Summer 2018

Immune Function and Metabolism of Hibernating North American Bats with White-Nose Syndrome

Briana Nicole Anderson
Missouri State University, Anderson0220@live.missouristate.edu

IMMUNE FUNCTION AND METABOLISM OF HIBERNATING NORTH AMERICAN BATS WITH WHITE-NOSE SYNDROME

A Masters Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Briana Anderson

August 2018

IMMUNE FUNCTION AND METABOLISM OF HIBERNATING NORTH AMERICAN BATS WITH WHITE-NOSE SYNDROME

Biology

Missouri State University, August 2018

Master of Science

Briana Anderson

ABSTRACT

White-nose syndrome (WNS) causes substantial mortality in certain species of hibernating North American bats. The responsible agent is *Pseudogymnoascus destructans (Pd)*, a fungus which causes physiological complications such as increased arousals and energy depletion during the hibernation season. Tricolored bats (*Perimyotis subflavus*) and northern long-eared bats (*Myotis septentrionalis*) suffer extensive WNS mortality, while gray bats (*Myotis grisescens*) and big brown bats (*Eptesicus fuscus*) are infected, but mortality is rarely observed. It is hypothesized that there is a difference in immune responses and/or hibernation metabolism between these bat species, resulting in this interspecific variation in disease severity. To test these hypotheses, experiments were conducted at both the cellular and whole-bat level. Tricolored bats were infected with *Pd* and half were treated with an anti-inflammatory to mute any immune response. Data from measurements of torpor energetics did not support the hypothesis, but an immune response was observed in mid-hibernation, based on white blood cell counts. Also, wing tissue fibroblasts from the four species listed above were infected with *Pd*, and RNA-seq analysis revealed interspecific differences in gene expression in response to *Pd*. This study could aid in establishing treatment and conservation strategies for North American bats. In addition, a cell culture method has been pioneered that will allow researchers to address a myriad of immunological questions, such as which western bat species might be most susceptible to WNS as it spreads westward.

KEYWORDS: white-nose syndrome, energetics, immunology, fibroblasts, RNA-seq

This abstract is approved as to form and content

_____

Tom Tomasi, PhD
Chairperson, Advisory Committee
Missouri State University

IMMUNE FUNCTION AND METABOLISM OF HIBERNATING NORTH AMERICAN BATS WITH WHITE-NOSE SYNDROME

By

Briana Anderson

A Masters Thesis
Submitted to the Graduate College
Of Missouri State University
In Partial Fulfillment of the Requirements
For the Degree of Master of Science, Biology

August 2018

Approved:

_____
Tom Tomasi, PhD

_____
Christopher Lupfer, PhD

_____
Day Ligon, PhD

_____
Julie Masterson, PhD: Dean, Graduate College

ACKNOWLEDGEMENTS

I would like to thank the following people for their support during the course of my graduate studies. For relentless guidance and encouragement, I would like to thank my committee members: Drs. Tom Tomasi, Christopher Lupfer, and Day Ligon. For research funding, I would like to thank the Missouri State University Graduate College and Department of Biology, Sigma Xi Scientific Research Society, and the American Society of Mammalogists. For assistance with wing biopsy and bat collection, I would like to thank Environmental Solutions and Innovations, Inc., Dr. Lynn Robbins, the Missouri Department of Conservation, Blake Sasse (Arkansas Game and Fish Commission), and Dr. Anna Doty. For research assistance, I would like to thank Keslie Naffa, Tricia Blankenship, Tyler Remick, Natalie Smith, Abbigale Mabary, Angeline Rodriguez, and my Fall 2017 Genetics Section 004. For inspiration, motivation, and relentless support, I would like to thank my mom, Christina, Alesha, Christine, and Sally.

I dedicate this thesis to my mom, Janice Hagerty.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

OVERVIEW

Among the Orders of extant mammals, Chiroptera is the second most biologically diverse with at least 1,100 known species dispersed across all continents except Antarctica (Simmons and Conway 2003). The life history of Chiroptera is unique in that bats are the sole mammals with true volant abilities, and bats experience an unusually long lifespan with slow reproduction relative to similarly sized mammals (Barclay and Harder 2003). These exceptional traits, paired with an almost global distribution, result in wide ecological variety among bats, particularly in the case of diet; the diversity of food preferences in bat species includes insects, fruits, nectar, seeds, fish, frogs, small mammals, and blood (Kunz et al. 2011).

Value of Bats

Mutualistic population interactions are observed between frugivorous or nectarivorous bats and their associated plants (Kunz et al. 2011). By consuming nectar, bats aid in pollination of over 500 angiosperm species, including the plant responsible for commercial tequila: *Agave tequiliana* (Kunz et al. 2011). Frugivorous bats contribute to reforestation by dispersing the seeds of over 500 plant species in the Neotropics (Lobova et al. 2009), many of which are necessary for primary forest succession (Muscarella and Fleming 2007). Consequently, nectarivorous and frugivorous bats have key roles in maintaining the genetic diversity of plant populations (Kunz et al. 2011).

Over two-thirds of all extant bat species and most of the 47 species in the United States are either obligate or facultative insectivores, consuming a variety of taxa

including moths, beetles, flies, ants, leaf hoppers, and true bugs (Kunz et al. 2011). To quantify the value of bat insectivory, the volume of insects consumed on a nightly basis can be estimated. Captive little brown bats (*Myotis lucifugus*) and big brown bats (*Eptesicus fuscus*) were observed to consume 25% of their body weight each night (Coutts et al. 1973). With artificial enclosures in the field, Reiskind and Wund (2009) observed that northern long-eared bat (*M. septentrionalis*) consumption of mosquitos, a common vector of mammalian disease, reduced mosquito oviposition by 32% compared to bat-free enclosures (Reiskind and Wund 2009). It was estimated that one colony of 150 big brown bats in Indiana consumes around 1.3 million pests per year (Whitaker 1995).

Herbivorous insects damage an estimated 25 – 50% of crops around the world (Pimentel et al. 1991), and insectivorous bats serve as natural pest control. One species which has been studied extensively for its economic value in North America is the Mexican free-tailed bat (*Tadarida brasiliensis*). Top-down suppression of problematic insect populations has been observed with this species (Kunz et al. 2011). By a maternity colony of one million of these bats, an estimated 8.4 metric tons of insects could be consumed in one night (Kunz et al. 1995). The consumption of corn earworm moths by Mexican free-tailed bats in southern Texas has been calculated to save 1-2 pesticide applications per year (Cleveland et al. 2006). If one pesticide application costs $25 per hectare of land, farmers save $100,000 for every 4,000 hectares (Cleveland et al. 2006). The total estimated value of bats in this region of Texas ranges from $5 to $70 per hectare, with an average of $30 per hectare (Cleveland et al. 2006). Assuming the accuracy of these estimates, and extrapolating them to all of the cropland in United States, the overall pest-suppression value of bats equates to $22.9 billion per year (Boyles

et al. 2011). This includes the avoided cost of pesticide applications but does not include the invaluable environmental benefits of applying less harmful chemical pesticides (Boyles et al. 2011). With the detected range expansion of Mexican free-tailed bats into southeastern portions of the United States (McCracken et al. 2018), the economic value of this species is likely to increase.

Conservation Concerns

The value of bat ecosystem services is evident throughout the world, yet around 15% of all bat species are threatened (IUCN Red List), and most sources of population decline in bats are of anthropogenic origin. For example, the introduction of invasive taxa such as rats, snakes, and birds is responsible for niche competition or unnatural predation of several island-dwelling bat species (Welch et al. 2017). Prior to 2000, the intentional killing of bats by humans caused the majority of multiple mortality events—events in which 10 or more bats in a colony died within one year. Motives for killing include hunting for meat, protecting fruit crops, protecting livestock from vampire bat bites, eradication from buildings, and fear of rabies (O'Shea et al. 2015). Recent extreme heat waves in Australia have caused the deaths of thousands of flying foxes due to hyperthermia, and the frequency of these occurrences is expected to increase with climate change (Welbergen et al. 2008). Anthropogenic activity such as deforestation, agricultural development, and urbanization has disrupted roosting habitat and foraging opportunities for bats worldwide (Mickleburgh et al. 2002). As in other long-lived taxa, the risk of bioaccumulation of environmental contaminants is also high in bats; researchers detected the presence of polybrominated diphenyl ethers, caffeine, warfarin,

thiabendazole, and others—all contaminants of emerging concern that could pose negative effects on bat life history—in carcasses of four North American bat species in 2010 (Secord et al. 2015).

Of all sources of multiple mortality events in bats, two have caused the overwhelming majority of bat fatalities in North America since 2000: deaths associated with wind turbines and white-nose syndrome (O'Shea et al. 2015). An estimated 650,000 to 1.3 million North American bats have died at wind energy facilities from 2000 – 2011 (Arnett and Baerwald 2013). The exact cause of mortality is unknown, but several ideas have been proposed. For example, the decompression hypothesis states that rapid air pressure reduction surrounding wind turbine blades causes pulmonary barotrauma, and a study of almost 200 bats killed at wind turbine facilities suggested barotrauma as the cause of death versus direct contact with turbine blades (Baerwald et al. 2008). The combined effects of the above sources of mortality, paired with the K-selected life history of bats, could lead to rapid depletion of bat populations around the world. Specifically regarding temperate North American cave-hibernating bats, the most dangerous risk to these populations in recent years is white-nose syndrome (WNS).

White-nose Syndrome

The probable first location of the WNS pathogen infecting bats in North America was at Howe's Cave, New York, in 2006 (Blehert et al. 2009). By 2018, the pathogen had radiated to 36 states and seven Canadian provinces, primarily in the midwestern and northeastern portions of the United States (USGS 2018, Figure 1). While most of these states and provinces are eastern or midwestern, the pathogen was also detected in

Washington state in 2016 (Lorch et al. 2016). Pathogenic symptoms of WNS have been confirmed in ten North American bat species, two of which were already listed as federally endangered (gray bat [*M. grisescens*] and the Indiana bat [*M. sodalis*]), and a third was listed as threatened as of April 2015 (northern long-eared bat) (USFWS 2015). Another species, the tricolored bat (*Perimyotis subflavus*), was under consideration for federal listing as of December 2017 due in part to reports of colony crashes caused by WNS (USFWS 2017). The total death toll in the six years from 2006 – 2012 was estimated to range from 5.7 – 6.7 million bats, and another five to seven million bats have probably died from WNS in the subsequent six years as the disease spread to more hibernacula (USFWS 2012). However, the lack of population data prior to WNS arrival makes it difficult to accurately calculate a death toll.

The responsible disease agent of white-nose syndrome is a psychrophilic fungus, formerly classified as *Geomyces destructans* (Gargas et al. 2009) but reclassified in 2013 as *Pseudogymnoascus destructans* (*Pd*) (Minnis and Lindner 2013). *Pd* grows optimally at 5 – 10ºC (Blehert et al. 2009) and ceases growth above 19ºC (Verant et al. 2012). The fungal hyphae invade the epidermis of the hibernating bat, replacing hair follicles and sebaceous glands; to release spores, fruiting bodies extend above the skin surface, resulting in a visible white growth on the bat's muzzle, ears, and wings (Blehert et al. 2009). The primary mechanism of *Pd* spread is bat-to-bat contact, but humans are also a likely vector. Samples of *Spinturnix* mites, an ectoparasite exclusive to bat wings, tested positive for *Pd* DNA, suggesting a third method of transmission as the mites transfer from bat to bat (Lučan et al. 2016).

Molecular evidence suggests that *Pd* has long been present throughout Europe

(Leopardi et al. 2015, Frick et al. 2015). It is probable that *Pd* was introduced anthropogenically to North America (Leopardi et al. 2015). By comparing genomic loci of European *Pd* isolates to North American isolates, multiple polymorphisms were observed in the European isolates while no variation was observed in the North American isolates, suggesting that *Pd* in North America is less divergent and was introduced recently from a single location (Leopardi et al. 2015). Photographic evidence of *Pd* growth on bats has been recorded in Europe since 1995 (Martínková et al. 2010). Virulent symptoms of white-nose syndrome have been observed in bats sampled in the Czech Republic (Bandouchova et al. 2014), yet no mass mortality events in Europe have been observed (Martínková et al. 2010). Similarly, the presence of *Pd* in caves and growing on bats has been confirmed in China, with diagnostic symptoms of WNS confirmed via histology. However, as in Europe, no mass mortality events due to WNS have been reported. Sequencing demonstrated that the fungus in China matches the North American strain of *Pd*, suggesting a comparable anthropogenic introduction of *Pd* to China (Hoyt et al. 2016). High exposure rates have also been detected throughout Russia with low disease severity and no mass mortality events, suggesting a similar phenomenon to that in China (Kovacova et al. 2018).

Since the identification of the WNS epizootic, a key priority has been to understand the fungus and determine potential treatment strategies. Members of the genus *Pseudogymnoascus* are thought to be primarily saprotrophic, securing nutrients via chemoheterotrophic digestion of decaying organic matter (Reynolds and Barton 2014). A comparison to several congeners demonstrated that *Pd* has reduced saprotrophic enzyme activity and greater ability to produce enzymes that aid in tissue invasion and adhesion,

suggesting that it may have evolved pathogenic capabilities while losing some saprotrophic ability (Reynolds and Barton 2014). Hypothetically, *Pd* could persist in a cave ecosystem without a bat host if it could practice saprotrophy and effectively compete with other members of the cave microbiome, or if it had spores that could lie dormant between hibernation seasons. *Pd* congeners were able to utilize significantly more substrates and grow faster at higher temperatures *in vitro* compared to *Pd*, suggesting that *Pd* is a more specialized fungus that may not be able to effectively outcompete other cave fungi, at least in warmer microhabitats (Wilson et al. 2017). However, qPCR detection of *Pd* DNA on cave floors, walls, and ceilings suggests the existence of a *Pd* reservoir. Hence, the fungus could persist through the summers in the absence of bats, as fungal spores are able to withstand conditions more extreme than found in hibernacula, and remain viable for extended periods of time (Zhang et al. 2014).

A variety of naturally occurring microbes have been shown to inhibit *Pd* growth and potentially serve as biocontrol agents—a method of treatment that could pose less of a threat to cave environments. The *Pd*-suppressing activity of over 300 microbial isolates showed that over 15% completely inhibited *Pd* growth; thus there is an array of bacteria, filamentous fungi, and yeast species that could be used as potential biocontrol treatments (Micalizzi et al. 2017). For example, a strain of *Trichoderma polysporum*, a psychrotolerant fungus isolated from William Preserve Mine in New York, inhibited *Pd* growth *in vivo* but did not inhibit growth of other common cave fungi, suggesting its potential effectiveness for impeding *Pd* without disrupting native cave fungi (Zhang et al. 2015). Commercially available cold-pressed terpeneless orange oil has also been shown to inhibit *Pd* growth for up to six months *in vitro* without negative effects on other

environmental microbes (Boire et al. 2016). Potential fumigation agents to use against *Pd* include 1-octen-3-ol (mushroom alcohol), an EPA-approved volatile organic compound that inhibited *Pd* growth as dilute as 5 ppm (Padhi et al. 2017). Based on an annotated genome, *Pd* was found to be genetically sensitive to UV-C light, lacking various DNA repair pathway genes that promote survival in the presence of this light (Palmer et al. 2018). Ergo, a variety of methods could be used to treat WNS, but successful *in vitro* treatments may not be as effective in the field. To date, few field trials have been completed.

Amidst the various treatment options, possibilities arise from the skin of infected bats. Of 133 bacterial morphotypes isolated from the skin of 40 North American bats of four species, two strains of *Pseudomonas* bacteria from big brown bats were capable of significantly reducing *Pd* growth *in vitro* (Hoyt et al. 2015). The most inhibitory strain, Pf1, was assessed in an *in vivo* study on little brown bats (Cheng et al. 2016). Bats simultaneously inoculated with *Pd* and Pf1 showed lower WNS severity and higher survival rates compared to bats solely inoculated with *Pd* (Cheng et al. 2016). However, the experimental group that was treated with Pf1 three weeks prior to *Pd* inoculation had significantly higher disease severity, suggesting the importance of timing with regards to treatment plans (Cheng et al. 2016). *Streptomyces* bacteria from the skin of several western bat species was also shown to inhibit *Pd* growth *in vitro* (Hamm et al. 2017). Studying the skin microbiome of WNS-affected bats shows promise for identifying potential treatment strategies and understanding interspecific differences in disease severity; however, the high variability and range of factors that can change the skin

microbiome on a day-to-day basis can demote this method relative to other areas of research (Hamm et al. 2017).

Hibernation and WNS

Another component of WNS that merits investigation is the effect of *Pd* infection on bats' hibernation physiology. As endotherms, birds and mammals have the ability to maintain a constant high body temperature within a range of ambient temperatures. At cool temperatures, endotherms use energetically costly mechanisms for generating metabolic heat, a strategy that allows a more active lifestyle in a wider variety of habitats. Smaller endotherms have larger surface area to volume ratios, meaning they have a larger surface over which heat could be lost to the environment; therefore, smaller endotherms must generate more endogenous heat than larger endotherms (gram-per-gram) to maintain a constant body temperature. This could become problematic in a temperate climate, where winters limit potential energy resources and ambient temperatures are low. Beyond behavioral thermoregulation such as microhabitat selection, it may not be feasible for a small endotherm to maintain constant body temperature in a cold habitat without a stable energy source. For some, it becomes critical to abandon endothermy for the duration of the winter and conserve energy with the use of torpor.

Torpor can be defined as a physiological reduction in body temperature and metabolic rate, followed by a marked cessation or slowing of bodily functions such as heart rate, breathing rate, cellular respiration, digestion, and mobility. (Geiser 2004). However, thermoregulation is not abandoned during torpor (Speakman and Thomas 2003). At the onset of torpor, the hypothalamic set point (T_{set}) for body temperature is

downregulated as ambient temperatures decrease below the thermoneutral zone; this is followed by a substantial decrease in metabolic rate (Geiser 2004). The animal's body temperature typically decreases to within a degree of ambient temperature, causing metabolic rate to decrease even further below basal metabolic rate ($MR_{B,}$ the energetic requirements of homeostasis), which is referred to as torpid metabolic rate (MR_T). If ambient temperature decreases below T_{set}, the animal will re-initiate thermoregulation via non-shivering thermogenesis to maintain body temperature at or above T_{set} (Geiser 2004).

Torpor can be expressed in daily bouts of heterothermy or for extended periods up to several months (hibernation). Multiple taxa utilize torpor as an energy-saving mechanism, including hummingbirds, nightjars, rodents, bats, bears, insectivores, and primates. Animals use daily torpor in times of limited food resources, in preparation for energetically costly events such as migration or pregnancy, or in response to energetically limited climates such as deserts (Geiser 2004). By practicing hibernation over the course of the winter, an animal can save over 85% of the energy it would have spent if it remained euthermic (Geiser 2004, Figure 2). Thus, torpor is an adaptation that could increase chances of survival and reproductive success in bats (Stawski et al. 2014).

Hibernation is not a constant torpid state; instead, bouts of torpor are interrupted by brief periods of euthermia, termed "arousals" (Geiser 2004, Figure 3). The underlying cause of arousals is unknown, but many reasons have been proposed, including evaporative water loss, accumulating metabolic wastes, and sleep deprivation (Thomas and Geiser 1997). Over the course of hibernation, arousals can account for up to 90% of the energy expenditure (Thomas et al. 1990, Day and Tomasi 2014). Thus, a hibernator may adjust the timing and frequency of arousals to maximize energy savings throughout

the winter. The optimization hypothesis refers to the balance between the benefits and costs of torpor (Humphries et al. 2003). While a hibernator benefits from the reduced energy requirements of abandoning endothermy, the costs of torpor could become detrimental. These include increased predation risk due to immobility, ceased mitosis and protein synthesis, dehydration, compromised ionic balance, accumulation of sleep-debt, and accumulation of reactive oxygen species (Humphries et al. 2003). Therefore, the optimization hypothesis states that, rather than utilize the deepest torpor for the longest amount of time possible, an animal may avoid some of the costs by minimizing torpor (Humphries et al. 2003). However, this may only be a possibility for animals with large fat stores, whereas an animal that enters hibernation in poor body condition may instead use deep torpor (Humphries et al. 2003). Animals that will have a high energy demand upon termination of hibernation, such as migration or reproduction costs, may also maximize torpor use to save as much energy as possible for these other functions. Because reproductive costs are generally higher in female mammals, the "thrifty female" hypothesis has been proposed to accommodate observed gender differences in hibernation strategies (Jonasson and Willis 2011).

In support of the optimization hypothesis, free-ranging little brown bats with lower body mass were found hibernating in colder sections of a cave (Boyles et al. 2007). In addition, injection of captive big brown bats with mercaptoacetate, a drug that simulates energy limitation, caused bats to choose significantly colder microclimates for hibernation compared to controls (Boyles et al. 2007). An optimum ambient temperature exists at which torpid bats spend the least amount of energy during hibernation. In hibernating Indiana bats, those hibernating at 4ºC would spend less the energy required to

hibernate at 2 ºC, 6ºC or 8ºC, indicating that 4ºC could be the optimal hibernation temperature for this species (Day and Tomasi 2014).

Bats may also facilitate energy savings during hibernation by practicing arousals that are less energetically costly. Arousal cascades have been observed in groups of free-ranging little brown bats, during which the arousal of one bat is followed by arousals in neighboring bats (Czenze and Willis 2015). These pseudo-synchronized arousals could facilitate passive rewarming in a cluster of hibernating bats, allowing energy savings for some bats toward the end of hibernation when fat stores have substantially decreased (Czenze and Willis 2015). Heterothermic arousals have also been observed, during which the bat's body temperature rises to euthermic levels, then quickly decreases to a shallow torpor (Willis 2017). Also, "cold arousals" were recently described, during which bats were visibly active without rewarming to euthermic temperatures (Mayberry et al. 2018). It is possible that varying torpor and arousal strategies, including optimization of the energy budget and microclimate selection, influence disease severity when suffering from WNS.

