Constitutive Immune Function Responds More Slowly to Handling Stress than Corticosterone in a Shorebird

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ABSTRACT

Ecological immunologists are interested in how immune function changes during different seasons and under different environmental conditions. However, an obstacle to answering such questions is discerning the effects of biological factors of interest and investigation artifacts such as handling stress. Here we examined handling stress and its effects on constitutive (noninduced) immune function via two protocols on captive red knots (*Calidris canutus*). We investigated how constitutive immunity responds to handling stress, how quickly these changes take place, and the practical implications for researchers interested in sampling baseline immune levels. We found that *Staphylococcus aureus* and *Candida albicans* killing increased with handling stress while total leukocyte and lymphocyte concentrations decreased. However, although corticosterone increased significantly and rapidly in response to handling stress, none of the 10 measures of constitutive immunity that we tested differed significantly from baseline withinn20 or 30 min of capture. Thus, researchers interested in baseline immune function should sample animals as soon as possible after capture, but studies in species not easily sampled in less than 3 min (such as red knots) could still yield useful results.

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Introduction

Ecological immunologists are interested in how immune function changes during different seasons and under different environmental conditions (reviewed in Lee 2006). However, an obstacle to answering such questions is separating the effects of stress due to the biological factors of interest and investigation artifacts such as handling stress. Stress and immune function are linked through complex interactions between the neuroendocrine and immune axes (McEwen et al. 1997). This complexity means that the stress response suppresses some forms of immunity while enhancing others and that different stressors have differing effects (Apanius 1998). Therefore, different stressors, such as acute and unpredictable stress (i.e., capture and handling or predator attack) and predictable life cycle events that result in increased allostatic load (i.e., reproduction or migration; McEwen and Wingfield 2003), must be defined and considered separately. Furthermore, different facets of the immune system such as constitutive or induced or general or specific immunity must also be distinguished.

Field and experimental conditions often result in a certain amount of time between animal capture and blood sampling; therefore, determining the effect of acute handling stress on baseline immune function is particularly relevant. Like the well-known corticosterone response (for which a baseline sample must be obtained within 2–3 min after capture; Wingfield et al. 1995; Romero and Reed 2005), immunity may also change rapidly in response to acute stress. Few researchers have examined this question in wild birds and existing studies have focused on nonmigratory species (Davis 2005; Matson et al. 2006; Millet et al. 2007). Information on migratory species is lacking, and red knots (*Calidris canutus*) are an excellent model system for examining

immune function in long-distance mi- grants (Buehler and Piersma 2008).

We examined the acute stress of capture and handling and its effects on constitutive (noninduced) immune function in captive red knots. We chose constitutive immunity because it can be measured from a single blood sample and can be tested repeatedly on the same individual. This repeated-measures de-sign and a captive approach allowed us to maximize our chances of detecting changes in immune function by examining within-individual changes and greatly decreased the sample sizes needed. We conducted two protocols, the first using stag- gered sampling to avoid resampling the same individual more than twice in a day and the second using repeated sampling (more than twice a day) to compare different time points to a single baseline. The two experiments also differed in that protocol 1 was performed in the spring when the birds were not in wing molt, and protocol 2 was performed in the fall when the birds were in the final stages of wing molt. We measured 10 variables of constitutive immune function by quantifying microbial killing abilities (Millet et al. 2007), circulating cellular immunity (Campbell 1995), and complement and natural antibodies (Matson et al. 2005). In protocol 1, we also measured hematocrit and corticosterone, a stress hormone that increases rapidly in response to acute stress (Wingfield et al. 1995). We asked (1) How do different measures of constitutive immunity respond to the handling stress? (2) How quickly do these changes take place? and (3) What are the practical implications of these changes when researchers are interested in baseline immune levels?

Material and Methods

Animals

Nineteen adult red knots of the subspecies *Calidris canutus islandica* were captured during fall migration or wintering in the Dutch Wadden Sea (53°31'N, 6°23'E) and were brought into captivity at the Royal Netherlands Institute for Sea Re- search (NIOZ). At capture, the birds were ringed, weighed, and aged as older than 2 yr (Prater et al. 1977), and sexes were later determined using molecular techniques (Baker et al. 1999). The birds were housed in aviaries (1.5 m # 4.5 m # 2.3 m) with a quarter of the aviary floor covered by an artificial sand flat flushed by saltwater and a tray of freshwater for drinking and bathing. The birds had free access to food (Trouvit trout food pellets, Vervins, France; or mud snails *Hydrobia ulvae*). Bird handling was carried out under the auspices of the Animal Experiment Committee (DEC; protocol NIOZ.05.01).

