JOURNAL OF Evolutionary Biology

doi: 10.1111/j.1420-9101.2012.02543.x

Independence among physiological traits suggests flexibility in the face of ecological demands on phenotypes

D. M. BUEHLER*†, F. VÉZINA‡, W. GOYMANN§, I. SCHWABL§, M. VERSTEEGH¶, B. I. TIELEMAN¶ & T. PIERSMA¶**

*Ecology and Evolutionary Biology, University of Toronto, Toronto, Ontario, Canada
†Natural History, Royal Ontario Museum, Toronto, Ontario, Canada
‡Département de Biologie, Chimie et Géographie, Groupe de recherche sur les environnements nordiques BORÉAS, Centre d'études nordiques, Université du Québec à Rimouski, Rimouski, Québec, Canada
§Abteilung für Verhaltensneurobiologie, Max-Planck-Institut für Ornithologie, Seewiesen, Germany
¶Animal Ecology Group, Centre for Ecological and Evolutionary Studies, University of Groningen, Groningen, The Netherlands
**Department of Marine Ecology, Royal Netherlands Institute for Sea Research (NIOZ), Texel, The Netherlands

Keywords:

basal metabolic rate;
constitutive immune function;
corticosterone;
flexible phenotype;
haematocrit;
pace-of-life;
shorebirds.

Abstract

Phenotypic flexibility allows animals to adjust their physiology to diverse environmental conditions encountered over the year. Examining how these varying traits covary gives insights into potential constraints or freedoms that may shape evolutionary trajectories. In this study, we examined relationships among haematocrit, baseline corticosterone concentration, constitutive immune function and basal metabolic rate in red knot *Calidris canutus islandica* individuals subjected to experimentally manipulated temperature treatments over an entire annual cycle. If covariation among traits is constrained, we predict consistent covariation within and among individuals. We further predict consistent correlations between physiological and metabolic traits if constraints underlie species-level patterns found along the slow-fast pace-of-life continuum. We found no consistent correlations among haematocrit, baseline corticosterone concentration, immune function and basal metabolic rate either within or among individuals. This provides no evidence for constraints limiting relationships among these measures of the cardiovascular, endocrine, immune and metabolic systems in individual red knots. Rather, our data suggest that knots are free to adjust individual parts of their physiology independently. This makes good sense if one places the animal within its ecological context where different aspects of the environment might put different pressures on different aspects of physiology.

Introduction

Organismal traits can show amazing flexibility, with physiological characteristics from gut size, to metabolic rate, to immune function showing as much variation as behaviour (Piersma & van Gils, 2011). This phenotypic flexibility allows animals to adjust their physiology to diverse environmental conditions and can potentially influence evolution through selection operating on suites of flexible but linked traits with downstream consequences for the genome (West-Eberhard, 2003), and/or, through the accumulation and release of cryptic genetic variation (reviewed by Pfennig *et al.*, 2010). Therefore, studying how flexible traits relate to each other – how they covary – adds a layer of complexity needed to gain insight into the constraints or freedoms that may shape evolutionary trajectories (Lande & Arnold, 1983).

Trait covariances, for example those among morphological traits, can have profound evolutionary implications (Lande & Arnold, 1983) because they affect how populations respond to selection by constraining responses, generating trade-offs, or otherwise shaping evolutionary trajectories (Roff & Fairbairn, 2007).

Correspondence: Deborah Buehler, Office of the Vice President, Research 12 Queen's Park Crescent, Toronto, ON, M5S 1S8.
Tel.: +1 416 978 3205; fax: +1 416 946 3707; e-mail: d.buehler@utoronto.ca

Therefore, the way that physiological traits covary at the individual level may be relevant for testing hypotheses about how these same traits covary at the species level. For example, the pace-of-life hypothesis (Ricklefs & Wikelski, 2002) argues that observed correlations between demographic and metabolic traits along a slow-fast continuum (Promislow & Harvey, 1990) might indicate that individual responses to different environments are limited by physiological mechanisms. In other words, different physiological systems are intricately linked and thus constrained (i.e. the endocrine system, the immune system and the metabolic system; Dhabhar *et al.*, 1995; Lochmiller & Deerenberg, 2000; Speakman, 2005; Landys *et al.*, 2006; Ardia *et al.*, 2011). If such constraints underlie the slow–fast continuum seen at the species level, then physiological traits should covary in the same way at the species and individual levels (Lande, 1979). This line of thinking also predicts consistent correlations among physiological traits, and between these traits and metabolic traits (i.e. basal metabolic rate, BMR) at the individual level.

Thus, at the individual level, consistent correlations among physiological traits are predicted if functional interactions among suites of traits limit trait evolution – functional constraint (Schwenk & Wagner, 2001). However, traits may also be negatively correlated in some environments or parts of the year due to trade-offs necessitated by the allocation of limited resources (Ardia *et al.*, 2011). Finally, phenotypic flexibility may allow organisms to adjust individual aspects of their physiology independently. This may reflect the fact that an organism's overall physiology must serve multiple functions, with each physiological system responding to environmental conditions particular to its function (Piersma & van Gils, 2011). This freedom to adjust individual aspects of physiology might be important in environments with, or during times of the year when, incompatible demands result in physiological conflicts (Ramenofsky & Wingfield, 2006; Vézina *et al.*, 2010, 2012).

Though seemingly clear-cut, these hypotheses may be difficult to tease apart. Consider a simplified and hypothetical example of two physiological traits (A and B) over the annual cycle (Fig. 1a). The scenarios detailed in the figure show that relationships between the traits in different periods of the year (columns represent Jan, May and Aug) could indicate: (a1) true functional constraint; (a2) negative relationships during periods of scarcity (trade-offs) and positive relationships during periods of abundance even though traits are independent; (a3) negative or positive relationships due to parallel or opposite responses to the environment (Ricklefs, 2000) even thought traits are independent; or (a4) a consistent lack of correlations indicating independent traits responding to independent aspects of the environment. Importantly, the negative correlations in column one (Jan) in a1–a3 could be interpreted as constraint if studied in isolation, but only a1, where the traits are correlated in the same way in all months, represents true functional constraint. This complexity highlights the importance of sampling traits in different environments and over time.

At the individual level, physiological syndromes (akin to behavioural syndromes reviewed by Bell, 2007) may exist if physiological traits are consistently correlated within an individual over time and across environments. The scenarios detailed in Fig. 1b show that relationships between traits measured repeatedly in different individuals (columns represent birds 1–3) could indicate: (b1) physiological syndromes caused by functional constraint; (b2) consistent syndromes within an individual over time, but because at least two relationships are possible in the population, no evidence for constraints that operate consistently in all individuals (i.e. if they exist, they are not universal); (b3 and b4) no syndromes or functional constraint. The pattern in b2 may also be caused by ecological constraints (e.g. individuals using different habitats). In b3, the strategy of 'no relationship' is not a syndrome because the relationship is not consistent over time (dots in the data could represent multiple samplings within the individual, and all possible combinations are present).

Red knots *Calidris canutus* (Linnaeus 1758) of the northerly wintering subspecies *C. c. islandica* (hereafter knots) are medium-sized (100–200 g) long-distance migrant shorebirds. Because they live well in captivity and have well-studied annual cycles and physiology (Piersma, 2007; Buehler & Piersma, 2008; Piersma & van Gils, 2011), we used them as a model to investigate covariation among different physiological traits over time and among individuals. Although many aspects of knot physiology are flexible, in this study, we focus on four traits: (i) haematocrit to represent the cardiovascular system, (ii) baseline corticosterone to represent the endocrine system, (iii) BMR to represent metabolism and (iv) aspects of constitutive (noninduced) immune function to represent the immune system. Haematocrit is the proportion of red blood cells per total blood volume and, along with haemoglobin concentration and oxygen affinity, determines blood oxygen-carrying capacity. Increased haematocrit is associated with increased oxygen-carrying capacity during increased workload in migrating birds (Bairlein & Totzke, 1992; Piersma *et al.*, 1996; Prats *et al.*, 1996), including associations between haematocrit and premigratory mass gain in knots (Piersma *et al.*, 2000a; D.M. Buehler, unpublished); and with cardiovascular responses accompanying changes in body temperature in lizards (Snyder, 1977) and frogs (Withers *et al.*, 1991). Thus, haematocrit represents an easily obtainable and widely measured aspect of the caridiovascular system's ability to deliver oxygen. Corticosterone is a widely measured glucocorticoid hormone involved in the onset and regulation of migratory movements and is elevated in association with migration and arrival at