One of the first assessments of deceased WNS bats indicated that the majority had no visible fat reserves, suggesting death from starvation (Blehert et al. 2009). Several studies have since been conducted to assess the effects of WNS on the physiology and behavior of afflicted bats, and to pinpoint the cause of fat depletion. As *Pd* invades the wing membrane, it digests the skin as colonies grow, replacing muscle fibers, elastin, and glands (Cryan et al. 2010). Histopathological observations have shown that dense cup-like erosions form as the hyphae invade the tissue; these symptoms have become the

"gold standard" for diagnosing WNS and perhaps the cause of physiological changes during torpor that result in death (Meteyer et al. 2009).

A study of over 500 bats at six hibernacula indicated that bats with more severe WNS symptoms exhibited more frequent arousals (Reeder et al. 2012). *Pd*-infected little brown bats have been observed to have significantly higher torpid metabolic rates (Janicki 2010) and higher evaporative water loss compared to uninfected controls (McGuire et al. 2017). A doubly-labelled water study of captive little brown bats showed that infected bats exhibited respiratory acidosis and utilized significantly more fat over the course of hibernation (Verant et al. 2014). Wing lesions in infected bats exhibit water and solute loss, suggesting that infected bats experience exacerbated dehydration, which could stimulate additional arousals in order to rehydrate (Cryan et al. 2013a). The blood of infected little brown bats had decreased blood sodium, increased hematocrit, and reduced glucose levels, suggesting dehydration and starvation (Warnecke et al. 2013). A disease progression model has been hypothesized: as *Pd* invades the epidermis, MR_T increases, and CO_2 accumulates in tissues, resulting in respiratory acidosis; as wing damage worsens, bats arouse more frequently to normalize blood pH, which causes fat depletion and dehydration (Verant et al. 2014).

A behavioral study of captive little brown bats demonstrated that infected bats reduced clustering behavior and self-isolated as the hibernation season progressed (Wilcox et al. 2014). Observations from the same study indicated that synchronized arousals increased in frequency in infected bats, suggesting that behavioral disturbance from nearby roosting bats can worsen disease severity (Turner et al. 2015). Perhaps to save energy as WNS severity worsened, infected captive little brown bats in another

study were significantly less active, with reduced grooming behavior and no increase in drinking, compared to uninfected bats (Bohn et al. 2016).

WNS-infected little brown bats had higher mortality: a) at warmer temperatures (10^{o}C versus 4^{o}C); b) when entering hibernation with a lower body condition; c) when inoculated with lower doses of *Pd*; and d) if male (Johnson et al. 2014). The gender differences in mortality may support the thrifty female hypothesis, whereby females enter hibernation with significantly higher body condition compared to males and young-of-the-year (Jonasson and Willis 2011). Therefore, the likelihood of mortality may be dependent on multiple biotic and abiotic factors, which may also explain differences in mortality between species.

Among the ten North American species confirmed with white-nose syndrome, markedly different mortality rates have been observed. Estimates range from 30% to 99% mortality for local populations of little brown bats in the northeast, leading to predicted regional extirpations (Frick et al. 2010). Four years after the arrival of *Pd* in Virginia, 95% declines were observed in northern long-eared bats (Reynolds et al. 2016). In two hibernacula in Illinois, the second winter of *Pd* infection resulted in 95 – 99% mortality for northern long-eared bats, 81-88% mortality for little brown bats, and 47 – 73% mortality for tricolored bats (Langwig et al. 2015). It appears that these three species are among the most vulnerable to white-nose syndrome.

In contrast, several cave-hibernating species do not appear vulnerable to WNS, including big brown bats, gray bats, Virginia big-eared bats *(Corynorhinus townsendii virginianus*), and eastern small-footed bats *(Myotis leibii*). In a comparative study, infected big brown bats had no wing lesions and maintained significantly higher body fat

in mid-hibernation compared to infected little brown bats (Frank et al. 2014). The big brown bats in this study maintained torpor bout lengths within their normal range, and the population increased in number while little brown bat numbers declined by 99.6% (Frank et al. 2014). Another species which does not experience declines due to WNS is the gray bat (Flock 2014). In portions of Tennessee, gray bats were observed to leave hibernacula periodically throughout the winter and actively forage, likely a result of insect prey availability and warmer winter climates relative to higher latitudes (Bernard and McCracken 2017). These environmental conditions could produce an energetically relaxed hibernation period for gray bats which could promote survival if infected with *Pd* (Bernard and McCracken 2017).

There are several proposed explanations for the dearth of WNS mortality in certain bat species, despite confirmed diagnostic symptoms. The lipids in the wing epidermis of big brown bats had over twice the average myristic and oleic acid levels compared to lipids of little brown bats (Frank et al. 2016). Myristic acid significantly decreased *Pd* growth *in vitro* at temperatures conducive to big brown bat hibernation, and pentadecanoic acid from bat white adipose tissue decreases *Pd* growth *in vitro*, but only at higher incubation temperatures; this suggests that the protective characteristics of free fatty acids in the wing membranes is influenced by temperature (Frank et al. 2016, Ingala et al. 2017). Furthermore, levels of these lipids in the wing epidermis of little brown bats decrease as hibernation progresses, which would decrease their ability to inhibit *Pd* growth (Ingala et al. 2017). Infected little brown bats had significantly more *Pd* skin lesions compared to big brown bats after 13 weeks of infection (Moore et al. 2018). In the same study, infected big brown bats had significantly longer torpor bouts than control

bats, suggesting that *Pd* does not influence the arousal frequency of this species (Moore et al. 2018). However, bats in this study were simultaneously infected with another unidentified fungus, and the results may have been influenced by the presence of concurrent fungal infections throughout the study.

Behavioral hibernation strategies could also explain interspecific variation in WNS disease severity. In one study, fungal loads at the end of the hibernation period increased significantly with average roosting temperatures (Langwig et al. 2016). Species hibernating at higher temperatures, including little brown bats and northern long-eared bats, had higher fungal loads and higher mortality rates (Langwig et al. 2012, 2016). Hence, bats choosing cooler microclimates for hibernation may be less likely to experience the physiological complications of high fungal loads late in hibernation (Langwig et al. 2016). The species with highest observed mortality rates (northern long-eared, little brown, and tricolored bats) had the fastest increases in prevalence and higher fungal loads than species with observed resistance or tolerance to WNS (big brown and gray bats) (Frick et al. 2017). These patterns have yet to be explained with certainty.

Recapture studies showed that a small number of little brown bats from a colony in New York survived WNS infection and that wing damage healed over the course of the active season (Dobony et al. 2011). Little brown bat populations began to stabilize four years after the arrival of WNS in the northeast (Langwig et al. 2012). Explanations for the survival of certain little brown bat individuals are lacking, but one study showed that survivors from New York did not arouse as frequently as bats that died, and survivors had significantly lower skin temperatures compared to dying bats (Lilley et al. 2016). While the little brown bat is a species that generally hibernates in large clusters,

surviving individuals were observed to change behavior and hibernate solitarily (Langwig et al. 2012). It would appear that hibernation behavior, microclimate selection, and potential physiological mechanisms combine to permit some individuals in susceptible species to survive WNS. The role of the immune system in this matter needs further investigation.

Immunology and WNS

During a cutaneous fungal infection on a euthermic animal, pathogen-associated molecular patterns (PAMPs) on fungal cell walls are detected by pattern recognition receptors (PRRs) on host epithelial cells such as fibroblasts. Such PRRs include toll-like receptors (primarily TLR_4 and TLR_2) and C-type lectin receptors (CLRs) (Biegańska 2014). This activates pro-inflammatory cytokine production (TNF-α, IL-1, etc.) that signals the recruitment of immune cells such as neutrophils and macrophages from the bloodstream, which are primarily responsible for the phagocytosis of fungi (Biegańska 2014). Neutrophil levels in the blood will spike during the first 48 hours of an infection, causing the neutrophil-lymphocyte ratio to increase. Neutrophil levels will usually normalize, followed by a spike in lymphocyte levels around five days post-infection which lowers the neutrophil-lymphocyte ratio. The cytokines interleukin-17A (IL-17A) and IL-22 activate a Th17 response that can also aid in killing the fungus. Beta-defensins, cathelicidins, and histatins belong to another group of proteins called antimicrobial peptides; they are produced by epithelial cells and have notable anti-fungal activity in mammals (Biegańska 2014). Most importantly, the high body temperatures of endothermic mammals create an unsuitable environment for most fungal pathogens

(Biegańska 2014).

A marked reduction in immune function during hibernation has been observed in multiple species, likely as a result of lowered body temperature and metabolic rate. This reduction includes a decline in the numbers of circulating leukocytes, margination of neutrophils to blood vessel walls, decreased production of lymphocytes, and lowered complement activity (Bouma et al. 2010, 2013). The extent of depressed immune function appears to be inversely proportional to torpid body temperature; for example, lower temperatures will further decrease numbers of circulating leukocytes (Sahdo et al. 2013). Upon a periodic arousal from hibernation, immune function appears to be restored as the number of circulating leukocytes increases to euthermic levels (Bouma et al. 2010). Injecting hibernating golden-mantled ground squirrels with lipopolysaccharide (to mimic a bacterial infection) resulted in longer arousal duration and higher arousal body temperatures compared to controls, suggesting that a function of arousals may be to restore immune function (Prendergast et al. 2002).

There is a paucity of research concerning the immunology of bats. Injection of lipopolysaccharide into euthermic short-tailed fruit bats (*Carollia perspicillata*) caused significant increases in white blood cells, weight loss, and mean reactive oxygen metabolites compared to controls (Schneeberger et al. 2013). Euthermic fish-eating myotis (*Myotis vivesi*) injected with lipopolysaccharide experienced an increase in resting metabolic rate of 140-185% and higher body temperature compared to controls (Otálora-Ardila et al. 2016). The majority of bat immune system studies pertain to the antiviral responses of fruit bats serving as asymptomatic reservoirs for over 100 viruses, including Hendra virus, Nipah virus, SARS, Marburg virus, and Ebola virus (Baker et al. 2012).

Though it is generally accepted that the immune system is largely shut down during torpor, a few observations of an immune response to *Pd* infection during hibernation have been noted. Plasma from hibernating little brown bats in WNS-affected sites was able to activate complement proteins on a significantly larger scale against *E. coli* infection compared to bats at unaffected sites, but it was significantly less capable of combating infection with *Candida albicans* fungus (Moore et al. 2011). Blood smears from hibernating little brown bats showed that total circulating leukocytes was significantly higher in bats from WNS-affected sites (Moore et al. 2013). European bats experienced infiltration of neutrophils in cases of deep fungal invasion of the epidermis, but no cases of fungal invasion of the dermis were reported; this suggests that a protective immune response could prevent intense fungal invasion that could lead to physiological complications (Wibbelt et al. 2013). Examination of RNA expression in the lung tissue of infected little brown bats showed 8 to 14-times higher concentrations of RNA for IL-10, IL-23, TNF-α, and cathelicidin in *Pd*-infected bats, as well as neutrophilic inflammation in the lungs (Rapin et al. 2014). Blood samples from little brown bats showed high titers of anti-*Pd* antibodies, but low titers were observed in European species; this suggests that an antibody-mediated response to *Pd* may be insufficient at protecting bats and may even be detrimental (Johnson et al. 2015). In contrast, a local inflammatory response in the wings associated with increased RNA expression of IL-6 and IL-1β in infected little brown bats was correlated with lower rates of mass loss (Lilley et al. 2017). This suggests a protective Th17 response during hibernation is maintained in the wings, but a systemic inflammatory Th17 response may be the cause of disrupted thermoregulation and mortality (Lilley et al. 2017). A febrile

response was recently observed in *Pd*-infected little brown bats, during which arousal temperatures were higher compared to uninfected bats (Mayberry et al. 2018).

Necropsies of post-hibernation bats with WNS showed high concentrations of neutrophils in the wing tissue, suggesting that an immune response is activated after infected bats emerge from hibernation in spring (Meteyer et al. 2011). Wing damage and erosion were observed along with signs of inflammation (Meteyer et al. 2011). These observations indicate that a phenomenon similar to immune reconstitution inflammatory syndrome (IRIS) may be occurring in WNS bats, during which an over-zealous immune response to *Pd* infection post-hibernation is detrimental to the host (Meteyer et al. 2012).

Transcriptomics data further support the notion that immune function is not entirely suppressed during torpor in bats. RNA-sequencing of bat wing tissue from infected and uninfected little brown bats showed that WNS caused upregulated gene expression related to inflammation, immune responses, wound healing, metabolism, and oxidative stress (Field et al. 2015). To compare susceptible and tolerant species, RNA-sequencing of bat wing tissue from the little brown bat and the greater mouse-eared bat, a tolerant European species, indicated that the European species does not upregulate immune response genes when infected with *Pd*, and infected bats showed no symptoms of WNS: no change in body mass, no visible fungal growth, and no cupping erosions in the wings (Davy et al. 2017). These studies highlight the importance of comparing tolerant and susceptible species, as well as the wealth of knowledge that can be gained from RNA-sequencing.

Questions and Hypotheses

Despite the extensive amount of research that has been conducted to understand WNS, many questions remain unanswered. A more thorough investigation is needed of the physiological mechanisms underlying observed differences between sick and healthy bats, and among species, including arousal frequency, torpor metabolic rate, immune response to infection, and other genetic differences. The overwhelming majority of studies investigating WNS thus far have focused on little brown bats. The results of these studies may not directly explain the effects of WNS on other species hard-hit by the disease, such as tricolored bats and northern long-eared bats. Studying a variety of North American bat species both resistant and vulnerable to WNS could aid in establishing species-specific conservation and preventative and/or treatment plans.

There is a general lack of knowledge regarding bat immune systems, and an enhanced understanding of the bat immune response to *Pd* infection could supply substantial insight regarding species-specific disease ecology. Previous studies have implied that an immune response is mounted against *Pd* in little brown bats during hibernation, but it remains unclear whether or not the immune response benefits and protects the bat host, or is detrimental. The underlying cause of increased arousals and elevated torpid metabolic rate during infection is also unknown. I proposed a disease progression model in which *Pd* invasion initiates an innate immune response which is responsible for elevated MR_T, increased arousal frequency, and eventual mortality (Figure 4). Portions of my research pertaining to hibernation energetics during WNS are discussed in the next chapter. Portions pertaining to the immune response to *Pd*-infection,

interspecific differences in disease severity, and the development of a cellular model to study WNS are discussed in the subsequent chapter.

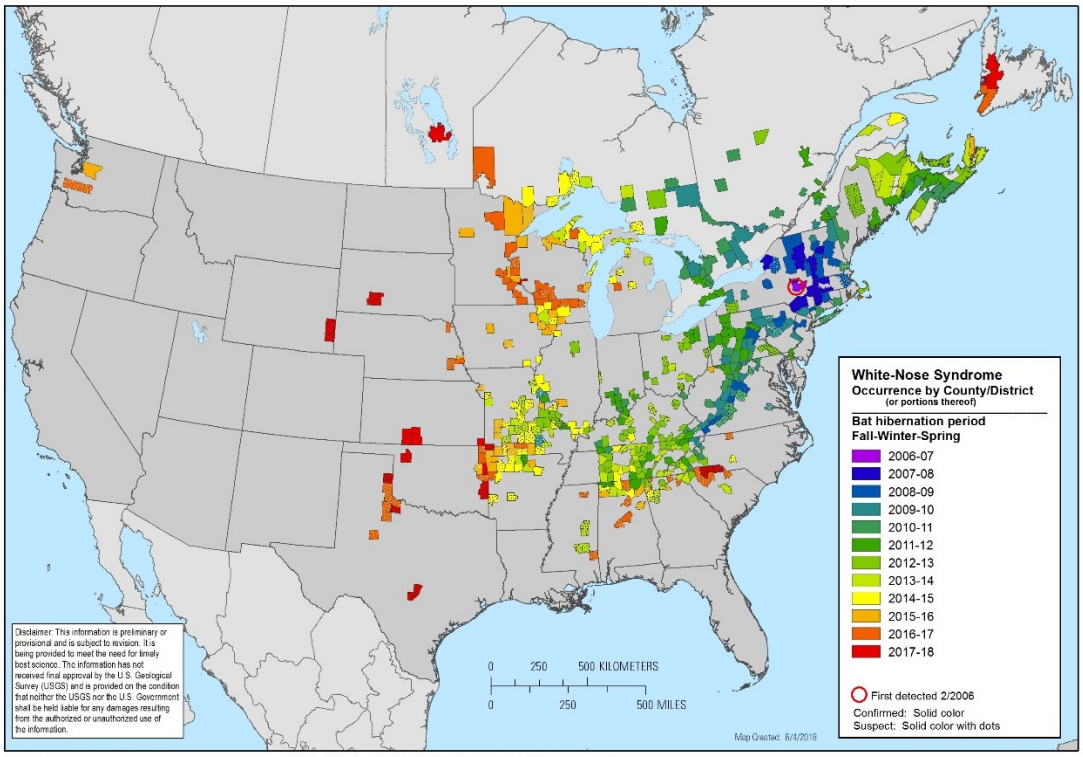

Figure 1. Map of WNS occurrence by county/district. Colors represent year of first occurrence since 2006. Published 06-01-2018 by United States Geological Survey.

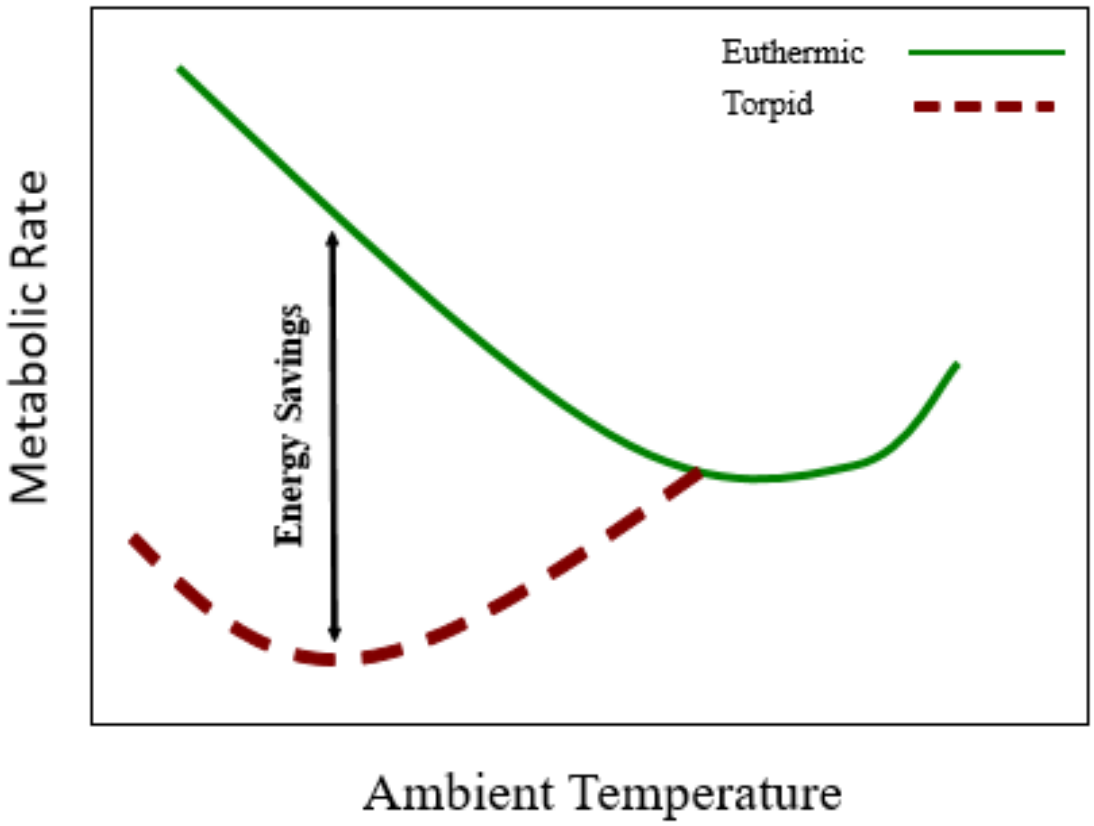

Figure 2. Mammalian model of metabolic rate at a range of ambient temperatures. The difference between metabolic rate while euthermic (green line) versus torpid (red dashed line) indicates energy that the animal can save by entering torpor.

