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No simple answers for ecological immunology: relationships among immune indices at the individual level break down at the species level in waterfowl

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Understanding immune function in the context of other life-history traits is crucial to understand the evolution of life histories, at both the individual and species levels. As the interest in assessing immune function for these comparative purposes grows, an important question remains unanswered: can immune function be broadly characterized using one or two simple measures? Often, interpretation of individual assays is ambiguous and relationships among different measures of immune function remain poorly understood. Thus, we employed five protocols to measure 13 variables of immune function in ten species of waterfowl (Anseriformes). All assays were based on a single blood sample subdivided into leukocyte (blood smear) and plasma (frozen until analysis) components. All assays were run using samples from every individual, and a nested analysis was used to partition variation/covariation at the levels of species and individuals within species. We detected positive correlations between functionally related measures of immunity within species, but these were absent from comparisons between species. A canonical correlation analysis revealed no significant relationships between the plasma and leukocyte assays at the levels of both individual and species, suggesting that these measures of immunity are neither competitive nor synergistic. We conclude that one measure of each assay type may be required to maximally characterize immune function in studies of a single species, while the same is not true in studies among species.

Keywords: Anseriformes; antioxidants; comparative immunology; leukocyte profiles; natural antibodies; plasma immunity

1. INTRODUCTION

Whether comparing physiological or life-history parameters within or among species, comparative biologists have measured immune function to test ideas about the evolution of immune responses to disease-causing organisms. Assuming that higher immunological responses better mitigate the effects of pathogens, but also impose greater costs (i.e. energetically, autoimmunologically, etc.), the evolved magnitude and variability of responses will depend on the balance between the disease environment and the costs of development, maintenance and use of immunological defence mechanisms, perhaps also taking into consideration expected life span.

The immune system is complex, comprising numerous distinct, but interacting components; thus, immune function can be quantified in many ways. On one hand, this complexity impedes attempts to comprehensively characterize immune function and understand its evolution and development; on the other hand, it has given rise to a wide variety of immunological measures applied to individuals in natural populations. Among these are assays designed to measure specific (Hasselquist *et al.* 1999) and non-specific (Matson *et al.* 2005) antibody titres, mitogen-driven lymphocyte proliferation (Leshchinsky & Klasing 2001*a*), PHA-induced swelling (Stadecker *et al.* 1977) and bacteria killing (Tieleman *et al.* 2005; Matson *et al.* in press).

Despite the desire to measure immunity as a trait of an individual, and the apparent availability of the tools to do so, the value of any particular assay is ambiguous. Immune responses depend on the type of disease organism (virus, bacteria, multi-cellular parasite, cancer, etc.) and other modifiers (dose/intensity, virulence, route, prior exposure; Power *et al.* 1998; Goldsby *et al.* 2000), and correlations between various indices of immune function and resistance to specific diseases appear to be generally pathogen-dependent (Adamo 2004). Moreover, small reductions of some aspects of immune function can lead to significant increases in disease susceptibility, while larger reductions in other aspects of immune function seem to have little effect (Keil *et al.* 2001). Thus, a strong argument for simultaneous measurement of multiple immune parameters has been put forward (Keil *et al.* 2001; Adamo 2004). Of course, most comparative studies of immune function are subject to numerous logistical limitations. For example, studies quantifying immune function in free-living birds are constrained by the stress that results from

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capture and handling, the unreliability of recapture, prohibitions on terminal studies, the lack of specialized (species-specific) reagents, the small body size of many study species, and the confounding effects that can result from repeated immunological challenges within individuals. Even so, in most studies, multiple measures of immune function can be made.

Measuring many parts of the immune system simultaneously allows one to determine whether immune responsiveness can be treated as a single variable comprising correlated responses of many components. Alternatively, different parts of the immune system may have been subject to diverging selection and, therefore, have to be evaluated separately. In general, the relationships underlying multiple measures of immune function are poorly understood, especially when working with species not normally used as biomedical models. Studies measuring multiple immune variables tend to focus on individual species (e.g. Luster *et al.* 1992; Keil *et al.* 2001; Leshchinsky & Klasing 2001*b*); including many species in an analysis would permit exploration of relationships among immune variables at both the species and individual levels.

