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Bottlenecks, budgets and immunity

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Limited access to food and physiological trade-offs in a long-distance migrant shorebird part II: constitutive immune function and the acute phase response

Deborah M. Buehler, Francisco Encinas-Viso, Magali Petit, François Vézina, B. Irene Tieleman and Theunis Piersma

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 time access to food. We separated birds into two experimental groups receiving either 6h or 22h food access and measured constitutive immune function. After 3 weeks we induced an acute phase response and, after a week of recovery, switched the groups to opposite food treatments and measured constitutive immune function again. We found little effect of food treatment on constitutive immune function, suggesting 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 down-regulated 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 down-regulation of night-time 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 (Norris and Evans 2000; Sheldon and Verhulst 1996). Immune function can be thought of in terms of maintenance (constitutive immunity) and use (induced immunity), and can be further divided into innate (non-specific) 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 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 libitum* food restriction lymphocyte proliferation was suppressed (Hangalapura et al. 2005), in yellow-legged gulls, *Larus cachinnans* enduring 33% of *ad libitum* food restriction wing web swelling to phytohaemagglutinin (PHA) was decreased (Alonso-Alvarez and Tella 2001), and in deer mice *Peromyscus maniculatus* enduring 70% of *ad libitum* food restriction secondary antibody production was decreased (Martin et al. 2007c). Data are now needed for the effects of energy constraint on both constitutive and induced immunity to gain 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 libitum* 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, during migration they encounter feast and famine conditions, and the *C. c. islandica* subspecies, which winters in the northern hemisphere, may face days of fasting if estuaries freeze (Buehler and Piersma 2008; Piersma 2007). In a previous experiment on red knots, Buehler et al. (2008a) manipulated energy expenditure to study immune function trade-offs. They found little effect of increased energy expenditure on constitutive immune function and suggested that *ad libitum* food access may have allowed birds to maintain baseline levels of immunity even when living at winter-like ambient temperatures (Vézina et al. 2006; Buehler et al. 2008a). 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. 2006a), we examine multiple indices of constitutive and induced immunity and perform principle component analyses (PCA) 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, thus 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 section, metabolic and behavioral adjustments are examined (Vézina et al. submitted) and here 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 6h or 22h of *ad libitum* food access and measure constitutive immune function. After 3 weeks of treatment we inject LPS to induce an acute phase response and, after a week of recovery, we switch the groups to opposite food treatments and measure constitutive immune function again. If immune maintenance is affected by food availability then we predict a decrease in constitutive immune function in the 6h food treatment that will be reversed when the birds return to 22h food access. If immune use is affected by food availability then we predict a decrease in acute phase response symptoms in birds in the 6h food treatment during LPS injection.

MATERIALS AND METHODS

Animals

Red knots of the subspecies *C. c. 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 two years (Prater et al. 1977). Sexes were later determined using molecular sexing (13 females, 11 males; Baker et al. 1999). The birds were housed in indoor aviaries (1.5m x 4.5m x 2.3m) with a quarter of the aviary floor covered by an artificial sand flat flushed by salt water and a tray of fresh water for drinking and bathing.

The experiment took place 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. 2008a). Throughout the experiment, photoperiod was similar for all birds and followed the natural cycle of the northern Netherlands. Ambient temper-

ature was held constant at $12.7^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ (room temperature, ca. 8°C under the lower critical temperature; Wiersma and Piersma 1994). Bird handling and all experimental protocols were carried out under the auspices of the Animal Experiment Committee of the Royal Netherlands Academy of Sciences (KNAW DEC; protocol NIOZ.07.01).

