Interrelations among Immune Defense Indexes Reflect Major Components of the Immune System in a Free-Living Vertebrate

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ABSTRACT

Understanding the relationships among immune components in free-living animals is a challenge in ecoimmunology, and it is important not only for selecting the immune assays to be used but also for more knowledgeable interpretation of results. In this study, we investigated the relationships among six immune defense indexes commonly used by ecoimmunologists and measured simultaneously in individual free-living tree swallows. Three main axes of variation in immune function were identified using a principal components analysis, representing variation in T-cell, B-cell, and innate immunity. Measures within each axis tended to be positively correlated among individuals, while measures in different axes were uncorrelated. A trade-off between T-cell function and B-cell function became apparent only when variation among individuals in body condition, age, and general quality was taken into account. Interestingly, the level of natural antibodies, a component of innate immunity, showed the strongest association with components of acquired B-cell function, possibly reflecting a common underlying genetic mechanism, as has been documented in poultry. Our results indicate that despite the complexity of the immune system, important insights can be gained by using the currently available assays but in a more comprehensive approach than has generally been used in the field of ecoimmunology.

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Introduction

Ecological immunology, or ecoimmunology, focuses on understanding immunological variation in the context of the ecology and life history of organisms (Sheldon and Verhulst 1996; Norris and Evans 2000; Schmid-Hempel 2003). One of the major challenges of this relatively new field of biology has been to work with nonmodel organisms, often in their natural environments, and to simultaneously assess immune function without oversimplifying the inherent complexity of the immune system. Much progress has been made in the field since its inception in the early 1990s (Lee 2006; Martin et al. 2006*c*), and ecoimmunological studies using a single measure of immune function as an index of general "immunocompetence" are increasingly rare. This has come as a result of both the repeated emphasis on the shortcomings associated with such an approach (Norris and Evans 2000; Zuk and Stoehr 2002; Adamo 2004) and the development of new assays that are amenable for use in wild animals in the field (e.g., Matson et al. 2005; Millet et al. 2007; reviewed in Boughton et al. 2011). Despite this clear advancement in ecoimmunology, a major remaining challenge is to understand whether and how different aspects of immune defense relate to each other in wild animals (Blount et al. 2003; Matson et al. 2006*a*; Buehler et al. 2011). This knowledge is important not only for aiding ecoimmunologists in deciding what immune measures and assays to use to answer a given question but also, and more important, for incorporating this understanding into the interpretation of ecological variation in immune defense.

The vertebrate immune system is classically divided into two main arms, innate (or nonspecific) immunity and acquired (or adaptive) immunity, with each of these arms composed of both cellular and humoral (i.e., soluble) components (Roitt et al. 1998). Innate immunity is the first line of defense against invading pathogens, and some of its effectors are phagocytic cells (e.g., monocytes, macrophages, heterophils), natural killer cells, natural antibodies (NAbs), and the complement system. NAbs have low specificity, are constitutively produced, and can neutralize or kill a wide range of pathogens either directly or indirectly via activation of the complement system (Ochsenbein and Zinkernagel 2000). The complement system consists of a group of proteins that upon activation form a lytic complex resulting in the lysis of pathogens and chemotactic peptides that recruit other immune cells to the site of infection. On the other hand, acquired immunity is the second line of defense and is mediated by lymphocytes, which upon activation proliferate mitotically to form an army of cells that can more effectively destroy the invaders (Roitt et al. 1998). Acquired humoral responses are mediated by B-lymphocytes (B-cells)

Table 1: Immune defense variables measured simultaneously in free-living tree swallows and their common classification according to the arm/component of the immune system they are intended to assess

Immune arm, immune component	Description	Variable measured	Assay
Acquired:			
T-cell function (cellular)	Proliferation of lymphocytes in response to the T-cell mitogens ConA and PHA	(1) ConA SI, (2) PHA SI	In vitro lymphocyte proliferation
B-cell function (humoral)	Proliferation of lymphocytes in response to the B-cell mitogen LPS	(3) LPS SI	In vitro lymphocyte proliferation
B-cell function (humoral)	Antibody production in response to SRBC challenge	(4) Anti-SRBC-Abs	In vivo challenge/ hemagglutination
Innate:			
Humoral	Levels of NAbs[a] constitutively circulating in plasma	(5) NAbs	Hemagglutination/ hemolysis
Humoral	Ability of circulating complement proteins to lyse cells	(6) Lysis	Hemagglutination/ hemolysis

Note. The last column indicates the assay (technique) used to obtain each variable. Abs, antibodies; ConA, concanavalin A; LPS, lipopolysaccharide; SRBC, sheep red blood cells; NAbs, natural antibodies; PHA, phytohemagglutinin; SI, stimulation index.

