1987) using GLMs with fixed effects and covariates.

To gain insight into relationships between different measures of immune function we performed principle component analyses (PCA). We included only one time point for each killing measure (*E. coli* after 10 min, *C. albicans* after 60 min and *S. aureus* after 120 min) and excluded total leukocyte concentration since it is the sum of the differential concentrations. Eosinophil concentrations were also excluded due to a large number of 0 values. For the leukocyte concentrations we used the transformed data (though transformed and untransformed produce the same result). To account for the nested and repeated measures structure of our data, we performed the PCA on correlation matrixes with the data partitioned into within-individual (between-month) and among-individual levels following the procedure described in (Matson et al. 2006a). We used varimax rotation to maximize contrasts of variable loadings between factors (n.b. with this rotation, component scores calculated from the rotated loadings can be correlated; Joliffe 1995). We also tested the saliency criteria for these loadings (Cliff and Hamburger 1967), and saved scores for components with eigenvalues > 1 for further analysis. SPSS v 14.0 (2005) was used for univariate comparisons and we created the correlation matrices and ran the PCA using STATISTICA 7 (2004).

RESULTS

Patterns over the annual cycle

Body mass and molt

Body mass and molt data confirm previous findings that red knots in captivity retain fattening and molt schedules (Jenni-Eiermann et al. 2002). Birds fattened in early May, mass peaked in late May, and birds lost mass in June and re-stabilized mass in July (Fig 5.1A, Table 5.1). Molt data show prealternate molt (breast, back and head feathers) climaxed during April and May. Prebasic molt (all body, wing and tail feathers) peaked from July until September, but low levels of wing molt extended until January (Fig 5.1B, Table 5.1).

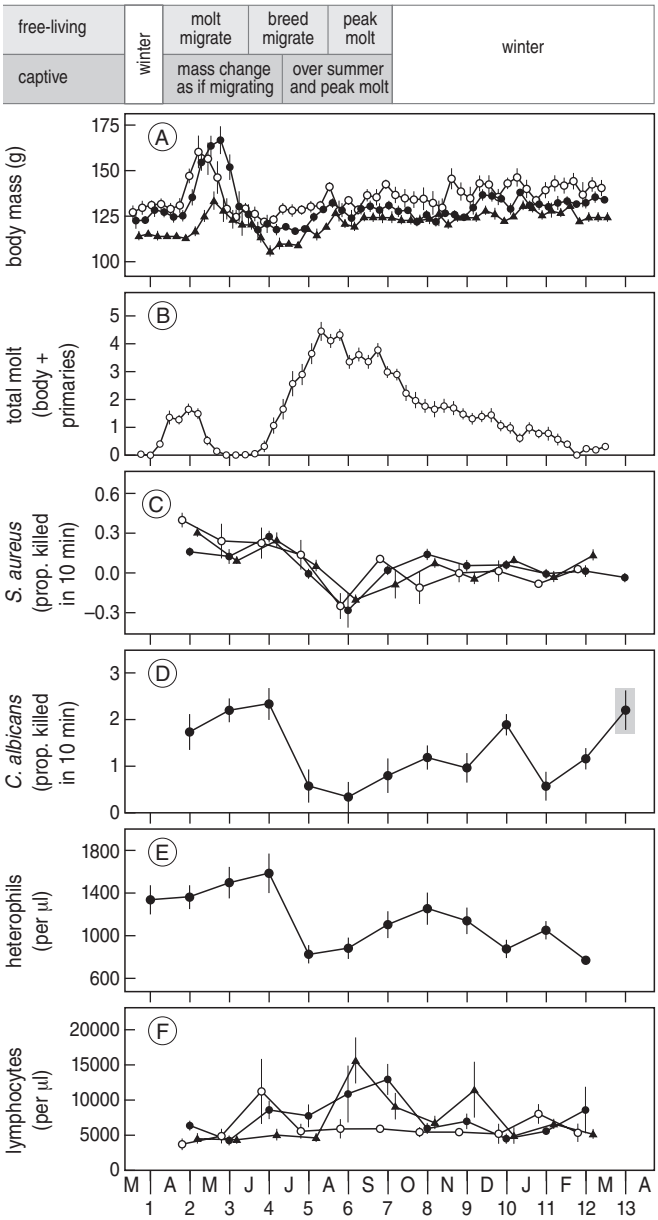

Figure 5.1. Trends in (A) body mass (B) molt and (C) to (J) constitutive immunity throughout the annual cycle. Where significant treatment by month interactions were detected, cold = open circles, variable = filled circles and warm = triangles. For all other variables the general trend is presented. For (E) to (H) statistics were performed on transformed values, but the raw data are shown for ease of interpretation. Symbols represent means and error bars show one SE. Due to repeated measures, error bars can only be used to infer statistical differences between treatments in (A), (C), (F) and (H), but not month to month differences. The annual cycle stages for free-living (Buehler & Piersma 2008) and captive birds (Jenni-Eiermann et al. 2002) are given above the graphs. Numbers under the months correspond to the labels in Fig 2C and boxed points in month 13 represent variable birds only.