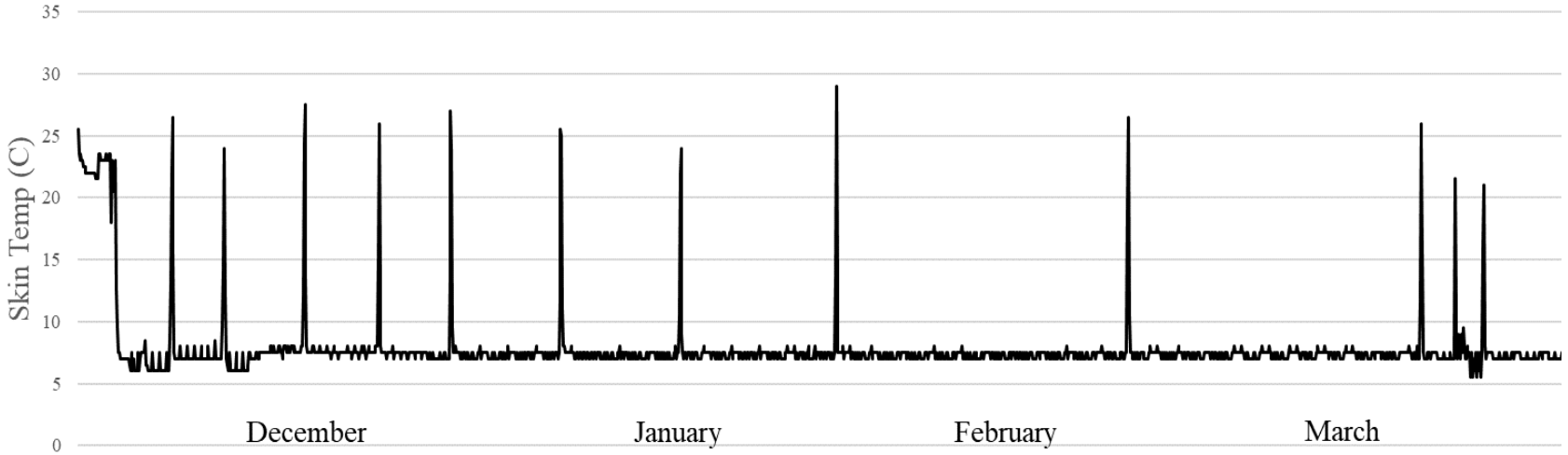

Figure 3. Example of torpor/arousal cycles for a single bat during hibernation. Skin temperature is indicative of core temperature; thus, skin temperature readings of 10ºC or higher represent a brief arousal from deep torpor, during which the bat maintains a steady temperature of about 7ºC.

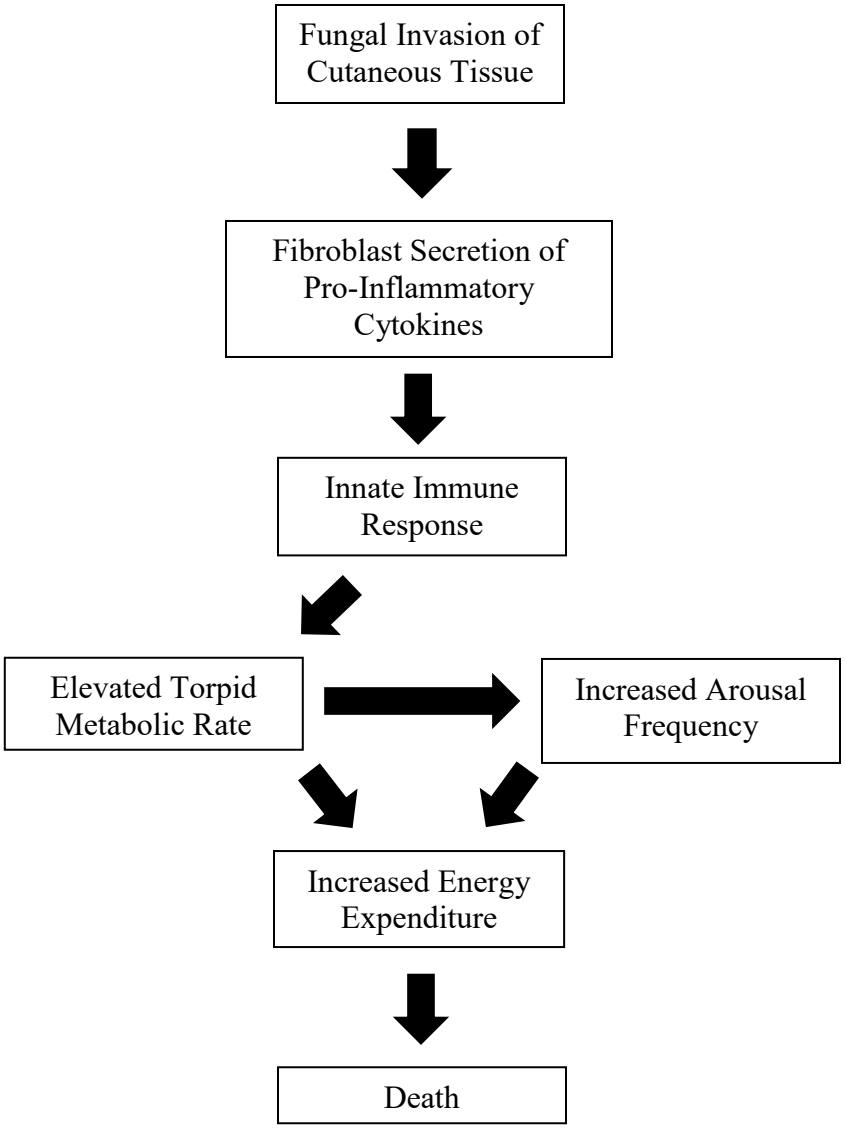

Figure 4. Proposed model of WNS disease progression for a susceptible bat species during hibernation.

ENERGETICS

Introduction

North American bats provide extensive ecological service through insectivory, consuming tons of insects that would otherwise become costly agricultural pests or vectors of mammalian disease (Kunz et al. 2011). Recent calculations have estimated the economic value of North American insectivorous bats to be $22.9 billion annually with regards to agriculture (Boyles et al. 2011). In addition, the nutrients associated with bat guano contribute to the sustainability and stability of cave ecosystems, which lack primary productivity (Culver and Pipan 2009).

The disease known as white-nose syndrome (WNS) has caused substantial mortality for certain species of hibernating North American bats since its introduction to New York in 2006 (Blehert et al. 2009). Susceptible species suffer high mortality rates from WNS, including little brown bats (*Myotis lucifugus*), tricolored bats (*Perimyotis subflavus*), and northern long-eared bats (*M. septentrionalis*). In contrast, high mortality rates have not been observed in other cave-hibernating species, including big brown bats (*Eptesicus fuscus*) and gray bats (*M. grisescens*). The responsible agent for this disease is *Pseudogymnoascus destructans* (*Pd*), a fungus which poses physiological complications for the hibernating bat (Verant et al. 2014). Little is currently known about how the bat's immune system responds to *Pd* infection, and how this might relate to other physiological manifestations of WNS during hibernation. A more complete understanding of these topics can provide insight for potential conservation strategies as WNS continues to spread in North America, posing extirpation risks for multiple bat species.

Previous studies have shown that bats with WNS arouse about twice as often as healthy bats (Reeder et al. 2012). The energy required for these arousals contributes greatly to body fat depletion, which may be the proximal cause of WNS mortality. An elevated torpid metabolic rate (MR_T) could produce the observed shortening of torpor bouts, similar to the increase in MR_T associated with hibernating at sub-optimal temperatures (Dzurick and Tomasi 2008; Janicki 2010; Day and Tomasi 2014; McGuire et al. 2017). This relationship begs the question of what would cause the elevation of MR_T in *Pd*-infected bats.

Upon the invasion of the epidermis by a novel pathogen, the "innate" immune system normally responds quickly in an attempt to clear the invader, followed by the "adaptive" immune response (T cells and antibodies). Leukocytes (white blood cells) such as macrophages and neutrophils are key innate first responders to the site of infection, and are also crucial for producing cytokines (proteins secreted by the cell which serve as a means of communication between neighboring cells and immune cells). Binding of cytokines to a receptor protein on an immune or epithelial cell surface induces signaling pathways to enhance an immune response, such as recruiting leukocytes to the site of infection, and promoting the adaptive immune response. Important cytokines include tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6), inflammatory cytokines which induce a state of local inflammation that can facilitate rapid clearance of the pathogen.

A key parameter for monitoring an organism's immune profile is the neutrophil-lymphocyte ratio (N:L) in the blood. Within the first 48 hours of infection, the N:L will spike as neutrophils are produced in high numbers during the innate immune response.

Neutrophil numbers will usually normalize after two days, and lymphocyte numbers will begin to increase five days post-infection during the initiation of the adaptive immune response. At this time, the N:L will decrease. There is no standard baseline measurement for a healthy mammalian N:L, because it has been determined for very few species. However, the calculated N:L for healthy adults humans averages around 1.65, with a normal range of 1 – 3 (Forget et al. 2017). In a study of helminth-infected big brown bats, the N:L ranged from about 1 – 3.5 (Warburton et al. 2016). However, there are no published data on the N:L of a torpid bat.

In mammals, hibernation generally reduces the organism's innate and adaptive immune functions (Bouma et al. 2010). For the hibernating bat infected with *Pd*, however, variable immune responses are shown that are not typical in other models of mammalian hibernation. For torpid little brown bats in WNS-affected sites, total circulating leukocytes were higher and total antioxidant power was lower compared to torpid bats from non-affected hibernacula (Moore et al. 2013). Hibernating little brown bats exposed to *Pd* also experience changes in expression of genes which promote inflammation, wound healing, and metabolism (Field et al. 2015). In addition, a local inflammatory response is seen at the site of the infection which includes increased gene expression of inflammatory cytokines; however, no recruitment of leukocytes to the site of infection is observed, and these bats are not protected by the immune responses that occur (Field et al. 2015). These studies were all conducted on little brown bats, so the ability to extrapolate these studies to the immune response to *Pd* infection in any other North American species is unclear.

The tricolored bat (*Perimyotis subflavus*, previously *Pipistrellus subflavus*), is a vespertilionid that can be distinguished from *Myotis* species by distinctly tricolored hairs—dark at the base, light in the middle, and dark at the tip (Fujita and Kunz 1984). This species is relatively small; autumn mass averages 7.5 g for males and 7.9 g for females, and spring mass averages 4.6 g and 5.8 g respectively (Fitch 1966). Tricolored bats are distributed across much of northeastern and central North America, including all of Missouri. Uniquely, tricolored bats tend to hibernate individually (McNab 1974) and select microclimates with constant ambient temperatures (Fitch 1966). It is generally reported that this species is among the first to enter hibernation and the last to emerge from hibernacula (La Val and La Val 1980). Tricolored bats are relatively hard-hit by WNS, with 47 – 73% mortality observed in multiple midwestern hibernacula (Langwig et al. 2015). The United States Fish and Wildlife Service has initiated the process for potentially listing this species as federally threatened or endangered (USFWS 2017). Declines have been observed in Missouri since 2013 (Colatskie and Elliot 2016). No captive hibernation studies have been published that assess the effects of WNS on this species.

I hypothesize that the increase in arousals observed in WNS bats (which contribute the most to depletion of body fat during hibernation) is due to an elevated metabolic rate while torpid, and that this elevated metabolic rate is due to an upregulated immune response to *Pd* exposure. If correct, an anti-inflammatory treatment which lowers the elevated immune response could allow the bats normally devastated by WNS to conserve enough energy to survive the hibernation season by decreasing metabolic expenditures (fewer arousals) and sustaining fat reserves. Also, a decreased metabolic

rate and fewer arousals would support the assumed link between metabolism and arousal frequency in the bat's winter energy budget. To test these hypotheses, torpid metabolic rates, torpor/arousal cycles, and leukocyte counts were measured in *Pd*-infected tricolored bats treated with an anti-inflammatory agent, meloxicam.

Methods

Meloxicam, a non-steroidal anti-inflammatory drug, was the veterinarian-recommended treatment for this study (M. Stafford, personal communication), although it has been shown to inhibit biofilm formation of other fungal species, including *Candida albicans*, at certain doses (Alem and Douglas 2004). Therefore, to ensure that any effect of meloxicam was due to its anti-inflammatory effect on the bat rather than a direct antifungal effect on *Pd*, a preliminary test was conducted to determine which meloxicam dose would avoid the latter but promote the former. Meloxicam concentrations of 0.01, 0.05, 0.10, 0.50, and 1.00 µg/mL in DMSO were added to Sabouraud dextrose agar (SDA) plates. Three plates of each meloxicam dose were inoculated with *Pd* spores (10, 100, 1000 cfu) and three with *Pd* hyphae (10, 100, 1000 cfu). After incubation (11ºC for 7 days), fungal growth was observed on all plates, but was inhibited at the two highest meloxicam concentrations. Since 0.1 µg/mL was a veterinarian-recommended effective topical anti-inflammatory dosage in other mammals (M. Stafford, personal communication), this was selected as the dosage for this study. Because meloxicam would be administered to the bats while torpid, it was assumed that the drug would be absorbed, metabolized, and excreted much slower than in a euthermic animal. However, no research was available to indicate the proper dosing frequency for a torpid mammal.

Therefore, the decision was to dose the bats twice (on the day of capture and after six weeks) while already disturbed for other purposes. Thus, unnecessary disturbances could be avoided.

Hibernating tricolored bats (n = 38) were captured by hand from Long Cave in McDonald County, Missouri near the beginning of the hibernation season (30 November). This study was approved by the Missouri State University IACUC (17-008.0, 11-29-2016), and collection permits were provided to Tom Tomasi by the Missouri Department of Conservation. Bats were transported individually in cloth bags to Missouri State University, weighed (to 0.1 grams), and sexed.

To check for the presence of fluorescing *Pd* growing on the skin at the start of the study, a photograph was taken of each wing membrane over a UV light. Also, each bat was rubbed with a sterile, dry swab across the wing membrane, muzzle, and ears. Swabs were stored at -80ºC. A modified yeast colony PCR protocol was used to assess for the presence of *Pd* DNA on the swabs. Briefly, swabs were centrifuged in sterile water then incubated at 99ºC in the presence of 0.02M NaOH. The *Pd* primers (Forward 5'-CTTTGTTTATTACACTTTGTTGCTTT-3' and Reverse 5'-CCGTTGTTGAAAGTTTTAACTATTATAT 3') were used under the following thermal cycler conditions: three minutes of primary denaturation (95ºC), 34 cycles of denaturation (30 seconds at 95ºC), annealing (30 seconds at 67ºC), and extension (two minutes at 72ºC), and a final extension step for ten minutes at 72ºC. Samples were evaluated on an agarose gel with ethidium bromide.

All 38 bats were inoculated with *Pd* fungal spores (1,000 spores in 100 µL saline) by pipetting onto the two wing membranes. Bats were divided into two treatment groups.

Control bats (n = 18) received just the *Pd* inoculation, and experimental bats (n = 20) received *Pd* inoculation plus a dosage of meloxicam (2.5 µg in 25 µL) pipetted onto the two ears for cutaneous absorption.

Twenty-seven temperature-sensitive dataloggers (modified iButtons™; Lovegrove 2009) were set to record temperature every 30 minutes, and they were attached to the backs of bats with surgical cement after trimming a small section of fur. Skin temperature readings in torpid and active bats accurately correlate with rectal temperatures (Barclay et al. 1996); therefore, skin temperature readings can reliably indicate changes in core body temperatures. For the remaining 11 bats, identification was achieved with a wing band attached to the forearm (n=5) or trimming a unique section of fur (n=6).

Wing tissue biopsies (4-mm diameter) were taken from half the bats in each treatment and stored at -80° C. A second biopsy was collected from four of those bats (two per treatment) for histological analysis and stored at room temperature in formalin. Blood samples (approximately 200 µL) were taken via cardiac puncture under isoflurane anesthesia from the same 19 bats. Blood samples were split into two tubes (100 µL each) and frozen at -80° C for: a) PCR analysis to assess cytokine expression; or b) assessment of various leukocyte numbers via flow cytometry.

Bats were placed by treatment into respective plastic-screen cages (20 liters) within an environmental chamber. Both chambers were maintained at 8° C and at least 85% relative humidity with installed misting systems. Water dishes were kept at the bottom of the cages to provide water *ad libitum*. A camera was positioned at the top of each cage, facing down, to visualize bats within the chamber and allow monitoring

without disturbance. If bats appeared dead or moribund (on the floor of the cage for an extended period of time), they were removed.

In mid-January (mid-hibernation), blood and wing tissue samples were collected from the bats not sampled the day of capture, as described above, and all the bats in the experimental group were dosed again with meloxicam. Also, in mid-January, bats were moved to individual 50 mL open-flow respirometry chambers for analysis of torpid metabolic rate. The inside of each chamber (n = 10) was lined with plastic mesh netting to provide a roosting surface for the bat, and these were placed in a refrigerated cabinet set to 8+/-1ºC. An air pump was used to provide continuous air flow through each metabolic chamber when not being measured. When metabolic rate was being measured for a bat, air from the metabolic chamber was instead pulled through a CO_2 and H_2O scrubber (soda lime and silica gel, respectively), by a flow controller set to 25 mL/min (Sable Systems), and sent to an oxygen analyzer calibrated to 20.95 % oxygen (Sable Systems, FC-10a). Bats were acclimated to the metabolic chamber for at least 12 hours prior to metabolic measurements to ensure return to torpor. ExpeData™ software sampled the percentage of oxygen in the system every 30 seconds, for a long enough period to record four breathing bouts per bat (see below). If a bat appeared aroused (over five bouts per hour), data for this bat were not used in subsequent analyses. Bats were weighed before being returned to environmental chambers.

Measurements of MR_T were also conducted for all surviving bats at the end of the experiment (late March, i.e., late hibernation), using the methods described above. Blood and wing tissue biopsies were then taken from all bats using methods described above.

Final mass was recorded for each bat, and photographs were taken under UV light to detect the presence of *Pd* invasion of the wings. All bats were then euthanized.

Oxygen consumption data were converted to torpid metabolic rates in ExpeData™ (Sable Systems, Las Vegas, NV) software. Bats were apneic for long periods of time, followed by a breathing bout. Measurements were made over three apneic/breathing cycles. The sum volume (mL) of oxygen consumed during each breathing bout was divided by the total time interval (minutes) for each measurement period to obtain metabolic rate values for each bat (expressed as mL O_2/hr).

Temperature-sensitive dataloggers were removed from each bat after euthanasia, and temperature recordings were scanned visually for arousal bouts (temperature readings) above 10° C. Long arousals corresponding to disturbance (e.g. transferring bats to the metabolic chambers) were not included. The duration of each arousal was measured with 0.5-hour resolution, as well as the time between arousals (torpor bouts). For each bat, number of arousals, average arousal duration, average torpor bout duration, and average torpor temperature were calculated.

Blood samples (approximately 100 µL) obtained in November and January were analyzed via flow cytometry. Samples were diluted in 1 mL of PBS and filtered through a 40 µm cell strainer to remove clots. November samples were processed in an Accuri C6 Flow Cytometer, and January samples were processed in an Attune NxT Acoustic Focusing Cytometer. The size and density of blood cells were measured, and were visually identified as red blood cells, neutrophils, lymphocytes, or macrophages.

The effect of meloxicam treatment on torpor/arousal characteristics, percent mass loss, neutrophil-leukocyte ratios, and percentage of leukocytes in the blood was tested

using a one-way analyses of variance (ANOVA). The effect of hibernation stage and meloxicam treatment on MR_T was tested using a two-way analysis of variance (ANOVA). Difference in percent survival between groups was assessed using a Wilcoxon rank test. Significance was established at $\alpha = 0.05$. Statistical analyses were performed using Minitab 18 or GraphPad Prism.

Results

Based on colony PCR of the swabs obtained on the day of capture, two bats appeared to be positive for the presence of *Pd*, both of which had been assigned to the meloxicam treatment group. Of the 38 tricolored bats that were captured for this study, 26 individuals survived to the end of the hibernation experiment. Survival was lower in the control group (55.6% with most of the mortality in the last two weeks) compared to the meloxicam group (80.0%), but the difference was not significant ($p = 0.149$, Figure 5). Percent mass loss over the course of the experiment also did not differ between meloxicam and control groups ($p = 0.96$, Figure 5). Torpid metabolic rate (MR_T) was not significantly affected by treatment group ($p = 0.561$) or stage of hibernation ($p = 0.199$) (Figure 6). However, the trend was for meloxicam bats to have higher average MR_T (0.273 ± 0.27 mL O_2/hour in January, 0.076 ± 0.01 mL O_2/hour in March) than the control bats (0.195 ± 0.07 mL O_2/hour in January, 0.072 ± 0.01 mL O_2/hour in March), and the average MR_T for both groups decreased from January to March (Figure 6).

Temperature-sensitive dataloggers supplied arousal frequency data for 24 bats throughout the hibernation experiment. On average, fewer arousals were observed in the meloxicam group (5.5 ± 0.51, n = 15) compared to the control group (6.1 ± 0.66, n = 9).

However, the difference was not significant ($p = 0.464$, Figure 7). Meloxicam bats spent less time aroused (108.41 ± 4.36 min) compared to control bats (123.12 ± 5.37 min), and the difference approached significance ($p = 0.057$, Figure 7). Consistent with this, meloxicam bats averaged more time in a torpor bout (261.47 ± 24.25 hr or 10.89 days) compared to control bats (231.08 ± 20.14 hr or 9.63 days), but the difference was not significant ($p = 0.415$, Figure 7). Lower average torpor temperatures were observed in the meloxicam group (6.0 ± 0.14° C) compared to the control group (6.5 ± 0.22° C), but the difference was not significant ($p = 0.081$, Figure 7).

Using flow cytometry, the ratio of neutrophils to lymphocytes (N:L) was calculated for each sample. The N:L was not significantly different between meloxicam and control groups in early hibernation ($p = 0.772$) or mid-hibernation ($p = 0.204$) (Figure 8). The percentages of blood cells that were lymphocytes, neutrophils, and macrophages were also calculated for each blood sample. In early hibernation, none of the percentages of leukocytes were significantly different between treatments. In mid-hibernation, percent neutrophils was significantly lower ($p = 0.029$), and percent macrophages was nearly significantly higher ($p = 0.054$) in meloxicam bats compared to control bats (Figure 8). The percentage of lymphocytes was not different between treatments in mid-hibernation ($p = 0.535$) (Figure 8).

