Limited Access to Food and Physiological Trade-Offs in a Long-Distance Migrant Shorebird. II. Constitutive Immune Function and the Acute-Phase Response

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ABSTRACT

In response to unbalanced energy budgets, animals must allocate resources among competing physiological systems to maximize fitness. Constraints can be imposed on energy availability or energy expenditure, and adjustments can be made via changes in metabolism or trade-offs with competing demands such as body-mass maintenance and immune function. This study investigates changes in constitutive immune function and the acute-phase response in shorebirds (red knots) faced with limited access time to food. We separated birds into two experimental groups receiving either 6 h or 22 h of food access and measured constitutive immune function. After 3 wk, we induced an acute-phase response, and after 1 wk of recovery, we switched the groups to the opposite food treatment and measured constitutive immune function again. We found little effect of food treatment on constitutive immune function, which suggests that even under resource limitation, a baseline level of immune function is maintained. However, birds enduring limited access to food suppressed aspects of the acute-phase response (decreased feeding and mass loss) to maintain energy intake, and they downregulated thermoregulatory adjustments to food treatment to maintain body temperature during simulated infection. Thus, under resource-limited conditions, birds save energy on the most costly aspects of immune defense.

Introduction

In situations of energetic constraint, where energy availability is limited or energy expenditure is increased, animals must balance demands on competing physiological systems by allocating resources to maximize fitness (King 1974; Willmer et al. 2000). In response to changing energy balance, animals can reallocate resources via metabolic adjustments such as downregulation of nighttime metabolic rate (e.g., Bautista et al. 1998; Deerenberg et al. 1998; Wiersma et al. 2005) or via trade-offs with other vital functions such as somatic self-repair (Wiersma and Verhulst 2005) or immune function (e.g., Sheldon and Verhulst 1996; Verhulst et al. 2005).

The immune system is important for survival, but maintaining and using that system carries energetic and immunopathological costs (Klasing 2004). Because of this combination of importance and cost, trade-offs between immune function and other costly activities (locomotion, reproduction, thermoregulation, etc.) have been predicted during times of energy constraint (Sheldon and Verhulst 1996; Norris and Evans 2000). Immune function can be thought of in terms of maintenance (constitutive immunity) and use (induced immunity), and these can be further divided into innate (nonspecific) and acquired (specific) branches (Schmid-Hempel and Ebert 2003). Aspects of induced-innate immunity, such as inflammation and fever during the acute-phase response, are considered to be particularly costly (Klasing 2004). Previous studies on the effect of energy constraint on immune function have focused on induced acquired immunity. For example, in chickens enduring 40% of ad lib. food restriction, lymphocyte proliferation was suppressed (Hangalapura et al. 2005); in yellow-legged gulls *Larus cachinnans* enduring 33% of ad lib. food restriction, wing-web swelling in response to to phytohemagglutinin was decreased (Alonso-Alvarez and Tella 2001); and in deer mice *Peromyscus maniculatus* enduring 70% of ad lib. food restriction, secondary antibody production was decreased (Martin et al. 2007). Data are now needed on the effects of energy constraint on both constitutive and induced immunity in order to gain

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insight into the energetic challenges posed by immune maintenance and use.

Previous studies examining adjustments to energy constraint manipulated energy balance by restricting food consumption to some percentage of ad lib. or by increasing foraging effort (e.g., Bautista et al. 1998; Wiersma et al. 2005). However, in the wild, food may become temporarily unavailable, independent of overall quantity or foraging effort. For example, in red knots *Calidris canutus*, a shorebird species specializing on intertidal prey, birds face daily time limitations on food availability due to tides and wind conditions (van Gils et al. 2006). Furthermore, they encounter feast and famine conditions during migration, and the *Calidris canutus islandica* subspecies, which winters in the northern hemisphere, may face days of fasting if estuaries freeze (Piersma 2007; Buehler and Piersma 2008). In a previous experiment on red knots, Buehler et al. (2008*a*) manipulated energy expenditure in order to study immune function trade-offs. They found little effect of increased energy expenditure on constitutive immune function and suggested that ad lib. food access may have allowed birds to maintain baseline levels of immunity even when experiencing winterlike ambient temperatures (Vézina et al. 2006; Buehler et al. 2008*a*). Here we limit time access to food and examine the other side of the energy-budget equation: energy availability.

Because single assays of immune function are difficult to interpret and different immune indices are often uncorrelated (Adamo 2004; Matson et al. 2006*a*), we examine multiple indices of constitutive and induced immunity and perform principal component analyses (PCAs) to examine relationships between the indices. We quantify constitutive immunity by measuring microbial-killing ability, leukocyte concentrations, levels of complement and natural antibodies, and baseline haptoglobin. To examine induced immunity, we mimic bacterial infection with lipopolysaccharide (LPS) to induce an acute-phase response. We focus on the acute-phase response as an index of induced immune function and examine it in the context of limited food access. However, in the context of constitutive immune function, LPS injection can also be seen as a treatment, and so we perform our constitutive immune assays before and after LPS injection to examine the effect of LPS on constitutive immune indices from a mechanistic standpoint.

This study is the second of a two-section experiment examining physiological trade-offs in red knots faced with limited time access to food. In the first part, metabolic and behavioral adjustments are examined (Vézina et al. 2009, in this issue), and in this article we examine the effects of limited time access to food on constitutive immune function (lower-cost immune maintenance) and on the acute-phase response (higher-cost immune use). We separate birds into two experimental groups receiving either 6 h or 22 h of ad lib. food access and measure constitutive immune function. After 3 wk of treatment, we inject LPS to induce an acute-phase response and, after 1 wk of recovery, we switch the groups to opposite food treatments and measure constitutive immune function again. If immune maintenance is affected by food availability, we predict a decrease in constitutive immune function in the 6-h food treatment that will be reversed when the birds return to 22 h of food access. If immune use is affected by food availability, we predict a decrease in acute-phase response symptoms in birds in the 6-h food treatment during LPS injection.