We employed five protocols to measure the constitutive levels of 13 variables of immune function in ten species of waterfowl (Anseriformes). Our study was conducted using captive animals; however, we worked within the typical constraints of field-based comparative immunology: all assays were completed using a single blood sample collected upon capture (because repeated sampling is often impossible) and reagents that are not species specific. All assays and analyses were based on the single blood sample being subdivided into leukocyte (blood smear) and plasma (frozen until analysis) components. This distinction between leukocyte and plasma samples, which is employed from this point forward, is a division based on sample types and statistical constraints, not traditional immunological terminology (i.e. cell-mediated and humoral immunity).

The order Anseriformes is globally distributed and comprises about 150 species. As a group, waterfowl are relatively uniform with respect to anatomy and physiology. Nonetheless, our sample of goose and duck species varies considerably with respect to distributional range (from tiny islands to entire continents) and habitat (freshwater, marine, and *terra firma*; Lack 1974). Because these birds naturally occupy such a wide variety of environments (in the sense of geography and, presumably, pathogen exposure), we expected waterfowl to exhibit enough variation in immune function to make analyses of correlations among different functions feasible.

We compared measures of plasma and leukocyte immunity on two levels—among species and among individuals within species—to determine whether interspecific patterns of variation, presumably representing evolved differences, parallel or can be extrapolated from intraspecific patterns. To avoid confounding factors such as variable environmental conditions the samples were all collected from captive individuals housed in one location at a single point in time. All birds were fed species-appropriate diets *ad libitum*; all were housed in shared open-air facilities; and all were exposed to the same ambient temperatures, light : dark cycles and pathogen and parasite milieu.

2. MATERIAL AND METHODS

(a) *Subjects and samples*

Between the 16th and 20th of September 2003, blood samples (~1 ml) were drawn from the medial metatarsal veins of 61 birds representing 10 species in 4 genera (*Anas rubripes*, North American black duck, NABD; *Anas laysanensis*, Laysan teal, LATE; *Anas georgica spinicauda*, Chilean pintail, CHPT; *Anas georgica georgica*, South Georgia pintail, SGPT; *Branta canadensis leucopareia*, Aleutian Canada goose, ALCG; *Branta sandvicensis*, nene or Hawaiian goose, NENE; *Cairina moschata*, muscovy duck, MUSC; *Cairina scutulata*, white-winged wood duck, WWWD; *Dendrocygna autumnalis*, black-bellied tree duck, BBTD; *Dendrocygna arborea*, Cuban tree duck, CUTD). All birds were housed in mixed species aviaries in Scotland Neck, NC, USA (36.1° N, −77.42° W). All birds were at least six months of age with the majority being adults (48 after-hatch year, 5 hatch-year and 8 unknown age). Not all birds were definitively sexed, but within a subset of individuals, sex ratios were comparable among species.

At collection, several drops of blood were used to make smears for leukocyte enumeration. The remaining blood was centrifuged and the plasma collected; in total, the plasma assays require ~135 µl of plasma. All samples were provided by Sylvan Heights Waterfowl and all work was approved by the animal care committees at UC Davis and UM Saint Louis.

(b) *Blood smear evaluation*

A single blood smear from each individual was evaluated by conducting differential counts and estimating the overall white blood cell (WBC) concentration (Bounous & Stedman 2000). From these data, concentrations (μl^{-1}) of heterophils, lymphocytes, monocytes, eosinophils and basophils were estimated (Bounous & Stedman 2000). All blood smears were evaluated blind to species by a single veterinary diagnostic laboratory technician (AVL Veterinary Clinical Laboratory; St Louis, MO).

(c) *Plasma sample analyses*

(i) *Haemolysis/haemagglutination titres*

We assessed innate humoral immunity by using a haemolysis–haemagglutination (HL–HA) assay to characterize natural antibody- (NAb-) mediated agglutination and lysis of exogenous red blood cells (RBCs) as described by Matson *et al.* (2005). Both lysis and agglutination are recorded as the negative $\log_2$ of the last plasma dilution exhibiting each function (i.e. a dilution of 1 : 8 is scored as 3). Lysis reflects the interaction of NAbs and lytic enzymes (e.g. complement); agglutination results only from NAb activity. Because the effect of RBC source is unknown, we assayed all samples using exogenous RBCs from two sources: (i) pooled rabbit RBCs (as in Matson *et al.* 2005, RBA050; HemoStat Laboratories; Dixon, CA) and (ii) RBCs from a single Rainbow trout (*Oncorhynchus mykiss*; 9999; BioSure; Grass Valley, CA).

