Summer 2019

White-Nose Syndrome and Immune Responses in a Resistant Bat Species (Eptesicus fuscus)

Keslie Skye Naffa
Missouri State University, Keslie1994@live.missouristate.edu

WHITE-NOSE SYNDROME AND IMMUNE RESPONSES IN A RESISTANT BAT SPECIES (*EPTESICUS FUSCUS*)

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Keslie Naffa

August 2019

WHITE-NOSE SYNDROME AND IMMUNE RESPONSES IN A RESISTANT BAT SPECIES (*EPTESICUS FUSCUS*)

Biology

Missouri State University, August 2019

Master of Science

Keslie Naffa

ABSTRACT

White-nose syndrome (WNS) has had a large negative impact on bat populations across eastern North America since its arrival in 2006. Bats affected by WNS appear to die of starvation, possibly due to the increased arousals during hibernation when there is no food present to replace the energy used to arouse. During hibernation, the bat's immune system should be suppressed. However, once a bat of a susceptible species is exposed to the fungus that causes WNS, *Psuedogymnoascus destructans* (*Pd*), the immune system seems to respond, potentially causing an elevation in metabolic rate, which may cause the bat to arouse more often. I hypothesize that resistant bats do not mount an immune response; however, if they do mount an immune response, I hypothesize that bats inoculated with *Pd* and treated with an anti-inflammatory treatment will not respond to the infection, ultimately preserving fat reserves and lowering metabolic expenditures during hibernation. I tested these hypotheses by inoculating a species that does not suffer from high WNS mortality (big brown bats: *Eptesicus fuscus*), with *Pd*. Meloxicam was given to one of the three treatment groups in order to suppress the immune system. Metabolic rate during torpor, via oxygen consumption, was measured in addition to arousal/torpor bout patterns, the latter utilizing temperature-sensitive dataloggers. To quantify expression of four immune-function genes (NLRP10, CD200, ICAM5, and TNFRSF21), gene activity was measured via RT-qPCR on tissue and blood samples taken from each bat pre- and post- hibernation. These genes were chosen based on a prior study that showed differences in these genes between susceptible and resistant species. There were no significant differences found across treatment groups for gene expression, nor energetic data; however, hibernation did suppress NLRP10 expression, and blood samples consistently had higher gene expression than tissue samples, thus indicating these genes may be expressed at low levels in some tissues. It is imperative that we continue investigating the differences between susceptible and resistant bat species as WNS is advancing westward throughout the country.

KEYWORDS: immune system, hibernation, torpor, WNS, white-nose syndrome, inflammation, *Eptesicus fuscus*, *Pseudogymnoascus destructans*, *Pd*

WHITE-NOSE SYNDROME AND IMMUNE RESPONSES IN A RESISTANT BAT SPECIES (*EPTESICUS FUSCUS*)

By

Keslie Naffa

A Master's 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 2019

Approved:

Tom Tomasi, Ph.D., Thesis Committee Chair

Christopher Lupfer, Ph.D., Committee Member

Day Ligon, Ph.D., Committee Member

Julie Masterson, Ph.D., Dean of the Graduate College

ACKNOWLEDGEMENTS

I would like to thank the following people for their support during the duration of my graduate studies. For undying encouragement and support, I thank my committee members: Drs. Tom Tomasi, Christopher Lupfer and Day Ligon. For research funding, I would like to thank Missouri State University Graduate College and Department of Biology. For the aid in bat collection and processing, I thank the Missouri Department of Conservation. For research assistance, I thank Tricia Blankenship, Susan Anderson, Meagan Rippee-Brooks, Abbigale Brown, and Christopher Bogart. For endless motivation, support, and much needed laughs, I thank Savanna and Lilly, Danielle, Ciera, Meagan, Mora, and Winston. I thank my grandmother, Bonnie, my grandfather, Mansour, and my step-father, Steve, for providing me with the motivation I needed during the trying times. For unconditional love and continuous inspiration, I thank my extraordinary mother, Dena- “to the moon and back, always.”

I dedicate this thesis to my best friend and better half, Jacob Inman, for providing unconditional encouragement, love, and support, even during the most undeserving of times. I am forever grateful for you and your devotion to our goals.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The Order Chiroptera represents more than 1,200 known species of bats that are distributed across the globe and demonstrate a wide variety of foraging techniques, diet specializations, reproductive behaviors, and habitat diversity (Jones et al. 2005; Kunz et al. 2011; Wilson 2019). They account for approximately 20% of all living mammalian species (Hill 1984) and include 19 different families. *Vespertilionidae*, the largest bat family in the world, includes over 400 insectivorous bat species. Vespertilionids are well established on all continents except Antarctica and hold great physiological diversity (Birkett et al. 2014). The International Union for Conservation of Nature (IUCN) reports 7 vespertilionid species critically endangered, 16 endangered, and 26 vulnerable (IUCN Red List 2019). With the continuous addition of species to the threatened list, it is imperative to continue research and educational outreach regarding bats and their value, in order to create management plans to aid in the conservation of these animals.

Bat Value

The natural resources and ecological services that bats provide to our society are extremely important. Bats are one of the world's leading groups of pollinators, and they are considered one of the most efficient forms of natural insect control (Kunz et al. 2011). A colony of 150 insectivorous bats can consume up to 1.3 million insects per season (Whitaker 1995), which saves agriculturalists time and money by not having to treat their crops with environmentally un-safe pesticides. Mexican free-tailed bats (*Tadarida brasiliensis*) are a critically important source of natural pest control. A maternity colony of one million bats can consume an estimated 8.4 metric tons of insects per night, which is equivalent to one individual

consuming half of its body weight per night (Kunz and Fenton 2003). It is estimated in the United States that insectivorous bats have an economic value of approximately $22.9 billion annually in by reducing the need for chemical insect control alone (Boyles et al. 2011). This estimation does not include the consumption of forest pests, which aid harvesters of lumber, nor does it include the human health aspects of avoiding the ingestion of potentially harmful pesticides or a decrease in insect-borne diseases. Therefore, the estimate of the economic importance of bats is clearly an underestimate.

In Mexico, *Agave tequilana*, the plant from which we derive tequila, was known to have a very small genetic diversity in cultivated fields, leaving them vulnerable to disease that may lead to widespread crop failure. With the help of bat pollination, and bat-friendly agricultural practices, genetic diversity has been increased (Trejo-Salazar et al. 2016). At one *Agave* farm, progressive *Agave* farmers are allowing 5% of their crops to flower, which feeds an estimated 89 individual bats per night (Trejo-Salazer et al. 2016). In addition to providing bats food, pollination as a result of bat nectar consumption has increased genetic diversity via pollination of *Agave* flowers throughout many *Agave* croplands across Mexico (Trejo-Salazer et al. 2016). An increase in genetic diversity is not only beneficial to the fitness of the species, but also acts as a form of job security for many *Agave* farmers in Mexico's rural areas, since their entire crop is less likely to be killed by an emerging disease or climate change issues. If this practice was implemented across Mexico, it could provide food for over two million bats per month across the country during the flowering season, in addition to aiding in economic growth (Trejo-Salazer et al. 2016).

Conservation Implications

Fungal diseases have had a devastating impact on multiple taxa, both plant and animal, across the globe. The increase in fungal pathogens are due to both environmental and non-environmental factors. Global warming is an environmental factor that is allowing fungi to flourish in areas that were not well-suited habitats several years ago. Because the majority of fungi are typically found in moist, temperate environments, the warming of the earth and the change in habitats are allowing fungal pathogens to inhabit new areas, which means they could be inhabiting new hosts (Konkel 2017). These shifts in temperature could drive the evolution of more heat-tolerant fungal pathogens (Konkel 2017). In addition, drug resistance has also been a factor in increasing fungal pathogens. A large amount of agricultural fungicides are no longer providing protection against fungal pathogens due to increased resistance (Konkel 2017). These fungal pathogens have been devastating to several populations and are increasingly becoming more virulent (Konkel 2017).

A fungi, known as rice blast (*Magnaporthe oryzae*), can cause losses of up to 30% of a grain field in a given season (Talbot 2003), which can inhibit agricultural yield and disrupt the ecological balance of the area. Among animal taxa, frog (McCallum 2005), bee (Fisher et al. 2012), and bat (Blehert et al. 2009) populations have all been declining due to invasive fungal diseases that affect the animals' environment and immune systems. Chytrid fungus in amphibians has decimated populations globally. This fungus infects keratinized cells of the epidermis, but the mechanism of how this fungus becomes fatal is unknown (Ouellet et al. 2005). Chalkbrood is a fungal disease in bee populations that affects the developing broods (Flores et al. 2005). It causes mummification of the infected individual, causing population declines and decreased honey production. In social animals such as bees, individual and social immune

responses may be produced to create a warning to other colony members (Armitage et al. 2011). Physiological, organizational, and behavioral defenses allow colonies to form social immunity by preventing attack and dispersal of pathogenic agents (Richter et al. 2012). Bats also face a high risk of contracting a fungal disease known as white-nose syndrome (WNS).

Bat populations of some species in North America have been declining rapidly for several decades. Habitat destruction (Rabinowitz and Tuttle 1980; Medellin 2003), wind energy farms (Arnett et al. 2008), and WNS (Blehert et al. 2009) are the primary causes for this decline. Bats typically select specialized habitats for foraging and roosting, such as caves and mines (Brooks and Ford 2006); however, due to cave and mine destruction for agriculture and development, populations that previously inhabited these areas are displaced. They then select areas to roost where they are seen as pests and/or are being forced to roost in areas of increased vulnerability to predators (Mering and Chambers 2014). However, scientists and civilians are now creating artificial roosts, where natural roosts have been destroyed, to aid in population management and to increase bat-mediated seed dispersal and pest control (Mering and Chambers 2014). In addition, bat mortalities have now surpassed bird mortalities in regions where wind turbines are abundant (Barclay et al. 2007). An estimated 1.3 million bats were killed by wind turbines from 2000-2012 (Arnett and Baerwald 2013). While conservationists and environmentalists are advocating for renewable energy resources to take over as primary energy sources, they are also fighting to reduce bird and bat mortalities by implementing new strategies to help these animals avoid the areas and to engineer safer designs for wind turbines (Baerwald et al. 2009; Zimmerling and Francis 2016). In addition, WNS has been the leading cause of bat population decline since its arrival in the US in 2006 (Blehert et al. 2009). Some species have

suffered from exceedingly high rates of mortality (90-99%) in several populations across eastern North America (Blehert et al. 2009).