Material and Methods

Animals

Red knots of the subspecies *Calidris canutus islandica* were captured with mist nets in the Dutch Wadden Sea (53°31′N, 6°23′E) in September 2006 and were brought into captivity at the Royal Netherlands Institute for Sea Research (NIOZ). At capture, the birds were ringed, weighed, and aged as older than 2 yr (Prater et al. 1977). Sex was determined later using molecular sexing methods (13 females, 11 males; Baker et al. 1999). The birds were housed in indoor aviaries (1.5 m × 4.5 m × 2.3 m); a quarter of the aviary floor was covered by an artificial sand flat flushed by salt water, and a tray of freshwater was provided for drinking and bathing.

The experiment occurred from mid-January until the end of March 2007, during a period of stable body mass and when the birds were not molting (Jenni-Eiermann et al. 2002; Buehler et al. 2008*a*). Throughout the experiment, photoperiod was similar for all birds and followed the natural cycle of the northern Netherlands. Ambient temperature was held constant at 12.7° ± 0.5°C (room temperature, ~8°C below the lower critical temperature; Wiersma and Piersma 1994). Bird handling and all experimental protocols were performed under the auspices of the Animal Experiment Committee of the Royal Netherlands Academy of Sciences (protocol NIOZ.07.01).

Experimental Design

Birds ($n = 24$) were divided into experimental and control groups ($n = 12$) that were balanced for sex, with two replicates ($n = 6$) to control for social-group effects. All birds were comparable in terms of structural body size (i.e., no difference between groups in principal component (PC) 1 reflecting variations in lengths of bill, total head, tarsus, and tarsus plus toe ANOVA $P = 0.9$; Vézina et al. 2009). Although all birds were healthy at baseline (day 0), over the course of the experiment a total of five birds showed signs of illness or infection. Four birds developed inflammation of the wings or feet, and one bird showed an elevated white blood cell count. These birds were left in their cages to keep group sizes consistent; however, their immune measurements were not included in the data set (leaving $n = 19$, 9 females, 10 males).

We manipulated access to food following Reneerkens et al. (2007) using a predictable feeding regime (i.e., food was available at the same times every day). Birds had access to food (Trouvit trout food pellets, Vervins, France) either 6 h a day (0900–1100 hours and 1300–1700 hours) or 22 h a day (22 h vs. 24 h of food a day did not result in significant changes in body mass [Vézina et al. 2009] or constitutive immunity [D. M. Buehler, unpublished data]). To minimize differences in digestive state (fed or fasted) during blood sampling, food was

removed from all cages at 1100 hours (1 h before blood sampling) and was left out until 1300 hours to allow time for cage cleaning.

The experiment was divided into periods (baseline, block 1, LPS and recovery, and block 2; Fig. 1). During block 1, group A experienced the 6-h food treatment and group B experienced the 22-h food treatment. The treatments remained the same during the induction of the acute-phase response and a week of recovery (LPS and recovery period). Then, following a design similar to Wiersma et al. (2005), we switched the groups such that group A was provided 22 h of food access and group B experienced the 6-h food treatment (block 2). Blood samples were collected six times over the duration of the experiment: at baseline, day 2, day 15 (2 wk after treatment began), day 22 (after LPS injection), day 29 (2 d after food treatment switch), and day 42 (2 wk after switch).

Blood Sampling

All blood samples were taken at 1200 hours, within 12 min of entering an aviary (mean ± SD, 5.4 ± 2.9 min), and were spaced at least 1 wk apart to minimize carryover effects. A previous experiment on captive red knots has shown that samples taken within 30 min of entering an aviary reflect baseline values for microbial killing, leukocyte concentrations, and levels of complement and natural antibodies (Buehler et al. 2008*a*). Similarly, correlative data on haptoglobin concentrations show that samples taken within 25 min of entering an aviary reflect baseline values (D. M. Buehler, unpublished data). To perform the sampling, we thoroughly sterilized the area around the brachial vein with 70% ethanol and collected about 400 μL of blood into presterilized, heparinized capillary tubes (capillary tubes were individually packaged and sterilized under ultraviolet light). Immediately after sampling we made blood smears, and we transported the remaining blood in sterile boxes to the laboratory for processing within 1 h of sampling.

Hematocrit

We measured hematocrit by centrifuging 25 μL 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.

Measuring Immune Function

Constitutive Immunity

Microbial-Killing Abilities. The microbial-killing assay measures the functional capacity to limit microbial infection (Tieleman et al. 2005; 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, BaseClear, KI-L046-M). To gain a broad understanding of microbial killing, we use three microorganisms: *E. coli*, a Gram-negative strain of bacteria killed mainly by soluble blood components (Merchant et al. 2003; Millet et al. 2007); *Candida albicans*, a yeastlike fungus; and *Staphylococcus aureus*, a Gram-positive strain of bacteria killed mainly by cellular blood components (Millet et al. 2007). For each microorganism, we diluted the blood in CO_2-independent media (18045-054, Invitrogen) to a volume of 200 μL 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). The dilution was optimized to a concentration of approximately 200 colony-forming units per 75 μL of diluted blood-bacteria mixture. We incubated the mixture at 41°C (*E. coli* for 10 min, *C. albicans* for 60 min, *S. aureus* for 120 min) and spread 75 μL of it onto agar plates in duplicate. We then stored the plates upside-down at 36°C and counted the number of colonies per plate the following day. We quantified the number of microorganisms in the inoculation using control plates (200 μL of media and 20 μL of bacteria suspension only, plated immediately without incubation) and calculated microbial-killing capacity as 1 mi-

