5-15-2007

Comparative Immune Function in Wild Birds

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A dissertation presented to the
Graduate School of Arts and Sciences of the
University of Missouri-St. Louis
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

January 2006

Saint Louis, Missouri

Dissertation Abstract

Over the last several decades, interest in quantifying immune function in comparative studies of wild animals has grown appreciably. Now, the field of ecological immunology is undergoing a transition, and "second generation" studies are being designed and carried out. With a greater appreciation of the complexity of immune systems, these second generation studies are commonly distinguished from their antecedents by making comparisons using multiple assays and including multiple species. I worked to advance this transition by developing novel approaches to comparative immunology, exploring the interrelationships among indices of immune function, and applying multiple indices to a question of comparative avian evolution.

First, I worked to develop individual methodologies that would be broadly applicable given the numerous limitations of field-based immunology. I present methodological details on two assays—a hemolysis-hemagglutination assay and a bacteria killing assay, and I report on intra- and inter-specific comparisons using both. Relatedly, using ten species of waterfowl, I examine how these and other indices correlate at both the individual and species levels.

Next, with an interest in developing a better understanding of the evolutionary forces molding immune function, I set out to broadly compare immune function in 15 phylogenetically matched pairs of bird populations from North America and from the islands of Hawaii, Bermuda, and the Galápagos. If immune defenses were costly, populations from relatively disease-free, oceanic islands are expected to exhibit attenuated immune function in response to reduced pathogen and parasite pressure. In fact, many island animals exhibit this postulated "island syndrome," one facet of which is

increased susceptibility to disease. After employing three protocols to measure eight indices of immune function, I found no support for my hypothesis. Rather than evidence of depauperate parasite communities and inherent costs of immune defenses selecting for reduced immune function, I found that several indices were elevated in island birds. I suggest that life on islands is accompanied by an apparent reorganization of the relative importance of various immune components.

Finally, in collaborative efforts with investigators here and at other institutions, I apply the hemolysis-hemagglutination assay to address a variety of questions across three diverse avian study systems.

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Acknowledgments

To my family, friends, colleagues, collaborators, and committee-members,

I say thank you.

Chapter 1

A hemolysis-hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds.

Published: K. D. Matson, R. E. Ricklefs & K. C. Klasing.

Developmental and Comparative Immunology. 2005; 29:275-286.

Summary: Methods to assess immunocompetence requiring only a single sample are useful in comparative studies where practical considerations prevent holding or recapturing individuals. The assay for natural antibody (NAb)-mediated complement activation and red blood cell (RBC) agglutination described here, requiring ~100 µL of blood, is highly repeatable. The effects of complement deactivation, 2-mercaptoethanol (2-ME), age, and lipopolysaccharide (LPS)-induced sickness response were examined to validate comparisons among diverse avian species. Complement deactivation by heating significantly reduces lysis and treatment with 2-ME reduces both lysis and agglutination. Lysis and agglutination both increase with age in chickens; LPS treatment does not influence these variables in 11-week-old chickens. In a comparison of 11 species, both lysis (0.0 to 5.3 titers) and agglutination (1.8 to 8.0 titers) vary significantly among species. Accordingly, this assay can be used to compare constitutive innate humoral immunity among species and with respect to age, sex, and experimental treatments within populations.

1. Introduction

A subset of immune defenses can be categorized as both constitutive and innate [1]. Constitutive innate immunity provides the first-line of protection against invading microbes. Among these defenses are two interrelated humoral components: natural antibodies (NAbs) and complement. On one hand, NAbs serve as a recognition molecule capable of opsonizing invading microorganisms and initiating the complement enzyme cascade, which ends in cell lysis [2]. On the other hand, levels of complement and the expression of its receptors by B cells are positively correlated with NAb diversity and B-1 cell number [2]. This interaction of NAbs and complement is an important link between innate and adaptive immunity [2-4].

Details of the role of complement have been elucidated through decades of research on the large group of proteins that make up the system. Because complement deficiencies are associated with a range of infectious and non-infectious diseases, including lupus and arthritis, assessment of the complement system has become commonplace in humans [5], as well as in a range of domestic animals [6-8], including ducks [9], chickens [10, 11], and turkeys [12]. In non-domestic animals, hemolytic activity associated with complement has been identified in higher vertebrates [13] as well as lower vertebrates [including 3 species of fish, 14].

Natural antibodies are unique among immunoglobulin molecules because their presence does not require previous exposure to a particular antigen. Circulating NAbs have been described in naïve animals, including those raised in germ-free environments [4, 15, 16]. Additionally, NAbs are encoded directly by the germ-line genome [15, 17, 18] and do not require somatic hypermutation and recombination during ontogenesis as in the case of the adaptive antibody repertoire [19, 20].

Natural antibodies react with various affinities to a wide variety of epitopes on macromolecular and particulate antigens including foreign red blood cells (RBCs),

bacteria, viruses, and toxins [15, 21, 22]. In mammals, the main sources of NAbs are peritoneal $CD5^+$ B-1 cells, a subset of the antibody-producing B cells [23]. Most NAb molecules are pentameric IgM, but some IgG (monomeric) and IgA (dimeric) forms have been reported [17]. A variety of functions have been proposed for NAbs, including directly controlling novel bacterial and viral disease challenges, enhancing antigen presentation and initiating specific responses of B and T cells, regulating self reactive B and T cells, and clearing damaged or transformed cells [4, 24].

Because a large fraction of all antibody-secreting B cells are located in gut-associated lymphoid tissue [25], and many of these are peritoneal B-1 cells that constitutively secrete NAbs, these molecules may be important in clearing commensal microflora that leak across the gut epithelial barrier [26]. While NAbs also might play an immunoregulatory role, the "evolutionary important physiological role for enhancing survival of the host seems to be in early resistance against infection" [4]. For example, NAbs against the parasite *Plasmodium lophurae* have been identified in White Leghorn chickens (*Gallus domesticus*) [27]. Also, NAbs at naturally occurring concentrations have been shown to kill bacteria and spirochetes *in vitro* [15, 22] and to promote clearance of bacterial cell wall components (e.g., lipopolysaccharides) *in vivo* [26].

Assessment of immune function in free-living vertebrates is emerging as an important tool in evolutionary and ecological research. The types of assays that can be employed with wild animals, however, are constrained by the stress that results from capture and handling, the unreliability of recapturing animals, the prohibition of terminal studies, the lack of specialized reagents, and the small size of many study species. Conventional assays of B-cell function, such as lymphocyte phenotyping and specific antibody responses to vaccinations, are usually not possible. The levels of NAbs and complement in small blood samples taken at single capture events may provide important information about immunocompetence. The direct, genotype-dependent

expression of NAb genes makes the assessment of innate humoral immunity particularly appealing for addressing evolutionary questions. As the first line of defense against initial infection, germ-line encoded NAb genes are subject to natural selection. Indeed, NAb levels in chickens have responded over 20 generations of artificial selection on the primary antibody response [28]. Also, NAbs should be less sensitive than acquired antibody responses to short-term variations in environmental conditions, nutritional status, or stress levels [e.g. 29]. Moreover, NAb production appears to represent a functionally distinct [though genetically related 28] component of humoral immunity in which the cells producing NAbs are not influenced by experimental infection and initiation of a specific antibody response [30].

Here, we describe a highly repeatable assay for characterizing NAb-mediated complement activation and RBC agglutination titers that is useful for comparing innate humoral immunity among species and with respect to age, sex, and experimental treatments within populations. This assay uses a single small blood sample (roughly 100 µL, which can be safely drawn from birds as small as 10 g) collected upon capture. This uncomplicated sampling makes the method ideal for comparative immunological studies requiring numerous samples from small birds, and where recapture is difficult.

2. Materials and Methods

2.1. Subjects and Samples

Plasma samples were collected from both captive and free-living birds. The captive species were jungle fowl (*Gallus gallus*, ~3 years old, *n*=4, all males), Cobb broiler chicken (*Gallus domesticus*, four 2-week-old chicks and twenty 11-week-old chicks, all males), mallard duck (*Anas platyrhynchos*, ~6 months old, 2 females, 3 males, *n*=5), Japanese quail (*Coturnix coturnix japonica*, ~1 year old, all males, *n*=6), American kestrel (*Falco sparverius*, average 11 years old, sexes unknown, *n*=5), and zebra finch (*Taeniopygia guttata*, ages unknown, all female, *n*=4). The wild species were mourning

dove (*Zenaida macroura*, ages and sexes unknown, *n*=4), house sparrow (*Passer domesticus*, ages and sexes unknown, *n*=4), common grackle (*Quiscalus quiscula*, ages and sexes unknown, *n*=6), gray catbird (*Dumetella carolinensis*, ages and sexes unknown, *n*=11), and waved albatross (*Phoebastria irrorata*, ages unknown, 7 males, 6 females, 1 unknown sex, *n*=14). Blood samples were collected using heparinized syringes or heparinized microcapillary tubes. With one exception, blood samples were kept on ice for periods less than 1 hour until centrifugation, after which the plasma fraction was removed and frozen for future analysis. Due to field constraints, the waved albatross blood samples remained un-centrifuged and at ambient temperatures for periods not exceeding 6 hours, at which point the plasma was collected and frozen.

Blood collection protocols for all captive birds were approved by the UC Davis Campus Committee on Animal Care and Use. The work with all wild birds was approved by the UM Saint Louis Institutional Animal Care and Use Committee.

Plasma enriched in IgM for use as a positive control was collected from 3 adult Leghorn chickens. Each chicken was subcutaneously injected with 50 µL of whole rabbit blood in Alsever's solution (HemoStat Laboratories #RBA050, Dixon, CA) in 4 places in the pectoral region. After approximately 90 hours, when specific IgM levels had increased but specific IgY levels had not, blood from each chicken was collected in heparinized syringes. The blood was centrifuged, and plasma was collected, pooled, and frozen for later use. Prior to freezing, a portion of the pooled plasma was heated to 56°C in order to deactivate complement.

2.2. General Hemolysis-Hemagglutination Assay

The assay is carried out in 96-well (8 rows by 12 columns) round (U) bottom assay plates [Corning Costar #3795, see 31]. 25 µL of eight plasma samples are pipetted into columns 1 and 2 of the plate and 25 µL of 0.01 *M* phosphate buffered saline (PBS; Sigma #P3813, St Louis, MO) are added to the columns 2 through 12. Using a

multi-channel pipetter the contents of the column 2 wells are serially diluted (1:2) through column 11. This results in dilutions ranging from 1 to 1/1024 and 25 µL in every well. The 25 µL of PBS only in column 12 serves as a negative control. For the assay itself, 25 µL of a 1% rabbit blood cell suspension is added to all wells, effectively halving all plasma dilutions. Each plate is then sealed with ParafilmM (Pechiney Plastic Packaging, Neenah, WI) and covered with a polystyrene plate lid. Plates are gently vortexed for 10 seconds prior to incubation during which they are floated in a 37°C water bath for 90 minutes.

Upon completion of the incubation, the long axis of each plate is tilted to a 45° angle for 20 minutes at room temperature in order to enhance visualization of agglutination. Plates are then scanned (full size image at 300 dpi) using the positive transparency (top-lit) setting of a flatbed scanner (Microtek Scanmaker 5900). Afterward, plates are kept at room temperature for an additional 70 minutes and scanned for a second time to record maximum lytic activity. From the digitized images, lysis and agglutination are scored for each sample. Lysis reflects the interaction of complement and NAbs, whereas agglutination results from NAbs only. Both variables are recorded as the negative $\log_2$ of the last plasma dilution exhibiting each behavior, i.e., column 9 is a score of 9 [32] (see Fig. 1). Half scores between two titers are recorded when the termination of lysis or agglutination is intermediate or is ambiguous [32].

2.3. Rabbit Blood Cell Suspension

Because chickens exhibit high levels of natural hemagglutinins for rabbit RBCs compared to RBCs from 4 other mammals [33], this assay was developed to use a commercially available suspension of whole rabbit blood in Alsever's solution (HemoStat Laboratories #RBA050, Dixon, CA; supplied as 50% whole blood, 50% Alsever's). The RBCs were washed 4 times with PBS (ca. 275 x *g* for 5 minutes). The hematocrit was

checked in duplicate using capillary tubes and the RBCs were adjusted to a final cell concentration of 1% in PBS. At 25 µL per well, each assay plate requires 2.4 mL of the prepared 1% cell suspension. To ensure that the 1% cell suspension remains well mixed, the suspensions were vortexed immediately prior to addition to the assay plate. Fresh cell suspensions were prepared daily.

2.4. Plasma Treatments

Two plasma treatments are referred to throughout this manuscript: heat and 2-mercaptoethanol (2-ME). Heat-treated plasma was de-complemented at 56°C for 30 minutes [34]. 2-ME-treated plasma had 2-ME added to a final concentration of 0.1 *M* in order to break up polymeric immunoglobulins (primarily IgM) [34]. 2-ME-treated plasma samples were incubated at 37°C for 30 minutes [32].

2.5. Assay Repeatability, Rabbit Blood Age Effects, and Scorer Effects

To examine assay repeatability, 16 assays using the Leghorn positive control plasma pool were run over a 2-day period when the rabbit blood was freshest (days 1 and 2). Two individuals (KM and A Scheuerlein) scored these assays to test for scorer effects on lysis and agglutination titers. Further, to examine effects of storage time of the rabbit blood in Alsever's solution, 4 additional assays were run on days 6, 10, and 15 after receipt of the shipment of fresh blood. Every assay plate contained 4 repeats of both unheated and heated plasma.

LPS-Induced Sickness Response

Fifteen 11-week-old male Cobb broiler breeder chicks were injected subcutaneously with 1 mg lipopolysaccharide (LPS; Sigma #L7261, St Louis, MO) from *Salmonella typhimurium* per kilogram body mass. At 4, 8, and 16 hours post injection, groups of 5 chickens were bled from the jugular vein. One mL of blood was collected in heparinized microcentrifuge tubes from each chicken. Control samples were collected in a similar manner from 5 non-injected 11-week-old male Cobb broiler breeder chicks.

2.7 Statistical Analyses

Within- and among-assay variation was calculated from 4 repeats for each of two types of plasma (heated and unheated pooled chicken plasma) run in 16 different assay plates. Within-assay variation is reported as the mean, minimum, and maximum values of the standard deviations (SD), standard errors (SE), and coefficients of variation (CV) (n=4 for each type of plasma on each plate) across the 16 plates, whereas among-assay variation is reported as the SD, SE, and CV of the 16 intra-plate means. Variation due to rabbit blood storage time was tested using a linear regression on the 5 daily means for lysis and agglutination with unheated and heated plasma during the 15-day test period. A one-sample *t*-test was used to assess the difference between scorers.

Variation in mean lysis and agglutination titers was tested independently using univariate general linear models (SPSS, Release 9.0.0, 1998). To test the effect of species and plasma treatment, we used a GLM with species and plasma treatment as fixed factors, individual as a random factor, and the interaction between species and plasma treatment. The effects of chick age and interval following LPS injection were analyzed separately for unheated and heated plasma using GLMs with only a single fixed factor. Post-hoc multiple comparisons were made using Tukey's test.

3. Results and Discussion

3.1. *Assay Design: Repeatability, Scorer Effects, and Rabbit Blood Storage Effects*

Within- and among-assay variation was calculated for lysis and agglutination titers using both unheated and heated plasma pools. Across all plates, the agglutination titer averaged 10.0 for unheated plasma and 9.8 for heated plasma and the lysis titer averaged 3.9 for unheated plasma. Lysis was absent from the heated plasma. The mean within-assay variation (SD) was ± 0.2 titers for agglutination and ± 0.1 titers for lysis (Table 1). The mean among-assay variation (SD) was ± 0.4 titers for agglutination and ± 0.3 titers for lysis (Table 2). The CV within and among assays is frequently

reported, but standard deviations are more meaningful when comparing values on a logarithmic scale because the mean value, by which the SD is divided to obtain the CV, is arbitrary and can be 0 or negative.

The assay data revealed a small, but significant scorer effect. Samples were scored blindly, and one individual recorded significantly lower values than the other. The mean difference was 0.2 titers (S.D. = 0.5; n = 128, $p<0.001$) for agglutination and 0.04 titers (S.D. = 0.2; n = 128, $p<0.001$) for lysis. The smaller difference between scorers for lysis is likely due to the fact that the endpoint for lysis is clearer than that of agglutination.

The rabbit blood cells as packaged by the supplier are given an expiration date of 2 weeks after collection and shipment. The storage time of the blood cells (up to 15 days) did not significantly affect the outcomes of the assays with either heated or unheated plasma (Table 3). Thus, it appears that one batch of rabbit blood cells can be safely used until its expiration date.

Due to concerns regarding the anti-complementary effects of heparin, lysis titers in plasma (collected in heparinized microcapillary tubes) and serum were compared in 3 species. No significant effects were found (unpublished data). Further, to ensure lytic activity was not limited by the concomitant serial dilution of endogenous divalent cations, the effects on lysis of 2 different dilutants (the standard PBS dilutant and a dilutant with Mg^{+2} and Ca^{+2}) were compared in 2 species. Again, no significant effects of were found (unpublished data). Across species, the functional importance of heparin interference and of endogenous cationic concentration on *in vitro* complement activation is not well understood, and, therefore, both warrant further investigation.

3.2. *Plasma Treatments*

The effects of heating plasma to deactivate complement and treating plasma with 2-ME to disaggregate IgM were tested in 5 species: mourning dove, Japanese quail,

mallard duck, jungle fowl, and 11-week-old Cobb chicken. Because the interaction between species and plasma treatment was significant for both lysis [$F(8,38) = 48.3$, $p<0.001$] and agglutination [$F(8, 38) = 61.8$, $p<0.001$], the effect of plasma treatment was examined independently for each species. Thus, data for each species were analyzed using plasma treatment as a fixed factor and individual as a random factor in a repeated-measures GLM. Simple contrasts were conducted to compare lysis and agglutination values for both heated plasma and 2-ME-treated plasma with values for untreated plasma.

A significant decrease in agglutination titer was observed when plasma from 11-week-old Cobb chicks was heated ($p=0.001$) or treated with 2-ME ($p<0.001$; Fig. 2). Significant reductions in agglutination were exhibited in jungle fowl ($p<0.001$), mallard ducks ($p<0.001$), and Japanese quail ($p=0.001$) only when the plasma was treated with 2-ME (Fig. 2). Neither heat nor 2-ME significantly reduced the low levels of agglutination observed in mourning doves (Fig. 2). Lysis was significantly reduced in both heated and 2-ME treated plasma in 11-week-old Cobb chickens, jungle fowl, mallard ducks, and Japanese quail ($p<0.001$ for both treatments for each species; Fig. 2). Because lysis is absent from untreated mourning dove plasma, no comparisons were made.

Plasma samples were heated to deactivate complement and then scored for lysis to ensure that complement, and not a heat stable serum factor (e.g. an acute phase protein), was responsible for the lysis titers that were determined in untreated plasma. Only a small (< 1 $\log_2$ unit) amount of lytic activity remained in heat-treated plasma from Cobb chickens and mallards and none occurred in most species. This indicates that all, or almost all, of the lysis observed with unheated plasma was due to complement. Additionally, the lysis titer was almost always below the agglutination titer, indicating that immunoglobulin was not limiting for measurement of complement levels. The exception was the common grackle, where the lysis and agglutination titers were similar. Thus, it is

not possible to determine whether the measured complement titer in this species was accurate because it may have been constrained by NAb levels. One potential side effect of the heat inactivation of complement is a significant reduction in agglutination titers (e.g. 11-week-old Cobb chicken samples). In this case, some NAb fraction appears to be denatured by the heat treatment.

We added 2-ME to plasma samples to break polymeric NAbs into monomeric units, such that a common currency could be used to compare agglutination across species. However, the ability of the immunoglobulin fragments to agglutinate is questionable [34], and the ME-resistant titers more likely represent IgY molecules that cross-react with rabbit RBCs. Though 2-ME treatment reduces titers by one-half or more, the interspecific pattern of agglutination seen with 2-ME resistant antibodies parallels the pattern of agglutination with untreated plasma and provides little additional information. One notable exception, jungle fowl plasma, exhibits a more severe reduction in agglutination titers. This reduction suggests that jungle fowl may rely more on polymeric forms of natural antibodies than the other species. In addition to reducing overall agglutination levels, 2-ME treatment totally eliminates all hemolytic activity. This elimination of lysis could result from the destruction of disulfide bonds in complement components or from the inability of 2-ME-resistant antibodies to initiate the complement cascade.

Many Passeriformes (songbirds) and Apodiformes (swifts and hummingbirds) are too small (<20 g) to collect sufficient blood to test all plasma treatments. In such cases, untreated plasma should be the highest priority because it provides the most information (i.e., lysis and agglutination titers).

3.3. Effects of Age in Young Birds

With respect to lysis and agglutination in unheated and heated plasma, 11-week-old Cobb broiler chicks exhibited significantly higher titers than 2-week-old chicks (Fig.3

2). In unheated plasma, lysis titers increased from 0.3 ± 0.3 (SE) in 2-week-old birds to 4.0 ± 0.3 in 11-week-old birds [$F(1, 7) = 79.5$, $p<0.001$] and agglutination titers increased from 1.5 ± 0.3 to 8.0 ± 0.3 [$F(1, 7) = 219.1$, $p<0.001$]. In heated plasma, lysis titers increased from 0.0 ± 0.0 in 2-week-old birds to 0.8 ± 0.2 in 11-week-old birds [$F(1, 7) = 12.4$, $p=0.010$] and agglutination titers increased from 0.0 ± 0.0 to 7.0 ± 0.3 [$F(1, 7) = 381.1$, $p<0.001$]. Seto and Henderson [33] found low levels of NAbs in embryos and in chicks up to 20 days of age. In their experiment, NAb levels increased rapidly during the next several weeks and reached a plateau at around 12 weeks of age. The low level of agglutination by plasma from young chicks indicates that maternal IgY contributes little to the NAb titer as measured with RBC agglutination. The timing of the increase and plateau of NAbs follows a time course that is similar to that of plasma IgM levels, further implicating this form as the responsible Ig.

Changes in immune function in developing birds are expected, but the generality of this result across species is unknown. The rates of increase of circulating natural antibody levels likely depend on the development period and vary over the altricial-precocial development spectrum. For practical purposes, these results suggest the need to limit comparisons to birds of similar age groups (e.g. hatchling, fledgling, hatch year or older) whenever possible.

3.4. Effects of LPS-induced Sickness Response

Lysis and agglutination titers were not greatly affected by LPS injection in 11-week-old Cobb chicks (Fig. 4). Only in one case was a significant effect shown: lysis in heated plasma decreased from 0.8 ± 0.2 in the control birds to 0.0 ± 0.0 in the 4, 8, and 16 hour birds [$F(3, 16) = 16.0$, $p<0.001$]. The nonsignificant decreasing trend in agglutination with time may, in fact, be the result of NAbs binding and clearing LPS, as suggested by Reid [26].

Because some acute phase proteins are likely able to lyse RBCs in a complement-dependent manner, one might predict higher levels of lysis in birds undergoing an acute phase response. On the other hand, the lack of substantial changes in lysis and agglutination titers after LPS injection may be less surprising in light of the finding of Baumgarth *et al.* [i.e. that infection does not affect NAb levels, 30] and our finding that vaccination with keyhole limpet hemocyanin has no effect on either titer (unpublished data). Nevertheless, that the acute sickness response with its symptomatic anorexia, elevated acute phase proteins, and periodic hypo- and hyperthermia did not significantly affect lysis or agglutination is noteworthy, particularly in relation to the tradeoffs that are made between other physiological stresses and immunocompetence [e.g. 29]. Thus, with wild birds it can be assumed that short-term fluctuations in health status, which are difficult to determine when a bird is in hand, do not strongly affect these measures of innate immunity.

3.5. *Inter-Species Comparisons*

Because the interaction of species and heat treatment was significant in the case of lysis [$F(10,61) = 37.7$, $p<0.001$] and agglutination [$F(10,61) = 2.5$, $p=0.012$], and because the effects of heat treatment were documented in a subset of the species in this study, we examined the effect of species independently within each plasma treatment type. The data for heated and unheated plasma were analyzed using a GLM with species as a fixed factor.

Lysis titers in unheated plasma (Fig. 2A) ranged from 0.0 ± 0.0 (SE) in the mourning dove to 5.3 ± 0.3 in the common grackle [$F(10,57) = 38.9$, $p<0.001$]. Agglutination titers for unheated plasma (Fig. 2A) ranged from 1.8 ± 0.7 in the mourning dove to 8.0 ± 0.3 in the 11-week-old Cobb chickens [$F(10,57) = 8.4$, $p<0.001$]. Lysis titers in heated plasma (Fig. 2B) ranged from 0.0 ± 0.0 in 9 different species to 0.8 ± 0.2 in the 11-week-old Cobb chickens [$F(10,57) = 10.8$, $p<0.001$]. Agglutination titers for

heated plasma (Fig. 2B) ranged from 1.6 ± 1.0 in the mourning dove to 7.4 ± 0.4 in the jungle fowl [$F(10,57) = 9.3$, $p<0.001$].

With the range in agglutination titers observed in the 11 species, we conclude that different species rely on the constitutive innate humoral branch of the immune system to differing degrees. The level of this reliance may reflect the underlying differences in the biology or life history of each species. Furthermore, the differences across species may represent tradeoffs with other branches of the immune system, so that birds with high NAb levels may rely less on induced and/or specific responses. This, however, does not appear to be the case *within* the humoral branch. Chickens artificially selected for high or low primary antibody responses exhibited parallel changes in NAb levels (and disease resistance) [28, 35, 36]. Thus, despite a functional partitioning, constitutive innate and induced specific humoral immunities appear to be genetically related to one another and to disease resistance within a species. The extent of these relationships across bird species, such that NAb variables could be measured as a proxy for overall humoral immunity, remains to be examined.

In addition to the variation in agglutination across species, the 11 study species also varied with respect to average lysis titers. When the two variables are examined together, the species can be placed in three groups based on the amount of complement (lysis) relative to NAb (agglutination). In the low group, lysis titers are less than 5% of total agglutination titers; in the medium group, they are 40 to 65%; and in the high group, greater than 95%. Both overall agglutination and lysis titers could vary to balance the robustness of other branches of the immune system; the particular pattern of immune defenses might reflect differences in the life history and ecology of species, but resolving these relationships will require carefully designed experiments and broader comparative studies.

In birds, the total fitness costs of maintaining NAbs and complement are not well understood. In mammals, however, pathological autoimmunity appears to be related to anomalous NAb production in some cases [37-39]. Ultimately, the degree of investment in innate humoral immunity should reflect a number of factors, including life span and the need for long-term memory, exposure to pathogenic organisms in the environment, and the coevolutionary responses of pathogens to a host's immune response.

Use of the assay described in this paper for characterizing constitutive innate humoral immunity has clear advantages over several methods currently employed to assess immune function in comparative and experimental studies. The most common assay of immune system function in the ecological literature, the swelling to a subcutaneous injection of phytohemagglutinin (PHA), quantifies nonspecific cellular immunity [40, 41]. This technique requires measurements over a 24-hour period. Moreover, the PHA response does not represent a single clearly definable immune phenomenon but a suite of responses that results in swelling. A second technique, involving specific antibody responses following vaccinations by particular antigens [such as sheep RBCs, 42, and keyhole limpet hemocyanin, 43], measures specific, humoral immunity. As this technique requires either recapturing individuals or holding individuals over periods of up to 30 days, the tests are logistically complicated and the results are confounded by other physiological phenomena such as stress responses or changes in breeding status. However, if the conjecture of Kohler *et al.* [44] that NAb levels against particular pathogens approximate the specific antibody response to those same pathogens is true across avian species, then minor modifications of our assay (i.e. replacement of RBCs with specific pathogens) could permit a comparative study of adaptive immunity.

4. Conclusions

The hemolysis-hemagglutination assay described here can be effectively used to characterize and quantify constitutive innate humoral immunity in birds. The assay is highly repeatable and the results are unambiguous. Agglutination and lysis titers vary significantly between species. In chickens, agglutination and lysis titers were affected by age, but were not affected by an LPS-induced sickness response. When plasma sample volume is limited, we recommend untreated plasma for use in this assay, as this option is most informative.

Acknowledgements

The authors thank the three anonymous reviewers who provided helpful comments and the thoughtful reviews. The authors thank A Scheuerlein and I Tieleman at UM Saint Louis for their assistance with the statistical analysis and assay scoring and for their comments on the manuscript. The authors wish to thank B Humphrey, E Koutsos, and R Peng at UC Davis for their assistance, particularly with sample collection from the captive species. The authors also thank K Huyvaert, P Parker, and J Merkel for providing the waved albatross plasma samples. Invaluable assistance has also provided by the Lubbock's at Sylvan Heights Waterfowl II and by D Kersting and his associates at Bird Medicine and Surgery. This research was supported in part by the National Science Foundation (IBN-0212587), the Saint Louis Zoo (FRC #03-13), an American Ornithologists' Union Graduate Research Award, the International Center for Tropical Ecology, the Saint Louis Audubon Society, the Webster Groves Nature Study Society, and the Explorer's Club.

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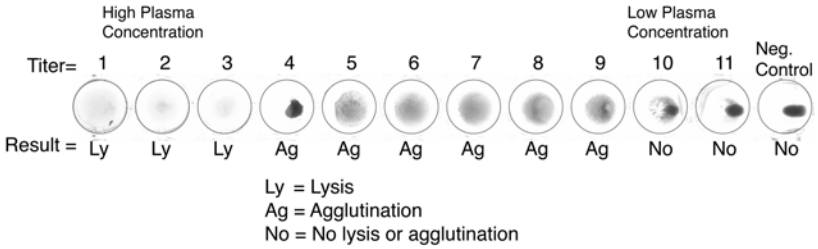

Fig. 1. An example scan. Scan shows results of the hemolysis-hemagglutination assay using serially diluted plasma from one 11-week-old Cobb broiler chicken (*Gallus domesticus*). Titers 1 through 3, show hemolysis. Titer 4 shows a tight or compact form of agglutination, while titers 5 through 9 show a more flocculent form of agglutination. In addition to the negative control (PBS only), the clear dripping in titers 10 and 11 demonstrate a lack of lysis and agglutination. This example scan would be given a lysis score of 3 and an agglutination score of 9.

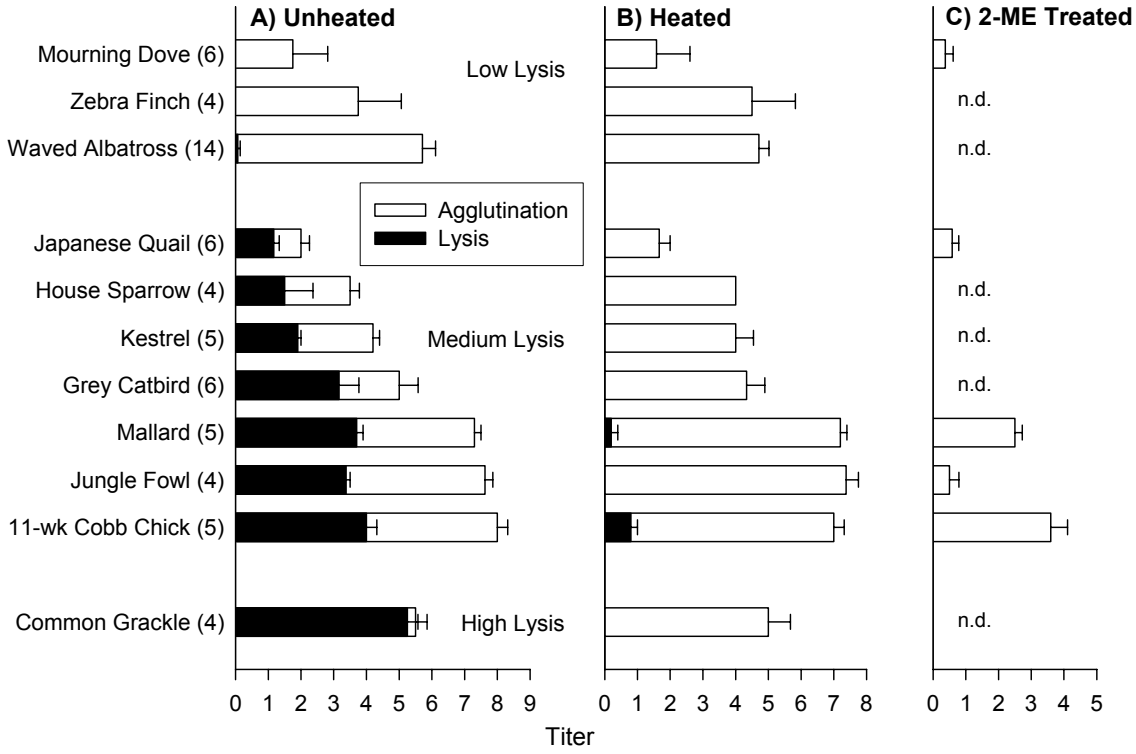

Fig 2. Lysis and agglutination titers in unheated (A), heated (B), and 2-ME treated (C) plasma from 11 species of birds. Filled bars represent lysis; open bars, agglutination. Error bars represent SE. Numbers in parentheses indicate number of individuals per species. Due to plasma volume limitations, not all species were treated with 2-ME (no data, n.d.)

