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Pathophysiology and recovery of myotis lucifugus affected by white nose syndrome

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PATHOPHYSIOLOGY AND RECOVERY OF *MYOTIS LUCIFUGUS* AFFECTED BY WHITE NOSE SYNDROME

by

NATHAN WILLIAM FULLER

B.S. Shippensburg University of Pennsylvania, 2007

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Approved by

First Reader

Michael D. Sorenson, Ph.D.
Professor of Biology
Boston University, Department of Biology

Second Reader

Paul M. Cryan, Ph.D.
Research Biologist
United States Geological Survey

Third Reader

Frederick E. Wasserman, Ph.D.
Professor of Biology
Boston University, Department of Biology

“We can’t stop here. This is bat country.”

--Raoul Duke

DEDICATION

I dedicate this work to my mentor Thomas Kunz, whose passion for bats defined a field and inspired a generation of bat scientists and conservationists. I owe him more thanks than will fit on this page.

I also dedicate this work to my parents. To my mother, whose passion for biology and the environment, paired with her willingness (or perhaps encouragement?) to let me do ***stupid*** things shaped me into the 30 year old nutcase I am today. To my father, who slogged through his job at Kellogg's for 30 years so his family could thrive. Whatever pain I've felt throughout graduate school surely doesn't compare.

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PATHOPHYSIOLOGY AND RECOVERY OF MYOTIS LUCIFUGUS AFFECTED BY WHITE NOSE SYNDROME

NATHAN W. FULLER

Boston University Graduate School of Arts and Sciences, 2016

Major Professor: Michael Sorenson, Professor of Biology

ABSTRACT

Critical to our understanding of wildlife diseases is the recovery phase, a period during which individuals clear infections and return to normal patterns of behavior and physiology. Most research on effects of white nose syndrome (WNS), an emerging fungal disease in bats, has focused on the pathophysiology of winter mortality and the effects of WNS on hibernating populations. The period immediately following emergence from hibernation has received little attention, but is a critically important time for survivors of the disease. During this time, survivors face significant physical and physiological challenges as they migrate to summer habitats, potentially begin gestation in the case of reproductive females, and begin to recover from wing damage caused by the fungus, which can be extensive and may greatly increase the energetic cost of flight. In this study, I (1) test the hypothesis that free-ranging bats heal from WNS-induced wing damage, (2) determine how WNS-induced wing damage changes skin surface lipid profiles on free-ranging bats, and (3) describe the temporal process of disease recovery in a colony of captive bats, including analyses of body mass, wing damage, pathogen load, skin surface lipid profiles, and histopathological metrics of WNS. I find that bats can quickly heal from wing damage in the wild and appear healthy as early as mid-July in New England.

Analysis of skin surface lipids does not reveal any striking differences between bats with wing damage and those without, although there are trends towards lower total surface lipids and increased levels of cutaneous cholesterol in bats with severe wing damage. Finally, I show that within 40 days of emerging from hibernation, bats quickly clear the fungal infection and gain body mass, undergoing rapid healing of wing damage and changes in skin surface lipid composition. Bats depend on their wings for a variety of vital processes including physiological regulation, locomotion and feeding. To fully understand the consequences of WNS and develop actionable management strategies, it is important to consider the long-term effects of this disease. My study helps fill critical knowledge gaps and will aid in the future conservation and management of affected bat species.

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LIST OF ABBREVIATIONS

BMI ..... Body Mass Index

FAME ..... Fatty acid methyl ester

FFA ..... Free fatty acid

GAM ..... Generalized additive model

GC ..... Gas chromatograph

GLP ..... Glycerophospholipid

H ..... Healthy

HPTLC ..... High performance thin-layer chromatograph

IT ..... Ion trap

LOD ..... Limit of detection

LOQ ..... Limit of quantification

MS ..... Mass spectrometer

NC ..... Necrotic

PAS ..... Periodic acid-Schiff

PCA ..... Principal component analysis

PC1 ..... First principal component

PC2 ..... Second principal component

Pd ..... *Pseudogymnoascus destructans*

SE ..... Sterol ester

sNC ..... sub-necrotic

SP ..... Spotted

SSL..... Skin surface lipid

TAG .....Triacylglycerides

TMS .....Trimethylsilyl

UV..... Ultraviolet

WDI..... Wing damage index

WE ..... Wax ester

WNS.....White nose syndrome

INTRODUCTION

Emerging infectious diseases represent one of the greatest modern threats to biodiversity. In particular, novel fungal diseases appear to have become more prevalent in recent years (Fisher et al. 2012), affecting a wide range of hosts including invertebrates (Kim and Harvell 2004), reptiles (Allender et al. 2015; Guthrie et al. 2015), amphibians (Rosenblum et al. 2010; Voyles et al. 2009; Weldon et al. 2004), and mammals, including humans (Blehert et al. 2009; Fisher et al. 2012). While scientists have made great strides in understanding the mechanisms of many new diseases, the broader, ecological impacts of disease are not well understood due to the challenges of such studies. In particular, sub-lethal effects of disease represent an understudied component of disease cycles. Much of what is known about sub-lethal effects is from predator-prey interaction literature. In particular, trophic cascades can result from behaviorally induced sub-lethal effects in which prey foraging ability is reduced dramatically by reduced foraging efficacy of predators, or perceived predation threats (Bowerman et al. 2010; Peckarsky et al. 1993; Sheriff et al. 2009).

From its initial observation in New York State during the winter of 2007, white nose syndrome (WNS) has spread throughout karst areas of eastern North America (Blehert et al. 2009; Gargas et al. 2009). WNS has been confirmed in 9 species of bats in 26 states and 5 Canadian provinces. The causative agent of WNS, a psychrophilic fungus *Pseudogymnoascus destructans (Pd),* is endemic to Eurasia, where it infects several species of hibernating bats (Leopardi et al. 2015; Puechmaille et al. 2010; Wibbelt et al. 2010; J. Hoyt, pers. comm.). However, European bats do not appear to suffer ill effects

from infection, an intriguing contrast to the severe effects observed in some North American species (Puechmaille et al. 2012). The most heavily impacted species is the once common little brown bat (*Myotis lucifugus)* (Frick et al. 2010; Langwig et al. 2012), which is the focus of this dissertation. Populations of this once nearly ubiquitous species have declined approximately 70% in areas affected by WNS for more than three years (Brooks 2011; Dzal et al. 2010; Turner et al. 2011).

When WNS first appeared, it was unclear whether mass die offs observed in caves in New York State were isolated incidents or a widespread phenomenon, but the disease quickly spread to neighboring caves in New England (Blehert et al. 2009). In each case, mass mortality or unexplained population declines in hibernacula were associated with a white fungus growing on the exposed skin surfaces of hibernating bats (Meteyer et al. 2009). Researchers determined that dead or dying bats at the cave entrance were emaciated and dehydrated, two symptoms that suggested bats were losing fat stores at a faster than usual rate (Reeder et al. 2012). Pioneering research using body temperature loggers then determined that bats with WNS were in fact arousing from hibernation three to five times more often than bats in unaffected hibernacula (Reeder et al. 2012).

Not all species of bat are equally susceptible to WNS. Little brown bats are the most heavily impacted species based on total mortality (Frick et al. 2010; Langwig et al. 2012; Turner et al. 2011; Turner and Reeder 2009), but this species shares hibernacula with a number of other hibernating bat species including endangered species such as Indiana bats (*Myotis sodalis*), grey bats (*Myotis grisescens*), and Virginia big-eared bats (*Corynorhinus townsendii virginianus*), and other more common species including the

northern long-eared bat (*Myotis septentrionalis*), the eastern small footed bat (*Myotis leibii*), and the big brown bat (*Eptesicus fuscus*). While *Pd* has been found on all of these species, only some suffer significant mortality due to WNS (*M. septentrionalis, M. sodalis*), whereas others (*E. fuscus, M. leibii, M. grisescens*) experience lower relative mortality while still being exposed to *Pd* (Langwig et al. 2012). A number of studies have sought to determine the nature of these differential effects, including analysis of surface lipid profile differences among species (Frank et al. 2014), investigations cutaneous proteins that may alter the ability of the fungus to proliferate (M. Moore, pers. comm.), or studies examining differences in hibernation microclimates between species (Langwig et al. 2012; Langwig et al. 2015; Langwig et al. 2015). Many of these research efforts are ongoing.

What drives increased arousal frequency in WNS bats is unknown; however, some hypotheses have been offered. Early suggestions focused on environmental contaminants that may have blocked bats from gaining sufficient energy stores for hibernation, or reduced insect populations in a way that prevented bats from getting enough of the right kinds of fat (e.g. polyunsaturated fatty acids) to survive hibernation (C. Frank, pers.comm). While mercury contamination is common in bats, it was not apparently connected to WNS mortality (Yates et al. 2014). Early thinking also suggested that affected bats were being infected by an opportunistic pathogens due to an already weakened state or lacked sufficient fat to survive hibernation (J. Reichard, pers. comm.). Overall, these hypotheses suggested that bats were suffering from ill effects caused by changes to their environment.

Research aimed at determining the cause of WNS did not support the environmental contaminant hypothesis. Rather, it became apparent that a new pathogen was the cause of WNS. Studies showed that fat stores in bats entering hibernation in WNS affected hibernacula were the same as in bats from the same sites prior to WNS (J Reichard pers. comm). Moreover, histopathological studies revealed that affected bats had widespread lesions on exposed skin surfaces, presumably caused by fungal activity (Cryan et al. 2010; Lorch et al. 2011; Meteyer et al. 2009; Reichard and Kunz 2009). Finally, studies demonstrated that WNS was caused by *Pd* infection and that a genetically similar fungus had been found associated with bats in Europe (Leopardi et al. 2015; Lorch et al. 2011; Puechmaille et al. 2010).

That researchers determined the ultimate cause of mortality (i.e., identification of a novel pathogen) does not mean that the proximate causes (i.e., the mechanisms of disease), and thus the potential targets for intervention, have been uncovered. However, recent studies have offered new approaches to this question by examining the pathophysiology of WNS. Cryan et al. (2010) were the first to suggest that changes in hibernation physiology could be the main driver of mortality. They postulate that the extensive distribution of wing lesions caused by *Pd* alter affected bats' ability to regulate evaporative water loss. Extensive erosion of the wing membranes and perhaps even wicking action by fungal hyphae drawing moisture from wing tissue, combined with naturally high levels of evaporative water loss, could result in a state of dehydration in hibernating bats (Cryan et al. 2010). The need to balance body water has been suggested to be one of the main reasons why hibernating mammals arouse from hibernation

(Thomas and Cloutier 1992). In fact, studies have shown that when hibernating mammals, including bats, are treated with diuretics it causes an increase in arousal frequency (Ben-Hamo et al. 2013; Cryan et al. 2013; Nemeth et al. 2010). An alternative but related hypothesis suggests that hibernation metabolic rate is increased by some mechanism related to *Pd* infection (Verant et al. 2014; L.P. McGuire, unpublished data). Higher metabolic rates result in greater energy consumption and higher CO_2 production; however, because of the unique metabolic conditions of hibernation, bats are unable to expel excess CO_2, which builds and causes acidosis and thus more frequent arousals (Verant et al. 2014). Such a cascade establishes a positive feedback of greater energy consumption due to frequent arousals, and increased water loss, which may lead to dehydration. Whatever the mechanism, it is becoming apparent that physiological disruption typifies WNS and drives premature consumption of energy stores and ultimately death. (Lorch et al. 2011; Reeder et al. 2012; Verant et al. 2014; Warnecke et al. 2012; Willis 2015; Willis et al. 2011).

Survivors of the disease, however, are also likely to be infected with *Pd*. In the weeks following arousal from hibernation, wings of affected bats develop additional wing damage that is not present during hibernation (Meteyer et al. 2012; Meteyer et al. 2011). Injuries can be minimal, resulting in small areas of discoloration, or can be as serious as large scale membrane loss or wing perforations. However, the dominant type of damage from WNS is areas of inflammatory crusting and flaking and subsequent wound contraction and scarring, which may affect wing membrane flexibility, and thus may cause changes to flight ability (Meteyer et al. 2012). Bat wings are highly elastic and

pliable, which is vital to proper wing function (Cheney et al. 2015; Findley et al. 1972; Studier 1972). Depending on the extent and location of damaged areas, flapping mechanics and aerodynamic cleanliness (a measure of airfoil efficiency) could be altered due to reduced membrane flexibility and reduced smoothness of flight surfaces (Bullen and McKenzie 2009; Bullen and McKenzie 2008). In addition, in areas where *Pd* has deeply eroded wing tissue, small wing hairs, which provide bats with important flight information (speed, direction of airflow, etc.) could be lost or made nonfunctional (Marshall et al. 2015; Sterbing-D'Angelo et al. 2011).

To fully grasp how WNS could lead to broader ecological effects, it is important that we build our understanding in three key areas. First, we need to establish the role of bats in an ecosystem. This information can be inferred from the decades of research on bat roosting and feeding ecology. Overall, however, our knowledge is lacking and should be the focus of future research. Second, we must develop robust population models that allow us to estimate how many bats have been lost to WNS. Such estimations have been made, and population decline and/or recovery models exist (Frick et al. 2010; Frick et al. 2015; Thogmartin et al. 2012; Thogmartin et al. 2013). However, these models are likely inadequate because the starting population of bats before WNS was never rigorously measured. Last, we need to understand in sufficient detail the individual-level processes and sub-lethal effects on surviving bats. number of researchers have looked into these matters, including healing and recovery studies (Fuller et al. 2011; Meteyer et al. 2011), documentation of interannual survival (Dobony et al. 2011; Reichard et al. 2014), multi-season disease monitoring (Langwig et al. 2012; Langwig et al. 2015; Langwig et al.

2015), disease modeling (Frick et al. 2010; Wilder et al. 2011), immunological studies (Moore et al. 2013; Moore et al. 2011), and behavioral studies (Reeder et al. 2012; T. Lilley, pers. comm).

The goal of my dissertation is to advance our understanding of recovery following WNS. In this study, I (1) test the hypothesis that free-ranging bats heal from WNS-induced wing damage, (2) determine how WNS-induced wing damage changes skin surface lipid profiles on free-ranging bats, and (3) describe the temporal process of disease recovery in a colony of captive bats, including analyses of body mass, wing damage, pathogen load, skin surface lipid profiles, and histopathological metrics of WNS. I find that bats can quickly heal from wing damage in the wild and appear healthy as early as mid-July in New England. Analysis of skin surface lipids does not reveal any striking differences between bats with wing damage and those without, although there are trends towards lower total surface lipids and increased levels of cutaneous cholesterol in bats with severe wing damage. Finally, I show that within 40 days of emerging from hibernation, bats quickly clear the fungal infection and gain body mass, undergoing rapid healing of wing damage and changes in skin surface lipid composition. Bats depend on their wings for a variety of vital processes including physiological regulation, locomotion and feeding. To fully understand the consequences of WNS and develop actionable management strategies, it is important to consider the long-term effects of this disease. My study helps fill critical knowledge gaps and will aid in the future conservation and management of affected bat species.

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CHAPTER ONE

FREE-RANGING LITTLE BROWN MYOTIS (MYOTIS LUCIFUGUS) HEAL FROM WING DAMAGE ASSOCIATED WITH WHITE-NOSE SYNDROME.

ABSTRACT

White-nose syndrome (WNS) is having an unprecedented impact on hibernating bat populations in the eastern United States. While most studies have focused on widespread mortality observed at winter hibernacula, few have examined the consequences of wing damage that has been observed among those bats that survive hibernation. Given that WNS-related wing damage may lead to life-threatening changes in wing function, we tested the hypothesis that the reduced relative abundance of little brown myotis (*Myotis lucifugus*) observed with severe wing damage, as the summer progresses, is due to healing of wing tissue. Initial photographs of captured and recaptured adult females were examined for total damage and healing rates were calculated for each category of wing damage index (WDI = 0-3). We found that bats with severe wing damage were able to heal to a lower WDI score within a time period as short as two weeks. Bats with more severe wing damage, and thus more wing area to heal, had higher healing rates than did individuals with less damage. We also found a significant relationship between body condition and WDI for adult female bats captured in the early weeks of the active season. Our results support the hypothesis that some bats can heal from severe wing damage, and thus may not experience increased mortality during the active season associated with reduced functions of damaged wings. We urge researchers and wildlife managers to use

caution when interpreting WDI data, especially during the latter months of the active season.

INTRODUCTION

The normal structure and function of wings is vital to the survival of bats. Not only do bats rely on their wings for flight and feeding, but these thin, highly vascularized structures also facilitate physiological functions such as gas exchange (Herreid et al. 1968; Makanya and Mortola 2007), water balance (Kallen 1964; Bassett 1980; Thomas and Cloutier 1992), and thermoregulation (Reichard et al. 2010). It is suspected that white-nose syndrome (WNS), an emerging fungal disease of hibernating bats (Blehert et al. 2009), is responsible for some wing damage in bats from the northeastern U.S. Because bats depend on their wings for aerial locomotion (dispersal, foraging, and migration), predator avoidance, and homeostatic regulation of temperature and water balance, it is important to understand whether these structures are able to heal and regain their normal functions following severe damage.

Histopathological examination of wing tissue from bats affected by WNS indicates that skin lesions are associated with a psychrophilic fungus, *Pseudogymnoascus destructans* (*Pd*), the putative causative agent of WNS (Gargas et al. 2009). The hyphae of *Pd* invade hair follicles as well as sweat and sebaceous glands, which may become filled with the proliferating hyphae and conidia (Charturvedi et al 2011). The fungus can then further invade the underlying connective tissue and capillary beds, severely eroding this tissue and causing infarctions (Meteyer et al. 2009; Cryan et al 2010). The resulting

necrotic tissue is prone to tearing or may slough, resulting in loss of wing surface area (Reichard and Kunz 2009).

When WNS emerged in the northeastern U.S. in 2006 researchers began to record evidence of abnormal wing damage in bats with and without symptoms of WNS, including perforations, scarring, and necrosis (Figure 1.1; Reichard and Kunz 2009). Interestingly, the prevalence of severe wing damage declines as the active season progresses, raising questions about the ultimate fate of bats with extensive wing damage (Reichard and Kunz 2009). Some of these bats could be dying because of complications resulting from poor wing condition. Alternatively, others could undergo intensive healing of their wing membranes, reducing the likelihood that severe wing damage will be observed later in the season.

Current understanding of healing in wing membranes is based on only two studies of free-ranging bats (Davis 1972; Weaver et al. 2009), although other studies have focused on wing healing in captive bats subjected to experimental damage (Church and Warren 1968; Iverson et al 1974; Faure et al. 2009). Free-ranging bats naturally incur damage through interactions with objects in the environment or as a consequence of failed predation attempts (Davis 1968). Despite the sometimes severe nature of initial wounding, most injuries to wing tissue will heal given sufficient time. For example, free-ranging pallid bats (*Antrozous pallidus*) healed from relatively large 15 mm^2 wounds to 1 mm^2 wounds in less than 33 days (Davis 1972). Both captive and free-ranging bats may be subjected to human-induced wing damage resulting from wing biopsies collected by researchers for genetic studies (e.g. Worthington-Wilmer and Barratt 1996; Turmelle et

al. 2011), dietary analysis (e.g. Sullivan et al. 2006; Cryan and Diehl 2009), or from injuries associated with marking bats (Kunz and Weise 2009). However, there is convincing evidence that bats exhibit an ability to recover from such injuries with minor negative impact on survivorship (Worthington-Wilmer and Barratt 1996; Faure et al. 2009; Weaver et al. 2009).

The primary objective of the present study was to test the hypothesis that bats are capable of healing following severe wing damage that is associated with WNS. We predicted that recaptured bats would show signs of recovery, with greater healing rates among individuals with more damage and thus more area to heal. A secondary objective was to evaluate the relationship between body condition and WDI. We tested this relationship in very early pregnancy, before a noticeable fetus is present, to remove a potential bias that could be introduced by body mass gained by females during pregnancy. This sampling protocol also provides an opportunity to assess how this relationship might vary with time following the appearance of *Pd* in a new region. We predicted that bats with high WDI would be in poorer body condition, based on derived body mass indices (BMI = length of forearm/mass).

METHODS

Study site

This study was conducted between 13 May and 10 August of 2009 at two maternity colonies of *M. lucifugus* in New England (Framingham, Massachusetts and Milford, New Hampshire). These maternity colonies are located in barns that are used to shelter

livestock and to store hay and varied household items. *Myotis lucifugus* is the most common species at these locations along with small numbers of *Eptesicus fuscus*, *M. septentrionalis*, and *Perimyotis subflavus*. The surrounding habitat is mixed hardwood forest, light residential and some agriculture. Both sites are located near wetlands and waterways (Sudbury River in Framingham, MA and Nashua River in Milford, NH). In both colonies, *M. lucifugus* females and pups form clusters during the day along the ridgepole of the barn and depart and return through the main doors or from openings between side-boards and eaves. These are the same colonies sampled by Reichard and Kunz (2009) in the development of the WDI, and thus are ideal for investigating the dynamics of wing healing within the affected range of WNS.

Trapping and field measurements

Each colony was trapped at bi-weekly intervals using two portable, double frame harp traps (0.9 m wide by 1.0 m high or 1.5 m wide by 1.9 m high). The traps were placed side by side in front one of the main open doors of the barns, while the second main entrances were closed and other large passageways were obstructed by plywood or cotton sheets to encourage bats to depart from a single portal (Kunz et al. 2009). Traps were left standing for approximately one hour or until no bats were captured over a ten-minute period. Each captured bat was placed into a clean, individually marked cotton bag and placed inside a heated holding container for later processing. Only adult bats were used in this study; juveniles were sexed and noted in the total number captured but were immediately released outside the building.

Sex, age, reproductive condition, length of forearm and body mass were recorded (Brunet-Rossinni and Wilkinson 2009; Racey 2009). Bats were banded with 2.9 mm, individually numbered, lipped alloy bands (Porzana Ltd, UK; Kunz and Weise 2009). Wings and uropatagia were transilluminated using a portable light box (GloBox Lightbox, Artograph, Inc., Delano MN) and assigned a WDI score following methods described by Reichard and Kunz (2009; Figure 1.1).

Quantifying recovery of wings

Both wings and the uropatagium of each bat were transilluminated and photographed with a digital camera (Fujifilm FinePix S700) by extending the wing and leg away from the body so that the leg was fully extended in its natural position and the forearm and propatagium were perpendicular to the axis of the body. Photographs were taken with the camera's automatic macro setting and no flash. A metric ruler or another object of known dimensions was included in each image for scale (Figure 1.1).

All photographs were characterized for the proportion of total damaged wing area using ImageJ (v. 1.43u, National Institutes of Health). First, the scale of each photograph was established using a scaling item (i.e. metric ruler or the radius of a US penny). Next, the entire pixel area of each wing was established by outlining the visible wing area. When part of the total wing area was occluded by the handler, we estimated this area and included it in the total. Wing area for each wing was summed for each bat (total wing

area). Finally, the damaged area was outlined and the total damaged area for each wing was summed for each bat (total damaged area of wings). We considered damage as one or more of the following conditions: discoloration (i.e. white areas, abnormal brown areas, black spots, red areas), tears, holes, flaking, necrosis, receded wing margins, and missing tissue (Figures 1.1 and 1.2). Healing was defined as a reduction in total damaged area between initial capture and recapture date, and was usually identified as a change from the above criteria to uniformly colored and structured tissue. Healing rate was calculated by dividing the total area of wing tissue that healed by the number of days between initial capture and recapture.

Statistical analyses

Statistical analyses of healing rate required that some of the recaptured bats be removed due to lack of photographs, or poor quality images that could not be used in our analysis. Of 37 recaptured bats, 29 were included in the analysis of healing rate. Groupings by wing damage were based on the initial WDI and resulted in the following sample sizes: n = 5 for WDI = 0; n = 10 for WDI = 1; n = 12 for WDI = 2; and n = 2 for WDI = 3. Average healing rates among WDI cohorts were compared using nonparametric statistical analyses.

The relationship between BMI and WDI was calculated using a subset of the total sample. To account for the effect of reproductive condition (i.e. body mass gained during pregnancy) on BMI, only female bats that were captured between 13 May 2009 and 27

May 2009 were included in this analysis. This time period was selected to avoid confounding effects of growing fetuses in pregnant bats; colonies first consisted of palpably pregnant bats on 27 May. We assumed that most females captured were pregnant between 13 May and 27 May, and that the small fetus during this period did not have a measurable effect on body mass or BMI. All statistical analyses were performed using JMP v. 9.0.0 (SAS Institute Inc., S. Cary, NC).

RESULTS

We captured a total of 324 bats (including 71 that had been banded in previous years) and recaptured 37. Many of the recaptured adult females were observed in lactation after 9 June. The greatest occurrence of pregnant females was on 27 May at the Massachusetts site (n = 46) and on 3 June at the New Hampshire site (n = 19). Throughout our study, only ten adult females showed no apparent signs of reproduction. Among these individuals, seven had BMI values that were lower than the average for all individuals captured between 13 May and 27 May (average BMI = 0.21). The relative abundance of adult females with different WDI scores varied with time (Pearson's $\chi^2 = 96.4$, $p < 0.001$, $N = 291$; Figure 1.3). The relative abundance of the most severe wing damage (WDI = 2 or 3) was higher in early summer (13 May – 10 June) than late summer (18 June – 6 August). In May, the relative abundance of bats with WDI = 2 and 3 was almost 50% of the total number captured. After 4 June, the relative abundance of bats with WDI = 2 was less than 0.25 and after 10 July no bats were observed with WDI = 3 or 2, except for one individual on August 6. The abundance of moderate to severe wing damage was greatest

during the time period coinciding with early to late pregnancy (13 May – 27 May; Figure 1.2).

Body Mass Index (BMI) varied among bats with different WDI scores (Kruskal-Wallis test, $\chi^2 = 11.66$, *d.f.* = 3, $p = 0.0086$) captured between 13 May 2009 and 27 May 2009 (Figure 1.4). Bats with WDI = 3 or 2 had significantly lower BMI than bats with WDI = 0 or 1 (pairwise comparisons using Wilcoxon Method).

Healing rate among recaptured bats varied within the grouped WDI measures (Figure 1.5). Healing rates among bats with moderate to severe wing damage (WDI = 2 or 3) were significantly greater than in bats with lesser damage (WDI = 0 or 1; Kruskal-Wallis test, $\chi^2 = 16.729$, *d. f.* = 2, $p = 0.0002$). Bats with an initial capture WDI of 0 (n = 5) had an average healing rate of 0.010 cm^2/day. Bats with an initial WDI score of 1 (n = 10) healed at an average rate of 0.232 cm^2/day. Half of these individuals healed to level 0. The group containing bats with an initial score of 2 (n = 12) had an average healing rate of 0.750 cm^2/day. Of these, eight individuals healed to level 1 and four healed to level 0. Due to the small sample size of bats with WDI = 3 and unusual healing patterns within this group, an average healing rate could not be calculated and compared. However, both of the individuals in this group showed extensive healing, reaching levels 2 and 1. The maximum healing rate observed in this study was 1.293 cm^2/day in a bat that repaired 37.498 cm^2 of damaged tissue in 29 days, transitioning from level 2 to level 0 (Figure 1.6).

