PART III

Immune function in free-living birds

CHAPTER 9

Captive and free-living red knots exhibit differences in non-induced immunity suggesting different immune strategies in different environments

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ABSTRACT

Experiments on captive animals, in which conditions can be controlled, are useful for examining complex biological phenomena such as immune function. Such experiments have increased our understanding of immune responses in the context of trade-offs and pathogen risk. However, few studies have examined how captivity itself affects immune function. We used microbial killing, leukocyte concentrations and complement-natural antibody assays to examine non-induced (constitutive) immunity in captive and free-living red knots *Calidris canutus*. Univariate and multivariate analyses indicated that captive and free-living birds differed in their immune strategies. Captive birds showed reduced *S. aureus* killing, *C. albicans* killing, heterophils, and eosinophils. In a principal component analysis, the affected variables fell onto a single axis, that reflected phagocytosis and inflammation based immunity. We discuss possible reasons for this result in an immune cost and protective benefit framework.

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INTRODUCTION

Experiments on captive animals, in which conditions can be carefully controlled, are useful for examining complex biological phenomena such as immune function. For example, experiments on wild birds recently taken into captivity have been important for understanding ecological questions such as how immune function differs in birds with differing reproductive (e.g. Casto et al. 2001), or migration (e.g. Mendes et al. 2006) strategies. Immune defences may play an important role in mediating life-history trade-offs, and measures of immune function have been used to address ecological and evolutionary questions about adaptations of animals to different environmental conditions (reviewed in Lee 2006). However, in the wild, numerous environmental factors act simultaneously, making it difficult to identify which factors influence immune variables, and necessitating experiments on captive animals. Yet, captivity itself presents animals with a different environment and little is known about whether or how this affects immune function.

Captive conditions can differ from wild conditions in many ways and here we focus on two that could affect immune function: energy balance and pathogen risk. In terms of energy balance, resource availability is often high in captivity because free access to food is provided, whereas energy expenditure is often low because housing conditions restrict activity. In contrast, in the wild, food availability can be unpredictable and food must be actively obtained. These differences may be important for immune function because maintaining and using an immune system carries energetic costs (Klasing 2004). Because of these costs, trade-offs between immunity and other costly physiological processes or activities (i.e. growth, reproduction, migration,) have been predicted under conditions of resource limitation (Sheldon and Verhulst 1996, Piersma 1997, Lochmiller and Deerenberg 2000, Norris and Evans 2000). Captive birds, without resource limitations, could be released from trade-offs that are inevitable in the wild. Thus, one might predict that immune function in captive birds might reflect more costly strategies than in wild birds.

Conditions in captivity and the wild may also differ in pathogen risk. Pathogen risk is expected to influence immune function since immune investment is a balance between costs and benefits, and investment is wasted in an environment without pathogens to defend against (Schmid-Hempel and Ebert 2003). Indeed, certain measures of immune function have been shown to correlate with pathogen risk (e.g. Lindström et al. 2004, Martin et al. 2007a). Unfortunately, pathogen risk is difficult to quantify, and studies comparing captive and free-living animals have focused on the transfer of specific diseases from domesticated captives to free-living animals (i.e. Deem et al. 2005), or on common diseases of captivity (Fujita and Kageyama 2007), rather than on overall pathogen risk. Nevertheless, housing of captive animals is cleaned regularly, presumably to decrease exposure to pathogens or at least to limit pathogen diversity (USGS 1999). Thus, one might predict that immune function may be down-regulated or focused on more specific immune defences in captivity because fewer pathogens would be encountered and fewer pathogens would be novel.

The immune system can be divided into innate (non-specific) and acquired (specific) arms and further divided into constitutive (non-induced) and induced branches

(Schmid-Hempel and Ebert, 2003). Constitutive levels of innate immunity can be measured from a single capture because an immune response is not induced prior to measurement. Such assays are ideal for studies on free-living animals and the assays we chose are all non-induced in this methodological sense. We used three techniques: microbial killing (Tieleman et al. 2005, Millet et al. 2007), leukocyte concentrations (Campbell 1995) and complement-natural antibody assays (Matson et al. 2005). Microbial killing measures the capacity to limit microbial infection and is a functional measure of immunity, thus is expected to be flexible in different conditions (Millet et al. 2007). Leukocyte concentrations provide information on circulating immune cells which can be used as an indicator of health (Campbell 1995). Differential leukocyte counts are also 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). The complement cascade and natural antibodies link innate and acquired immunity and provide the first line of defense against spreading infections, including viruses (Ochsenbein and Zinkernagel 2000).