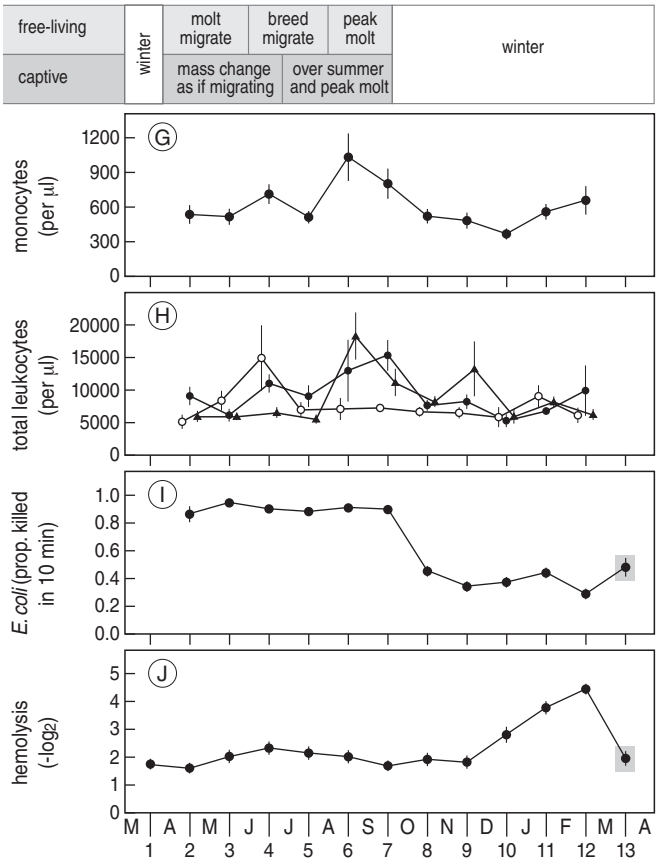

Table 5.1. Categorization of months based on monthly mass means, total molt means and Jenni-Eiermann et al. (2002).

	Month	Mean body mass ± SE	Mass description	Mean total molt ± SE	Total molt description	Category
1	March	123.1 ±1.8	Stable	0.10 ±0.17	Latent	Overwintering (B)
2	April	124.4 ±1.3	Mass gain	1.20 ±0.18	Body molt	Migration (E)
3	May	144.9 ±1.3	Peak mass	0.61 ±0.12	Latent	Migration (F)
4	June	121.2 ±1.0	Mass loss	0.35 ±0.10	Latent	Migration (G)
5	July	119.8 ±1.2	Lowest and rebound	2.74 ± 0.11	Wing and molt increasing	Over-summering (H)
6	August	127.4 ±1.1	Stable	4.01 ±0.10	Peak wing and body molt	Prebasic molt (A)
7	September	129.6 ±1.2	Stable	3.31 ±0.12	Wing and body molt decreasing	Prebasic molt (A)
8	October	127.6 ±1.2	Stable	1.97 ±0.11	Wing molt decreasing	Overwintering (B)
9	November	129.7 ±1.1	Stable	1.59 ±0.10	Wing molt decreasing	Overwintering (B)
10	December	132.7 ±1.4	Stable	1.22 ±0.13	Wing molt decreasing	Overwintering (B)
11	January	133.3 ±1.2	Stable	0.85 ±0.11	Latent	Overwintering (B)
12	February	132.9 ±1.0	Stable	0.38 ±0.10	Latent	Overwintering (B)

Table 5.2. Repeatability of immune function in red knots over the annual cycle (after Lessells & Boag 1987). Repeatability was calculated using models with fixed effects and covariates from individuals for which we had data for all 12 months (n = 27; 11 months for microbial killing). Because covariates differed for different immune indices, error degrees of freedom differ.

Response	Individual Repeatability				
	r	SE	df	F	*P*
Microbial Killing (proportion killed)					
S. aureus after 60 min	0.150	0.043	22,235	3.26	< 0.001
S. aureus after 120 min	0.216	0.053	22,235	4.72	< 0.001
C. albicans after 30 min	0.101	0.033	22,255	2.43	< 0.001
C. albicans after 60 min	0.097	0.032	22,258	2.68	< 0.001
E. coli after 10 min	0.400	0.070	22,258	9.10	< 0.001
E. coli after 20 min	0.469	0.072	22,258	11.64	< 0.001
White blood cell parameters (per μl)					
Total WBC	0.168	0.046	22,264	3.44	< 0.001
Heterophils	0.362	0.068	22,286	7.69	< 0.001
Eosinophils	0.061	0.024	22,286	1.79	0.020
Lymphocytes	0.196	0.050	22,264	3.94	< 0.001
Monocytes	0.135	0.040	22,286	2.85	< 0.001
Thrombocytes	0.049	0.021	22,286	1.61	0.043
Plasma parameters (log2)					
Hemolysis	0.291	0.063	22,286	5.92	< 0.001
Hemagglutination	0.107	0.034	22,286	2.44	< 0.001