Discussion

The results of this study do not support the hypothesis that *Pd*-infected bats dosed with an anti-inflammatory agent would experience milder WNS symptoms such as fewer arousals and lower torpid metabolic rates compared to *Pd*-infected controls. While the

majority of meloxicam bats survived to the end of the experiment, only slightly more than half of the control bats survived. Differences between treatments were in the predicted directions, but they were not large enough to be statistically significant. Meloxicam bats tended to arouse less frequently, and arousals tended to be shorter compared to control bats. However, the dataloggers did not supply a high temporal resolution, thus the arousal duration measurements may not be accurate. Meloxicam bats hibernated at a cooler average temperature than controls, suggesting that meloxicam bats used deeper torpor to save more energy during the hibernation season. However, there is uncertainty in these measurements due to microhabitat differences within the same cage and a lack of datalogger calibration. If meloxicam, with its anti-inflammatory properties, was able to successfully hinder an upregulated immune response to *Pd* infection during torpor, it could explain the trends observed in this study: less mortality, fewer arousals, and lower torpid body temperatures.

A small sample size could explain the lack of statistically different findings. A power analysis revealed that with the standard error observed among the individual bats within each treatment in the torpid metabolic rate data set, a sample size of over 150 bats per treatment group would have been needed to achieve significant differences. Only 38 bats were captured for this study due to conservation constraints. Tricolored bats are particularly affected by WNS mortality, so it was important to refrain from removing a substantial portion of the population for this study. Of the 38 bats, only 27 temperature-sensitive dataloggers were able to be fitted to bats, and not all dataloggers successfully captured data, thus lowering the sample size even further for arousal frequency measurements. On the other hand, significant values would have been obtained with our

sample size if the variation was substantially lower (half) among the individual bats within each treatment. The variation observed could be biologically relevant, or it could be associated with temperature fluctuations in the both the hibernation chambers and metabolic chambers, as a difference in ambient temperature of 1 – 2^{o} C may impact the hibernation energetics of bats (Day and Tomasi 2014; Dzurick and Tomasi 2008). Ambient temperatures were regulated by the environmental chambers/cabinet but were not independently monitored throughout this study.

Metabolic rates while torpid (MR_T) were measured for each bat on two occasions to supply mid-hibernation and end-hibernation data. It was predicted that meloxicam-treated bats, with a hypothesized muted immune response to *Pd* infection, would have lower MR_T compared to *Pd*-infected controls, which would experience an elevation in MR_T due to an upregulated immune response to *Pd* infection. Although the difference between the two groups was not significant, the meloxicam bats tended to have a higher average MR_T compared to controls during both mid-hibernation and late hibernation measurements. This raises the question of whether or not meloxicam effectively downregulated an immune response to *Pd* infection. Also, both treatment groups had lower MR_T in late hibernation compared to mid-hibernation. This difference could be an artifact of temperature fluctuations in the chambers, or it could represent a hibernation strategy in which bats will conserve as much energy as possible toward the end of hibernation when fat stores are lowest.

The neutrophil-lymphocyte ratio is frequently utilized in the medical field to indicate inflammation or stages of infection. A low N:L, caused by elevated lymphocytes and/or reduced neutrophils in the blood sample, would indicate a later stage of infection

during which the adaptive immune response has been initiated. The meloxicam group tended to have a lower N:L compared to the control group in mid-hibernation. This observation, paired with the significantly lower neutrophil numbers in meloxicam bats in mid-hibernation, suggests that meloxicam lowered the circulation of neutrophils. The anti-inflammatory properties of meloxicam include the inhibition of cyclooxygenases, which prevents an inflammatory response. These results suggest that meloxicam may have successfully reduced the torpid bats' immune responses. However, the lack of an uninfected control group prevents the determination of a "normal" N:L during hibernation.

Without an uninfected control group for comparison, it is also difficult to determine whether a significant response to *Pd* was activated. Further, more frequent blood sampling would have provided a clearer timeline of any immune responses. However, minimizing disturbances during hibernation was deemed a higher priority than obtaining as many samples as possible. The observed differences in percent or N:L ratio between early and mid-hibernation samples could be due to the differences in machines used to analyze each set of blood samples. There is also a potential effect of storage time. The early hibernation blood samples were analyzed on the same day of collection, whereas the mid-hibernation samples were stored at -80^{o} C for over a year before analysis. These factors could be sources of error, but the extent has not been determined. To measure the expression of various cytokines and more effectively determine whether an immune response to *Pd* was activated, qPCR of blood and wing tissue samples will occur at a later date.

The meloxicam dosage or number of doses may not have been sufficient to elicit an anti-inflammatory response in hibernating bats, even if effective pharmaceutically. The calculated dose was based on the highest concentration of meloxicam that would not inhibit *Pd* growth *in vitro*, then adjusted for the average weight of the bats. Doses were applied topically to the ears on two occasions, the first day of the experiment and during mid-hibernation following metabolic rate measurements. These may not have been the most efficient methods for dosing the bats. Taking into consideration the reduced metabolic rate, circulation, and body temperature of a hibernating bat, a different dose of meloxicam or a different number of applications may have been necessary. It may have been more effective to apply meloxicam directly to the wing membranes, at the epicenter of *Pd* infection. However, applying meloxicam directly to *Pd*-infected tissue may have inhibited the growth of *Pd* rather than (or in addition to) downregulated the immune response. Further trials are necessary to determine the appropriate dose of meloxicam, number of applications, and proper application method for a hibernating bat.

The primary determinant explaining the lack of significance in these results could be the effectiveness of *Pd* inoculation in causing WNS. Each bat was inoculated with viable *Pd* spores in the same manner practiced by researchers in similar hibernation experiments, yet none of the bats exhibited severe symptoms of WNS by the end of the experiment. No visible white fungus was observed growing on the wings, ears, or muzzle of any bats. Four months post-inoculation, ultraviolet light revealed very small areas of fluorescence that may have been fungal growth on the wing membranes, but no large areas on the wings appeared to be affected by *Pd*. Histological observations of wing tissue samples taken at the end of hibernation for a separate study did not reveal the

diagnostic cupping erosions used as the "gold standard" for WNS diagnosis (Meteyer et al. 2009). Why the inoculations in this study failed to cause more severe *Pd* infections is hard to explain, but without a severe WNS response, the data remain inconclusive.

Twelve years after the initial discovery of white-nose syndrome in New York, *Pd* continues to spread throughout the eastern and central North America. An understanding of the physiological response to *Pd* infection is vital for determining which species will experience high mortality and demand priority with conservation and management plans. Successful mitigation of wildlife disease requires incorporating all three aspects of the 'disease triangle': host, pathogen, and environment (Scholthof 2007). Understanding the response of the host to the pathogen is a key component of combating wildlife disease, establishing efficacious conservation plans, and preventing/slowing spread to additional populations.

References

Alem, M. A. S., and L. J. Douglas. 2004. Effects of aspirin and other nonsteroidal anti-inflammatory drugs on biofilms and planktonic cells of *Candida albicans*. Antimicrobial Agents and Chemotherapy 48:41-47.

Barclay, R. M. R., M. C. Kalcounis, L. H. Crampton, C. Stefan, M. J. Vonhof, L. Wilkinson, and R. M. Brigham. 1996. Can external radiotransmitters be used to assess body temperature and torpor in bats? Journal of Mammalogy 77:1102-1106.

Blehert, D. S., et al. 2009. Bat white-nose syndrome: an emerging fungal pathogen? Science 323:227-227.

Bouma, H. R., H. V. Carey, and F. G. M. Kroese. 2010. Hibernation: the immune system at rest? Journal of Leukocyte Biology 88:619-624.

Boyles, J. G., P. M. Cryan, G. F. McCracken, and T. H. Kunz. 2011. Economic importance of bats in agriculture. Science 332:41-42.

Colatskie, S., and A. Elliott. 2016. Summary of the 2015/2016 Missouri bat hibernacula surveys and white-nose syndrome disease surveillance effort. Missouri Department of Conservation.

Culver, D. C., and T. Pipan. 2009. The biology of caves and other subterranean habitats. 2nd ed. Oxford University Press. Oxford, United Kingdom.

Day, K. M., and T. E. Tomasi. 2014. Winter energetics of female Indiana bats (*Myotis sodalis*). Physiological and Biochemical Zoology 87:56-64.

Dzurick C., and T. Tomasi. 2008. Ambient temperature and the hibernation energy budget of the endangered Indiana bat (*Myotis sodalis*). Pp. 381-390 in Hypometabolism in animals: hibernation, torpor and cryobiology (B. G. Lovegrove and A. E. McKechnie, eds). University of KwaZulu-Natal, Pietermaritzburg, South Africa.

Field, K. A., J. S. Johnson, T. M. Lilley, S. M. Reeder, E. J. Rogers, M. J. Behr, and D. M. Reeder. 2015. The white-nose syndrome transcriptome: activation of anti-fungal host responses in wing tissue of hibernating little brown myotis. PLoS Pathogens 11:1-30.

Fitch, J. H. 1966. Weight loss and temperature response in three species of bats in Marshall County, Kansas. Search 6:17-24.

Forget, P., C. Khalifa, J. Defour, D. Latinne, M. Van Pel, and M. De Kock. 2017. What is the normal value of the neutrophil-to-lymphocyte ratio? BMC Research Notes 10:1-4.

Fujita, M. S., and T. H. Kunz. 1984. *Pipistrellus subflavus*. Mammalian Species 228:1-6.

Janicki, A. 2010. The effect of white-nose syndrome on torpid metabolic rates of *Myotis lucifugus*. M.S. thesis, Missouri State University. Springfield, Missouri.

Kunz, T. H., E. Braun de Torrez, D. Bauer, T. Lobova, and T. H. Fleming. 2011. Ecosystem services provided by bats. Annals of the New York Academy of Sciences 1223:1-38.

Langwig, K. E., et al. 2015. Invasion dynamics of white-nose syndrome fungus, midwestern United States, 2012-2014. Emerging Infectious Diseases 21:1023-1026.

La Val, R. K., and M. L. La Val. 1980. Ecological studies and management of Missouri bats with emphasis on cave-dwelling species. Terrestrial Sere Missouri Department of Conservation 8:1-53.

Lovegrove, B. G. 2009. Modification and miniaturization of Thermochron iButtons for surgical implantation into small animals. Journal of Comparative Physiology B: Biochemical, Systems, and Environmental Physiology 179:451-458.

McGuire, L. P., H. W. Mayberry, and C. K. R. Willis. 2017. White-nose syndrome increases torpid metabolic rate and evaporative water loss in hibernating bats. American Journal of Physiology—Regulatory, Integrative and Comparative Physiology 313:680-686.

McNab, B. K. 1974. The behavior of temperate cave bats in a subtropical environment. Ecology 55:943-958.

Meteyer, C. U., et al. 2009. Histopathologic criteria to confirm white-nose syndrome in bats. Journal of Veterinary Diagnostic Investigation 21:411-414.

Moore, M. S., J. D. Reichard, T. D. Murtha, M. L. Nabhan, R. E. Pian, J. S. Ferreira, and T. H. Kunz. 2013. Hibernating little brown myotis (*Myotis lucifugus*) show variable immunological responses to white-nose syndrome. PLoS ONE 8:1-9.

Reeder, D. M., et al. 2012. Frequent arousal from hibernation linked to severity of infection and mortality in bats with white-nose syndrome. PLoS ONE 7:1-10.

Scholthof, K. G. 2007. The disease triangle: pathogens, the environment and society. Nature Reviews Microbiology 5:152.

United States Fish and Wildlife Service (USFWS). 2017. Evaluation of a petition to list the tricolored bat as an endangered or threatened species under the act. Federal Register 82:60364.

Verant, M. L., C. U. Meteyer, J. R. Speakman, P. M. Cryan, J. M. Lorch, and D. S. Blehert. 2014. White-nose syndrome indicates a cascade of physiologic disturbances in the hibernating bat host. BMC Physiology 14:1-10.

Warburton, E. M., C. A. Pearl, and M. J. Vonhof. 2016. Relationships between host body condition and immunocompetence, not host sex, best predict parasite burden in a bat-helminth system. Parasitology Research 115:2155-2164.

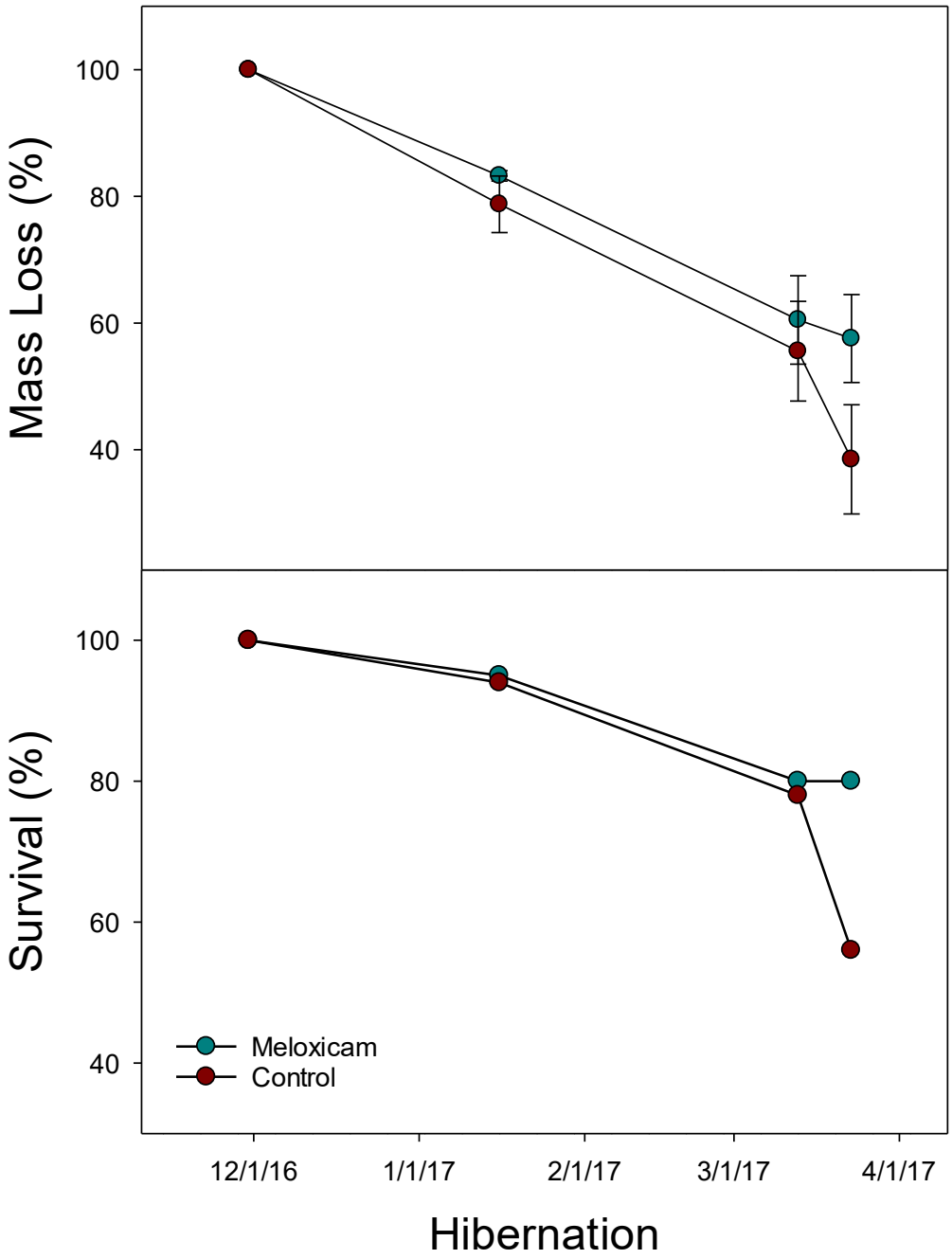

Figure 5. Trends over the course of hibernation for control and meloxicam groups. Mass (g) for each bat was measured on four occasions: early hibernation (November 30th), mid-hibernation (January 16th), and late-hibernation (March 13th and March 23rd). Top: mass loss (mean ± SE) was not significantly different between treatment groups ($p = 0.96$). Bottom: number of individuals in each treatment group that survived to each time point in the study. Meloxicam group = 80% survival, control group = 55.6% survival ($p = 0.149$).

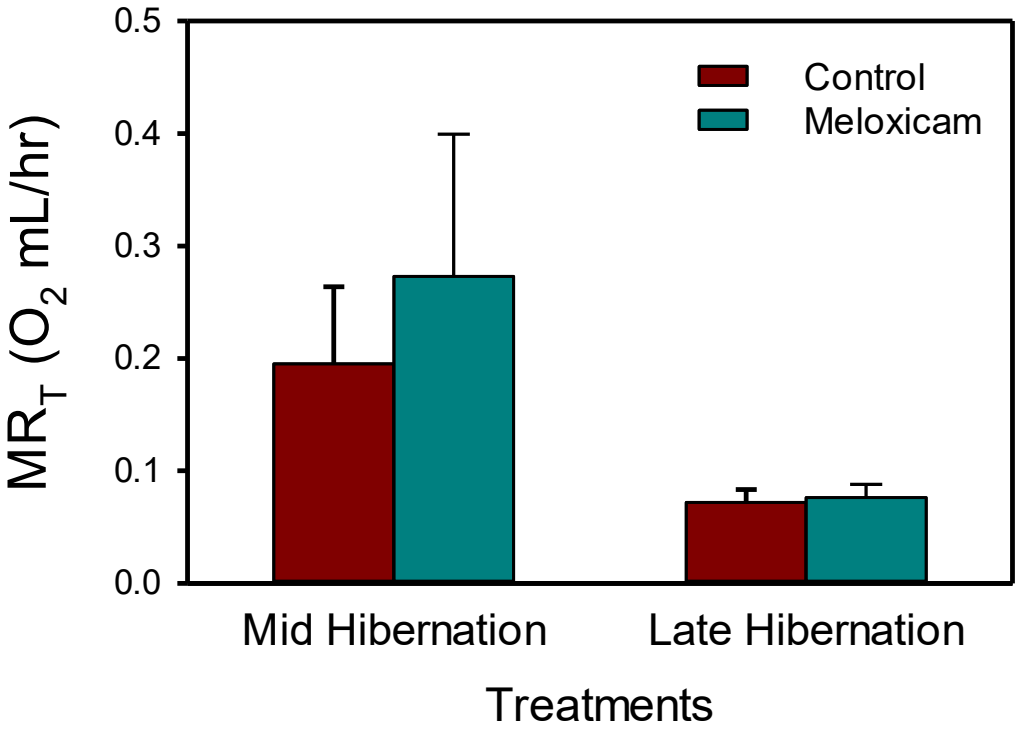

Figure 6. Metabolic rate (mean ± SE) while torpid (MR_T) for each treatment group for two measurement periods: mid-hibernation (January) and late-hibernation (March). Metabolic rates were not significantly affected by treatment groups ($p = 0.561$) or date ($p = 0.199$).

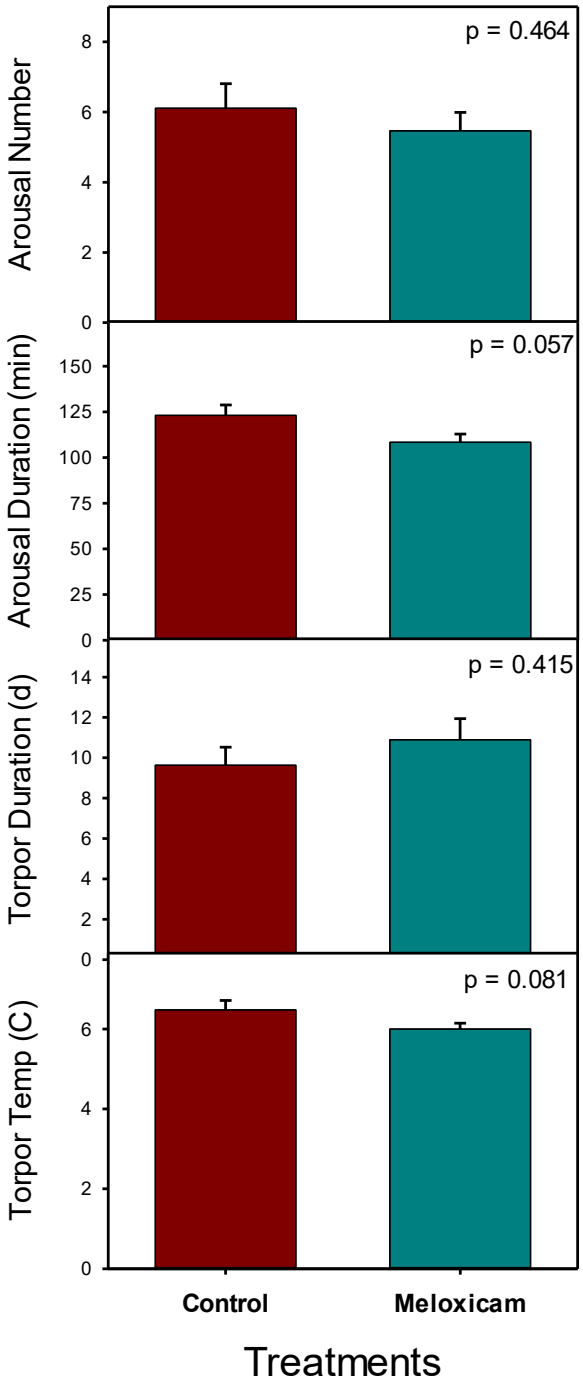

Figure 7. Average (± SE) number of arousals, arousal duration, torpor bout duration, and skin temperature while torpid for meloxicam-treated and control groups.