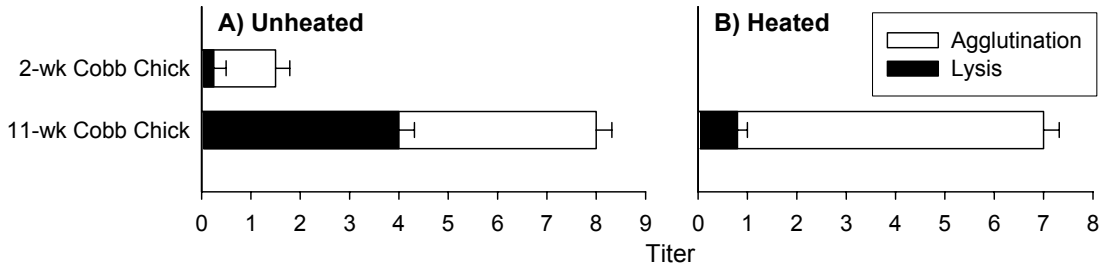

Fig 3. Lysis and agglutination titers in unheated (A) and heated (B) plasma from four 2-week-old and five 11-week-old Cobb broiler chickens (*Gallus domesticus*). Filled bars represent lysis; open bars, agglutination. Error bars represent SE.

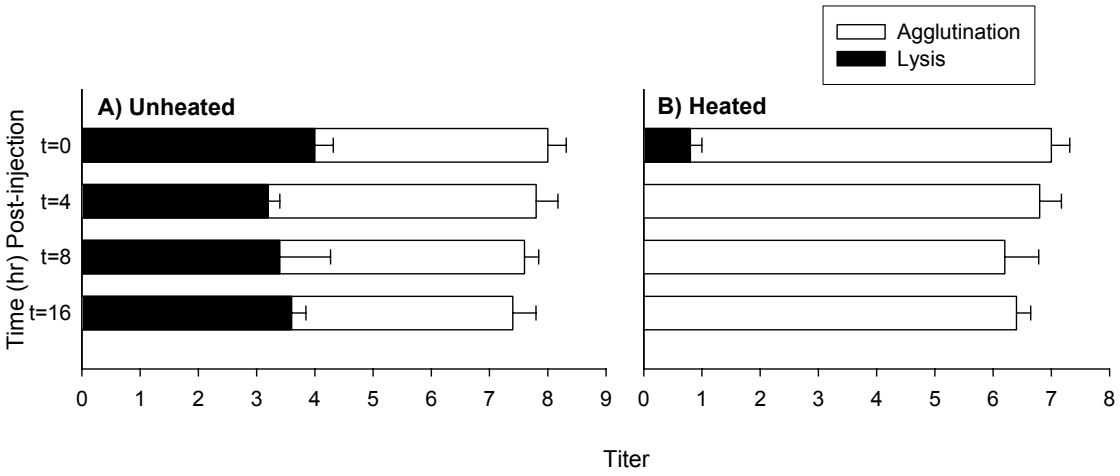

Fig 4. Lysis and agglutination titers in unheated (A) and heated (B) plasma from 11-week-old Cobb broiler chickens (*Gallus domesticus*). Each group was composed of 5 different birds. Filled bars represent lysis; open bars, agglutination. Error bars represent SE.

				SD				SE				CV			
Plasma Treatment	Variable	n_{sample}	Mean	Mean	SD	High	Low	Mean	SD	High	Low	Mean	SD	High	Low
Chicken Pos. Std., Unheated	Agglutination	4	10.0	0.2	0.3	0.9	0.0	0.1	0.1	0.4	0.0	2.0	2.5	8.1	0.0
Chicken Pos. Std., Heated	Agglutination	4	9.8	0.2	0.2	0.5	0.0	0.1	0.1	0.3	0.0	2.1	2.2	5.7	0.0
Chicken Pos. Std., Unheated	Lysis	4	3.9	0.1	0.1	0.5	0.0	0.0	0.1	0.2	0.0	2.0	3.9	13.2	0.0
Chicken Pos. Std., Heated	Lysis	4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	.	.	.	.

Table 1. Within-assay variation reported as the mean, minimum, and maximum values of the standard deviations (SD), standard errors (SE), and coefficients of variation (CV). Across the 16 plates, n=4 for each type of plasma on each plate.

Plasma Treatment	Variable	n_{plate}	Mean	SD	SE	CV
Chicken Pos. Std., Unheated	Agglutination	16	10.0	0.5	0.1	4.7
Chicken Pos. Std., Heated	Agglutination	16	9.8	0.4	0.1	4.2
Chicken Pos. Std., Unheated	Lysis	16	3.9	0.3	0.1	7.0
Chicken Pos. Std., Heated	Lysis	16	0.0	0.0	0.0	.

Table 2. Among-assay variation reported as the standard deviations (SD), standard errors (SE), and coefficients of variation (CV) of the 16 within-assay-plate means for each type of plasma.

Plasma Treatment	Variable	Beta	d.f.	*t*	*p*
Chicken Pos. Std., Unheated	Agglutination	-0.097	3	-0.168	0.877
Chicken Pos. Std., Heated	Agglutination	0.521	3	1.058	0.368
Chicken Pos. Std., Unheated	Lysis	-0.719	3	-1.793	0.171
Chicken Pos. Std., Heated	Lysis	.	.	.	.

Table 3. Results of linear regressions on the 5 daily means for lysis and agglutination with unheated and heated plasma during the 15-day test period. The storage time of the blood cells did not significantly affect the outcomes of the assays with either heated or unheated plasma.

Chapter 2

Capture stress and the bactericidal competence of blood and plasma in five species of tropical birds.

In press: K. D. Matson, B. I. Tieleman & K. C. Klasing.

Physiological and Biochemical Zoology.

Summary: In wild birds, relatively little is known about intra- or inter-specific variation in immunological capabilities and even less is known about the effects of stress on immune function. Immunological assays adaptable to field settings and suitable for a wide variety of taxa will prove most useful for addressing these issues. We describe a novel application of an in vitro technique that measures the intrinsic bacteria-killing abilities of blood. We assessed the capacities of whole blood and plasma from free-living individuals of five tropical bird species to kill a non-pathogenic strain of *E. coli* before and after the birds experienced an acute stress. Killing invasive bacteria is a fundamental immune function, and the bacteria-killing assay measures constitutive, innate immunity integrated across circulating cell and protein components. Killing ability varied significantly across species, with common ground doves exhibiting the lowest levels and blue-crowned motmots, the highest levels. Across species, plasma killed bacteria as effectively as whole blood, and higher concentrations of plasma killed significantly better. One hour of acute stress reduced killing ability by up to 40%. This assay is expected to be useful in evolutionary and ecological studies dealing with physiological and immunological differences in birds.

Introduction

Relatively little is known about the immunological capabilities of free-living birds, and even less is known about how stress impacts immune system function across species. Furthermore, the immune assays most commonly applied to comparative ecological and evolutionary questions are fraught with limitations relating to methodology and interpretation. Thus, with a growing interest in comparative studies of immune function, new assays free of these restrictions are needed. We propose that measuring the in vitro bacteria-killing ability of whole blood and blood plasma represents a novel method for quantifying and comparing immune function when addressing questions of ecology and evolution. This assay measures the integrated effects of multiple components of innate immunity. As such, the bacteria-killing assay overcomes many of the limitations commonly associated with measuring immune function. In particular, unlike some other assays, the results are easily interpretable: a higher in vitro bacteria-killing ability equates with a greater capacity of the subject to limit infection by the particular species or strain used in the assay. Furthermore, the assay does not require species-specific reagents, and, because the assay measures the constitutive, or standing, ability of an individual to kill bacteria, handling time is minimized and stress effects are controlled. We measured the bacteria-killing abilities of 5 species of tropical birds found in open and edge habitats. Additionally, we conducted an experiment to determine the immediate effects of acute stress from capture, handling, and restraint on bacteria-killing ability.

Immunological assays developed for particular model species (e.g. chickens) are often of little use for comparative studies as a result of the need for species-specific reagents. Prohibitions on terminal studies and limitations because of the small body size of many birds further restrict comparative studies of avian immune function. Despite these impediments, a diverse set of studies spanning disciplines (e.g. behavioral

ecology (Kilpimaa et al. 2004) and life history evolution (Tella et al. 2002)) compare immunological capacities both within and among species.

Researchers most commonly rely on two methods to assess immune function. The first method uses color change (ELISA) or agglutination to quantify specific antibodies produced following vaccination with a novel, noninfectious antigen (e.g. KLH (Hasselquist et al. 1999)). Measurement of specific antibody titers quantifies a well-defined branch of the immune system: inducible, adaptive, humoral immunity. Often considered to evaluate "cell-mediated immunity," the second method measures swelling following a subcutaneous injection of phytohemagglutinin (PHA) (Stadecker et al. 1977; Goto et al. 1978). Triggering a broad suite of non-specific reactions, injection of PHA integrates widely over numerous immune components. Widely integrative assays are advantageous in some situations, particularly in comparative studies where measurement of multiple individual components are constrained logistically, but because the relationship between PHA-induced swelling and fundamental immune functions (i.e. preventing infection) is unclear, the interpretation of the PHA-swelling results can be challenging. Further, the induced nature of both measures necessitates repeated sampling (over 24 hr for PHA and over several days for specific antibodies). Associated with this repeated sampling are the often-overlooked effects of stress associated with repeated capture (or captivity) and handling. Difficult to control, these stresses likely confound outcomes and may account for the conflicting results reported within and between studies (e.g. (Deerenberg et al. 1997; Ilmonen et al. 2003)).

Chronic stress is generally considered immunosuppressive (Raberg et al. 1998). Circulating stress hormones elicit a wide variety of responses from the immune system. Empirical data and experimental results suggest that the functional effects of stress depend on a number of factors that vary among study organism (e.g., birds (Gross and Siegel 1973; Regnier et al. 1980; Gross and Siegel 1983; Ilmonen et al. 2003; Kushima

et al. 2003), mammals (Keller et al. 1983; Endresen et al. 1991) and fish (Peters et al. 1991; Demers and Bayne 1997)). Within birds, stress effects depend on the branch of the immune system and the type of defense measured (e.g., white blood cell profiles (Gross and Siegel 1983; Ilmonen et al. 2003; Kushima et al. 2003), specific antibody responses (Gross and Siegel 1973; Regnier et al. 1980; Ilmonen et al. 2003), and hypersensitivities (Ilmonen et al. 2003)). The nature of the applied stressor (e.g., nutritional (Klasing 1988), thermal (Regnier et al. 1980), reproductive (Deerenberg et al. 1997; Ilmonen et al. 2003), or psychological (Gross and Siegel 1973; Endresen et al. 1991; Kushima et al. 2003)) and any modifiers (e.g., duration (Dhabhar and McEwen 1997), severity and novelty) also influence the effects of stress on immune function (Dohms and Metz 1991). While widely viewed as immunosuppressive, in some instances stress can also enhance immunity (Endresen et al. 1991; Dhabhar and McEwen 1996; Demers and Bayne 1997).

The immune system is complex, and the relative contribution of different components (e.g. cellular versus humoral) to different species during periods of homeostasis and stress is unclear. Nonetheless, the recognition and destruction of invading bacteria are clearly fundamental functions of organisms' immune systems, but due to the diversity of immune response mechanisms, species may accomplish this function by different means (Roitt 1997). The assay we describe here assesses constitutive innate immune function. Because this form of immunity, which includes standing anatomical, physiological, phagocytic, and inflammatory barriers, depends neither on an individual's history of exposure to pathogens nor on the somatic rearrangement of the genes that encode antibodies (as with adaptive immunity) (Roitt 1997), the evolved response of a population to pathogens should be more directly revealed by innate immune mechanisms. Making inter-specific predictions that relate

particular immune functions to other parameters (e.g. life history variables), however, is not easy given the paucity of data.

As a measure of innate immunity, bacteria killing integrates cytological (Keusch et al. 1975) and serological (Merchant et al. 2003) immune components. While avian nonlymphoid cells exhibit varying abilities to phagocytize pathogens, phagocytosis by heterophils (Harmon 1998) and macrophages (Qureshi 1998) is particularly important for defense against infection by bacteria. In the blood plasma, a number of soluble proteins also play key roles in limiting infection. Natural antibodies serve as non-specific recognition molecules with the ability to limit early microbial infection (Ochsenbein et al. 1999). The complement enzyme cascade can lyse targeted cells by way of a membrane-attack complex endproduct (Esser 1994) or through protein byproducts (Nordahl et al. 2004). Lysozyme, another plasma component, exhibits bactericidal capacity through an enzymatic digestion of cell-wall structural carbohydrates (Selsted and Martinez 1978). Also, though generally considered an induced response, some acute phase proteins, such a mannose-binding protein, may be constitutively produced at concentrations high enough to enhance (via complement fixation or opsonization) the capacity of blood to kill bacteria (Roitt 1997). Thus, to fully address questions of comparative biology, this multi-faceted nature of immune defenses combined with intrinsic and extrinsic differences among study populations or species may necessitate the use of multiple species or strains of microorganisms or require other methodological considerations.

Materials and Methods

Subjects and Samples

Between 26 March 2004 and 28 April 2004, we captured blue-crowned motmots (BCMM, *Momotus momota*, *n*=6), blue-gray tanagers (BGTA, *Thraupis episcopus*, *n*=13), crimson-backed tanagers (CBTA, *Ramphocelus dimidiatus*, *n*=9), clay-colored

robins (CCRO, *Turdus grayi*, *n*=24), and ruddy ground-doves (RUGD, *Columbina talpacoti*, *n*=14) in mist nets in Gamboa, Panama. We bled birds sterilely from a wing vein two times: once within 3 minutes of the subject's first striking the net (t_0) and once 60 minutes later (t_{60}). During the 60-minute period between bleeds, we held all birds in small fabric bags in an air-conditioned room (~21 ^{o}C). Plasma samples collected within 3 minutes reflect baseline levels of the stress hormone corticosterone, while samples collected after one hour typically show significantly increased levels (Wingfield et al. 1982). The volume of the two blood draws never exceeded 1.0% of a bird's body mass. After creating a sterile zone around the wing vein by saturating the area with 70% ETOH and allowing it to air-dry for approximately 20 seconds, we collected blood into sterile heparinized capillary tubes before it had a chance to escape the sterilized area. We used sterilized clay cards to plug all capillary tubes and airtight plastic containers for transporting the cards and tubes back to the lab. For use in parametizing assay variables, we also collected blood from 3-week-old Cobb chicks (*Gallus domesticus*) utilizing similar sterile techniques. All protocols were approved by the animal care committees at UC Davis or UM Saint Louis.

Bacteria-killing Assay

The use of fresh whole blood and plasma necessitated that we initiate assays immediately after sample collection over the same 34-day period of bird capture. In sterile 1.5 mL tubes, we diluted plasma (10 µL and 20 µL) and whole blood (20 µL) from each individual at both time points to a final volume of 200 µL using CO_2 independent media (#18045; Gibco-Invitrogen, Carlsbad, CA) plus 4mM L-Glutamine and 5% heat-inactivated fetal calf serum. To each diluted plasma and blood sample, we added a 20-µL aliquot containing about 600 colony-forming units (CFUs) from an *Escherichia coli* working culture. This *E. coli* (ATCC # 8739) culture was prepared from lyophilized pellets ($3.1x10^7$ CFUs per pellet; Epower Microorganisms #0483E7; Microbiologics, St

Cloud, MN), which were reconstituted according to the instructions provided by the manufacturers. In order to ensure all cultures contained the correct number of CFUs, we regularly plated sub-samples on tryptic-soy agar plates and counted the resulting colonies.

The final suspensions (220 µL total; diluted whole blood or plasma plus bacteria) were incubated at 41^{o}C for 30 minutes during which the processes of the bacterial culture (growth and division) and immune components (stasis and killing) were allowed to interact. After incubation, we removed and briefly (~5 s) vortexed the samples. In duplicate, we pipetted 75-µL aliquots onto two agar plates and spread the mixture evenly over the surface of the agar. Following a brief drying period (~20 minutes), we covered and inverted the plates and incubated them overnight at ambient temperature (~27^{o}C). The next day we counted the number of viable colonies and determined the proportion of colonies killed by comparison to control plates, which were made by diluting bacteria in media alone. To avoid contamination, we made use of a portable laminar flow hood (Airclean 600, Airclean Systems, Raleigh, NC) at all stages requiring a sterile environment.

In conjunction with the development of this protocol, we also measured the effect of whole blood concentration on bacteria killing using samples collected from chickens. A 1:4 dilution was made in addition to 1:10 and 1:20 dilutions as above. Additionally, we heat de-complemented subsamples of chicken plasma (Delhanty and Solomon 1966). Killing abilities of heated and unheated plasma samples were compared in order to quantify the contribution of complement and other heat-labile proteins.

Statistical Analyses

The differences in the number of viable bacteria after incubation and the number in the initial inoculums are expressed as the proportion killed. After first rounding up all negative proportions to zero, we arcsine transformed [arcsine (square root (proportion

killed))] all data prior to statistical analysis (Sokal and Rohlf 1998). To facilitate interpretation, however, we present some data as untransformed percentages.

To analyze the variation in the transformed data set, we estimated a repeated-measures general linear model (SAS 9.0) with species (5 sp), sample type (3 types: 10 µL plasma, 20 µL plasma, 20 µL blood), and stress time point (0, 60 minutes) as independent variables. We included in the analysis only individuals for which all 6 values (3 sample types at 2 stress time points) had been measured (BCMM, *n*=4; BGTA, *n*=10; CBTA, *n*=6; CCRO, *n*=14; and RUGD, *n*=8). This criterion only minimally impacted sample size; but when a particular, more focused analysis resulted in an increased sample size and a change in the significance level, we report both results.

Results

Among Species Comparisons

Bacteria-killing ability varied widely among our 5 study species and depended on sample type (Figure 1). Because 2-way and 3-way interactions between species, sample type, and stress time point (all $p<0.011$) had significant effects on transformed proportion of bacteria killed, we examined the effect of species within each plasma treatment type separately at t_0. The transformed proportion of bacteria killed using 10 µL plasma, 20 µL plasma, and 20 µL blood was analyzed using a univariate GLM with species as a fixed factor. In all cases the variation among species was significant. Using 10 µL plasma, the percentage of bacteria killed ranged from 4.9% ± 0.5% (SE) in the RUGD to 99.1% ± 0.0% in the BCMM [$F(4,37) = 15.7$ $p<0.0001$]. Using 20 µL plasma, the percentage of bacteria killed ranged from 64.3% ± 2.7% in the RUGD to 99.6% ± 0.1% in the BCMM [$F(4,37) = 3.8$ $p=0.011$]. Using 20 µL whole blood, the percentage of bacteria killed ranged from 7.7% ± 1.2% in the BGTA to 99.2% ± 0.1% in the BCMM [$F(4,37) = 15.9$ $p<0.0001$, Figure 1].

Sample Type Comparisons

Bacteria killing varied more among sample types in some species than in others (Figure 1). Again, because of the significant 2- and 3-way interactions identified above, this variation among sample types was examined independently for each species at t_0. The transformed proportion of bacteria killed by each sample type within a species was analyzed using a repeated-measures GLM. The bactericidal ability of the 3 sample types varied significantly in all species (BGTA [$F(2,18) = 43.2$ $p<0.0001$], CBTA [$F(2,10) = 18.5$ $p<0.001$], CCRO [$F(2,26) = 9.2$ $p=0.001$], and RUGD [$F(2,14) = 23.2$ $p<0.0001$]), except the BCMM [$F(2,6) = 1.0$ $p=0.432$, Figure 1]).

Post-hoc comparisons revealed that within-species differences among sample types are driven by differences in plasma concentration, but not by the addition of the cellular component. Pair-wise tests of the killing ability of 10 µL plasma and 20 µL blood showed no significant effects in any species. When comparing 10- and 20-µL plasma samples the same tests revealed significant differences in all species (BGTA [$F(1,9) = 61.3$ $p<0.0001$], CBTA [$F(1,5) = 44.9$ $p=0.001$], CCRO $F(1,13) = 16.6$ $p=0.001$], and RUGD [$F(1,7) = 25.4$ $p=0.002$]), except the BCMM [$F(1,3) = 5.8$ $p=0.095$].

Effects of Stress

The magnitudes of the observed stress effects vary by species and by sample type. Therefore, the effect of holding time on transformed proportion of bacteria killed was analyzed using a repeated-measures GLM within each sample type and independently for each species. In 3 of the 5 species, the stress of capture and handling had significant negative impacts in one or more sample type (Figure 2A). No significant effects were seen in the BCMM or in the CCRO.

In the CBTA, stress from capture resulted in significant reductions in the bactericidal abilities of 10 µL plasma [$F(1,5) = 12.8$ $p=0.016$], 20 µL plasma [$F(1,5) = 8.1$ $p=0.036$], and 20 µL blood [$F(1,5) = 13.6$ $p=0.014$]. With 20 µL blood, however, the

stress effect on bacteria killing became marginally insignificant when one individual, originally excluded from the main analysis due to incomplete sampling, was included in a one-way analysis [$F(1,6) = 5.1$ $p=0.065$].

The BGTA showed a significant decline in killing ability due to capture stress only in the 20-µL plasma sample [$F(1,9) = 29.2$ $p<0.001$]. The decrease in the bactericidal abilities of the RUGD were marginally insignificant for 20 µL plasma [$F(1,7) = 5.3$ $p=0.054$] and 20 µL blood [$F(1,7) = 4.8$ $p=0.065$]. In the case of 20 µL plasma, the stress effect on bacteria killing became significant when four individuals, originally excluded from the main analysis due to incomplete sampling, were included in a one-way analysis [$F(1,11) = 8.5$ $p=0.014$].

Other Methodological Considerations

Varying assay parameters resulted in direct and predictable changes in the outcome of the assay. Increasing the concentration of chicken whole blood increases the proportion of the bacteria killed. With a 30-minute incubation, a 1:4 dilution killed 98.0% (±1.1% SD, n=3); a 1:10 dilution, 92.1% (±1.4% SD, n=3); and a 1:20 dilution, 83.9% (±2.5% SD, n=3). Additionally, complement and other heat-labile proteins appear to account for much of the killing abilities of plasma. Following a 15-minute incubation, heat de-complemented chicken plasma killed only 12.6% (±5.6% SD, n=3) of bacteria, while intact untreated plasma killed 64.6% (±4.0% SD, n=3).

Discussion

Among Species Differences

Our results show substantial and significant variation in the ability of 5 bird species to kill a single strain of bacteria. Similar work has demonstrated striking differences in the capacity of human and alligator (*Alligator mississippiensis*) serum to control *E. coli* (Merchant et al. 2003). This variation suggests that different species employ this particular branch of the immune system to differing degrees for preventing and

controlling *E. coli* infections. Because of the observed inter-specific variation, the bacteria-killing assay is expected to be well suited for comparative studies of ecology and evolution. Though, depending on the study design, inclusion of multiple species or strains of microorganisms may be required to fully characterize innate immunity.

Within birds, for example, bacteria-killing ability is predicted to relate to where a particular species falls on the slow-fast continuum of life-history variation and correlate with other variables like rates of reproduction and development (Ricklefs and Wikelski 2002). While our small sample size precludes a proper analysis, it does appear as if this immune measure relates to generalized life-history syndromes. Specifically, at the fast end of the spectrum the ruddy ground-dove fledges at about 12 days (Skutch 1983) and has the lowest bacteria-killing ability, whereas at the slow end of the spectrum the blue-crowned motmot fledges at about 31 days (Skutch 1983) and exhibits the highest bacteria-killing ability. Furthermore, a follow-up study that increases the number of species (but measures bacteria-killing using whole blood only) demonstrates bacteria killing and mass-corrected basal metabolic rates are inversely related, again suggesting slow pace of life is associated with high bacteria-killing abilities. (Tieleman et al. 2005).

Exposure is another factor that could affect bacteria-killing abilities. Acquired immunity and the production of specific IgY would result from previous exposure to the exact strain used in this assay (Roitt 1997) and would likely increase killing ability. Alternatively, exposure to other strains of *E. coli* (and even to other enteric bacteria species) generates and maintains circulating levels of cross-reactive nonspecific (or natural) antibodies in the form of IgM (Reid et al. 1997). If prior exposure is anticipated to be a problem, then birds with specific IgY could be indentified by deactivating the background IgM with mercaptoethanol (Delhanty and Solomon 1966; Van Der Zijpp and Leenstra 1980) before use in the assay. All birds are commonly exposed to many different strains of *E. coli* (e.g. in their commensal microflora and diet), but the specific

strain used in this assay has evidently not been previously isolated from birds (Biosis database search, June 2005).

Condition-dependent fluctuations in bacteria-killing abilities brings to light the benefits of applying this assay to the same individuals over time. Adding this longitudinal aspect to the experimental design would contribute to the overall understanding of this measure but would not entirely allow differentiation between maximum capacity and current response, a problem common to all functional assays.

Though subject to logistical constraints (e.g. blood draw volume limitations, physiological effects of multiple captures, etc), using the sum of these techniques to measure the abilities of birds to kill a range of microorganisms (e.g. different strains of *E. coli*, *Staphylococcus* spp. and *Saccharomyces* spp.) will result in the most complete picture of this functional response. Resulting from inter-specific differences in both host immune strategies and microorganism defense strategies, complete correlation among all measures is not expected.

Sample Type Differences

In this experiment, 10 µL plasma was equally effective as 20 µL whole blood at killing this particular strain of *E. coli*. Within a subset of the study subjects, the hematocrit averaged 50.6% (±6.1% SD, n=42). Thus, 20 µL whole blood contains approximately 10 µL plasma. Across all 5 species, a linear regression showed that the killing ability of 10 µL plasma significantly explained 77% of the variation in the killing ability of 20 µL whole blood [R^2=0.772; $F(1,40) = 135.5$ $p<0.0001$] (Figure 3). In the resulting model, the slope did not differ significantly from 1.0 and the intercept did not differ significantly from 0.0. Thus, it is plasma and its protein constituents that are apparently responsible for the killing capacity measured when using whole blood. Moreover, the analysis of the chicken plasma samples point specifically to heat-labile proteins and suggest that one or more components of complement may be responsible for lysing the bacteria. Similarly,

because the bacteria-killing ability is inhibited by both heating and preincubating with proteolytic enzymes, complement-like proteins are thought to contribute to the antibacterial capacities of alligator serum (Merchant et al. 2003).

In some cases, cellular effects have been shown to be critical for innate bacterial resistance and for the bactericidal activity of blood (Davies et al. 1981; Hanski et al. 1991). This difference in cellular effects might be related to the pathogenicity of the assay microorganism. Studies involving a range of bacteria (including *E. coli*) and study species (including mammals, birds, and fish) reveal an inverse association between microbial pathogenicity and serum resistance (Joens and Nuessen 1986; Magarinos et al. 1994; Mellata et al. 2003). Thus, the lack of a cellular contribution to killing likely stems from using a non-pathogenic strain of *E. coli*. It is expected use of pathogenic strains would result in the cellular component contributing more to overall killing ability. An equivalent assay developed for clinical application illustrates that leukocyte bactericidal activity can be quantified when the bacteria used are limited to serum-resistant strains (Keusch et al. 1975). However, that plasma exhibits bactericidal qualities means the assay as presently described is freed of even the most basic limitations imposed by cell culture (e.g. maintaining viable leukocytes), which is beneficial when conducting field-based studies.

Plasma concentration also affected killing capacity; 20 µL plasma resulted in substantially better killing than 10 µL, except in the BCMM where 10 µL plasma was sufficient to kill virtually all bacteria. The increase in killing ability that results from doubling plasma concentration is significantly and inversely correlated with the transformed proportion of bacteria killed by 10 µL plasma [R^2=0.945; $F(1,3) = 51.8$ p=0.0055] (Figure 4). Analysis of the chicken whole-blood samples reveals a similar result; the bacteria-killing abilities increase in conjunction with a 5-fold increase in

concentration. Concentration-dependent effects have also been shown in the antimicrobial capacity of alligator serum (Merchant et al. 2003).

With the 5 tropical bird species, the differences between 10- and 20-μL samples highlight important differences related to where each species falls on a saturation curve. The killing abilities of high responders, in this case the BCMM, have reached a plateau, killing practically 100% with only 10 μL plasma. In contrast, the killing abilities of low and medium responders are on the increasing part of the curve and, as such, rise significantly. As a result of this pattern, in comparative studies, the use of multiple plasma concentrations might be required for identifying differences between species. In fact, when blood-draw volume is not limiting, the bacteria-killing assay can be easily extended to include a series of dilutions of whole blood and plasma (or even a series of incubation periods) to tease apart subtle differences. With immunologically complex systems (e.g. blood), though, quantification of bacteria killing using a serial dilution technique will reflect the limiting component, be it complement, antibody or phagocytosis.

Effects of Stress

Significant stress effects were seen in 3 of the 5 species and found across all 3 sample types (Figure 2A). When we examined each sample type individually, we found that bacteria killing at t_0 affected the amount of stress-induced difference ($t_{60} - t_0$) in bacteria killing. If 10 μL plasma killing is used as an example, one can see maximized stress effects when t_0 bacteria killing falls in the middle of the response range (e.g. CBTA, Figure 2B). Similar relationships are seen with the other two sample types. As with the concentration effects, the magnitude of the stress effects depend on a species' position on a conceptual curve. Stress appears to reduce the effective plasma concentration of bacteria-killing components, and taken together, concentration and stress effects suggest an S-shaped dose-response curve. At one end of the spectrum, the low

responders (RUGD, and to a lesser extent, BGTA) are so poor at killing bacteria that little room exists for stress effects of any consequence. At the other end of the spectrum, the high responders (BCMM and, to a lesser extent, CCRO) kill with such efficacy that the effects of stress are slight and insignificant, as measured by the present assay. As a result, with 10 µL plasma we found a significant difference in only one species, CBTA, which kills about half of the bacteria culture using the t_0 sample. Thus, it appears that when addressing questions of intra-specific variation, a modification of the assay so the final dilution kills approximately 50% of the bacteria will offer the greatest sensitivity.

A number of factors could explain effects of acute stress on plasma-dependent bacteria killing. Stress can induce leakage of gut-associated bacteria across the gut epithelium (Saunders et al. 1994). If plasma proteins important for bacteria lysis also work to opsonize and facilitate the clearance of invasive enteric bacteria (e.g. natural antibodies; (Reid et al. 1997)), then a reduction in circulating levels of unbound proteins would likely result. In addition, the physical stress from capture and the initial bleed might cause tissue trauma and hemolysis. Clearance of these damaged cells involves the same plasma constituents and would again lower the levels available to prevent infection in vivo and kill bacteria in vitro. By adding biologically relevant concentrations of lysed endogenous red blood cells to the reaction mixtures prior to incubation or by using different individuals for t_0 and t_{60} sample collections, the contribution of this mechanism could be tested. Regardless of cause, the apparent result is a reallocation of plasma proteins important for bacteria lysis from the circulatory system to the interstitial fluid and lymph system. The observed stress-induced decrease in plasma-mediated innate constitutive immunity has important health implications. One or more acute stresses could immunocompromise an individual, and microbes that are ordinarily

non-pathogenic could result in an infection and elicit a more energetically costly immune response (e.g. the acute phase response).