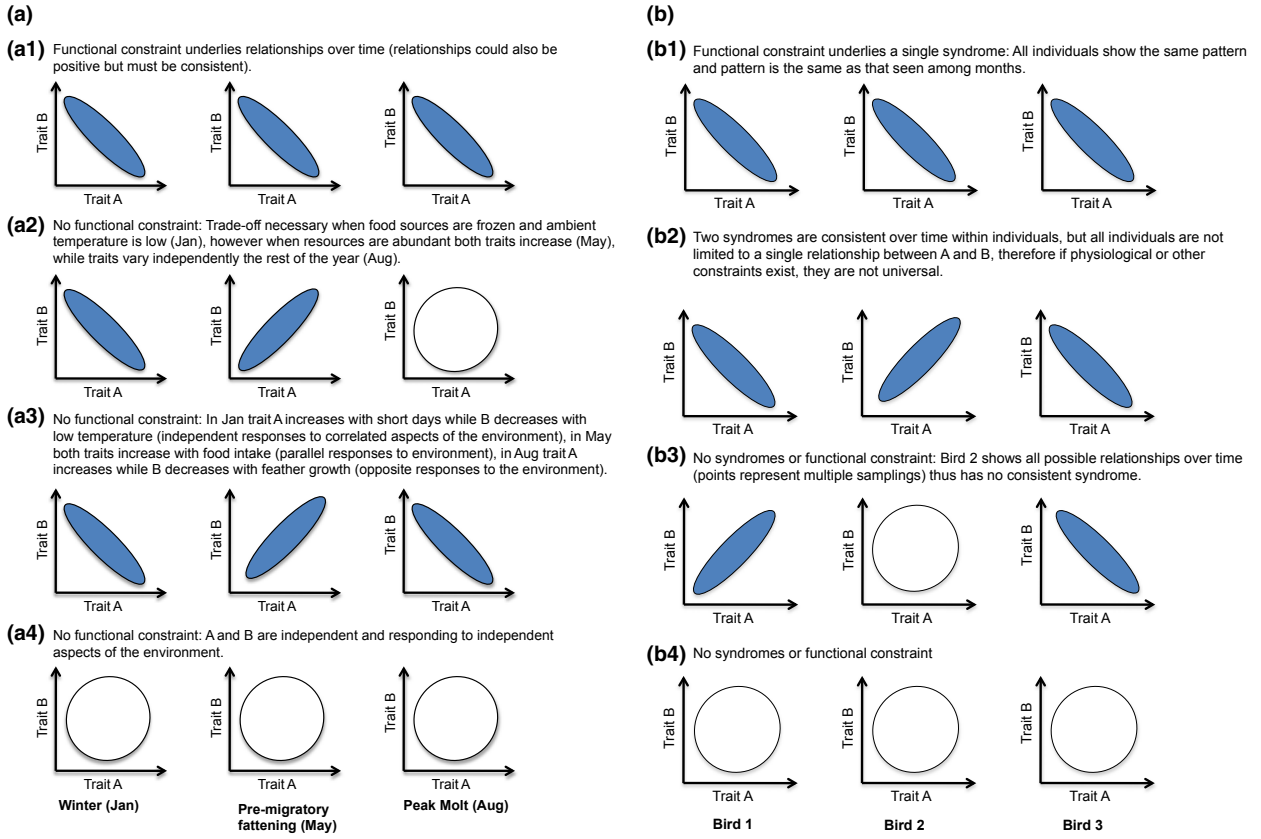

Fig. 1 Hypothetical scenarios illustrating how physiological traits A and B might covary both over time (a) and among individuals (b). Data clouds are represented by ellipses, where the tilt, narrowness and colour of the ellipse represent the direction, strength and significance of the correlations. In A points in the data cloud are individuals, and each graph in a row represents a month in the annual cycle (Jan, May or Aug). In B points in the data cloud represent repeated samplings of the same individual, and each graph in a row represents a different individual.

the breeding grounds in knots (Piersma *et al.*, 2000b; Reneerkens *et al.*, 2002; Landys *et al.*, 2004). Corticosterone may therefore be correlated with other physiological processes known to vary in association with migration including haematocrit (Piersma *et al.*, 1996, 2000a; Landys-Ciannelli *et al.*, 2002), immune function (Buehler *et al.*, 2008b) and BMR (Vézina *et al.*, 2011).

In this study, we describe annual variation in haematocrit and baseline corticosterone and combine these data with previously published datasets of immune function (Buehler *et al.*, 2008b) and BMR (Vézina *et al.*, 2011) measured in the same individuals. These birds were part of a yearlong experiment in which we exposed them to cold, warm (thermoneutral) or variable (tracking seasonal conditions) temperatures to manipulate thermoregulatory costs and to uncouple seasonal changes from physiological adjustments to ambient temperature (see Buehler *et al.*, 2008b; Vézina *et al.*, 2011 for details). Indices of immune function were chosen to cover a range of protective functions including the functional capacity of blood to limit microbial infection (Tieleman *et al.*, 2005; Millet *et al.*, 2007), concentrations of circulating immune cells and levels of complement and natural antibodies. These immune indices are seasonally variable but also repeatable characteristics of individual birds (Buehler *et al.*, 2008b); furthermore, they assay an evolutionarily important branch of the immune system that provides broad spectrum and immediate protection against invaders. We used these combined data on haematocrit, corticosterone, immune function and BMR to look at covariation among traits over time and at the individual level. If functional constraints give rise to physiological syndromes, then we predict consistent covariation within and among individuals. Furthermore, if functional constraints underlie species-level patterns found along the slow-fast pace-of-life continuum, then we predict consistent correlations between physiological and metabolic traits.

Materials and methods

Birds and experimental treatments

Knots were captured in the Dutch Wadden Sea (53°31′N, 6°23′E) in August and September 2004. We ringed, weighed and aged (all > 2 years; Prater *et al.*, 1977) birds at capture, and sex was later determined using molecular techniques (Baker *et al.*, 1999). The birds were then brought into captivity at the Royal Netherlands Institute for Sea Research (NIOZ) where they had at least 1 month to acclimate to captivity before the start of measurements. The birds had free access to food (mudsnails *Hydrobia ulvae*) and fresh water for drinking, and we set photoperiod to track the seasonal changes in day length in the northern Netherlands.

We randomly assigned birds (total $n = 30$, 21 females, 9 males) to three treatment groups in identical indoor aviaries ($4.5 \times 1.5 \times 2.3$ m high). Birds in the variable treatment ($n = 12$) were divided equally between two aviaries ventilated with outdoor air and maintained at outdoor temperature (mean temperature of 15.4 ± 4.8 °C). Birds in the warm treatment ($n = 12$) were also divided between two aviaries and were maintained at 24.7 ± 1.8 °C. Finally, birds in the cold treatment ($n = 6$) were kept in a single aviary at 4.9 ± 1.2 °C (see Buehler *et al.*, 2008b and Vézina *et al.*, 2006 for details). All groups were similar in terms of sex ratio and morphometrics (Vézina *et al.*, 2006).

Over the course of the experiment, three individuals died (all female, two from the cold treatment and one from the warm treatment). Two of the causalities were replaced meaning that a total of 32 individuals were studied during the course of a year. We include all 12 months and 32 individuals in our univariate analysis of corticosterone concentration and haematocrit. However, we use only 11 months (because we lack microbicidal capacity data for 1 month) and the 27 birds for which we have data for all months for our analysis of covariation.

Measuring haematocrit, corticosterone and immune function

We took blood samples to assess haematocrit, baseline levels of corticosterone and immune function every month from March 2005 to March 2006 (mean ± SD = 10 : 44 ± 5 min). Because both corticosterone concentration and aspects of immune function can be affected by capture and handling, we took samples for corticosterone analysis within 2 to 4 min of entering an aviary (Wingfield *et al.*, 1995) and samples for assays of immune function within 20 min of entering an aviary (Buehler *et al.*, 2008a).

Haematocrit data were obtained 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. Similarly, we obtained plasma for corticosterone and plasma-based assays of immune function by centrifuging blood samples for 12 min at 12 000 *g*.

Plasma for corticosterone analysis was stored at −80 °C and then shipped on dry ice to the Max Planck Institute for Ornithology, Andechs. Corticosterone concentrations were determined by direct radioimmunoassay (Goymann *et al.*, 2006), using antibody obtained from Esoterix Endocrinology (Calabasas Hills, CA, USA). Extraction efficiency was 87.7 ± 4.6% (± SD), as calculated from trace amounts of tritiated hormone added to each sample (NET 399; Perkin Elmer, Rodgau, Germany). Due to the large number of samples, two assay runs were needed. Intra-assay coefficients of variation (CV) were 10.7% and 10.6%, and inter-assay CV was 12.2%. The limits of detection were 6.6 and 6.1 pg per tube in each assay run (all samples were well above this limit).

