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FIELD INVESTIGATION OF INNATE IMMUNITY IN PASSERINE BIRDS IN SUBURBAN CHICAGO, ILLINOIS, USA

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ABSTRACT: The innate immune system is the first line of defense against pathogens, and it plays a fundamental role in coordinating a protective immune response in birds. Although many studies have evaluated avian immune responses in the laboratory, many fewer studies to date have done so in a field setting. To gain insight into interspecific differences in immune function in wild birds, we used a field-deployed in vitro microbicidal assay to measure constitutive innate immunity of whole blood collected from three common passerines in suburban Chicago, Illinois, in 2009. Data from one microbe, *Escherichia coli* 8739, revealed that American Robins (*Turdus migratorius*) had significantly lower bactericidal capacity than House Sparrows (*Passer domesticus*) or Gray Catbirds (*Dumetella carolinensis*). Bactericidal capacity for *E. coli* 8739 tended to be lower for birds infested with chewing lice than those without chewing lice, and male birds had lower microbicidal capacity than females in the case of *Staphylococcus aureus*. This study demonstrates the potential for field-deployable eco-immunologic tools to inform infectious disease ecology research.

Key words: Disease resistance, ecologic immunology, microbicidal capacity, passerine birds.

INTRODUCTION

Parasite-host interactions are mediated by the host's immune system and immune evasion by parasites (Sadd and Schmid-Hempel, 2009). The vertebrate immune system is multifaceted, including innate as well as adaptive components. The trade-off of energetic costs associated with immune function is additionally influenced by tolerance, the ability of the host to limit the health impact of a parasite (Schneider and Ayres, 2008). Hosts invest in different strategies to recognize and kill parasites, and likewise, parasites are capable of immune evasion and subsequent spread within the host (Schmid-Hempel, 2008). An understanding of these complex interactions is central to the field of ecologic immunology and is of intrinsic interest to the study of infectious disease ecology (Hawley and Altizer, 2010).

Traditionally, the host response to an infectious agent has been quantified in a controlled environment, and the ability of a pathogen to successfully invade, replicate, and shed (i.e., quantification of an infectious period) determines the ability of a host to serve as a reservoir (i.e., host competence; Komar et al., 2003; Reisen et al., 2003b). In arthropod-borne disease systems, for example, additional quantification of vector-host contact rates along with host competence allows the importance of particular host species in contributing to the transmission of a pathogen to be quantified (Kilpatrick et al., 2006; Kent et al., 2009). However, a limitation of laboratory-based studies is that captive birds differ physiologically and immunologically from their wild counterparts (Buehler et al., 2008b). Once in captivity, birds undergo physiologic stress (Cockrem and Silverin, 2002) and cease typical behaviors such as foraging. Captive birds also have restricted activity, reduced energy expenditure, and reduced exposure to pathogens, all of which are known to influence immune function.

We attempted here to complement laboratory-based infection studies with an in vitro immune function assay that can be performed in the field on wild birds. The assay employs a series of blood-based microbicidal assays, which represent an ecologically relevant index of comparative

immune function among species of birds (Millet et al., 2007). In particular, the assay measures different humoral and cellular components of constitutive innate immunity, which represents an organism's first defense against invading pathogens (Matson et al., 2005). Microbicidal assays have been used to study differences in constitutive innate immunity among vertebrate species (Matson et al., 2006a, b) and under different environmental conditions (Allen et al., 2009; Zimmerman et al., 2010), physiologic conditions (Forsman et al., 2010), and infection profiles (Wilcoxen et al., 2010b; Beechler, pers. comm.).

We tested the ability of whole avian blood to kill several microbes, each of which evaluates a different component of the immune system (Millet et al., 2007; Buehler et al., 2010). Previous studies have demonstrated that in vitro bactericidal capacity is a significant predictor of susceptibility of humans to a bacterial infection (Keusch et al., 1975). Townsend et al. (2010) demonstrated that inbred nestling American Crows (*Corvus brachyrhynchos*) had a lower body condition index, lower bactericidal capacity (i.e., innate immunocompetence), and higher rates of death due to disease. Additionally, Wilcoxen et al. (2010b) showed that Florida Scrub-jays (*Aphelocoma coerulescens*) with a higher bactericidal capacity (*Escherichia coli* strain 8739) were more likely to survive an epizootic of eastern equine encephalitis virus. Using a different measure of innate immunity, the hemagglutination assay, Parejo and Silva (2009) determined that individual Eurasian Kestrels (*Falco tinnunculus*) with higher levels of complement (see discussion) were less prone to be parasitized by *Haemoproteus* and collectively demonstrated that innate immunity is linked to survival in a wild bird. These previous studies demonstrate the potential for immunologic assays to predict susceptibility to an infectious agent in nature.