WNS and Big Brown Bats

The responsible pathogenic agent of WNS is *Pseudogymnoascus destructans* (*Pd*) (Field et al. 2015), previously referred to as *Geomyces destructans* (Lorch et al. 2011). *Pd* is a psychrophilic (cold-loving) fungal pathogen that poses physiological threats such as increased frequency of arousal and resulting depletion of fat during the hibernation season (Verant et al. 2014). The fungus was first observed at Howe's Cave, New York in 2006 (Blehert et al. 2009) and has since spread westward throughout the eastern and central United States and Canada, and reaching small, isolated sections of California and Washington state (Lorch et al. 2016; Fig. 1). The presence of *Pd* has been observed on 18 species of bats in North America: eastern small-footed bats (*Myotis leibii*), Indiana bats (*M. sodalis*), gray bats (*M. grisescens*), little brown bats (*M. lucifugus*), northern long-eared bats (*M. septentrionalis*), southeastern myotis (*M. austroriparius*), Yuma myotis (*M. yumanensis*), big brown bat (*Eptesicus fuscus*), cave myotis (*Myotis* velifer), silver-haired bat (*Lasionycteris noctivagan*s), Virginia big-eared bat (*Corynorhinus townsendii virgianus*), eastern red bat (*Lasiurus borealis*), Rafinesque's big-eared bat (*Corynorhinus rafinesquii*), Ozark big-eared bat *(Plecotus towndendii ingens*), Townsend's big-eared bat *(Corynorhinus townsendii*), western small-footed bat (*Myotis ciliolabrum*), Mexican free-tailed bat (*Tadarida brasiliensis*), and tri-colored bat (*Perimyotis subflavus*) (Moore et al. 2013; Bat Conservation International 2018). Although *Pd* spores and/or hyphae have been observed in these species, it is not always an indication of inevitable mortality. Some species are relatively resistant to WNS, while others are known to be extremely susceptible

(Davy et al. 2017). It has been suggested that bat populations in Europe most likely coevolved with *G. destructans*, enabling resistance to the fungus via residual microbes (Wibbelt et al. 2010). European populations have had relatively little-known mortality compared to populations in North America. *Pd* has also been found in several sites in northeastern China, as well as in high rates throughout Russia (Hoyt et al. 2016; Kovacova et al. 2018), without any mass mortality events documented. In addition to questioning the genetic differences between European bat populations and North American populations, this also raises the question of how WNS is being transmitted between land masses. Marine vessels have been a vehicle of assisted migration of bats to Alaska and Canada (Voute 1980; Wright and Moran 2011). Furthermore, human-assisted spread of *Pd* has contributed to the rapid dispersal of the fungus to and within North America. The lack of decontamination efforts by cavers have likely increased the speed and range of fungal spread (Ballmann et al. 2017).

Pd grows at temperatures between 2-18°C (Verant et al. 2012), with an optimal growth temperature of 5-10°C (Blehert et al. 2009), which coincides with typical ambient temperatures of bat hibernacula. When fungal spores germinate, they develop into filaments of fungal cells, called hyphae, that become embedded in the epidermis of the hibernating bat skin. The hyphae become a large network, which then extends under the skin surface, leading to the generation of white fruiting bodies on the cutaneous membranes (skin) of the face and wings of the bats (Boyles and Willis 2010), causing microscopic erosions in tissue that serves as a quantitative index of disease severity (Reeder et al. 2012). Symptoms of the syndrome include an increased number or arousals (Reeder et al. 2012), low fat reserves (Britzke et al. 2010; Courtin et al. 2010), increased evaporative water loss (Willis et al. 2011), and increased metabolic rate (Anderson 2018; McGuire et al. 2017). *Pd* can also be spread through bat-to-bat or environment-

to-bat contact within and between hibernacula (Meyer et al. 2016); additionally, ectoparasitic *Spinturnix* mites, are found exclusively on bat wings and have tested positive for *Pd*, suggesting an additional mode of transmission as mites travel from bat-to-bat (Lučan et al. 2016).

Big brown bats have suffered relatively little mortality since the arrival of WNS in North America (Langwig et al. 2012). In sites that have been previously exposed to *Pd*, big brown bat populations remain stable, whereas little brown bat populations have declined dramatically (Frank et al. 2014). Little brown bats suffer from more severe membrane lesions compared to big brown bats (Moore et al. 2018). Big brown bats have a body mass of 11-25 grams (Kurta and Baker 1990) and have a wide geographical range, spanning from northern Canada to Mexico (Parker et al. 1997). In the northern part of their range, these bats typically hibernate in extremely cold microclimates, not only in caves and mines, but also in tree holes, rock crevices, and man-made structures. Big brown bats typically arouse more often than little brown myotis during the hibernation period, with torpor bouts lasting 3.3 ± 13.3 days (Halsall et al. 2012) and 20.88 ± 6.22 days (Jonasson and Willis 2012; Ehlman et al. 2013), respectively. This could explain why severe population declines have not occurred in big brown bats (Turner et al. 2011; Frank et al. 2014), suggesting more euthermic periods could aid in fighting WNS. However, this has never been tested in whole bats, only in wing tissue (Field et al. 2015). Big brown bats' arousal frequencies do not seem to be influenced by *Pd* presence (Moore et al. 2018).

Besides a differing immune response, another factor that might provide protection from WNS is the fatty acid composition on skin membranes. *E. fuscus* have higher amounts (compared to *M. lucifugus*) of myristic, palmitoleic, and oleic acids (fatty acids present on cutaneous membranes) that inhibit the growth of *Pd in vitro* (Frank et al. 2016). It has been suggested that fatty acids on the skin are providing protection against membrane deterioration,

creating a barrier that doesn't allow the pathogen to infiltrate the tissue (Ingala et al. 2017), and requiring little or no immune response to the fungus. A third factor that might confer WNS resistance is the skin microbial community. Microbes such as *Pseudomonas* have also been shown to act as a probiotic on *in vitro* bat skin that may outcompete *Pd* (Hoyt et al. 2015). Due to big brown bats being relatively resistant to WNS, they have received less attention, and little is known about how their immune system might actually respond to *Pd*.

Hibernation

During hibernation, mammals go through periods of torpor, which consist of a controlled decrease of body temperature (T_b) and associated physical inactivity (Carey et al. 2003). Bats are heterotherms that can reduce T_b to save energy (Dunbar and Tomasi 2006). Their hibernation seasons usually consist of long bouts of torpor (10-20 days) separated by brief arousals that last 2-3 hours (Spurrier and Dawe 1973; Lyman et al. 1982; Fig. 2). During an arousal, the animal's metabolism increases, heightening T_b (Day and Tomasi 2014) and causing bats to utilize stored fat reserves. Thus, with more arousals, the fat reserves are depleted more quickly. Mitochondria in brown adipose tissue utilize a modified electron transport chain in order to produce heat to raise the T_b during an arousal. In addition, the ATP generated from glycolysis, oxidative phosphorylation, and lipid catabolism are utilized to increase oxygen consumption and T_b. In a population of healthy bats, individuals will typically go through seasonal and sexual variation in metabolism and thermoregulation. Females will gain more weight prior to hibernation to ensure some energy is available for reproduction the following spring (Richardson et al. 2018). To conserve energy, bats at the end of hibernation appear to use barometric pressure within the

hibernacula to predict the weather and associated changes in abundance of insects outside of the roost without having to investigate first-hand (Paige 1995).

Similar to the decreased rate of other physiological processes during hibernation, the immune system is often suppressed. The production of antibodies (McKenna and Musacchia 1968) and the lipopolysaccharide (LPS) response are generally limited in most hibernating animals (Maniero 2000; Prendergast et al. 2002). Animals infected with psychrophilic bacteria during hibernation could have an increased risk of severe infection due to the lack of an immune response (Luis and Hudson 2006). Arousals may be an opportunity to fight off pathogens due to the suppression of the immune system while in torpor. Therefore, both immune function and the thermogenesis of arousals may contribute to the depletion of energy reserves (Canale and Henry 2011).

Immune Function

In response to a pathogen invasion, the immune system will initiate immune defenses, beginning with cell signaling and resulting in phagocytosis and destruction of the pathogen (Shoman and Levitz 2005; Blanco and Garcia 2008). The initial response against a pathogen is formed by the "innate" immune system, which is always present and responsive. The innate immune system is responsible for the detection and defense against "non-self" pathogens by recognition of the proteins and surface molecules of foreign biota. Once the immune system detects a pathogen, it will begin its defense by secreting chemical messengers to attract leukocytes (white blood cells), such as macrophages and neutrophils, to the site of infection. These leukocytes are crucial to the production of cytokines, which are more proteins released for cell-to-cell communication during immune responses. Pro-inflammatory cytokines will recruit

other nearby leukocytes, which intensifies the immune response and upregulates specific genes, such as NLRP10 or CD200 (defined below).

At this time, the "adaptive" immune system will usually start producing antibodies against the pathogen. These antibodies can remain present for several years in the body and can continue to fight off the pathogen during subsequent exposures. In little brown bats, anti-*Pd* antibodies have been observed, which suggests that antibody-mediated responses to *Pd* may not provide adequate protection against WNS (Johnson et al. 2015).

In order to bolster our knowledge of how the immune system responds against *Pd*, it is important to explore all viable options, and distinguish between competing hypotheses. Some species suffer extensive mortality due to WNS, while others appear to be resistant. In spite of the immune system being suppressed during torpor and hibernation, a partial (albeit insufficient) immune response to *Pd* exposure may still occur in at least little brown bats (Moore et al. 2013). This elevates torpid metabolism (Janicki 2010; McGuire et al. 2017), possibly causing the observed increase in arousals (Reeder et al. 2012). WNS mortality seems to stem from emaciation (Britzke et al. 2010), which may be due to the increased energetic cost during torpor and the increased number of arousals.

Differences in WNS mortality could be explained by variations in the immune response. One possible explanation for WNS mortality is that, upon exposure to *Pd*, the immune system of susceptible bat species initiates an inflammatory response, increasing energy expenditures during hibernation, leading to a depletion of previously stored fat reserves. However, the immune response is not sufficient to rid the bat of the fungus and thus the immune response is indirectly detrimental to the survival of the bat. In a susceptible species, this suggests that an anti-inflammatory treatment, such as meloxicam (mel), would suppress the immune response,

allowing the bats to preserve fat reserves instead of arousing to fight off a pathogen. If a WNS-resistant species, such as big brown bats, are surviving the WNS epidemic because they do not try to mount an immune response, it is expected that an anti-inflammatory treatment will have no effect on them. Susceptible bats have been shown to differentially express immune-function genes, in response to *Pd*, which resistant species do not (Anderson 2018). That study was conducted via cell culture analysis through transcriptomics and was used to identify the specific genes I have selected. Investigating the immune function of resistant bats will aid in understanding the distinction between resistant and susceptible bat immune responses.

Research Questions

I focused on the following metabolic and immune regulatory genes to understand how the immune system in big brown bats responds to *Pd* exposure during hibernation. These genes are:

- NOD-like receptor family pyrin-10 (NLRP10) – This gene plays a key role in the innate immune system. In addition, it has been suggested that this gene is also a negative regulator of inflammation and cellular apoptosis. It has also been implicated in the immune response to fungal infection in mice.

- Intercellular adhesion molecule 5 (ICAM5) – This adhesion gene is crucial to the immune-nervous system interactions during an infection. It is known to bind to leukocytes during inflammation and immune responses.

- Cluster of differentiation 200 (CD200) – This gene plays a role in immunosuppression and anti-tumor activity. It is distributed across the cell surface as a glycoprotein, which can interact with receptors and mediate the immune response.