(ii) *Bactericidal competence*

We assessed anti-microbial activity of plasma in a bacteria-killing assay similar to one previously described (Matson *et al.* in press). Because different strains vary in their susceptibility to killing by plasma, we used two bacterial strains: *Escherichia coli* (ATCC 8739) and *Staphylococcus aureus* (ATCC 6538). The final suspensions (220 µl total composed of 190 µl

Table 1. Species samples sizes, means, standard deviations and the results of a GLM testing for an effect of species on the 13 variables. (Species are abbreviated as follows: NABD, North American black duck, *A. rubripes*; LATE, Laysan teal, *Anas laysanensis*; CHPT, Chilean pintail, *Anas georgica spinicauda*; SGPT, South Georgia pintail, *Anas georgica georgica*; ALCG, Aleutian Canada goose, *Branta canadensis leucopareia*; NENE, nene or Hawaiian goose, *Branta sandvicensis*; MUSC, muscovy duck, *Cairina moschata*; WWWD, white-winged wood duck, *Cairina scutulata*; BBTD, black-bellied tree duck, *Dendrocygna autumnalis*; CUTD, Cuban tree duck, *Dendrocygna arborea*).

		NABD (6)		LATE (8)		CHPT (8)		SGPT (8)		ALCG (7)		NENE (8)	
	variable	mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.
plasma	trout: lysis (titres)	3.2	0.8	3.3	0.7	2.9	0.7	2.6	0.5	2.1	0.4	2.3	0.5
plasma	trout: agglut (titres)	4.3	1.0	5.4	0.9	4.2	0.5	3.9	0.4	5.2	0.6	4.5	1.2
plasma	rabbit: lysis (titres)	4.8	0.4	4.8	0.5	4.8	0.5	4.0	0.5	4.0	0.6	4.5	0.5
plasma	rabbit: agglut (titres)	7.7	1.0	10.0	0.8	7.8	1.3	6.1	0.5	8.2	0.8	7.1	1.2
plasma	*E. coli* (% control)	51.7	88.4	18.5	12.0	47.9	44.3	48.7	57.2	49.8	35.6	130.8	125.1
plasma	*S. aureus* (% control)	110.7	22.0	125.3	21.3	114.2	15.6	121.0	15.6	106.8	9.5	111.2	10.1
plasma	haptaglobin (mg ml^{-1})	0.0813	0.0161	0.1770	0.0869	0.1127	0.0356	0.1128	0.0388	0.1271	0.0650	0.0992	0.0493
plasma	TEAC (mmol l^{-1} Trolox equiv.)	0.5899	0.2670	0.6796	0.2185	0.3419	0.1485	0.5317	0.0721	0.4342	0.1704	0.5354	0.0975
leukocyte	lymphocyte (µl^{-1})	6595	1901	7154	2476	3310	1163	3122	701	6826	1192	10 391	6157
leukocyte	heterophil (µl^{-1})	4139	6051	2467	1189	2943	2407	3927	2055	1195	398	1644	728
leukocyte	monocyte (µl^{-1})	389	188	307	342	86	77	88	145	228	137	144	169
leukocyte	eosinophil (µl^{-1})	111	271	69	128	97	171	0	0	23	60	21	60
leukocyte	basophil (µl^{-1})	0	0	116	230	16	45	0	0	0	0	0	0