Discussion

Healing, reproducing and surviving with damaged wings

We offer evidence that some bats are capable of rapid healing of their wing membranes and may in fact survive the effects of WNS-associated wing damage. We demonstrate healing among 29 of 37 (78%) recaptured bats. Of the remaining eight bats that were recaptured, four showed a decline in WDI, but we were unable to document these changes due to lack of digital images. The remaining four bats that showed no evidence of healing had low initial WDI scores (0 or 1). Our study demonstrates the value of mark-recapture data and thus reveals unexpected resilience to wing injuries among free-ranging *M. lucifugus* subjected to damage associated with WNS.

A hypothesis posited by Reichard and Kunz (2009) states that the reduction in relative abundance of moderate to severe wing damage late in the active season was due to increased mortality from predation or starvation resulting from reduced flight maneuverability and foraging efficiency of these individuals. In support of this hypothesis, they reported only two recaptured bats during the summer of 2008 that had improved wing conditions and observed numerous dead bats in and nearby their study colonies. While we offer evidence to the contrary, we cannot fully reject this hypothesis. Recapture rates for the present study were similar to that of past studies on *M. lucifugus* (0.10 – 0.35; Frick et al. 2010b), but were low (~0.10). Thus, we cannot account for bats that were not recaptured, and it is possible that some or all of these bats did not survive.

We observed a faster healing rate among bats with moderate to severe wing damage (WDI = 2 or 3) than in bats with lesser damage (WDI = 0 or 1). This result matches previous studies of wing healing studies in free-ranging and captive bats (Church

and Warren 1968; Iverson et al 1974; Davis 1972; Faure et al 2009; Weaver et al. 2009) and patterns of mammalian wound healing in general (Singer and Clark 1999). Cutaneous wound healing generally progresses as follows: clotting, inflammation, reepithelialization, wound contraction, and angiogenisis (Singer and Clark 1999). Within each step there is a complex interaction of gene regulation, cell migration, and cytokine secretions (i.e. epidermal growth factors) that promotes cell proliferation, microbial clearing, and tissue restructuring (Martin 1997). The accelerated healing we observed in bats with severely damaged wing tissues likely represents rapid epithelialization paired with wound contraction (Mannik et al 2010). However, bats with lighter damage, including spotting and lack of pigmentation, had most likely progressed past epithelialization since their membranes were generally intact. In these individuals, we were most likely documenting changes due to melanocyte repopulation, a process that happens in the final stages of wound healing (Cox et al 1989).

The cellular mechanisms of wing-membrane healing in bats are not known, nor is there information on whether wings of bats undergo full regeneration, such as what has been observed in ear tissue of other mammals (Williams-Boyce and Daniel 1985). Whether bats regenerate wing tissue completely, including replacement of hair follicles and sebaceous glands, as reported for skin tissue of mice and rabbits (Breedis 1954; Lu and Ghazizadeh 2005; Mannik et al 2010), is an important component to our understanding of how wing damage affects the physiology of bats during hibernation (Cryan et al. 2010). If bat wings do not regenerate completely, leaving them with poorly functioning skin features (e.g., hair follicles, sebaceoous glands, and sweat glands), then

survivors of one hibernation season may suffer progressively reduced fitness as their wings become more compromised with each successive winter of damage followed by intensive healing in the active season.

In addition to the direct effects of wing damage on flight aerodynamics and wing physiology, rapid wing tissue healing may reflect a greater allocation of nutrients to wound healing in those bats that have severe wing damage. Rapid healing of small wounds in bats is likely an adaptive process and is usually beneficial, considering that bats regularly incur minor injuries (Davis 1968). However, the level of damage that occurs in WNS-affected regions is uncommon in free-ranging bats (Davis 1968) and there has likely been little selective pressure on bats to develop a healing response to severe wing damage. While healing to this degree increases the probability of short-term survival by facilitating flight, long-term fitness may be compromised when individuals allocate more nutrients to healing than they normally would. Wound healing is energetically expensive to mammals and depends considerably upon the status of the immune system (Lee 2006). Thus, bats may experience energetic trade-offs by allocating more energy to regenerating lost tissue rather than to other important processes such as reproducing, mounting an immune response to challenges other than wound healing, or migration (Bernardo and Agosta 2005). A trade-off between reproduction and immune function has been shown in a number of vertebrate taxa (French et al. 2007; French et al. 2009) and was also recently identified in vespertilionid bats (M.S. Moore pers. comm). If limited energy resources are allocated to repairing wing tissue, then bats may face increased risks to survival and reproduction during the early weeks of the active season, a

trade-off that might be exacerbated by low ambient temperatures (increasing thermoregulatory costs) and reduced insect availability during the spring in New England (Hoying and Kunz 1998).

Our results suggest that WNS-associated wing damage to *M. lucifugus* may not overtly impact reproductive success during the post-partum period. Most of the adult female bats that we captured after the parturition period showed signs of lactation, although offspring of these individuals were not identified. Some individuals, including the bat with the worst wounding pattern observed in this study (Figure 1.2), transitioned into lactation and post-lactation between initial capture and recapture, suggesting that their pups were likely carried to term. The bat illustrated in Figure 1.2 also reached stages of lactation and post-lactation at the same time as many of the other females captured from the same colony during the present study. Overall, the females comprising this colony reached lactation and post-lactation during the same period that is typical of *M. lucifugus* for this region, although reproductive timing varies latitudinally and is highly dependent on precipitation (Frick et al 2010b). Alternatively, individuals whose offspring do not thrive may also be observed in a post-lactating state after the death of a pup. It is unknown if the pups in question developed normally and survived until weaning and beyond. Further research is needed to assess the impact of WNS on the reproductive success of *M. lucifugus*, including the timing of reproduction, sex ratio of offspring, post-natal growth rates of pups, and long-term survival of individuals born to mothers with badly damaged wings.

While we were unable to directly assess how wing damage affects post-natal growth and survival of pups, we can make suggestions about the effect of wing damage on adult females during pregnancy. A female little brown myotis during late pregnancy carries a fetus that can weigh up to 25% of her own mass immediately before the onset of parturition and is not particularly effective at capturing prey, presumably because of increased wing loading (measured as the mass carried per unit of wing area) and decreased flight maneuverability (Kurta and Kunz 1987). Thus, a pregnant female's net energy intake during late stages of pregnancy is low and can increase significantly after parturition and during lactation (O'Farrell and Studier 1976; Anthony and Kunz 1977; Kurta et al. 1989). The loss of overall surface area and flexibility of wing tissue damaged by WNS during hibernation will further increase wing loading by affecting total surface area of healthy tissue, thus altering flight aerodynamics and reducing overall maneuverability. Pregnant bats with heavy wing damage may suffer more negative consequences (i.e. declining body condition, decreased thermoregulatory capacity, and impaired water balance) from wing damage than non-pregnant females or adult males.

Implications for WNS research

WNS is having an unprecedented impact on hibernating bat populations in the eastern United States (Frick et al. 2010a) and the actual mechanism of mortality remains elusive. However, a hypothesis recently proposed by Cryan et al. (2010) suggests that damaged wing membranes may play a significant role in the death of these bats during hibernation. As the hyphae of *Pd* invade and erode wing tissue, specifically the sebaceous glands that

produce secretions that aid in waterproofing, wings may lose their ability to regulate water balance. Consequently, affected bats may be forced to arouse from torpor more frequently because of their need to replenish body water that is lost to the environment (Cryan et al. 2010, Nementh et al 2010). If this postulated mechanism is indeed the cause of mortality among bats with WNS, then healing of wing tissue during the active season represents a life-saving process to the few bats that survive hibernation after being infected with *Pd*. Additional research is needed to understand the physiology of healthy and damaged wing tissue during the hibernation and active season.

Records of WDI are invaluable in establishing a baseline level of wing damage in a region and may also be used to assess the lasting impact of WNS into the active season. For example, large-scale studies offer the potential to determine the relationship between the occurrence of wing damage and regions of *Pd* infection (Francl et al 2011). It is tempting, however, to diagnose "confirmed presence" of WNS in a region where a higher frequency of wing damage from *Pd* is observed, but wing condition alone is not a diagnostic tool to confirm WNS (Meteyer et al. 2009; Reichard and Kunz 2009). Until a temporal model of wing membrane healing and a reliable field test of *Pd* presence are developed, factors such as date of observation must be considered before attempting to correlate wing damage with WNS. We note that very little damage was discernable on most individuals by mid- to late-summer (i.e. during post lactation; Figure 1.2); any damage observed in or after late July should be carefully scrutinized for cause.

Researchers may attempt to correlate intensity of *Pd* infection (i.e. fungal load) with degree of wing damage. Again, some evidence supports this hypothesis, but these observations must be validated with histopathological analysis to demonstrate fungal penetration of wing tissue (Meteyer et al. 2009). Given the fragility of wing tissue and the gregarious nature of most hibernating bats, a number of other sources of wing damage could be misinterpreted as damage from *Pd.* Assessing baseline occurrence of WDI in geographic regions that are unaffected by WNS will permit researchers to identify new patterns of wing damage in bat populations that incur wing damage more regularly. For example, gleaning bats in the southwestern US frequently encounter cactus spines while foraging and it is not uncommon for their wings to incur significant tears from these interactions (Davis 1968).

Limitations

One trend revealed from comparing the 2008 study (Reichard and Kunz 2009) and our 2009 findings is the notable decrease in total captures in 2009. We offer two possible explanations for this pattern. First, the hibernating populations at the two major known hibernacula closest to these colonies (Aeolus Cave, East Dorset, VT and Chester Mine, Chester, MA) incurred large-scale mortality due to WNS. The hibernating population at Chester Mine before the winter of 2008/2009 was estimated to be 8,000-10,000 individuals, but has since declined to just 116 bats by mid winter in 2009/2010 (T. French, pers. comm.). The population at Aeolus Cave before it was infected with *Pd* may have been as high as 300,000 individuals (Trombulak et al. 2001) but the majority of the

bats hibernating in the accessible portions of this site had died by late January 2009 (S. Darling, pers. comm.). Second, the decline in some summer maternity colonies between 2008 and 2009 may be the result of disturbance from previous research that was conducted at these sites (Zhao et al. 2004; Schulz et al. 2006; Townsend et al. 2008). Bats tend to avoid or abandon colonies that are trapped too often (Kunz et al. 2009) and thus may have largely abandoned these sites in the summer of 2009 following previous capture efforts. We can further illustrate the decline at these colonies using the Schnabel Method (Schnabel 1938) for estimating population size based on mark-recapture data of adult female bats. Using Reichard and Kunz's (2009) data for summer of 2008, we calculated total occupancy of bats in the MA site to be 4570 individuals, while the total at the NH site was 657 individuals. Unfortunately, these numbers appear to be greatly inflated compared to direct emergence counts at these colonies. Notwithstanding, these values illustrate the point that bats were sufficiently numerous in 2008 that the probability of recapturing an individual bat was extremely low, which will bias Schnabel estimations. In the summer of 2009, we calculated the population of bats in the MA site to be 281 individuals and 41 individuals in the NH site using the same trapping and population estimation procedures. Although this value may still overestimate colony size because of inherit limitations and assumptions of mark-recapture methods (O'Shea et al. 2004), the difference between years is striking, showing a nearly 94% population decline at each colony.

Limitations in our study are common among other investigations of bat ecology. First, we sampled only a subset of the population by trapping at two *M. lucifugus*

maternity colonies in New England. Because male bats tend to use alternate and often unidentified summer roosts, our sampling protocol does not account for male bats that were affected by WNS during hibernation. Second, as is common in most mark-recapture studies, recapture rates were low (~0.10; O'Shea et al. 2004). This trend raises questions regarding the ultimate fate of the individuals banded early in the season when wing and body conditions were poorest. Lastly, the measure used for assessing BMI did not compensate for body mass gained during very early pregnancy (i.e. prior to 13 May). Our criteria for excluding bats from BMI analysis attempted to eliminate this factor, yet females in the early stages of pregnancy may have contributed to slightly higher average BMI compared to nonreproductive individuals.

CONCLUSION

This study represents important evidence that some bats that incur wing damage during hibernation (presumably from exposure to WNS, in this instance) are able to heal rapidly and may also successfully reproduce in spite of such damage. However, while this evidence is a positive note among mostly negative trends in WNS research, it is important that conservation efforts for bats focus on year-round strategies both during hibernation and throughout the active season. With the severe declines in summer populations observed in this and other studies (Dzal et al. 2010; Brooks 2011), and the prediction of regional extinction of *M. lucifugus* within 16-20 years (Frick et al. 2010a), the few maternity colonies that remain represent vital islands of reproduction and genetic variation. If human interventions, such as increased utility-scale wind-energy

development (Kunz et al. 2007; Arnett et al. 2008) and “pest control” practices threaten the viability of these colonies, then local extinction of populations of *M. lucifugus* may occur sooner than predicted. Further research is needed to assess the long-term impacts of WNS on summer colonies of bats in the northeastern U.S. and the effect of wing damage on their reproductive success, foraging ability, flight maneuverability, physiological functions of wings, and energetic costs of healing.

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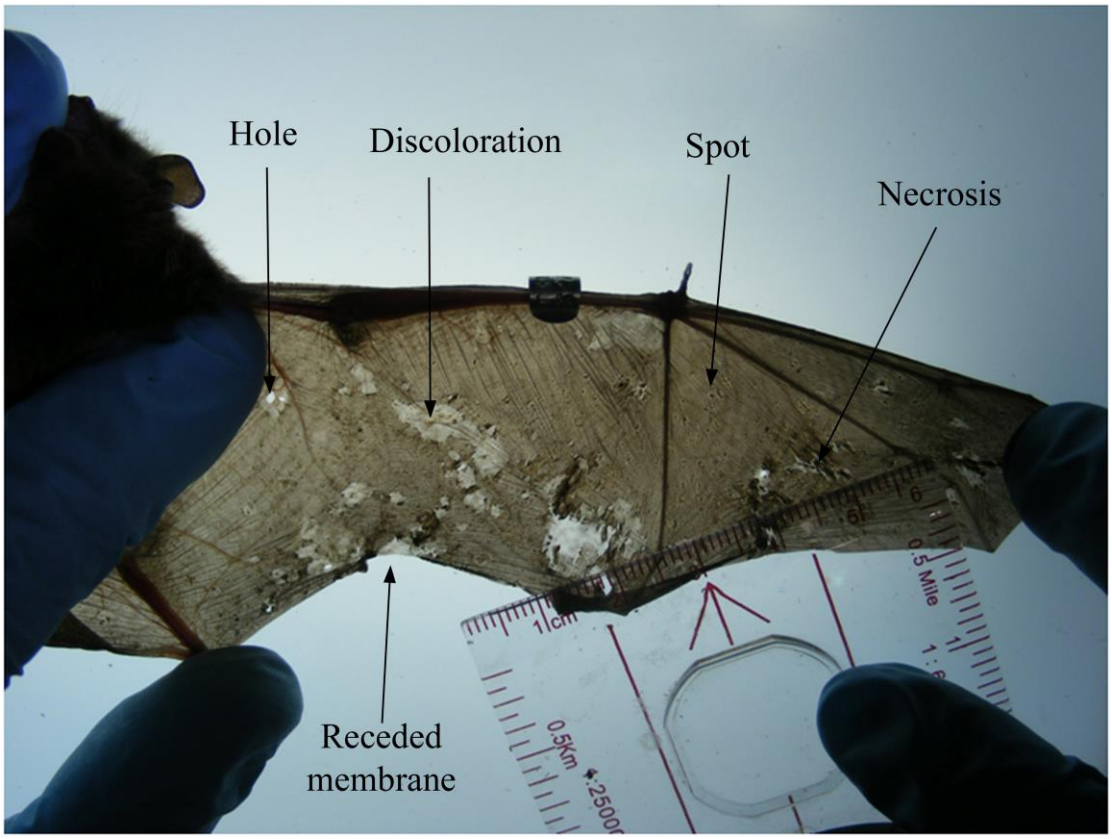

Figure 1.1. Wing damage was defined following Reichard and Kunz (2009) and included the following criteria: discoloration, tears, holes, flaking, necrosis, receded wing margins, and missing tissue.

Figure 1.2. Photograph of a transilluminated wing of a little brown myotis showing the most extensive healing recorded in this study. The highlighted region shows an area of lost wing tissue that healed over a period of time extending from 10 June 2009 (A) to 6 August (B) 2009 (57 days). This particular bat was captured in lactation on 23 June 2009 and was observed in post-lactation on 6 August 2009.

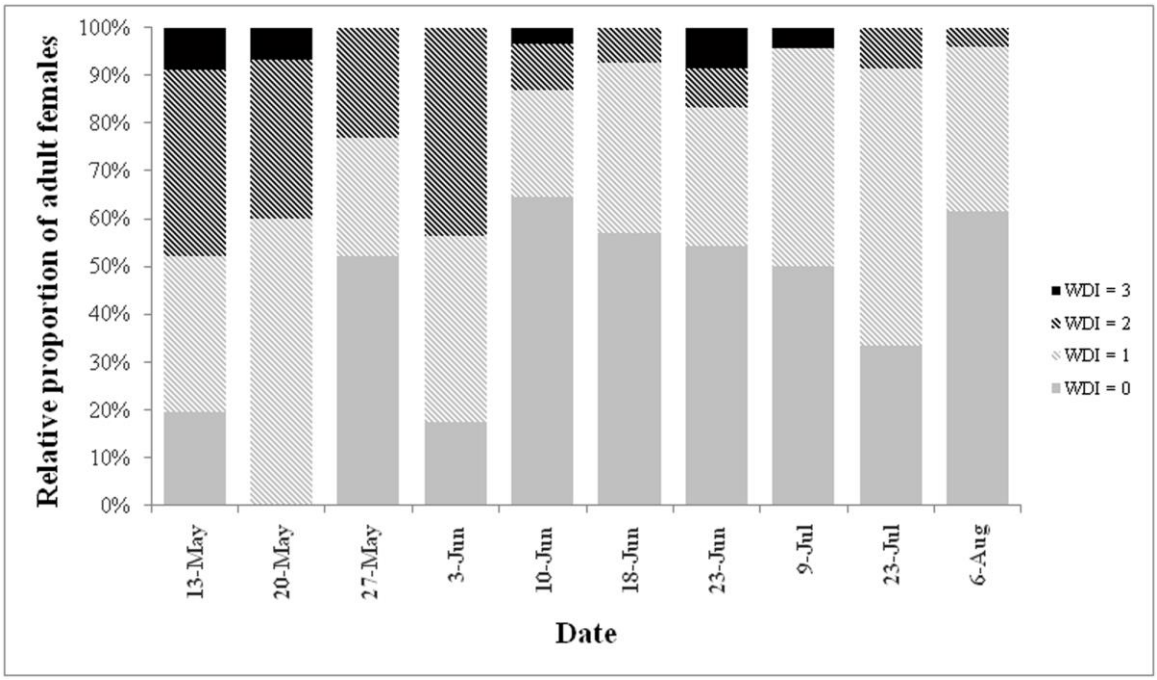

Figure 1.3. Relative proportion of adult female *M. lucifugus* with various degrees of wing damage recorded at two summer maternity colonies in New England. Trapping events that yielded fewer than 5 individuals were excluded from this analysis.

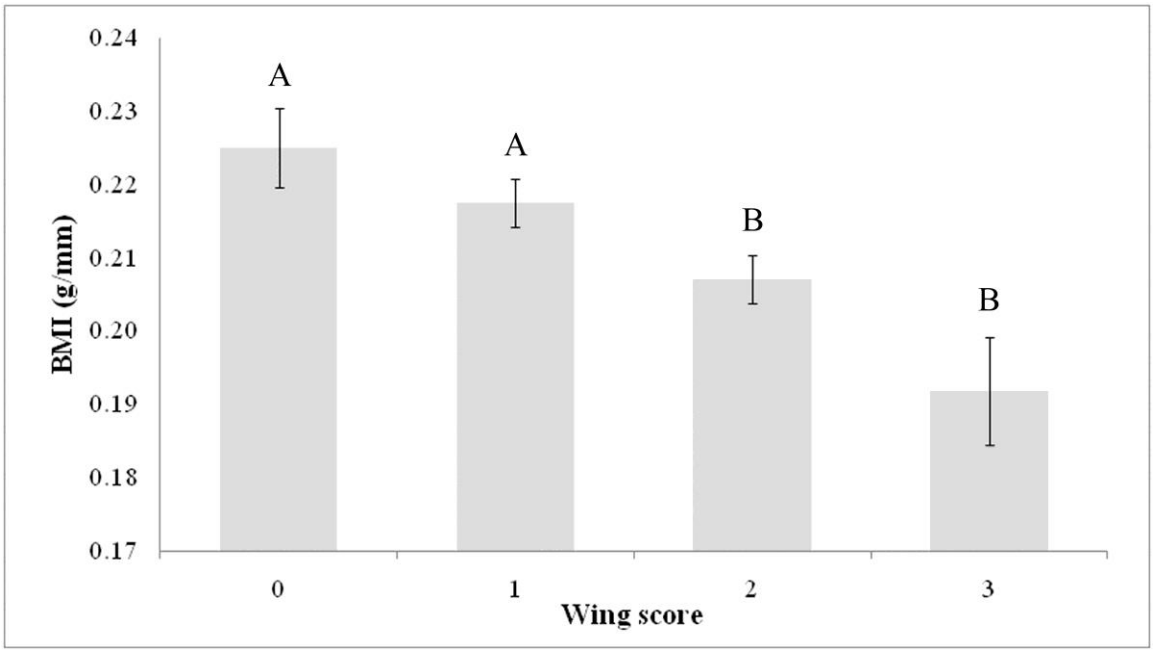

Figure 1.4. Mean BMI [BMI = M_b (g) / length of forearm (mm)] of adult female *Myotis lucifugus* with different wing damage index (WDI) scores at summer maternity colonies in northeastern US from 13 May to 27 May 2009. BMI was calculated before visibly or palpably pregnant females were observed at study colonies to control for gain in body mass during pregnancy. Bars labeled with similar letters are not significantly different. Error bars are standard errors.

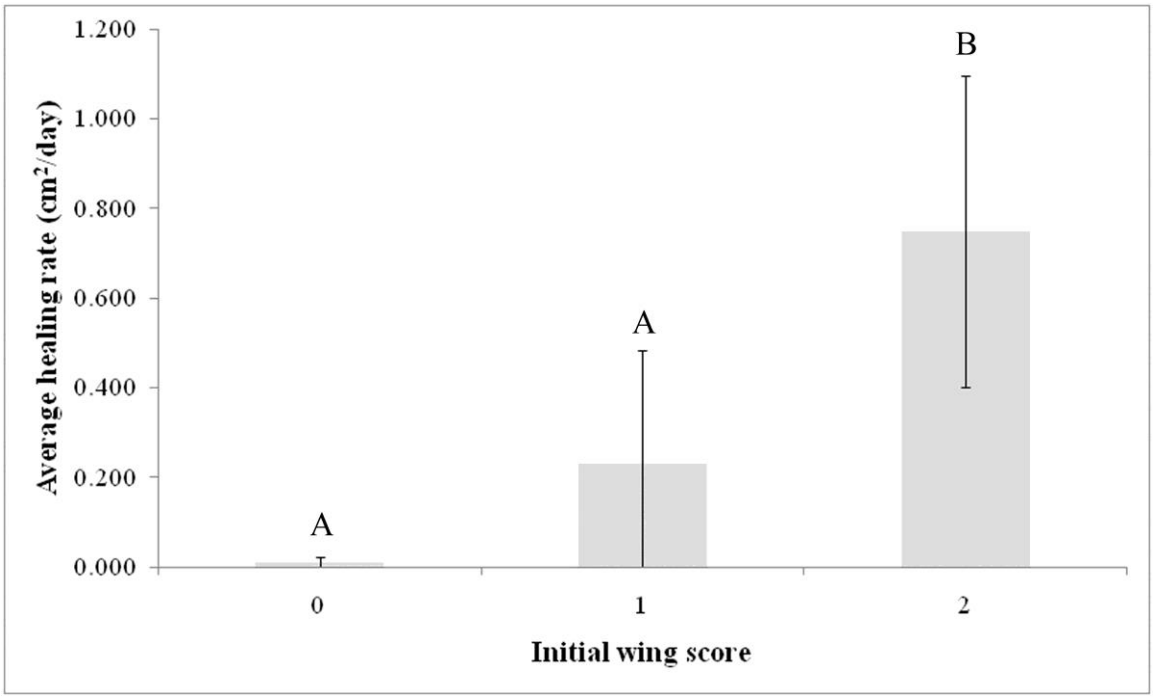

Figure 1.5. Average healing rates (cm^2 per day) of wings of adult female *M. lucifugus* at two summer maternity colonies in New England. Bars labeled with similar letters are not significantly different. Error bars are standard errors.

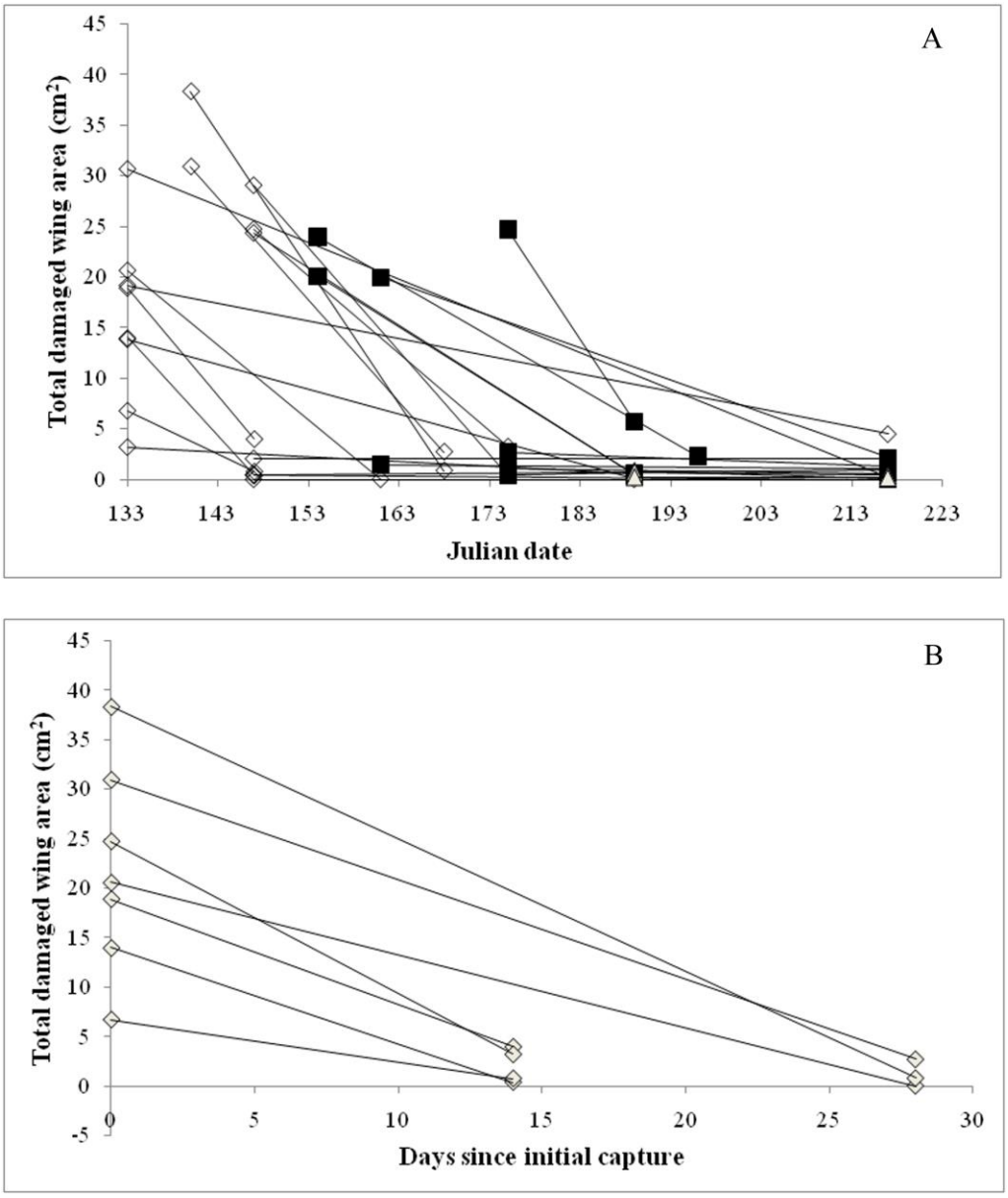

Figure 1.6. Healing rates of adult female *M. lucifugus*. Each connected pair of points represents an individual bat. A. Healing rates of each recaptured bat that was included in photographic wing analysis. Markers represent month of initial capture. May = open diamonds, June = closed squares, July = shaded triangles. B. Healing rates of bats that were initially captured between 13 May 2009 and 27 May 2009.

