Roosting ecology and variation in adaptive and innate immune system function in the Brazilian free-tailed bat
(*Tadarida brasiliensis*)

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Abstract Bats have recently been implicated as reservoirs of important emerging diseases. However, few studies have examined immune responses in bats, and even fewer have evaluated these responses in an ecological context. We examined aspects of both innate and adaptive immune response in adult female Brazilian free-tailed bats (*Tadarida brasiliensis*) at four maternity roosts (two natural caves and two human-made bridges) in south-central Texas. Immune measurements included in vitro bactericidal ability of whole blood and in vivo T cell mediated response to mitogenic challenge. Bactericidal activity in *T. brasiliensis* varied with roosting ecology, but appears to be sensitive to colony-level effects. Blood from females living at one cave had significantly lower bactericidal ability than blood from females at three other sites. T cell mediated response in this species was associated with variation in roost ecology, with females from two caves having greater responses than females from two bridges. T cell mediated response and bactericidal activity were negatively correlated with one another within individuals that were tested for both. Variation in immunological response of *T. brasiliensis* is important for understanding the influence of the environment on the frequency and distribution of immunologically competent individuals and for understanding disease-host dynamics in this and other colonial species.

Keywords Artificial roosts · Bactericidal ability · Bats · Immune response · Phytohaemagglutinin

Abbreviations

- BKA Bacterial killing ability
- FBS Fetal bovine serum
- PBS Phosphate buffered saline
- PHA Phytohaemagglutinin

Introduction

The emergence in wildlife of infectious diseases, such as AIDS, Ebola, West Nile virus, SARS, and Hantaviruses, highlight the need to understand the ecology of reservoir hosts to disease, and the interactions of immunological response and ecological variation. Recently, bats have become a focus of attention as possible reservoirs of several emerging pathogens, including SARS-like viruses (Li et al. 2005), and other Corona viruses (Dominguez et al. 2007; Gloza-Rausch et al. 2008), Nipah and Hendra viruses (Joharra et al. 2001; Halpin et al. 2000), Ebola virus (Leroy et al. 2005), Herpes virus (Wibbelt et al. 2007), and others (reviewed in Calisher et al. 2006). Bats also have long been recognized as reservoir hosts, and vectors to humans and domestic animals, of
rabies virus (Messenger et al. 2003; Constantine 1967) and related lyssaviruses (Fraser et al. 1996; Botvinkin et al. 2003). However, little is known about variation in immune function and susceptibility to disease in free-ranging bats (Calisher et al. 2006; Dobson 2005; but see Christe et al. 2000). Improving our understanding of variation in immune function among individuals and populations of bats is important for understanding the role of bats as potential vectors of emerging diseases (Messenger et al. 2003; Dobson 2005).

Bats possess a variety of life-history traits that are likely to affect their immune function and the role of bats as reservoirs and vectors of disease (Calisher et al. 2006). Many species are colonial, although considerable variation in colony size exists (Kunz 1982; O’Shea and Bogan 2003). The Brazilian free-tailed bat (Tadarida brasiliensis), for example, roosts in some of the largest aggregations of mammals on earth, with several thousand to several million individual bats estimated to form maternity colonies in caves and under highway bridges (Davis et al. 1962; McCracken 2003; Keeley and Keeley 2004; Betke et al. 2008). Colonial living brings the possibility of increased exposure to infectious pathogens, with direct links to immune defense and protection. Relationships have been shown between coloniality and immune responsiveness in several avian species, although evidence is conflicting. For example, Tella et al. (2001) found that T cell mediated immune response in fledglings of the Magellanic penguin (Spheniscus magellanicus) was negatively correlated with colony size, whereas Møller et al. (2001), examining immunity at the interspecific level, found that highly colonial swallows and martins, have higher levels of T and B cell response compared to less social songbirds.