Conclusions

Avian blood and plasma can both be effectively used in a functional assay that quantifies bacteria-killing capacities in vitro. Using the assay as described, we found significant differences between species, significant decreases in some species following acute stress, and no differences between equivalent concentrations of plasma and whole blood. By using additional species or strains of microorganisms, eliminating nonspecific antibodies, varying incubation period, or extending the range of dilutions, this assay could be optimized for use in a wide variety of intra- and inter-specific studies where quantification of immune function is required.

Acknowledgements

The authors thank members of the Ricklefs' lab at UMSL, particularly R Ricklefs and A Scheuerlein, and two anonymous reviewers for their feedback and assistance with this manuscript. The authors also thank J Bennett at UCD for her work developing the assay. This research was supported in part by the National Science Foundation (IBN-0212587), the Saint Louis Zoo (FRC #03-13), the American Ornithologists' Union, the Saint Louis Audubon Society, the Webster Groves Nature Study Society, the Explorer's Club, the International Center for Tropical Ecology, and the University of Missouri.

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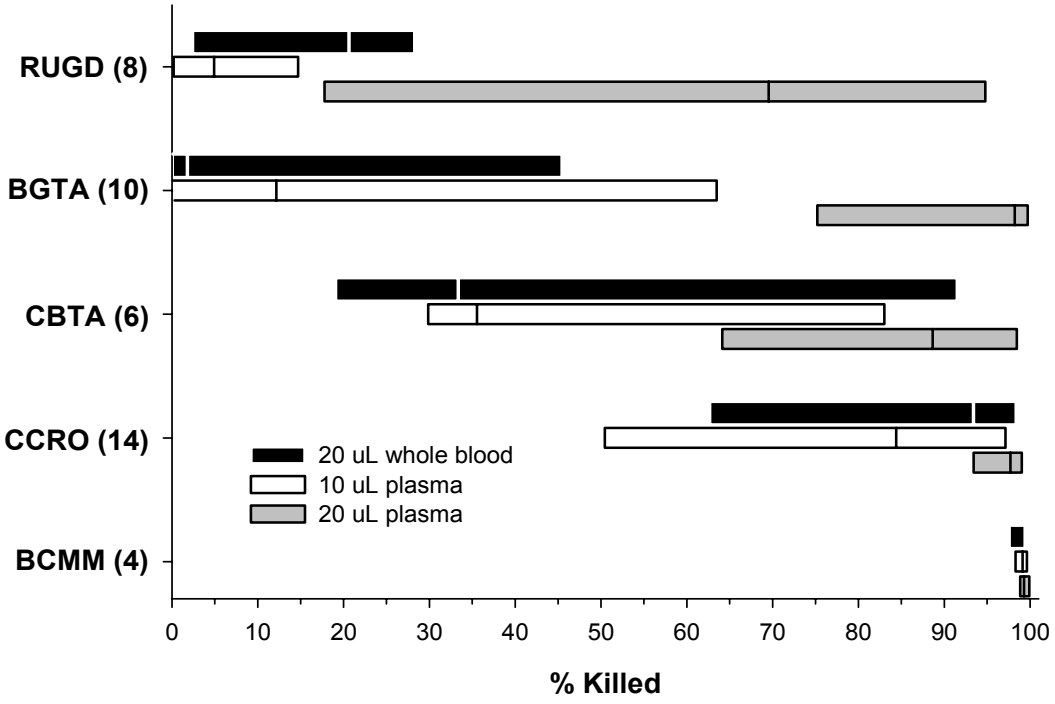

Figure 1

Box plots show among species and among sample-type variation in the bacteria-killing abilities (untransformed percentages) of 5 species of birds (BCMM = blue-crowned motmot, *Momotus momota*; BGTA = blue-gray tanager, *Thraupis episcopus*; CBTA = crimson-backed tanager, *Ramphocelus dimidiatus*; CCRO = clay-colored robin, *Turdus grayi*; and RUGD = ruddy ground-dove, *Columbina talpacoti*). The low end of the range identifies the 25th percentile; the high end, the 75th percentile; and the intermediate point, the median. The numbers in parentheses indicate the sample sizes

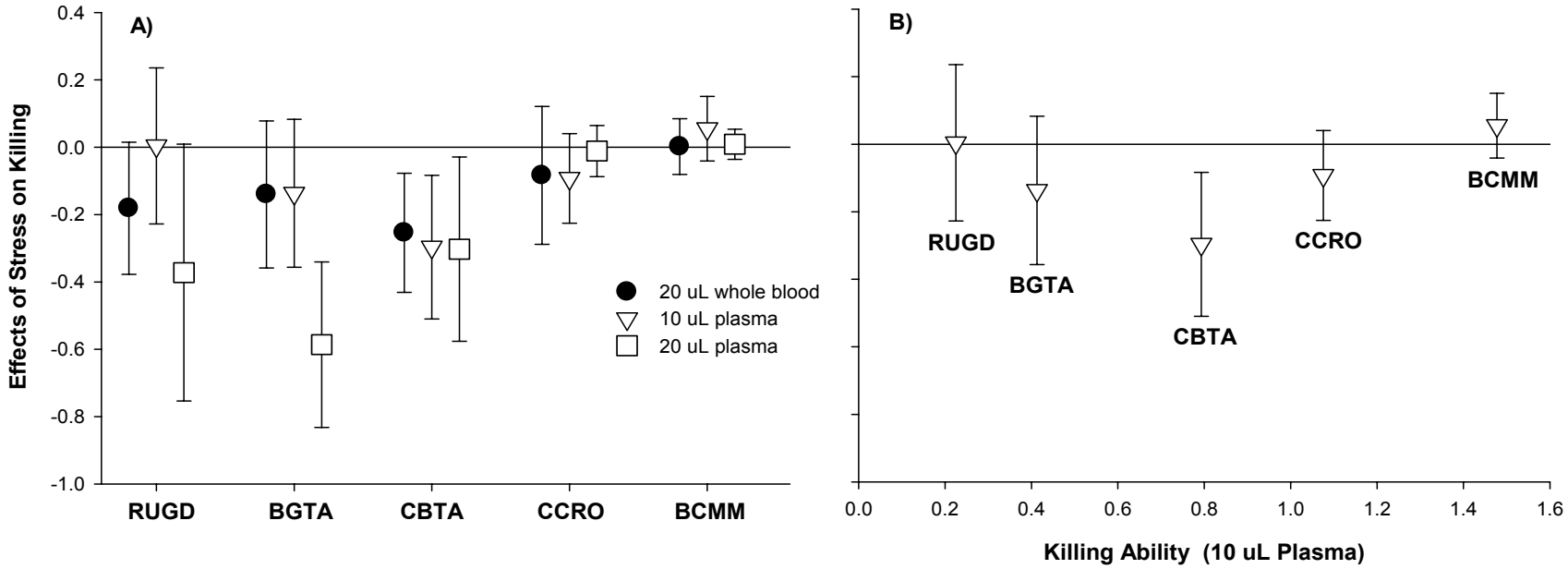

Figure 2

Stress from capture and holding generally results in subtly reduced in vitro bacteria-killing abilities. The magnitude of the observed stress-effects depends on species and sample type (A) and on pre-stress ability to kill bacteria (B). In both (A) and (B), the y-axes represent the differences in the pre- and post-stress bacteria-killing abilities (transformed proportions) and the error bars indicate 95% confidence intervals. Those with error bars that do not cross the horizontal at 0 show significant depressive effects of acute stress on immune function.

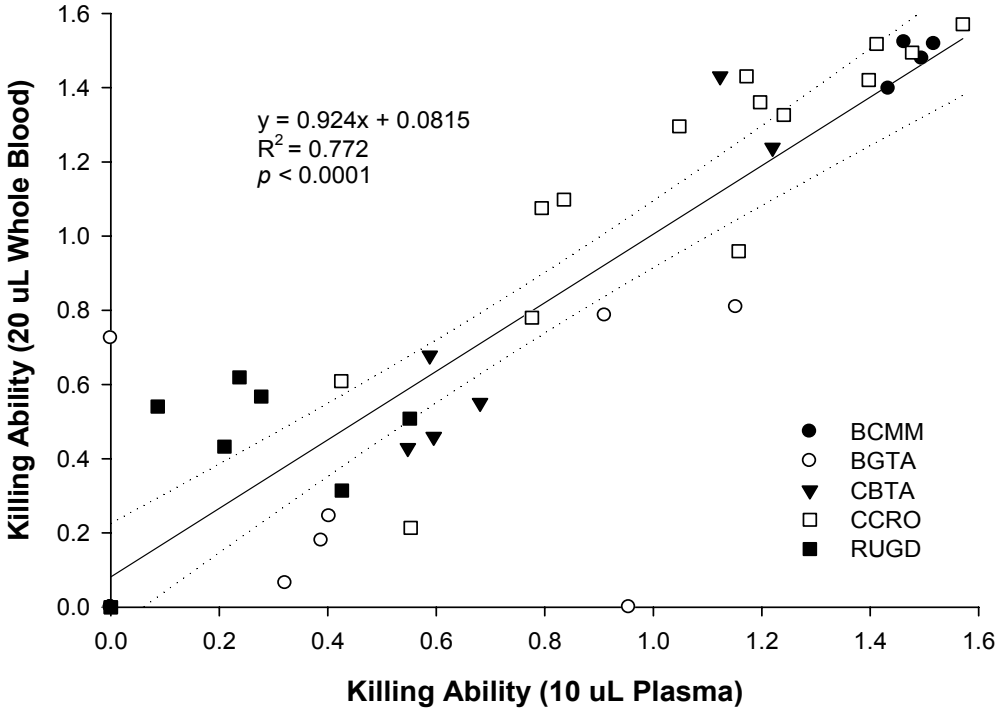

Figure 3

A linear regression with 95% confidence intervals shows a 1:1 relationship between the killing abilities (transformed proportions) of 10 µL plasma and 20 µL whole blood. Symbols represent individual birds of the five species included in the study.

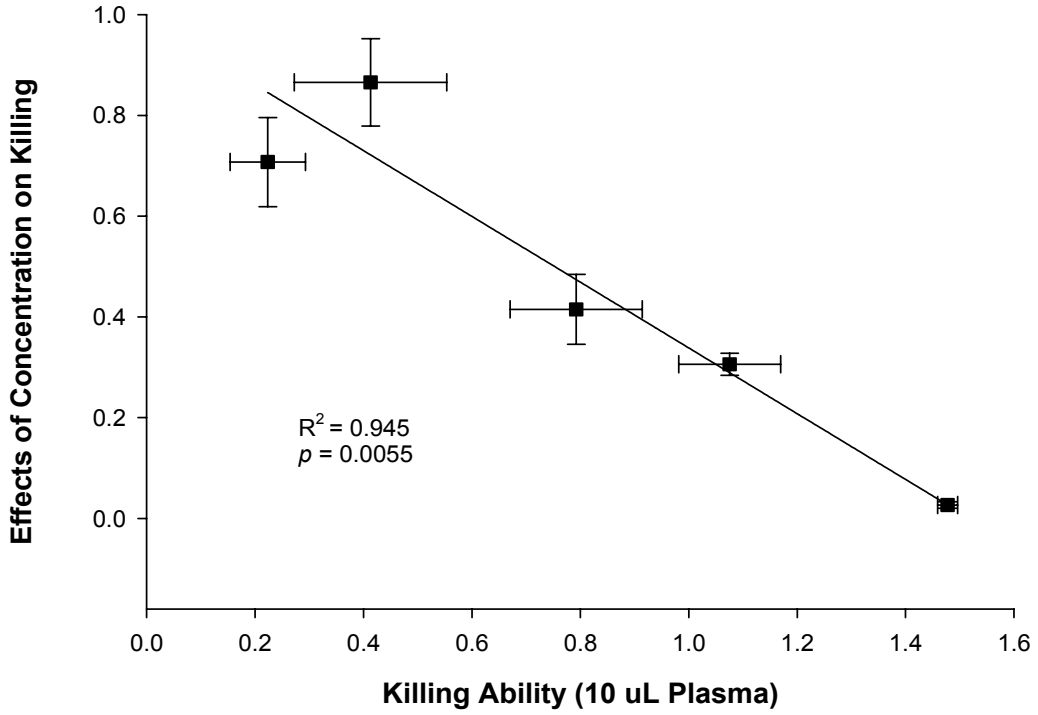

Figure 4

Doubling the amount of plasma used in the bacteria-killing assay to 20 µL results in an increase in killing ability (y-axis, transformed proportions) that is indirectly related to killing ability of 10 µL plasma (x-axis, transformed proportions). This relationship shows that each of the 5 study species has a unique saturation curve, which is defined by the intrinsic capacity of a species' plasma to kill bacteria.

Chapter 3

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

In press: K. D. Matson, A. A. Cohen, K. C. Klasing, R. E. Ricklefs & A. Scheuerlein. *Proceedings of the Royal Society of London - Series B: Biological Sciences.*

Summary: Understanding immune function in the context other life history traits is crucial to understanding 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.

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 defense 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 titers, mitogen-driven lymphocyte proliferation (Leshchinsky & Klasing 2001a), PHA-induced swelling (Stadecker et al. 1977), and bacteria killing (Matson et al. in press; Tieleman et al. 2005).

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) (Goldsby et al. 2000; Power et al. 1998), 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 (Adamo 2004; Keil et al. 2001). 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 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. (Keil et al. 2001; Leshchinsky & Klasing 2001b; Luster et al. 1992)); 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. Materials and Methods

2.1. 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^{o}N, -77.42^{o}W). All birds were at least 6 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 ~135uL 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.

2.2. 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 (Feldman et al. 2000). From these data, concentrations (#/μL) of heterophils, lymphocytes, monocytes, eosinophils, and basophils were estimated (Feldman et al. 2000). All blood smears were evaluated blind to species by a single veterinary diagnostic laboratory technician (AVL Veterinary Clinical Laboratory; St Louis, MO).

2.3. Plasma Sample Analyses

<u>Hemolysis/Hemagglutination Titers</u>

We assessed innate humoral immunity by using a hemolysis-hemagglutination (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: 1) pooled rabbit RBCs (as in (Matson et al. 2005), #RBA050; HemoStat Laboratories; Dixon, CA) and 2) RBCs from a single Rainbow trout (*Oncorhynchus mykiss*; #9999; BioSure; Grass Valley, CA).

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 CO_2-independent media (#18045; Gibco-Invitrogen; Carlsbad, CA), 10 µL plasma, and 20 µL bacteria) were incubated at 41^{o}C for 30 minutes 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^{o}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.

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 level, but concentrations increase with inflammatory responses, which result from infection or

trauma. Hp complexes and removes heme, thereby preventing the heme 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) of Hp in all plasma samples.

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 TEAC (Trolox Equivalent Antioxidant Capacity) 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 analog) and a negative control, which are then used to calculate antioxidant levels in units of mmol/L Trolox equivalents.

2.4. 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

3.1. 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 (df=9, F=5.19, P<0.0001; Table 1). Lymphocyte concentration varied from 2336/μL in CUTD to 10391/μL 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).

3.2. Principal Component Analysis

Individual-level analysis

With the individual-level analysis of plasma variables, we identified 4 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 2A). 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 3 PCs with eigenvalues > 1 that cumulatively account for 79% of the total variation (Table 2B). PC1, the eosinophil/heterophil axis, accounts for 32%; PC2, the monocyte axis, 25%; and PC3, the lymphocyte/basophil axis, 22%.

Species-level analysis

We identified 3 PCs with eigenvalues >1 among plasma immunological assays at the species level (Table 3A), 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 3 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 3 PCs that cumulatively account for 84% of the total variation (Table 3B). 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.

3.3. 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 1A). 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 1B).

4. Discussion

4.1. 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 defense against novel pathogens. Lymphocytes mediate the adaptive-antibody and the cell-mediated responses, which are pathogen specific but have little value in the early defense against novel pathogens. Agglutination titers of exogenous RBCs are indicative of levels of natural antibodies, which facilitate initial pathogen recognition and initiate adaptive immune responses, while lysis titers 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 heme 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).

4.2. 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.

4.3. 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 (Fig. 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 nonlymphoid 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 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.

4.4. 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, 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.

Acknowledgements

We thank the Lubbock's at Sylvan Heights Waterfowl for providing the samples on which this research was based. We also thank A. Doust and I. Jiménez for providing insight on the statistical analyses and three anonymous reviewers for offering thorough reviews and constructive criticisms. This research was supported in part by the National Science Foundation (IBN-0212587), Saint Louis Zoo (FRC #03-13), American Ornithologists' Union, Saint Louis Audubon Society, Webster Groves Nature Study Society, the International Center for Tropical Ecology, and the University of Missouri. A. Cohen is supported by a predoctoral fellowship from the Howard Hughes Medical Institute.

		NABD (6)		LATE (8)		CHPT (8)		SGPT (8)		ALCG (7)		NENE (8)	
	Variable	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Plasma	Trout: lysis (titers)	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 (titers)	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 (titers)	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 (titers)	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)	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 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)	6595	1901	7154	2476	3310	1163	3122	701	6826	1192	10391	6157
Leukocyte	Heterophil (#/µL)	4139	6051	2467	1189	2943	2407	3927	2055	1195	398	1644	728
Leukocyte	Monocyte (#/µL)	389	188	307	342	86	77	88	145	228	137	144	169
Leukocyte	Eosinophil (#/µL)	111	271	69	128	97	171	0	0	23	60	21	60
Leukocyte	Basophil (#/µL)	0	0	116	230	16	45	0	0	0	0	0	0

		MUSC (5)		WWWD (8)		BBTD (2)		CUTD (1)		Effect of Species (df=9)		
	Variable	Mean	SD	Mean	SD	Mean	SD	Mean	SD	F	*P*	R^2
Plasma	Trout: lysis (titers)	2.4	0.5	2.8	0.8	2.5	0.7	3.0	.	2.69	0.012	0.322
Plasma	Trout: agglut (titers)	4.9	0.2	5.9	1.3	4.5	0.7	8.0	.	5.64	<0.0001	0.499
Plasma	Rabbit: lysis (titers)	4.0	0.7	3.8	0.9	3.5	0.7	5.0	.	3.41	0.0024	0.376
Plasma	Rabbit: agglut (titers)	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)	0.1126	0.0464	0.0849	0.0214	0.0973	0.0610	0.0826	.	2.13	0.044	0.273
Plasma	TEAC (mmol/L 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)	4158	1019	4661	2089	3102	653	2336	.	5.19	<0.0001	0.478
Leukocyte	Heterophil (#/µL)	5756	4215	2526	973	2112	933	704	.	1.64	0.13	0.224
Leukocyte	Monocyte (#/µL)	195	229	133	185	286	31	160	.	1.72	0.11	0.233
Leukocyte	Eosinophil (#/µL)	276	319	92	107	0	0	0	.	1.48	0.18	0.207
Leukocyte	Basophil (#/µL)	36	80	114	123	0	0	0	.	1.58	0.15	0.218

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, *Anas 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*.

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

Table 2: Individual level principal components retained according to the Kaiser criterion for plasma (A) and leukocyte (B) data sets. Underlined 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

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

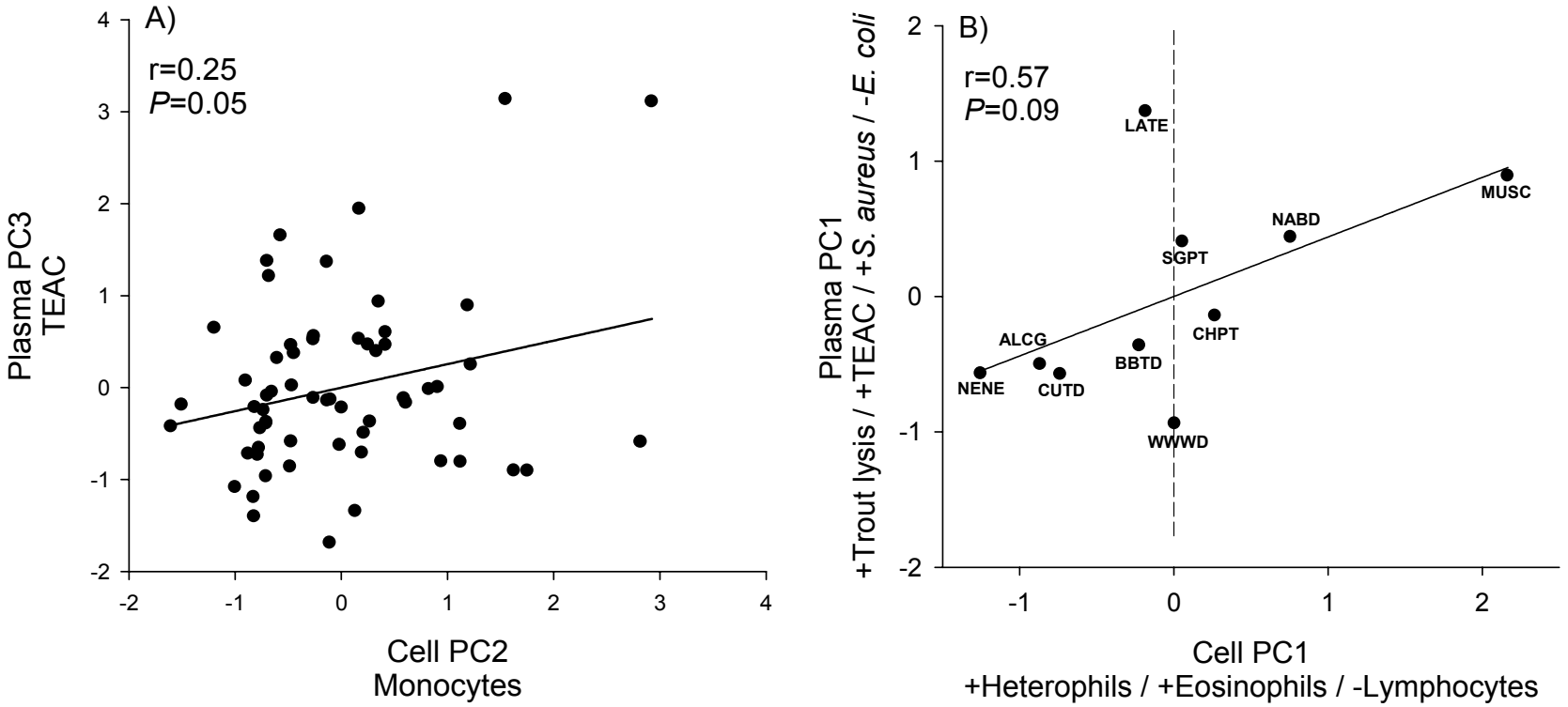

Figure 1: Dominant correlations between plasma and leukocyte PCs at the individual (A) and species (B) level.

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Chapter 4

Immune function in continental and insular birds: is there an "island syndrome"?

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Proceedings of the Royal Society of London - Series B: Biological Sciences.

Summary: If immune defenses were costly, populations from relatively disease-free, oceanic islands would exhibit attenuated immune function in response to reduced pathogen and parasite pressure. Many insular animals exhibit a postulated "island syndrome," which includes increased susceptibility to disease. In some cases, insular populations have declined when they failed to resist infection by introduced pathogens. I measured several indices of immune function in 15 phylogenetically matched pairs of bird populations from North America and from the islands of Hawaii, Bermuda, and the Galápagos. The insular populations included endemics, natives, and recent introductions. I employed three protocols to measure eight indices of immune function: hemolysis, hemagglutination, concentration of haptoglobin, and concentration of five leukocyte types. Immune responses were not attenuated in insular birds, and several indices, including the concentration of plasma haptoglobin, were elevated. Thus, I find no support for the hypothesis that depauperate parasite communities and the inherent costs of immune defenses select for reduced immune function. Instead, I suggest that life on islands leads to an apparent reorganization of immune function, which is defined by increases in defenses that are innate and inducible.

1. Introduction

Biologists widely believe that insular avifaunas are particularly vulnerable to introduced diseases, and numerous examples of increased susceptibility to specific diseases exist in both wild and captive populations of insular animals (Jarvi et al. 2001; Van Riper III & Scott 2001; Wikelski et al. 2004). In the Hawaiian Islands, for example, populations of many native bird species have declined, in some cases to extinction, owing to introductions of two pathogens (avian pox virus, *Avipoxvirus* spp., and the malaria parasite *Plasmodium relictum*) and a vector (southern house mosquito, *Culex quinquefasciatus*) (Jarvi et al. 2001; Van Riper III & Scott 2001). Recent reports from the Galápagos Archipelago documenting the establishment of *C. quinquefasciatus* (Whiteman et al. 2005) and characterizing avian pox there (Thiel et al. 2005), raise concerns that the Galápagos avifauna could meet a similar fate (Wikelski et al. 2004).

The immunological and evolutionary foundations of reduced resistance and increased susceptibility in insular populations are poorly understood and have only recently begun to be investigated (e.g. (Jarvi et al. 2001)). Ostensibly, these changes represent one aspect of an "island syndrome" of reduced interspecific (in this case, host-parasite) interactions in the simplified ecological communities of islands (Blumstein & Daniel 2005; Hochberg & Moller 2001). Attenuated parasite and pathogen pressure on islands might weaken selective pressures that maintain immune system function (Frankham 1997; Jarvi et al. 2001; Van Riper III & Scott 2001), leading to diminished immune function as an evolutionary response to the energetic (Martin et al. 2002), autoimmunological (Råberg et al. 1998), and survival (Hanssen et al. 2005) costs of immune system development, maintenance, and use. Even without these fitness costs, reduced benefits of immunologically-relevant genetic diversity might result in the loss of this genetic diversity through mutation or drift in small insular populations (Frankham 1997).

Some studies suggest that parasite communities on small, isolated islands are depauperate (Wikelski et al. 2004), although the extent of this phenomenon is unclear. Positive taxa-area relationships have been found in microbial communities (Bell et al. 2005; Horner-Devine et al. 2004), and avian blood parasites are reduced on, or even absent from, some islands (Steadman et al. 1990; Super & van Riper III 1995). In any case, on distant islands small populations of potential hosts could suffer immunological consequences of founder effects and inbreeding depression independently of disease threats and immune system costs (Frankham 1997).

The immune system has many components, each with its own inherent costs and protective value. The mix of components likely reflects an array of factors including the nature of the disease threat (e.g. epidemic interval, (Harding et al. 2005)). For instance, frequent and repeated exposure to several common antigens might favor highly specific immunological strategies that maximize pathogen control and minimize collateral damage (Segel & Bar-Or 1999). Conversely, infrequent exposure to a broader range of rarer antigens might favor responses that maximize the speed of the initial response. Thus, the equilibrium between costs and benefits of immune responses should produce varied immunological strategies both within and among species. Indeed, numerous indices of immune function have been shown to vary among individuals of the same species (e.g., with stress: (Råberg et al. 1998)) and among species (Matson et al. In press; Matson et al. 2005; Mendes et al. In press; Tieleman et al. 2005). Moreover, in waterfowl, a multivariate analysis of immune function revealed that the measured indices vary independently and inconsistently among species (Matson et al. In press). Such variation likely reflects some level of environmental optimization via phenotypic plasticity (Ricklefs & Wikelski 2002), but the common garden design in Matson et al. (In press) suggests evolutionary differences due to genotype-environment interactions are seemingly important as well. To date, few comparative studies have investigated

environmental effects on immune responses in free-living vertebrates (cf. (Mendes et al. In press)). Such studies would provide insights into the cost-benefit balance of immune function if individual responses varied consistently in relation to disease threat.

In this study, comparisons were made between 15 phylogenetically matched pairs contrasting birds in North America with close relatives in Hawaii, Bermuda, and the Galápagos Islands. The insular taxa included endemics, natives, and recent introductions. Blood plasma was used in two assays to measure agglutination and lysis of rabbit red blood cells and baseline concentrations of the acute phase protein haptoglobin (Hp). Blood smears were used to estimate concentrations of five types of leukocytes. These indices were selected to probe a variety of protective functions that have a range of costs of use. Agglutination titers are indicative of levels of natural antibodies (NAbs), which facilitate initial pathogen recognition and initiate acquired immune responses. Lysis titers are indicative of complement and other circulating lytic enzymes. Hp offers protection against harmful end products of the immune response, namely heme from damaged host cells and free radicals from phagocytes. Among leukocytes, lymphocytes mediate the acquired antibody and the cell-mediated responses, which are pathogen-specific but of little value in early defense against novel pathogens; the other cell types mediate innate immunity, the primary defense against novel pathogens.

I sought to use these indices to examine how life on distant, oceanic islands has molded the evolution of immune defenses. Assuming that immune defenses incur costs, birds with evolutionary histories on oceanic islands would exhibit reduced immune function if the relative threats of disease-causing microorganisms were reduced. Accordingly, compared to continental populations, insular populations would exhibit reduced hemolysis and hemagglutination titers, lower plasma concentrations of Hp, and depressed circulating concentrations of leukocytes. However, because the relationships

among different measures of immune function are complex and poorly understood (Matson et. al. in press PRSLB), measurable reductions might be limited to indices of functions that are costly or under strong genetic influence, and hence, those that are most responsive to selection or other evolutionary forces, such as mutation and drift.

2. Materials and Methods

2.1. Subjects and Samples

I collected small blood samples from 516 individual birds representing 25 species in 17 genera (Appendix, Table 1). In some cases, samples from multiple populations (e.g. on different islands or from different seasons) were collected within species (Appendix, Table 1).

After using a needle to puncture the brachial vein, blood was drawn into heparinized microcapillary tubes. At collection, I used several drops of blood to make smears for leukocyte enumeration. The remaining blood was centrifuged and the plasma collected. After centrifugation, plasma samples were frozen at -20ºC or below until analysis. All work was approved by the animal care committee at the University of Missouri-Saint Louis (#W01-12).

2.2. Immune Assays

Innate humoral immunity was assessed by using a hemolysis-hemagglutination (HL-HA) assay to characterize 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). Hp, an acute phase protein found in a wide range of taxa including birds (Delers et al. 1988), was quantified (mg/mL) by following the “manual method” instructions provided with a commercially available assay kit (#TP801; Tri-Delta Diagnostics, Inc.; Morris Plains, NJ). A single blood smear from each individual was evaluated blindly to species by a single veterinary diagnostic laboratory technician (AVL

Veterinary Clinical Laboratory; St Louis, MO). From differential counts (percentage of heterophils, lymphocytes, monocytes, eosinophils, and basophils) and estimations of overall leukocyte concentration (Feldman et al. 2000), concentrations of each leukocyte type were estimated. In addition to the original methodological publications, all assays used in the present study have been summarized previously (Matson et al. In press) and extended methodologies are appended.

2.3. Statistical Analyses

I summarized the raw data for each immune variable by calculating means and standard deviations for each population and used both univariate parametric (general linear model, GLM) and non-parametric (Kruskal-Wallis, KW) tests (SPSS v13.0) to investigate the effects of population (samples collected from a species in a specific place and at a specific time) within each genus. All GLMs identifying a significant effect of population were followed with Tukey's post hoc tests to identify homogenous subsets (SPSS v13.0).

Separately within each genus, I calculated means of insular and continental populations. This process resulted in single insular and continental units, eliminated pseudo-replication, and provided a conservative estimation of island-continent differences. Simple means and means weighted by the square root of the sample size (i.e. the number of individuals sampled per population) were generated. These means were used to test by pair-wise comparisons (paired samples T-test and Wilcoxon signed ranks test) the effect of island-status on immune function. Coefficients of variation (CVs) were used to summarize variation among populations and genera; all CVs were corrected for sample size (Sokal & Rohlf 1995).

