Resistance to White-Nose Syndrome in bat populations of the Northern Forest: exploring the critical disease-genotype-microbiome link

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Description (2 sentences):

We observed a change in fungal community composition among little brown bats in White-Nose Syndrome (WNS) affected versus unaffected populations, with WNS status (e.g., positive-WNS versus negative-WNS) and cave location helping to predict fungal community composition. We developed methods to characterize genes of the Major Histocompatibility Complex (MHC), which play important roles in the immune response of individuals, and observed a high number of MHC II DRB alleles in all populations regardless of WNS status.

Project Summary (non-technical; 350-400 words)

Cave-dwelling bat populations in North America are facing declines and the threat of regional extinction due to White Nose Syndrome (WNS), an invasive fungal disease caused by *Pseudogymnoascus destructans* (Pd; Minnis and Lindner 2013; Drees et al. 2017). Much research is focusing on bat recovery and adaptation to WNS because some of the originally infected populations are now showing signs of stabilizing. However, how host genetics and microorganisms may be influenced by their environment, how the two factors interrelate, and what their synergistic effect may be on WNS susceptibility is understudied. The relationship among host genetics and microbiome and the location of resistant populations are of considerable value to the management of bat species and populations. If resistant or adapted bat populations can be protected from additional threats such as habitat degradation, they may survive and help re-populate the reduced bat populations. Our objectives were to isolate potentially disease resistant genotypes of little brown bats (*Myotis lucifugus*) to help explain the relationships among fungal communities, host genotype, and the resistance to WNS. We characterized baseline mycobiomes from multiple locations spanning WNS-positive and -negative geographic regions. In addition, we characterized genes of the MHC, which play important roles in the immune response of individuals. As hypothesized, disease progression and sampling site were the major predictors of fungal community composition. We also found a high number of MHC II DRB alleles per population, average 20.8 $\pm$ 9.1, range 11 - 33, similar to reported diversity from other locations and bat species. This result may indicate lack of selection pressure or that selection maintained diversity through a bottleneck at this particular MHC gene, but spatial variability of the MHC II DRB alleles has not been fully analyzed at the time of this report. Our preliminary findings of spatial differences in the mycobiome community relative to WNS status in combination with potential spatial differences in diversity of the MHC alleles will allow analysis into the complex relationship between an individual's fungal load and immune response to WNS disease.

Acknowledgments:

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Background and Justification:

WNS, bat decline, and recovery

White-Nose Syndrome is a devastating fungal disease that has killed millions of cave dwelling bats and has spread nationally over the last decade (Frick et al. 2010). Although considerable knowledge exists about bat and fungal genetic diversity, less is known about the complex interactions among a host's immune response to disease based on their mycobiome and genetic make-up. Stabilizing bat populations with individuals that survive persistent exposure suggest there may be some genetic resistance to WNS (Langwig et al. 2017), and that the host mycobiome may contribute to this resistance (Boyles et al. 2011; Hoyt et al. 2015). If this complex interaction can be better understood and characterized, we may be able to identify selection pressures and develop new approaches to help conserve and restore remnant, adapted populations to help repopulate landscapes.

Extensive research effort has uncovered abiotic environmental factors that may influence the transmission and severity of WNS and other fungal diseases (Cryan et al. 2013), but little attention has been paid to potential genetic resistance (or adaptation) to disease. The degree of resistance to disease may depend in part on the genetic make-up of the individual host and affected population, which influences disease severity (Savage and Zamudio 2011; Robinson et al. 2012). There are several reported signs suggesting bat populations may be able to adapt to WNS if given enough time. First, Pd exists on European bats without causing mass mortality, indicating European bat species may have evolved resistance to infection (Warnecke et al. 2012; Wibbelt et al. 2010). Second, the bat populations in New York where Pd and WNS were first reported in 2006 are now showing signs of stabilizing (NYDEC 2012). Third, the survival and reproduction of individuals repeatedly exposed to WNS (Dobony et al. 2011), suggest some bats may be resistant to WNS. However, without identifying and protecting resistant individuals or populations, bats will incur a high extinction risk because of their reduced population sizes post-epidemic, the potential for reinfection, and the added effects of other pressures. If WNS-resistant genotypes are identified, threats in areas harboring these individuals can be reduced thereby aiding the natural expansion of genetically resistant bats needed for population recovery (O'Brien and Everman 1988; Thogmartin et al. 2013). For example, habitat loss and degradation of foraging areas around hibernacula, or loss of maternal roosts where females genetically resistant to WNS raise young that can pass on this resistance, can be monitored and reduced.