Protocol 1: Staggered Sampling

Protocol 1 took place in April 2006 (*n*=12, 8 females, 4 males, captured August 2004 to February 2005), just before pre-migratory fattening and when none of the birds were molting. Baseline was defined as within 3 min of entering the aviary (Piersma et al. 2000*b*), and the birds were left undisturbed at least the entire night and the morning preceding sampling. We followed the standardized handling-stress protocol outlined in Wingfield et al. (1995) and examined multiple time points (baseline, 20 min, 60 min, and 120 min) using a balanced repeated-measures design to neutralize the order in which the time point treatments were administered. Pairs of birds (one from each aviary holding six birds) were randomly assigned to one of six sequences (e.g., 20 min, 60 min, 120 min or 120 min, 20 min, 60 min). To avoid sampling the same individual several times in 1 d, protocol 1 was designed so that each bird was bled only twice (baseline and a given time point) on each sampling day. Six days were left between each sampling day for any given individual to minimize carry-over effects (which are likely to be small; see Piersma et al. 2000*a*). Therefore, the entire sampling protocol lasted 18 d with each bird experiencing all three time points.

On entering the aviary, we captured the bird in a net, sterilized the area around the brachial vein with 70% ethanol, and collected about 300 mL of blood into presterilized, heparinized capillary tubes (Fisher Emergo). After blood sampling, we weighed the bird and then placed it into an individual keeping box (0.4 m # 0.6 m # 0.3 m) at room temperature and without food or water until it was time to take the next blood sample. Keeping individuals in boxes and out of their normal environment mimicked prolonged periods of capture and handling. Immediately after each blood sampling session, 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.

Protocol 2: Repeated Sampling

Protocol 2 was carried out in October 2005 (*n*=7, 4 females and 3 males, captured October 1995) when the birds were in the final stages of wing molt. We sampled one bird a day at four sampling times (baseline, 30 min, 90 min, and 150 min). Blood samples were obtained as discussed earlier, but only about 200 mL of blood

were taken because several small samples were needed to compare several time points to a single baseline.

Measuring Immune Function

We quantified microbial killing abilities (Tieleman et al. 2005; Millet et al. 2007), circulating cellular immunity (Campbell 1995), and complement and natural antibodies (Matson et al. 2005). These assays have been described extensively in the orig- inal methodological articles, and we have included an extended immune methodology in Appendix A. Briefly, the microbial- killing assay measures the capacity of blood to kill microorganisms in vitro, and we used three strains: *E. coli* ATCC 8739, *Candida albicans* ATCC 10231, and *Staphylococcus aureus* ATCC 6538 (MicroBioLogics, St. Cloud, MN). Higher "killing" equates with a greater capacity to limit infection by the particular strain of microorganism used in the assay (Millet et al. 2007). Circulating cellular immunity was measured using leukocyte concentrations that provide a description of circulating cellular immunity via differential concentrations of phagocytes and lymphocytes (Campbell 1995). Complement and natural antibodies were measured using a hemolysis-hemagglutination assay in which lysis and agglutination of rabbit red blood cells is quantified using serial dilution of plasma (Matson et al. 2005).

Hematocrit

We measured hematocrit by centrifuging 25 mL of blood in a capillary tube for 12 min at 12,000 g and reading the relative proportion of red blood cells to total volume.

Corticosterone

Plasma was obtained by centrifuging blood samples for 12 min at 12,000 g. The plasma was then stored at - 80°C before it was shipped on dry ice to the Max Planck Institute for Ornithology, Andechs. Corticosterone concentrations were determined by direct radioimmunoassay following Goymann et al. (2006), and the antibody was obtained from Esoterix Endocrinology, Calabasas Hills, California. Extraction efficiency ($\pm$SD), as calculated from trace amounts of tritiated hormone added to each sample, was 87.2% $\pm$ 4.4% (Perkin Elmer, NET 399), and the average limit of detection was 6.2 pg per tube (all samples were well above this limit). To minimize variance, all samples were analyzed within one assay with an intra-assay coefficient of variation (CV) of 6.7%.