3. Results

3.1. *Hemagglutination/Hemolysis*

I measured agglutination and lysis titers in all 516 individuals, which belonged to 59 populations (mean = 8.7 indiv/pop, s.d. = 5.8) representing species, island-continent

status, location and time (month/breeding stage) of sample collection, and captivity status of the individuals (Appendix, Tables 1, 2A, and 2B). Comparisons were made within and among genera. No significant effects of season were detected in agglutination or lysis in the four genera that were sampled during different seasons (continental *Cardinalis*, *Dumetella*, *Vireo*, *Zenaida*). Similarly, no effects of captivity were seen in the two genera for which samples were collected from both wild and captive individuals (continental *Cardinalis* and *Dumetella*). Overall, univariate tests identified significant effects of population on agglutination in four genera (*Anas*, *Buteo*, *Columba*, and *Sturnus*, Appendix, Table 2A) and on lysis in seven genera (*Anas*, *Branta*, *Dendroica*, *Passer*, *Sialia*, *Sturnus*, and *Vireo*, Appendix, Table 2B). With the exceptions of *Sturnus* agglutination and *Vireo* lysis, the results of the GLM and KW tests were similar. These effects of population suggested the need to use weighted means when collapsing populations within each genus; however, in all cases weighted and unweighted means were highly correlated (all $r > 0.95$) and did not differ significantly (all $p > 0.5$). Nonetheless, I use weighted means in further analyses.

When all populations and all genera were included, pair-wise tests indicated no significant difference in agglutination (paired samples T-test, $t = 1.4$, $df = 14$, $p = 0.2$; Wilcoxon signed ranks test $p = 0.1$) or lysis (paired samples T-test, $t = -0.4$, $df = 14$, $p = 0.7$; Wilcoxon signed ranks test $p = 0.8$) between insular and continental forms (Table 1). Limiting the assessment to comparisons between *in situ* native continental populations and *in situ* native or endemic insular populations did not change this result for agglutination (paired samples T-test, $t = 0.6$, $df = 8$, $p = 0.6$; Wilcoxon signed ranks test $p = 0.5$) or lysis (paired samples T-test, $t = 0.0$, $df = 8$, $p = 1$; Wilcoxon signed ranks test $p = 0.9$).

3.2. Haptoglobin

I measured Hp concentration in 209 individuals, which were divided, in a similar manner as above, into 33 populations (mean = 6.3 indiv/pop, s.d. = 4.8; Appendix, Tables 1 and 3). Within genera among continental populations, effects of captivity and season were examined. No effects of captivity were seen in *Cardinalis*, but captive *Dumetella* had higher plasma Hp concentrations than free-living ones ($p < 0.05$). In Zenaida, no effects of season were observed. Overall, univariate tests identified significant effects of population on Hp concentration in four genera (*Anas*, *Columbina*, Dendroica, and *Dumetella*; Appendix, Table 3). With *Anas* and *Columbina*, the results of the GLM and KW tests were similar; with *Dendroica* and *Dumetella* only the GLM identified significant effects (both $p < 0.04$).

As with the agglutination and lysis variables, I conducted pair-wise analyses using the weighted means. When all populations and all genera were included, pair-wise tests revealed significantly higher plasma Hp concentrations in insular forms (paired samples T-test, t = 5.7, df = 8, $p < 0.0005$; Wilcoxon signed ranks test $p = 0.007$, Table 1). Limiting this analysis to comparisons between *in situ* native continental populations and *in situ* native or endemic insular populations did not change this result (paired samples T-test, t = 7.2, df = 4, $p = 0.002$; Wilcoxon signed ranks test $p = 0.04$).

3.3. Leukocyte Concentrations

Concentrations of five leukocyte types were estimated from 107 blood smears from individuals in three genera, which were subdivided into 14 populations (mean = 7.6 indiv/pop, s.d. = 6.5, Appendix, Tables 1 and 4). About 40% of the smears (n = 44) were reported to have some smudged cells. Smudged cells can affect the estimation of leukocyte concentrations; however, because the presence of smudge cells did not significantly affect overall leukocyte concentration (*Cardinalis* , $F(1, 35) = 0.7$, $p = 0.42$; *Dumetella*, $F(1, 33) = 2.2$, $p = 0.15$; *Zenaida*, $F(1, 14) = 1.8$, $p = 0.21$) or create a significant interaction between smudge status and population (*Cardinalis* , $F(4, 35) = 0.2$,

$p = 0.96$; *Dumetella*, $F(2, 33) = 2.4$, $p = 0.11$; *Zenaida*, $F(2, 14) = 0.9$, $p = 0.42$), data from all smears were included in the analysis.

No significant effects of population were detected for any of the five types of leukocytes in *Zenaida* doves. In *Cardinalis*, a significant effect of population was found in heterophil concentration using both GLM and KW (both $p < 0.02$). A Tukey's post-hoc test revealed that this effect was driven by island-continent status rather than captivity status or seasonal differences. Among *Dumetella* populations, significant effects of population appeared in concentrations of heterophils, lymphocytes, and monocytes, with GLM and KW tests agreeing in the cases of lymphocytes and monocytes (all $p < 0.04$). With heterophils and monocytes, the highest concentrations were in the Bermuda and captive St. Louis populations; with lymphocytes, the highest concentration was in the wild, autumn St. Louis population.

As in the case of the other measures, weighted and unweighted means of cellular concentrations were similar for insular and continental populations within each genus, but significant effects of population necessitated the use of weighted means when pooling samples for the overall island-continent comparisons, which revealed no significant pattern in any of the five leukocyte types (Table 2). Despite the lack of significance, consistently across all three genera of birds surveyed for leukocytes, insular populations had higher circulating concentrations of heterophils (by an average of 50%) and eosinophils (by an average of 114%) than continental populations. On average, monocyte and basophil concentrations were also higher in insular populations, but elevatations were only observed in *Cardinalis* and *Dumetella*. Overall lymphocyte concentration averaged 5% lower in insular populations, with only *Cardinalis* exhibiting a higher concentration.

4. Discussion

The hypothesis that immune function might be attenuated in insular faunas is rooted in the ideas that islands have impoverished parasite communities and that immune functions incur physiological costs. The results of this study do not point to any overall attenuation in immune responses associated with island life. Instead, the results identify significantly higher concentrations of plasma Hp and suggest elevations in two leukocyte types in insular birds. In some cases, the absence of consistent differences between islands and continents reveal more about the indices themselves than about broader patterns associated with island life. Nonetheless, the observed patterns provide novel perspectives on, and raise new questions about, the evolutionary lability of immune function.

4.1. Evidence of Reduced Genetic Variability?

NAbs react with various affinities to a wide variety of epitopes on bacteria, viruses, and toxins (Ochsenbein & Zinkernagel 2000). Evolutionarily, NAbs are encoded directly by the germ line genome (Ochsenbein & Zinkernagel 2000) and respond to selection (Parmentier et al. 2004). Developmentally, the presence of NAbs does not require previous antigenic exposure and they have been described in naïve (antigen- and germ-free) animals. An important role of these molecules is early resistance against infection (Ochsenbein & Zinkernagel 2000). (In contrast, acquired antibodies are highly specific, require antigenic stimulation, and depend on somatic gene rearrangement.) Hemagglutination and hemolysis revealed no overall differences between insular and continental birds. The absence of a reduction in these indices in insular birds suggests that the broad benefits of NAbs and lytic enzymes, regardless of parasite environment, outweigh the costs of maintenance. This absence could also suggest no overarching differences exist between the parasite communities of islands and continents.

Hemagglutination and hemolysis titers appear to be relatively stable within species, regardless of short-term health status (Matson et al. 2005). The titers,

however, are far from invariant—differing significantly among bird species (Matson et al. In press; Matson et al. 2005) and in some cases significantly (e.g. up to 2.4 $\log_2$ units in *Columba*, see Appendix, Table 2A) within individual island-continent pairs. Compared to continental titers, insular agglutination titers were higher in some comparisons and lower in others. The lack of a consistent result suggests that within-pair differences result from population-specific genetic differences (e.g., lack of diversity from founder effects, inbreeding, or drift in insular populations; (Frankham 1997)) rather than weakened natural selection, physiological costs of immune function, or simplified insular pathogen communities. Hemagglutination titers have been shown to differ significantly, but not predictably, among populations of a naturally inbred species of bird exhibiting different levels of heterozygosity (Whiteman et al. In press). Additionally, on average, more-inbred populations show less within-population variability in agglutination than less-inbred populations (Whiteman et al. In press). Similarly, on average and compared to continental populations, insular populations show lower within-population CVs for both agglutination (island = 34%; continent = 43%) and lysis (island = 68%; continent = 86%).

4.2. A shift in the balance of immune function?

Interactions between the innate and acquired branches of the immune system can affect the evolution of immune function. Models of the evolution of interacting immune responses suggest acquired immunity (i.e. specific antibodies to a disease) can reduce selection pressure on the evolution of innate resistance traits (Harding et al. 2005). Consequently, where the loss of genetic variability impairs one or more components of immunity, a shift in the functional balance could result in greater reliance on other components.

In the case of Hp, the island-continent analysis showed significantly higher levels in insular populations. Hp works to complex and remove heme, thereby preventing the heme from serving as a nutrient for pathogens and from initiating deleterious oxidation

reactions (Dobryszycka 1997). Hp normally circulates at low levels, but concentrations increase during inflammatory responses, which result from infection or trauma. Increased Hp means that insular populations have 1) higher baseline levels, 2) higher response levels, or 3) larger proportions of individuals responding to challenges at any one time. Although all three options suggest a more intense or more prominent acute phase response (APR) in insular populations, with non-repeated measurements the cause cannot be distinguished definitively. However, because the first and third causes both result in decreased variation, the slightly higher mean within-population CV of Hp in islands (island = 49%; continent = 45%) suggests that higher response levels are, at least in part, the cause.

Given the energetic costs of APRs (e.g. anorexia and hyperthermia, but also increased resting metabolic rates, cf. (Martin et al. 2002)) and the direct relationship between energetic and fitness costs (Deerenberg & Overkamp 1999), an intensification would be unusual, but elevated Hp could signify an impairment of other components and an associated shift in immune defense strategy. Indeed, Hp appears to have a modulatory role in the T-helper-1 (generally cell-mediated immunity) and T-helper-2 (generally humoral immunity) balance (Arredouani et al. 2003). With this hypothesized shift, tradeoffs between increases in one component and decreases in another are expected; however, island-continent differences in Hp and differences in agglutination or lysis were not correlated (both $p > 0.3$). Comparing additional measures, such as cytotoxic lymphocyte responses and antibody or MHC diversity, between insular and continental populations might reveal such tradeoffs.

The island-continent analysis also revealed that concentrations of heterophils and eosinophils were consistently elevated in insular birds. Both leukocyte types are involved in innate immunity. Heterophils are phagocytes that are important in early control of bacterial infection; eosinophils, though poorly undertood, are thought to be

involved with both parasitic infection and allergic reaction. Conversely, concentrations of lymphocytes—leukocytes mainly involved with acquired humoral immunity— were elevated on islands in only one of the three genera (*Cardinalis*) and were reduced on average in insular populations. While no significant island-continent patterns were identified, a statistically more powerful design incorporating additional comparisons should be pursued.

As with Hp, the causes of leukocyte elevation are unknown, but these increases similarly suggest a shift in immune defense strategy, seemingly favoring innate as opposed to acquired responses. Innate responses might dominate if systems of acquired humoral immunity and immunological memory are less important (e.g. due to epidemiological properties of islands) or dysfunctional. Insular populations might be compensating for some aspects of reduced genetic diversity or immune system quality through the upregulation of these innate non-lymphoid cells. Alternatively, elevated non-lymphoid cell (and Hp) concentrations raise the possibility that islands have intensified, rather than reduced, disease risks.

4.3. Islands: different from continents?

With evidence of an apparent shift in the mix of immune function towards components that are innate and inducible, this study of phylogenetically matched pairs of bird populations from North America and from oceanic islands documents systematic differences in immune function. The significantly higher concentrations of plasma Hp and the elevated levels of heterophils and eosinophils in insular populations, however, provide no obvious support for the notion that islands have impoverished parasite communities, an idea that is fundamental to the hypothesis of immune function attenuation. The precise causes of the observed immunological shift are unknown and require further investigation. Moreover, a number of other wide-ranging questions remain unanswered as well. For example, do generalizable differences in disease

susceptibility between continental and insular faunas exist and can these differences be measured? Or is each insular population uniquely defended against disease threats as a result of genetic and stochastic processes related to small population sizes and limited geographic ranges?

The extent to which insular taxa, as a whole, exhibit increased disease susceptibility compared to continental taxa is uncertain. Sustaining longer lasting and more lethal infections, various native Hawaiian birds are particularly susceptible to malaria (Jarvi et al. 2001; Van Riper III & Scott 2001). However, at least one Hawaiian native species—the thrush (*Myadestes obscurus*)—is able to produce antibodies against and survive *Plasmodium* infection (Atkinson et al. 2001). Conversely, continental taxa can also suffer the effects of introduced or newly emergent diseases. For example, following the 1999 arrival of West Nile Virus to North America, some corvid and owl populations declined as the result of their high susceptibility, the causes of which are poorly understood (Gancz et al. 2004). Of 30 emergent infectious diseases affecting wildlife, all but one (avian malaria in Hawaii) primarily affect continental areas, and the three diseases found in continental birds are associated with high mortality (Daszak et al. 2000).

4.4. Future Directions

Methodologically, the protocols used in this analysis have clear advantages over other commonly employed immune indices. In particular, nonspecific cellular response to phytohemagglutinin (e.g. (Martin et al. 2002)) or specific antibody response to vaccination (e.g. (Hasselquist et al. 1999)) require repeated capture or holding of birds over periods ranging from one to 30+ days, raising the possibility of confounding effects from stress responses and other physiological consequences of captivity. The measurements used here require only a single blood sample collected upon capture. While this approach is ideal for comparative immunological studies where large sample

sizes are needed, measurements made before and after immunological challenges are required to completely characterize immune systems and, accordingly, to identify any changes in immune functions associated with island life, particularly if these changes involve the innate/acquired balance.

The specificity of acquired humoral responses to vaccination complicates broad characterization of island-continent differences. Circumventing this specificity by instead characterizing the acute phase response through measurement of changes in acute phase protein concentrations, basal metabolic rates (BMR), and behavior represents one alternative. Macromolecular or particulate antigens with abundant epitopes (e.g. whole killed bacteria) can trigger acute phase responses, which are energetically costly (Martin et al. 2002), ensuring significant impacts on fitness. Measuring the in vitro ability of blood to kill a range of microorganisms is another alternative, given this index's broad relevancy to innate immunity, simple interpretation, and known associations with metabolism (Tieleman et al. 2005).

In addition to comparative immunology, a broad understanding of parasite-driven evolution of immune function will also require investigations in population genetics and immunogenetics. A better characterization of communities of disease-causing organisms, including the diversity and abundance of unicellular and multicellular pathogens and parasites, among different environments is also essential.

Acknowledgements

I thank R. Ricklefs, K. Klasing, and B. Addison for providing feedback on this manuscript and A. Scheuerlein for assisting with haptoglobin quantification. For their field assistance I thank, K. Lee (Hawaii), J. Beadell and S. Young (Bermuda), and the UMSL/St Louis Zoo Galápagos bird health survey team, in particular J. Bollmer. I also thank L. Spinney, M. Eicholtz, E. Wheeler, A. Cohen, and T. Ryder for assistance

collecting samples from continental birds and Sylvan Heights Waterfowl, St. Louis Bird Rehab, Three Rings Ranch, and Conservation Research Foundation for providing samples. This research was supported by the National Science Foundation (IBN-0212587), St. Louis Zoo (FRC #03-13), American Ornithologists' Union, St. Louis Audubon Society, Webster Groves Nature Study Society, International Center for Tropical Ecology, and University of Missouri.

	Agglutination (titers)		Lysis (titers)		[Haptoglobin] (mg/mL)	
Genus	Isl	Cont	Isl	Cont	Isl	Cont
Anas	**8.1**	7.7	4.4	**4.8**	**0.23**	0.15
Branta[3]	**7.5**	7.4	4.2	**4.9**	**.**	**.**
Buteo[2]	**8.7**	7.6	**0.3**	0.1	**.**	**.**
Cardinalis	6.4	**6.5**	**0.8**	0.3	**0.23**	0.19
Carpodacus/Hemignathus[3]	**3.4**	3.3	0.0	0.0	**0.12**	0.07
Columba	**6.5**	4.1	0.0	0.0	**.**	**.**
Columbina	**4.9**	4.1	0.0	0.0	**0.13**	0.04
Dendroica[2]	**4.3**	3.7	0.0	**0.6**	**0.17**	0.11
Dumetella[1]	**4.6**	4.4	**3.3**	3.1	**0.45**	0.42
Mimus/Nesomimus[2]	**5.0**	4.7	**1.6**	1.0	**.**	**.**
Passer	**6.4**	5.7	**0.7**	0.0	**0.09**	0.08
Sialia[1]	3.4	**4.2**	0.0	**1.2**	**0.16**	0.12
Sturnus	3.9	**5.1**	0.4	**3.1**	**.**	**.**
Vireo[2]	5.7	**5.8**	**2.5**	0.6	**.**	**.**
Zenaida[3]	4.8	4.8	0.0	0.0	**0.12**	0.079
Mean	5.6	5.3	1.2	1.3	0.19	0.14
SD	1.7	1.5	1.6	1.8	0.11	0.11
t	1.4		-0.4		5.7	
df	14		14		8	
p	0.2		0.7		<0.0005	

[1] Native to islands; included in analysis limited to in situ island natives/endemics.

[2] Endemic to islands; included in analysis limited to in situ island natives/endemics.

[3] Some populations endemic to islands; included in analysis limited to in situ island natives/endemics the weighted means of only those populations: Agglutination, *Branta* = 7.3, *Hemignathus* = 4.8, *Zenaida* = 3.6; Lysis, *Branta* = 3.9; Haptoglobin, *Hemignathus* = 0.12, *Zenaida* = 0.12.

Table 1: Paired t-tests of the effect of island-continent status on three indices of immune function: agglutination titer, lysis titer, and haptoglobin concentration. Mean values for each location within each genus are weighted by the square root of the sampled population sizes.

	[Heterophil] (no.*10^3/uL)		[Lymphocyte] (no.*10^3/uL)		[Monocyte] (no.*10^3/uL)		[Eosinophil] (no.*10^3/uL)		[Basophil] (no.*10^3/uL)		[Total Leukocyte] (no.*10^3/uL)	
Genus	Isl	Cont	Isl	Cont	Isl	Cont	Isl	Cont	Isl	Cont	Isl	Cont
Cardinalis	**3.10**	0.95	**4.70**	3.64	**0.55**	0.27	**0.037**	0.027	**0.005**	0.000	**8.39**	4.92
Dumetella	**2.48**	1.93	3.32	**4.25**	**0.52**	0.29	**0.125**	0.083	**0.034**	0.006	6.49	**6.55**
Zenaida	**2.63**	2.59	4.16	**4.97**	0.27	**0.30**	**0.073**	0.000	0.000	0.000	7.12	**7.85**
Mean	2.74	1.82	4.06	4.29	0.45	0.29	0.078	0.037	0.013	0.002	7.33	6.44
SD	0.32	0.83	0.70	0.67	0.15	0.02	0.044	0.042	0.018	0.003	0.97	1.47
t	1.4		-0.4		1.7		2.3		1.3		0.7	
df	2		2		2		2		2		2	
p	0.3		0.8		0.2		0.1		0.3		0.6	

Table 2: Paired t-tests of the effect of island-continent status on heterophil, lymphocyte, monocyte, eosinophil, basophil, and total leukocyte concentration. Mean values for each location within each genus are weighted by the square root of the sampled population sizes.

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Expanded Materials and Methods

Hemagglutination/Hemolysis Analysis

I assessed innate humoral immunity by using a hemolysis-hemagglutination (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). This HL-HA assay requires a 50-µL sample of blood plasma per individual, which is serially diluted along the long axis of a 96-well microtiter plate. Diluted plasma samples are incubated with exogenous RBCs. Assays were randomized and run blindly with respect to sample; digitized images produced by scanning plates at assay completion were randomized with respect to plate, plate location, and sample, and were scored blindly for both maximum lytic activity and agglutination. 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.

To the protocol described by Matson et al. (2005), I made two minor modifications. First, I used plates processed for improved hydrophilic qualities (Corning Costar #3798, instead of #3795). Second, for all steps requiring phosphate buffered saline (PBS), I used Dulbecco's PBS (#D8662; Sigma; St Louis, MO). Because this formulation includes $MgCl_2*6H_2O$ and $CaCl_2*2H_2O$ in addition to the basic ingredients, its use counteracts any effects of plasma serial dilution on endogenous divalent cation concentration. The sources of the exongenous RBCs were farm-raised rabbits; whole blood was collected on heparin as a byproduct for purposes of biological research (#RBA050; HemoStat Laboratories; Dixon, CA).

Haptoglobin Quantification

Haptoglobin (Hp) is an acute phase protein found in a wide range of taxa including birds (Delers et al. 1988). I 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) of Hp in all plasma samples. This colorimetric assay is based on the binding properties of Hp and haemoglobin and the peroxidase activity of free haemoglobin. I performed the assay at room temperature in 96-well microtiter plates using 7.5 µL of plasma. Absorbance was recorded at 630 nm five minutes after reaction initiation using a microplate reader (VERSAmax; Molecular Devices; Sunnyvale, CA). I serially diluted the calibrator (provided at a known concentration of 2.0 mg/mL) with diluent to generate a standard curve for use in calculating concentrations from absorbance values. A positive control (pooled plasma samples from house sparrows, *Passer domesticus*) was run in duplicate in every plate, and Hp concentrations were standardized among plates based on the mean with-plate positive control value.

Blood Smear Evaluation

A single blood smear from each individual was evaluated by conducting differential counts and estimating the overall leukocyte concentration (Feldman et al. 2000). Differentials were determined by counting individual cell types until a cumulative total of 100 leukocytes was reached. Total leukocyte concentrations were estimated by averaging the number of leukocytes in ten microscope fields at high power and multiplying this mean value by 2000 to approximate the number per uL (Feldman et al. 2000). From these data, concentrations (#/µL) of heterophils, lymphocytes, monocytes, eosinophils, and basophils were estimated (Feldman et al. 2000). All blood smears were evaluated blind to species by a single veterinary diagnostic laboratory technician (AVL Veterinary Clinical Laboratory; St Louis, MO).

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Appendix Tables 1 – 4

Table 1 provides information relevant to the species origins and sample sources for the 59 populations that were sampled for this study, and each population is assigned an ascension number. Referenced by these ascension numbers, population sample sizes, means, standard deviations, standard errors, general linear model and Kruskal-Wallis statistics, Tukey's homogeneous subsets, and insular and continental means and weighted means are included in Tables 2A (agglutination), 2B (lysis), 3 (haptoglobin), and 4 (leukocytes).

Appendix, Table 1

			Origin			Sample Source				
Pop. #	Genus	Species	Isl/Cont	Loc	Native, Intro, Endem	*In/Ex situ*	Loc	Captive/Wild	Sample Mo	Breeding Season
01	***Anas***	***georgica***[1]	Island	South Georgia Island	Endemic (subsp)	Ex	North Carolina	Captive	9	Post-Breed
02	***Anas***	***laysanensis***	Island	Laysan Island	Endemic (sp)	Ex	North Carolina	Captive	9	Post-Breed
03	***Anas***	***georgica***[1]	Cont	South America	Native	Ex	North Carolina	Captive	9	Post-Breed
04	***Anas***	***rubripes***	Cont	North America	Native	Ex	North Carolina	Captive	9	Post-Breed
05	***Branta***	***sandvicensis***	Island	Hawaii (Hawaii)	Native	In	Hawaii	Captive	6	Post-Breed
06	***Branta***	***sandvicensis***	Island	Hawaii	Native	Ex	North Carolina	Captive	9	Post-Breed
07	***Branta***	***hutchinsii***[1]	Island	Aleutian Islands, Alaska	Endemic (subsp)	Ex	North Carolina	Captive	9	Post-Breed
08	***Branta***	***canadensis***	Cont	Illinois	Native	In	Illinois	Wild	6	Post-Breed
09	***Buteo***	***galapagoensis***	Island	Galapagos (Espanola)	Endemic (sp)	In	Espanola	Wild	5-6-7-8	Breed
10	***Buteo***	***galapagoensis***	Island	Galapagos (Fernandina)	Endemic (sp)	In	Fernandina	Wild	5-6-7	Breed
11	***Buteo***	***galapagoensis***	Island	Galapagos (Isabela)	Endemic (sp)	In	Isabela	Wild	5-6-7-8	Breed
12	***Buteo***	***galapagoensis***	Island	Galapagos (Marchena)	Endemic (sp)	In	Marchena	Wild	5-6-7-8	Breed
13	***Buteo***	***galapagoensis***	Island	Galapagos (Pinta)	Endemic (sp)	In	Pinta	Wild	5-6-7	Breed
14	***Buteo***	***galapagoensis***	Island	Galapagos (Santiago)	Endemic (sp)	In	Santiago	Wild	5-6-7	Breed
15	***Buteo***	***galapagoensis***	Island	Galapagos (Santa Fe)	Endemic (sp)	In	Santa Fe	Wild	5-6-7-8	Breed
16	***Buteo***	***solitarius***	Island	Hawaii (Hawaii)	Endemic (sp)	In	Hawaii	Captive (Rehab)	6	Breed
17	***Buteo***	***swainsoni***	Cont	Idaho	Native	In	Idaho	Wild	7-8	Breed
18	***Cardinalis***	***cardinalis***	Island	Bermuda	Introduced	In	Bermuda	Wild	5-6	Breed
19	***Cardinalis***	***cardinalis***	Island	Hawaii (Hawaii)	Introduced	In	Hawaii	Wild	6	Breed
20	***Cardinalis***	***cardinalis***	Island	Hawaii (Oahu)	Introduced	In	Oahu	Wild	6	Breed
21	***Cardinalis***	***cardinalis***	Cont	Missouri	Native	In	Missouri	Wild	2	Winter
22	***Cardinalis***	***cardinalis***	Cont	Missouri	Native	In	Missouri	Wild	4-5	Breed
23	***Cardinalis***	***cardinalis***	Cont	Missouri	Native	In	Missouri	Wild	8-9-10	Post-Breed
24	***Cardinalis***	***cardinalis***	Cont	Missouri	Native	In	Missouri	Captive (Rehab)	8-9-10	Post-Breed
25	***Columba***	***livia***	Island	Bermuda	Introduced	In	Bermuda	Wild	5-6	Breed
26	***Columba***	***livia***	Island	Galapagos (Isabela)	Introduced	In	Isabela	Wild	7	Breed
27	***Columba***	***livia***	Cont	Missouri	Introduced	In	Missouri	Captive (Rehab)	9	Post-Breed
28	***Columbina***	***passerina***	Island	Bermuda	Non-Native	In	Bermuda	Wild	5-6	Breed
29	***Columbina***	***Talpacoti***	Cont	Panama	Native	In	Panama	Wild	3-4	Breed

30	***Dendroica***	***petechia***[1]	Island	Galapagos (Isabela)	Endemic (subsp)	In	Isabela	Wild	7	Post-Breed
31	***Dendroica***	***petechia***[1]	Island	Galapagos (Santa Cruz)	Endemic (subsp)	In	Santa Cruz	Wild	7	Post-Breed
32	***Dendroica***	***petechia***	Cont	Michigan	Native	In	Michigan	Wild	7	Breed
33	***Dumetella***	***carolinensis***	Island	Bermuda	Native	In	Bermuda	Wild	5-6	Breed
34	***Dumetella***	***carolinensis***	Cont	Missouri	Native	In	Missouri	Wild	4-5	Breed
35	***Dumetella***	***carolinensis***	Cont	Missouri	Native	In	Missouri	Wild	8-9	Post-Breed
36	***Dumetella***	***carolinensis***	Cont	Missouri	Native	In	Missouri	Captive (Rehab)	8-9	Post-Breed
37	***Hemignathus***	***virens***	Island	Hawaii (Hawaii)	Endemic (gen)	In	Hawaii	Wild	6	Breed
38	***Carpodacus***	***mexicanus***	Island	Hawaii (Hawaii)	Introduced	In	Hawaii	Wild	6	Breed
39	***Carpodacus***	***mexicanus***	Island	Hawaii (Oahu)	Introduced	In	Oahu	Wild	6	Breed
40	***Carpodacus***	***mexicanus***	Cont	New Jersey	Native	In	New Jersey	Wild	3	Pre-Breed
41	***Nesomimus***	***parvulus***	Island	Galapagos (Genovesa)	Endemic (gen)	In	Galapagos	Wild	7	Post-Breed
42	***Mimus***	***polyglottos***	Cont	Missouri	Native	In	Missouri	Captive (Rehab)	9	Post-Breed
43	***Passer***	***domesticus***	Island	Bermuda	Introduced	In	Bermuda	Wild	5-6	Breed
44	***Passer***	***domesticus***	Island	Hawaii (Hawaii)	Introduced	In	Hawaii	Wild	6	Breed
45	***Passer***	***domesticus***	Island	Hawaii (Oahu)	Introduced	In	Oahu	Wild	6	Breed
46	***Passer***	***domesticus***	Cont	Missouri	Introduced	In	Missouri	Wild	5-6-7	Breed
47	***Sialia***	***sialis***	Island	Bermuda	Native	In	Bermuda	Wild	5-6	Breed
48	***Sialia***	***sialis***	Cont	Illinois	Native	In	Illinois	Wild	5	Breed
49	***Sturnus***	***vulgaris***	Island	Bermuda	Introduced	In	Bermuda	Wild	5-6	Breed
50	***Sturnus***	***vulgaris***	Cont	Missouri	Introduced	In	Missouri	Wild	4-5	Breed
51	***Vireo***	***griseus***[1]	Island	Bermuda	Endemic (subsp)	In	Bermuda	Wild	5-6	Breed
52	***Vireo***	***griseus***	Cont	Missouri	Native	In	Missouri	Wild	4-5	Breed
53	***Vireo***	***griseus***	Cont	Missouri	Native	In	Missouri	Wild	8-9	Post-Breed
54	***Zenaida***	***macroura***	Island	Bermuda	Introduced	In	Bermuda	Wild	5-6	Breed
55	***Zenaida***	***galapagoensis***	Island	Galapagos (Genovesa)	Endemic (sp)	In	Genovesa	Wild	7	Post-Breed
56	***Zenaida***	***galapagoensis***	Island	Galapagos (Santa Cruz)	Endemic (sp)	In	Santa Cruz	Wild	7	Post-Breed
57	***Zenaida***	***macroura***	Cont	Missouri	Native	In	Missouri	Wild	1	Winter
58	***Zenaida***	***macroura***	Cont	Missouri	Native	In	Missouri	Wild	4	Breed
59	***Zenaida***	***macroura***	Cont	Missouri	Native	In	Missouri	Wild	6	Breed

1 Endemic subspecies: #01 A. g. georgica, #03 A. g. spinicauda, #07 B. h. leucopareia, #30 and #31 D. p. aureolla, #51 V. g. bermudianus.