- Tumor necrosis factor receptor superfamily member 21 (TNFRSF21) – This gene plays a role in T-helper cell activation, which is directly related to immune response.

In summary, all of these genes play an essential role for both the innate and adaptive immune system (National Center for Biotechnology Information Database). Differential expression of these genes will indicate whether the bats are activating their immune system. I

hypothesized that because big brown bats are relatively resistant to WNS, exposure to *Pd* would not change the expression of these genes. Consistent with the belief that resistance is due to a lack of an immune response, I further hypothesized that inoculated bats of a WNS-resistant species treated with meloxicam would not express differences in gene regulation compared to the *Pd*-inoculated group. Gene expression was measured via RT-qPCR from wing and blood tissue taken during initiation and termination of this experiment.

In addition to gene expression, measurements of metabolic rate and T_b were made to evaluate differences in arousals and torpor duration among treatment groups. Due to the WNS-resistant nature of this species, I hypothesized that bats exposed to *Pd* would not display any differences in torpor/arousal patterns. Furthermore, I hypothesized that the *Pd*-inoculated bats treated with the anti-inflammatory drug would also not display any differences from inoculated bats without the anti-inflammatory agent. To test these hypotheses, torpor duration, arousal cycles, and O_2 consumption were measured in *Pd*-exposed big brown bats with and without the meloxicam, the anti-inflammatory agent, and compared to control bats.

METHODS

In January 2018, 30 male hibernating big brown bats were collected by hand from a *Pd*-positive mine hibernaculum in Unity Village, Missouri. Bats were transported in sterilized cloth bags to Missouri State University where they were weighed and assigned to treatment groups. Capture of these bats was permitted by the Missouri Department of Conservation (permit #17622 to Thomas Tomasi). All animal welfare and care techniques were approved through the Missouri State University Institutional Animal Care and Use Committee (IACUC) under protocol 18.017.

All bats were tested for *Pd* presence on their muzzle, ears, and wing membrane using a sterile swab. All swabs were stored at -80°C until PCR according to the DreamTaq polymerase protocol by ThermoFisher Scientific (cat. no. K1072; Lot: LT-02241) could be completed. Following PCR with the use of *Pd* primers (Table 1), swab samples were then evaluated via agarose gel using ethidium bromide to verify the presence of the *Pd* sequence. Results of this PCR demonstrated that all bats tested negative for *Pd* at the beginning of the study.

The bats were randomly assigned into three treatment groups (n=10 per treatment) and placed in environmental chambers maintained at 8°C, 85% relative humidity, and 0L:24D photo period until April 2018, the end of their natural hibernation season. Water was added to a glass dish at the bottom of each chamber when needed. In order to observe the bats without disturbance, IR cameras were positioned inside each environmental chamber. Cameras were checked twice daily to ensure any apparently dead or distressed bats were removed in a timely manner.

Pd spores were placed along the lateral aspect of the body, on the wing margins of bats in the two treatment groups. The first treatment group was inoculated with $1x10^5$ CFUs (colony

forming units) of *Pd*, plus 2.5 µg meloxicam in 25 µL dimethyl sulfoxide (DMSO) on the posterior side of the ears. This is approximately 0.15 µg/g bat, a veterinarian-recommended effective anti-inflammatory concentration for topical use (M. Stafford, personal communication). The second treatment group received the same inoculation of *Pd*, plus 25 µL DMSO (vehicle control) along the posterior aspect of the ears. The final treatment group served as a control (i.e. no *Pd* inoculation, DMSO, or meloxicam). Subsequent dosing of 25µL DMSO (vehicle) or meloxicam occurred approximately every two weeks, on the posterior aspect of the ears, until hibernation ended. Because meloxicam has been shown to inhibit biofilm formation of other fungal species (Alem and Douglas 2004), a preliminary test was conducted to determine which meloxicam dose would directly inhibit *Pd* growth (Anderson 2018). In that study, 0.10 µg/mL showed no fungal inhibition (in the *in vitro* growth medium). It should be noted however, that the effect of torpor on the dosage/metabolism of meloxicam has not been previously studied.

Temperature-sensitive dataloggers (AlphaMach iButtons^{TM}; DS2422), modified to reduce mass (Lovegrove 2009), were set to record temperature (to 0.5ºC) every 20 minutes and were attached to the back of each bat using surgical cement to identify torpor/arousal cycles throughout winter.

During the middle (8 January 2018) and end (15 March 2018) of the hibernation season, metabolic rates while torpid were calculated as a rate of O_2 consumption. These measurements were conducted in 50 mL open-flow metabolic chambers. Chambers were lined with plastic mesh to provide a surface for attachment for the bat and were placed in a small refrigerator that was maintained at 8 ± 2°C. Continuous air flow through each metabolic chamber was achieved by an air pump when the bat's metabolism was not being measured. During metabolic rate measurements, air was pulled through these chambers via a flow controller maintained at

25mL/min (Sable Systems). Soda lime and silica gel removed carbon dioxide and water, respectively, prior to passing through the oxygen analyzer (Sable Systems, FC-10a) with room air set as 20.95% oxygen. All bats were acclimated to the metabolic chamber for at least 12 hours prior to measurements to ensure torpor was established. ExpeData^{TM} was used to sample the excurrent percent oxygen every second until at least three full apneic/breathing cycles were recorded (Fig. 3).

Dataloggers were removed from each bat post-euthanasia or prior to release, and temperature data were analyzed for arousal bouts. Arousals were defined as any three consecutive (equaling one hour) temperature recordings above 15°C. To obtain metabolic rates for each bat (displayed as mL O_2/hr), the sum volume (mL) oxygen consumed per breathing cycle was divided by the total time (hours) of each cycle. O_2 consumption data from mid- and late- hibernation were averaged to obtain one value per bat. Arousal durations were measured as the total amount of time (minutes) above 15°C. Arousals that correspond to the disturbance of opening the chamber for bat removal were not included.

Ultraviolet (UV) light photographs were taken to identify the presence of any *Pd* growth on each wing membrane at the beginning (day 0) and end (day 77) of the study. Photo analyses were completed using ImageJ software (Rasband 1997-2018). The percentage of area infected was computed by outlining the area (pixels) of the whole bat, then determining the percent of area infected by outlining the area (pixels) of the fluoresced/infected regions of the bat wing membrane.

Wing tissue biopsies (3-mm diameter) and blood samples (~20-50µL via femoral vein) were also taken from the bats upon capture to establish the baseline immune function, and upon completion, to measure differentially expressed genes. All samples were stored at -80°C.

Surviving bats in the control treatment were released at the site of capture in early April after one week of feeding and monitoring. All other bats were then euthanized to avoid releasing *Pd*-exposed bats into the wild. Survival rates were calculated as the number of days alive from point of capture to point of experiment termination (77 days).

Blood and tissue samples were analyzed for gene expression of NLRP10, CD200, ICAM5, and TNFRSF21 via RT-qPCR according to the manufacturer's protocol (DyNAmo HS SYBR Green: ThermoFisher Scientific; cat. no. F410L; lot: 00672146) following RNA isolation using TRIzol (Invitrogen; cat. no. AM97381). Primers for the genes of interest were created based on transcriptomics data (Anderson 2018) via CLC Genomics Workbench. The annealing temperatures were confirmed via PCR and gel electrophoresis (Table 1) and used under the following thermal cycler conditions: three minutes of primary denaturation (95°C), 40 cycles of denaturation (30 seconds at 95°C), annealing (30 seconds at the specified temperature per Table 1), and extension (1 minute at 72°C), with a final extension step (10 minutes at 72°C).

Gene expression was determined by the ΔΔCT method based on the number of PCR cycles compared to a standard curve. I did a linear regression of the cycle threshold values from the standards. This line was then used to interpolate the number of copies of RNA for each individual bat sample. The four genes of interest for each sample were also normalized against the expression of RPS8 (ribosomal protein subunit 8) after determining the RPS8 expression by the ΔΔCT method based on the number of PCR cycles compared to its own standard curve. This was completed to eliminate any differences in RNA concentration due to variability of RNA extraction efficiency or sample handling. The normalized data were then log10 transformed to minimize the data distribution. RNA concentrations below the level of detection were assigned a relative value of 0.01 ($\log_{10} = -2.00$).

Treatment effects on rates of oxygen consumption, total arousal time (hr), number of arousals, and total mass loss (g) were tested via one-way analyses of variance (ANOVAs). Because the control bats were not exposed to the fungus, the effect of meloxicam was assessed by comparing the *Pd*-inoculated bats to the *Pd* + mel bats, via ANOVA, and infection severity was assessed via UV photos. Survival rates were analyzed via a Log-rank test. Quantitative gene expression (relative number of RNA copies) between treatment groups were analyzed via one-way ANOVA and Tukey's post-hoc test when the ANOVA was significant. Comparisons of tissue type (blood vs. wing tissue) and hibernation duration (January vs. March) were also made with two-sample T-tests. Significance was established at $\alpha = 0.05$. Statistical analyses were performed using Minitab 18. Data are presented as mean ± SE.

RESULTS

Data from temperature-sensitive dataloggers (Fig. 4; Table 2) were analyzed for 28 bats throughout the hibernation study and were compared among treatment groups; two bats did not wear dataloggers due to equipment limitations. We should note that some dataloggers failed to communicate with the software/hardware post-experiment, mostly in the *Pd* + mel treatment, which reduced the sample size for this treatment to 5 bats.

The number of arousals did not differ among the treatment groups ($p = 0.340$). Consistent with the number of total arousals, the average total arousal duration did not differ among treatments ($p = 0.395$). O_2 consumption (mL/min) also did not differ significantly among treatment groups ($p = 0.089$), though *Pd* + mel bats did trend toward a higher average O_2 consumption than *Pd* bats or control bats (Fig. 4; Table 2). On average, mass loss did not differ between treatments ($p = 0.145$; Fig. 4; Table 2). Overall, across all groups, mass loss (%) did not change with number of arousals ($p = 0.820$; Fig. 5). There was no relationship between torpid O_2 consumption and number of arousals ($p = 0.104$).

UV analyses of infected wing area revealed no differences between treatments (Table 2). The survival rates, through the 77 days of the study, for the control, *Pd*, and *Pd* + mel bats were 80%, 70%, and 50%, respectively (Fig. 6; Table 2).

Based upon gene expression data, the random assignment of bats was successful; 7 of 8 of the measures in January (blood and wing samples), showed no difference between treatment groups. However, CD200 January wing samples did show a difference between treatment groups prior to treatment initiation (Table 3; Figs. 7-10). Bats in the control treatment showed a decrease in gene expression from January to March in NLRP10 ($p < 0.0005$) and CD200 ($p < 0.0005$), but

not in ICAM5 ($p = 0.085$) or TNFRSF21 ($p = 0.585$). When all genes and treatments are combined, expression is lower in March than in January ($p < 0.0005$).