Statistics

Before performing statistical comparisons, all data were tested for normality using one-sample Kolmogorov-Smirnov, and histograms of each variable were examined visually. When data were not normally distributed, we transformed them. Leukocyte concentrations were right skewed, and these data were log (base 10) transformed. After transformation, all variables conformed to normality both when testing the data themselves and when testing the residuals generated by parametric models. Eosinophil concentrations were very low (on average <1% of the full differential) and were thus not included in statistical comparisons.

Protocol 1 had different baselines for each time point (between first stress and blood sample), and we first tested to see whether the baselines themselves differed. Baselines did not differ for corticosterone (P=0.3) or any immune measure (all P's 1 0.25; see App. B for individual baselines). Thus, we averaged the baselines for each bird and used a linear mixed model with time between first stress and blood sample (Tstress) as a fixed factor and "bird" as a random factor (Quinn and Keough 2002). This method allowed us to compare multiple time points, to control for the repeated-measures nature of the study, to run various different covariates, and to allow for missing values. However, to be rigorous, we also performed tests be- tween each individual baseline and its 20-min sampling point (App. C). For these tests, we also performed power analyses using the program G*Power to ensure that our power to detect significant changes between baseline and 20 min was moderate to high (Faul et al. 2007; App. C).

When different time points were sampled on different days (protocol 1), we included the number of colonies on the inoculate control, the ratio of incubated over inoculate control, and the number of days since resuspending the microorganisms as covariates for microbial killing (see App. A for details). Co- variates were sequentially removed from the models when not statistically significant at the 0.05 level. We used SPSS version 14.0 for all statistical comparisons, and significance is reported using both the conventional a=0.05 and a sequential Bonferroni correction (Rice 1989). All descriptions of the data in the text report mean $\pm$ SD. Graphs represent mean $\pm$ SE for clarity.

Results

Body Mass, Hematocrit, and Corticosterone

We found no effect of T_{stress} on body mass (Table 1). Thus, we do not think that changes in immune function due to handling stress are confounded by concurrent effects on body mass. Hematocrit tended to decrease as T_{stress} increased (Table 1), but as the trend only neared significance after 120 min ($P=0.06$), this was not likely the result of repeated bleedings. We found a strong, immediate, and significant effect of T_{stress} on corticosterone (Table 1; Fig. 1), indicating that our protocols successfully stimulated capture and handling stress.

Immune Function

Staphylococcus aureus killing increased in response to handling stress in both protocols, but in neither case was the response immediate (Fig. 2a; Table 1). *Candida albicans* killing also tended to increase (Fig. 2b); however, this change was only significant in protocol 2 and then only by 150 min. In contrast, both total leukocyte and lymphocyte concentrations decreased with handling stress in protocol 1, but again the response was not immediate, occurring only after 20 min (Fig. 2c, 2d). In protocol 2, total leukocyte and lymphocyte concentrations also tended to decrease, but the trends were not significant after sequential Bonferroni correction (Table 1). Thrombocytes increased with handling stress but only in protocol 2 and only by 150 min (Fig. 2e; Table 1). *E. coli* killing, monocyte concentrations, hemolysis, and hemagglutination were not significantly affected by handling stress (Table 1).

Discussion

Constitutive Immunity and Handling Stress

Even after 2.5 h, *E. coli* killing was not affected by handling stress. This is interesting as two other studies have found weak but significant changes in *E. coli* killing after 30 or 60 min of handling stress in certain species (Matson et al. 2006; Millet et al. 2007). Matson et al. (2006) found decreases in *E. coli* killing in three of five species of tropical passerines and suggest that the lack of effect in the other two species might be because these species lay very high or very low on an S-shaped dose response curve. However, the red knots in this study had an average baseline killing of 53.6% ± 23.5%, falling in the middle of the dose response curve, thus an effect of stress should have been detectable. Millet et al. (2007) found a significant negative correlation between E. coli killing and corticosterone measured 30 min after capture. It is important to note, however, that both Matson et al. (2006) and Millet et al. (2007) examined microbial killing of tropical birds that live in different environments and have life histories different from those of red knots. As data on microbial killing from more species become available, it may be possible to examine whether differing responses to handling stress reflect different life histories.