[a]NAbs have alternatively been classified as acquired or integrative defenses rather than innate.

and the specific antibodies that these cells produce and are effective against extracellular pathogens. Conversely, acquired cell-mediated responses are mediated by a subset of T-lymphocytes (T-cells) and are effective against intracellular pathogens and tumor cells (Roitt et al. 1998).

Earlier ecoimmunological studies focused on variation in the acquired arm of the immune system, particularly measuring the T-cell-mediated skin-swelling response to phytohemagglutinin (PHA) and the B-cell-mediated humoral response to foreign antigens (e.g., sheep red blood cells [SRBC]). As pointed out by Forsman et al. (2008), the full range of relationships (positive, negative, or nonexistent) has been documented between these two immune defense components in studies of free-living birds (the most widely studied group among vertebrates). More recently, aspects of the other arm of the immune system (e.g., levels of NAbs and complement-mediated lysis) have been integrated into ecoimmunology studies. As was the case for acquired immunity, positive (e.g., Møller and Haussy 2007; Buehler et al. 2008; Sparkman and Palacios 2009), negative (e.g., Parejo et al. 2007), and nonexistent (e.g., Mauck et al. 2005; Mendes et al. 2006; Palacios et al. 2009) patterns have been described between these two components in free-living animals. These diverse results highlight the intricacy of the immune system and suggest that a more comprehensive approach might be necessary to interpret the observed relationships. We suggest that such an approach is to measure components of both the innate and acquired arms of the immune system simultaneously in the same individuals and incorporate more than one measure for a given arm/component.

In this study we explore the relationships among six measures of immune defense representing three major components of the immune system (i.e., T-cell function [acquired], B-cell function [acquired], and innate humoral function) measured simultaneously in individual free-living tree swallows *Tachycineta bicolor*, using three immunological assays (i.e., techniques; table 1). Using this array of immune variables, we focused on addressing the following questions: (1) Can a single immune measure provide an adequate index to a given immune component? Although currently few ecoimmunological studies use a single measure of immune function to index overall immunocompetence of individuals, many studies do use a single immune measure as an index to the overall competence of a given immune component. Here we test the implicit assumption of this approach that different measures of a given component are positively associated to one another such that using any one of those measures can give an adequate index to that immune component. For instance, under this assumption, we would expect to find a positive correlation between in vitro B-cell proliferation and in vivo antibody production by B-cells, given that they are both intended to assess B-cell function (i.e., acquired humoral immunity). (2) Is there evidence of trade-offs within and/or between the innate and acquired arms of the immune system? Although the existence of trade-offs between immune function and other costly functions (e.g., reproduction) has been demonstrated in ecoimmunological studies, evidence for trade-offs within the immune system of individuals remains scant (Martin et al. 2006*b*). In the case of a trade-off within the innate and/or the acquired arms of the immune system, we would expect negative associations between different immune components within a given arm (e.g., T-cell proliferation vs. B-cell proliferation in the acquired arm). On the other hand, in the case of a trade-off between the innate and acquired immune arms, we would expect negative associations between components of different arms (e.g., T-cell proliferation vs. complement activity). Because individual differences in body condition (e.g., Saino et al. 1997; Navarro et al. 2003), age (e.g.,

Haussmann et al. 2005; Palacios et al. 2007; Sparkman and Palacios 2009), and/or other aspects of quality (e.g., clutch initiation date in tree swallows; Hasselquist et al. 2001; Ardia 2005) can affect immune variables and potentially obscure trade-offs, we also tested and controlled for the effects of these variables in our analyses.

Material and Methods

Field Sampling

Tree swallows are small (~20-g) aerial insectivores widely distributed throughout North America and members of the family Hirundinidae (Robertson et al. 1992). We collected the data used in this study as part of a study on immunosenescence in tree swallows conducted in 2005 (Palacios et al. 2007) in a tree swallow nest-box population in Tompkins County, New York (42°29′N, 76°27′W). During the breeding season (May–June), we monitored nests daily to determine clutch initiation date. On June 3, between 0500 and 1300 hours, we captured 45 females in the late-incubation (last 4 d) or early-nestling (first 4 d) period of breeding. Females ranged from 1 to 10 yr of age, as determined by their banding history. We knew exact ages for 26 females, and for the remaining, age was a minimum estimate based on each female's plumage in the year she was first banded. We collected blood (~200 μL) for the three immunological assays by jugular venipuncture (see Palacios et al. 2007 for details). Females were then weighed to the nearest 0.1 g and head-bill length (a measure of structural body size) was measured to the nearest 0.1 mm to estimate size-corrected body mass, an index of body condition (Schulte-Hostedde et al. 2005). Before their release, we injected females intraperitoneally with 100 μL of a 2% suspension of SRBC (HemoStat Laboratories, Dixon, CA) in sterile phosphate-buffered saline. Females were recaptured 8 d later and a second blood sample (~60 μL) was obtained to determine anti-SRBC antibody production.