Our study took place in suburban Chicago, Illinois, USA, in an area of high, seasonal West Nile virus (WNV) transmission (Hamer et al., 2008; Ruiz et al., 2010). We investigated constitutive innate immunity in three avian species, the American Robin (*Turdus migratorius*), the House Sparrow (*Passer domesticus*), and the Gray Catbird (*Dumetella carolinensis*), which contribute differentially to the amplification of WNV in the study region (Hamer et al., 2009). Additionally, we investigated the relationship between microbicidal capacity and host factors such as sex and age, condition indices, WNV antibody status, and ectoparasite burden.

MATERIALS AND METHODS

The southwest suburban Chicago, Illinois, study region included three residential and four seminatural sites (urban green spaces; Hamer et al., 2008; 87°44′W, 41°42′N), and field work was conducted from June through July 2009. Field personnel were equipped with rapid bleeding kits to obtain a blood sample by jugular venipuncture within 5 min of a bird being captured in a mist-net. Birds that remained in the nets for longer than 5 min were excluded, given that acute stress following capture can significantly decrease microbial killing capacity (Matson et al., 2006b), although the relationship between killing ability and the duration of handling stress has been inconsistent in previous studies (Matson et al., 2006b; Buehler et al., 2008a; Rubenstein et al., 2008). Great care was taken to obtain a sterile blood sample, which was collected in an insulin syringe. Following collection, a portion of the sample was transferred to a sterile heparinized microcapillary tube for the killing assay. Prior to release, birds were aged as hatch year (HY) or after hatch year (AHY) according to plumage, iris color, and palate, and sex was determined when possible based on plumage or the presence of a brood patch or cloacal protuberance (Pyle, 1997). We gave each bird a score for body condition (poor, fair, good, or excellent) using an index based on the musculature lateral to the keel and fat deposits around the neck (Kaiser, 1993). We also calculated a body condition index (BCI) as measured by residuals from an ordinary least squares (OLS) regression of body mass against wing chord (Schulte-Hostedde et al., 2005; Ishak et al., 2010). We scored the presence or absence of chewing lice (Mallophaga) visible on the primary feathers by naked eye when

backlit (e.g., held to the sky). Blood samples were later tested for WNV and WNV antibodies using reverse transcriptase–polymerase chain reaction (RT-PCR) and an inhibition-enzyme-linked immunosorbent assay (ELISA), respectively, as previously described (Hamer et al., 2008).

We followed a previously tested microbicidal killing assay protocol (Tieleman et al., 2005; Millet et al., 2007) with some modifications. The microbes used in this study included *Escherichia coli* ATCC# 8739, *E. coli* ATCC #51813, *Staphylococcus aureus* ATCC#6538, and *Candida albicans* ATCC#10231 (Microbiologics, Inc., St. Cloud, Minnesota, USA). *E. coli* 8739 is killed primarily by plasma components of the immune system, while the other microbes are likely killed by cellular components of the innate immune system (Millet et al., 2007). Microbes were rehydrated in sterile phosphate-buffered saline (PBS) to create a working stock solution, which resulted in approximately 250 colonies per blood sample. Working stocks were made every week to ensure microbe survival.

Blood samples were processed within 45 min of capture. Sample incubation began in the field utilizing a hot plate connected to a vehicle battery. Blood was diluted 1:10 with L-glutamine–enriched CO_2 media and incubated at 40 C for various durations according to microbial species to achieve maximum killing. Controls of microbes and media were incubated under identical conditions. Blood samples and controls were then plated in duplicate on Tryptic Soy Agar plates. Plating was done in a field laboratory using an inverted translucent container to maintain an environment that was as aseptic as possible. If contamination was detected in any controls, experimental results were discounted for that trial. Tryptic Soy Agar plates were incubated at room temperature for 24 hr for bacterial species and for 48 hr in the case of *C. albicans*. After incubation, microbial colonies were counted and compared to control values, and microbicidal capacity was quantified as the proportional reduction in microbial growth relative to controls (1 − duplicate mean on experimental plates/duplicate mean on control plates), and values are presented as unadjusted means for each species±standard error.