To address the question of any *Pd* effect on gene expression, I compared January (pre-*Pd*) to March (post-*Pd*) wing samples, which showed no difference in the expression of any genes of interest (Table 4). Similarly, in March, gene expression was not different in *Pd* bats compared to controls (Table 4). To address the question of whether mel alters gene expression of bats exposed to *Pd*, I compared January (pre-treatment) to March (post-treatment) bat samples, which only revealed a difference in NLRP10 expression (Table 4). Gene expression in *Pd* + mel bats did not differ from expression in *Pd* bats (Table 4). Expression across all treatment groups in March did not differ throughout any genes of interest (Table 3). I also found that gene expression was higher in blood than in wing samples (Table 3; $p \leq 0.001$ for all four genes).

DISCUSSION

The results from the energetics section of this study support the hypotheses that neither *Pd* inoculation, nor an anti-inflammatory agent, would cause differences in WNS symptoms, such as arousal duration or O_2 consumption, in big brown bats. Meloxicam bats trended toward more mass loss, more time aroused (number of arousals, and longer arousal duration), and higher metabolic rates in torpor, which together suggest that meloxicam may harm the bats. However, individually, these parameters did not differ between treatments and there is no direct evidence that meloxicam effected mortality.

Bats inoculated with *Pd* (with or without meloxicam) demonstrated severe damage to the wing membrane, which is typically observed in susceptible species like little brown bats (Meteyer et al. 2009). This level of severity has not been observed in *Pd*-exposed big brown bats. While the infection severity was higher in meloxicam bats, the survival rates among treatment groups did not differ significantly.

The control bats were not infected with *Pd*, but their expression levels for all genes of interest still decreased with time, indicating that expression was inhibited by hibernation progression, not the *Pd* infection. This could occur if, for example, the suppression of gene expression was based on levels of body fat. An additional variable could be the physiological state of the bats when samples were collected. Bats were aroused for several hours prior to first sample collection (January), during transport from their hibernation site to the laboratory, whereas bats were fairly torpid during second sample collection (March). This is consistent with the findings that immunosuppression takes place during torpor bouts (McKenna and Musacchia 1968; Luis and Hudson 2006). Another possible explanation is that RPS8 (normalizing gene)

increased as hibernation progressed, but this seems unlikely due to the physiological depression that occurs during torpor. In addition, gene expression of RPS8 is consistent across multiple tissue types in euthermic birds (Carvalho et al. 2019), but this has not been tested during torpor. January samples were taken prior to exposure to *Pd*, which may indicate that these bats express these genes at low levels consistently.

Expression of CD200 in January wing samples was different across treatment groups, which posed a peculiar question of why there would be any difference prior to *Pd* exposure, but no significant difference post-exposure in March. The cause of this significant difference is unknown and could be a Type II statistical error.

Gene expression was higher in January blood samples than in January and March wing samples. This is likely due to the density of leukocytes (cells with nuclei), and thus active immune system genes, in the blood compared to other types of tissues, which contain a larger variety of cell types. A dilution effect takes place when these immune system cells, such as macrophages, are mixed with other cells, such as normal epithelial cells, in skin tissue that do not participate in immune system function.

Other than CD200 in January wing samples, all other gene expression findings support my hypothesis. Due to the WNS-resistance of big brown bats, I hypothesized there would not be significant differences in gene expression across treatment groups. Instead of upregulating immune genes, their immune systems would remain downregulated, as typically observed during hibernation. The combination of infection severity, observed by UV photos, and the scarcity of gene expression differences among treatment groups supports my hypothesis that big brown bats do not seem to mount an immune response during hibernation when exposed to *Pd*.

Limitations to this study include a limited sample size due to collecting permit limitations based on conservation measures. In addition, the refrigerator that housed the metabolic chambers displayed a $\pm$2°C shift from the desired temperature, and this range of 4°C may have affected the measured metabolic rates of these bats. Statistical analyses for gene expression may have yielded some differences if March blood samples were also included. Without March blood samples, meloxicam's influence on the ability to mount an immune response was based solely on wing tissue gene expression data. Blood samples were taken in March, but extremely low RNA concentration levels after extraction prevented these samples from being utilized. As conservation constraints should remain present, it is apparent that blood provides an accurate representation of gene expression; however, wing tissue sampling should be considered as it seems less invasive.

Since the debut of WNS in North America in 2006, an estimated 5.7–6.7 million bats had died by 2012 (USFWS 2012), with a likely additional 6 million bats dying in the next 6-year period (2012–18). As WNS continues to spread west, it is important to understand the physiological and genetic differences between resistant and susceptible species. If there is an understanding of how resistant species are maintaining their vigilance against *Pd*, there is potential for both increasing the resistance of susceptible species and identifying the susceptibility of western bat populations before the fungus presents itself in those areas. While it is imperative to implement additional conservation measures, such as caving regulations and educational awareness, it is also crucial that we continue investigating the differences between susceptible and resistant bat species as WNS is advancing westward throughout the North American continent.

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.

ANDERSON, B. A. 2018. The immune function and metabolism of hibernating North American bats with white-nose syndrome. M.S. thesis, Missouri State University. Springfield, Missouri, USA.

ARMITAGE, S. A. O., J. F. BROCH, H. F. MARIN, D. R. NASH, AND J. J. BOOMSMA. 2011. Immune defense in leaf-cutting ants: a cross-fostering approach. Evolution 65:1791-1799.

ARNETT, E. B., ET AL. 2008. Patterns of bat fatalities at wind energy facilities in North America. The Journal of Wildlife Management 72:61-78.

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., J. EDWORTHY, M. HOLDER, AND R. M. R. BARCLAY. 2009. A large-scale mitigation experiment to reduce bat fatalities at wind energy facilities. The Journal of Wildlife Management 73:1077-1081.

BALLMANN, A. E., M. R. TORKELSON, E. A. BOHUSKI, R. E. RUSSELL, AND D. S. BLEHERT. 2017. Dispersal hazards of *Pseudogymnoascus destructans* by bats and human activity at hibernacula in summer. Journal of Wildlife Diseases 53:725-735.

BARCLAY, R. M. R., E. F. BAERWALD, AND J. C. GRUVER. 2007. Variation in bat and bird fatalities at wind energy facilities: assessing the effects of rotor size and tower height. Canadian Journal of Zoology 85:381-387.

BAT CONSERVATION INTERNATIONAL. 2018. White-nose syndrome species and locations. http://www.batcon.org/our-work/regions/usa-canada/white-nose-syndrome/species-and-locations

BIRKETT, K. M., K. S. WEIDMAN, AND Y. WOO. 2014. Vespertilionidae (On-line). Animal Diversity Web. https://animaldiversity.org/accounts/Vespertilionidae/. Accessed 1 January 2019.

BLANCO, J. L., AND M. E. GARCIA. 2008. Immune response to fungal infections. Veterinary Immunology and Immunopathology 125:47-70.

BLEHERT, D. S., ET AL. 2009. Bat white-nose syndrome: an emerging fungal pathogen? Science 323:227.

BOYLES, J. G. AND C. K. R. WILLIS. 2010. Could localized warm areas inside cold caves reduce mortality of hibernating bats affected by white-nose syndrome? Frontiers in Ecology and the Environment 8:92-98.

BOYLES, J. G., P. M. CRYAN, G. F. MCCRACKEN, AND T. H. KUNZ. 2011. Economic importance of bats in agriculture. Science 332:41-42.

BRITZKE, E. R., P. SEWELL, M. G. HOHMANN, R. SMITH, AND S. R. DARLING. 2010. Use of temperature-sensitive transmitters to monitor the temperature profiles of hibernating bats affected with white-nose syndrome. Northeast Naturalist 17:239-246.

BROOKS, R. T. AND W. M. FORD. 2006. Introduction to the special section: bat habitat use in eastern North American temperate forests: site, stand, and landscape effects. The Journal of Wildlife Management 70:1171-1173.

CANALE, C. I. AND P. Y. HENRY. 2011. Energetic costs of the immune response and torpor use in a primate. Functional Ecology 25:557-565.

CAREY, H. V., M. T. ANDREWS, AND S. L. MARTIN. 2003. Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. Physiological Reviews 83:1153-1181.

CARVALHO, A. V., N. COUROSSE, S. CROCHET, AND V. COUSTHAM. 2019. Identification of reference genes for quantitative gene expression studies in three tissues of Japanese Quail. Genes 10:1-12.

COURTIN, F., W. B. STONE, G. RISATTI, K. GILBERT, AND H. J. VAN KRUININGEN. 2010. Pathologic findings and liver elements in hibernating bats with white-nose syndrome. Veterinary Pathology 47:214-219.

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.

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

DUNBAR, M. B. AND T. E. TOMASI. 2006. Arousal patterns, metabolic rate, and an energy budget of eastern red bats (*Lasiurus borealis*) in winter. Journal of Mammalogy 87:1096-1103.

EHLMAN, S. M., J. J. COX, AND P. H. CROWLEY. 2013. Evaporative water loss, spatial distributions, and survival in white-nose syndrome-affected little brown myotis: a model. Journal of Mammalogy 94:572-583.

FIELD, K. A., ET AL. 2015. The white-nose syndrome transcriptome: activation of anti-fungal hose responses in wing tissue of hibernating little brown myotis. PLoS pathogens 11:e1005168.

FISHER, M. C., ET AL. 2012. Emerging fungal threats to animal, plant and ecosystem health. Nature 484:186-194.

FLORES, J. M., I. GUTIEREZ, AND R. ESPEJO. 2005. The role of pollen in chalkbrood disease in *Apis mellifera*: transmission and predisposing conditions. Mycologia 97:1171-1176.

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:e0113958.

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 destructants*, the etiological agent of white-nose syndrome (WNS). PLoS One 11:0153535.

HALSALL, A. L., J. G. BOYLES, AND J. O. WHITAKER, JR. 2012. Body Temperature patterns of big brown bats during winter in a building hibernaculum. Journal of Mammalogy 93:497-503.

HILL, J. J. 1984. Bats: a natural history. Austin: A University Press.

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 the *Pseudogymnoascus destructans*, the causative agent of white-nose syndrome. PLoS One 10:0121329.

HOYT, J. R., ET AL. 2016. Widespread bat white-nose syndrome fungus, northeast China. Emerging Infectious Diseases 22:140-142.

INGALA, M. R., R. E. RAVENELLE, J. J. MONRO, AND C. L. FRANK. 2017. The effects of epidermal fatty profiles, 1-oleglycerol, and triacylglycerols, on the susceptibility of hibernating bats to *Psuedogymnoascus destructans*. PLoS One 12:e0187195.

INVITROGEN. 2016. TRIzol reagent user guide. ThermoFisher Scientific. https://assets.thermofisher.com/TFS-Assets/LSG/manuals/trizol_reagent.pdf

IUCN. 2019. The IUCN Red List of Threatened Species. Version 2018-2. http://www.icunredlist.org. Accessed 1 January 2019.

JANICKI, A. F. 2010. Effects of white-nose syndrome on winter energetics of little brown myotis (*Myotis lucifugus*). M.S. thesis, Missouri State University. Springfield, Missouri, USA.

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. 2012. Hibernation energetics of free-ranging little brown bats. Journal of Experimental Biology 215:2141-2149.