CHAPTER TWO

SURFACE LIPID PROFILES OF BATS WITH WING DAMAGE CAUSED BY WHITE NOSE SYNDROME

ABSTRACT

White nose syndrome (WNS) is a cutaneous fungal disease of hibernating bats that causes severe wing membrane damage. In surviving individuals, skin damage may change skin surface lipid (SSL) chemical composition or abundance. As SSL are the first barrier against infectious microbes, they function as part of the innate immune system. Thus structural damage may impair immune function and decrease integumentary suppleness and elasticity. Given the extent of wing damage in some bats affected by WNS, we tested the hypothesis that bats with severe wing damage have altered SSL profiles compared to bats with visibly undamaged wing tissue. During the early active season (May to early June) of 2012, we used SebuTape® indicators to collect SSL from *Myotis lucifugus* with and without wing damage. We used high performance thin layer chromatography, gas chromatography/mass spectrometry, and a fluorescent sterol assay to determine SSL composition and broad lipid class ratios for each wing damage category. We found trends of greater free sterol but fewer free fatty acids in wing damaged tissue, but results were not statistically significant. These preliminary results are the first test of sub lethal WNS effects on damaged wing tissue function from wild populations and represent valuable efforts for WNS disease biomarker identification.

While these results do not clarify wing damage roles from WNS in SSL profiles, our study suggests interesting trends for further studies in laboratory infected captive individuals.

INTRODUCTION

Emerging infectious diseases are among the greatest modern threats to biodiversity, causing range reductions and population declines in affected species (Daszak et al. 2000). Among emerging wildlife diseases, pathogenic fungi are among the most devastating and are becoming increasingly prevalent (Fisher et al. 2012). One example is white nose syndrome (WNS), a fungal disease of hibernating bats in North America (Blehert et al. 2009; Lorch et al. 2011). The disease is caused by a fungal pathogen *Pseudogymnoascus destructans* (*Pd*), which causes a cutaneous infection on wings and other exposed skin surfaces of hibernating bats (Minnis and Lindner 2013). Mortality at affected winter colonies are often above 70% and summer populations are correspondingly reduced, as shown by acoustic surveys and evidence from summer maternity roosts (Brooks 2011; Dzal et al. 2011; Reichard et al. 2014). While mortality at hibernacula is quite high, small proportions of affected bats survive. There is little evidence that survivors suffer mortality after arriving at summer maternity sites (Fuller et al. 2011). Instead, surviving bats captured at summer maternity roosts show evidence of WNS (i.e., poor body condition and wing damage) but recover quickly from injuries (Fuller et al. 2011; Meteyer et al. 2011; Reichard and Kunz 2009).

Histopathological studies and visual examination reveals damage caused by *Pd* infection can be quite extensive, thus affecting physiology and the maintenance of

homeostasis (Cryan et al. 2010). Fungal hyphae grow across integumentary surfaces and gather in diffuse foci where the fungus erodes epidermal tissue and infiltrates the dermis, leading to deep wounds on wing surfaces (Meteyer et al. 2009). While pilosebaceous units are not specifically targeted during hyphal invasion, evidence suggests skin appendages (e.g., hair follicles or sebaceous glands) may facilitate dermal infiltration by providing a point of entry into the skin. Epidermal wounds may disrupt the cutaneous water barrier by damaging the lipid rich stratum corneum. Such disruption could result in increased evaporative water loss, which is thought to be directly linked to WNS mortality (Cryan et al. 2010; Warnecke et al. 2013; Willis et al. 2011). While the role of wing lesions as a contributor to WNS mortality has garnered much attention, sub lethal wing damage effects have received little focused study. Wing damage has been shown to negatively affect flight behavior of birds (Swaddle and Witter 1997; Swaddle et al. 1996), insects (Dukas and Dukas 2011; Higginson and Barnard 2004), and is thought to have a similar effects in bats (Voigt 2013). However, subtle changes in physiological function of wings during healing have not been studied. Specifically, changes in skin surface lipid (SSL) profiles during wound healing remain to be elucidated.

SSLs are a complex mixture of molecules comprised of sebaceous gland secretions, epidermal cellular lipids, and their products from microbial or environmental decomposition. SSLs are the initial external barrier against infectious microbes, thus functioning as part of the primary innate immune response (Grice and Segre 2011). SSLs also lubricate hair and integumentary surfaces aiding in skin elasticity and suppleness. Changes to SSL composition may lead to epidermal pathologies, such as scarring

alopecia, folliculitis, and seborrheic dermatitis (Ro and Dawson 2005). Epidermal and cuticular lipid profiles have been studied in many animal taxa, including insects (Gołębiowski et al. 2008), birds (Thomas et al. 2010), and mammals (Nicolaides et al. 1968). Among mammals, SSL profiles vary according to taxonomic grouping and ecological niche.

Normal flexibility and elasticity of wing membrane is vital for proper aerodynamic functioning and flight kinematics. Changes in SSL composition associated with sub lethal wing damage may contribute to morphological changes in wing tissue, such as reducing flexibility and causing skin to peel and flake. Large patches of stiffened, flaky skin on wing surfaces reduces aerodynamic cleanliness, i.e. wing efficiency. A smooth airfoil (i.e., an undamaged bat wing) will experience low turbulence over its surface and thus produce lift more efficiently, whereas an airfoil with a non-uniform surface (i.e., a damaged bat wing) will produce vortices and eddies, generate lift poorly, and thus reduce flight efficiency (Bullen and McKenzie 2007). Such changes to flight aerodynamics and flapping kinematics could cause affected bats to expend additional energy during flight. During a period of low prey availability and existing high energetic costs, additional energetic demands may result in unsuccessful reproduction or additional mortality (Anthony and Kunz 1977).

Bat SSL profiles have only recently been described. Bat SSLs vary little among North American bat species but are highly unique among mammals (Nicolaides et al. 1968; Pannkuk et al. 2014). Bat SSLs are predominantly cholesterol, with moderate amounts of free fatty acids (FFAs), sterol esters (SEs), and wax esters (WEs) (Pannkuk et

al. 2013; Pannkuk et al. 2012). Other lipid groups found in lower amounts include glycerolipids (e.g., triacylglycerides [TAGs]), squalene, ceramides, glycerophospholipids (GLPs), and sphingolipids (Pannkuk et al. 2013).

Given the damage that *Pd* causes during infection, and that sebeacous glands are often filled with fungal hyphae, sebaceous glands on damaged wing tissue are likely nonfunctional. Specifically, WE and TAG output may be dramatically reduced during WNS infection due to sebaceous glands' role as the primary source of these lipids. In addition, epidermal cholesterol synthesis increases when the cutaneous water barrier is disrupted (Wu-Pong et al. 1994). Thus, cholesterol synthesis should be stimulated by wing damage and wound healing.

The purpose of this study was to characterize and compare SSL profiles in active season bats with badly damaged wing tissue (presumably from WNS) and healthy wing tissue. We hypothesized that wing damage due to WNS would stimulate the synthesis of cutaneous cholesterol, thus increasing the proportion of free sterol in SSL. We also expected to collect reduced amounts of WEs and TAGs from bats with severe damage due to loss of function of sebaceous glands. Finally, we hypothesized that FFA acid profiles would vary between bats with wing damage and those without. Understanding the changes in SSL profiles in bats with WNS aids in developing WNS disease 'signatures', biomarker discoveries, and diagnostic advancements. Additionally, SSL profiles may predict species specificity or therapeutic targets, thus contributing to disease management strategies.

MATERIALS AND METHODS

Field sites and bat capture

Surface lipid samples were collected from 245 bats at two sites in Massachusetts (Pepperell and Princeton) and three sites in New Hampshire (Charlestown, Milford, and Peterborough). All roosts are man-made structures that have hosted substantial *M. lucifugus* colonies for at least 10 years. Harp traps were erected at the main exit portal or inside the roost prior to sunset. Bats were removed from the harp trap bag immediately after capture and placed into clean holding bags. Bagged bats were placed into a heated fabric cooler until processing to prevent torpor use and facilitate release. The cooler was heated by a medical heating pad set to medium heat. For each bat we recorded body mass (± 0.1 g), forearm length (± 0.1 mm), and reproductive condition. Both ventral wing surfaces of each bat were photographed using a digital camera (Sony Cybershot model DSC-H55; Sony Corporation, Tokyo, Japan) on automatic settings without flash. A white fluorescent light box was used to transilluminate wings, making damage more apparent. We recorded wing damage index (Reichard and Kunz 2009) and another wing assessment method (see below). Each bat was fitted with a uniquely numbered, lipped aluminum alloy band (Porzana Ltd, East Sussex, UK). Individuals typically were released within one hour of capture. Decontamination guidelines for WNS were strictly followed.

Wing damage assessment

The wing damage index (WDI) methodology developed by Reichard and Kunz (2009) is a rapid and effective means by which to describe wing damage, when used appropriately. However, it is less useful when applied to bats that are captured early in

the active season (throughout May and the first two weeks of June), which is when wing damage is most prevalent and severe, but is also not easily categorized by the WDI scaling metrics. For example, within the first week of the active season, wing damage is little more than small regions of discoloration, which correspond to microscopic lesions (see Chapter 4 of this dissertation and Turner et al. 2014). These regions, which are unaccounted for by WDI score, will become scabs or inflammatory crusts that will eventually form holes or white discoloration. Moreover, the current interpretation of WDI scoring includes a heavy bias toward white discoloration and holes because this is the most common kind of damage seen when the majority of bat field research is conducted. The resulting WDI score thus does not include much usable information about the extent of necrotic tissue. To put it more simply, wing damage assessment using WDI is meant to be a "quick and dirty" field technique that describes wing damage of all kinds. For this chapter, a metric for WNS-specific wing damage and recovery was necessary.

Bats were assigned a broad wing damage category based on WDI and a qualitative assessment of appearance. Bats with extensive crusting and flaking (broadly WDI = 2 and 3) were labeled as necrotic (NC; Figure 2.1A). Bats with less extensive but similar damage (mostly WDI = 2) were sub-necrotic (sNC; Figure 2.1B). Bats with discoloration, but overall normal wing structure (WDI = 1 and 2) were labeled as spotted (SP; Figure 2.1C). Bats with little to no wing damage (WDI = 0) were referred to as healthy (H; Figure 2.1D).

Lipid Sampling

Surface lipids were collected as previously described (Pannkuk et al. In Review). Sebutape® Indicator Strips (cat# S232; CuDerm Corporation, Dallas, TX) were pressed onto the ventral portion of the plagiopatagium for 1 min to minimize stress to the animal (Pagnoni et al. 1994). Tape samples were placed into 3:2 chloroform:methanol (HPLC grade; Fisher Scientific, U.S.A.) with 0.5% butylated hydroxytoluene (to prevent oxidation of fatty acids) in a glass vial with a Teflon® lined cap (Law et al. 1995). Sebutape® was removed within 12 hrs to prevent tape polymer breakdown. Samples were stored in a -20°C freezer until they were shipped on dry ice overnight to the Arkansas Biosciences Institute (Jonesboro, AR), and stored at -20°C until analysis.

Thin-layer Chromatography

Solvent was evaporated under N_2 and lipid residue was reconstituted in 50 µl 3:2 chloroform:methanol for high performance thin-layer chromatography (HPTLC). HPTLC was performed using EMD (Darmstadt, Germany) silica gel plates (20 cm x 10 cm x 200 µm). Plates were washed with 3:2 chloroform:methanol and activated for 10 min at 120°C. Squalene, triolein, cholesteryl palmitate, and stearyl palmitate were used as standards (Nu-check Prep Inc., Elysian, MN). Seventeen lanes were spotted as 8.0 mm bands and 15.0 mm from the edge with a CAMAG Linomat 5 auto-applicator (Wilmington, NC). Seven lanes were a standard serial dilution and the remaining 10 lanes were experimental samples. Non-polar lipids were separated with one run of isooctane:ethyl ether (95:5 v/v) to the top. Plates were sprayed with 3% cupric acetate in 8% sulfuric acid, charred for 20 min (180°C), imaged with a CAMAG TLC Scanner 3, and analyzed with winCAT scanner 3 software (#027.6315).

Gas Chromatography/Mass Spectrometry

Free fatty acid analysis

Fatty acids were analyzed by esterifying free fatty acids to fatty acid methyl esters (FAMEs). FAMEs were prepared by reconstituting in 0.2 ml toluene, 1.5 ml methanol, and 0.3 ml methanol-35% HCl. Samples were mixed and heated overnight at 45°C to complete the transesterification. The next morning, 1.0 ml ultrapure H_2O and 1.0 ml hexane were added and tubes were vortexed for one minute. The top organic layer (with FAMEs) was removed and evaporated under a stream of N_2. Remaining lipid residue was reconstituted in 50.0 µl hexane and placed in a GC vial with a 0.25 ml conical glass insert (Agilent cat.# 5183-2085).

Major FAMEs (i.e., >1% of total) were arcsin transformed and compared between NC and H samples using a Wilcoxon Rank Sum (Mann-Whitney U) test (JMP Pro ver. 11, SAS Institute Inc., Cary, NC).

Lipid Derivatization for Cholesterol Trimethylsilyl (TMS) Ester Analysis

Lipid samples were analyzed for cholesterol amount following previously described methods (Pannkuk et al. 2013). Briefly, sample solvent was evaporated under N_2 and dry lipid residue was heated at 60°C for 30 minutes with 200 µl hexamethyldisilazane (HMDS):trimethylchlorosilane (TMCS):pyridine (3:1:9 v/v/v, Sigma-Aldrich, St. Louis, MO, product # 33038).

GC/MS Settings

FAMEs and TMS cholesterol esters were analyzed on a Varian (Santa Clara, CA) 450-Gas chromatograph (GC) unit equipped with Agilent Durabond HP-88 column (60 m

x 0.25 mm, with a 0.20 µm film thickness) and a Varian CP-8400 autosampler coupled to an ion trap (IT) Varian 240-MS/4000 Mass spectrometer (MS). Operating conditions for GC injector temperatures, transfer line temperatures, EI-MS, manifold, transfer-line, and trap temperatures were performed as previously described (Pannkuk et al. 2014; Pannkuk et al. 2013). Oven temperature program for FAME analysis was 100°C for 1 min, then 100°C to 200°C at 5°C/min; 200°C to 250°C at 20°C/min; and held at 250°C for 1 min. Oven temperature program for TMS esters was 140°C for 2 min, then 140°C to 250°C at 2°C/min; and held at 250°C for 8 min. Both samples were injected as 1.0 µl splitless injections (9.8 ml/min split flow, carrier gas helium, 0.7 ml/min flow rate, 24.0 psi initial pressure, 8.0 min solvent delay). Data was acquired with Varian Workstation Software (version 6; Walnut Creek, CA). Target peaks were identified by reference to an authentic standard and matching electron ionization spectra to the NIST/EPA/NIH Mass Spectral Library (NIST 11) and the NIST Mass Spectral Search Program (Version 2.0f) (Gaithersburg, MD).

Free Sterol vs. Sterol Ester Ratio Assay

Quantitative amounts of free sterol vs. esterified sterol between groups were detected by a fluorometric coupled enzyme assay according to the manufacturer's instructions (Sigma # **MAK043).**

RESULTS

Capture results

We captured 434 bats and collected lipids from 245 individuals. The majority of these bats were adult females. Some juveniles were sampled, but were not included in the analysis because of age-dependent differences in SSL profiles (cite). The number of bats assigned to each wing damage category are as follows: 29 NC, 14 sNC, 102 SP, and 100 H. The severe classification were more prevalent during May and June, whereas the lower damage categories were common later in the season.

Due to the low volume of lipid collected by SebuTape® indicators, samples had to be split to adequately replicate each of the following analyses. Given that this study was partly an exploratory analysis of SSL profiles of bats with WNS, it was necessary to complete an array of analyses to build a more comprehensive picture of SSL profiles.

Thin-layer Chromatography

Thin-layer chromatography of wing SSL showed different patterns from the two damage categories. Healthy tissue had higher amounts of total non-polar lipid than necrotic samples; however, the specific class remains to be elucidated. Healthy tissue indicated presence of non-polar lipid; however, it was below the limit of quantification (LOQ) and scanning densitometry was unable to determine total lipid amount. Non-polar lipid from necrotic tissue was below the limit of detection (LOD) in the three of four samples. One sample was slightly above the LOD. Both damage categories showed a band of unidentified lipid, which we hypothesize are likely wax diesters or triesters. The standard resulted in incomplete separation of the non-polar lipid (e.g., sterol esters, squalene, and wax esters) band (Fig. 2).Considering the LOD for SE and squalene is ~40 ng and ~15 ng for wax esters it is likely all three compound classes contribute to the band

detected in experimental samples (CAMAG app. note #A-89.1). Due to the low total lipid amounts collected by Sebutape® indicators, HPTLC was not sensitive enough to differentiate between lipid classes.

Gas Chromatography/Mass Spectrometry

Fatty Acid Methyl Ester Analysis

There were no differences in the amount of free fatty acids from healthy and necrotic tissues. There were trends for reduced 16:0, 18:0, and 18:2, while 18:1 and 20:0 increased, but no changes were significant (Table 1).

Cholesterol Trimethylsilyl Esters

Total lipid was derivatized as TMS esters and sterol amount was determined for healthy and necrotic tissue. There was no significant difference between sterol amounts from necrotic tissue (1.79±0.6 ng/µl) and healthy tissue (0.38±0.10 ng/µl).($z = 2.78$, $df = 4$, $P=0.18$).

Free Sterol vs. Sterol Ester Ratio Assay

Healthy tissue (n=9), spotted (n=10), subnecrotic (n=5), and necrotic (n=8) were assessed for total free sterol and SE with a fluorescent assay (Fig. 5). Differences in amount of free sterol and SE were not statistically significant (Wilcoxon Rank Sum test; $z = 5.316$, $df = 3$, $P = 0.15$; $z = 2.18$, $df = 3$, $P = 0.53$, respectively).

DISCUSSION

This is the first field-based assessment of changes in wing SSL composition during the healing process after WNS infection. The goal of this study was to test the

hypothesis that wing damage from WNS affects post-hibernation SSL profiles on presumably infected free-ranging bats and to identify possible lipid biomarkers for assessment of wing damage associated with WNS. We found no support for our hypotheses. However, we have provided baseline information on SSL composition of WNS-affected bats. While significant differences were not detected in this study, possible diagnostic techniques were established for further study with higher sample sizes. Sample size was limited because our study was focused on the first month of the active season, a time when colony population sizes are small and sensitive to frequent trapping, but the only time when the most severe class of wing damage is observed (Kunz et al. 2009; Reichard and Kunz 2009).

Given the existing evidence that skin damage can cause changes in SSL composition, it is slightly paradoxical that we did not observe highly significant SSL profile changes in this study. Lipid sampling technique may have played a role in our ability to detect SSL differences in damaged and healthy tissue. SebuTape® is an effective way to gather lipid samples for biochemical analysis, allowing for rapid sample collection without harsh solvent application directly to a target's skin (Pannkuk et al. 2013). This is important when sampling damaged tissue or thin skin membranes that can absorb harmful compounds. SebuTape® also allows for more consistent sampling as each indicator tab samples approximately 1 cm^2. While we made every attempt to sample an area of damage that was similar in size to the indicator pad and representative of the overall pattern of wing damage on each individual, it is possible that damaged tissue and healthy tissue were sampled together, thus providing a non-representative SSL sample. In

addition, SebuTape® Indicator tabs are not adhesive and thus lipid absorption occurs slowly. Another product, SebuTape® Strips have an adhesive backing and thus collect much more of the FFA component from the target tissue (Chapter 4).

Bats begin to heal WNS-damaged wing tissue within a day of emergence from hibernation (Fuller et al. 2011; Meteyer et al. 2011). In natural conditions, a hibernating bat colony takes several weeks to completely disperse from a hibernaculum. Additionally, maternity colonies are comprised of individuals that have come from several different hibernacula, sometimes from great distances (insert distance here), each of which with its own characteristics that can alter the timing of emergence (Norquay et al. 2013). The result is that a sample of bats emerging from a maternity roost on any given night will be comprised of individuals that have left hibernation at different times, and hence be at different stages of wing healing. While there are strong correlations between date and wing condition, with a distinct peak in wing damage in late May (Reichard and Kunz 2009), we cannot discount extensive healing that may have occurred between emergence from hibernation and capture at the maternity roost, and that some bats with what appears to be severe wing damage may be in the final stages of recovery.

Our study suggests that wing SSL profiles may be altered during WNS infection. Such changes could have implications for bats in the early active season, such as increased energetic costs associated with flight and healing. We also provide the first study comparing SSL of bats with WNS wing damage to healthy bats. These data build our knowledge on possible disease biomarkers during WNS progression and may be useful for explaining species-specific disease patterns. Trends revealed by our sampling

of wing SSLs in post-WNS bats must be confirmed by further study. Ideally, a captive longitudinal study in which inoculated or naturally infected bats are monitored throughout the active season would further elucidate our results. Furthermore, broad lipid class contributing to FAME proportions should be determined.

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Table 2.1. Comparisons of fatty acid methyl ester proportions of *Myotis lucifugus* with healthy (*n*=5) and severe (*n*=4) wing damage (Means and S.E.)

Lipid Class	***Healthy***	***Necrotic***	***P-value***	***Z-value***
16:0	0.30 ± 0.02	0.25 ± 0.03	0.33	-0.86
18:0	0.44 ± 0.03	0.41 ± 0.04	0.62	-0.37
18:1	0.06 ± 0.01	0.10 ± 0.04	0.54	0.61
18:2	0.02 ± 0.01	0.01 ± <0.01	0.90	0.12
20:0	0.17 ± 0.03	0.21 ± 0.09	1.00	0.00

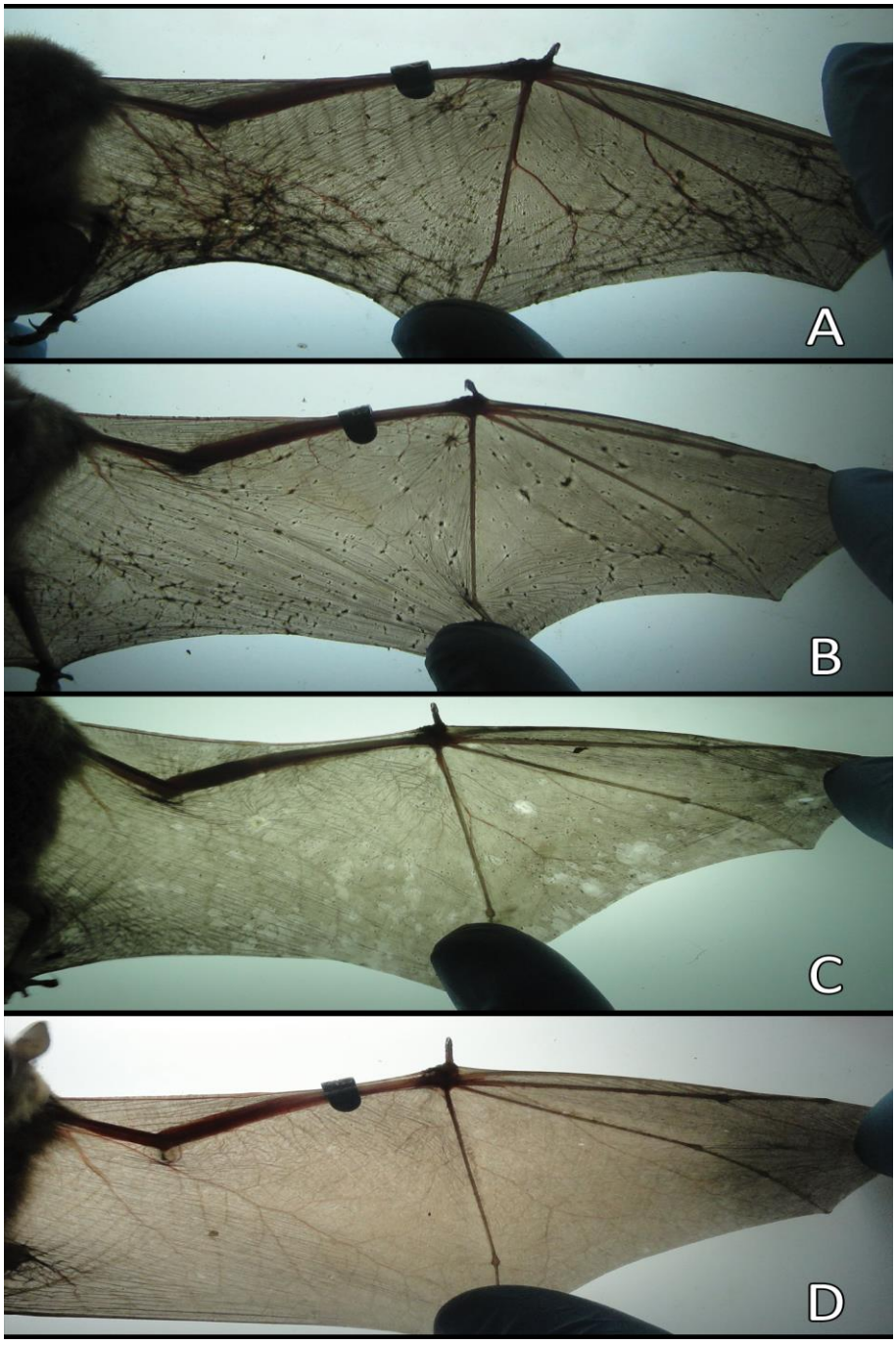

Figure 2.1. Digital photographs of characteristic little brown bat (*Myotis lucifugus*) wing condition. For this study, wing damage was defined in broad damage categories. A. necrotic (NC); B. subnecrotic (sNC); C. spotted (SP); D. Healthy (H).