Immune function

Repeatability and variability of individual immune measures

Immune function varied throughout the annual cycle at both the among- and within-individual levels. Among-individual differences explained 5 to 47% of total variation, depending on the index, and were significantly repeatable in all measures over the annual cycle (Table 5.2). At the within-individual level, all immune indices except hemagglutination varied significantly over months (Table 5.3).

Co-variation of immune variables among- and within-individuals

Among-individuals, PCA revealed three components with eigenvalues > 1 accounting for 70% of the total variability in the data (Table 5.4A). *S. aureus* killing, *C. albicans* killing, heterophil concentrations and hemolysis were positively correlated with PC1, *E. coli* killing, hemagglutination, and monocyte concentrations were positively correlated with PC2 (Fig 5.2A; monocytes also salient on PC3) and lymphocytes and thrombocytes were positively correlated with PC3. PC1 and PC2 scores were significantly correlated (Pearson r = 0.763, $P < 0.001$; after omitting the outlier, r = 0.655, $P < 0.001$; Fig 5.2B) as were PC2 and PC3 scores (r = 0.273, $P = 0.035$). PC1 and PC3 scores were not correlated (r = 0.173, $P = 0.186$).

Table 5.3. Immune function in red knots over the annual cycle. Effect sizes are generalized eta-squared (η_G^2; Olejnik & Algina 2003). Significance at 0.05 is in italic and at 0.01 in bold. Degrees of freedom vary among indices because in some birds we did not obtain the full set of immune indices from every bird in every month. Statistics for interactions are in the text.

Response	Among-individuals												Within -individuals			
	Treatment - fixed				Cage(Treatment) - random				Bird(Cage(Treat)) - random				Month - fixed			
	df	F	*P*	η_G^2	df	F	*P*	η_G^2	df	F	*P*	η_G^2	df	F	*P*	η_G^2
Microbial Killing (proportion killed)																
S.aureus 60 min	2,2	0.71	0.58	< 0.01	2,25	0.44	0.65	< 0.01	26,278	3.31	< **0.01**	**0.18**	10,278	11.6	< **0.01**	**0.24**
S.aureus 120 min	2,2	0.89	0.53	0.01	2,26	0.49	0.62	0.01	26,258	4.14	< **0.01**	**0.18**	10,258	18.2	< **0.01**	**0.31**
C.albicans 30 min	2,2	6.50	0.14	0.02	2,25	0.24	0.78	< 0.01	26,278	2.82	< **0.01**	**0.19**	10,278	2.88	< **0.01**	**0.07**
C.albicans 60 min	2,2	2.28	0.29	<0.01	2,25	0.13	0.88	< 0.01	26,278	2.66	< **0.01**	**0.18**	10,278	3.87	< **0.01**	**0.10**
E.coli 10 min	2,2	0.49	0.72	< 0.01	2,26	0.13	0.88	< 0.01	26,279	9.31	< **0.01**	**0.13**	10,279	130.1	< **0.01**	**0.71**
E.coli 20 min	2,2	0.32	0.76	< 0.01	2,26	0.33	0.72	< 0.01	26,281	11.80	< **0.01**	**0.22**	10,281	77.8	< **0.01**	**0.57**
White blood cell parameters (per μl)																
Total leukocytes	2,2	0.19	0.84	< 0.01	2,25	2.07	0.15	0.03	26,285	3.03	< **0.01**	**0.17**	11,285	4.38	< **0.01**	**0.10**
Heterophils	2,2	0.73	0.93	< 0.01	2,26	2.94	0.7	0.07	26,307	6.55	< **0.01**	**0.28**	11,307	8.27	< **0.01**	**0.15**
Eosinophils	2,2	0.82	0.55	0.01	2,25	1.15	0.33	0.01	26,219	1.60	*0.04*	*0.12*	11,219	6.39	< **0.01**	**0.21**
Lymphocytes	2,2	0.40	0.72	< 0.01	2,25	1.07	0.36	0.02	26,285	3.50	< **0.01**	**0.19**	11,285	4.64	< **0.01**	**0.11**
Monocytes	2,2	0.08	0.92	< 0.01	2,25	4.79	***0.02***	***0.06***	26,305	2.35	< **0.01**	**0.15**	11,305	2.65	< **0.01**	**0.07**
Thrombocytes	2,2	0.70	0.59	0.02	2,24	4.87	***0.02***	***0.04***	26,307	1.47	0.07	0.09	11,307	4.53	< **0.01**	**0.12**
Plasma parameters (-log2)																
Hemolysis	2,2	7.50	0.14	0.07	2,25	0.35	0.71	0.01	26,310	5.57	< **0.01**	**0.20**	11,310	25.6	< **0.01**	**0.38**
Hemagglutination	2,2	4.40	0.13	0.01	2,25	0.08	0.92	0.01	26,311	2.05	**0.01**	**0.17**	11,311	1.27	0.24	0.04

Table 5.4. Principal component loadings after varimax rotation (A) among-individuals and (B) within-individuals. Bold faced loadings are the highest loading for a measure across the PCs and underlined loadings meet the saliency criteria for that PC.