Bats sometimes roost in proximity to urban and agricultural landscapes (Horn and Kunz 2008), and occasionally within human dwellings. In south-central Texas, T. brasiliensis uses both natural and man-made structures as roosts, including caves, bridges, buildings, and bat houses (Kunz and Reynolds 2003; McCracken 2003; Sgro and Wilkins 2003). Roosts can differ in structure, capacity and possible quality (Lausen and Barclay 2006; Neubaum et al. 2006). Given the recognized role of bats in disease transmission, it is important to understand variation in their immune condition in relation to ecological variables, particularly for those species roosting in close proximity to humans.

Innate immunity establishes an early line of defense against invading pathogens and inhibits the progression of infection. In contrast, the adaptive immune responses are induced upon antigen processing and have greater importance for clearing infections (Goldsby et al. 2003). The innate and adaptive arms of the immune response interact to protect individuals, although their competence may vary among individuals (Blount et al. 2003). Most previous research on the effects of ecological conditions on immune function has focused primarily on the adaptive immune response (but see Tieleman et al. 2005 and Blount et al. 2003), by estimating T cell proliferation in response to mitogenic challenge (Tella et al. 2001; Tella et al. 2002). Evaluation of multiple components of immune response is needed to develop a more complete understanding of immune competence in free-ranging animals.

In the present study, we evaluate aspects of both the innate and adaptive arms of the immune system in T. brasiliensis over a period of 5 months at four maternity colonies (two natural caves, two man-made bridges) in south-central Texas. Bactericidal activity of whole blood in culture, one aspect of the innate immune response, primarily measures complement-mediated cytotoxicity (Merchant et al. 2003), which is a major factor in defense against viruses (Blue et al. 2004). T cell mediated response, which contains aspects of both innate and adaptive immune components, is also important in clearance of viruses, including rabies virus (Hooper et al. 1998). This study was designed to characterize responses of T. brasiliensis to two immune challenges. We postulated that variation in both the innate and adaptive arms of the immune system would be affected by differences in roosting ecology as it relates to roost environment and colony size.

Materials and methods

Animal sampling

This study was conducted in south-central Texas from May to September of 2005. Bactericidal activity and T cell infiltration were assessed in free-ranging adult female Brazilian free-tailed bats (T. brasiliensis), captured at two natural caves (Frio Cave and Davis Cave) and two large pre-caste concrete highway bridges (Seco Creek Bridge and East Elm Creek Bridge). Periods of data collection corresponded to female reproductive stages: pregnancy (May–June); lactation (June–July); post-lactation/non-reproductive (August–September). Earlier studies indicated that mating in T. brasiliensis occurs prior to spring migration (Davis et al. 1962; Cockrum 1969), though recent evidence suggests that mating also occurs in Texas in March and April (Keeley and Keeley 2004). Outside of the mating season, however, many of these roosts are occupied by non-territorial maternity colonies, comprised mostly of females. Thus, we have focused on adult females in the present study.
Approximately 15 bats were captured on a given night at each site, most of which were sampled within 2–4 days in each sampling period, for a total of 327 bats. Both immunological assays were not always performed on each bat, due to temporal constraints of the specific protocols. Body mass, reproductive condition (non-reproductive, pregnant and lactating) and age class (adult or juvenile; Anthony 1988) were determined for each individual. Juveniles were not included in the analyses, due to low sample sizes (n = 20) over the entire study period. Bats were held for up to 14 h for the immunological assays described below and then released at the site of capture. All individuals received a site-specific tattoo on their wings to prevent re-sampling. Tattoos on wings provide effective markings on these bats (Lollar and Schmidt-French 1998) that can last up to 3 years. All capture, handling and experimental procedures were approved by the University of Tennessee Animal Care and Use Committee (#890) to G.F.M. and Boston University Animal Care and Use Committee (05–012) to T.H.K., and under Texas Parks and Wildlife Department permit SPR-0305-058 to T.H.K.

