Hibernating Little Brown Myotis (*Myotis lucifugus*) Show Variable Immunological Responses to White-Nose Syndrome

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Abstract

White-nose syndrome (WNS) is an emerging infectious disease devastating hibernating North American bat populations that is caused by the psychrophilic fungus *Geomyces destructans*. Previous histopathological analysis demonstrated little evidence of inflammatory responses in infected bats, however few studies have compared other aspects of immune function between WNS-affected and unaffected bats. We collected bats from confirmed WNS-affected and unaffected sites during the winter of 2008–2009 and compared estimates of their circulating levels of total leukocytes, total immunoglobulins, cytokines and total antioxidants. Bats from affected and unaffected sites did not differ in their total circulating immunoglobulin levels, but significantly higher leukocyte counts were observed in bats from affected sites and particularly in affected bats with elevated body temperatures (above 20°C). Bats from WNS-affected sites exhibited significantly lower antioxidant activity and levels of interleukin-4 (IL-4), a cytokine that induces T cell differentiation. Within affected sites only, bats exhibiting visible fungal infections had significantly lower antioxidant activity and levels of IL-4 compared to bats without visible fungal infections. Overall, bats hibernating in WNS-affected sites showed immunological changes that may be evident of attempted defense against *G. destructans*. Observed changes, specifically elevated circulating leukocytes, may also be related to the documented changes in thermoregulatory behaviors of affected bats (i.e. increased frequencies in arousal from torpor). Alterations in immune function may reflect expensive energetic costs associated with these processes and intrinsic qualities of the immunocapability of hibernating bats to clear fungal infections. Additionally, lowered antioxidant activity indicates a possible imbalance in the pro- versus antioxidant system, may reflect oxidative tissue damage, and should be investigated as a contributor to WNS-associated morbidity and mortality.

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Introduction

Successful resistance against pathogen invasion involves the coordinated elevation of multiple innate and adaptive immune mechanisms, some of which may be particular to the type of invading organism. Although there is no single method to assess the overall elevation of immune response in reaction to pathogen invasion, quantifying the relative concentrations of immune function components (e.g. circulating leukocytes, immunoglobulin and cytokines) may provide evidence of which mechanisms have been activated, especially when compared to corresponding levels observed in healthy individuals of the same species. This process is complicated when the ecology of a newly emerging infectious disease is difficult to study (e.g. host and pathogen are adapted to relatively extreme conditions, host is widespread and free-ranging) and further challenged when little is known about the immune systems of affected species. These difficulties may result in slow elucidation of the role of immune responses in the pathogenesis of a particular disease because methods must be modified and validated for testing affected species and baseline data must be collected at the same time disease processes are being investigated.

Unfortunately, a devastating disease emerging in North American hibernating bat species known as white-nose syndrome (WNS) [1] has caused the deaths of an estimated 5.7 to 6.7 million bats [2] and is threatening at least one species with regional extinction [3,4]. Characteristics associated with the syndrome include low fat reserves [1,5,6] to emaciation [7], increased...
frequency in arousal from torpor [7,8] and atypical behavior such as roosting or flying outside of hibernacula in mid winter (Alan Hicks, pers. comm.). Additionally, severe wing damage is observed primarily early in the active season [9,10]. However, the diagnostic and most notable symptom of WNS is a cutaneous fungal infection that manifests itself as a white, filamentous growth on the muzzle, and powdery growth on the surfaces of ears, wings and tail membranes of hibernating bats [1,6]. The agent causing this characteristic fungal infection and the cause of the syndrome is a recently identified psychrophilic fungus named *Geomyces destructans* [11,12], which is adapted to the cold and humid conditions typical of hibernacula (i.e. site of hibernation) and proliferates optimally at a temperature range of 5–10°C [1]. *G. destructans* causes cupping erosions and ulceration of epidermal tissues, destruction of underlying connective tissue, and invasion of sebaceous and apocrine glands, as well as hair follicles [6]. However, according to an early study, bats with extensive fungal invasion collected during the hibernation period demonstrated scant presence of inflammation in the skin [6]. In contrast, bats exhibiting wing damage and sampled outside of affected hibernacula in early spring showed evidence of cutaneous cellular immune responses with suppurrative dermatitis and serocellular crusts containing fungal hyphae [6]. To date, presence of *G. destructans* has been confirmed on nine species of bats in the family Vespertilionidae (*Myotis lucifugus*, *M. septentrionalis*, *M. leibii*, *M. sodalis*, *M. velifer*, *M. griseus*, *M. austroriparius*, *Eptesicus fuscus*, *Perimyotis subflavus*; [1,11,13]), all of which hibernate for a significant fraction of the year [14]. *G. destructans* has also been observed or isolated from multiple bat species in Europe [15,16,17,18] and recent studies confirmed several European bats with lesions characteristic of WNS [19]. Notably, no major mortality events have been observed in Europe and Warneke et al. suggest *G. destructans* may have been recently introduced to North America from this region [7]. Although some aspects of bat immune function have been described [20,21,22,23], there is a general lack of knowledge regarding their responses particularly to pathogen invasion [24,25,26,27,28,29]. Additionally, virtually nothing is known about how bat immune responses vary with season and the use of daily and/or seasonal torpor.