Experimental design

Birds ($n = 24$) were divided into experimental and control groups ($n = 12$) balanced for sex and 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 1 reflecting variations in length of bill, total head, tarsus and tarsus plus toe ANOVA $P = 0.9$; Vézina et al. submitted). 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 measures were not included in the dataset (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 everyday). Birds had access to food (Trouvit trout food pellets, Vervins, France) either 6h a day (9:00 to 11:00 and 13:00 to 17:00) or 22h a day (22h versus 24h of food did not result in significant changes in body mass (Vézina et al. submitted) 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 11:00 (1h before blood sampling) and was left out until 13:00 to allow time for cage cleaning.

The experiment was divided into sections (baseline, block 1, LPS and recovery, and block 2; Figure 7.1). During block 1, group A experienced the 6h food treatment and group B the 22h food treatment. The treatments remained the same during the induction of the acute phase response and a week of recovery (LPS and recovery). We then switched the groups following a design similar to (Wiersma et al. 2005) such that group A returned to 22h food access and group B experienced the 6h food treatment (block 2). Blood samples were taken 6 times over the experiment: at baseline, day 2, day 15 (2 weeks after treatment began), day 22 (after LPS injection), day 29 (2 days after food treatment switch) and day 42 (2 weeks after switch).

Blood Sampling

All blood samples were taken at 12:00, within 12 min of entering an aviary (mean \pm SD = 5.4 ± 2.9) and were spaced at least one week apart to minimize carry-over 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. 2008c). Similarly, correlative data on haptoglobin concentrations show that samples taken within 25 minutes of entering an aviary reflect baseline values (D. M. Buehler, unpublished data). At sampling we thoroughly sterilized the area around the brachial vein with 70% ethanol and then collected about $400\mu\text{l}$ of blood into pre-sterilized, heparinized capillary

tubes (capillary tubes were individually packaged and sterilized under UV light). Immediately after sampling, we made blood smears and transported the remaining blood in sterile boxes to the laboratory for processing within an hour of sampling.

Hematocrit

We measured hematocrit by centrifuging 25 μ l of blood in a capillary tube for 12 min at 12000 \times 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 (Millet et al. 2007; Tieleman et al. 2005). 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). To gain a broad understanding of microbial killing we use three microorganisms: *Escherichia coli*, a gram negative strain of bacteria killed mainly by soluble blood components (Merchant et al. 2003; Millet et al. 2007); *Candida albicans*, a yeast-like fungus; and *Staphylococcus aureus*, a gram positive strain of bacteria killed mainly by cellular blood components (Millet et al. 2007). For each micro-organism we diluted the blood in CO₂-independent media (#18045-054, Invitrogen) to a volume of 200 μ l and added 20 μ l 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 μ l of diluted blood–bacteria mixture. We incubated the mixture at 41°C (*E. coli*: 10 min, *C. albicans*: 60 min, *S. aureus*: 120 min) and spread 75 μ l onto agar plates in duplicate. We then stored 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 with control plates (200 μ l of media and 20 μ l of bacteria-suspension only, plated immediately without incubation) and calculated microbial killing capacity as one minus 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, Germany), randomized and counted blind to treatment by a single researcher (FEV) at 1000X magnification with oil immersion. The first 100 leukocytes seen were classified as heterophils, eosinophils, lymphocytes or monocytes following 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 manufacturers instructions (No. 5877; Becton Dickinson).

Complement and natural antibodies

Complement and natural antibodies provide a first line of defense against spreading infections via cell lysis, and link innate and acquired immunity (Ochsenbein and Zinkernagel 2000). 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 two to row 11 and left the 12th row as a negative control (PBS only). We then added 25 μl of 1% of rabbit red blood cell suspension to all wells and incubated the plates at 37°C for 90-min. After incubation, plates were tilted at a 45° angle and were scanned (Epson Perfection 4990 scanner) for agglutination after 20 minutes and lysis after 90 minutes. The scans were randomized with respect to sample origin, plate, and location within the plate and were scored blindly for lysis and agglutination by a single observer (DMB) using the criteria outlined in Matson et al. (2005).