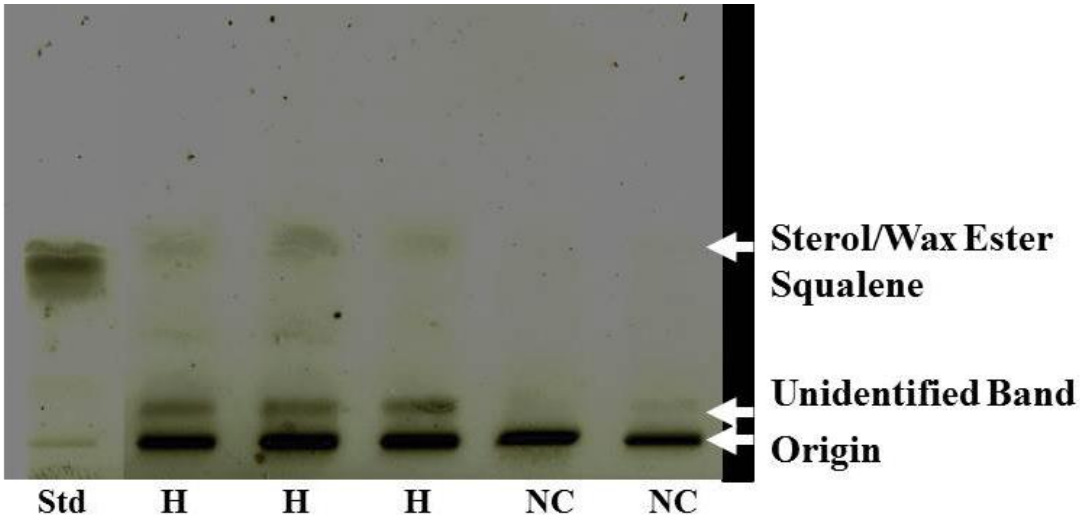

Figure 2.2. High performance thin-layer chromatogram of total lipid extracted from little brown bat (*Myotis lucifugus*) wing tissue (Std=standard, H=healthy, NC=necrotic).

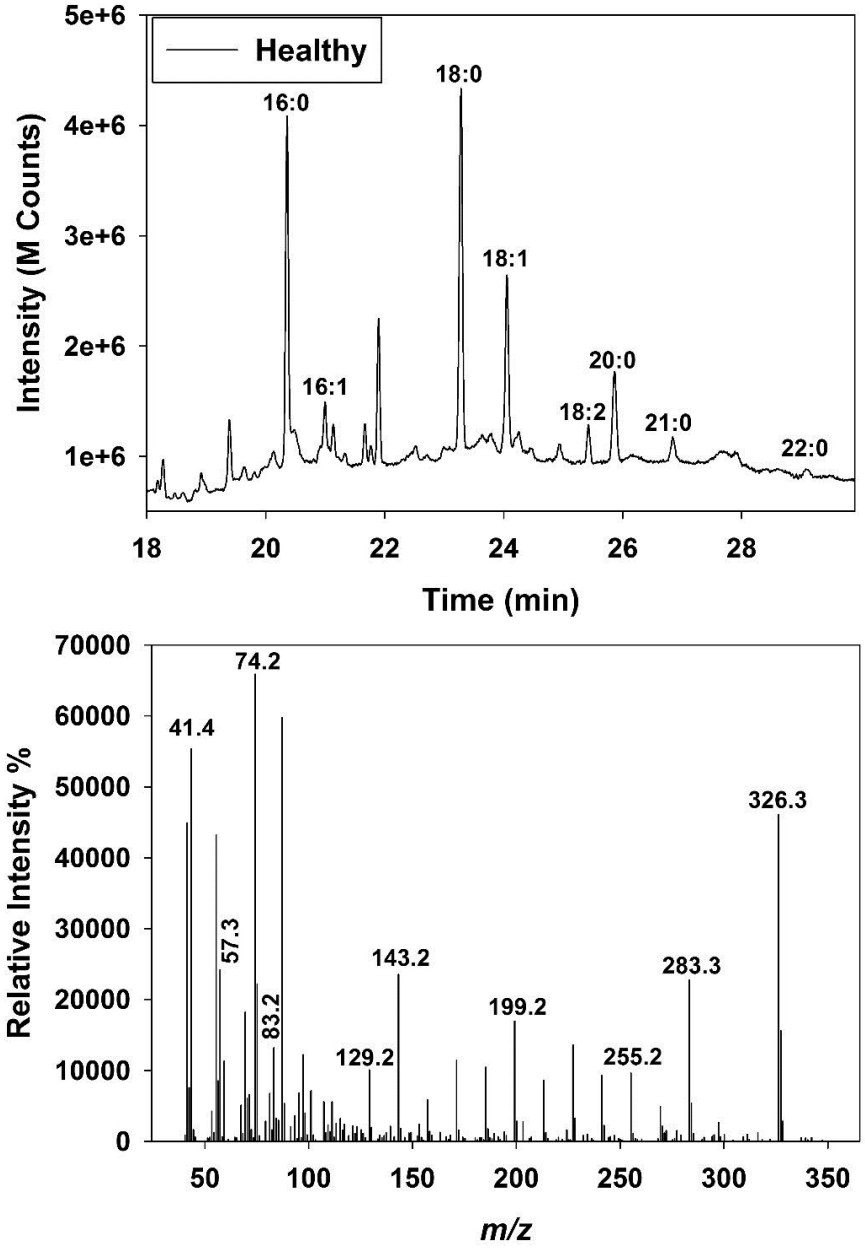

Figure 2.3. Total ion current obtained for *Myotis lucifugus* healthy tissue sebaceous lipid and mass spectrum for 20:0 fatty acid methyl ester.

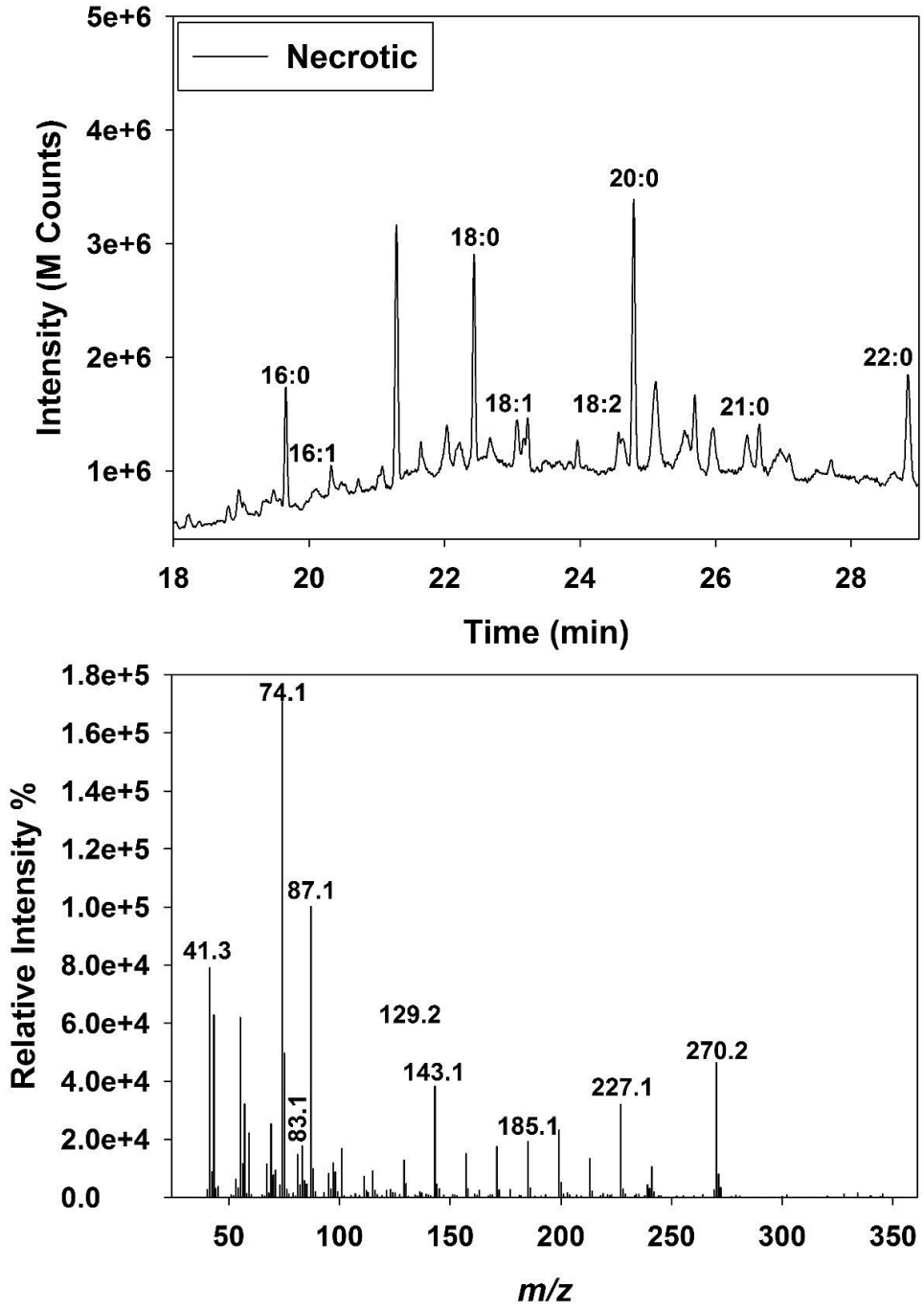

Figure 2.4. Total ion current obtained for *Myotis lucifugus* necrotic tissue sebaceous lipid and mass spectrum for 16:0 fatty acid methyl ester.

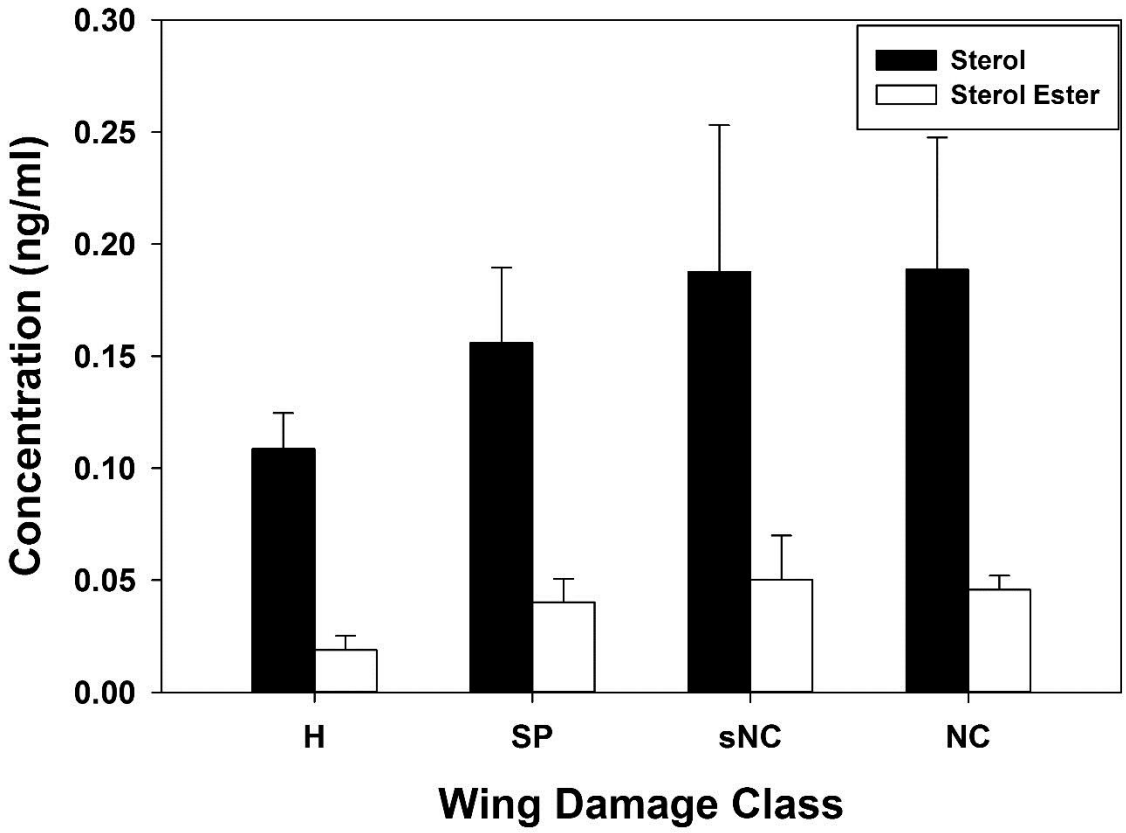

Figure 2.5. Total free vs. esterified sterol in little brown bat (*Myotis lucifugus*) wing tissue (H=healthy, SP=spotted, sNC=subnecrotic, NC=necrotic).

CHAPTER THREE

CHARACTERIZATION OF HEALING PROCESSES IN A CAPTIVE COLONY OF LITTLE BROWN MYOTIS (MYOTIS LUCIFUGUS) RECOVERING FROM SUBLETHAL INJURY FROM WHITE NOSE SYNDROME

ABSTRACT

White nose syndrome (WNS) is thought to have killed at least 6 million bats since 2006 and has spread halfway across North America. The most heavily impacted species is the little brown myotis (*Myotis lucifugus*), populations of which has declined precipitously in the last decade. While understanding population fluctuations and mortality resulting from WNS, it is equally important that researchers consider remnant bat populations, and what processes occur in surviving bats. I observed a captive colony of little brown myotis as they recovered from natural manifestations of WNS. Supportive care was provided to this colony, including food, water, electrolyte and vitamin supplements. I quantified recovery by monitoring the following morphological and physiological variables: body mass, body temperature, wing damage amount (number of lesions) and extent (total damaged area), skin surface lipid profiles, fungal load, and collected wing biopsies for histopathlogical analysis. I found that recovering bats gain body mass quickly after emerging from hibernation. Wing damage analysis revealed four visually distinct forms of wing damage. Wing damage visible with UV illumination is most widespread at the beginning of recovery, whereas wing damage visible under white fluorescent light illumination is not visually apparent until after bats have been euthermic for several days, and rises to peak levels several weeks later. Skin surface lipids, meanwhile, transition from a signal typical

of *Pd* (dominated by unsaturated fatty acids) to a signal more typical of bats (dominated by saturated fatty acids). Histopathology and qPCR analysis of fungal load reveal that fungus is rapidly cleared within a week from emergence, yet remains detectable on wing tissue for long periods after emergence from hibernation. I conclude that recovery is a complex process that may lead to increased energetic demands during the initial weeks of the active season due to changes in flight mechanics, wing aerodynamics, reduced foraging efficiency, and increased energetic investment in healing processes. I provide the first detailed description of recovery phase processes and discuss ways that recovery research may inform future research into the broader effects of WNS.

INTRODUCTION

From its initial observation in New York State during the winter of 2006/7, white nose syndrome (WNS) has spread throughout karst areas of eastern North America (Blehert et al. 2009; Gargas et al. 2009). WNS has been confirmed in 9 species of bats in 26 states and 5 Canadian provinces. The causative agent of WNS, a psychrophilic fungus *Pseudogymnoascus destructans (Pd),* is endemic to Eurasia, where it infects several species of hibernating bats during the winter (Leopardi et al. 2015; Puechmaille et al. 2010; Wibbelt et al. 2010). However, European bats do not suffer ill effects from infection, an intriguing difference between North American bats and European bats (Puechmaille et al. 2012). The most heavily impacted species is the once common little brown bat (*Myotis lucifugus)* (Frick et al. 2010; Langwig et al. 2012), which is the focus of this chapter. Populations of this once nearly ubiquitous species have declined

approximately 70% in areas affected by WNS for more than 3 years (Brooks 2011; Dzal et al. 2010; Turner et al. 2011).

Disruptions of normal physiological processes during hibernation, such as higher than usual evaporative water loss, are hypothesized to be the source of increased mortality associated with white nose syndrome (Cryan et al. 2010; Verant et al. 2014; Warnecke et al. 2013). During hibernation, *Pd* erodes wing tissue in susceptible species such as little brown myotis. Fungal DNA is detected on bats very soon after the start of hibernation, which implies that skin lesions appear soon after hibernation begins (Langwig et al. 2015). These lesions perforate the epidermis, and can cause damage to the underlying dermal layer (Meteyer et al. 2009). Combined, such damage may disrupt the cutaneous water barrier, such that affected bats lose a relatively large volume of body water during periods of inactivity. As a result, bats may arouse from hibernation more often than normal to drink. However, water in caves most likely does not contain high concentrations of electrolytes or minerals and repeated consumption of unenriched water may result in hypotonic dehydration, which exacerbates the need to arouse and balance body chemistry. An alternative but related hypothesis suggests that hibernation metabolic rate is increased by some mechanism related to *Pd* infection (Verant et al. 2014; L.P. McGuire, unpublished data). Higher metabolic rates result in greater energy consumption and higher CO_2 production; however, because of the unique metabolic conditions of hibernation, bats are unable to expel excess CO_2, which builds and causes acidosis and thus more frequent arousals. Such a cascade establishes a positive feedback of greater energy consumption due to frequent arousals, and increased water loss, which

may lead to dehydration. Whatever the mechanism, it's becoming apparent that physiological disruption typifies WNS and drives premature consumption of energy stores and ultimately death. (Lorch et al. 2011; Reeder et al. 2012; Verant et al. 2014; Warnecke et al. 2012; Willis 2015; Willis et al. 2011).

While a majority of little brown bats with WNS die, a small proportion survive (Dobony et al. 2011; Fuller et al. 2011; Reichard and Kunz 2009; Reichard et al. 2014). It is currently unknown what traits contribute to the survival of these individuals. Hypotheses range from behavioral adaptations, to genetically conferred resistance, to luck. Survivors of the disease, however, are also likely to be infected with *Pd*. The majority of bats in affected hibernacula test positive for *Pd* (Langwig et al. 2015), and many individuals show signs of developing wing damage (Turner et al. 2014). In the weeks following arousal from hibernation, wings of affected bats develop additional wing damage that is not present during hibernation (Meteyer et al. 2012; Meteyer et al. 2011), such as flaky and necrotic spots. Some individuals have small holes in their flight membranes. Others show signs of large holes or dramatic loss of flight membranes (Fuller et al. 2011; Reichard and Kunz 2009).

The effects of wing damage on an individual bat's overall health will likely depend on the nature and extent of damage. While loss of flight membrane surface area is the most striking form of damage, extensive membrane loss from WNS is not common and can heal quite quickly (Church and Warren 1968; Davis 1968; Davis 1972; Fuller et al. 2011; Iversen et al. 1974; Meteyer et al. 2011; Pollock et al. 2015; Weaver et al. 2009). Instead, the dominant type of damage from WNS is areas of inflammatory

crusting and flaking and subsequent wound contraction and scarring, which affect wing membrane flexibility, and thus may cause changes to flight (Meteyer et al. 2012). Bat wings are highly elastic and pliable, which is vital to proper wing function (Cheney et al. 2015; Crowley and Hall 1994; Findley et al. 1972; Studier 1972; Swartz et al. 1996). Depending on the extent and location of damaged areas, flapping mechanics and aerodynamic cleanliness (a measure of airfoil efficiency) could be altered due to reduced membrane flexibility and reduced smoothness of flight surfaces (Bullen and McKenzie 2009; Bullen and McKenzie 2008; Swartz and Konow 2015). In addition, in areas where *Pd* has deeply eroded wing tissue, small wing hairs that provide bats with important flight information (speed, direction of airflow, etc.) could be lost or made nonfunctional (Chadha et al. 2012; Dickinson 2010; Marshall et al. 2015; Sterbing-D'Angelo et al. 2011). Skin glands that secrete lipids onto the skin surface are also vital to proper wing function because they lubricate and moisturize wing tissue. Non-functional wing glands may exacerbate the negative consequences of wing damage and delay healing. Past studies have shown that bats with WNS have surface lipid profiles that are dissimilar to those of healthy bats (Pannkuk et al. 2015). However, despite the likely fitness implications of these effects, they have not been subjected to detailed study.

Much of the existing literature on non-lethal injury and recovery in free-ranging animals focuses on acute damage, such as limb loss, or autotomy, such as strategic tail loss. Such injuries are common in nature, and can be ecologically significant. For example, there is evidence that failed predation attempts may result in limb deformities in frogs (Bowerman et al. 2010). Such effects have long-term consequences for fitness and

survival due to reduced mobility. Studies on chronic, long-lasting injury often focus on irreparable injury, such as wing wear in bumblebees or feeding apparatus wear in insects (Arens 1990; Cartar 1992; Roitberg et al. 2005). Bats with WNS provide an interesting model through which to understand sub-lethal injuries. Wing damage is an acute injury in that it appears suddenly and increases in severity quickly. The effects of wing damage may be chronic, however, in that the healing process may take over a month, may not reach completion, and can recur each year that a bat hibernates within an infected hibernaculum.

While wing damage may have direct effects on flight mechanics, additional consequences of WNS with negative fitness effects may also exist. WNS has been shown to alter levels of inflammatory promoter cytokines (Moore et al. 2013) that are known to induce sickness behaviors, such as anti-social behavior, anorexia, or lethargy (Johnson 2002), which may play a role in the recovery and long-term survival of affected individuals. Upon emergence from hibernation, little brown bats make regional migrations that can cover Up to several hundred kilometers (Davis and Hitchcock 1965; Fenton 1969; Norquay et al. 2013). Few studies have explored these regional migrations, but there is little evidence that bats will cease migratory movements to feed (McGuire et al. 2012; J. Chenger, pers. comm.). Thus, migrations must be powered solely by stored fat reserves. Bats with WNS have little fat left by the end of hibernation (Reeder et al. 2012), and this may either increase mortality for migrating individuals or perhaps preclude the normal post-hibernation migration. Those that survive until arrival at a summer roost must quickly gain fat during a time when ambient temperatures and insect

abundance may be low (Anthony and Kunz 1977). Sickness behaviors triggered by latent immune function may prevent bats from roosting normally or feeding effectively. A previous study on roosting ecology of tropical bats suggests that bats which roost in groups have stronger immune response than those that roost singly (Schneeberger et al. 2013), likely because social animals such as bats are stressed when separated from roostmates. Thus, when bats exhibit sickness behaviors that cause them to roost alone rather than with other members of a colony, they may be less capable of clearing *Pd* infection or opportunistic infections by other microbes. In addition, roosting alone leads to increased thermoregulatory needs and potentially more torpor use, which can stunt immune responses.

To better understand how bats recover from WNS, I characterized the healing process in wild caught bats that had been naturally infected with *Pd* and were thought to have experienced at least one hibernation season with WNS. Bats were captured in the field but rehabilitated in a controlled, captive setting and observed for 40 days post-emergence from hibernation. I monitored changes in mass, wing damage, fungal load, and skin surface lipid profiles. I collected wing biopsies to monitor changes in lesion structure and infection through histopathological examination. Finally, I attached skin temperature loggers to each individual to determine activity levels and incidence of torpor during the recovery phase. This study seeks to further elucidate an understudied portion of bat life history, (i.e., what happens in the weeks immediately following hibernation) and provides critical information about an unknown phase of WNS that we must understand before comprehensive conservation efforts can be employed. The

primary goal was to describe changes in wing damage, mass, and signals of infection (*Pd* detected on skin and surface lipid profiles). I also sought to determine when bats appear to be free of effects from WNS following emergence from hibernation.

Methods

I collected male little brown bats (*M. lucifugus*) from sites in Canada that were WNS-positive for 3-5 years. I collected from the Hunt Mine (Renfrew County, Ontario; Fenton 1969; positive for WNS in 2010/2011), Laflèche Cave (Argenteuil County, Québec; positive for WNS in 2009/2010), and High Rock Cave (Québec; positive for WNS in 2010-2011) at the end of hibernation (April 28, 29, 30, respectively) (Fig 1). Prior to collecting bats, a survey was conducted to establish a population estimate of each cave. As stipulated in our permitting agreements, no more than 10% of the total hibernating population was removed from each cave. This amounted to 50, 5, and 4 bats from Hunt Mine, Laflèche Cave and High Rock Cave, respectively. Of all bats handled, less than 1% were female, suggesting that most females had already departed from the hibernacula (Fenton 1969; van Schaik et al. 2015). Thus, my pre-collection surveys provided conservative estimates of hibernating population sizes.

I removed torpid bats from their roost by hand, noting the size of the cluster in which the bat roosted. Female bats were immediately returned to their original cluster. I placed bats into new, individually numbered cloth holding bags and moved to the entrance of the hibernaculum to process the animals away from the hibernating colony to minimize disturbance. I collected the following measurements for all bats: mass (±0.1 g),

forearm length (±0.1 mm), wing damage index score (Reichard and Kunz 2009) (including a digital photograph of each wing), hibernating cluster size, and whether collected bats showed visual signs of *Pd* infection (i.e., white fungal growth on exposed skin surfaces of tail and wing membranes and ears). It is not possible to determine age at that time of year. To confirm *Pd* infection, I collected swabs for qPCR analysis (details below).

Each bat was fitted with a uniquely numbered skin temperature data logger (iButton). Data loggers were removed from their original metal casings and coated in plastic to reduce mass (Reeder et al. 2012). I trimmed a small patch of fur in the intrascapular dorsal region and affixed the iButton with ostomy cement (Osto-Bond®, Montreal Ostomy Inc., Vaudreuil-Dorion, QC, Canada; Figure 2). I held the bat in a rolled cotton bag to allow the cement to set. My collaborator (LPM) calibrated iButtons by placing them in a temperature controlled cabinet and gradually decreasing the temperature in 5°C increments. For each increment, iButtons were allowed to equilibrate overnight and the temperature was changed the next morning. Recorded temperatures were compared to known cabinet temperatures and a standard curve was made for each iButton and individually applied as a correction.

After I fully processed each bat, I placed two or three individuals in a uniquely labeled cotton bag. Bats are less stressed during transport when they are allowed to cluster with other individuals (C. Willis pers.comm). I placed the bags into biosecure, hepa-filtered animal carriers (Taconic Transit Cage™, Taconic Biosciences, Inc., Hudson, NY, USA) stored in a temperature and humidity controlled cabinet during

transport to the University of Winnipeg. Upon arrival at Winnipeg, bats were removed from their holding bags, weighed again, and banded. Bats lost 1.54 ± 0.42 g mass during transport. Twenty-five bats died during transport, likely due to stress related to long-distance transport and an already weakened state due to WNS. Surviving bats were released into a flight chamber with a natural light cycle for the region (11:13 light:dark). The flight chamber (2.24m length, 1.01 m wide x 2.42 m high) was constructed of aluminum and fine mesh porch screening. The chamber contained a free-standing, wooden bat box (44 cm x 6.3 cm x 60 cm) containing an integrated heating coil, which maintained an inside temperature of 30°C. To provide a variety of roosting locations, a bundle of towels was suspended on the wall opposite from the bat box. Ambient conditions for the room were maintained at 18°C and 60% relative humidity. This arrangement allowed bats to use facultative daily torpor over a range of temperatures and roosting substrates. We also provided a heated environment to provide bats with an area in which *Pd* would not propagate (i.e., temperatures greater than 20°C, (Verant et al. 2012)). During the initial days of the study, bats were hand fed mealworms, offered mealworms from petri dishes and offered water from droppers. Bats were also offered Pedialyte® (Abbott Nutrition, Abbott Park, Il, USA) and Nutrical® (Tomlyn Veterinary Science, Fort Worth, TX, USA) as supplements. I monitored consumption and hand feeding was reduced to facilitate the transition to self-feeding. Once the colony was self-feeding, mealworms and water were provided *ad libitum*. An additional 17 bats died during the initial five days in captivity. Four bats died between Day 5 and the end of the

study. This mortality was likely due to latent effects of WNS, as bats were fed by hand and provided with food, water, and a heated roost.