We used data on aspects of constitutive (noninduced) immune function published by Buehler *et al.* (2008b). These data comprise three general protocols measuring microbicidal capacity, leucocyte concentrations and complement-like and natural antibody activity. Microbicidal capacity measures the functional capacity of blood to limit microbial infection (Tieleman *et al.*, 2005; Millet *et al.*, 2007). This assay is commonly known as the bacteria killing assay (BKA), but we stick to microbicidal capacity because we also measure killing of yeast. Differential leucocyte concentrations provide information on circulating immune cells (Campbell & Ellis, 2007) and are useful in their relationship to functional immune assays such as microbicidal capacity (Buehler *et al.*, 2008b). Complement-like activity and natural antibodies provide a first line of defence against spreading infections and link innate and acquired immunity (Ochsenbein & Zinkernagel, 2000). Here, we focus on the microbicidal capacities of whole blood against *E. coli* (after 10 min incubation), *C. albicans* (after 60 min) and *S. aureus* (after 120 min); circulating concentrations of heterophils, lymphocytes and monocytes; and haemolysis and hemagglutination titres assaying complement-like and natural antibody activity against rabbit red blood cells.

Measuring metabolic rate

We used data on BMR published by Vézina *et al.* (2011). Briefly, BMR was measured on two birds from the same aviary each day over 15 consecutive days. The order in which specific birds were measured was randomly chosen, and the same order was kept for the duration of the experiment. We removed food from the aviary at 17h00 the day before measuring BMR and the next morning at 10h00 placed the two birds to be measured in a holding box with water but without food (food was returned to the other birds in the aviary). Measurements began at 16h00 and lasted until 9h00 the following morning. Birds were weighed before and after BMR sessions, and average mass was used in the analysis. We

define BMR as the oxygen consumption/energy expenditure of a resting post-absorptive knot measured at night and at thermoneutrality (see Vézina *et al.*, 2006, 2007 for setup and methods). In our analyses, we examine both whole-organism BMR and mass-corrected BMR (the residuals of a linear model between log BMR and log body mass), the latter of which is a predictor of tissue-level processes.

Statistical analyses

We performed all statistical analyses using the R programming environment (R Development Core Team, 2011) and report packages used in specific analyses below. We used visual examination of histograms to check data and model residuals for normality, and log10 transformed corticosterone and leucocyte concentrations for all analyses.

Haematocrit and corticosterone among treatments and over the annual cycle

We used nested general linear models (lme in lme4 and nlme) to investigate how haematocrit and baseline corticosterone vary between treatments (main effect of treatment) and over the annual cycle (main effect of month). We also examined whether any differences between the treatments depended on the time of year (month × treatment) and whether there were sex effects (main effect of sex, month × sex). Cage and individual bird were included in all models as random effects. We used similar nested general linear models to generate residuals (corrected for the effects of treatment, cage and month) for use in principle component analysis. For univariate analyses examining how treatment and season affect immune measures (including immune PCs) and metabolism, we refer the reader to Buehler *et al.* (2008b) and Vézina *et al.* (2011), respectively.

Bivariate correlations to examine covariation over time and among individuals

Bivariate correlations provide a simple way to look at covariation among variables across multiple samplings (or groups) without assuming that relationships among variables are the same across samplings (Buehler *et al.*, 2011). We calculated Pearson correlation coefficients within and among birds as described by Buehler *et al.* (2011) and present these correlations as ellipses (Murdoch & Chow, 1996) modified to include representation of both *r*- and *P*-values (using ellipse, lattice, Matrix, Hmisc; A. Cohen, pers. com.). Rather than presenting a matrix with all pairwise correlations for each month or individual, we reorganized the plots to show BMR in columns (one column for each month or bird) and the other physiological traits in rows. These plots allow us to see how measures of immune function, corticosterone and haematocrit covary with metabolic rate and to gauge the consistency and strength of correlations over time and among individuals. We also present mean correlation coefficients (the mean of 27 individuals per month or the mean of 11 months per individual), *t*-tests, 95% confidence intervals and ranges for all trait pairs as a supplementary table (excluding immune index pairs that are presented by Buehler *et al.*, 2011). To determine thresholds for statistical significance while accounting for multiple comparisons, we employ two methods of correction: sequential Bonferroni correction (Rice, 1989) and an approach based on False Discovery Rates (Pike, 2011).

Principal component analysis (PCA) to examine covariation among individuals

We were also interested in how metabolic rate, corticosterone and haematocrit covary with immune function, and we used PCA to reduce the number of immune variables to a few orthogonal components. We calculated principal component scores using varimax rotation (psych), for consistency with Buehler *et al.* (2008b) using the residuals of general linear models to take into account multiple sampling (each bird is represented 11 times). Essentially this means we pooled months within each bird assuming that relationships among immune variables were similar across months (an assumption proven reasonable by Buehler *et al.*, 2011). We did not pool individuals within months for a similar analysis because the assumption that relationships among immune variables are the same among individuals is not valid (Buehler *et al.*, 2011).

Results

Variation in haematocrit and corticosterone among treatments and over time

Similar to the previously reported effects of treatment on body mass (Vézina *et al.*, 2007; Buehler *et al.*, 2008b), birds in the cold treatment had consistently higher haematocrit than birds in the warm treatment (compare Fig. 2a,c), although this effect did not reach statistical significance ($F_{2,2} = 3.11$, $P = 0.24$). Similarly, males had consistently higher haematocrits than females, except in September when levels in females approached those in males (month × sex: $F_{11,301} = 1.95$, $P = 0.03$). Also like body mass, birds in the variable treatment tended to have higher haematocrit (approaching values in the cold treatment) during the winter months, and lower values in summer months (approaching values in the thermoneutral treatment; Fig. 2a,c). Again, this interaction was not statistically significant (month × treatment: $F_{11,291} = 1.17$, $P = 0.28$) and may have been masked by an overall pattern of higher haematocrit in winter months and lower haematocrit in summer for all treatments ($F_{11,313} = 10.61$, $P < 0.0001$).

We found no significant effects of thermal treatments or sex on corticosterone concentration, either as a main effect (treatment: $F_{2,2} = 0.75$, $P = 0.57$, sex: $F_{1,26} = 2.09$, $P = 0.16$) or depending on the time of year (treatment × month: $F_{22,230} = 0.41$, $P = 0.99$, sex × month: $F_{11,241} = 1.69$, $P = 0.08$). However, corticosterone did vary significantly among months over the year ($F_{11,252} = 3.95$, $P < 0.0001$) with a pronounced peak in late April to May coinciding with peak body mass in preparation for migration (Fig. 2b,c). This peak in corticosterone concentration seems present in the warm and variable treatments, but not in the cold treatment. Nevertheless, differences between these groups in May and June did not reach statistical significance (May $F_{2,2} = 0.58$, $P = 0.63$; June $F_{2,2} = 0.69$, $P = 0.59$).

Consistency of intra-individual inter-seasonal correlations over time

We used bivariate correlations to examine the consistency of intra-individual inter-seasonal correlations at the month level. Figure 3 shows Pearson correlations between whole animal BMR (in columns) and mass-corrected BMR (rBMR), immune indices, corticosterone concentration and haematocrit (in rows). We include rBMR so that the strong and consistent correlation between BMR and rBMR can serve as a point of reference for the other correlations. BMR and rBMR are correlated because when the majority of variation in BMR is not related to mass (e.g. in this study mass explained < 50% of variation in BMR on average), these measures represent nearly the same variable because rBMR is BMR with variation due to mass removed (Tieleman *et al.*, 2009). Correlations in Fig. 3 are depicted as ellipses with tilt indicating the direction, narrowness the strength, and shading the significance of the relationship.