Innate immune function: bactericidal ability

Bacterial killing ability of the bat’s whole blood against *Escherichia coli* was measured to represent one aspect of the innate immune response. This technique has been used successfully as an in vitro assay of innate immune function in free-ranging animals (Tieleman et al. 2005). A small amount of whole blood (6 μl) was collected in sterile heparinized capillary tubes via venopuncture (Kunz and Nagy 1988) within 2 h of capture from each bat. Although Matson et al. (2006) found a decrease in bactericidal ability of the blood of several species of birds at this length of time post-capture, preliminary data on *T. brasiliensis* shows no reduction in bactericidal ability up to 2 h post-capture. The blood was diluted to 1:50 in RPMI-1640 media (Roswell Park Memorial Institute), supplemented with 5% Fetal Bovine Serum (FBS). The *E. coli* (ATCC 8739; Microbiologics, USA) solution was diluted to 1:1,000 using sterile phosphate buffered saline (PBS). A subcutaneous injection of phytohaemagglutinin (PHA-P #L8754; Sigma, USA) was administered to assess T cell infiltration in a total of 163 individual bats. Injections were administered in the interfemoral membrane (uropatagium) below the knee at the point of contact with the leg. Prior to injection, the area was measured with a digital micrometer (Mitutuyo #293-230, Japan). The experimental area on each bat was injected with 0.05 ml of 3 mg/ml PHA in PBS. As a control, the contralateral side was then injected with 0.05 ml of PBS. Swelling at the sites of injection was subsequently measured at 12 hours post-challenge. Measurements of PHA swellings in the uropatagium of bats are very similar to the more common measurements made in the wing web of birds. The swelling produces thickened cellular infiltrate between the two layers of skin adjacent to the leg, thus our measurements are comparable in execution to those conducted in birds. Although bactericidal ability of blood was not tested in every bat, all bats were bled within 3 min of capture, for a companion study on stress hormones, therefore all bats in our study were bled once prior to PHA challenge. To ensure consistency in assessing the PHA test, one of us (L.C.A.) performed all injections, and another (A.S.T.) made all measurements. Care was taken to conduct repeatable, blind measurements with each swelling measured twice and averaged. Following Navara et al. (2005), the unit-less index below was used to determine the swelling response to the mitogenic challenge standardized against the control response:

\[
\text{BKA index} = -1 \times \left( \frac{\text{blood plate } 60 - \text{blood plate } 0}{\text{control } 60 - \text{control } 0} \times 100 \right)
\]

This index assigned large positive values when bats had blood with high cytotoxic activity and negative values when blood had little or no cytotoxic activity against the bacteria. The index method controls for bacterial die-off occurring within an assay that is unrelated to the blood’s bactericidal ability. Only data from assays where there was less than a 15% difference between the 0 and 60 min controls were used. Three individuals whose bactericidal indices were over three standard deviations lower than the mean of the remaining individuals were excluded from the data set, because these values deviated from the normal distribution of bactericidal ability found in our samples. In the final analyses, bactericidal indices were derived from 89 individual adult female bats.

Adaptive immune function: T cell mediated immune response
Indices greater than one designate bats with marked T cell infiltration to the injection area, whereas smaller index values indicate that little or no response was observed. One individual with an index value that was over three standard deviations beyond the normal range of indices was excluded from the data set, resulting in analyses of T cell infiltration for 162 individual adult female bats.

While this technique has been widely used in avian immunology to assess T cell mediated response (Bonforte et al. 1972; Tella et al. 2001), recent studies have cautioned that the proper identification of cellular components present in the swelling is necessary to accurately interpret the response (Kennedy and Nager 2006; Martin et al. 2006a). In a separate group of *T. brasiliensis* (*N* = 18), we analyzed PHA treated and control tissues with biopsies of the swellings taken 6–21 h post-challenge. We documented significantly larger numbers of lymphocytes (mean cell count = 6), as well as heterophils (mean cell count = 35), in PHA-challenged tissues that were not observed in any numbers in control tissues, demonstrating that specific cellular infiltration had occurred in response to the PHA challenge. We found that measurements of the swellings at 12-h post-injection capture the maximal swelling response of individual bats, and represented when increased lymphocyte and heterophil infiltration occurred (Turmelle and Mendonça, unpublished data). The immune response to PHA challenge in bats suggests that both innate and adaptive components are responsible for local swelling at the injection site, as was found in birds by Martin et al. (2006a). Because PHA elicits T-lymphocyte response at the site of injection, this method is a good overall measure of cell-mediated immune function in this species.

