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Capture Stress and the Bactericidal Competence of Blood and Plasma in Five Species of Tropical Birds

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ABSTRACT

In wild birds, relatively little is known about intra- or interspecific 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 nonpathogenic 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 exhibiting 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 affects 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 that are 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. Thus, the bacteria-killing assay overcomes many of the limitations commonly associated with measuring immune function. In particular, unlike some other assays, its 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 five 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 (enzyme-linked immunosorbent assay) or agglutination to quantify specific antibodies produced following vaccination with a novel, noninfectious antigen (e.g., keyhole limpet hemocyanin; 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 nonspecific reactions, injection of PHA

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integrates widely over numerous immune components. Widely integrative assays are advantageous in some situations, particularly in comparative studies where measurements 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 h 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 probably 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 (Råberg 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 organisms (e.g., birds [Gross and Siegel 1973, 1983; Regnier et al. 1980; 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 of 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, stress can also enhance immunity in some instances (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 vs. 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 because of 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. However, making interspecific predictions that relate particular immune functions to other parameters (e.g., life-history variables) 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 nonspecific 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 end product (Esser 1994) or through protein by-products (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 as 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 multifaceted 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.

Material and Methods

Subjects and Samples

Between March 26 and April 28, 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 in a sterile manner from a wing vein two times: once within 3 min of the subject's first striking the net (t_0) and once 60 min later (t_{60}). During the 60-min period between bleeds, we held all birds in small fabric bags in an air-conditioned room (~21°C). Plasma samples collected within 3 min reflect baseline levels of the stress hormone corticosterone, while samples collected after 1 h 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 s, we collected blood into sterile

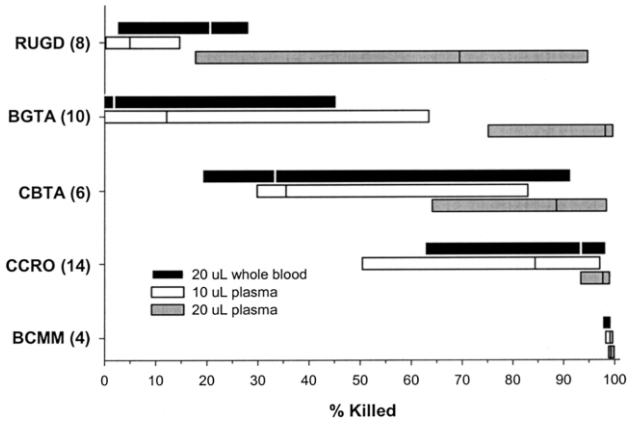

Figure 1. Box plots show among species and among sample-type variation in the bacteria-killing abilities (untransformed percentages) of five species of birds (BCMM = blue-crowned motmot, *Momotus momota*; BGTA = blue gray tanager, *Thraupis episcopus*; CBTA = crimson-backed tanager, *Ramphocelus dimidiatus*; CCRO = clay-colored robin, *Turdus grayi*; and RUGD = ruddy ground dove, *Columbina talpacoti*). The low end of the range identifies the twenty-fifth percentile, the high end is the seventy-fifth percentile, and the intermediate point is the median. The numbers in parentheses indicate the sample sizes.

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 parameterizing assay variables, we also collected blood from 3-wk-old Cobb chicks (*Gallus domesticus*), utilizing similar sterile techniques. All protocols were approved by the animal care committees at the University of California, Davis, or the University of Missouri–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-d 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 (catalog no. 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 *E. coli* working culture. This *E. coli* (ATCC 8739) culture was prepared from lyophilized pellets (3.1×10^7 CFUs per pellet; Epower Microorganisms no. 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 subsamples 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°C for 30 min during which the processes of the bacterial culture (growth and division) and immune components (stasis and killing) were allowed to interact. 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 min), we covered and inverted the plates and incubated them overnight at ambient temperature (~27°C). The next day we counted the number of viable colonies and determined the proportion of colonies killed by comparison with 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 that required a sterile environment.

In conjunction with the development of this protocol, we also measured the effect of whole blood concentration on killing bacteria, 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-decomplemented 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 between 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 before 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 ($n = 5$), sample type (three types: 10 μL plasma, 20 μL plasma, 20 μL blood), and stress time point (0, 60 min) as independent variables. We included in the analysis only individuals for which all six values (three sample types at two 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 affected sample size; however, 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 five study species and depended on sample type (Fig. 1). Because two-way

