Innate Immunity in Free-Ranging African Buffalo (*Syncerus caffer*): Associations with Parasite Infection and White Blood Cell Counts

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ABSTRACT

Mammalian immunology has been studied in great detail in laboratory animals, but few of the tools and less of the insight derived from these studies have been placed in the context of natural, outbred wildlife populations subject to variable environments. We investigated patterns of innate immunity in free-ranging African buffalo in relation to host traits (age, reproductive status, body condition, white blood cell counts) and disease status (bovine tuberculosis [BTB], gastrointestinal nematodes, coccidia, ticks). We evaluated and used an in vitro assay measuring bactericidal competence of blood to assess a component of innate immunity in 200 female buffalo captured at Kruger National Park, South Africa, in June/July and October 2008. Animals with BTB had higher bactericidal competence of blood. Animals with higher neutrophil counts had higher bactericidal competence, whereas animals with lower lymphocyte counts had higher bactericidal competence. This pattern was driven by animals captured at the end of the dry season (October) and may be evidence of immune polarization, whereby individuals are unable to upregulate multiple components of immunity simultaneously. Bactericidal competence did not vary with host pregnancy status, body condition, age, lactation, tick infestation, nematode egg count, or coccidia oocyst count. Overall, we demonstrate that the bactericidal competence assay is practical and informative for field-based studies in wild bovids. Our results also show a correlation between bactericidal competence and bovine tuberculosis infection and reveal possible functional polarizations between different types of immune response in a free-ranging mammal.

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Introduction

Parasites and pathogens affect the fitness of most free-living organisms and as such represent a major selection pressure on their hosts (Grenfell and Dobson 1995). As a result, hosts must protect themselves against parasitic exploitation by investing resources in immune defenses. Ecoimmunology investigates the costs of immunity, optimal strategies for effective immune defense in the face of multiple demands on host resources, and plasticity in these strategies due to variable host environments. Despite lively interest in this emerging field (Martin et al. 2006, 2008, 2011; Fenton et al. 2008; Schulenberg et al. 2009; Demas et al. 2011), there is still a lack of knowledge about variation in immunological capabilities in wild mammalian populations (Sadd and Schmid-Hempel 2009). Empirical studies in ecoimmunology have focused largely on birds (e.g., see Alonso-Alvarez and Tella 2001; Grasman 2002; Friedl and Edler 2005; Lee et al. 2008; Wilcoxen et al. 2010) and invertebrates (e.g., Moret and Schmid-Hempel 2001; Moret and Siva-Jothy 2003; Cremer and Sixt 2009), extending strong foundations in life-history theory in these taxa. Transferring these approaches to free-living mammal species has only recently begun (see, e.g., Allen et al. 2009; Ezenwa et al. 2011*b*; Graham et al. 2011; Jackson et al. 2011).

The immune response is a complex, interdependent web of factors often divided into innate and acquired arms of immunity. Innate immunity provides an immediate initial line of defense against invading pathogens and directs subsequent acquired immune responses (Tizard 2004). Innate immunity is thought to be developmentally less costly than adaptive immunity, but its upregulation carries substantial energetic and immunopathological costs (Klasing 2004; Martin et al. 2008). Innate immunity is mediated by a number of serological and cytological effector mechanisms. The main serological components are complement mediated killing, natural antibodies, antimicrobial peptides, soluble acute phase proteins, and lysozyme activity, whereas cytological components include neutrophil, macrophage, and natural killer-cell-mediated killing of pathogens (Tizard 2004). Previous studies have looked at patterns of innate immunity in free-ranging wildlife, predominantly in birds, using a variety of assays, including the bactericidal competence assay (reviewed in Boughton et al. 2011; Demas et al. 2011). Several studies have demonstrated indi-

vidual variation in bactericidal capability related to environmental parameters (Martin et al. 2008; Allen et al. 2009). For instance, bats in caves had decreased bactericidal ability compared to bats roosting under bridges (Allen et al. 2009), while body condition had no effect on bactericidal competence. Although innate immune variation is often associated with environmental and demographic variables, genetic variation has also been shown to be an important predictor of innate immune capability (Forsman et al. 2008; Wilcoxen et al. 2010; Ardia et al. 2011). Several studies have also noted seasonal patterns in innate immune capability in a range of species (reviewed in Martin et al. 2008). Other studies have found interspecific variation in bactericidal ability (Matson et al. 2006; Martin et al. 2007; Millet et al. 2007), sometimes along a life-history gradient (Tieleman et al. 2005).