Mycobiome

Host-microbe symbioses and the efficacy of biocontrol efforts are context dependent (Daskin and Alford 2012), and host genotype may be regarded as an important part of that context. The mechanisms underlying the relationship of host genetics to the skin microbiome, which is the microbial community most relevant to WNS due to the disease's destruction of bat skin tissue (especially the wings and ears) (Blehert et al. 2009), are not well understood (de Koning et al. 2012). However, this knowledge is particularly important to northern forests where preserving the ecosystem benefits provided by bats is of particular concern and doing so requires a clear picture of the natural defenses of bats to WNS to develop biological control measures and effectively manage their recovery post-epidemic.

Goal

Our goal was to determine the variability of the mycobiome and class II MHC gene of the little brown bat (*Myotis lucifugus*) across WNS-positive and WNS-negative sites across the northeast and midwestern United States. We hypothesized a change in the mycobiome community composition and a reduction in MHC variants in WNS affected areas because of the potentially selective influence of WNS (Hartle and Clark 2007). However, natural selection can also preserve high levels of variability in bat populations carrying MHC alleles resistant to Pd (i.e. in affected but living individuals), given a potential selective advantage in the presence of WNS (Hartle and Clark 2007).

Identifying the distribution of resistant populations will help to protect critical habitat around these populations to enhance survival and reproductive success of surviving individuals. In addition, finding hibernating bat populations that are resistant or have adapted to WNS may be especially critical to repopulating bat populations across northern forests.

Methods

We used a polymerase chain reaction (PCR) based approach to isolate MHC immune system alleles by contrasting the genetic composition of WNS-negative populations to those surviving following the WNS epidemic, and applied a molecular approach with high-throughput amplicon sequencing (HTAS) to determine the mycobiome composition among these same sampling locations. For this report, we specifically describe changes in the allelic diversity at the MHC DRB II location, and community composition of fungi in the mycobiome using the ITS2 DNA barcode region.

Bat Tissue and Mycobiome Sampling

We collected wing tissue and mycobiome samples from 155 bats collected from 10 caves or abandoned mines. Because two caves in Wisconsin (Bay City Mine and Maiden Rock Mine) are less than five miles apart, the locations may represent a single site from an inheritable standpoint. This may also be the case for the two hibernacula in Pennsylvania (Glen Lyon and Treschow). Thus, from an inheritable standpoint, we have eight locations representing three negative-WNS and five positive-WNS geographic populations (Figure 1).