Although little is known about the mechanisms involved in skin immune responses in bats [30], there is a probable set of responses activating against this fungal pathogen if bats respond to invading fungi using similar mechanisms as other taxa. Specifically regarding invasion through the skin, these mechanisms should include phagocytosis by resident and recruited innate immune cells (i.e. macrophages, neutrophils), the respiratory burst, edema, vascular reaction and an increase in acute-phase proteins [31]. Activation of complement proteins may also occur in the stratum vascular reaction and an increase in acute-phase proteins [31]. We observed that these alterations are also related to hibernation stage (i.e. early, mid-, or late winter) and body condition. In the current study, we continued with a similar line of research using multiple measures of immune function to test the following hypotheses: (1) that WNS-affected bats are elevating immune parameters presumably in response to *G. destructans*, (2) that immune responses are positively related to body temperature, or state of torpor versus arousal, and (3) that immune responses are positively related to body condition, or available energy stores. Testing these hypotheses are critical for understanding the pathogenesis of WNS since attempted but unsuccessful immune responses against *G. destructans* may contribute to mortality in itself and lead to depletion of critical energy stores that further reduce the health status of WNS-affected bats.

**Methods**

We used blood-based methods that could be applied to samples stored frozen because the logistics of collecting bats both within and outside the affected region precluded our ability to test fresh tissues. We also focused on the most efficient use of blood samples, since even when using terminal sampling methods only very small volumes (~150 μL whole blood) can be collected from animals of this size (6-9 g), especially while they are torpid. Using each of these methods, we tested blood from the same individuals used in our previous study and compared little brown myotis captured from WNS-affected and unaffected sites throughout the 2008–2009 hibernation period.

**Ethics Statement**

Capture, handling and sample collection protocols for this study were reviewed and approved by the Boston University IACUC (protocol #08-022) and the US Fish and Wildlife Service Disinfection Protocol for Bat Studies was used for all collections. Authorized state biologists with whom we worked directly permitted all sample collections for our work in New Jersey, New York, and Pennsylvania. Protocols were permitted in Massachusetts (Permit #167.08 SCM), Michigan (Permit #SC620) and Vermont (10 VSA Section 5408: Thomas H. Kunz, 2008–09). Bats were collected by hand from roost substrates, individually placed in cloth bags, and sacrificed by decapitation.

**Collection and Sampling Methods**

Adult female little brown myotis were collected from the following affected sites during the winter of 2008–2009: William’s Hole Six Mine, Ulster County, New York State on December 17, 2008 (n = 18); Aeolus Cave, Bennington County, Vermont on 18 November, 2008, 31 January, 2009, and 27 March, 2009 (n = 58); Chester Mine, Hampden County, Massachusetts on 20 November, 2008, 2 February, 2009 (n = 37); Hibernia Mine, Morris County, New Jersey on 13 January, 2009, 16 March, 2009 (n = 38). Adult female little brown myotis were collected from the
unaffected CS&M Mine, Lawrence County, Pennsylvania on 21 January 2009, 17 March 2009 (n = 36) and Jones Adit/Vulcan Mines, Dickinson County, Michigan on 25 January 2009 (n = 19). For the purposes of this study we considered November and December as early hibernation, January and February as mid-hibernation, and March as late hibernation.

On each sampling date, 10 adult female little brown myotis were collected, six each corresponding to the following body temperature categories: 1) torpor: <10°C, 2) rewarming phase: 10–20°C and 3) euthermic: >20°C. Arousal from torpor to achieve these Tₜₙ’s was assisted using an electric heating pad and insulated bag. Rectal Tₐ was measured using a small thermocouple (Omega HH82A, Stamford, CT) inserted before and after individuals were sacrificed by decapitation. Once a bat reached the desired temperature it was sacrificed immediately, except in the case of bats sampled near 37°C, which were held for no longer than 30 minutes before sacrifice. Because hibernating bats naturally undergo passive rewarming from arousing or euthermic bats roosting adjacent to them [42] it is unlikely this manipulation introduced an additional stressor. General life history data were collected, including body mass (g) and length of forearm (mm) and all bats were visually inspected for fungal infections and wing damage [9]. Individuals that exhibited obvious signs of fungal growth were defined as symptomatic; individuals that did not exhibit such signs were considered asymptomatic for the purposes of our comparisons. Blood was collected into 70 mm heparinized capillary tubes and centrifuged for 3 minutes to separate plasma from formed elements. Hematocrit was estimated using a card style hematocrit reader. Separately, plasma and red blood cell fractions were frozen on dry ice or in a dry nitrogen dewer during field collection and transportation, then stored at −80°C until assays were performed.