Here, we build on this information, using the bactericidal competence assay to evaluate the degree to which host traits and infection status are correlated with innate immune function in a free-ranging bovid population. Before assessing the ecological questions, we first evaluated the bactericidal assay for use in African buffalo. We asked, first, how bactericidal competence of blood and plasma compare because plasma contains only serological effectors of the innate immune response, whereas whole blood contains both cellular and serological components; second, whether there is evidence for a dose-dependent bactericidal effect of buffalo blood and plasma; and third, whether individual buffalo varied in innate immunity as measured by the assay. The assay was adapted for field-based use in birds by Tieleman et al. (2005), has subsequently been used in numerous other field studies of birds (e.g., Matson et al. 2006; Hasselquist 2007; Millet et al. 2007; Forsman et al. 2008) and mammals (e.g., Allen et al. 2009), and is reviewed by Demas et al. (2011) and Boughton et al. (2011). The in vitro bactericidal assay used here measures the ability of whole blood (i.e., serological and cytological innate effectors) or plasma (i.e., serological components only) to kill bacterial cells. As such, the assay provides an easily interpreted functional measure of an innate immune response to a specified bacterial pathogen; hosts with high in vitro bactericidal ability should be better able to limit infection by the microorganisms used in the assay.

Using the bactericidal competence assay, we investigated patterns of innate immunity in relation to host traits (age, reproductive status, body condition) and disease status (bovine tuberculosis [BTB], gastrointestinal nematodes, coccidia, ticks). The importance of connecting disease profiles to immune profiles has recently been emphasized in the literature (Hawley and Altizer 2010), but data from free-living host populations are sparse. We also looked for evidence of within-immune-system polarization, comparing lymphocyte counts to bactericidal competence. Lymphocytes are white blood cells that function within the adaptive immune system, helping to mount antibody-mediated (B cell) and cell-mediated (T cell) responses (Tizard 2004). Because hosts subject to variable environments may exhibit plasticity in their allocation to different immune components—for example, innate versus adaptive, constitutive versus inducible—according to their costs and effectiveness in combating different infectious challenges (Lochmiller and Deerenberg 2000; Klasing 2004; Houston et al. 2007; Bradley and Jackson 2008; Fenton et al. 2008; Martin et al. 2008), we expected that hosts with limited resources may allocate their resources to different forms of immune response.

Material and Methods

Study Site and Population

Kruger National Park (KNP) is located in northeastern South Africa and comprises almost 19,000 km^2, with a buffalo population of approximately 30,000 (Cross et al. 2009). Adult female African buffalo (*Syncerus caffer*) were captured in the southern portion of KNP, as part of a larger study on parasite interactions in free-ranging buffalo. Young (age 2–5 yr) female buffalo were targeted for capture because of the needs of the larger study. The first 100 buffalo were captured at the end of the wet season, between June 23 and July 5, 2008 (Lower Sabie herd). The second 100 buffalo were captured at the end of the dry season, between October 1 and 8, 2008 (Crocodile Bridge herd). Animals were chemically immobilized with M99 (etorphine hydrochloride) and ketamine by darting from a helicopter. Following data collection, immobilization was reversed using M5050 (diprenorphine). All immobilizations were performed by South African National Parks (SANParks) veterinarians and game capture staff, and all procedures were approved by Oregon State University, University of Montana, and SANParks Institutional Animal Care and Use Committees.

Demographic Parameter Assessment

After immobilization, demographic data were collected, including age, body condition, pregnancy, and lactation status. Pregnancy was assessed by rectal palpation, which has a nearly 100% sensitivity rate after 51 d of gestation in Egyptian buffalo (*Bos bubalis*; Aly et al. 2011), while lactation was evaluated via manual milking of all four teats (Jolles et al. 2005). Age was assessed from incisor emergence patterns for buffalo 2–5 yr old and from tooth wear of the first incisor for buffalo 6 yr of age and older (Jolles 2007). Body condition was measured by visually inspecting and palpating four areas on the animal where fat is stored in buffalo: ribs, spine, hips, and base of tail. Each area was scored from 1 (very poor) to 5 (excellent), and a body condition score was calculated as the average of all four areas. This index is correlated with the kidney fat index (Ezenwa et al. 2009), and similar body condition indices have been used in other studies of African buffalo (Prins 1996; Caron et al. 2003).