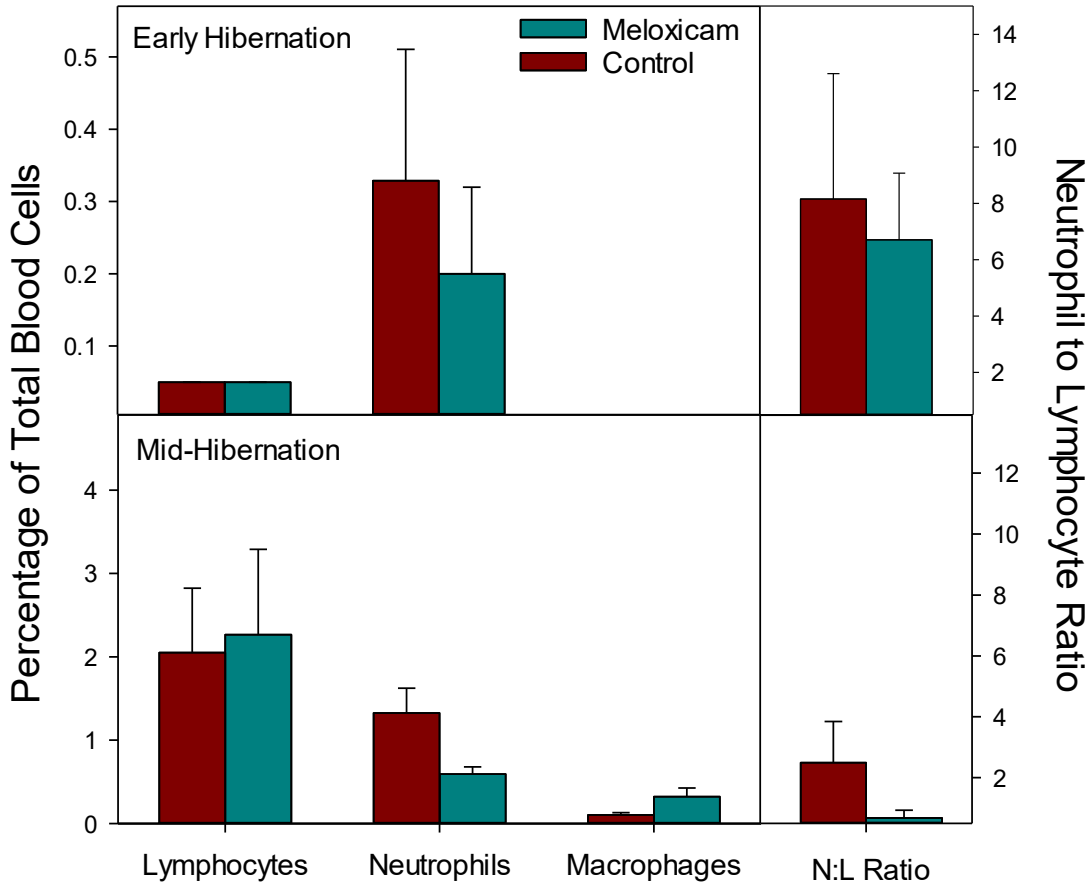

Figure 8. Average percent (± SE) of leukocytes (lymphocytes, neutrophils, and macrophages) out of the total number of blood cells (left) and neutrophil-lymphocyte ratios (right) for both treatment groups in early and mid-hibernation. There were no significant differences between treatments in early hibernation for any leukocyte. In mid-hibernation, meloxicam bats had significantly lower neutrophils compared to control bats ($p = 0.029$). The neutrophil to lymphocyte ratio was not different between treatment groups in early or mid-hibernation.

FIBROBLASTS

Introduction

Among the Orders of extant mammals, Chiroptera is the second most biologically diverse with at least 1,100 known species dispersed across all continents except Antarctica (Simmons and Conway 2003). Over two-thirds of extant bat species are either obligate or facultative insectivores, including the majority of the 47 bat species in the United States (Kunz et al. 2011). Some species are capable of consuming up to 25% of their body mass in insect prey each night, resulting in tremendous top-down suppression of insect populations (Coutts et al. 1973, Kunz et al. 2011). The value of the natural pest control services provided by insectivorous bats in the United States has been estimated to be around $22.9 billion per year (Boyles et al. 2011), a valuation that is likely underestimated because it does not include the downstream environmental benefits of reduced pesticide applications, the impact of bat insectivory on other non-crop plants such as timber, or the reduced spread of insect borne diseases (Reiskind and Wund 2009). Bats also supply a substantial amount of nutrients to cave ecosystems, which lack primary productivity (Culver and Pipan 2009). Thus, the ecosystem services and economic value of insectivorous bats in the United States must be preserved.

One of the largest threats to bat populations in North America at present is white-nose syndrome (WNS), first discovered in a New York cave in 2006 (Blehert 2009). The etiological agent behind this disease is a psychrophilic fungus, *Pseudogymnoascus destructans* (*Pd*), which invades the epidermis and dermis of cave-hibernating bats (Blehert et al. 2009). Over the course of twelve years, the fungal pathogen has spread to

34 states and five Canadian provinces, primarily in northeastern and central regions of North America (USGS 2018). A 2012 assessment culminated with an estimation of 5.7 to 6.7 million deaths caused by WNS, although exact mortality rates are hard to estimate with the dearth of pre-WNS population estimates for many species (USFWS 2012). Given its rate of spread, it would be reasonable to assume that in the subsequent six-year period, a similar number of additional bats have died from WNS. A strong understanding of WNS—including pathogenic strategies of the fungus, the influence of environmental factors, and the response of the bat host—is necessary for proper implementation of conservation plans and disease mitigation strategies.

Bats and many other endothermic species utilize torpor, an energy-saving mechanism during which body temperature and metabolic rate are physiologically depressed. This is accompanied by a cessation or slowing of bodily functions such as cardiac output, digestion, mobility, and cellular respiration (Geiser 2004). By practicing extended torpor over the course of the winter (hibernation), animals can save over 85% of the energy that would be spent had they remained euthermic (Geiser 2004). Hibernation is intermittently disrupted by brief returns to euthermia (arousals), the underlying cause of which is unknown. Arousals can account for up to 90% of the total energy expenditure during hibernation (Thomas et al. 1990). Animals likely maximize energy savings throughout winter by entering hibernation with sufficient fat stores, selecting proper microclimates, and budgeting the frequency of arousals (Humphries et al. 2003).

White-nose syndrome is associated with a variety of physiological disturbances during hibernation. As *Pd* invades the epidermal surfaces of the wing membranes, ears, and muzzle, the fungal hyphae form diagnostic cutaneous lesions and cupping erosions

that can be identified via histology (Meteyer et al. 2009). Increased severity of fungal invasion leads to significantly shorter torpor bouts; the concomitant increase in the frequency of arousals is hypothesized to be a main cause of mortality, as arousals quickly consume the bat's winter energy budget (Reeder et al. 2012). Bats with WNS also have higher torpid metabolic rates (MR_T), another increase in energy expenditure during hibernation (McGuire et al. 2017) and this could explain the shorter torpor bouts. Another potential explanation for the frequent arousals focuses on *Pd*-associated erosion of the wing tissue increasing water and solute loss, causing bats to arouse in attempts to rehydrate (Cryan et al. 2013). This hypothesis is supported by observations of increased rates of evaporative water loss in WNS bats (McGuire et al. 2017). Observations of blood chemistry showed respiratory acidosis, hypovolemia, decreased blood sodium and glucose levels, and increased hematocrit—all indicating physiological changes that could culminate in a disruption of hibernation and subsequent mortality (Warnecke et al. 2013, Verant et al. 2014). However, many factors involved in the effect of *Pd* on the bat host, including the generation of an immune response, have not yet been thoroughly investigated.

A marked reduction in immune function has been observed during hibernation in various mammals, likely a result of reduced body temperature and metabolic rate (Bouma et al. 2010, 2013). Injecting hibernating golden-mantled ground squirrels with lipopolysaccharide resulted in longer arousal durations and higher arousal temperatures, suggesting that a primary function of arousals could be to restore immune function (Prendergast et al. 2002). However, there is a general paucity of research concerning the immunology of bats.

An immune response to *Pd* infection may be activated in the hibernating bat, though whether or not it can partially or fully clear the pathogen is unclear. *Pd*-infected bats experienced increased complement activity in the blood (Moore et al. 2011), higher numbers of total circulating leukocytes in the blood (Moore et al. 2013), and higher RNA expression of interleukin (IL)-10, IL-23, tumor necrosis factor-alpha (TNF-α), and cathelicidin as well as neutrophilic inflammation in the lung tissue (Rapin et al. 2014). Increased RNA expression of IL-6 and IL-1β in wing tissue was correlated to lower rates of mass loss, suggesting a protective Th17 response in infected bats (Lilley et al. 2017). A febrile response has also been observed, based on higher arousal temperatures in infected bats compared to controls (Mayberry et al. 2018). RNA-sequencing of wing tissue from infected and uninfected bats revealed upregulated gene expression involved in inflammation, immune responses, wound healing, metabolism, and oxidative stress (Field et al. 2015). After emergence from hibernation, some bats' immune systems have been reported to generate an over-zealous response that may be detrimental to the host: wing tissue membranes become flooded with neutrophils alongside signs of inflammation, suggesting a phenomenon similar to immune reconstitution inflammatory syndrome (IRIS) (Meteyer et al. 2011, 2012).

All of the aforementioned WNS observations pertain to studies on the little brown bat (*Myotis lucifugus*), one species that was particularly afflicted by the disease. Once widely distributed and common throughout the eastern United States, WNS has caused collapses of little brown bat populations ranging from 30% to 99%, leading to predictions of regional extirpations (Frick et al. 2010). However, hibernaculum surveys have revealed that not all species respond similarly to *Pd*. Ten bat species have been observed

to have symptoms of WNS so far. Of these, the little brown bat, tricolored bat (*Perimyotis subflavus*), and northern long-eared bat (*Myotis septentrionalis*) have experienced substantial declines to date (Langwig et al. 2015). Three other species, the eastern small-footed bat (*Myotis leibii*), big brown bat (*Eptesicus fuscus*) and gray bat (*Myotis griscesens*) are among those that do not appear to experience declines from WNS (Flock 2014, Frick et al. 2017). Mechanisms of disease resistance have been studied only in the big brown bat. In one study, big brown bats studied in the field had no signs of *Pd* growth, higher fat content mid-hibernation, and no increase in arousal frequency compared to little brown bats at the same hibernaculum (Frank et al. 2014). Bacteria isolated from the skin of big brown bats inhibited *Pd* growth *in vitro* (Hoyt et al. 2015) and lowered WNS severity when applied to little brown bats simultaneously with *Pd* inoculation (Cheng et al. 2016). Cutaneous fatty acids in big brown and little brown bat tissue, including myristic and oleic acids, inhibited *Pd* growth *in vitro* at temperatures above 5° C and 10° C respectively, but not at lower temperatures tested (Frank et al. 2016, Ingala et al. 2017). Thermoregulatory patterns, including longer torpor bouts and selection of colder microhabitats, of infected big brown bats compared to uninfected controls suggested beneficial energy-saving mechanisms (Moore et al. 2018). However, the role of the immune system in WNS resistance has not yet been investigated in big brown bats or other North American species except little brown bats.

As the range of *Pd* continues to extend further west, and spreads from a recently infected site in Washington state (Lorch et al. 2016), more bat species are likely to be negatively affected. Conservation strategies and disease mitigation plans are most successful when the interactions between the pathogen, the host, and the environment are

fully understood. RNA-sequencing of wing tissue from the greater mouse-eared bat (*Myotis myotis*), a European species that has not experienced WNS mortality, revealed that this species does not upregulate immune response genes when infected with *Pd* as little brown bats do (Davy et al. 2017). It is possible that the variation in disease severity among North American bat species is due to differences in immune response during hibernation. Studies of little brown and big brown bats support this prediction, but it is unclear whether or not the conclusions derived from these two species can be applied to other species such as northern-long eared bats or tricolored bats. Thus, studying the immune response of all affected species could aid in the generation of susceptible and resistant immune profiles.

Bats maintained in the laboratory for WNS studies often must be euthanized at the conclusion of the research. As populations of multiple species have experienced substantial declines, captive studies may further exacerbate the negative effects of WNS. Therefore, non-invasive—or at least non-lethal—study techniques should be developed and implemented so that WNS studies can continue without causing additional damage to populations. In this regard, a wealth of information can be gained from tissue samples. Obtaining a wing tissue biopsy or blood sample from bats in the field requires only a small amount of handling time, minimal stress, and the bat can be released upon completion. One 4-mm wing biopsy will be 90% healed within 27 to 41 days in euthermic wild big brown bats (Pollock et al. 2016). Transcriptomics (RNA-sequencing) of tissue samples can reliably supply immense amounts of information, and this method has been used in various wildlife immunology studies, including shrimp (Robalino et al. 2007), zebrafish (Stockhammer et al. 2009), and rainbow trout (Koskinen et al. 2004).

Transcriptomes have been compiled for vampire bats (Francischetti et al. 2013) and Jamaican fruit bats (Shaw et al. 2012), and the two transcriptomic studies of little brown bats and greater mouse-eared bats in response to *Pd* infection have expanded understanding of the disease by identifying several genes expressed in response to *Pd* infection (Field et al. 2015, Davy et al. 2017). Therefore, comparative RNA-sequencing could be utilized to explain the resistance of species such as big brown and gray bats, as well as the role of the immune response in disease severity.

The development of cell cultures from a single tissue sample can serve to supply an extensive amount of material for experimentation. An array of in-depth questions can be explored with cellular models, and this technique would greatly reduce the reliance of researchers on whole-animal studies (Crameri et al. 2009). Immortalized cell lines from 20 different organs have been established from Pteropodid bats, allowing in-depth exploration of the susceptibility of these bats to Hendra and Nipah viruses (Crameri et al. 2009). Cell lines have also been established from greater mouse-eared bats, African fruit bats, Serotine bats, and others to study susceptibility to different viruses (Biesold et al. 2011, Virtue et al. 2011, Mourya et al. 2013, He et al. 2014a, b, and Eckerle et al. 2014). No cell lines have been established to study susceptibility to *Pd* infection.

I have established wing tissue-derived fibroblast cell lines from four North American bat species to expand the field of WNS knowledge beyond that of little brown bats and to develop a method that could potentially reduce dependence on captive bat studies. Two WNS-susceptible and two WNS-resistant species were chosen for study:

1) The tricolored bat is distributed across eastern north and central North America (Fujita and Kunz 1984). This species weighs 4 – 8 grams and tends to hibernate singly (Fujita and Kunz 1984). It is generally reported that tricolored bats are the first to enter hibernation and the last to emerge in the spring (La Val and La Val 1980). Substantial

declines due to WNS have caused the United States Fish and Wildlife Service to start an investigation in 2017 whether or not this species should be listed as federally threatened or endangered.

2) The northern long-eared bat is distributed across the northeastern and central portions of the United States into central Canada (Caceres and Barclay 2000). This species weighs 5 – 8 grams and tends to hibernate in small numbers; however, numbers may be underestimated because these bats prefer to hibernate in deep cave crevices (Whitaker and Rissler 1992). Mortality rates due to WNS led to the listing of this species as federally threatened in April 2015 (USFWS 2015).

3) The big brown bat is widely distributed across most of North America, Central America, and as far south as northwestern South America (Kurta and Baker 1990). This species weighs 11 – 23 grams and either hibernates in small clusters or individually. They are usually observed in cooler microclimates within hibernacula (Kurta and Baker 1990).

4) The gray bat's distribution is restricted to cave regions in the central United States, and most are confined to nine caves during hibernation (Crozier and Mengak 2012). This species weighs 7 – 16 grams, uniquely inhabits caves year-round, and has very specific hibernaculum requirements. Gray bats are susceptible to human disturbance, and substantial declines led to its listing as an endangered species in 1976 (Crozier and Mengak 2012). Gray bats tend to migrate long distances between summer habitats and winter hibernacula, making them a potential vector of *Pd* transfer between caves. Both the big brown and gray bat appear to be resistant or tolerant to WNS.

The purposes of this study were to provide insight into the role of the immune response to *Pd* infection, explain interspecific differences in disease severity, and expand the field of WNS knowledge to species other than little brown and big brown bats. Established cell lines for each species were infected with *Pd*, and RNA-sequencing was conducted to establish the type and magnitude of their responses to infection. This study presents a new method of investigating bats that does not require captive studies or euthanasia. Knowledge from this study can be applied to form species-specific management or treatment plans, and the cell line method could be implemented for western bat species to predict their susceptibility to WNS before *Pd* arrives in their range.

Methods

Sample Collection. Wing tissue samples were obtained from bats captured by harp net at the Ozark Plateau National Wildlife Refuge in eastern Oklahoma (November 2016). Species sampled were the tricolored bat, gray bat, and northern long-eared bat. Gray bat and northern long-eared bat samples were also obtained by harp net capture at Sequiota Park in Springfield, MO (October 2017). Big brown bat samples were obtained during a hibernaculum survey at Unity Village in Lee's Summit, MO (January 2018). Tricolored bat samples were also collected from Mountain Home, AR (February 2018). Permission for these collections was obtained from the Missouri Department of Conservation, Arkansas Game and Fish Commission, and United States Fish and Wildlife Service.

Upon capture, one biopsy was collected from each wing using a 3 or 4 mm biopsy punch. Samples were collected using sterile forceps and transferred to a 1.5 mL microcentrifuge tube (pooled by species) containing 1.0 mL of Dulbecco's Modified Eagle Medium (DMEM). Samples were stored on ice for no more than 24 hours for transport to Missouri State University.

Fibroblasts. To collect wing-derived fibroblasts, wing biopsies were stripped to the cellular level by incubating at 37ºC for three hours with Collagenase type II. Fibroblasts were then manually isolated through a 40 µm cell strainer and plated with 50% FBS/50% DMEM. Antimicrobials were added to prevent growth of bacteria and fungus that may have been on the wings upon capture: ampicillin (100 µg/mL); penicillin and streptomycin (50 µg/mL); gentamycin (20 µg/mL); kanamycin (100 µg/mL); and

aureobasidin A (0.5 µg/mL). Cells were allowed to grow in the same medium at 37ºC in cell culture flasks until confluent.

To store fibroblasts for further experimentation, all growth media was removed, and the flask was incubated at 37ºC for 10 minutes with 2.5 mL 0.25% Trypsin and 0.1% EDTA in HBSS to detach adherent cells. Then, 10 mL of DMEM was added to the flask and thoroughly mixed. Cells and media were centrifuged for seven minutes at 400 x *g*. The supernatant was discarded, and freezing media (90% fetal bovine serum, 10% pure dimethyl sulfoxide) was mixed thoroughly with the cell pellet before being stored in 0.5-1 mL aliquots at -80ºC until needed.

Infection Experiments. Once fast-growing cell cultures had been established for each species, infection experiments were conducted to determine any immune responses of the fibroblast cells. Fibroblasts of each species were counted with a hemocytometer and diluted to equal concentrations (at least 500,000 cells/mL DMEM), and then transferred into four wells of a 6-well plate. Cells were given 24 hours to incubate at 37ºC within the plate to establish a monolayer. For the infection, two wells (duplicates) were uninfected, and two were infected with 20 µL of *Pd* hyphae (dissolved in PBS) and 20 µL of *Pd* spores (3.925×10^6 spores/mL). Infections were conducted at 37ºC for 12 hours. If not proceeding directly to RNA isolation after the infection period, the media was removed, replaced with Trizol, and plates were frozen at -80º C until isolation. (See Table 1)

RNA Isolation. Following infection experiments, total RNA was isolated and purified from the fibroblasts (Qiagen's miRNeasy Mini Kit). The protocol combines phenol/guanidine-based lysis of samples with silica-membrane-based purification.

QIAzol Lysis Reagent was used to homogenize cells via syringe and needle. Chloroform was then added to separate organic and aqueous phases via centrifugation. RNA was then transferred to a spin column, and ethanol was used to wash the RNA to remove contaminants and purify the RNA sample, which was ultimately dissolved in RNase-free water and stored at -80ºC.

Transcriptomics. Following RNA isolation, the quality and concentration of purified RNA was determined (Agilent Bioanalyzer 2100). Samples with a total RNA concentration above 50 ng/µL and RNA Integrity Number (RIN) above 6.8 were sequenced (Novogene). Library preparation procedures (250 – 300 base pair insert non-directional cDNA library) were performed by Novogene. RNA-seq was performed on an Illumina NovaSeq platform with 150 paired-end base pair reads obtained (20 million raw reads per sample). Four samples (two uninfected, two *Pd*-infected) were sequenced for each of the following species: gray bat, northern long-eared bat, tricolored bat, and big brown bat.

The processing and analyses of paired-end reads from all samples were performed on CLC Genomics Workbench (v11.0.1). Paired-end reads were combined and trimmed by quality scores (limit 0.05) and ambiguous nucleotides (maximum: 2). Fifteen 5' terminal nucleotides were trimmed from each read to remove adapters, and samples were filtered for reads between 15 and 1,000 base pairs. Using the RNA-seq Analysis tool, all trimmed reads were first mapped to the *Pd* genome and annotations (ENSEMBL genome 20631-21) to remove *Pd* sequences. All unmapped reads were then mapped to the little brown bat genome and annotations (ENSEMBL). All default Read Alignment settings were used for both mappings.

Using the Differential Expression (DE) for RNA-Seq tool, differential gene expression was tested for all 16 samples. This tool uses a multi-factorial analysis to generate a separate general linear model for each gene while assuming a negative binomial distribution, similar to EdgeR and DESeq. The number of differentially expressed genes between replicate pairs was calculated based on an FDR-corrected *p*-value of 0.001 to filter out DE due to natural variation between replicates. DE was tested due to treatment (infected or uninfected) while controlling for species, and comparisons were made between all group pairs. The analysis results were manually filtered to remove all genes that were not differentially expressed ± two-fold or higher, as well as all genes with an FDR-corrected *p*-value greater than 0.05. These genes were further assessed via gene group functional profiling, using a tool that performs statistical enrichment analysis to determine overrepresentation of Gene Ontology terms (g:GOSt function on g:profiler). The little brown bat was selected to provide the reference organism annotations.

