Tennessee Winter Bat Population and White-nose Syndrome Monitoring Report for 2019-2020

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These surveys could not be conducted with such a high level of effort or as geographically widespread without the assistance of numerous partners and volunteers. Because the majority of caves and winter sites occur on private lands in Tennessee, the number of surveys would be greatly reduced without the support, assistance, and willingness of private landowners. Without the partner, volunteer and landowner support, we would not be able to understand the distribution of winter bat populations and effects of white-nose syndrome in Tennessee.

Acronyms

AAFB……..……..Arnold Air Force Base

FORT……Fort Campbell Military Installation

MTSU.....Matthew Grisnik and Dr. Donald Walker

NPS……...……..National Park Service

TDEC…….Tennessee Department of Environment and Conservation

TNC…….The Nature Conservancy of Tennessee

TVA……..……Tennessee Valley Authority

TWRA…….….Tennessee Wildlife Resources Agency

UoS..... Sewanee: The University of the South

USFWS……..United States Fish and Wildlife Service

USFS .....United States Forest Service

UTK……University of Tennessee at Knoxville

Species Codes

CORA……... *Corynorhinus rafinesquii*

EPFU…….. *Eptesicus fuscus*

LANO……..… *Lasionycteris noctivagans*

MYAU……...… *Myotis austroriparius*

MYGR…….. *Myotis grisescens*

MYLE……... *Myotis leibii*

MYLU……...…. *Myotis lucifugus*

MYSE……...…. *Myotis septentrionalis*

MYSO…… *Myotis sodalis*

MYsp……...…..Unknown Myotis

PESU……... *Perimyotis subflavus*

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UTK……Ash Cable, Carlin Frost, Mallory Tate, and Dr. Emma Willcox

Executive Summary

During the 2019-2020 monitoring season, field signs of white-nose syndrome (WNS) were observed in 22 of the 96 (22.9%) caves surveyed, but many of the caves surveyed have previously been confirmed WNS positive. One new county, Moore, was confirmed suspect during the monitoring period. WNS and its causal fungal pathogen *Pd* can now be found in 57 of the 78 (73%) counties containing caves and is considered widespread in Tennessee.

The 2019-2020 winter field season was an "off" year for significant bat species and surveys were not performed at priority *Myotis grisescens* (gray bat) and *Myotis sodalis* (Indiana bat) sites. Surveys for these species will be performed during the 2020-2021 winter field season and partners and cooperators will again try to conduct surveys at all priority *M. grisescens* and *M. sodalis* priority sites.

Observations of *Perimyotis subflavus* (tri-colored bat) decreased 28.11% between the 2018-2019 and 2019-2020 winter field seasons. Since the 2009-2010 winter survey period, observations of *P. subflavus* have declined 46.23%. *Myotis lucifugus* (little brown bat) observations decreased 13.59% when comparing years non-priority caves are surveyed. Further analyses were not performed since this monitoring period was not a priority cave survey period. *M. septentrionalis* (Northern long-eared bat) equaled a previous all-time low during this monitoring period as only 2 individuals were observed across all surveys statewide. Winter observations of *M. septentrionalis* have declined 99.4% since 2010.

Positive trends in observations of the big brown bat are occurring as observations for this species increased during the 2019-2020 winter survey period. The percent change in observations for big brown bats is 83.43% since intensive cave surveys began in 2010. Few significant Rafinesque's big-eared bat sites were surveyed during the 2019-2020 field season and analyses were not performed for this species.

In collaboration with TWRA, TDEC, TVA, and TNC, 565 total skin swabs were collected from 8 bats species across 38 counties in Tennessee between 2016 - 2019. A total of 404 out of 565 bats tested positive (71.5%) for white-nose syndrome. Unfortunately, white-nose syndrome is widespread in Tennessee and detected in nearly all counties sampled. *P. subflavus*, which is being petitioned by USFWS for listing as an endangered species, was the most heavily sampled species and showed high prevalence of white-nose syndrome across the state. To date, 355 bacterial strains have been isolated and tested for anti-fungal activity against the white-nose pathogen. 111 strains of bacteria slow the growth of the white-nose pathogen and one isolate shows potential as a biocontrol agent. High-throughput DNA sequencing was used to characterize the skin microbiome of the bat and determined that it correlates with white-nose disease status, suggesting the importance of the skin microbiome in disease resistance. Both disease monitoring and understanding the role of the microbiome in bat host health will help inform management decisions for this economically and ecologically important group of organisms.

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Introduction

This report summarizes data collected by all cooperating agencies and partners in Tennessee during the winter of 2019-2020.

Historical survey work within the state of Tennessee was conducted to monitor the success of conservation efforts for endangered bats in Tennessee. This was accomplished by state and federal agencies and non-governmental groups conducting winter bat hibernaculum censuses. This work occurred on a bi-annual basis or staggered every three years depending on the species involved and the availability of personnel. At one-point, selected sites were monitored annually to establish a dataset that would allow trend analysis of populations. These efforts were disbanded in 2015 because of potential negative impacts as a result of repeated visitation. Historical surveys have generally focused on two of three endangered species of bat found in Tennessee, *Myotis sodalis* (Indiana bats) and *M. grisescens* (gray bats). No winter occurrences of the third species of endangered bat, *Corynorhinus townsendii virginianus* (Virginia big-eared bat), are known from Tennessee. A list of all bat species for Tennessee and their regulatory designations can be found in Table 1.

Beginning in 2009 with the concern of bat population declines due to white-nose syndrome (WNS), there was increased awareness to not only continue monitoring the status of endangered species, but to also assess the numbers and health of the common species of cave hibernating bats. Prior to the occurrence of white-nose syndrome (WNS), there was very limited information available on bat hibernacula and winter population trends for once common species of cave hibernating bats, that include: *M. lucifugus*, (little brown bat[1]), *M. septentrionalis* (Northern long-eared bat[2]), *M. leibii* (Eastern small-footed bat), *Eptesicus fuscus* (big brown bat), *Perimyotis subflavus* (tri-colored bat[1]), and *C. rafinesquii* (Rafinesque's big-eared bat). Because of the paucity of data for these species, assessing trends of winter populations of bats and WNS caused mortality has been difficult.

Initially, a tiered monitoring approach was developed and implemented during early monitoring efforts with each tier having varying levels of effort. This approach allowed survey effort to be adjusted to each cave minimizing potential impacts to hibernating bats, while allowing for the objectives of winter monitoring to be met. A description of the tiered monitoring system can be found in Lamb and Wyckoff (2010) and Flock (2014). As the need to gather data for all species increased, complete censuses of bat populations found within all sites surveyed was implemented in lieu of the tiered monitoring approach.

[1] Both *Myotis lucifugus* and *Perimyotis subflavus* were listed as threatened within Tennessee by TWRA in August 2018.

[2] *Myotis septentrionalis* was listed as threatened by the USFWS April 2, 2015 because of severe declines attributed to WNS (USFWS 2015).

Table 1. Conservation status with year of designation and occurrence of WNS for Tennessee bat species (species of greatest conservation need are in bold). D – Deemed in Need of Management; 1 – Global and Subnational Ranks; S – Species in which Pd has been detected, but not WNS confirmed in the state (Bernard et al. 2015); TN – Species that have tested WNS positive in Tennessee (Campbell 2017).

Common Name	Scientific Name	Global Rank[1]	State Rank[1]	Federal Protection	State Protection	WNS Confirmed	*Pd* Positive
Rafinesque's big-eared bat	***Corynorhinus rafinesquii***	G3G4	S3		D[1983]		Yes[S]
Virginia big-eared bat	***Corynorhinus townsendii virginianus***	G3G4T2	SNR	E[1979]	E[1979]		Yes
Big brown bat	*Eptesicus fuscus*	G5	S5			Yes	
Silver-haired bat	*Lasionycteris noctivagans*	G3G4	S4S5				Yes[S]
Eastern red bat	*Lasiurus borealis*	G3G4	S5				Yes[S]
Hoary bat	*Lasiurus cinereus*	G3G4	S5				
Seminole bat	*Lasiurus seminolus*	G5	SNR				
Southeastern bat	***Myotis austroriparius***	G4	S3			Yes	
Gray bat	***Myotis griesecens***	G4	S2	E[1976]	E[1976]	Yes[TN]	
Eastern small-footed bat	***Myotis leibii***	G4	S2S3		D[1983]	Yes	
Little brown bat	***Myotis lucifugus***	G3	S5		T[2018]	Yes[TN]	
Northern long-eared bat	***Myotis septentrionalis***	G1G2	S4	T[2015]	T[2015]	Yes[TN]	
Indiana bat	***Myotis sodalis***	G2G3	S1	E[1967]	E[1967]	Yes	
Evening bat	*Nyctieius numeralis*	G5	S5				
Tri-colored bat	***Perimyotis subflavus***	G2G3	S5		T[2018]	Yes[TN]	
Brazilian free-tailed bat	*Tadarida brasiliensis*	G5	SNR				

D - Deemed in Need of Management

[1] - Global and subnational ranks are obtained from NatureServe.org.

[S] - Species in which *Pd* has been detected in Tennessee, but not WNS confirmed in the state (Bernard et al. 2015)

[TN] - Species that have tested WNS Positive in Tennessee (Campbell 2017)

WNS and its causal fungal pathogen *Pseudogymnoascus destructans* (*Pd*) were first recorded in Tennessee in the winter of 2010 (Figure 1). Since 2010, *Pd* has been histopathological confirmed[3] on bats in 50 counties and genetic material of *Pd* has been located on bats in four counties in Tennessee (Figure 2). More than seventy-three percent of the counties with caves in Tennessee (78) have been confirmed WNS positive or suspect. Appendix A lists all confirmed or suspect sites and the species from which samples were collected in Tennessee. A list of all species in which *Pd* has been diagnostically confirmed or detected can be found at https://www.whitenosesyndrome.org/about/bats-affected-wns.

Figure 1. Progression of WNS has occurred quickly in Tennessee since being discovered in 2010. A single cave in Moore County was designated WNS suspect during the 2019-2020 monitoring period. The monitoring period includes caves surveyed from January 2020 through March 2020.

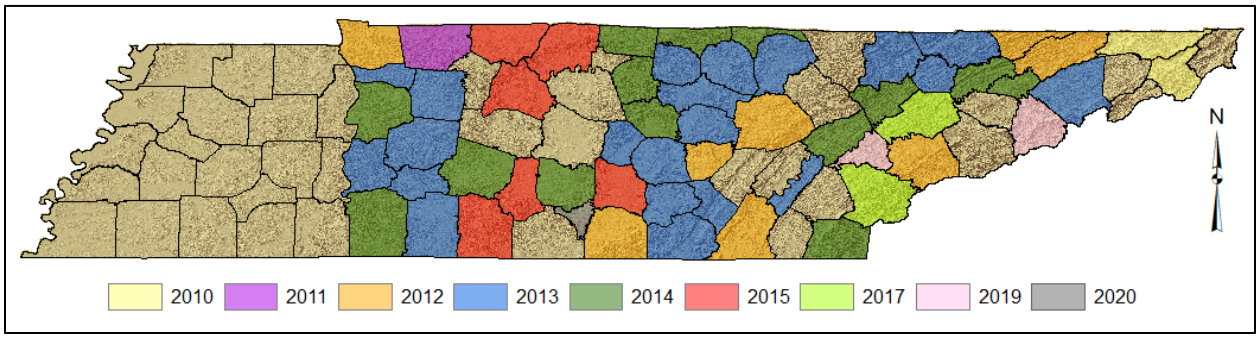
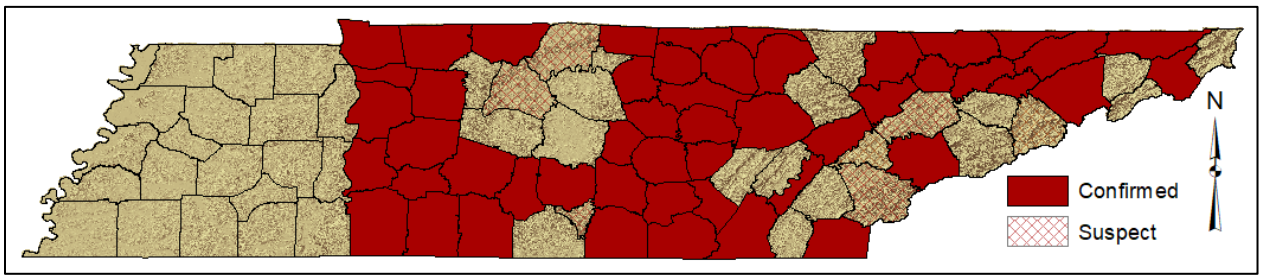


Figure 2. Most cavernous counties in Tennessee have been designated WNS confirmed and currently four counties are WNS.