These ellipse plots clearly show a lack of correlations, either positive or negative, between metabolism and immune function, corticosterone or haematocrit over the annual cycle (Fig. 3a; see Table 1 for abbreviations used in the figures, tables and text). Furthermore, the few correlations found were not consistent over time (e.g. Hct was positively correlated with BMR in April during

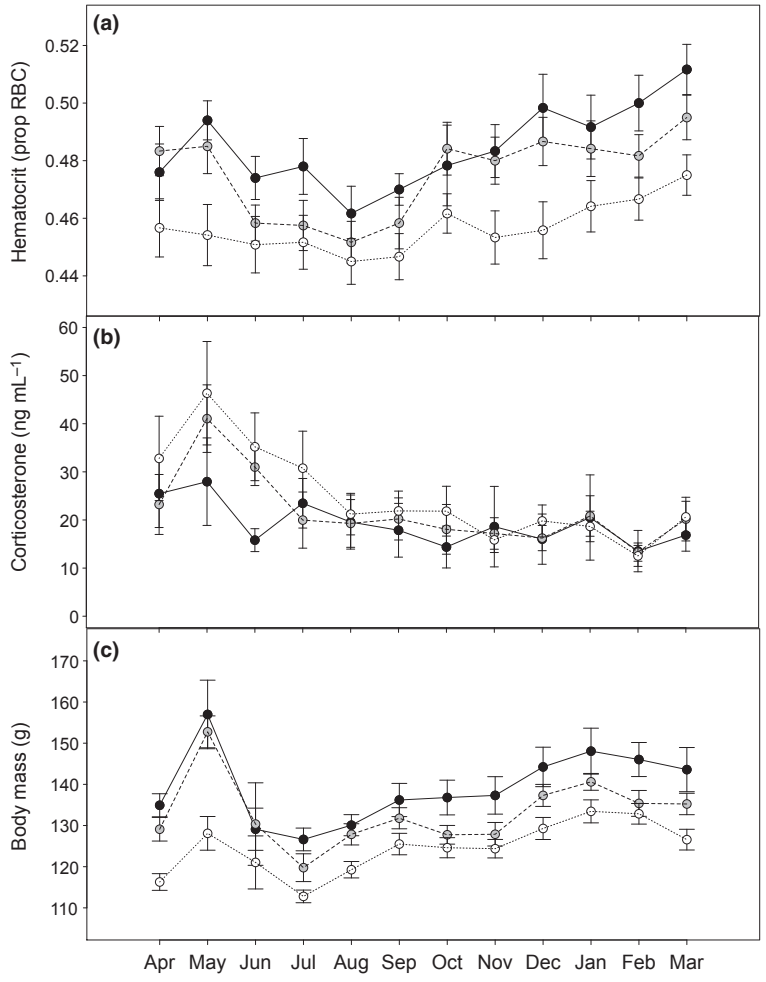

Fig. 2 Haematocrit (a), corticosterone concentration (b) and body mass (c) measured in red knots over the annual cycle. Dots and error bars represent mean and standard error per month in the warm (white), variable (grey) and cold (black) treatments. See text for statistics.

Table 1 Abbreviations used in tables and figures.

Physiological variables	
BMR	Basal metabolic rate
rBMR	Residual (mass-corrected) BMR
Hct	Haematocrit
Cort	Corticosterone concentration
Het	Heterophil concentration
Lym	Lymphocyte concentration
Mon	Monocyte concentration
MCSa	Microbicidal capacity against *S. aureus*
MCCa	Microbicidal capacity against *C. albicans*
MCEc	Microbicidal capacity against *E. coli*
Lys	Complement-mediated lysis
Agg	Natural antibody-mediated agglutination

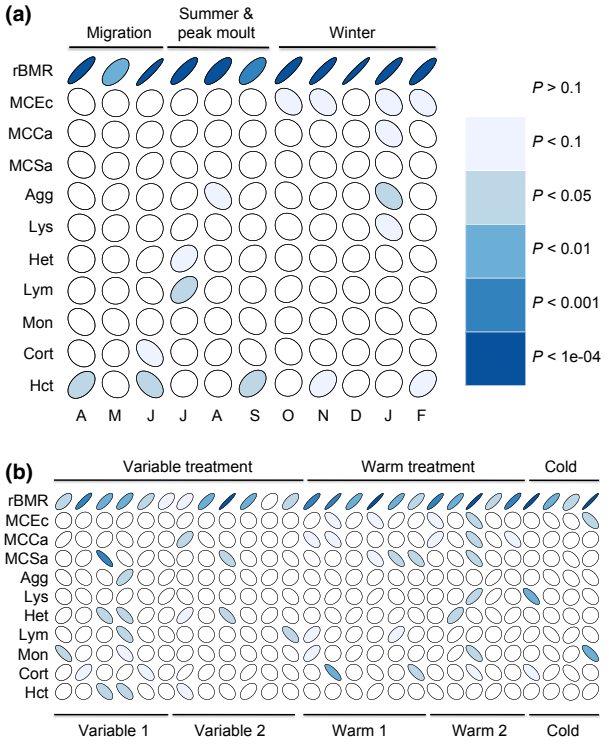

Fig. 3 Pearson correlations between BMR (columns) and mass-corrected BMR, corticosterone concentration, haematocrit and immune indices (rows) over an annual cycle (a, 11 columns per months) and in individuals (b, n = 27 columns per birds). Each ellipse represents a correlation from (a) 27 birds within a given month, or (b) 11 months within a given bird. Right-tilted ellipses indicate positive correlations and left-tilted ellipses negative correlations. The narrowness of ellipses is proportional to r-values and the shading to P-values (see legend).

premigratory mass gain and negatively correlated during June when birds are losing mass). T-tests for mean correlation coefficients differing from zero confirmed this lack of consistent relationships for all trait pairs (Table 3a). The lack of consistent correlations appears to be due to actual heterogeneity in the correlations over the annual cycle (and across individuals, see below), as detected by a nested model of correlation structure (*sensu* Cohen & McGraw, 2009), despite the limited sample size.

Consistency of inter-individual correlations

We used bivariate correlations and PCA analysis to examine the consistency of inter-individual correlations (averaged across seasons) at the bird level. The bivariate correlations in Fig. 3b show that the general pattern at the individual level is also a lack of correlations between metabolism and other physiological traits. Again, where correlations existed, they varied among individuals (e.g. MCSa is negatively correlated with BMR in some but not all individuals). T-tests revealed that among individuals, more correlations differed significantly from zero than when examining intra-individual patterns over time (Table 3a vs. 3b). BMR and rBMR were negatively correlated with MCSa, a pattern driven by negative correlations between these traits in some but not all individuals (Fig. 3b). In addition, Hct was negatively correlated with MCEc and positively correlated with Lys when correlation coefficients were averaged over all individuals; however, the range of correlations includes zero in both cases (Table 3b).

Not surprisingly, PCA analysis on a subset of the immune data presented by Buehler *et al.* (2008b) gave very similar loadings (Table S1) with Lym and Mon falling together on PC1, Het, MCSa and MCCa falling on PC2, and MCEc, Lys and Agg falling on PC3. Plotting metabolic rate, corticosterone and haematocrit against these principal components revealed no strong positive or negative relationships (Fig. 4) and no statistically significant correlations (Table 2). However, the plots in Fig. 4 do reveal substantial differences among individuals in the multivariate levels of these traits. This suggests that a substantial proportion of variance in multivariate physiology may be at the individual level.

Discussion

This study explored relationships among haematocrit, baseline corticosterone concentration, constitutive immune function and BMR in knot individuals. Here, we briefly discuss variation in haematocrit and corticosterone among treatments and over the annual cycle and then consider our main finding of no evidence for functional constraints limiting relationships among these aspects of the cardiovascular, endocrine, immune and metabolic systems in red knots.

Haematocrit and corticosterone among treatments and over the annual cycle

Haematocrit varied throughout the year according to seasonal variation in temperature. Not surprisingly, this