	Response	PC1	PC2	PC3
(A)	*S.aureus* killing (proportion killed)	**0.870**	0.067	-0.178
	C.albicans killing (proportion killed)	**0.907**	0.152	0.102
	E.coli killing (proportion killed)	0.149	**0.872**	-0.104
	Heterophils (per μl)	**0.835**	0.242	0.001
	Lymphocytes (per μl)	0.178	-0.311	**0.808**
	Monocytes (per μl)	0.186	**0.610**	0.523
	Thrombocytes (per μl)	-0.243	0.239	**0.734**
	Hemolysis (-log2)	**0.542**	0.337	0.099
	Hemagglutination (-log2)	0.415	**0.654**	0.022
	Totals			
	Variance (%) per component	32.1	21.3	17.0
	Cumulative variance (%)	32.1	53.4	70.4

	Response	PC1	PC2	PC3
(B)	*S.aureus* killing (proportion killed)	-0.429	**0.780**	0.113
	C.albicans killing (proportion killed)	-0.351	**0.810**	-0.018
	E.coli killing (proportion killed)	0.290	0.178	**0.776**
	Heterophils (per μl)	0.040	**0.730**	0.458
	Lymphocytes (per μl)	**0.836**	-0.401	0.192
	Monocytes (per μl)	**0.852**	0.019	0.252
	Thrombocytes (per μl)	**0.788**	-0.088	-0.051
	Hemolysis (-log2)	0.496	**0.714**	-0.133
	Hemagglutination (-log2)	0.044	0.033	**-0.952**
	Totals			
	Variance (%) per component	29.8	27.9	20.6
	Cumulative variance (%)	29.8	57.7	78.3

Within-individuals (among-months) PCA also revealed three components with eigenvalues > 1 accounting for 78% of the total variability in the data (Table 5.4B). Lymphocytes, monocytes and thrombocytes were positively correlated with PC1; *S. aureus* killing, *C. albicans* killing, heterophil concentrations and hemolysis were positively correlated with PC2; *E. coli* killing was positively correlated and hemagglutination was negatively correlated with PC3.

Changes in immune function over the annual cycle

Changes in immune indices corresponded to physiological changes that knots experience over a captive annual cycle (Fig 5.1). *S. aureus* killing, *C. albicans* killing and heterophil concentrations peaked during mass change and dropped during peak molt (Fig 5.1C, D and E). Conversely, lymphocyte and monocyte concentrations showed a