Statistics

The bactericidal indices among all individuals included in the final data set were normally distributed. However, T cell responses among individuals were not normally distributed, and thus the data were log-transformed to fit a normal distribution prior to statistical analysis. Non-transformed values, means ± standard error, of bactericidal and T cell indices are presented. Indices for either assay did not differ statistically between reproductive classes (BKA, *P* = 0.374; PHA, *P* = 0.961). Thus, all reproductive classes were combined prior to analyses of the data sets.

For both immune indices, a nested mixed ANOVA model, with one covariate (general linear model; using JMP 5.0.1), was used for comparison of roost type. By roost type we refer to the structure of the roost (bridge or cave), whereas by colony we refer to the location at which the individual bats were sampled (Frio Cave, Davis Cave, Seco Creek Bridge and East Elm Creek Bridge). Because we were only able to sample four colonies, a nested design was used (comparing roost types, with colony nested within roost type) to examine if there is significant variation in roost types beyond variation due to differences among colonies alone. Roost type, and colony nested within roost type were independent variables and body condition, a ratio of body mass to right forearm length, was a covariate in the overall model. We also treated colony as a random effect (i.e., we are interested in generalizing to other colonies of Brazilian free-tailed bats); roost type (bridge or cave), a fixed effect, was tested over colony nested within roost type. Because colony size varies with roost type (the majority of cave colonies, including those studied here, have more bats living in them than bridge colonies), we were unable to directly test for the effects of population size on immune function, but we will explore this hypothesis in our discussion below. Lastly, we tested for a linear association between bactericidal ability and the log-transformed T cell response data, within individuals subjected to both assays (*n* = 74), using Pearson’s product-moment correlation (*z* = 0.05).

Results

Innate immune function: bactericidal ability

The overall model of roost type, colony within roost type and body condition significantly explained variation in bacterial killing ability (*P* = 0.03, *F* = 2.88[3,88], $R^2 = 0.12$). Within the model, colony (within roost type) was the only significant predictor of the variation in bactericidal ability (*P* < 0.05; Fig. 1a). Blood from females at Davis Cave had significantly lower killing ability (20.19 ± 4.32) than the three other colonies; Frio Cave (35.46 ± 4.60), Seco Creek Bridge (44.31 ± 7.59), and East Elm Creek Bridge (38.68 ± 6.73). Other variables including body condition (*P* = 0.77) and roost type [bridge (41.15 ± 5.15) and cave (27.35 ± 3.22); *P* = 0.06] were not significant predictors of bactericidal ability in female bats, although roost type borders on significance. There were no interactions between roost type or colony nested within roost type and body condition.

Adaptive immune function: T cell mediated immune response

The overall model of roost type, colony within roost type and body condition significantly explained variation in
Within the model roost type and colony (within roost type) were both significant predictors of PHA response \( (P < 0.001 \text{ and } P = 0.02, \text{ respectively}) \). Females from caves \( (1.68 \pm 0.07) \) exhibited a greater T cell response than those living in bridges \( (1.05 \pm 0.12) \). Females from Davis Cave \( (1.54 \pm 0.08) \) and Frio Cave \( (1.90 \pm 0.10) \) had larger responses than those at the bridge colonies, East Elm Creek \( (1.14 \pm 0.21) \) and Seco Creek \( (1.01 \pm 0.15; \text{ Fig. } 1b) \). Variation in T cell response was not explained by body condition \( (P = 0.33) \). Again, there were no interactions between roost type or colony nested within roost type and body condition.

**Combined immune function**

Within individual females, there was a weak but significant negative correlation between bactericidal ability and T cell response to PHA \( (\text{Pearson's } \rho = -0.190, P = 0.05) \). Females that produced larger swellings in response to PHA had blood with lower bactericidal ability (Fig. 2).

**Discussion**

This study demonstrates that individual *T. brasiensis* differ in their ability to mount functional immunological responses, and that roosting ecology significantly impacts immune function. We also provide evidence of a negative association between innate and adaptive immune responses within individuals.