JONES, K. E., O. R. P. BININDA-EMONDS, AND J. L. GITTLEMAN. 2005. Bats, clocks, and rocks: diversification and patterns in Chiroptera. Evolution 59:2243-2255.

KONKEL, L. 2017. Fungal diseases are on the rise. Is environmental change to blame? Accessed June 7, 2019. https://ensia.com/features/19036/

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., AND M. B. FENTON. 2003. Bat Ecology. University of Chicago Press, Chicago, Illinois.

KUNZ, T. H., E. BRAUM DE TORREZ, D. BAUER, T. LOBOVA, AND T. H. FLEMING. 2011. Ecosystem service 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 the novel fungal disease, white-nose syndrome. Ecology Letters 15:1050-1057.

LORCH, J. M., ET AL. 2011. Experimental infection of bats with *Geomyces destructans* causes white-nose syndrome. Nature 480:376-378.

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.

LUCAN, R. K., ET AL. 2016. Ectoparasites may serve as vectors for the white-nose syndrome fungus. Parasites & Vectors 9:16.

LUIS, A. D. AND P. J. HUDSON. 2006. Hibernation patterns in mammals: a role for bacterial growth? Functional Ecology 20: 471-477.

LYMAN, C. P., J. S. WILLIS, A. MALAN, AND L. C. H. WANG. 1982. Hibernation and torpor in mammals and birds. Academic Press, New York. Chapter 2: 12-31.

MANIERO, G. D. 2000. The influence of temperature and season on mitogen-induced proliferation of ground squirrel lymphocytes. Life in the Cold, eds G. Heldmaier and M. Klingenspor 493-503.

MCCALLUM, H. 2005. Inconclusiveness of chytridiomycosis as the agent in widespread frog declines. Conservation Biology 19:1421-1430.

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. Regulatory, Integrative and Comparative Physiology 313:R680-R686.

MCKENNA, J. M. AND X. J. MUSACCHIA. 1968. Antibody formation in hibernating ground squirrels (*Citellus tridecemlineatus*). Proceedings of the Society for Experimental Biology and Medicine 129: 720-724.

MEDELLIN, R. A. 2003. Diversity and conservation of bats in Mexico: research priorities, strategies, and actions. Wildlife Society Bulletin 31:87-97.

MERING, E. D. AND C. L. CHAMBERS. 2014. Thinking outside of the box: review of artificial roosts for bats. Wildlife Society Bulletin 38:741-751.

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

MEYER, A. D., D. F. STEVENS, AND J. C. BLACKWOOD. 2016. Predicting bat colony survival under control targeting multiple transmission routes of white-nose syndrome. Journal of Theoretical Biology 409:60-69.

MOORE, M. S., ET AL. 2013. Hibernating little brown myotis (*Myotis lucifugus*) show variable immunological responses to white-nose syndrome. PLoS One 8:e59876.

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 188:163-176.

NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION DATABASE, U.S. National Library of Medicine. Accessed 1 January 2019. https://www.ncbi.nlm.nih.gov.

OUELLET, M., I. MIKAELIAN, B. D. PAULI, J. RODRIGUEZ, AND D. M. GREEN. 2005. Historical evidence of widespread chytrid infection in North America amphibian populations. Conservation Biology 19:1431-1440.

PARKER, D. I., B. E. LAWHEAD, AND J. A. COOK. 1997. Distributional limits of bats in Alaska. Arctic 50:256-265.

PAIGE, K. N. 1995. Bats and barometric pressure: conserving limited energy and tracking insects from the roost. Functional Ecology 9:463-467.

PRENDERGRAST, B. J., D. A. FREEMAN, I. ZUCKER, AND R. J. NELSON. 2002. Periodic arousal from hibernation is necessary for initiation of immune response in ground squirrels. American Journal of Physiology, Regulatory Integrative and Comparative Physiology 282:1054-1062.

RABINOWITZ, A. AND M. D. TUTTLE. 1980. Status of summer colonies of the endangered gray bat in Kentucky. The Journal of Wildlife Management 44:955-960.

RASBAND, W.S. 1997-2018. ImageJ. U. S. National Institutes of Health. Bethesda, MD, USA. https://imagej.nih.gov/ij/.

REEDER, D. A. M., ET AL. 2012. Frequent arousal from hibernation linked to severity of infection and mortality in bats with white-nose syndrome. PLoS One 7:e38920.

RICHARDSON, C. S., T. HEEREN, AND T. H. KUNZ. 2018. Seasonal and sexual variation in metabolism, thermoregulation, and hormones in the big brown bat (*Eptesicus fuscus*). Physiological and Biochemical Zoology 91:705-715.

RICHTER, J., S. HELBING, S. ERLER, AND H. M. G. LATTORFF. 2012. Social context-dependent immune gene expression in bumblebees (*Bombus terrestris*). Behavioral Ecology and Sociobiology 66:791-796.

SHOMAN, S., AND S. M. LEVITZ. 2005. The immune response to fungal infections. British Journal of Haematology 129:569-582.

SPURRIER, W. A., AND A. R. DAWE. 1973. Several blood and circulatory changes in the hibernation of the 13-lined ground squirrel, *Citellus tridecemlineatus*. Comparative Biochemistry and Physiology Part A: Comparative Physiology 44:267-282.

TALBOT, N. J. 2003. On the trail of a cereal killer: exploring the biology of *Magnaporthe grisea.* Annual Review of Microbiology 57:177-202.

TREJO-SALAZAR, R. E., L. E. EGUIARTE, D. SURO-PINERA, AND R. A. MEDELLIN. 2016. Save our bats, save our tequila: industry and science join forces to help bats and *Agaves*. Natural Areas Journal 36:523-530.

TURNER, G. G., D. M. REEDER, AND J. T. H. COLEMAN. 2011. A five-year assessment of mortality and geographic spread of white-nose syndrome in North American bats, with a look at the future. Update of white-nose syndrome in bats. Bat Research News 52:13-27.

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.batcon.org/pdfs/USFWS_WNS_Mortality_2012_NR_FINAL.pdf

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:e46280.

VERANT, M. L., C. U. METEYER, J. R. SPEAKMAN, P. M. CRYAN, J. M. LORCH, AND D. S. BLEHERT. 2014. White-nose syndrome initiates a cascade of physiologic disturbances in the hibernating bat host. BMC Physiology 14:10.

VOUTE, A. M. 1980. First recorded transatlantic bat transport. Bat Research News 23:16-18.

WHITAKER JR. W. O. 1995. Food of the big brown bat *Eptesicus fuscus* and maternity colonies in Indiana and Illinois. The American Midland Naturalist 134:346-360.

WIBBELT, G., ET AL. 2010. White-nose syndrome fungus (*Geomyces destructans*) in bats, Europe. Emerging Infectious Diseases 16:1237-1243.

WILLIS, C. K. R., A. K. MENZIES, J. G. BOYLES, AND M. S. WOJCIECHOWSKI. 2011. Evaporative water loss is a plausible explanation or mortality of bats from white-nose syndrome. Integrative and Comparative Biology 51:364-373.

WILSON, D. E. 2019. Bat. Encyclopedia Britannica, Inc. Accessed February 16, 2019. https://www.britannica.com/animal/bat-mammal.

WRIGHT, S. K. AND J. R. MORAN. 2011. Ocean-going vessels: a possible conduit for the introduction of white-nose syndrome fungus (*Geomyces destructans*) into bats in Alaska. Northwestern Naturalist 92:133-135.

ZIMMERLING, J. R. AND C. M. FRANCIS. 2016. Bat mortality due to wind turbines in Canada. The Journal of Wildlife Management 80:1360-1369.

Table 1. Primer sequences for all primers used in this study were based on prior transcriptomics data (Anderson 2018). Primers were originally tested via PCR and then analyzed on an agarose gel using ethidium bromide.

Primer	Primer Sequence	Annealing Temperature (°C)
NLRP10 forward	GACATCTTCATGGCCTACGTC	53
NLRP10 reverse	CGGAAGCTGTAGAACTTCTTGATG	53
CD200 forward	GAGCTGGGACTTCAGAACACAACC	55
CD200 reverse	TTCCTGACCCAGCGACCTTCCAG	55
ICAM5 forward	GCACCGCGGCCAATGTCCAG	53
ICAM5 reverse	GGGGCCATATTCCACCGTGATGG	53
TNFRSF21 forward	AATGCTACCTGCGCTCCCCATA	55
TNFRSF21 reverse	TCATTGATGTCAAAATGCTTGTGCG	55
RS8 forward	CTCGGGACAACTGGCACAAG	57
RS8 reverse	TCAGCTTGGCCCCCTTCTTGC	57
Pd forward	CTTTGTTTATTACACTTTGTTGCTTT	67
Pd reverse	CCGTTGTTGAAAGTTTTAACTATTATAT	67

Table 2. Energetics data from big brown bats during hibernation, inoculated with *Pd*, with or without meloxicam (anti-inflammatory). P-values are based on a one-way analysis of variance (ANOVA). Data are presented as mean ± SE (n).

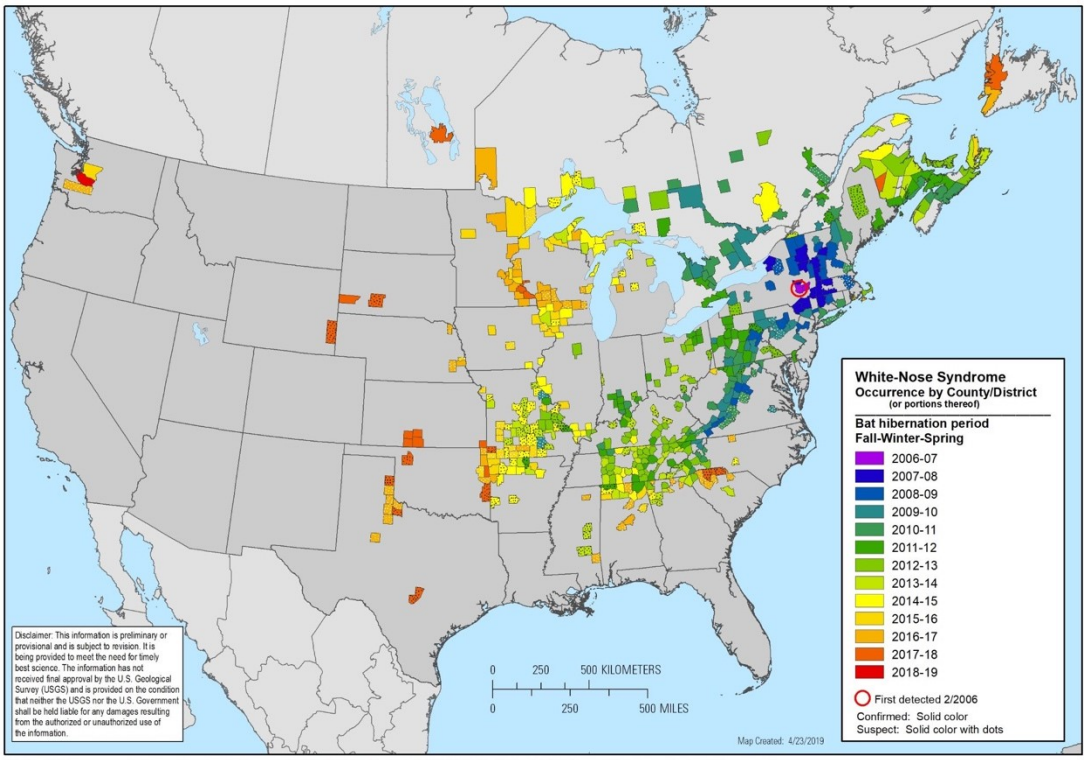
Parameter	Control	*Pd*	*Pd* + mel	P-value
Mass loss (g)	3.08 ± 0.26 (9)	3.42 ± 0.28 (9)	4.17 ± 0.64 (10)	0.145
# of arousals	6.40 ± 0.51 (5)	8.10 ± 0.82 (9)	8.40 ± 0.92 (10)	0.340
Avg. arousal duration (hr)	15.40 ± 1.90 (5)	13.26 ± 1.59 (9)	21.77 ± 6.41 (10)	0.395
O_2 consumption (mL/min)	0.004 ± 0.00 (5)	0.004 ± 0.00 (7)	0.01 ± 0.01 (4)	0.089
Avg. infection score (%)		17 ± 2 (7)	23 ± 6 (7)	0.357
Avg. study survival (days)	76.2 ± 0.6 (10)	74.7 ± 1.5 (10)	75.9 ± 0.5 (10)	0.513