Blood Smear Preparation and Analysis

We analyzed blood smears to quantify total circulating leukocytes and to detect potential changes in concentrations of circulating leukocytes as they migrate from other tissues through the periphery to the site of infection. A small sample (~5 µL) of fresh whole blood was used to prepare a single blood smear for each individual. Our initial attempts to prepare blood smears directly after sacrifice failed due to the extreme humidity in caves and mines. We therefore held blood samples in an ice bath until we exited hibernacula and reached drier conditions, which delayed preparation of smears for no longer than 8 hours after sacrifice at some sites. After preparation, blood smears were air-dried for 30 minutes and fixed in 100% methanol. A methylene blue – eosin based stain kit (Hema-3 Stain, ThermoFisher Scientific 22-122-911) comparable to the Wright-Giemsa method [43] was used to differentially stain leukocytes and, after another period of drying, the solution turned yellow. The underside of the plate was wiped with a lint-free cloth and absorbance was measured at 450 nm using a microtiter plate reader (Bio-Rad Laboratories Inc., Model # 680). To standardize results from multiple assays, mean absorbances were multiplied by their dilution factor and a coefficient of variation, which was calculated for each assay using the interassay variation in quality control samples [43]. Percent differences between sample duplicates were all within 10%, except four samples that had percent differences greater than 10% but less than 15%. Interassay variation was 13.7%.

Total Antioxidant Power

We measured total antioxidant activity to estimate the maintenance of antioxidants in the system. We conducted this analysis to test for the capacity of this defense system to protect the self from the destructive effects of free-radical production. This destruction could either happen during the respiratory burst or during the massive increases in mitochondrial respiration that one required visualizing 95 fields before a single leukocyte was observed. To the best of our knowledge there is no defined correction factor to convert average number of leukocytes per field to leukocytes per µL in bats. We therefore express total leukocyte counts as mean number cells per field.
occur during the rewarming phase, which notably occurs more frequently in WNS-affected bats [7,8]. A colorimetric assay kit (Oxford Biomedical Research #TA02d) based on the ability of antioxidants to convert Copper (II) to Copper (I) was used to estimate total antioxidant power (TAP) or the collective activity of all antioxidants in the red blood cell fraction of blood samples. For each sample, erythrocyte volume was measured to the nearest microliter using a standard metric ruler, equating each millimeter in capillary tube length to one microliter in erythrocyte volume. Erythrocytes were then expelled from their capillary tubes using a bulb assembly, and lysed by diluting each sample 1:10 in ice-cold deionized water. Because the chromogenic reagent used in the assay kit exhibits an absorption maximum at 450 nm [44] and hemoglobin absorbs wavelengths between 340 and 600 nm [45], hemoglobin was precipitated by adding 0.25 volumes 98% ethanol and 0.15 volumes chloroform to each diluted sample. Following the addition of ethanol and chloroform, samples were vortexed for approximately 1 min and then centrifuged at 10,000 rpm for 10 min at 4°C. The clear supernatant was collected and frozen at −80°C for no more than 1 week, then assayed following the kit protocol without modification. Absorbance was measured using a microtiter plate reader (Bio-Rad Laboratories Inc., Model # 680). Net absorbance values were calculated by subtracting the mean of each sample or standard’s reference measurements from the mean of each sample or standard’s corresponding final absorbance measurements. TAP values were initially expressed in terms of μM uric acid equivalents using each sample’s net absorbance divided by the value for the slope of the uric acid standard curve. Values were further converted into μM copper reducing equivalents (CREs) by multiplying each uric acid equivalent by a given constant, 2189 μM.