Immunological Assays and Variables Measured

In Vitro Lymphocyte Proliferation. We used a whole-blood mitogenic stimulation assay (Cunnick et al. 1994) that we optimized for use in tree swallows. Details on this and the remaining immune assays described below can be found in Palacios et al. (2007). We tested for proliferation of lymphocytes by exposing blood samples to two standard T-cell mitogens, phytohemagglutinin (PHA) and concanavalin A (ConA), and the B-cell mitogen lipopolysaccharide (LPS from *Escherichia coli*), which stimulate mitosis in lymphocytes. Proliferation was quantified by the amount of [^{3}H]-thymidine incorporated by the dividing cells during an incubation step. We expressed the proliferative responses of lymphocytes as a stimulation index (SI) calculated as the ratio between the counts per minute (cpm) of mitogen-stimulated samples and the cpm for nonstimulated samples for each individual. With this assay we measured three immune variables: PHA SI, ConA SI, and LPS SI (table 1).

In Vivo Challenge with SRBC. We quantified specific antibody production by B-lymphocytes in response to immunization with SRBC using a hemagglutination assay (e.g., Ardia 2003; Palacios et al. 2007). Individual plasma samples were serially twofold diluted and exposed to SRBC, and agglutination of erythrocytes by antibodies was visually determined. As expected, none of the preimmunization plasma samples (collected on June 3) caused hemagglutination. For plasma samples collected 8 d after immunization, titers of specific antibodies against SRBC (anti-SRBC Abs) are expressed as the $\log_2$ of the highest dilution factor of plasma that showed hemagglutination. We recorded half scores between two titers when the termination of hemagglutination was intermediate.

NAbs and Complement Activity. We assessed the levels of NAbs in plasma and the ability of complement to cause lysis of rabbit red blood cells (RRBC), using a hemagglutination-hemolysis assay (Matson et al. 2005). This assay was performed on plasma samples collected on June 3 (i.e., before immunization with SRBC), given that it was meant to measure constitutive levels of the innate components in circulation. As with the hemagglutination assay described above, individual plasma samples were serially twofold diluted and exposed to RRBC, and agglutination of erythrocytes was determined. Samples were next allowed to stand at room temperature and occurrence of lysis was determined. Hemagglutination of RRBC is caused by NAbs, while lysis is caused by the interaction of NAbs and complement proteins (i.e., NAbs activate the complement cascade that results in formation of pores on cells, leading to their lysis). Titers are expressed as the $\log_2$ of the highest dilution factor of plasma that showed each type of response. When the termination of hemagglutination or lysis was intermediate, we recorded half scores between the two highest titers.

Statistical Analyses

Immune function variables were not normally distributed and were therefore $\log_{10}$ transformed before statistical analysis. Sample sizes differ among analyses because not all variables could be measured in all individuals. We used simple correlations to assess the effects of individual variation in age, body condition, and clutch initiation day on immune responses. We then used multiple regressions to obtain residuals of each immune function variable after correction for these factors. We performed further statistical analyses on both the uncorrected data and the residuals of immune function.

Two statistical approaches were used to explore the relationships among immune function variables. First, simple Pearson correlations were used to determine pairwise correlations among immune function variables. Then, principal components analysis (PCA), followed by factor rotation, was used to further explore the relationships among the different aspects of immune function. Rotation of the principal components (PCs) can facilitate the interpretation of the resulting linear combination of variables. We tested the performance of both an orthogonal rotation (varimax) and an oblique rotation (ob-

limin) in explaining the structure of our data. Varimax rotation maximizes the contrast between variable loadings within each axis (i.e., high loadings become higher, while small loadings become smaller) but constrains the axes to remain orthogonal (i.e., uncorrelated) to each other. Oblique rotation does not have the latter constraint, therefore allowing the assessment of correlation among axes. We used the Kaiser (1960) criterion and retained only PCs with eigenvalues >1 for interpretation. All statistical analyses were performed using JMP software (SAS Institute, Cary, NC).