Table 3. Descriptive statistics and p-values for treatment differences for all genes of interest. All treatment groups were tested against each other within each parameter for all four genes. P-values are based on a one-way analysis of variance (ANOVA). Data are presented as the log10 mean ± SE (n). The asterisk indicates statistical significance ($p < 0.05$).

Parameters	Control	*Pd*	*Pd* + mel	P-Value
NLRP10				
January Blood	2.420 ± 0.159 (10)	2.624 ± 0.124 (9)	2.479 ± 0.224 (7)	0.658
January Wing	1.595 ± 0.328 (7)	0.762 ± 0.606 (6)	1.965 ± 0.419 (5)	0.217
March Wing	-1.634 ± 0.281 (7)	-0.641 ± 0.621 (5)	-1.722 ± 0.237 (4)	0.173
CD200				
January Blood	0.766 ± 0.341 (10)	1.436 ± 0.146 (9)	0.930 ± 0.524 (7)	0.413
January Wing	0.651 ± 0.346 (7)	-1.170 ± 0.534 (6)	0.393 ± 0.685 (5)	*0.043
March Wing	-1.232 ± 0.392 (7)	-0.318 ± 0.600 (5)	0.112 ± 0.619 (4)	0.214
ICAM5				
January Blood	1.080 ± 0.371 (10)	1.059 ± 0.605 (9)	1.445 ± 0.598 (7)	0.818
January Wing	0.093 ± 0.622 (7)	-1.585 ± 0.415 (6)	-0.168 ± 0.833 (5)	0.153
March Wing	-1.321 ± 0.349 (7)	-0.849 ± 0.487 (5)	0.215 ± 0.411 (4)	0.072
TNFRSF21				
January Blood	0.302 ± 0.320 (10)	1.092 ± 0.198 (9)	0.404 ± 0.449 (7)	0.176
January Wing	-0.363 ± 0.344 (7)	-0.050 ± 0.420 (6)	0.182 ± 0.615 (5)	0.692
March Wing	-0.729 ± 0.579 (7)	-0.643 ± 0.390 (5)	-0.279 ± 0.617 (4)	0.841

Table 4. Selected comparisons of wing tissue across time and treatments for gene expression. P-values are based on one-way analysis of variance (ANOVA) for all genes of interest. The asterisk indicates statistical significance ($p < 0.05$).

	NLRP10	CD200	ICAM5	TNFRSF21
Jan. *Pd* vs. Mar. *Pd*	0.143	0.315	0.276	0.336
Mar. Control vs. Mar. *Pd*	0.138	0.221	0.440	0.619
Jan. *Pd* + Mel vs. Mar. *Pd* + Mel	*< 0.0005	0.776	0.716	0.618
Mar. *Pd* vs. Mar. *Pd* + Mel	0.185	0.636	0.151	0.151



Citation: White-nose syndrome occurrence map - by year (2019). Data Last Updated: 4/23/2019. Available at: https://www.whitenosesyndrome.org/resources/map.

Figure 1. WNS occurrence map, per county/district, (updated 04/23/2019) illustrating the origin of the fungus in the North America and the spread westward since 2006. https://www.whitenosesyndrome.org/static-spread-map/april-23-2019

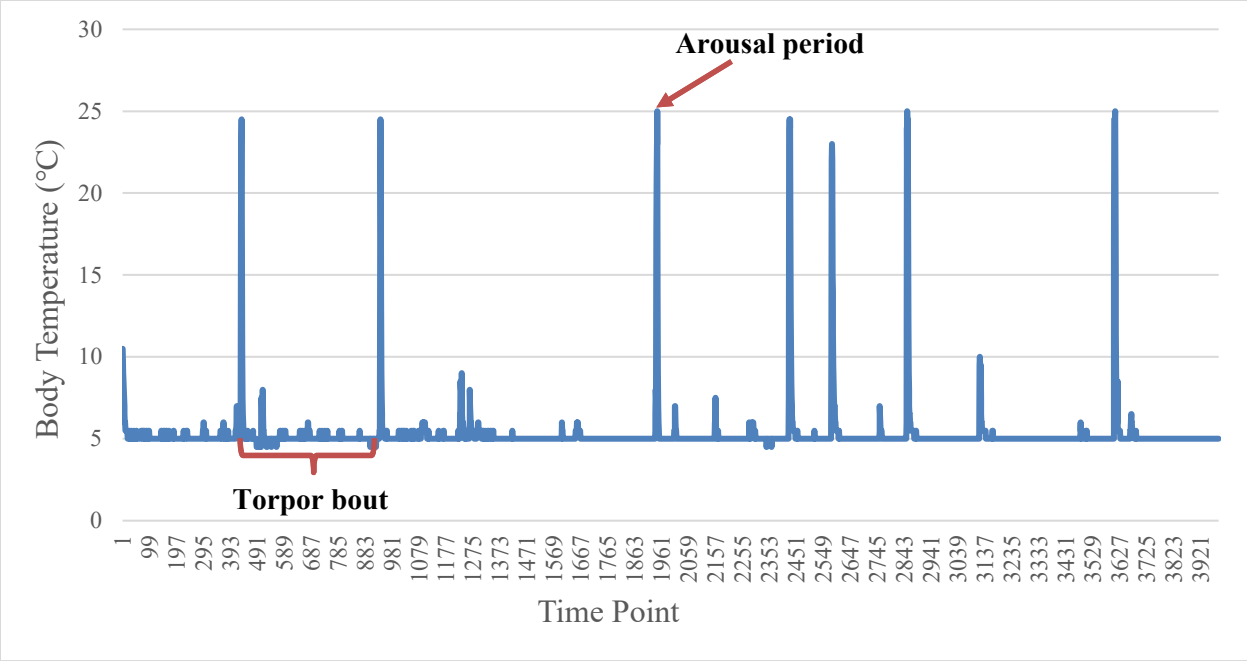


Figure 2. Body temperature (ºC) can vary through torpor and arousal cycles. Torpor, a physiological depression, conserves stored energy. During an arousal period, body temperature will increase quickly, depleting fat reserves.

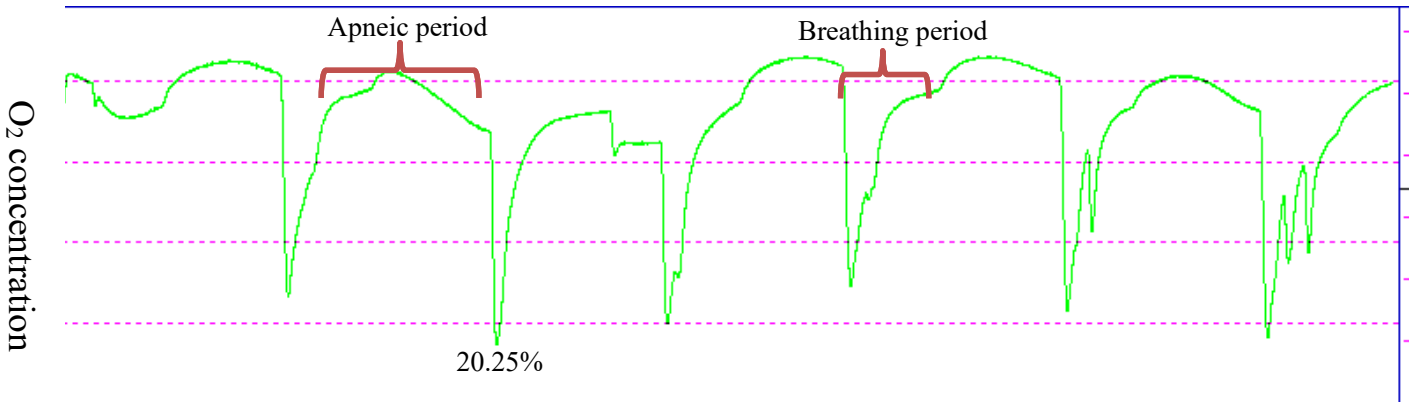


Figure 3. O_2 consumption was measured via ExpeData^{TM} every second until at least three apneic/breathing cycles were accomplished. The apneic periods are shown as the relatively stable periods around baseline. The breathing periods are shown as the decrease in oxygen consumption, indicating the bat is utilizing oxygen present in the chamber. During apneic periods, the O_2 concentration hovered around 20.95% oxygen; the breathing periods decreased the O_2 concentration to approximately 20.26%.