Circulating Levels of Various Cytokines

A bead-based immunofluorescence assay developed for detecting mouse analytes (Luminex Inc., Austin, TX) and using multiplex cytokine kits and reagents (R&D Systems, Minneapolis, MN) was used to estimate plasma levels of the following cytokines: IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, and TNF-α. These cytokines were chosen to detect aspects of the fever, acute phase, inflammatory and anti-inflammatory responses (IL-1β, IL-6, TNF-α, and IL-10) as well as T cell subset responses (T41/1: IFN-γ, IL-2, IL-12p70; T41/2: IL-4, IL-6, IL-10 and IL-13). This method employs a sandwich immunoassay-based protein array system containing dyed microspheres conjugated with a monoclonal antibody specific for a target protein. Plasma samples were thawed, diluted to 1:4, and assayed in duplicate. Following the kit protocol, antibody-coupled beads were first incubated with the plasma sample then with biotinylated detection antibody before finally being incubated with streptavidin-phycocerythrin. A broad sensitivity range of standards (R&D Systems), ranging between 7.09 and 50,300 pg/mL depending on the cytokine were used to increase sensitivity. Captured bead-complexes for both samples and standards were detected using a Luminex 200 array reader at the Harvard Medical School Department of Genetics Biopolymers Facility. This instrument uses fluorescent bead-based technology with a flow-based dual laser detector and real-time digital signal processing that allows for the simultaneous detection of multiple cytokines in a single sample.

Statistical Analysis

Total immunoglobulin levels (total Ig) were normally distributed (Shapiro-Wilks test p = 0.79). We used a parametric mixed model ANOVA/ANCOVA for this dataset and tested the following independent variables for significance: site, perpetual date, hibernation stage (i.e. early, mid, late hibernation) for samples collected from the affected Aelous Cave only, Tb, hematocrit, body mass index (BMI; log body mass (g)/log length of forearm (mm)), site type categorized as WNS-affected versus unaffected, and individuals categorized as symptomatic versus asymptomatic (from affected sites only and based on the presence or absence of visible cutaneous fungal infections characteristic of WNS). We tested for interactions and used a nested design to test for differences in total Ig between affected and unaffected sites beyond individual site differences. We used Type IV sums of squares to account for uneven sample sizes between groups and an unbalanced design with missing treatments (e.g. no unaffected bats were sampled during early hibernation). Comparisons of interest were extracted using simple contrasts and each contrast was examined for significance. We used Levene’s test of unequal variance and Brown-Forsythe and Welch’s tests to assess significance when Levene’s p < 0.05. To further determine differences and relationships between immune response and variables included in the reduced model, we used Tukey’s post hoc analysis.

Total leukocyte counts (Shapiro-Wilks test p<0.001), TAP levels (Shapiro-Wilks test p<0.05) and cytokine levels (IL-4; Shapiro-Wilks test p<0.001) were not normally distributed. Because transformations did not improve normality for these measures, statistical analysis was conducted using non-parametric tests including the Kruskal Wallis test, the Mann-Whitney U test, and the Spearman’s Rho test. Two extreme values were excluded from TAP analysis. SPSS (16.0.2, 2008) was used for all analyses with α = 0.05.

Results

Test statistics for all analyses are indicated in Table 1. For greater detail, including distribution of samples across groups, see Table S1.

Total Circulating Leukocytes

Total circulating leukocyte counts (WBC) were significantly higher in bats from affected sites compared with bats from unaffected sites. Within affected sites, WBC did not vary between symptomatic and asymptomatic bats. Across all samples, WBC did not vary depending on hibernation stage and also did not vary depending on hibernation stage within groups representing bats from affected or unaffected sites. Hematocrit was not significantly related to WBC across all samples or within groups representing bats collected from WNS-affected and unaffected sites respectively. Tb was marginally correlated with WBC in bats in affected from collected affected sites, but was not correlated with WBC in bats from unaffected sites. To investigate this possible relationship further, we categorized Tb based on temperatures that were indicative of torpor, the rewarming phase, or euthermia. WBC was significantly related to temperature category specifically in affected bats, but not in bats from unaffected sites. In affected bats, WBC was highest in individuals with Tb>20°C (Figure 1). WBC did not vary depending on BMI in bats from affected or unaffected sites. This lack of a relationship held true in each group throughout the entire hibernation period as well as within each hibernation stage (i.e. early, mid-, late).

Total Immunoglobulins

The reduced model including site and hibernation stage significantly explained variation in total immunoglobulins (Ttg; N = 154; F2,151 = 7.228; p<0.001; R2 = 0.26; adjusted R2 = 0.22). Whether a site was affected by WNS did not significantly predict...
TAP did not correlate with hematocrit. BMI was significantly and negatively correlated with TAP when all hibernation stages were analyzed together. However, correlate with TAP levels in bats from affected or unaffected sites bats from affected and unaffected sites separately. BMI did not correlate with Tb.