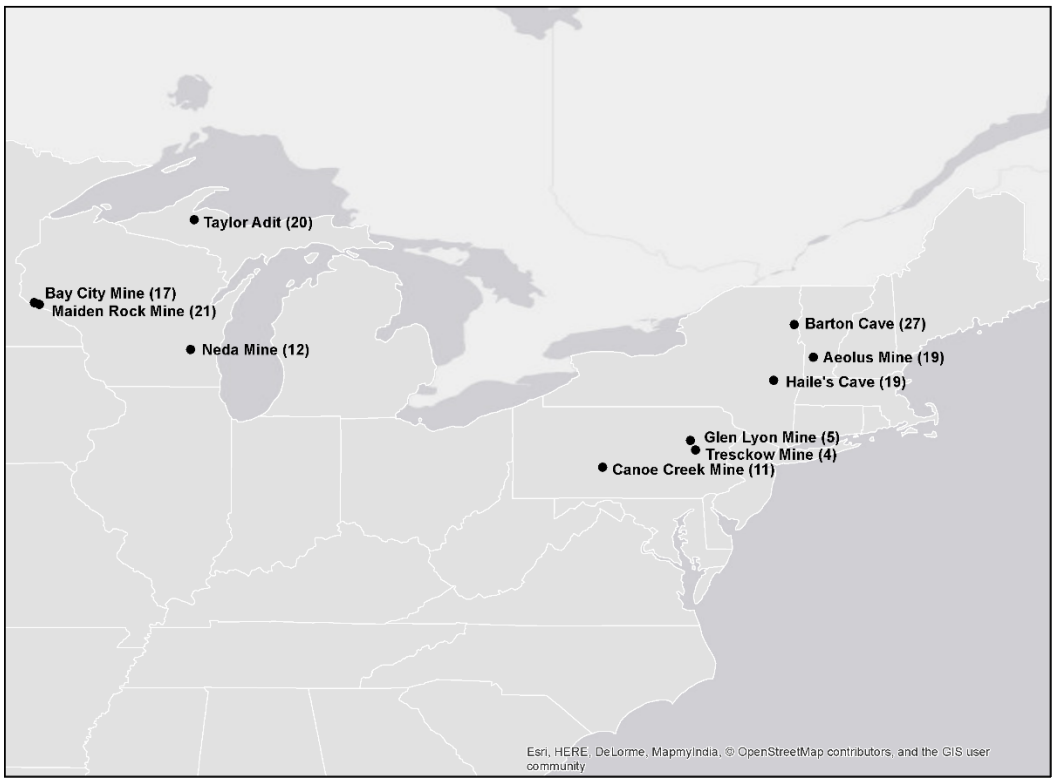

Figure 1. Approximate locations of the 10 hibernaculum where bats were sampled are marked by black dots. Because several hibernaculum are within close proximity and may represent a single population

from an inheritability standpoint for MHC, 8 study sites represent 5 northeastern sites that are positive for WNS (post-WNS) and 3 Midwestern sites that are negative for WNS (neg-WNS).

Bats were captured in cooperation with the corresponding state natural resource agency, and in conjunction with a planned hibernaculum visit or sampling effort. All bats were sampled April – May, 2014, except for Neda Mine in Wisconsin, which was repeatedly sampled 2014 and 2015 to secure enough individuals. In addition, Taylor Adit in the Upper Peninsula of Michigan, was sampled in November 2014 due to accessibility issues related to heavy snows prior to spring emergence. Thus, bats were sampled primarily but not exclusively before spring emergence. Bats were retrieved from cave walls by experienced bat handlers. All bat capture work was done in accordance to Forest Service IACUC 2016-001 by permitted individuals when caves were on Forest Service lands, and otherwise by permitted individuals within each State Agency.

For each bat, we recorded sex, relative age, and reproductive condition, body mass, and morphological measurements; wing tissue samples and wing swabs were collected for genetic and mycobiome analyses, which is described below. Two tissue samples, one from each wing, were collected per bat using a 2 or 4 mm sterile biopsy punch. Punches were taken from individual bats with a Reichard wing damage score of less than 3, as 3 indicates heavy damage from WNS. Bats with a wing score of 2 (moderate damage) were sampled when sufficient non-necrotic tissue was present. Wing tissue biopsies were taken from an area near the body wall within the lower half of the wing membrane or uropatagium. These locations have been demonstrated to have faster healing rates and are less disruptive to flight aerodynamics (Faure et al. 2009). Tissue samples were placed in sterile dry tubes or tubes containing RNALater solution, which is a nontoxic tissue storage reagent. To obtain mycobiome samples, a sterile cotton swab was used to gently swab both sides of the muzzle and the dorsal surface of each distended wing three to five times. The swab was rotated with each stroke and taken from the body towards the outer margin of the patagium. The tip of the swab was placed in a sterile vial. All blood and mycobiome samples were kept cold until they could be processed in the laboratory.

Laboratory methods – DNA isolation and other pre-processing.