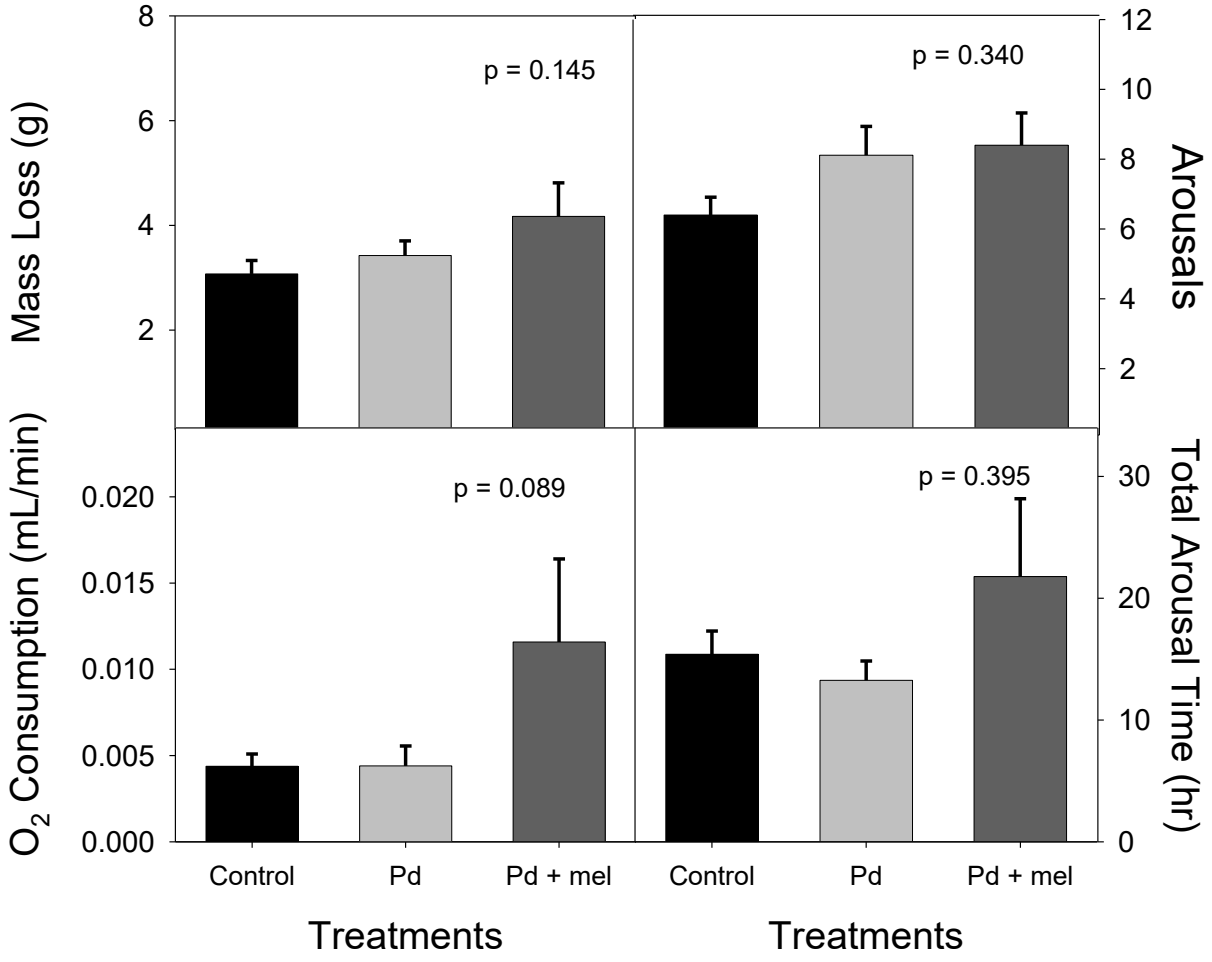


Figure 4. Energetics data from big brown bats during hibernation, inoculated with Pd, with or without meloxicam (anti-inflammatory). P-values are based on a one-way analysis of variance (ANOVA). The torpid O_2 consumption rates and total arousal time were highest in *Pd* + mel, which is consistent to a higher mass loss in this treatment. However, the among treatment values were not significantly different.

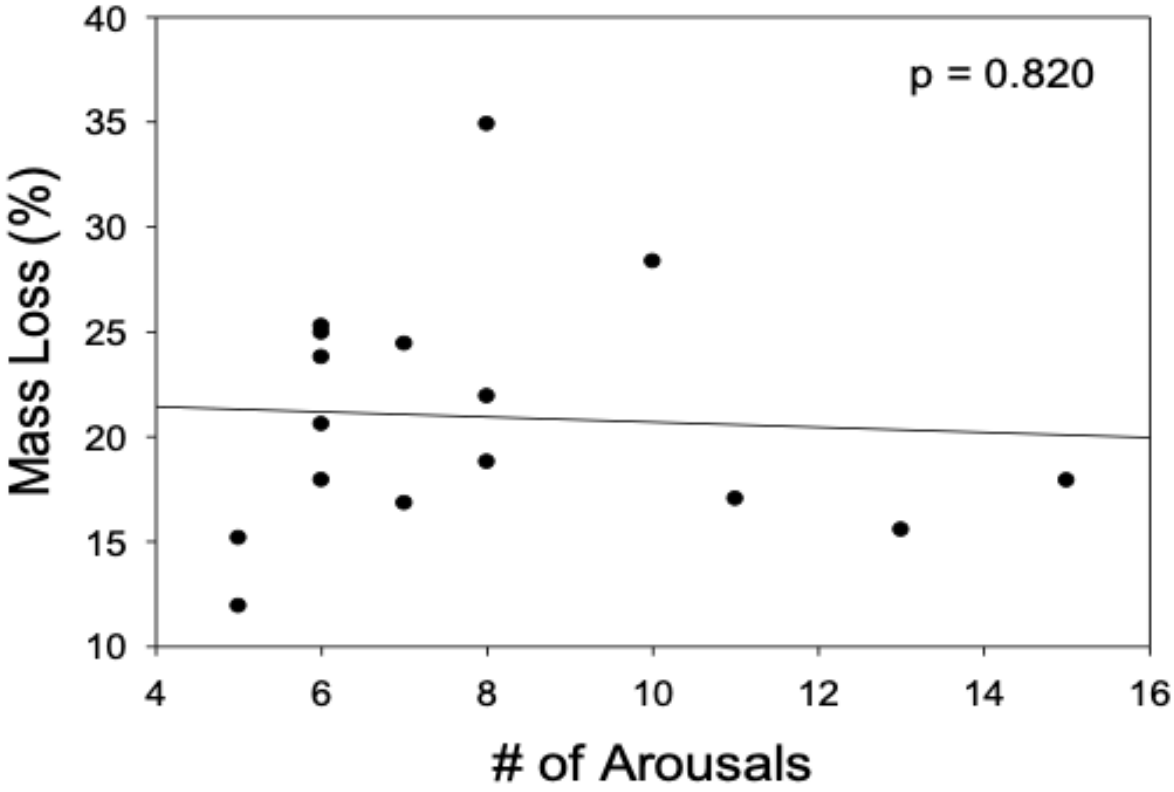


Figure 5. Mass loss (g) and total number of arousals had a positive relationship. The increase of arousals correlated with a lower overall mass loss for each individual.

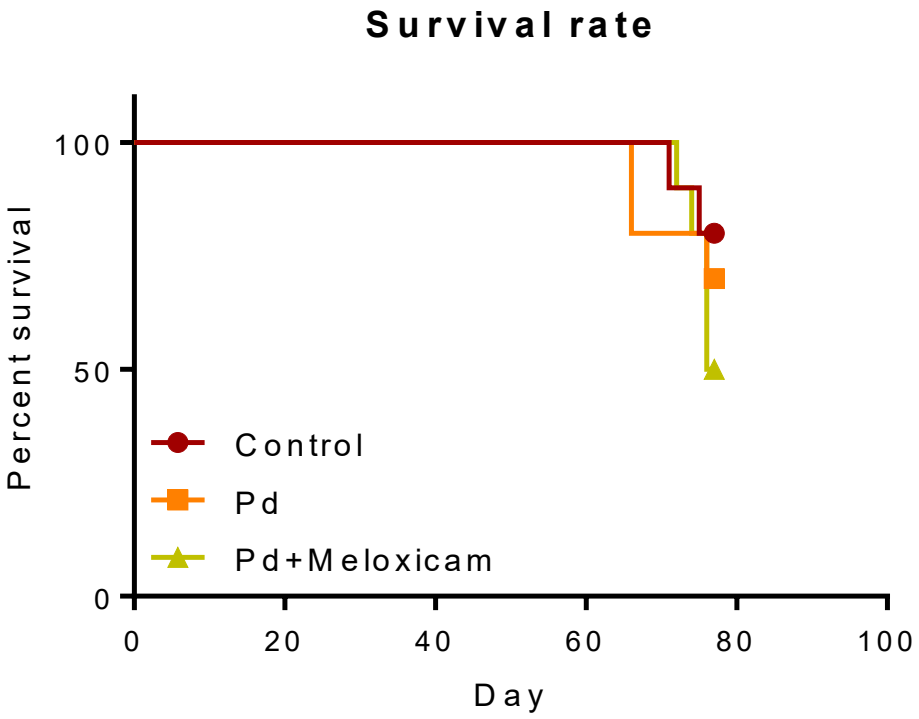


Figure 6. Survival per treatment groups is displayed as percent survived, over 77 days.

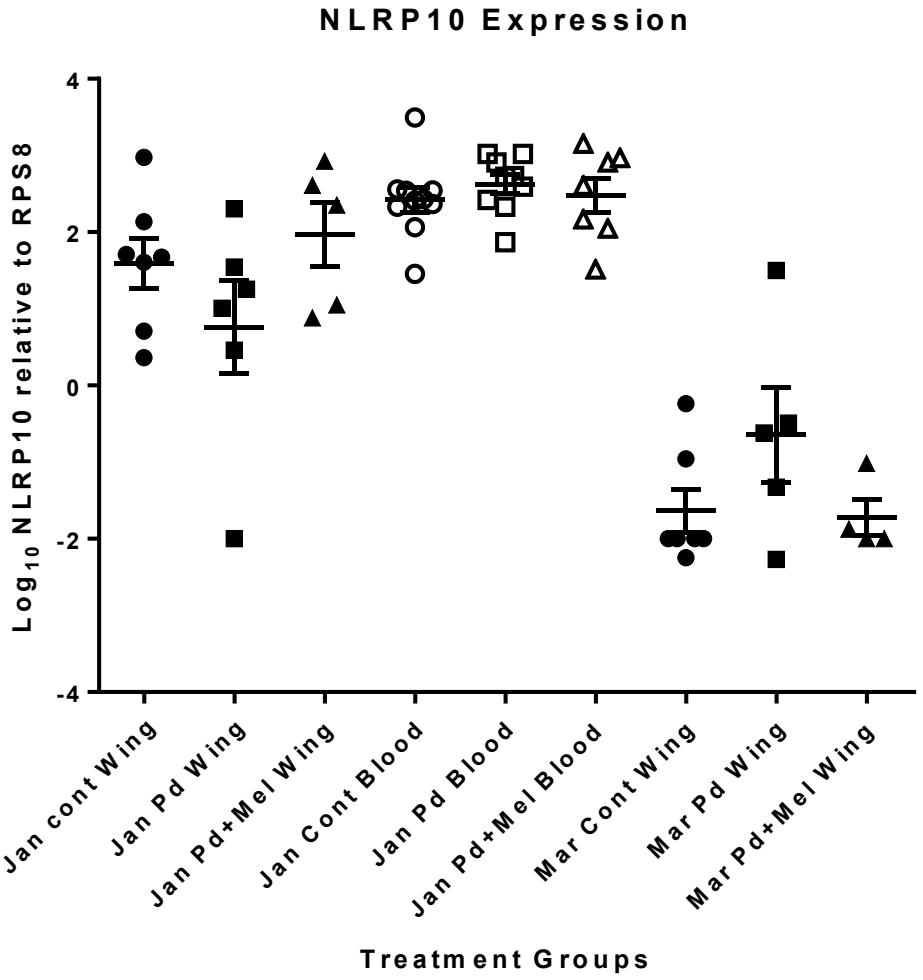


Figure 7. NLRP10 expression data, per month, treatment, and sample type (wing or blood), are displayed as log10 gene expression relative to RPS8 (normalizing gene). Circles = control animals; squares = bats in the Pd treatment; triangles = bats in the Pd + mel treatment group. Closed symbols = wing samples; open circles = blood samples. NLRP10 expression across treatment groups were not found to be different, except for January vs. March *Pd* + mel treatments. Blood samples also showed a higher NLRP10 expression than wing samples.