Sample Collection

Blood was taken from each animal via jugular venipuncture between 30 and 60 min after darting and collected in sterile EDTA for white blood cell counts and differentials and in lithium-heparinized tubes for BTB diagnostics and bactericidal competence assays. All samples were placed on ice within 5

min of collection in a cooler for transportation back to the laboratory. Plasma was collected from heparinized tubes after centrifugation for 10 min at 5,000 *g* to ensure the separation of cytological components. Plasma (100 μL) was pipetted from the top of the tube for use in the bactericidal assay. Fecal samples were collected rectally from each animal and placed on ice within 5 min for transport back to the laboratory. Total time between sample collection and sample testing or storage in appropriate conditions was never greater than 8 h and typically ranged between 4 and 6 h.

Infection Status Assessment

Fecal egg and oocyst counts were used to assess gastrointestinal nematode and coccidia prevalence and load. Counts were performed using a modified McMaster method (Ezenwa 2003), and all fecal samples were processed within 8 h of collection. In livestock, nematode fecal egg counts can reflect both the number of worms in the host and the fecundity of those worms (Stear et al. 1994; Cabaret et al. 1998); however, this has not been investigated in African buffalo, so we cautiously refer only to fecal egg counts when discussing the results pertaining to worms. Tuberculosis infection status was determined using a standard whole-blood gamma interferon assay protocol (Wood and Jones 2001; Schiller et al. 2009). In brief, this assay is performed by comparing the in vitro IFNγ response to *Mycobacterium bovis* antigen (bovine tuberculin) to the IFNγ response to an avian tuberculin antigen and background IFNγ levels in the absence of antigenic stimulation. This assay has been optimized for use in African buffalo (Michel et al. 2011), and blood cells from buffalo infected with *M. bovis* show a pronounced spike in IFNγ production in response to bovine but not avian tuberculin, whereas bovine tuberculin challenge does not induce IFNγ production in the blood of unexposed animals (Michel et al. 2011). We implemented the gamma interferon assay with the BOVIGAM enzyme-linked immunosorbent assay kit (Prionics), which has a sensitivity of 86% and a specificity of 92% in African buffalo (Michel et al. 2011). Tick burden was assessed by counting the number of adult and nymphal ticks located in three body areas where tick density is highest in buffalo—inguinal, axillary, and perianal regions (Anderson et al., unpublished data).

White Blood Cell Counts

White blood cell counts were performed on whole blood on the day of capture, within 8 h of blood collection, using an automated impedance cell counter (model ABC-VET) to determine the number of white blood cells, red blood cells, and platelets in each sample. Differential counts of white blood cells to determine the fractions of each type of white blood cell (eosinophils, neutrophils, basophils, monocytes, and lymphocytes) were performed manually from a blood smear by a single trained observer. All counts are reported as cells per milliliter.

Bactericidal Competence Assay

We tested bactericidal competence of whole blood (June and October) and plasma (June only). In all cases, testing was done on the day of sample collection and within 8 h. Following methods similar to those outlined by Matson et al. (2006), we diluted whole-blood (5, 10, and 20 μL) or plasma (5, 10, and 20 μL) samples from each individual in a broth containing CO_2-independent media (Gibco-Invitrogen, Carlsbad, CA), 4 mM L-glutamine, and 5% heat-inactivated fetal calf serum for a total volume of 220 μL. No components of the media contained any mediators that result in death of bacteria. A 20-μL aliquot containing 600 colony-forming units (CFUs) of nonpathogenic *E. coli* (ATCC 8739) was added to each diluted plasma and blood sample. The bacterial 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 manufacturer's instructions. The *E. coli* strain we used is a laboratory strain that the buffalo have not encountered in the wild. Therefore, previous exposure is unlikely, and acquired immunity should not play a significant role in killing the bacteria.

The resulting mixtures of bacteria, media, and diluted whole blood or plasma (220 μL total) were vortexed for 3 s and incubated at room temperature for 30 min. This period allowed for the bacteria and blood components to interact. Samples were again briefly vortexed to establish a uniform mixture of the suspension. Following this, 75-μL aliquots were pipetted onto tryptic soy agar (TSA) plates, and the mixture was spread uniformly over the surface of the agar. All samples were plated in duplicate. In addition, each day, five controls were plated containing only bacteria and broth on TSA, from which we established reference numbers of colonies for the corresponding samples. Plates were allowed to dry for a period of 20 min, after which they were inverted and placed in an incubator at 37°C. After 24 h, the number of viable colonies was counted, and plate quality was assessed by checking for contamination, desiccation, or poor spreading of sample.