Table 1: Summary of changes in body mass, corticosterone, and immune function due to handling stress

Variable	T_{stress} Response	T_{stress}	P	Bird	P
Protocol 1:					
Body mass (g)	No change	$F_{3, 33} = .39$	.764	$F_{11, 33} = 14.41$	**<.001**
Hematocrit	No change	$F_{3, 33} = 2.36$	.089	$F_{11, 33} = 3.99$	**.001**
Corticosterone (ng/mL)	Increases by 20 min	$F_{3, 32} = 11.66$	**<.001**	$F_{11, 32} = 1.58$	.152
Staphylococcus aureus (prop. killed)	Increases by 60 min	$F_{3, 30} = 8.60$	**<.001**	$F_{11, 30} = 1.74$	.112
Candida albicans (prop. killed)	No change	$F_{3, 30} = .95$	.427	$F_{11, 30} = 1.55$	.167
E. coli (prop. killed)	No change	$F_{3, 31} = 2.14$	.116	$F_{11, 31} = 9.84$	**<.001**
Total leukocytes (per mL)	Decreases by 60 min	$F_{3, 32} = 6.18$	**.002**	$F_{11, 32} = 1.41$	.215
Heterophils (per mL)	No change	$F_{3, 33} = 1.40$	.261	$F_{11, 33} = 4.01$	**.001**
Lymphocytes (per mL)	Decreases by 60 min	$F_{3, 32} = 6.24$	**.002**	$F_{11, 32} = 1.06$	.421
Monocytes (per mL)	No change	$F_{3, 29} = 1.87$	.157	$F_{11, 29} = 1.46$	.20
Thrombocytes (per mL)	No change	$F_{3, 33} = 1.218$	.319	$F_{11, 33} = 2.06$	.054
Hemolysis ($\log^2$)	No change	$F_{3, 32} = 1.01$	.399	$F_{11, 32} = 2.40$	***.026***

		T_{stress}		Bird	
Hemagglutination (log^2)	No change	$F_{3, 32} = 1.43$	.252	$F_{11, 32}$ p 6.12	<**.001**
Protocol 2:					
S. aureus (prop. killed)	Increases by 90 min	$F_{3, 17} = 13.92$	<**.001**	$F_{6, 17} = 5.07$	**.002**
C. albicans (prop. killed)	Increases by 150 min	$F_{3, 16} = 4.28$	*.021*	$F_{6, 16} = 3.21$	*.029*
E. coli (prop. killed)	No change	$F_{3, 17} = 1.03$	.403	$F_{6, 17} = 13.05$	<**.001**
Total leukocytes (per mL)	Decreases by 90 min	$F_{3, 16} = 3.41$	*.043*	$F_{6, 16} = 1.43$	.262
Heterophils (per mL)	Trend for increase by 90 min	$F_{3, 16} = 3.13$	.055	$F_{6, 16} = 2.76$	*.048*
Lymphocytes (per mL)	Decreases by 90 min	$F_{3, 16} = 3.46$	*.041*	$F_{6, 16} = 1.41$	.270
Monocytes (per mL)	No change	$F_{3, 14} = 1.73$	.207	$F_{6, 14} = .901$	.521
Thrombocytes (per mL)	Increases by 150 min	$F_{3, 16} = 7.91$	**.002**	$F_{6, 16} = 1.42$	.383

Note. Sampling time ("T_{stress}") is a fixed factor, and individual bird ("Bird") is a random factor. Statistical significance at a=0.05 is shown in italic boldface; after sequential Bonferroni correction (Rice 1989) is shown in roman boldface; prop. =proportion.

Both *Staphylococcus aureus* and *Candida albicans* killing in- creased after 60 or 90 min of handling stress. To our knowledge, the effect of handling stress on microbial killing against these two strains has not been studied. *Staphylococcus aureus* killing is generally carried out through phagocytosis (Matson et al. 2006; Millet et al. 2007), and Millet et al. (2007) found a significant decrease in the ability of monocytes to phagocytose *S. aureus* after 30 and 60 min of handling stress. However, the phagocytosis assay described in Millet et al. (2007) examines phagocytosis by macrophages not heterophils (which are washed off before assaying). Heterophils are the most numerous phagocytes in birds (Janeway et al. 2004), and during acute stress, as lymphocytes are redeployed to the lymph system (Dhabhar et al. 1995), heterophils increase proportionally in the peripheral blood. This may explain the discrepancy between our results and those of Millet et al. (2007). If *S. aureus* and *C. albicans* killing are achieved mainly by heterophil phagocytosis, then killing may increase even though monocyte phagocytosis is decreased.