We explored differences in microbicidal efficiency of American Robin, Gray Catbird, and House Sparrow blood using one-way analysis of variance (ANOVA). We used a post hoc test (Tukey's honestly significant difference [HSD]) to compare the means among species. We then used a linear mixed model to identify the relationship among microbicidal capacities using species nested within site as random factors and sex, age, condition, parasites, WNV antibody status, and the residuals of the BCI as fixed factors. Model residuals were assessed for normality, and we adjusted *S. aureus* killing capacity using a square-root (x+0.5) transformation to achieve normality. Model selection criteria included a backward elimination of fixed effects explaining little variation. The random variables of species and site were removed from the model when they explained little variance and the linear model had a lower Akaike information criterion value than the mixed model. All statistical tests were conducted in R v2.11.1 statistical programming language (R Development Core Team, 2008).

RESULTS

We analyzed blood samples from 25 American Robins, 14 Gray Catbirds, and 15 House Sparrows (Table 1). All samples were negative for WNV based on RT-PCR results, and four were antibody-positive for WNV antibodies (two robins, one catbird, and one sparrow). All antibody-positive birds were adults, and, given the time of sample collection (early in the WNV transmission season), most seroconversions probably occurred in a previous year. The frequency of chewing lice infestation was highest for American Robins, followed by Gray Catbirds, and House Sparrows (60%, 42.9%, and 0%, respectively).

We observed a significant difference in microbicidal activity among bird species for *E. coli* strain 8739 (F=17.80, df=51, P<0.001) but not *E. coli* strain 51813, *S. aureus*, and *C. albicans* (F=2.22, df=40, P<0.121; F=0.69, df=49, P<0.507; F=1.32, df=42, P<0.278; respectively; Fig. 1). Gray Catbird and House Sparrow blood had significantly greater bactericidal capacity when assayed with *E. coli* strain 8739 (86.7%±4.28 bactericidal capacity and 93.9%±2.16, respectively) than did American Robin blood (55.1%±5.81). The random factor of species explained 64.8% of the variation in *E. coli* strain 8739 killing capacity, and the main effect of ectopar-

TABLE 1. Sample size, body mass (SE), sex, age, and percent infestation with chewing lice of birds collected in southwest suburban Chicago, 2009.

Species	N	Body mass (g) (SE)	Sex			Age		Parasites (%)
			M	F	U	AHY[a]	HY[b]	
American Robin (*Turdus migratorius*)	25	77.0 (1.6)	8	5	12	14	11	60.0
Gray Catbird (*Dumetella carolinensis*)	14	38.0 (2.6)	1	3	10	12	2	42.9
House Sparrow (*Passer domesticus*)	15	27.4 (0.8)	6	4	5	10	5	0.0

[a] AHY = after-hatch-year bird.

[b] HY = hatch-year bird.

asites was marginally nonsignificant with a negative coefficient ($F=3.43$, df=1,50, $P=0.070$; Table 2), indicating that bactericidal capacity was lower for birds infested with chewing lice than those without chewing lice (60.1%±6.89 and 83.6%±3.56, respectively). Sex was a significant predictor of *S. aureus* killing capacity ($F=4.64$, df=2,48, $P=0.014$), with males having lower capacity than females (8.6%±3.84 and 12.32%±3.83, respectively). No measured variables explained variance in *E. coli* strain 51813 or *C. albicans* killing capacity.

DISCUSSION

In this study, we evaluated the constitutive innate immune function of wild birds using an in vitro microbicidal assay. We found that American Robins had lower bactericidal capacity than Gray Catbirds or House Sparrows for *E. coli* 8739, but no significant differences among species were found for the other microbes. It has been suggested that *E. coli* killing is primarily accomplished by plasma components, while phagocytosis is the main mechanism underlying killing of *C. albicans* (Tieleman et al., 2010). Additionally, *E. coli* 8739 is more susceptible to plasma killing components than to cellular components (Millet et al., 2007). The complement system includes numerous proteins and cell surface receptors that aid pathogen recognition and clearance. Complement activation plays a critical role in antiviral immunity as well (Avirutnan et al., 2008). In murine models, mice lacking individual components of the three pathways of complement activation had marked increases in WNV susceptibility, for example (Mehlhop et al., 2005; Mehlhop and Diamond, 2006). Our observation of a lower *E. coli* 8739 microbicidal capacity in American Robins compared to Gray Catbirds is consistent with the observations that these two hosts show different competence for WNV and *Borrelia* infection (Mather et al., 1989; Richter et al., 2000; Ginsberg et al., 2005; Kilpatrick et al., 2007). We speculate that the plasma components of the constitutive innate immune function are responsible for the differences in competence among species exposed to these pathogens. Future research on the relevance of these findings to protection from naturally circulating pathogens would be warranted.