Haptoglobin

Haptoglobin is an acute phase protein that binds iron (haem) to keep it from providing nutrients to pathogens and offers protection against harmful end products of the immune response (Delaers et al. 1988). Haptoglobin was quantified in mg/ml following the 'manual method' instructions provided with a commercially available assay kit (#TP801; Tri-Delta Diagnostics, Inc., Morris Plains, NJ). We pipetted 7.5 μl of plasma into rows 2 to 11 and 7.5 μl of prepared calibrators (0-2mg/ml) into rows 1 and 12 of 96-well plates. We then added 100 μl of diluted haemoglobin (Reagent 1), followed by 140 μl of chromogen/substrate solution (Reagent 2) to each microwell using a multi-channel pipette. We incubated the plates for 5 minutes 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) 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 in their treatments for 3 weeks (Figure 7.1). We subdivided the 6h and 22h treatments into injected and uninjected groups, balanced for sex (each cage had 3 injected and 3 uninjected birds). Injected birds received 500 μl of 0.25mg/ml LPS in saline (Sigma L 7261, source strain *Salmonella typhimurium* ATCC 7823) intra-peritoneally for a dosage of 1mg/kg. This dosage was the same as those used to elicit responses in Japanese quail, *Coturnix coturnix japonica* (Koutsos and Klasing 2001) and pigeons, *Columba livia* (K. Matson, pers. comm. 2006). Uninjected birds received the same handling procedure as injected birds, but the skin on their abdomen was not broken (Koutsos and Klasing 2001).

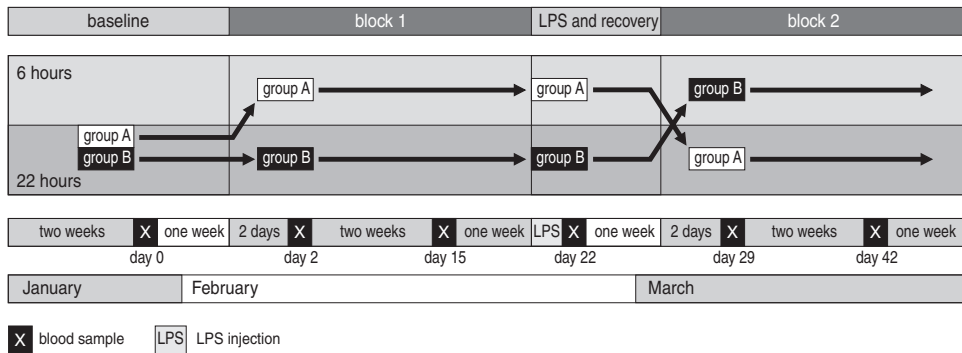


Figure 7.1. The experimental set-up. We separated birds into two experimental groups receiving either 6h or 22h of food *ad libitum* and measured immune function 2 and 15 days after the treatment began (block 1). After 3 weeks we induced the acute phase response with lipopolysaccharide (LPS) and measured immune function (day 22). We then switched the food treatments and measured immune function 2 days and 2 weeks after the switch (days 29 and 42; block 2) after the switch. Group A ($n = 8$) experienced 6h then 22h food and group B ($n = 11$) 22h then 6h food.

We measured body mass and body temperature (cloacal insertion of a high accuracy thermocouple, Omega 450 ATT, calibrated against a certified mercury thermometer) at injection and 5h and 17h after injection. We also took blood samples to examine changes in constitutive immunity one week before (day 15) and 17 hours after injection (day 22). 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 took place at 19:00 in the evening. To monitor sickness behavior, we took 30min videos of each cage at 9:30 the day of injection and again at 9:30 the day after injection. The timing of these observations (10h before and 14h 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 individual observations, and all observations were made by a single researcher (FEV). 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 1-sample Kolmogorov–Smirnov tests and visual examination of histograms to test for normality. Leukocyte and haptoglobin data were log10 transformed (due to 32% 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 at 6h in block 1 and 22h in block 2, and group B at 22h in block 1 and 6h in block 2, see Figure 7.1) and in all cases we considered the effect of social group as cage nested in group. We first looked for significant group by 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 carry-over effects from the LPS injections in block 2.