Monitoring and analysis

We visually monitored bat health and condition daily. Various measurements and samples were taken at different intervals as follows and as detailed in Table 1:

Body temperature

iButton data loggers were removed from the bats 14 days into the study. Body temperature data were downloaded from functional iButtons using OneWire Viewer (ver. 2.1, Maxim Integrated, San Jose, CA, USA).

Body Mass

I recorded body mass (±0.1g) twice daily during the first two weeks of captivity. These frequent measurements were made to ensure that bats were gaining or at least sustaining body mass and to inform interventions (e.g., increased monitoring, isolation, or euthanasia), if needed. Bats that lost mass were placed in isolation and hand fed until they began to gain mass. After this initial period, I recorded body mass every other day, allowing me to monitor recovery while also limiting disturbance. For data analysis and plotting I used the mean value on days with two body mass measurements.

Wing photos

I captured digital photographs to document changes in wing damage during the recovery process. I photographed bats with white fluorescent illumination 15 times (Table 1) and with UV illumination 8 times (Table 1) following techniques established by Reichard and Kunz (2009), and modified by Fuller et al. (2011) for visible "white" light (approx. 390-700 nm) and by Turner et al. (2014) for the ultraviolet (UV) spectrum (<400 nm). I held bats over the light source with wings outstretched and photographed the trans-illuminated ventral surface using a tripod-mounted digital camera (Sony Cybershot, model DSC-H55; Sony Corporation, Tokyo, Japan). A 2 s shutter delay was implemented to allow the camera to stabilize before each image was captured. Each photo contained a label identifying the bat and an object to provide scaling information (see Figures 3 and 4). For white fluorescent light, I used a segment of a ruler that was glued to the surface of the light box. For UV light, I used an object that would not fluoresce but could be easily seen and measured- a Canadian $1 coin (26.5 mm), a metal washer (38.1 mm), or a histology cassette sponge (25.0 mm).

I documented four visually distinct kinds of damage based on photo analysis. Damage that was apparent using white fluorescent illumination was categorized as either black lesions or white spots (Figure 5). Illumination with UV light revealed areas of orange fluorescence and teal fluorescence (Figure 6). In some cases, the different types of lesions overlapped, Black, orange, and teal lesions were sometimes associated with one another; however this association was not necessarily robust. Orange lesions often existed unassociated with other lesions. Teal lesions were more often associated with black lesions, but this was not always the case. White damage, however, developed after black

lesions and often began to appear as a halo of discoloration before black lesions were no longer apparent. To determine the amount (number of lesions) and extent (total damaged area) of each type of wing damage, I used image analysis software (ImageJ, ver 1.47; United States National Institutes of Health; http://imagej.nih.gov/ij) following techniques established by Fuller et al. (2011). To maintain consistency among photos I assessed damage to the plagiopatagium, which was always well illuminated and not obscured by researcher fingers.

I traced lesions manually using a Wacom Co, Ltd (Kazo, Saitama, Japan) Bamboo tablet and stylus (model CTH-460) and recorded results in ImageJ's region of interest (ROI) manager. Wound tracing was done by a single observer (NWF) to maintain consistency in identifying and highlighting damage. I recorded the number of lesions and area of damage for each photo. I calculated the average damage from both wings for analysis, or used a single wing when image quality on the opposite wing was not sufficient for analysis.

Black, teal, and orange wing damage was typically associated with small point lesions that did not dramatically vary in size over time. Therefore analysis of black, teal, and orange damage was based on the number of lesions. White damage was associated with larger areas which grew over time and often ran together, making precise counting difficult. Therefore analysis of white damage was based on total area.

qPCR measurement of fungal load

To determine the number of infected individuals and to characterize fungal load (amount of pathogen), I collected six swabs from each bat for qPCR analysis of fungal DNA, once at capture and then five more times at subsequent time points (Table 1) coincident with histological sampling. I followed standard swabbing protocols e.g., (Janicki et al. 2015; Langwig et al. 2012) and stored swabs in RNA Later at -20°C. Swabs were sent to Dr. Jeff Foster at Northern Arizona University for qPCR analysis. Briefly, samples and standards were extracted using DNeasy Blood and Tissue extraction (Qiagen, Valencia, CA). The extraction was modified for fungal DNA by adding lyticase during lysis. Cycle threshold (Ct) values above 40 was considered a positive *Pd* detection. The mean fungal load from both replicates was used in all analyses (Muller et al. 2013; Shuey et al. 2014). Fungal load was calculated from a standard curve:

$$(fungal\ load = 10^{\frac{22.04942 - observed\ C_t\ value}{3.34789}})$$

derived from a serial dilution of standard *Pd* isolate 20631-21. All samples were run in duplicate.

Surface lipids

I collected lipids following Pannkuk et al. (2014). Samples were collected five times from each individual (Table 1). Briefly, I selected a location on the plagiopatagium by targeting an area of UV fluorescence. I affixed a strip of SebuTape® (CuDerm Corporation, Dallas, Texas, USA), a lipid-binding polymer that is used to collect and store surface lipid samples. The SebuTape® was left adhered to the wing for 1 minute

and then removed with forceps. I placed the strip into a 4 ml cryovial with a Teflon® lined cap (Fisher Scientific, Pittsburgh, PA, USA) containing approximately 2 ml of 3:2 chloroform:methanol with 0.1% butylated hydroxytoluene (VWR International, Radnor, PA) to prevent oxidation of lipids. The vials were then held at -20°C for 12 h after which I removed the SebuTape® strips and stored the samples at -20°C for up to 30 days. Samples were shipped on dry ice overnight to the Arkansas Biosciences Institute (Jonesboro, AR), and stored at -20°C prior to analysis.

Fatty acids were analyzed by esterifying free fatty acids to fatty acid methyl esters (FAMEs) by reconstituting in 0.2 ml toluene, 1.5 ml methanol, and 0.3 ml methanol-35% HCl. Samples were mixed and heated overnight at 45°C to complete the transesterification. The next morning, 1.0 ml ultrapure H_2O and 1.0 ml hexane were added and tubes were vortexed for 1 min. The top organic layer (with FAMEs) was removed and evaporated under a stream of N_2. Remaining lipid residue was reconstituted in 50.0 µl hexane and placed in a gas chromatography vial with a 0.25 ml conical glass insert (Agilent cat.# 5183-2085).

FAMEs were quantified on a Varian (Santa Clara, CA) 450-Gas chromatograph (GC) unit equipped with an Agilent Durabond HP-88 column (60 m x 0.25 mm, with a 0.20 µm film thickness) and a Varian CP-8400 autosampler coupled to an ion trap (IT) Varian 240-MS/4000 Mass spectrometer (MS). Operating conditions for GC injector temperatures, transfer line temperatures, EI-MS, manifold, transfer-line, and trap temperatures were performed as previously described (Pannkuk et al. 2014; Pannkuk et al. 2013). The oven temperature program for FAME analysis was 100°C for 1 min, then

100°C to 200°C increasing at 5°C/min; 200°C to 250°C at 20°C/min; and then held at 250°C for 1 min. Samples were injected as 1.0 µl splitless injections (9.8 ml/min split flow, carrier gas helium, 0.7 ml/min flow rate, 24.0 psi initial pressure, 8.0 min solvent delay). Data were acquired with Varian Workstation Software (version 6; Walnut Creek, CA). Target peaks were identified by reference to an authentic standard and matching electron ionization spectra to the NIST/EPA/NIH Mass Spectral Library (NIST 11) and the NIST Mass Spectral Search Program (Version 2.0f, Gaithersburg, MD). Proportions of major fatty acids (>1% of total) were arcsin square root transformed for analysis.

Histopathology

I developed a novel method to conduct histopathological analysis of wing tissue biopsies, rather than lethal sampling required for analysis of the full wing. I collected wing biopsies from each bat twice, once from each wing and at an interval of one to two weeks apart. For example, the left wing of individual 0809 was sampled on May 9 and the right wing on May 27. The subset of bats sampled on each sampling day was determined randomly. I located a suitable biopsy location by illuminating the wing with UV light from the dorsal side (Turner et al. 2014). I targeted bright areas of fluorescence (Fig 7). To prevent contraction of biopsied tissue, the dorsal side of the wing was lightly moistened with water using a cotton swab to adhere a small nitrocellulose filter (0.22 µm pore size; Millipore Corporation, cat# GSWP 013 00). The tissue was then excised using a 6 mm biopsy punch (Tru-Punch^{TM}, Sklar Corp., West Chester, PA, USA) and placed into a round histology cassette containing histology sponges. A stainless steel washer in

the cassette caused the cassette to sink when placed in a vial containing 10% formalin. Biopsies were fixed in formalin for at least 48 h, but some remained in fixative for up to 2 weeks. I dehydrated, cleared, and embedded biopsies with paraffin using standard procedures (Meteyer et al. 2009; Reeder et al. 2012; Warnecke et al. 2012). I aligned biopsies in embedding molds so that the resulting sections would be lateral transects of tissue. I sectioned samples in groups of four 7μm thickness sections at 100μm intervals until the remaining sample was too thin to provide meaningful sections. As sections were cut from the block, they were floated in warm water and picked up with a Fisherbrand™ SuperFrost™ Plus (Fisher Scientific, cat# 12-550-15) slide, using as many slides as necessary per sample. Slides were allowed to air dry before being placed onto a slide warmer overnight. I modified an established protocol for Periodic acid-Schiff (PAS) staining for *Pd* (Meteyer et al. 2009). Instead of hematoxylin and eosin counterstain as used in other studies, I counterstained with light green (Electron Microscopy Sciences, cat# 17920). Light green provides more contrast that makes the magenta coloration of PAS-positive regions more apparent.

I used a compound light microscope (Model CM E, Leica Microsystems, Buffalo, NY, USA) to evaluate each slide for three variables: amount of fungus, number of cupping erosions, and proportion of inflammatory crust on the skin surface. From each set of 4 sections, I selected the most suitable (e.g. flatness, stain clarity, no air bubbles) for further evaluation. I determined amount of fungus by visually dividing the section into 10 roughly equal portions and counting the number that contained fungal hyphae. I counted the number of cupping erosions, a diagnostic feature of WNS (Meteyer et al.

2009; Meteyer et al. 2011), present on each section. Cupping erosions were defined by loss of epidermis and clear infiltration of fungal hyphae into the dermis, leading in some cases to perforations of the wing. I quantified inflammatory crusts by estimating the proportion of the section in which inflammatory crust was the major surface feature. Identification of these features following established methodology (Meteyer et al. 2009). Due to low sample sizes and zero-inflated data resulting from infrequent sampling of individuals, I did not perform statistical analyses on the histology data.

Statistical analyses

All statistical analyses were performed in R (ver. 3.1.1). Given non-linear relationships and violation of independence due to repeated measures, conventional statistical techniques (i.e., MANOVA or multiple regressions) were not appropriate. Thus, I used generalized additive models (GAMs) to describe patterns in my data, including individual as a random effect. In all cases, I included day of measurement (time) as the smoothing term. I attempted to add other terms to the models following an information theory approach to select the best model. For each variable (i.e., mass, black lesions, orange lesions, teal lesions, white damage, and fungal load), I added additional combinations of variables to examine the effect on model strength. These combinations included ‘base’ (all forms of wing damage plus fungal load and mass), ‘UV’ (orange and teal lesions), ‘fungus’ (orange lesions and load), ‘early wing damage’ (orange, teal, and black lesions), ‘late wing damage’ (white damage area), ‘mass’ (mass only), and ‘time’ (time only). The variable in question was removed from the model when necessary (e.g., orange lesions

were removed from ‘UV’ for the orange lesion model). In all cases, no models explained more variation than time, or suggested biologically irrelevant effects (e.g., surface lipids predicting body mass) and thus their inclusion in the models was not justified. Thus, time alone was used for all models. Furthermore, the goal of this study was to provide descriptive analysis of recovery events. Thus, complex statistical analyses to identify predictors of recovery are not needed.

To determine differences between study days, I used pairwise Kruskal-Wallis tests. I compared all days to the initial measurement day for each variable. The initial measurement day was not the same for each variable; see Table 1 for starting days. I made these comparisons because this study is an exploratory examination of the WNS healing and recovery timeline, and comparing subsequent days to the initial day allowed me to determine times of peak damage quantitatively.

I used principal component analysis (PCA) to elucidate the relative contributions of a number of variables to the temporal patterns revealed in my analysis. I used PCA in three different ways. First, I used PCA for data reduction in skin surface lipid profiles, including all lipids that comprised more than 1% of the total lipids for each sample. Similarly, I used PCA for data reduction of wing-damage variables. The four classes of wing damage (teal lesion count, orange lesion count, black lesion count, and white damage area) were included in this second PCA. The final PCA included body mass, *Pd* load, and the individual scores for principal components 1 and 2 for both the lipid and wing-damage PCAs. Including all raw data in a single PCA would result in over-

emphasis of lipid variables (7 of 13 variables total) and wing damage variables (4 of 13 variables). The final PCA can be considered an integrated measure of healing.

RESULTS

Body Temperature

Data on body temperature are not reported because the iButton failure rate was greater than 50%, which resulted in only 5 usable data sets. Variation in individual body temperature profiles was high, thus no general conclusions about torpor use could be made.

Mass

Individuals gained body mass rapidly during the study and plateaued at approximately 9 g on Day 24. Mean mass gain was 3.8 ± 0.77 g. Daily mass was significantly higher than arrival mass after Day 3 (Kruskal-Wallis Test, $H = 8.0134$, $df = 1$, $p = 0.0046$) of the study. On Day 17 a lighting malfunction, in which the flight chamber lights did not turn off overnight, resulted in bats not feeding normally for one evening and thus resulted in a loss approximately 1 g each (mean loss 1.3 ± 0.25 g). I modeled mass gain using generalized additive models, using time as a smoothing term. The smoothing term was highly significant ($p < 0.0001$) and explained 72.6% of the variation observed in mass increase (Fig 8).

Wing damage

Each type of visually distinct wing damage had a unique temporal trend (Figs. 9 and 10). Black, orange, and teal lesions each began at high levels and declined by the end of the study. Peak abundance for orange and teal lesions was at the start of the study (Day 5), whereas black lesions were in low abundance at the start of the study, and rapidly increased to peak abundance two weeks into the study. White discoloration was not apparent at the start of the study and was not present in high abundance until after peak abundance of black lesions. This trend implies that white damage is a result of black lesions and healing processes related to black lesion clearance.

For both orange and teal lesions, abundance of lesions was highest on the first day of the study, in this case Day 5. Orange lesion count on Day 5 was significantly higher than Day 7 – 40 (Kruskal-Wallis test, $H = 7.0726$, $df = 1$, $p = 0.0078$). Teal lesion count on Day 5 was not significantly different from Days 7 or 14 (Kruskal-Wallis test, $H = 0.0097$, $df = 1$, $p = 0.9214$; $H = 3.3901$, $df = 1$, p 0.0656, respectively), but was significantly higher on Day 5 than on Day 16 (Kruskal-Wallis test, $H = 6.244$, $df = 1$, $p = 0.0125$), and each day thereafter. The highest orange lesion count was 167 for individual 0813 on Day 5. The lowest orange lesion count was zero for all bats on Day 40. Peak teal lesion count was 74 for individual 0812 on Day 5. Minimum teal lesion count was zero for all bats on Day 40 (Figure 9).

Black lesions were common early in the study, but rapidly decreased in abundance after Day 25, whereas white damage was largely not present until Day 20 of the study and was most abundant a month into the study. Black lesion count on Day 5 was significantly lower than Days 14 (Kruskal-Wallis test, $H = 6.0636$, df, = 1, p =

0.0138) and 16 (Kruskal-Wallis test, H = 9.7287, df = 1, p = 0.0018). Day 5 was not significantly different from Days 20 (Kruskal-Wallis test, H = 2.8038, df = 1, p = 0.0940), 22 (Kruskal-Wallis test; H = 0.1304, df = 1, p = 0.718), and 24 (Kruskal-Wallis test, H = 0.7858, df = 1, p = 0.3754). Lesion count on Day 5 was significantly higher than Day 25 (Kruskal Wallis test, H = 6.4264, df = 1, p = 0.0112 and each day thereafter. Maximum black lesion count was 1232 from individual 0810 on Day 14. Minimum lesion count was 6.5 from the same individual on Day 40. White damage area on Day 5 was significantly lower than all days except Days 7 (Kruskal-Wallis test, H = 2.1841, df = 1, p = 0.1394) and 40 (Kruskal-Wallis test, H = 0.7948, df = 1, p = 0.3726). Maximum white damage area was 10.16 cm^2 on Day 30 from individual 0814. Minimum white damage area was zero for several individuals on Days 5 and 40 (Figure 9).

Generalized additive models with time after emergence as a smoothing term were used to describe temporal patterns in wing damage. In all cases, the smoothing term was highly significant ($p < 0.0001$). However, the deviance explained by the model for teal (38.2%) and white wing lesions (32.7%) was lower than the deviance explained for black (65.1%) and orange lesions (57.2%) (Figs 9 and 10).

Principal components 1 (PC1) and 2 (PC2) explained 72% of the total variance (47% and 25% respectively) in wing damage variables. The first principal component included black, teal, and orange lesion count, all of which loaded strongly negative, and white area, which loaded strongly positive. This component likely represents temporal trends in wing damage, such as black, teal, and orange lesion abundance occurring early during healing, and white lesions being more extensive during late healing. The second

principal component (PC2) had a strong negative loading for black lesion count, and positive loadings for teal and orange lesion counts, and white damage area. The remaining variation captured by PC2 is likely due to the nonlinear temporal pattern in black lesion abundance. Black lesions peak on Day 16, a time when other types of damage are near zero (Table 2).

qPCR measurement of fungal load

All bats captured for this study had visual signs of WNS at the capture site (e.g., white tufts of fungal growth on exposed skin surfaces such as muzzle, ears, and wing membranes and swabs taken at the time of capture tested positive for *Pd* based on a PCR test (Muller et al. 2013). PCR results indicate that most individuals remained infected throughout the study, albeit at considerably lower levels than at capture or within a week of emergence. Five days after emergence, and each day thereafter, the study colony's mean infection intensity was significantly lower than at capture (Kruskal-Wallis test, $H = 8.1592$, $df = 1$, $p = 0.0042$). By Day 14, infection intensity was essentially zero. A generalized additive model with time as a smoothing term was highly significant ($p < 0.0001$) and explained 43.9% of the deviance (Figure 12).

Surface lipids

The most common skin surface FFAs were palmitic, stearic, oleic, and linoleic acid, which were present in varying proportions over time. PCA revealed patterns in temporal changes. Early in recovery, unsaturated FFAs (e.g., oleic, linoleic, α-linoleic, and gondoic

acids) were more common, but they quickly declined. Within two weeks, unsaturated FFAs were replaced by saturated FFAs (e.g., palmitic and stearic fatty acids), a shift reflected in PC1 by positive loadings on saturated FFAs, and negative loadings for unsaturated FFAs. Concurrent with a shift from saturated to unsaturated FFAs, short chain surface lipids (e.g., mainly 18C fatty acids) dominated early on in recovery and gradually gave way to long chain FFAs (e.g.,20+ C chain fatty acids). This pattern was reflected in PC2; as chain length increased, factor loading increased from strongly negative to strongly positive. PC1 and PC2 explained 82% of the total variance in lipid changes (61% and 21%, respectively) (Table 3, Figure 13).

Histopathology

Histopathological examination revealed dramatic and rapid changes in tissue structure and presence of fungus over time. Presence of fungus closely matched results from qPCR of wing swabs. Fungus was widely present in all individuals at the time of capture. No bats were free of fungus. The amount of fungus gradually decreased over time, with some bats clear of visible fungus by day 25 and only one bat retaining fungus to day 40. Fungus was mainly present in diffuse patches on the epidermis. Later sections (e.g., Days 25 and 40) showed small fungal foci that were not interconnected and sometimes included only a single hyphal fragment. Cupping erosions were not common in the samples I analyzed. No more than one cupping erosion was observed in a given sample, and no erosions were observed beyond day 14. This pattern is likely due to my sampling protocol that specifically targeted single points of fluorescence, which may have been

single cupping erosions (Turner et al. 2014) Inflammatory crusting was present on all but one individual until day 40. Crusts were less prevalent on day 7 than day 14 and 25. Crusts were often associated with or found to encase large foci of hyphae. Below the crusts, new epidermis formed in a manner resembling hyperkeratosis (Ginn et al. 2007). Wing perforations resulted from areas where hyphal clumps had formed erosions in the membrane, but were also rare and did not arise until after Day 25. Opposite sides of the perforation showed evidence of rapid cell proliferation and hyperkeratosis. I saw no evidence for specificity in fungal invasion; skin appendages such as glandular features and hair follicles did not appear to be particularly susceptible to the fungus (Figure 14).

Integrated PCA

Principal components 1 and 2 of the overall PCA explained 70% of total variance (46% and 24%, respectively). PC1 had high positive loadings on mass, damage timing (PC1 from damage PCA), and FFA saturation (PC1 from lipid PCA). Fungal load as measured by qPCR was negatively loaded, along with black wing damage (PC2 of damage PCA). FFA chain length loaded at approximately zero. PC2 had strong negative loadings on black wing damage and FFA chain length (PC2 from damage and lipid PCAs, respectively). Damage timing and fungal load had equal negative loadings, while mass and FFA saturation were approximately zero. These results suggest that PC1 represents body size and other traits related to fungal infection that have a monotonic relationship with time, while PC2 explains additional, non-linear progression of healing (Figure 15). This conclusion is supported by the clustering of individual data points by sampling day

in a plot of PC2 versus PC1 scores (Figure 16). Early in the study, there was more overlap between sampling days. Days 25 and 40 were not as discretely separated as the previous three sampling days (Table 4, fig 15 & 16).

DISCUSSION

By monitoring physical and physiological changes of *Pd*-infected bats for 40 days, I have shown that bats undergo rapid recovery from WNS. Body mass increased rapidly in the initial two weeks after emergence from hibernation before stabilizing. I identified four unique types of wing damage, all presumably the result of fungal activity. Wing damage visible under ultraviolet illumination is most widespread within 14 days of emergence but quickly dissipates. Wing damage that is visible with white fluorescent light follows two opposing patterns. Black lesions increased from Day 5 to a peak at Day 16, followed by a rapid decline. White discoloration was minimal during the initial two weeks after emergence but reached peak levels nearly one month after emergence. I also used qPCR to document infection intensity during the recovery period and showed that, while infection levels varied widely when bats were captured, all individuals were qPCR positive for fungal DNA. Fungal loads dropped to nearly undetectable levels within a week of emergence from hibernation. However, all individuals retained a background infection until day 40. There were clear transitions in surface lipid profiles over time, shifting from unsaturated to saturated fatty acids, and with increasing fatty acid chain length over time. Histopathological examination of wing lesions showed evidence of

fungal infection in all individuals, but the most intense infections cleared quickly. Cupping erosions were not common, whereas inflammatory crusts were widespread. Principal components analysis suggested that recovery is characterized by parallel and likely interrelated changes in mass, fungal load, wing damage, and saturation of skin surface lipids. Concurrently, wing damage that was not visible during hibernation suddenly develops after bats have been out of hibernation for approximately one week, similar to past accounts (Meteyer et al. 2012; Meteyer et al. 2011). While I did not directly measure immune activation, past studies have shown a sudden increase in metabolic activity within one week of emergence (D. Reeder, pers. comm.). This spike is most likely the result of a sudden and exuberant immune response, which may be the source of WNS related wing damage (Meteyer et al. 2012). Without a direct measurement of immune function, such as bactericidal ability or white blood cell counts, it is not possible to determine immune response. However, my overall PCA, which could be considered an integrated measurement of recovery based on the variables measured in this study, points to evidence of such a spike in bat-mediated recovery processes (note the peak around Day 14 on Figure 16). Overall, it appears that 'recovery' from WNS is in reality a period in which affected bats experience a marked decline in health before returning to a more regular state of health.

Mass gained and feeding behaviors

Bats gained mass at a steady pace during the initial captive period, stabilizing at a mean of ~9 g after day 20. Such rapid weight gain was likely due to several factors. First, bats were hand fed to satiation twice a day at the start of the study. For many bats, this approached 15 – 20 mealworms (~1.5 to 2 g) per feeding. Bats were then allowed to freely use the flight chamber, including a heated wooden artificial roost. Somewhat surprisingly, and especially in the initial two weeks of the study, many individuals roosted outside of the heated box, instead roosting behind a towel on the far side of the flight chamber, or between the side of the box and the screen of the flight chamber. Thus, while these individuals chose to roost in areas that were protected from open areas by a physical barrier, many individuals roosted in areas that were exposed to the ambient temperatures of the containment facility (~18 °C), rather than roosting in a relatively warmer roost (~30 °C). When handled, it was apparent that many individuals were regularly using daily torpor. Male little brown bats are known to use different summer roosts than female little brown bats (Kunz 1982). This is thought to be an energy conservation strategy. Indeed, because our study animals were provided food *ad libitum*, did not need to fly to forage each evening (most crawled to the food dish), and could eat to satiation, some bats became quite fat. Individual 0827, which began the study at a dangerously low mass, eventually grew to approximately 13 g, nearly twice the size of a free-ranging male little brown bat in this area (Fenton and Barclay 1980). It is possible, however, that bats recovering from WNS are using shallow torpor as an adaptive healing strategy. Evidence suggests that bats will mount an immune response to *Pd* while in hibernation (Field et al. 2015; Moore et al. 2013). While immune response of torpid bats

has not been measured, it is likely that immune activation following emergence would allow bats to mount a stronger immune response than during hibernation while reducing metabolic rate significantly. Thus, daily torpor usage could be an adaptive strategy in which sick bats can fight infection at lower energetic cost.

Bats did not self-feed immediately at the start of this study. Self-feeding within the colony began between day 10 and 12 (with some individual variation). Such a pattern may explain rapid mass increase in most individuals around day 10 (Figure 8.). The overall trend (smoothing line in Figure 8), however, is more important than changes among individual days. Bats emerge from hibernation at approximately 5 – 6 g (mean of 6.0 for this study) and immediately undertake a migration to roosting sites (). The energetic strategy that bats use during this time is unclear, but they may regularly use daily torpor and may go a day or more without feeding due to poor feeding conditions (rain storms, cold weather, low insect abundance, etc.) (Anthony and Kunz 1977). Eventually, improving spring conditions allow bats to feed freely each night. Thus, the feeding regime realized in the lab may be roughly representative of natural feeding habits, with the notable exception that bats were not required to fly and maneuver for food items.