Results

Simple correlations showed that age explained significant variation in the two T-cell proliferation variables, with older individuals having lower stimulation indexes than younger ones (PHA SI: $r = -0.50$, $P = 0.0006$, $n = 43$; ConA SI: $r = -0.42$, $P = 0.0047$, $n = 43$). Body condition explained significant variation in B-lymphocyte proliferation (LPS SI), with individuals in better body condition having lower stimulation indexes than those in poorer condition ($r = -0.39$, $P = 0.009$, $n = 43$). Clutch initiation date, an indicator of quality in tree swallows, explained significant variation in the levels of NAbs, with individuals that started breeding later (i.e., lower-quality individuals) showing lower levels of circulating NAbs than earlier breeders ($r = -0.45$, $P = 0.0074$, $n = 34$).

Pairwise correlations between immune function variables using the uncorrected data showed three significantly positive correlations (table 2): (1) between T-lymphocyte proliferation in response to PHA (PHA SI) and in response to ConA (ConA SI; fig. 1*a*), (2) between B-lymphocyte proliferation in response to LPS (LPS SI) and the titer of NAbs (fig. 1*b*), and (3) between the titers of NAbs and anti-SRBC Abs (fig. 1*c*). Results were somewhat different when pairwise comparisons were performed using the residuals of immune function (i.e., after correction for age, body condition, and clutch initiation date). Although the positive correlation between PHA SI and ConA SI remained significant (table 2; fig. 2*a*), the positive correlations between LPS SI and NAbs and between NAbs and anti-SRBC Abs were only marginally significant ($0.1 < P < 0.05$) when using the residuals of immune function (table 2). Interestingly, analysis of the residuals also showed a significantly negative correlation between ConA SI and anti-SRBC Abs, suggesting a trade-off between those variables (table 2; fig. 2*b*).

PCA followed by varimax rotation conducted on the uncorrected data yielded three PCs that cumulatively accounted for ~74% of the total variance in immune variables (table 3). PC 1 showed high positive loadings for PHA SI and ConA SI, thus interpreted as an axis of T-cell function. PC 2 showed high positive loadings for LPS SI and NAbs and intermediate positive loadings for anti-SRBC Abs, so it was interpreted as an axis of B-cell function. Finally, PC 3 showed high positive loading for lysis and intermediate negative loading for anti-SRBC Abs and was interpreted as an axis of primarily innate immunity. Oblique rotation resulted in axes nearly indistinguishable from those obtained after the orthogonal rotation (data not shown), indicating that the axes of immune function described above are indeed uncorrelated with each other. When PCA was conducted on the residuals of immune function variables, again three PCs were identified that cumulatively explained ~74% of the total variance in the data. One main difference, however, emerged in comparison to the analysis on uncorrected data. While in the uncorrected data analysis, anti-SRBC Abs had intermediate loadings in PCs 2 and 3, in the residual analysis, this variable showed high negative loading in PC 1, which opposed the high positive loadings for PHA SI and ConA SI on this axis (table 3), again supporting a trade-off between the measures of T-lymphocyte proliferation and production of specific antibodies by B-cells. Other loadings on PCs 2 and 3 were similar between analyses (table 3).

Discussion

Our study of the relationships among immune variables measured simultaneously in free-living tree swallows yields important insights into the nature of these relationships. Three main axes of variation in immune function were identified by the PCA when analyzing the uncorrected data, the first one representing variation in T-cell function, the second variation in B-cell function, and the third mainly variation in innate immunity as indicated by complement activity. The analysis using the residuals of immune function after correction for individual differences in age, body condition, and clutch initiation date suggested the existence of a trade-off within the acquired arm of the immune system. Below we discuss our major findings and their implications in the context of our original questions and relate them to knowledge from other work (mainly in poultry) on the relationships among these indexes of immune function.

Can a Single Immune Measure Provide an Adequate Index to a Given Immune Component?

The PCA on uncorrected data supported three main axes separating the six immune variables into those related to T-cell function, B-cell function, and innate immune function. Together with the fact that variables within each axis tended to be positively associated (although some pairwise correlations did not reach statistical significance), this finding provides support to the approach of using a single measure from each axis to characterize each of these three immune components. For instance, the two measures of T-cell function (PHA SI and ConA SI) were strongly positively correlated to each other (and not to the remaining variables), such that investigators could potentially select only one of these two measures as an index to T-cell function (acquired cellular immunity) without losing much information. An exception, however, is provided by the observation that levels of NAbs were more closely related to the two measures of acquired B-cell function than to the other measure of innate humoral immunity (i.e., complement-mediated lysis).

