Hibernation Activity and the Susceptibility of Southeastern Bats to *Pseudogymnoascus destructans*, the Causal Agent of White-Nose Syndrome

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Degree

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ABSTRACT

Past research in the southeastern United States suggests that bats are regularly leaving hibernacula throughout winter. Of the bats captured during winter outside of cave hibernacula more than 50% of bats were negative for *Pseudogymnoascus destructans* (*Pd*), the causal agent of white-nose syndrome (WNS). In addition, of the bats we captured that were *Pd* positive, pathogen load and prevalence varied considerably among species. Episodic activity and foraging during winter raises body temperature, which should activate the immune system, possibly retarding fungal growth, and resulting in repeated low-level exposures to the *Pd* pathogen that could lead to disease immunity. Although it is established that bats in the southeastern U.S. are active outside cave hibernacula during winter, the possible impacts on susceptibility to infection by *Pd* and the epizootiology of WNS both within and among species have not been investigated. Differences in winter activity that may account for species-specific differences in WNS disease susceptibility, particularly in more southern latitudes, require further investigation. To investigate these issues, we captured active bats outside of hibernacula for five winters (2012/13, 2013/14, 2015/16, 2016/17, and 2017/18) to track capture rates and dynamics of *Pd* from initial invasion through establishment in five cavernicolous bat species ranging in *Pd* susceptibility. Additionally, we monitored the winter activity of four of these bat species throughout the hibernation seasons of 2016/17, 2017/18, and 2018/19 to delineate similarities and differences in winter activity regimes within and among species. We found that capture rates declined starkly in three out of five study species, which were the three species most susceptible to WNS included in my study. Dynamics of *Pd* shifted as time passed, although species maintained similar levels to susceptibility to *Pd* from initial invasion into early establishment. Lastly, various characteristics of activity of hibernating bats (i.e. torpor and arousal skin temperatures, torpor bout and arousal frequency, activity length, and activity frequency) in the southeast U.S. varied significantly and could likely explain some variation of susceptibility to *Pd* observed in target species.

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LIST OF ABBREVIATIONS

GSMNP: Great Smoky Mountains National Park

NPS: National Park Service

Pd: *Pseudogymnoascus destructans*

PIT: Passive Integrated Transponder

TNC: The Nature Conservancy

TWRA: Tennessee Wildlife Resources Agency

U.S.: United States

USFWS: United States Fish and Wildlife Service

USGS: United States Geological Survey

WMA: Wildlife Management Area

WNS: White-nose Syndrome

CHAPTER 1:
GENERAL INTRODUCTION

BACKGROUND AND JUSTIFICATION

White-Nose Syndrome in North American Bats

In the 21st Century, the disease white-nose syndrome (WNS) has had devastating impacts on North American bat populations (Frick et al. 2017). The psychrophilic fungus *Pseudogymnoascus destructans* (*Pd*) is the causative agent of the disease and was first documented in Howe's Cave, New York, United States (U.S.) in 2006. Since its discovery, the disease has killed over 6-million cave hibernating bats across the eastern and midwestern U.S. and Canada and spread to at least 33 states and 5 Canadian provinces (U.S. Fish and Wildlife Service [USFWS] 2012, USFWS 2016, U.S. Geological Survey [USGS] 2016, Texas Parks and Wildlife Department [TPWD] 2017). White-nose syndrome was detected in Tennessee in the winter of 2009/10, and has now been confirmed in 52 of the 78 counties in the state (66.7%; Campbell 2016). Humans are thought to have played a role in introducing this invasive pathogen to North America from Europe, where *Pd* has persisted in bat populations for potentially millennia (Wibbelt et al. 2010, Kunz et al. 2011, Puechmaille et al. 2012, Warnecke et al. 2012).

As of 2019, twelve species of bats are known to be affected by WNS, with an additional six species known to carry *Pd* (Bernard et al. 2015, TPWD 2017, Washington Department of Fish and Wildlife [WDFW] 2019). The Indiana bat (*Myotis sodalis*) and the gray bat (*Myotis grisescens*) are two federally endangered species affected by the disease. The Indiana bat was federally listed in 1967 due to its limited use of available hibernacula, susceptibility to winter disturbance from humans entering hibernacula, and small range of summer habitat (USFWS 1999). It has since remained listed because of continued habitat loss and the subsequent invasion of WNS, which has decimated populations (Turner et al. 2011). The gray bat was federally listed as endangered in 1976 due to its marked population declines (down 76% from historical numbers), year-round use of 5% of all known available caves within its range, and its susceptibility to disturbance via humans and environmental factors (e.g., flooding and natural alterations; Tuttle 1979, USFWS 1982). Although populations have rebounded and more caves have been found that are used by gray bats, the species has remained federally listed as endangered due to potential dangers from WNS (USFWS 2009). In 2015, the northern long-eared bat (*Myotis septentrionalis*) was the first species to be listed as federally threatened as a result of dramatic declines from WNS (USFWS 2015). Additionally, the big brown bat

(*Eptesicus fuscus*), cave bat (*Myotis velifer*), eastern small-foot bat (*Myotis leibii*), little brown bat (*Myotis lucifugus*), long-legged bat (*Myotis volans*), southeastern bat (*Myotis austroriparius*), tri-colored bat (*Perimyotis subflavus*), western long-eared bat (*Myotis evotis*), and Yuma bat (*Myotis yumanensis*) have all been confirmed to contract WNS (Blehert et al. 2009, whitenosesyndrome.org 2019). The tri-colored bat has been petitioned for federal listing and may receive protection soon (Center for Biodiversity & Defenders of Wildlife 2016). Another eight species/sub-species of North American bats have been confirmed *Pd* positive, but have not displayed symptoms of WNS: eastern red bat (*Lasiurus borealis*), Mexican free-tailed bat (*Tadarida brasiliensis*), Ozark big-eared bat (*Corynorhinus townsendii ingens*), Rafinesque's big-eared bat (*Corynorhinus rafinesquii*), silver-haired bat (*Lasionycteris noctivagans*), Townsend's big-eared bat (*Corynorhinus townsendii*), Virginia big-eared bat (*Corynorhinus townsendii virginianus*), and western small-footed bat (*Myotis ciliolabrum*; Bernard et al. 2015, TPWD 2017, whitenosesyndrome.org 2019).

Pseudogymnoasucus destructans grows best at temperatures ranging from 12°C to 15.8°C, with extreme lower and upper limits of 0°C and 19.8°C, respectively (Blehert et al. 2009, Verant et al. 2012). The fungus erodes the epidermal tissue of hibernating bats, creating lesions on the skin, muzzle, forearms, and wing membranes (Cryan et al. 2010). Even in mild cases, erosion of the wing membranes can occur and is characterized by cup-like structures filled with *Pd* hyphae (Meteyer et al. 2009). The fungal colonization of the epidermis leads to disruption of physiological processes that preserve homeostasis, including thermoregulation and water-electrolyte balance, leading to respiratory acidosis, hypotonic dehydration, and increased fat metabolism in certain *Myotis* species (Cryan et al. 2010, Warnecke et al. 2012, Warnecke et al. 2013, Cryan et al. 2013, Verant et al. 2014). These physiological interruptions frequently lead to an increase in energetically-demanding torpor arousals throughout winter (Reeder et al. 2012). Additionally, bats exhibit unusual and erratic behaviors, including increased emergence from hibernacula during the daytime and cold temperatures and roosting in abnormal hibernation sites (i.e., outside of cave entrances; Foley et al. 2011, McAlpine et al. 2011, Carr et al. 2014). When compounded, these behaviors deplete fat reserves that generally cannot be replenished during winter when prey sources are often low. Death eventually follows as a result of starvation, dehydration, and exposure (Cryan et al. 2010). Bats that survive to spring emergence may exhibit

clinical signs of WNS long after hibernation ends and may even have remaining *Pd* infection until mid-summer (Meteyer et al. 2009, Carpenter et al. 2016).

As a result of WNS emergence, researchers have documented changes in bat community composition. *Myotis* species have been disproportionately impacted by WNS, especially northern long-eared bats and little brown bats, which are now threatened with regional extinction as a result of the disease (Frick et al. 2010, Turner et al. 2011). Researchers found extensive local extinctions in 69% of hibernacula of northern long-eared bats, possibly the species most negatively affected by WNS (Langwig et al. 2012, Frick et al. 2015). Frick et al. (2010) predicted a 99% decrease in eastern little brown bat populations, which was later corroborated (Langwig et al. 2012, Frick et al. 2015). Frick et al. (2015) also estimated a 10-fold decrease in the abundance of all bats at hibernacula following the introduction of WNS. Indiana bats are thought to be facing extinction across their range within a century due to the synergistic effects of WNS and wind energy development, factors that when isolated would have been unlikely to cause extinction (Thogmartin et al. 2013, Erickson et al. 2016). Langwig et al. (2012) predicted that tri-colored bats, another species heavily impacted by WNS, would stabilize at ~6 bats per hibernacula, following a significant population decrease, and face potential regional extirpation (Langwig et al. 2012, Frick et al. 2015). Winter hibernacula surveys in Tennessee show dramatic post-WNS declines in little browns, northern long-eared, and tricolored bats, with many historical hibernacula showing 90–98% decreases across the state since 2009/10 (Campbell 2016, Carpenter 2017). Similar trends are being seen elsewhere, with studies examining pre- and post-WNS changes in summer capture rates (Moosman et al. 2013, Francl et al. 2012, Powers et al. 2015, Reynolds et al. 2016) and acoustic data (Ford et al. 2011, Brooks 2011). While some species are now seeing local persistence post-WNS, populations will be depressed for the foreseeable future despite this survival (Daszak et al. 2000, Langwig et al. 2016, Bernard et al. 2017, Frick et al. 2017). The overall declines exhibited by many southeastern *Myotis* species emphasize the importance of prioritizing protection of critical winter and summer habitat for bats, as well as conserving migration connections for metapopulation movements (O'Shea et al. 2003, Thogmartin et al. 2013, Erickson et al. 2016).

Winter Ecology of North American Bats

Torpor, or a metabolically reduced state, is a mechanism used by bats to conserve energy during energetically stressful times of the year. There are generally two types employed by mammals (Geiser and Ruf 1995). Daily torpor occurs when an organism has a minimum body temperature (T_b) below normal homeothermic T_b that lasts less than 24 hours (Hudson 1973). Hibernation torpor is characterized by a significant drop in T_b and metabolic processes that lasts from days to weeks (Hudson 1973, Geiser and Ruf 1995, Humphries 2005). By reducing body temperature to near ambient levels (T_a), mammals in torpor can reach minimum metabolic rates that are <5% of basal metabolic rate (2°C–8°C for bats; Kayser 1963, Barnes 1989, Thomas et al. 1990). Hibernation is employed by many bat species in North America, for 4–9 months out of the year, in order to avoid energetically stressful times when prey resources are limited due to seasonal changes (Wang 1989, Humphries et al. 2005). Of the 47 bat species occurring in North America, 25 have been observed using some form of torpor, and 21 of those are known to hibernate (Cryan et al. 2010).

Hibernating mammals that spend a majority of their time during the hibernation season in torpor occasionally arouse to normothermic T_b. These arousals are thought to be the main drain on stored energy reserves (costing ~90%), despite only accounting for 1% of hibernation time (French 1985, Thomas et al. 1990, Geiser 2004). They allow animals to raise T_b to normal euthermic levels by increasing their metabolic rate (Hayward et al 1966, Wang 1978, Brack and Twente 1984, Thomas et al. 1990). With bats, the process of raising T_b to euthermic levels can take up to 45 minutes, and arousal bouts can last 1–2 hours (Thomas et al. 1990, Reeder et al. 2011). Internally, arousal is caused by heat that is generated from brown adipose tissue stored in the intrascapular brown fat, liver, and muscles, as well as passive warming (Hayward and Ball 1966, Foster and Frydman, 1978, Thomas et al. 1990). Flying decreases the length of time it takes to arouse from torpor, suggesting the flight muscles may play a role in thermogenesis (Willis and Brigham 2003).

In bats, the purposes behind arousal are not fully understood, although some hypotheses do exist (Thomas et al., 1992; Humphries et al., 2005). The "metabolism hypothesis" theorizes that the accumulation of metabolic wastes becomes so high that animals must arouse to reestablish homeostatic conditions (Lyman et al. 1982). The "biological clock hypothesis" proposes that torpor-arousal rhythm is determined by a biological clock that may potentially be

T_b dependent (Willis 1982). The "water balance hypothesis" states that hibernators face a water deficit due to excessive evaporative water loss, which results in a high concentration of electrolytes in the body that force animals to arouse and drink (Fisher and Manery 1967). Many bat species hibernating in caves choose areas with relative humidity levels from 60–100%, which, coupled with their high surface areas (wings) for evaporative water loss, could put them at high risk of dehydration (Kallen 1964, Davis 1970, Thomas et al. 1992, Cryan et al. 2010). Occasional arousals may also enable the immune system to return to fully operational levels and fight any pathogens introduced during hibernation (Prendergast et al. 2002). Additionally, feeding, copulating, and roost switching have been observed during arousals, although they may not be the initial cause of arousal (Davis 1970, Avery 1985, Brigham 1987, Tidemann 1982, Speakman and Racey 1989, Thomas and Geiser 1997, Luis and Hudson 2006, Ben-Harno et al. 2013).

In the Northeastern U.S., arousal from torpor and subsequent winter activity of bats at cave hibernacula has been attributed to the effects of WNS (Blehert et al. 2009, Turner et al. 2011, Foley et al. 2011, Reeder et al. 2012). Past research states that the physiological effects of WNS in bats may cause these increased arousals, likely stemming from an interruption of homeostasis or attempts to mount an immune response to the fungal invasion (Cryan et al. 2010, Puechmaille et al. 2011, Reeder et al. 2012). However, this may not be the case in the Southeastern U.S. The results of studies in Tennessee indicate that in this region, where winters are less severe and prey are available, bats are regularly arousing from torpor and leaving hibernacula on warmer winter nights to forage, as evidenced by guano collection (Bernard et al. 2017). Of 871 active bats captured outside Tennessee hibernacula during the winters of 2012/13 and 2013/14, 50% were negative for *Pd*, despite coming from infected caves (Bernard et al. 2017). This indicates southeastern bats are arousing from torpor and emerging from caves in winter, regardless of *Pd* and WNS status, at least in the years immediately following invasion of *Pd* and WNS emergence. In addition, of the bats captured that were *Pd* positive, pathogen load (the amount of fungus on a bat) and prevalence (the number of individuals within a swabbed populations that have measurable *Pd* loads) varied considerably among species, with Indiana bats, little brown bats, northern long-eared bats, and tri-colored bats having higher *Pd* loads and prevalence than big brown and gray bats over the two years examined. Less than 18% of all gray bats captured had detectible *Pd* DNA compared to more than 60% of Indiana bats and 70% of

northern long-eared bats. Fungal loads on captured bats were also lower than those of torpid bats in the northeast during the same times of year (Bernard et al. 2017). Further long-term comparison of *Pd* loads and prevalence in relation to winter torpor and emergence behaviors may highlight currently unknown mechanisms affecting differences in susceptibility to *Pd* within and among species. Differences in winter ecology and behavior (i.e., torpor and emergence patterns) may account for species-specific differences in *Pd* loads and prevalence and susceptibility, and ultimately WNS manifestation, particularly in more southern latitudes.

OBJECTIVES

The goal of our study was to investigate the dynamics of *Pd* on bats (i.e., eastern small-footed bat, gray bat, Indiana bat, northern long-eared bat, and tri-colored bat) active during hibernation over time, following pathogen invasion and subsequent establishment, and to explore whether hibernation behavior might influence a species' *Pd* susceptibility. Our objectives were to:

1) Assess *Pd* loads (the amount of *Pd* fungus on a bat), *Pd* prevalence (the number of bats within a swabbed population that have measurable *Pd* loads), and winter capture rates of our target bat species over time as an indicator of *Pd* susceptibility (high susceptibility: high *Pd* load and prevalence, low susceptibility: low *Pd* load and prevalence) and the effect *Pd* has had on winter populations.
2) Compare torpor/arousal bouts and winter activity patterns of our target bat species, including:
    a) Frequency and length of torpor and arousal bouts.
    b) Frequency, duration, and timing of emergence from cave hibernacula.
3) Determine the potential effect torpor and winter emergence patterns may have on *Pd* susceptibility (i.e., *Pd* loads and prevalence) of our target bat species.

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CHAPTER 2:

DYNAMICS OF *PSEUDOGYMNOASCUS DESTRUCTANS* ON BATS ACTIVE DURING HIBERNATION FOLLOWING PATHOGEN INVASION AND SUBSEQUENT ESTABLISHMENT

My consistent use of "we" throughout this chapter is in reference to my collaborators. I was the primary contributor to this work, which involved the following tasks: (1) development of project design and all data collection, (2) statistical analysis, (3) gathering and interpretation of relevant literature, and (4) all writing. E.V. Willcox and R.F. Bernard advised on project design and assisted with editing, J.M. Zobel advised on statistical analysis.