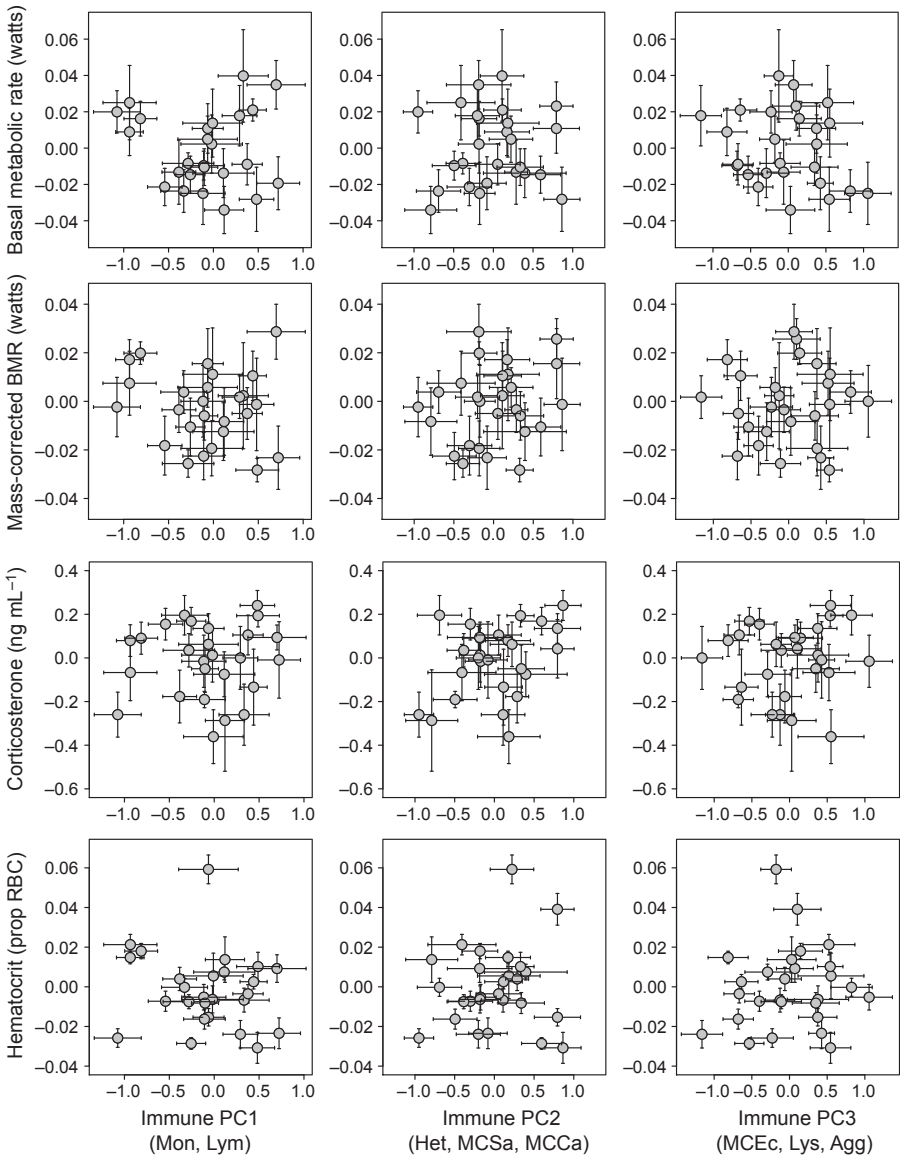

Fig. 4 Correlations between BMR, mass-corrected BMR, corticosterone concentration and haematocrit (rows) and principle components of immune function (see Table S2 for loadings). Data points represent the mean ± SE of 11 monthly measurements taken in individual birds (n = 27 points). See Table 2 for statistics.

pattern was most pronounced in the variable treatment where haematocrit was higher during the winter months, approaching the cold-acclimated birds, and lower in summer months, approaching the thermoneutral treatment (see trend in Fig. 2a). Furthermore, birds in the cold treatment had higher haematocrit than birds in the warm treatment (see trend in Fig. 2a) consistent with previous studies examining the effect of temperature on haematocrit (Kubena *et al.*, 1972; Fair *et al.*, 2007). That these patterns associated with treatments did not reach statistical significance may have been due to small samples sizes, and we recommend that more numerous and sex-balanced samples be used in future studies.

Haematocrit also showed a small peak during premigratory mass gain (most pronounced in the cold and variable treatments where mass gain was also largest; Fig. 2a,c). Increased haematocrit implies more red blood cells and higher oxygen-carrying capacity and might occur in conjunction with circannually programmed muscle hypertrophy in preparation for migration (Dietz *et al.*, 1999). Associations between haematocrit and premigratory mass gain have been found in captive knots (Piersma *et al.*, 2000a); and in free-living knots

Table 2 Pearson correlations between BMR, mass-corrected BMR, corticosterone concentration and haematocrit, and principle components of immune function (see Table S1 for loadings).

	Immune Function	*r*	*P*-value
Basal metabolic rate (watts)	PC1	0.01	0.98
	PC2	−0.03	0.90
	PC3	−0.28	0.16
Mass-corrected BMR (watts)	PC1	0.12	0.55
	PC2	0.22	0.28
	PC3	0.03	0.89
Corticosterone (ng mL^{-1})	PC1	0.11	0.60
	PC2	0.35	0.07
	PC3	0.10	0.61
Haematocrit (prop RBC)	PC1	0.14	0.47
	PC2	0.07	0.72
	PC3	0.08	0.68

($t = 2.764$, $P = 0.007$, D. M. Buehler, unpublished) and bar-tailed godwits *Limosa lapponica* (Piersma *et al.*, 1996; Landys-Ciannelli *et al.*, 2002).

Plasma corticosterone concentration peaked in late April to early May, coinciding with peak migratory mass gain (Fig. 2b,c), a pattern found previously in knots (Piersma *et al.*, 2000b; Landys *et al.*, 2004). Differences between the temperature treatments were largest during this peak period with birds in the warm and variable treatments showing pronounced peaks, whereas birds in the cold treatment lacked the peak altogether (Fig. 2b). This general pattern suggests that annual variation in corticosterone is not dependent on photoperiod alone and that ambient temperature may also play a role. Furthermore, the link between increased corticosterone and hyperphagia appears to be uncoupled in our data because birds in the warm treatment had the highest corticosterone peak but gained the least mass (Fig. 2b,c).

No evidence for functional constraint among physiological systems

If functional constraints limit the range of relationships possible among physiological systems, then we expected consistent patterns of covariation among the physiological traits we measured (resembling Fig. 1a1,b1). Further more, if physiological syndromes exist, then we expected physiological traits to covary consistently within and among individuals (Fig. 1b1,b2). Our results are not consistent with either hypothesis. The ellipse plots clearly show a lack of correlations, either positive or negative, between immune function, corticosterone, haematocrit and BMR (Fig. 3), and the few correlations that do exist are not consistently present across months or individuals. For example, Hct is positively correlated with BMR in April and September, negatively correlated in June and uncorrelated the rest of the year; and MCSa is negatively correlated with BMR in some, but not all individuals (akin to Fig. 1b3). Table 3b shows a few additional relationships when averaging across all individuals, namely a negative relationship between Hct and MCEc and a positive relationship between Hct and Lys. However, looking at the range of possible correlations reveals that some individuals show no correlation or even the opposite pattern (all ranges include zero except BMR–rBMR). This indicates the same lack of consistency as can be seen visually in Fig. 3b for correlations between BMR and MCSa.

Testing the simplified hypotheses presented in Fig. 1 (other than a1 and b1) will require further experiments. For example, manipulation of access to resources to test for trade-offs (a2) combined with measurements of multiple physiological variables, and a multivariate analysis of how variables covary in the experimental groups. Nevertheless, our findings clearly indicate inconsistent correlation structures within-species for physiological variables both across seasons and among individuals. This is concordant with similar findings for immune variables (Matson *et al.*, 2006; Buehler *et al.*, 2011) and antioxidant measures (Cohen & McGraw, 2009).

We further predicted consistent correlations between physiological and metabolic traits if functional constraints underlie species-level patterns found along the slow-fast Pace-of-Life continuum; however, we found no such correlations. This result agrees with a study carried out on stonechats *Saxicola torquata*, which found that neither corticosterone concentration or two of three PCs of immune function covaried with mass-corrected BMR (rBMR) (Versteegh, 2012), the one PC that did covary with metabolism included Lys and Agg and showed a negative relationship. Another study, on housewrens *Troglodytes aedon*, found a negative relationship between MCEc and rBMR at the individual level (Tieleman *et al.*, 2005). Both the stonechat and housewren studies showed that patterns of covariation among immune, endocrine and metabolic traits differed between the levels of species, subspecies and individual birds. Furthermore, relationships more complex than a single pace-of-life axis have been found between antioxidants and individual life-history traits at the interspecies level (Cohen *et al.*, 2008). Taken together, these results suggest that the phenomenon of a single pace-of-life axis does not result from universal physiological constraints (*contra* Lande, 1979; Ricklefs & Wikelski, 2002).

It is important to remember, however, that just because some physiological measures are not consistently correlated, it does not follow that all are not. We only measured one trait in each of the cardiovascular, endocrine and metabolic systems and only one branch of immune function. Relationships among physiological, metabolic and demographic traits exist at the species level, and our findings at the individual level do not contradict this. However, our findings, and those of others (Tieleman *et al.*, 2005; Cohen *et al.*, 2008; Vers-

Table 3 Mean correlation coefficients (in increasing order) between physiological variables measured in red knots (see Table 1 for abbreviations). The mean correlation coefficient over the annual cycle (a) is calculated from 11 correlations (months) each with $n = 27$ birds. The mean correlation coefficient among birds (b) is calculated from 27 correlations (birds) each with $n = 11$ months. Unadjusted *P*-values are presented, and bold text denotes correlations significantly different from zero using thresholds determined by sequential Bonferroni correction (Rice, 1989) and all three False Discovery Rate methods (Pike, 2011). Only 38 of 66 possible pairs are shown here; pairs of immune variables (e.g. Het-MCSa or Lym-Mon) are reported by Buehler *et al.* (2011).