With over 10,000 caves in Tennessee and 20% of the known caves in the United States (The Nature Conservancy of Tennessee n.d.), conducting annual surveys of all caves or of all winter bat populations in Tennessee is not a realistic and feasible approach, and not one considered by the WNS Advisory Council of Tennessee. A significant effort is made each year by all state and federal agencies, non-governmental groups and individuals to perform as many winter surveys as possible. Because of the density of caves throughout the state, less than 1% of the caves are visited each year. As a result of this, any conclusions or predictions concerning the

[3] During monitoring efforts, a site cannot be confirmed positive for the presence of WNS until histologic investigations reveal *Pd* has infected the tissues of bats. Suspect sites through 2014 are sites which test PCR positive for the presence of *Pd* and this designation is not removed until histology reports reveal tissue infections. Since 2014, the criteria used to classify WNS suspect sites has changed to minimize the need to euthanize bats and can be found at https://www.whitenosesyndrome.org/resource/revised-case-definitions-white-nose-syndrome-11252014.

spread of WNS across Tennessee and its effect on the bat population should take survey effort into consideration.

In all years, surveys are conducted in a manner allowing strict adherence to the USFWS WNS Decontamination protocols (https://www.whitenosesyndrome.org/topics/decontamination). Decontamination has been a high priority in all years to minimize the potential of surveys aiding the spread of *Pd* across the state. As a result of this priority, the number of caves visited per day is limited based on geography, personnel, and maintaining adequate supplies of decontaminated equipment. Despite the large number of caves in Tennessee and issues surrounding decontamination, efforts have helped to identify new bat hibernacula and to allow changes of winter bat populations to be tracked.

Methods

The 2019-2020 winter cave surveys were conducted between January 6, 2020 and March 13, 2020. As manpower allows, extending the survey effort through April 1st, as this is typically later in the season for winter surveys, allows for further development of WNS symptoms as observed during 2009-2010 surveys (Holliday 2012). Objectives of surveys conducted during the 2019-2020 field season fell into the following three categories with considerable overlap with the last two.

WNS Surveillance

Although a majority of the cavernous counties are WNS confirmed or suspect, surveys are still conducted to determine the presence of WNS at all sites. There are countless caves across the state that still appear to be WNS negative despite county-level WNS designations. Surveys are implemented to gauge the presence of WNS on a site level because of the lack of uniformity of its progression across the state. As a result of this lack in uniformity, monitoring impacts of WNS on winter bat populations on a site by site basis is necessary.

Because of the need to increase knowledge of wintering populations of bat species not listed, complete censuses of all bats observed in caves was implemented. This approach was different from the tiered monitoring approach used in previous years. In the event cooperators deemed presence within the cave was creating unnecessary disturbance to wintering bats, estimates of large clusters of bats were made to decrease the length of time surveyors were in the cave.

WNS Mortality Monitoring

Selected caves previously confirmed or suspected WNS positive were visited to assess the level of mortality that may have occurred since prior visits (Samoray 2011). In order to collect the best data possible under survey conditions, a full census of all bats observed within the caves was conducted. Several of the sites selected for mortality monitoring (Lamb and Wyckoff 2010) were visited again during the 2019-2020 field season to continue these efforts.

Two methods have been used at these sites to assess mortality: repeated, annual visits to count all bats or banding of all bats to assess survivorship at sites previously determined to be WNS positive. It should be noted, of the sites previously selected for these efforts in Lamb and Wyckoff (2010), monitoring efforts have been reduced or not occurred annually as a result of manpower concerns, potential impacts from repeated disturbance, eliminating visitation at sites in which severe declines have occurred to the wintering bat populations, or the bat populations declining to critically low levels or levels too low to make these efforts a viable option.

Bat Population Monitoring

Because historic survey efforts were focused on monitoring endangered *M. sodalis* and *M. grisescens*, there is a paucity of data pertaining to other cave hibernating species in Tennessee. A continued goal of the 2019-2020 surveys was to identify new sites which serve as hibernacula for non-listed, but WNS affected bats. These species include: *P. subflavus*, *M. septentrionalis*, *M. lucifugus*, and *M. leibii*. Several of the sites visited during this period have been visited during previous survey years. Despite these repeated visits, full censuses of bats observed in the caves were performed. Several sites not previously surveyed, were visited during this period and, again, complete surveys of all bats were performed. Methods detailed by Holliday (2012) were used to select these new sites to determine if they harbor cave hibernating bats.

2020 Statewide Results

Ninety-six (96) caves were visited across 42 counties during the winter of 2019-2020. This is the fourth highest number of caves visited in Tennessee during any WNS monitoring period since surveys began in 2009-2010. WNS field signs were observed in 22 caves. One new county, Moore, has been designated as suspect as WNS field signs were observed on bats within the cave and the proximity of the county to other WNS confirmed counties. The results of all caves surveyed can be found in Appendix B.

Almost 1,700 bat observations were made during the surveys. *P. subflavus* constituted over 68% of the observations and this species was observed in 81% of all caves surveyed. *C. rafinesquii* comprised almost 12% of the total bat observations. Unfortunately, less than 1% of the total observations were of *M. septentrionalis*. Declines in some species were observed yet again during the 2019-2020 winter monitoring period and the most concerning of these declines was for *M. septentrionalis* given only 2 individuals were observed.

The 2019-2020 monitoring period was not a priority count year for *M. grisescens* and *M. sodalis* and no surveys were performed at priority sites for these species. Because surveys were not performed at priority sites, analyses and discussion for *M. grisescens*, and *M. sodalis* were omitted from this report. Surveys at these priority sites will occur during the 2020-2021 field season. Discussion regarding *M. lucifugus* is limited in this report because the majority of observations for this species are made during priority count years.

Because of the lack of historic data for bat species not typically monitored, the 2009-2010 winter survey period was used as the base for which comparisons of current bat numbers could be made. Although this is not a preferred method for reasons that include equal survey effort between sites and across years, difficulty in observing cryptic species, addition or discovery of significant bat sites, and movement of bats across sites within and between survey years, it is the best dataset to make comparisons for assessing potential declines of these bats as the result of WNS.

Table 2. Percent increase or decrease for species observed between 2010 and 2020.

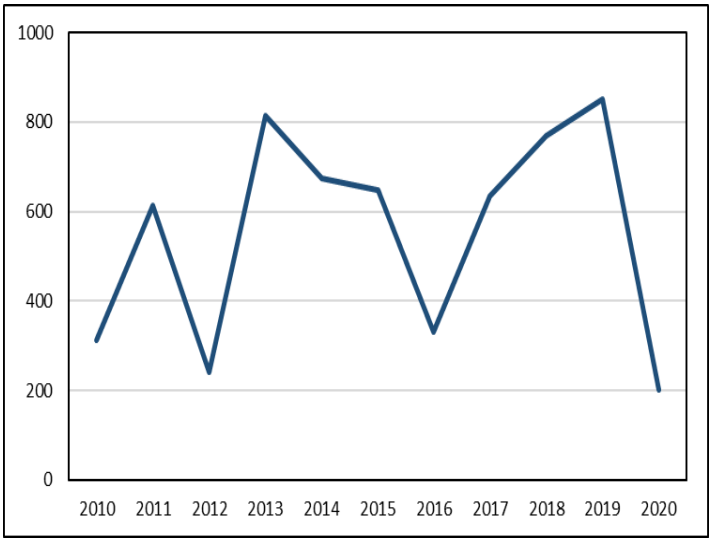
	CORA	EPFU	MYLE	MYLU	MYSE	PESU
2010 (n)	313	28	5	2,075	292	2,159
2020 (n)	201	169	4	89*	2	1,161
% Decline	-35.78%	503.57%	-20.00%	-95.71%	-99.32%	-46.23%

* - Priority sites were not surveyed during the 2019-2020 survey period.

Corynorhinus rafinesquii

Winter populations of *C. rafinesquii* appear stable despite the presence of WNS at many sites. Presence of *Pd* has been detected on this species using real-time PCR methods at winter sites in Tennessee (Bernard et al. 2015). Winter counts have exceeded over 600 individuals since 2013 when most priority sites are surveyed. The impact of survey effort has on observations is apparent for this species given the reduced observations made in 2012, 2016, and 2020 when only a portion of priority sites were surveyed (Figure 3). Survey effort for this species has not been equal across all years and this is because of the limited number of sites and the sensitivity of the species to repeated visitation increasing the difficulty in assessing trends for the species.

Figure 3. Total annual observations of *C. rafinesquii* since 2010.

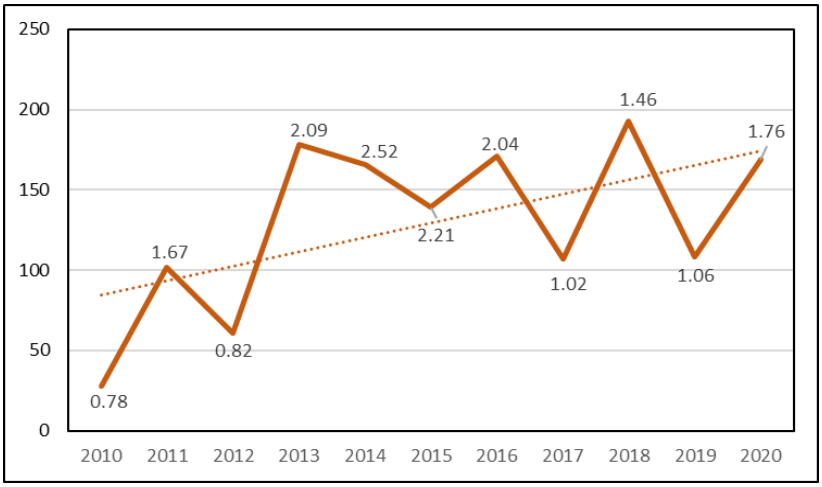


Eptesicus fuscus

The number of *E. fuscus* observed annually has increased since the 2009-2010 winter survey period and this is most likely attributed to increased survey effort. During the 2009-2010 winter monitoring, 36 caves were surveyed compared to the 96 caves surveyed during the 2019-2020 winter. The average number of individual *E. fuscus* observed during each cave surveyed was 1.76 during 2019-2020 compared to just 0.82 individuals per cave surveyed in 2009-2010 (Figure 4).

It appears numbers for this species are trending upward during the winter, but due to the low number of observations through the years it is difficult to determine if the trend is statistically significant. Observations for this species may be difficult to make because of roost preferences or selection during the winter. Many of the observations made during the winter are in plain sight or open areas of caves; however, if *E. fuscus* select roosts such as rock crevices, as observed by Neubaum et al. (2006), observations within caves may become problematic. Also, in other portions of the species range, the use of man-made structures during the winter (Whitaker Jr. and Gummer 2000) may indicate winter surveys should include nontraditional sites. Diagnostic symptoms of WNS have been documented in this species (Blehert et al. 2009).

Figure 4. Annual total observations statewide of *E. fuscus* during annual cave surveys are represented by the line. Annual average individuals observed per cave are indicated along the graph.



Myotis leibii

Observations of this species are extremely limited and have never exceeded 24 in any given year since 2009. The most sites this species has been observed at in any year was 8 (2019), making it difficult to ascertain whether populations of this species are stable, increasing or declining. Similar to *E. fuscus*, it is likely the roosting preferences of this species lead it to be under surveyed annually. In contrast with other cave-roosting bats, *M. leibii* chooses roosts on the cave floor, under talus, or in cracks or crevices within the substrate (Erdle and Hobson 2001). Admittedly, these roosts are under surveyed during the winter, as assessing these areas would increase the time of surveys, visitation, and increase disturbance to other roosting bats. Despite the lack of survey effort for this species, there is still concern WNS may impact this species given diagnostic symptoms have been observed in *M. leibii* (https://www.whitenosesyndrome.org/about/bats-affected-wns).

Myotis lucifugus

Numbers of *M. lucifugus* have mirrored the cyclical surveys conducted for *M. sodalis*, as these two species are often observed within the same hibernacula; however, there are sites within the state where the two species do not occur together. Only 89 total individuals were observed during cave surveys for this monitoring period, but this was not a priority count year. Total observations for this species declined 13.59% since the 2017-2018 survey period, the last monitoring period in which priority caves were not surveyed.