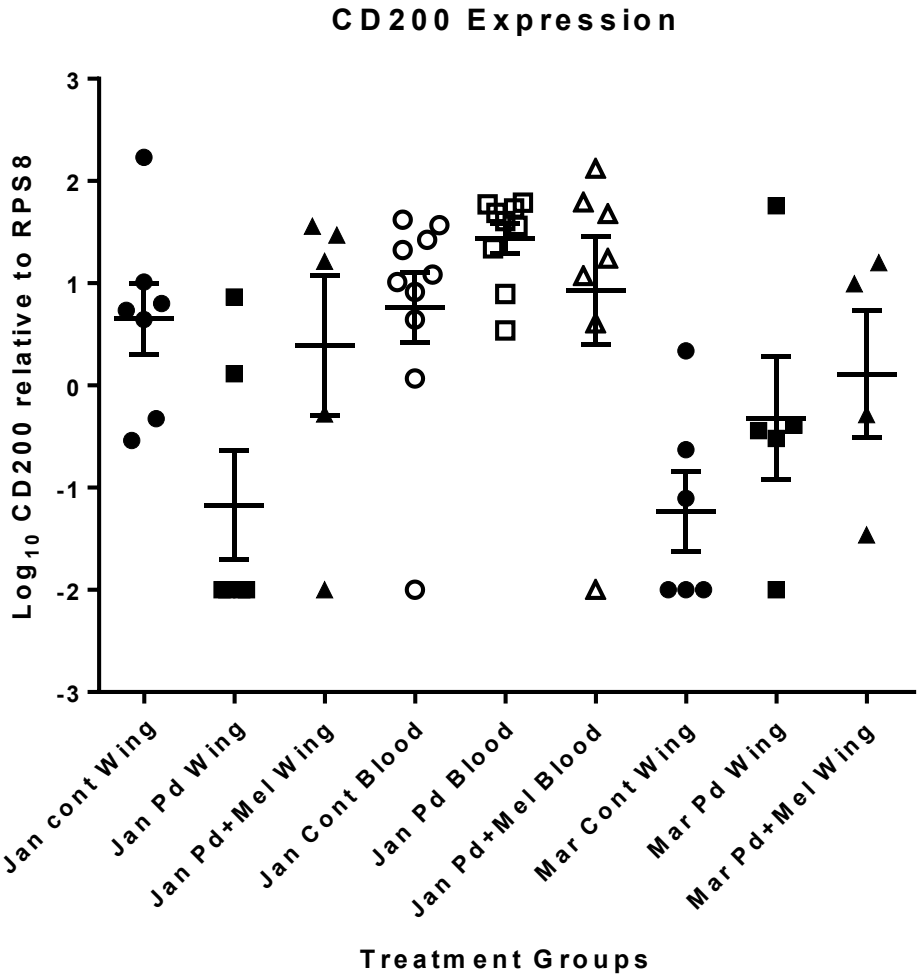


Figure 8. CD200 expression data, per month, treatment, and sample type (wing or blood), are displayed as log10 gene expression relative to RPS8 (normalizing gene). Circles = control animals; squares = bats in the Pd treatment; triangles = bats in the Pd + mel treatment group. Closed symbols = wing samples; open circles = blood samples. January wing tissue, across treatment groups, was different for CD200 expression. Expression in blood samples was also higher than wing samples.

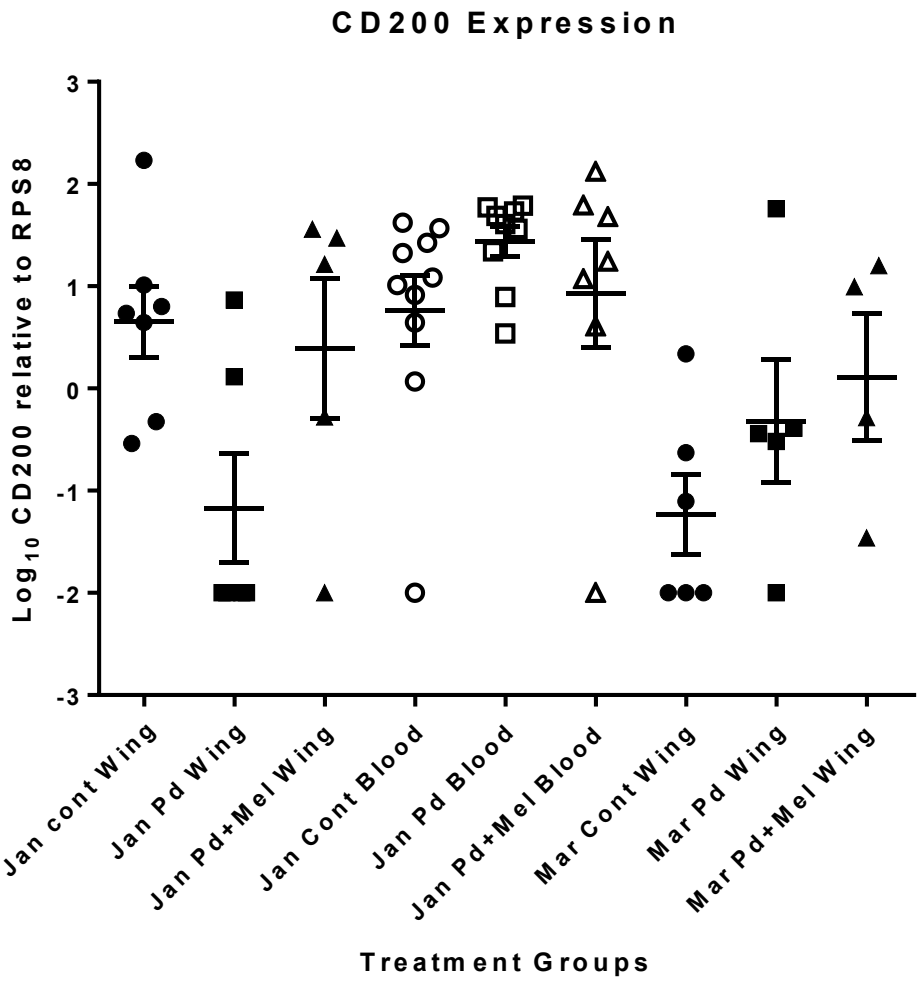


Figure 9. ICAM5 expression data, per month, treatment, and sample type (wing or blood), are displayed as log10 gene expression relative to RPS8 (normalizing gene). Circles = control animals; squares = bats in the Pd treatment; triangles = bats in the Pd + mel treatment group. Closed symbols = wing samples; open circles = blood samples. There are no differences in ICAM5 expression across treatment groups; however, blood samples did show higher expression than wing samples.

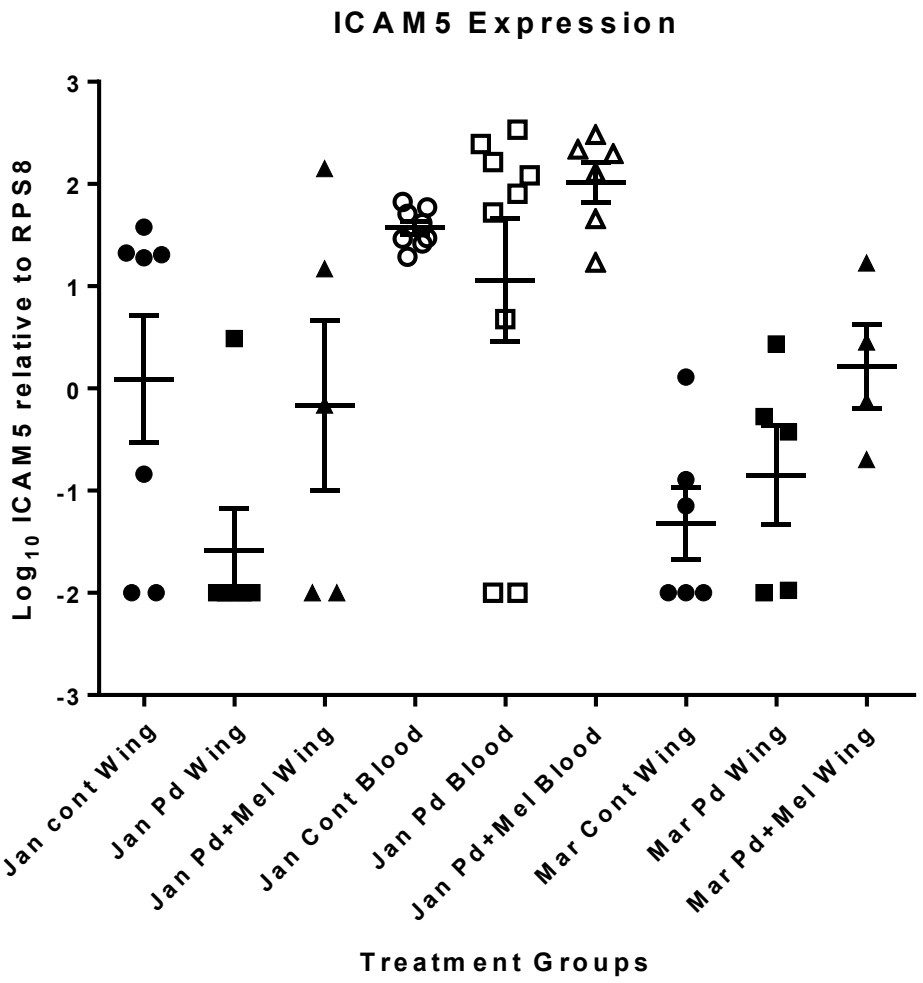


Figure 10. TNFRSF21 expression data, per month, treatment, and sample type (wing or blood), are displayed as log10 gene expression relative to RPS8 (normalizing gene). Circles = control animals; squares = bats in the Pd treatment; triangles = bats in the Pd + mel treatment group. Closed symbols = wing samples; open circles = blood samples. The distribution of gene expression throughout treatment groups is fairly limited. Though the blood samples did contain higher expression compared to wing samples, those data are not significant.

APPENDIX

Application to Use Live Vertebrate Animals

PI: Thomas E Tomasi Page: 1 of 12

Dept: Graduate College

IACUC ID: 18-017.0

Title: Immune Function and Metabolism of Tricolored and Big Brown Bats with WNS - II

Species: Bat (Two bat species)

Application Type: Amendment

Multiple Species No

Total Animal Number: 60 (Non-ORC - Caught in Wild)

Office Use Only

IACUC ID: 18-017.0-A

Renewal

Date: 12/2020

.

Submission History for Amendment:

04/03/2018 - Submitted

04/04/2018 - Under Review

04/11/2018 - Approved

04/11/2018 - Complete

12/10/2020 - Renewal Date

Approval