NAbs are considered components of the innate immune arm

Table 2: Pairwise Pearson correlations between measures of immune function ($\log_{10}$ transformed) of female tree swallows

	ConA SI	PHA SI	LPS SI	Anti-SRBC Abs	NAbs	Lysis
ConA SI:						
r		*.676*	.040	−.180	−.015	−.150
P		*<.001*	.795	.325	.936	.424
n		*43*	43	32	33	31
PHA SI:						
r	*.580*		−.044	−.094	.070	.011
P	*<.001*		.780	.611	.699	.952
n	*43*		43	32	33	31
LPS SI:						
r	.198	.138		.247	**.381**	−.073
P	.204	.378		.172	**.029**	.696
n	43	43		32	**33**	31
Anti-SRBC Abs:						
r	**−.354**	−.266	.228		**.398**	−.127
P	**.048**	.142	.210		**.040**	.544
n	**32**	32	32		**27**	25
NAbs:						
r	−.058	.118	.317	.370		−.060
P	.750	.514	.072	.058		.744
n	33	33	33	27		31
Lysis:						
r	−.050	.125	−.204	−.089	−.111	
P	.787	.503	.272	.674	.546	
n	31	31	31	25	31	

Note. Correlations above the diagonal were calculated on uncorrected data, while those below the diagonal were obtained using the residuals of immune function after correction for age, body condition, and clutch initiation date. Significant relationships ($P < 0.05$) are depicted in bold, while significant correlations after Bonferroni correction for multiple comparisons are shown in italics. Abs, antibodies; ConA, concanavalin A; LPS, lipopolysaccharide; SRBC, sheep red blood cells; NAbs, natural antibodies; PHA, phytohemagglutinin; SI, stimulation index. *r*, Pearson's ρ; *P*, *P* value; *n*, sample size.

because their occurrence in plasma does not require previous antigen stimulation and they can be found even in animals raised in germ-free environments (Baumgarth et al. 1999; Matson et al. 2005; Zhou et al. 2007). They are, however, produced by a subset of B-cells. Most NAbs are produced constitutively by peritoneal B1 lymphocytes, while specific antibodies are produced by B2 lymphocytes only upon activation by antigen (Ochsenbein and Zinkernagel 2000). Studies in poultry indicate the existence of a positive genetic correlation between the levels of NAbs in plasma and antibody responses to specific antigens (Parmentier et al. 2004; Cotter et al. 2005). Namely, selection of chickens for high antibody responses to SRBC leads to parallel changes in the levels of NAbs against RRBC (Cotter et al. 2005), the same two immune measures we used in tree swallows in this study. Therefore, the correlation of NAbs with the axis of B-cell function rather than with the axis of innate humoral immunity in tree swallows can be reconciled if we consider their origin and possible genetic link with specific antibody responses. This correlation, however, suggests that ecoimmunologists should be cautious about using NAbs as the only measure of innate immune function, particularly in studies intended to contrast the two arms. In fact, many of the functions of NAbs actually serve as a link between the innate and acquired arms of the immune system (Ochsenbein and Zinkernagel 2000).

Regarding the PCA axis attributed primarily to T-cell function, lymphocyte proliferation responses to the two T-cell mitogens were strongly positively correlated, being the tightest relationship among any pair of immune responses measured in this study. This likely results, at least in part, from the fact that both these responses are indexes of the ability of T-lymphocytes to proliferate upon activation by mitogens and were measured by the same assay; however, this might not be the whole story. Responses to PHA and ConA are not always correlated in chickens (e.g., Morrow and Abplanalp 1981), suggesting that these mitogens might activate different (independent) subsets of the T-lymphocyte population. Indeed, although our data show that these responses are positively correlated among adult female tree swallows, the ontogeny of these responses in tree swallow nestlings appears to be uncoupled, with development of the PHA response lagging behind that of the ConA response (Palacios et al. 2009), a phenomenon that has