Appendix, Table 2A

AGGLUTINATION
(titers)

Pop. #	Genus	Origin Isl/Cont	N	Mean	SD	SE	df	K-W Chi^2	Sig	ANOVA F	Sig	Tukey	Mean (Indiv)	Weighted Mean	Isl/Cont
01	***Anas***	Island	8	6.1250	0.5175	0.1830						a			
02	***Anas***	Island	8	10.0000	0.8018	0.2835						c	8.1	**8.1**	I
03	***Anas***	Cont	8	7.8125	1.3076	0.4623						b			
04	***Anas***	Cont	6	7.6667	0.9832	0.4014	3	21.028	**0.000**	22.759	**0.000**	b	7.8	**7.7**	C
05	***Branta***	Island	5	7.3000	1.3964	0.6245									
06	***Branta***	Island	8	7.0625	1.2082	0.4272									
07	***Branta***	Island	7	8.2143	0.7559	0.2857							7.5	**7.5**	I
08	***Branta***	Cont	26	7.4038	1.2886	0.2527	3	4.450	0.217	1.213	0.317		7.4	**7.4**	C
09	***Buteo***	Island	3	6.9167	1.0104	0.5833						a			
10	***Buteo***	Island	15	9.3333	1.6165	0.4174						a			
11	***Buteo***	Island	3	9.5833	1.4216	0.8207						a			
12	***Buteo***	Island	5	10.3500	0.8588	0.3841						a			
13	***Buteo***	Island	7	8.6429	1.5736	0.5948						a			
14	***Buteo***	Island	8	9.0625	1.6298	0.5762						a			
15	***Buteo***	Island	5	7.1500	0.7826	0.3500						a			
16	***Buteo***	Island	5	8.1000	2.9026	1.2981						a	8.8	**8.7**	I
17	***Buteo***	Cont	21	7.5952	2.4628	0.5374	8	19.409	**0.013**	2.274	**0.033**	a	7.6	**7.6**	C
18	***Cardinalis***	Island	2	7.7500	0.0000	0.0000									
19	***Cardinalis***	Island	22	6.5000	1.3093	0.2791									
20	***Cardinalis***	Island	5	5.2500	1.7766	0.7945							6.4	**6.4**	I
21	***Cardinalis***	Cont	9	6.8333	0.7395	0.2465									
22	***Cardinalis***	Cont	11	6.3182	1.3652	0.4116									
23	***Cardinalis***	Cont	10	6.5500	0.8563	0.2708									
24	***Cardinalis***	Cont	3	6.0000	1.7321	1.0000	6	9.689	0.138	1.398	0.232		6.5	**6.5**	C
25	***Columba***	Island	8	5.5313	1.8049	0.6381						ab			
26	***Columba***	Island	5	7.8000	0.5701	0.2550						b	6.4	**6.5**	I
27	***Columba***	Cont	7	4.0714	3.2968	1.2461	2	6.032	**0.049**	3.863	**0.041**	a	4.1	**4.1**	C
28	***Columbina***	Island	15	4.8667	2.4014	0.6200							4.9	**4.9**	I
29	***Columbina***	Cont	6	4.1250	2.4622	1.0052	1	0.125	0.724	0.403	0.533		4.1	**4.1**	C

30	***Dendroica***	Island	8	4.0000	0.4629	0.1637								
31	***Dendroica***	Island	8	4.5625	1.5910	0.5625						4.3	**4.3**	I
32	***Dendroica***	Cont	5	3.7000	2.2804	1.0198	2	2.153	0.341	0.574	0.573	3.7	**3.7**	C
33	***Dumetella***	Island	13	4.5769	2.0901	0.5797						4.6	**4.6**	I
34	***Dumetella***	Cont	17	4.4706	1.9880	0.4822								
35	***Dumetella***	Cont	19	4.7368	2.1945	0.5035								
36	***Dumetella***	Cont	2	3.5000	3.5355	2.5000	3	0.812	0.847	0.217	0.884	4.6	**4.4**	C
37	***Hemignathus***	Island	2	4.7500	0.3536	0.2500								
38	***Carpodacus***	Island	16	3.5938	1.1138	0.2785								
39	***Carpodacus***	Island	16	2.8438	1.4913	0.3728						3.3	**3.4**	I
40	***Carpodacus***	Cont	4	3.2500	0.8660	0.4330	3	6.721	0.081	1.862	0.155	3.3	**3.3**	C
41	***Nesomimus***	Island	8	5.0000	2.0000	0.7071						5.0	**5.0**	I
42	***Mimus***	Cont	8	4.6875	1.0670	0.3772	1	0.102	0.750	0.152	0.702	4.7	**4.7**	C
43	***Passer***	Island	26	6.3654	1.3119	0.2573								
44	***Passer***	Island	13	6.5769	1.6627	0.4611								
45	***Passer***	Island	6	6.1250	1.4296	0.5836						6.4	**6.4**	I
46	***Passer***	Cont	12	5.6875	1.7027	0.4915	3	2.486	0.478	0.840	0.478	5.7	**5.7**	C
47	***Sialia***	Island	7	3.3571	1.7491	0.6611						3.4	**3.4**	I
48	***Sialia***	Cont	11	4.1818	1.8203	0.5489	1	0.305	0.581	0.904	0.356	4.2	**4.2**	C
49	***Sturnus***	Island	10	3.8500	1.1797	0.3731						3.9	**3.9**	I
50	***Sturnus***	Cont	7	5.0714	1.5392	0.5818	1	4.113	**0.043**	3.446	0.083	5.1	**5.1**	C
51	***Vireo***	Island	13	5.6538	3.6764	1.0197						5.7	**5.7**	I
52	***Vireo***	Cont	5	4.4000	4.0373	1.8055								
53	***Vireo***	Cont	3	7.6667	5.7735	3.3333	2	1.531	0.465	0.613	0.553	5.6	**5.8**	C
54	***Zenaida***	Island	10	6.0500	2.6609	0.8415								
55	***Zenaida***	Island	5	3.2000	1.6047	0.7176								
56	***Zenaida***	Island	1	4.5000	.	.						5.1	**4.8**	I
57	***Zenaida***	Cont	3	5.6667	2.2546	1.3017								
58	***Zenaida***	Cont	1	3.0000	.	.								
59	***Zenaida***	Cont	6	4.9583	2.0762	0.8476	5	5.705	0.336	1.241	0.327	5.0	**4.8**	C

Appendix, Table 2B

LYSIS (titers)		Origin						K-W		ANOVA			Mean	Weighted	
Pop. #	Genus	Isl/Cont	n	Mean	SD	SE	df	Chi^2	Sig	F	Sig	Tukey	(Indiv)	Mean	Isl/Cont
01	***Anas***	Island	8	4.0000	0.5345	0.1890						a			
02	***Anas***	Island	8	4.7500	0.4629	0.1637						b	4.4	**4.4**	I
03	***Anas***	Cont	8	4.7500	0.4629	0.1637						b			
04	***Anas***	Cont	6	4.7500	0.4183	0.1708	3	9.954	**0.019**	4.868	**0.008**	b	4.8	**4.8**	C
05	***Branta***	Island	5	3.9000	0.7416	0.3317						a			
06	***Branta***	Island	8	4.5000	0.5345	0.1890						ab			
07	***Branta***	Island	7	4.0000	0.5774	0.2182						a	4.2	**4.2**	I
08	***Branta***	Cont	26	4.8846	0.4540	0.0890	3	16.197	**0.001**	8.792	**0.000**	b	4.9	**4.9**	C
09	***Buteo***	Island	3	0.0000	0.0000	0.0000									
10	***Buteo***	Island	15	0.3833	0.8497	0.2194									
11	***Buteo***	Island	3	0.0000	0.0000	0.0000									
12	***Buteo***	Island	5	0.4000	0.8944	0.4000									
13	***Buteo***	Island	7	0.2857	0.7559	0.2857									
14	***Buteo***	Island	8	0.6250	1.4079	0.4978									
15	***Buteo***	Island	5	0.0000	0.0000	0.0000									
16	***Buteo***	Island	5	0.2000	0.4472	0.2000							0.3	**0.3**	I
17	***Buteo***	Cont	21	0.0714	0.3273	0.0714	8	6.202	0.625	0.675	0.712		0.1	**0.1**	C
18	***Cardinalis***	Island	2	0.0000	0.0000	0.0000									
19	***Cardinalis***	Island	22	1.0455	1.4712	0.3137									
20	***Cardinalis***	Island	5	0.9000	1.7464	0.7810							0.9	**0.8**	I
21	***Cardinalis***	Cont	9	0.0000	0.0000	0.0000									
22	***Cardinalis***	Cont	11	0.0000	0.0000	0.0000									
23	***Cardinalis***	Cont	10	0.5000	1.0801	0.3416									
24	***Cardinalis***	Cont	3	1.3333	2.3094	1.3333	6	10.231	0.115	1.679	0.143		0.3	**0.3**	C
25	***Columba***	Island	8	0.0000	0.0000	0.0000									
26	***Columba***	Island	5	0.0000	0.0000	0.0000							0.0	**0.0**	I
27	***Columba***	Cont	7	0.0000	0.0000	0.0000	2	1.000	0.000	.	.		0.0	**0.0**	C
28	***Columbina***	Island	15	0.0000	0.0000	0.0000							0.0	**0.0**	I
29	***Columbina***	Cont	6	0.0000	0.0000	0.0000	1	1.000	0.000	.	.		0.0	**0.0**	C

30	***Dendroica***	Island	8	0.0000	0.0000	0.0000						a			
31	***Dendroica***	Island	8	0.0000	0.0000	0.0000						a	0.0	**0.0**	I
32	***Dendroica***	Cont	5	0.6000	0.8216	0.3674	2	6.737	**0.034**	4.571	**0.025**	b	0.6	**0.6**	C
33	***Dumetella***	Island	13	3.2692	1.1835	0.3282							3.3	**3.3**	I
34	***Dumetella***	Cont	17	2.9412	1.6191	0.3927									
35	***Dumetella***	Cont	19	3.7105	1.1939	0.2739									
36	***Dumetella***	Cont	2	2.0000	2.8284	2.0000	3	3.863	0.277	1.483	0.231		3.3	**3.1**	C
37	***Hemignathus***	Island	2	0.0000	0.0000	0.0000									
38	***Carpodacus***	Island	16	0.0000	0.0000	0.0000									
39	***Carpodacus***	Island	16	0.0000	0.0000	0.0000							0.0	**0.0**	I
40	***Carpodacus***	Cont	4	0.0000	0.0000	0.0000	3	1.000	0.000	.	.		0.0	**0.0**	C
41	***Nesomimus***	Island	8	1.5625	1.4985	0.5298							1.6	**1.6**	I
42	***Mimus***	Cont	8	1.0000	1.3093	0.4629	1	0.974	0.324	0.639	0.437		1.0	**1.0**	C
43	***Passer***	Island	26	0.3654	0.8192	0.1607						a			
44	***Passer***	Island	13	0.6154	1.1022	0.3057						ab			
45	***Passer***	Island	6	1.5833	1.9600	0.8002						b	0.6	**0.7**	I
46	***Passer***	Cont	12	0.0000	0.0000	0.0000	3	7.325	0.062	3.720	**0.017**	a	0.0	**0.0**	C
47	***Sialia***	Island	7	0.0000	0.0000	0.0000						a	0.0	**0.0**	I
48	***Sialia***	Cont	11	1.2273	1.4554	0.4388	1	5.139	**0.023**	4.867	**0.042**	b	1.2	**1.2**	C
49	***Sturnus***	Island	10	0.3500	0.9443	0.2986						a	0.4	**0.4**	I
50	***Sturnus***	Cont	7	3.0714	1.4268	0.5393	1	8.856	**0.003**	22.602	**0.000**	b	3.1	**3.1**	C
51	***Vireo***	Island	13	2.5000	1.9149	0.5311						a	2.5	**2.5**	I
52	***Vireo***	Cont	5	0.1000	0.2236	0.1000						a			
53	***Vireo***	Cont	3	1.1667	2.0207	1.1667	2	4.826	0.090	3.771	**0.043**	a	0.5	**0.6**	C
54	***Zenaida***	Island	10	0.0000	0.0000	0.0000									
55	***Zenaida***	Island	5	0.0000	0.0000	0.0000									
56	***Zenaida***	Island	1	0.0000	.	.							0.0	**0.0**	I
57	***Zenaida***	Cont	3	0.0000	0.0000	0.0000									
58	***Zenaida***	Cont	1	0.0000	.	.									
59	***Zenaida***	Cont	6	0.0000	0.0000	0.0000	5	1.000	0.000	.	.		0.0	**0.0**	C

Appendix, Table 3

[HAPTOGLOBIN]
(mg/mL)

Pop. #	Genus	Origin Isl/Cont	N	Mean	SD	SE	df	K-W Chi^2	ANOVA Sig	ANOVA F	ANOVA Sig	Tukey	Mean (Indiv)	Weighted Mean	Isl/Cont
01	***Anas***	Island	8	0.1838	0.0635	0.0224						ab			
02	***Anas***	Island	8	0.2838	0.1373	0.0485						b	0.234	**0.234**	I
03	***Anas***	Cont	8	0.1725	0.0602	0.0213						ab			
04	***Anas***	Cont	6	0.1300	0.0283	0.0115	3	8.330	**0.040**	4.268	**0.014**	a	0.154	**0.153**	C
18	***Cardinalis***	Island	2	0.1800	0.0141	0.0100									
19	***Cardinalis***	Island	22	0.2659	0.2462	0.0525									
20	***Cardinalis***	Island	5	0.2000	0.0985	0.0440							0.249	**0.234**	I
23	***Cardinalis***	Cont	11	0.1582	0.0502	0.0151									
24	***Cardinalis***	Cont	3	0.2600	0.1997	0.1153	4	4.136	0.388	0.649	0.631		0.180	**0.193**	C
28	***Columbina***	Island	7	0.1286	0.0219	0.0083						b	0.129	**0.129**	I
29	***Columbina***	Cont	6	0.0383	0.0147	0.0060	1	9.176	**0.002**	72.911	**0.000**	a	0.038	**0.038**	C
30	***Dendroica***	Island	4	0.2425	0.1053	0.0527						b			
31	***Dendroica***	Island	4	0.1050	0.0624	0.0312						a	0.174	**0.174**	I
32	***Dendroica***	Cont	6	0.1100	0.0569	0.0232	2	5.253	0.072	4.652	**0.034**	ab	0.110	**0.110**	C
33	***Dumetella***	Island	13	0.4454	0.1583	0.0439						ab	0.445	**0.445**	I
35	***Dumetella***	Cont	20	0.3390	0.1478	0.0838						a			
36	***Dumetella***	Cont	2	0.6650	0.3748	0.2650	2	5.352	0.069	4.511	**0.019**	b	0.369	**0.417**	C
37	***Hemignathus***	Island	2	0.1200	0.0424	0.0300									
38	***Carpodacus***	Island	4	0.1150	0.0603	0.0301									
39	***Carpodacus***	Island	4	0.1275	0.0386	0.0193							0.121	**0.121**	I
40	***Carpodacus***	Cont	4	0.0725	0.0150	0.0075	3	4.322	0.229	1.314	0.324		0.073	**0.073**	C
43	***Passer***	Island	6	0.0650	0.0451	0.0184									
44	***Passer***	Island	2	0.0700	0.0283	0.0200									
45	***Passer***	Island	4	0.1375	0.0556	0.0278							0.090	**0.091**	I
46	***Passer***	Cont	9	0.0833	0.0472	0.0157	3	4.464	0.216	2.057	0.144		0.083	**0.083**	C

47	***Sialia***	Island	7	0.1643	0.0877	0.0332						0.164	**0.164**	I
48	***Sialia***	Cont	7	0.1229	0.0647	0.0245	1	1.209	0.272	1.011	0.335	0.123	**0.123**	C
54	***Zenaida***	Island	10	0.1220	0.0781	0.0247								
55	***Zenaida***	Island	5	0.1180	0.0726	0.0325								
56	***Zenaida***	Island	1	0.1100	.	.						0.120	**0.119**	I
57	***Zenaida***	Cont	2	0.0477	0.0039	0.0325								
58	***Zenaida***	Cont	1	0.0596	.	.								
59	***Zenaida***	Cont	6	0.1050	0.0704	0.0287	5	3.210	0.668	0.458	0.803	0.087	**0.079**	C

Appendix, Table 4

[HETEROPHIL] (no.*10^3/uL)		Origin							ANOVA			Mean	Weighted	
Pop. #	Genus	Isl/Cont	n	Mean	SD	SE	df	K-W Chi^2	Sig.	F	Sig.	Tukey	(Indiv)	Mean	Isl/Cont
18	***Cardinalis***	Isl	2	7.1300	4.6810	3.3100						b			
19	***Cardinalis***	Isl	22	2.0418	1.8308	0.3903						a			
20	***Cardinalis***	Isl	5	2.7720	2.4757	1.1072						a	2.52	**3.10**	I
22	***Cardinalis***	Cont	3	0.7300	0.2166	0.1250						a			
23	***Cardinalis***	Cont	11	1.1136	0.6449	0.1944						a			
24	***Cardinalis***	Cont	3	0.8633	0.8223	0.4748	5	14.027	**0.015**	4.766	**0.002**	a	1.00	**0.95**	C
33	***Dumetella***	Isl	10	2.4810	1.6264	0.5143						ab	2.48	**2.48**	I
34	***Dumetella***	Cont	8	1.4138	0.6344	0.2243						a			
35	***Dumetella***	Cont	20	1.5600	1.0213	0.2284						a			
36	***Dumetella***	Cont	2	4.1500	3.8467	2.7200	3	3.847	0.278	3.469	**0.026**	b	1.69	**1.93**	C
54	***Zenaida***	Isl	9	3.4589	1.8162	0.6054									
55	***Zenaida***	Isl	5	1.9000	1.1594	0.5185									
56	***Zenaida***	Isl	1	1.7600	.	.							2.83	**2.63**	I
59	***Zenaida***	Cont	6	2.5850	1.4507	0.5923	3	2.976	0.395	1.243	0.325		2.59	**2.59**	C

[LYMPHOCYTE]

Pop. #	Genus	Origin Isl/Cont	n	Mean	SD	SE	df	K-W Chi^2	Sig.	ANOVA F	Sig.	Tukey	Mean (Indiv)	Weighted Mean	Isl/Cont
18	***Cardinalis***	Isl	2	5.405	1.775	1.2550									
19	***Cardinalis***	Isl	22	4.793	4.520	0.9636									
20	***Cardinalis***	Isl	5	4.056	1.940	0.8677							4.71	**4.70**	I
22	***Cardinalis***	Cont	3	2.450	0.611	0.3528									
23	***Cardinalis***	Cont	11	4.334	1.193	0.3598									
24	***Cardinalis***	Cont	3	3.513	0.782	0.4517	5	7.807	0.167	0.342	0.884		3.86	**3.64**	C
33	***Dumetella***	Isl	10	3.3240	1.8735	0.5924						a	3.32	**3.32**	I
34	***Dumetella***	Cont	8	3.1350	1.1650	0.4119						a			
35	***Dumetella***	Cont	20	5.0200	1.8285	0.4089						a			
36	***Dumetella***	Cont	2	4.0400	1.0889	0.7700	3	9.995	**0.019**	3.427	**0.027**	a	4.45	**4.25**	C
54	***Zenaida***	Isl	9	4.6544	1.3958	0.4653									
55	***Zenaida***	Isl	5	4.0800	1.5881	0.7102									
56	***Zenaida***	Isl	1	2.8400	.	.							4.34	**4.16**	I
59	***Zenaida***	Cont	6	4.9717	2.9316	1.1968	3	1.372	0.712	0.427	0.736		4.97	**4.97**	C

[MONOCYTE]

Pop. #	Genus	Origin Isl/Cont	n	Mean	SD	SE	df	K-W Chi^2	Sig.	ANOVA F	Sig.	Tukey	Mean (Indiv)	Weighted Mean	Isl/Cont
18	***Cardinalis***	Isl	2	0.6150	0.4031	0.2850									
19	***Cardinalis***	Isl	22	0.6650	1.0278	0.2191									
20	***Cardinalis***	Isl	5	0.2540	0.0750	0.0336							0.59	**0.55**	I
22	***Cardinalis***	Cont	3	0.0700	0.1212	0.0700									
23	***Cardinalis***	Cont	11	0.3873	0.2576	0.0777									
24	***Cardinalis***	Cont	3	0.2367	0.1721	0.0994	5	8.394	0.136	0.630	0.678		0.30	**0.27**	C
33	***Dumetella***	Isl	10	0.5150	0.4217	0.1334						a	0.52	**0.52**	I
34	***Dumetella***	Cont	8	0.1075	0.1156	0.0409						a			
35	***Dumetella***	Cont	20	0.3480	0.2320	0.0519						a			
36	***Dumetella***	Cont	2	0.4450	0.4455	0.3150	3	8.579	**0.035**	3.130	**0.037**	a	0.29	**0.29**	C
54	***Zenaida***	Isl	9	0.2444	0.3091	0.1030									
55	***Zenaida***	Isl	5	0.3060	0.1585	0.0709									
56	***Zenaida***	Isl	1	0.2500	.	.							0.27	**0.27**	I
59	***Zenaida***	Cont	6	0.2950	0.3190	0.1302	3	0.632	0.889	0.067	0.977		0.30	**0.30**	C

[EOSINOPHIL]
(no.*10^3/uL)

Pop. #	Genus	Origin Isl/Cont	n	Mean	SD	SE	df	K-W Chi^2	Sig.	ANOVA F	Sig.	Tukey	Mean (Indiv)	Weighted Mean	Isl/Cont
18	***Cardinalis***	Isl	2	0.0000	0.0000	0.0000									
19	***Cardinalis***	Isl	22	0.0382	0.0844	0.0180									
20	***Cardinalis***	Isl	5	0.0580	0.1297	0.0580							0.039	**0.037**	I
22	***Cardinalis***	Cont	3	0.0533	0.0924	0.0533									
23	***Cardinalis***	Cont	11	0.0155	0.0513	0.0155									
24	***Cardinalis***	Cont	3	0.0233	0.0404	0.0233	5	1.813	0.874	0.328	0.893		0.024	**0.027**	C
33	***Dumetella***	Isl	10	0.1250	0.1822	0.0576							0.125	**0.125**	I
34	***Dumetella***	Cont	8	0.1725	0.2159	0.0763									
35	***Dumetella***	Cont	20	0.0325	0.0675	0.0151									
36	***Dumetella***	Cont	2	0.0650	0.0919	0.0650	3	3.612	0.307	2.220	0.103		0.072	**0.083**	C
54	***Zenaida***	Isl	9	0.1089	0.2011	0.0670									
55	***Zenaida***	Isl	5	0.0340	0.0760	0.0340									
56	***Zenaida***	Isl	1	0.0500	.	.							0.080	**0.073**	I
59	***Zenaida***	Cont	6	0.0000	0.0000	0.0000	3	4.081	0.253	0.759	0.532		0.000	**0.000**	C

[BASOPHIL]

Pop. #	Genus	Origin Isl/Cont	n	Mean	SD	SE	df	K-W Chi^2	Sig.	ANOVA F	Sig.	Tukey	Mean (Indiv)	Weighted Mean	Isl/Cont
18	***Cardinalis***	Isl	2	0.0000	0.0000	0.0000									
19	***Cardinalis***	Isl	22	0.0087	0.0297	0.0063									
20	***Cardinalis***	Isl	5	0.0000	0.0000	0.0000							0.007	**0.005**	I
22	***Cardinalis***	Cont	3	0.0000	0.0000	0.0000									
23	***Cardinalis***	Cont	11	0.0000	0.0000	0.0000									
24	***Cardinalis***	Cont	3	0.0000	0.0000	0.0000	5	2.230	0.816	0.377	0.862		0.000	**0.000**	C
33	***Dumetella***	Isl	10	0.0340	0.0943	0.0298							0.034	**0.034**	I
34	***Dumetella***	Cont	8	0.0000	0.0000	0.0000									
35	***Dumetella***	Cont	20	0.0120	0.0537	0.0120									
36	***Dumetella***	Cont	2	0.0000	0.0000	0.0000	3	3.156	0.368	0.537	0.660		0.008	**0.006**	C
54	***Zenaida***	Isl	9	0.0000	0.0000	0.0000									
55	***Zenaida***	Isl	5	0.0000	0.0000	0.0000									
56	***Zenaida***	Isl	1	0.0000	.	.							0.000	**0.000**	I
59	***Zenaida***	Cont	6	0.0000	0.0000	0.0000	3	0.000	1.000	.	.		0.000	**0.000**	C

[TOTAL LEUKOCYTE] (no.*10^3/uL)		Origin							ANOVA			Mean	Weighted	
Pop. #	Genus	Isl/Cont	n	Mean	SD	SE	df	K-W Chi^2	Sig.	F	Sig.	Tukey	(Indiv)	Mean	Isl/Cont
18	***Cardinalis***	Isl	2	13.1500	6.8589	4.8500									
19	***Cardinalis***	Isl	22	7.5455	7.2152	1.5383									
20	***Cardinalis***	Isl	5	7.1400	4.2741	1.9114							7.86	**8.39**	I
22	***Cardinalis***	Cont	3	3.4333	0.5132	0.2963									
23	***Cardinalis***	Cont	11	5.8455	1.2817	0.3864									
24	***Cardinalis***	Cont	3	4.6333	1.4640	0.8452	5	10.841	0.055	0.980	0.442		5.21	**4.92**	C
33	***Dumetella***	Isl	10	6.4900	3.2220	1.0189							6.49	**6.49**	I
34	***Dumetella***	Cont	8	4.8250	1.8242	0.6450									
35	***Dumetella***	Cont	20	6.9700	2.5567	0.5717									
36	***Dumetella***	Cont	2	8.7000	3.1113	2.2000	3	5.410	0.144	1.750	0.174		6.51	**6.55**	C
54	***Zenaida***	Isl	9	8.4667	3.0830	1.0277									
55	***Zenaida***	Isl	5	6.3200	2.2073	0.9871									
56	***Zenaida***	Isl	1	4.9000	.	.							7.51	**7.12**	I
59	***Zenaida***	Cont	6	7.8500	4.3029	1.7567	3	2.313	0.510	0.681	0.576		7.85	**7.85**	C

Chapter 5

Disease ecology in the Galápagos Hawk (*Buteo galapagoensis*): host genetic diversity, parasite load and natural antibodies.

In press: N. K. Whiteman, K. D. Matson, J. L. Bollmer & P. G. Parker.

Proceedings of the Royal Society of London - Series B: Biological Sciences.

Summary: An increased susceptibility to disease is one hypothesis explaining how inbreeding hastens extinction in island endemics and threatened species. Experimental studies show that disease resistance declines as inbreeding increases, but data from *in situ* wildlife systems are scarce. Genetic diversity increases with island size across the entire range of an extremely inbred Galápagos endemic bird, providing the context for a natural experiment examining the effects of inbreeding on disease susceptibility. Extremely inbred populations of Galápagos hawks had higher parasite abundances than relatively outbred populations. We found a significant island effect on constitutively produced natural antibody (NAb) levels and inbred populations generally harboured lower average and less variable NAb levels than relatively outbred populations. Furthermore, NAb levels explained abundance of amblyceran lice, which encounter the host immune system. This is the first study linking inbreeding, innate immunity and parasite load in an endemic, *in situ* wildlife population and provides a clear framework for assessment of disease risk in a Galápagos endemic.

1. INTRODUCTION

Extinctions of island endemics account for 75% of animal extinctions and 90% of bird extinctions (Myers 1979; Reid & Miller 1989). Several synergistic key factors may be responsible for this high extinction rate, including introduction of exotic animal and human predators (Blackburn *et al.* 2004), habitat destruction (Rolett & Diamond 2004), demographic stochasticity (Drake 2005), and inbreeding in island endemics and threatened species (Frankham 1998; Spielman *et al.* 2004a).

The interaction of disease agents with genetically depauperate (Pearman & Garner 2005) and isolated populations is one hypothesis explaining how inbreeding facilitates extinction in small populations (de Castro & Bolker 2005). Parasites evolve more quickly than hosts, so host antiparasite adaptations are perpetually obsolete (Hamilton *et al.* 1990; Lively & Apanius 1995). Consequently, genetically uniform host individuals (Acevedo-Whitehouse *et al.* 2003) and populations (Spielman *et al.* 2004b) are more susceptible to parasitism than genetically diverse hosts. Studies of model laboratory systems (Arkush *et al.* 2002), captive wildlife (Cassinello *et al.* 2001), and free-ranging domesticated animal populations (Coltman *et al.* 1999) support this claim, although other studies do not (Trouvé *et al.* 2003). Scant evidence of this phenomenon exists from *in situ* native wildlife populations (Meagher 1999), and no study has examined the effects of inbreeding on parasite load and innate, humoral immunity across bird populations in the wild (Keller & Waller 2002). The intact endemic avifauna of the Galápagos Islands provides a unique opportunity to examine disease ecology and will provide insight into the impact of invasive disease agents that may enter the ecosystem (Lindström *et al.* 2004; Thiel *et al.* 2005).

The Galápagos hawk (*Buteo galapagoensis*), an endemic raptor threatened with extinction (2004 IUCN Red List), breeds on eight islands within the Galápagos National Park, and has been extirpated from several others (Fig. 1). Island size and genetic

diversity are positively related and between-island population structure is high, rendering it an appealing model system in which to examine the effects of inbreeding on disease severity (Bollmer *et al.* 2005). The basic biology of its two chewing louse species (Insecta: Phthiraptera), an amblyceran (*Colpocephalum turbinatum*) and an ischnoceran (*Degeeriella regalis*), has been described (Whiteman & Parker 2004a, b). Thus, we examined the response of each parasite lineage to variance in host inbreeding, using population-level heterozygosity values from the eight island populations of *B. galapagoensis* and one population of the sister species (*B. swainsoni*; Reising *et al.* 2003).

We also examined the relationship between immunological host defences, island-level inbreeding effects, and parasite abundance. To assess immunological host defences, we quantified non-specific natural antibody (NAb) titres within seven populations of *B*. *galapagoensis*. Quantification of NAbs has several conceptual and methodological advantages over other methods used to assess immune response of wild vertebrates (Matson *et al.* 2005). NAbs are a product of the innate, humoral immune system and their production is constitutive (stable over time and generally not induced by external antigenic stimulation). Encoded by the germ-line genome, NAbs are present in antigenically naïve vertebrates (; Ochsenbein & Zinkernagel 2000), form a large percentage of the serum immunoglobulin (Kohler *et al.* 2003), are capable of recognizing any antigen, and prime the adaptive immune response (Adelman *et al.* 2004). In chickens, NAbs reacting to ectoparasite-derived antigens have been identified (Wikel *et al.* 1989) and in lines artificially selected for either high or low levels of specific antibodies, specific and natural antibody levels covary (Parmentier *et al.* 2004). NAb response is hypothesized to predict the strength of the adaptive immune response (Kohler *et al.* 2003). Thus, NAbs form a functional link between the innate and acquired parts of the humoral immune system (Lammers *et al.* 2004).

Inbreeding may negatively impact phytohemagglutinin (PHA) induced swelling within wild bird populations (Reid *et al.* 2003), and reductions in population size reduce overall within-population genetic variation, including variation at loci of immunological import in vertebrates (Miller & Lambert 2004). Since variation in NAb levels responds to artificial selection in chickens (Parmentier *et al.* 2004), it is reasonable to predict that variation in NAb levels will covary with variation in wild bird population genetic diversity. However, the impact of natural microevolutionary processes on circulating levels of NAbs is unknown in wild vertebrates.

Amblyceran lice (e.g., *C. turbinatum*) directly encounter host immune defences because they feed on blood and living skin (Marshall 1981). Conversely, bird ischnocerans (e.g., *D. regalis*) generally feed on the keratin of feathers and dead skin (Marshall 1981) and mainly encounter the mechanical host defences (e.g., preening). Feeding by ectoparasites on skin and blood elicits immune responses (Wikel 1982) that vary from cell-mediated (Prelezov *et al.* 2002) to humoral (i.e., antibodies; Pfeffer *et al.* 1997) and from innate (Wikel *et al.* 1989) to acquired (Ben-yakir *et al.* 1994). Host antibodies reduce louse fecundity and survivorship, and regulate population growth rate (Ben-yakir *et al.* 1994). Across bird species, variation in PHA-induced swelling was directly related to amblyceran but not ischnoceran species richness (Møller & Rózsa 2005). However, whether NAbs regulate ectoparasite populations, and louse populations in particular, is unknown.

We measured host inbreeding, parasite abundance and NAb response, and made three predictions: (1) at the island-level, higher inbreeding results in lower average humoral immune response relative to outbred populations; (2) also at the island-level, higher inbreeding results in reduced variation in humoral immune response relative to outbred populations; and (3) birds with high humoral immune responses harbour fewer parasites (amblyceran lice) relative to birds with lower immune responses.