Tissue samples:
Tissue was extracted from plagiopatagium membranes with 2 x 2 mm or 1 x 4 mm sterile biopsy punches (Wilmer and Barratt 1996). A biopsy was selected from a region of the wing with few or no visible blood vessels to reduce bleeding; one biopsy was collected from each wing. Tissue was stored in 2.0 mL tubes and shipped on ice packs to the US Forest Service Northern Research Station in Rhinelander, WI. To kill *P. destructans* or other potentially infectious agents, shipping containers and contents were decontaminated with 10% bleach solution (National WNS decontamination protocol v 06.25.2012; https://www.whitenosesyndrome.org/topics/decontamination). Paper and any material that could not withstand bleach treatment were UV irradiated for 10 minutes. Once received, samples were stored at -20° C (tissue in dry tubes had 100% ethanol added prior to freezing). DNA was extracted using the DNeasy® Blood and Tissue Kit (Qiagen, Valencia, CA), following the manufacturer's protocols (tissue stored in RNALater was washed in lysis buffer prior to extraction). PCR and sequencing details of these samples are described by Palmer et al. (2016).

Swab samples:
All swab samples were processed at the USFS NRS Center for Forest Mycology Research in Madison, Wisconsin. The swabs were transported to the lab on ice packs. Once received, 300 ul of filter-sterilized

cell lysis solution (CLS) was added to each swab, and the swabs were then stored at -20° C. DNA was extracted from the swabs following Lindner and Banik (2009) with modifications for swabs described by Jusino et al. (2016). Following extraction, fungal DNA from the swabs was amplified with fungal specific primers ITS4 (White et al. 1990) and ITS7 (Ihrmark et al. 2012), which target the ITS2 region of the ITS fragment. Our ITS primers were modified for metabarcoding by adding a unique barcode sequence and an Ion Torrent Xpress A adapter sequence on each forward (ITS7) primer and an Ion Torrent Xpress trP1 adapter on the reverse (ITS 4) primer. PCR amplification and sequencing on the Ion Torrent platform followed Palmer and Jusino et al. (2018). The swab DNA was also subjected to real time PCR assay (qPCR) of the fungal IGS region using a probe specific to *Pseudogymnoascus destructans* following the protocol in Muller et al. (2013). WNS-positive status was determined based on cycle threshold (CT) values, the number of cycles required for the fluorescent signal to exceed background.

Post-processing and Analysis

For the MHC, our bioinformatics approach is described in Palmer et al. (2016). Briefly, we followed the genotyping method in Lighten et al. (2014) where an excel macro for genotyping was used under the assumption of up to 5 loci (10 alleles): in each sample the 10 most abundant variants were selected, and then their depths were fit to the expected depths in two models CNV (copy number variation) and DOC (depth of coverage) to estimate the number of different putative alleles in each sample. To test the possibility of a larger number of alleles, we also generated a new excel macro by expanding the original macro up to 6 loci (12 alleles). We found that results were the same using '5 loci' and '6 loci' macros. We also tested two other methods which identified different putative alleles due to different pre-processing methods; however, the numbers of different alleles per sample were consistent (average 1.86 and 1.60), suggesting high reliability of final genotyping.

For the mycobiome, we used the AMPtk pipeline to bioinformatically process the fungal ITS data, following the methods described in Palmer and Jusino et al. (2018). The bioinformatics were parameterized with a fungal mock community. The fungal operational taxonomic units (OTUs) that resulted from the bioinformatics steps were then put into a community matrix, and downstream analyses were performed on that matrix.

Fungal community analyses:
All fungal community analyses were performed on a modified raup-crick distance matrix (Chase et al. 2011), using the raup function in the vegan package of R (R Core Team 2017; Oksanen et al. 2017). We performed a principal coordinate analysis on our distance matrix to visualize the fungal communities using the PCoA function in the ape package of R (Paradis and Schliep 2018). We also identified the most positively and negatively correlated OTUs along the first two PCoA axes, and those are provided in the visualization (Figure 2) and in table format (Table II). We then performed permutational multivariate analysis of variance (PERMANOVA) using the adonis function in the vegan package to test for overall differences in the mycobiomes of bats across our 10 different sites and 3 different disease statuses (positive, negative, or borderline, as determined from the qPCR results). We used the betadisper function in the vegan package to test for homogeneity of multivariate dispersion between sites and different disease statuses.