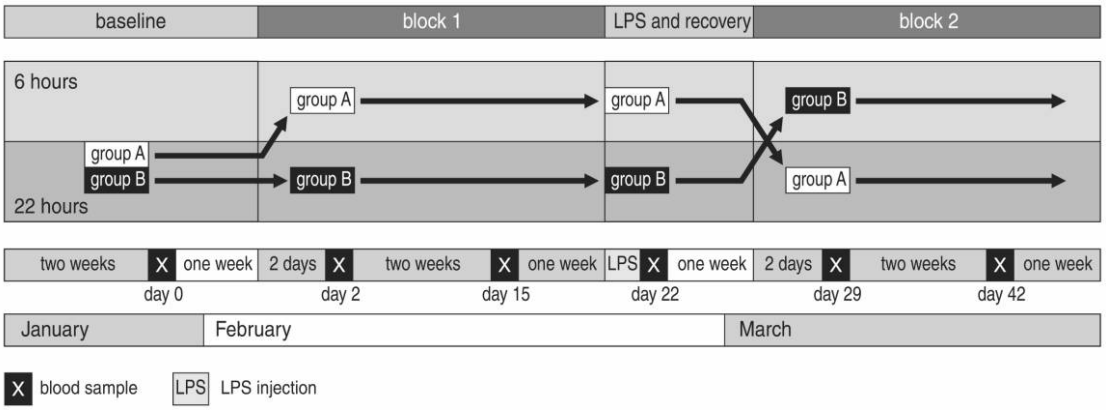

Figure 1. Experimental setup. We separated birds into two experimental groups that received either 6 h or 22 h of food ad lib., and then we measured immune function 2 d and 15 d after the treatment began (block 1). After 3 wk we induced the acute-phase response with lipopolysaccharide (LPS) and measured immune function (day 22). We then switched the food treatments and measured immune function 2 d and 2 wk after the switch (days 29 and 42, respectively; block 2): group A ($n = 8$) experienced 6 h then 22 h of food, and group B ($n = 11$) experienced 22 h then 6 h of food.

nus the experimental-plate colony count divided by the control-plate colony count.

Circulating Cellular Immunity. Leukocyte concentrations provide information on circulating immune cells and can be used as an indicator of health (Campbell 1995). Blood smears were stained (Giemsa stain, Sigma-Aldrich, Munich), randomized, and counted blind to treatment by a single researcher (F.E.-V.) at ×1,000 magnification with oil immersion. The first 100 leukocytes observed were classified as heterophils, eosinophils, lymphocytes, or monocytes following the techniques in Campbell (1995). Basophils were extremely rare (<0.5%) and were therefore not included in the counts. While counting the first 100 leukocytes, thrombocytes were 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 manufacturer's instructions (5877; Becton Dickinson).

Complement and Natural Antibodies. Complement and natural antibodies provide a first line of defense against spreading infections via cell lysis, and they link innate and acquired immunity (Ochsenbein and Zinkernagel 2000). We performed the assay as described by Matson et al. (2005). We pipetted 25 μL of plasma into the first and second rows of 96-well plates. Using Dulbecco's PBS (Mauck et al. 2005), we serially diluted the plasma from row 2 to row 11 and left the twelfth row as a negative control (PBS only). We then added 25 μL of 1% 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 scanned (Epson Perfection 4990 scanner) for agglutination after 20 min and for lysis after 90 min. 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 (D.M.B.) using the criteria outlined in Matson et al. (2005).

Haptoglobin. Haptoglobin is an acute-phase protein that binds iron (heme) to prevent it from providing nutrients to pathogens and offers protection against harmful end products of the immune response (Delers et al. 1988). Haptoglobin was quantified (mg mL^{-1}) following the manual method instructions provided with a commercially available assay kit (TP801; Tri-Delta Diagnostics, Morris Plains, NJ). We pipetted 7.5 μL of plasma into rows 2–11 and 7.5 μL of prepared calibrators (0–2 mg mL^{-1}) into rows 1 and 12 of 96-well plates. We then added 100 μL of diluted hemoglobin (reagent 1) followed by 140 μL of chromogen/substrate solution (reagent 2) to each microwell using a multichannel pipette. We incubated the plates for 5 min at room temperature and then read absorbance at 630 nm using a microplate reader (Molecular Devices Spectra Max 340). To facilitate calculation of haptoglobin concentration in the plasma, we generated a calibration curve by plotting absorbance (630 nm) versus haptoglobin concentration (mg mL^{-1}) in the calibrators.

Acute-Phase Response

The acute-phase response is associated with changes in body temperature (hyperthermia in larger birds or hypothermia in small passerines), the secretion of acute-phase proteins from the liver, and sickness behaviors including reduced food intake, body-mass loss, and reduced activity (Owen-Ashley and Wingfield 2007). We induced an acute-phase response after the birds had been exposed to their treatments for 3 wk (Fig. 1). We subdivided the 6-h and 22-h treatments into injected and uninjected groups that were balanced for sex (each cage had three injected and three uninjected birds). Injected birds received 500 μL of 0.25 mg mL^{-1} LPS in saline (Sigma L 7261, source strain *Salmonella typhimurium* ATCC 7823) intraperitoneally for a dosage of 1 mg kg^{-1}. This dosage was identical to those used to elicit responses in Japanese quail *Coturnix coturnix japonica* (Koutsos and Klasing 2001) and pigeons *Columba livia* (K. Matson, personal communication, 2006). Uninjected birds received the same handling procedure as injected birds, but the skin on their abdomen was not broken (Koutsos and Klasing 2001).