Blood from female bats at Davis Cave had lower bacterial killing ability compared to females at Frio Cave and the two bridge sites (Fig. 1a). This trend is also present when roost types are compared; suggesting that there are inherent differences between bridges and caves that likely affect immunity. Although this result is not linked to body condition, there may be additional factors that affect innate immunity which are not included in our model. One possible factor could be differences in ectoparasitism among the colonies surveyed. High parasite loads affect immune function adversely in a variety of vertebrates (Sheldon and Verhulst 1996; Möller et al. 1999). Examination of infestations of ectoparasitic mites on *T. brasiensis* at these same sites show that individuals at Davis Cave, the site with the lowest mean BKA index, had the highest degree of mite cover, followed by individuals at Frio Cave and then at bridge sites (Turmelle 2005). Although these types of immune responses are not directly related, we suggest that
higher parasitism of bats in the colony at Davis Cave may reflect a weakened immune system overall; however, the exact causative mechanisms are currently unknown. Research on the relationships between ectoparasitism and immune responses in *T. brasiliensis* is in progress.

The lower T cell responses in females occupying bridges compared to their cave-dwelling counterparts indicates that adaptive immune response is also influenced by roost type (Fig. 1b). This supports our hypothesis that environmental differences may exist between roost types. Bats living in bridges may experience physiological stress associated with living in man-made roosts that negatively impact adaptive immune responses. As reviewed by Nelson et al. (2000) immune suppression associated with stress hormones has been demonstrated in several species of vertebrates. Notwithstanding, evidence linking stress hormone levels and immune system function in the Brazilian free-tailed bat is currently lacking. Alternatively, this pattern may also reflect differences in colony size between the roost types. While our model does not directly test for the effects of colony size on swelling response to PHA, a post-hoc test shows a positive correlation ($P < 0.0001$, $F = 19.79(3,161)$, $R^2 = 0.11$; Fig. 3) between mean PHA response and the best current estimates of colony size (Betke et al. 2008). Because colony size varied with roost type, and the effects of both variables may be working in combination, additional research will be needed to tease apart these effects. Similarly, in an interspecific study Møller et al. (2001) found that highly colonial swallows and martins had more robust T cell responses compared to less social songbirds, and they suggested that higher levels of parasitism observed in larger colonies may cause individuals to allocate more resources to combat infection. Tella et al. (2001), found the opposite relationship, examining colonies of the Magellanic penguin (*Spheniscus magellanicus*), with larger colonies having reduced T cell responses compared to smaller ones. The authors point out that density dependent food limitations and crowding negatively affected body condition and immunocompetence in fledglings. Since we found PHA response to increase with colony size, it is likely that these bats may not be particularly sensitive to crowding and food limitations.

Because the adaptive arm of the immune system responds to chronic infection (Klasing 2004), greater investment in immunity is expected in organisms threatened by a larger number of pathogens (Read and Allen 2000). Evidence suggests that bats in caves have higher ectoparasite loads than those roosting beneath bridges (Turmelle 2005). Differences in exposures to other pathogens may also vary by roost type. For example, once the spores of *Histoplasma capsulatum* and other fungi that grow in the accumulated guano below bats become airborne they can infect lungs and mucosal membranes (McMurray and Russel 1982). Resistance to *H. capsulatum* infection in mammals is dependent on a cellular immune response primarily mediated by T cells (Cain and Deepe 1998; Deepe 1994). One critical determinant of the course of infection is the inflammatory response evoked in response to host-pathogen interactions. The inability to evoke the appropriate inflammatory response can lead to disease progression. Compared to bats roosting in caves, bats roosting under bridges may be less exposed to fungi due to smaller accumulations of guano and greater air circulation between the guano and the roosting bats. Thus, we expect that higher pressures from parasites and fungi in caves may result in cave-roosting bats investing more energy in mounting memory-related, adaptive, resistance to the pathogens they commonly face. These findings are in contrast with those of Christe et al. (2000) who found that reproductive females of *Myotis myotis* had lower T cell responsiveness and higher mite loads than non-reproductive females, and that during lactation immunocompetence was positively correlated with body mass. In the current study, we found no evidence for differences in T cell response due to body condition or reproductive stage. Moreover, we found evidence for a positive relationship between ectoparasitic mite loads and T cell response, although this pattern may be indirect owing to differences in roosting condition in bridge and cave-roosting bats.