Wing damage

This study identified four visually distinct of wing damage within our bat colony, visible under white fluorescent light and UV light. The types of wing damage familiar to most WNS researchers include wounds that are apparent under white fluorescent illumination

(black and white in this study) (Moore et al. 2013; Reichard and Kunz 2009). Additional forms of wing damage visible under UV illumination (teal and orange in this study) were recently detailed, yet it is more difficult to determine their cause. Wing damage visible under UV illumination is thought to be the result of an interaction between *Pd* and the host and often presents as a yellow-orange region (Turner et al. 2014). Past studies have shown that this fluorescence is closely associated with cupping erosions, and can be widespread (Turner et al. 2014). However, my study did not find a clear association between UV lesions and cupping erosions, nor were lesions as widespread. Both orange and teal lesions appeared in discrete clusters.

The difference between my study and the past study on UV-visible wing damage may be explained by differences in timing between the two studies. Whereas the single published account of UV wing damage focused on bats in natural habitats during hibernation, when fungus is most active and bat-mediated processes are at their lowest levels, my study focused on the period after hibernation and on bats being given supportive care, when fungal action is at its lowest due to warm temperatures and bat-mediated recovery processes, such as grooming and immune response, are likely at peak levels. Thus, orange lesions (bat-fungus interactions) in our study may have been uncommon because fungus was already nearly cleared from the wing surface by bat grooming and/or kept from growing by high ambient temperatures. I hypothesize that teal lesions, which are a novel observation from this study, are points of active inflammation or some other bat-mediated process that drives clearance of fungus and the initial phases of healing. Unfortunately, due to tissue processing needed for histopathology, it was not

possible to directly relate a teal lesion to corresponding wing microstructure, thus further study is needed to determine the origin of teal lesions found in my study. Alternatively, differences in coloration could be due to the level at which fungus has infected bat tissue. Infections by *Pd* manifest in two ways, as a cutaneous surface infection or as a deeper dermal infection. Thus, it is possible that orange wing damage represents surface infections where fungus is present in small amounts and has not penetrated into skin layers. Teal lesions, on the other hand, might become evident when fungus has penetrated more deeply into the skin and infiltrated the dermis. Historical works have shown that lymphatic nodes are widespread in bat wings and that lymphatic fluid quickly infiltrates wounds (Cliff and Nicoll 1970; Dongaonkar et al. 2009). It is possible, then, that teal lesions are evidence of lymphatic and inflammatory fluids infiltrating lesions (Meteyer et al. 2012). If this is indeed the mechanism causing fluorescence, then it may also mean that bats are still suffering increased fluid losses during recovery as lymph is no longer contained in lymphatic nodes.

The differences in UV wing damage between my study and Turner et al. (2013) may also be explained by infection history at the study sites. Bats used in my study came from hibernacula that had been affected by WNS for 3 years. Anecdotal evidence and unpublished accounts suggest that winter mortality at WNS sites declines rapidly after three years of infection (C. Herzog pers. comm.; C. Butchowski pers. comm.). This suggests that prior experience with *Pd* may influence the effects of fungal infection on individual bats in subsequent years. It is possible that bats that have survived serial *Pd* infections ameliorate the effects of WNS by preventing widespread infection on wing

surfaces. Instead, infections occur in small, discrete pockets on the wing surface, thus expressing the patterns seen in this study

Both orange and teal wing lesions declined rapidly with time. Prevalence of orange lesions closely resembled the time course of infection intensity, though there was some lag between reduced fungal loads and an associated drop in orange lesions. Teal wing damage was far more variable as compared to orange. While this type of damage also declined rapidly, there was significant variation among individuals. However, like orange, teal lesions were not apparent by the end of the study.

Wing damage visible using white fluorescent illumination (i.e., black lesions and white areas) showed opposite patterns of prevalence. At the start of the study, black lesions were beginning to form on wing surfaces and increased in number and distribution over time. By the end of the study, black lesions were nearly cleared from all individuals. Black lesions are likely the remains of inflammatory cell proliferation at lesions caused by *Pd* (Meteyer et al. 2011). Indeed, histopathlogical examination of these lesions reveal areas that are PAS positive and show cell clusters. I suggest that the peak of black wing damage occurs after the most vigorous inflammatory response to fungal infection has subsided. This hypothesis implies that most inflammatory action occurs within the first 10 days after emergence from hibernation when bats are warmed and supportive care is provided in captivity. Evidence from field studies support the hypothesis that bats heal quickly from WNS damage. Fuller et al. (2011) found that bats could reduce the total area of wing damage by half in as little as two weeks. Field-based manipulations, in which wing biopsy healing was observed in a colony of bats, show that

closure of wing perforations occurs within a month of initial damage (Faure et al. 2009; Pollock et al. 2015; Weaver et al. 2009). However, no field studies have examined healing rates among bats in late spring, immediately following emergence from hibernation. Thus, it is difficult to infer exactly what healing patterns free-ranging bats will show.

Black lesions may also reflect immune system processes for clearing fungus that has penetrated more deeply into tissue layers and that cannot be cleared by grooming. Histology from this study and others shows clumps of hyphae within black lesions (Meteyer et al. 2011). However, if fungus is cleared by this mechanism, it may then also serve as a mode of transmission from hibernacula to summer colonies or from infected hibernacula to uninfected hibernacula. Bats in this study would often shed skin tissue onto the floor and walls of the flight chamber likely due to bats grooming and scratching crusts from their wing surfaces. Evidence suggests that the reservoir for *Pd* is environmental, thus this process of broadcast transmission could play a major role in movement of the disease to susceptible individuals. A vital further study would be to collect crusts from healing bats and attempt to culture *Pd* from them, which would answer whether the fungus is viable after being encased by immune cells and remaining in the environment for some time.

White damage is the most familiar variety of wing damage, as first reported Reichard and Kunz (2009). Reichard and Kunz's (2009) wing damage index (WDI), which defines wing damage severity on a scale of 0 -3 based mostly on proportion of the wing membrane affected by damage, is heavily based on the prevalence of white

discoloration. At the start of the study, white discoloration was almost non-existent. Two weeks after hibernation ended, white discoloration became more prevalent, often/typically in association with black lesions, forming a halo of white around black lesions. As black lesions cleared from wing surfaces, or sometimes prior to black lesion clearance, white discoloration developed in these regions. White discoloration is likely the result of tissue contraction around wounds (Bonaccorso and Smythe 1972; Church and Warren 1968; Faure et al. 2009; Weaver et al. 2009); however it may also be the result of epidermal tissue, and thus skin pigment, being shed in response to a minor *Pd* infection. Thus, these areas of damage grow and combine with adjacent areas of discoloration, sometimes covering the majority of wing surface, as was seen in this study and others (Fuller et al. 2011; Reichard and Kunz 2009).

This study and others show that wing damage patterns are nonlinear (Ceballos-Vasquez 2014; Church and Warren 1968; Davis 1972; Faure et al. 2009; Fuller et al. 2011; Pierce and Mark 2011; Pollock et al. 2015; Weaver et al. 2009). During hibernation, there was very little visible wing damage on affected bats, but wing damage became very apparent soon after hibernation, and increased dramatically before declining slowly. These patterns draw into question the appropriateness of using the WDI early in the recovery period. The index does not include criteria for evaluating sparsely distributed inflammatory crusts, instead taking into account larger areas of sloughing tissue, which has been assumed to represent necrotic tissue. If one were to use the WDI to evaluate wing damage on bats in late spring, many would be placed in the most severe category (WDI = 3) inappropriately because widely distributed black lesions would be

defined as necrotic tissue. Wing damage related to WNS on free-ranging bats is not apparent in most cases after mid-June (Fuller et al. 2011). Given that most WNS wing damage is seen when bats are captured in May and June, the time period phase in which white damage dominates, bats have already been recovering for several weeks by this point. My study suggests this switch point occurs among captive bats nearly three weeks after emergence from hibernation, however this period could be longer for free-ranging bats. Given these complications, I suggest that a more temporally sensitive wing damage scale, weighted by timing and types of damage, be developed to more accurately evaluate WNS-induced wing damage.

Outside of hibernation, wing damage may have adverse effects on long-term individual survival and fitness. Given that flight is fundamental to bat survival, it follows that even low levels of wing damage could generate adverse effects. Few studies have examined the effects of wing damage on flying animals, and only one study has attempted to quantify how damage changes the energetic costs of flight in bats (Voigt 2013). Using stable isotope techniques, Voigt (2013) showed that energetic cost decreased in bats with wing damage. While this seems counterintuitive, the author suggested that reduced flight costs could result from adaptive behaviors in damaged bats, such as making fewer abrupt flight maneuvers (Voigt 2013). Wing damage could also disrupt the flow of important sensory information during flight. Bat wings have numerous sensory hairs (1 hair per mm^2) which provide information on flight speed and direction (Chadha et al. 2012; Marshall et al. 2015; Sterbing-D'Angelo et al. 2011). When these

hairs are damaged or lost, flight patterns change; bats make wider turns and fly faster, thus increasing the energetic cost of flight (Sterbing-D'Angelo et al. 2011).

Research on insects has shown that wing damage significantly impacts foraging and survivorship. For example, honeybees with damaged wings will undertake foraging bouts of longer duration but show a significant decline in food delivery rates (Dukas and Dukas 2011; Higginson and Barnard 2004; Higginson et al. 2011). These bees also experience higher mortality with increasing age. This suggests that wing damage reduces foraging success by hampering flight maneuverability, increasing energetic flight cost and predation risk. Thus, honeybees appear to compensate by limiting their time away from the hive at the cost of foraging profitability (Dukas and Dukas 2011; Higginson and Barnard 2004; Higginson et al. 2011). Studies using simulated primary feather molt in birds have shown that reduced wing area affects body mass and flight performance. Birds in mid-molt, when wing area is most greatly reduced and wing loading is highest, show significantly lower body mass than birds with complete feathers, which may be due to reduced foraging success or compensation for increased wing loading (Swaddle and Witter 1997; Swaddle et al. 1996). Molt also results in reduced flight maneuverability and slower takeoff speed, driven by a loss in kinetic energy gain per wingbeat (Swaddle 1999). Reduced flight maneuverability and changes to takeoff dynamics likely cause birds to have lower foraging success and suffer greater mortality, as their burst flight abilities decline dramatically. When one considers the above examples in the context of WNS, it becomes apparent that wing damage from this disease may be having dramatic sub-lethal effects on bat flight aerodynamics and mechanics, which point to an increase in

the energetic cost of flight. Increased energetic demand immediately after hibernation, a time when bats are experiencing high energetic demands of thermoregulation and reproduction (for females), may increase mortality among bats who are least successful hunters or have especially low fat stores. The potential for such additional mortality is a topic that has received no attention from the WNS community, except for brief mentions as untested hypotheses (Meteyer et al. 2012). I argue that active season mortality is of equal importance as winter mortality and, while it may be difficult, should studied intensely.

qPCR analysis of fungal load

A qPCR-based measure of fungal load declined rapidly after hibernation ended. This pattern may be explained by extensive grooming by bats, thus removing detectable surface particles of the fungus. Fungal load declined rapidly in this captive colony. Fungal DNA was undetectable from three individuals on Day 7, while the remaining individuals had Ct scores near the threshold of detection (between 36.6 and 40). Following Day 7, all individuals retained consistently low fungal loads. However, in several cases, individuals would regain fungal loads after having undetectable levels (e.g., Day 14; Figure 12). This pattern could be explained by a number of factors. First, bats rotated roosting areas, rotating between the heated roost, an area beside the heated roost, and behind some towels that were hung on the wall of the flight chamber.. To determine whether bats could be contacting fungus from the environment, we collected environmental swab samples from inside the flight chamber in typical roosting areas. By

the end of the study, these roosting locations returned fungal loads of similar magnitude to swabs from bats during the first week of the study. Thus, bats may have been subjected to ongoing exposure to *Pd* from the environment. This mode of transmission is thought to be a major factor influencing infection probability and *Pd* persistence in hibernacula (Langwig et al. 2015; Lorch et al. 2013).

Additional exposure to *Pd* might have been mediated by colony-mate interactions. After the first week, the bats mostly roosted as a colony in the bat box or in small groups in other locations around the flight chamber. However, there were some cases in which single individuals did not register as being infected, despite the rest of the colony carrying fungal DNA. For example, one bat carried no fungus on Days 14 and 25, but obtained fungus before sampling occurred on Day 40. These individuals may have been more effective groomers or simply did not encounter another individual or roost surface with fungal particles. I handled bats with clean gloves and they did not share cotton bags when they were being processed (e.g., mass measurements and wing photos), and all handling equipment was either discarded or cleaned with hydrogen peroxide solution after handling. Thus, these individuals must have gained fungus from its roostmate or an environmental source.

Another possibility is that bats were continually shedding small amounts of fungus that had infiltrated tissue more deeply than fungus that was shed earlier in the study. Small islands of hyphae could have remained on wing surfaces, in addition to inflammatory crusts potentially containing hyphae, which may be viable in the right conditions. Indeed, comparisons with histopathology confirm that most bats had fungus

present on their wings, even at the end of 40 days. This conclusion would suggest that the qPCR protocol was adequately sensitive to the presence of fungus. However, these results may not necessarily mean bats are 'infected' by *Pd* because there is no means by which to determine whether the presence of fungal DNA infers viability of fungus. These results simply reveal whether fungus is present.

Skin surface lipids

I found that skin surface lipid profiles changed dramatically over time. Principal component analysis revealed that these changes were driven mostly by a transition from unsaturated free fatty acids to saturated free fatty acids, and by a shift from short chain to long chain free fatty acids. The profiles in this study closely matched those observed in previous studies that examined the surface lipid components of captive little brown bats with WNS, but did not address changes during recovery (Pannkuk et al. 2014; Pannkuk et al. 2013). These studies used portions of wing tissue that had been excised after termination of controlled *Pd* infection experiment. Thus, the profiles published therein reflect lipid profiles from hibernation more so than recovery, but are comparable, especially in the early period of my study. Early samples (Days 5 and 7) showed some difference from previous studies. Most notably, I detected higher amounts of unsaturated free fatty acids. Mammals cannot synthesize fatty acids with odd numbers of unsaturated carbons, thus these are considered essential fatty acids, oleic (18:1) and α-linoleic acids (18:3). Considering that these acids were present in high concentrations during the early time points, I suspect that their source was fungal tissue that was collected from skin

surfaces during lipid sampling. A comparison of GC/MS spectra from surface lipids of bats in this study to a published account of *Pd* lipid profile (Pannkuk et al. 2014), lends support to this hypothesis. Spectra from *Pd* show relatively high levels of 18:1, 18:2, and 18:3 fatty acids, and lower levels of 16:0 and 18:0. Early samples from bats show similarly high levels of 18:1 and 18:2 fatty acids and high concentrations of 16:0 and 18:0 fatty acids (Figure. 17). At the end of the study, fatty acid profiles had undergone pronounced change so that 18:0 was then the dominant free fatty acid (as is typical for bats), and unsaturated fatty acids had declined to lower levels. I hypothesize that this transition was driven by loss of a fungal signal in the lipid samples, which was due to effective grooming by the bats.

Another explanation for the changes observed in lipid profiles is bats shifting to post-hibernation physiology. During hibernation, many processes are down regulated, including immune responses and glandular activity (Bouma et al. 2010; Sisk 1957). In a study of wing gland activity, Sisk (1957) found that glandular activity declined dramatically during the winter. In late summer and early fall, wing glands become filled with excess secretion, which is expelled during hibernation but not replenished until summer (Sisk 1957). Bat glandular secretions are comprised of many different free fatty acids and other compounds, such as proteinaceous secretions, enzymes, and glycogen (during hibernation), but are dominated by saturated free fatty acids (Pannkuk et al. 2014; Sisk 1957). Thus, more saturated fatty acid on the skin surface may signify reactivation of dormant sebaceous glands or healing/regeneration of damaged glands. In addition, microbial activity serves to convert saturated fatty acids to unsaturated fatty acids, and to

cleave long chain fatty acids into short chain fatty acids (Alford et al. 1964; Ward and Singh 2005). During hibernation, microbial activity may transform fatty acids at a faster rate than semi-dormant sebaceous glands replace them. Thus, short chain, unsaturated fatty acids may dominate hibernation lipid profiles, whereas post-hibernation profiles would be dominated by long chain, saturated fatty acids, similar to the patterns suggested by my principal component analysis (Table 3; Figure 13).

Frank et al. (2014) suggest that surface lipids may be one driving factor for differential infection rates and mortality among affected bat species. The hypothesis is that since surface lipids are the initial line of defense against microbial infection, different concentrations of lipids could prevent *Pd* from infecting and infiltrating skin tissue. This is an intriguing idea, but it is not supported by field data. Frank et al. (2014) use laboratory conditions to test the antimicrobial activity of free fatty acids, using concentrations of lipid that would rarely if ever occur in a natural situation (Pannkuk et al. 2014; Pannkuk et al. 2013; Pannkuk et al. 2015; Pannkuk et al. 2013). Thus, while there may be some background antimicrobial activity in a natural scenario, these findings will not apply to natural systems because low concentrations of lipids will not have a similar effect as lipid concentrations that are much higher than natural cases. In addition, laboratory studies culture fungus on substrates that are enriched with the target free fatty acid. In a natural system, there are many more kinds of free fatty acids on the skin surface, plus a number of other compounds and microbes. Simply put, a petri dish in a laboratory is not an acceptable substitute for bat wings. Another hypothesis suggests that because bat surface lipid profiles vary interspecifically, it is possible that lipid profiles

may prevent exuberant infections on less susceptible species. Aside from the low concentration of lipid on skin surfaces, this argument is probably not the most parsimonious explanation for differential susceptibility. Given that all species roost in different climatic gradients, and *Pd* grows optimally in a small range of temperatures and humidity levels, the simplest answer is the interplay between roost environments and fungal growth preferences. Surface lipids likely have a small overall effect, and thus may serve better as biomarkers for disease and hibernation than as a WNS panacea.

Histopathology

Using a novel, non-lethal technique for histopathological examination, I also tracked the microscopic changes of WNS wing lesions over 40 days of recovery. Histopathology is considered the standard for WNS diagnostics (Blehert et al. 2009; Cryan et al. 2010; Meteyer et al. 2009; Meteyer et al. 2012; Meteyer et al. 2009; Reeder et al. 2012; Turner et al. 2014). However, a drawback of using histopathology is that entire wings are needed, and thus bats must be euthanized. For a disease with more than 70% mortality at affected sites, sacrificing bats is not an ideal approach for diagnostics. Nonlethal diagnostic, techniques, such as examination of wings with UV light, have proven to be quite effective for diagnostics (Bandouchova et al. 2015; Turner et al. 2014). My technique is also an effective means by which to examine wing lesions in detail, as well as to examine other cutaneous features, without the need for euthanasia.

In general, histopathology confirmed observations made using UV light examination and qPCR swab data. Fungus was visible in all samples, though by day 40

was present in low densities. Many areas of fluorescence were apparent on wings under UV illumination and some of these regions were revealed to be cupping erosions when examined with histology. However, cupping erosions were rare and were not widely apparent after the first sampling period. Black wing damage excised for histopathology was a clear indicator of crusted over inflammatory cells. One drawback of this technique was that cupping erosions were not found in great quantity on the wings I examined. UV examination showed widespread fluorescence, which Turner et al. (2014) suggest signal cupping erosions. However, none of my histpathology samples scored higher than one cupping erosion. This finding was likely due to my sampling protocol that targeted a single point of fluorescence, or because there were few areas to sample by the end of the study.. Thus, my non-lethal histopathology technique may not be appropriate if the goal is to examine the full distribution of cupping erosions and other forms of damage on wing surfaces. Thus, WNS severity cannot be quantified in a similar way to other studies, i.e., I did not quantify spatial spread of fungus, nor did I examine how widespread cupping erosions are. However, when combined with detailed wing damage photos using both UV and white fluorescent illumination, it may possible to infer severity

IMPLICATIONS FOR WNS RESEARCH

White nose syndrome is among the worst wildlife diseases in recorded history, and its continued spread across North America will put more bat species at risk. Already, mortality due to WNS has led to northern long-eared bats being listed as a threatened species under the Endangered Species Act, and there are similar petitions to list other

bats, including the once nearly ubiquitous little brown bat. While we have made progress toward understanding transmission dynamics and the mechanism of disease, our broader knowledge of potential long-term consequences of the disease is lacking. Only a small number of studies have examined the effects of WNS outside of hibernation. While hibernation is understandably an important phase to study, since it is when the majority of mortality occurs, post-hibernation recovery patterns should receive at least equal attention from the WNS research community because it is the surviving individuals that are most important to the potential recovery of affected populations.. I provide the first account of patterns of post-hibernation fungal load on recovering bats and how a fungal reservoir accumulates within a roost, potentially reinfecting individuals that had previously cleared infection. Such a pattern could have far-reaching implications for transmission of *Pd* to uninfected regions and individuals. I also provide the most detailed account of the temporal trends of the development of, and recovery from, wing damage following hibernation. Combined, the results from this study provide the most detailed record of WNS recovery, and could be used to better understand the lingering effects of WNS on survivors. These few individuals represent the remaining vestiges of bat populations that must successfully reproduce to maintain, or someday increase, the populations of affected bat species. Current management practices and disease transmission models are inadequate because they do not emphasize the active summer phase. Results from this study show that late spring/early summer is a dynamic time for bats with WNS. While it is understandably difficult to target conservation efforts to summer habitats, given that bats are cryptic during this time, a paradigm shift in research

efforts toward understanding long-term effects on life history is needed to fully understand this disease.

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Table 3.1. Timeline of sampling events. Body temperature loggers began to spontaneously release from some individuals at approximately day 10. During a larger sampling event on Day 14, remaining attached loggers were removed

Study day	***Mass***	***UV Photos***	***White light photos***	***Pd Swab***	***Surface lipids***	***Histopathology***	***Body temperature***
Capture	x		x	x			x
0	x						x
1	x						x
2	x						x
3	x						x
4	x						x
5	x	x	x	x	x	x	x
6	x						x
7	x	x	x	x	x	x	x
8	x						x
9	x						(x)
10	x						(x)
11	x						(x)
12	x						(x)
14	x	x	x	x	x	x	(x)
16	x	x	x				
17	x						
18	x						
20	x	x	x				
22	x	x	x				
24	x		x				
25		x	x	x	x	x	
26	x		x				
28	x		x				
30	x		x				
32	x		x				
34	x		x				
36	x		x				
38	x						
40	x	x	x	x	x	x	
Total sampling events	**29**	**8**	**16**	**6**	**5**	**5**	**12 - 17**

Table 3.2. Factor loadings of principal components 1 and 2 of wing damage patterns of recovering little brown bats. The first principal component is related to timing of damage (early vs. late), while the second reflects patterns in black lesions.

Damage Type	***PC1 (Timing)***	***PC2 (Black lesions)***
Black lesions	-0.373	-0.747
White area	0.504	0.317
Teal lesions	-0.566	0.304
Orange lesions	-0.535	0.409
Proportion of total variance explained	0.474	0.247

Table 3.3. Factor loadings of principal components 1 and 2 of free fatty acids (FFA) taken from the skin surface of recovering little brown bats. The first principal component represents FFA saturation, while the second reflects carbon chain length.

Free Fatty Acid Type	***PC1 (Saturation)***	***PC2 (Chain Length)***
Palmitic acid (16:0)	0.200	-0.607
Stearic acid (18:0)	0.375	-0.285
Oleic acid (18:1)	-0.390	-0.094
Linoleic acid (18:2)	-0.413	0.051
α-linoleic acid (18:3)	-0.372	0.1685
Arachidic acid (20:0)	0.341	0.254
Gondoic acid (20:1)	0.021	0.561
Heneicosylic acid (21:0)	0.345	0.258
Lignoceric acid (24:0)	0.348	0.253
Proportion of total variance explained	0.618	0.206

Table 3.4. Factor loadings of principal components 1 and 2 of an integrated measure of WNS recovery. The first principal component likely represents timing of recovery events and rapid gains in body mass, while the second reflects processes related to immune response and wing healing.

Recovery variable	***PC1***	***PC2***
Mass	0.546	-0.094
Infectious load	-0.358	-0.193
Damage timing (PC1)	0.491	-0.210
Black lesions (PC2)	-0.154	-0.658
Saturation (PC1)	0.552	0.049
Chain length (PC2)	0.062	-0.689
Proportion of total variance explained	0.462	0.235

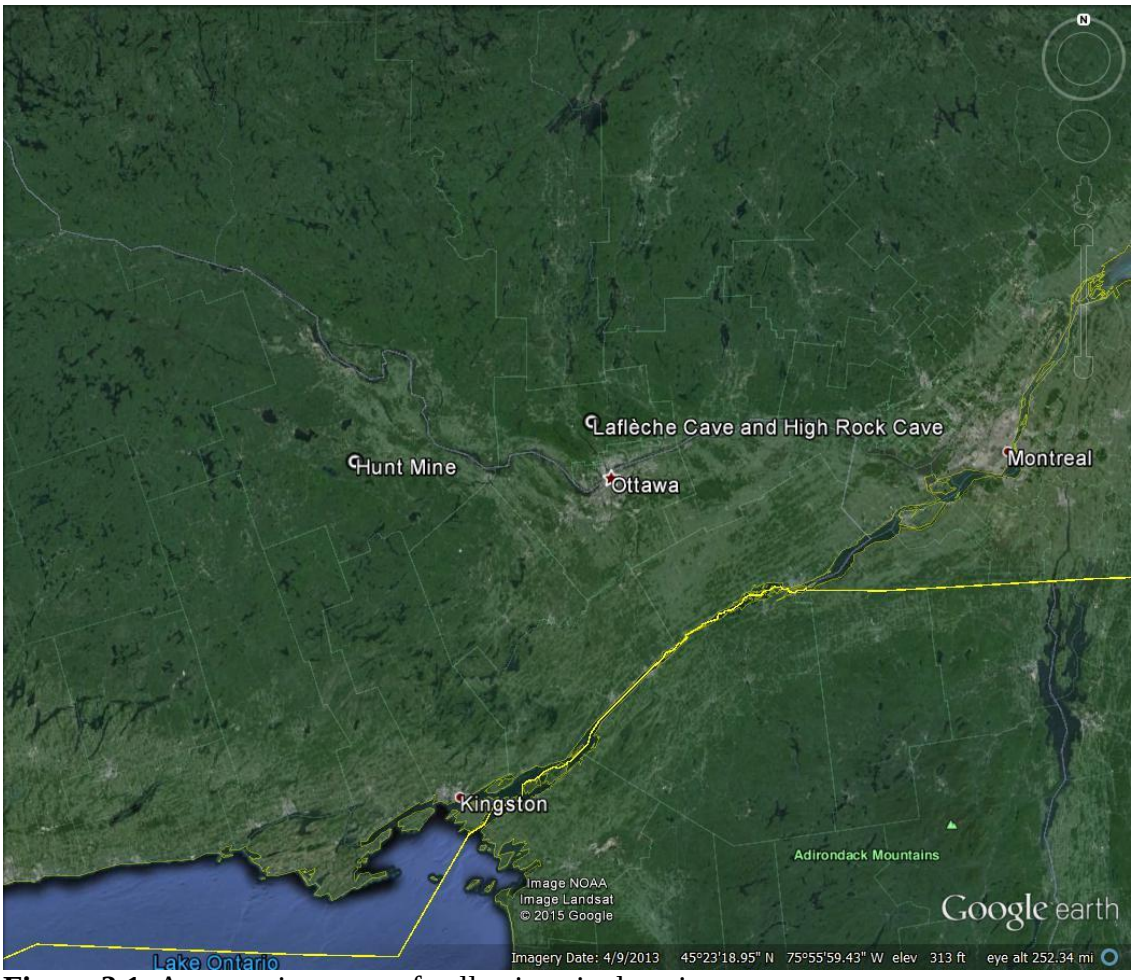


Figure 3.1. An overview map of collection site locations

Figure 3.2. Handling and processing immediately after capture from study site. This photograph displays attachment of body temperature loggers.