Despite this species once occurring in large numbers at winter sites in northern portions of its range (Davis and Hitchcock 1965) and populations in Tennessee constituting a small portion of the overall population (Kunz and Reichard 2010), the decline of *M. lucifugus* within the state resemble those modeled by Frick et al. (2010), in which a 99% chance of regional extinction of the species was possible. Conservation and recovery efforts for *M. lucifugus* will prove both challenging and difficult given the declines observed in Tennessee.

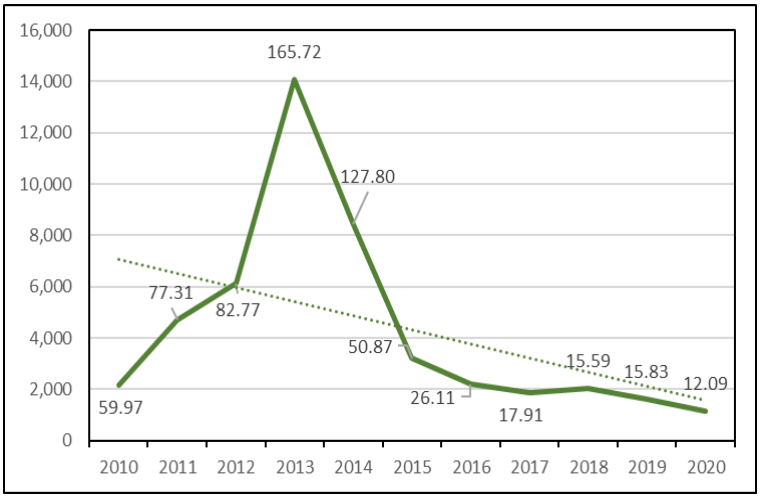
Myotis septentrionalis

Historically, observations of *M. septentrionalis* have been low as it was recorded anecdotally while conducting surveys for species with more significant designations. During 2009-2010, surveyors collected data with increased emphasis on this species. *M. septentrionalis* displays roost preferences similar to those of *E. fuscus* and *M. leibii*, roosting in cracks and crevices of the cave substrate likely leading to it being under surveyed across all years. Since 2012, winter populations of *M. septentrionalis* have declined precipitously; only 2 individuals were observed in 2020 (Table 2). Although the lack of observations can be attributed to roosting preferences of the species, such a drastic decline in the number of observations across multiple winters indicates WNS is having detrimental impacts to *M. septentrionalis*. Given the decrease in observations and known WNS impacts, there is high cause of concern for this species in the state.

Perimyotis subflavus

P. subflavus was one of the most commonly encountered solitary roosters within caves during the winter, being observed in 80% or more caves surveyed annually. Sadly, this species is no longer observed at historic densities and its numbers at sites have declined significantly over the past three years. As with other species, numbers peaked in 2013, but have declined at an alarming rate since. Observations decreased 28.11% from 1,651 (2018-2019) to 1,161 (2019-2020). Along with the decrease in total in observations, the number of *P. subflavus* observed during each cave survey has declined significantly since the 2009-2010 monitoring period. During 2009-2010, the average number of *P. subflavus* observed per cave survey was 59.97, however, the average number of individuals observed during 2019-2020 cave surveys was 12.09.

Figure 5. Annual total observations statewide of *P. subflavus* during annual cave surveys are represented by the line. Annual average individuals observed per cave are indicated along the graph.



WNS Mortality / Bat Population Monitoring

Numerous sites across the state have been visited annually or multiple times since the widespread, multi-species focused survey efforts began in 2009-2010. Table 3 illustrates the observed declines at sites surveyed in 2009-2010 or 2010-2011, visited a minimum of 4 times between 2009-2010 and 2019-2020, and were surveyed during the 2019-2020 field season. While there were some sites in which increases for *P. subflavus* were observed, observations for this species at the majority of sites are declining, many of which exceed 70%. Declines have occurred at all sites for all species, except *C. rafinesquii* and *P. subflavus* at Little Bat Cave and *E. fuscus* at Grindstaff Cave. Although roost switching occurs by bats throughout the winter, it is evident WNS is greatly impacting winter bats in Tennessee, especially *M. lucifugus*, *M. septentrionalis*, and *P. subflavus*. Some bat researchers and biologists believe WNS is causing extirpation of species from sites.

Table 3. The percent change in observations of 4 species of bats in Tennessee. Percentages in red indicate declines at sites between 2009-2010 and 2019-2020.

	CORA	EPFU	MYSE	PESU
Bridgewater Cave	-	+	-	**-68.75%**
Coriolis Cave	+	-	**100.00%**	**-65.39%**
Grindstaff Cave	-	137.50%	-	+
Indian Cave	-	**100.00%**	-	**-45.24%**
Keith Cave	-	-	-	**-94.22%**
Little Bat Cave	57.51%	+	-	400.00%
Marble Bluff Cave	-	+	-	**-82.61%**
Measles Gulf Cave	**-50.00%**	**-66.67%**	-	**-100.00%**
Norris Dam Cave	-	+	-	**-30.95%**
Oaks Cave	-	**100.00%**	-	**-70.00%**
Worley's Cave	-	+	-	**-88.10%**

Conclusions

With each year of survey effort, the impact of WNS to winter bats in Tennessee becomes clearer. During the past three years, large declines of *M. lucifugus*, *M. septentrionalis*, and *P. subflavus* have been made, and these declines are even more apparent when assessing WNS impacts at individual winter sites. Unfortunately, the declines are magnified by the increased effort it now takes researchers, biologists and consultants to captures these species on the landscape during summer months. Despite the widespread declines being observed at many winter sites, there are winter bat populations stable or trending upward at some sites. Biologists are cautiously optimistic populations at these sites will maintain as such given similar increases have been observed at sites prior to declines.

White-nose syndrome disease monitoring of bats in Tennessee, spatiotemporal patterns of the bat host microbiome and biocontrol of *Pseudogymnoascus destructans*

Bats are both economically and ecologically important for crop-pest management and ecosystem function. Bats are estimated to provide farmers with 3.7 billion dollars/year in pest management in North America by consuming a diet of insects known to cause agricultural losses (Boyles et al. 2011). Unfortunately, the fungus *Pseudogymnoascus destructans* (hereafter, *Pd*) was introduced into North America, and has caused the disease called white-nose syndrome (hereafter, WNS), which has led to massive population declines of bats. Understanding the host and geographic distribution of WNS in Tennessee is critical to inform conservation management decisions for bat species and develop effective treatments to alleviate this disease.

All organisms on planet Earth have an assemblage of microorganisms called the microbiome colonizing their skin. The microbiome is known to act as the first line of protection against pathogenic organisms. Since white-nose syndrome is a skin disease, severity of *P. destructans* infections is partially dependent on host-microbiome-pathogen interactions on the bat's skin. Recent work has determined a correlation between the presence of anti-fungal bacteria on bats and resistance to infection by *P. destructans*. During this project, we studied the synergistic to antagonistic host-microbiome-pathogen interactions between the white-nose pathogen and bat skin microbiome. We have isolated a type of bacteria that could be further developed as a biocontrol treatment of white-nose syndrome of bats. Work within this project has been separated into three sections including 1) patterns of WNS across TN, 2) spatiotemporal patterns of the bat skin microbiome, and 3) ability of the bat skin microbiome to protect the host against fungal pathogenicity.

To briefly summarize the complete report, in collaboration with TWRA, TDEC, TVA, and TNC we have collected 565 total skin swabs from 8 bats species across 38 counties in Tennessee. A total of 404 out of 565 bats tested positive (71.5%) for white-nose syndrome. Unfortunately, the white-nose fungus is widespread in TN and detected in nearly all counties sampled. The Tri-colored bat, which is being petitioned by USFWS for listing as an endangered species, was the most heavily sampled and showed high prevalence of white-nose syndrome across Tennessee. To date, we have isolated 355 bacterial strains and tested them for anti-fungal activity against the white-nose pathogen. We found that 111 strains of bacteria slow the growth of the white-nose pathogen and one isolate shows potential as a biocontrol agent. We used high-throughput DNA sequencing to characterize the skin microbiome of the bat and determined that it correlates with white-nose disease status, suggesting the importance of the skin microbiome in disease resistance. Both disease monitoring and understanding the role of the microbiome in bat host health will help inform management decisions for this economically and ecologically important group of organisms.

Purpose and Objectives

- Characterize the bat, cave soil, and roost microbiome.
- Determine the spatiotemporal changes in the bat and cave microbiome.
- Correlate *Pd* infection severity (fungal load) and health of bat populations with microbial communities in caves and on bat skin.
- Determine if the resident skin bacteria have antifungal activity against *Pd*.
- Quantify *Pd* loads on bats.
- Characterize anti-*Pd* bacteria for future *in vivo* (animal based) testing and development of a biocontrol agent for WNS.

Important notes about results

It is important to remember that these samples were not collected following a survey protocol, meaning there was not equal effort put into sampling across species (i.e. *M. grisescens* were sampled every other year) and as a result we cannot say one species is more or less impacted by *Pd*. Additionally, all observed bats were not sampled, and therefore, this introduces bias. More specifically, we sampled bats opportunistically, resulting in low hanging and accessible individuals being swabbed, which can skew estimates as some bats change roosting behavior based on infection status.

Table 4. A total of 11 species of bats were included in this project. Certain species were targeted as surveyors anticipated their presence during cave surveys. Non-target species were species in which presence within cave was known to vary and samples were taken as they were observed. The number of samples taken for each species is indicated by the number for each designation.

	CORA	EPFU	LANO	MYAU	MYGR	MYLE	MYLU	MYSE	MYSO	NYHU	PESU
Target	8	-	-	-	33	-	12	2	12		434
Non-target	-	62	0	0	-	2	-	-	-	0	-

Host and spatiotemporal distribution of white-nose syndrome in Tennessee

A total of 565 skin swabs from 8 bats species across 38 counties in Tennessee were collected (Figure 6). A total of 404 out of 565 bats tested positive (71.5%) for white-nose syndrome (Table 5). White-nose syndrome is widespread in TN and detected in nearly all counties sampled (Table 6, Figure 7). The only county that is currently determined WNS negative is Hawkins county, however, this may be a false negative as we only sampled five bats from this county. Most of our sampling was opportunistic (see statement above) and targeted the bat species EPFU, MYGR, and PESU. Sample sizes are greatest for these species because they were the most numerous and accessible within caves. The Tri-colored bat (PESU), which is being petitioned by USFWS for listing as an endangered species, showed high prevalence of white-nose syndrome across Tennessee (Figure 8). Approximately 6.5% of Big brown bats (EPFU) tested positive for *Pd* from four counties in Tennessee (Figure 9). This is in line with other studies that have found EPFU to be resistant to WNS, resulting in few positive individuals during surveys (Frank et al., 2014). Although sample sizes were small, several species of *Myotis* (MYSO, MYSE, MYLU) showed high numbers of positive individuals (58 – 100% tested; Table 5). Yearly trends (2017-2019) for Tri-colored bats (PESU) were similar in that 85 – 93% of individuals were WNS positive (Figures 10-13). Interestingly, all bats (n = 38) from four different species sampled during the summer were *Pd* negative indicating an interesting disease trend in Tennessee (Table 7). Decreased levels of

summer infection have been documented before and linked to summer cave use in males (Carpenter et al., 2016).

Figure 6. Sampling for white-nose syndrome from bats in a Tennessee cave.

Table 5. Species sampled and determined as positive or negative for white-nose syndrome using qPCR.

Species	# Sampled	% of Total	# Pd Positive	# Pd Negative	% Positive	% Negative
CORA	8	1.4%	1	7	12.5%	87.5%
EPFU	62	11.0%	4	58	6.5%	93.6%
MYGR	33	5.8%	1	32	3.0%	97.0%
MYLE	2	40.0%	0	2	0.0%	100.0%
MYLU	12	2.1%	12	0	100.0%	0.0%
MYSE	2	40.0%	2	0	100.0%	0.0%
MYSO	12	2.1%	7	5	58.3%	41.7%
PESU	434	76.8%	377	57	86.9%	13.1%
Total	**565**	**-**	**404**	**161**	**71.5%**	**28.5%**

Table 6. Counties sampled and determined as positive or negative for white-nose syndrome of all bat species using qPCR.