Statistical Analysis

Only plates with the quality rating of good (i.e., not desiccated, contaminated, or poorly spread) were included in analyses. The proportion of colonies killed was calculated as

$$[\mu(\text{no. colonies on control plates}) - \mu(\text{no. colonies on the experimental plates})]/\mu(\text{no. colonies on control plates}).$$

The mean proportion of colonies killed was calculated from replicate plates. The average number of colonies on the control plates was used as an offset term to account for daily variation in control colony number because the number of bacterial colonies added to samples was not consistent from one day to the next. Ratio data were arcsine transformed, but for ease of visualization, graphs are presented with the original ratios. To evaluate the differences between sample types (plasma vs. blood), paired *t*-tests with a Bonferroni correction were per-

formed between similar concentrations of plasma and blood. In addition, we assessed the pairs for equal variance using a Levene's test. To assess whether increasing concentrations of blood and plasma had an effect on the proportion of colonies killed, a one-way ANOVA was performed. To assess whether bactericidal competence of whole blood correlated with environmental, demographic, or disease parameters, a generalized linear model using a quasi-Poisson distribution and log-link function was performed with the untransformed data. The quasi-Poisson distribution is appropriate for independent variables representing a difference in counts when those counts are overdispersed, as was the case here.

We included sample processing day in the model to statistically account for daily variation such as laboratory conditions and time to sample processing. Our full model included the following main effects: day, season/herd, age, condition, pregnancy, lactation, BTB status, fecal egg count (worms), fecal oocyst count (coccidia), tick burden, neutrophil count per milliliter, lymphocyte count per milliliter, eosinophil count per milliliter, monocyte count per milliliter, as well as all two-way interaction terms with season (e.g., season × age, season × condition, etc.) and age (age × condition, age × pregnancy, etc.). Backward selection was performed by eliminating any variable that did not cause a drop in deviance of greater than 5% from the full model to the reduced model. This method is similar to using Akaike's Information Criteria (AIC), but a drop in deviance criteria is a more appropriate method for overdispersed count data than AIC.

Results

Assay Optimization: Sample Type Comparison

For a given sample volume, bactericidal competence of plasma was higher than that of whole blood (fig. 1) for June captures ($n = 90$). However, when exploring our data for associations between host traits and innate immunity, we found that variability in blood bactericidal competence, but not plasma bactericidal competence, was explained by some of the host traits we measured (table 1 for blood; no significant associations for plasma). Plasma bactericidal competence was thus less informative than bactericidal competence of whole blood in the context of the host traits examined here. This difference between sample types was not due to higher variance in blood bactericidal competence overall, as compared to plasma (Levene's test, $F = 2.191$, $P = 0.14$). In our October sampling, we therefore used whole-blood samples only, and below we present results for whole-blood bactericidal competence rather than plasma.

Assay Optimization: Dose Dependence of Bactericidal Blood Activity

The proportion of colonies killed by buffalo blood was dose dependent: the more blood added to the bacterial broth, the fewer colonies were able to establish (one-way ANOVA, $n = 186$; $F = 2{,}346.189$, $P < 0.0001$; fig. 2). Samples containing 20 μL of blood showed very low bacterial growth, with an average of 76.3% of colonies killed compared to control samples containing no blood, limiting variability in bactericidal competence between individual buffalo. This reduced variability made the 20-μL concentration less informative for evaluating demographic and disease patterns. Samples containing 5 μL of blood resulted in highly variable bactericidal competence, with many sample plates not differing from, or even exceeding, control plates in bacterial growth. Samples treated with 10 μL of blood showed intermediate variability in bactericidal competence, and when we tested initial associations between bactericidal ability and host traits, we found the 10-μL concentration to be the most informative for detecting demographic and disease patterns. Thus, for all further analyses, the 10-μL blood volume was used as the response variable.

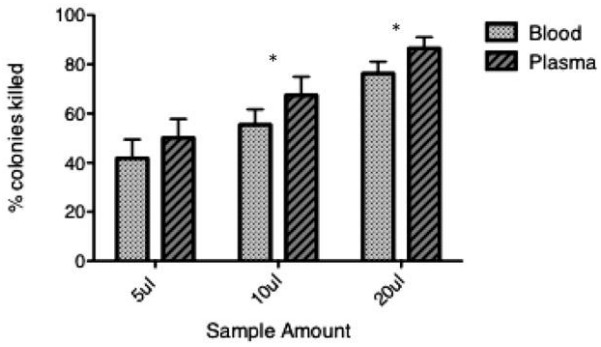

Figure 1. Mean percentage of colonies killed, with standard error, by sample type and concentration for June captures only ($n = 90$). Blood concentration (blood 5, blood 10, blood 20) is given according to the volume of blood (5, 10, or 20 μL, respectively) contained in each 220 μL of bacterial broth. Significant differences were seen between blood 10 and plasma 10 (paired t-test, $t = 2.54$, $P = 0.0119$) and between blood 20 and plasma 20 (t-test, $t = 2.79$, $P = 0.0059$) but not between blood 5 and plasma 5 (t-test, $P = 0.119$). Asterisks indicate significant differences.