Given differences between captive and wild conditions, the question of whether or how captivity affects immune function is important to put experiments conducted on captive animals into better ecological perspective and to provide insight into the relationship between environmental context and immunity. Our study investigates whether differences in immune function exist between captive and free-living red knots *Calidris canutus* (Linnaeus 1758) by comparing birds recently taken into captivity (< 1 year in captivity) with free-living birds. We consider two predictions: first, more costly immune strategies in captive birds because they have regular access to food and are potentially released from trade-offs; or second, down-regulated immune function in captive birds because they are exposed to fewer or a smaller variety of pathogens.

MATERIALS AND METHODS

Birds and sampling

Red knots are medium-sized shorebirds (100 to 200 g) that acclimate well to captive conditions (Piersma and Ramenofsky 1998), and retain natural mass change and moult cycles in captivity (Jenni-Eiermann et al. 2002, Reneerkens et al. 2007). This indicates that any differences in immune function between captive and free-living knots should represent acclimation to captive conditions rather than cessation of normal physiological rhythms in captivity.

We studied a total of 27 red knots of the subspecies *C. c. islandica* (as determined using the criteria in Nebel et al. 2000), captured in the Dutch Wadden Sea (53ºN, 5ºE). Birds were ringed and weighed at capture and sexes were later determined using molecular techniques (Baker et al. 1999). Birds also were aged on the basis of plumage characteristics (Prater et al. 1977), and to minimize age effects, we used only adults

(older than two years) for both free-living and captive samples. Furthermore, because some measures of non-induced immunity can be affected by current infection, we assessed body mass, condition of the feathers, leukocyte counts, and the skin of the feet, carpal and keel bones for signs of inflammation (bumblefoot) to verify that birds (captive and free) were healthy.

Captive birds

Birds in the captive sample (n = 12) were captured in August and September 2004 (6 females, 3 males) or February 2005 (1 female, 1 male) and kept at the Royal Netherlands Institute for Sea Research (NIOZ). These birds were part of a larger study examining the effect of ambient temperature on immune function over the annual cycle (Buehler et al. 2008a). For this study we only consider data from birds in the variable temperature treatment, which was set up as a control group (ambient temperature mirroring outdoor conditions). The birds were housed in aviaries 1.5 m by 4.5 m by 2.3 m with smooth walls (no mesh) and a smooth floor that was constantly flushed with filtered salt water. A quarter of the aviary floor was covered by an artificial sand flat and also flushed by salt water, and a tray of fresh water was provided for drinking and bathing. The birds had free access to mud snails *Hydrobia ulvae* which can make up a large proportion of their natural diet (van Gils et al. 2003). We cleaned organic waste and flushed the aviary floors with fresh water daily. At weekly intervals, we removed the birds for weighing and moult score while the aviaries were disinfected using bleach.

Free-living birds

We sampled free-living red knots (n = 15) in early September 2005 (7 females, 2 males) and late July 2006 (4 females, 2 males). The birds were captured in mistnets opened and monitored at night on the rising and falling tides.

Blood Sampling

Blood sampling of both captive and free-living birds occurred in the period corresponding to southward migration (July, August and September) to minimize seasonal differences between groups. Captive birds were sampled in July and August 2005 and samples were taken within 13 minutes of entering the aviary (mean ± SD = 5.5 ± 4.1). Free-living birds were sampled in September 2005 and July 2006, immediately upon removal from mistnets. We checked the nets every five to 15 minutes meaning that the absolute longest a bird could hang in a net was 15 minutes, thus all samples were taken within 20 min of the bird hitting the net (mean ± SD = 14.2 ± 4.8). Time-series experiments show no change in any of our immune indices within 20 min of capture (Buehler et al. 2008c) thus we assume that both of our samples represent baseline immune function. However, as a further precaution we also included the time between capture and blood sampling in our statistical analyses.

For both captive and free-living samples, we collected 300 to 600 μl blood into presterilized heparinized capillary tubes (Fisher Emergo) after sterilizing the area around the brachial vein with 70% ethanol. Immediately after sampling we made two blood

smears and the remainder of the blood was transported in sterilized plastic boxes to the laboratory and processed within an hour of sampling.