County	Total Samples	# Pd Positive	% Positive	County	Total Samples	# Pd Positive	% Positive
Anderson	5	5	100%	Knox	22	14	64%
Bedford	18	13	72%	Lawrence	9	8	89%
Campbell	34	28	82%	Loudon	14	14	100%
Cannon	17	15	88%	Macon	5	5	100%
Carter	25	8	32%	Marion	19	12	63%
Claiborne	2	2	100%	Meigs	34	31	91%
Clay	7	7	100%	Monroe	7	7	100%
Cocke	10	10	100%	Montgomery	19	7	37%
Coffee	5	1	20%	Perry	5	5	100%
Dekalb	6	5	83%	Putnam	10	3	30%
Fentress	45	33	73%	Roane	20	20	100%
Franklin	84	64	76%	Robertson	3	3	100%
Grainger	10	7	70%	Smith	3	3	100%
Greene	2	2	100%	Sullivan	8	4	50%
Grundy	15	0	0%	Sumner	15	11	73%
Hamblen	3	1	33%	Union	31	24	77%
Hawkins	5	0	0%	Van Buren	5	1	20%
Hickman	3	3	100%	Warren White	6	5	83%
Jackson	5	4	80%	White	22	15	68%

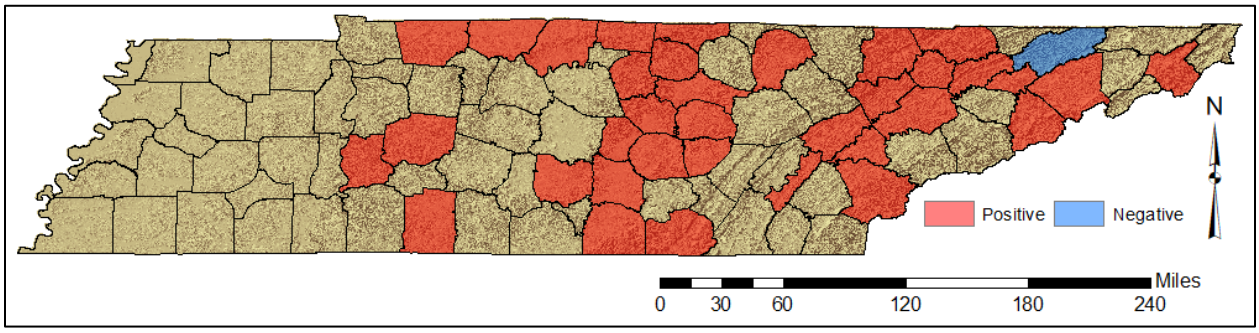


Figure 7. Geographic distribution of white-nose syndrome in Tennessee from samples taken during winter surveys between December 2016 and March 2019. The only county without positive tests was Hawkins County. Unfortuantely, Hawkins County was represented by only five samples and could represent a false negative for white-nose syndrome.

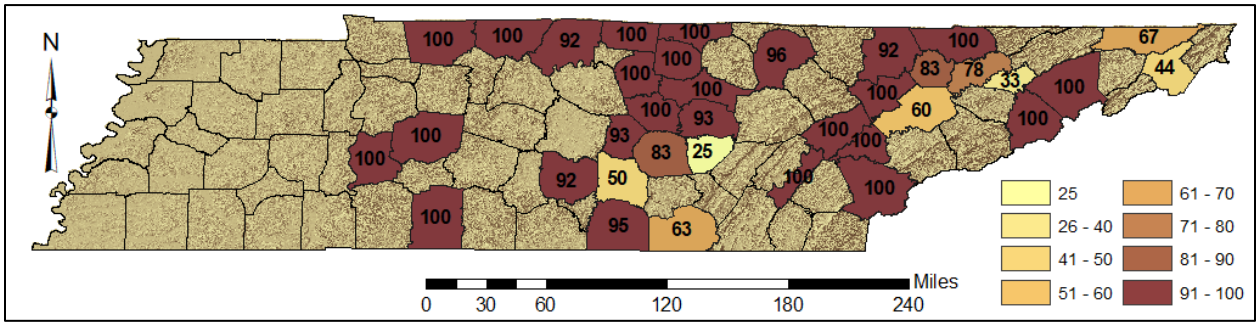


Figure 8. Percentage of positive Tri-colored bats (PESU) within each sampled county. Samples were collected during winter sampling from December 2016 to March 2019.

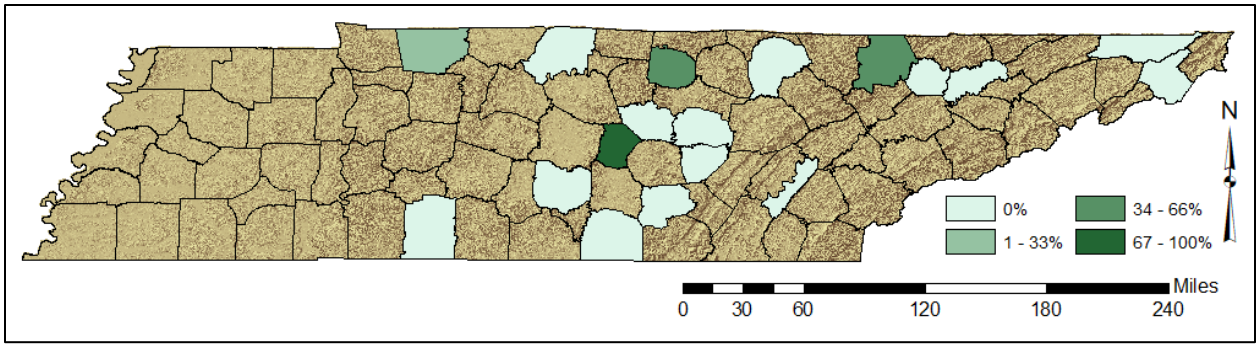


Figure 9. Percentage of positive Big brown bats (EPFU) within each sampled county. Samples were collected from December 2016 to March 2019.

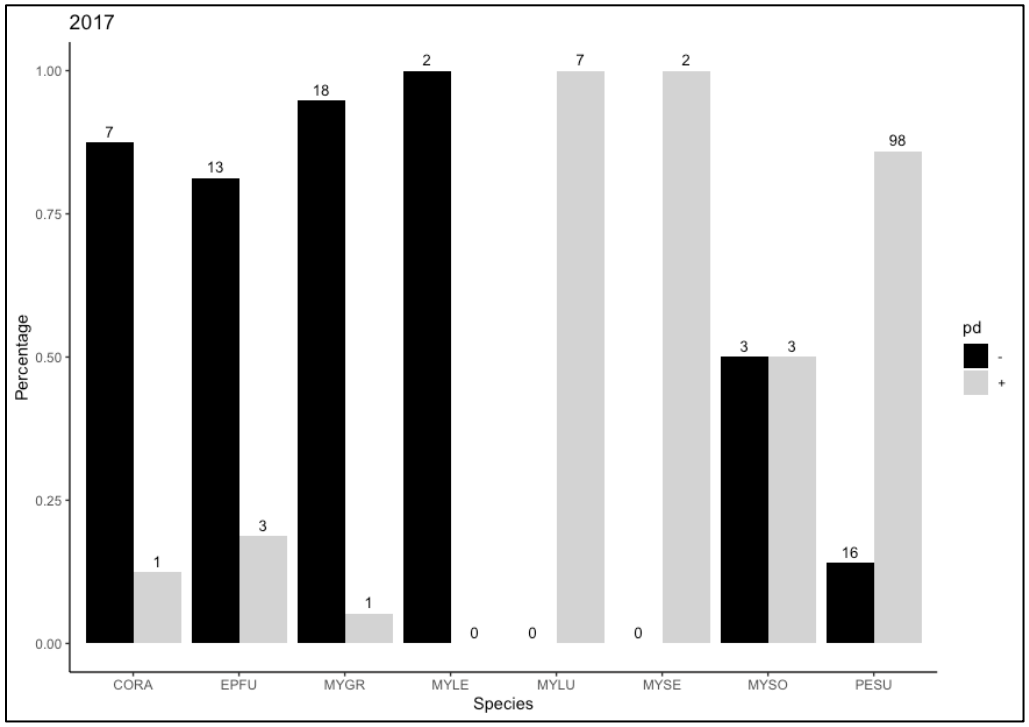


Figure 10. 2017 sampling season of *Pd* status by bat species. Percentages of samples for each bat species collected over the three-year period that were positive (gray bar) or negative (black bar) for *Pd*.

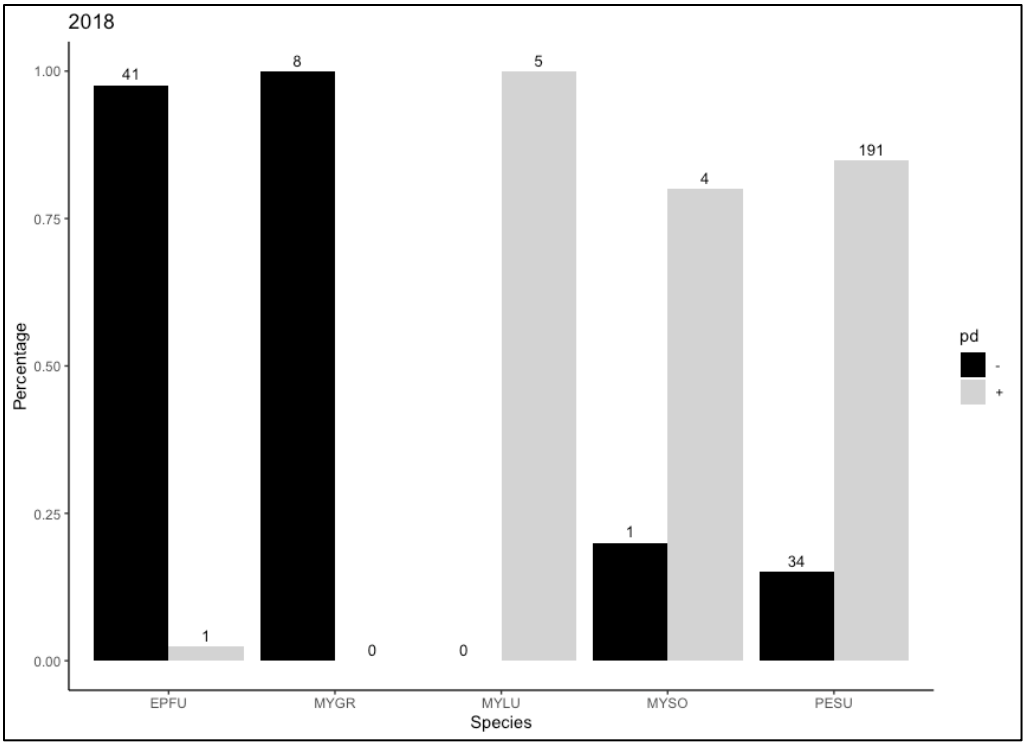


Figure 11. 2018 sampling season of *Pd* status by bat species. Percentages of samples for each bat species collected over the three-period that were positive (gray bar) or negative (black bar) for *Pd*.