To examine whether food treatment affected the LPS-induced acute phase response, we tested the treatment by 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 principle component analyses (PCA). 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 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. (2006a). 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 v 14.0 (2005) or JMP v 5 (2002) for all statistical comparisons, but 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 by day interaction $F_{4,67} = 95.3$, $P < 0.001$; see Figure 1 and Table 2 in Vézina et al. submitted for a week-by-week analysis). During block 1, birds in group A showed a rapid decline in body mass after exposure to the 6h treatment, whereas birds in group B showed no significant change (Figure 6.1 in Vézina et al. submitted). Furthermore, during block 2, birds in group B, now experiencing the 6h food treatment, showed a pattern of body mass loss very similar to birds in group A during block 1 (Figure 1 in Vézina et al. submitted). This pattern indicates that our 6h treatment produced a negative energy balance (Vézina et al. submitted) and changes in hematocrit further demonstrate this point (group by day interaction $F_{4,67} = 5.71$, $P = 0.001$). During block 1, hematocrit in birds in group A

(6h treatment) showed a significant decrease by day 15, whereas hematocrit in birds in group B (22h treatment) did not differ from baseline (Figure 7.2A). During block 2, birds in group B (6h treatment) showed a significant decrease in hematocrit by day 29, whereas hematocrit in birds in group A (22h treatment) returned to baseline levels (Figure 7.2A).

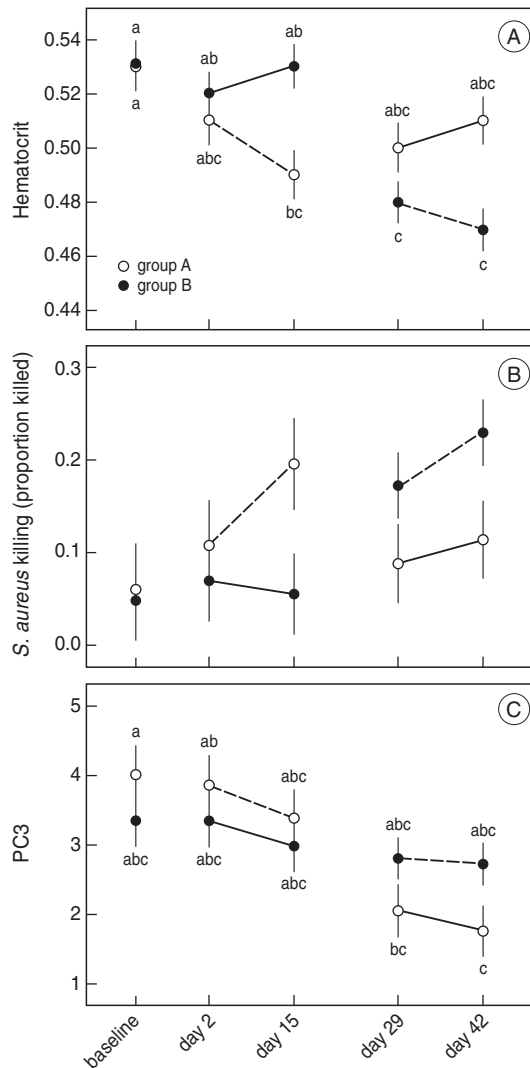


Figure 7.2. Trends over the whole experiment in variables with statistically significant group by day interactions: (A) hematocrit (group*day: $F_{4,67} = 5.71$, $P = 0.001$), (B) *S. aureus* killing (group*day: $F_{4,67} = 3.42$, $P = 0.01$) and (C) PC3 (group*day: $F_{4,66} = 2.66$, $P = 0.04$). Symbols represent least squared means and bars \pm one 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 ($n = 8$, open circles) and group B ($n = 11$, closed circles). Dashed lines indicate 6h food access and solid lines 22h food access.