Because blood samples in captive birds were taken during the day and free-living birds were caught at night, we ran a repeated measures study (analysed using GLM to account for covariates) in which we sampled nine birds (5 females, 4 males) at 10:30 and 02:30, times which represented our captive (10:38 ± 5 min) and free-living (02:00 ± 85 min) sampling times. To avoid resampling the birds on the same day, we followed a rotating design in which each bird was sampled once a day and then allowed a day of recovery. Immune function did not differ with time of sampling (Appendix 1), which is not surprising since red knots can be active at night in captivity (T. Piersma, unpublished data) and are known to feed at night in the wild (e.g. Sitters et al. 2001, van Gils et al. 2005c, Leyrer et al. 2006, Rogers et al. 2006).

Measuring immune function

Microbial killing capacity of whole blood

The microbial-killing assay is a functional measure of the capacity of blood to kill microorganisms *in vitro* and measures immunity integrated across circulating cell and plasma protein components. We use three microorganisms: *Escherichia coli*, a strain of gram negative bacteria; *Candida albicans*, a strain of yeast-like fungi; and *Staphylococcus aureus*, a strain of gram positive bacteria. The use of several microorganisms gives a broad understanding of microbial killing because their killing relies on different mechanisms. *S. aureus* and *C. albicans* are killed mainly by cells via phagocytosis (Millet et al. 2007), whereas *E. coli* killing relies mainly on soluble blood components (Merchant et al. 2003, Millet et al. 2007).

We followed the basic procedure outlined in Millet et al. (2007) and performed the assay in a sterile working environment (a dead air box equipped with a UV Air Cleaner, Base Clear BV, KI-L046-M). For both captive and free-living birds samples were transported to the lab in pre-sterilized capillary tubes sealed with pre-sterilized clay in pre-sterilized Tupperware containers, and blood was processed within one hour of collection. Briefly, for each micro-organism we diluted the blood in CO_2-independent media (#18045-054, Invitrogen) and added 20 μl of microorganism suspension reconstituted from lyophilized pellets (*E. coli* ATCC # 8739, *C. albicans* ATCC #10231, *S. aureus* ATCC # 6538: MicroBioLogics, St Cloud, MN) to a concentration of approximately 200 colonies per 75 μl of diluted blood–bacteria mixture. The mixture was incubated at 41° C (*E. coli*: 10 min., *C. albicans*: 60 min., *S. aureus*: 120 min.) and 75μl was spread onto agar plates in duplicate. The plates were stored upside down at 36° C, and the number of colonies per plate was counted the following day.

For all strains we calculated the microbial killing capacity as one minus the number of colonies on blood plates relative to the number of colonies on inoculate control plates (200 μl of media mixed with 20 μl of microorganism without incubation). We used the inoculate for our calculations because it reflects the initial situation at the time that the blood starts to act, mimicking the biology of a bird responding to a pathogen.

Leukocyte concentrations

Leukocyte concentrations provide a description of circulating cellular immunity. Blood smears were randomized and counted blind to treatment using the criteria in Campbell (1995) 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. Basophils were extremely rare (< 0.5%) and were therefore not included in the counts. 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).

Hemolysis-hemagglutination assay

Complement and other lytic enzymes lyse extracellular pathogens and work with natural antibodies to facilitate initial pathogen recognition and initiate acquired immune responses (Ochsenbein and Zinkernagel 2000). We performed the assay as described by Matson et al. (2005). Hemolysis reflects complement action from the amount of haemoglobin released from lysed rabbit red blood cell and the hemagglutination reflects the interaction between natural antibodies and the antigens on rabbit red blood cells. Hemolysis and hemagglutination were quantified by serial dilution. 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 plates were tilted at a 45° angle and then digitally scanned (Epson Perfection 4990 scanner) for agglutination after 20 min and lysis after 90 min. The scans were randomized with respect to sample origin, plate, and location within the plate and were scored blindly by a single researcher (DMB) for lysis and agglutination using the criteria outlined in Matson et al. (2005).

Statistics

All data and residuals of parametric models were tested for normality using 1-sample Kolmogorov–Smirnov tests and histograms were examined visually. *E. coli* killing data were left-skewed and were squared to achieve normality. Leukocyte concentrations were right-skewed and were logarithmically (base 10) transformed. After transformation all variables conformed to normality.