Total leukocyte and lymphocyte concentrations in red knots decreased after 60 min handling stress. This result is consistent with a similar experiment on house finches (*Carpodacus mexicanus*), showing that total leukocytes decreased over a 1-h interval, both in birds bled at baseline and after 60 min and in birds bled only after 60 min (Davis 2005). Our results are also consistent with the general dynamics of leukocyte redistribution during acute stress, in which heterophil traffic is in- creased and lymphocyte traffic is reduced in the bloodstream (Dhabhar et al. 1995). This redistribution of cells enables mobilization of heterophils through the blood to form a first line of defense and the redeployment of lymphocytes to the lymph system where they receive antigens released from sites of infection (Dhabhar et al. 1995).
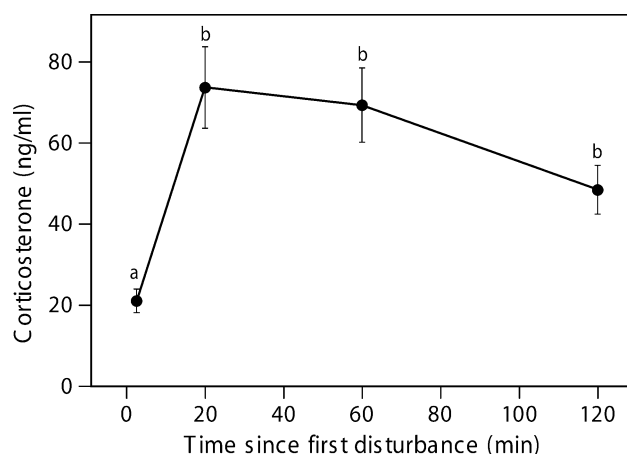

Figure 1. Corticosterone response to capture stress stimulated using the standard capture-stress protocol in protocol 1. Sampling times, which differ significantly from one another (Tukey's post hoc tests), are indicated by different letters.
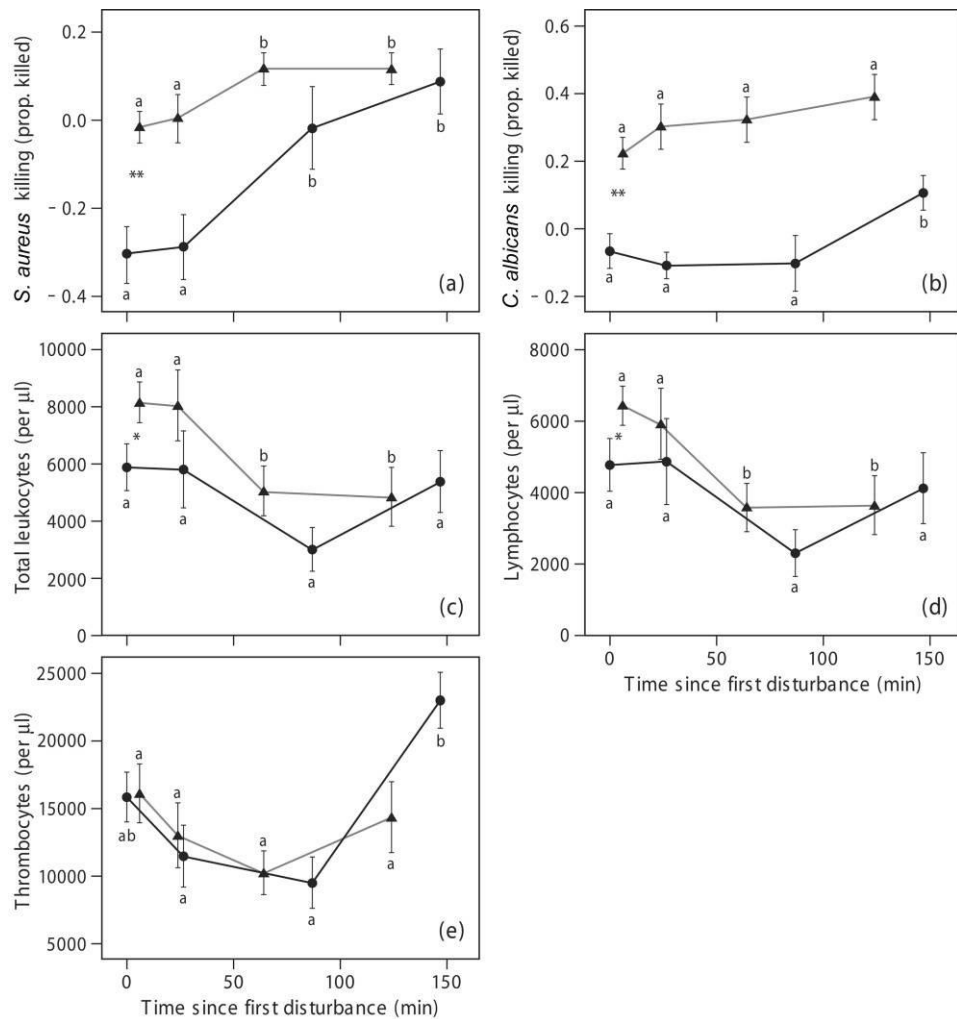
Figure 2. The response of different measures of constitutive immunity to capture stress. Triangles indicate protocol 1 (n=12), circles indicate protocol 2 (n=7), and error bars show ±1 SE. For leukocyte concentrations, statistics were performed on transformed values, but raw data are shown here for ease of interpretation. Sampling times, which differ significantly from one another (Tukey's post hoc tests), are indicated by different letters, and significant differences between the two protocols at baseline are indicated as follows: one asterisk, $P<0.05$; two asterisks, $P<0.01$. Complete statistics are shown in Table 1.