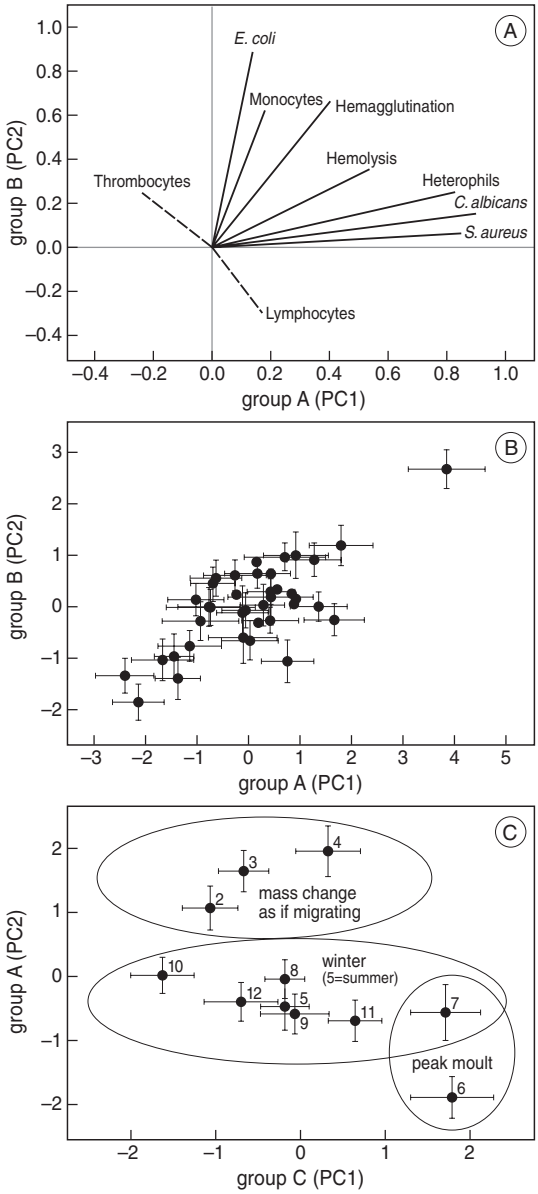

Figure 5.2. Relationships among immune indices based on principal components analysis among-individuals. Axes labels correspond to the alphabetical groups in Table 5. In (a) vectors are the PC1 and PC2 loadings of each measure (dashed vectors are best explained by PC3). Vector length indicates the strength of the relationship and the angle between two vectors gives the degree of correlation (adjacent = highly correlated, orthogonal (90°) = uncorrelated, and opposite (180°) = negatively correlated). In (b) the relationship between PC1 and PC2 scores among-individuals is shown and points (mean ± SE) represent individual birds. In (c) relationships between PC1 and PC2 scores within-individuals indicate redistribution of immune strategies over the annual cycle. Points represent months (mean ± SE) and numbers correspond to the months in Fig 1. Annual cycle stages are based on Jenni-Eiermann et al. (2002) and Table 5.1.

small increase during mass change, but peaked during peak molt (Fig 5.1F and G). Total leukocyte concentrations (the sum of all other leukocyte types) closely matched the pattern in lymphocytes because lymphocytes made up on average 74% of the total leukocyte count (Fig 5.1H).

E. coli killing and hemolysis also changed over the annual cycle; however, these changes did not occur during mass change or molt (Fig 5.1I and J). *E. coli* killing dropped between September and October and then rebounded modestly by April 2006 (boxed point in Fig 5.1I). In contrast, hemolysis increased from December through February and then trended back towards original levels by April 2006 (boxed point in Fig 5.1J).

We plotted monthly mean scores from PC1 and PC2 to further examine annual patterns among immune measures (Fig 5.2C). These scores cluster into three physiologically-relevant periods: (1) mass change (gain, loss and re-stabilization associated with migration) (2) peak wing and prebasic molt, and (3) winter (see Jenni-Eiermann et al. 2002 and Table 5.1 for categorization). During mass change PC2 scores are high, whereas during peak molt PC2 scores are low and PC1 scores are high. In month 5, an "over-summering" transition month between mass change and peak molt for captive red knots, PC2 scores are intermediate.

Effects of temperature treatments

Body mass and molt

Body mass was significantly affected by temperature treatment. Cold birds were consistently heavier than warm birds; variable birds were intermediate (outside the fattening period; Tukey post-hoc Cold > Variable > Warm, $P < 0.001$). A significant week by treatment interaction ($F_{104,1379} = 3.26$, $P < 0.001$) resulted from variable birds gaining more pre-migratory mass than cold birds or warm birds (Fig 5.1A, see Vézina et al. 2007). Molt was not affected by treatment (Molt: $X^2 = 2.33$, df = 2, $P = 0.31$).

Immune function

Annual cycle effects (reflected by month in Table 5.3) dominated over the effect of our experimental manipulations of thermoregulatory costs (treatment in Table 5.3). Significant month by treatment interactions were found only for *S. aureus* killing after 120 min ($F_{20,258} = 1.83$, $P = 0.02$, $\eta_p^2 = 0.09$), lymphocyte concentrations ($F_{20,285} = 1.86$, $P = 0.01$, $\eta_p^2 = 0.10$) and total leukocyte concentrations ($F_{20,285} = 1.98$, $P < 0.01$, $\eta_p^2 = 0.13$). With *S. aureus* killing, the interaction, which was driven by significantly lower killing ability in the variable treatment in April ($F_{2,3} = 30.97$, $P = 0.008$), is clearly less important than the overall temporal trends (Fig 5.1C). For lymphocyte and total leukocyte concentrations, cold birds peaked in June and July during lowest mass; warm and variable birds peaked in August and September during peak molt (Fig 5.1 F and H); however, Tukey post-hoc tests revealed no significant treatment effect in any month. No other immune measures showed significant treatment effects (Table 5.3) or significant treatment by month interactions (all p's > 0.10).

DISCUSSION

This study explored immune function over the annual cycle of red knots and examined how ambient temperature influenced this pattern. Our results established the repeatability of immune indices at the level of individual birds and showed that immune indices co-varied similarly among- and within-birds over time, suggesting functional "immune strategies". We discuss these strategies in terms of their possible costs and protective benefits at the among- and within-individual levels. We then discuss how these strategies shift during different stages of the annual cycle in terms of physiological trade-offs and predicted pathogen pressure in the wild. Finally, we discuss the relative lack of temperature effect on these patterns.