Pair of variables	Mean *r* over the year	*t*-value	d.f.	*P*-value	95% confidence interval	Range
(a)						
BMR-MCEc	−0.135	−1.999	10	0.0735	−0.279 to 0.016	−0.277 to 0.078
BMR-Mon	−0.133	−3.283	10	0.0083	−0.220 to −0.043	−0.426 to 0.213
BMR-Cort	−0.101	−1.571	10	0.1473	−0.240 to 0.042	−0.397 to 0.280
rBMR-MCCa	−0.090	−1.384	10	0.1966	−0.232 to 0.055	−0.376 to 0.198
BMR-MCCa	−0.068	−1.150	10	0.2768	−0.197 to 0.063	−0.297 to 0.240
BMR-Lys	−0.066	−1.203	10	0.2566	−0.186 to 0.056	−0.241 to 0.300
Hct-MCSa	−0.065	−0.701	10	0.4994	−0.266 to 0.141	−0.378 to 0.292
BMR-Agg	−0.043	−0.526	10	0.6104	−0.220 to 0.137	−0.358 to 0.255
BMR-MCSa	−0.038	−0.737	10	0.4779	−0.153 to 0.077	−0.417 to 0.270
rBMR-Mon	−0.038	−0.903	10	0.3878	−0.131 to 0.056	−0.249 to 0.306
rBMR-MCSa	−0.035	−0.639	10	0.5375	−0.154 to 0.086	−0.230 to 0.274
Cort-MCSa	−0.035	−0.599	10	0.5623	−0.162 to 0.094	−0.319 to 0.159
rBMR-Cort	−0.030	−0.423	10	0.6813	−0.186 to 0.128	−0.344 to 0.330
rBMR-MCEc	−0.023	−0.385	10	0.7083	−0.153 to 0.108	−0.293 to 0.329
Cort-Hct	−0.017	−0.223	10	0.8278	−0.188 to 0.154	−0.506 to 0.432
Hct-Agg	−0.009	−0.149	10	0.8848	−0.143 to 0.125	−0.245 to 0.442
rBMR-Het	−0.003	−0.042	10	0.9670	−0.148 to 0.142	−0.225 to 0.413
Hct-MCCa	0.006	0.079	10	0.9390	−0.158 to 0.169	−0.410 to 0.339
BMR-Het	0.014	0.224	10	0.8273	−0.126 to 0.153	−0.321 to 0.211
Cort-MCCa	0.016	0.258	10	0.8016	−0.121 to 0.152	−0.490 to 0.358
BMR-Lym	0.023	0.374	10	0.7164	−0.112 to 0.156	−0.304 to 0.323
rBMR-Lym	0.033	0.563	10	0.5856	−0.098 to 0.163	−0.325 to 0.397
rBMR-Lys	0.041	0.793	10	0.4463	−0.074 to 0.155	−0.388 to 0.336
Hct-MCEc	0.048	0.745	10	0.4732	−0.096 to 0.190	−0.287 to 0.484
Cort-Mon	0.049	0.557	10	0.5898	−0.145 to 0.239	−0.149 to 0.404
rBMR-Agg	0.050	0.629	10	0.5437	−0.126 to 0.222	−0.342 to 0.459
Hct-Lys	0.054	0.983	10	0.3488	−0.069 to 0.176	−0.313 to 0.655
Hct-Het	0.055	0.813	10	0.4352	−0.095 to 0.203	−0.303 to 0.317
Cort-Agg	0.056	0.995	10	0.3432	−0.070 to 0.180	−0.322 to 0.343
Cort-Het	0.075	1.053	10	0.3170	−0.083 to 0.229	−0.220 to 0.497
rBMR-Hct	0.096	1.181	10	0.2650	−0.085 to 0.270	−0.244 to 0.248
Cort-Lym	0.106	1.563	10	0.1491	−0.045 to 0.253	−0.305 to 0.410
Cort-Lys	0.115	2.113	10	0.0607	−0.006 to 0.232	−0.465 to 0.435
Hct-Mon	0.117	2.213	10	0.0513	−0.001 to 0.231	−0.466 to 0.404
BMR-Hct	0.124	1.446	10	0.1787	−0.067 to 0.306	−0.373 to 0.398
Cort-MCEc	0.135	1.573	10	0.1467	−0.056 to 0.316	−0.126 to 0.364
Hct-Lym	0.162	3.483	10	0.0059	0.059–0.261	−0.105 to 0.317
BMR–rBMR	**0.854**	**11.777**	**10**	**< 0.0001**	**0.774–0.907**	**0.573–0.954**
(b)						
Hct-MCEc	**−0.403**	**−6.217**	**26**	**< 0.0001**	**−0.514 to −0.279**	**−0.749 to 0.256**
rBMR-MCSa	**−0.299**	**−4.045**	**26**	**0.0004**	**−0.434 to −0.151**	**−0.789 to 0.350**
BMR-MCSa	**−0.289**	**−3.761**	**26**	**0.0009**	**−0.430 to −0.134**	**−0.849 to 0.382**
rBMR-Cort	−0.231	−2.564	26	0.0165	−0.401 to −0.047	−0.934 to 0.523
rBMR-MCCa	−0.187	−2.555	26	0.0168	−0.329 to −0.037	−0.798 to 0.574
rBMR-Het	−0.162	−1.897	26	0.0690	−0.327 to 0.014	−0.728 to 0.614
rBMR-Hct	−0.157	−2.114	26	0.0443	−0.302 to −0.004	−0.651 to 0.791
BMR-Cort	−0.135	−1.459	26	0.1566	−0.316 to 0.056	−0.646 to 0.476
BMR-MCEc	−0.103	−1.381	26	0.1790	−0.252 to 0.050	−0.843 to 0.442
rBMR-Lys	−0.097	−1.436	26	0.1630	−0.233 to 0.042	−0.777 to 0.597
BMR-MCCa	−0.081	−0.996	26	0.3284	−0.243 to 0.086	−0.758 to 0.416
BMR-Mon	−0.076	−0.928	26	0.3620	−0.240 to 0.092	−0.477 to 0.650
BMR-Hct	−0.074	−1.133	26	0.2674	−0.206 to 0.060	−0.729 to 0.443

Table 3 (*continued*)

Pair of variables	Mean *r* over the year	*t*-value	d.f.	*P*-value	95% confidence interval	Range
Hct-Agg	−0.064	−0.944	26	0.3541	−0.200 to 0.075	−0.760 to 0.721
Hct-Mon	−0.054	−0.708	26	0.4853	−0.210 to 0.103	−0.594 to 0.719
rBMR-MCEc	−0.046	−0.630	26	0.5344	−0.194 to 0.104	−0.698 to 0.610
BMR-Agg	−0.045	−0.733	26	0.4702	−0.168 to 0.081	−0.688 to 0.663
BMR-Het	−0.041	−0.493	26	0.6261	−0.208 to 0.129	−0.569 to 0.823
Hct-Lym	−0.019	−0.280	26	0.7817	−0.159 to 0.122	−0.732 to 0.477
Hct-Het	−0.015	−0.204	26	0.8398	−0.159 to 0.131	−0.614 to 0.557
Cort-Lys	−0.013	−0.173	26	0.8642	−0.165 to 0.140	−0.709 to 0.632
Hct-MCSa	−0.013	−0.173	26	0.8643	−0.163 to 0.138	−0.666 to 0.672
rBMR-Agg	−0.012	−0.181	26	0.8580	−0.146 to 0.123	−0.548 to 0.803
BMR-Lys	−0.007	−0.090	26	0.9289	−0.157 to 0.144	−0.579 to 0.733
Hct-MCCa	0.007	0.078	26	0.9387	−0.164 to 0.176	−0.703 to 0.750
BMR-Lym	0.008	0.106	26	0.9161	−0.149 to 0.165	−0.531 to 0.462
Cort-Hct	0.010	0.189	26	0.8514	−0.100 to 0.121	−0.794 to 0.883
Cort-Agg	0.010	0.143	26	0.8878	−0.139 to 0.160	−0.710 to 0.503
rBMR-Mon	0.019	0.235	26	0.8158	−0.144 to 0.181	−0.739 to 0.591
Cort-Lym	0.045	0.531	26	0.6001	−0.128 to 0.216	−0.779 to 0.583
Cort-MCSa	0.063	0.792	26	0.4358	−0.101 to 0.224	−0.686 to 0.580
Cort-Mon	0.071	0.709	26	0.4849	−0.134 to 0.269	−0.752 to 0.687
Cort-MCCa	0.121	1.403	26	0.1724	−0.056 to 0.290	−0.595 to 0.772
rBMR-Lym	0.132	2.304	26	0.0295	0.014 to 0.247	−0.680 to 0.680
Cort-Het	0.152	2.022	26	0.0536	−0.003 to 0.300	−0.603 to 0.507
Cort-MCEc	0.193	2.417	26	0.0230	0.029–0.346	−0.262 to 0.755
Hct-Lys	**0.269**	**4.672**	**26**	**0.0001**	**0.153–0.378**	**−0.571–0.825**
BMR–rBMR	**0.826**	**14.851**	**26**	**< 0.0001**	**0.767–0.871**	**0.426–0.958**

teegh, 2012), suggest that these patterns can arise without the presence of functional constraints if different aspects of the environment put different pressures on different aspects of an individual's physiology.