We measured body mass and body temperature (via cloacal insertion of a high accuracy thermocouple [Omega 450 ATT] calibrated against a certified mercury thermometer) at injection and 5 h and 17 h after injection. We also collected blood samples to examine changes in constitutive immunity 1 wk before (day 15) and 17 h after (day 22) injection. The timing of immune sampling followed that used in Matson et al. (2005) and Millet et al. (2007). In order to keep blood-sampling times consistent with the rest of the experiment, LPS injections were performed at 1900 hours. To monitor sickness behavior, we took 30-min videos of each cage at 0930 hours the day of injection and again at 0930 hours the day after injection. The timing of these observations (10 h before and 14 h after injection) is similar to that used by Bonneaud et al. (2003). Video cameras were placed outside the cages and were focused through a one-way mirror so that they did not interfere with the birds' activity. Before the experiment began, we individually marked each bird with color rings to allow for individual observations, and all observations were made by a single researcher (F.E.-V.). Behaviors were scored as feeding (eating and drinking), activity (flying and walking), and self-care (preening and bathing). Time budgets were obtained by scoring the number of seconds the birds performed the behaviors during the observation period and any time not feeding, active, or self-caring was classified as rest.

Statistics

Before performing statistical comparisons, we used one-sample Kolmogorov-Smirnov tests and visual examination of histograms to test for normality. Leukocyte and haptoglobin data were $\log_{10}$ transformed (because 32% of eosinophil counts resulted in zero values, eosinophils were excluded from further analysis), hemolysis was squared, and hemagglutination was square-root transformed. After transformation, all variables and the residuals of the models were normally distributed.

To examine treatment effects over the course of the experiment, while taking into account the fact that we inverted the food-access treatment at the midpoint of the experiment, we performed a general linear model with group and day as main effects and social group (cage) and bird as random effects. The group variable took into account the sequence of the treatments (i.e., group A exposed to 6 h during block 1 and 22 h during block 2, and group B exposed to 22 h during block 1 and 6 h during block 2; see Fig. 1), and in all cases we considered the effect of social group as cage nested in group. We first looked for significant group × day interactions, which indicated that the groups responded differently under the two food treatments. We then used Tukey post hoc tests to determine the significance of treatment effects and to examine the timing and reversibility of within-individual effects. For all analyses, we included LPS (injected or uninjected) as a covariate to take into account any carryover effects from the LPS injections performed during block 2.

To examine whether food treatment affected the LPS-induced acute-phase response, we tested the treatment × LPS interaction in a general linear model with food treatment and LPS (injected and uninjected) as main effects and cage as a random effect. We used the same model to examine the effect of LPS injection on constitutive immunity, temperature, body mass, and behavior by testing for an LPS effect after the LPS treatment.

To gain insight into relationships between measures of constitutive immunity, we performed PCAs. Total leukocyte concentrations were excluded (since they are the sum of the differential concentrations), and we used transformed data for this analysis (both transformed and untransformed data produce the same result). To take into account the repeated-measures structure of the data, we used an among-bird correlation matrix following the procedure described in Matson et al. (2006*a*). We used varimax rotation to maximize the contrasts of the variable loadings, 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 version 14.0 (2005) or JMP version 5 (2002) for all statistical comparisons, but we created the correlation matrix for the PCA using Statistica 7 (2004).

Results

Effects of Food Treatment

Body Mass and Hematocrit. Our food treatments clearly affected body mass (group × day interaction, $F_{4,67} = 95.3$, $P < 0.001$; see Fig. 1 and Table 2 in Vézina et al. [2009] for a week-by-week analysis). During block 1, birds in group A showed a rapid decline in body mass after exposure to the 6-h treatment, whereas birds in group B showed no significant change (Fig. 1 in Vézina et al. 2009). Furthermore, during block 2, birds in group B, which were then experiencing the 6-h food treatment, showed a pattern of body-mass loss that was very similar to that in birds in group A during block 1 (Fig. 1 in Vézina et al. 2009). This pattern indicates that the 6-h treatment produced a negative energy balance (Vézina et al. 2009), and changes in hematocrit further demonstrate this point (group × day interaction, $F_{4,67} = 5.71$, $P = 0.001$). During block 1, hematocrit in birds in group A (6-h treatment) showed a significant decrease by day 15, whereas hematocrit in birds in group B (22-h treatment) did not differ from baseline levels (Fig. 2*A*). During block 2, birds in group B (6-h treatment) showed a significant decrease in hematocrit by day 29, whereas hematocrit in birds in group A (22-h treatment) returned to baseline levels (Fig. 2*A*).

Constitutive Immunity. PC analysis identified four PCs with eigenvalues >1 that cumulatively accounted for 75.9% of the total variation in constitutive immunity (Table 1). Loadings on these PCs showed that *Staphylococcus aureus* killing, *Candida albicans* killing, and heterophils correlated with PC1 (23.3% of total variation); *E. coli* killing, hemolysis, and hemagglutination correlated with PC2 (22.3% of total variation); lymphocytes and monocytes correlated with PC3 (17.1% of total variation); and thrombocytes and haptoglobin correlated with PC4 (13.2% of total variation).