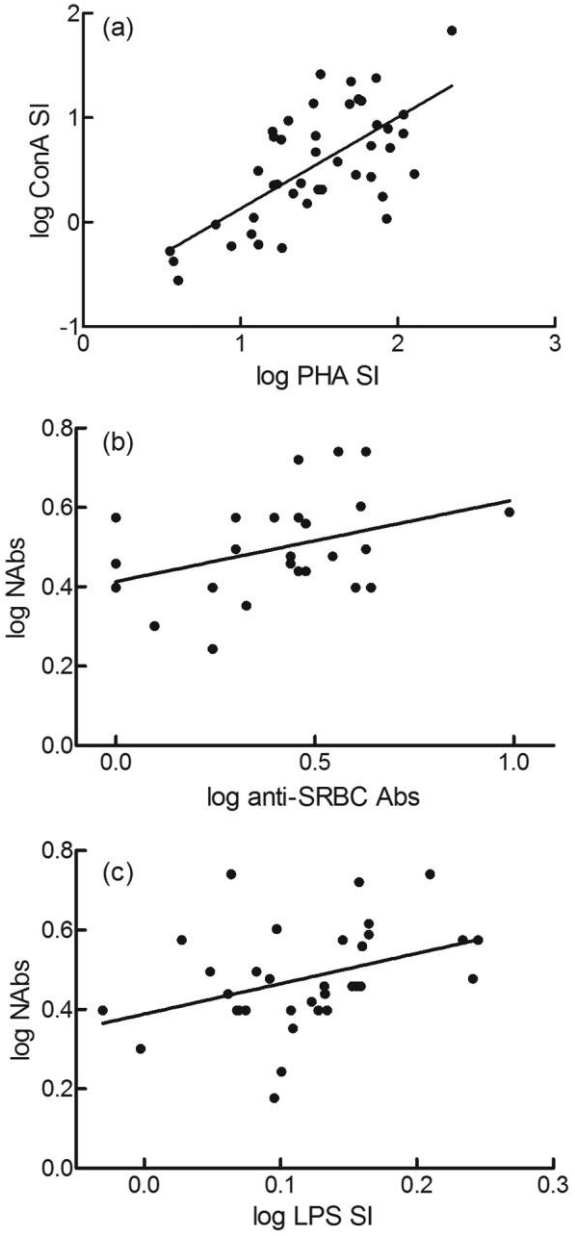

Figure 1. Simple pairwise correlations between immune function variables in free-living tree swallows (uncorrected immune measures). *a*, In vitro lymphocyte proliferation in response to phytohemagglutinin (PHA) and concanavalin A (ConA). *b*, Anti–sheep red blood cell antibody (anti-SRBC Abs) response and natural antibody (NAbs) titer. *c*, In vitro lymphocyte proliferation in response to lipopolysaccharide (LPS) and NAbs titer. Statistics and sample sizes provided in table 2. SI, stimulation index.

also been documented in poultry (Suresh et al. 1993). Thus, although in most cases using one of the two measures might suffice, the inclusion of both may provide greater insights.

An interesting venue for future research is the relationship between in vitro and in vivo measures of T-cell function. The in vivo skin-swelling response to PHA has been the most widely used assay in ecoimmunology since the inception of the field. Early ecoimmunological studies interpreted this response as an index to T-cell function. Although T-cells do mediate this inflammatory response (Goto et al. 1978), much of the swelling observed at the site of injection is due to infiltration by innate immune cells such as heterophils and basophils (Stadecker et al. 1977; McCorkle et al. 1980; Martin et al. 2006*a*). Given the complexity of the PHA skin-swelling response and the widespread use of this technique to assess immune function in wild animals, it would be important to determine whether variation in this response among individuals is generally related to the axis of T-cell function or the axis of innate immune function or whether it constitutes a separate axis of integrated cutaneous immune function. We have shown that tree swallows in our study population show immunosenescence in both the in vivo (skin swelling; Haussmann et al. 2005) and in vitro (T-cell proliferation, Palacios et al. 2007) responses to PHA, suggesting that the smaller swellings observed in older individuals might indeed be caused by the lower ability of their T-lymphocytes to proliferate upon stimulation (Palacios et al. 2007). These