2. METHODS

(a) *Host sampling*

We live-captured a total of 211 *Buteo* hawk individuals on eight of the Galápagos Islands (*n* =202 *B. galapagoensis*; Fig. 1) and near Las Varillas, Córdoba, Argentina (n =9 *B. swainsoni*; Whiteman & Parker 2004a), from May-August 2001 (Islas Española, *n* =8; Isabela, *n* =25; Marchena, *n* =26; Santa Fe, *n* =13), May-July 2002 (Isla Santiago, *n* =58), January 2003 (Argentina, *n* =9), and May-July 2003 (Islas Fernandina, *n* =28; Pinta, *n* =31; Pinzón, *n* =10). Birds were sampled following Bollmer *et al.* (2005) from multiple locations throughout each island. The University of Missouri-St. Louis Animal Care Committee and the appropriate governmental authorities approved all procedures and permits.

(**b) *Parasite sampling***

We quantitatively sampled parasites from birds via dust ruffling with pyrethroid insecticide (non-toxic to birds; Zema® Z3 Flea and Tick Powder for Dogs, St. John Laboratories, Harbor City, California; Whiteman & Parker 2004a, b). Dust-ruffling provides excellent measures of relative louse intensity (Clayton & Drown 2001).

© ***Blood Collection***

From each bird, we collected two 50 μl blood samples via venipuncture of the brachial vein for genetic analyses. Samples were immediately stored in 500 μl of lysis buffer (Longmire *et al.* 1988). For immune assay, whole blood samples were collected from a subsample of birds (*n* = 46) in heparinized tubes, centrifuged in the field and plasma was stored in liquid nitrogen. Due to logistical constraints, no plasma was collected from the Pinzón population of *B. galapagoensis* or from *B. swainsoni*.

(d) *Innate humoral immunity*

We used the general hemolysis-hemagglutination assay protocol (Matson *et al.* 2005) with two minor modifications (we used plates from Corning Costar #3798, instead of #3795 and Dulbecco's PBS, #D8662, Sigma, St Louis, MO). Sample sizes from Galápagos hawk island populations were as follows: Española, *n* =3; Fernandina, *n* =15; Isabela, *n* =3; Marchena, *n* =5; Pinta, *n* =7; Santa Fe, *n* =5; Santiago, *n* =8. In each plate, we ran the assay on six hawk samples and two positive controls (pooled chicken plasma, #ES1032P, Biomeda, Foster City, CA). Using digitized images of the assay plates, all samples were blindly scored twice to individual, plate number and position. To demonstrate positive standard reliability, assay variation never exceeded 6.8% and 5.6% coefficient of variation (in all cases, CV was calculated using the sample size correction; Sokal & Rohlf 1995) for agglutination titres among and within plates, respectively. Mean NAb agglutination titres and CV were then calculated for each island population from which plasma was collected. CV is a useful measure in studies such as these, since island population means varied widely and CV is dimensionless and relatively stable compared to standard deviation (Snedecor & Cochran 1989).

(e) *DNA fingerprinting*

To determine island-level population genetic diversity, we performed phenol-chloroform DNA extraction on a subset of hawks from each population comprising a total of 118 individuals (Galápagos hawks: Española, *n* =7; Fernandina, *n* =20; Isabela, *n* =10; Marchena, *n* =20; Pinta, *n* =10; Pinzón, *n* =10; Santa Fe, *n* =10; Santiago, *n* =23; Swainson's hawks: *n* =8), followed by multi-locus minisatellite (VNTR) fingerprinting using the restriction endonuclease *Hae III* and Jeffreys' probe 33.15 (Jeffreys *et al.* 1985) and following procedures described elsewhere for birds generally (Parker *et al.* 1995) and Galápagos hawks (Bollmer *et al.* 2005). Estimates of island-level population genetic diversity were obtained by calculating multilocus VNTR heterozygosity values (referred to as *H*; Stephens *et al.* 1992) for each island population and for the population

of Swainson's hawks using GELSTATS v.2.6 (Rogstad & Pelikan 1996). These markers yield an excellent measure of relative genetic diversity in small, isolated vertebrate populations (Gilbert *et al.* 1990; Stephens *et al.* 1992; Parker *et al.* 1998; Bollmer *et al.* 2005) but do not measure individual heterozygosity values.

A large study on Galápagos hawk population genetics (Bollmer *et al.* 2005) used the same multilocus minisatellite markers to estimate population genetic diversity (and included all of the individuals genotyped here). Bollmer *et al.* (2005) strongly supports the pattern of genetic diversity that we found among these hawk populations. Nearly 90% of the variation in hawk population genetic diversity was explained by island area, and the latter correlates with hawk population size (Bollmer *et al.* 2005). The four smallest islands with hawk populations had the highest reported levels of minisatellite uniformity of any wild, relatively unperturbed bird species.

As in Bollmer *et al.* (2005), we randomly selected individuals sampled within each population to assess the relative amount of genetic diversity within each population. We prioritized samples from adults in territorial breeding groups (groups are comprised of unrelated adults; Faaborg *et al.* 1995). On Isla Pinzón, we sampled only from non-territorial birds from multiple geographic locales because we were unable to capture adults there. However, these birds were likely offspring of multiple breeding groups given that many were of the same age cohort (based on plumage characteristics), and that hawks usually produce only one offspring per breeding attempt. Moreover, marked, non-territorial birds disperse from the natal territory following fledging and roam over their entire natal islands (de Vries 1975; Faaborg 1986; Bollmer *et al*. 2005). To ensure that our sampling of birds was not biased by the possible presence of within-island population genetic structure, we sampled and multilocus genotyped birds from multiple geographic locales. For example, on Islas Española and Santiago (which harbour hawk populations with among the lowest and highest genetic diversity,

respectively), we sampled territorial birds from the extreme eastern and western portions of the islands (Fig. 1). On the smaller islands, we sampled birds from a greater proportion of island area than on the larger islands (Fig. 1). Due to the low genetic diversity within the four smallest hawk populations (Española, Santa Fe, Pinzón, and Marchena), sampling from relatively fewer individuals on the smallest islands was sufficient to characterize their population genetic diversity (Bollmer *et al.* 2005). Bollmer *et al.* (2005) found only four multilocus genotypes within Isla Santa Fe in the 15 birds sampled from both multiple years and geographic locations throughout the island (the entire population of hawks on Santa Fe is likely to be ~30 birds). Bollmer *et al.* (2005) further found that populations from Islas Santa Fe, Española, Pinzón, and Marchena were all relatively inbred compared to more variable (but still inbred) populations from Islas Pinta, Fernandina, Isabela and Santiago. Our samples from Swainson's hawks (n =8) and from Isla Isabela (n =10) were small relative to the larger Galápagos hawk population sample sizes, yet both were relatively outbred based on H estimated from the minisatellites. Given this, our estimation of relative genetic diversity within each hawk population sampled is representative of the standing genetic diversity within each population and is not an artifact of sampling bias or within-population genetic structure.

(f) *Statistical analyses*

For all statistical analyses except the overall comparison of prevalence between louse species which utilized Quantitative Parasitology v.2.0 (Reiczigel & Rózsa 2001), louse abundance data were $ln + 1$ transformed and Stephen's heterozygosity values were arcsine square root transformed to meet assumptions of normality.

We performed a Pearson's correlation analysis in SPSS v.11.0 (2004) to assess the strength of the relationship between host population genetic diversity (H) and average host population parasite abundance from nine hawk populations (eight *B. galapagoensis* and one *B. swainsoni*). The correlation analyses were one-tailed given

our *a priori* predictions about the direction of the relationship between the variables. We then examined the relationship between average louse abundance and *H* for the eight Galápagos hawk populations to determine if the relationship was being driven by the relatively outbred Swainson's hawks.

Next, we examined the relationship between innate humoral immunity (NAb agglutination titres) and *H* on the entire subset of individuals (*n* =46) for which plasma was collected. The relationship between average island NAb agglutination titres and *H* was not linear. Thus, we used the GLM procedure in SPSS to determine if there was a significant effect of island-level *H* (a fixed factor) on NAb agglutination titres (the dependent variable) instead (Española, *n* =3; Fernandina, *n* =15; Isabela, *n* =3; Marchena, *n* =5; Pinta, *n* =7; Santa Fe, *n* =5; Santiago, *n* =8).

Finally, we performed a GLM analysis in SPSS using a subset of data that included all 43 birds sampled for both plasma and parasites to determine if antibodies and louse abundances were correlated. In order to control for the effect of island inbreeding we used the GLM procedure as in the preceding analysis (NAb agglutination titres of the 43 hawks dependent on island as a fixed factor) except that louse abundance for each of the 43 individuals was included as a covariate in the model (Española *n* =3; Fernandina *n* =14; Isabela *n* =3; Marchena *n* =5; Pinta *n* =7; Santa Fe *n* =4; Santiago *n* =7). One analysis was performed for each louse species. A scatterplot of the louse abundance data and NAb agglutination titres was created to show the relationships between the two variables before the analyses and individuals were labeled as either inhabiting a relatively inbred (Española, Marchena or Santa Fe) or outbred (Fernandina, Isabela, Pinta or Santiago) island (Fig. 3).

3. RESULTS

(a) *Parasite collections*

We collected a total of 14,843 individuals of the louse *C. turbinatum* and 2,858 individuals of the louse *D. regalis* from 199 Galápagos hawks sampled for lice. These lice typically occur on no other birds in the Galápagos, but have been reported from mainland *Buteo swainsoni* (Whiteman & Parker 2004a). Overall prevalence (across islands) of *C. turbinatum* (97.5%) was higher than that of *D. regalis* (85.4%; Fisher's exact test, $p < 0.001$); both louse species occurred in all 8 host populations.

We collected a total of 17 individuals of *C. turbinatum*, 22 individuals of *Laemobothrion maximum* and 11 individuals of a *Kurodaia* sp. from the nine Swainson's hawks. These three species abundances were pooled and constitute the amblyceran lice from Swainson's hawks; *C. turbinatum* was the only amblyceran collected from Galápagos hawks. No *Degeeriella* were collected from the nine Swainson's hawks.

(b) *Assessment of population genetic diversity*

Untransformed values of *H* for each host population are shown in Figure 1. Individuals from the smallest island-populations of the Galápagos hawk had the highest reported levels of minisatellite uniformity of any wild, unperturbed bird species and these results are consistent with those of Bollmer *et al.* (2005). As in Bollmer *et al.* (2005), we found >50% of all bands were fixed within these populations (Santa Fe, 13/16 bands fixed; Española, 10/16 bands fixed; Pinzón, 11/20 bands fixed; Marchena, 11/18 bands fixed). The four most inbred populations contained multiple individuals or sets of individuals that were genetically identical at all loci, whereas no identical individuals were found within the four larger islands populations or within Swainson's hawks (Bollmer *et al.* 2005).

© *Effects of genetic diversity and other host factors on parasite load*

Among *Buteo* populations (*n* =208 total individuals sampled for lice by population: Española, *n* =8; Fernandina, *n* =28; Isabela, *n* =25; Marchena, *n* =26; Pinta, *n* =31; Pinzón, *n* =10; Santa Fe, *n* =13; Santiago, *n* =58; Swainson's hawks *n* =9),

average amblyceran louse abundance within populations and *H* were significantly and negatively related across populations (Fig. 2A; *C. turbinatum*; Pearson's $r = -0.949$, $n = 9$, $p < 0.0001$; *D. regalis*; $r = -0.854$, $n = 9$, $p < 0.01$). When limited to the eight Galápagos hawk island populations only, similar negative relationships were found: *C. turbinatum* ($r = -0.875$, $n = 8$, $p < 0.01$) and *D. regalis* ($r = -0.69$, $n = 8$, $p < 0.05$).

(d) *Innate antibody levels, genetic diversity and parasite load*

We found a significant (and non-linear) effect of island on average NAb agglutination titres (Fig. 2B; one-way ANOVA; $n = 46$, $F_{6, 39} = 3.41$, $p < 0.01$). The Marchena population, the third most inbred population, exhibited the highest average titre and Española and Santa Fe, the most inbred populations, exhibited the lowest (Fig. 2B). The more outbred island populations had intermediate NAb titres. The variance in NAb titres was lower within the inbred populations than the more outbred populations (Fig. 2B). The CV of the inbred populations (Santa Fe, Española, Marchena) was 12% within and 25.5% among islands, whereas the CV of the more outbred islands (Fernandina, Isabela, Pinta, Santiago) was 17.8% within and 4.7% among islands. Furthermore, *C. turbinatum* abundance was negatively related to NAb agglutination titres (marginally significant) when individual birds were considered (controlling for the effects of island in a GLM; corrected model $F_{7, 35} = 4.05$, $p < 0.01$; island effect $F = 2.50$, $p < 0.05$, *C. turbinatum* abundance parameter estimate $\beta = -0.342$, $F = 4.10$, $p = 0.05$; Fig. 3). The scatterplot yielded a triangular pattern whereby birds with low NAb titres consistently harboured high *C. turbinatum* abundances, but birds with high NAb titres harboured both low and high louse abundances. As predicted, no significant relationship was found between the ischnoceran, feather-feeding *D. regalis* and NAb agglutination titres (controlling for the effects of island in a GLM; corrected model $F_{7, 35} = 3.01$, $p <$

0.05; island effect $F = 2.60$, $p < 0.05$, *D. regalis* abundance parameter estimate $\beta = -0.259$, $F = 1.68$, $p > 0.05$).

4. DISCUSSION

We have shown that variation in host population genetic diversity is correlated negatively with average parasite load and positively with variation in NAb levels across populations of the Galápagos Hawk. Smaller, more inbred host populations had higher parasite loads, lower average immune responses (generally) and lower variation in within-population immune response than more outbred populations. NAb levels were negatively correlated with the abundance of a skin and blood feeding amblyceran louse, further linking inbreeding, immune response and parasite burden.

As a result of lower within-population genetic variability and lower and less variable within-population NAb levels, most of the peripheral, inbred and highly differentiated island populations of the Galápagos hawk are vulnerable to disease agents. This result may not be surprising, but few studies have evaluated this relationship in wildlife populations. These populations contained more among-island variability in NAb levels than the larger island-populations, possibly due to the strong effects of genetic drift (Spielman *et al.* 2004b; Pearman & Garner 2005) or local coevolutionary dynamics (Thompson 1999). Protection of the highly differentiated peripheral hawk populations should be prioritized as the variation they contain is essential for the long-term viability of this species (Lesica & Allendorf 1995). Conversely, the large amount of within-population genetic and immunological variation within the largest hawk island populations is also important from a conservation perspective. Since tradeoffs exist between the humoral and cellular immune response (Lindström *et al.* 2004), these populations may be better able to respond to multiple invasions of pathogens than the smaller, more isolated populations. Notably, breeding populations within three large islands (Islas Floreana, San Cristóbal and Santa Cruz) are

now likely extinct (Bollmer *et al.* 2005; in press) and each of these is geographically proximal to one or several of the most inbred island populations. Thus, if metapopulation dynamics were operating in this system (Thompson 1999; Templeton *et al*. 2001), the potential for the introduction of novel alleles (e.g., resistance alleles) by recurrent gene flow among populations has now been reduced given that only eight out of eleven island populations remain intact. Thus, managers of the Galápagos National Park may consider restricting travel to the smallest island populations of the hawk, given that invasive avian disease vectors have established within several human-inhabited islands that serve as a base of operations for the tourism industry (Whiteman et al. 2005).

As a potential mechanism underlying the relationship between host genetic diversity and average parasite load, we showed that NAb agglutination titres were negatively related to abundance of native parasites that fed on skin and blood (*C. turbinatum*), although the correlational nature of this analysis and its marginal significance, after correcting for the effects of island, indicate that this result be accepted with caution and requires confirmation. However, strength of the PHA-induced immune response in birds was directly related to amblyceran species richness, indicating that amblycerans and their avian hosts are engaged in coevolutionary arms races (Møller & Rózsa 2005). Thus, our finding of a potential relationship between host immune response and amblyceran, but not ischnoceran abundance at the individual host level, is in accord with this macroevolutionary trend.

The influence of another unmeasured factor correlating with population genetic diversity may also explain the results, although we know of no such factor. Nearly 90% of the variation in hawk genetic diversity is explained by island size and these hawk populations are genetically isolated from one another (Bollmer *et al.* 2005; in press). Given that larger island populations typically had lower parasite loads, a simple

relationship between host population size and parasite load is unlikely here (Lindström *et al.* 2004). Specific mechanisms underlying the relationship between *H* and disease susceptibility may include the exposure of deleterious recessive alleles (Keller & Waller 2002), the fixation of slightly deleterious alleles through genetic drift (Johnson & Seger 2001), other microevolutionary processes associated with founder events and maintenance of small population sizes over time, or a combination of these. Generalized inbreeding depression may also lead to physical and behavioral changes that affect preening efficiency and this may be particularly germane for *D. regalis*, which mainly encounters mechanical host defences (Clayton *et al.* 1999; Whiteman & Parker 2004b).

Extinction and disease ecology are "by their nature cryptic and difficult to study in natural communities" (de Castro & Bolker 2005). Clearly, however, this information is of basic biological interest and offers insight into how populations will respond to invasions of alien pathogens, which is underway in most previously isolated ecosystems. Future studies examining host immunogenetics, parasite population genetics and transmission dynamics are necessary for fully assessing the threat of pathogens to this island endemic.

5. ACKNOWLEDGEMENTS

NKW and PGP were supported by a Dissertation Enhancement Grant from the National Science Foundation (NSF; INT-030759), grants from the Field Research for Conservation Program (FRC) of the Saint Louis Zoo, International Center for Tropical Ecology (UM-St. Louis), Sigma Xi, and funds from the E. Desmond Lee and Family Fund's Collaborative Vision in Zoological Studies. KDM was supported by grants from the NSF (IBN-0212587) and the FRC from the Saint Louis Zoo. For the Galápagos sampling and permits, we thank the Servicio Parqué Nacional de Galápagos and the Estación Científica Charles Darwin (Dr. David Wiedenfeld), Isla Santa Cruz, Galápagos,

Ecuador and Dr. Tjitte de Vries and students, Pontificia Universidad Catolica del Ecuador for help with fieldwork. TAME provided discounted roundtrip air-travel within Ecuador. For sampling in Argentina, we thank José H. Sarasola and Dr. Juan J. Negro (Estación Biológica de Doñana, Seville, Spain), Mark Beilstein (University of Missouri-St. Louis) and Donna Eakman (Bothel, WA), and for collection and export permits, Dr. Sandra Aliscioni (Instituto de Botánica Darwinion, Buenos, Aires, Argentina). We thank Dale H. Clayton (University of Utah), Kirk C. Klasing (University of California-Davis), Robert J. Marquis (University of Missouri-St. Louis), Robert E. Ricklefs (University of Missouri-St. Louis), Alex Scheuerlein (University of Missouri-St. Louis), and two anonymous reviewers for helpful comments on a previous version of the manuscript.

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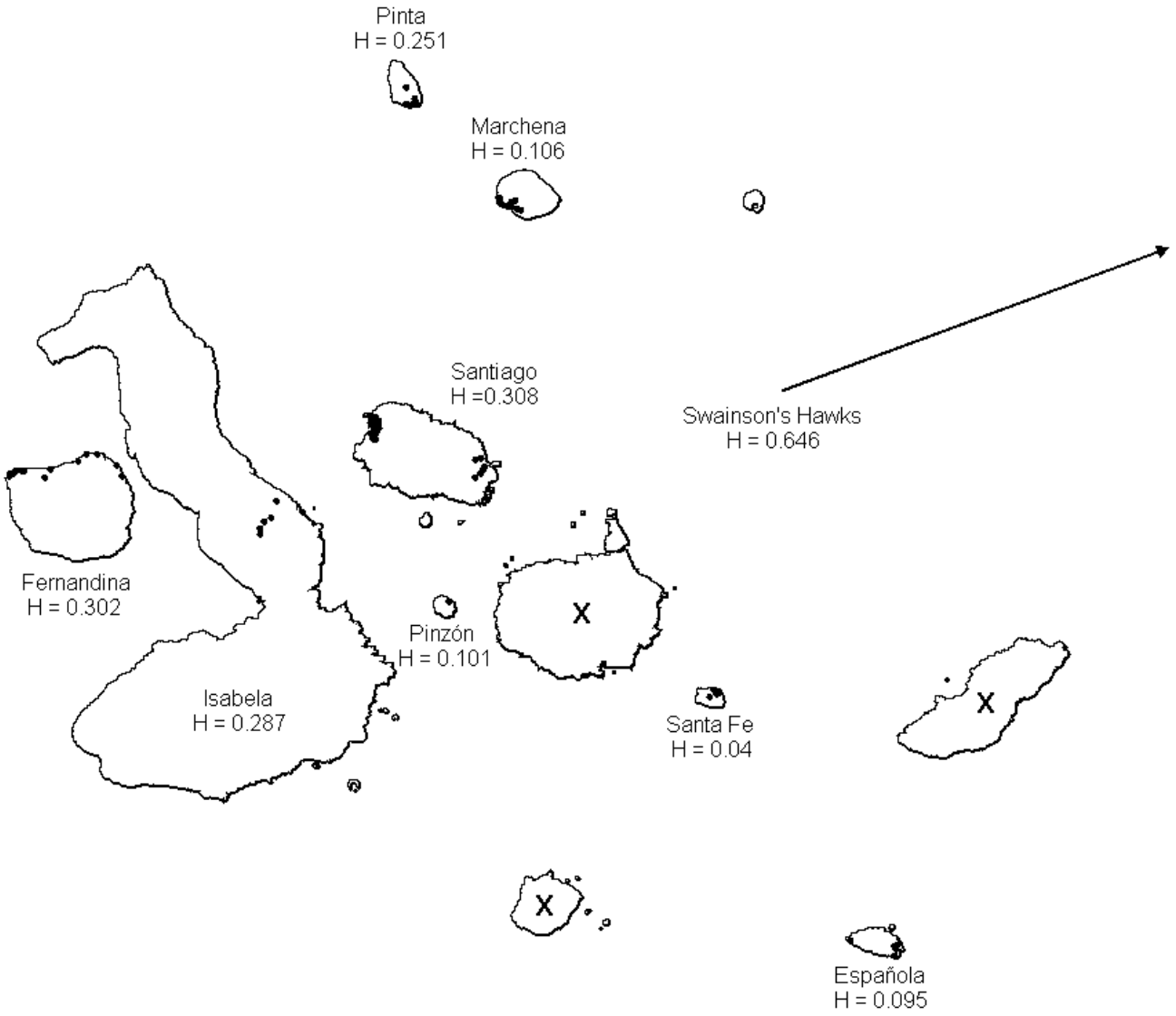
FIGURE LEGENDS

Figure 1. Map of the Galápagos Archipelago, located ~1000 km west of mainland Ecuador, South America. Extant breeding island populations of the Galápagos hawk (*Buteo galapagoensis*) are named, followed by estimates of island population genetic diversity (*H*; Stephens heterozygosity values) calculated from multilocus minisatellite data. Small black dots within islands indicate sampling localities. An estimation of *H* from the mainland Swainson's hawk (the putative sibling species of *B. galapagoensis*) was included for comparative purposes. Extinct island populations of *B. galapagoensis* are indicated by an "X" (there is no evidence indicating hawks have ever inhabited Isla Genovesa located in the northeastern part of the archipelago).

Figure 2. Scatterplot of two disease susceptibility variables vs. estimated host population genetic diversity (heterozygosity) values. **(A)** Louse abundance vs. host population genetic diversity. Closed circles = average amblyceran abundance ± 95% confidence intervals (*Colpocephalum turbinatum*, *Laemobothrion maximum*, and *Kurodaia* sp.; $r = -0.949$, $n = 9$, $p < 0.0001$). Open circles = average ischnoceran abundance ± 95% confidence intervals (*Degeeriella regalis*; $r = -0.854$, $n = 9$, $p < 0.01$). Dyads with heterozygosity values > 0.9 represent a mainland *B. swainsoni* population and the remaining values represent eight island populations of *B. galapagoensis*. Island populations reading left to right are as follows: Santa Fe, Española, Pinzón, Marchena, Pinta, Isabela, Fernandina, Santiago; **(B)** Average agglutination titres (NAbs) ± SDM from 46 *B. galapagoensis* individuals vs. estimated host population genetic diversity (the relationship between NAb agglutination titres and genetic diversity was not linear, although significant differences existed in average NAb agglutination titres among island-populations, one-way ANOVA: $F_{6,39}$, $p < 0.01$). Island populations reading left to right are as follows: Santa Fe, Española, Marchena, Pinta, Isabela, Fernandina, Santiago.

Figure 3. Negative linear relationship between *Colpocephalum turbinatum* abundance and natural antibody (NAb) titres. The regression line through the raw data (uncorrected for island) is shown ($\beta = -0.355$, $p < 0.01$). The relationship was marginally significant after controlling for the effects of island and other host factors ($\beta = -0.342$, $p = 0.05$). Open circles = individuals from more inbred island populations (Española, Marchena, Santa Fe), solid circles = individuals from more outbred island populations (Fernandina, Isabela, Pinta, Santiago).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Santiago
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Swainson's Hawks
H = 0.646
Fernandina
H = 0.302
Pinzón
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Isabela
H = 0.287
Santa Fe
H = 0.04
Española
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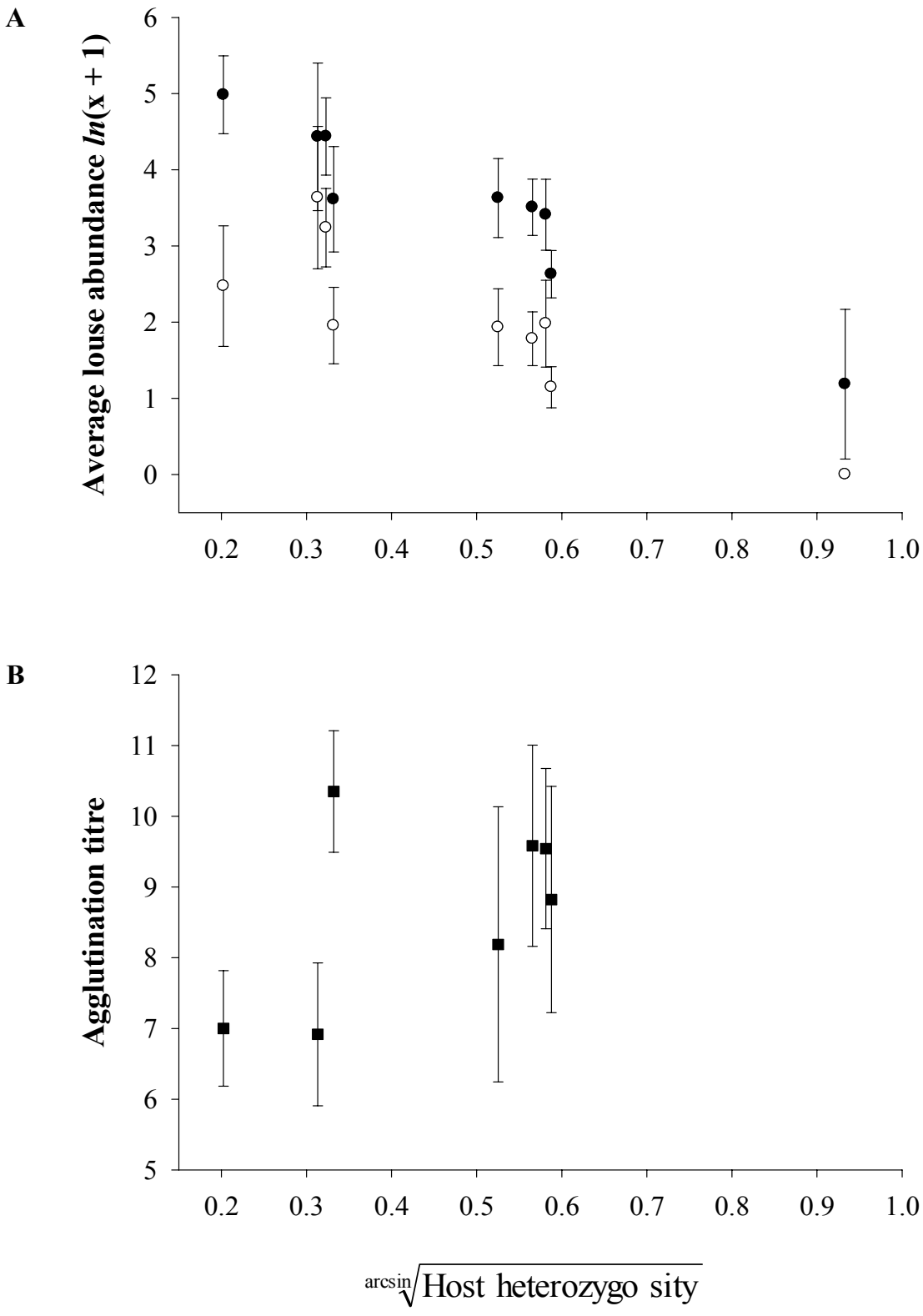
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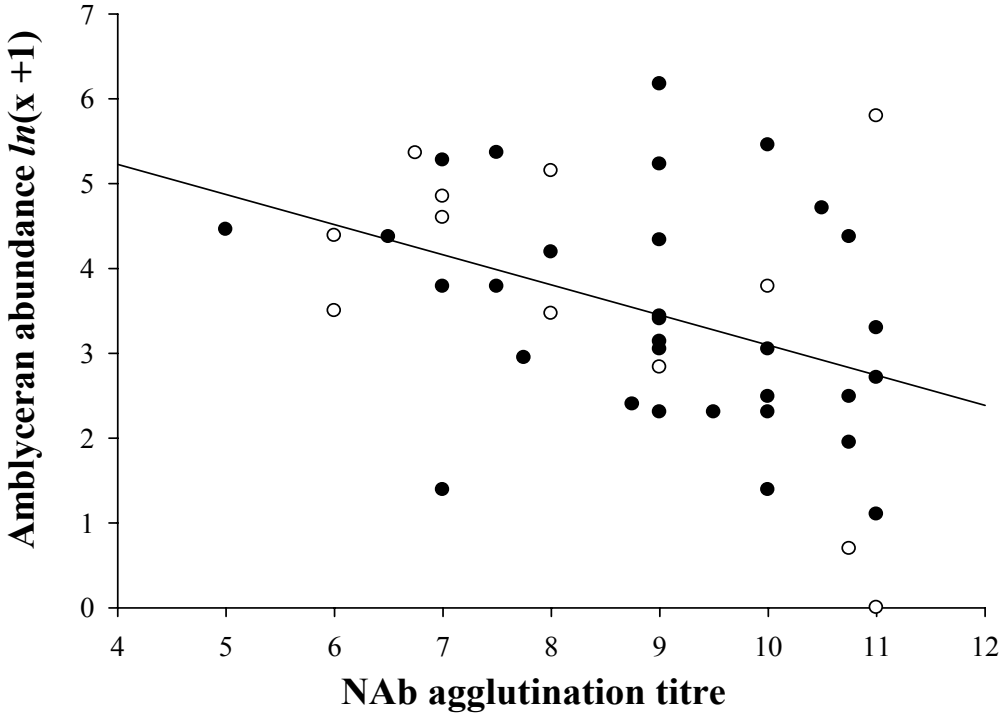
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Chapter 6

Increase in the constitutive innate humoral immune system in Leach's storm petrel (*Oceanodroma leucorhoa*) chicks is negatively correlated with growth rate.

In press: R. A. Mauck, K. D. Matson, J. Philipsborn & R. E. Ricklefs.

Functional Ecology.