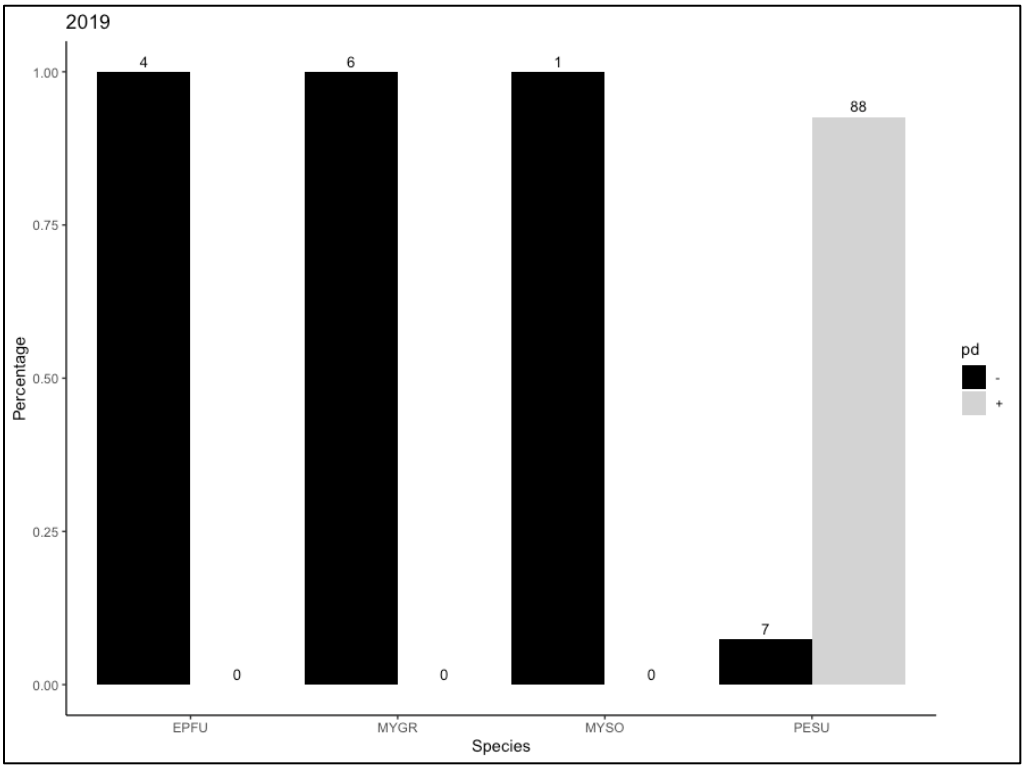


Figure 12. 2019 sampling season of *Pd* status by bat species. Percentages of samples for each bat species collected over the three-year period that were positive (gray bar) or negative (black bar) for *Pd*.

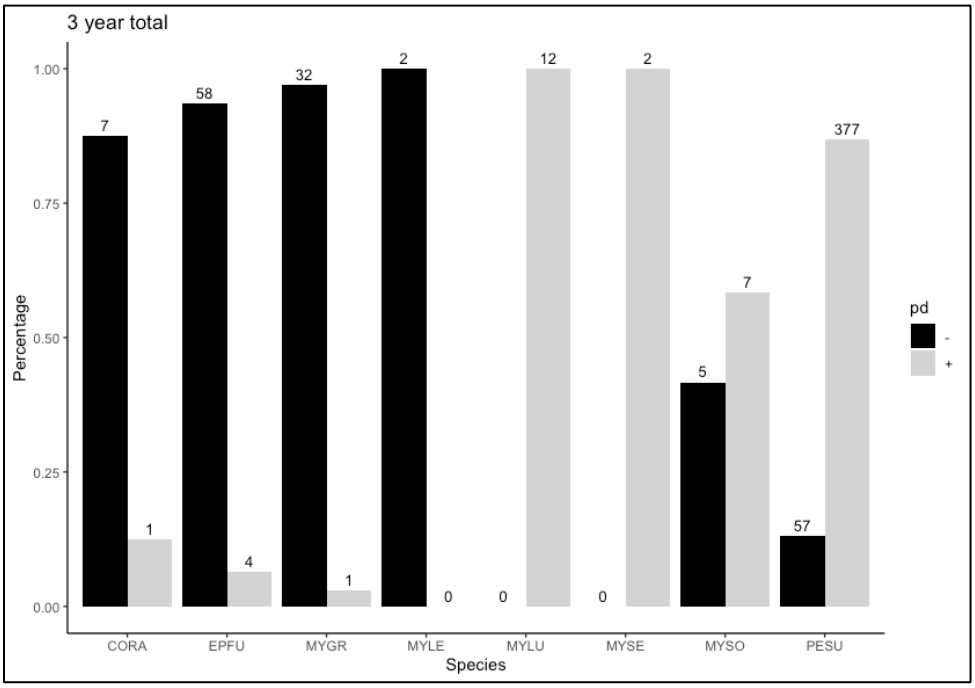


Figure 13. Three-year summary of *Pd* status by bat species. Percentages of samples for each bat species collected of the three-year period that were positive (gray bar) or negative (black bar) for *Pd*.

Table 7. Summer sampling and detection of *Pd* from bats in Fentress, Franklin, and Grundy counties. All bats sampled tested negative for *Pd*.

Species	# Sampled	# Pd Positive	# Pd Negative
CORA	4	0	4
EPFU	21	0	21
MYGR	8	0	8
PESU	5	0	5
Total	**38**	**0**	**38**

The bat microbiome and white-nose syndrome

Microbial assemblage *variation* and average assemblage *structure* are two critical terms to understand for the interpretation of microbiome analyses. Microbial assemblage variation (*betadisper* analysis) describes the *degree of variation* in assemblage composition across a gradient (time) or factor (space). Microbial assemblage structure describes how the *average* community structure might change across a gradient or factor (Anderson et al., 2011). Permanova results explain *average* assemblage structure and nMDS ordinations are a visual display of community *structure* and dissimilarity between skin swab samples. Both microbial assemblage structure and variation are important components of the bat microbiome and were analyzed across spatial and temporal gradients within Tennessee. More specifically, the microbiomes of all bat species were compared across the winter hibernation season of

three years (2017-2019) and one summer season when bats were active on the landscape. Given the small sample size during the summer, only winter hibernation samples were further analyzed across factors including, ecoregion, watershed, geological rock type, and cave site (Fig. 14A–B). The winter hibernation season consisted of 324 microbiome samples in 38 counties across all three years.

We observed an interesting seasonal trend in microbial assemblage structure and variation. The variation in bat skin assemblages increased during the winter for MYGR and PESU relative to the summer (Figure 15). The assemblage structure collectively for all bat species also showed seasonal trends in the skin microbiome (Figure 16). This is particularly interesting when comparing these results to the absence of *Pd* on bat skin during summer months (Table 7). Seasonal shifts in structure and variation in the bat skin microbiome might correlate with the absence of *Pd* during summer months, or alternatively changes in host behavior/habitat use. Species like Gray bats (MYGR) roost in caves year-round and, therefore, are likely constantly exposed to the psychrophilic (cold-loving) and cave dwelling fungus, *Pd.* It is particularly interesting that a shift in skin microbiome structure of MYGR correlates with the absence of *Pd* during summer months. In addition, a decrease in microbiome variation during summer months could indicate the convergence of bat skin microbial assemblages on a beneficial/protective community of microorganisms. Often times dysbiosis (disruption) of the microbiome is correlated with increased variation between microbial assemblages (Blanquer et al., 2016; Glasl et al., 2016). Patterns in the skin microbiome of winter hibernating PESU and MYGR (Figure 15) with *Pd* infection might explain trends of increased assemblage variation. In fact, when measuring this in PESU positive/negative bats, we observed an increase in assemblage variation (Figure 17A) and a difference in average community structure (Figure 17B; Table 8) in positive individuals suggesting disease related dysbiosis of the microbiome. Furthermore, fungal presence is correlated with changes to the average assemblage structure (permanova; $p<0.001$; Table 9) and variation (betadisper; $p<0.001$; Table 10) in the microbiome when all bat species were analyzed collectively. From a quantitative perspective, the amount of *Pd* or severity of infection (fungal load) correlated with the microbial assemblages of *Pd* positive and negative Tricolored bats (PESU; Figure 18). Lastly, average assemblage structure for all species together differed across both year (permanova; $p<0.001$) and *Pd* status by year (permanova; $p<0.029$), suggesting different effects of *Pd* on the microbiome across years.

In order to determine additional factors descriptive of the bat microbiome, we compared different broad to fine spatial scales and geological rock type to describe the variation and structure of microbial assemblages (see comparisons in Figure 14). Bat host species was descriptive of average assemblage structure (permanova; $p<0.001$) and variation (betadisper; $p<0.001$) in the microbiome (Tables 9 – 10; Figure 19). More specifically, variation in assemblages differed across broad (Ecolevel 3) to fine (Ecolevel 4, HUC6) scale spatial comparisons (betadisper; $p<0.001$) collectively for all bat species (Table 10). The only exception to this trend was at the broadest spatial scale of comparison in HUC2 (Table 10; betadisper; $p>0.05$). Interestingly, when testing for differences in space/rock type across bat species, the microbial assemblage structure was similar (Table 9; bat host interaction effects), suggesting that similar spatial factors are at play in structuring bat species-specific microbiomes. Taken together, we concluded that *variation* within the bat microbiome exists across spatial scales finer than HUC2 (broadest scale; Figure 19A), however, the *average* assemblage structure collectively for all bats is similar across fine to broad spatial scales.

We made similar comparisons independently for each EPFU and PESU to determine if factors predictive of *all* bat microbiomes (previous paragraph) were also consistent for each species. For PESU, ecoregion 3 was descriptive of average structure of the microbiome (permanova; $p<0.05$) suggesting differences across space, however, there was limited variation (betadisper; $p>0.05$) within each of the ecoregions (Table 8, 10). A different trend was observed for Big-brown bats (EPFU) as ecoregion 3, HUC6, and rock type were all explanatory of skin microbial assemblage structure and variation (Figure 20).

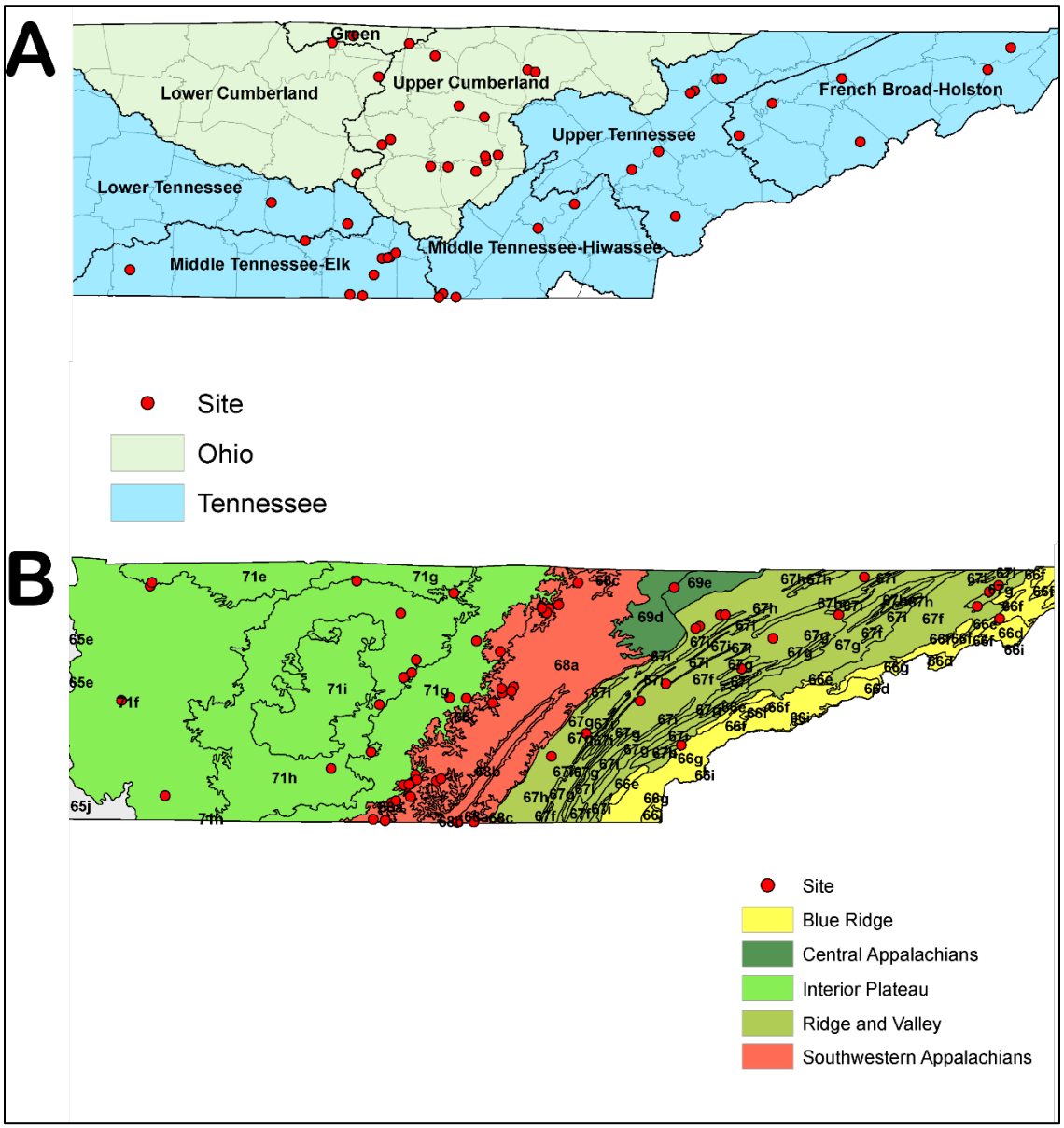


Figure 14. A. Spatial comparisons of river basins - colors show HUC2, Ohio and Tennessee river basins; HUC6 drainages are labeled with text and delineated with bold black lines (e.g. Upper Tennessee). **B.** Spatial comparisons across landscapes - Ecoregion level 3. (Eco3 – broad spatial scale) shown as colored regions (e.g. Blue Ridge) and numbers (e.g. 71h) representative of ecoregion level 4 (Eco4 – fine spatial scale). Caves are shown as red dots.