Within affected sites, symptomatic bats had significantly lower total Ig compared with bats from unaffected sites. Total Ig varied throughout the hibernation period (early: 0.93 ± 0.200 corrected OD, n = 13; mid: 2.115 ± 0.225 corrected OD, n = 13; late: 1.777 ± 0.193 corrected OD, n = 16) and was highest during mid-hibernation as compared with early (p = 0.003) and late hibernation (p = 0.001). We observed no difference in TIg between visibly symptomatic and asymptomatic bats within affected sites and no relationship between TIg and BMI, hematocrit, or T

### Total Antioxidant Power

Bats from WNS-affected sites exhibited significantly lower total antioxidant power (TAP) compared to bats from unaffected sites. Within affected sites, symptomatic bats had significantly lower TAP compared to asymptomatic bats. Across samples collected only from Aelous Cave, TAP did not vary depending on hibernation stage. Across all samples, T

### Circulating Cytokines

Of the nine cytokines that we attempted to detect using the multiplex cytokine array with mouse specific reagents, IL-4 was the only analyte that was detected with any consistency. We therefore focused on this cytokine alone in our analyses. Circulating levels of IL-4 were greater in bats collected from unaffected sites compared with bats from affected sites. Within affected sites only, IL-4 levels were significantly lower in bats displaying visible fungal infections characteristic of the syndrome. IL-4 did not differ depending on hibernation stage in bats collected from Aelous Cave. Across all samples, IL-4 did not differ depending on BMI, nor did it vary depending on BMI when we analyzed bats from affected and unaffected sites separately. Across all samples, IL-4 levels did not correlate with T

### Discussion

Our observations of increased WBC and decreased TAP suggest an inflammatory response is stimulated in some WNS-affected bats [46,47,48]. These results apparently contradict those of Meteyer et al. [6], where little inflammation was observed in the cutaneous tissues of infected bats sampled during hibernation. However, our
show alterations in this cytokine suggesting that T cell responses are potentially induced.

**Total Circulating Leukocytes**

We estimated extremely low leukocyte population counts in hibernating little brown myotis across our sample set and observed significantly higher WBC in WNS-affected bats sampled at temperatures corresponding to euthermia ($T_b > 20^\circ C$) compared with rewarming ($T_b < 20^\circ C$) and torpor ($T_b < 10^\circ C$). This change in circulating leukocytes did not correlate with hematocrit, suggesting it was not related to hydration status of the individuals sampled, nor was a similar pattern observed in samples from unaffected bats. We did not observe differences in WBC between visibly symptomatic and asymptomatic bats within affected sites. It seems that, if obvious signs of fungal infection were an indication of infection status, we would have observed some indication of this in cell counts. One possible explanation is that individuals without visible evidence of fungal infections may have groomed all external fungus from their skin during more recent arousals from torpor. Alternatively, these individuals may have been in the earlier stages of fungal infection before hyphae and conidia were manifested and before additional leukocytes could be produced or recruited.

We were unable to conduct differential leukocyte counts because of the extremely low white blood cell populations present on blood smears and because of other factors likely associated with hibernation physiology (e.g. viscosity of blood), or possibly suboptimal field conditions (e.g. extreme humidity). A similar difficulty was encountered by Krutzsch and Hughes [41] when they compared hematological values in active and torpid *Tadarida brasiliensis* and *Myotis velifer*. They were able to quantify total circulating leukocytes, but were unable to perform differential counts, and observed an overall decrease in circulating leukocytes in torpid bats compared with active bats. Bouma et al. [50] also observed significant decreases in leukocyte populations in hibernating European ground squirrels (*Spermophilus citellus*), which returned to euthermic summer levels 1.5 hours after arousal. In unaffected (i.e. control) bats, we did not observe elevated WBC associated with euthermia as would be expected. Our samples were collected within 30 minutes of animals reaching a $T_b$ indicative of euthermia, which may have been too soon to detect a return to normothermic values. However, bats typically rewarm to euthermia more rapidly than other hibernators and may have arousal bouts under 90 minutes [51] making it difficult to predict changes in circulating leukocytes given the extent to which they differ from other taxa.

**Total Circulating Immunoglobulins**

The role of antibody immunity in controlling invasive fungal infections has been difficult to determine in any taxon [52,53]. Support for the protective aspect of antibodies has been equivocal, and a number of studies have produced contradictory results [54]. Nonetheless, there is a body of evidence showing that antibodies prevent the adherence of fungi to tissue surfaces, neutralize toxins produced by fungi, function as opsonins, and mediate antibody-dependent cellular cytotoxicity [53].