Relationships among immune indices

We examined relationships among nine immune indices with a range of protective benefits and hypothesized energetic and immunopathology costs (Table 5.5). Principal component analyses identified three axes (PCs) comprised of the same indices; however, the ordering of the PCs differed within- and among-individuals. For clarity we define these axes as group A, B and C (Table 5) and refer to them as such throughout the discussion.

Group A comprised *S. aureus* killing, *C. albicans* killing, heterophils and hemolysis (Table 5.4A, B). *S. aureus* and *C. albicans* killing are primarily the result of phagocytosis (Millet et al. 2007) and heterophils are the most numerous phagocytic cells in birds (Janeway et al. 2004). Heterophil phagocytosis produces harmful reactive oxygen and nitrogen species (Splettstoesser and Schuff-Werner 2002) and energy requirements may be heightened by their high turnover rates (Janeway et al. 2004). Complement, which mediates lysis, is linked to inflammation (high immunopathology costs) and must be tightly regulated to prevent host tissue damage (Janeway et al. 2004). Thus, we associate group A with more costly immunity in terms of collateral damage to the host (immunopathology cost) and energy requirements when a host encounters a pathogen and the constitutive immunity we measured is put to use (Table 5.5, Klasing 2004, Buehler et al. 2008b). Functionally, these indices are associated with protection from extracellular bacteria and yeast infections. Thus, this strategy (Group A) may be favored under conditions where diversity of these pathogens is high and encounters with new strains are frequent (Schmid-Hempel and Ebert 2003).

Group B comprised *E. coli* killing and natural antibodies (Table 5.4A, B). *E. coli* killing relies on soluble components in blood plasma (Matson et al. 2006b; Millet et al. 2007) rather than more costly phagocytosis (Splettstoesser and Schuff-Werner 2002). Natural antibodies link innate and acquired immune systems (Ochsenbein and Zinkernagel 2000). These molecules exhibit weak affinities and low specificities, but are important for initiating acquired antibody responses, which are highly specific and unlikely to be self-reactive. Thus, relative to group A, we interpreted group B as moderate in terms of immune cost (Table 5.5). Functionally, *E. coli* killing represents the ability to control or prevent infection by some gram-negative bacteria, and natural antibodies are a constitutive component of the acquired immune system providing a first line of defense against pathogen attack (Ochsenbein et al. 1999). Interestingly, hemol-

Table 5.5. Summary and clarification of the nine independent indices measured and hypothesized costs and classifications for the groupings identified by principal components analysis. [1] Millet et al. (2007) [2] Janeway et al. (2004) [3] Splettstoesser & Schuff-Werner (2002) and [4] Matson et al. (2005).

Group	Specific Index	Description and Function	Strategy	Hypothesized Cost	Among-bird	Within-bird
A	*S. aureus* killing *C. albicans* killing Heterophils Hemolysis	Bacteria defense - mostly phagocytosis [1] Yeast defense - mostly phagocytosis [1] Short-lived phagocytes associated with inflammation [2, 3] Complement mediated lysis during inflammation [4]	Constitutive innate immunity (phagocytosis and inflammation)	High - high energy costs due to short lived cells and high immunopathology costs due to inflammation	PC1	PC2
B	*E. coli* killing Hemagglutination	Bacteria defense - little phagocytosis [1] Natural antibody mediated clumping of invaders [4]	Constitutiv innate immunity (soluble factors)	Moderate - lower energy and immunopathology costs for killing by soluble factors rather than phagocytosis	PC2	PC3
C	Lymphocytes Monocytes Thrombocytes	Basis of specific induced immunity [2] Long-lived phagocytes and antigen presenting cells for specific immunity [2] Important for blood clotting [2]	Constitutive *potential* for induced acquired immunity	Low - low energy costs due to longer lived cells and low immunopathology costs due to less host damage	PC3	PC1

ysis did not correlate with group B. Complement and other lytic proteins are also soluble and have correlated with natural antibodies in other analyses (Matson et al. 2006a, Buehler et al. 2008b). The inclusion of monocytes in group B at the among-individual level is also puzzling; however, because monocytes meet the saliency criteria for group C among-individuals and clearly fall into group C within-individuals (Table 5.4B) we discuss them with group C below.

Group C comprised lymphocytes, monocytes and thrombocytes (Table 5.4A, B). Lymphocytes form the basis of cell-mediated (T-cell) and antibody-mediated (B-cell) acquired immunity (Janeway et al. 2004). Antibody-mediated immunity in particular is considered low cost due to its high efficiency and high specificity, which lowers energy and immunopathology costs (Klasing 2004). Monocytes mature into long-lived macrophages and antigen-presenting-cells important for initiating acquired immune responses, and thrombocytes are important for blood coagulation (Janeway et al. 2004). Thus, relative to groups A and B, we interpreted group C as lower cost because of its association with acquired immunity (Table 5.5). Functionally, lymphocyte and monocyte based immunity might be favored in conditions where energy demands are high or where pathogen pressure is associated with reoccurring infections rather than with novel pathogens (Schmid-Hempel and Ebert 2003).