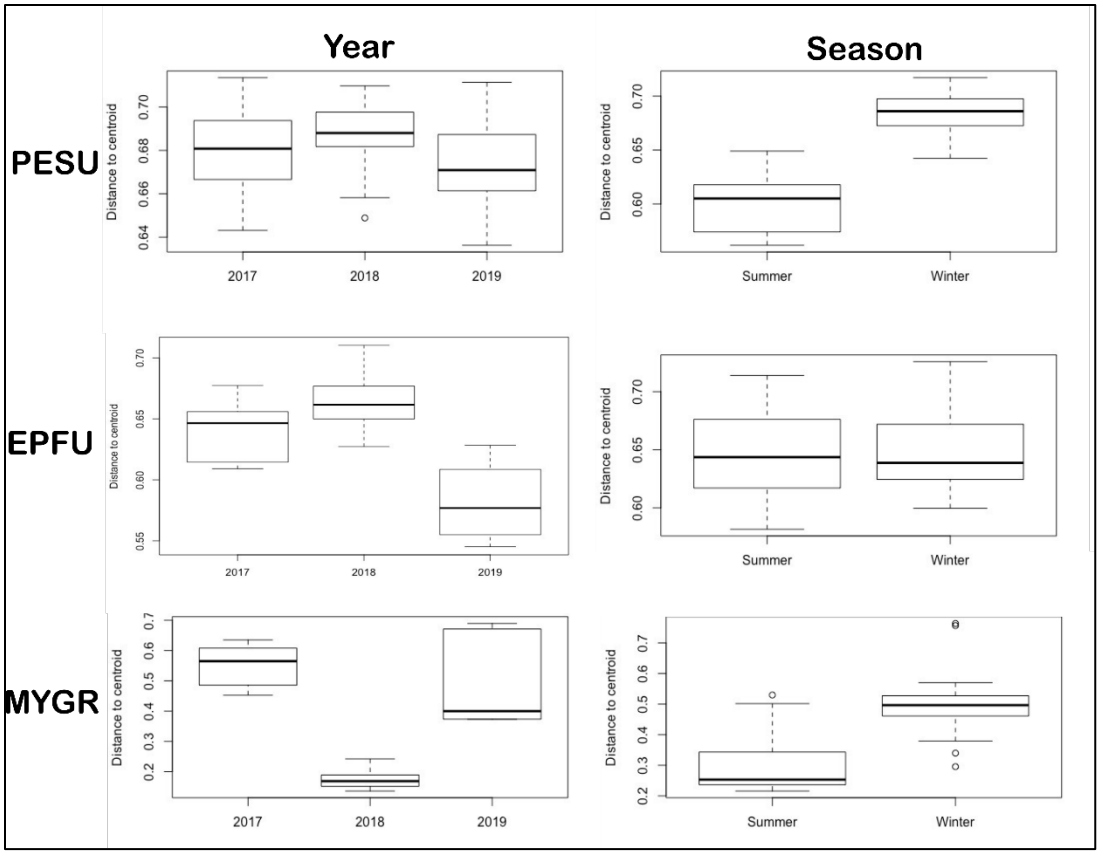


Figure 15. Temporal variation in bat skin microbiome across both years (2017–2018) and seasons (summer, winter). All comparisons (except EPFU season) of microbial assemblage variation were statistically significant ($p < 0.001$; see Table 7). Both PESU and EPFU show a similar trend across years with an increase in variation during 2018, whereas, MYGR shows the inverse trend. MYGR and PESU show a similar trend of increasing variation in the microbiome during winter months. This suggests that the bat skin microbiome is most variable during winter months and more conserved during summer months.

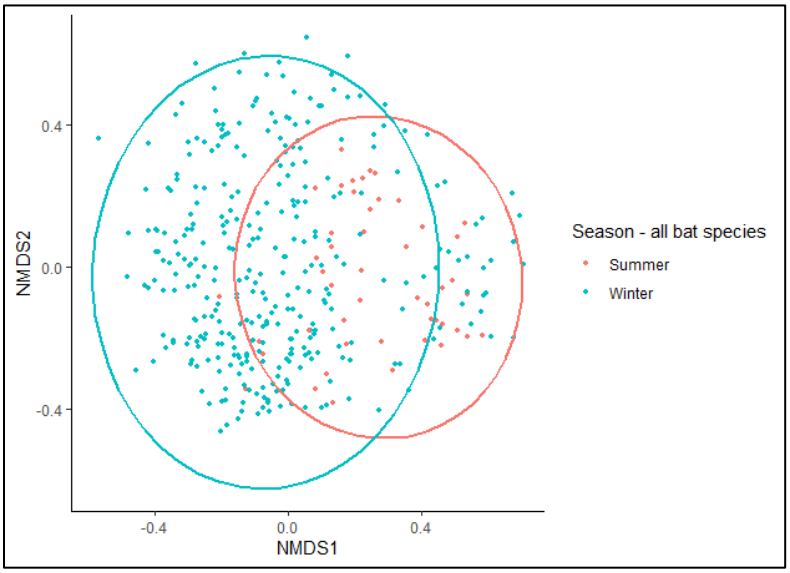


Figure 16. Microbial assemblage structure for all bat species across winter and summer seasons. These patterns suggest that seasonal change in microbiome structure correlate with disease status given that all bats sampled during the winter were *Pd* negative.

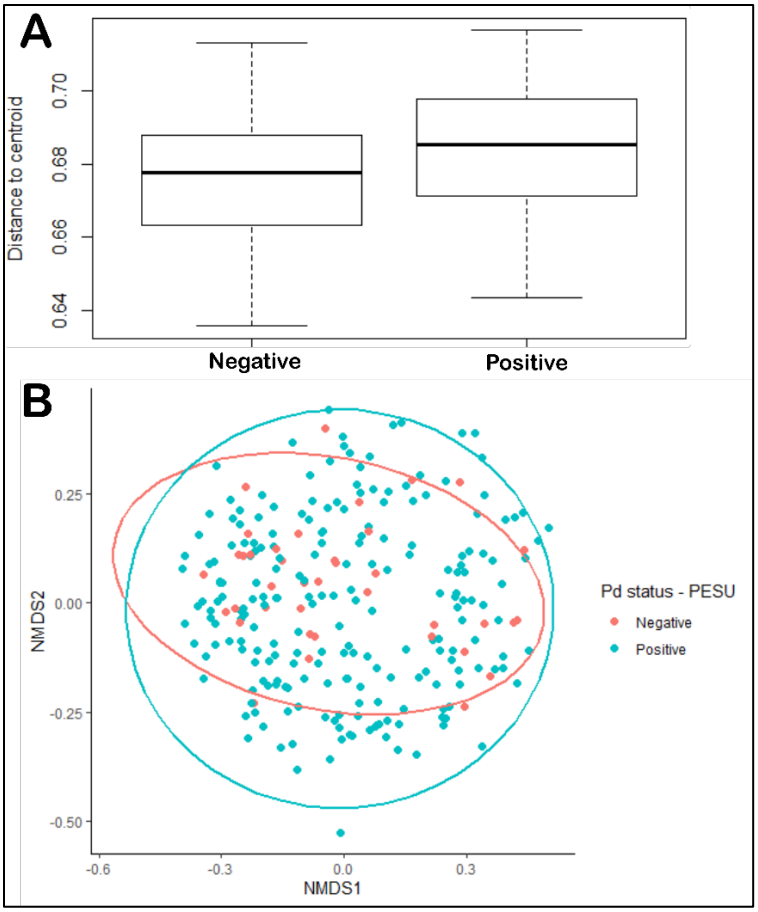


Figure 17. Variation (A) and structure (B) of microbial assemblages for PESU (Tricolored bats) by *Pd* status for samples taken during winter months from 2017 - 2019.

Table 8. Summary of the average microbiome assemblage structure based on PERMANOVA analyses for *independent* bat species during winter hibernation season. Factors are shown independently for each bat species to determine if *Pd*, space, time and geological rock type are predictive of assemblage structure. Bold values are statistically significant and R^2 values show the amount of variation accounted for in the model by each factor.

	PESU		EPFU	
Factor	**R^2**	**Pr(>F)**	**R^2**	**Pr(>F)**
Pd status	0.005	**0.050**	–	–
Ecoregion 3	0.012	**0.038**	0.130	**0.048**
HUC6	–	0.410	0.168	**0.034**
Rock	–	0.320	0.196	**0.035**
Site	–	0.908	–	0.087
Year	0.012	**0.001**	0.045	**0.033**

Table 9. Summary of the average microbiome assemblage structure based on PERMANOVA analyses for all bat species during winter hibernation season. Factors predictive of the microbiome are shown independently and also using an interaction term to tease out the effect of bat host species and the effect of *Pd* independent of bat species across space, time, and geological rock type. Bold values are statistically significant and R^2 values show the amount of variation accounted for in the model by each factor.

Factor	Df	Sums of squares	Mean Squares	F Model	R^2	Pr(>F)
Pd status	1	1.31	1.31	3.13	0.009	**0.001**
Species	6	10.01	1.67	3.98	0.066	**0.001**
Year	1	0.77	0.77	1.84	0.005	**0.001**
Eco Region 3	4	2.64	0.66	1.57	0.017	0.989
HUC6	6	4.09	0.68	1.63	0.027	0.794
Site	49	27.94	0.57	1.36	0.183	0.956
Rock	6	3.92	0.65	1.55	0.026	0.753
Effect of bat host species						
Species:Pd	3	0.77	0.26	0.61	0.005	0.991
Species:Year	6	2.65	0.44	1.05	0.017	0.389
Species:Eco Region 3	3	1.22	0.41	0.96	0.008	0.289
Species:HUC6	7	2.99	0.43	1.02	0.020	0.252
Species:Rock	5	2.09	0.42	1.00	0.014	0.291
Effect of Pd regardless of bat species						
Pd:Species	3	0.77	0.26	0.61	0.005	0.988
Pd:Year	1	0.50	0.50	1.20	0.003	**0.029**
Pd:Eco Region 3	2	0.87	0.43	1.03	0.006	0.402
Pd:HUC6	6	2.75	0.46	1.09	0.018	0.159
Pd:Rock	5	2.26	0.45	1.08	0.015	**0.055**

Table 10. Variation in the winter bat skin microbial assemblages across host, geographic space, geological rock type, year, site, and Pd status. Values in bold text indicate statistically significant variation in all bat species or individually in PESU, EPFU, and MYGR. Both time and space were found to correlate with variation in the bat skin microbiome for all bat species, however, there were also bat species specific effects.