Results and Discussion

Major Histocompatibility Complex

Much of the granting period was spent in developing and finalizing Next Gen sequencing methods to characterize MHC II DRB alleles. In total 137 MHC samples were successfully genotyped using the method in Lighten et al. (2014) and 50 putative MHC alleles were identified among these samples. After receiving feedback on our initial genotyping method (see methods - *Post-processing and Analysis*), we compared our findings with other approaches used to reduce the massive amount of next-generation sequencing data processed for individual genotypes and deal with artifacts (Xueling and Latch 2018). Because MHC loci are highly variable and may have multiple alleles, similar sequencing reads can be different true alleles, making the clustering algorithm used initially by Palmer et al. (2016) less suitable for MHC analysis. Thus, we compared the initial findings that clustered similar reads (Lighten et al. 2014) with other methods: (1) pre-processed the raw sequencing data using the program jMHC to keep all different sequence variants separately (Stuglik et al. 2011), and (2) pre-processed data were genotyped using an alternative method modified from Sommer et al. (2013) that considers amplification efficiency and allelic dropout to improve confidence in allele scoring. Comparison of the methods showed the CNV-DOV methods (initial and 1 above) identified different putative alleles due to the different pre-processing methods; however, the numbers of different alleles per sample were consistent (average 1.86 and 1.6), suggesting high reliability of genotyping.

For the final data set, five samples were excluded due to the inconsistent and ambiguous results from CNV and DOC models. Fifteen blood samples could not be successfully genotyped. The number of putative MHC alleles per sample varied from 1 to 6 (average 1.85), and most samples had only 1 identified putative allele (Table 1). Real-Monroy et al. (2014) found 3-5 alleles per individual in *Artibeus jamaicensis*. Schad et al. (2012) found a single MHC class II DRB locus in genus *Noctilio*. Mayer and Brunner (2007) found 1-3 different alleles per individual in *Saccopteryx bilineata*, but Schad et al. (2012) suggest up to 10 loci in *Saccopteryx bilineata*. Our results from the three methods (initial, 1 and 2) fall within the range of these previous works. There was a high number of alleles per population, and initial results do not indicate much spatial variability (Table 1); however, diversity data (total # alleles) presented below have not been corrected for unequal sample sizes across populations nor has a spatial analysis been completed.

Table 1. Summary of allelic diversity by population for MHC data. For each State, data include the number of geographic populations, number of samples sequenced, the average number (range) of different alleles per sample, the total number of different alleles in the population, and the number of private alleles found only in that population. Note: diversity data (total # alleles) have not been corrected for unequal sample sizes across States.

Population (# geographic populations)	N	Average # alleles/ sample (range)	Total # alleles	# private alleles
NY (2)	35	1.85 (1-4)	27	7
PA (2)	14	1.71 (1-4)	11	2
VT (1)	15	2.13 (1-4)	14	1
MI (1)	17	1.59 (1-3)	19	4
WI (2)	51	1.84 (1-6)	33	13
Total	132	1.85	50	27

Mycobiome

High through-put amplicon sequencing (HTAS) of the fungal ITS region was used to characterize the baseline mycobiomes of *M. lucifugus* from WNS-positive and -negative locations by examining how the fungal community composition changes with site and WNS status. We detected 2,205 fungal OTUs from the 154 little brown bat swabs that we processed. On the 102 Pd-positive (n = 91) or Pd-borderline bats (n = 11), we detected 1808 OTUs, with an average of 49 fungal OTUs per bat. We detected 796 OTUs on Pd-negative bats (n=52), with an average of 32 fungal OTUs per bat. Our PERMANOVA results (Figure 2) demonstrate that fungal community composition and multivariate dispersion varies across sampling sites (adonis $r^2 = 0.88$, pseudo F = 121.67, $p< 0.0001$; betadisper F = 11.15, p = 0.001), and also varies with disease status (adonis $r^2 = 0.46$, pseudo F = 65.0, $p< 0.0001$; betadisper F = 4.67, p = 0.016). WNS status was highly correlated with geographic location.