		MUSC (5)		WWWD (8)		BBTD (2)		CUTD (1)		effect of Species (d.f. = 9)		
	variable	mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.	F	p	R^2
plasma	trout: lysis (titres)	2.4	0.5	2.8	0.8	2.5	0.7	3.0	.	2.69	0.012	0.322
plasma	trout: agglut (titres)	4.9	0.2	5.9	1.3	4.5	0.7	8.0	.	5.64	<0.0001	0.499
plasma	rabbit: lysis (titres)	4.0	0.7	3.8	0.9	3.5	0.7	5.0	.	3.41	0.0024	0.376
plasma	rabbit: agglut (titres)	9.3	0.8	8.3	1.2	9.0	1.4	11.0	.	9.78	<0.0001	0.633
plasma	*E. coli* (% control)	12.4	8.2	131.0	123.8	190.3	80.5	26.0	.	2.58	0.016	0.313
plasma	*S. aureus* (% control)	130.1	25.8	123.2	9.4	168.3	34.7	112.4	.	3.13	0.0045	0.356
plasma	haptaglobin (mg ml^{-1})	0.1126	0.0464	0.0849	0.0214	0.0973	0.0610	0.0826	.	2.13	0.044	0.273
plasma	TEAC (mmol l^{-1} Trolox equiv.)	0.6717	0.5755	0.3104	0.0771	0.2962	0.0402	0.3897	.	2.46	0.021	0.303
leukocyte	lymphocyte (µl^{-1})	4158	1019	4661	2089	3102	653	2336	.	5.19	<0.0001	0.478
leukocyte	heterophil (µl^{-1})	5756	4215	2526	973	2112	933	704	.	1.64	0.13	0.224
leukocyte	monocyte (µl^{-1})	195	229	133	185	286	31	160	.	1.72	0.11	0.233
leukocyte	eosinophil (µl^{-1})	276	319	92	107	0	0	0	.	1.48	0.18	0.207
leukocyte	basophil (µl^{-1})	36	80	114	123	0	0	0	.	1.58	0.15	0.218

CO_2-independent media (18045; Gibco-Invitrogen; Carlsbad, CA), 10 µl plasma and 20 µl bacteria) were incubated at 41 °C for 30 min during which the processes of the bacterial culture (growth and division) and immune components (stasis and killing) were allowed to interact. Afterwards, we briefly vortexed the suspensions, and we pipetted and spread 75 µl aliquots onto two agar plates. Plates were incubated overnight at room temperature (~25 °C). The next day we counted the number of viable colonies and determined the percentage of colonies in these experimental plates compared to control plates, which were made by diluting bacteria in media alone.

(iii) *Acute phase protein concentration*

Haptoglobin (Hp) is an acute phase protein found in a wide range of species including birds (Delers *et al.* 1988). Under normal conditions Hp circulates at low levels but concentrations increase with inflammatory responses, which result from infection or trauma. Hp complexes and removes haeme, thereby preventing the haeme from serving as a nutrient for pathogens and from initiating deleterious oxidation reactions (Dobryszycka 1997). We followed the 'manual method' instructions provided with a commercially available assay kit (TP801; Tri-Delta Diagnostics, Inc.; Morris Plains, NJ) to quantify the concentration (mg ml^{-1}) of Hp in all plasma samples.

(iv) *Antioxidant capacity*

Antioxidants are an important physiological mechanism for protection against free radical damage, and, as such, are related to immune function. Specifically, antioxidants quench free radicals that originate from the respiratory burst of phagocytes during an inflammatory response (Mates & Sanchez-Jimenez 1999). We used a modified version of the Trolox Equivalent Antioxidant Capacity (TEAC) assay to measure antioxidant capacity in all plasma samples (Miller *et al.* 1993). This technique works by measuring spectrophotometrically the change in quantity of a standard free radical, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), in the presence of a sample of unknown antioxidant capacity. The change in absorbance over time is standardized relative to a positive standard (Trolox, a water-soluble vitamin E analogue) and a negative control, which are then used to calculate antioxidant levels in units of mmol l^{-1} Trolox equivalents.

(d) *Statistical analyses*

A general linear model (PROC GLM; SAS 9.1 2002–2003; Cary, NC) was used to test for the effect of species on variation in each of the measures of the immune system (table 1). Furthermore, we calculated the variance/covariance matrix among species and among individuals within species (PROC NESTED; SAS). To gain insight into the underlying structure and relationships within both branches of the immune system and at both the individual and the species levels, we extracted the principal components (PCs, PROC FACTOR; SAS) separately for both plasma and leukocyte measures using correlation matrices generated from the nested analysis output. We used varimax rotation to maximize the contrasts of the variable loadings between the factors, restricting further analysis to factors with eigenvalues >1 (Kaiser 1960). Finally, we scored (PROC SCORE) independently the plasma and leukocyte principal components at both the species and individual levels. We performed a canonical correlation analysis (PROC CANCORR) using these new scored-factor data sets to explore how the plasma and leukocyte variables relate to each other.