Table 7.1. 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	PC4
<i>S. aureus</i> (prop. killed)	<u>0.922</u>	0.108	-0.033	-0.237
<i>C. albicans</i> (prop. killed)	<u>0.802</u>	-0.086	0.111	0.016
<i>E. coli</i> (prop. killed)	<u>0.471</u>	<u>0.793</u>	-0.188	-0.099
Heterophils (per μ l)	<u>0.702</u>	0.088	-0.057	0.238
Lymphocytes (per μ l)	-0.096	0.124	<u>0.862</u>	0.286
Monocytes (per μ l)	0.115	-0.163	<u>0.904</u>	-0.127
Thrombocytes (per μ l)	0.248	0.317	0.130	<u>0.650</u>
Hemolysis (log2)	0.007	<u>0.864</u>	-0.159	0.219
Hemagglutination (log2)	-0.151	<u>0.799</u>	0.234	-0.170
Haptoglobin (mg/ml)	-0.140	-0.221	0.011	<u>0.776</u>
Totals				
Variance (%) per component	23.3	22.3	17.1	13.2
Cumulative variance (%)	23.3	45.6	62.7	75.9

CONSTITUTIVE IMMUNITY

Principal component analysis identified four PCs with eigenvalues > 1 that cumulatively accounted for 75.9% of the total variation in constitutive immunity (Table 7.1). Loadings on these PCs showed that *S. aureus* killing, *C. 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).

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 's > 0.17) confirming homogeneity of group composition. However, upon analyzing the blood smears (after the experiment was completed) we found that group A had significantly higher lymphocytes concentrations ($F_{1,2} = 16.7$, $P = 0.04$) and marginally higher total leukocyte concentrations ($F_{1,2} = 13.2$, $P = 0.06$) than group B. Group A also had higher PC3 scores than group B at baseline ($F_{1,2} = 14.9$, $P = 0.02$). Thus, for these measures we examined the effect of limited time access to food using both the difference before and after treatment (to control for differences at baseline) and absolute differences between the groups during the 6h and 22h 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 by day interaction $F_{4,67} = 3.42$, $P = 0.013$). In block 1, *S. aureus* killing increased in group A (6h treatment), but remained constant in group B (22h treatment; Figure 7.2B). During block 2 this pattern reversed, *S. aureus* killing increased in group B (6h treatment) and returned to baseline in group A (22h treatment; Figure 7.2B). 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, only PC3 (lymphocytes and monocytes) showed a statistically significant group by day interaction ($F_{4,66} = 2.66$, $P = 0.04$). However, Tukey post-hoc analysis indicated that this interaction was caused by a significant drop in PC3 from baseline to day 42 in group A only (Figure 7.2C); a different pattern from the reversible food treatment effect seen in hematocrit and *S. aureus* killing.

ACUTE PHASE RESPONSE – SICKNESS BEHAVIOR AND BODY TEMPERATURE

Injected and un.injected birds did not differ significantly on any temperature or behavioral indices (all P 's > 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 (Figure 7.3A to C). For feeding behavior, injected birds in the 22h treatment showed a trend for decreased feeding relative to un.injected birds, whereas injected birds in the 6h treatment showed a trend for increased feeding relative to un.injected birds (Figure 3A; LPS*Treat: $F_{1,13} = 8.01$, $P = 0.01$). Furthermore injected birds in the 6h treatment fed more than both injected and un.injected birds in the 22h treatment. For body mass, injected birds in the 22h treatment showed a trend for mass loss compared with un.injected birds whereas injected and un.injected birds both lost mass in the 6h treatment (Figure 7.3B; LPS*Treat: $F_{1,13} = 4.85$, $P = 0.04$). For body temperature, no significant difference was found between injected and un.injected birds in the 22h group, but in the 6h group injected birds increased or held body temperature steady, whereas un.injected birds decreased temperature (Figure 7.3C; 6h after injection LPS*Treat: $F_{1,13} = 14.38$, $P = 0.002$ and 17h after injection LPS*Treat: $F_{1,13} = 6.07$, $P = 0.03$).