Summary:

1. Using a simple technique for assessing constitutive innate immune function recently adapted for use in wild populations, we characterize changes in avian immune system development by repeated measurements of individuals over the period of nestling growth in a wild population of Leach's storm-petrels (*Oceanodroma leucorhoa*).
2. We measured levels of natural antibodies (NAb) during the early, middle, and late phases of storm-petrel development and related these levels and NAb rate of change to mass and wing length growth. We used natural variation in nestling growth to assess the influence of nutritional status on the development of innate immunity.
3. NAb levels increased over the first fifty days of chick development, however, rate of increase was inversely proportional to wing growth.
4. Initial titer levels were inversely proportional to rate of change in NAb levels over the initial 50 days of immune development. This suggests that individuals with low initial NAb levels accelerate immune development to reach adult levels, whereas individuals with high initial levels do not.
5. As in previous studies, our results demonstrate an inverse relationship between growth rate and development of components of the avian immune system. While such a relationship is consistent with the idea that immune

function development involves trade-offs, the processes involved are more complex than simple energy allocation.

Introduction

Immune function is increasingly seen in the context of life-history trade-offs (Martin et al., 2001; Norris & Evans, 2000; Schmid-Hempel, 2003; Sheldon & Verhulst, 1996; Tella et al., 2002). The ability to respond effectively to parasites and pathogens has been shown to have fitness benefits in terms of body condition, survival, and reproductive success (e.g., Alonso-Alvarez & Tella, 2001; Christe et al., 2001; Horak et al., 1999; Johnsen et al., 2000; Råberg & Stjernman, 2003; Svensson et al., 2001). With benefits come costs; resources allocated to the immune system are not available for other functions. For example, experimentally elicited immune responses have been shown to decrease avian nestling growth (Alonso-Alvarez & Tella, 2001; Brommer, 2004; Fair et al., 1999; Nilsson, 2003; Soler et al., 2003a; Whitaker & Fair, 2002), degrade adult body condition (Alonso-Alvarez & Tella, 2001; Sanz et al., 2004), and increase metabolic rate (Ots et al., 2001).

Many studies (reviewed in Tella et al., 2002) have employed the phytohemagglutinin (PHA) skin test to assess non-specific cellular immunity (commonly referred to as cell-mediated immunity, or CMI). Although the PHA response involves proliferation of T-cells, PHA also attracts other immune cells, including basophils and heterophils, to the injection site and causes inflammation (Smits et al., 1999). Response to PHA is typically positively correlated with nestling body condition or food availability, sometimes mediated through an effect of brood size or season (e.g., Dubiec & Cichon, 2001; Hoi-Leitner et al., 2001; Merino et al., 2000; Westneat et al., 2004).

Much recent work on immune function in developing birds has focused on measuring induced immunological responses, a subset of immune function that involves adaptive humoral immunity (e.g., antibody responses to specific antigens) and cellular immunity (e.g., changes in leukocyte profiles, often after a specific or nonspecific challenge). Less attention has been paid to constitutive immune function in wild

populations. An important humoral component of constitutive, innate immunity are natural antibodies (NAb), which represent a first-line of defense against pathogens (Ochsenbein & Zinkernagel, 2000).

Unlike adaptive Ab, NAb are directly encoded by germ-line genes in the absence of somatic rearrangement (Avrameas, 1991; Boes, 2000; Ochsenbein & Zinkernagel, 2000) and, by definition, are the only class of immunoglobin molecules whose concentration in blood is not dependent on previous exposure to specific antigens (Boes, 2000; Pereira et al., 1986). NAb serve a number of functions, including direct immediate control of novel bacterial and viral challenges, initiation of the complement enzyme cascade that results in cell lysis, regulation of self-reactive B and T cells, and clearance of damaged or transformed cells (Boes, 2000; Carroll & Prodeus, 1998; Ochsenbein & Zinkernagel, 2000; Reid et al., 1997). Selection experiments with chickens suggest that changes in NAb levels parallel changes in primary antibody responses and general disease resistance (e.g., Parmentier et al., 2004) and might be genetically linked with specific humoral responses (Cotter et al., 2005).

Laboratory studies of chickens show that the immune system of newly hatched birds is inefficient and poorly developed, then becomes stronger with age (Apanius, 1998). However, little is known about the development of constitutive innate humoral immune function in the wild, particularly about the influence of nutrition and environment on the rate of development.

Recently, Matson et al. (2005) described a highly repeatable assay for innate humoral immunity based on NAb-mediated complement activation and red blood cell agglutination. Because the assay requires only a single, small (~100μL) blood sample, it is suitable for use in wild populations of small birds. Unlike the PHA test, which assesses non-specific cellular immunity involving an array of responses, the NAb assay measures a single, well-characterized component of the innate immune response. Moreover,

measurement of NAb does not induce an immune response *in situ*, and so, unlike the PHA test, natural antibody titers of an individual can be measured independently and repeatedly over time, allowing the investigator to quantify the development of immune function in young birds and changes in immune function in response to natural or experimental treatments.

In this paper, we present a longitudinal study of NAb concentrations in chicks of Leach's storm-petrels (*Oceanodroma leucorhoa*) over the postnatal growth period and in relation to the chicks' rate of mass gain and wing growth. Storm-petrels are particularly appropriate for investigating the effects of nutrition on immune function development because food provisioning varies widely and chicks are readily accessible through the entire development period. Characteristic of the avian order Procellariiformes, storm-petrels are long-lived, breeding up to 35 years, and lay a single egg each year (Huntington et al., 1996). Petrels dig their nests in underground burrows where male and female members of a pair share incubation during a 40- to 44-day incubation period (Gross, 1935; Huntington et al., 1996). Once hatched, the nestling is brooded for about 5 days, after which it remains alone in the burrow for another 55 to 65 days before fledging, physically indistinguishable from adult individuals. During development, a nestling is fed during brief, nocturnal visits by both parents. Due the unpredictable nature of the pelagic resource, there is great variation in chick provisioning by adults and, therefore, in chick nutritional status (Ricklefs et al., 1985; Ricklefs & Schew, 1994). Rate of increase in wing length and maximum chick mass during development ($x = 82.5 \pm 6.1$ SD, range 67 – 97 g) reflect the total food delivered to the chick during development (Mauck & Ricklefs, 2005). We measured naturally occurring antibody levels during the early, middle, and late phases of storm-petrel development and related these levels and NAb rate of change to mass and wing length growth. We used natural variation in nestling growth to assess the influence of nutritional status on the development of innate

immunity.

Methods

Study population

We conducted this study at a breeding colony of about 2,000 pairs of Leach's storm-petrels at the Bowdoin Scientific Station on Kent Island, New Brunswick, Canada (44°35' N, 66°45' W) near the mouth of the Bay of Fundy. During the 2003 breeding season, we monitored chick growth in 13 burrows on Kent Island. At approximately the same time each day, we measured chick wing length to the nearest mm, tarsus length to the nearest 0.1mm, and chick mass to the nearest 0.1g with a portable electronic balance (OHAUS, Inc.). Two of the thirteen chicks were not measured daily and were measured only on the days on which blood samples were collected.

Measuring immune response

We collected three blood samples from each chick, which represented early (day 19-21), middle (day 33-35), and late (day 49-51) phases of chick development. For each sample, we used heparinized microcapillary tubes to collect approximately 100µL of whole blood from the brachial vein. Samples were kept without refrigeration for less than two hours before centrifugation and plasma removal. We froze the resulting blood plasma at -20ºC for storage and transport.

We assessed innate humoral immunity by characterizing NAb-mediated rabbit red blood cell agglutination titers as described by Matson et al. (2005), with two minor modifications. First, for all steps requiring phosphate buffer saline (PBS), we used Dulbecco's PBS (#D8662, Sigma, St Louis, MO). Because this formulation includes $MgCl_2 \cdot 6H_2O$ and $CaCl_2 \cdot 2H_2O$ in addition to the basic PBS ingredients, its use counteracted any effects of plasma serial dilution on endogenous divalent cation concentration. Second, we used plates processed for improved hydrophilic qualities (Corning Costar #3798, instead of #3795). Assays were randomized and run blindly with

respect to sample. Digitized images produced by this protocol were also randomized with respect to plate, plate location, and sample, and were scored blindly for both lysis and agglutination of rabbit red blood cells. Lysis titers reflect the interaction of NAb and complement; agglutination results only from NAb activity. In our samples, 90-minute lysis titers showed almost no variation with 34 out of 35 samples scoring 0.0 (0.07 ± 0.07 SE titers), which were similar to scores reported by Matson et al. (2005) for the related waved albatross (*Phoebastria irrorata*). Accordingly, we used only agglutination titers for our analyses of immune function in storm-petrel chicks.

Of the 39 samples from 13 chicks, four samples were not usable due to insufficient volume of blood plasma. Thus, we were able to analyze early, middle, and late samples for nine chicks, early and middle samples for two chicks, and middle and late samples for two chicks.

Statistical analyses

We used several derived variables to compare chick development and immune function. For each chick, we calculated the rate of increase (slope) of the wing (WLS; mm d^{-1}) during the linear phase of wing growth (20-50 days of age) and maximum mass attained during the nestling period (MaxMass; g). As an index of chick body condition at maximum mass (BCI_{max}), we divided MaxMass by tarsus length (TL). For each chick, we used the slope of the least squares regression of agglutination titer versus age to characterize change in NAb activity during chick development (NAbSlope). As an index of chick body condition on day 50 (BCI_{50}), we divided chick mass by tarsus length (TL). For all derived variables, the distribution of values did not differ from normal.

To determine whether NAb activity increased with chick age, we used a one-sample, one-sided *t*-test of NAbSlope against the null hypothesis that mean NAbSlope was less than or equal to zero.

To investigate how chick growth and environment influenced rate of immune function development, we used an information-theoretic approach (Burnham & Anderson, 1998) to investigate models of the relationship between NAbSlope and four independent variables representing seasonal effect (Julian hatch date of the chick), food delivered to the nest (MaxMass), and chick growth rate (WLS), as well as initial NAb level (NAb_{20}) to account for any effect of starting point on immune function development. As suggested by Burnham and Anderson (1998) for observational studies, this approach allowed us to rank models and select a "best" model using Akaike's information criterion (AIC). We constructed all possible combinations of seasonal effect, food delivered to the nest, chick growth rate, and initial NAb levels. In this way, we constructed 15 models with these four variables (Table 1). We used the JMP (SAS Institute) statistical package's stepwise regression function to calculate AIC_c values for each model. We then calculated Akaike model weights ($\square_m$) to rank models, and Akaike parameter weights ($\square_p$) to rank individual variables with respect to their explanatory power. When one model and one parameter emerged as a particularly important factor, we used linear regression to test the post-hoc hypothesis that chick growth rate influenced the rate of immune function development. We then used multiple linear regression to assess the effect of the initial NAb level on immune development while holding chick growth rate constant.

To compare our data with studies reporting immune function in relation to body condition at a single point near the end of chick development, we regressed the agglutination titer on day 50 against chick body condition (BCI) on day 50. To investigate the generality of the relationship between agglutination titer and BCI, we repeated the analysis for day 20 and day 35 samples.

Results

Agglutination titers increased with chick age (Fig. 1). Mean change in Agglutination titer over time (0.06 ± 0.02 SE titers day^{-1}) was significantly greater than zero (one-sample, one-sided t-test, *df*=12, *T*=2.92, *P*=0.006).

The best model (Table 1) for predicting the rate of increase in NAb (NAbSlope) contained only chick growth rate (WLS). This simple model was superior to the models containing only Initial (NAb_{20}) level (ΔAIC_c = 1.74) or both $NAb_{20}$and WLS (ΔAIC_c = 1.70). Models with ΔAIC_c less than 2.0, however, are often considered equivalent (Burnham & Anderson, 1998) and, therefore, NAB_{20} must be considered a useful predictor of NAbSlope. Since models with a ΔAIC_c greater than 4 are considered implausible (Burnham & Anderson, 1998), inclusion of only WLS and NAB_{20} may be sufficient to explain variation in immune function development rate. As indicated by their low Akaike parameter weights (Table 2), total food delivered to the chick (MaxMass) and season (JD) probably have little predictive power. The primary importance of chick growth rate as a predictor of change in agglutination titers over the first fifty days of chick development is indicated by the difference in Akaike parameter weights between WLS and all other variables (Table 2).

Linear regression of NAbSlope against WLS (the “best” model) revealed a negative correlation (-0.14 ± 0.06 SE titers d^{-1}/mm d^{-1}) between NAb rate of increase and chick growth rate (R^2 = 0.34, *df* = 12, *F* = 9.03, *P* = 0.009; Fig. 2).

Linear regression of NAbSlope against both WLS and NAB_{20} (R^2 = 0.69, *df* = 8, *F* = 5.69, *P* = 0.036) had little effect with respect to WLS (-0.11 ± 0.04 SE titers d^{-1}/mm d^{-1}, *P*=0.02) and revealed a negative correlation between NAbSlope and initial NAb level (-0.02 ± 0.008 SE titers d^{-1}/ NAB_{20} score, *P*=0.03).

Linear regression revealed a negative correlation (-1.99 ± 0.54 SE titers/BCI) between NAbScore and body condition index on day 50 (R^2 = 0.60, *df* = 10, *F* = 13.65, *P*

= 0.005). BCI_{50} and chick mass on day 50 are highly correlated (r = 0.77). Therefore, to allow easier interpretation of immune function and chick nutritional state on day 50, we used chick mass rather than BCI in to show this relationship graphically (Fig. 3). There was no correlation between body condition and agglutination score on day 20 (r = 0.17) or day 35 (r = 0.06).

Discussion

Our results add to the literature demonstrating an inverse relationship between particular components of immune function and growth rate of individuals in wild populations; it is the first, however, to directly measure development rate of the immune system itself in a natural population. As expected, NAb activity increased over the first fifty days of chick development (Fig. 1), however, the rate of increase was inversely proportional to wing growth rate (Fig. 2). For every mm d^{-1} increase in wing growth rate, innate immune function development decreased by –0.14 titers d^{-1}. This result is consistent with the idea that development of the innate immune system involves trade-offs, though the nature of those trade-offs is unclear.

At the same time, initial titer levels were inversely proportional to rate of change in NAb levels over the initial 50 days of immune development (-0.02 ± 0.008 SE titers d^{-1}/ NAB_{20} score). This suggests that individuals starting with low NAb levels accelerate immune development to reach adult levels, whereas individuals starting with high levels do not. Indeed, variation in NAb levels decreases through early (CV=164.0), middle (CV=93.4), and late (CV=58.8) development. Incubating adults show even less variation (CV=23.0; Mauck et al., unpubl.). Possibly, initial NAb levels set a rate of development for this component of immune function that, in turn, imposes a cost in terms of chick growth.

Our results are consistent with studies that imposed an immunological cost on developing chicks, resulting in slower growth rates (Brommer, 2004; Soler et al., 2003b;

Swain & Johri, 2000; Tsiagbe et al., 1987), although other studies have failed to find such a cost (e.g., Fair et al., 1999; Whitaker & Fair, 2002). The relationship between immune function and other life-history traits is not well understood (Norris & Evans, 2000; Owens & Wilson, 1999). In particular, it is not clear that simple maintenance of immune function is costly in the absence of infection (Klasing, 1998b; Kraaijeveld & Godfray, 1997; Webster & Woolhouse, 1999), though maintenance is apparently costlier for the innate immune system than for the adaptive immune system (Råberg et al., 2002). There is little doubt, however, that mounting an immune response is energetically costly due to both direct and indirect metabolic requirements of both the innate and adaptive systems (e.g., Demas et al., 1997; Lochmiller & Deerenberg, 2000; Nilsson, 2003; Råberg et al., 2002). Unlike many previous studies, we investigated immune function development rather than immune function response – i. e., having an immune system vs. using an immune system. Although we did not measure energetic costs directly, nestling growth rate is certainly linked to energy availability. Procellariiform growth and development is thought to be energetically expensive compared to other altricial and semi-altricial bird species (Hodum & Weathers, 2003; Ricklefs et al., 1980). It is, therefore, possible that the apparent growth-immune function trade-off observed here can be explained by energetics.

It is doubtful, however, that simple energy allocation is wholly responsible for the negative relationship (Fig. 2) between rate of innate immune function development and chick growth. A simple energy allocation model would predict an effect of food delivery on rate of immune development. Typical of all storm-petrels, chicks gain mass irregularly to a maximum late in the nestling period, followed by rapid decline before fledging (Huntington et al., 1996; Mauck & Ricklefs, 2005). The excess mass is primarily lipids accumulated as insurance against an uncertain resource (Ricklefs & Schew, 1994). If immune development is a simple matter of energy allocation, then we should have seen

a positive effect of MaxMass on immune development, since the heaviest chicks have a net energy surplus. We did not (Table 1, 2). The simple energy allocation model would also predict a positive correlation between BCI and agglutination score at any age. We found no correlation between BCI and agglutination score during early (r = 0.17) and mid (r = 0.06) development and a strong negative correlation late in development (r = 0.77). Apparently, stored energy alone did not enable rapid immune function development in these nestling storm-petrels. Although our results certainly suggest a trade-off, it is evidently more complex than a simple allocation of energy, perhaps also involving other critical resources or control mechanisms.

Our results stand in contrast to the many studies that have measured immune function at the end of chick development and found that chicks in better body condition are better able to respond to immune challenges (e.g., Hoi-Leitner et al., 2001; Horak et al., 1999; Saino et al., 2002; Tella et al., 2001). We found a negative relationship between body size and innate immune function near the end of the growth period (Fig. 3) and it is not clear why. The irregular pattern of petrel provisioning is such that chicks lose weight on 43.8% of days during development; on a finer scale, some chicks gain as much as 20g in 24 h, or lose weight on as many as 6 consecutive (x = 3.0 ± 0.9 SD) days (RAM, unpubl.). It is tempting, therefore, to see petrel chick provisioning as a natural parallel to experiments by Klasing (1998a) in which domestic chicken nestlings were either force-fed or deprived of food. In those experiments, food-deprived (light) chicks showed increases in some aspects of immune response and over-fed (heavy) chicks showed decreased responses. Although fifty-day old storm-petrel chicks show a similar pattern (Fig. 3), the lack of any relationship between mass and agglutination score during early (r = 0.17) and mid (r = 0.06) development suggest this is not an adequate explanation for our result.

Not surprisingly, our measures of innate immune function increased with time (Fig. 1). The extensive literature on galliform birds clearly demonstrates an increase in the concentration of natural antibodies over the first six months of age in chickens (Apanius, 1998). In validating their NAb measurement protocol, Matson et al. (2005) tested Cobb broiler chickens and found that NAb levels increased over time to adult levels by 11 weeks of age. Storm-petrels differed from chickens in this respect because the NAb levels of 50-day old chicks (2.06 ± 0.36 SE titers) were only 60% of those observed in incubating adult petrels (3.42 ± 0.18 SE titers; Mauck et al., unpublished data). Structural growth in petrels is completed before fledging, usually by day 55-60 (Mauck & Ricklefs, 2005; Ricklefs et al., 1980). On average, day 50 chicks in this study had achieved 78% of their completed wing growth. Thus, innate immune system development appears to lag behind structural development in storm-petrels.

Using this simple technique for assessing constitutive innate immune function in wild populations, we have characterized changes in avian immune system development by repeated measurements of individuals over the period of nestling growth in a wild population. As in previous studies, our results demonstrate an inverse relationship between growth rate and development of the avian immune system. While such a relationship is consistent with the idea that immune function development involves trade-offs, the processes involved are certainly more complex than simple energy allocation.

Acknowledgements

We thank N.T. Wheelwright and C.E. Huntington for hospitality and advice at the Bowdoin Scientific Station, from which this paper is Contribution No. 171. We thank two anonymous reviewers for helpful comments. This research was supported by the Kenyon College Summer Science Scholars Program, the Horatio B. Ebert Foundation, and from a grant from the National Science Foundation (IBN-0212587) to RER. KDM is additionally supported by the Saint Louis Zoo (FRC #03-13), American Ornithologists'

Union (Graduate Research Award), International Center for Tropical Ecology, Saint Louis Audubon Society, and Webster Groves Nature Study Society.

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Figure Legends

1. Change in immune function (agglutination score) during storm-petrel chick development during the early (day 20), middle (day 35) and late (day 50) phases of nestling growth. Black circles represent mean values at each phase. Error bars are SE. The dotted line represents the mean regression line for change in agglutination titer with age for *N* = 13 chicks.
2. Change in rate of immune function development as a function of chick growth rate. Solid line represents least squares fitted line for *N* = 13 chicks.
3. Immune function (agglutination score) as a function of chick mass on day 5 for *N* = 11 storm-petrel chicks.

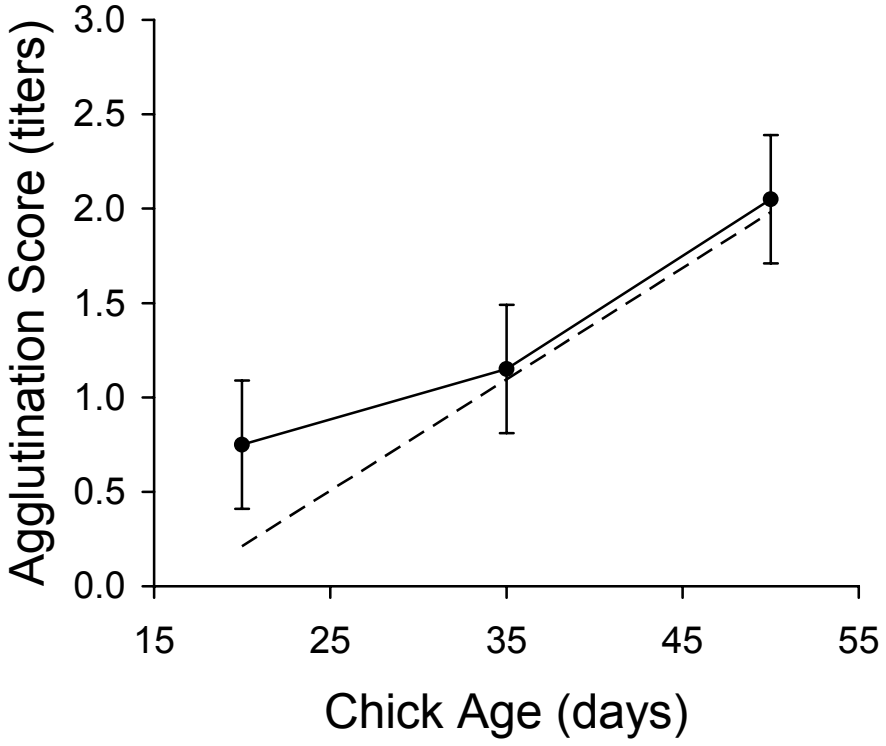
Agglutination Score (titers)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
15
25
35
45
55
Chick Age (days)

Figure 1

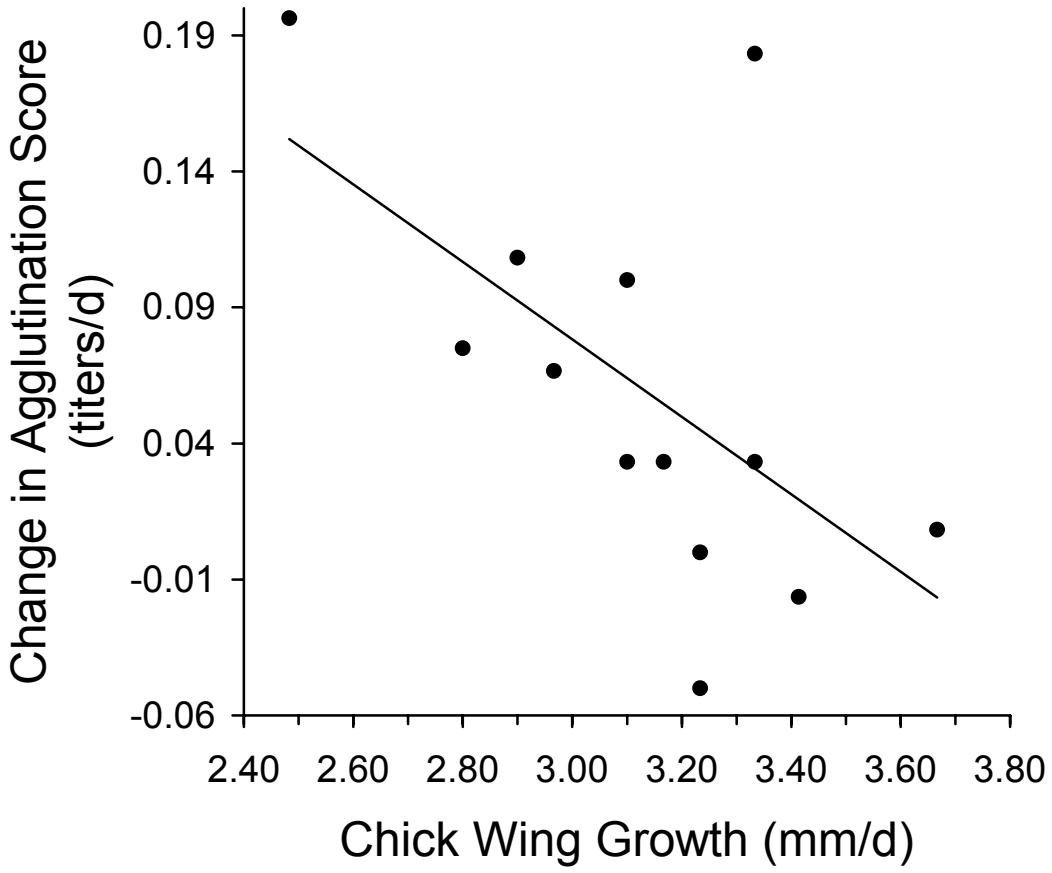
Change in Agglutination Score
(titers/d)
0.19
0.14
0.09
0.04
-0.01
-0.06
2.40
2.60
2.80
3.00
3.20
3.40
3.60
3.80
Chick Wing Growth (mm/d)

Figure 2

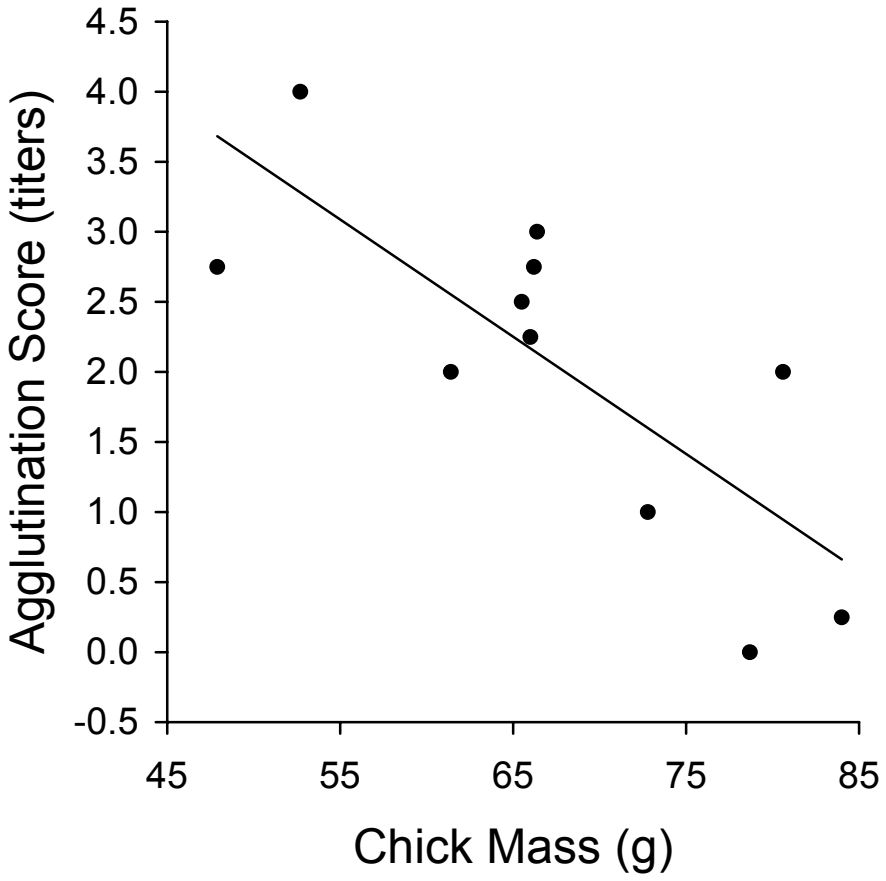
Agglutination Score (titers)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
45
55
65
75
85
Chick Mass (g)

Figure 3

Table 1. All possible models involving seasonal effect (Julian Hatch Date of chick; JD), food delivery to nest (Maximum Mass attained by chick; MaxMass), chick growth rate (Wing Length Slope between day 20 and day 50; WLS) and initial titer (NAb_{20}). Models are ranked by ΔAIC_c after Burnham and Anderson (Burnham & Anderson, 1998). #Par represents the number of parameters included in the model.

Rank	Variables included	#Par	AIC	AIC_c	ΔAIC_c	ω_i
1	WLS	1	-57.96	-55.29	0.00	0.411
2	WLS, NAb_{20}	2	-58.59	-53.59	1.70	0.175
3	NAb_{20}	1	-56.21	-53.55	1.74	0.172
4	WLS, JD	2	-56.33	-51.33	3.96	0.057
5	WLS, MaxMass	2	-56.06	-51.06	4.23	0.050
6	MaxMass	1	-52.84	-50.18	5.11	0.032
7	JD	1	-52.30	-49.63	5.66	0.024
8	NAb_{20}, JD	2	-54.63	-49.63	5.66	0.024
9	MaxMass, NAb_{20}	2	-54.23	-49.23	6.06	0.020
10	WLS, MaxMass, NAb_{20}	3	-57.18	-48.61	6.68	0.015
11	WLS, NAb_{20}, JD	3	-56.61	-48.04	7.25	0.011
12	MaxMass, JD	2	-51.32	-46.32	8.96	0.005
13	WLS, MaxMass, JD	3	-54.33	-45.76	9.53	0.003
14	MaxMass, NAb_{20}, JD	3	-52.66	-44.09	11.20	0.002
15	Full Model	4	-55.45	-41.45	13.84	0.000

Table 2. Akaike variable weights ($\square_I$) calculated after Burnham and Anderson (Burnham & Anderson, 1998) from all possible models (Table 1) involving these four variables. *N* = 13 for all variables, except MaxMass and Initial Titer (NAb_{20}) for which *N* = 11.

Variable	X ± SE	$\square_i$
WLS	3.13 ± 0.08 mm d^{-1}	0.721
NAb_{20}	0.75 ± 0.4 g	0.418
JD	260.8 ± 2.8 d	0.126
MaxMass	81.6 ± 2.5 g	0.126

Chapter 7

Variation in the innate and acquired arms of the immune system among five shorebird species.

In press: L. Mendes, T. Piersma, D. Hasselquist, K. D. Matson & R. E. Ricklefs.

Journal of Experimental Biology.

Summary: To contribute to an understanding of the evolutionary processes that shape variation in immune responses, we compared several components of the innate and acquired arms of the immune system in five related, but ecologically diverse, migratory shorebirds (ruff Philomachus pugnax Linnaeus, 1758; ruddy turnstone Arenaria interpres Linnaeus, 1758; bar-tailed godwit Limosa lapponica Linnaeus, 1758; sanderling Calidris alba Pallas, 1764; red knot C. canutus Linnaeus, 1758). We used a hemolysis-hemagglutination assay in freeliving shorebirds to assess two of the innate components (natural antibodies and complementmediated lysis), and a modified quantitative enzyme–linked immunosorbent assay in birds held in captivity to assess the acquired component (humoral antibodies against tetanus and diphtheria toxoid) of immunity. Ruddy turnstones showed the highest levels of both innate and acquired immune responses. We suggest that turnstones could have evolved strong immune responses because they scavenge among rotting organic material on the seashore, where they might be exposed to a particularly broad range of pathogens. Although ruffs stand out among shorebirds in having a high prevalence of avian malaria, they do not exhibit higher immune response levels. Our results indicate that relationships between immune response and infection are not likely to follow a broad general pattern but instead depend on type of parasite exposure, among other factors.