3. RESULTS

(a) *Species means*

All eight measures of plasma immunity varied significantly among species (table 1). Four species consistently held the highest rank in one or more variables (LATE: trout-lysis, Hp, TEAC; CUTD: trout-agglutination, rabbit-lysis, rabbit-agglutination; ALCG: *S. aureus*; MUSC: *E. coli*). Similarly, four species held the lowest rank in one or more variables (BBTD; Rabbit-Lysis, *S. aureus*, *E. coli*, TEAC; SGPT: trout-agglutination, rabbit-agglutination; ALCG: trout-lysis; NABD: Hp).

Of the five leukocyte types, only lymphocytes varied significantly among species (d.f. = 9, $F = 5.19$, $p < 0.0001$; table 1). Lymphocyte concentration varied from 2336 μl^{-1} in CUTD to 10 391 μl^{-1} in NENE. Interestingly, the single CUTD also had the lowest concentrations of heterophils, eosinophils (tied with SGPT and BBTD), and basophils (tied with NABD, SGPT, ALCG, NENE, and BBTD).

(b) *Principal component analysis*

(i) *Individual-level analysis*

With the individual-level analysis of plasma variables, we identified four PCs with eigenvalues >1 that cumulatively account for 75% of the total variation. The patterns of loadings on these PCs after a varimax rotation revealed that these axes parallel the four plasma assay classes: PC1 represents HL–HA; PC2, bacteria-killing; PC3, TEAC; and PC4, Hp (table 2*a*). The HL–HA axis accounts for 32% of the total variation, the most of any axis. Analysis of leukocyte variables at the individual level resulted in three PCs with eigenvalues >1 that cumulatively account for 79% of the total variation (table 2*b*). PC1, the eosinophil/heterophil axis, accounts for 32%; PC2, the monocyte axis, 25%; and PC3, the lymphocyte/basophil axis, 22%.

(ii) *Species-level analysis*

We identified three PCs with eigenvalues >1 among plasma immunological assays at the species level (table 3*a*), but the loading patterns are less clear-cut and do not parallel the assay classes as with the individual level analysis. PC1 correlates positively with trout lysis, *S. aureus* killing, and TEAC, but negatively with *E. coli* killing. PC3 correlates positively with rabbit lysis (and to a lesser extant Hp); but negatively with *S. aureus* killing. Hp loads strongest on PC3, but its variation is dispersed over all three PCs, and it does not meet the saliency criteria (Cliff & Hamburger 1967; Pennell 1968) for any single PC. Notably, both rabbit and trout agglutination load exclusively on PC2. In total, the three PCs account for 77% of the total variation. Analysis of leukocyte variables at the level of species results in three PCs that cumulatively account for 84% of the total variation (table 3*b*). Accounting for 40% of the variation, PC1 correlates positively to eosinophil and heterophil concentrations and negatively to lymphocyte concentration. PC2, the monocyte axis and PC3, the basophil axis, account for 24% and 20% of the variation, respectively.

(c) *Canonical correlation analysis*

The canonical correlations did not detect any significant relationships between the plasma and leukocyte immune measures at either the species or individual level. The first canonical correlation at the level of individuals nested within species was 0.178, which did not differ significantly from zero ($F=1.31$, $p=0.22$) using Wilk's lambda test. The first canonical correlation at the level of species is also not significantly different from zero regardless of whether an unweighted model (1.095; $F=0.67$, $p=0.72$) or a model weighted for the number of individuals per species (1.286; $F=0.87$, $p=0.59$) is used. However, at the level of individuals nested within species, we detected a positive correlation between monocyte concentration (leukocyte PC2) and TEAC (plasma PC3; $r=0.25$, $p=0.05$, see figure 1*a*). At the level of species, no bivariate correlations are significant; the first PCs of the leukocyte (+heterophil/+eosinophil/−lymphocyte) and plasma (+trout lysis/+TEAC/+*S. aureus*/−*E. coli*) analyses are positively, but not significantly, correlated ($r=0.57$, $p=0.09$, see figure 1*b*).