Because our free-living birds were caught in September 2005 and July 2006 we examined each immune measure over three groups (captive, free-living 2005 and free-living 2006) coded as a fixed factor "captivity" in a general linear model. Where "captivity" was significant we used Tukey tests to determine the significance of group differences. We included sex in our models as a fixed factor (models including and excluding sex produced the same result), and body mass and time between capture and blood sampling as covariates. For microbial killing we also included the number of colonies on the inoculate control as a covariate. Covariates were sequentially removed from the models when not statistically significant.

To gain insight into the complex relationships among measures of immunity (Matson et al. 2006b) we performed a principle component analysis. We excluded total leukocyte concentrations from the analysis since they are the sum of the differential concentrations and thus caused problems with co-linearity. We used varimax rotation to maximize the contrasts of the variable loadings between factors, tested the saliency criteria for these loadings (Cliff and Hamburger 1967), and saved scores for components with eigenvalues > 1 for further analysis (Kaiser 1960).

We used SPSS 14.0 for all statistical comparisons. We report mean ± SD in the text and mean ± SE in the graphs.

RESULTS

Captive birds showed lower *S. aureus* killing ($F_{2,23} = 14.92$, $P < 0.001$), *C. albicans* killing ($F_{2,23} = 19.95$, $P < 0.001$), heterophil concentrations ($F_{2,24} = 7.59$, $P = 0.003$) and eosinophil concentrations ($F_{2,29} = 6.68$, $P = 0.006$) compared with free-living birds (Fig. 9.1). We found no statistically significant difference for total leukocyte count ($F_{2,24} = 1.72$, $P = 0.20$), lymphocytes ($F_{2,24} = 1.34$, $P = 0.28$) or monocytes ($F_{2,24} = 0.30$, $P = 0.74$; Fig. 9.1). For *E. coli* killing ($F_{2,23} = 6.14$, $P = 0.007$), hemolysis ($F_{2,24} = 22.47$, $P < 0.001$) and hemagglutination ($F_{2,24} = 6.32$, $P = 0.006$) differences between the free-living groups were larger than differences among captive and free-living groups (Fig. 9.1). Free-living individuals caught in 2005 killed more *E. coli* than those caught in 2006, but showed less lysis and agglutination.

The principal component analysis identified three PCs with eigenvalues > 1 that cumulatively accounted for 77% of the total variation. The patterns of loadings on these PCs, revealed that *S. aureus* killing, *C. albicans* killing, heterophil count and eosinophil count correlated with PC1 (29.6% of total variation) and lymphocytes and monocytes correlated with PC2 (24.9% of total variation; Table 9.1, Fig. 9.2A). Paralleling the univariate analysis, captive and free-living birds differed significantly on PC1 ($F_{2,24} = 16.68$, $P < 0.001$) with captive birds significantly lower (captive versus Sep05, $P < 0.001$, captive vs. Jul06, $P = 0.008$; Fig. 9.2B), but did not differ on PC2 ($F_{2,24} = 0.303$, $P = 0.74$; Fig 2b). *E. coli* killing, hemolysis and hemagglutination correlated with PC3 (22.0% of total variation; Table 9.1).

DISCUSSION

We found that captivity reduced certain measures of immune function, but not others, suggesting that birds may use different immune strategies, representing "optimum" immunity, in different environments (Schmid-Hempel and Ebert 2003). Here we discuss our results, by first considering the results of the principal component analysis, then by examining the costs and benefits of the immune measures reduced by captivity, and finally by proposing a hypothesis about why captive and free-living birds may use different strategies.