Figure 3.3. A representative white fluorescent light illuminated wing photograph with scaling items.

Figure 3.4. A representative UV light illuminated wing photograph with scaling item (Canadian $1 coin).

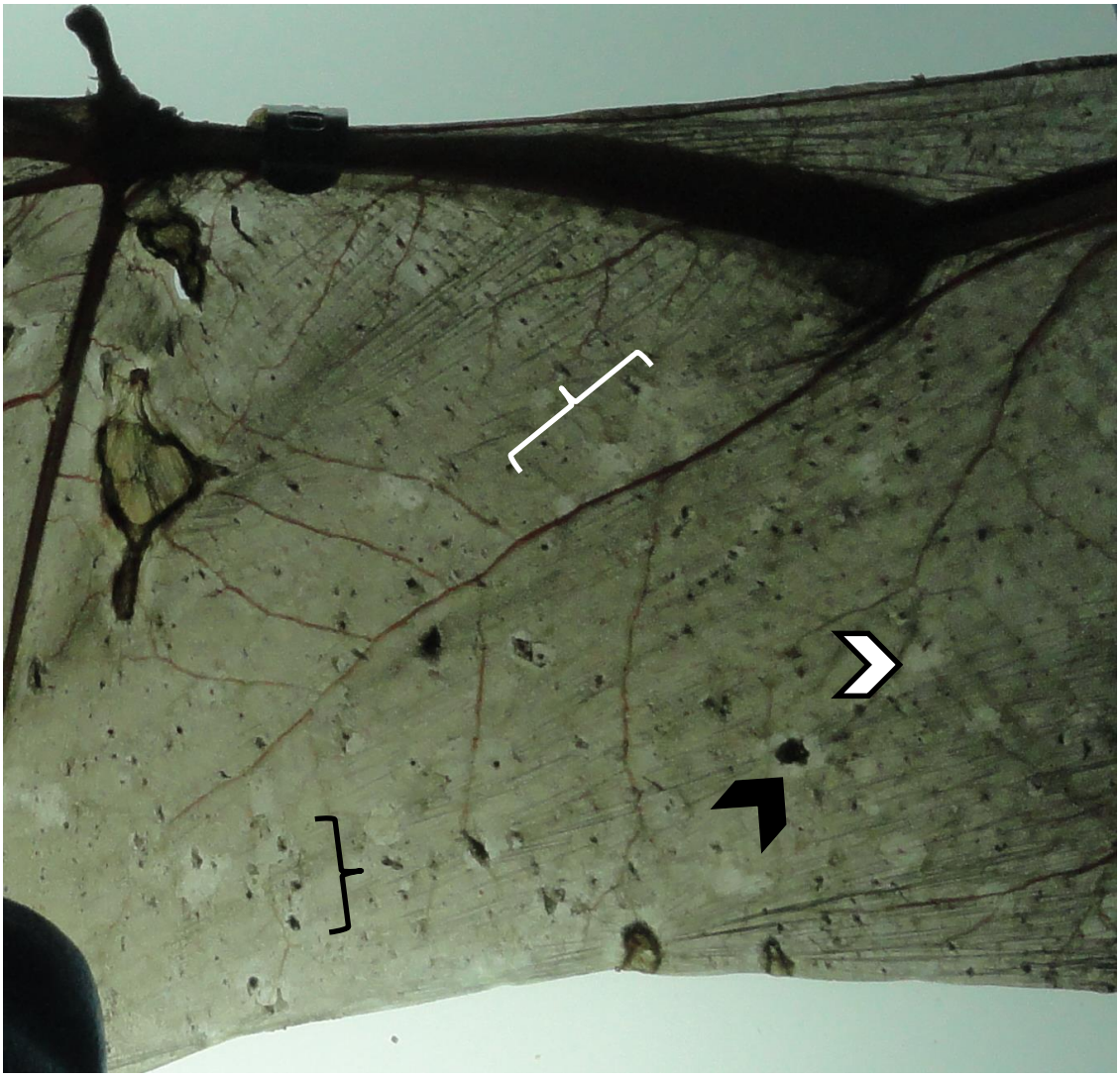


Figure 3.5. A magnified photograph of wing damage illuminated with white fluorescent lighting detailing white damage (white arrowhead and bracket) and black lesions (black arrowhead and bracket).

Figure 3.6. A magnified photograph of wing damage illuminated with ultraviolet lighting, detailing teal lesions (teal arrowhead) and orange lesions (orange arrowhead).

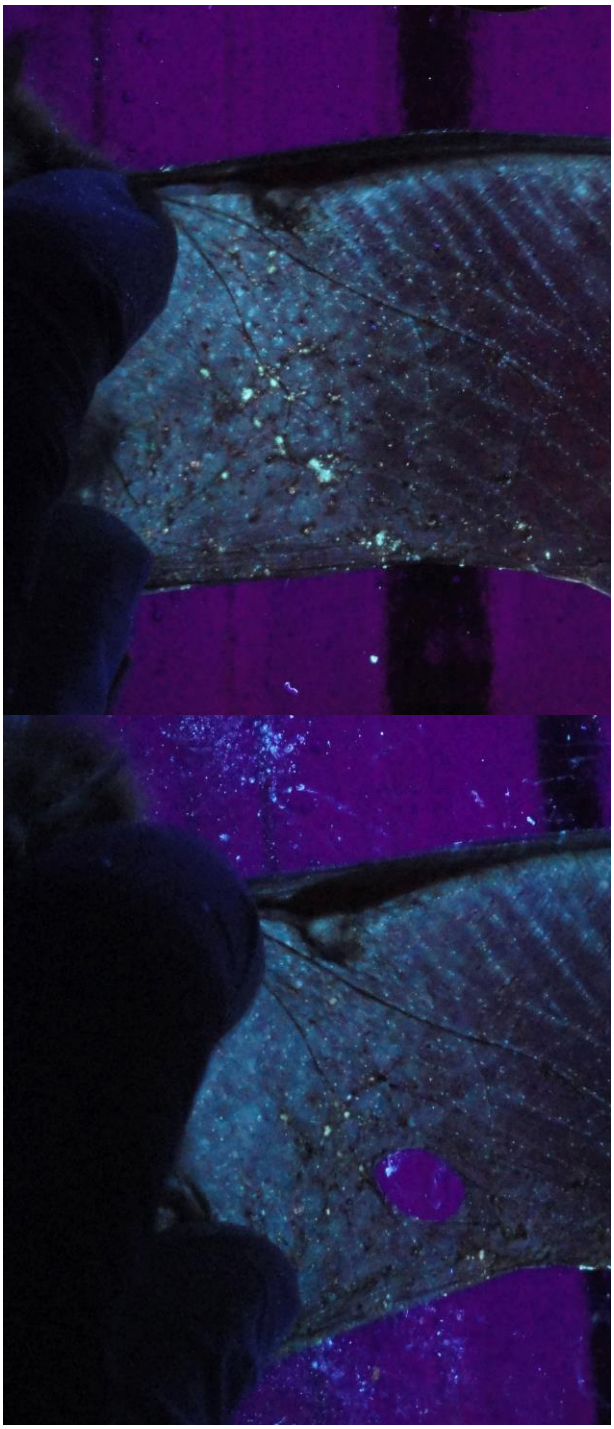


Figure 3.7. An example of a tissue biopsy that was collected using UV illumination to target an appropriate location. Note the large area of teal fluorescence in the left-hand photograph that was excised before the right-hand photograph was taken.

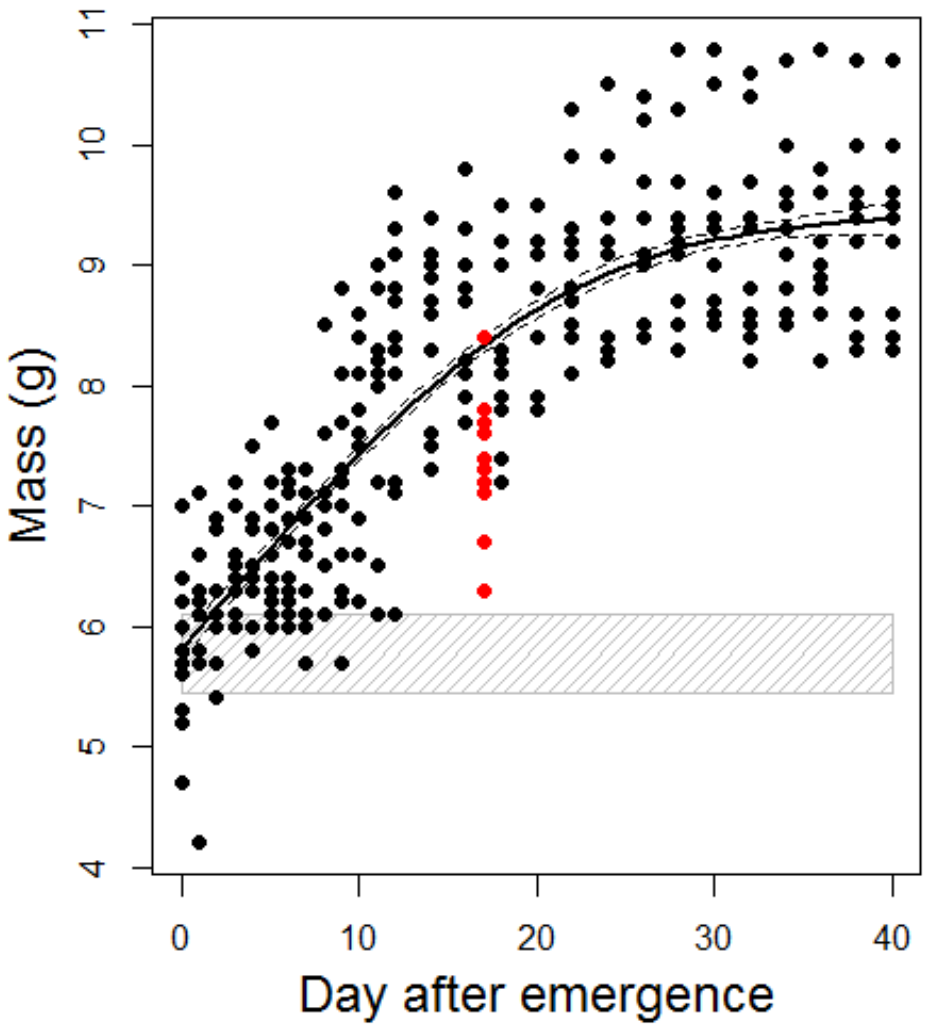


Figure 3.8. Scatter plot of daily mean body mass of little brown bats recovering from WNS. The red points show a sudden decline in mass due to a lighting malfunction on day 17, after which bats regained mass rapidly. The shaded gray box denotes the interquartile range of mass from Day 0. Bats gained mass so quickly that mean body mass was significantly higher than Day 0 by Day 3 ($\chi^2 = 8.0135$, d.f. = 1, p = 0.0046). The smoothing curve was determined using generalized additive modeling with time as a smoothing factor and individual ID as a random effect.

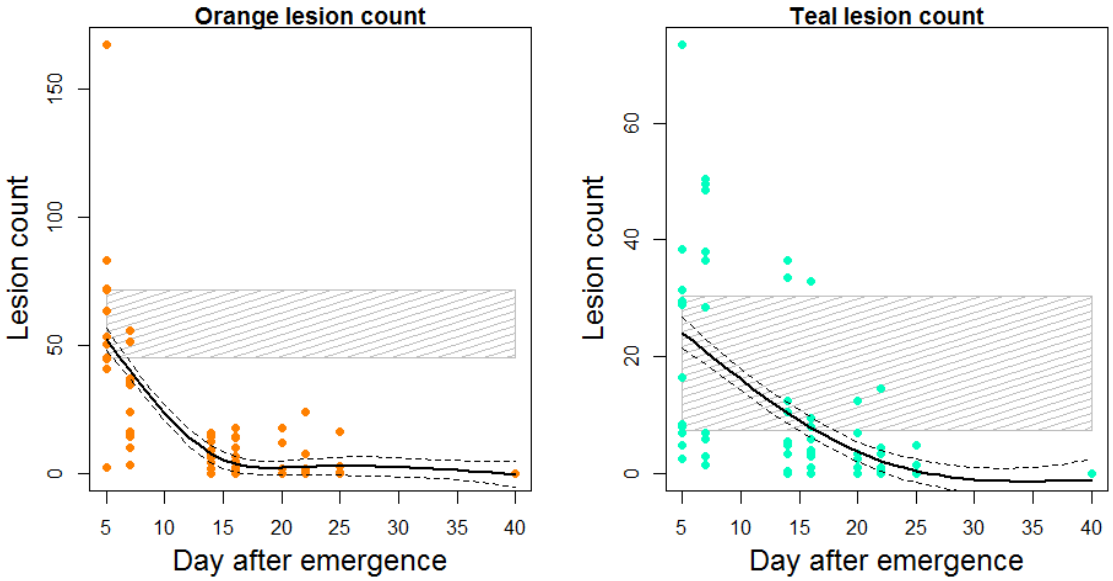


Figure 3.9. Scatter plot of count of wing lesions visible under ultraviolet illumination on little brown bats recovering from WNS. Orange lesion count was significantly lower on Day 7 than on Day 5 ($\chi^2 = 7.0726$, d.f. = 1, p = 0.0078). Teal lesion count was significantly lower on Day 16 than on Day 5 ($\chi^2 = 6.244$, d.f. = 1, p = 0.0125). The shaded gray boxes denote the interquartile ranges of lesion count from Day 5. Smoothing curves were determined using generalized additive modeling with time as a smoothing factor and individual ID as a random effect.

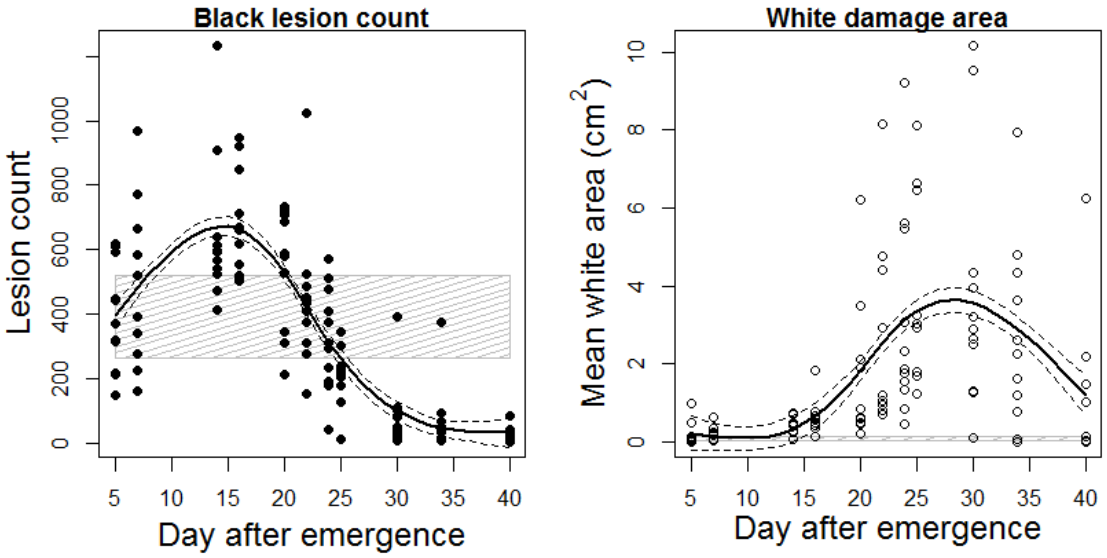


Figure 3.10. Scatter plot of count of wing lesions visible under white fluorescent light on little brown bats recovering from WNS. Black lesion count was significantly higher on Day 14 than on Day 5 ($\chi^2 = 9.7287$, d.f. = 1, p = 0.0138) and remained significantly higher until Day 20. Lesion count was then significantly lower than Day 5 on Day 25 ($\chi^2 = 6.4264$, d.f. = 1, p = 0.0112). White damage area was significantly higher on Day 14 than on Day 5 ($\chi^2 = 6.3949$, d.f. = 1, p = 0.0114). The shaded gray boxes denote the interquartile ranges of lesion count (black damage) and lesion area (white damage) from day 5. Smoothing curves were determined using generalized additive modeling with time as a smoothing factor and individual ID as a random effect.

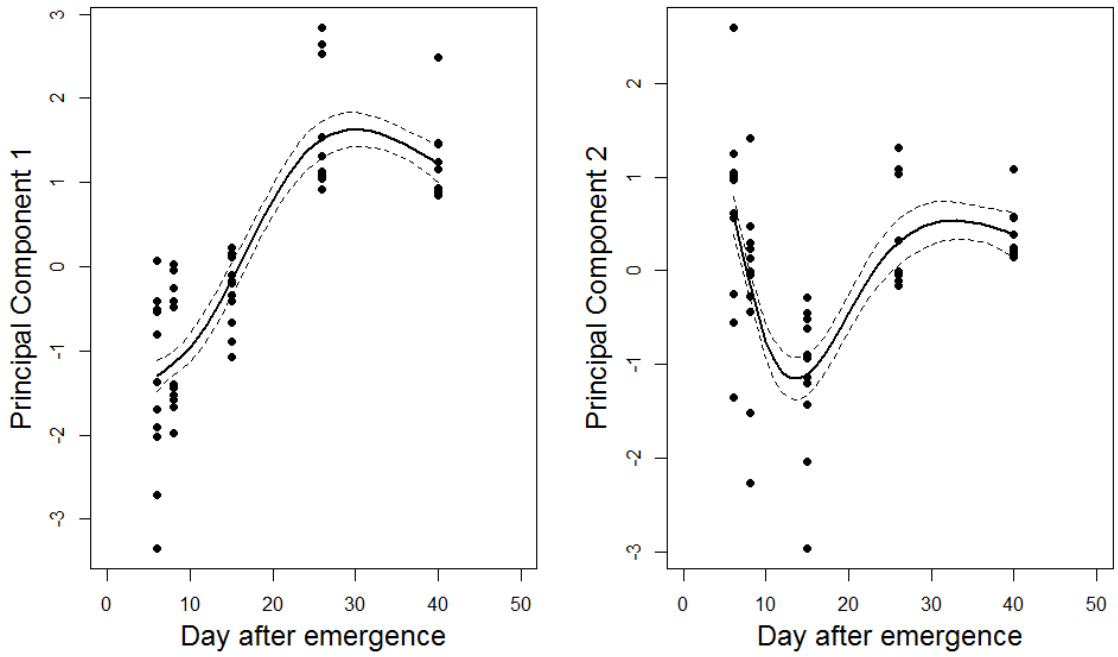


Figure 3.11. The first two principal components wing damage patterns plotted over time. Smoothing curves were determined using generalized additive modeling with time as a smoothing factor and individual ID as a random effect.

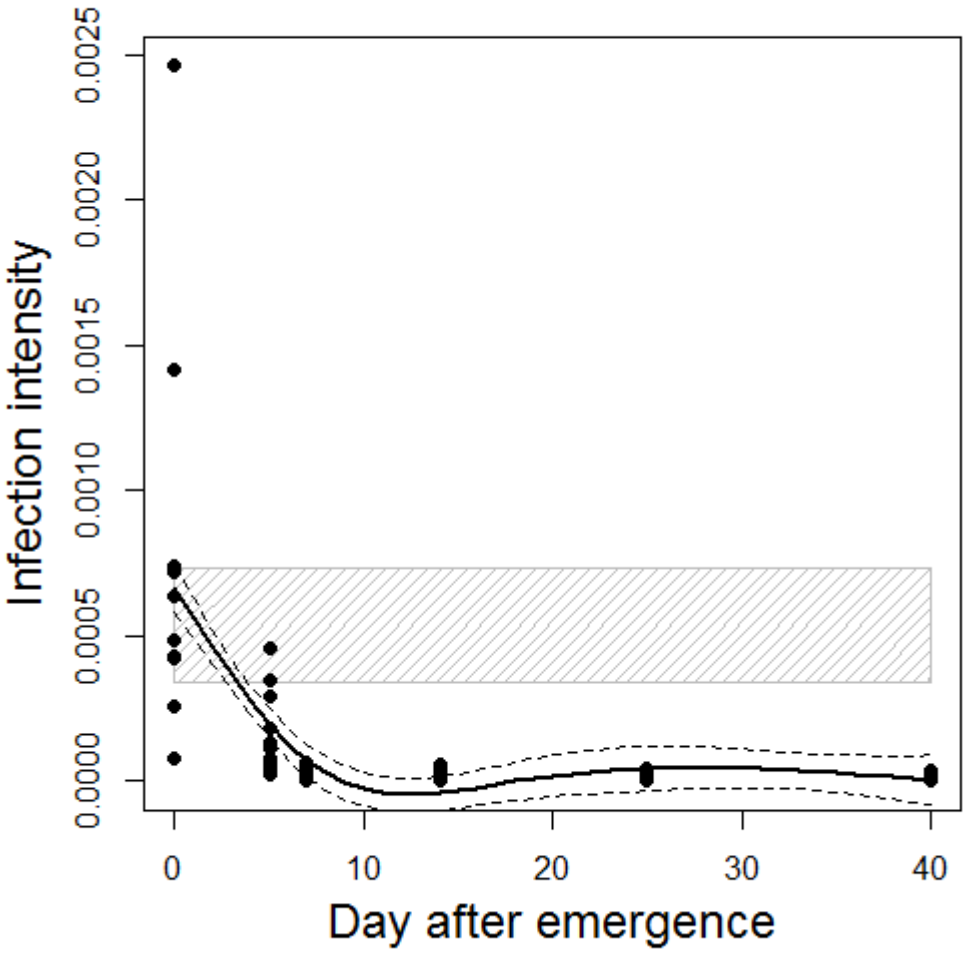


Figure 3.12. Temporal plot of fungal load of *Psuedogymnoascus destructans* on little brown bats recovering from WNS. The shaded gray box denotes the interquartile range of infection intensity from Day 0 (capture). Fungal load was significantly lower than Day 0 by Day 5. The smoothing curve was determined using generalized additive modeling with time as a smoothing factor and individual ID as a random effect.