The decrease in total leukocyte and lymphocyte concentrations that we measured may also have been caused by repeated bleedings. However, we feel that this is unlikely because concentrations did not decrease significantly between baseline and 20 min but only after 60 min. Furthermore, concentrations did not decrease more in protocol 2 than in protocol 1 even though the birds were bled four times in protocol 2 and only twice in protocol 1. Finally, in protocol 2, after 150 min (thus after four bleedings), leukocyte concentrations tended to increase (trend between 90 and 150 min in Fig. 2c and 2d) rather than de- creasing further as would be predicted.

Constitutive Immunity and Corticosterone

We found a strong effect of handling stress on corticosterone (Fig. 1) and as expected this effect occurred within 20 min of entering the aviaries. Similar baseline levels and increases in corticosterone due to handling stress have been found in both captive (Landys et al. 2004) and free-living red knots (Reneerkens et al. 2002). In contrast to the immediate response of corticosterone, immune measures appear to respond more slowly to handling stress. No measure of constitutive immune function was significantly different from baseline within 20 to 30 min of handling stress (Table 1; App. C).

A Comparison of the Protocols

The two protocols showed similar patterns in terms of the effect of handling stress on different measures of constitutive im- munity (Table 1), indicating that both handling-stress protocols induced stress, and that in both April (without molt) and Oc- tober (with some wing molt) red knots respond to handling stress in a similar manner. We expected that if the responses to the protocols differed at all, then protocol 2 would be more stressful because the birds were sampled repeatedly within a 2.5-h period. The steeper increases in *S. aureus* killing and *C. albicans* killing indicate that this may be the case, at least for some measures (Fig. 2*a*, 2*b*). However, because baselines dif- fered for *S. aureus* and *C. albicans* killing, the intensity of the response may also be affected by how close the birds were to their maximum response at baseline. Thrombocytes are im- portant for blood coagulation (Janeway et al. 2004) and in- creased more steeply in protocol 2 than in protocol 1 (Fig. 2*e*), perhaps because we had inflicted four small wounds by 150 min.

The difference in baselines between the two protocols for *S. aureus* and *C. albicans* killing is interesting and is most likely due to seasonal changes in immune function over the annual cycle. We performed protocol 1 in April when the birds were not molting and protocol 2 in October during wing molt. Both *S. aureus* and *C. albicans* killing were lower in protocol 2, consistent with a study examining immune function over the annual cycle in knots, which found that *S. aureus* and *C. albicans* killing decreased during molt (Buehler et al. 2008).

Practical Implications for Immune Sampling

None of the 10 measures of constitutive immunity that we tested differed significantly from baseline within 20 or 30 min of handling stress, and this result was verified using two different protocols at two different times of the year and with different statistical methods. In contrast and as expected, corticosterone increased much more rapidly.