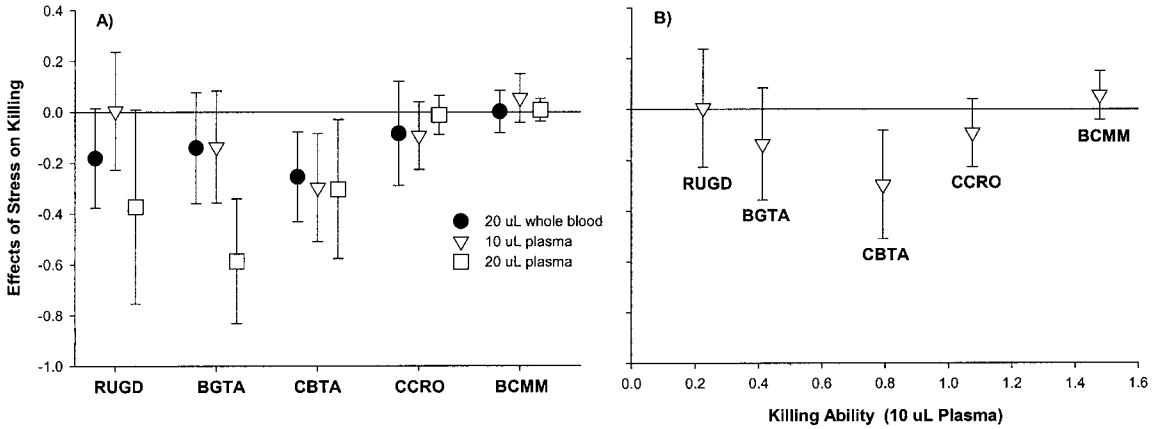

Figure 2. Stress from capture and holding generally results in subtly reduced in vitro bacteria-killing abilities. The magnitude of the observed stress effects depends on species and sample type (*A*) and on prestress ability to kill bacteria (*B*). In both *A* and *B*, the *Y*-axes represent the differences between the pre- and poststress 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.

and three-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 generalized linear model (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% $\pm$ 0.5% (SE) in the RUGD to 99.1% $\pm$ 0.0% in the BCMM ($F = 15.7$, df $= 4,37$, $P < 0.0001$). Using 20 μL plasma, the percentage of bacteria killed ranged from 64.3% $\pm$ 2.7% in the RUGD to 99.6% $\pm$ 0.1% in the BCMM ($F = 3.8$, df $= 4,37$, $P = 0.011$). Using 20 μL whole blood, the percentage of bacteria killed ranged from 7.7% $\pm$ 1.2% in the BGTA to 99.2% $\pm$ 0.1% in the BCMM ($F = 15.9$, df $= 4,37$, $P < 0.0001$; Fig. 1).

Sample Type Comparisons

Bacteria killing varied more among sample types in some species than in others (Fig. 1). Again, because of the significant two- and three-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 three sample types varied significantly in all species (BGTA, $F = 43.2$, df $= 2,18$, $P < 0.0001$; CBTA, $F = 18.5$, df $= 2,10$, $P < 0.001$; CCRO, $F = 9.2$, df $= 2,26$, $P = 0.001$; and RUGD, $F = 23.2$, df $= 2,14$, $P < 0.0001$), except the BCMM ($F = 1.0$, df $= 2,6$, $P = 0.432$; Fig. 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. Pairwise 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, $F = 61.3$, df $= 1,9$, $P < 0.0001$; CBTA, $F = 44.9$, df $= 1,5$, $P = 0.001$; CCRO, $F = 16.6$, df $= 1,13$, $P = 0.001$; and RUGD, $F = 25.4$, df $= 1,7$, $P = 0.002$), except the BCMM ($F = 5.8$, df $= 1,3$, $P = 0.095$).

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 three of the five species, the stress of capture and handling had significant negative effects in one or more sample type (Fig. 2*A*). 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 ($F = 12.8$, df $= 1,5$, $P = 0.016$), 20 μL plasma ($F = 8.1$, df $= 1,5$, $P = 0.036$), and 20 μL blood ($F = 13.6$, df $= 1,5$, $P = 0.014$). With 20 μL blood, however, the stress effect on bacteria killing became marginally insignificant when one individual, originally excluded from the main analysis as a result of incomplete sampling, was included in a one-way analysis ($F = 5.1$, df $= 1,6$, $P = 0.065$).

The BGTA showed a significant decline in killing ability due to capture stress in only the 20-μL plasma sample ($F = 29.2$, df $= 1,9$, $P < 0.001$). The decrease in the bactericidal abilities

of the RUGD were marginally insignificant for 20 μL plasma ($F = 5.3$, df $= 1,7$, $P = 0.054$) and 20 μL blood ($F = 4.8$, df $= 1,7$, $P = 0.065$). 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 ($F = 8.5$, df $= 1,11$, $P = 0.014$).