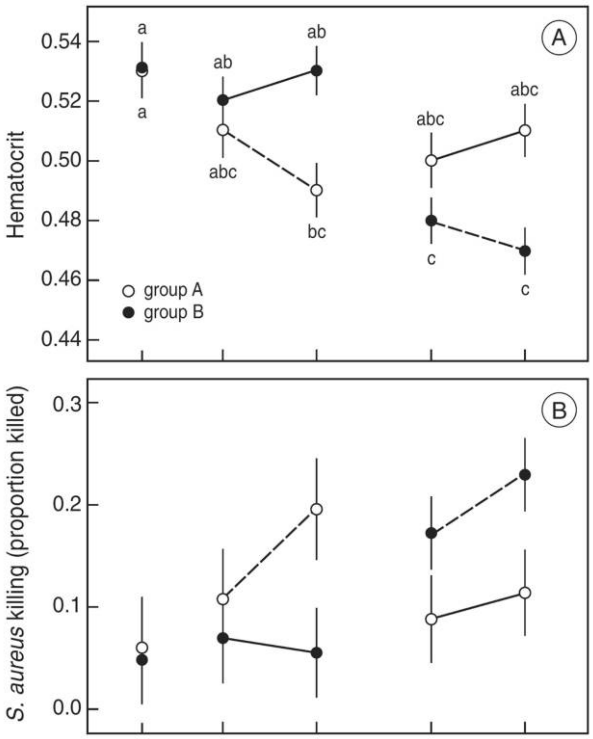

Figure 2. Trends in variables with statistically significant group × day interactions over the whole experiment: hematocrit (*A*; group × day: $F_{4,67} = 5.71$, $P = 0.001$) and *S. aureus* killing (*B*; group × day: $F_{4,67} = 3.42$, $P = 0.01$). Symbols represent least squares means, and bars are ±1 SE. Different letters indicate significant Tukey post hoc tests for between-group and within-bird differences. Where no letters appear, no significant post hoc differences were found. Group A ($n = 8$) is represented by open circles, and group B ($n = 11$) is represented by filled circles. Dashed lines indicate 6 h of food access, and solid lines indicate 22 h of food access.

Table 1: Principal component (PC) loadings after varimax rotation

Response	PC1	PC2	PC3	PC4
Staphylococcus aureus (proportion killed)	**.922**	.108	−.033	−.237
Candida albicans (proportion killed)	**.802**	−.086	.111	.016
E. coli (proportion killed)	.471	**.793**	−.188	−.099
Heterophils (per μL)	**.702**	.088	−.057	.238
Lymphocytes (per μL)	−.096	.124	**.862**	.286
Monocytes (per μL)	.115	−.163	**.904**	−.127
Thrombocytes (per μL)	.248	.317	.130	**.650**
Hemolysis ($\log^2$)	.007	**.864**	−.159	.219
Hemagglutination ($\log^2$)	−.151	**.799**	.234	−.170
Haptoglobin (mg mL^{-1})	−.140	−.221	.011	**.776**
Totals:				
Variance (%) per component	23.3	22.3	17.1	13.2
Cumulative variance (%)	23.3	45.6	62.7	75.9

Note. Values in bold are the highest loadings for a measure across the PCs; underlined values are loadings that meet the saliency criteria for that PC.

During the baseline period, groups A and B and social groups (cages) did not differ significantly with respect to microbial killing, complement, natural antibodies, or hematocrit (all P values >0.17), confirming homogeneity of group composition. However, after analyzing the blood smears (after the experiment was completed), we found that group A had significantly higher lymphocyte concentrations ($F_{1,2} = 16.7$, $P = 0.04$) and marginally higher total leukocyte concentrations ($F_{1,2} = 13.2$, $P = 0.06$) than did group B. Thus, for these measures, we examined the effect of limited time access to food using the differences both before and after treatment (to control for differences at baseline) and absolute differences between the groups during the 6-h and 22-h treatments. Both analyses gave the same results.

Only one index of constitutive immunity, *S. aureus* killing, was affected by limited time access to food (group × day interaction: $F_{4,67} = 3.42$, $P = 0.013$). During block 1, *S. aureus* killing increased in group A (6-h treatment) but remained constant in group B (22-h treatment; Fig. 2*B*). During block 2, this pattern was reversed: *S. aureus* killing increased in group B (6-h treatment) and returned to baseline levels in group A (22-h treatment; Fig. 2*B*). However, Tukey post hoc tests showed that between-treatment and within-bird differences did not reach statistical significance. Considering all indices of constitutive immunity together using PCA scores indicated no significant group × day interactions ($P > 0.10$).

Acute-Phase Response: Sickness Behavior and Body Temperature. Injected and uninjected birds did not differ significantly in terms of any temperature or behavioral indices (all P values >0.30) before LPS injection (day 15), confirming homogeneity of injection-group composition. LPS and food treatment had interactive effects on feeding behavior, body mass, and body temperature (Fig. 3*A*–3*C*). In terms of feeding behavior, injected birds in the 22-h treatment showed a trend of decreased feeding relative to uninjected birds, whereas injected birds in the 6-h treatment showed a trend of increased feeding relative to uninjected birds (Fig. 3*A*; LPS × treatment: $F_{1,13} = 8.01$, $P = 0.01$). Furthermore, injected birds in the 6-h treatment fed more than did both injected and uninjected birds in the 22-h treatment. In terms of body mass, injected birds in the 22-h treatment showed a trend for mass loss compared with uninjected birds, whereas injected and uninjected birds both lost mass in the 6-h treatment (Fig. 3*B*; LPS × treatment: $F_{1,13} = 4.85$, $P = 0.04$). In terms of body temperature, no significant difference was observed between injected and uninjected birds in the 22-h group, but in the 6-h group, injected birds increased or maintained body temperature, whereas uninjected birds displayed a decrease in body temperature (Fig. 3*C*; 6 h after injection, LPS × treatment: $F_{1,13} = 14.38$, $P = 0.002$; 17 h after injection, LPS × treatment: $F_{1,13} = 6.07$, $P = 0.03$).

LPS injection, but not treatment, affected activity and resting behaviors during the acute-phase response. Injected birds in both food treatments decreased activity (LPS: $F_{1,14} = 7.72$, $P = 0.01$; treatment: $F_{1,2} = 0.13$, $v = 0.75$; Fig. 3*D*) and showed a trend for increased rest (LPS: $F_{1,14} = 4.23$, $P = 0.06$; treatment: $F_{1,2} = 0.004$, $P = 0.95$).