ABSTRACT

White-nose syndrome (WNS) is an infectious disease of cave hibernating bats caused by the fungal pathogen, *Pseudogymnoascus destructans* (*Pd*). Since its introduction in 2006, the disease is estimated to have killed more than 6 million bats. In the southeastern US, bats arouse from torpor and are active outside of caves during the hibernation season regardless of *Pd* status, at least in the short-term post-WNS emergence. While studies have looked at load and prevalence of *Pd* on hibernating bats several years post-invasion, no one has examined these metrics for free-ranging bats that are regularly active outside caves during the hibernation season for longer than 1–2 years post pathogen invasion. Understanding variation in load and prevalence of pathogens within and among host species populations following initial invasion and later establishment can be used to understand potential long-term impacts of a disease. Therefore, the goal of our study was to explore the dynamics of *Pd* on bats active during hibernation (October 1–April 30) over the course of five seasons. Through a long-term capture effort during the hibernation seasons of 2012/13–2017/18, we determined the prevalence and load of *Pd* on active bats, their capture rates, variation within and among species, and variation among hibernation seasons and hibernation periods. The eastern small-footed bat (*Myotis leibii*), the smallest myotid in the region, continued to be captured throughout the study and was one of two species of *Myotis* that experienced low *Pd* loads and prevalence (the other being the gray bat, *M. gricescens*), suggesting some form of resistance or tolerance to the fungus. In contrast, active Indiana bats (*M. sodalis*), northern-long eared bats (*M. septentrionalis*), and tri-colored bats (*Perimyotis suflavus*) maintained generally high *Pd* loads and prevalence over time and their capture rates declined, often precipitously, over the course of the study.

INTRODUCTION

The mortality events caused by the introduction of novel pathogens into naive wildlife populations have been implicated as contributing factors in the decline of many species (Berger et al. 1998, Daszak et al. 2000, Dobson and Foufopoulos 2001, van Riper et al. 2008). Long-term research has provided insight into how host populations react to novel pathogen introductions, such as seen with Tasmanian devil facial tumor disease in Australia and chytrid fungus in amphibian populations across the world (McCallum 2012, Lips 2016). Observing differences in the dynamics of newly introduced pathogens compared to the characteristics of established disease-causing agents allows researchers to understand how wildlife communities may survive with pathogen presence in the long-term (Strayer et al. 2006, Crowl et al. 2008). In some cases, initial infections yield high mortality rates that reduce population size (Härkönen and Heide-Jorgensen 1990, McCallum et al. 2009, Smith et al. 2006). This is exemplified with the chytridiomycosis invasion and subsequent infection of many amphibian populations throughout Australia and the Americas, especially in the Neotropics (Lips et al. 2006, Skeratt et al. 2007, Lips 2016). Chytridiomycosis is thought to have contributed to the decline of 501 amphibian species worldwide, making it one of the most devastating wildlife pathogens in modern history (Scheele et al. 2019). Of these, 60 species are showing initial signs of recovery, 5–13 years after initial invasion (Perez et al. 2014, Voyles et al. 2018, Scheele et al. 2019). Additionally, the once common American chestnut (*Castanea dentata*) was decimated following the introduction of the chestnut blight, *Cryphonectria parasitica,* and is now reduced to dispersed young understory trees that never mature and reproduce (Gravatt 1949; Paillet 2002; Rigling 2018). After the invasion of a pathogen and the subsequent initial mortality of hosts, at-risk populations may shift in a variety of ways, including local extirpation or extinction, range reductions or shifts, or, ultimately, development of tolerance or resistance (Strayer et al. 2006, Frick et al. 2017, Daversa et al. 2018). While a pathogen may infect a number of hosts within its expanding range, host species will likely experience a variety of outcomes (Harvell et al. 1999, Lips et al. 2006, Smith et al. 2006). Understanding variation in the prevalence and severity of pathogens within and among host species populations following initial invasion and later establishment can be used to understand potential long-term impacts on affected species and their populations.

White-nose syndrome (WNS) is an infectious disease of cave hibernating bats caused by the psychrophilic fungal pathogen, *Pseudogymnoascus destructans* (*Pd*). The disease was identified in New York, United States (U.S.), in 2007 and has since spread throughout much of temperate North America, killing an estimated 6-million cave hibernating bats (USFWS 2012, Frick et al. 2015). Currently, twelve species of bats have been affected by the disease, with an additional six species known to carry *Pd* (Bernard et al. 2015, Texas Parks and Wildlife Department [TPWD] 2017, Washington Department of Fish and Wildlife [WDFW] 2019). The populations of numerous bat species have been dramatically altered by the pathogen, with many once-common species exhibiting catastrophic population declines across much of their range (Frick et al. 2015).

Since the emergence of WNS, there have been regional changes in bat community composition (Langwig et al. 2012, Frick et al. 2015). Recent studies estimate a 10-fold decrease in the abundance of numerous bat species at hibernacula following the introduction of WNS (Frick et al. 2015). Declines in summer bat capture rates (Francl et al. 2012, Moosman et al. 2013, Powers et al. 2015) and acoustic activity (Brooks 2011, Ford et al. 2011, Dzal 2011, Bernard 2015) have been observed post-WNS across the eastern U.S. A number of *Myotis* species have been found to be particularly susceptible to *Pd*, including the northern long-eared bat (*Myotis septentrionalis*) and little brown bat (*M. lucifugus*), both of which have been hypothesized to face regional extirpation due to WNS in some areas (Langwig et al. 2016, Frick et al. 2017). The northern long-eared bat, a species severely affected by WNS, has been extirpated from 69% of hibernacula and has been listed as threatened under the Endangered Species Act due to the impacts of the disease on this species declines (Langwig et al. 2012, Frick et al. 2015, U.S. Fish and Wildlife Service [USFWS], 2015). While there were early predictions of dramatic declines in little brown bat populations in the eastern portion of their range, some populations appear to have stabilized at a fraction of historical levels which may be promising for the long-term persistence of the species (Langwig et al. 2016, Dobony and Johnson 2018). Indiana bats (*M. sodalis*) have seen severe declines across their range due to WNS, and may face a reduction of over 86% of their total population within the next 50 years (Thogmartin et al. 2013). This species is estimated to be facing potential extinction within a century due to the synergistic effects of WNS and wind energy development across their range, factors that, when isolated, would have been unlikely to cause range-wide extinction (Thogmartin et al. 2013,

Erickson et al. 2016). Researchers predicted the tri-colored bat (*Perimyotis subflavus*), another species heavily impacted by WNS, would likely stabilize at very low numbers of bats per hibernaculum following significant population declines, however the long-term stability of the species is still unknown (Langwig et al. 2012, Frick et al. 2015, Hayman et al. 2016). While some species now appear to be persisting in the presence of the disease, populations will be depressed for the foreseeable future (Frick et al. 2015, Langwig et al. 2016, Bernard et al. 2017).

Several other species of cave hibernating bats appear less susceptible to *Pd* and have not experienced as severe declines due to WNS. These species include the big brown bat (*Eptesicus fuscus*), eastern small-footed bat (*M. leibii*), gray bat (*M. grisescens*), and Rafinesque's big-eared bat (*Corynorhinus rafinesquii*), all of which will hibernate in caves during winter, but have not experienced major population changes due to the disease (Frank et al. 2014, Bernard et al. 2015, Frick et al. 2017, Pettit and O'Keefe 2017). While the mechanisms behind variation in susceptibility to *Pd* are still unclear, these species have managed to resist or tolerate the fungus and WNS in hibernacula for multiple years (Langwig et al. 2016).

In the northeastern U.S., bats arouse from torpor during the hibernation season and emerge from hibernacula due to the effects of *Pd* infection (Reeder et al. 2012). However, in the southeastern U.S., bats arouse from torpor and are active outside of caves during the hibernation season regardless of *Pd* status, at least in the short-term post-WNS emergence (Bernard and McCracken 2017, Bernard et al. 2017). In addition, for these active bats, pathogen load (the amount of fungus on a bat) and prevalence (the number of individuals within a swabbed population with measurable *Pd* loads) varies considerably among species (Bernard et al. 2015, Bernard et al. 2017). Studies have looked at load and prevalence of *Pd* on hibernating bats several years post-invasion and establishment (Frick et al. 2017). However, the only study to examine these metrics for free-ranging bats regularly active outside caves during the hibernation season was conducted for just 1–2 years immediately after pathogen invasion (Bernard et al. 2017). Episodic activity and foraging during winter raises body temperature, which may activate the immune system, possibly retarding fungal growth and resulting in repeated low-level exposures to the *Pd* pathogen that could lead to disease immunity (Prendergast et al. 2002). Winter foraging may also provide bats hibernating in southern latitudes with energy not available to more northern bat populations, helping them avoid or fight off *Pd* infection (Bernard et al. unpublished). Although it is established that bats in the southeast U.S. are active and foraging

outside cave hibernacula during winter (Bernard et al. 2017), the possible effects of activity on the dynamics of *Pd*, both within and among species, have not been investigated beyond the period of initial pathogen invasion. Long-term data that highlight how organisms respond to pathogens once they have become established are uncommon but can provide important information for the management of susceptible species. Analyzing these data provide researchers the ability to better understand disease dynamics and develop mitigation strategies that may aid species survival (McCallum 2012). Exploring variation in *Pd* load and prevalence, both within and among active species, following initial invasion and once established may help researchers and natural resource managers understand how local populations might persist with the pathogen.

The goal of our study was to explore the dynamics of *Pd* on bats active during hibernation (October 1–April 31) over the course of five years. We focused on five target bat species known to be active throughout the hibernation season in the Southeast and that exhibit varying degrees of susceptibility to *Pd*: Eastern small-footed bat, gray bat, northern long-eared bat, Indiana bat, and tri-colored bat (Langwig et al., 2016, Bernard et al. 2017, Frick et al., 2017). We analyzed data collected by Bernard et al. (2017) from the hibernation seasons of 2012/13 and 2013/14, following initial *Pd* invasion, as well as new data collected in 2015/16, 2016/17, and 2017/18, after *Pd* establishment.

Our objectives were to 1) determine if *Pd* load and prevalence vary within seasons and over years since *Pd* invasion for species that exhibit varying susceptibility to *Pd* and 2) establish whether capture rates vary within seasons and over years since *Pd* invasion for species that exhibit varying susceptibility to *Pd*.

METHODS

Study Area

Our study was conducted at four caves in East Tennessee: Blount Cave, Campbell Cave, Hawkins Cave, and Warren Cave (Figure 2.1). Blount Cave is located in Great Smoky Mountains National Park (GSMNP) and managed by the National Park Service (NPS). Prior to WNS, Blount Cave was the largest known Indiana bat hibernaculum in the state. A population census conducted in February 2019 indicated Indiana bat numbers at this site have declined from

~8,000 to ~750 since WNS was confirmed present in the colony in the winter of 2009/10. Eastern small-footed bats and tri-colored bats are also encountered at this cave. Hawkins Cave, managed by the Tennessee Wildlife Resources Agency (TWRA), is one of the largest gray bat hibernacula in the state, with estimated populations of over 350,000 as of January 2019. This cave also contains a small hibernating population of Indiana bats and low numbers of a few other bat species. Campbell Cave, managed by TWRA and The Nature Conservancy (TNC), contains approximately 1,000 bats, including ~60 Indiana bats and numerous tri-colored and eastern small footed-bats. Warren Cave, also managed by TWRA and TNC, is a gray bat hibernaculum, housing a population similar to Hawkins Cave. Hibernacula in Tennessee were confirmed positive for *Pd* as early as the winter of 2009/10 (Blount Cave). Hawkins Cave was confirmed in the winter of 2010/11, with Campbell Cave and Warren Cave both confirmed in the winter of 2012/13. These years of confirmation were corroborated by real-time PCR (Muller et al. 2013) or histopathology for *Pd* detection (Meteyer et al. 2009).

Bat Capture

We captured bats emerging from caves during the hibernation season (October 1–April 30) of 2012/13, 2013/14 (Bernard et al., 2017), 2015/16, 2016/17, and 2017/18 using mist nets (Avinet Inc., Dryden, NY; mesh diameter: 75/2, 2.6m high, 4 shelves, 4–9 m wide). We netted up to 4 times per month at each cave on nights when there was no rain and temperatures were above 0°C. We opened nets 30 min before civil sunset and kept them open for 5 hours, or until temperatures dropped below 0°C. In months when no bats were captured during the first netting attempt, we made additional netting attempts. After capture, we placed bats in paper bags and in an insulated cooler with a heat source (HotHands®, Dalton, Georgia, U.S.). During the hibernation season, bats were held for no longer than 30 minutes. For all bats captured, we recorded species, sex, right forearm length (mm), and body mass (g) and applied a uniquely numbered 2.4 mm or 2.9 mm metal forearm band (Porzana, Ltd., Icklesham, East Sussex, UK). We transilluminated the wing and tail membranes of captured bats with an ultraviolet (UV) light (wavelengths 385–390 nm) to look for the yellow-orange fluorescence indicative of *Pd* colonization and WNS manifestation (Turner et al. 2014). To determine *Pd* load and prevalence of each individual captured, we collected fungal samples using a sterile, polyester-tipped epidermal swab dipped in deionized water. We rubbed the epidermal swab across the right

forearm and muzzle of each bat five times each (Langwig et al. 2015) and then placed the swab in a 2 ml microtube filled with RNAlater® tissue stabilization solution (Life Technologies, Grand Island, NY). We stored epidermal swab samples at 4°C until they were shipped to the Foster lab at Northern Arizona University and the University of New Hampshire for analysis. All bats were released at the site of capture. While mist netting, followed decontamination procedures outlined by the U.S Fish and Wildlife Service (Shelley et al. 2013). Capture, handling, and sample collection protocols were approved by the University of Tennessee Institutional Animal Care and Use Committee (IACUC 2253-0317), as developed by the American Society of Mammalogists (Sikes et al. 2016) and authorized under scientific collection permits from the USFWS (TE35313B-3), NPS (GRSM-2018-SCI-1253), TWRA (3742) and TDEC (2009-038).

Epidermal swab samples were analyzed by the Foster Lab using quantitative PCR (qPCR) assays developed by Muller et al. (2013). As per Bernard et al. (2017), they extracted fungal DNA from samples using DNEasy 96 Blood & Tissue kits (Qiagen Inc., Valencia, CA). They tested all samples, as well as negative control wells distributed across each PCR plate, for the presence of *Pd* DNA using a Real-Time PCR assay targeting the intergenic spacer (IGS) region of the rRNA gene complex. They ran all plates in duplicate with a quantified standard of isolate *Pd* 20631-21. They considered any reaction that crossed the threshold baseline in fewer than 40 cycles on either plate positive for *Pd* DNA. Average *Pd* load in nanograms (ng) was then calculated in each sample based on the cycle threshold (Ct) value and a generated standard curve based on serial dilutions and an equation described by Langwig et al. (2015) and Bernard et al. (2015). We considered a sample to be positive for the fungus (*Pd*+) if at least one of the two replicates had Ct values of less than 40. Otherwise, a sample was considered negative for the fungus (*Pd*-). As our study subjects were active bats, we opted to use a more liberal cut off for positive designation in order to ensure detection of *Pd* even after extensive activity (i.e., arousal, grooming, and flight; Mosher et al. 2018).

Data Analysis

We log transformed fungal load data prior to analysis to meet normality and homogeneity of variance assumptions (Conover 1999, Zar 1999). We used generalized linear mixed models (using the lme4 package (Bates et al. 2015) in the R statistical package (R Core Team 2017) to

compare *Pd* load (family Gaussian) within and among species and among hibernation seasons (2012/13–2017/18; time) and hibernation periods (i.e., early [October 1–November 30], mid [December 1–February 28], and late [March 1–April 30]), with cave hibernacula as a random effect. We followed this with post-hoc tests using least square means comparisons with no adjustment (i.e., Fisher's LSD test) due to small sample size (using the emmeans package (Lenth 2019) in R).

We calculated *Pd* prevalence as the number of *Pd*+ individuals of a species captured divided by the total number of individuals of that species captured. We used a generalized linear model fitted in R with Type III sum of squares (using the car package (Fox and Weisberg 2011) in R) to compare *Pd* prevalence data (family binomial) within and among species and among hibernation seasons and hibernation periods, followed by least square means comparisons with no adjustment. Due to small sample size, we were only able to include one interaction term in our *Pd* prevalence models. As we were primarily interested in understanding changes in *Pd* prevalence over time, we chose to include a species * hibernation season rather than species * hibernation period interaction.

We calculated capture rates by dividing the total number of individuals of a species captured during a netting session by the total number of net hours for the session. Net hours were determined by multiplying the length and width of open nets (m^2) by the number of hours nets were open each night, resulting in captures/net (m^2)/hour. We used a Kruskal-Wallis test in R, followed by Mann-Whitney U tests with a Bonferroni adjustment to compare capture rates of individual bat species among hibernation seasons and hibernation periods at their principal cave hibernacula. Caves where >50 individuals were recorded during pre-WNS hibernacula surveys (i.e., survey year 2009/10 and prior) were considered principal hibernacula for each species (Bernard et al., 2017; USFWS, 2007). Hawkins and Warren caves were considered principal hibernacula for gray bats, Campbell cave was classified as a primary hibernaculum for eastern small-footed bats, and Campbell and Blount caves were assessed as principal hibernacula for Indiana bats, northern long-eared bats, and tri-colored bats.