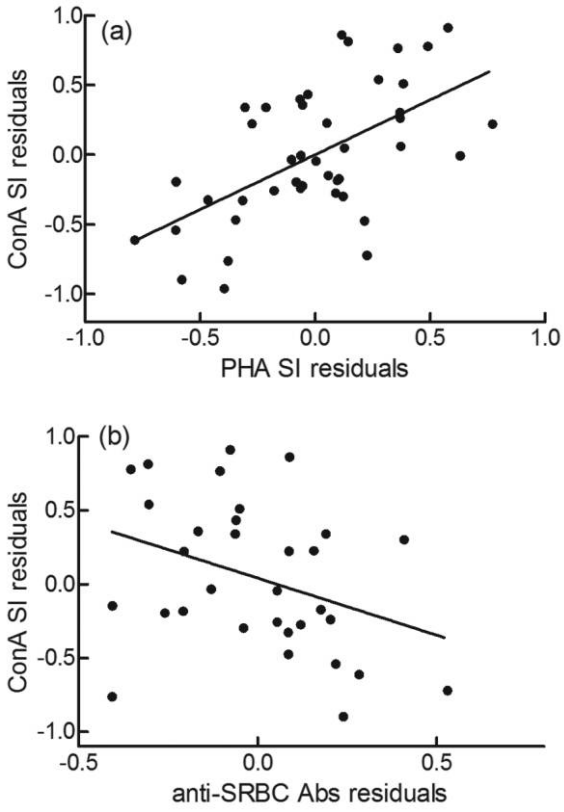

Figure 2. Simple pairwise correlations between immune function variables in free-living tree swallows (residuals of immune measures corrected for age, body condition, and clutch initiation date). *a*, In vitro lymphocyte proliferation in response to phytohemagglutinin (PHA) and concanavalin A (ConA). *b*, Anti–sheep red blood cell antibody (anti-SRBC Abs) response and in vitro lymphocyte proliferation in response ConA. Statistics and sample sizes provided in table 2. SI, stimulation index.

Table 3: Results of principal components analysis of measures of immune function ($\log_{10}$ transformed) of female tree swallows

	Uncorrected data			Residuals		
Variable	PC 1	PC 2	PC 3	PC 1	PC 2	PC 3
ConA SI	**.90**	−.09	−.11	**.86**	−.07	−.04
PHA SI	**.87**	.11	.06	**.75**	.16	.37
LPS SI	−.05	**.77**	.14	−.04	**.79**	−.25
Anti-SRBC Abs	−.26	**.52**	−.49	**−.74**	.28	.09
NAbs	.17	**.85**	−.11	−.11	**.88**	.12
Lysis	−.11	.07	**.90**	.03	−.10	**.93**
Variance (%)	27.9	27.0	18.3	30.9	25.1	18.3

Note. PCs either were calculated on uncorrected data or were obtained using the residuals of immune function after correction for age, body condition, and clutch initiation date. Depicted are the factor loadings after varimax rotation of the principal components (PCs). Values in boldface indicate the highest loading for each immune variable across PCs. Percentage of variance explained by each factor is shown at the bottom of the table. Abs, antibodies; ConA, concanavalin A; LPS, lipopolysaccharide; SRBC, sheep red blood cells; NAbs, natural antibodies; PHA, phytohemagglutinin; SI, stimulation index.

responses, however, were not measured simultaneously in the same individuals; thus, further research in this area is warranted.

A third axis of immune variation we identified in tree swallows, interpreted as an axis of innate immune function, reflected individual variation in complement-mediated lysis. As determined by the hemagglutination-hemolysis assay, lysis of red blood cells is the result of the interaction of NAbs and complement proteins (Matson et al. 2005). As such, one might expect that the levels of lysis would depend on the levels of NAbs present. However, this does not need to be the case, because a few NAbs molecules might be sufficient to activate the complement cascade, resulting in cell lysis. Indeed, we found that NAbs and lysis were not correlated with each other in tree swallows, and they appeared to constitute independent axes of variation in immune function among individuals. Nevertheless, other studies have reported positive (e.g., Matson et al. 2006*a*; Møller and Haussy 2007; Buehler et al. 2008; Sparkman and Palacios 2009), negative (e.g. Parejo et al. 2007), and no significant correlations (e.g., Mauck et al. 2005; Mendes et al. 2006) between these two immune defense measures. Thus, further study is necessary to elucidate why the relationship between NAbs and complement-mediated lysis, both measured in the same assay and widely used by ecoimmunologists, is so variable. One approach could be to assess the levels of complement (or complement activity) by using an independent assay that does not include the confounding of NAbs levels (e.g., Kai et al. 1985) and compare this to levels obtained through the hemagglutination-hemolysis assay. In addition, future studies would benefit from the incorporation of additional aspects of innate humoral immune function (e.g., levels of lysozyme or other antimicrobial proteins, bacterial killing capacity of plasma), as well as aspects of innate cellular immune function (e.g., phagocytosis, production of reactive oxygen species by heterophils), especially as techniques for assessing these components become amenable for use by ecoimmunologists in the field (e.g., Tieleman et al. 2005; Matson et al. 2006*b*; Millet et al. 2007; Papp and Smits 2007).