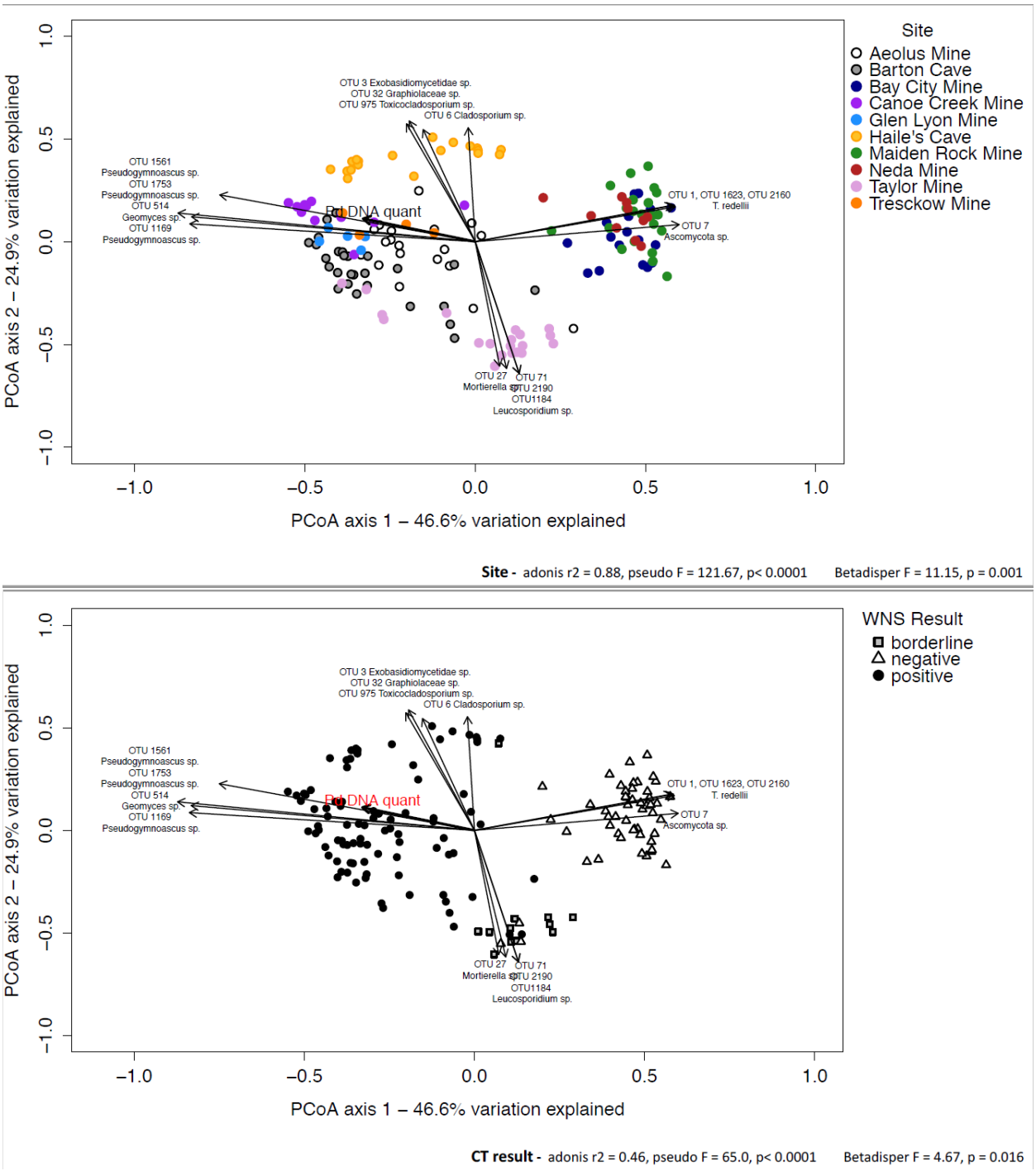

Figure 2. Principle coordinate analysis (PCoA) of Operational Taxonomic Unit (OTU) by site (top panel) and by infection status (bottom panel) of bat mycobiome collected from 10 locations. Axis 1 explained 46.6% of the variance, and axis 2 explained 24.9% of the variance.