RESULTS

Pseudogymnoascus destructans Dynamics

We transilluminated the wings and tail membranes of 784 individuals of our target bats species to look for UV fluorescence, with 13.90% (n = 109/784) fluorescing and considered UV positive (UV+; Table 2.1). Of these UV+ individuals, tri-colored bats comprised 36.70% (n = 40/109), northern long-eared bats 33.94% (n = 37/109), Indiana bats 25.68% (n = 28/109), gray bats 2.75% (n = 3/109), and eastern small footed bats 0.92% (n = 1/109; Table 2.1).

We swabbed 1,277 individuals of our five target bat species for *Pd*. Of the bats swabbed, 37.19% were *Pd*+ by Real-Time PCR analysis (Table 2.2). *Pseudogymnoascus destructans* loads were highest for tri-colored bats (-2.431 ± .084 $\log_{10}$ng [$\bar{x}$ ± SE], n = 111) and northern long-eared bats (-2.458 ± 0.102 $\log_{10}$ng, n =133) and lowest for eastern small-footed bats (-4.586 ± 0.088 $\log_{10}$ng, n = 75) and gray bats (-4.957 ± 0.061 $\log_{10}$ng, n = 74). Indiana bats had intermediate *Pd* loads (-3.186 ± 0.131 $\log_{10}$ng, n = 77).

A bat species * hibernation season interaction had an effect on *Pseudogymnoascus destructans* loads ($P = 0.022$; Table 2.3). Within species, post hoc tests indicated Indiana bat *Pd* loads were higher during 2017/18 than 2012/13, 2013/14, and 2015/16 ($P \leq 0.047$), but similar to loads in 2016/2017 ($P = 0.092$; Table 2.3). Tri-colored bat *Pd* loads were lower in 2015/16 ($P < 0.001$) than in all other seasons, during which they were similar ($P \geq 0.150$). Eastern small-footed bat, gray bat, and northern long-eared bat *Pd* loads were similar across all seasons ($P \geq 0.051$; Table 2.3). Among species, post hoc tests indicated eastern small-footed bat and gray bat *Pd* loads were similar ($P \geq 0.094$) and lower than those of Indiana bats, northern long-eared bats, and tri-coloreds in 2012/13, 2013/14, 2015/16, and 2016/17 ($P \leq 0.008$). The exception was 2017/2018, when *Pd* loads on Indiana bats were similar to those of eastern small-footed bats and gray bats ($P \geq 0.181$) and lower than northern long-eared bats and tri-colored bats ($P \leq 0.011$; Table 3). During 2013/14 and 2015/16, northern long-eared bat *Pd* loads were higher than those for tri-colored bats ($P < 0.011$), but similar to those for Indiana bats ($P \geq 0.061$). However, in 2012/13 and 2016/17 Indiana bat, northern long-eared bat, and tri-colored bat *Pd* loads were similar ($P > 0.464$; Table 2.3).

A species * hibernation period interaction also had an effect on *Pd* loads ($P < 0.001$; Table 2.4). Within species, eastern small-footed bat, Indiana bat, and tricolored bat *Pd* loads

were lower in early hibernation than in mid or late hibernation (P <0.0471). Northern long-eared bat *Pd* loads were greater in mid hibernation than early or late hibernation (P <0.001; Table 2.4). Gray bat *Pd* loads were similar across all hibernation periods ($P \geq 0.485$; Table 2.4). Among species, post hoc tests indicated that, in early hibernation, eastern small-footed bat and gray bat *Pd* loads were lower than those for Indiana bats and tricolored bats ($P \leq 0.023$), but similar to those of northern long-eared bats ($P \geq 0.060$). In mid and late hibernation, eastern small footed and gray bat *Pd* loads were lower than those of Indiana bats, northern-long eared bats, and tri-colored bats ($P \leq 0.001$). While, in mid hibernation, *Pd* loads for eastern small-footed bats and gray bats were similar ($P = 0.067$), by late hibernation, eastern small-footed bat *Pd* loads were higher than those for gray bats ($P = 0.003$). In early hibernation, *Pd* loads were similar for Indiana bats and tri-colored bats ($P = 0.892$). However, in mid hibernation *Pd* loads were higher for Indiana bats than for northern long-eared bats and tri-colored bats ($P \leq 0.049$), and by late hibernation they were higher for tri-colored bats than for Indiana bats and northern long-eared bats ($P \leq 0.001$).

Pseudogymnoascus destructans prevalence was highest for northern long-eared bats (76.88%, n = 133/173) and tri-colored bats (66.87%, n = 11/166) and lowest for gray bats (16.12%, n = 7/490; Table 2.4). Indiana bat and eastern small-footed bat had intermediate *Pd* prevalence (36.49%, n = 77/211 and 31.65%, n = 75/237, respectively; Table 2.4).

A species * hibernation season interaction had an effect on *Pd* prevalence ($P = 0.040$, Table 2.5). Within species, post hoc tests suggested eastern small-footed bat *Pd* prevalence was lower in 2015/16 than in any other season (P <0.011; Table 2.5). Gray bat *Pd* prevalence was higher in 2013/14 and 2017/18 than in any other season (P <0.037; Table 2.5). For both these species prevalence never exceeded 50%. *Pseudogymnoascus destructans* prevalence in northern long-eared bats was lower in 2012/13 than 2013/14 (P <0.001). *Pseudogymnoascus destructans* prevalence for this species was >75% in all but the first hibernation season. *Pseudogymnoascus destructans* prevalence in tri-colored and Indiana bats was similar across seasons (P >0.147), despite appearing considerably lower in 2015/16 and 2017/18 for Indiana bats and 2017/18 for tri-colored bats (Table 2.5, Figure 2.2). Among species, post hoc tests indicated *Pd* prevalence in gray bats was lower than for all other species during 2012/13 and 2013/14 (P <0.001; Table 2.5). *Pseudogymnoascus destructans* prevalence in eastern small-footed bats was lower than for Indiana bats, northern long-eared bats, and tri-colored bats in these same seasons (P <0.001).

Beyond 2012/13 and 2013/14, post hoc tests generally suggest *Pd* prevalence for all species was similar.

Capture Numbers and Rates

Over the course of the study, we captured 1,670 bats over 134 nights of mist netting (11,832.5 net*hours), with 1,505 (90.12%) individuals from the five target species. Of the target species captured, gray bats comprised 34.02% (n = 512), Indiana bats 22.39% (n = 337), eastern small-footed bats 17.6% (n = 265), northern long-eared bats 13.42% (n = 202) and tricolored bats 12.55% (n = 189; Table 2.6). Although Indiana bats were the second most frequently captured species, no individuals were captured from November–March of 2016/17 and 2017/18. Across all caves, species had an effect on capture rate (P <0.001). Gray bat capture rates were greater than for any other species (Table 2.7). Of the remaining species, northern long-eared bats had the next greatest capture rate, followed by eastern small-footed bats and tri-colored bats. Capture rates for Indiana bats were lower than for all other species (Table 2.7).

Hibernation season had an effect on capture rate of northern long-eared bats at Campbell Cave and tri-colored bats at Blount Cave (Table 2.8). Post hoc comparisons indicated capture rates of northern long-eared bats were lower at Campbell Cave in 2015/16 and 2017/18 than in 2012/13 ($P \leq$0.026), declining by $\geq$99.46% (Figure 2.3; Table 2.8). While hibernation season was not found to have an effect on the capture rate of northern long-eared bats at Blount Cave, similar rates of decline were observed from 2012/13 to 2015/16 (98.73%; Figure 3, Table 9). Post hoc comparisons showed no differences in capture rates among seasons for tri-colored bats at Blount cave ($P \geq$0.075), despite them declining by 98.97% from 2012/13 to 2013/14 (Figure 2.3, Table 2.8). Tri-colored bat capture rates at Blount Cave remained low through 2017/2018. Although hibernation season was not found to have an effect on capture rates of tri-colored bats at Campbell Cave, declines were also observed. Tri-colored bat capture rates were lowest in 2015/2016, declining by 78.15% from 2012/2013, and did not recover in later study seasons (Table 2.8, Figure 2.3).

Hibernation season had no effect on eastern small-footed bat, gray bat, or Indiana bat capture rates at principle hibernacula (Table 2.8). Capture rates for eastern small-footed bats and gray bats did remain relatively stable across seasons (Figure 2.3). However, despite there being no effect of season, Indiana bat capture rates at Blount Cave were 82.16% lower in 2013/2014

than in 2012/2013 and remained low through 2017/2018 (Table 2.8, Figure 2.3). At Campbell Cave the capture rate for Indiana bats declined to 0 in 2015/2016, although it increased slightly in later seasons (Table 2.8, Figure 2.3).

There was also an effect of hibernation period on capture rate of species at their principal hibernaculum (Table 2.9). There was no effect of hibernation period on capture rates of eastern small-footed bats or northern long-eared bats at their principal hibernacula (Table 2.9). Capture rates for gray bats at Hawkins Cave were lower in the mid-hibernation period than in the early or late hibernation periods ($P < 0.009$; Table 2.9). Tri-colored bat capture rates at Blount Cave were higher in the late hibernation period than in the early hibernation period ($P = 0.049$; Table 2.9). Capture rates of Indiana bats at both Blount Cave and Campbell Cave varied significantly ($P < 0.03$; Table 2.9). At both caves, capture rates of Indiana bats were higher in the early hibernation period than in the late hibernation period ($P \leq 0.05$; Table 2.9).

DISCUSSION

Our study is the first to examine *Pd* dynamics on active bats for at least five years, post pathogen invasion and establishment. Our results suggest that fungal load and prevalence of *Pd* on active bats varies within and among species, and among hibernation seasons and periods. Additionally, they indicate the capture rates for highly susceptible species (i.e., Indiana bats, northern long-eared bats, and tri-colored bats) decline precipitously the longer a hibernaculum is *Pd* positive.

Pseudogymnoascus destructans loads on gray bats and eastern small-footed bats were similar and did not tend to vary over time. In addition, *Pd* loads for these two species were consistently much lower than for the other three target species examined. Further, *Pd* prevalence for both species was lower than for the other species, never exceeding 50%. These low *Pd* loads and prevalence indicate gray bats and eastern small-footed bats are less susceptible to the fungus than Indiana, northern long-eared, and tri-colored bats. Combined with their relatively consistent capture rates throughout the study, our results suggest a resistance or tolerance to the fungus that has been present since initial pathogen invasion, similar to observations of hibernating bats (Frick et al. 2017).

The large body size of gray bats could explain their potential resistance to or tolerance of *Pd*, given that they enter hibernation with more fat reserves that can help stave off starvation (Kunz et al. 1998, Verant et al. 2014, Cheng et al. 2018). Studies conducted on a similar large-bodied European species, *M. myotis*, show that immune and biological response to disease were major drivers in tolerance to *Pd*, as opposed to microclimate (Davy et al. 2017). Given their similarity in size to gray bats, this could provide a possible explanation for tolerance or resistance in this species.

In contrast to gray bats, eastern small-footed bats were the smallest *Myotis* species included in this study (~4 grams). Unlike gray bats, eastern small-footed bat *Pd* prevalence appeared to fluctuate over time. A possible explanation for these changes in *Pd* prevalence in later hibernation seasons could be changes in weather conditions that promoted use of non-traditional hibernacula where microclimates and lack of other *Pd* infected bats inhibited the growth and transmission of *Pd* (Best and Jennings 1997, Moosman et al. 2015, Moosman et al. 2017). Thermal refugia in talus slopes, rock outcroppings, and similar substrates could be optimal hibernation sites during warmer winter periods. The microclimates in these non-traditional hibernacula are likely too cold for *Pd* growth (Moosman et al. 2015, Moosman et al. 2017). In addition, the chance of *Pd* transmission is probably lower in these alternative roosts due to the reduced potential for an uninfected bat to occupy the same crevice as an infected bat (Moosman et al. 2017, Verant et al. 2018). In warm winters, like those seen in Tennessee, eastern small-footed bats could use these crevices as roosts for hibernation more frequently than caves, possibly reducing their contact with environmental *Pd* spores (Langwig et al., 2012; Moosman et al., 2015).

P. destructans loads in Indiana bats and tri-colored bats remained stable until the last year of the study at which point loads decreased. Prevalence trends varied across hibernation seasons for these two species, ranging from just over 10% to complete saturation. Additionally, capture rates declined severely for both species over the five hibernation seasons at important historical hibernacula. The fluctuations in loads and prevalence seen in both species is likely tied to the decline in capture rates observed in both species. Over time, captures of Indiana bats and tri-colored bats were low to nonexistent during mid-hibernation and almost all swabbed individuals from these species in later seasons were captured in early hibernation [i.e. October–November]. Bats swabbed during this period exhibit lower mean loads and prevalence compared to bats

swabbed in later hibernation periods (Langwig et al. 2016, Frick et al. 2017). Given that most Indiana bats and tri-colored bats swabbed in later years of this study were captured in the early hibernation period, when loads and prevalence are lowest, the apparent reduction in prevalence and load seen in these species during this study could be explained simply by when the majority of active bats were sampled.

Regardless of shifts in *Pd* dynamics in both active Indiana and tri-colored bats over time, the decline in populations of both species at historical hibernacula is apparent. Both species exhibited precipitous declines the longer *Pd* had been present at a hibernaculum, similar to findings in studies of hibernating bats (Langwig et al. 2012). Given the consistently high fungal loads and potential explanations for fluctuations in prevalence mentioned above, it is likely that active Indiana bats and tri-colored bats have not developed resistance or tolerance to *Pd* (Frick et al. 2017). While the mechanisms behind continued susceptibility remain unclear, potential explanations exist for these two species. For example, for tri-colored bats, their small body size and roosting preferences within the optimal growth range of *Pd* (T_a: 9°C - 12°C and >80% RH) stand out (Briggler and Prather, 2003; Verant et al. 2012, Langwig et al. 2016). By hibernating in areas that promote *Pd* growth, tri-colored bats are subjected to enhanced *Pd* growth each season compared to roosts in drier, colder areas (Langwig et al. 2016). While studies show some susceptible species select different microclimates post-WNS, recent research in the southeast recorded tri-colored bats continuing to select roosts in areas with optimal conditions for *Pd* growth (Boyles et al. 2007, Lilley et al. 2016, Johnson et al. 2016, Sirajuddin 2018). Although some research suggests tri-colored bats developing tolerance to *Pd* in certain parts of their range, more research is needed in southern regions to see if this could be a population-wide trend (Frick et al. 2017).

In comparison, plausible explanations regarding susceptibility to *Pd* in Indiana bats heavily focus on clustering behavior and sensitivity to disease severity (Langwig et al. 2012, Langwig et al. 2016, Frick et al. 2017). Indiana bats are known to create tight clusters consisting of up to hundreds of individuals within hibernacula, and in colonies exhibiting these tight clusters disease transmission rates can be high (Barbour and Davis 1969, Getz and Pickering 1983, Langwig et al. 2012). Neighboring individuals may increase the opportunity for contact with fungal spores, this increasing fungal load and prevalence within highly gregarious species (Langwig et al. 2012). Additionally, individuals forming these tight clusters have been found to

arouse simultaneously with neighboring bats, thus potentially reducing the energetic demand of thermogenesis (Czenze et al. 2013). Although this potential reduction in energetic demands during torpor could be helpful in managing fat reserves, Indiana bats are still seeing high mortality due to *Pd* infection (Frick et al. 2017). Additionally, while some changes in clustering behavior have been recorded since WNS invasion, the degree of change is currently not enough to reduce disease transmission rates significantly (Langwig et al. 2012). In lieu of behavioral changes, it is possible that this species faces heightened innate sensitivity to *Pd* infection, which could explain continued susceptibility and population declines for long periods of time after establishment (Langwig et al. 2016, Frick et al. 2017).

Fungal loads were higher on northern long-eared bats than on all other focal species swabbed and also increased over time. Additionally, prevalence of *Pd* on active northern long-eared bats never fell below 70%. Overwinter capture rates of this species declined at all principal hibernacula. In terms of *Pd* dynamics, our results for northern long-eared bats show similar trends seen by Frick et al (2017). Looking at capture rates, our data shows similar trends reported for northern long-eared bats during summer (Reynolds et al. 2016). Within their range, populations of northern long-eared bats have crashed due to WNS and their inability to cope with or manage *Pd* growth (Langwig et al. 2012, Frick et al. 2015, Reynolds et al. 2016). It is possible that their small body size and roosting preference within the optimal *Pd* growth range, similar to tri-colored bats, could explain their inherent inability to function with *Pd* infection (Cryan et al. 2010, Langwig et al. 2012 Verant et al. 2012, Frick et al. 2017). Although encountered sparingly throughout this study, no more than 5 individuals were ever captured each year across all sites after the significant decline from 2012/13 to 2015/16. We see that northern long-eared bat populations are continuing to decline several years after *Pd* invasion, although perhaps populations could be stabilizing at a mere fraction of their historic numbers, similar to what has been predicted for other species highly susceptible to WNS (Langwig et al. 2016). While northern long-eared bat winter populations are difficult to survey given their roosting preferences, the fraction of active bats that are still encountered during winter could hint that this species may be persisting undetected within hibernacula (Caceres and Barclay 2000).