A recent study focusing on understanding the relationships among innate humoral immune components in waterfowl found that indexes measured by a given assay (e.g., hemagglutination-hemolysis) were positively correlated among individuals, while indexes measured by separate assays (e.g., hemagglutination-hemolysis vs. bacterial killing) were independent of each other (Matson et al. 2006*a*). In contrast, we found that in tree swallows the association of immune variables was dependent not on the assay type (except perhaps in the case of T-cell function) but rather on functional characteristics of the immune aspects measured. For instance, the PCA axis of B-cell function was composed of the in vitro B-lymphocyte proliferation response to LPS, the in vivo production of antibodies in response to SRBC challenge, and the levels of NAbs produced by B1 B-cells, all measured through independent assays. This disparity between studies may be due to species-specific differences in the relationships among immune variables (Buehler et al. 2011) and/or could be related to the fact that this study included broader aspects of immune function (i.e., from both the acquired and innate arms), while the study in waterfowl focused on relationships only within the innate arm. An additional factor that could have contributed to differences in results might be related to data analysis. The analysis by Matson et al. (2006*a*) pooled data from various waterfowl species. A recent reanalysis of those data showed that species actually differed in the relationships among their immune responses, such that pooling of the data across species might be misleading (Buehler et al. 2011). In any case, this highlights the need for further research in order to understand how and why immune components vary in their relationships to each other and the ecological implications of this variation.

Is There Evidence of Trade-Offs within and/or among Different Arms of the Immune System?

Ecological immunologists have clearly demonstrated that components of immune function are often traded off against other costly activities such as growth and reproduction (reviewed in Sheldon and Verhulst 1996; Norris and Evans 2000; Schmid-Hempel 2003). The existence of trade-offs within the immune system of an individual, however, is only moderately supported (Martin et al. 2006*b*). We found no evidence that tree swallows show a trade-off between components of the innate and acquired arms of the immune system. On the other hand, our results provide evidence for a trade-off within the acquired arm of the immune system. In particular, we found a negative relationship between the humoral response by B-cells to SRBC challenge (B-cell function) and proliferation of T-lymphocytes in response to ConA (T-cell function). This trade-off, however, was apparent only when controlling for individual differences in age, body condition, and clutch initiation date. When the uncorrected data were analyzed, immune variables were always

positively associated within each of the three axes of immune function identified, such that individuals showing stronger responses for one immune measure also showed stronger responses for the other measures within the same axis. These results are in accordance with the notion that given the costs of immune function (reviewed in Lochmiller and Deerenberg 2000), better-quality individuals, or those in better body condition, can allocate a larger proportion of their resources to immune defense and vice versa. However, given a limited amount of resources available for immune defense, a relatively high allocation to T-cell function can come at the cost of a relatively low allocation to B-cell function and vice versa. In addition, these two aspects of acquired immune function might show a trade-off because of their cross-regulation by different T helper (Th)–cells (Mosmann and Fowell 2002): antibody-mediated responses by B-lymphocytes are orchestrated by Th2-cells, while cell-mediated responses by T-cells are orchestrated by Th1-cells, and these two types of Th-cells can downregulate each other (Mosmann and Moore 1991).

Only a few studies have explored the relationships among immune measures commonly assessed by ecoimmunologists, and results from these studies do not generally support trade-offs between components (either within or between arms) measured simultaneously (or in close temporal proximity) in the same individuals (e.g., Saks et al. 2003; Lindstrom et al. 2004; Matson et al. 2006*a*; Roulin et al. 2007; Forsman et al. 2008, but see Horak et al. 2006; Martin et al. 2006*b*). Not all studies, however, have corrected for differences among individuals tha might obscure trade-offs. In particular, the age of individuals is rarely known in free-living animals. More studies are therefore needed before we can determine how common and unde what circumstances trade-offs within the immune system are apparent in free-living animals, as well as the mechanisms mediating these trade-offs.

Conclusion

Understanding the relationships among different immune de fense components in free-living animals is a major current challenge in ecoimmunology (Matson et al. 2006*a*; Forsman et al. 2008; Buehler et al. 2011). Our present work suggests that significant insights can be gained by means of a more com prehensive approach than has previously been employed (i.e., incorporating more than one component of each arm of the immune system) in ecoimmunological studies. This approach allowed us to identify three main axes of immune function variation among individuals that reflect major components of the immune system: T-cell function, B-cell function, and innate immune function. We also found that a trade-off between T cell function and B-cell function is apparent when controlling for confounding variation among individuals, as is expected from the cross-regulation of these two types of immune responses and the costs of immune function. Our results reemphasize the notion that using a single measure of immune function is unlikely to serve as a general index of an individual's immunocompetence (Norris and Evans 2000; Adamo 2004). An adequate characterization, however, might be attained by measuring one immune variable from each of the three axes identified in this study. Thus, our results highlight that by using current assays available to ecoimmunologists, the immune system is comprehensible despite its complexity (Martin et al. 2006*c*).

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