Our data are from captive birds. One may argue that captive birds are accustomed to handling stress and that free-living birds may be affected more quickly. However, we think this is unlikely because the captive birds in this experiment showed an immediate and significant increase in corticosterone, indicating that they were indeed experiencing acute stress. Nevertheless, to test this definitively we would have had to run a capture and handling stress experiment on wild birds, and such an experiment, with adequate sample sizes, was not achieved. Correlational data from free-living red knots, however, show no significant relationship between microbial killing or leukocyte concentrations (the measures most affected in captive birds) for capture and handling times 20 min or less (D. M. Buehler, unpublished data).

Our results indicate that, in red knots, the indices of constitutive immunity that we measured do not change within capture and handling times of less than 20 min. Thus, although birds should be sampled as soon as possible after capture, studies of baseline immune function in species not easily sampled in less than 3 min (such as red knots) should still yield useful results.

Acknowledgments

Appendix A

Supplementary Immune Methods

Microbial killing capacity of whole blood. 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) using three microorganisms: *E. coli*, a Gram negative bacteria; *Candida albicans*, a yeastlike fungi; and *Staphylococcus aureus*, a Gram positive bacteria. For each microorganism, we

diluted the blood in CO_2-independent media (18045-054, Invitrogen) and added 20 mL 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 mL 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 75 mL 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 inoculates control plates (200 mL of media mixed with 20 mL of microorganism without incubation).

Statistical considerations for microbial killing. Microbial killing was determined as the proportion of inoculated colonies killed after a strain-specific incubation time. This measure might be affected by factors that vary on a day-to-day basis, such as the initial number of microbes (the inoculation concentration), the intrinsic microbial population trends during incubation (growth or death), and the microbial-suspension age (time since resuspension of the lyophilized pellets). In protocol 2, where different time points were measured on different days, we thus included covariates to control for inoculation concentration, microbial-suspension age, and intrinsic microbial population trends during incubation in our statistical models.

These covariates were determined during a 15-d pilot study conducted in July and August 2005. Each day, we prepared three control suspensions of each microbe (200 mL of media and 20 mL of microbial suspension) without blood. We plated the first immediately, without incubation, to determine day- to-day variation in the number of microbes inoculated. We incubated the second and third suspensions for two strain- specific incubation periods (10 and 20 min for *E. coli*, 30 and 60 min for *C. albicans*, and 60 and 120 min for *S. aureus*) to determine variation in intrinsic microbial population growth/ death. We also examined the effects of microbial-suspension age by tracking changes related to the number of days since resuspension of the lyophilized pellet.

The number of E. coli colonies on the nonincubated control plates varied from 93 to 266 (mean $\pm$ SD=176.1 $\pm$ 42.3; CV=24%) over the 15-d period. Cultures tended to grow after 10 and 20 min of incubation; however, growth was not significant (growth on 14 of 15 d, F2, 42=3.00, P=0.06). Microbial-suspension age contributed significantly to the number of plated colonies with a general increase for the first 2 d and then a decrease to 7 d (F10, 34=2.73, P=0.01).

The number of C. albicans colonies on the nonincubated control plates varied from 148 to 261 (mean $\pm$ SD=190.6 $\pm$ 32.5; CV=17%) over the 15-d period. Incubation time (F3, 56=4.03, P=0.012) significantly affected the number of colonies plated, and on average there was a 13% increase in cell number after 30 min, a 6% increase after 60 min, and a 2.4% decrease after 120 min of incubation. Microbial-suspension age also significantly affected the number of colonies plated (F10, 49=3.24, P=0.003) with colony numbers decreasing for the first 3 d, increasing to 5 d, and then decreasing again.

The number of S. aureus colonies on the nonincubated control plates varied from 132 to 253 (mean $\pm$ SD=193.7 $\pm$ 38.7; CV=20%) over the 15-d period, and on average there was a 10% increase after 60 min and a 6% increase after 120 min (F2, 42 p 0.874, P p 0.425). Microbial-suspension age contributed significantly (F_{10}, $34=4.67$, $P<0.001$) to the number of colonies on plates showing a general decrease in colonies as pellet age increased, with the steepest drop in the first 4 d.

Given the results of this pilot study, we included the inoculation concentration and the number of days since pellet re-suspension as covariates in our statistical analysis for all three strains. Furthermore, because *C. albicans* and *S. aureus* have 1- or 2-h incubation times, we controlled intrinsic microbial population trends during incubation using the ratio of control plate counts (i.e., in the absence of blood) before and after incubation (where a ratio greater than one signifies growth and less than one death). Because correlations between this ratio and the initial number of microbes added might result in collinearity problems (i.e., the number of microbes added might contribute to whether growth or death occurs), we calculated tolerance for these covariates. In all cases, tolerances were 10.8 and thus well above the 0.1 collinearity cutoff (Quinn and Keough 2002, p. 128).