4. DISCUSSION

(a) *Differences among species*

The immune system defends against pathogens and is essential for life; mounting an immune response, however, can divert host resources from other important activities like reproduction. Each component of the immune system has its own inherent costs and protective value, and the final mix of components is likely to be related to an animal's life history. In this study, 13 indices of immunity were selected to probe a wide variety of protective functions that have a range of costs of use. Heterophils and monocytes mediate innate immunity—the primary defence against novel pathogens. Lymphocytes mediate the adaptive-antibody and the cell-mediated responses, which are pathogen specific but have little value in the early defence against novel pathogens. Agglutination titres of exogenous RBCs are indicative of levels of natural antibodies, which facilitate initial pathogen recognition and initiate adaptive immune responses, while lysis titres are indicative of the level of complement and other circulating lytic enzymes. Bacterial killing activity of plasma results from the integrated activities of antibodies and accessory proteins like complement. Haptoglobin and antioxidants (TEAC) offer protection against harmful end products of the immune response, namely haeme from damaged host cells and free radicals from phagocytes.

Comparisons of species means revealed a significant effect of species in nine of the 13 variables. In general, plasma measures had lower coefficients of variation (CV) than leukocyte measures. Correspondingly, all eight plasma variables, but only one leukocyte type, varied significantly among species. Across species, the four HL–HA variables had consistently low intra-specific CV, which averaged 16%, the lowest of any assay class. The mean intra-specific CV for the bacteria killing was 53%, but this differed greatly between bacterial strains (*E. coli*, 91%; *S. aureus*, 14%). Intra-specific CV of Hp and TEAC averaged 36 and 34%, respectively. Lymphocyte concentration, the only WBC type to vary significantly among species, had the lowest mean intra-specific CV (32%) of the five types (the other four types averaged 120%). We hypothesize that the measures of immune function with the lowest CV (all HL–HA variables and *S. aureus* killing) are under strong genotypic influence reflecting strong stabilizing selection. In contrast, high intra-specific CVs are indicative of more important phenotypic effects, broad reaction norms and temporal variability in individual condition (e.g. current health status).

(b) *Relationships among immune variables*

When considering the species that rank highest or lowest for measures of plasma immunity, it becomes apparent that extreme (high or low) responses of species may be limited to a single assay class (high, CUTD; low, SGPT) or may comprise different assay classes (high, LATE; low, BBTD). The principal components analyses help us understand these inter-variable relationships while concurrently partitioning variation at the individual/species level and reducing the number of variables for subsequent analyses. Compared to the analysis at the individual level, which results in PCs that mirror the plasma-assay classes, the principal component analysis of plasma variables at the species level reveals a more complex picture. Specifically, the highest loadings of variables within assay types are distributed across PCs, and, in some cases, PCs cannot be easily described by a single assay type. Comparisons made at higher taxonomic levels (e.g. family or order) will probably reveal different relationships among immune variables yet again.

The more complex pattern of correlations among indices of immune function at the level of species may be a sign of overlap and redundancy in different functional components of the immune system, but further studies specifically designed to address this point are required. It is likely that evolutionary pressures from pathogens have driven the immune systems of different species in many different directions and that similar levels of protection against pathogens can be accomplished by different combinations of protective systems, thereby providing a degree of unpredictability from the pathogen perspective.

(c) *Competition or synergism between plasma and leukocyte immunity?*

While the canonical correlation analysis did not identify a significant relationship between the plasma and leukocyte data sets at either the individual or the species levels, the dominant correlations between the leukocyte and plasma PCs are positive (figure 1).

Across individuals, we identified a positive trend between monocyte concentration (leukocyte PC2) and TEAC (plasma PC3; $r=0.25$, $p=0.05$). Monocytes are phagocytic cells that produce free radicals and are associated with inflammation (Mates & Sanchez-Jimenez 1999). Thus, it is not surprising that the concentrations of these two inducible factors should correlate at the level of individual. Incongruously, concentrations of other non-lymphoid phagocytic cells (e.g. heterophils) do not correlate with TEAC.