Birds with Symptoms of Infection. Over the course of the experiment, a total of five birds showed signs of illness or infection. Of the five, four displayed inflammation of the foot or wing and became ill during the 6-h food-access treatment (three in group A and one in group B) and one had an elevated leukocyte count and became ill when exposed to the 22-h food-access treatment after 3 wk of exposure to 6 h of food access. Of the three birds in group A that became ill during the 6-h food-access treatment, two recovered when they received 22 h of food access. Analysis of samples from the foot and wing lesions indicated bumblefoot and avian pox infections (T. Kuiken, personal communication). Bumblefoot is caused by *S. aureus* bacteria (although not the strain used in our assay), and avian pox is caused by several strains of avipoxvirus (U.S. Geological Survey 1999).

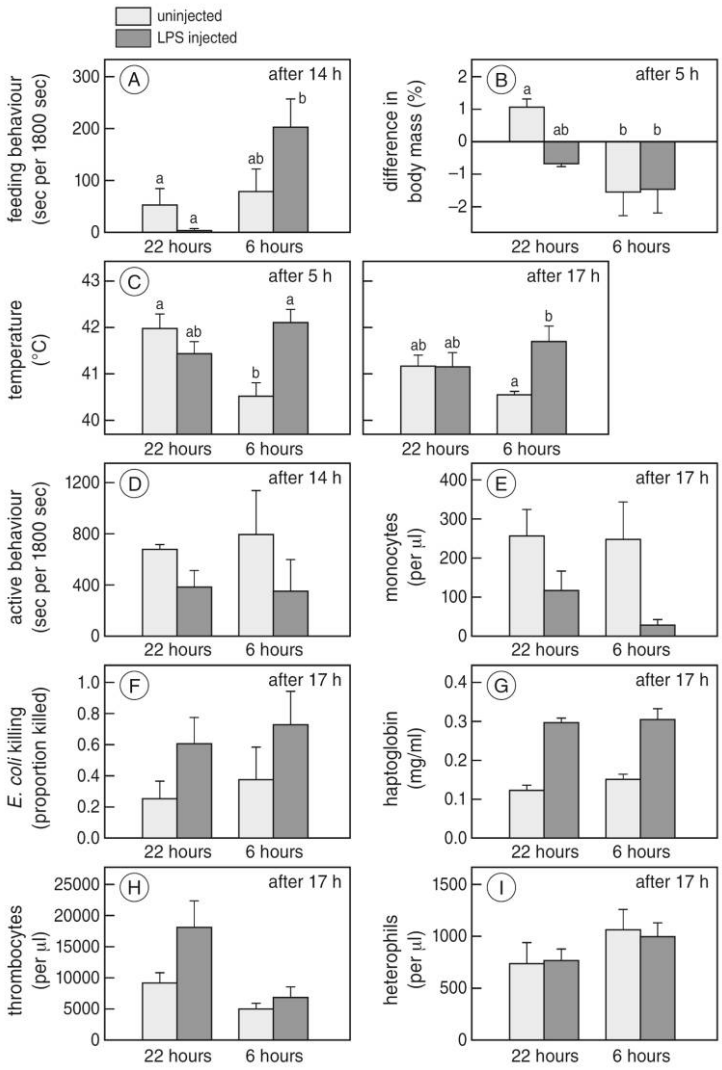

Figure 3. Effect of limited time access to food on the lipopolysaccharide (LPS)-induced acute-phase response: feeding (*A*), body mass (*B*), temperature (*C*), activity (*D*), monocytes (*E*), *E. coli* killing (*F*), haptoglobin (*G*), thrombocytes (*H*), and heterophils (*I*). *Gray bars* = uninjected birds (22 h: $n = 5$; 6 h: $n = 4$); *black bars* = LPS-injected birds (22 h: $n = 6$; 6 h: $n = 4$). Bars are least squares means, and error bars are ± 1 SE. In *A–C*, where the food treatment × LPS interaction was significant, different letters indicate significant Tukey post hoc tests and interaction statistics are given in the text. For *D–I*, LPS and treatment main effects are presented in the text.

LPS Effects on Constitutive Immunity. Injected and uninjected birds did not differ significantly in any of the constitutive immune indices (all P values >0.160) before LPS injection (day 15). However, by chance, injected birds in the 6-h group had higher hematocrit than did uninjected birds; thus, we analyzed the change in hematocrit before and after injection. We found that LPS injection resulted in a 10% drop in hematocrit in injected birds but no change in hematocrit in uninjected birds (LPS: $F_{1,14} = 13.80$, $P = 0.002$).

LPS injection affected several measures of constitutive immunity, inducing a decrease in monocytes (LPS: $F_{1,14} = 8.78$, $P = 0.01$; treatment: $F_{1,2} = 0.14$, $P = 0.74$; Fig. 3*E*) but increases in *E. coli* killing (LPS: $F_{1,14} = 5.788$, $P = 0.03$; treatment: $F_{1,2} = 1.04$, $P = 0.41$) and haptoglobin concentration (LPS: $F_{1,14} = 105.1$, $P < 0.001$; treatment: $F_{1,2} = 0.88$, $P = 0.44$; Fig. 3*F–3G*). LPS also induced an increase in thrombocytes (LPS: $F_{1,14} = 7.88$, $P = 0.01$), and the data indicate a stronger effect in the 22-h treatment, although neither the interaction nor the treatment effect are statistically significant (treatment × LPS: $F_{2,13} = 0.54$, $P = 0.82$; treatment: $F_{1,2} = 7.30$, $P = 0.11$; Fig. 3*H*). Food treatment, but not LPS, affected heterophils, which were higher in the 6-h treatment (both injected and uninjected) than in the 22-h treatment after LPS injection (Fig. 3*I*; treatment: $F_{1,2} = 15.53$, $P = 0.04$; LPS: $F_{1,14} = 0.10$, $P = 0.75$). Analysis using the PC scores showed a significant LPS-induced increase in PC4 (LPS: $F_{1,14} = 29.31$, $P < 0.001$; treatment: $F_{1,2} = 13.6$, $P = 0.03$) and trends for increases in PC1 (LPS: $F_{1,14} = 3.46$, $P = 0.08$; treatment: $F_{1,2} = 0.23$, $P = 0.67$) and decreases in PC3 (LPS: $F_{1,2} = 3.17$, $P = 0.09$; treatment: $F_{1,2} = 0.47$, $P = 0.56$). No treatment × LPS interactions were

significant for individual immune indices ($P < 0.8$) or for PC scores ($P > 0.30$).