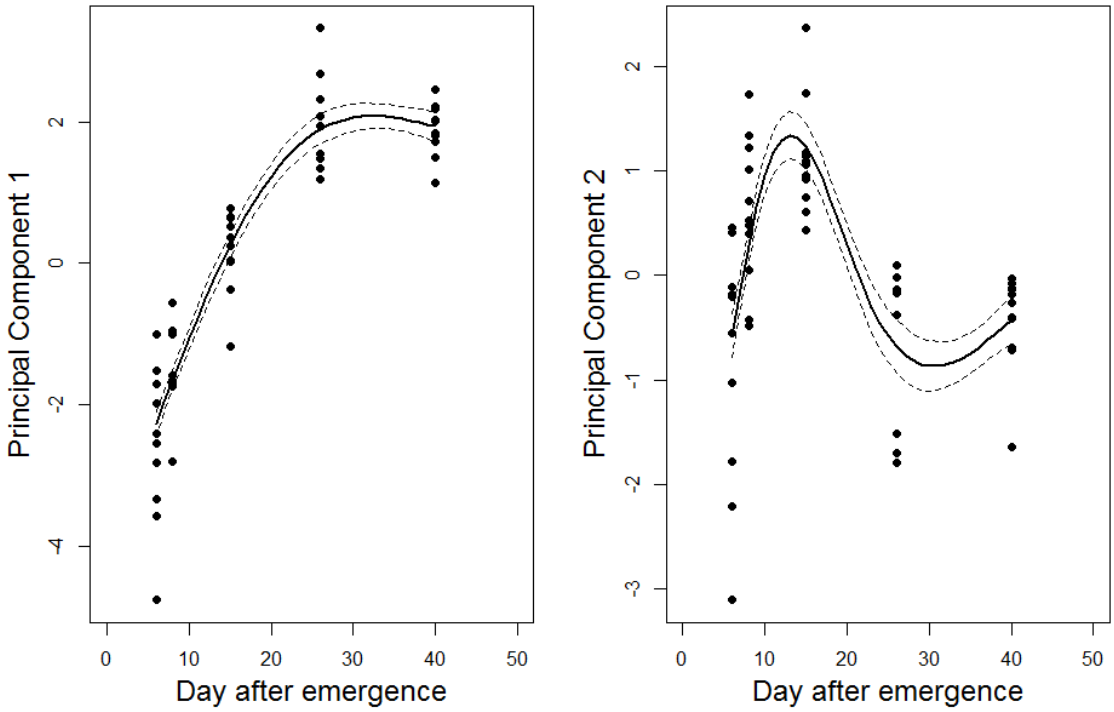
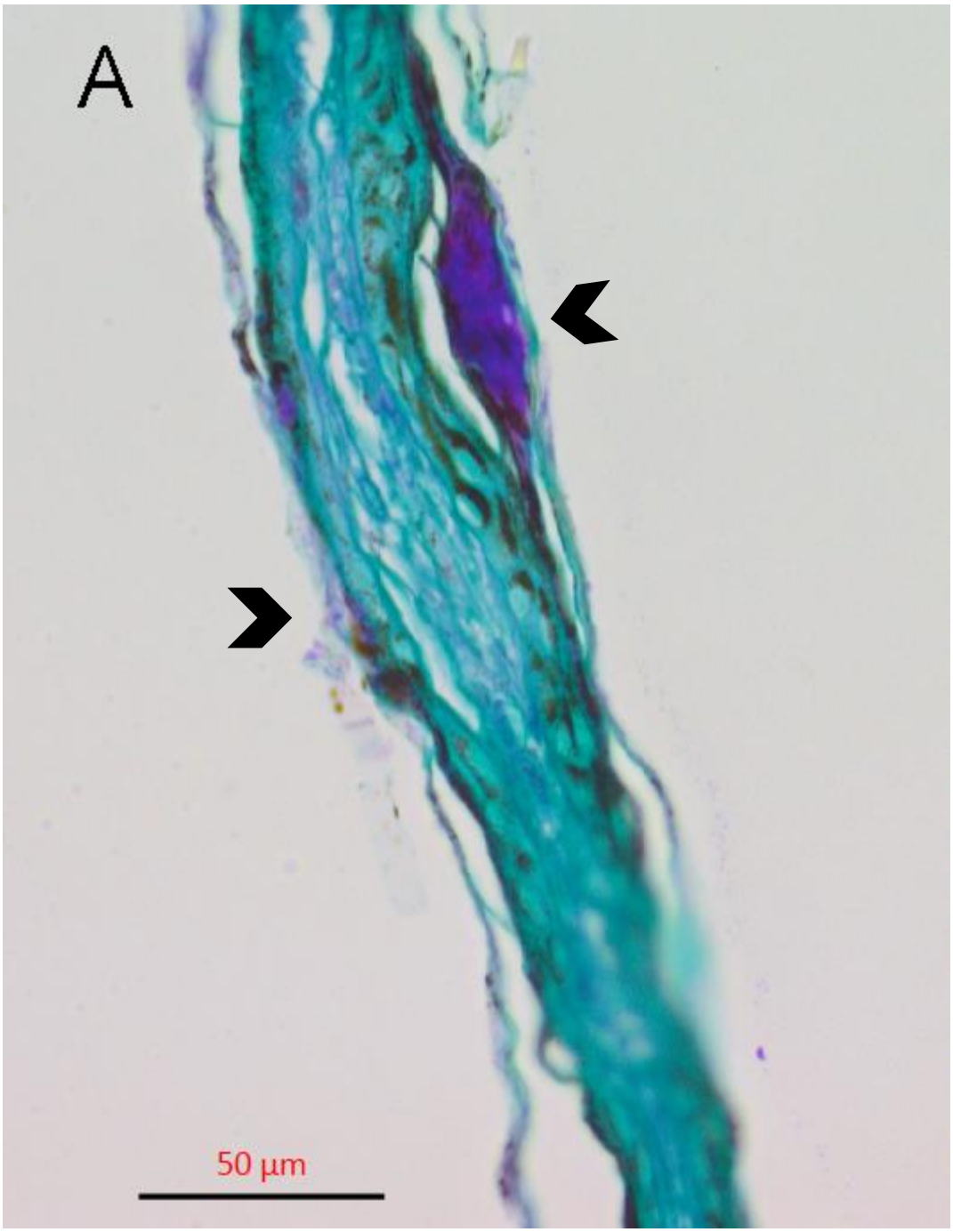
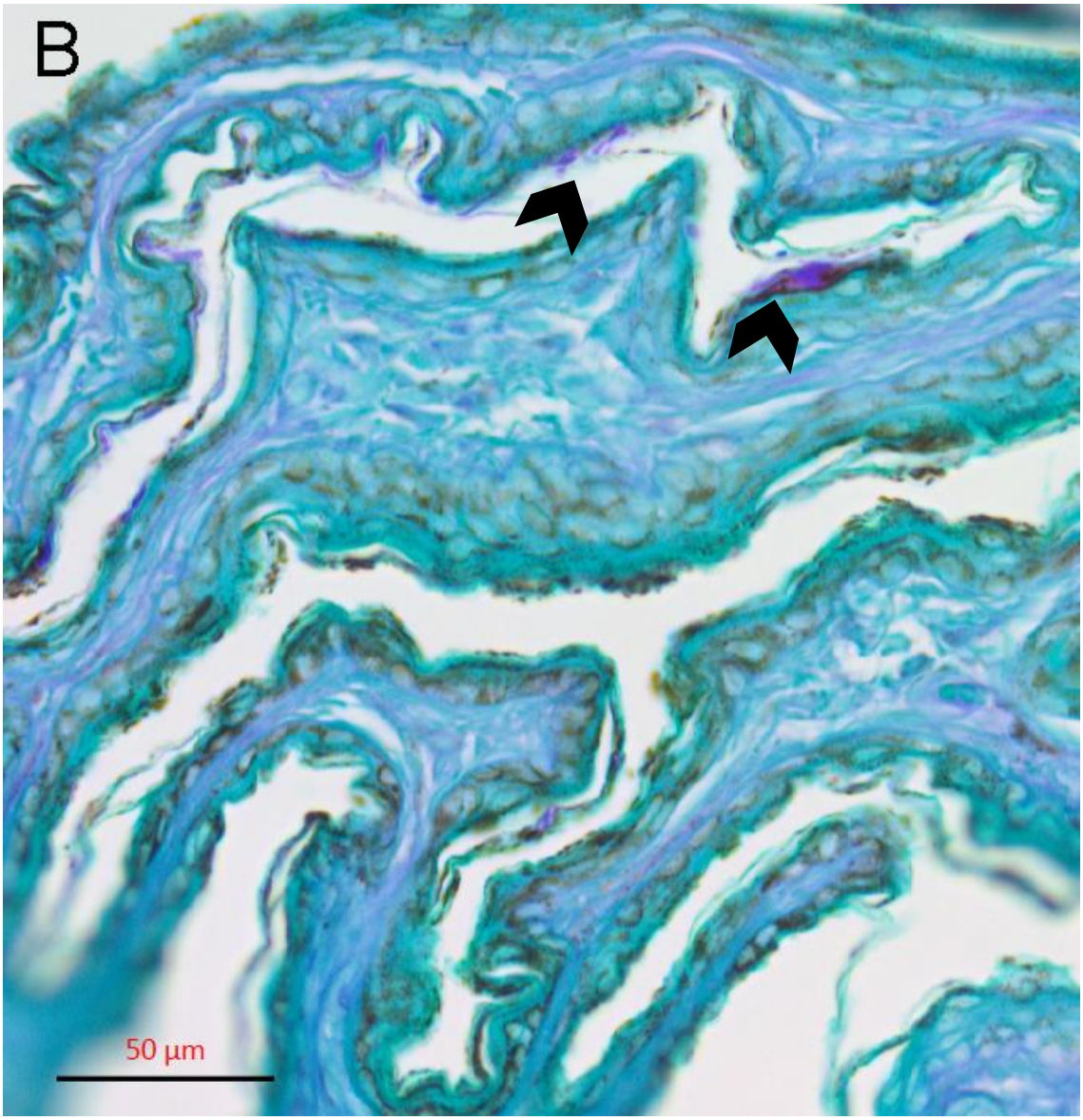
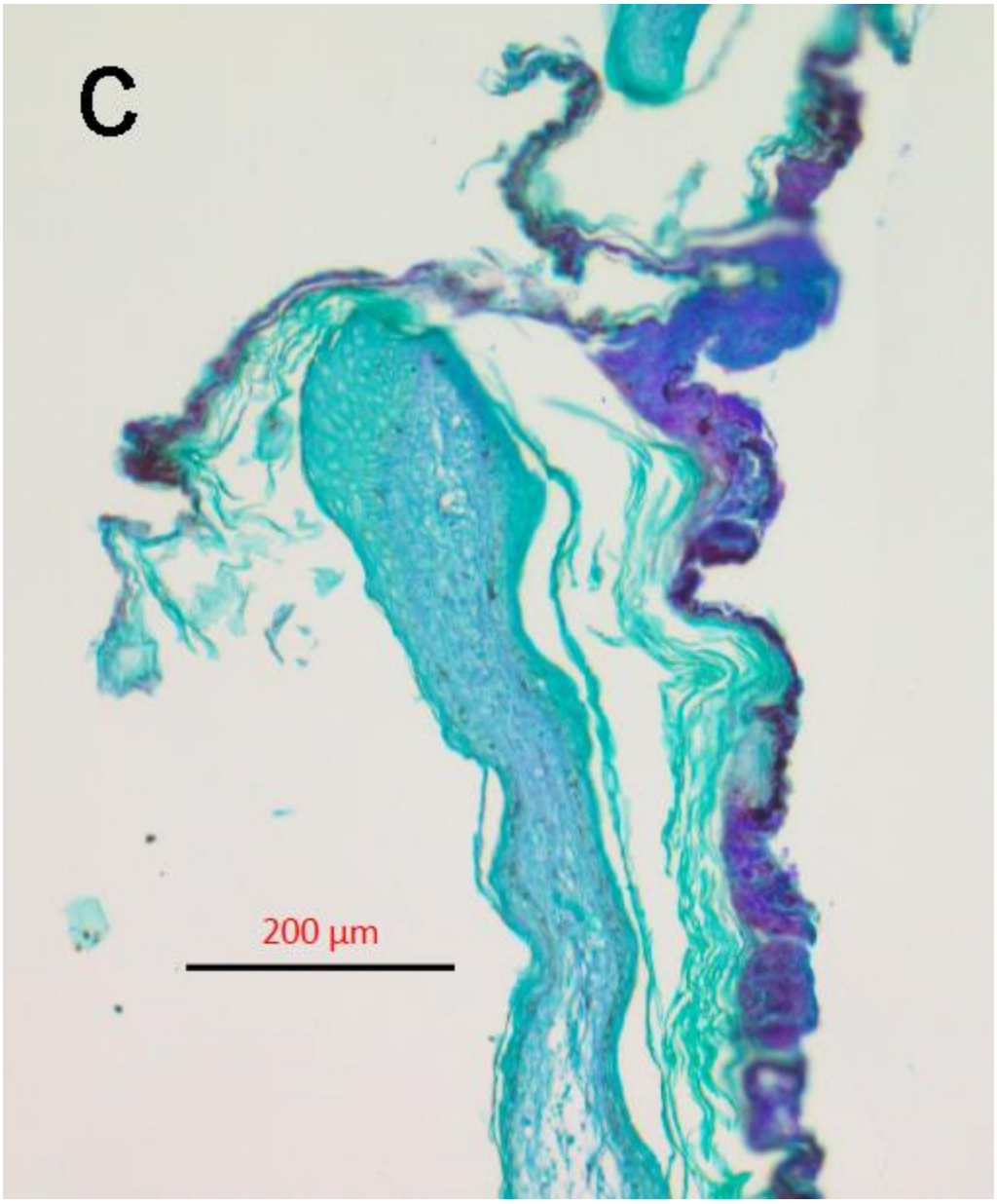


Figure 3.13. The first two principal components of skin surface lipid patterns plotted over time. Smoothing curves were determined using generalized additive modeling with time as a smoothing factor and individual ID as a random effect.


A
50 µm


B
50 µm


C
200 µm

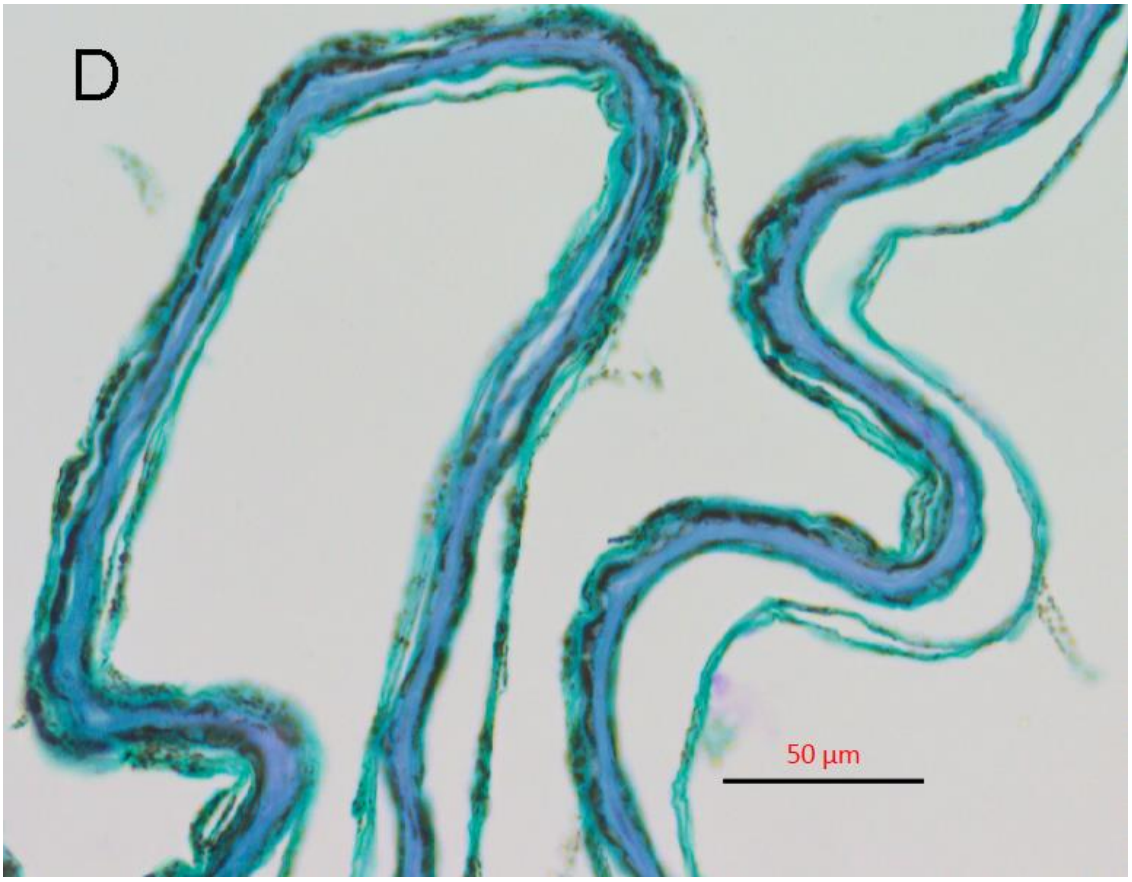


Figure 14. Representative histopathology photos from each sampling event. During the early phases of recovery (A and B), fungus is clearly visible on wing surfaces (arrows). Later, perforations show evidence of rapid healing (C). By Day 40, wing skin appears structurally similar to normal bat wing skin (D).

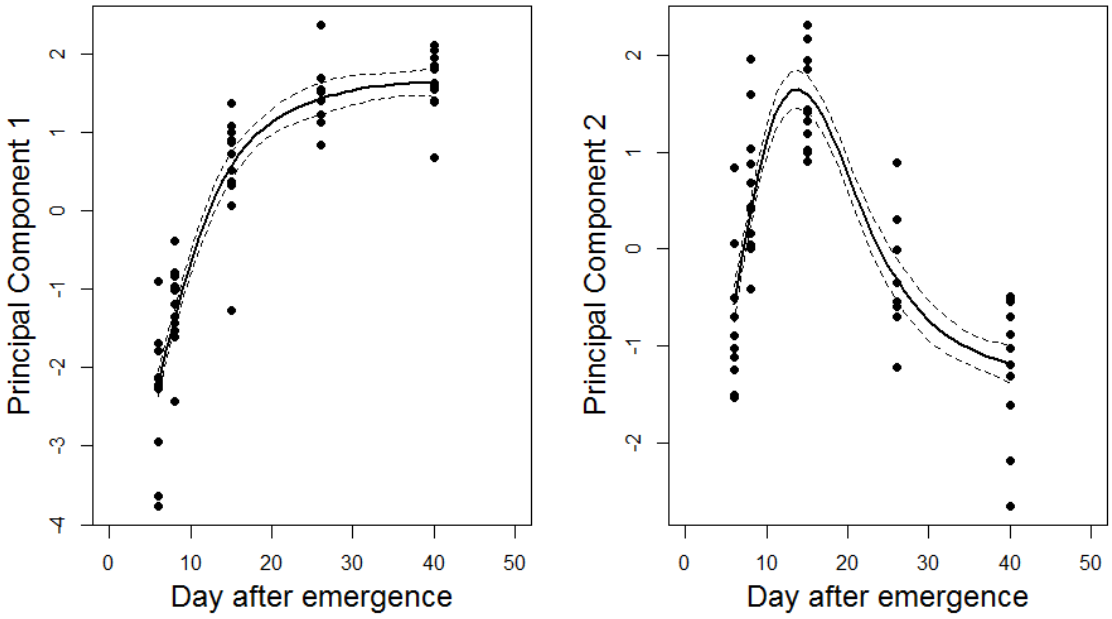


Figure 3.15. The first two principal components of a principal components analysis using all variables from this study plotted temporally.

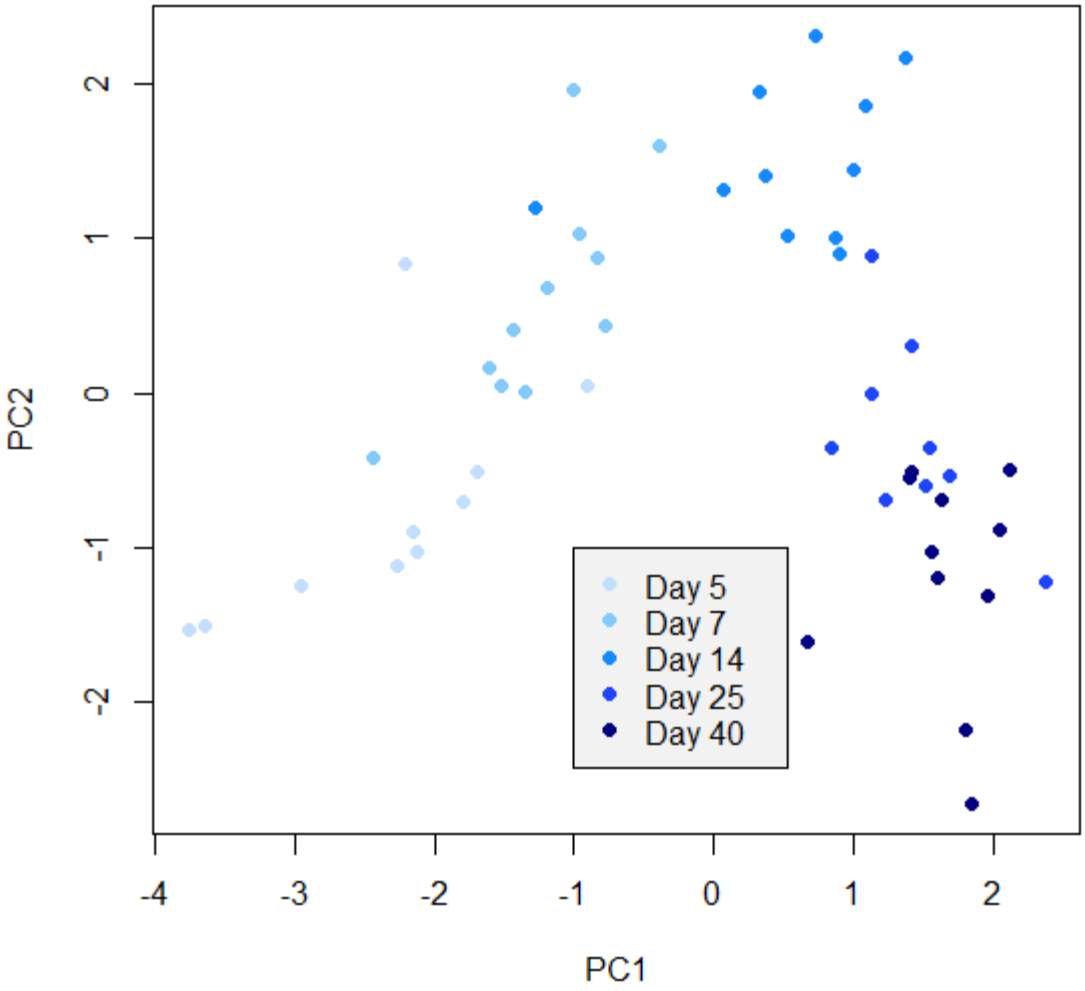


Figure 3.16. Biplot of PC1 and PC2 scores from the integrated PCA. Data points are color coded by sample day. Early samples clustered well by day. However, samples from later days did not cluster as tightly, which is consistent with my findings that bats from day 25 and day 40 have undergone significant recovery and may be indistinguishable based on measurements presented in this study.

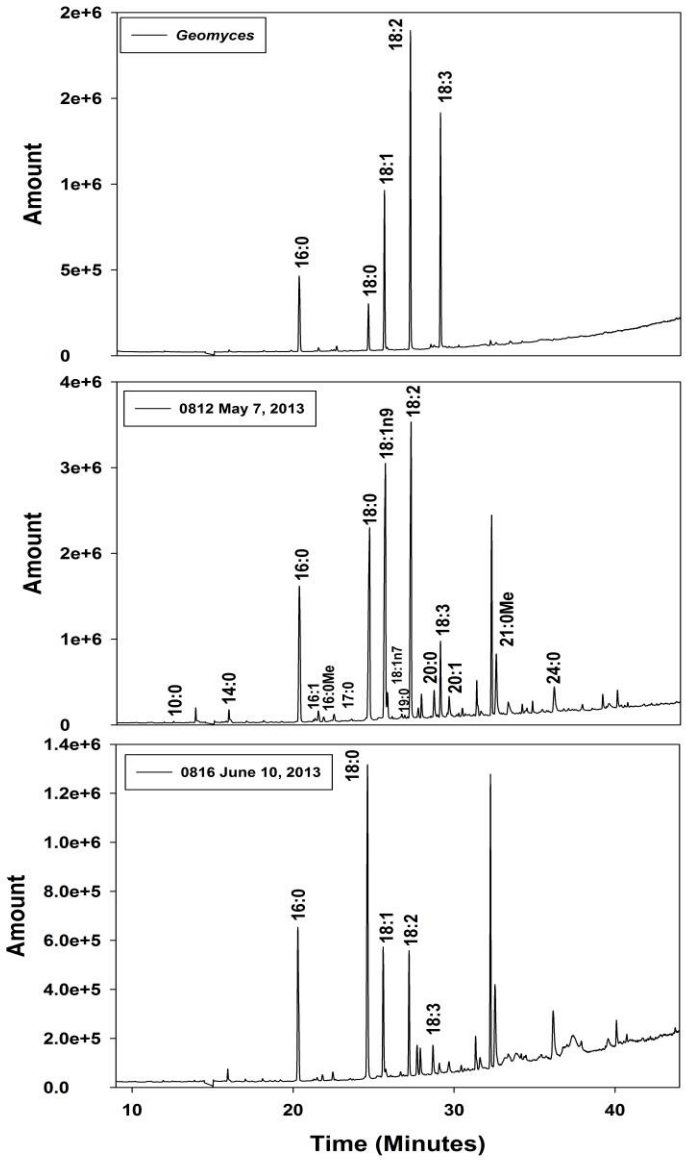


Figure 3.17. Representative GC/MS spectra from *Pd* (A), and two recovering bats. Early recovery phase bats (B) showed surface lipid spectra that closely resembled signals from *Pd.* Later during the recovery phase, bats showed signals that were similar to recorded levels from past studies on bats regions without WNS (C).

CONCLUSIONS AND FUTURE DIRECTIONS

My research is the first to characterize in detail the processes that recovering bats undergo immediately following emergence from hibernation. While the main focus of my study has been directed toward WNS, my research also sheds light on a period of bats' lives that has received little focused study. The initial several weeks following emergence from hibernation are difficult to study because bats are transient during that time (Davis and Hitchcock 1965). However, this time period is valuable to study, especially in the context of WNS. Individuals that survive the winter with WNS must emerge in spring, travel to summer roosts and begin to recover at a time when ambient temperature is still cold and food is scarce (Anthony and Kunz 1977). They may also immediately face a rapidly re-mobilized immune response which may cause wing damage likely to reduce foraging ability and increase energetic costs (Bullen and McKenzie 2007; Bullen and McKenzie 2009; Meteyer, et al. 2012). This spring recovery bottleneck may be especially severe for females that are already energetically constrained because they depend on stored energy to initiate spring reproduction. Thus, it is critical that WNS researchers focus efforts to understand how such a secondary bottleneck can further harm dwindling bat populations and how those effects may be mitigated.

The next steps in WNS research and funding are now moving toward development of treatments and 'cures' of WNS. However, this goal may prove to be extremely challenging. Fungal pathogens, by virtue of their complexity and close evolutionary relationships (compared to bacteria and viruses) to their hosts, are extremely hard to treat (Fisher, et al. 2012). When such limitations are confounded with the

difficulty of trapping wildlife reliably, most treatment options may eventually be ruled out due to impracticality. For example, it has been suggested that hibernacula should be treated with anti-fungal sprays (C. Cornelison, pers. comm). This treatment is inappropriate in two ways: 1) non-target spraying of sensitive cave ecosystems will have unknown impacts on native cave species, and 2) multiple applications of a spray will disrupt normal hibernation processes of bats. Other treatments call for applications of probiotics or other direct application treatments to hibernating clusters (T. Cheng, pers, comm; M. Vonhof, pers. comm.). However, such a treatment requires bats to be handled regularly for multiple applications. In the end, treatments may be a promising opportunity; however it seems most likely that WNS represents a dramatic selection event in which resistance may arise from the remnant populations of survivors. Historical records of bat populations in Europe suggest that bat species have not existed in the same densities as North American species (Puechmaille, et al. 2011). Such a pattern, coupled with the fact that European bats become infected by *Pd* but do not suffer mass mortality (Puechmaille, et al. 2012), may suggest that such a selection event could have occurred in Europe prior to written records on bat populations. Perhaps North America's bat populations will come to reflect what is currently observed in Europe.

Future studies should not only seek to further our understanding of the disease ecology of WNS, but also incorporate research into the sublethal effects of WNS. First, I would identify the specific effects of wing damage on flight, following the model of a series of papers on the effect of molt on bird flight, including navigation of obstacle fields and takeoff efficiency (Swaddle 1999). When birds lose flight feathers to molt, the

amount of power gained per wingbeat is reduced, which reduces the angle at which they leave the ground (Swaddle and Witter 1997). While bats do not take flight from the ground, they do pull out of freefall when leaving the roost. Thus, measuring time and angle to 'recover' from freefall (i.e., when vertical velocity is zero), can reveal some aspects of the aerodynamic effects of WNS. In addition, obstacle fields can reveal whether bats with wing damage have reduced ability to navigate narrow spaces and sharp angles effectively. Another experimental trial would be to examine turning maneuvers in bats with wing injuries and if their flight patterns resemble those found in previous studies of wing damage and wing hair removal (Cheney, et al. 2015; Sterbing-D'Angelo, et al. 2011).

Second, more studies should be conducted to understand the energetic cost of wing damage for flying bats. One study has attempted to understand these effects, but did not specifically examine WNS, rather bats with large areas of missing wing membrane (Voigt 2013). To fully understand the energetic costs of flight, it will be important to recreate the conditions that bats with wing damage experience. Voigt's study may show a greater effect of wing damage on energetic costs because that nature of wing damage that he studied (loss of membrane) would likely change wing aerodynamics and flapping mechanics. Wing damage from WNS may not show a large effect, given that wing surface area is not often drastically reduced, just altered. However, wings that are scabbed and dry may not be as flexible as normal wings and thus could dramatically alter flapping mechanics (Bullen and McKenzie 2002; Bullen and McKenzie 2007; Bullen and McKenzie 2009; S. Swartz. pers. comm.).

Third, to better understand the ecological impacts of WNS, the effect of wing damage on foraging range and efficiency should be determined. With the rapid advancements in the field of movement ecology, this question is quite tractable. Small GPS tags, passive monitoring stations, and satellite tracking have advanced to the point where researchers can track bats for an entire season (Fahr, et al. 2015; McGuire, et al. 2012). Monitoring bats' movements during the healing phase would uncover whether injured animals forage for longer periods and over longer distances. Information such as this, will also play into understanding the additional energetic costs that bats experience due to WNS.

While the nature of scientific research into WNS falls more under largely exploratory, applied, and management questions, WNS has greatly advanced our knowledge of fungal diseases and bat hibernation physiology. I expect that research will continue on this trajectory, despite many researchers shifting efforts toward treatment strategies rather than the basic biology questions that remain unanswered. My suggestion for the future of WNS research is for funding agencies to dial back their efforts to develop a wide spectrum of potential cures, and instead focus on several promising options, while using the remaining funds to build our understanding of bat physiology, behavior, and ecology, as it pertains to WNS.

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