Immune function as an individual trait

Our immune indices co-varied similarly among- and within-birds over time suggesting functional "immune strategies" at multiple levels of organization. This idea is supported by a study describing species-specific immune strategies in mammals (Martin et al. 2007b) and by a study describing similar co-variation among constitutive immune indices in captive and free-living red knots (Buehler et al. 2008b). We found that the proportion of variability explained by each strategy differed among- and within-individuals (Table 5.4A and B). Higher cost indices (Table 5.5) explained more variability among-birds suggesting that individuals are best distinguished by more costly immunity. Furthermore, the positive correlation between group A and B indices among-birds (Fig 5.2B), indicates that individuals maintain either high or low levels of more costly immune strategies. It is also notable that *E. coli* killing and natural antibody levels are positively correlated among-individuals (Table 5.4A), but negatively correlated within-individuals (Table 5.4B). This indicates, like the relationship between group A and B strategies, that individuals maintain either high or low soluble constitutive defenses, but that across the year there is an antagonistic relationship between *E. coli* killing and natural antibody levels.

Redistribution of immune function in different annual cycle stages

Over the annual cycle, limited resources must be allocated to immune defense, predicting an "optimal" portfolio of defenses (Schmid-Hempel and Ebert 2003). Our results suggest that different strategies may be used during different periods of the year (Fig 5.2C).

The period of mass change in captive birds is associated with fattening and spring migration in the wild. Because fattening and migration are energy demanding activities,

trade-offs with immune function may be predicted when resources are limited. However, our results indicate that group A indices, which are associated with higher energy and immunopathology costs, where high during the period of mass change. This may be due to the fact that our birds had *ad libitum* access to food and were not experiencing resource limitation. Studies manipulating food availability are underway to examine this possibility (D. M. Buehler et al. submitted, chapter 7 of this thesis) and studies on free-living birds will be necessary to test whether trade-offs occur under resource limited conditions in the wild. From a disease perspective, fattening and spring migration are considered high risk (Buehler and Piersma 2008). During migration birds pass through a variety of environments where they might encounter novel pathogens (Møller and Erritzøe 1998) and during stopovers they feed in dense flocks facilitating disease transmission (Altizer et al. 2006). Thus, during this period, the protective benefits of group A indices might outweigh their costs.

The period of body mass stabilization in captive birds is associated with over-summering or breeding in the wild. During this period we found an abrupt drop in group A indices (month 5 in Figs 5.1C, D, E and 5.2B), which may represent a transition period between mass change and peak molt in captive birds. From a disease perspective, in the wild, once the knots establish a breeding territory they are widely dispersed. Thus, transmission risk and the change of encountering novel pathogens may be decreased (Buehler and Piersma 2008).

Peak molt occurs at approximately the same time in captive and free-living knots (Fig 5.1). During molt, feathers must be synthesized by keratinocytes (Haake & Sawyer 1986) and experiments have demonstrated that hydrogen peroxides, such as those generated during respiratory burst and phagocyte ingestion (Splettstoesser and Schuff-Werner 2002), inhibit keratinocyte proliferation (O'Toole et al. 1996). Furthermore, growing feathers are a potential cause of dermal inflammation (Silverin et al. 1999), which can be costly in terms of immunopathology (Råberg et al. 1998). Thus, immune strategies that counter infection with less phagocytosis should be favored during molt. Our results support this idea as we found a clear shift from group A to group C indices during peak molt (Fig 5.2C) indicating a decrease in phagocytosis-based defenses and a greater role for lymphocytes and monocytes.

Looking at the annual cycle as a whole our data suggest two main strategies. Group A indices represent defenses that are necessary during periods of high pathogen pressure, but that are costly enough to warrant down-regulation when pathogen pressures are lower and the costs of these defenses outweigh their benefits. Conversely, Group C indices represent relatively low cost immunological functions that may be necessary year-round. To fully test the ideas presented here, studies on free-living birds and induced immune responses will also be required.

No trade-off between constitutive immunity and thermoregulatory costs

Contrary to predictions that ambient temperature might affect immune function in knots, this study suggests temperature in itself is relatively unimportant to overall annual variation. Higher thermoregulatory costs alone do not seem to necessitate trade-offs between constitutive immune function and thermoregulation. Nevertheless,

cold birds did respond physiologically to low temperatures. They were heavier than warm birds (Fig 5.1) and exhibited 14% higher mass specific BMR than warm birds in February and March 2005 (Vézina et al. 2006). Exposure to cold for a year also led to larger spleen size in cold birds (100% heavier than warm birds, fresh mass, F. Vézina unpublished data 2006). The spleen is an important organ for the presentation and recognition of antigens in induced acquired immunity (Janeway et al. 2004, but see Smith and Hunt 2004), indicating that thermoregulatory costs may impact aspects of immunity that were not measured.