The presence of ectoparasites was a significant predictor of *E. coli* 8739 microbicidal activity, but when variation among species was accounted for in the mixed model, ectoparasite infestation was marginally nonsignificant. This result suggests that bactericidal capacity tended to be lower for birds infested with chewing lice, which is consistent with a meta-analysis showing that brood manipulation (i.e., increased reproductive effort) decreased immune responsiveness and in-

TABLE 2. Factors explaining variation in microbe killing ability in passerine birds in suburban Chicago, Illinois, 2009. A linear mixed model was used for *Escherichia coli* strain 8739 and 51813 killing capacity, and a linear model was used for *Staphylococcus aureus* and *Candida albicans* killing capacity. Model selection was by backwards elimination of nonsignificant variables, and values shown are from the point at which each variable was removed from the model.

Variable[a]	Coefficient	df	*F*	*P*
E. coli 8739 killing capacity				
Intercept	0.783	1,51	42.246	<0.001
Ectoparasite	−0.128	1,50	3.432	0.070
Sex (female)	−0.117	2,48	1.451	0.245
Age (HY)	−0.059	1,47	0.362	0.551
WNV sero-status	0.048	1,46	0.162	0.690
Condition (poor)	0.140	2,44	0.288	0.751
BCI residuals	0.000	1,43	0.018	0.893
E. coli 51813 killing capacity				
Intercept	0.264	1,30	8.036	0.008
Sex (female)	0.163	2,28	0.964	0.394
WNV sero-status	−0.099	1,27	0.289	0.595
Condition (poor)	−0.141	2,25	0.426	0.658
BCI residuals	0.003	1,24	0.133	0.718
Age (HY)	−0.240	1,23	0.261	0.614
Ectoparasite	−0.049	1,22	0.097	0.758
S. aureus killing capacity				
Sex (female)	−0.047	2,48	4.643	0.014
WNV sero-status	0.027	1,48	0.438	0.511
Ectoparasite	0.009	1,47	0.134	0.716
BCI residuals	−0.001	1,46	0.080	0.779
Age (HY)	0.007	1,45	0.004	0.948
Condition (poor)	0.001	2,43	0.013	0.987
C. albicans killing capacity				
Condition (poor)	−0.125	2,42	1.562	0.222
BCI residuals	0.006	1,41	1.511	0.226
WNV sero-status	−0.068	1,40	0.347	0.559
Sex (female)	0.099	2,38	0.528	0.594
Age (HY)	−0.026	1,37	0.267	0.609
Ectoparasite	0.046	1,36	0.231	0.634

[a] HY = hatch-year bird, WNV = West Nile virus, BCI = body condition index.

creased hematozoan parasitemia (Knowles et al., 2009). Additionally, Whiteman et al. (2006) found that ectoparasite abundance on the Galapagos Hawk (*Buteo galapagoensis*) was negatively correlated with natural antibody titers, as measured by the hemolysis-hemagglutination assay. The lack of correlation between constitutive innate immunity and condition indices observed in this study is consistent with previous studies in birds (Palacios et al., 2009; Forsman et al., 2010), which suggest that the innate branch of the immune system is less sensitive to body condition than adaptive immunity. Additionally, anthropogenic food availability to urban birds is well known (Chamberlain et al., 2009), which might suggest that the birds measured here were not energetically limited, resulting in a minimal trade-off with immune function.