Given that bats largely disappeared during later years of the study, fungal load and prevalence of *Pd* estimates for highly susceptible species (i.e., Indiana bats, tri-colored bats, and northern long-eared bats) may be skewed towards characteristics of *Pd* load and prevalence seen

in early hibernation (~October) when most individuals of these species were captured. However, results from this study provide further evidence that WNS continues to negatively impact populations of highly susceptible bat species well into pathogen establishment in the southeastern U.S. This research demonstrates that capture rate decreases in susceptible species in the region began within three years of initial *Pd* invasion. Continued study of bat activity during the hibernation period in the southeast could also provide insight on the mechanism by which species with stable rates of activity during the winter are coping compared to others.

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APPENDIX 1

Tables

Table 2.1. Number of bats (no.) transilluminated with ultraviolet (UV) light that fluoresced (UV positive [UV+]) and did not fluoresce (UV negative [UV-]) at four cave hibernacula in Tennessee over five hibernation seasons (October 1–April 30), 2012/13–2017/18. Fluorescence indicates infiltration of the wings or tail membrane by *Pseudogymnoascus destructans* the fungal agent of white-nose syndrome (WNS), and WNS manifestation.

	UV Status														
	2012/13			2013/14			2015/16			2016/17			2017/18		
Species	UV+	UV-	Total	UV+	UV-	Total	UV+	UV-	Total	UV+	UV-	Total	UV+	UV-	Total
Eastern s*mall-footed bat* (*Myotis leibii*)	0	12	12	1	49	50	0	61	61	0	47	47	0	13	13
Gray bat (*Myotis grisescens*)	1	3	4	2	150	152	0	113	113	0	38	38	0	20	20
Indiana bat (*Myotis sodalis*)	5	4	9	12	10	22	7	25	32	4	0	4	0	25	25
Northern long-eared bat (*Myotis septentrionalis*)	6	42	48	26	25	51	3	2	5	1	0	1	1	1	2
Tri-colored bat (*Perimyotis subflavus*)	1	7	8	5	26	31	15	0	15	16	1	17	3	1	4

Table 2.2. Number of bats (no.) captured at four cave hibernacula in Tennessee found positive (*Pd*+) and negative (*Pd*-) for *Pseudogymnoascus destructans*, the fungal agent of white-nose syndrome, over five hibernation seasons (October 1–April 30), 2012/13–2017/18.

	Pd Status														
	2012/13			2013/14			2015/16			2016/17			2017/18		
Bat Species	*Pd*+	*Pd*-	Total	*Pd*+	*Pd*-	Total	*Pd*+	*Pd*-	Total	*Pd*+	*Pd*-	Total	*Pd*+	*Pd*-	Total
Eastern s*mall-footed bat* (*Myotis leibii*)	19	24	43	23	28	51	6	56	62	22	25	47	5	29	34
Gray bat (*Myotis grisescens*)	22	106	128	32	120	152	9	104	113	7	42	59	9	29	38
Indiana bat (*Myotis sodalis*)	38	11	49	14	8	22	8	24	32	4	0	4	13	91	104
Northern long-eared bat (*Myotis septentrionalis*)	79	29	108	45	9	54	4	1	5	1	0	1	4	1	5
Tri-colored bat (*Perimyotis subflavus*)	55	16	71	20	12	32	15	0	15	16	1	17	5	26	31

Table 2.3. Mean loads (log_{10}ng) of *Pseudogymnoascus destructans*, the fungal agent of white-nose syndrome, on bats captured at four cave hibernacula in Tennessee over five hibernation seasons (October 1–April 30), 2012/13–2017/18.

	Pd Load ($\bar{x} \pm$ SE)[a, b]				
Species	2012/2013	2013/2014	2015/2016	2016/2017	2017/2018
Eastern small-footed bat (*Myotis leibii*)	-4.426 ± 0.193$_{A:1}$	-4.619 ± 0.191$_{A:1}$	-4.467 ± 0.152$_{A:1}$	-4.796 ± 0.106$_{A:1}$	-4.258 ± 0.461$_{A:1}$
Gray bat (*Myotis grisescens*)	-4.868 ± 0.151$_{A:1}$	-4.912 ± 0.091$_{A:1}$	-5.177 ± 0.044$_{A:1}$	-5.289 ± 0.035$_{A:1}$	-4.864 ± 0.181$_{A:1}$
Indiana bat (*Myotis sodalis*)	-2.642 ± 0.111$_{A:2}$	-3.107 ± 0.292$_{A:2,3}$	-2.99 ± 0.376$_{A:2,3}$	-2.862 ± 0.287$_{A,B:2}$	-5.079 ± 0.060$_{B:1}$
Northern long-eared bat (*Myotis septentrionalis*)	-2.469 ± 0.133$_{A:2}$	-2.579 ± 0.183$_{A:2}$	-2.148 ± 0.274$_{A:2}$	-1.367 ± 0.000$_{A:2}$	-1.473 ± 0.306$_{A:2}$
Tri-colored bat (*Perimyotis subflavus*)	-2.349 ± 0.093$_{A:2}$	-2.348 ± 0.238$_{A:3}$	-2.558 ± 0.197$_{B:3}$	-2.475 ± 0.222$_{A:2}$	-3.142 ± 0.909$_{A:2}$

[a] $\bar{x} \pm$ SE in the same row followed by the same uppercase letter not significantly different (P >0.05).
[b] $\bar{x} \pm$ SE in the same column followed by the same number not significantly different (P >0.05).

0

Table 2.4. Mean loads (log_{10}ng) of *Pseudogymnoascus destructans*, the fungal agent of white-nose syndrome, on bats captured at cave hibernacula in Tennessee during three hibernation periods (i.e., early [October 1–November 30], mid [December 1–February 28], and late [March 1–April 30]), 2012/13–2017/18.

	Pd Fungal Load ($\bar{x} \pm$ SE)[a, b]		
Species	Early Hibernation	Mid Hibernation	Late Hibernation
Eastern small-footed bat (*Myotis leibii*)	-5.021 ± 0.260$_{A:1}$	-4.501 ± 0.129$_{B:1}$	-4.611 ± 0.133$_{B:1}$
Gray bat (*Myotis grisescens*)	-5.172 ± 0.042$_{A:1}$	-4.801 ± 0.160$_{A:1}$	-4.985 ± 0.067$_{A:2}$
Indiana bat (*Myotis sodalis*)	-4.369 ± 0.256$_{A:2}$	-2.577 ± 0.261$_{B:2}$	-2.755 ± 0.098$_{B:3}$
Northern long-eared bat (*Myotis septentrionalis*)	-4.665 ± 0.343$_{A:1,2}$	-1.977 ± 0.088$_{B:3}$	-2.959 ± 0.177$_{C:3}$
Tri-colored bat (*Perimyotis subflavus*)	-3.114 ± 0.338$_{A:2}$	-2.389 ± 0.097$_{B:3}$	-2.214 ± 0.109$_{B:4}$

[a] $\bar{x} \pm$ SE in the same row followed by the same uppercase letter not significantly different (P >0.05).
[b] $\bar{x} \pm$ SE in the same column followed by the same number not significantly different (P >0.05).

1

Table 2.5. Prevalence (%) of *Pseudogymnoascus destructans*, the fungal agent of white nose syndrome, on five bat species captured at four cave hibernacula in Tennessee over five hibernation seasons (October 1–April 30), 2012/13–2017/18.

	Prevalence (%)[a, b]				
Species	2012/2013	2013/2014	2015/2016	2016/2017	2017/2018
Eastern small-footed bat (*Myotis leibii*)	$44.19_{A:1}$	$45.10_{A:1}$	$9.68_{B:1}$	$46.81_{A:1}$	$14.71_{A:1}$
Gray bat (*Myotis grisescens*)	$17.19_{A:2}$	$21.05_{B:2}$	$7.96_{A:1,2}$	$11.86_{A:2}$	$23.68_{B:1}$
Indiana bat (*Myotis sodalis*)	$77.55_{A:3}$	$63.63_{A:3,4}$	$25.00_{A:2}$	$100.00_{A:1,2}$	$12.50_{A:2}$
Northern long-eared bat (*Myotis septentrionalis*)	$73.15_{A:3}$	$83.33_{B:4}$	$80.00_{A,B:2}$	$100.00_{A,B:1,2}$	$80.00_{A,B:1,2}$
Tri-colored bat (*Perimyotis subflavus*)	$77.46_{A:3}$	$62.50_{A:3}$	$100.00_{A:2}$	$94.12_{A:1,2,3}$	$16.13_{A:2}$

[a] $\bar{x} \pm$ SE in the same row followed by the same uppercase letter not significantly different ($P > 0.05$).
[b] $\bar{x} \pm$ SE in the same column followed by the same number not significantly different ($P > 0.05$).

2

Table 2.6. Number of individuals (no.) of five bat species captured while mist netting outside four cave hibernacula in Tennessee over five hibernation seasons (October–April), 2012/13–2017/18.

Species	Cave Hibernacula	Number Captured (no.)				
		2012/2013	2013/2014	2015/2016	2016/2017	2017/2018
Eastern small-footed bat	Blount	0	1	4	4	2
(*Myotis leibii*)	Campbell	46	35	61	44	45
	Hawkins	0	0	0	0	0
	Warren	5	15	3	-	-
Gray bat	Blount	0	0	0	0	0
(*Myotis grisescens*)	Campbell	0	0	0	0	0
	Hawkins	95	80	58	79	28
	Warren	34	73	65	-	-
Indiana bat	Blount	43	37	31	95	96
Myotis sodalis	Campbell	5	6	0	0	14
	Hawkins	4	2	1	3	0
	Warren	0	0	0	-	-
Northern long-eared bat	Blount	17	4	1	3	2
Myotis septentrionalis	Campbell	110	48	1	0	1
	Hawkins	0	1	2	0	2
	Warren	7	2	1	-	-
Tri-colored bat	Blount	44	4	7	10	17
Perimyotis subflavus	Campbell	19	21	8	9	22
	Hawkins	0	1	0	0	0
	Warren	19	7	1	-	-

Table 2.7. Capture rates (captures/m^2/hr) of five bats species from mist netting conducted at four cave hibernacula in Tennessee during the hibernation season (October 1–April 30), 2012/13–2017/18.

Bat Species	Capture Rate (captures/m^2/hr; $\bar{x} \pm$ SE)[a]
Eastern small-footed bat (*Myotis leibii*)	$0.041 \pm 0.009_A$
Gray bat (*Myotis grisescens*)	$0.253 \pm 0.068_B$
Indiana bat (*Myotis sodalis*)	$0.029 \pm 0.009_C$
Northern long-eared bat (*Myotis septentrionalis*)	$0.059 \pm 0.019_D$
Tri-colored bat (*Perimyotis subflavus*)	$0.031 \pm 0.008_E$

[a] $\bar{x} \pm$ SE followed by the same uppercase letter not significantly different (P >0.05).

Table 2.8. Capture rates (captures/m^2/hr) of five bat species from mist netting conducted at principal hibernacula in Tennessee (i.e., where >50 individuals of a species were recorded during pre-white-nose syndrome hibernacula surveys [2009/10]) over five hibernation seasons (October 1–April 31), 2012/13–2017/18.

Species	Cave Hibernacula	Capture Rate (captures/m^2/hr; $\bar{x} \pm$ SE)[a]					*P*
		2012/2013	2013/2014	2015/2016	2016/2017	2017/2018	
Eastern small-footed bat (*Myotis leibii*)	Campbell	0.178 ± 0.078	0.158 ± 0.074	0.180 ± 0.071	0.128 ± 0.074	0.069 ± 0.029	0.671
Gray bat (*Myotis grisescens*)	Hawkins	0.530 ± 0.309	0.246 ± 0.147	0.422 ± 0.244	0.332 ± 0.196	0.091 ± 0.055	0.927
	Warren	0.024 ± 0.018	0.089 ± 0.055	0.311 ± 0.302	-	-	0.408
Indiana bat (*Myotis sodalis*)	Blount	0.269 ± 0.127	0.048 ± 0.027	0.059 ± 0.042	0.062 ± 0.059	0.052 ± 0.051	0.101
	Campbell	0.034 ± 0.034	0.025 ± 0.026	0.000 ± 0.000	0.000 ± 0.000	0.012 ± 0.012	0.801
Northern long-eared bat (*Myotis septentrionalis*)	Blount	0.157 ± 0.147	0.006 ± 0.004	0.002 ± 0.002	0.002 ± 0.002	0.005 ± 0.003	0.177
	Campbell	$0.557 \pm 0.177_{A}$	$0.281 \pm 0.140_{A}$	$0.003 \pm 0.003_{BC}$	$0.000 \pm 0.000_{AC}$	$0.001 \pm 0.001_{BC}$	<0.001
Tri-colored bat (*Perimyotis subflavus*)	Blount	$0.246 \pm 0.069_{A}$	$0.005 \pm 0.004_{A}$	$0.013 \pm 0.011_{A}$	$0.008 \pm 0.003_{A}$	$0.014 \pm 0.017_{A}$	0.019
	Campbell	0.119 ± 0.073	0.112 ± 0.064	0.026 ± 0.014	0.028 ± 0.006	0.032 ± 0.013	0.987

[a] $\bar{x} \pm$ SE in the same row followed by the same uppercase letter not significantly different (P >0.05).

Table 2.9. Capture rates (captures/m^2/hr) of five bat species from mist netting conducted at principal hibernacula in Tennessee (i.e., where >50 individuals of a species were recorded during pre-white nose syndrome hibernacula surveys [2009/10]) over three hibernation periods: early (October 1–November 30), mid- (December 1 – February 28), and late (March 1 – April 30), during 2012/13–2017/18.

Species	Cave Hibernacula	Capture Rate (captures/m^2/hr; $\bar{x} \pm$ SE)[a]			*P*
		Early Hibernation	Mid Hibernation	Late Hibernation	
Eastern small-footed bat (*Myotis leibii*)	Campbell	0.091 ± 0.019	0.169 ± 0.052	0.144 ± 0.054	0.831
Gray bat (*Myotis griscecens*)	Hawkins	$0.188 \pm 0.098_A$	$0.056 \pm 0.019_B$	$1.081 \pm 0.230_A$	<0.001
	Warren	0.037 ± 0.026	0.006 ± 0.002	0.423 ± 0.286	0.085
Indiana bat (*Myotis sodalis*)	Blount	$0.163 \pm 0.064_A$	$0.030 \pm 0.021_A$	$0.089 \pm 0.060_{A,B}$	0.029
	Campbell	$0.068 \pm 0.036_A$	$0.000 \pm 0.000_A$	$0.000 \pm 0.000_{A,B}$	0.005
Northern long-eared bat (*Myotis septentrionalis*)	Blount	0.004 ± 0.002	0.048 ± 0.042	0.002 ± 0.002	0.416
	Campbell	0.287 ± 0.178	0.139 ± 0.071	0.179 ± 0.119	0.255
Tri-colored bat (*Perimyotis subflavus*)	Blount	$0.007 \pm 0.004_A$	$0.047 \pm 0.030_A$	$0.056 \pm 0.029_{A,B}$	0.015
	Campbell	0.129 ± 0.074	0.033 ± 0.022	0.068 ± 0.027	0.148

[a] $\bar{x} \pm$ SE in the same row followed by the same uppercase letter not significantly different (P >0.05).