Independence among physiological systems may enable responses to changing and sometimes conflicting ecological demands

Our data suggest that relationships among haematocrit, corticosterone, immune function and metabolic rate are independent at the individual level and that knots are free to adjust aspects of each trait to the demands of their environment. A case in point is the manner in which our temperature treatments affected aspects of metabolism (Vézina *et al.*, 2006, 2007, 2011), but not aspects of constitutive immune function (Buehler *et al.*, 2008b), or relationships between metabolism and immune function (Fig. 3b). Ambient temperature has known effects on metabolism (Tieleman *et al.*, 2004; Vézina *et al.*, 2011), but might only affect immune function if low temperatures elevate energy expenditure enough to limit available resources and necessitate a trade-off. Because our birds had free access to food during this study, conditions may not have been severe enough to warrant trade-offs. In fact even under limited access to food, constitutive immune function remains unchanged although knots adjust aspects of the more costly acute phase response (Buehler *et al.*, 2009). The lack of consistent correlations between corticosterone and immune function (Fig. 4 and Table 2) further suggests that neither our temperature treatments nor predictable aspects of the annual cycle resulted in stress sufficient to influence the aspects of immune function we measured. Had we manipulated an environmental factor that impacts the immune system (e.g. pathogen pressure; Guernier *et al.*, 2004; Buehler *et al.*, 2008c; Horrocks *et al.*, 2011), or the endocrine system (e.g. environmental predictability; Bassett *et al.*, 1973; Wingfield & Kitaysky, 2002) we might have seen effects on immune function and corticosterone rather than, or in addition to, an effect on metabolism.

This raises the point that our approach cannot detect constraints in circumstances that we did not test. For instance, as mentioned above, we do not find associations between corticosterone and immune function, though predictions and evidence for the effect of different stressors on different aspects of immune function abound in the literature (reviewed by Dhabhar, 2009). This does not mean that corticosterone does not impact immune function in red knots, but rather that in certain circumstances, corticosterone is not correlated with certain immune parameters. Further experimental manipulations will be needed to address the possibility that constraints differ under different circumstances or in different types of individuals.

We tested the idea that physiological variables should be consistently correlated if they are subjected to inherent, unmovable functional constraints. Instead, we found

independence among physiological traits; independence that makes sense if one places the animal firmly within its environment where constraints might differ under different circumstances. Although each physiological system may function as a cohesive module (i.e. symmorphosis Weibel, 2000; Piersma & van Gils, 2011), these systems must flexibly 'cooperate' to maximize fitness in different ecological contexts (Schlichting & Smith, 2002). Our findings highlight the notion that ecological as well as functional constraints should be considered when examining patterns of covariance among phenotypic traits.

Acknowledgments

We thank Y. Verkuil for helpful discussions and B. Sheldon, A. Cohen and anonymous reviewers for improvements on earlier drafts. Financial support came from grants to DMB from the Natural Science and Engineering Council of Canada (NSERC-PDF) and the Netherlands Organization for Scientific Research (NWO-Rubicon); to TP from The Royal Netherlands Institute for Sea Research; to BIT from NWO and a Rosalind Franklin fellowship from the University of Groningen; and to FV from NSERC and NWO.

References

Ardia, D.R., Parmentier, H.K. & Vogel, L.A. 2011. The role of constraints and limitation in driving individual variation in immune response. *Funct. Ecol.* **25**: 61–73.

Bairlein, F. & Totzke, U. 1992. New aspects on migratory physiology of trans-saharan passerine migrants. *Ornis Scand.* **23**: 244–250.

Baker, A.J., Piersma, T. & Greenslade, A.D. 1999. Molecular vs. phenotypic sexing in red knots. *Condor* **101**: 887–893.

Bassett, J.R., Cairncross, K.D. & King, M.G. 1973. Parameters of novelty, shock predictability and response contingency in corticosterone release in the rat. *Horm. Behav.* **10**: 901–907.

Bell, A.M. 2007. Future directions in behavioural syndromes research. *P. Roy. Soc. B Bio.* **274**: 755–761.

Buehler, D.M. & Piersma, T. 2008. Travelling on a budget: predictions and ecological evidence for bottlenecks in the annual cycle of long-distance migrants. *Philos. T Roy. Soc. B* **363**: 247–266.

Buehler, D.M., Bhola, N., Barjaktarov, D., Goymann, W., Schwabl, I., Tieleman, B.I. *et al.* 2008a. Constitutive immune function responds more slowly to handling stress than corticosterone in a shorebird. *Physiol. Biochem. Zool.* **81**: 673–681.

Buehler, D.M., Piersma, T., Matson, K. & Tieleman, B.I. 2008b. Seasonal redistribution of immune function in a shorebird: annual-cycle effects override adjustments to thermal regime. *Amer. Nat.* **172**: 783–796.

Buehler, D.M., Piersma, T. & Tieleman, B.I. 2008c. Captive and free-living red knots *Calidris canutus* exhibit differences in non-induced immunity that suggest different immune strategies in different environments. *J. Avian Biol.* **39**: 560–566.

Buehler, D.M., Encinas-Viso, F., Petit, M., Vézina, F., Tieleman, B.I. & Piersma, T. 2009. Limited access to food and physiological trade-offs in a long distance migrant shorebird part II: constitutive immune function and the acute-phase response. *Physiol. Biochem. Zool.* **82**: 561–571.

Buehler, D.M., Versteegh, M., Matson, K.D. & Tieleman, B.I. 2011. One problem, many solutions: simple statistical approaches help unravel the complexity of the immune system in an ecological context. *PLoS ONE* **6**: e18592.

Campbell, T.W. & Ellis, C.K. 2007. *Avian and Exotic Animal Hematology and Cytology*, 3rd edn. Wiley-Blackwell, Ames, IA.

Cohen, A.A. & McGraw, K.J. 2009. No simple measures for antioxidant status in birds: complexity in inter- and intraspecific correlations among circulating antioxidant types. *Funct. Ecol.* **23**: 310–320.

Cohen, A., McGraw, K., Wiersma, P., Williams, J., Robinson, W., Robinson, T. *et al.* 2008. Interspecific associations between circulating antioxidant levels and life-history variation in birds. *Am. Nat.* **172**: 178–193.

Dhabhar, F.S. 2009. A hassle a day may keep the pathogens away: the fight-or-flight stress response and the augmentation of immune function. *Int. Comp. Biol.* **49**: 215–236.

Dhabhar, F.S., Miller, A.H., McEwen, B.S. & Spencer, R.L. 1995. Effects of stress on immune cell distribution. Dynamics and hormonal mechanisms. *J. Immunol.* **154**: 5511–5527.

Dietz, M., Piersma, T. & Dekinga, A. 1999. Body building without power training: endogenously regulated pectoral muscle hypertrophy in confined shorebirds. *J. Exp. Biol.* **202**: 2831–2837.

Fair, J., Whitaker, S. & Pearson, B. 2007. Sources of variation in haematocrit in birds. *Ibis* **149**: 535–552.

Goymann, W., Trappschuh, M., Jensen, W. & Schwabl, I. 2006. Low ambient temperature increases food intake and dropping production, leading to incorrect estimates of hormone metabolite concentrations in European stonechats. *Horm. Behav.* **49**: 644–653.

Guernier, V., Hochberg, M.E. & Guégan, J.-F. 2004. Ecology drives the worldwide distribution of human diseases. *PLoS Biol.* **2**: e141.

Horrocks, N.P.C., Matson, K.D. & Tieleman, B.I. 2011. Pathogen pressure puts immune defence into perspective. *Int. Comp. Biol.* **51**: 563–576.

Kubena, L.F., May, J.D., Reece, F.N. & Deaton, J.W. 1972. Hematocrit and hemoglobin of broilers as influenced by environmental temperature and dietary iron level. *Poultry Sci.* **51**: 759–763.

Lande, R. 1979. Quantitative genetic-analysis of multivariate evolution, applied to brain – body size allometry. *Evolution* **33**: 402–416.

Lande, R. & Arnold, S.J. 1983. The measurement of selection on correlated characters. *Evolution* **37**: 1210–1226.

Landys, M.M., Piersma, T., Ramenofsky, M. & Wingfield, J.C. 2004. Role of the low-affinity glucocorticoid receptor in the regulation of behavior and energy metabolism in the migratory red knot *Calidris canutus islandica*. *Physiol. Biochem. Zool.* **77**: 658–668.