Table 2: OTUs correlated with PCoA axis 1 and 2 in Figure 2.

OTU number	identity	correlated WNS status	correlated geographic location	PCoA axis correlation	correlated site(s)
1561	*Pseudogymnoascus sp*.	positive	Northeast	PCoA axis 1	Canoe Creek Mine, Glen Lyon Mine, Barton Cave, Taylor Mine, Tresckow Mine, Aeolus Mine
1753	*Pseudogymnoascus sp*.	positive	Northeast	PCoA axis 1	Canoe Creek Mine, Glen Lyon Mine, Barton Cave, Taylor Mine, Tresckow Mine, Aeolus Mine
514	*Geomyces sp.*	positive	Northeast	PCoA axis 1	Canoe Creek Mine, Glen Lyon Mine, Barton Cave, Taylor Mine, Tresckow Mine, Aeolus Mine
1169	*Pseudogymnoascus sp.*	positive	Northeast	PCoA axis 1	Canoe Creek Mine, Glen Lyon Mine, Barton Cave, Taylor Mine, Tresckow Mine, Aeolus Mine
3	*Exobasidiomycetidae sp.*	positive	Northeast	PCoA axis 2	Haile's Cave
32	*Graphiolaceae sp.*	positive	Northeast	PCoA axis 2	Haile's Cave
975	*Toxicocladosporium sp.*	positive	Northeast	PCoA axis 2	Haile's Cave
6	*Cladosporium sp.*	positive	Northeast	PCoA axis 2	Haile's Cave
27	*Mortierella sp.*	borderline / positive	Upper Michigan	PCoA axis 2	Taylor Mine
71	*Leucosporidium sp.*	borderline / positive	Upper Michigan	PCoA axis 2	Taylor Mine
2190	*Leucosporidium sp.*	borderline / positive	Upper Michigan	PCoA axis 2	Taylor Mine
1184	*Leucosporidium sp.*	borderline / positive	Upper Michigan	PCoA axis 2	Taylor Mine
1	*Trichophyton redellii*	negative	Wisconsin	PCoA axis 1	Maiden Rock Mine, Bay City Mine, Neda Mine
1623	*Trichophyton redellii*	negative	Wisconsin	PCoA axis 1	Maiden Rock Mine, Bay City Mine, Neda Mine
2160	*Trichophyton redellii*	negative	Wisconsin	PCoA axis 1	Maiden Rock Mine, Bay City Mine, Neda Mine
7	*Ascomycota sp.*	negative	Wisconsin	PCoA axis 1	Maiden Rock Mine, Bay City Mine, Neda Mine

Implications in the Northern Forest Region

This study describes microbiome community composition and adaptive allelic diversity (i.e. bat immune system) for little brown bats across a geographic gradient and time-since-infection. Although initial findings do not suggest much spatial pattern in the diversity of the MHC DRB II locus, there are some spatial differences indicating a change in fungal community composition along a gradient from WNS positive to WNS negative status, with variation among sites also influencing community composition. This work broadly supports Avena et al. (2016) who reported that skin microbiomes of four species of bats contain site specific microbial species with key host-specific taxa. Thus, if further analysis indicates these predictors (WNS status, site, MHC diversity) to be significant, the information can be used to develop strategies for protecting and restoring potentially WNS resistant populations and their habitat surrounding hibernaculum in which these populations are found, thus helping efforts to re-populating northern forests with little brown bats. Also, because disease status correlates positively with fungal communities, mycobiome patterns may be useful as predictive markers of disease progression (Cui et al. 2013).