Figures

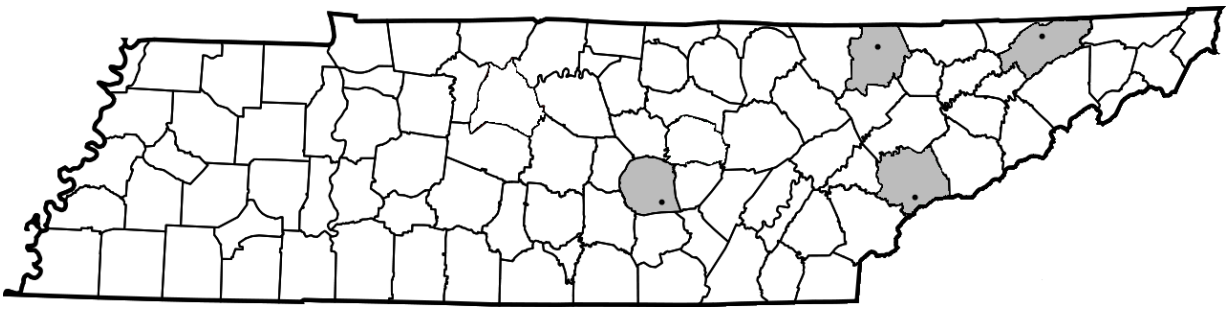


Figure 2.1. Tennessee state map showing the location of four cave hibernacula used in a study examining *Pseudogymnoascus detructans* load and prevalence and capture rates of five bat species over five hibernation seasons (October 1–April 30), 2012/13–2017/18. Study counties are depicted in gray, with a black dot (•) showing the approximate location of each cave hibernacula

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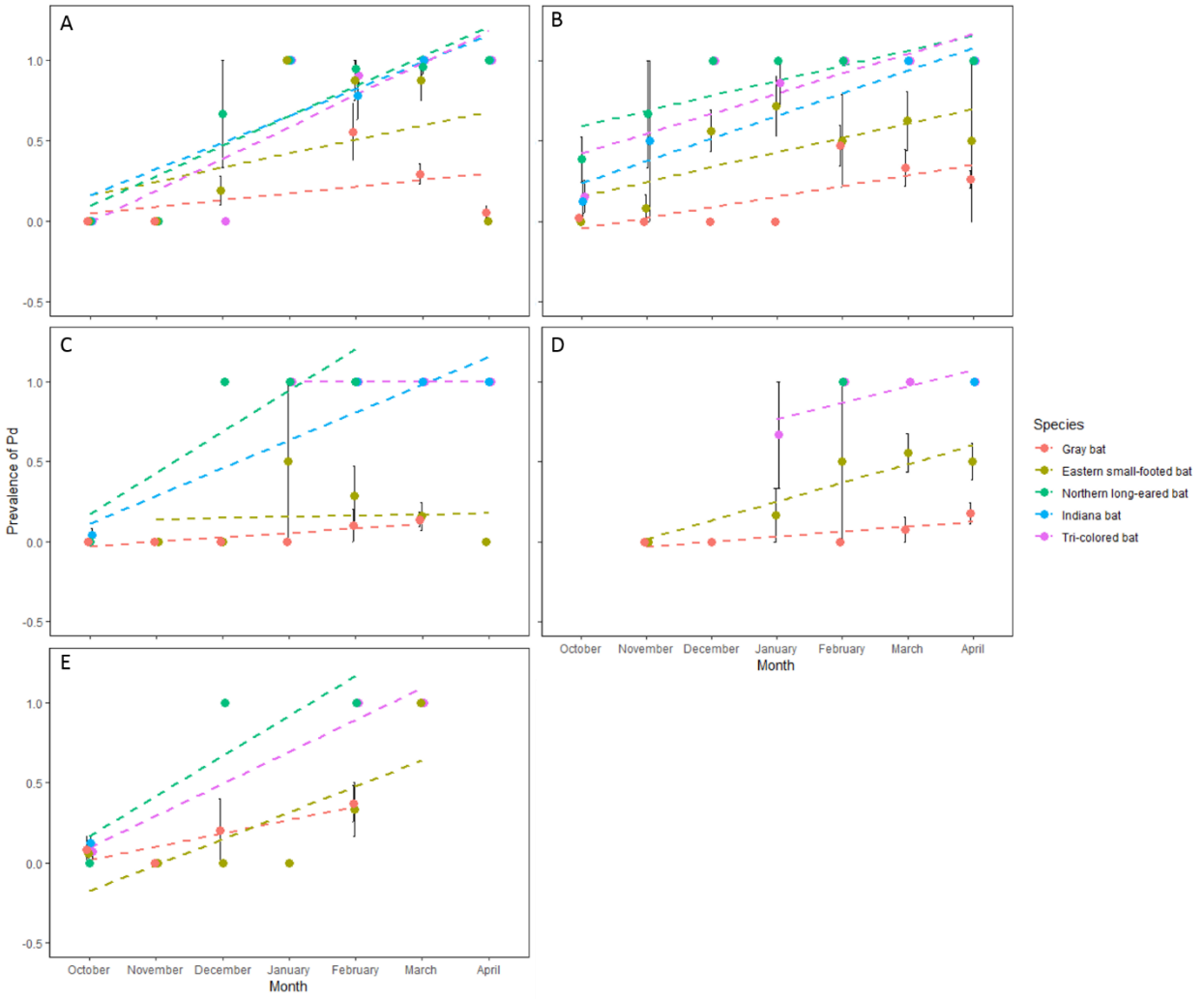


Figure 2.2. Prevalence (%) trends for *Pseudogymnoascus destructans*, the causal agent of white-nose syndrome, for five bats species captured at principal hibernacula in Tennessee (i.e., where >50 individuals of a species were recorded during pre-white nose syndrome hibernacula surveys [2009/10]) over five hibernation seasons (October 1–April 30), 2012/13–2017/18. A: 2012/13; B: 2013/14; C: 2015/16; D: 2016/17; E: 2017/18.

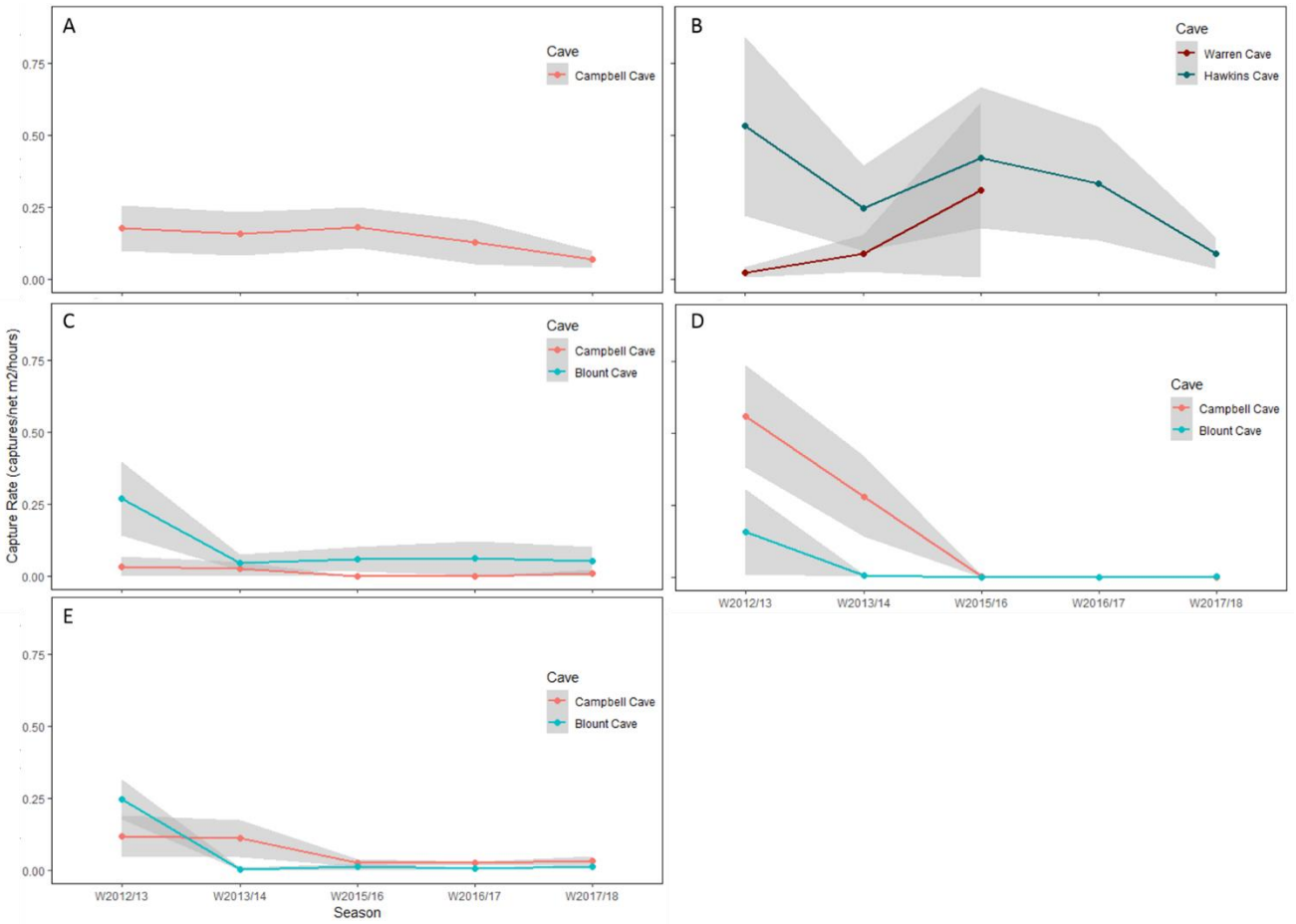


Figure 2.3. Mean capture rates (captures/m^2/hr) for five bats species from mist netting conducted at principal hibernacula in Tennessee (i.e., where >50 individuals of a species were recorded during pre-white nose syndrome hibernacula surveys [2009/10]) over five hibernation seasons (October 1–April 31), 2012/13–2017/18. A: eastern small-footed bat; B: gray bat; C: Indiana bat; D: northern long-eared bat; E: tri-colored bat. Standard error of species capture rates are represented in gray.

CHAPTER 3:

HIBERNATION BEHAVIOR OF FOUR BAT SPECIES WITH DIFFERING SUSCEPTIBILITY TO *PSEUDOGYMNOASCUS DESTRUCTANS* DURING WINTER

My consistent use of "we" throughout this chapter is in reference to my collaborators. I was the primary contributor to this work, which involved the following tasks: (1) development of project design and all data collection, (2) statistical analysis, (3) gathering and interpretation of relevant literature, and (4) all writing. E.V. Willcox and R.F. Bernard advised on project design and assisted with editing, J. M. Zobel advised on statistical analysis.

ABSTRACT

Bat species exhibit varying susceptibility to *Pseudogymnoascus destructans* (*Pd*), the causative agent of white-nose syndrome. The influence winter activity (i.e., emergence and torpor behaviors) may have on susceptibility has yet to be investigated. We deployed temperature-sensitive transmitters on four bat species (*Myotis grisescens, M. leibii, M. sodalis,* and *Perimyotis subflavus)* to monitor torpor bouts and implanted passive integrated transponder (PIT) tags subcutaneously to monitor activity at cave entrances. Radio transmitters were detected by receivers and antennas deployed in caves. Passive integrated transponder tags were detected by antennae surrounding cave entrances. We deployed 82 transmitters and implanted 1,347 PIT tags. Mean torpid skin temperature was lowest for *M. grisescens* at 13.33°C and highest for *M. leibii* at 18.73°C. Mean torpor bout length was lowest for *M. leibii* at 37.667 ± 26.891 hours and highest for *M. sodalis* at 260.667 ± 41.332 hours. *Myotis sodalis* had the greatest range in torpor bout length (range: 81–491 hours). PIT tag detections indicated *M. leibii* were active throughout hibernation (November–February), with an average of 74.22 ± 10.62% of tagged individuals detected at cave entrances each month. Of the 531 PIT-tagged bats active during winter, only 12.6% (n = 67) were detected at a cave entrance more than once/night, with the majority being *M. leibii*. The length of time between detections in the same night ranged from 0.17 to 10.46 hours, with a mean activity length of 0.87 hours.

INTRODUCTION

The disease white-nose syndrome (WNS), caused by the fungus *Pseudogymnoascus destructans (Pd)*, has killed approximately 6-million cave hibernating bats since its discovery in New York, United States (U.S.) in 2006 (Frick et al. 2017), and has since spread through much

of temperate North America (whitenosesyndrome.org 2019). As the fungus infiltrates the skin tissues of bats during hibernation, WNS manifests with a variety of associated effects, including dehydration, interrupted metabolic activities, starvation, and winter emergence and exposure (Reeder et al. 2012, Cryan et al. 2013, Verant et al. 2014). White-nose syndrome does not always end in mortality, with many affected bats successfully emerging in spring (Reichard et al. 2014, Maslo et al. 2015). However, many sick bats that emerge are still suffering from the effects of WNS, including emaciation, dehydration, a reversal of immune reconstitution inflammatory syndrome, and overall poor physiological condition (Fuller et al. 2011, Meteyer et al. 2012). Poor body condition and stress can negatively affect reproduction efforts in spring and summer, thus reducing yearly recruitment in species already suffering population declines due to WNS (Davy et al. 2016, Wilcox and Willis, 2016).

As of 2019, twelve species of bats are known to be affected by WNS, with an additional six species known to carry *Pd* (Bernard et al. 2015, TPWD 2017, Washington Department of Fish and Wildlife [WDFW] 2019). Some bat species have been more heavily affected by, and appear more susceptible to, WNS than other species hibernating in the same caves (Chapter 2; Frick et al. 2015, Langwig et al. 2016, Bernard et al. 2017). The endangered Indiana bat (*Myotis sodalis*) and tri-colored bat (*Perimyotis subflavus*) have experienced population declines across their range, with counts at hibernacula decreasing by nearly 80% to 94%, respectively (Langwig et al. 2015). Due to these declines, the tri-colored bat was petitioned for federal listing under the Endangered Species Act (Center for Biological Diversity 2016). Conversely, several other cave-hibernating species have maintained relatively stable populations following *Pd* invasion (Frick et al. 2017, Pettit and O'Keefe 2017). The endangered gray bat (*Myotis grisescens*) has been documented with *Pd*, although no deleterious effects of the disease have been observed in the species (Frick et al. 2017). The eastern small-footed bat (*Myotis leibii*), similar in size and behavior to the tri-colored bat (Best and Jennings 1997), has not experienced many negative population effects since the introduction of *Pd* (Langwig et al. 2012, Frick et al. 2017).

Differences in *Pd* loads and prevalence among species provide an additional indication of WNS susceptibility (Chapter 2; Bernard et al. 2017, Frick et al. 2017). Studies on torpid bats indicate that little brown bats (*M. lucifugus*), northern long-eared bats (*Myotis septentrionalis*) and tri-colored bats have extremely high fungal loads and prevalence, with Indiana bats usually exhibiting slightly lower loads but similarly high prevalence (Langwig et al. 2015, Langwig et al.

2016, Frick et al. 2017). Active Indiana bats, little brown bats, northern long-eared bats, and tri-colored bats mirror this trend, albeit with slightly lower Pd loads, possibly due to disruptions from activity, especially following initial *Pd* invasion (Chapter 2; Bernard et al. 2017). Alternatively, big brown bats (*Eptesicus fuscus*), gray bats, eastern small-footed bats, and Rafinesque's big-eared bats (*Corynorhinus rafinesquii*) all exhibit low *Pd* loads and prevalence, among both torpid and active individuals (Chapter 2; Johnson et al. 2012, Bernard et al. 2015, Langwig et al. 2015, Langwig et al. 2016, Bernard et al. 2017, Frick et al. 2017). The mechanisms underlying certain species low susceptibility to WNS are currently not understood. However, understanding the reasons for differences in susceptibility to WNS among affected species could help researchers better manage the disease and mitigate population collapse.

Multiple explanations for differences in susceptibility to WNS among species have been studied, such as microclimate preference, clustering behavior, skin microbiome, and winter activity. First, microclimate preferences appear to be similar for species that have been highly impacted by *Pd*, yet differ for species that have not experienced major population effects (Langwig et al. 2012). Species that have not seen dramatic declines from *Pd*, however, also share similar microclimate choices (Langwig et al. 2012). The most highly susceptible species typically select hibernation roosts that coincide with the optimal growth range of *Pd* (ambient temperature (T_a): 9°C–12°C and >80% relative humidity), allowing the fungus to colonize tissue more quickly than if bats roosted in cooler locations (Langwig et al. 2012, Verant et al. 2012). Less susceptible species select roosts with lower temperatures and relative humidity, outside of the fungus' preferred range (Langwig et al. 2012). Second, variation in roosting preference (i.e., solitary versus clustering behaviors) of species may play a role in determining WNS susceptibility (Langwig et al. 2012). In recent studies, the prevalence of *Pd* did not differ between small and large populations of clustering species; whereas, in solitary roosting species, such as tri-colored bats and northern long-eared bats, declines were less dramatic in the small populations, suggesting transmission of *Pd* may be frequency-dependent for some species and density-dependent in others (Lorch et al. 2011, Langwig et al. 2012). Differences in transmission dynamics affect how many individuals of a species are likely to be infected with a pathogen, thus resulting in different incidences of disease within a species (McCallum et al. 2001, Langwig et al. 2012). These differences among bat species could result in *Pd* infecting colonies at different rates even within the same hibernacula (Langwig et al. 2012). Third, bat microbiomes are also

thought to have a significant effect on a species' susceptibility to WNS, although specifics have yet to be discovered (Hoyt et al. 2015, Avena et al. 2016). Various bacterial communities on bat skin exhibited anti-fungal properties that reduced the spread of *Pd* (Hoyt et al. 2015). Bacteria isolated from big brown bat microbiomes had a greater inhibitory impact on *Pd* spread than that of bacteria cultured from little brown bat microbiomes, but more research is needed to understand how these microbiomes compare to those of other WNS susceptible species (Hoyt et al. 2015). Additionally, microbiomes on bats with and without *Pd* are significantly different in terms of diversity, leading researchers to believe that microbiome could be an important factor in determining persistence within afflicted bat species (Lemieux-Labonté 2017). Finally, periodic winter activity has been hypothesized as influencing susceptibility to *Pd* (Johnson et al. 2012, Moosman et al. 2015, Bernard et al. 2017, Reynolds et al. 2017). Repeated arousals throughout hibernation may allow bats to groom more frequently, likely removing fungal spores in the process and reducing *Pd* loads (Brownlee-Bouboulis and Reeder, 2013). Activities during these arousals likely include drinking and foraging, which could combat some of the major effects of WNS infection like dehydration and starvation by enabling individuals to supplement as needed (Verant et al. 2014, Reynolds et al. 2017).