Our observations of total Ig (TIg) in little brown myotis show that bats hibernating in sites affected by WNS have TIg levels no different from bats hibernating in unaffected sites. This suggests that affected bats are capable of maintaining similar TIg levels throughout the hibernation period as compared with controls. Further research needs to be performed to determine whether or not little brown myotis are capable of producing anti-*G. destructans* antibodies. These studies should be conducted during the active
season when there are fewer constraints on the immune system before being pursued during the hibernation period.

Total Circulating Antioxidants

Upon detection of microbial intruders, phagocytes of the host’s innate immune system increase their consumption of molecular oxygen through the activity of NADPH oxidase. This oxidative burst results in the production of metabolites that propagate strongly anti-microbial free radicals such as various reactive oxygen and nitrogen species [48,55]. To protect the self, antioxidants are produced by the host and neutralize the damaging effects of free-radical production during the inflammatory process [56,57]. Tissue concentrations of these free-radical scavengers are known to vary depending on infection status [58,59,60] and disease state [61,62,63]. When the rate of free radical production exceeds the antioxidant capacity of an organism, this imbalance, known as oxidative stress, results in damage to DNA, proteins, and lipids [48,64]. Another component of the anti- / pro-oxidant system as it may relate to WNS involves the extreme fluctuations in oxygen consumption of bats while they periodically transition from deep torpor to euthermia [65]. During arousals, the rapid rates of oxygen consumption in brown adipose tissue (BAT) promote the generation of reactive oxygen species [66], which easily oxidize lipid molecules [67]. It is possible that antioxidants are relatively depleted in WNS-affected bats because of sustained immune responses against G. destructans that may start before bats enter deep hibernation or may commence during interbout arousals. Increased frequency of arousals probably also leads to reductions in antioxidants as they neutralize the additional free radicals. These two mechanisms in combination could present a situation where the production and replacement of antioxidants cannot occur in sufficient amounts to balance the system. Moreover, during hibernation, protein synthesis is slowed [38], therefore potentially inhibiting the replenishment of certain antioxidants. Since WNS-affected bats exhibit limited fat reserves [1,5,6,7], these animals probably have diminished ability to produce additional proteins compared with unaffected bats. One additional possibility is that antioxidants are being shunted from the periphery to susceptible tissues in WNS-affected bats to protect these tissues against oxidation [68]; however, this would likely be a temporary response that may not be protective throughout hibernation.

Because we did not concurrently measure products of lipid peroxidation, such as malonaldehyde, it is difficult to determine if little brown myotis hibernating in WNS-affected sites are indeed experiencing oxidative stress. These measures should be made to assess the relative degree of oxidative stress that affected bats may be experiencing; and the ability of phagocytes to engulf G. destructans and produce intracellular and extracellular reactive oxygen and nitrogen species should also be tested.

Circulating Cytokines

We observed consistent results in only one of the analytes detected (IL-4) and it is difficult to tell if negative results associated with other cytokines of interest are due to a lack of cross-reactivity between mouse-specific reagents and bat cytokines or an absence of specific cytokines in circulation. Nevertheless, our results show that bats hibernating in WNS-affected sites have significantly lower IL-4 levels compared with bats from unaffected sites. Additionally, within affected sites only, visibly symptomatic bats showed lower IL-4 levels than asymptomatic bats. There may be a polarization of T lymphocyte responses in favor of Th1 responses in infected bats given that inhibition of IL-4 is known to decrease the activation of the Th2 subset [69]. Additionally, if visible signs of fungal infections are any indication of infection status, visibly symptomatic bats may be in a later stage of infection and immune activation. It is possible that infected individuals are attempting resistance through T cell mediated responses, which would result in activation of phagocytes at the site of infection. However, without the measurement of additional cytokines, particularly IFN-γ, it is difficult to know if the appropriate stimuli for activation of the Th1 subset are present. This could be an extremely important avenue of further inquiry because Th1 responses promote effective fungal clearance (i.e. memory responses) and increase inflammation in other taxa, whereas Th2 responses are inhibitory to the control of fungal infections [34].

Conclusions

While the aspects of immune response measured in this study are an important selection of responses that might occur against G. destructans, it is evident that these processes respond to this disease to different degrees and even in different directions. Underlying mechanisms and the biological meaning of the observed changes are yet to be described. Thus, future studies should identify and test responses specifically against G. destructans (e.g. measure anti-G. destructans antibodies) in addition to the measurement of general immune parameters (e.g. total circulating leukocytes). Additionally, controlled captive studies that measure immune responses combined with measures of infection (i.e. histology) over a time course could provide clearer evidence of what capability little brown myotis have for successful immunological resistance against G. destructans. Regardless, this study demonstrates that hibernating little brown myotis are capable of certain immunological responses to WNS and suggests that investigating the role of oxidative tissue damage in WNS pathogenesis is warranted.