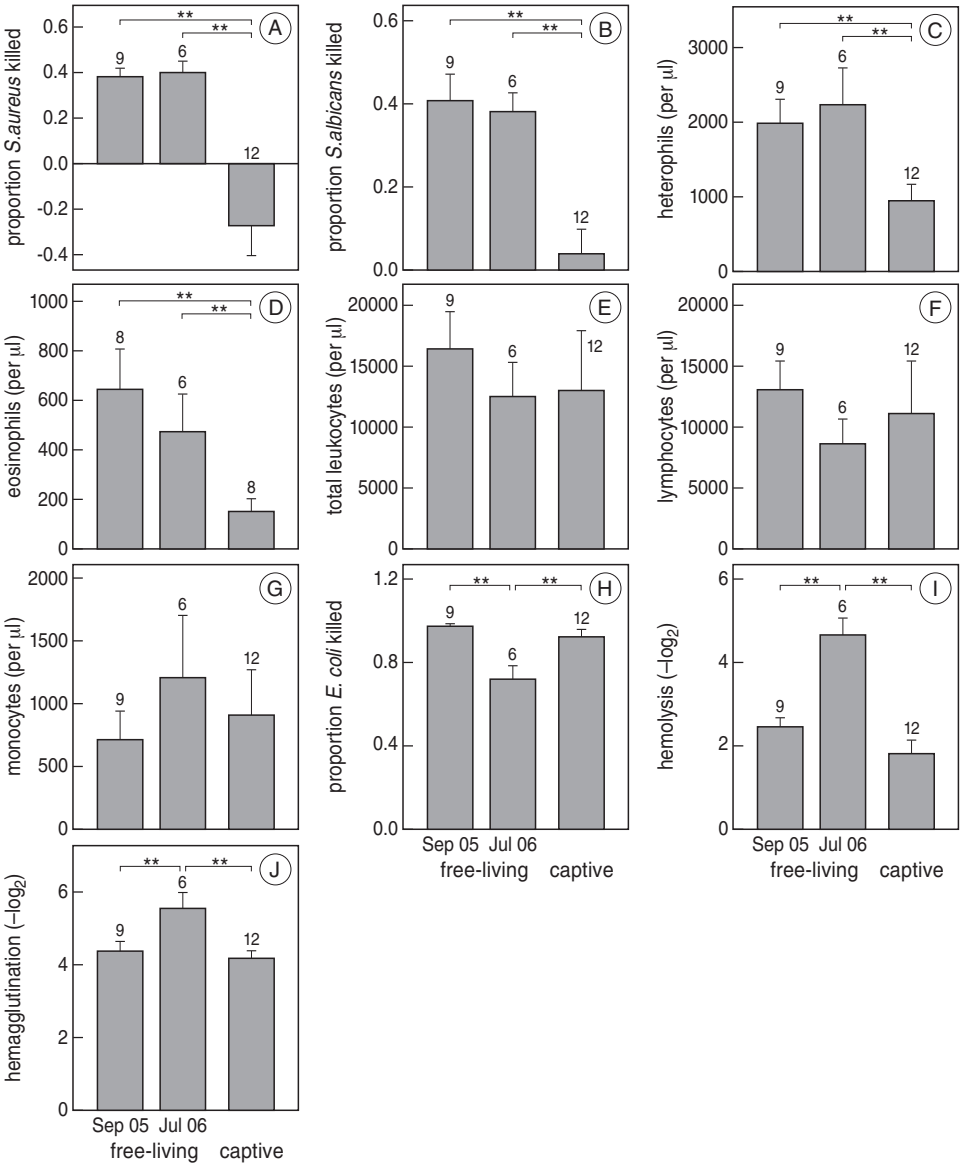

Figure 9.1. Immune function in captive versus free-living red knots. For *E. coli* killing and leukocyte concentrations statistics were performed on transformed values, but raw data are shown here for ease of interpretation. Bars indicate mean ± SE and sample sizes are indicated above the bars (lower for eosinophil count because zero values are not included). Significance from post-hoc Tukey tests is presented here as $P < 0.05$ (*) and $P < 0.01$ (**).

Table 9.1. Principal component loadings after varimax rotation. 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
Microbial killing (proportion killed)			
S. aureus killing	**0.578**	0.491	0.373
C. albicans killing	**0.884**	-0.027	0.195
E. coli killing	0.174	-0.190	**-0.849**
Leukocyte parameters (per μl)			
Heterophils	**0.605**	0.561	0.361
Eosinophils	**0.809**	0.277	-0.067
Lymphocytes	0.208	**0.901**	-0.034
Monocytes	-0.009	**0.863**	0.075
Plasma parameters (log2)			
Hemolysis	0.492	0.047	**0.707**
Hemagglutination	0.462	-0.126	**0.664**
Totals			
Variance (%) per component	29.6	24.9	22.0
Cumulative variance (%)	29.6	54.5	76.6