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: $F_{1,2} = 0.13$, $P = 0.75$, Figure 7.3D) 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 6h food access (three in Group A and one in Group B) and one had an elevated leukocyte count and became ill upon returning to 22h food after three weeks on 6h food. Of the three birds in Group A that became ill during 6h food, two recovered when back on 22h food. Analysis of samples from the foot and wing lesions indicated bumblefoot and avian pox infections (T. Kuiken, pers. comm.) 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 (USGS 1999).

LPS effects on constitutive immunity

Injected and un.injected birds did not differ significantly on any constitutive immune indices (all P 's > 0.160) before LPS injection (day 15). However, by chance, injected

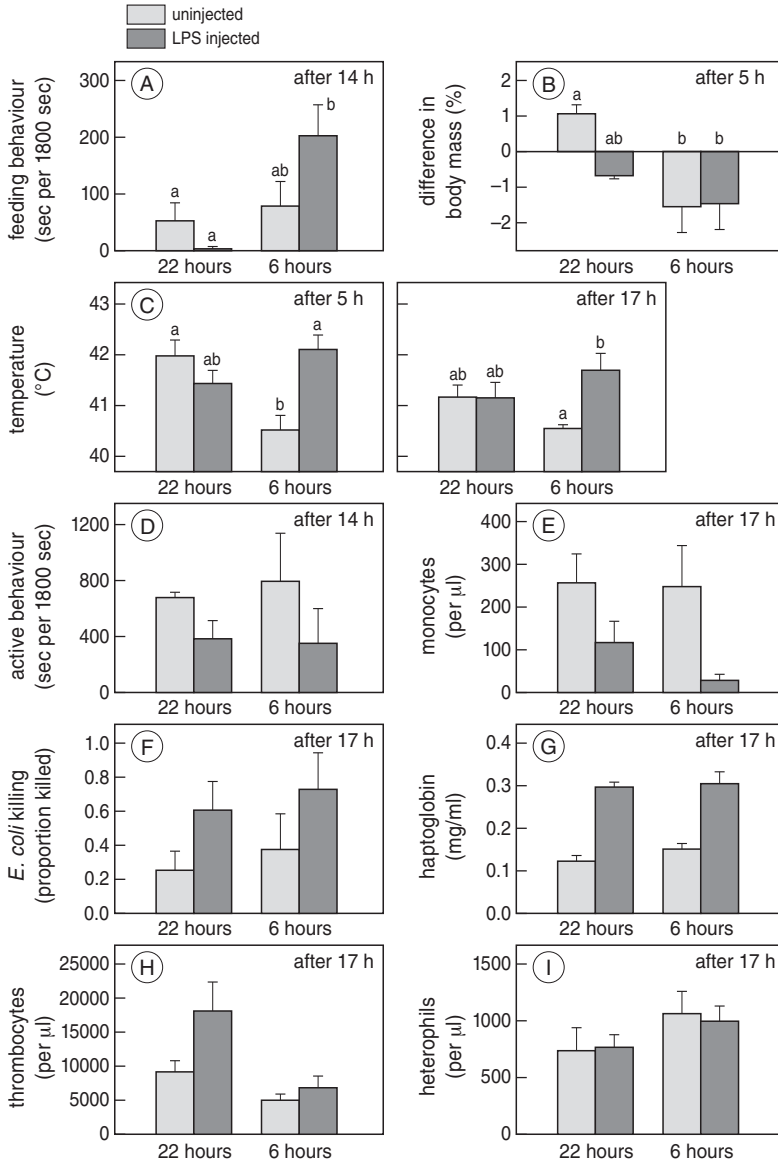


Figure 7.3. The effect of limited time access to food on the lipopolysaccharide (LPS) induced acute phase response: (A) feeding (B) body mass (C) temperature (D) activity (E) monocytes (F) *E. coli* killing (G) haptoglobin (H) thrombocytes (I) heterophils. Light grey bars = uninjected (22 hours, n = 5, 6 hours, n = 4); dark grey bars = LPS injected (22 hours, n = 6, 6 hours, n = 4). Bars are least squared means and error bars ± one SE. In (A) to (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) to (I) LPS and treatment main effects are presented in the text.