Supporting Information

Table S1 Statistics for all outcome variables used in this study, including total circulating leukocytes, total circulating immunoglobulins, total antioxidant power, and IL-4. (XLSX)

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Author Contributions

Conceived and designed the experiments: MSM JDR THK. Performed the experiments: MSM TDM MLN REP JSF. Analyzed the data: MSM. Wrote the paper: MSM JDR THK.
References

1. Blehert DS, Hicks AC, Behr M, Meteyer CU, Berlozi-Sizer BM, et al. (2009) Bat white-nose syndrome: an emerging fungal pathogen? Science 323: 227–228.

2. United States Fish and Wildlife Service (2012) News Release: North American bat death toll exceeds 5.5 million from white-nose syndrome. http://www.fws.gov/whitenosesyndrome/pdf/WNS_Mortality_2012_NR_FINAL.pdf. Accessed 4 May 2012.

3. Frick WF, Pollock JF, Hicks AC, Langewig KE, Reynolds DS, et al. (2010) An emerging disease causes regional population collapse of a common North American bat species. Science 329: 679–682.

4. Langewig KE, Frick WF, Bried JT, Hicks AC, Kunz TH, et al. (2012) Sociality, density-dependence and microclimates determine the persistence of populations suffering from a novel fungal disease, white-nose syndrome. Ecology Letters 15: 1050–1057.

5. Courin F, Stone WB, Risatti G, Gilbert K, Van Krainingen HJ (2010) Pathogenic findings and liver elements in hibernating bats with white-nose syndrome. Veterinary Pathology 47: 214–219.

6. Meteyer CU, Buckles EL, Blehert DS, Hicks AC, Green DE, et al. (2009) Histopathologic criteria to confirm white-nose syndrome in bats. Journal of Veterinary Diagnostic Investigation 21: 411–414.

7. Warneke L, Turner JM, Bollinger TK, Lorch JM, Misra V, et al. (2012) Geomyces destructans, the white-nose syndrome fungus, in bats from the Czeh Republic. Emerg Infect Dis 18: 154–162.

8. Gargas A, Trest MT, Christensen M, Volk TJ, Blehert DS (2009) Geomyces destructans sp. nov. associated with bat white-nose syndrome. Mycologia 101: 141–154.

9. Turner GG, Reeder DM, Coleman JTH (2011) A five-year assessment of white-nose syndrome affected sites. PLoS One 6(11): e27430. doi:10.1371/journal.pone.0027430.

10. Turmelle AS, Jackson FR, Green D, Mc Cracken GF, Rupprecht CE (2010) Host immunity to repeated rabies virus infection in big brown bats. Journal of General Virology 91: 2560–2566.

11. Sulkin SE, Allen R (1974) Virus infections in bats. Monographs in Virology 8: 1–103.

12. Tesh RB, Schmedau JR, JD (1966) Experimental infection of North American insectivorous bats (Tadarida brasiliensis) with Histoplasma capsulatum. American Journal of Tropical Medicine and Hygiene 15: 544–551.

13. Greer DL, McMurray DN (1981) Pathogenesis of experimental histoplasmosis in the bat, Artibeus lituratus. American Journal of Tropical Medicine and Hygiene 30: 653–659.

14. Turmelle AS, Ellison JA, Mendoza MT, McCracken GF (2010) Histological assessment of cellular immune response to the phytohemagglutinin skin test in Brazilian free-tailed bats (Tadarida brasiliensis). Journal of Comparative Physiology B 180: 1155–1164.

15. Kupper TS, Fulhbrigge RC (2004) Immune surveillance in the skin: mechanisms and clinical consequences. Nature Reviews Immunology 4: 211–222.

16. Speeth C, Rambusch G, Würzner R, Lau-Flor C (2008) Complement and fungal pathogens: an update. Mycoses 51: 477–496.

17. Takeda K, Kaisho T, Akira S (2003) Toll-like receptors. Annual Reviews of Immunology 21: 353–376.

18. Romani L (2011) Immunity to fungal infections. Nature Reviews 11: 273–288.

19. Shoham S, Levitz SM (2005) The immune response to fungal infections. British Journal of Haematology 129: 569–582.

20. Moore MS, Reichard JD, Murtha TD, Zahedi B, Fallier RM, et al. (2011) Specific alterations in complement protein activity of little brown myotis (Myotis lucifugus) hibernating in white-nose syndrome affected sites. PLoS One 6(11): e27430. doi:10.1371/journal.pone.0027430.