Results from our study have shown a negative correlation between PHA response and bactericidal ability within individual bats (Fig. 2). Forsman et al. (2008) found similar results, and showed that humoral bactericidal activity was negatively related to cutaneous immune activity (PHA

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**Fig. 3** Mean PHA index (response to PHA injection at 12 h) as a function of estimated colony size. Colonies estimates in order from largest to smallest are Frio Cave, Davis Cave, Seco Creek Bridge and East Elm Creek Bridge. The positive correlation between colony size and mean PHA Index was significant ($P < 0.0001$, $F = 19.79(3,161)$, $R^2 = 0.11$). Sample sizes for Frio and Davis Caves and Seco Creek and East Elm Creek Bridges are $n = 50$, 76, 24, 12, respectively.
assay) in house wrens. Moreover, Martin et al. (2006b) found that some immune responses can negatively affect other recent immunological activity in female white-footed mice, when examining simultaneous wound healing and cutaneous immune response. Our data suggests that individuals may not be able to maximally activate all aspects of immunity owing to competing costs and the variety of strategies associated with a multiple-component immune system (Klasing 2004). T cell mediated inflammation aids in the clearance of viruses (Hooper et al. 1998); therefore individuals at greater risk of coming in contact with viruses, such as rabies in bats, may be selected to invest more into adaptive immune defenses, potentially at the expense of reduced innate immune responsiveness.

In addition to Rabies and other lyssaviruses, Brazilian free-tailed bats may be incidental hosts to various arboviruses, including the flaviviruses (Family Flaviviridae, Genus Flavivirus) Rio Bravo Virus (RBV; Constantine and Woodall 1964), St Louis encephalitis (SLE; Allen et al. 1970; Herbold et al. 1983), and West Nile Virus (WNV; Davis et al. 2005; Pilipski et al. 2004), and the alphaviruses (Family Togaviridae, Genus Alphavirus) Eastern Equine Encephalitis (EEE), and Western Equine Encephalitis (WEE) (Constantine 1970). The infection cycle for these arboviruses occurs primarily between birds and arthropods, with incidental infection of mammals generally not producing significant viremia to act as amplifying hosts or facilitate transmission. Enzootic foci for arboviruses are generally swampy areas, drainage, or irrigated agricultural land, with infection occurring primarily during the summer months. Both risk factors are relevant for roosting aggregations of Brazilian free-tailed bats, particularly colonies that roost over standing water (bridges) or near irrigated agricultural lands (both caves and bridges). Infection with flaviviruses or alphaviruses should induce a viral neutralizing antibody response that confers lifelong immunity against the specific virus responsible for infection. In the context of our data, we would predict that bats which are unable to mount strong T cell mediated antibody responses would be more likely to succumb to viral infection. In subclinical cases, bats with lower T cell mediated response may develop limited protection against future or related exposures.

Determining the ecological factors that predict variation in the ability of individual Brazilian free-tailed bats to exhibit functional immunological responses is important to understand disease dynamics and population health in this and other species of colonial animals. A number of variables can affect the ability of organisms to combat pathogens and viral infections, and this study is a first step toward understanding susceptibility related to immunocompetence. A companion paper investigating links between roosting ecology and disease exposure (Rabies virus) is currently in review. Evidence from this current study suggests that immune responsiveness and presumably disease susceptibility are linked to the roosting ecology of the host. We predict that the immunotypic composition of a colony will influence pathogen transmission and persistence in free-ranging bats (Dimitrov et al. 2006, 2007).

Energetically costly aspects of immunological defense may also impose tradeoffs and energetic limitations for other seasonal behaviors and life-history functions that are important for survival. From this perspective the emerging field of ecoimmunology has contributed to our deeper understanding of the role of immunological competence in population regulation and the evolution of coloniality (Lochmiller 1996). The implications of such studies are further enhanced by examining species that are known to play a role in infectious disease transmission.

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