Factor	All bats	PESU	EPFU	MYGR
Pd status	**0.001**	**0.006**	–	–
Host species	**0.001**	–	–	–
Year	**0.001**	**0.001**	**0.034**	0.244
Eco Region 3	**0.001**	0.836	**0.001**	**0.011**
Eco Region 4	**0.001**	**0.001**	**0.001**	**0.045**
HUC2	0.615	0.372	**0.037**	0.546
HUC6	**0.001**	**0.001**	**0.001**	0.16
Site	**0.001**	**0.001**	**0.001**	0.059
Rock	**0.001**	**0.001**	**0.001**	0.097

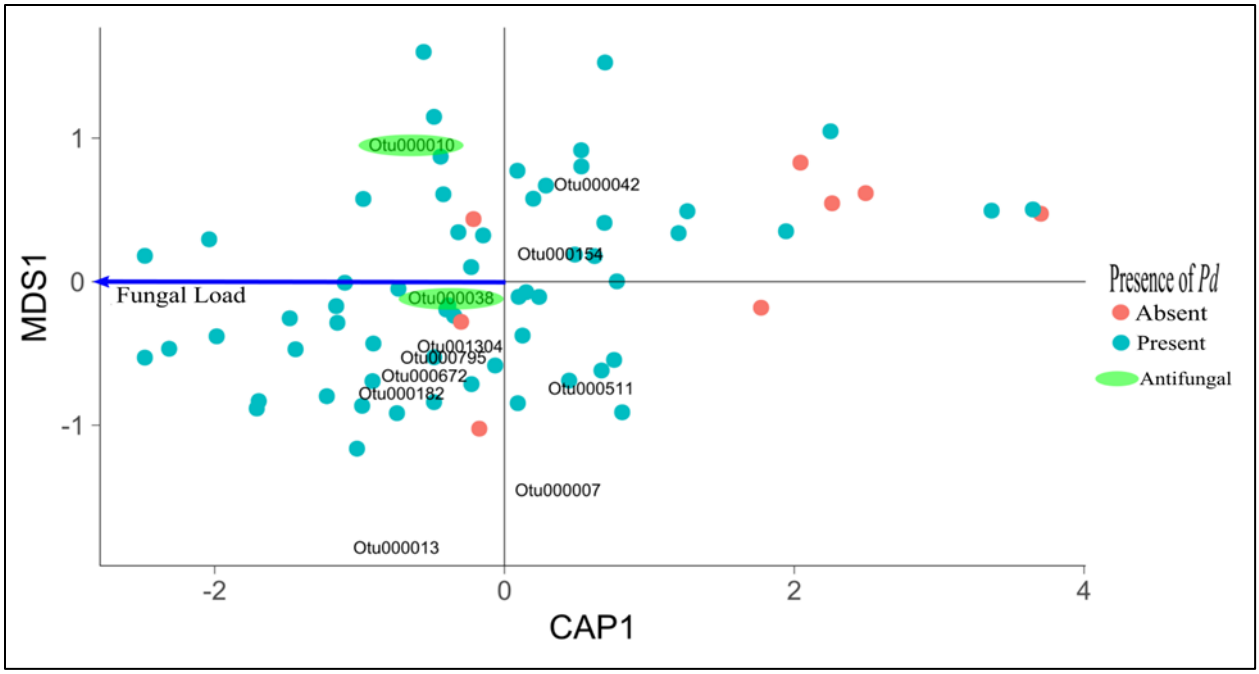


Figure 18. Pd fungal load correlates with microbial assemblage of *Pd* positive and negative PESU (Tricolored bats). Colored circles represent the microbiota of *Pd* positive (blue) and *Pd* negative (red) PESU. Light green ovals around OTU labels indicate a bacterial isolate that was found *in vitro* to inhibit the growth of *Pd*. The blue vector shows the direction of increasing *Pd* fungal load from qPCR results and the relationship with bat cutaneous microbial assemblages and *Pd* load. These data are from a single sampling year – 2017. Figure from Grisnik M et al., The cutaneous microbiota of bats has in vitro antifungal activity against the white nose pathogen. FEMS Microbiology Ecology. 2020 Feb;96(2):fiz193.

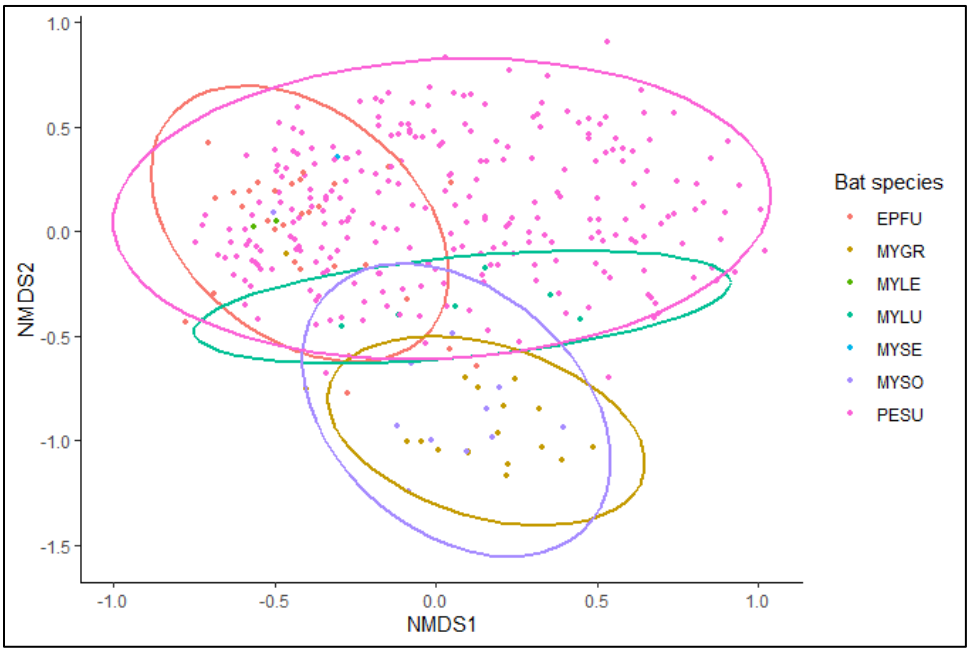


Figure 19. Host species is predictive of the structure of the bat skin microbiome. nMDS ordination of Bray-Curtis values with 95% confidence ellipses shown for all species except for MYLE and MYSE due to small sample sizes.

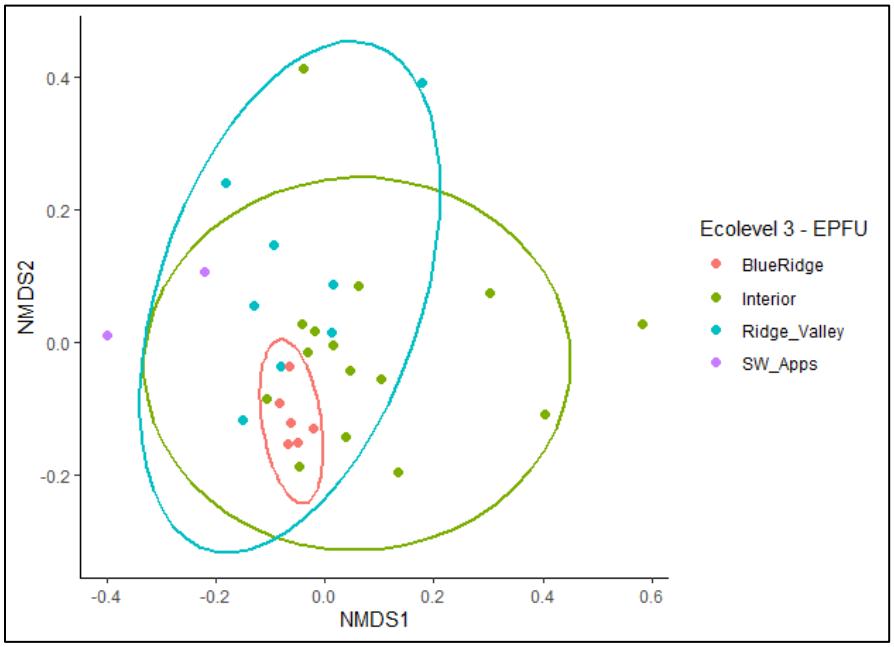


Figure 20. Assemblage structure based on broad spatial comparisons at ecolevel 3 within Tennessee are predictive of the structure of skin microbial assemblages of *E. fuscus*.

Protective nature of the bat skin microbiome against fungal pathogenicity

Three main factors influence disease outcome as described by the disease triangle (Scholthof, 2007). A susceptible host, virulent pathogen, and favorable environment must be present for disease to manifest (McNew, 1960). The composition of the microbiome community has implications for host health, and its role in pathogen resistance is currently an area of active research (Cho and Blaser 2012, Grice et al., 2011). Bacterial species composing the microbiome can influence pathogenic or transient invaders through competition, producing antifungal compounds (Cornelison et al., 2014, Rollins-Smith 2009), or immunomodulation (Reid et al., 2011). White-nose syndrome (WNS) is an infectious fungal disease caused by *Pseudogymnoascus destructans* (hereafter *Pd*) that has killed millions of bats since being introduced to the USA in 2006 (Frick et al., 2016). Alternative strategies are required to mitigate the impact of this fungus on bat populations. To date, mitigation strategies for bats infected with *Pd* such as the application of chemical fungicides and non-native fungistatic bacteria into the cave ecosystem have shown only limited success (Hoyt et al., 2019). Several studies have identified antifungal bacterial species in the cutaneous microbiome of bats (Cheng et al., 2016, Cornelison et al., 2014, Hoyt et al., 2015). Bats that are white nose positive typically have a microbiome enriched with bacterial species producing antifungal activity (Lemieux-Labonte et al., 2017, Grisnik et al., 2020). The identification of antifungal bacteria within the bat microbiome has led to an interest in using the microbiome to treat white nose syndrome (WNS) of bats caused by *Pd*. The objective of this project was to identify bacterial species capable of inhibiting the growth of *Pd* found within the bat and the cave microbiome. The long-term goal of this effort is to create an effective and environmentally safe biological treatment for WNS.

One of the major risks involved in using antifungal bacteria to treat WNS is the potential introduction of a harmful, or novel, strain of bacteria into a fragile cave environment, thus disrupting the ecosystem. Therefore, to minimize potential adverse collateral effects to a cave ecosystem, it is important that any candidate treatment be native to the cave environment. Candidate treatments found in this study were found on both bat skin and in the cave environment. We used high-throughput amplicon sequencing to characterize the structure of the bat skin, roost, and cave soil microbial assemblages. We determined that the bat skin assemblage structure is largely different compared to the cave environment, however, there is some overlap between the microbiomes (Figure 21). We isolated 355 living bacterial strains and tested them for antifungal activity against the white-nose pathogen. We found that 111 strains of bacteria slow the growth of the white-nose pathogen and twelve isolates occur both on the bat skin and in the cave environment (Table 11). One isolate shows strong antifungal activity and would be a top candidate as a biocontrol agent (Figure 22). We then compared DNA sequences from the living antifungal isolates to the high-throughput sequencing data used to characterize the microbiome to determine a tentative source (e.g. cave environment) for antifungal taxa. We found that sample type had similar richness, or number of antifungal taxa (LMM: $\chi^2(2) = 2.28$, $p > 0.05$) across bat skin, roost, and the cave environment (Figure 23, Grisnik et al., 2020). We then performed a similar comparison, however, instead of analyzing richness, we observed relative abundance of antifungal taxa and found that the bat skin is enriched for antifungal bacteria relative to the roost or soil environment (Figure 24; LMM ($\chi^2(2) = 28.09$, $p \leq 0.05$; Grisnik et al., 2020). Lastly, we compared antifungal richness between disease positive and negative individuals and found a statistical difference in antifungal bacterial richness (Figure 25; LMM, $\chi^2(1) = 4.88$, $p \leq 0.05$; Grisnik et al., 2020). Collectively, these results suggest that antifungal bacteria occur on both the bat skin and in the cave environment, bat skin is enriched in antifungal taxa relative to the environment, and *Pd* negative bats have more antifungal microbes relative to *Pd* positive bats. One bacterial isolate (Figure 22) has strong antifungal activity and was also found in the cave environment making it a good candidate for a biological treatment of WNS.