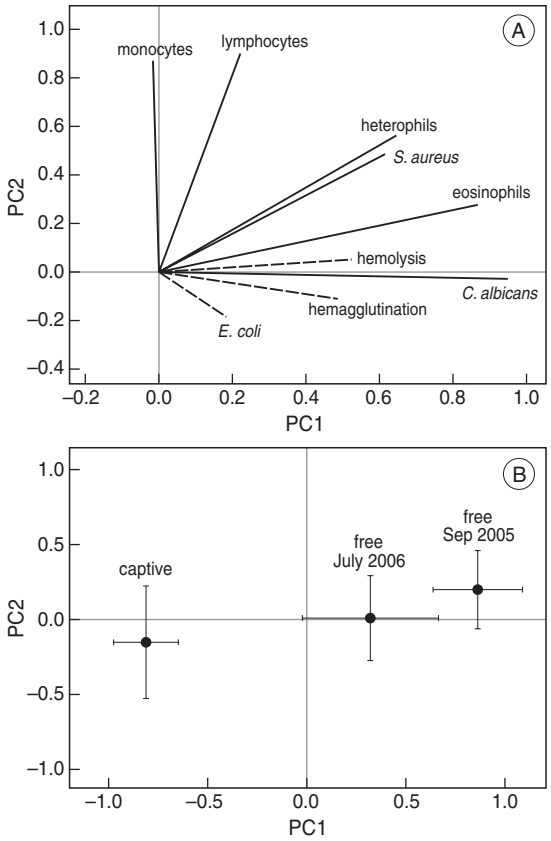

Figure 9.2. (A) Relationships between immune measures. The axes represent the first two components of a principal component analysis. Vectors are the loadings of each immune measure and the length of the vector indicates the strength of the relationship (the R2 value) or how much of the variation in an immune measure is explained by the two axes. Dashed vectors are measures best explained by PC3 (see Table 9.1 for component loadings). The angle between two vectors gives the degree of correlation between them. Adjacent vectors are highly correlated with each other, orthogonal (90°) vectors are uncorrelated, and vectors pointing in opposite directions (180°) are negatively correlated. (b) The groupings of principal component scores (captive birds, free-living birds caught in September 2005 and free-living birds caught in July 2006). For each group the mean ± SE of PC1 is plotted against the mean ± SE of PC2. Captive and free-living birds are distinguished on PC1 but not on PC2.

The groupings found in the principal component analysis closely match those found at both the individual level and within birds over the annual cycle in Buehler et al. (2008a). Our principal component analysis indicated that *S. aureus* killing, *C. albicans* killing, heterophil concentrations and eosinophil concentrations correlated with PC1 and were lower in captive than in free-living knots. Lymphocyte and monocyte concentrations correlated with PC2 and did not differ between captive and free-living knots. *E. coli* killing, hemolysis and hemagglutination correlated with PC3 and did not differ between captive and free-living birds, but separated free-living birds sampled in 2005 from those sampled in 2006, perhaps indicating environmental differences (e.g. food, temperature, rainfall) between years. *E. coli* killing differs from *S. aureus* and *C. albicans* killing because like hemolysis and hemagglutination, it relies mainly on soluble blood components rather than phagocytosis (Merchant et al. 2003, Millet et al. 2007).

The measures of immune function affected by captivity, *S. aureus* and *C. albicans*killing, heterophils and eosinophils, are associated with immediate and non-specific immunity. *S. aureus* and *C. albicans* killing are carried out mainly through phagocytosis (Millet et al. 2007) and heterophils are the most numerous phagocytic cells in birds (although thrombocytes and monocytes are also phagocytes, Campbell 1995). In terms of cost, heterophil phagocytosis may have high immunopathology costs due to the large amount of reactive oxygen and nitrogen species that they produce during particle ingestion (Splettstoesser and Schuff-Werner 2002). Energy costs may also be high due to the high turnover rates of heterophils (Janeway 2004). In terms of benefits, these measures are associated with protection from bacteria and yeast, pathogens with high replication rates and substantial damage potential if not stopped quickly. These measures of immune function were hypothesized by Buehler et al. (2008a) to represent an immune strategy necessary during periods of high pathogen pressure, but costly enough to warrant down-regulation when pathogen pressures are lower.

We considered two predictions regarding immune function in captive and free living red knots. First, more costly immune strategies in captive birds because they have regular access to food and a lower workload, potentially releasing them from trade-offs, and second down-regulated immune function in captive birds because they are exposed to fewer or a smaller variety of pathogens. In light of the immune cost and protective benefit discussion above, our results do not support the idea that captives are released from trade-offs, because immune function in wild birds reflected more costly strategies than in captive birds. However, our results do support the proposal that immune function may be down-regulated in captivity. In the wild, where encounters with bacteria, yeasts and other novel pathogens are high, the protective benefits of immediate, non-specific immune function might outweigh the costs, and immune function represented by PC1 might be favoured (Schmid-Hempel and Ebert 2003). This may be especially true during migration when large aggregations of birds make disease transmission easier (Altizer et al. 2006). However, in captivity where, at least with captive knots, cleaning regimes are likely to keep all but a few common diseases of captivity at bay, the costs of immediate, non-specific immune function might outweigh the benefits (Schmid-Hempel and Ebert 2003).