Landys, M.M., Ramenofsky, M. & Wingfield, J.C. 2006. Actions of glucocorticoids at seasonal baseline versus stress-related levels in the regulation of periodic life processes. *Gen. Comp. Endocr.* **148**: 132–149.

Landys-Ciannelli, M.M., Jukema, J. & Piersma, T. 2002. Blood parameter changes during stopover in a long-distance migra-

tory shorebird, the bar-tailed godwit *Limosa lapponica taymyrensis*. *J. Avian Biol.* **33**: 451–455.
Lochmiller, R.L. & Deerenberg, C. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* **88**: 87–98.
Matson, K.D., Cohen, A.A., Klasing, K.C., Ricklefs, R.E. & Scheuerlein, A. 2006. No simple answers for ecological immunology: relationships among immune indices at the individual level break down at the species level in waterfowl. *P. Roy. Soc. B Bio.* **273**: 815–822.
Millet, S., Bennet, J., Lee, K.A., Hau, M. & Klasing, K.C. 2007. Quantifying and comparing constitutive immunity across avian species. *Dev. Comp. Immunol.* **31**: 188–201.
Murdoch, D. & Chow, E. 1996. A graphical display of large correlation matrices. *Am. Stat.* **50**: 178–180.
Ochsenbein, A.F. & Zinkernagel, R.M. 2000. Natural antibodies and complement link innate and acquired immunity. *Immunol. Today* **21**: 624–630.
Pfennig, D.W., Wund, M.A., Snell-Rood, E.C., Cruickshank, T., Schlichting, C.D. & Moczek, A.P. 2010. Phenotypic plasticity's impacts on diversification and speciation. *Trends Ecol. Evol.* **25**: 459–467.
Piersma, T. 2007. Using the power of comparison to explain habitat use and migration strategies of shorebirds worldwide. *J. Ornithol.* **148**: S45–S59.
Piersma, T. & van Gils, J. 2011. *The Flexible Phenotype. A Body-Centered Integration of Ecology, Physiology and Behaviour*. Oxford University Press, Oxford.
Piersma, T., Everaarts, J.M. & Jukema, J. 1996. Build-up of red blood cells in refueling bar-tailed godwits in relation to individual migratory quality. *Condor* **98**: 363–370.
Piersma, T., Koolhaas, A., Dekinga, A. & Gwinner, E. 2000a. Red blood cell and white blood cell counts in sandpipers (*Philomachus pugnax, Calidris canutus*): effects of captivity, season, nutritional status and frequent bleedings. *Can. J. Zool.* **78**: 1349–1355.
Piersma, T., Reneerkens, J. & Ramenofsky, M. 2000b. Baseline corticosterone peaks in shorebirds with maximal energy stores for migration: a general preparatory mechanism for rapid behavioral and metabolic transitions? *Gen. Comp. Endocr.* **120**: 118–126.
Pike, N. 2011. Using false discovery rates for multiple comparisons in ecology and evolution. *Method Ecol. Evol.* **2**: 278–282.
Prater, A.J., Marchant, J.H. & Vuorinen, J. 1977. *Guide to the Identification and Aging of Holarctic Waders*. British Trust for Ornithology, Tring, UK.
Prats, M.-T., Palacios, L., Gallego, S. & Riera, M. 1996. Blood oxygen transport properties during migration to higher altitude of wild quail, *Coturnix coturnix coturnix*. *Physiol. Zool.* **69**: 912–929.
Promislow, D.E.L. & Harvey, P.H. 1990. Living fast and dying young: a comparative analysis of life-history variation among mammals. *J. Zool.* **220**: 417–437.
R Development Core Team. 2011. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, http://www.R-project.org, Vienna, Austria.
Ramenofsky, M. & Wingfield, J.C. 2006. Behavioural and physiological conflicts in migrants: the transition between migration and breeding. *J. Ornithol.* **147**: 135–145.
Reneerkens, J., Morrison, R.I.G., Ramenofsky, M., Piersma, T. & Wingfield, J.C. 2002. Baseline and stress-induced levels of the corticosterone during different life cycle substages in a shorebird on the High Arctic breeding grounds. *Physiol. Biochem. Zool.* **75**: 200–208.
Rice, W.R. 1989. Analyzing tables of statistical tests. *Evolution* **43**: 223–225.
Ricklefs, R.E. 2000. Density dependence, evolutionary optimization, and the diversification of avian life histories. *Condor* **102**: 9–22.
Ricklefs, R.E. & Wikelski, M. 2002. The physiology/lifehistory nexus. *Trends Ecol. Evol.* **17**: 462–468.
Roff, D.A. & Fairbairn, D.J. 2007. The evolution of trade-offs: where are we? *J. Evol. Biol.* **20**: 433–447.
Schlichting, C.D. & Smith, H. 2002. Phenotypic plasticity: linking molecular mechanisms with evolutionary outcomes. *Evol. Ecol.* **16**: 189–211.
Schwenk, K. & Wagner, G.P. 2001. Function and the evolution of phenotypic stability: connecting pattern to process. *Am. Zool.* **41**: 552–563.
Snyder, G.K. 1977. Influence of temperature and hematocrit on blood viscosity. *Am. J. Physiol.* **220**: 1667–1672.
Speakman, J.R. 2005. Body size, energy metabolism and lifespan. *J. Exp. Biol.* **208**: 1717–1730.
Tieleman, B.I., Williams, J.B. & Visser, G.H. 2004. Energy and water budgets of larks in a life history perspective: parental effort varies with aridity. *Ecology* **85**: 1399–1410.
Tieleman, B.I., Williams, J.B., Ricklefs, R.E. & Klasing, K.C. 2005. Constitutive innate immunity is a component of the pace-of-life syndrome in tropical birds. *P. Roy. Soc. B Bio.* **272**: 1715–1720.
Tieleman, B.I., Versteegh, M.A., Helm, B. & Dingemanse, N.J. 2009. Quantitative genetics parameters show partial independent evolutionary potential for body mass and metabolism in stonechats from different populations. *J. Zool.* **279**: 129–136.
Versteegh, M.A. 2012. *Physiology in a Life History Perspective*. PhD thesis, University of Groningen, Groningen, The Netherlands.
Vézina, F., Jalvingh, K.M., Dekinga, A. & Piersma, T. 2006. Acclimation to different thermal conditions in a northerly wintering shorebird is driven by body mass-related changes in organ size. *J. Exp. Biol.* **209**: 3141–3154.
Vézina, F., Jalvingh, K.M., Dekinga, A. & Piersma, T. 2007. Thermogenic side effects to migratory predisposition in shorebirds. *Am. J. Physiol. – Reg I.* **292**: 1287–1297.
Vézina, F., Dekinga, A. & Piersma, T. 2010. Phenotypic compromise in the face of conflicting ecological demands: an example in red knots *Calidris canutus*. *J. Avian Biol.* **41**: 88–93.
Vézina, F., Dekinga, A. & Piersma, T. 2011. Shorebirds' seasonal adjustments in thermogenic capacity are reflected by changes in body mass: how preprogrammed and instantaneous acclimation work together *Int. Comp. Biol.* **51**: 394–408.
Vézina, F., Williams, T.D., Piersma, T. & Morrison, R.I.G. 2012. Phenotypic compromises in long-distance migrating shorebirds: consequences of coping with cold during the life-stage transition from migration to reproduction in the High Arctic. *Funct. Ecol.* **26**: 500–512.
Weibel, E.R. 2000. *Symmorphosis: On Form and Function in Shaping Life*. Harvard University Press, Cambridge, MA.
West-Eberhard, M.J. 2003. *Developmental Plasticity and Evolution*. Oxford University Press, New York, NY.
Wingfield, J.C. & Kitaysky, A.S. 2002. Endocrine responses to unpredictable environmental events: stress or anti-stress hormones? *Int. Comp. Biol.* **42**: 600–609.

Wingfield, J.C., O'Reilly, K.M. & Astheimer, L.B. 1995. Modulation of the adrenocortical responses to acute stress in arctic birds: a possible ecological basis. *Am. Zool.* **35**: 285–294.
Withers, P.C., Hillman, S.S., Hedrick, M.S. & Kimmel, P.B. 1991. Optimal hematocrit theory during activity in the bullfrog (*Rana catesbeiana*). *Comp. Biochem. Physiol. A* **99**: 55–60.

Supporting information

Additional Supporting Information may be found in the online version of this article:
Table S1 Principal component loadings (after varimax rotation) among individuals (monthly measures pooled). The highest loading for a measure across the components is shown in bold.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be reorganized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

Data deposited at Dryad: doi: 10.5061/dryad.4q6f1

Received 1 March 2012; revised 18 April 2012; accepted 23 April 2012