birds in the 6 hour group had higher hematocrit than 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 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: $F_{1,2} = 0.14$, $P = 0.74$, Figure 7.3E), 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: $F_{1,2} = 0.88$, $P = 0.44$; Figure 7.3F to G). 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 22h treatment, although neither the interaction nor the treatment effect are statistically significant (treatment*LPS: $F_{2,13} = 0.54$, $P = 0.82$; Treatment: $F_{1,2} = 7.30$, $P = 0.11$; Figure 7.3H). Food treatment, but not LPS, affected heterophils, which were higher in the 6h treatment (both injected and uninjected) than in the 22 hour group after LPS injection (Figure 7.3I, Treatment $F_{1,2} = 15.53$, $P = 0.04$; LPS: $F_{1,14} = 0.10$, $P = 0.75$). Analysis using the PC scores showed trends for LPS induced increases in PC1 (LPS: $F_{1,14} = 4.50$, $P = 0.05$; Treatment: $F_{1,2} = 0.02$, $P = 0.89$) and PC4 (LPS: $F_{1,2} = 3.98$, $P = 0.06$; Treatment: $F_{1,2} = 0.004$, $P = 0.95$) and decreases in PC3 (LPS: $F_{1,2} = 4.46$, $P = 0.05$; Treatment: $F_{1,2} = 0.23$, $P = 0.68$). No treatment*LPS interactions were significant for either individual immune indices ($P > 0.8$) or for PC scores ($P > 0.75$).

DISCUSSION

This study investigated constitutive immune function and the acute phase response in red knots faced with limited time access to food. Our 6h food treatment led to a clear energy deficit, producing reversible adjustments in body mass, feeding behavior (Vézina et al. submitted), and hematocrit (an indicator of nutritional status; Campbell 1995). Nevertheless, we found little affect 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 measured only *S. 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 n.s.). Even when all indices of constitutive immunity were considered together, none of the PCs were significantly affected by food treatment. PC3 decreased over the course of the experiment in group A regardless of food treatment, whereas PC3 scores remained constant in group B, indicating that the effect was not driven by our by food treatments. 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 (Buehler and Piersma 2008; Piersma 2007; van Gils et al. 2006). 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. submitted). A similar situation is seen in the maintenance of mass independent basal metabolic rate during limited food access (Vézina et al. submitted).

On the other side of the energy budget equation, Buehler et al. (2008a) manipulated energy expenditure by making birds live at winter-like 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; Vézina et al. submitted); 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 during the 6h treatment. This result may be a mechanistic response to the stress of limited food availability. Our 6h treatment led to a clear energy imbalance (Vézina et al. submitted) 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. Since 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 6h and 22h 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), usually seen 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 22h treatment tended to decrease feeding and lose mass relative to uninjected birds, injected birds in the 6h treatment tended to increase feeding and lost approximately the same amount of mass as uninjected birds. Because body mass was taken when birds in the 6h 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 6h-birds is consistent with the energy limitation hypothesis (Owen-Ashley and Wingfield, 2007) stating that the acute phase response may be suppressed if reductions in energy reserves caused by sickness behavior result in body mass values 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 was slowed via behavioral adjustments including vastly increased feeding during periods when food was available (Vézina et al. submitted). Thus, during the acute phase response, birds in the 6h 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 6h group to save energy (i.e. if an interactive effect was present). Instead, our data indicate no significant response in the 22h treatment and decreased temperature in uninjected birds in the 6h treatment (Figure 7.3C). In many bird species hypothermia is used as a strategy to conserve energy during periods of restricted food availability (Hainsworth et al. 1972; MacMillen and Trost 1967; Rashotte and Henderson 1988). We measured body temperature at times when birds in the 6h treatment did not have access to food. Thus, the lower body temperature seen in the uninjected birds is likely reflecting an energy conserving strategy in response to limited food access. However, in the injected birds this energy conserving strategy may have been over ridden by the acute phase response.