Episodic activity and foraging during winter raise body temperature, which activates the immune system, possibly halting *Pd* growth and potentially resulting in milder infections throughout hibernation. In the northeastern U.S. and Canada, winter emergence of bats from cave hibernacula and large-scale mortality on the landscape has been attributed to the effects of WNS (Blehert et al. 2009, Foley et al. 2011, Turner et al. 2011, Frick et al. 2016). However, in the southeastern U.S., less than 50% of bats captured outside of cave hibernacula during winter were positive for *Pd*, despite emerging from infected sites (Chapter 2; Bernard et al. 2017). This suggests that, in the southeastern U.S., bats remain active outside of cave hibernacula throughout winter, regardless of *Pd* and WNS status, and may be taking advantage of less severe winters and prey availability to forage (Bernard et al. 2017), as evidenced by guano collection (Bernard et al. unpublished). Milder winters and consistent prey availability could help WNS survivors in these southern climates persist and allow populations to rebound more quickly from the effects of WNS than in more northern latitudes, where winters are longer and harsher.

The goal of our study was to explore characteristics of hibernation behavior, i.e. torpor, arousal, and activity around cave entrances, of four target bat species in relation to differences in

susceptibility to *Pd*. The objectives of our study were to 1) investigate the frequency and duration of torpor and arousal bouts, and 2) determine the frequency and duration of activity around cave entrances within and among our target species. We selected four species that range across the *Pd* susceptibility spectrum (high, medium, and low) for this study. The high susceptibility species we examined were tri-colored bats. Indiana bats were considered as medium to high susceptibility, and eastern small-footed and gray bats were classified as low susceptibility species (Chapter 2; Langwig et al. 2012, Langwig et al. 2016, Bernard et al. 2017, Frick et al. 2017).

In Chapter 2, we explored the pathogen dynamics and capture rates of northern long-eared bat; however, due to the population decline of the species we were unable to capture enough individuals to include them in this portion of the study. Additionally, the data collection from this study demonstrated that bat activity decreased abruptly after the first week of November. Given this pattern, we opted to denote hibernation as November 1–March 31 for this portion of our research (Johnson et al. 2012).

METHODS

Study Area

Our study was conducted at five cave hibernacula in East Tennessee: Blount Cave, Campbell Cave, Hawkins Cave, and White Cave 1 and 2 (Figure 3.1). Blount Cave is located in Great Smoky Mountains National Park (GSMNP) and managed by the National Park Service (NPS). Prior to WNS, Blount Cave was the largest known Indiana bat hibernaculum in the state. A population census conducted in February 2019 indicated Indiana bat numbers at this site have declined from ~8,000 to ~750 since it was confirmed WNS positive in the winter of 2009/10. Eastern small-footed bats and tri-colored bats are also encountered at this cave. Hawkins Cave, managed by the Tennessee Wildlife Resources Agency (TWRA), is one of the largest gray bat hibernacula in the state, with estimated populations of over 350,000 as of January 2019. This cave also contains a small hibernating population of Indiana bats and low numbers of a few other bat species. Campbell Cave, managed by TWRA and The Nature Conservancy (TNC), contains approximately 1,000 bats, including ~60 Indiana bats and numerous tri-colored and eastern small footed-bats. Warren Cave, also managed by TWRA and TNC, is a gray bat hibernaculum,

housing a population similar in size to Hawkins Cave. Hibernacula in Tennessee were confirmed positive for *Pd* as early as the winter of 2009/10 (Blount Cave). Hawkins Cave was confirmed in the winter of 2010/11, with Campbell Cave and Warren Cave both confirmed in the winter of 2012/13. These years of confirmation were corroborated by real-time PCR (Muller et al. 2013) or histopathology for *Pd* detection (Meteyer et al. 2009).

Bat Capture

During fall swarm (September 1–October 31), spring emergence (April 1–April 30), and hibernation (November 1–March 31) of 2016/17, 2017/18, and 2018/19, we used mist nets (Avinet Inc., Dryden, NY; mesh diameter: 75/2, 2.6m high, 4 shelves, 4–9 m wide) and harp traps (Bat Conservation and Management, Inc., Carlisle, PA) to capture bats flying at cave entrances. During fall swarm, we mist-netted and harp-trapped at each cave 1–3 times per week, while during the hibernation season we mist netted 1–4 times per month. We mist-netted and harp-trapped on nights when there was no rain and temperatures were above 0°C (32°F). We opened mist nets and harp traps 30 minutes before civil sunset and left them open for 2–5 hours, or until temperatures fell below 0°C. For all individuals captured, we recorded age, sex, right forearm length (mm), and body mass (g). Each bat captured was fitted with a unique 2.4 mm or 2.9 mm (depending on species) forearm band (Porzana, Ltd., Icklesham, East Sussex, UK). During winter, bats were held for no longer than 30 minutes in individual bags in an insulated cooler with a heat source (HotHands®, Dalton, Georgia, U.S.). All bats were released at the site of capture.

Frequency and Length of Torpor and Arousal Bouts

To determine length of torpor and arousal bouts and arousal frequency of our target bat species during hibernation (November 1–March 31; Objective 1), we applied 0.27 g or 0.32 g temperature-sensitive VHF radio transmitters (LB-2X, Holohil Systems Ltd., Isanti, Ontario, Canada) to individuals captured during monthly mist-netting events at the entrance of Blount, Campbell, and Hawkins Caves in 2016/17, 2017/18, and 2018/19. Additionally, in 2016/17 and 2018/19, we entered Blount, Campbell, Hawkins, and White Cave 1 and 2 with TWRA, TNC, and NPS and applied transmitters to hibernating bats during biannual endangered species surveys. We attached transmitters to bats just below the shoulder blades in the interscapular

region, away from concentrations of brown adipose tissue, using surgical adhesive (Perma-Type, Plainville, CT; Johnson et. al. 2012). For eastern small-footed and tri-colored bats, the transmitter never exceeded 7% of body weight (Aldridge and Brigham 1988), while for gray bat and Indiana bat transmitter load never exceeded 5% of body weight, per USFWS permit specifications.

The signal (i.e., pulse rate) of transmitters was detected and recorded by data-logging radio-telemetry receivers (R4500SD, Advanced Telemetry Systems, Inc., Isanti, MN) and dipole antennas (Model 13861, Advanced Telemetry Systems, Inc.) connected to an external power source. Depending on the size and configuration of each cave, 1–3 antennas were deployed inside, with an additional antenna deployed outside each cave to provide additional coverage inside and outside each site. Receivers for all antenna were located outside each cave to minimize disturbance during weekly equipment checks. Antennas were deployed in September of each study year, prior to the start of the hibernation season, to reduce disturbance to hibernating bats. Data were downloaded from each receiver weekly, when possible, and at a minimum biweekly.

The manufacturer calibrated each transmitter and provided a unique polynomial equation that was used to convert transmitter pulse rate to bat skin temperature (T_{sk}; Britzke et al. 2010). We used T_{sk} as a proxy for body temperature to determine periods of torpor and arousal (Barclay et al. 1996). We considered a bat in torpor when its T_{sk} was <22°C and in an arousal bout when its T_{sk} was >22°C if it eventually reached a Tsk > 28°C (Park et al. 2000, Turbill and Geiser 2008). Past studies have shown that bats typically exhibit active arousals if T_{sk} surpasses 22ºC until temperatures reach 34–38°C, given that bats will thermoregulate during this period (Park et al. 2000). We did not include individuals that exhibited passive arousals (i.e., slowly increased T_{sk} over a period of time >100 minutes with no rise to normothermia (34–38°C) in calculations of arousal bout length (Sirajuddin 2018). We defined torpor bout length as the period between two arousal bouts, when T_{sk} remained below 22°C for >30 minutes (Reeder et al. 2012) and calculated torpor bout length to the nearest minute. We calculated mean torpor temperature and mean arousal temperature for each torpor and arousal bout. To allow us to compare among species and control for differences in transmitter lifespans, we calculated a torpor bout index, i.e., the number of torpor bouts/number of days the transmitter was active. We used the same method to calculate an arousal frequency index, i.e., the number of arousals/number of days the

transmitter was active (Sirajuddin 2018). We characterized activity during arousals as 1) departed the cave indefinitely, 2) departed the cave and returned, 3) moved location in cave, 4) no movement, or 5) arrival/transmitter attachment. We selected these possible activities based on movement (or lack thereof) of the transmitter signal between antenna placed within and outside of the cave. "No movement" meant that the transmitter was consistently picked up on the same antenna and there was no gap in data collection. "Departed the cave" was evidenced by movement of the transmitter among antenna and then loss of transmitter signal. "Arrival/transmitter attachment" was signified when a bat with a transmitter was picked up on antenna at hibernacula either for the first time (just after initial transmitter attachment) or upon reentry into the cave.

Frequency and Duration of Activity

To determine activity during hibernation (November 1– March 31; Objective 2), we implanted bats captured at Blount, Campbell, and Hawkins caves with a uniquely identifiable 12 mm passive integrated transponder (PIT) tag (HDX12 Preloaded, Biomark, Inc., Boise, ID) during fall swarm (August 1–October 31) and spring staging (April 1–April 30) of 2016/17, 2017/18, and 2018/19. During fall swarm, bat activity at caves is high, as individuals are mating and foraging to prepare for hibernation (Davis and Hitchcock 1965, Fenton 1969). Although bat activity is high during this period, individuals encountered at a site may not always use the site during hibernation (van Schaik et al. 2015). To increase the chances of tagging bats that would remain at the hibernacula over winter, we increased netting effort during this period of each year. We implanted PIT tags subcutaneously, just below the shoulder blades in the interscapular region (O'Shea et al. 2010, Britzke et al. 2012, Johnson et al. 2012.) using an implant gun (MK25, Biomark, Inc., Boise, ID). We only implanted PIT tags in bats with a body mass ≥4.5 g and no obvious health issues to reduce potential stress from handling of bats preparing for hibernation.

Passive integrated transponder tags are activated when they come in proximity of an associated antenna and reader, thereby providing an opportunity to collect long-term monitoring and movement information without the need for recapture or additional stress to animals (Gibbons and Andrews 2004, Rigby et al. 2011). Each cave entrance was surrounded by a 15-meter long PIT tag cable antenna attached to a PIT tag reader/data-logger (IS1001 Cord Antenna System, Biomark, Inc., Boise, ID) with an external power source (Figure 3.2). We placed the PIT

tag reader outside Blount, Campbell, and Hawkins caves to minimize disturbance during equipment checks. We downloaded data from each receiver weekly, when possible, and at a minimum biweekly. Data was then summarized as the number and identity of tagged bats passing through the sensor field each day.

Due to technical difficulties, our PIT tag systems did not run continuously throughout hibernation. Therefore, we categorized each night the PIT tag reader was running as "useable" or "not useable." A "useable night" was one during which the PIT tag system was operational for >2 hours post-sunset, in order to record emergence (Johnson et al. 2012, Schwab and Mabee 2014, Bernard and McCracken 2017, Reynolds et al. 2017). We determined the mean number of bats active/night at each hibernacula by dividing the number of bats detected on the PIT tag reader during a month by the number of "useable nights" the PIT tag antenna system was operational. We then extrapolated the mean number of bats active/night to 30 days to give an estimate of the total number of bats anticipated to have been active per month if the PIT tag reader had been 100% operational. Finally, we divided the number of bats active per month by an estimate of the number of PIT tagged bats remaining at each site at the onset of hibernation (November 1) to determine monthly activity frequency for each target species and all species combined. The number of PIT tagged bats remaining at each site at the onset of hibernation was determined as the number of PIT tagged bats detected during October (i.e., the end of fall swarm). We used this estimate, instead of the total number of PIT tagged bats at each cave, as the latter includes bats that may not hibernate in the cave and does not consider dropped PIT tags or natural or WNS-induced mortality over the course of the study (Horton and Letcher 2008, Johnson et al. 2012).

Our PIT tag antenna systems could not be used to determine the direction in which a bat was flying when it was detected (i.e., into or out of the cave), only that it was active at the cave entrance. Therefore, we determined the time between activity events for each individual and calculated the mean activity length for each of our target species. We excluded any activity events that occurred less than 10 minutes apart or greater than 24 hours apart to reduce recording 1) occasions when bats were repeatedly moving in and out of the antenna range and 2) times when bats likely employed torpor outside of the cave (Jonasson and Willis 2012). Activity recordings were set to a 24-hour day starting at noon to capture all bats that emerged throughout a night (between 12:00-12:00 pm; Johnson et al. 2012).

While mist netting and harp trapping, we followed decontamination procedures outlined by the U.S. Fish and Wildlife Service (Shelley et al. 2013). Capture, handling, sample collection, radio-transmitter application, and PIT tagging protocols were approved by the University of Tennessee Institutional Animal Care and Use Committee (IACUC 2253-0317), as developed by the American Society of Mammalogists (Sikes et al. 2016) and authorized under scientific collection permits from the USFWS (TE35313B-3), NPS (GRSM-2018-SCI-1253), TWRA (3742), and TDEC (2009-038).

Data Analysis

We log transformed all torpor and arousal data prior to analysis to meet normality and homogeneity of variance assumptions (Conover 1999, Zar 1999). We used linear mixed-effects models (using the lme4 package (Bates et al. 2015) in the R statistical program (R Core Team 2017)) to compare torpor bout length, arousal bout length, torpor temperature, arousal temperature, torpor bout index, and arousal bout index among species, with individual bat as a random effect. We included a bat as a random effect in order to correct for variation within individuals, as most bats had multiple torpor bouts and arousal events within the life of a transmitter (Czenze and Willis 2015, Sirajuddin 2018). We followed this with post-hoc testing using least square means comparisons (using the emmeans package (Lenth 2019) in R).

We transformed activity length data prior to analysis using a negative quadratic root (Osborne 2010) and used a linear model to compare activity length among species. In our activity frequency dataset, we applied a positive quadratic root transformation (Osborne 2010). For analysis, we ran a linear mixed-effects model with activity frequency as the response variable and species and month as fixed effects. Year was considered a random effect due to the addition of a new site (Campbell cave) in the second year of this study and to compensate for variation between sites. We used least square means comparisons for our post-hoc testing of both our activity length and activity frequency models with no adjustment. We opted for no adjustment (i.e., Fisher's LSD test) given our limited dataset.

RESULTS

Bat Captures

From August–October and April of 2016/17–2018/19, we captured 1,612 bats representing nine species. Of these, 1,480 were individuals of our four target species. Gray bats accounted for 52.6% of captures (n = 779/1480), followed by Indiana bats (27.1%, n = 401/1480). We captured 200 tri-colored bats (13.5% of captures) and 100 eastern small-footed bats (6.8% of captures).

Frequency and Length of Torpor and Arousal Bouts

We deployed 82 temperature-sensitive radio transmitters and successfully recorded T_{sk} data from 21 individuals (Table 3.1). Indiana bats had the longest mean torpor bout length (260.67 ± 41.33 hours), whereas eastern small-footed bats had the shortest (37.67 ± 26.89 hours). However, species had no effect on mean torpor bout length (P = 0.051; Table 3.2, Figure 3.3).

Eastern small-footed bats had the highest mean torpor T_{sk} (18.57 ± 0.20 °C), whereas gray bats had the lowest mean torpor T_{sk} (13.72 ± 0.60 °C). Species had an effect on mean torpor T_{sk} (P = 0.007; Figure 3.4). Post-hoc tests indicated eastern small-footed bats had a higher mean torpor T_{sk} than tri-colored bats (14.62 ± 0.49 °C; P = 0.048), and gray bats had a lower mean torpor T_{sk} compared to eastern small-footed bats (P = 0.007) and Indiana bats (16.48 ± 0.79 °C; P = 0.024).

Eastern small-footed bats had the highest torpor bout index (0.654 ± 0.346) and arousal frequency index (1.231 ± 0.769), whereas Indiana bats had the lowest indices (torpor bout index: 0.097 ± 0.015; arousal frequency index: 0.117 ± 0.016). There was an effect of species on both torpor bout index ($P \leq 0.0495$) and arousal frequency index ($P \leq 0.043$; Table 3.3). Post hoc tests suggested eastern small-footed bats had a greater torpor bout index and arousal frequency index than Indiana bats ($P \leq 0.0121$). However, the torpor bout index and arousal bout index of eastern small-footed bats were similar to that of gray bats ($P \geq 0.091$) and tri-colored bats ($P \geq 0.315$).