Host Traits and Disease Status as Predictors of Bactericidal Competence

We assessed whether host age, reproductive status (pregnancy and lactation), body condition, white blood cell counts, and disease status (gastrointestinal nematodes, coccidia, tuberculosis status, tick burden) were correlated with bactericidal competence (see table 2 for summary data on the dependent variables). For all analyses, we controlled for capture period (June/July: Lower Sabie herd or October: Crocodile Bridge herd) and day. Capture period had a significant effect on bactericidal competence, with competence being higher in the October samples than the June/July samples (table 1). We also detected day-to-day variation in bactericidal competence (ANOVA, $F = 7.88$, $P \leq 0.0001$). This day-to-day variation was due primarily to variation in the number of CFUs per pellet and was accounted for statistically in all further analyses (see appendix).

Of the host traits and infection parameters examined, tu-

Table 1: Effect of demographic, environmental, and disease variables on bactericidal capability of blood

Variable	Estimate	*F* value	SE	*P* value
Day	−.148	−3.537	.042	.0005*
Capture period (July)	−1.523	−4.017	.380	<.0001*
Tuberculosis (positive)	.286	2.003	.143	.047*
Neutrophil count	.152	2.464	.062	.015*
Lymphocyte count	−.117	−2.129	.055	.035*
Lymphocyte count and capture period interaction	.148	1.722	.086	.087
Neutrophil count and capture period interaction	−.349	−1.942	.180	.054

*$P < 0.05$.

berculosis status, neutrophils, and lymphocytes showed significant associations with innate immunity as measured by bactericidal competence (table 1). Animals with BTB tended to have higher bactericidal blood competence. Animals with higher neutrophil counts had higher blood bactericidal competence, but we detected a negative correlation between lymphocyte counts and blood bactericidal competence driven by the October sampling of animals (fig. 3 and interaction effects in table 1). Lactation, age, body condition, nematode fecal egg counts, coccidia oocyte counts, tick burden, reproductive status, total white blood cell counts, basophils, eosinophils, and monocytes did not predict blood bactericidal competence.

Discussion

Assay Optimization: Sample Type Comparison

Plasma was more effective at killing bacteria than the same amount of whole blood, suggesting that the serological components of the innate response are important in the killing response. We attribute the difference in bactericidal competence between blood and plasma to a greater concentration of immunologically active components (such as complement) in plasma as compared to whole blood, which contains numerous noneffector cells such as red blood cells (Tizard 2004). This is similar to the findings of Matson et al. (2006), where the killing ability of 20 μL of blood was equivalent to that of 10 μL of plasma. An alternative explanation is that the strain of *E. coli* used (ATCC 8739) is more susceptible to the serological components than the cytological components because typically, bacterial killing of the ATCC 8739 strain is considered complement dependent (Millet et al. 2007). Despite the increased killing ability of plasma, no host traits or infection variables accounted for the observed variation in percentage of bacteria killed, whereas some of these variables were predictive of whole-blood bactericidal competence. One could argue that this is confounded by capture period, such that all correlations observed between whole blood and bactericidal ability were driven by the October captures and the lack of associations for plasma could be due to the fact that these samples were collected in the June/July capture session. However, although the white blood cell correlations with bactericidal competence were driven by the October captures, the correlation between BTB and whole-blood bactericidal ability was present for both capture periods. Previous studies have used whole blood (e.g., Tieleman et al. 2005; Millet et al. 2007; Buehler et al. 2008) or plasma (Forsman et al. 2008), but only Matson et al. (2006) evaluated the use of plasma and blood in the same study. In contrast to our results, Matson et al. (2006) found that both plasma and blood varied in the same direction with their parameters; however, they were comparing bactericidal ability among species rather than among individuals of a single species as in our study.