Across species, leukocyte PC1 (+heterophil/+eosinophil/−lymphocyte) correlates positively, but not significantly, with plasma PC1 (+trout lysis/+TEAC/+*S. aureus*/−*E. coli*; $r=0.57$, $p=0.09$). The complex natures of both PC axes, however, obscure this relationship. Of the nine bivariate correlations between the three leukocyte and three plasma PCs, there are no significant

Table 2. Individual level principal components retained according to the Kaiser criterion for (*a*) plasma and (*b*) leukocyte data sets. (Italicized factor loadings meet the saliency criterion. Bold-faced factor loadings are the highest loading for each variable across PCs).

(*a*)

plasma	assay	variable	PC1	PC2	PC3	PC4
	HL–HA	trout: lysis	***0.89***	−0.09	0.03	−0.13
		trout: agglut	***0.77***	−0.17	0.15	−0.24
		rabbit: lysis	***0.64***	−0.19	−0.10	*0.37*
		rabbit: agglut	***0.76***	0.19	−0.08	0.18
	bacteria killing	*S. aureus*	*0.29*	***0.60***	*0.53*	0.02
		E. coli	−0.25	***0.87***	−0.15	−0.01
	acute phase protein	haptoglobin	−0.08	−0.08	***0.90***	0.03
	antioxidant	TEAC	−0.02	0.01	0.05	***0.93***
		% variance	32.1	16.0	14.0	12.9

(*b*)

leukocyte	cell type	PC1	PC2	PC3
	eosinophil	***0.89***	−0.15	0.07
	heterophil	***0.77***	*0.50*	−0.10
	monocyte	0.01	***0.90***	0.05
	lymphocyte	−0.23	0.28	***0.78***
	basophil	*0.33*	*−0.30*	***0.70***
	% variance	31.6	24.6	22.3

negative correlations (all $r<0.2$), and, therefore, no evidence that these immune functions are competitive. The positive correlation between first PCs of the leukocyte and plasma analyses is driven by the low and high values for leukocyte PC1; where leukocyte PC1 equals zero, a diverse group of species (representing three of the four genera), span an even greater range for plasma PC1. While these two apparent relationships between leukocyte PC1 and plasma PC1 further suggest uncoupling between these components of immune function, interestingly, no single species combines high leukocyte with low plasma abilities or vice versa.

(**d**) *Conclusions*

When exploring a wide variety of protective functions, the complexity of immune systems becomes evident. The magnitude, breadth, and consistency of responses across assays vary among species. The relationships of different measures among individuals within species co-vary in a manner that reflects assay types, but these correlations break down at the level of species.

Thus, two striking negative results of this study are the lack of correlation between variables arising from different assays, even at the individual level, and the lack of correlation among variables even of the same assay-type at the species level. The former suggests that there are no strong constraints, synergisms, or trade-offs of the systems being measured by these assays; the latter indicates that not only is the immune system as a whole highly complex, but the sub-systems measured by these assays are also complex and not always subject to simple interpretation. Furthermore, the absence of correlations between the plasma and leukocyte data at both the individual and species levels also implies a lack of constraints, synergisms or trade-offs.

These findings can help direct future studies. Some studies may benefit from a broad quantification of immune function. In these cases, one measure of each plasma assay type (e.g. trout lysis, *E. coli* killing, haptoglobin, TEAC) along with quantification of WBC concentrations could be used to maximally characterize immune function in studies of a single species. This is not the case in studies among species, but here inclusion of plasma measures and leukocyte concentrations will add robustness. In other cases, maximum characterization of immune function may not be possible or of interest. For example, when studying energetic trade-offs a 'costly' immune response may be more appropriate to measure than a 'cheap' response. Regardless of whether the result of uncontrollable logistical constraints or intentional experimental design, however, researchers measuring only one parameter of immune function should be careful not to overstate the broader immunological implications of their measurements.

The lack of correspondence between individual-level and species-level variation, even among variables like HL–HA, which apparently have a large genotypic component, suggests that on an evolutionary time scale selection on these immune measures may not be straightforward directional selection on widespread variation already existing in populations, and that more complex interactions may be involved. If this is the case, attempts to understand evolutionary variation in immune function may need to wait for better elucidation of how these immune variables respond to selection.