Eastern small-footed bats had the longest mean arousal bout length (103.75 ± 60.62 minutes), whereas Indiana bats had the shortest mean arousal bout length (66.94 ± 6.82 minutes). However, there was no effect of species on mean arousal bout length (P = 0.6548).

Eastern small-footed bats had the highest mean arousal T_{sk} (32.29 ± 0.67 °C) and Indiana bats had the lowest mean arousal T_{sk} (28.59 ± 0.38 °C). There was an effect of species on mean arousal T_{sk} ($P = 0.0067$; Figure 3.4). Eastern small-footed bats had a higher mean arousal T_{sk} than gray bats (29.01 ± 0.64 °C; $P = 0.012$) and Indiana bats ($P = 0.005$), but a similar mean arousal T_{sk} to tri-colored bats (30.33 ± 0.54 °C; $P = 0.171$). Mean arousal T_{sk} was similar for gray bats, Indiana bats, and tri-colored bats ($P \geq 0.099$). Activity during arousals varied, but 36.17% of arousals resulted in bats moving out of range of the antennas and returning within five hours (n = 17/47 arousal events). In 12.7% of arousal events, bats left the cave and were never recorded again during the life of the transmitter (n = 6/47). In 19.15% of arousal events there was no indication of movement within or out of the cave (n = 9/47).

Frequency and Duration of Activity

We implanted 1,465 PIT tags in nine species, with 1,347 of those in our four target species (males: 1,236, females: 111; Table 3.4). Useable PIT tag data were collected at our three study caves on 86% of nights between November 1–March 31 over three years (n=1039/1208). Eight of the nine species tagged were detected at some point during winter, including all four of our target species. Eastern red bats were the only species not detected on PIT tag readers during winter. Five hundred thirty-one individuals of our target species were active during November 1–March 31, 2016/17–2018/19. Of the 531 bats detected during hibernation, 67 individuals were recorded more than once per night. Indiana bats and tri-colored bats comprised 7.46% of these individuals, respectively (n = 5/67), 23.88% were gray bats (n = 16/67) and 61.19% were eastern small-footed bats (n = 41/67).

Eastern small-footed bats had the greatest activity frequency during hibernation, with a mean of 74.22 ± 10.62% of tagged individuals detected per month (Table 3.5). A mean of 26.02 ± 10.94% of tagged tri-colored bats were detected each month, whereas both Indiana bats and gray bats had less than 10% of tagged bats detected monthly throughout hibernation (Table 3.5).

A species*month interaction had an effect on activity frequency ($P \leq 0.001$; Table 3.5). Among species, post hoc tests indicated that eastern small-footed bats had a greater activity frequency than all other species in November and December ($P \leq 0.032$, Table 3.5). Eastern small-footed bats had higher activity frequency in January than gray bats or Indiana bats ($P \leq 0.008$), but not different from tri-colored bats ($P = 0.099$). In February and March, activity

frequency of eastern small-footed bats and tri-colored bats did not differ from one another ($P \geq 0.109$), but were greater than for gray bats and Indiana bats ($P \leq 0.016$). In December, February, and March, activity frequency of gray bats and Indiana bats did not differ from each other ($P \geq 0.099$). In November, Indiana bats had a higher activity frequency than gray bats ($P = 0.001$), however in January gray bats had a higher activity frequency than Indiana bats ($P = 0.008$).

Within species, eastern small-footed bat activity frequency was lowest in January ($P \leq 0.011$), and did not differ between December and January ($P = 0.150$). Activity frequency in tri-colored bats remained consistent in November and December and increased steadily over the course of the hibernation period (Figure 3.5, $P < 0.036$). The highest activity frequency of gray bats was in March (0.159 ± 0.0351, $P \leq 0.003$; Table 3.5), although there was no difference in gray bats among December, January, or February ($P \geq 0.089$). Within Indiana bats, November and March had the highest activity frequencies ($P \leq 0.016$), but this species was never detected in December or January (Table 3.5).

Mean activity length was 0.872 ± 0.085 hours (Figure 3.5). Indiana bats had the longest mean activity length (1.591 ± 0.633 hours), whereas eastern small-footed bats had the shortest mean activity length (0.792 ± 0.087 hours). There was a significant effect of species on activity length ($P = 0.038$; Table 3.6), but not of month ($P = 0.358$). Post-hoc testing showed only that eastern small-footed bats displayed a shorter activity length than gray bats ($P = 0.027$).

DISCUSSION

This study is the first to use PIT tag technology in conjunction with temperature-sensitive radio transmitters to study the hibernation behavior of multiple bat species with varying susceptibility to WNS. PIT tag and radio-transmitter data collected during our study indicate that there are variations in hibernation behavior among four cavernicolous bat species post-WNS invasion. Variations in torpor and arousal T_{sk}, torpor bout and arousal frequency indices, activity frequency, and activity length existed among species.

Eastern small-footed bats were the most active of the four focal species in this study. Eastern small-footed bats had the highest mean torpor and arousal T_{sk}, the highest torpor bout and arousal frequency indices, the longest mean arousal length, the shortest mean torpor bout

length, the shortest mean activity length, and the highest mean activity frequency of all species. Our data show that the eastern small-footed bats in our study employed short, shallow torpor bouts throughout winter and were active on the landscape throughout hibernation, with a decrease in activity in the coldest months (i.e., January). The consistent active hibernation behavior exhibited by this species could be tied to the variety of temperatures and roost types (i.e., talus slopes and rock crevices) used during hibernation as opposed to *Pd* infection (Barbour and Davis 1969, Fenton 1972, Best and Jennings 1997, Reeder et al. 2012, Moosman et al. 2015, Moosman et al. 2017). Eastern small-footed bats have been documented roosting solitarily at freezing temperatures and low humidity, conditions typically outside of the optimal ranges of *Pd* growth (12°C to 15.8°C; Best and Jennings 1997, Blehert et al. 2009, Verant et al. 2012). If this species is using such roosts throughout hibernation, this could potentially explain the low fungal loads and prevalence of *Pd* described in Chapter 2. Furthermore, by using non-traditional thermal refugia, eastern small-footed bats could be limiting their time in contact with environmental *Pd* spores found in traditional hibernacula, thus reducing the potential for *Pd* infection (Lorch et al. 2013, Moosman et al. 2017, Verant et al. 2018). Evidence of long arousals during winter in this species could provide extra insight into overwinter survivorship with WNS. Previous studies found evidence of foraging and guano production in eastern small-footed bats during winter in Tennessee (Bernard et al. unpublished data, Jackson et al. unpublished data). Our study demonstrates that even with *Pd* prevalence of <50%, eastern small-footed bats move in and out of hibernacula frequently for extended periods of time in winter, during which time foraging bouts could take place (Chapter 2; Jackson et al. unpublished data). Foraging during the hibernation season could provide calories important in surviving the energetic demands of hibernation and WNS infection (Thomas et al. 1990, Cryan et al. 2013, Verant et al. 2014). Lastly, the high activity frequency in this species, coupled with low *Pd* prevalence observed for several years, indicates that these observed behaviors in eastern small-footed bats may not be WNS-induced behavior, but rather established life history characteristics corroborated by historical accounts pre-WNS (Mohr 1936, Best and Jennings 1997).

Tri-colored bats exhibited varying activity frequencies across months in hibernation. Torpor bout lengths were short, torpor bout and arousal frequency indices were low, and mean activity length was over an hour. Tri-colored bats were highly active in later hibernation months (i.e., February and March), much earlier than PIT tagged gray bats and Indiana bats from this

study and much earlier and more frequent than seen in pre-WNS hibernation phenology data for this latitude (Fujita and Kunz, 1984). This trend happens to mirror the seasonal increase in *Pd* loads and prevalence described in Chapter 2, which could possibly explain some of this late hibernation behavior (Reeder et al. 2012, Bohn et al. 2016, Langwig et al. 2016, Bernard et al. 2017). As bats grow progressively sicker as hibernation continues, tri-colored bats could be exhibiting sickness behavior in the form of increased emergence from hibernacula (Reeder et al. 2012, Bohn et al. 2016, Langwig et al. 2016). While our data conflicts with other reports of tri-colored bat hibernation behavior in WNS-stricken areas, latitudinal variations in weather and environmental conditions could possibly explain regional differences (Sirajuddin 2018). Additionally, mean torpor T_{sk} in tri-colored bats during this study was within optimal *Pd* growth range (12°C to 15.8°C; Blehert et al. 2009, Verant et al. 2012). The continued use of microclimates within optimal *Pd* range could explain the *Pd* dynamics trends described in Chapter 2 and also the high activity frequency of tri-colored bats seen in later hibernation months (McAlpine et al. 2012, Reeder et al. 2012, Carr et al. 2014, Langwig et al. 2016, Bernard et al. 2017). In the case of tri-colored bats, activity at hibernacula entrances likely does not aid in reducing fungal infection like it does in eastern small-footed bats, potentially because the fungal loads and prevalence among active individuals are much higher and could be driving the high activity frequency seen in this study (Chapter 2; Reeder et al. 2012, Langwig et al. 2016, Bernard et al. 2017, Frick et al. 2017).

Indiana bats had highly varied and long torpor bout lengths, long arousal and activity lengths, and low torpor bout and arousal frequency indices. Data from radio-transmitters and PIT tag dataloggers show that this species aroused infrequently and had the lowest activity frequency of focal species. Indiana bats were active the first week of November, after which all activity stopped until late-February. The timing of entry into hibernation could explain some of the behaviors witnessed during winter. During early November, Indiana bats were highly active, on the same level as eastern small-footed bats. While phenology information for hibernation immergence varies, Indiana bats are believed to enter hibernation anywhere between mid-October and late-November according to pre-WNS data (Clawson et al. 1980, USFWS 2007). Given that Indiana bats were active outside of hibernacula so late, bats could be remaining active later in the year and spending extra time foraging and increasing fat stores prior to hibernation (Kunz et al. 1998, Frick et al. 2017, Cheng et al. 2019). Extra fat stores could explain the low

arousal frequency seen during the earlier months of hibernation in this study, in that the late entry time large fat reserves could reduce the need for multiple arousals (Thomas et al. 1990, Park et al. 2000). Additionally, the clustering aggregations created by this species during hibernation also reduce energy loss which could limit the need for arousals (Barbour and Davis 1969, Boyles et al. 2008). While this clustering behavior may be beneficial for energy management, it has been implicated as a major factor in susceptibility to *Pd* seen in Indiana bats (Verant et al. 2012, Langwig et al. 2016, Bernard et al. 2017). *Pd* dynamics in this species exhibit mid-range loads and high prevalence (Chapter 2; Langwig et al. 2016, Bernard et al. 2017, Frick et al. 2017). Large clusters created by this species during hibernation likely increase transmission rates given the close proximity of infected and uninfected bats (Langwig et al. 2012). Although this gregarious behavior has many benefits, it could negatively affect Indiana bats by increasing prevalence of *Pd* among hibernating and active individuals (Langwig et al. 2012, Frick et al. 2017).

Gray bats had long torpor bouts, high torpor bout and arousal frequency indices, long activity and arousal lengths, but low activity frequency. While data collected from temperature-sensitive radio transmitters suggests high activity rates throughout winter, data collected from PIT tag dataloggers gave lower estimates of activity during hibernation. Several studies conducted during winter in other locales within the range of gray bats describe this species as being highly mobile during fall and winter, congruent with our data from radiotransmitters (Hall and Wilson 1966, Elder and Gunier 1978, Gore et al. 2012). Additionally, capture rates described in Chapter 2 for gray bats were high and stable, with little variation over winter seasons. It is likely that our metrics for measurement of activity frequency via PIT tags in this species could be underreporting the rate of activity during hibernation. Regardless, gray bats show high activity levels during winter and also have low fungal loads and prevalence of *Pd*, as described in Chapter 2 (Hall and Wilson 1966, Elder and Gunier 1978, Bernard et al. 2017). It is interesting to note that the two species included in these studies, i.e. the eastern small-footed bat and the gray bat, with the lowest susceptibility to *Pd* also exhibit the highest levels of activity during winter. Additionally, high activity during hibernation could affect these *Pd* dynamics in a number of ways, as mentioned above. However, gray bats exhibit different hibernation behaviors than those seen in eastern small-footed bats, most notably their large aggregations that can contain thousands of bats per cluster (Tuttle 1979). Similar to eastern small-footed bats though, roost

switching during winter has been noted historically and could possibly affect *Pd* infection intensity (Langwig et al. 2016, Moosman et al. 2017). Additionally, earlier research has shown gray bats producing guano throughout winter, which is direct evidence of foraging and energy intake (Bernard et al, unpublished data). Gray bats, which are already the largest bat included in this study, could potentially be foraging and supplementing fat reserves with extra caloric intake, thus preparing themselves to endure the stresses of torpor and *Pd* infection better than lighter bats (Kunz et al. 1998, Cheng et al. 2019).

Here, we determined that various species exhibit different winter activity regimes in the southeastern U.S. post-WNS establishment. Unfortunately, a small sample size of radio-tagged tri-colored and eastern small-footed bats likely affected the significance of our results. More data is needed to elucidate what activities are occurring during hibernation, as well as the potential impact of these activities on susceptibility to WNS. Our data will help natural resource managers make informed management decisions regarding WNS-affected species in that we have established bats are active at cave entrances during hibernation. Cave and immediate area closures could reduce stress on bats active and roosting at hibernacula entrances, thus possibly affecting overwinter survivorship. Additionally, establishing what eastern small-footed bats and gray bats do during their arousals could help researchers infer how winter activity may help these species persist. Research similar to ours should be replicated in other areas of our focal species' ranges to see how our trends are reflected geographically. In doing so, researchers may better isolate how species may fare range-wide with WNS.

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APPENDIX 2

Tables

Table 3.1. Number of temperature sensitive radio transmitters applied to four target bat species, and the number of transmitters subsequently detected by radio telemetry receivers, as part of a study examining torpor behavior of bats during hibernation (November 1–March 31) of 2016/17–2018/19.

	Number of Transmitters (no.)			
	Applied		Detected	
Species	Male	Female	Male	Female
Eastern small-footed bat (*Myotis leibii*)	12	6	1	1
Gray bat (*Myotis grisescens*)	20	7	5	3
Indiana bat (*Myotis sodalis*)	8	11	0	9
Tri-colored bat (*Perimyotis subflavus*)	15	3	2	0

Table 3.2. Mean torpor bout length (hours) of four bat species tracked with temperature-sensitive radio transmitters at five cave hibernacula in Tennessee during hibernation (November 1–March 31) of 2016/17–2018/19.

Species	Torpor bout length (hours; $\bar{x} \pm$ SE)[a]
Eastern small-footed bat (*Myotis leibii*)	$37.667 \pm 26.891_{A}$
Gray bat (*Myotis grisescens*)	$162.133 \pm 48.791_{A}$
Indiana bat (*Myotis sodalis*)	$260.667 \pm 41.332_{A}$
Tri-colored bat (*Perimyotis subflavus*)	$86.300 \pm 44.650_{A}$

[a] $\bar{x} \pm$ SE in the same column followed by the same uppercase letter not significantly different ($P > 0.05$).

Table 3.3. Mean torpor bout index (no. of torpor bouts/no. of days a radio transmitter was detected) and arousal frequency index (no. of arousals/no. of days a radio transmitter was detected) of four bat species at five cave hibernacula in Tennessee during hibernation (November 1–March 31) of 2016/17–2018/19.

	Index ($\bar{x} \pm$ SE)[a]	
Species	Torpor Bout Index	Arousal Frequency Index
Eastern small-footed bat (*Myotis leibii*)	$0.654 \pm 0.346_{A}$	$1.231 \pm 0.769_{A}$
Gray bat (*Myotis grisescens*)	$0.364 \pm 0.203_{A,B}$	$0.595 \pm 0.355_{A,B}$
Indiana bat (*Myotis sodalis*)	$0.097 \pm 0.015_{B}$	$0.117 \pm 0.016_{B}$
Tri-colored bat (*Perimyotis subflavus*)	$0.282 \pm 0.134_{A,B}$	$0.389 \pm 0.167_{A,B}$

[a] $\bar{x} \pm$ SE in the same column followed by the same uppercase letter not significantly different ($P > 0.05$).

Table 3.4. Number of individuals (no.) of four bat species implanted with passive integrated transponder (PIT) tags at three cave hibernacula in Tennessee over three fall swarm (August–October) and spring staging seasons (April), 2016/17–2018/19.