Results

DE analysis was conducted for each pair of replicate samples to determine the extent of variability in gene expression between replicates. Significant differences were determined based on an FDR-corrected p-value < 0.001 and fold-change greater than ±2. For all replicate pairs, differentially expressed genes (of 19,728 mapped genes) were < 5% (Table 2). Because of the low number of replicates per group and the low variability between samples, the replicates for each sample were pooled for further DE analysis.

To determine the response of wing-tissue fibroblasts to *Pd* exposure, DE analysis was conducted between uninfected and infected samples of each species (Table 3). Of

those with a fold-change value greater than ±2 and an FDR-corrected *p*-value less than 0.05, two genes were significant between uninfected and infected gray bat samples. One of these genes is undefined, and the second gene, *SFTPC*, encodes an enzyme involved in surfactant production on cell membranes and was overexpressed (279-fold) by infected cells. There were no genes differentially expressed between big brown bat uninfected and infected samples based on the above parameters.

Uninfected and infected northern long-eared bat samples exhibited 14 genes with significant DE, all of which were overexpressed by infected samples. Of these, four are undefined, six have functions unrelated to immune or metabolic responses, and four genes have known metabolic functions or roles in wound repair: *ADH7* (alcohol dehydrogenase 7), *HMOX1* (heme oxygenase 1), *MMP3* (matrix metallopeptidase 3), and *DHRS7* (dehydrogenase reductase 7). For tricolored bat samples, 118 genes had significant DE between uninfected and infected samples, 31 of which are undefined. Twenty-two of the DE genes have known immune response or metabolic function (Table 3), including *SPP1* (secreted phosphoprotein 1), *MMP8* (matrix metallopeptidase 8), and *HAS2* (hyaluronan synthase 2).

DE analyses were also conducted for all species-pairs (uninfected and infected separately) to determine interspecific differences. The number of DE genes was similar for the uninfected and infected analyses of a species-pair, with the two *Myotis* species having the fewest differences (1,034 and 977 genes, respectively: Table 4). However, after characterizing these genes via statistical enrichment analysis (g:profiler), the biological functions of the DE genes appear to differ between uninfected and infected samples (compare examples in Figure 9). To visualize similarity between replicates as

well as interspecific differences in gene expression between samples, a heat map was generated which only includes genes with a fold-change greater than ±2 and an FDR-corrected *p*-value less than 0.05 (Figure 10). Hierarchical clustering of species indicates that northern-long eared bat and gray bat samples are genetically similar to each other compared to big brown bat and tricolored bat samples. Hierarchical clustering of genes indicates a distinction between the northern long-eared bat/gray bat cluster and the big brown bat/tricolored bat cluster.

Discussion

The main goals of this study were to investigate both the response of bat fibroblasts to *Pd* exposure and differences in this response between WNS-resistant and susceptible bat species. The data suggest that wing tissue fibroblasts from species resistant to WNS (big brown bat and gray bat) do not mount an immune response to *Pd* exposure. Big brown bat infected fibroblasts did not up- or down-regulate any genes compared to uninfected fibroblasts, while gray bat infected fibroblasts greatly overexpressed two genes compared to uninfected fibroblasts. However, these two genes do not represent an immune or metabolic response to infection.

In contrast, *Pd*-infected wing tissue fibroblasts from WNS-susceptible species (northern long-eared bat and tricolored bat) differentially expressed several genes compared to uninfected fibroblasts. These species overexpressed various metabolism and oxidative stress genes, suggesting energy expenditure in response to fungal infection. I hypothesized that WNS-susceptible species experience excess energy expenditure during hibernation due to elevated MR_T and increased arousal frequency. The up-regulation of

metabolism genes in bat fibroblasts from my two susceptible species supports this hypothesis but does not identify the cause or mechanism behind the elevated MR_T.

Another potential explanation for WNS disease severity relates to the involvement of an immune response to *Pd* infection. I hypothesized that WNS-susceptible species utilize more of their winter energy budget when infected due to an immune response to the fungus. Up-regulation of several immune and metabolism genes was observed in *Pd*-infected tricolored bat fibroblasts compared to uninfected. These genes code for proteins that function in wound healing, tissue repair, cytokine regulation, metabolism, adaptive immune response activation, inflammation, and responses to oxidative stress. These data suggest that an immune response to *Pd*-infection could be responsible for downstream energy expenditure during hibernation in tricolored bats with WNS. Transcriptome data from a previous study of infected and uninfected little brown bat wing tissue samples also support this hypothesis (Field et al. 2015), but the variety of immune and metabolism genes up-regulated in little brown bats was widely different from the genes observed in tricolored bats. However, the RNA sequenced in that dataset originated from wing tissue samples and not fibroblasts, which could explain the variation in gene expression.

An array of factors could influence the results observed in my dataset, and more RNA-seq data should be obtained before drawing firm conclusions. Samples from all four species were mapped to the little brown bat reference genome rather than creating a *de novo* assembly. An extensive portion of the little brown bat genome remains unclassified, so the functions of many differentially expressed genes observed in this dataset were unknown. Also, imperfect mapping to the little brown bat genome could affect the data. Around 71% of reads from northern long-eared bat and gray bat samples

mapped to the little brown bat genome, as they are congeners (*Myotis*). Samples from the big brown bat and tricolored bat, members of *Eptesicus* and *Perimyotis*, respectively, only exhibited a 63% match to the little brown bat genome. A more extensive set of DE genes may have been obtained with a *de novo* assembly for each species.

Additionally, the results of this study could have been influenced by the features of the infection experiments. The concentration of *Pd* spores and hyphae given to the infected samples may not have been high enough to elicit a realistic immune response. Also, the time allotted between inoculation and RNA-isolation (12 hours) may have been too short or too long. In the future, RNA-seq data should be compiled for infection experiments conducted with varying *Pd* concentrations and infection times. To differentiate a distinct immune response to *Pd* from a general immune response to infection, more experiments could be conducted with a variety of pathogens (LPS to mimic a bacterial infection, poly I:C to mimic a viral infection, and *Aspergillus* to include another fungal infection). Finally, the infection experiments for this study were all conducted at 37ºC, the optimal growth temperature for fibroblasts. This study should be repeated at varying temperatures, particularly those more conducive to bat hibernation and *Pd* growth.

This study was the first to establish viable wing tissue fibroblast cultures for North American insectivorous bat species and utilize them for research. Fibroblast cell lines from humans and mice are valuable sources of information in a variety of medical research fields. The cell lines that have been established for bats across the globe have been used to study the ability of these species to serve as reservoirs for a variety of pathogens, including Ebola and Hendra viruses (Crameri et al. 2009, He et al. 2014a,

etc.). A variety of research questions can be studied with cell lines, decreasing the necessity of taking animals from the wild and studying them in a laboratory setting. The majority of WNS studies require live bat experiments in the laboratory, which almost always require euthanasia of *Pd*-infected bats to prevent further spreading of the fungus. To establish cell lines such as those established in this study, less than five minutes of handling stress is required before the bat can be released, and the tissue biopsy wound is capable of healing without hindering the bat's daily activity. In the future, more cell lines should be established for North American insectivorous bats, particularly western species. After confirming that responses to *Pd* observed at the cellular level correspond to responses at the whole-bat level, cellular infection experiments can be utilized to predict the susceptibility of western species to WNS before the fungus arrives in those regions.

Considering the genetic differences between the four species in this study, it may not be feasible to assume that the data from *Pd*-infected little brown bats can pertain to other bat species. Excluding a few studies of the big brown bat, all laboratory investigations of WNS have been conducted on little brown bats. The results of this study suggest that WNS should be studied on a species-by-species basis, and conservation strategies should be created for individual species. The results of this study suggest variable responses to *Pd* infection at the wing tissue fibroblast level for four species of North American bats, with immunological and metabolic gene expression observed in susceptible species. Further studies with fibroblasts and comparisons to whole-bat research can verify that cellular investigations can replace certain laboratory studies that require euthanasia, promoting the conservation of valuable bat species in North America.

References

Biegańska, M. J. 2014. Two fundamentals of mammalian defense in fungal infections: endothermy and innate antifungal immunity. Polish Journal of Veterinary Sciences 17:555-567.

Biesold, S. E., et al. 2011. Type I interferon reaction to viral infection in interferon-competent, immortalized cell lines from the African fruit bat *Eidolon helvum*. PLoS ONE 6:1-11.

Blehert, D. S., et al. 2009. Bat white-nose syndrome: an emerging fungal pathogen? Science 323:227-227.

Bouma, H. R., H. V. Carey, and F. G. M. Kroese. 2010. Hibernation: the immune system at rest? Journal of Leukocyte Biology 88:619-624.

Bouma, H. R., et al. 2013. Reduction of body temperature governs neutrophil retention in hibernating and nonhibernating animals by margination. Journal of Leukocyte Biology 94:431-437.

Boyles, J. G., P. M. Cryan, G. F. McCracken, and T. H. Kunz. 2011. Economic importance of bats in agriculture. Science 332:41-42.

Caceres, M. C., and R. M. R. Barclay. 2000. *Myotis septentrionalis*. Mammalian Species 634:1-4.

Cheng, T. L., et al. 2016. Efficacy of a probiotic bacterium to treat bats affected by the disease white-nose syndrome. Journal of Applied Ecology 54:701-708.

Coutts, R. A., M. B. Fenton, and E. Glen. 1973. Food intake by captive *Myotis lucifugus* and *Eptesicus fuscus* (Chiroptera: Vespertilionidae). Journal of Mammalogy 54:985-990.

Crameri, G., et al. 2009. Establishment, immortalization, and characterization of pteropodid bat cell lines. PLoS ONE 4:1-9.

Crozier, S., and M. T. Mengak. 2012. Gray bat (*Myotis grisescens*). Warnell School of Forestry and Natural Resources Natural History Publication Series 12:1-8.

Culver, D. C., and T. Pipan. 2009. The biology of caves and other subterranean habitats. 2nd ed. Oxford University Press. Oxford, United Kingdom.

Davy, C. M., et al. 2017. The other white-nose syndrome transcriptome: tolerant and susceptible hosts respond differently to the pathogen *Pseudogymnoascus destructans*. Ecology and Evolution 7:7161-7170.

Eckerle, I., et al. 2014. Bat airway epithelial cells: a novel tool for the study of zoonotic viruses. PLoS ONE 9:1-9.

Field, K. A., J. S. Johnson, T. M. Lilley, S. M. Reeder, E. J. Rogers, M. J. Behr, and D. M. Reeder. 2015. The white-nose syndrome transcriptome: activation of anti-fungal host responses in wing tissue of hibernating little brown myotis. PLoS Pathogens 11:1-30.

Flock, B. 2014. Bat population monitoring and white-nose syndrome surveillance. Retrieved from http://www.tnbwg.org/Files/14-07%202014%20Bat%20Hibernacula%20Surveys%20and%20WNS%20Monitoring.pdf

Francischetti, I. M. B., T. C. F. Assumpção, D. Ma, Y. Li, E. C. Vicente, W. Uieda, and J. M. C. Ribeiro. 2013. The "vampirome": transcriptome and proteome analysis of the principal and accessory submaxillary glands of the vampire bat *Desmodus rotundus*, a vector of human rabies. Journal of Proteomics 82:288-319.

Frank, C. L., A. Michalski, A. A. McDonough, M. Rahimian, R. J. Rudd, and C. Herzog. 2014. The resistance of a North American bat species (*Eptesicus fuscus*) to white-nose syndrome (WNS). PLoS ONE 9:1-14.

Frank, C. L., M. R. Ingala, R. E. Ravenelle, K. Dougherty-Howard, S. O. Wicks, C. Herzog, and R. J. Rudd. 2016. The effects of cutaneous fatty acids on the growth of *Pseudogymnoascus destructans*, the etiological agent of white-nose syndrome. PLoS ONE 11:1-15.

Frick, W. F., et al. 2010. An emerging disease causes regional population collapse of a common North American bat species. Science 329:679-682.

Frick, W. F., et al. 2017. Pathogen dynamics during invasion and establishment of white-nose syndrome explain mechanisms of host persistence. Ecology 98:624-631.

Fujita, M. S., and T. H. Kunz. 1984. *Pipistrellus subflavus*. Mammalian Species 228:1-6.

Geiser, F. 2004. Metabolic rate and body temperature reduction during hibernation and daily torpor. Annual Review of Physiology 66:239-274.

He, X., T. Korytář, Y. Zhu, J. Pikula, H. Bandouchova, J. Zukal, and B. Köllner. 2014a. Establishment of *Myotis myotis* cell lines – model for investigation of host-pathogen interaction in a natural host for emerging viruses. PLoS ONE 9:1-10.

He, X., T. Korytář, J. Schatz, C. M. Freuling, T. Müller, and B. Köllner. 2014b. Anti-lyssaviral activity of interferons kappa and omega from the serotine bat, *Eptesicus sertotinus*. Journal of Virology 88:5444-5454.

Hoyt, J. R., T. L. Cheng, K. E. Langwig, M. M. Hee, W. F. Frick, and A. M. Kilpatrick. 2015. Bacteria isolated from bats inhibit the growth of *Pseudogymnoascus destructans*, the causative agent of white-nose syndrome. PLoS ONE 10:1-12.

Humphries, M. M., D.W. Thomas, and D. L. Kramer. 2003. The role of energy availability in mammalian hibernation: a cost-benefit approach. Physiological and Biochemical Zoology 76:165-179.

Ingala, M. R., R. E. Ravenelle, J. J. Monro, and C. L. Frank. 2017. The effects of epidermal fatty acid profiles, 1-oleoglycerol, and triacylglycerols on the susceptibility of hibernating bats to *Pseudogymnoascus destructans*. PLoS ONE 12:1-15.

Koskinen, H., et al. 2004. Response of rainbow trout transcriptome to model chemical contaminants. Biochemical and Biophysical Research Communications 320:745-753.

Kunz, T. H., E. Braun de Torrez, D. Bauer, T. Lobova, and T. H. Fleming. 2011. Ecosystem services provided by bats. Annals of the New York Academy of Sciences 1223:1-38.

Kurta, A., and R. H. Baker. 1990. *Eptesicus fuscus*. Mammalian Species 356:1-10.

Langwig, K. E., et al. 2015. Invasion dynamics of white-nose syndrome fungus, midwestern United States, 2012-2014. Emerging Infectious Diseases 21:1023-1026.

La Val, R. K., and M. L. La Val. 1980. Ecological studies and management of Missouri bats with emphasis on cave-dwelling species. Terrestrial Sere Missouri Department of Conservation 8:1-53.

Lilley, T. M., et al. 2017. Immune responses in hibernating little brown myotis (*Myotis lucifugus*) with white-nose syndrome. Proceedings of the Royal Society B 284:1-8.

Lorch, J. M., et al. 2016. First detection of bat white-nose syndrome in western North America. mSphere 1:e00148-16.

Mayberry, H. W., L. P. McGuire, and C. K. R. Willis. 2018. Body temperatures of hibernating little brown bats reveal pronounced behavioural activity during deep torpor and suggest a fever response during white-nose syndrome. Journal of Comparative Physiology B: Biochemical, Systems, and Environmental Physiology 188:333-343.

McGuire, L. P., H. W. Mayberry, and C. K. R. Willis. 2017. White-nose syndrome increases torpid metabolic rate and evaporative water loss in hibernating bats. American Journal of Physiology- Regulatory, Integrative and Comparative Physiology 313:680-686.

McNab, B. K. 1974. The behavior of temperate cave bats in a subtropical environment. Ecology 55:943-958.

Meteyer, C. U., et al. 2009. Histopathologic criteria to confirm white-nose syndrome in bats. Journal of Veterinary Diagnostic Investigation 21:411-414.

Meteyer, C. U., et al. 2011. Recovery of little brown bats (*Myotis lucifugus*) from natural infection with *Geomyces destructans*, white-nose syndrome. Journal of Wildlife Diseases 47:618-626.

Meteyer, C. U., D. Barber, and J. N. Mandl. 2012. Pathology in euthermic bats with white-nose syndrome suggests a natural manifestation of immune reconstitution inflammatory syndrome. Virulence 3:1-6.

Moore, M. S., J. D. Reichard, T. D. Murtha, B. Zahedi, R. M. Fallier, and T. H. Kunz. 2011. Specific alterations in complement protein activity of little brown myotis (*Myotis lucifugus*) hibernating in white-nose syndrome affected sites. PLoS ONE 6:1-11.

Moore, M. S., J. D. Reichard, T. D. Murtha, M. L. Nabhan, R. E. Pian, J. S. Ferreira, and T. H. Kunz. 2013. Hibernating little brown myotis (*Myotis lucifugus*) show variable immunological responses to white-nose syndrome. PLoS ONE 8:1-9.

Moore, M. S., et al. 2018. Energy conserving thermoregulatory patterns and lower disease severity in a bat resistant to the impacts of white-nose syndrome. Journal of Comparative Physiology B: Biochemical, Systems, and Environmental Physiology 188:163-176.

Mourya, D. T., R. J. Lakra, P. D. Yadav, P. Tyagi, C. G. Raut, A. M. Shete, and D. K. Singh. 2013. Establishment of cell line from embryonic tissue of *Pipistrellus ceylonicus* bat species from India and its susceptibility to different viruses. Indian Journal of Medical Research 138:224-231.

Pollock, T., C. R. Moreno, L. Sánchez, A. Ceballos-Vasquez, P. A. Faure, and E. C. Mora. 2016. Wound healing in the flight membranes of wild big brown bats. The Journal of Wildlife Management 80:19-26.

Prendergast, B. J., D. A. Freeman, I. Zucker, and R. J. Nelson. 2002. Periodic arousal from hibernation is necessary for initiation of immune responses in ground squirrels. American Journal of Physiology- Regulatory, Integrative, and Comparative Physiology 282:1054-1062.

Rapin, N., et al. 2014. Activation of innate immune-response genes in little brown bats (*Myotis lucifugus*) infected with the fungus *Pseudogymnoascus destructans*. PLoS ONE 9:1-7.

Reeder, D. M., et al. 2012. Frequent arousal from hibernation linked to severity of infection and mortality in bats with white-nose syndrome. PLoS ONE 7:1-10.

Reiskind, M. H., and M. A. Wund. 2009. Experimental assessment of the impacts of northern long-eared bats on ovipositing *Culex* (Diptera: Culicidae) mosquitoes. Journal of Medical Entomology 46:1037-1044.

Robalino, J., et al. 2007. Insights into the immune transcriptome of the shrimp *Litopenaeus vannamei*: tissue-specific expression profiles and transcriptomic responses to immune challenge. Physiological Genomics 29:44-56.

Robinson, M. D., and G. K. Symth. 2008. Small-sample estimation of negative binomial dispersion, with applications to sage data. Biostatistics 9:321-332.

Shaw, T. I., et al. 2012. Transcriptome sequencing and annotation for the Jamaican fruit bat (*Artibeus jamaicensis*). PLoS ONE 7:1-12.

Simmons, N. B., and T. M. Conway. 2003. Evolution of ecological diversity in bats. Pp. 493-579 in Bat ecology (T. H. Kunz and M. B. Fenton, eds.). University of Chicago Press. Chicago, Illinois.

Stockhammer, O. W., A. Zakrzewska, Z. Hegedus, H. P. Spaink, and A. H. Meijer. 2009. Transcriptome profiling and functional analyses of the zebrafish embryonic innate immune response to *Salmonella* infection. Journal of Immunology 182:5641-5653.

Supek, F., M. Bošnjak, N. Škunca, and T. Šmuc. 2011. REVIGO summarizes and visualizes long lists of gene ontology terms. PLoS ONE 6:1-9.

Thomas, D. W., M. Dorais, and J. Bergeron. 1990. Winter energy budgets and cost of arousals for hibernating little brown bats, *Myotis lucifugus*. Journal of Mammalogy 71:475-479.

United States Fish and Wildlife Service (USFWS). 2012. North American bat death toll exceeds 5.5 million from white-nose syndrome: [Arlington, Virginia, Office of Communications]
http://www.fws.gov/whitenosesyndrome/pdf/WNS_Mortality_2012_NR_ FINAL.pdf

United States Fish and Wildlife Service (USFWS). 2015. Endangered and threatened wildlife and plants; threatened status for the northern long-eared bat with 4(d) rule. Federal Register 80:17974-18033.

Verant, M. L., C. U. Meteyer, J. R. Speakman, P. M. Cryan, J. M. Lorch, and D. S. Blehert. 2014. White-nose syndrome indicates a cascade of physiologic disturbances in the hibernating bat host. BMC Physiology 14:1-10.

Virtue, E. R., G. A. Marsh, M. L. Baker, and L. F. Wang. 2011. Interferon production and signaling pathways are antagonized during henipavirus infection of fruit bat cell lines. PLoS ONE 6:1-8.

Warnecke, L., et al. 2013. Pathophysiology of white-nose syndrome in bats: a mechanistic model linking wing damage to mortality. Biology Letters 9:1-5.

Whitaker, Jr., J. O., and L. J. Rissler. 1992. Seasonal activity of bats at Copperhead Cave. Proceedings of the Indiana Academy of Science 101:127-135.

Table 1. Design of study with comparisons within a species (control vs. inoculated with *Pd*), and between species (resistant vs. susceptible). In all cases, RNA from wing fibroblast cells was isolated following treatment, sequenced, and analyzed for differential gene expression.