Another explanation for the limited effects of temperature treatments on measured immune indices may be that the *ad libitum* feeding regime allowed cold-treatment birds to compensate for increased energy expenditure via higher food intake (Vézina et al. 2006). Energy expenditure is only one component of the energy budget, and future experiments that simultaneously manipulate energy intake are needed to examine the effect of limited resources on immune function. Furthermore, future experiments manipulating other environmental factors that vary over the annual cycle (e.g. photoperiod and pathogen pressure) will help resolve the proximate causes of variation in immune function in wild birds.

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APPENDIX 5.1. SUPPLEMENTARY METHODS FOR MICROBIAL KILLING ASSAY

Choice of micro-organisms

By quantifying the capacity of whole blood to kill micro-organisms *in vitro*, the microbial killing assay measures innate immunity integrated across circulating cellular and plasma components (Millet et al. 2007). In order to minimize the effects of different antigen-exposure histories, we used three common microbial strains, none of which are highly pathogenic (*E. coli* ATCC 8739, *C. albicans* ATCC 10231, *S. aureus* ATCC 6538).

Escherichia coli, gram negative bacteria, are often commensal in the intestinal tract, but can cause infection in the respiratory tract in birds. *Candida albicans* are yeast-like fungi that can cause candidiasis in birds when ingested. *Staphylococcus aureus*, gram positive bacteria, are normal inhabitants of skin but can result in inflammation and infection if they enter a wound (United States Geological Survey 1999).

The *E. coli* strain we used is primarily killed by plasma components of the blood (Matson et al. 2006b; Millet et al. 2007), whereas cellular components of blood are needed for the effective killing of *C. albicans* and *S. aureus* (Millet et al. 2007). Nonetheless, we always used whole blood in order to ensure all assays equally integrated cellular and soluble immune components.

Optimal blood dilutions and incubation times for red knots

We performed pilot studies (in February 2005 for *E. coli* and *S. aureus* and March 2005 for *C. albicans*) to determine the optimal dilutions and incubation times for each strain. We found that on average a 1:10 blood:media dilution killed 62.3% of *E. coli* colonies after 10 min, 76.3% after 20 min and 94.0% after 30 min (n = 3). For *C. albicans* we found killing took longer; on average a 1:10 blood:media dilution killed 23 % after 30 min, 50% after 60 min, 62% after 120min, and 71% after 180 min (n = 2). Killing of *S. aureus* was the lowest, even after increasing the blood concentration. On average a 1:4 blood:media dilution killed only 15.7% of *S. aureus* after 60 min and 32% after 120 min. After 180 min of incubation, killing was back down to 31%, n = 3 indicating that increased incubation did not increase killing. From this pilot study we chose the blood dilutions and incubation times listed in the main text, which gave killing proportions nearest to the centre of the S-shaped dose response curve described in Matson et al. (2006b).

Variation in inoculation concentrations

We quantified the variation in the number of microorganisms added to diluted blood at the start of each assay. For each strain and from the same stock suspension, we produced eight replicate control suspensions (20μl microbial stock in 200μl media) and plated each control suspension in duplicate. Following standard procedure, we calculated the average of each pair of control plates. We found that the coefficient-of-variation among our 8 replicates was 5.8% for *E. coli* (average colonies ± SD: 515.7 ± 30.1, n = 8; for this trial we were targeting 500 colonies), 9.2% for *C. albicans* (167.0 ± 15.4, n = 8) and 9.2% for *S. aureus* (178.5 ± 16.4, n = 8).

Normal versus binomial treatment of microbial killing data

Because microbial killing capacity is calculated as a proportion, the data might better fit a binomial, rather than a normal, distribution. However, for *C. albicans* and *S. aureus* killing, the data were in fact better fit by a normal distribution because in many cases (*C. albicans*: 56% at 30min and 19% at 60min; *S. aureus*: 48% at 60min and 33% at 120min) the bacteria grew in the presence of blood producing "negative killing", or in other words more bacterial colonies with blood than without blood. Biologically, this indicates that the microorganisms actually benefited from the presence of the blood, possibly by using nutritive blood components as food.

This "negative killing" violates the assumption of a binomial distribution bounded by 0 and 1. For *E .coli*, only 3 out of 353 observations showed "negative killing" and when these three observations were set to 0 or excluded, the *E. coli* killing data were well approximated by either a binomial or a normal distribution. Thus, we analysed the *E. coli* killing data using both a GLM in SPSS 14 (2005) and a generalized linear model with a binomial distribution and log link function in MLWIN (Rasbash et al. 2004). The output of the two models was identical and because we examined all other response variables with the GLM we present only GLM output.