Leukocyte concentrations. We obtained differential leukocyte concentrations using blood smears. After staining, the smears were examined at 1,000# magnification with oil immersion, and the first 100 leukocytes were counted and classified as heterophils, eosinophils, lymphocytes, or monocytes. 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. Basophils were extremely rare (!0.5%) and were therefore not included in the counts. All counts were made in random order and blind to time point by D. Barjaktarov

for protocol 1 and N. Bhola for protocol 2. In combination with the blood smears, we obtained leukocyte concentrations using the indirect eosinophil Unopette method (Campbell 1995), following the manufacturer's instructions (No. 5877, Becton Dickinson).

Hemolysis-hemagglutination assay. Plasma was obtained by centrifuging blood samples for 12 min at 12,000 g and storing the separated plasma at -80°C until the assay was performed. We performed the assay as described by (Matson et al. 2005). Hemolysis and hemagglutination were quantified by serial dilution. We placed 25 mL of plasma in the first and second rows of a 96-well plate, and then from the second to the eleventh rows, we performed 10 1 : 2 dilutions using Dulbecco's PBS (Mauck et al. 2005). We then added 25 mL of 1% of rabbit red blood cells suspension to each well, and incubated the plates at 37°C for 90 min. After incubation, the plates were tilted at a 45° angle and were scanned for agglutination after 20 min and lysis after 90 min. Sample order was randomized and all samples were assayed blindly with respect to origin. The re- sulting images from each sample were randomized with respect to day, to plate, and to row and were scored blindly for lysis and agglutination by D. M. Buehler.

Appendix B

Table B1: Individual baselines for 20-, 60-, and 120-min sampling points for protocol 2

Variable	20 min		60 min		120 min	
	Mean	SD	Mean	SD	Mean	SD
Corticosterone (ng/mL)	25,753.0	13,589.7	24,971.8	22,817.4	17,177.2	7,320.2
Staphylococcus aureus (prop. killed)	-.03	.22	-.03	.20	-.01	.15
Candida albicans (prop. killed)	.26	.32	.29	.27	.20	.28
E. coli (prop. killed)	.45	.28	.45	.30	.53	.27
Total leukocytes (per mL)	12,980.0	8,992.8	9,704.4	6,325.5	10,595.7	7,947.4
Heterophils (per mL)	1,010.5	848.2	1,175.3	1,100.0	666.6	233.3
Lymphocytes (per mL)	11,012.3	8,464.9	7,829.1	5,424.2	8,821.3	7,237.4
Monocytes (per mL)	816.4	986.0	669.7	562.9	844.6	714.2
Thrombocytes (per mL)	25,314.6	20,399.7	21,677.6	20,509.4	17,786.1	10,775.2
Hemolysis ($\log_2$)	4.5	.7	4.6	1.3	5.2	1.3
Hemagglutination ($\log_2$)	2.0	1.2	2.0	.9	1.9	.9

Note. Baselines did not differ for corticosterone ($P=0.3$) or any immune measure (all P's>0.25). Thus, for statistical tests we averaged the baselines for each bird; prop.=proportion.

Appendix C

Table C1: Power analysis and tests between individual baseline and 20-min sampling points for protocol 2

Variable	F	df	P	Effect Size	Power
Staphylococcus aureus (prop. killed)	.77	1, 11	.814	.436	.536
Candida albicans (prop. killed)	1.40	1, 11	.262	.362	.400
E. coli (prop. killed)	1.16	1, 11	.303	.537	.713
Total leukocytes (per mL)	1.26	1, 11	.286	.420	.500
Heterophils (per mL)	<.01	1, 11	.980	.490	.632
Lymphocytes (per mL)	2.38	1, 11	.151	.525	.693
Monocytes (per mL)	1.48	1, 10	.252	.317	.320
Thrombocytes (per mL)	2.33	1, 11	.155	.506	.661
Hemolysis ($\log_2$)	1.21	1, 11	.295	.447	.555
Hemagglutination ($\log_2$)	4.81	1, 10	.053	.419	.503

Note. Using G∗Power (Faul et al. 2007).

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