		Number of PIT tags Deployed (no.)					
	Cave	2016/2017		2017/2018		2018/2019	
Species	Hibernacula	M	F	M	F	M	F
Eastern small-footed bat	Blount	1	0	4	0	5	0
(*Myotis leibii*)	Campbell	-	-	35	31	41	39
	Hawkins	0	0	0	0	0	0
Gray bat	Blount	0	0	0	0	0	0
(*Myotis grisescens*)	Campbell	-	-	0	0	0	0
	Hawkins	343	15	684	29	684	29
Indiana bat	Blount	185	11	300	13	310	13
Myotis sodalis	Campbell	-	-	27	3	29	3
	Hawkins	19	2	22	3	22	3
Tri-colored bat	Blount	11	2	35	7	39	8
Perimyotis subflavus	Campbell	-	-	62	9	99	15
	Hawkins	1	0	6	1	6	1

Table 3.5. Mean monthly activity frequency of four bat species detected entering and exiting three hibernacula in Tennessee from passive integrated transponder (PIT) tag detections during hibernation (November 1–March 31) 2016/17–2018/19.

	Activity Frequency ($\bar{x} \pm$ SE)[a, b]				
Species	November	December	January	February	March
Eastern small-footed bat (*Myotis leibii*)	$1.000 \pm 0.000_{A:1}$	$0.524 \pm 0.006_{A:1}$	$0.217 \pm 0.032_{A,B:1}$	$0.984 \pm 0.016_{A,B,C:1}$	$0.984 \pm 0.016_{A,B,C:1}$
Gray bat (*Myotis grisescens*)	$0.033 \pm 0.009_{A:2}$	$0.002 \pm 0.001_{B:2}$	$0.005 \pm 0.001_{A,B:2}$	$0.012 \pm 0.004_{A,B:2}$	$0.159 \pm 0.0351_{C:2}$
Indiana bat (*Myotis sodalis*)	$0.428 \pm 0.253_{A:3}$	$0.000 \pm 0.000_{B:2}$	$0.000 \pm 0.000_{B:3,}$	$0.011 \pm 0.007_{C:2}$	$0.0537 \pm 0.011_{D:2}$
Tri-colored bat (*Perimyotis subflavus*)	$0.024 \pm 0.024_{A:2}$	$0.024 \pm 0.024_{A:2,3}$	$0.063 \pm 0.032_{B:1,2}$	$0.487 \pm 0.275_{C:1}$	$0.703 \pm 0.248_{C:1}$

[a] $\bar{x} \pm$ SE in the same row followed by the same uppercase letter not significantly different (P >0.05).
[b] $\bar{x} \pm$ SE in the same column followed by the same number not significantly different (P >0.05).

Table 3.6. Mean activity length (hours) of four bats species from passive integrated transponder (PIT) tag detections at three cave hibernacula in Tennessee during the hibernation (November 1–March 31), 2016/17–2018/19.

Bat Species	Activity Length (hours; $\bar{x} \pm$ SE)[a]
Eastern small-footed bat (*Myotis leibii*)	$0.792 \pm 0.087_{A}$
Gray bat (*Myotis grisescens*)	$1.169 \pm 0.195_{B}$
Indiana bat (*Myotis sodalis*)	$1.591 \pm 0.633_{A,B}$
Tri-colored bat (*Perimyotis subflavus*)	$1.531 \pm 1.281_{A,B}$

[a]$\bar{x} \pm$ SE followed by the same uppercase letter not significantly different (P >0.05).

Figures

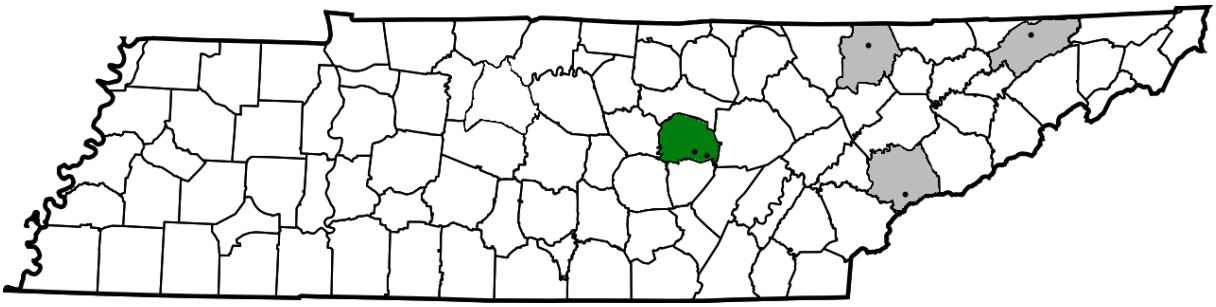


Figure 3.1. Tennessee state map showing the location of five cave hibernacula used in a study examining winter activity regimes of four species of cavernicolous bats during the hibernation (November 1–March 31), 2016/17–2018/19. Study counties with passive integrated transponder (PIT) tag systems and radio telemetry stations are depicted in gray. Counties in green only had radio telemetry systems. Black dots (•) shows the approximate location of each cave hibernacula.



Figure 3.2. 15-meter long passive integrated transponder (PIT) antennas attached to a PIT tag reader/data-logger (IS1001 Cord Antenna System, Biomark, Inc., Boise, ID) with an external power source. Each antenna system was constructed in a unique, cave-specific fashion in order to increase coverage and decrease obstruction in front of hibernacula entrances. Shown here are A.) Hawkins Cave, B.) Campbell Cave, and C.) Blount Cave.

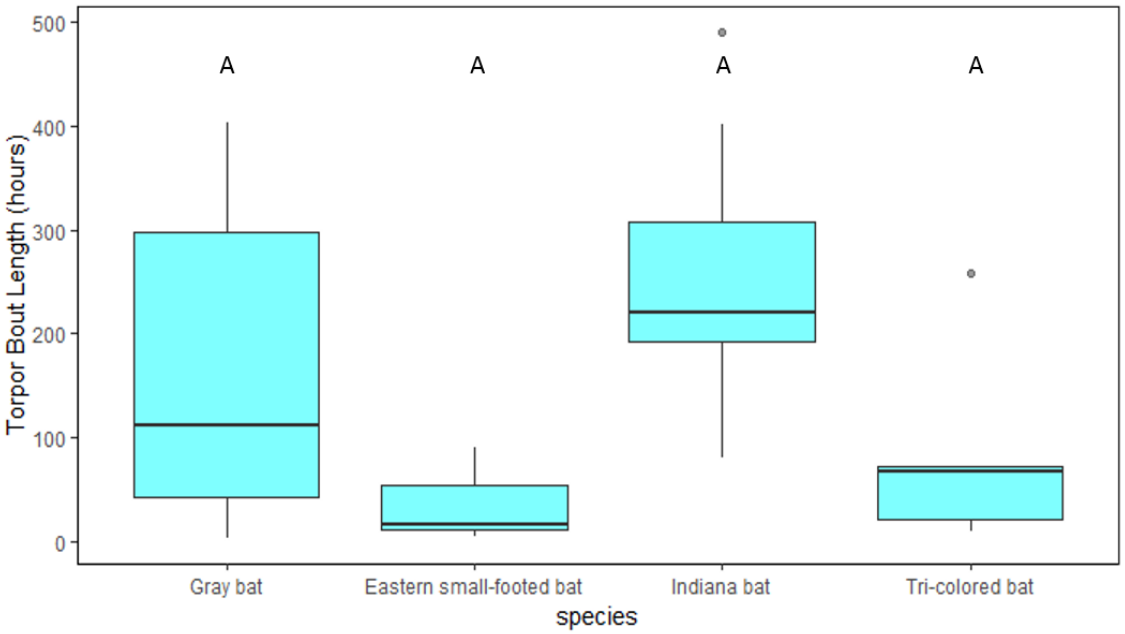


Figure 3.3. Mean torpor bout length (hours) of four bat species tracked with temperature-sensitive radio transmitters at five cave hibernaculum in Tennessee during hibernation (November 1 – March 31) of 2016/17–2018/19. Dots above plots represent outlying data. * Boxplot data followed by the same uppercase letter not significantly different (P >0.05).

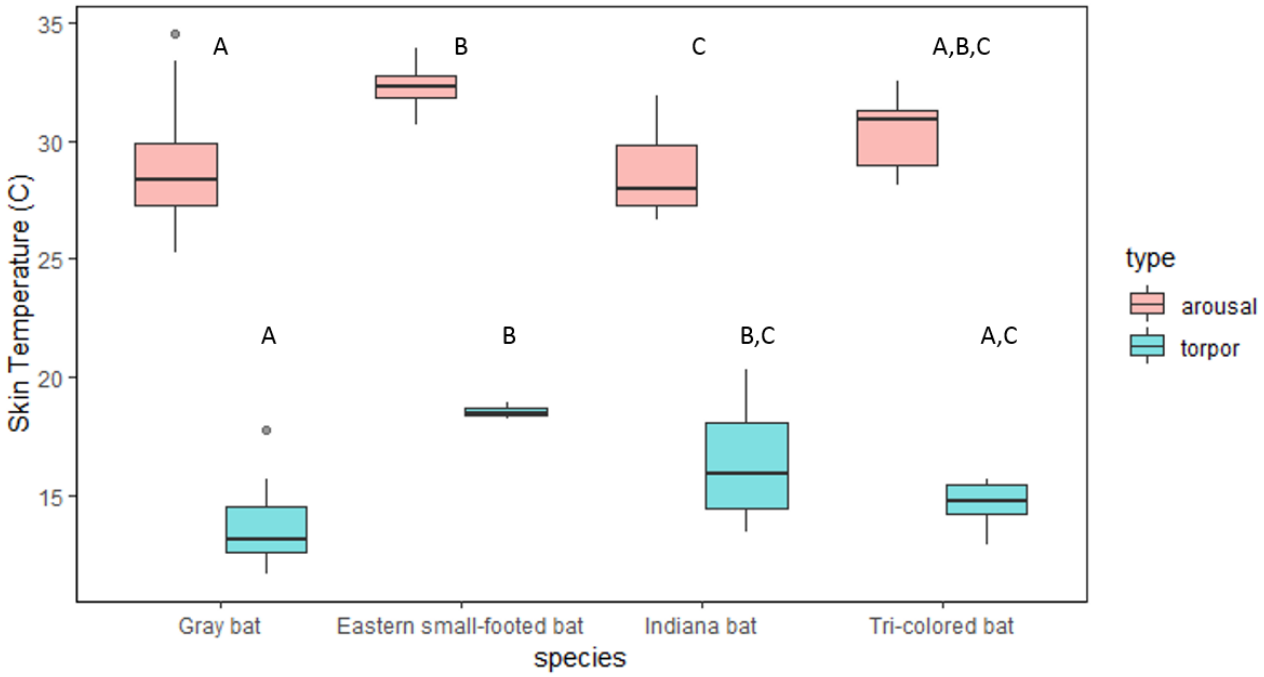


Figure 3.4. Mean skin temperatures (°C) during arousal events and torpor bouts of four target bat species tracked with temperature-sensitive radio transmitters at five cave hibernaculum in Tennessee during hibernation (November 1–March 31) of 2016/17–2018/19. Dots above plots represent outlying data.
*Boxplot data followed by the same uppercase letter not significantly different (P >0.05).

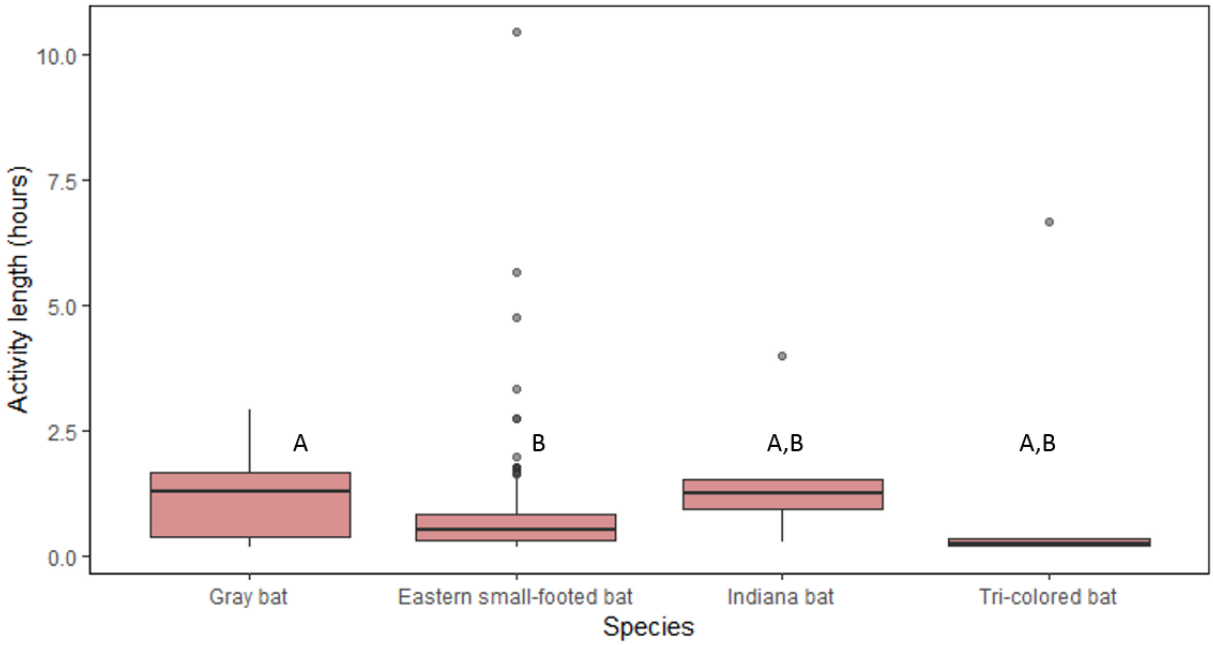


Figure 3.5. Mean activity length (hours) of four bat species as detected from Passive Integrated Transponder (PIT) tag detections at three cave hibernacula in Tennessee during the hibernation seasons of 2016/17–2018/2019. Dots above plots represent outlying data.
*Boxplot data followed by the same uppercase letter not significantly different (P >0.05).

CHAPTER 4:
CONCLUSIONS

In this thesis, we were interested in observing characteristics among bats living with *Pseudogymnoascus destructans* (*Pd*), the causal agent of white-nose syndrome (WNS). White-nose syndrome is a deadly fungal disease found in cave-roosting bats that has currently killed over 6-million bats in North America. We investigated the dynamics of *Pd*, with a focus on fungal load and prevalence, as well as the capture rates of five bat species from initial *Pd* invasion through early establishment at hibernaculum. Additionally, we characterized various aspects of hibernation activity (i.e., torpor and arousal bout lengths, skin temperatures during torpor and arousal, activity frequency, and activity length) of four species of cave-roosting bats in Tennessee.

We found that that three bat species, the Indiana bat, the northern long-eared bat, and the tri-colored bat, had very high susceptibility to *Pd* from invasion through early establishment, with slight fluctuations among years. These same species also saw severe declines in capture rates at principal hibernacula during this study. The other two species included in our study, the gray bat and the eastern small-footed bat, exhibited low susceptibility to *Pd* and stable capture rates throughout the study.

When looking at hibernation activity, we found different behavior among our four target bat species included in my study. Eastern small-footed bats had the highest activity frequency throughout hibernation, as well as high skin temperatures during torpor and arousal, short torpor bouts, and frequent arousals from torpor. Gray bats exhibited low activity frequency, low skin temperatures during torpor and arousal, long torpor bouts, and frequent arousals from torpor. Tagged Indiana bats were not active at hibernacula entrances from December–late February, had medium-range skin temperatures during torpor and arousal, had very long torpor bouts, and did not arouse frequently from torpor. Lastly, tri-colored bats had high activity frequency during February–March, medium-range skin temperatures during torpor and arousal, short torpor bouts, and frequent arousals from torpor.

Differences in hibernation activity could explain differences in susceptibility to *Pd*, however, more research is needed to explore these relationships. Species with low susceptibility to *Pd*, (i.e., the eastern small-footed bat and the gray bat) exhibited higher rates of activity during hibernation compared to species with high susceptibility to *Pd* (i.e., the Indiana bat and the tri-colored bat). Further exploration into the nuances of potential

relationships between susceptibility to *Pd* and hibernation activity could help researchers understand species' differences in susceptibility to *Pd* and ultimately develop mitigation strategies targeting *Pd* resistance.

VITA

Reilly Jackson was born in Puyallup, Washington where she lived before moving to Palo Alto, California in 2000. There, she lived in the Bay Area and initially began her interest in biological sciences by spending summers in the redwood forests and in the San Francisco Baylands. She moved to Knoxville, Tennessee in 2007 and became captivated by the diversity of the southeastern US. Although she started her college career at the University of California, Santa Cruz as a Marine Biology major, she dropped out after two quarters and took a field job in the Great Smoky Mountains National Park working with birds, salamanders, and people. After this first field job, she enrolled at the University of Tennessee, Knoxville to complete her undergraduate degree.

She graduated with a Bachelor's of Science in Wildlife and Fisheries Science, with a concentration on Wildlife Management from the University of Tennessee, Knoxville in 2016. She also earned a minor in Forestry. She was lucky enough to have multiple field opportunities studying bats beginning in 2013, which has since taken her all over the country and the world.

She began her Masters of Science degree under Dr. Emma Willcox in fall of 2016. After the completion of her Master's thesis defense, she plans to move to the University of Arkansas, Fayetteville to begin a study of disease and anthropogenic stressors of bats in Kenya.