Although we expected to observe reduced constitutive innate immune function in juvenile birds, we did not. Tieleman et al. (2010) observed that older Stonechats (*Saxicola torquata*) had superior microbicidal ability than did younger individuals, suggesting that avian immune

function may improve with age in some species. Similarly, Wilcoxen et al. (2010a) found that bactericidal capacity was higher for older Florida Scrub-jays. However, both of these studies observed improved bactericidal capacity after about 5 yr of age, a pattern that could result from natural culling of individuals with lower immunocompetence. A study comparing innate and adaptive immunity in juvenile and adult Tree Swallows (*Tachycineta bicolor*) found that innate defenses reached adult levels earlier than adaptive defenses (Palacios et al., 2009), which supports the lack of age-dependent microbicidal capacity in the current study.

The interpretation of assays used to measure immune defense components as they relate to pathogen exposure is challenging. The microbicidal assays we used only measure certain components of the immune system and do not represent the complex cascade of multiple components involved in the recognition, response, and resolution of infection with certain pathogens. Additionally, parasite immune evasion, a ubiquitous biologic phenomenon (Schmid-Hempel, 2008), further confounds our interpretation of immune assays, as does the immune defense mechanism of tolerance (Schneider and Ayres, 2008). Finally, previous biologic events can influence innate and humoral immunity. For example, the immunosuppressive effects of poor nutrition (Ezenwa, 2004; Smith et al., 2005), stress (Reisen et al., 2003a; Martin, 2009; Jankowski et al., 2010), and reproductive status (Deviche and Cortez, 2005) could influence these results. Finally, previous exposure to parasites or current infections of parasites not evaluated in our study could introduce confounding effects (Telfer et al., 2010).

Although we found only four birds that had been previously exposed to WNV, we suspect that the humoral (i.e., acquired) immunity obtained by these birds would have had little influence on the immune function measured by the microbicidal assays in our study. Alternatively, birds that survived a WNV exposure and produced antibodies might imply a stronger constitutive innate immune response, although we did not observe a difference with our limited sample size. Given the lower bactericidal capacity of robins, it is interesting to note that concurrent studies on robins in the same region show high prevalences of avian filarioid nematode infections (Hamer et al., unpubl. data) and Haemosporidian parasites (M. Medeiros, University of Missouri, St. Louis, pers. comm.).

The ultimate challenge of interpreting microbicidal capacity is to relate constitutive innate immunity to a host's susceptibility and competence to a specific disease agent (Hawley and Altizer, 2010). To fully examine the trade-offs between immune function and the threats posed by pathogens, it would be necessary to assay constitutive immunity, induced innate immunity, and helper-T-cell–mediated acquired immunity concurrently (Buehler et al., 2010). This is challenging because many components of the immune system cannot be measured from a single blood sample. Investigations of the genetic basis of immune function represent a promising direction that would complement efforts to understand immune function and resistance to disease. For example, suppression subtractive hybridization and cDNA microarrays have been utilized to explore genetic responses of House Finches (*Carpodacus mexicanus*) to infection by *Mycoplasma gallisepticum* (Wang et al., 2006), and Barakat et al. (2009) used next-generation sequencing to identify candidate genes in the American chestnut (*Castanea dentata*) responsible for resistance *to Cryphonectria parasitica*, the fungal agent of chestnut blight. Additional beneficial directions might include concurrent immunologic assays and pathogen infection studies in controlled environments. For example, Saks et al. (2006) used measures of immunocompetence during an experimental infection study of

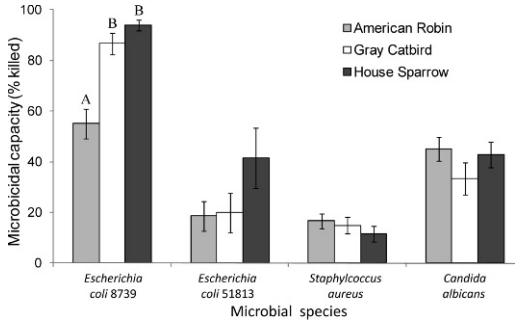

FIGURE 1. Bactericidal activity in American Robin, Gray Catbird, and House Sparrows in southwest suburban Chicago, 2009. Percent killed (±standard error) refers to the comparison of number of different microbes killed in whole blood compared to controls. Different letters above bars indicate statistically significant differences (Tukey's honestly significant difference post hoc test; $P<0.05$).

the Greenfinch (*Carduelis chloris*) with Coccidia. Although microbial killing assays such as the one we used may be crude by comparison to in vivo experimental infections, their relative ease, rapidity, and relevance to wild populations make them attractive tools for assaying immune function in wild birds when prudently applied.

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