21. Turmelle AS, Ellison JA, Mendoza MT, McCracken GF (2010) Histological assessment of cellular immune response to the phytohemagglutinin skin test in Brazilian free-tailed bats (Tadarida brasiliensis). Journal of Comparative Physiology B 180: 1155–1164.

22. Kupper TS, Fulhbrigge RC (2004) Immune surveillance in the skin: mechanisms and clinical consequences. Nature Reviews Immunology 4: 211–222.

23. Speeth C, Rambusch G, Würzner R, Lau-Flor C (2008) Complement and fungal pathogens: an update. Mycoses 51: 477–496.

24. Takeda K, Kaisho T, Akira S (2003) Toll-like receptors. Annual Reviews of Immunology 21: 353–376.

25. Romani L (2011) Immunity to fungal infections. Nature Reviews 11: 273–288.

26. Shoham S, Levitz SM (2005) The immune response to fungal infections. British Journal of Haematology 129: 569–582.

27. Moore MS, Reichard JD, Murtha TD, Zahedi B, Fallier RM, et al. (2011) Specific alterations in complement protein activity of little brown myotis (Myotis lucifugus) hibernating in white-nose syndrome affected sites. PLoS One 6(11): e27430. doi:10.1371/journal.pone.0027430.

28. Turmelle AS, Ellison JA, Mendoza MT, McCracken GF (2010) Histological assessment of cellular immune response to the phytohemagglutinin skin test in Brazilian free-tailed bats (Tadarida brasiliensis). Journal of Comparative Physiology B 180: 1155–1164.

29. Kupper TS, Fulhbrigge RC (2004) Immune surveillance in the skin: mechanisms and clinical consequences. Nature Reviews Immunology 4: 211–222.

30. Speeth C, Rambusch G, Würzner R, Lau-Flor C (2008) Complement and fungal pathogens: an update. Mycoses 51: 477–496.
56. Kelly SA, Havrilla CM, Brady TC, Abramo KH, Levin ED (1998) Oxidative stress in toxicology: established mammalian and emerging piscine model systems. Environmental Health Perspectives 106: 373–384.

57. Fridovich I (1995) Superoxide radical and superoxide dismutases. Annual Review of Biochemistry 64: 97–112.

58. Peterhans E (1997) Oxidants and antioxidants in viral diseases: disease mechanisms and metabolic regulation. Journal of Nutrition 127: 962S–965S.

59. Gharib B, Abdallahi OMS, Dessein H, Reggi MD (1999) Development of eosinophil peroxidase activity and concomitant alteration of the antioxidant defenses in the liver of mice infected with Schistosoma mansoni. Journal of Hepatology 30: 594–602.

60. Postma NS, Mommers EC, Eling WM, Zuidema J (1996) Oxidative stress in malaria: implications for prevention and therapy. Pharmacy World and Science 18: 121–129.

61. Ihara Y, Chuda M, Kuroda S, Hayabara T (1999) Hydroxyl radical and superoxide dismutase in blood of patients with Parkinson’s disease: relationship to clinical data. Journal of the Neurological Sciences 170: 90–95.

62. Mettutz PGH, Bartens C, Fischer M, Fridrich P, Steltzer H, et al. (1999) Antioxidant status in patients with acute respiratory distress syndrome. Intensive Care Medicine 25: 180–185.

63. Valentine JS, Deacette PA, Potter SZ (2005) Copper-zinc superoxide dismutase and amyotrophic lateral sclerosis. Annual Reviews of Biochemistry 74: 563–593.

64. Monaghan P, Metcalfe NB, Torres R (2009) Oxidative stress as a mediator of life history trade-offs: mechanisms, measurement, and interpretation. Ecology Letters 12: 75–92.

65. Lyman CP (1970) Thermoregulation and metabolism in bats. In: Wimsatt WA, editor. Biology of Bats. New York: Academic Press. 301–330.

66. Lopez-Torres M, Perez-Ocampo R, Barja de Quiroga G (1991) Aging in brown fat: antioxidant defenses and oxidative stress. Mechanisms of Ageing and Development 59: 129–137.

67. Halliwell B, Chirico S (1993) Lipid peroxidation: its mechanism, measurement, and significance. The American Journal of Clinical Nutrition 57: 715S–725S.

68. Tøien Ø, Drew KL, Chao ML, Rice ME (2001) Ascorbate dynamics and oxygen consumption during arousal from hibernation in Arctic ground squirrels. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 281: R572–R583.

69. Kindt TJ, Goldsby RA, Osborne BA (2007) Kuby Immunology. New York: WH Freeman and Company.