Discussion

This study investigated constitutive immune function and the acute-phase response in red knots faced with limited time access to food. Our 6-h food treatment led to a clear energy deficit, producing reversible adjustments in body mass, feeding behavior (Vézina et al. 2009), and hematocrit (an indicator of nutritional status; Campbell 1995). Nevertheless, we found little effect of food treatment on constitutive immune function, although aspects of the more costly acute-phase response were suppressed. Here we discuss these results and anecdotal evidence suggesting the importance of disease itself as an indicator of trade-offs. We also discuss the effect of LPS injection on constitutive immunity from a mechanistic standpoint.

Little Effect of Limited Time Access to Food on Constitutive Immune Function

Of the indices of constitutive immune function that we measured, only *Staphylococcus aureus* killing was affected by limited access to food, and the effect was not statistically significant at the between-group or within-bird levels (post hoc tests not significant). Even when all indices of constitutive immunity were considered together, none of the PCs were significantly affected by food treatment. These results suggest that constitutive immunity, which is constantly maintained and ready for immediate action against pathogen threats, is not traded off during periods of limited food availability. Maintaining basic immune function in the face of limited access to food makes sense, since the red knot lifestyle includes frequent periods of limited food availability (van Gils et al. 2006; Piersma 2007; Buehler and Piersma 2008). Rather than trading off constitutive immunity during periods of negative energy balance, birds seem to adjust their behavior and pay for deficits using energy stores (Vézina et al. 2009). A similar situation is observed in the maintenance of mass-independent basal metabolic rate during limited food access (Vézina et al. 2009).

On the other side of the energy-budget equation, Buehler et al. (2008*a*) manipulated energy expenditure by making birds live at winterlike ambient temperatures. They also found little effect on constitutive immune function. Thus, it appears that although acclimation to situations of increased energy expenditure and limited food access requires adjustments in body mass and feeding behavior (Vézina et al. 2006, 2009), a basic level of constitutive immunity remains robust within the natural range of temperatures and food availabilities experienced by red knots in the wild.

Contrary to the prediction that constitutive immunity would either decrease or not change in birds experiencing limited access to food, *S. aureus* killing increased in the 6-h treatment. This result may be a mechanistic response to the stress of limited food availability. Our 6-h treatment led to a clear energy imbalance (Vézina et al. 2009), and this energetic deficit and associated adjustments in feeding behavior likely caused physiological and social stress. During periods of acute stress, there is a redistribution of lymphocytes from the blood to the lymph system (detectable from 30 min after stress and lasting up to a few weeks if stress is continued; Dhabhar and McEwen 1997), leaving a higher proportion of circulating phagocytes. Because cellular components in whole blood are thought to be important in *S. aureus* killing (Millet et al. 2007), this relative increase in phagocytes may be connected to increases in phagocytosis-based killing.

The Acute-Phase Response and Energy Balance under Limited Time Access to Food

Significant LPS by food × treatment interactions were detected in several aspects of the acute-phase response, indicating that birds in the 6-h and 22-h treatments responded differently to the LPS challenge. Feeding behavior and patterns of mass loss suggest that anorexia (decreased feeding behavior; Owen-Ashley and Wingfield 2007), which is usually observed as part of the acute-phase response (Klasing 2004; Owen-Ashley and Wingfield 2007), was not present in food-restricted birds exposed to LPS. While injected birds in the 22-h treatment tended to decrease feeding behavior and lose mass relative to uninjected birds, injected birds in the 6-h treatment tended to increase feeding behavior and lost approximately the same amount of mass as did the uninjected birds. Because body mass was measured when birds in the 6-h treatment did not have access to food, it is not surprising that both injected and uninjected birds lost mass. The lack of anorexia in LPS-injected 6-h birds is consistent with the energy-limitation hypothesis (Owen-Ashley and Wingfield 2007), which states that the acute-phase response may be suppressed if reductions in energy reserves caused by sickness behavior result in body-mass values that are low enough to threaten survival. Our birds lost mass at a rapid rate during the first few days of limited food access, and this weight loss rate was slowed via behavioral adjustments that included vastly increased feeding during periods when food was available (Vézina et al. 2009). Thus, during the acute-phase response, birds in the 6-h treatment may not have been able to afford to suppress feeding activity.

Our body temperature data are intriguing, since we expected either fever (hyperthermia) in both food treatments (i.e., LPS effect alone) or the suppression of fever in injected birds in the 6-h group to save energy (i.e., if an interactive effect was present). Instead, our data indicate no significant response in the 22-h treatment and decreased temperature in uninjected birds in the 6-h treatment (Fig. 3*C*). In many bird species, hypothermia is used as a strategy to conserve energy during periods of restricted food availability (MacMillen and Trost 1967; Hainsworth et al. 1972; Rashotte and Henderson 1988). We measured body temperature at times when birds in the 6-h treatment did not have access to food. Thus, the lower body temperature observed in the uninjected birds likely reflects an energy-conserving strategy in response to limited food access.