Future directions

The complexity and massive data generated using Next Gen Sequencing for a highly diverse bat MHC gene and fungal mycobiome allowed initial description of community structure and MHC diversity, but precluded us from identifying potentially resistant populations (i.e., unique MHC II DRB allele in a stabilizing bat population) within the timeline of this grant. We will continue to discuss ways in which we might analyze our data to provide new insights into WNS as a potential selective force of genetic diversity at the MHC class II locus. We also plan to correlate the fungal community with our Pd load findings to gain more knowledge on how Pd influences mycobiome composition and diversity on bats.

List of products

1. Peer-reviewed publications
    a. Population genetic structure of MHC II DRB alleles and disease resistance in *Myotis lucifugus*. This manuscript is in preparation with expected finish date, December, 2019.
    b. Changes in mycobiome diversity and community composition under outbreak of white-nose syndrome. This manuscript is in preparation with expected finish date, September, 2019.
2. Other publications
    a. Palmer JM, Berkman LK, Marquardt PE, Donner DM, Jusino MA, and Lindner, DL (2016) Preliminary characterization of little brown bats (*Myotis lucifugus*) immune MHC II DRB alleles using next-generation sequencing. *PeerJ PrePrints* 4, e662v1. doi.org/10.7287/peerj.preprints.1662v1; DOI: 10.7287/peerj.preprints.1662v1/sup
3. Data Archives
    a. Palmer JM, Berkman LK, Marquardt PE, Donner DM, Jusino MA, and Lindner, DL (2016). Sequence data for 15 MHC IIDRB amplicons were deposited in the NCBI SRA. Accession number SRP067348 for manuscript: Preliminary characterization of little brown bats (*Myotis lucifugus*) immune MHC II DRB alleles using next-generation sequencing. Available at https://www.ncbi.nlm.nih.gov/sra
4. Conference presentations-Invited
    a. Berkman, Leah, Palmer, Jonathan, Lindner, Daniel, Marquardt, Paula, Donner, Deahn, and Frair, Jacqueline (2014). Revealing novel links among bat ecology, genetics, and disease to understand white-nose syndrome. SUNY ESF and Upstate Medical Center symposium on "From lab to landscape: integrated infectious disease research", Syracuse, NY.
    b. Donner, Deahn, Berkman, Leah, Marquardt, Paula, Frair, Jacqueline, and Lindner, Daniel (2014) Resistance to White-Nose Syndrome in bat populations of the Northern forest: Exploring the critical disease-genotype-microbiome link. White-Nose Syndrome Workshop, St. Louis, MO, September 9-12.
    c. Lindner, Daniel, Jusino, Michelle, Palmer, Jonathan, Banik, Mark, Marquardt, Paula, Donner-Wright, Deahn (2019) The mycobiome of bats: Critical information for understanding and managing white-nose syndrome. Mycological Society of America Conference, Minneapolis, MN, August 10-14.
5. Conference presentations-Offered
    a. Berkman, Leah, Palmer, Jonathan, Lindner, Daniel, Marquardt, Paula, Donner, Deahn, and Frair, Jacqueline (2014) Characterizing MHC diversity pertinent to disease resistance with application to bats with white-nose syndrome. National Conference of the Wildlife Society, Pittsburgh, PA, Oct 25-30.
    b. Berkman, L., Donner, D., Marquardt, P., Frair, J., Lindner, D., Prom, B. (2014) Revealing novel links among genotypes, White-Nose Syndrome, microbiome, and spatial variables in the northern forests. 3rd Science in the Northwoods Conference, Boulder Junction, WI, October 15-17.
6. Awards
    a. 2016 The highly esteemed US Forest Service *Wings Across the America's* – National Conservation Award: Research Partnership Glass Award. Donner, Deahn, Marquardt,

Paula, Heeringa, Brian, Eklund, Dan, Lindner, Dan, and Frair, Jaqueline. An Integrated, Multi-Scale Landscape Approach for Studying the Secondary Effects of White-nose Syndrome in Bats of the Upper Midwest. March 16. White-Nose Syndrome is one of the greatest conservation challenges of our time and the solutions developed from this line of science will provide critical input for the bat recovery program.

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