We put forth this idea as a hypothesis, but we acknowledge that this study does not fully test it since we did not measure pathogen pressure. Preliminary data examining pathogen pressure over a range of microbes in red knot habitats show about 135% more microbial colonies per gram mud in the Wadden Sea (n = 5) than in our cages (n = 4), and higher coliform density in the Wadden Sea (27.3 ± 30.4, n = 4) than in our cages (1.5 ± 1.5 colonies per Petrifilm^{3M}, n = 4; D. M. Buehler unpublished data). These data tentatively support the idea of higher pathogen pressure or pathogen diversity in the wild, but more data are needed.

Few studies have examined immune function in relation to pathogen pressure in captive and free-living animals. The studies we could find focused on nuisance diseases caused by captive conditions, which we argue would favour protection by induced and specific, rather than immediate and non-specific, immune function. For example, in chimpanzees *Pan troglodytes* the bacteria *Clostridium perfringens* was more prevalent in captive than in wild animals likely due to the high calorie and low fiber captive diet (Fujita and Kageyama 2007). In blue-fronted parrots *Amazona aestiva* antibody titers to *Salmonella pullorum* were higher in captive birds (Deem et al. 2005). Again this is not surprising as pet parrots in Bolivia are often in close contact with poultry that carry *Salmonella* and their higher antibody titers indicate that the disease is being fought mainly by specific immune function.

An alternative explanation for our results is the more conventional idea that captive birds are stressed and have depressed immune systems, However, we feel that this alternative is unlikely for red knots because they acclimate well to captive conditions (Piersma and Ramenofsky, 1998) and retain natural cycling of corticosterone in captivity (Piersma et al. 2000b).

We examined immune function using indices that could be taken from a single capture, thus we could not measure induced responses. Therefore, we encourage further research examining induced measures (e.g. specific antibody titres, acute phase responses) to explore differences in immune function between captive and free-living animals. Furthermore, we suggest further study of immune function using animals kept under different captive conditions, or the manipulation of pathogen pressure in captivity to build upon the ideas discussed here.

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Appendix 9.1. Summary of immune function measured at 10:30 and 02:30. Statistics were performed on transformed values for leukocyte measures but raw means are shown here for ease of interpretation. Eosinophils were very rare and are thus excluded from the analysis.

	Mean ± SD		Statistics	
Variable	10:30	2:30	$F_{1,8}$	*P*
Microbial killing *S. aureus* (proportion killed)	0.17 ± 0.34	0.07 ± 0.14	0.67	0.44
Microbial killing *C. albicans* (proportion killed)	0.15 ± 0.13	0.03 ± 0.14	3.93	0.08
Microbial killing *E. coli* (proportion killed)	0.51 ± 0.39	0.58 ± 0.33	0.44	0.53
Total leukocytes (per μl)	10745.4 ± 9856.3	13291.7 ± 8710.1	0.44	0.53
Heterophils (per μl)	988.1 ± 600.9	1089.5 ± 569.3	0.25	0.63
Lymphocytes (per μl)	9243.3 ± 9690.4	11374.5 ± 7966.8	0.41	0.54
Monocytes (per μl)	463.7 ± 242.7	763.4 ± 657.8	0.89	0.37
Thrombocytes (per μl)	22853.3 ± 22279.0	25563.9 ± 23990.8	0.36	0.56
Hemolysis (-log2)	3.10 ± 1.16	3.22 ± 0.56	0.33	0.58
Hemagglutination (-log2)	5.40 ± 0.82	5.33 ± 0.35	0.03	0.86
H:L ratio	0.17 ± 0.13	0.14 ± 0.09	0.16	0.70
Hematocrit (proportion RBC)	0.46 ± 0.05	0.45 ± 0.04	0.14	0.72
Young RBCs (proportion in 5000 RBCs)	0.04 ± 0.02	0.04 ± 0.01	0.94	0.36