	Source	**Control**	**Inoculated**
Resistant			
Gray bats (*Myotis grisescens*)	Sequiota Park, Springfield, MO	Fibroblasts (5^5 cells/well of a 35 mm culture plate) incubated at 37°C with DMEM + FBS/PS (1 mL) for 12 h Replicates = 2	Fibroblasts (5^5 cells/well of a 35 mm culture plate) incubated at 37°C with DMEM + FBS/PS (1 mL) and infected with *Pd* spores (25 µL) and *Pd* hyphae (25 µL) for 12 h Replicates = 2
Big brown bats (*Eptesicus fuscus*)	Unity Village, Lee's Summit, MO		
Susceptible			
Long-eared bats (*Myotis septentrionalis*)	Ozark Plateau National Wildlife Refuge, Colcord, OK		
Tricolored bats (*Perimyotis subflavus*)	Mountain Home, AR		

Table 2. Comparisons of replicates for each sample. The number of differentially expressed (DE) genes with a fold-change greater than ±2 and an FDR-corrected *p*-value <0.001 was divided by the total number of mapped genes (19,728) to determine percentages.

Replicate Pair	Number of DE Genes	Percentage of Total Mapped Genes
BBB Infected	19	0.09
BBB Uninfected	308	1.56
GREY Infected	942	4.77
GREY Uninfected	751	3.81
NLEB Infected	893	4.53
NLEB Uninfected	979	4.96
TRI Infected	1	0.005
TRI Uninfected	1	0.005

Table 3. Differentially expressed genes of interest between infected and uninfected samples of each species. Big brown bat samples did not exhibit differentially expressed genes. For each gene, the FDR-corrected *p*-value, fold-change from uninfected to infected (positive values represent overexpressed genes in infected samples), and general function of the gene product are included.

Gene	FDR-corrected *p*-value	Fold-Change	Product Function
Gray			
SFTPC	0.00036	279.26	Manufactures surfactant
NLE			
ADH7	1.25 E^{-10}	7.58	Metabolism of a wide variety of substrates
HMOX1	4.41 E^{-8}	7.95	Heme catabolism
MMP3	0.000158	2.96	Wound repair, breakdown of extracellular matrix
DHRS7	0.000158	2.44	Metabolism of many different compounds
Tricolored			
HAS2	< 0.05 E^{-16}	7.45	Wound healing, tissue repair, homing of leukocytes
SPP1	< 0.05 E^{-16}	3.69	Cytokine; upregulates expression of IFNγ and IL-12
PLAUR	< 0.05 E^{-16}	2.86	Influences degradation of extracellular matrix
CYP27B1	< 0.05 E^{-16}	2.24	Monooxygenase; catalyzes many reaction
PNP	< 0.05 E^{-16}	2.24	Catalyzes phosphorolysis of purine nucleosides
FOSL1	< 0.05 E^{-16}	2.11	Dimerizes to form AP-1 transcription factor complex
ERRFI1	< 0.05 E^{-16}	2.08	Induced during stress; mediates cell signaling
HIVEP3	< 0.05 E^{-16}	2.02	Regulates NFκB-mediated transcription
PDK4	< 0.05 E^{-16}	-2.05	Regulates glucose metabolism
TNFRSF21	< 0.05 E^{-16}	-2.06	Activates NFκB; T-helper cell activation and inflammation roles in mice
TP53I3	7.01 E^{-13}	2.08	Similar to oxidoreductases
DUSP5	1.7 E^{-10}	2.29	Negative regulation of MAP kinase superfamily
GRB14	2.0 E^{-7}	-2.63	Signaling pathways that regulate growth and metabolism
ISLR	3.15 E^{-7}	-2.43	Immunoglobulin superfamily containing leucine rich repeat
ITGA6	3.46 E^{-5}	2.30	Integrin; cell surface adhesion and signaling
RFX3	0.000193	-2.51	Influences HLA class II expression
P2RY6	0.001106	-2.14	May mediate inflammatory responses
IGSF10	0.002513	-11.71	Immunoglobulin superfamily member 10
BMF	0.009726	-2.73	Apoptotic activator
GPX3	0.019194	-2.62	Protects cells from oxidative damage
MMP8	0.029922	-2.25	Breakdown of extracellular matrix
RNF125	0.03055	2.67	May be a positive regulator of T-cell receptor signaling

Table 4. Differentially expressed genes between samples of uninfected species-pairs (top right) and samples of infected species-pairs (bottom left). DE criteria are based on FDR-corrected *p*-values < 0.05 and fold-changes greater than ±2.

		Comparisons Between Uninfected Cells			
		NLE	Gray	Tricolored	Big Brown
Comparisons Between *Pd*-Infected Cells	NLE		1034	4681	3647
	Gray	977		4849	4239
	Tricolored	4321	4529		3717
	Big Brown	4163	4631	3546	

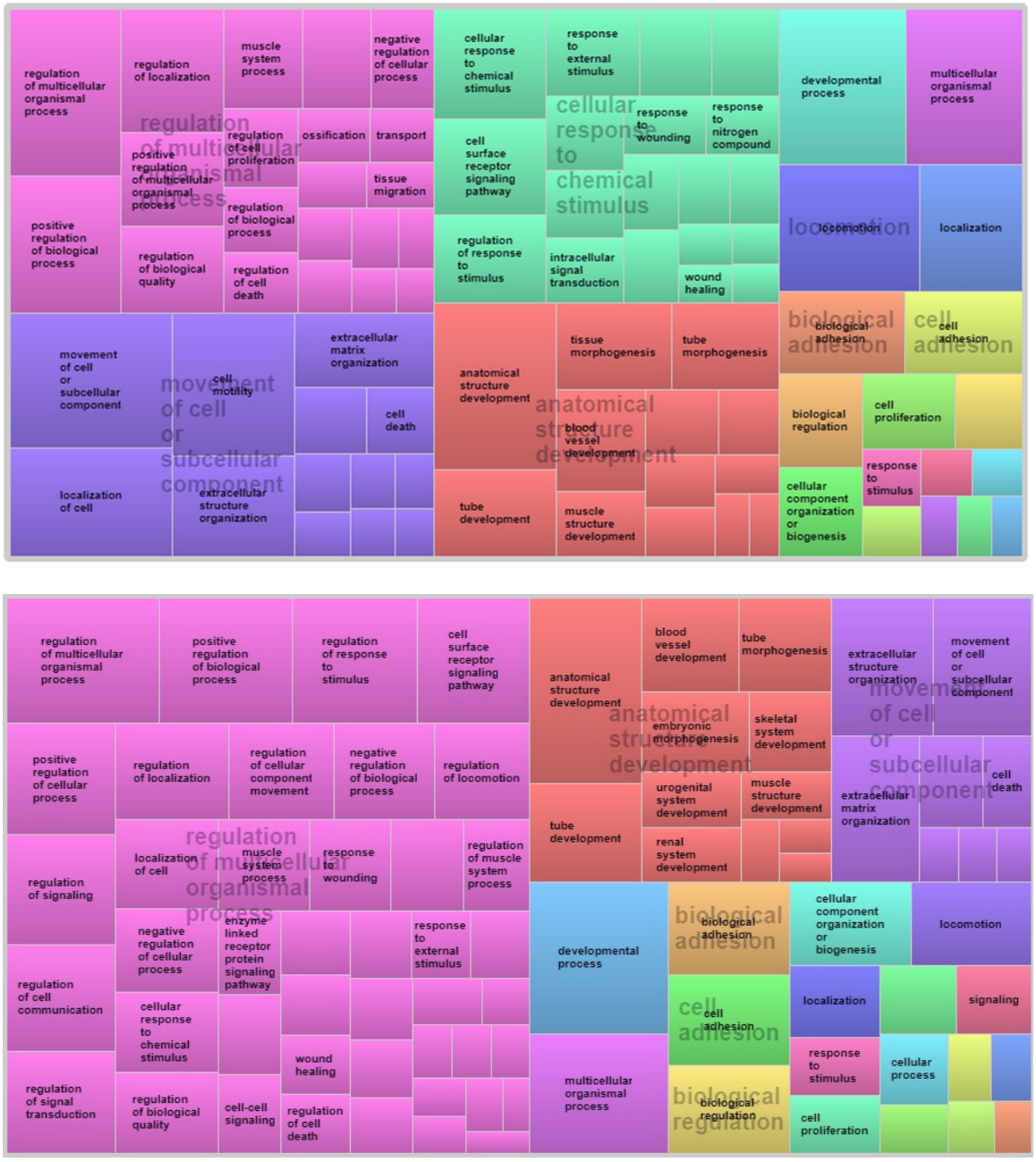

Figure 9. Tree maps of differentially expressed gene ontology terms between infected (top) and uninfected (bottom) samples of tricolored bat and big brown bat fibroblasts. Maps represent all genes with an FDR-corrected *p*-value < 0.05 and two-fold overexpression in tricolored bats. Maps were generated via REVIGO (Supek et al. 2011).

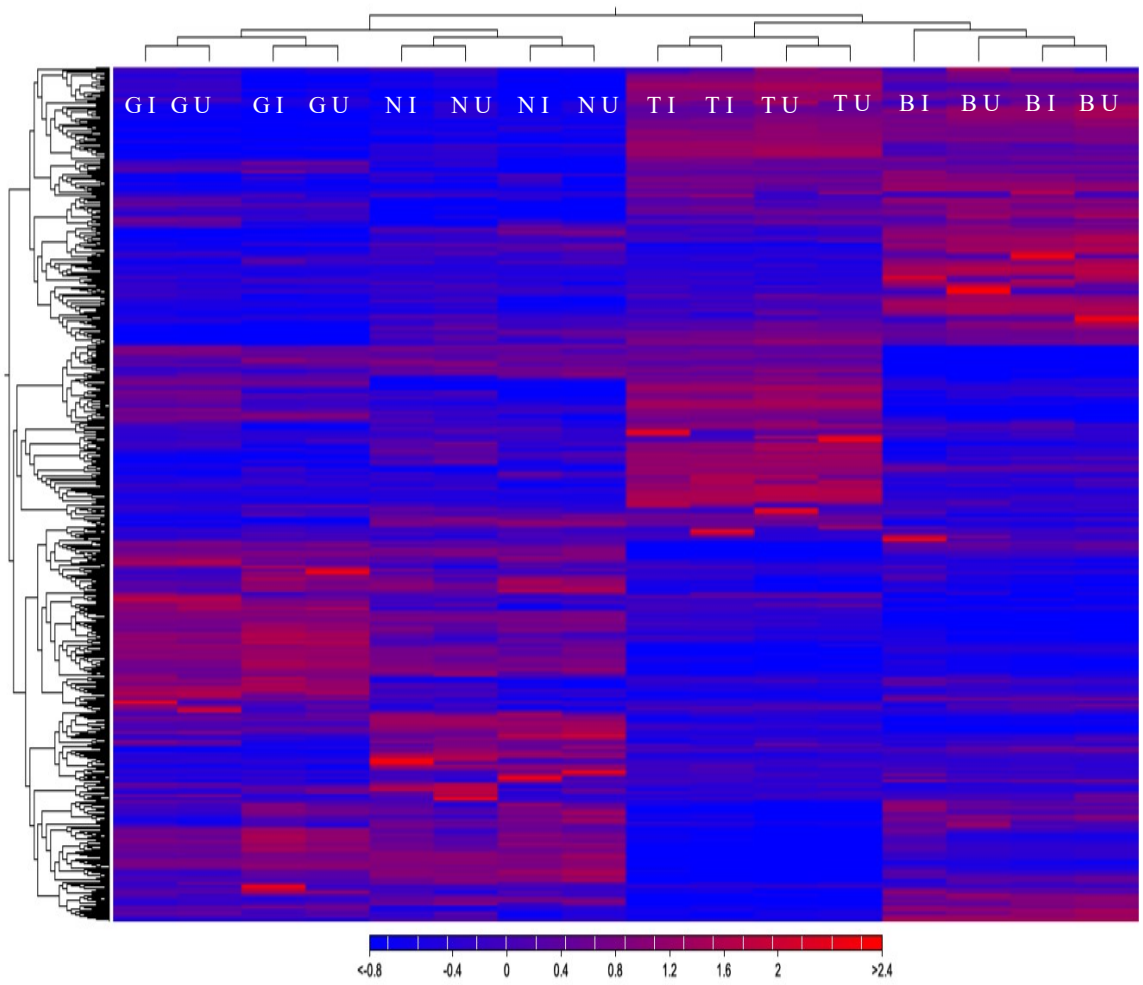

Figure 10. Heat map comparing gene expression between *Pd*-infected and uninfected fibroblast samples of four species including replicates. Columns indicate species (left to right: gray, northern long-eared, tricolored, and big brown), with both replicates for uninfected (U) and infected (I) samples. Columns (species) and rows (genes) were hierarchically clustered via complete linkage and calculations of Euclidean distance.

SUMMARY

The proposed WNS disease model for this study differs among bat species. For susceptible species such as the tricolored bat and northern long-eared bat, the model proposes an up-regulated immune response to *Pd* infection, resulting in elevated metabolic rates, which could cause the observed increase in arousal frequency. This excess energy expenditure during hibernation culminates with dehydration, emaciation, and potential mortality. The Energetics portion of this thesis attempted to determine if a susceptible species, the tricolored bat, experiences excess energy expenditure due to an immune response to *Pd*. By administering a dosage of meloxicam, an anti-inflammatory agent, to a subset of these bats, it was hypothesized that an up-regulated immune response would be prevented, allowing these bats to save energy during hibernation. Due to a lack of established WNS in either group, the data could not support the hypothesis.

Flow cytometry analysis of blood samples in early and mid-hibernation indicated that tricolored bats in both treatment groups (Energetics study) were in a late-stage response to infection, specifically referring to neutrophil:lymphocyte ratios. Also, the RNA-seq analysis of *Pd*-infected fibroblast samples suggests an overexpression of certain immunological and metabolism genes in infected fibroblasts of susceptible species. Previous mammalian hibernation studies have suggested that the immune system is muted or completely shut off during hibernation, but these results suggest that, in some species at least, torpid bats initiate an immune response to *Pd* infection.

The flow cytometry data from the Energetics study and the RNA-seq data from the Fibroblasts study indicate that interspecific variability in WNS disease severity could

be due to an immune response to *Pd*. Neither big brown bat nor gray bat fibroblasts exhibited much differential expression between infected and uninfected samples, suggesting a lack of response to *Pd*-infection in these WNS-resistant species.

Regarding the proposed WNS disease model, it was hypothesized that resistant species would not experience an up-regulated immune response to *Pd*, allowing them to conserve energy during the hibernation season. The results of RNA-seq analysis may support this model, but more research is necessary to confirm this. Another study of the effects of meloxicam on *Pd*-infected big brown bats has been conducted to provide a whole-bat comparison, but results have not yet been analyzed. Furthermore, the blood and wing tissue samples collected at various stages of hibernation from tricolored bats and the separate big brown bat study will undergo qPCR to determine expression levels of certain cytokines. Together, these studies could supply a broader understanding of interspecific differences in WNS severity, as well as the immune system's role in WNS.

The combination of bat research at the whole-animal and cellular level is essential for gaining a clearer understanding of WNS. After verifying that fibroblast studies can accurately predict the whole-bat response to infection, cellular methods can eventually replace some whole-bat laboratory studies that require euthanasia. In the future, fibroblast cell lines can be established for bat species across North America, and the susceptibility of western bat species to WNS can be predicted before the fungus arrives in those areas. Cellular and molecular techniques supply an extensive amount of data from a miniscule tissue biopsy, thus promoting the conservation of bat species while augmenting our knowledge of them. It is critical to adopt these methods as society becomes more aware of the value of North American bats, and as WNS continues to spread.

REFERENCES FOR OVERVIEW AND SUMMARY

Alem, M. A. S., and L. J. Douglas. 2004. Effects of aspirin and other nonsteroidal anti-inflammatory drugs on biofilms and planktonic cells of *Candida albicans*. Antimicrobial Agents and Chemotherapy 48:41-47.

Arnett, E. B., and E. F. Baerwald. 2013. Impacts of wind energy development on bats: implications for conservation. Pp. 435-456 in Bat evolution, ecology, and conservation (R. A. Adams and S. C. Pedersen, eds.) Springer-Verlag. New York, New York.

Baerwald, E. F., G. H. D'Amours, B. J. Klug, and R. M. R. Barclay. 2008. Barotrauma is a significant cause of bat fatalities at wind turbines. Current Biology 18:695-696.

Baker, M. L., T. Schountz, and L-F. Wang. 2012. Antiviral immune responses of bats: a review. Zoonoses and Public Health 60:104-116.

Bandouchova, H., et al. 2014. *Pseudogymnoascus destructans*: evidence of virulent skin invasion for bats under natural conditions, Europe. Transboundary and Emerging Diseases 62:1-5.

Barclay, R. M. R., M. C. Kalcounis, L. H. Crampton, C. Stefan, M. J. Vonhof, L. Wilkinson, and R. M. Brigham. 1996. Can external radiotransmitters be used to assess body temperature and torpor in bats? Journal of Mammalogy 77:1102-1106.

Barclay, R. M. R., and L. D. Harder. 2003. Life histories of bats: life in the slow lane. Pp. 209-253 in Bat ecology (T. H. Kunz and M. B. Fenton, eds.). University of Chicago Press. Chicago, Illinois.

Bernard, R. F., and G. F. McCracken. 2017. Winter behavior of bats and the progression of white-nose syndrome in the southeastern United States. Ecology and Evolution 7:1487-1496.

Biegańska, M. J. 2014. Two fundamentals of mammalian defense in fungal infections: endothermy and innate antifungal immunity. Polish Journal of Veterinary Sciences 17:555-567.

Biesold, S. E., et al. 2011. Type I interferon reaction to viral infection in interferon-competent, immortalized cell lines from the African fruit bat *Eidolon helvum*. PLoS ONE 6:1-11.

Blehert, D. S., et al. 2009. Bat white-nose syndrome: an emerging fungal pathogen? Science 323:227-227.

Bohn, S. J., et al. 2016. Evidence of 'sickness behaviour' in bats with white-nose syndrome. Behaviour 153:981-1003.

Boire, N., et al. 2016. Potent inhibition of *Pseudogymnoascus destructans*, the causative agent of white-nose syndrome in bats, by cold-pressed, terpeneless, Valencia orange oil. PLoS ONE 11:1-10.

Bouma, H. R., H. V. Carey, and F. G. M. Kroese. 2010. Hibernation: the immune system at rest? Journal of Leukocyte Biology 88:619-624.

Bouma, H. R., et al. 2013. Reduction of body temperature governs neutrophil retention in hibernating and nonhibernating animals by margination. Journal of Leukocyte Biology 94:431-437.

Boyles, J. G., M. B. Dunbar, J. J. Storm, and V. Brack, Jr. 2007. Energy availability influences microclimate selection of hibernating bats. Journal of Experimental Biology 210:4345-4350.

Boyles, J. G., P. M. Cryan, G. F. McCracken, and T. H. Kunz. 2011. Economic importance of bats in agriculture. Science 332:41-42.

Caceres, M. C., and R. M. R. Barclay. 2000. *Myotis septentrionalis*. Mammalian Species 634:1-4.

Cheng, T. L., et al. 2016. Efficacy of a probiotic bacterium to treat bats affected by the disease white-nose syndrome. Journal of Applied Ecology 54:701-708.

Cleveland, C. J., et al. 2006. Economic value of the pest control service provided by Brazilian free-tailed bats in south-central Texas. Frontiers in Ecology and the Environment 4:238-243.

Colatskie, S., and A. Elliott. 2016. Summary of the 2015/2016 Missouri bat hibernacula surveys and white-nose syndrome disease surveillance effort. Missouri Department of Conservation.

Coutts, R. A., M. B. Fenton, and E. Glen. 1973. Food intake by captive *Myotis lucifugus* and *Eptesicus fuscus* (Chiroptera: Vespertilionidae). Journal of Mammalogy 54:985-990.

Crameri, G., et al. 2009. Establishment, immortalization, and characterization of pteropodid bat cell lines. PLoS ONE 4:1-9.

Cryan, P. M., C. U. Meteyer, J. G. Boyles, and D. S. Blehert. 2010. Wing pathology of white-nose syndrome in bats suggests life-threatening disruption of physiology. BMC Biology 8:1-8.

Cryan, P. M., et al. 2013. Electrolyte depletion in white-nose syndrome in bats. Journal of Wildlife Diseases 49:398-402.

Cryan, P. M., C. U. Meteyer, J. G. Boyles, and D. S. Blehert. 2013a. White-nose syndrome in bats: illuminating the darkness. BMC Biology 11:1-4.

Culver, D.C., and T. Pipan. 2009. The biology of caves and other subterranean habitats. 2nd ed. Oxford University Press. Oxford, United Kingdom.

Czenze, Z. J., and C. K. R. Willis. 2015. Warming up and shipping out: arousal and emergence timing in hibernating little brown bats (*Myotis lucifugus*). Journal of Comparative Physiology B: Biochemical, Systems, and Environmental Physiology 185:575-586.

Day, K. M., and T. E. Tomasi. 2014. Winter energetics of female Indiana bats (*Myotis sodalis*). Physiological and Biochemical Zoology 87:56-64.

Davy, C. M., et al. 2017. The other white-nose syndrome transcriptome: tolerant and susceptible hosts respond differently to the pathogen *Pseudogymnoascus destructans*. Ecology and Evolution 7:7161-7170.

Dobony, C. A., R. E. Rainbolt, A. C. Hicks, R. L. von Linden, J. C. Okoniewski, and K. E. Langwig. 2011. Little brown myotis persist despite exposure to white-nose syndrome. Journal of Fish and Wildlife Management 2:190-195.