While all measures of immune function in this study were made using samples collected during a single capture event, inclusion of experimentally induced immune responses would potentially add a new dimension to a similar study. Though not possible in the current study,

Table 3. Species level principal components retained according to the Kaiser criterion for (*a*) plasma and (*b*) leukocyte data sets. (Italicized factor loadings meet the saliency criterion. Bold-faced factor loadings are the highest loading for each variable across PCs).

(*a*)

plasma	assay	variable	PC1	PC2	PC3
	HL–HA	trout: lysis	***0.60***	0.42	0.13
		trout: agglut	−0.19	***0.95***	−0.11
		rabbit: lysis	0.23	0.06	***0.88***
		rabbit: agglut	0.32	***0.87***	0.22
	bacteria killing	*E. coli*	***−0.81***	−0.06	−0.17
		S. aureus	***0.62***	0.36	*−0.61*
	acute phase protein	haptoglobin	0.45	0.26	**0.50**
	antioxidant	TEAC	***0.88***	−0.18	0.15
		% variance	38.8	22.2	16.4

(*b*)

leukocyte	cell type	PC1	PC2	PC3
	eosinophil	***0.85***	0.22	0.00
	heterophil	***0.89***	−0.13	−0.13
	monocyte	0.10	***0.91***	0.11
	lymphocyte	***−0.62***	*0.59*	−0.20
	basophil	−0.05	0.05	***0.98***
	% variance	39.7	23.9	20.4

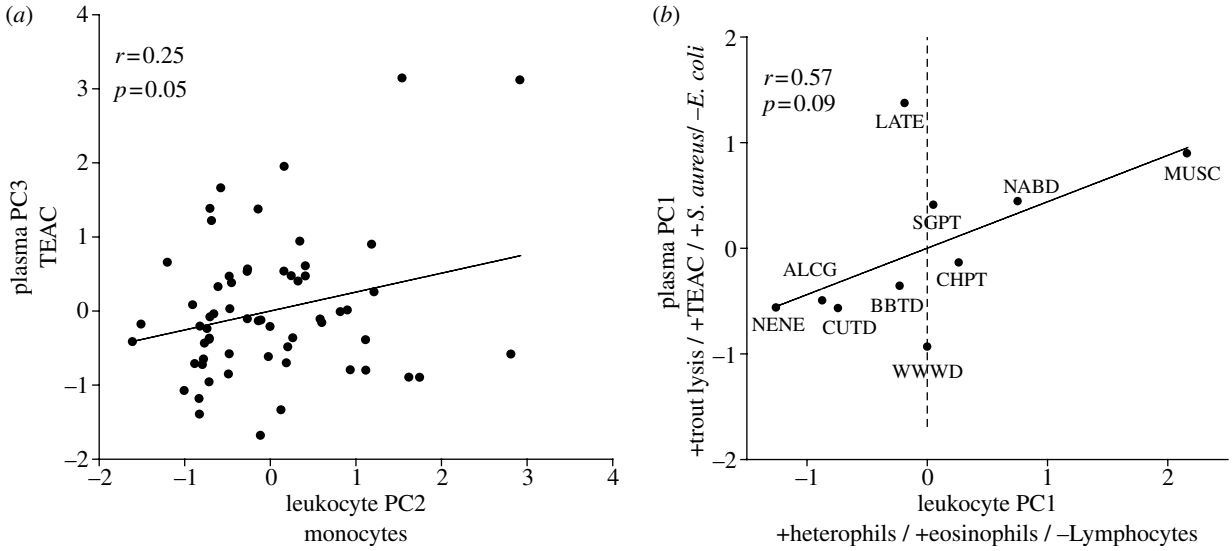

Figure 1. Dominant correlations between plasma and leukocyte PCs at (*a*) the individual and (*b*) species level.

incorporation of a disease resistance facet (e.g. through artificial infection) could help begin to unravel the connections between a broad slate of immune parameters and immune system functionality across species. Nonetheless, the current study highlights the complexity of immune systems and the uncoupled nature of many measures of immune function.

Ecological immunology is still a young field, but the toolbox of the field-based comparative immunologist is bigger and more diverse than ever. While the field is no longer in its infancy, the need is real for more basic research that examines intra- and inter-specific variation and that incorporates more species and more variables. More research on these fundamentals will promote efforts to delve deeper into questions of comparative immunology in general and evolution of immune function in specific.

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