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 bacteria killed. With a 30-min incubation, a 1 : 4 dilution killed 98.0% (±1.1% SD, $n = 3$), a 1 : 10 dilution killed 92.1% (±1.4% SD, $n = 3$), and a 1 : 20 dilution killed 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-min incubation, heat-decomplemented 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 five 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 interspecific variation, the bacteria-killing assay is expected to be well suited for comparative studies of ecology and evolution. However, 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 such as 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 RUGD fledges at about 12 d (Skutch 1983) and has the lowest bacteria-killing ability, whereas at the slow end of the spectrum, the BCMM fledges at about 31 d (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 that bacteria killing and mass-corrected basal metabolic rates are inversely related, again suggesting a 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 antibodies in the form of IgY would result from previous exposure to the exact strain used in this assay (Roitt 1997) and would probably 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 identified 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 fluctuation 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), 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. As a result of interspecific 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 five 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 ($R^2 = 0.772$, $F = 135.5$, df $= 1,40$, $P < 0.0001$; Fig. 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 that is measured when using whole blood. Moreover, the analysis of the chicken plasma samples points specifically to heat-

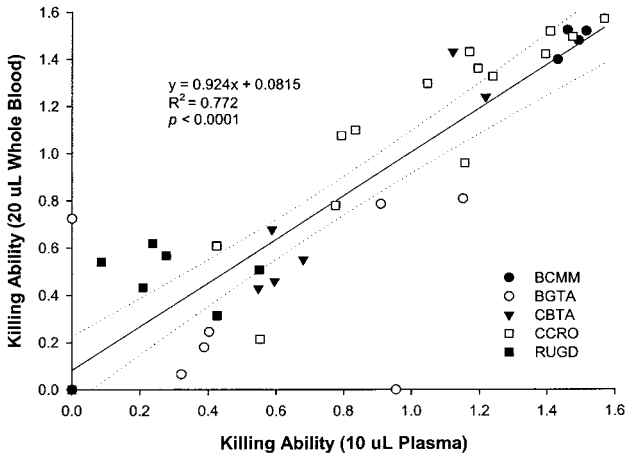

Figure 3. A linear regression with 95% confidence intervals shows a 1 : 1 relationship between the killing abilities (transformed proportions) of 10 μL plasma and 20 μL whole blood. Symbols represent individual birds of the five species included in the study.

labile proteins and suggests that one or more components of the complement enzyme cascade 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 probably stems from using a nonpathogenic strain of *E. coli*. It is expected that 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 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 ($R^2 = 0.945$, $F = 51.8$, df $= 1, 3$, $P = 0.0055$; Fig. 4). Analysis of the chicken whole-blood samples reveals a similar result; the bacteria-killing abilities increase in conjunction with a fivefold increase in concentration. Concentration-dependent effects have also been shown in the antimicrobial capacity of alligator serum (Merchant et al. 2003).

With the five 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 so 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), however, quantification of bacteria killing using a serial dilution technique will reflect the limiting component, whether it is complement, antibody, or phagocytosis.

Effects of Stress

Significant stress effects were seen in three of the five species and found across all three sample types (Fig. 2*A*). When we examined each sample type individually, we found that bacteria

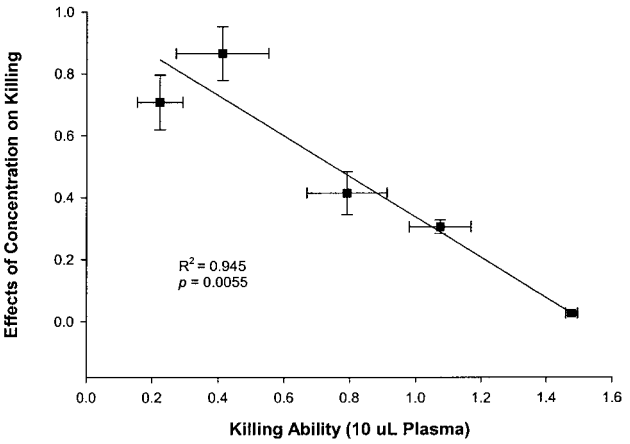

Figure 4. Doubling the amount of plasma used in the bacteria-killing assay to 20 μL results in an increase in killing ability (*Y*-axis, transformed proportions) that is indirectly related to killing ability of 10 μL plasma (*X*-axis, transformed proportions). This relationship shows that each of the five study species has a unique saturation curve, which is defined by the intrinsic capacity of a species' plasma to kill 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; Fig. 2*B*). Similar relationships are seen with the other two sample types. As with the concentration effects, the magnitude of the stress effects depends 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 intraspecific 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 probably 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 before 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 nonpathogenic 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 interspecific studies where quantification of immune function is required.

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