1. INTRODUCTION

The immune system is one of the most important defense mechanisms of vertebrates for protection against pathogens and parasites (e.g.; see Zuk and Stoehr, 2002; Schmid-Hempel, 2003). Besides the obvious benefits, immune responses also convey costs, including greater risk of autoimmune disease (Råberg et al., 1998; Finch and Crimmins, 2004) and the depletion of energy that could otherwise be used in other activities (Nelson et al., 2002). Such costs, which potentially even reduce survival (Hanssen et al., 2004), will mould the evolution of the immune defence (e.g. Råberg et al., 2000). Therefore, maximising parasite resistance must be balanced by minimising damage to the host (Råberg et al., 1998; Segel and Bar-Or, 1999). This benefit/cost balance should depend on environmental conditions. For instance, relative benefits will increase with parasite density or parasite diversity (Råberg, 2002), while in habitats with high rates of infection, repeated activation of the immune response might select for strategies that minimise the risk of collateral damage and place a premium on optimising the immune responses (Segel and Bar-Or, 1999). The balance between benefit and cost is likely to lead to variation in immune response, and indeed, within individuals of the same species, the immune function can vary with sex, age and season (Hasselquist et al., 1999; Duffy et al., 2000; Lorenzo and Lank, 2003; Nelson et al., 2002). In comparisons between species, immune response variation may also reflect the optimization of phenotype responses to the environment (Ricklefs and Wikelski, 2002); variation among species might thus represent phenotypic plasticity or genotype-environment interactions.

In vertebrates, the immune system consists of two arms, a non-specific, innate arm and a more specific, acquired arm (Male and Roitt, 2000; Doan et al., 2005). The innate immune system provides initial protection to a wide variety of foreign organisms. The acquired immune system confers delayed, but more specific, protection against foreign antigens; in the blood stream it acts through specific antibodies that attach to its

target pathogen. Higher levels of one component of the immune system need not imply greater overall resistance (Adamo, 2004); hence one should strive to assay the different parts of the immune system. In the present study, we collected several measurements of both the innate and the acquired (humoral) arm of the immune system.

Migratory shorebirds share many of the life-history traits that are thought to correlate with well-developed immune response, such as low reproductive rate and relatively long life span (Tella et al., 2002). However, this group of birds also varies with respect to migration strategy, habitat choice, and foraging style (Piersma, 2003). While migration strategies might affect immune response through competition for limited energy resources (Piersma, 1997; Møller and Erritzøe, 1998), habitat choice also can create differences in disease risk (Moore, 2002; Mendes et al., 2005). In effect, while positive relationships between disease risk and immune response have been found in several studies (Lindström et al., 2004; Apanius et al., 2000), the relationship between migration and immunity may prove to be more difficult to uncover.

In this study, we use a combination of immunological assays that measure different branches of the immune system (innate as well as acquired) in a comparative and experimental study of five related Scolopacidae, including four Arctic-breeding and coastal wintering species: red knot *Calidris canutus* Linnaeus, 1758; bar-tailed godwit *Limosa lapponica* Linnaeus, 1758; sanderling *Calidris alba* Pallas, 1764; and ruddy turnstone *Arenaria interpres* Linnaeus, 1758; and the temperate-breeding ruff *Philomachus pugnax* Linnaeus, 1758. Unlike the other species, the ruff is confined to freshwater wetlands year-round. Ruddy turnstones breed at more southerly latitudes than the other marine wintering species and they routinely scavenge among human and other refuse along seashores (Piersma et al., 1996). Among coastal shorebirds, ruddy turnstones seem to be particularly affected by wildlife diseases (Hansson, 2003), as are species using freshwater habitats in the tropics, such as ruff (Mendes et al., 2005).

2. MATERIAL AND METHODS

Wild shorebirds were caught along the East Atlantic flyway (Smit and Piersma, 1987; van de Kam et al., 2004). Coastal/marine shorebirds were caught at night with mistnets in the Parc National du Banc d'Arguin, northern Mauritania, ca. 20°N, 16°W, during November-December 2002 and in the western Wadden Sea, The Netherlands, 53º, 5ºE, between 1999 and 2002 during northward and southward migration, and also during winter. In addition, we captured birds during the day using so-called 'wilsternets' (see Jukema et al., 2001) in the meadows of the Dutch province of Fryslân (ca. 53º N, 5º 30'E) in April-May 2002. In total, we caught 54 red knots, 33 sanderlings, 15 ruddy turnstones, 8 bar-tailed godwits, and 12 ruffs. Birds captured with wilsternets were bled within ca. 10 min after capture, those captured in mistnets within ca. 3 hrs.

Individuals of all five species to be held in captivity were caught in The Netherlands during the nonbreeding season. Three species were caught with mistnets at night during southward migration in the western Wadden Sea (53º 16'N; 5º 08'E): 10 red knots of the African wintering subspecies *C. c. canutus* and 11 sanderlings in July-August 2001, and two sets of ruddy turnstones, the first group with 24 individuals during August 2001 and the second with 11 individuals during November 2002, after post-breeding moult in the Wadden Sea (Meltofte et al., 1994). Fourteen bar-tailed godwits and 10 ruffs were trapped with wilsternets in daytime during northward migration (Jukema et al., 2001). The bar-tailed godwits were caught in meadows on the island of Texel (53º 05'N, 4º 75'E) in May 2001, and the ruffs, in the province of Fryslân during April-May 2003. All birds were individually ringed, measured, weighed, and aged as being in their first year of life or older on the basis of plumage characteristics (Prater et al., 1977).

Measuring immune responses

We chose assays to examine both the innate and the acquired arms of the immune system. Innate immunity was investigated in free-living individuals by measuring two of

its most important components, i.e. natural antibodies and the complement cascade (Matson et al., 2005). Natural antibodies recognise and attach to invading organisms and are also responsible for initiating the complement cascade (Ochsenbein and Zinkernagel, 2000). The complement cascade recognises and kills extracellular foreign organisms (Wilson et al., 2002). To assess the acquired immune response, we challenged wild birds kept under identical aviary conditions with two antigens widely used in immunoecology studies, i.e. tetanus and diphtheria toxoid (inactivated toxin; e.g. Svensson et al., 1998; Råberg et al., 2003; Hanssen et al., 2004). In this study, we considered separately antibody binding before vaccination and after primary and secondary immune responses because these involve different mechanisms and molecules (Doan et al., 2005). In the humoral immune response, specific antibodies are responsible for neutralizing the intracellular pathogens by blocking cell binding/entry and preventing the spread of pathogenic organisms; they also neutralize toxins produced by bacteria such as diphtheria and tetanus (Roitt et al., 2000).

Hemolysis-hemagglutination assay in free-living shorebirds

A blood sample of ca. 160 μl was obtained by puncturing the brachial vein of wild shorebirds with a sterile 23-gauge needle; blood was collected in two 80 μl heparinized microhematocrit capillary tubes. Samples were stored on ice and were centrifuged for 10 minutes at 6.900 g within two hours. Plasma was stored at –20 ºC until analysis at the University of Missouri-St. Louis.

To estimate the levels of circulating natural antibodies and complement we used the hemolysis-hemagglutination assay described in detail by Matson et al. (2005). The agglutination reaction measures the interaction between natural antibodies and antigens, which results in blood clumping. The lytic reaction measures the amount of hemoglobulin released from the lysis of exogenous erythrocytes (e.g. rabbit), which is a function of the

amount of lytic complement proteins present in the sampled blood. In both cases, quantification is achieved by serial dilution of plasma samples and assessment of the dilution step at which either the agglutination or lysis reaction stopped. For this assay, we placed 25 μl of plasma in 6 of the 8 wells of the first row of a 96-well polysterene plate (8 columns by 12 rows) (Corning Costar # 3795). The same amount of 0.01 *M* sterile phosphate solution (PBS; Sigma #P3813, St Louis, MO) was set in the first well to serve as the negative control; 25 μl of plasma of a well known high responder (a chicken standard sample) was added to the last well as a positive control. Next, we used a multi-channel pipette to dilute with PBS all 6 plasma samples, the negative control and the positive standard sample up to 1:1024, through a set of ten 1:2 serial dilutions. After the addition of 25 μl of 1% of rabbit blood cell suspension to each well, each plate was sealed with a polystyrene plate lid. Plates were vortexed for 10 sec at a low speed, and set to incubate at 37 ºC for 90 min. After incubation plates were tilted at a 45º angle along their long axis for 20 min at room temperature, plates were scanned (Microtek Scanmaker 5900) using the positive transparency (top-lit) option and a full size image (300 dpi). We then quantified agglutination (which gives a measure of natural antibody levels) and complement-mediated lysis by assessing the dilution stage (on a scale from 1 to 12) at which these two reactions stopped (for further details, see Matson et al., 2005).

Humoral immune assays on wild birds held in captivity

With the exception of the 24 ruddy turnstones caught during August 2001 that were challenged with antigens five months after capture, all other birds were challenged within a month of capture.

To avoid the possibility of confounding effects of sex and age on the immune response, we attempted to restrict our experimental animals to adult females. Upon

capture we selected bar-tailed godwits with the longest bills (Piersma and Jukema, 1990), red knots and sanderlings with long bills and the clearest brood patches (Nebel et al., 2000), and small-sized ruffs (van Rhijn, 1991). There are no external criteria for distinguishing female ruddy turnstones, and therefore we determined sex by a molecular PCR-DNA technique verified for red knots (Baker et al., 1999), and tested for sex and age differences in the group with enough individuals to compare between sexes or ages, the first group of ruddy turnstones (9 males and 15 females; 10 adults and 14 juveniles). We found no differences in diphtheria antibody levels between males and females or between first year and older birds (sex: repeated measures ANOVA: $F_{1, 20}$=0.13; p=0.73; age: $F_{1, 20}$=0.29; p=0.60; sex*age: $F_{1, 20}$=1.22; p=0.28) or in tetanus antibody levels (sex: repeated measures ANOVA: $F_{1, 20}$=0.11; p=0.75; age: $F_{1, 20}$=0.63; p=0.44; sex*age: $F_{1, 20}$=0.42; p=0.52). Therefore, in the context of interspecific comparisons, sex and age differences in antibody production are probably negligible.

Birds were kept in single-species flocks in large aviaries at the Royal Netherlands Institute for Sea Research (NIOZ) under the ambient natural light: dark cycle. The size of the aviaries, which had running fresh water and seawater ranged from 1 m by 3 m and 2.5 m high, to 7 m by 7 m and 3.5 m high. Bar-tailed godwits, red knots, sanderlings and ruddy turnstones were fed trout pellets *ad libitum*, and ruffs also received mealworms *Tenebrio* sp. By two weeks after capture, body mass had stabilised and we presumed that birds had acclimated to captivity. At the time of testing, body masses as a percent of the level at capture were 81% ± 11% for bar-tailed godwits (mean capture mass = 316 g, n = 14), 87% ± 11% for red knots (mean = 137 g, n = 10), 86% ± 14% for sanderlings (mean = 52 g, n = 11), 90% ± 15% for the first group of ruddy turnstones (mean = 117 g, n = 24), 98% ± 14% for the second group of ruddy turnstones (mean = 114 g, n = 11), and 98% ± 8% for the ruffs (mean =108 g, n=10).

Primary immune responses were elicited through vaccination with 120 µl of the combined tetanus and diphtheria toxoid in the pectoral muscle with a 0.5 ml sterile syringe (see Hasselquist et al., 2001 for further details of procedures). Secondary immune responses were elicited through a second vaccination with 100 µl of the same vaccine combination. Blood samples were taken prior to the first injection, and with the exception of the second group of ruddy turnstone which were sampled one week later, at day 14 after the first injection and day 7 after the second injection, respectively (Feldman, 2000; Hasselquist et al., 1999, 2001; Owen-Ashley et al., 2004). Blood was centrifuged for 12 min at 6.900g and the plasma preserved at -30º C until analysis.

Antibody levels against tetanus and diphtheria toxoid were determined by using a modified quantitative enzyme–linked immunosorbent assay (ELISA, Hasselquist et al., 2001). Individual polysterene 96-well plates (Costar) were coated with either a diphtheria toxoid or with a tetanus toxoid (both diluted to 3 µg/ml with 0.15 M of carbonate buffer, at pH 9.6) and left to incubate overnight at 4º C. After washing three times with a buffer (0.01 M PBS with 0.05% Tween 20), all plates were blocked with 3% milk powder, diluted in the same buffer, for 2 h at room temperature. Plates were then washed twice and 100 µl of a 1:1600 diluted plasma sample was added (plasma was diluted in a 1:2 serial dilution with 1% milk powder mixed in PBS/Tween20) and left incubating overnight at 4º C. After three buffer washes, 100 µl of a 1:1000 diluted rabbit anti-passerine Ig antibody (produced against redwinged blackbird *Agelaius phoeniceus* antibodies; Hasselquist et al., 1999) was added to the wells and left to incubate for 1 h at 37º C. Plates were washed again two times and a diluted peroxidase-labelled goat anti-rabbit antibody (Cat. A 6154, Sigma) was added and incubated for 30 min at 37º C. Plates were washed twice and thereafter the substrate solution [200 µl of 0.2mM ABTS (Cat. A 1888, Sigma) and 80 µl of 30% H_2O_2 (diluted 1:40 in distilled H_2O) mixed in 20ml of citrate buffer (pH 4.0)] was added to achieve colour reaction. We used a Vmax

microplate reader (Molecular Devices, Sunnyvale, CA, USA) to read the kinetics of colour reactions at 405 nm every 30 s for 14 min. Calculation of antibody titers was based on the slope of the substrate conversion, in millioptical density units/min (mOD/min).

Statistical analysis

All samples from the specific antibody measurements were run in duplicate. Repeatability (intersample variability) was estimated as a percentage of the total variability; interplate variability was based on the series of diluted reference samples (1:600 to 1:76800) run on each plate. Intersample variability was 2% and interplate variability was 16%. We used the average values of the duplicate samples in all analyses. To account for interplate variation we adjusted all values to be comparable with a reference plate, using plasma from one red knot (known to be a high responder) as reference sample on all plates.

Natural antibody data were $\log_2$ transformed, to achieve normality (samples were 1:2 serial diluted). We tested for interspecific differences in natural antibody levels with analysis of covariance (ANCOVA), in which body mass entered as a covariate. Complement activity data was not normally distributed, and therefore we used Kruskal-Wallis (multiple species) and Kolmogorov-Smirnov tests (two species), to test for interspecific differences (Sokal and Rohlf, 1995).

Humoral antibody titers were $\log_{10}$-transformed to normalize the residuals (Sokal and Rohlf, 1995). We accounted for the unwanted variability caused by interspecific differences in body mass, by using an analysis of covariance (ANCOVA), in which body mass was entered as a covariate. Furthermore, to identify which species exhibited the highest antibody response, we performed a posthoc Tukey test.

To investigate whether immune responses exhibit a general pattern, we correlated the different immune measurements at the individual and the species level. We used the

parametric Pearson correlation coefficient to determine the relationships between complement activity and natural antibody levels (innate components) and between tetanus and diphtheria humoral response (acquired components). Because the innate and acquired measurements were taken in different individuals, we used Spearman rank correlations to see whether species average response values correlated among and between the two arms of the immune system. All tests were performed in SYSTAT 9 for Windows.

3. RESULTS

Natural antibodies and complement activity of wild birds

Natural antibodies levels only differed among species when we corrected for body mass (ANCOVA: species $F_{4,121}$= 1.41; P=0.23, body mass $F_{1,121}$= 1.24; P=0.27; species×body mass $F_{4,121}$= 2.63; P=0.04; Fig. 1). The level of complement activity varied significantly among species (Kruskal-Wallis U=43.36, df=4; p=0.00, Fig. 1). The non-parametric Kolmogorov-Smirnov test revealed that ruddy turnstones had the highest level of complement-mediated lysis (all species: $p<0.05$; see also Fig. 1).

Humoral immune assays on wild birds held in captivity

The two groups of ruddy turnstones differed with respect to diphtheria pre-vaccination antibody levels (ANCOVA: trial $F_{1,31}$= 6.40, p=0.02; body mass $F_{1,31}$= 2.06, p=0.16) and tetanus primary immune response (ANCOVA: trial $F_{1,31}$= 4.92, p=0.03; body mass $F_{1,31}$= 0.15, p=0.70), but not with respect to the primary immune response against the diphtheria toxoid (ANCOVA: trial $F_{1,31}$= 0.92, p=0.35; body mass $F_{1,31}$= 0.41, p=0.53), or the secondary immune response (ANCOVA: trial $F_{1,31}$= 0.84, p=0.37; body mass $F_{1,31}$= 0.13, p=0.73). The same was true for the pre-vaccination (ANCOVA: trial $F_{1,31}$= 0.29, p=0.60; body mass $F_{1,31}$= 0.27, p=0.61) and secondary antibody titers against the

tetanus antigen (ANCOVA: trial $F_{1,31}$= 0.05, p=0.82; body mass $F_{1,31}$= 0.22, p=0.65). Although the absolute magnitudes of these differences were small compared to the differences between the shorebird species (Fig. 2), we nonetheless included only the group of ruddy turnstones that were challenged within a month of capture in the interspecific analysis.

All species responded positively to vaccination by producing antibodies against the diphtheria toxoid (Repeated Measures ANOVA: ruff $F_{2,18}$= 17.96, p=0.00; ruddy turnstone $F_{2,16}$= 111.39, p=0.00; bar-tailed godwit $F_{2,26}$= 12.39, p=0.00; sanderling $F_{2,20}$= 8.93, p=0.00; red knot $F_{2,18}$= 10.11, p=0.00) and the tetanus toxoid (Repeated Measures ANOVA: ruff $F_{2,18}$= 26.37, p=0.00; ruddy turnstone $F_{2,16}$= 81.26, p=0.00; bar-tailed godwit $F_{2,26}$= 18.26, p=0.00; sanderling $F_{2,20}$= 14.44, p=0.00; red knot $F_{2,18}$= 23.92, p=0.00) (see also Fig.2).

Diphtheria antibody levels differed between species, even before vaccination (ANCOVA: species $F_{4, 49}$= 4.54, p=0.00; body mass $F_{1, 49}$= 0.07, p=0.79). The interspecific differences in diphtheria antibody levels widened during the primary (ANCOVA: species $F_{4, 47}$= 6.23, p=0.00; body mass $F_{1, 47}$= 0.09, p=0.77) and the secondary immune responses (ANCOVA: species $F_{4, 47}$= 16.92, p=0.00; body mass $F_{1, 47}$= 2.95, p=0.09). In contrast, tetanus antibody levels did not differ between species, either before vaccination (ANCOVA: species $F_{4, 49}$= 1.06, p=0.39; body mass $F_{1, 49}$= 0.29, p=0.59), or during the primary immune response (ANCOVA: species $F_{4, 47}$= 0.90, p=0.47; body mass $F_{1, 47}$= 0.06 p=0.82), but they did differ during the secondary immune response (ANCOVA: species $F_{4, 47}$= 9.94, p=0.00; body mass $F_{1, 47}$= 2.68, p=0.11). Post hoc Tukey tests revealed that the ruddy turnstone had (in the case of diphtheria), or developed (in the case of tetanus), higher antibody levels to the same amount of vaccine than the other species. Pre-vaccination, primary, and secondary antibody levels against

diphtheria and secondary antibody levels against tetanus did not differ among the other species (see also Fig. 2).

Relation between the different immune measurements

The two innate components measured in this study, i.e., natural antibody level and complement-mediated lysis, were not correlated (r= 0.09, n=127, p=0.17), but the two measurements of the acquired arm of the immune system (antibody titers against diphtheria and tetanus) were positively correlated during pre-injection (r= 0.66, n=44, p=0.00), primary response (r= 0.63, n=54, p=0.00) and especially secondary immune response (r= 0.82, n=55, p=0.00).

Even though the correlations between innate and acquired immune components were based on the data points for the five species and were never significant at the 5% level, there was a tendency for a positive correlation between natural and background antibodies against diphtheria and between complement activity and secondary tetanus antibody titers (Table 1).

4. DISCUSSION

Although we found considerable interspecific variation in both innate and humoral immune components, differences were most pronounced for complement-mediated lysis and primary and secondary humoral immune response. This result suggests that not all immune components are under the same pressure to be internally regulated. Indeed, the levels of natural antibodies varied little, even among species with such different body masses as the sanderling and the bar-tailed godwit. This is consistent with the idea that natural antibody production is largely independent of internal and external stimuli (Ochsenbein and Zinkernagel, 2000). However, although natural antibodies are present in relatively low densities, they play an important role in the initial recognition of foreign particles and they support subsequent defense by the complement cascade and the

acquired humoral response (Ochsenbein and Zinkernagel, 2000; Turner, 2000). Therefore, organisms may benefit by maintaining a minimum level of immunoglobulins, as these molecules likely convey benefits in terms of earlier detection of parasites. With respect to the innate immune system, we found no difference between the five shorebird species in natural antibody levels, whereas ruddy turnstones showed a higher complement system activity than the four other species. For the humoral responses of the acquired immune system, pre-injection, primary, and secondary antibody titers against diphtheria toxoid and secondary antibody titers against tetanus were higher in ruddy turnstones, whereas there were no differences in antibody responses between any of the other shorebird species.

The hemolysis-hemagglutination assay measurements of natural antibodies and complement activity were well within the range of values found for other bird groups (Matson et al., 2005). With respect to the ELISA assay of antibody levels against tetanus and diphtheria, we found that the primary and secondary antibody titers in all five shorebird species were significantly higher than pre-injection values. Hence, despite the ELISA being designed for passerine birds, it apparently works well also in shorebirds. Among all five shorebird species, antibody responses against diphtheria were lower than those against tetanus, which is in accordance with other studies on wild birds (e.g. Westneat et al., 2003; Owen-Ashley et al., 2004).

We did not find any correlation between the two innate components (natural antibody level and complement-mediated lysis), nor between innate and acquired components. This result underlines the problem of obtaining a ‘general’ measure of immunocompetence and emphasizes the importance of measuring different aspects of the immune system (Adamo, 2004; Matson et al, in press). There was a tendency for a relationship between natural antibodies and background antibody titers, which suggests

that they both might reflect the basic level of (polyclonal) natural antibodies in the circulation.

Ruddy turnstones stand out as high responders in three of the four immune measurements taken (complement mediated lysis, humoral responses to tetanus and diphtheria toxoid). This difference is not likely to be explained by phylogeny because turnstone's closest relatives (sanderling, red knot, and ruff) were as low responders as the more distantly related bar-tailed godwit (see Piersma et al., 1996). Thus, the high responder is embedded in a clade of low responders in our study, and presumably evolved from a low-response state. Furthermore, neither habitat choice per se, nor migration strategy can explain the exceptionally strong immune responses observed in the ruddy turnstone, since this species shares coastal wetlands and long-distance migration with other low responders, such as the bar-tailed godwit, the sanderling, and the red knot. Ruddy turnstones do stand out, however, by their scavenging habits. They often feed on decomposing food remains, including dead fish and mammals (Piersma et al., 1996), and as a consequence they are often found close to human settlements, e.g. in harbours, where they are likely to benefit from an abundance of such food items. This opportunistic feeding style might expose them to infections, particularly diseases that are transmitted by contaminated dead animals, e.g. Avian Cholera or Herpes virus (Friend and Franson, 2001). Indeed, in the eastern USA, ruddy turnstones carried 67.5% of Avian Influenza Virus (AIV) infections, even though they accounted only for 12.4% of 2162 individuals from 15 different shorebird species in a study by Hansson (2003).

We suggest that in the nonbreeding season ruddy turnstones might be exposed to a particularly broad range of disease organisms, and that they therefore require high responsiveness in several parts of the immune system. A similar conjecture was made for populations of the Darwin's finch *Geospiza fuliginosa*, in which islands with the highest prevalence of avian pox and feather mites supported host populations with the

highest natural and humoral immune responses (antibody levels) (Lindström et al., 2004).

It is perhaps surprising that ruffs exhibited low levels of immune response, as they occur in inland freshwater habitats where the likelihood of avian malaria infection is high (Mendes et al., 2005). This environment presumably would select ruffs to invest strongly in their immune systems (Piersma, 1997), but this hypothesis was not supported here. Note, however, that we did not measure cell-mediated immunity, a type of response known to be involved in the control of malaria parasites (Wakelin, 1998; Doan et al., 2005).

To the best of our knowledge, this is the first time that a suite of immune system measures has been applied to shorebirds in a comparative study of immunocompetence between species. In brief, our findings emphasize the need to study several immune components, preferably from different arms of the immune system when assessing "general immunocompetence." Furthermore, we suggest that the relationships between immune response and infection patterns are particular, rather than general, and depend strongly on the range and strength of exposures and the precise variety of parasite types.

Acknowledgements. Isabel Magalhães and Casper Kraan helped with the care of the captive birds. Shorebirds were captured by Bernard Spaans, Joop Jukema, Catharinus Monkel and Kees Oosterbeek. Dick Visser produced the final graphics. Wouter Vahl and anonymous referees gave valuable comments on the manuscript. This study was financed by PRAXIS XXI grant (BD/19939/99) from Fundação para a Ciência e Tecnologia (FCT), Portugal, to LM, by a PIONIER-grant of the Netherlands Organisation for Scientific Research (NWO) to TP, by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Formas), the Carl Trygger Foundation, and

the Crafoord Foundation to DH, by NSF grant IBN-0212587 and a Saint Louis Zoo grant FRC#03-13 to KDM and by NSF grants DEB-0089226 and IBN-0212587 to RER.

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Table 1. Spearman rank correlation coefficients (r_S) based on humoral immune response values, calculated from the species averages (n=5). All correlations were positive, but none were significant at the 5% confidence level; when $r_S>0.7$, then $0.05<p<0.1$.

	Diphtheria			Tetanus		
	background	primary	secondary	background	primary	secondary
Natural antibodies	0.7	0.1	0.1	0.5	0.4	0.3
Complement-mediated lysis	0.3	0.1	0.4	0.4	0.1	0.7

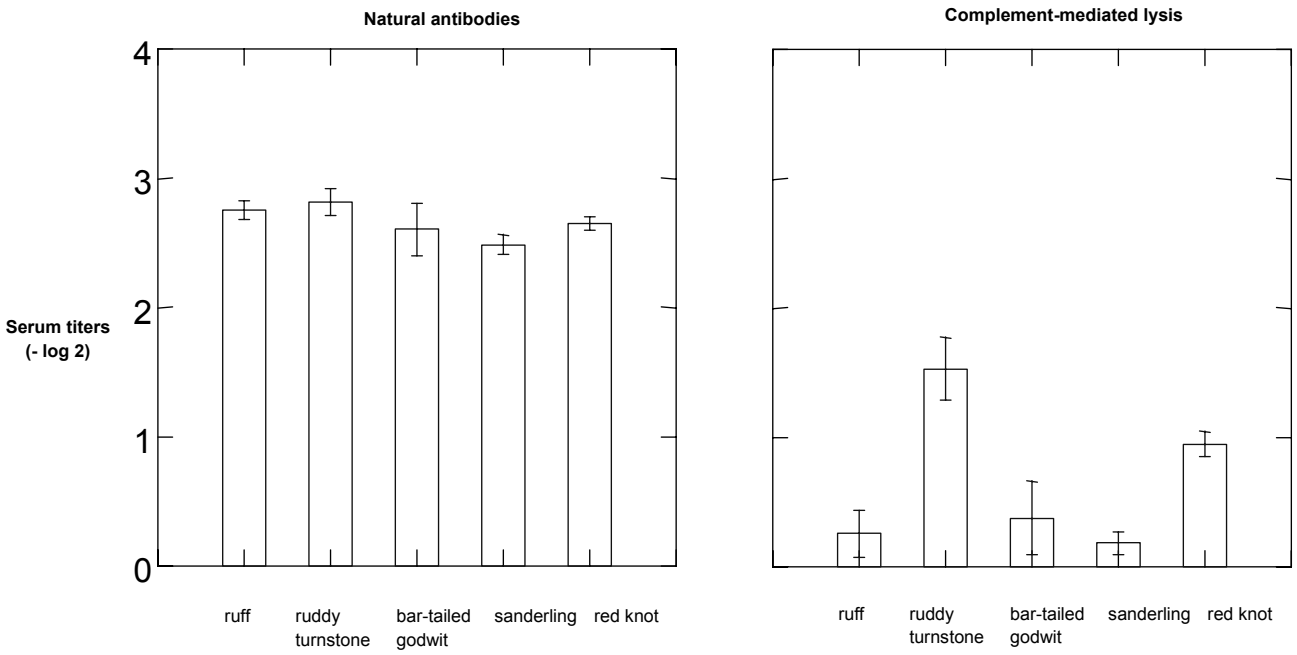

Fig. 1. Natural antibody levels and complement-mediated lysis in five species of shorebirds, estimated the $\log_2$ transformation of the score of the 1:2 serial dilution of the shorebirds sera. Natural antibodies level was calculated at the step agglutination stops and complement at the step at which lysis stops. Error bars represent standard errors.

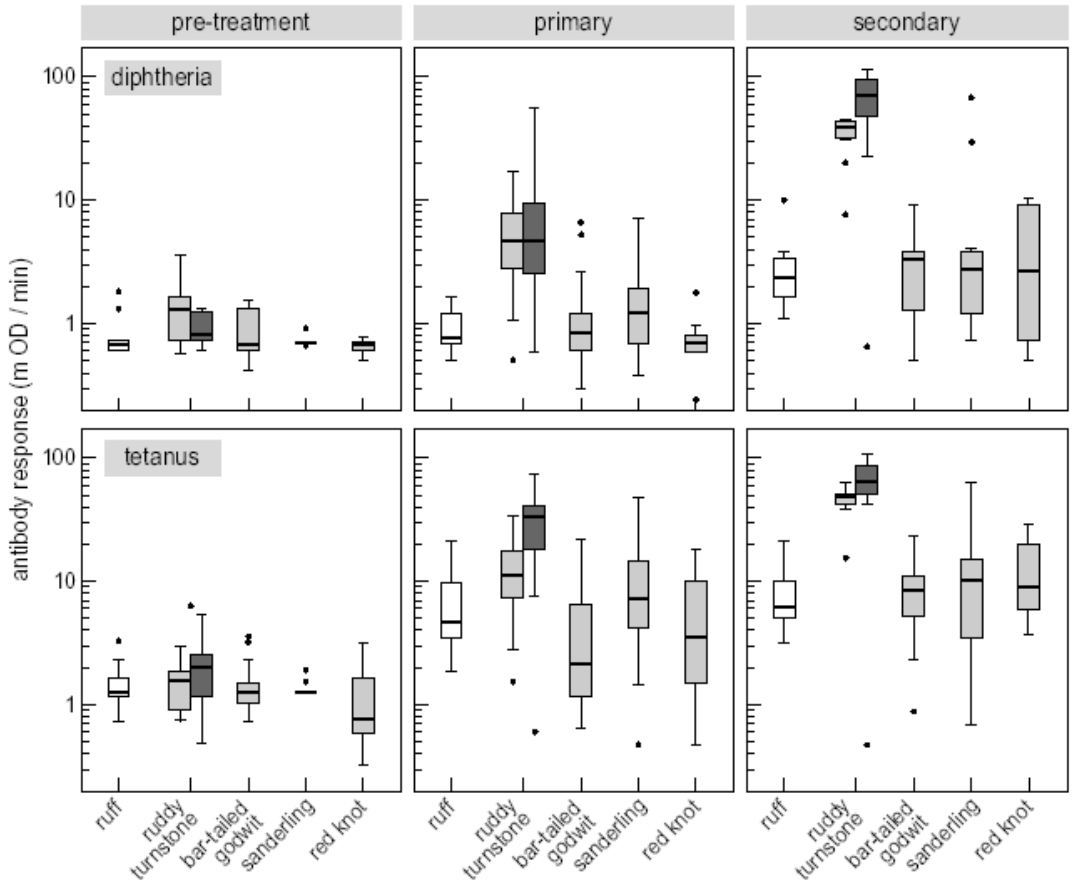

Fig. 2. Antibody titers before and after (repeated) vaccination with tetanus and diphtheria toxoids in five shorebird species. For the ruddy turnstone, the dark box represents the experiment where birds were tested five months after capture. The ruff, the only freshwater specialist, is indicated by a white box.