One limitation of the data presented here is that they are cross-sectional. We are thus unable to tease apart variation among individuals due to their current circumstances (e.g., reproductive status, condition, infections, the season when they were captured, etc.), as opposed to variation due to individual quality per se. It is possible that individual buffalo maintain relatively constant levels of complement and other proteins involved in plasma bactericidal activity but that each buffalo has a different set point for these relatively inexpensive defense

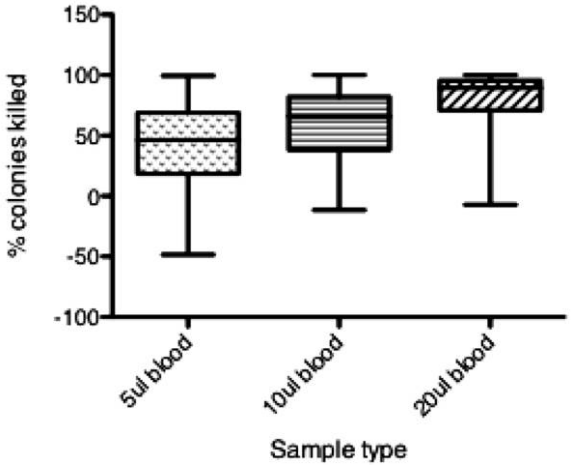

Figure 2. A box plot showing dose dependence in bactericidal activity of buffalo blood. Means are denoted with a solid line, the box shows the seventy-fifth percentile of data points, and the range is denoted with the lines extending from the box. The percentage of bacteria colonies killed increased with blood concentration. Blood concentration (blood 5, blood 10, blood 20) is given according to the volume of blood (5, 10, or 20 μL, respectively) contained in each 220 μL of bacterial broth.

Table 2: Summary information on 200 buffalo broken down by herd

	Lower Sabie (June/July capture period)	Crocodile Bridge (October capture period)
Pregnancy status	50.7% pregnant	63.1% pregnant
Lactation	20% lactating	3% lactating
Age (yr):		
Median	2	4.7
Mean	3.7	4
Body condition (mean)	4.2	2.9
Nematode egg count	30% infected with a mean burden of 2.07 eggs/g	69% infected with a mean burden of 4.44 eggs/g
Tick burden	100% infected with a mean of 136 ticks/individual	100% infected with a mean of 774 ticks/individual
Coccidia oocyst count	27% infected with a mean of 137 OPG	44% infected with a mean of 133 OPG
BTB status	10% infected	14% infected

Note. BTB = bovine tuberculosis; OPG = oocysts per gram.

compounds. By contrast, buffalo may regulate cytological components of innate immunity, which are more costly energetically and in terms of immunopathology (Klasing 2004; Tizard 2004; Buehler et al. 2008), according to available resources and concurrent energy demands. Longitudinal and genetic data on innate immunity in buffalo would help clarify why we are observing this difference between blood and plasma bactericidal competence. It would also be helpful to use different pathogens, in addition to *E. coli*, which may preferentially be targeted by different components of the innate immune response (serological vs. cytological), to further explore potential reasons for observed differences between blood and plasma bactericidal competence in buffalo.

Host Traits and Disease Status as Predictors of Bactericidal Competence

Capture period, tuberculosis status, neutrophils, and lymphocytes showed significant associations with innate host immunity in our study population of free-ranging African buffalo. Capture period was strongly correlated with bactericidal competence, with animals in the October capture having higher competence than those in the June/July capture. Unfortunately, this was confounded by geographical location of capture because we captured animals in the Lower Sabie herd in June/July and the Crocodile Bridge herd in October. We are thus unable to distinguish whether this variability in bactericidal competence assay is attributable to seasonal shifts in immunity or to differences in immunity between the two buffalo herds (e.g., based on resource availability, parasite exposure, or genetic background). For example, there is a striking difference in infection profile (see table 2) between the two seasons, with tick burden and nematode egg count being more prevalent in the October capture period (Crocodile Bridge herd), although neither tick burden nor nematode egg count correlated with bactericidal ability in this study. Tuberculosis status was weakly and positively correlated with bactericidal competence, such that those animals that were BTB positive had increased bactericidal competence. There is evidence that animals with acute BTB infection have upregulated immune responses, which may include innate immune responsiveness (Pollock et al. 2006). By contrast, chronically infected animals would be expected to suffer immune suppression, resulting in anergy (Raja 2004; Cross et al. 2009). Most buffalo acquire BTB infection between the ages of 2 and 5 (Cross et al. 2009), and the majority of our buffalo are young, with the range of BTB-infected animals ranging from 1 to 11 yr of age, with a median of 4 yr of age (entire population ranges are reported in table 2), so most of the BTB infections in these animals are recent. Concordantly, the immune system stimulation caused by BTB infection may result in an upregulated bactericidal competence in the BTB-positive animals in this study.