Table 2: Summary of hypothesized costs and immune strategies

Specific Index	Description and Function	Use Cost[a]	Strategy
Lymphocytes	Form the basis of specific immunity[b]	Low	Constitutive,[c] leukocytes associated with specific (acquired) immune defense
Monocytes	Long-lived phagocytes and presenting cells for specific immunity[b]		
Thrombocytes	Important for blood clotting[b]		
E. coli killing	Bacteria defense, mostly without phagocytosis[d]	Moderate	Constitutive,[c] soluble factors associated with nonspecific and immediate defense
Hemagglutination	Natural antibody mediated clumping of invaders[e]		
Hemolysis	Complement mediated lysis and opsinization[e]		
Staphylococcus aureus killing	Bacteria defense, mostly by phagocytosis[d]	High	Constitutive,[c] phagocytosis associated with nonspecific and immediate defense
Candida albicans killing	Yeast defense, mostly by phagocytosis[d]		
Heterophils	Short-lived phagocytes[b]		
Temperature	Kills invaders with heat[b]	Very high	Induced, inflammation and systemic nonspecific defense
Haptoglobin (induced)	Acute-phase protein[b]		
Decreased feeding	Starves invader[b]		

[a] Inferred using Klasing (2004) and Buehler et al. (2008*a*).
[b] Inferred using Campbell (1995) and Janeway et al. (2004).
[c] Groupings within constitutive immunity were inferred from the principal component analyses performed in this study and in Buehler et al. (2008*a*, 2008*b*).
[d] Inferred using Matson et al. (2006*b*) and Millet et al. (2007).
[e] Inferred using Matson et al. (2005).

However, in the injected birds, this energy-conserving strategy may have been overridden by the acute-phase response.

The differences in body temperature between injected and uninjected birds in the 6-h treatment was 1.5°C and 1°C after 5 h and 17 h, respectively (Fig. 3*C*). According to the equation $MR = c(T_b - T_a)$, where MR is metabolic rate, c is thermal conductance (0.045 W $°C^{-1}$ for red knots; Wiersma and Piersma 1994), T_b is body temperature, and T_a is ambient temperature (12.7° ± 0.5°C in the cages), and assuming that birds in the 6-h treatment remain hypothermic all day, uninjected birds saved about 0.07 W on thermoregulation. This suggests a 4.2% savings on overall daily energy expenditure (DEE; average baseline DEE is 1.66 W for group A; Vézina et al. 2009). This savings is relatively small, but the fact that it is traded off during the acute-phase response suggests that, in contrast to constitutive immunity, the acute-phase response represents an energetic challenge for red knots.

Susceptibility to Disease

Although we did not specifically test for it, anecdotal evidence suggests that birds facing limited access to food are more susceptible to disease. Four of the five birds that showed signs of inflammation or illness did so while enduring limited food availability (three in group A and one in group B), and of the three birds in group A, two recovered upon returning to 22 h of food availability. These data bring to light the important point that poor resource conditions can exacerbate relatively mild disease (Chandra and Chandra 1986). Thus, although animals exhibiting disease symptoms should be excluded from analysis of constitutive immunity (because they represent an immune response rather than immune maintenance), these individuals remain important for biologically relevant thinking about variation in immune function. In the future, experiments and monitoring of wild birds should include both measures of immune function and documentation of disease. Furthermore, where ethically possible, studies examining disease susceptibility or manipulating pathogen pressure will help our understanding of biologically viable levels of immune defense.

The Effect of LPS Injection on Constitutive Immune Indices

The injection of LPS increased *E. coli* killing and tended to decrease *Candida albicans* killing (although not statistically significantly [$P = 0.32$]; data not shown), which is similar to responses reported in chickens (Millet et al. 2007). In addition, monocytes decreased, possibly as they migrated out of the blood to the tissues (Dhabhar and McEwen 1997), and thrombocytes increased, especially in the 22-h group, perhaps in anticipation of blood clotting at the site of the injection wound (Janeway et al. 2004). Finally, concentrations of the acute-phase protein haptoglobin were nearly doubled in injected birds (Fig. 3*G*). Haptoglobin concentration increases during an acute-phase response because the liver increases secretion of acute-phase proteins in response to cytokines (Eckersall 1995). Thus, this strong increase in haptoglobin indicates the successful induction of an acute-phase response.

Optimal Immune Adjustments in Energetically Constrained Situations

The pathogen-thwarting benefits of immune defenses must be balanced against their energetic and immunopathological costs (Sheldon and Verhulst 1996; Råberg et al. 1998; Norris and Evans 2000; Schmid-Hempel and Ebert 2003), especially in energetically constrained situations. To illustrate the costs and benefits of immune indices measured in this study, we summarize constitutive immune groupings identified by PCA (this study; Buehler et al. 2008*a*, 2008*b*) with the acute-phase response (Table 2). We exclude thrombocytes and baseline haptoglobin because these indices were not measured in all studies. Table 2 shows that constitutive immune indices cluster into three different cost-benefit strategies: a low-cost strategy associated with lymphocytes and monocytes, a moderate-cost strategy composed of soluble factors (*E. coli* killing, hemolysis, and hemagglutination), and a high-cost strategy composed of phagocytosis-based defenses (*S. aureus* killing, *C. albicans* killing, and heterophils). However, despite these groupings, this study shows that a baseline level of constitutive immune function is constantly maintained, while aspects of the acute-phase response appear to be downregulated during resource limitation. This suggests that all constitutive immune strategies are of lower costs than the acute-phase response (fever, anorexia, and induced haptoglobin) and implies that in energetically constrained situations, birds optimize by saving energy on the most costly aspects of immune defense.

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Note: the scale on the x-axis of Figure 2 should read:

Baseline, Day 2, Day 15, Day 29 and Day 42