Dzurick, C., and T. Tomasi. 2008. Ambient temperature and the hibernation energy budget of the endangered Indiana bat (*Myotis sodalis*). Pp. 381-390 in Hypometabolism in animals: hibernation, torpor and cryobiology (B. G. Lovegrove and A. E. McKechnie, eds). University of KwaZulu-Natal, Pietermaritzburg, South Africa.

Eckerle, I., et al. 2014. Bat airway epithelial cells: a novel tool for the study of zoonotic viruses. PLoS ONE 9:1-9.

Field, K. A., J. S. Johnson, T. M. Lilley, S. M. Reeder, E. J. Rogers, M. J. Behr, and D. M. Reeder. 2015. The white-nose syndrome transcriptome: activation of anti-fungal host responses in wing tissue of hibernating little brown myotis. PLoS Pathogens 11:1-30.

Fitch, J. H. 1966. Weight loss and temperature response in three species of bats in Marshall County, Kansas. Search 6:17-24.

Flock, B. 2014. Bat population monitoring and white-nose syndrome surveillance. Retrieved from http://www.tnbwg.org/Files/14-07%202014%20Bat%20Hibernacula%20Surveys%20and%20WNS%20Monitoring.pdf

Forget, P., C. Khalifa, J. Defour, D. Latinne, M. Van Pel, and M. De Kock. 2017. What is the normal value of the neutrophil-to-lymphocyte ratio? BMC Research Notes 10:1-4.

Francischetti, I. M. B., T. C. F. Assumpção, D. Ma, Y. Li, E. C. Vicente, W. Uieda, and J. M. C. Ribeiro. 2013. The "vampirome": transcriptome and proteome analysis of the principal and accessory submaxillary glands of the vampire bat *Desmodus rotundus*, a vector of human rabies. Journal of Proteomics 82:288-319.

Frank, C. L., A. Michalski, A. A. McDonough, M. Rahimian, R. J. Rudd, and C. Herzog. 2014. The resistance of a North American bat species (*Eptesicus fuscus*) to white-nose syndrome (WNS). PLoS ONE 9:1-14.

Frank, C. L., M. R. Ingala, R. E. Ravenelle, K. Dougherty-Howard, S. O. Wicks, C. Herzog, and R. J. Rudd. 2016. The effects of cutaneous fatty acids on the growth of *Pseudogymnoascus destructans*, the etiological agent of white-nose syndrome. PLoS ONE 11:1-15.

Frick, W. F., et al. 2010. An emerging disease causes regional population collapse of a common North American bat species. Science 329:679-682.

Frick, W. F., et al. 2015. Disease alters macroecological patterns of North American bats. Global Ecology and Biogeography 24:741-749.

Frick, W. F., et al. 2017. Pathogen dynamics during invasion and establishment of white-nose syndrome explain mechanisms of host persistence. Ecology 98:624-631.

Fujita, M. S., and T. H. Kunz. 1984. *Pipistrellus subflavus*. Mammalian Species 228:1-6.

Gargas, A., M. T. Trest, M. Christensen, T. J. Volk, and D. S. Blehert. 2009. *Geomyces destructans* sp. nov. associated with bat white-nose syndrome. Mycotaxon 108:147-154.

Geiser, F. 2004. Metabolic rate and body temperature reduction during hibernation and daily torpor. Annual Review of Physiology 66:239-274.

Hamm, P. S., et al. 2017. Western bats as a reservoir of novel *Streptomyces* species with antifungal activity. Applied and Environmental Microbiology 83:1-10.

He, X., T. Korytář, Y. Zhu, J. Pikula, H. Bandouchova, J. Zukal, and B. Köllner. 2014a. Establishment of *Myotis myotis* cell lines – model for investigation of host-pathogen interaction in a natural host for emerging viruses. PLoS ONE 9:1-10.

He, X., T. Korytář, J. Schatz, C. M. Freuling, T. Müller, and B. Köllner. 2014b. Anti-lyssaviral activity of interferons kappa and omega from the serotine bat, *Eptesicus sertotinus*. Journal of Virology 88:5444-5454.

Hoyt, J. R., T. L. Cheng, K. E. Langwig, M. M. Hee, W. F. Frick, and A. M. Kilpatrick. 2015. Bacteria isolated from bats inhibit the growth of *Pseudogymnoascus destructans*, the causative agent of white-nose syndrome. PLoS ONE 10:1-12.

Hoyt, J. R., et al. 2016. Widespread bat white-nose syndrome fungus, northeastern China. Emerging Infectious Diseases 22:140-142.

Humphries, M. M., D.W. Thomas, and D. L. Kramer. 2003. The role of energy availability in mammalian hibernation: a cost-benefit approach. Physiological and Biochemical Zoology 76:165-179.

Ingala, M. R., R. E. Ravenelle, J. J. Monro, and C. L. Frank. 2017. The effects of epidermal fatty acid profiles, 1-oleoglycerol, and triacylglycerols on the susceptibility of hibernating bats to *Pseudogymnoascus destructans*. PLoS ONE 12:1-15.

Janicki, A. 2010. The effect of white-nose syndrome on torpid metabolic rates of *Myotis lucifugus*. M.S. thesis, Missouri State University. Springfield, Missouri.

Johnson, J. S., et al. 2014. Host, pathogen, and environmental characteristics predict white-nose syndrome mortality in captive little brown myotis (*Myotis lucifugus*). PLoS ONE 9:1-9.

Johnson, J. S., et al. 2015. Antibodies to *Pseudogymnoascus destructans* are not sufficient for protection against white-nose syndrome. Ecology and Evolution 5:2203-2214.

Jonasson, K. A., and C. K. R. Willis. 2011. Changes in body condition of hibernating bats support the thrifty female hypothesis and predict consequences for populations with white-nose syndrome. PLoS ONE 6:1-8.

Koskinen, H., et al. 2004. Response of rainbow trout transcriptome to model chemical contaminants. Biochemical and Biophysical Research Communications 320:745-753.

Kovacova, V., et al. 2018. White-nose syndrome detected in bats over an extensive area in Russia. BMC Veterinary Research 14:1-9.

Kunz, T. H., J. O. Whitaker, Jr., and M. D. Wadanoli. 1995. Dietary energetics of the insectivorous Mexican free-tailed bat (*Tadarida brasiliensis*) during pregnancy and lactation. Oecologia 101:407-415.

Kunz, T. H., E. Braun de Torrez, D. Bauer, T. Lobova, and T. H. Fleming. 2011. Ecosystem services provided by bats. Annals of the New York Academy of Sciences 1223:1-38.

Kurta, A., and R. H. Baker. 1990. *Eptesicus fuscus*. Mammalian Species 356:1-10.

Langwig, K. E., W. F. Frick, J. T. Bried, A. C. Hicks, T. H. Kunz, and A. M. Kilpatrick. 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.

Langwig, K. E., et al. 2015. Invasion dynamics of white-nose syndrome fungus, midwestern United States, 2012-2014. Emerging Infectious Diseases 21:1023-1026.

Langwig, K. E., et al. 2016. Drivers of variation in species impacts for a multi-host fungal disease of bats. Philosophical Transactions Royal Society B 371:1-9.

La Val, R. K., and M. L. La Val. 1980. Ecological studies and management of Missouri bats with emphasis on cave-dwelling species. Terrestrial Sere Missouri Department of Conservation 8:1-53.

Leopardi, S., D. Blake, and S. J. Puechmaille. 2015. White-nose syndrome fungus introduced from Europe to North America. Current Biology 25:217-219.

Lilley, T. M., et al. 2016. White-nose syndrome survivors do not exhibit frequent arousals associated with *Pseudogymnoascus destructans* infection. Frontiers in Zoology 13:1-8.

Lilley, T. M., et al. 2017. Immune responses in hibernating little brown myotis (*Myotis lucifugus*) with white-nose syndrome. Proceedings of the Royal Society B 284:1-8.

Lobova, T. A., C. K. Geiselman, and S. A. Mori. 2009. Seed dispersal by bats in the Neotropics. In memoirs of the New York Botanical Garden. New York Botanical Garden Press. Bronx, New York.

Lorch, J. M., et al. 2016. First detection of bat white-nose syndrome in western North America. mSphere 1:e00148-16.

Lovegrove, B. G. 2009. Modification and miniaturization of Thermochron iButtons for surgical implantation into small animals. Journal of Comparative Physiology B: Biochemical, Systems, and Environmental Physiology 179:451-458.

Lučan, R. K., H. Bandouchova, T. Bartonička, J. Pikula, A. Zahradníková, Jr., J. Zukal, and N. Martínková. 2016. Ectoparasites may serve as vectors for the white-nose syndrome fungus. Parasites and Vectors 9:1-5.

Mammals. The IUCN Red List of Threatened Species. Version 2017.3. www.iucnredlist.org. Accessed 2 July 2018.

Martínková, N., et al. 2010. Increasing incidence of *Geomyces destructans* fungus in bats from the Czech Republic and Slovakia. PLoS ONE 5:1-7.

Mayberry, H. W., L. P. McGuire, and C. K. R. Willis. 2018. Body temperatures of hibernating little brown bats reveal pronounced behavioural activity during deep torpor

and suggest a fever response during white-nose syndrome. Journal of Comparative Physiology B: Biochemical, Systems, and Environmental Physiology 188:333-343.

McCracken, G. F., et al. 2018. Rapid range expansion of the Brazilian free-tailed bat in the southeastern United States, 2008-2016. Journal of Mammalogy xx:1-9.

McGuire, L. P., H. W. Mayberry, and C. K. R. Willis. 2017. White-nose syndrome increases torpid metabolic rate and evaporative water loss in hibernating bats. American Journal of Physiology- Regulatory, Integrative and Comparative Physiology 313:680-686.

McNab, B. K. 1974. The behavior of temperate cave bats in a subtropical environment. Ecology 55:943-958.

Meteyer, C. U., et al. 2009. Histopathologic criteria to confirm white-nose syndrome in bats. Journal of Veterinary Diagnostic Investigation 21:411-414.

Meteyer, C. U., et al. 2011. Recovery of little brown bats (*Myotis lucifugus*) from natural infection with *Geomyces destructans*, white-nose syndrome. Journal of Wildlife Diseases 47:618-626.

Meteyer, C. U., D. Barber, and J. N. Mandl. 2012. Pathology in euthermic bats with white-nose syndrome suggests a natural manifestation of immune reconstitution inflammatory syndrome. Virulence 3:1-6.

Micalizzi, E. W., J. N. Mack, G. P. White, T. J. Avis, and M. L. Smith. 2017. Microbial inhibitors of the fungus *Pseudogymnoascus destructans*, the causal agent of white-nose syndrome in bats. PLoS ONE 12:1-15.

Mickleburgh, S. P., A. M. Hutson, and P. A. Racey. 2002. A review of the global conservation status of bats. Oryx 36:18-34.

Minnis, A. M., and D. L. Lindner. 2013. Phylogenetic evaluation of *Geomyces* and allies reveals no close relatives of *Pseudogymnoascus destructans*, comb. nov., in bat hibernacula of eastern North America. Fungal Biology 117:638-649.

Moore, M. S., J. D. Reichard, T. D. Murtha, B. Zahedi, R. M. Fallier, and T. H. Kunz. 2011. Specific alterations in complement protein activity of little brown myotis (*Myotis lucifugus*) hibernating in white-nose syndrome affected sites. PLoS ONE 6:1-11.

Moore, M. S., J. D. Reichard, T. D. Murtha, M. L. Nabhan, R. E. Pian, J. S. Ferreira, and T. H. Kunz. 2013. Hibernating little brown myotis (*Myotis lucifugus*) show variable immunological responses to white-nose syndrome. PLoS ONE 8:1-9.

Moore, M. S., et al. 2018. Energy conserving thermoregulatory patterns and lower disease severity in a bat resistant to the impacts of white-nose syndrome. Journal of

Comparative Physiology B: Biochemical, Systems, and Environmental Physiology 188:163-176.

Mourya, D. T., R. J. Lakra, P. D. Yadav, P. Tyagi, C. G. Raut, A. M. Shete, and D. K. Singh. 2013. Establishment of cell line from embryonic tissue of *Pipistrellus ceylonicus* bat species from India and its susceptibility to different viruses. Indian Journal of Medical Research 138:224-231.

Muscarella, R., and T. H. Fleming. 2007. The role of frugivorous bats in tropical forest succession. Biology Reviews 82:573-590.

O'Shea, T. J., P. M. Cryan, D. T. S. Hayman, R. K. Plowright, and D. G. Streicker. 2016. Multiple mortality events in bats: a global review. Mammal Review 46:175-190.

Otálora-Ardila, A., L. G. Herrera M., J. J. Flores-Martínez, and K. C. Welch, Jr. 2016. Metabolic cost of the activation of immune response in the fish-eating myotis (*Myotis vivesi*): the effects of inflammation and the acute phase response. PLoS ONE 11:1-14.

Padhi, S., I. Dias, and J. W. Bennett. 2017. Two volatile-phase alcohols inhibit growth of *Pseudogymnoascus destructans*, causative agent of white-nose syndrome in bats. Mycology 8:11-16.

Palmer, J. M., K. P. Drees, J. T. Foster, and D. L. Lindner. 2018. Extreme sensitivity to ultraviolet light in the fungal pathogen causing white-nose syndrome of bats. Nature Communications 9:1-10.

Pimentel, D., et al. 1991. Environmental and economic effects of reducing pesticide use. BioScience 41:402-409.

Pollock, T., C. R. Moreno, L. Sánchez, A. Ceballos-Vasquez, P. A. Faure, and E. C. Mora. 2016. Wound healing in the flight membranes of wild big brown bats. The Journal of Wildlife Management 80:19-26.

Prendergast, B. J., D. A. Freeman, I. Zucker, and R. J. Nelson. 2002. Periodic arousal from hibernation is necessary for initiation of immune responses in ground squirrels. American Journal of Physiology- Regulatory, Integrative, and Comparative Physiology 282:1054-1062.

Rapin, N., et al. 2014. Activation of innate immune-response genes in little brown bats (*Myotis lucifugus*) infected with the fungus *Pseudogymnoascus destructans*. PLoS ONE 9:1-7.

Reeder, D. M., et al. 2012. Frequent arousal from hibernation linked to severity of infection and mortality in bats with white-nose syndrome. PLoS ONE 7:1-10.

Reiskind, M. H., and M. A. Wund. 2009. Experimental assessment of the impacts of northern long-eared bats on ovipositing *Culex* (Diptera: Culicidae) mosquitoes. Journal of Medical Entomology 46:1037-1044.

Reynolds, H. T., and H. A. Barton. 2014. Comparison of the white-nose syndrome agent *Pseudogymnoascus destructans* to cave-dwelling relatives suggests reduced saprotrophic enzyme activity. PLoS ONE 9:1-11.

Reynolds, R. J., K. E. Powers, W. Orndorff, W. M. Ford, and C. S. Hobson. 2016. Changes in rates of capture and demographics of *Myotis septentrionalis* (northern long-eared bat) in western Virginia before and after onset of white-nose syndrome. Northeastern Naturalist 23:195-204.

Robalino, J., et al. 2007. Insights into the immune transcriptome of the shrimp *Litopenaeus vannamei*: tissue-specific expression profiles and transcriptomic responses to immune challenge. Physiological Genomics 29:44-56.

Sahdo, B., A. L. Evans, J. M. Arnemo, O. Fröbert, E. Särndahl, and S. Blanc. 2013. Body temperature during hibernation is highly correlated with a decrease in circulating innate immune cells in the brown bear (*Ursus actos*): a common feature among hibernators? International Journal of Medical Sciences 10:508-514.

Schneeberger, K., G. A. Czirják, and C. C. Voigt. 2013. Inflammatory challenge increases measures of oxidative stress in a free-ranging, long-lived mammal. Journal of Experimental Biology 216:4514-4519.

Scholthof, K. G. 2007. The disease triangle: pathogens, the environment and society. Nature Reviews Microbiology 5:152.

Secord, A. L., K. A. Patnode, C. Carter, E. Redman, D. J. Gefell, A. R. Major, and D. W. Sparks. 2015. Contaminants of emerging concern in bats from the northeastern United States. Archives of Environmental Contamination and Toxicology 69:411-421.

Shaw, T. I., et al. 2012. Transcriptome sequencing and annotation for the Jamaican fruit bat (*Artibeus jamaicensis*). PLoS ONE 7:1-12.

Simmons, N. B., and T. M. Conway. 2003. Evolution of ecological diversity in bats. Pp. 493-579 in Bat ecology (T. H. Kunz and M. B. Fenton, eds.). University of Chicago Press. Chicago, Illinois.

Speakman, J. R., and D. W. Thomas. 2003. Physiological ecology and energetics of bats. Pp. 430-490 in Bat ecology (T. H. Kunz and M. B. Fenton, eds.). University of Chicago Press. Chicago, Illinois.

Stockhammer, O. W., A. Zakrzewska, Z. Hegedus, H. P. Spaink, and A. H. Meijer. 2009. Transcriptome profiling and functional analyses of the zebrafish embryonic innate immune response to *Salmonella* infection. Journal of Immunology 182:5641-5653.

Supek, F., M. Bošnjak, N. Škunca, and T. Šmuc. 2011. REVIGO summarizes and visualizes long lists of gene ontology terms. PLoS ONE 6:1-9.

Thomas, D. W., M. Dorais, and J. Bergeron. 1990. Winter energy budgets and cost of arousals for hibernating little brown bats, *Myotis lucifugus*. Journal of Mammalogy 71:475-479.

Thomas, D. W., and F. Geiser. 1997. Periodic arousals in hibernating mammals: is evaporative water loss involved? Functional Ecology 11:585-591.

Turner, J. M., L. Warnecke, A. Wilcox, D. Baloun, T. K. Bollinger, V. Misra, and C. K. R. Willis. 2015. Conspecific disturbance contributes to altered hibernation patterns in bats with white-nose syndrome. Physiology and Behavior 140:71-78.

United States Fish and Wildlife Service (USFWS). 2012. North American bat death toll exceeds 5.5 million from white-nose syndrome: [Arlington, Virginia, Office of Communications]
http://www.fws.gov/whitenosesyndrome/pdf/WNS_Mortality_2012_NR_ FINAL.pdf

United States Fish and Wildlife Service (USFWS) 2015. Endangered and threatened wildlife and plants; threatened status for the northern long-eared bat with 4(d) rule. Federal Register 80:17974-18033.

United States Fish and Wildlife Service (USFWS). 2017. Evaluation of a petition to list the tricolored bat as an endangered or threatened species under the act. Federal Register 82:60364.

United States Geological Survey. 2018. White-nose syndrome occurrence map – by year (2018). Data Last Updated: 6/1/2018. Available at:
https://www.whitenosesyndrome.org/resources/map.

Verant, M. L., J. G. Boyles, W. Waldrep, Jr., G. Wibbelt, and D. S. Blehert. 2012. Temperature-dependent growth of *Geomyces destructans*, the fungus that causes bat white-nose syndrome. PLoS ONE 7:1-7.

Verant, M. L., C. U. Meteyer, J. R. Speakman, P. M. Cryan, J. M. Lorch, and D. S. Blehert. 2014. White-nose syndrome indicates a cascade of physiologic disturbances in the hibernating bat host. BMC Physiology 14:1-10.

Virtue, E. R., G. A. Marsh, M. L. Baker, and L. F. Wang. 2011. Interferon production and signaling pathways are antagonized during henipavirus infection of fruit bat cell lines. PLoS ONE 6:1-8.

Warburton, E. M., C. A. Pearl, and M. J. Vonhof. 2016. Relationships between host body condition and immunocompetence, not host sex, best predict parasite burden in a bat-helminth system. Parasitology Research 115:2155-2164.

Warnecke, L., et al. 2013. Pathophysiology of white-nose syndrome in bats: a mechanistic model linking wing damage to mortality. Biology Letters 9:1-5.

Welbergen, J. A., S. M. Klose, N. Markus, and P. Eby. 2008. Climate change and the effects of temperature extremes on Australian flying-foxes. Proceedings of the Royal Society of London B: Biological Sciences 275:419-425.

Welch, J. N., and C. Leppanen. 2017. The threat of invasive species to bats: a review. Mammal Review 47:277-290.

Whitaker, Jr., J. O. 1995. Food of the big brown bat *Eptesicus fuscus* from maternity colonies in Indiana and Illinois. American Midland Naturalist 134:346-360.

Whitaker, Jr., J. O., and L. J. Rissler. 1992. Seasonal activity of bats at Copperhead Cave. Proceedings of the Indiana Academy of Science 101:127-135.

Wibbelt, G., et al. 2013. Skin lesions in European hibernating bats associated with *Geomyces destructans*, the etiologic agent of white-nose syndrome. PLoS ONE 8:1-10.

Wilcox, A., et al. 2014. Behaviour of hibernating little brown bats experimentally inoculated with the pathogen that causes white-nose syndrome. Animal Behavior 88:157-164.

Wilson, M. B., B. W. Held, A. H. Freiborg, R. A. Blanchette, and C. E. Salomon. 2017. Resource capture and competitive ability of non-pathogenic *Pseudogymnoascus* spp. and *P. destructans*, the cause of white-nose syndrome in bats. PLoS ONE 12:1-18.

Zhang, T., et al. 2014. Mycobiome of the bat white nose syndrome affected caves and mines reveals diversity of local fungi and adaptation by the fungal pathogen *Pseudogymnoascus* (*Geomyces*) *destructans*. PLoS ONE 9:1-13.

Zhang, T., V. Chaturvedi, and S. Chaturvedi. 2015. Novel *Trichoderma polysporum* strain for the biocontrol of *Pseudogymnoascus destructans*, the fungal etiologic agent of bat white-nose syndrome. PLoS ONE 10:1-18.