There was a positive correlation between neutrophil count and bactericidal competence of whole blood. Neutrophils are one of the main effector cells of bacterial killing ability of whole blood, the other main cellular component being macrophages (Tizard 2004). The importance of the cytological component of blood in bactericidal competence has been demonstrated in several species, including mice (Hanski et al. 1991) and rats (Davies et al. 1981). Neutrophils had a stronger correlation with bactericidal competence in the October capture period than in the June/July capture period, as evidenced by the interaction term between neutrophil count and season of capture (table 1). Bactericidal competence was higher overall in animals captured in October than in July, perhaps increasing detectability of the effects of individual immune components on bactericidal competence. Alternatively, seasonal and/or herd variability in immune functioning may be causing the observed difference in the role of neutrophils in bactericidal activity.

There was a negative association between lymphocyte count and bactericidal competence of whole blood, suggesting possible immune polarization, with individual animals that have high bactericidal competence having lower lymphocyte counts. Lymphocytes are typically associated with an acquired immune response, whereas bactericidal competence is primarily medi-

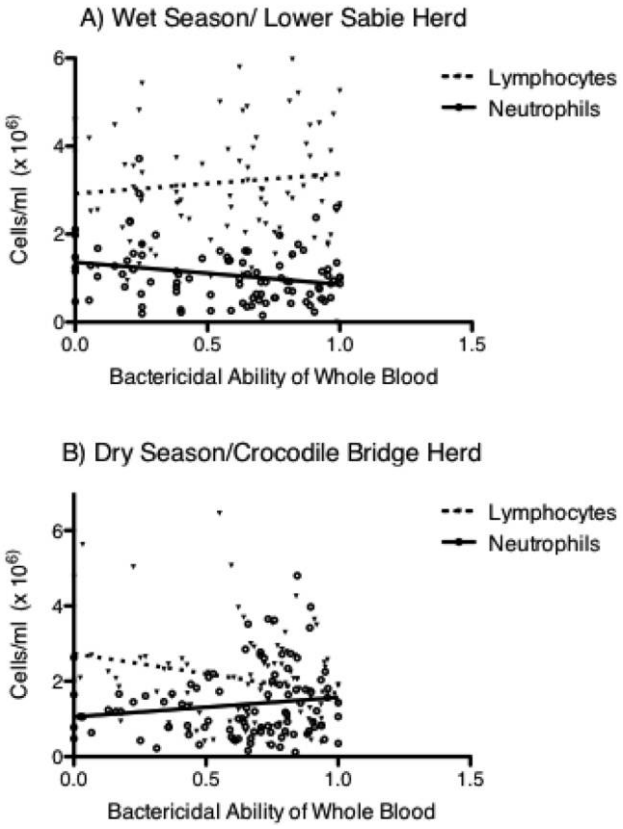

Figure 3. Correlation between lymphocytes per milliliter, neutrophils per milliliter, and bactericidal ability (% colonies killed) by season/herd. *A* shows that there were no significant correlations between lymphocytes or neutrophils and bactericidal ability (bactericidal competence assay) in the June/July capture period. However, *B* shows that lymphocytes were negatively correlated with bactericidal competence assay and positively correlated with neutrophils in the October capture period.

ated by an innate immune response. Our data are thus suggestive of polarization between innate and acquired immune responses, but additional functional measures of the acquired and innate response are needed to explore this concept.

Polarizations within the immune system can be caused by genetic or environmental factors and can be inherited (genetic or epigenetic) or functional (Graham et al. 2005; Betelli et al. 2007; Ardia et al. 2011). There are no published accounts of polarization between bactericidal and any other immune competence measure in mammals; however, other types of within-immune-system polarizations have been published. For example, many studies in mammals have found evidence suggesting polarization between two types of lymphocytes of the acquired immune system (TH1 vs. TH2; Morel and Oriss 1998; Graham 2008; Jolles et al. 2008; Ezenwa et al. 2011). Animals tend to be able to mount an effective TH1 or TH2 response, but not both simultaneously, because messenger molecules that upregulate TH1 immunity simultaneously downregulate TH2 pathways and vice versa (Abbas et al. 1996; Mosmann and Sad 1996). In invertebrates, polarizations between bactericidal ability and other immune responses have been documented. For instance, in a study of *Trichoplusia ni* larvae, there was polarization between bactericidal competence of hemolymph and hemocyte (a phagocytic immune cell) numbers (Freitak et al. 2007); however, whether this was due to a functional polarization or energetic constraints is unknown. Similarly, there are published accounts of polarizations between phenyloxidase activity and bactericidal competence in bumblebees (Moret and Siva-Jothy 2003). Once again, however, the mechanism behind this polarization is unclear.