The difference in body temperature between injected and uninjected birds in the 6h treatment was 1.5°C and 1°C after 5h and 17h, respectively (Figure 7.3C). According to the equation $MR = c(T_b - T_a)$, where MR is metabolic rate, c is thermal conductance (0.045W/°C for red knots; Wiersma and Piersma 1994), T_b is body temperature and T_a is ambient temperature (12.7°C ± 0.5°C in the cages), and assuming that birds in the 6h treatment remain hypothermic all day, uninjected birds saved about 0.07W on thermoregulation. This suggests a 4.2% saving on overall daily energy expenditure (DEE; average baseline DEE is 1.66W for group A; Vézina et al. submitted). This saving 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 22h food. 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.

Table 7.2. A summary of hypothesized costs and immune strategies: [1] inferred using Klasing (2004), [2] Janeway et al. (2004), [3] Matson et al. (2006b), Millet et al. (2007), [4] Matson et al. (2005), [5] T. Kuiken, pers. comm. Groupings within constitutive immunity from this study and Buehler et al. (2008a and b).

Specific Index	Description and Function	Use Cost [1]	Strategy
Lymphocytes	Form the basis of specific immunity [2]	Low	Constitutive, leukocytes associated with specific (acquired) immune defense
Monocytes	Long-lived phagocytes and presenting cells for specific immunity [2]		
Thrombocytes	Important for blood clotting [2]		
<i>E. coli</i> killing	Bacteria defense - mostly without phagocytosis [3]	Moderate	Constitutive, soluble factors associated with non-specific and immediate defense
Hemagglutination	Natural antibody mediated clumping of invaders [4]		
Hemolysis	Complement mediated lysis and opsinization [4]		
<i>S. aureus</i> killing	Bacteria defense - mostly by phagocytosis [1]	High	Constitutive, associated phagocytosis with non-specific and immediate defense
<i>C. albicans</i> killing	Yeast defense - mostly by phagocytosis [3]		
Heterophils	Short-lived phagocytes [2]		
Temperature	Kills invaders with heat [2]	High	Induced, inflammation and systemic non-specific defense
Haptoglobin (induced)	Acute phase protein [2]		
Decreased feeding	Starves invader while saving energy [2]		

The effect of LPS injection on constitutive immune indices

The injection of LPS increased *E. coli* killing and tended to decrease *C. albicans* killing (though not statistically significant $P = 0.32$, data not shown), 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 22h 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 (Figure 7.3G). 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 (Norris and Evans 2000; Råberg et al. 1998; Schmid-Hempel and Ebert 2003; Sheldon and Verhulst 1996) especially in energetically constrained situations. To illustrate the costs and benefits of immune indices meas-

ured in this study, we summarize constitutive immune groupings identified by PCA (this study, Buehler et al. 2008a and b) with the acute phase response (Table 7.2). We exclude thrombocytes and baseline haptoglobin because these indices were not measured in all studies. Table 7.2 shows that constitutive immune indices cluster into three different cost and benefit strategies: a low cost strategy associated with lymphocytes and monocytes; a moderate cost strategy comprised of soluble factors (*E. coli* killing, hemolysis and hemagglutination); and a high cost strategy comprised of phagocytosis-based defense (*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 down-regulated during resource limitation. This suggests that all constitutive immune strategies are lower cost 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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