The polarization we observed between lymphocyte count and bactericidal ability was driven by the animals captured in October; if we examine each capture period separately, polarization is detectable in the October sample but not the June/July sample (fig. 3). Our October 2008 capture fell toward the end of the dry season in KNP, when food resources for herbivores are sparse and grazers are likely to have used up much of their fat reserves (du Toit 2003; Bengis et al. 2003). Accordingly, the buffalo we sampled in October were in much worse body condition than those sampled in June/July (mean body condition, October = 2.9; June/July = 4.2; two-tailed *t*-test, $P < 0.001$). Perhaps buffalo under severe resource limitation are unable to maintain high bactericidal competence and high lymphocyte numbers simultaneously. Different types of immune responses incur different energetic costs and pathological costs, and they have differential spectrums of effect (Klasing 2004), so animals under resource restriction may respond to an immunological challenge differently than animals not under resource restriction. Indeed, there is accumulating evidence that the degree of TH1-TH2 polarization in mammals, for example, may vary with resource levels (Long and Nanthakumar 2004; Jolles et al. 2008; Ezenwa and Jolles 2011). Alternatively, pathogen exposure (Hawley and Altizer 2010) or genetic differences (Ardia et al. 2011) between buffalo from the Crocodile Bridge and Lower Sabie herds may be responsible for this immune polarization being detectable in one group but not the other. Longitudinal data on both groups of animals will be needed to clarify how herd membership and/or season relate to immune polarization. Nevertheless, this study reveals a striking negative association between lymphocyte count and bactericidal competence in a wild mammal population, which is suggestive of immune polarization. We also found evidence for modulation of innate immunity by concurrent BTB infection. Future work, focusing on longitudinal patterns of immunity, holds promise for disentangling host and environmental factors driving the immune variability we observed in this study system.

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APPENDIX

We recaptured 57 of the female buffalo in the Lower Sabie herd in Kruger National Park between June and July of 2009 to investigate (and subsequently mitigate) the causes of the high day-to-day variation observed in our 2008 samples. We hypothesized that some of the day-to-day variability in bacterial growth may be due to different numbers of colony-forming units present in each *E. coli* pellet (the pellets are certified to contain 1.1–9.9 $\times$ 10^7 colony-forming units (CFUs) of *E. coli*).

We therefore performed a the bactericidal assay using *E. coli* colonies maintained in the laboratory, instead of using a new *E. coli* pellet each day. To prepare the actively growing *E. coli* colonies, we reconstituted the pellet as above and plated 75 μL of bacteria broth on a tryptic soy agar (TSA) plate, which was then incubated for 24 h at 37°C. These cultures were used to create TSA slant cultures of bacteria that were maintained in the refrigerator. The day before an experiment was set to be run, refrigerated colonies of the two bacterial populations were used to inoculate individual fresh TSA plates and allowed to incubate overnight for use in the following day's bactericidal assay. Refrigerated colonies on slants were replaced weekly. In order to perform the assay, all bacterial dilutions were then created from the incubated plate, using the BD BBL Prompt Inoculation System (catalog no. 226306) and following manufacturer instructions, utilized in bacteriology laboratories to acquire a constant number of CFUs per milliliter. We mixed 100 CFUs of *E. coli* with the buffalo blood and plated as described above. Data were analyzed the same as the 2008 data.

The samples collected in the summer of 2009 with the modified bactericidal competence assay protocol showed no day-to-day variation (ANOVA, $F = 0.869$, $P = 0.61$) in the number of colonies on the control plates or in bactericidal activity of buffalo blood, supporting our hypothesis that variability in the concentration of CFUs between lyophilized *E. coli* pellets was a causal factor. For our 2008 samples, our best option remains to control for day-to-day variation in bacterial growth and bactericidal competence assay statistically, by including the number of colonies on control plates and day as variables in our statistical models. The 2009 experiments serve to explain this unwanted variability and have allowed us to modify our protocol in our subsequent work.

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