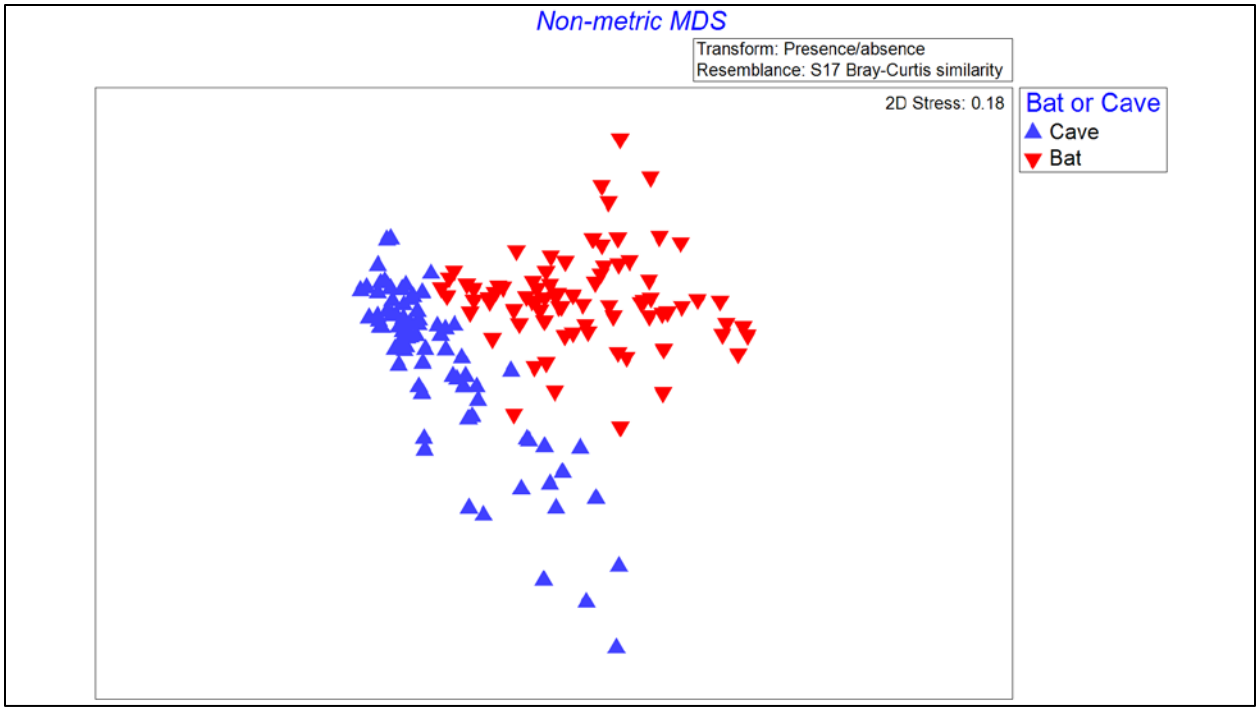


Figure 21. Comparison of bat (PESU) and cave microbiome assemblage structure using non-metric multidimensional scaling ordination of Bray-Curtis dissimilarity values. The bat skin microbial assemblages are mostly distinct from cave assemblages.

Table 11. Heat map showing the identities of antifungal bacteria isolated from bat (PESU) cutaneous swabs. The column labeled as "Isolate" is colored to indicate strength of anti-Pd activity with darker colors showing stronger activity. The last three column show the percent of samples each isolate was found in, more ubiquitous bacteria have darker shading indicating that they occur in/on numerous substrates or the bat host. The bacterial isolates listed in this table are tentative candidates for biocontrol agents of WNS. Figure from Grisnik M. et al., The cutaneous microbiota of bats has in vitro antifungal activity against the white nose pathogen. FEMS Microbiology Ecology. 2020 Feb;96(2):fiz193.

Isolate	Otu	Phylum	Class	Order	Family	*Genus*	Bats	Soil	Roost	Percent of samples
CCB1.4	Otu011978	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	*Arthrobacter*	3%	0%	0%	90%
CCB3.1	Otu001538	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Lysobacter*	6%	7%	11%	80%
CCB307.1	Otu000086	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	39%	64%	49%	70%
CCB307.9	Otu000053	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	*Agromyces*	58%	57%	51%	60%
CCB311.5	Otu000010	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	*Rhodococcus*	58%	18%	41%	50%
CCB313.6	Otu001968	Proteobacteria	Alpha proteobacteria	Rhizobiales	Rhizobiaceae	*Rhizobium*	4%	4%	3%	40%
CCB314.4	Otu003502	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Achromobacter*	6%	4%	0%	30%
CCB315.5	Otu000397	Proteobacteria	Alpha proteobacteria	Rhizobiales	Phyllobacteriaceae	*Aminobacter*	24%	43%	14%	20%
CCB33.13	Otu000647	Proteobacteria	Alpha proteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	15%	21%	16%	10%
CCB33.5	Otu003445	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	1%	0%	3%	1%
CCB36.2	Otu001121	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Rhodanobacteraceae	*Luteibacter*	9%	7%	5%	0%
CCB41.2	Otu000050	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	*Streptomyces*	30%	36%	49%	
CCB43.2	Otu003788	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	*Streptomyces*	3%	4%	5%	
CCB43.6	Otu008587	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	*Microbacterium*	1%	4%	8%	
CCB44.6	Otu000038	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	*Nocardia*	58%	7%	54%	isolate - zoi (cm)
CCB52.1	Otu005913	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	1%	0%	0%	3
CCB53.6	Otu004987	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	*Corynebacterium*	3%	0%	0%	2
CCB57.2	Otu000546	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	6%	0%	0%	1
										0.01

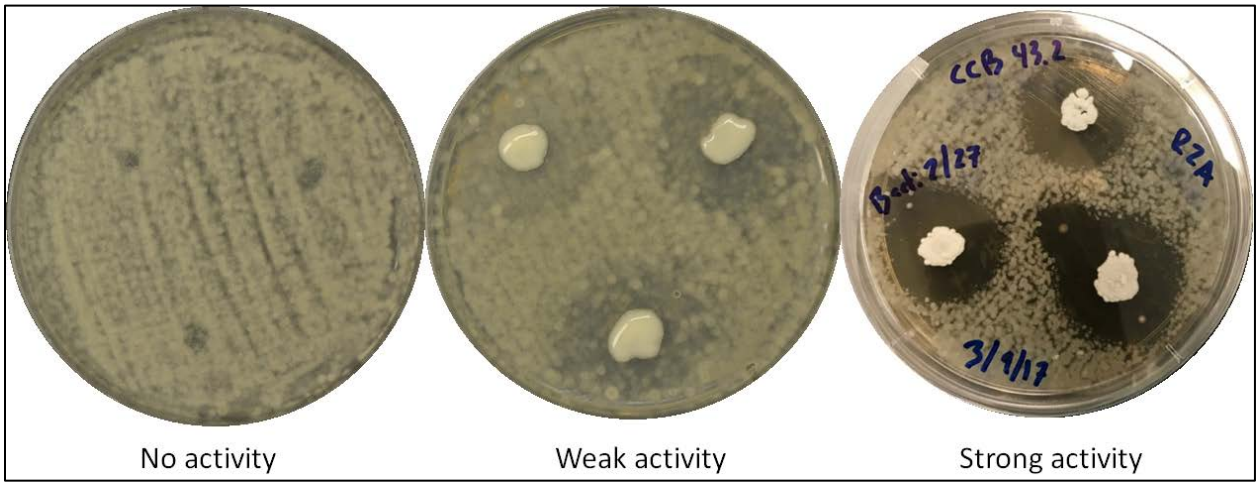


Figure 22. Petri plate assay showing tentative biocontrol agent for white-nose syndrome (CCB43.2). The Petri plate labeled as strong activity shows a species of bacteria from *P. subflavus* that stops the white-nose fungus from growing (zone of clearing around white dots).

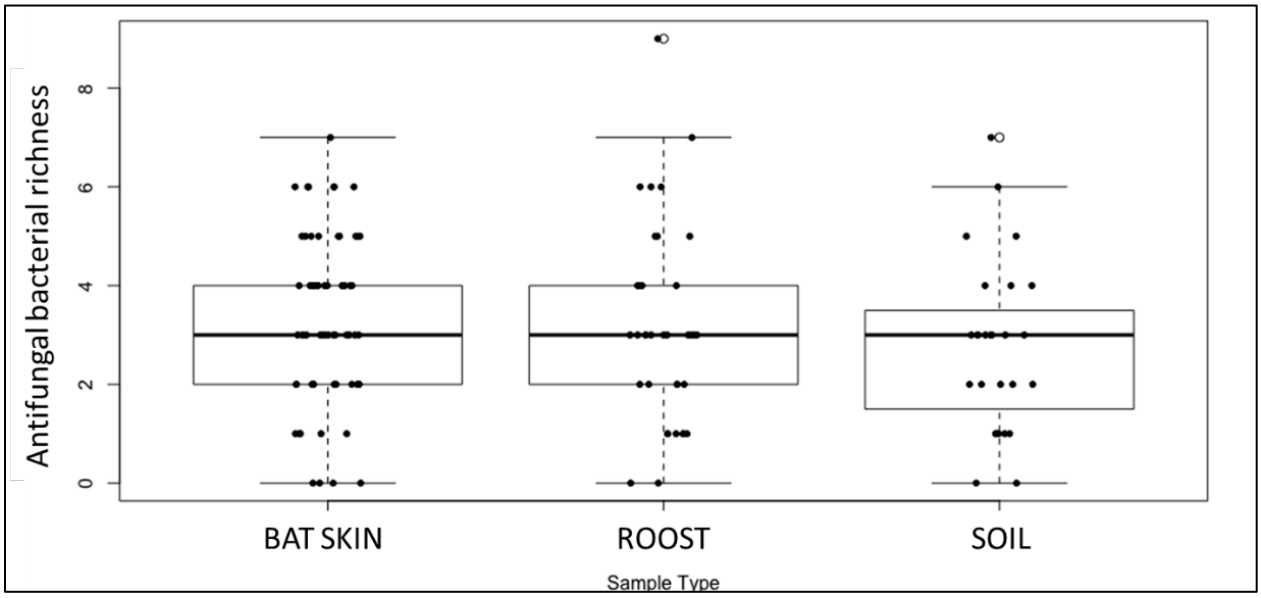


Figure 23. Comparison of DNA sequences from antifungal isolates to the high-throughput sequencing data to determine a tentative source for antifungal taxa found living on bat (PESU) skin. We found that sample type (bat skin, roost, soil) had similar richness of antifungal taxa (LMM □2 (2) = 2.28, p > 0.05). Figure from Grisnik M. et al., The cutaneous microbiota of bats has in vitro antifungal activity against the white nose pathogen. FEMS Microbiology Ecology. 2020 Feb;96(2):fiz193.

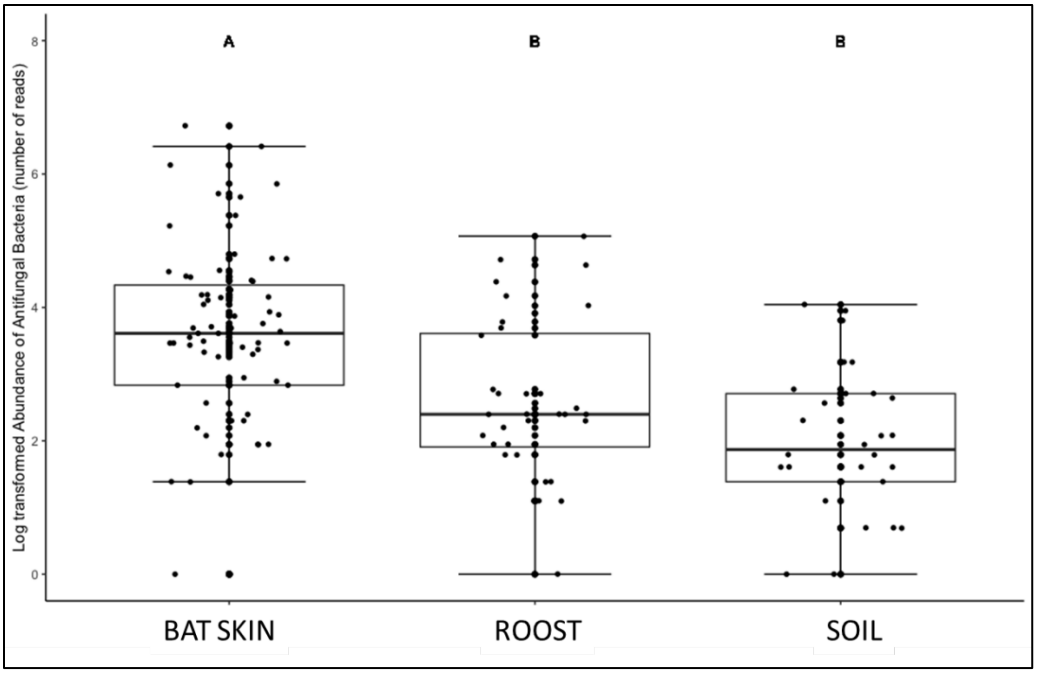


Figure 24. Abundance of antifungal taxa found on bat (PESU) skin compared to the roost or cave soil environment. Letters that differ (A, B) indicate statistically significant comparisons LMM (□2 (2) = 28.09, $p \leq 0.05$). Bat skin is enriched with antifungal taxa relative to the environment. Figure from: Grisnik M. et al., The cutaneous microbiota of bats has in vitro antifungal activity against the white nose pathogen. FEMS Microbiology Ecology. 2020 Feb;96(2):fiz193.

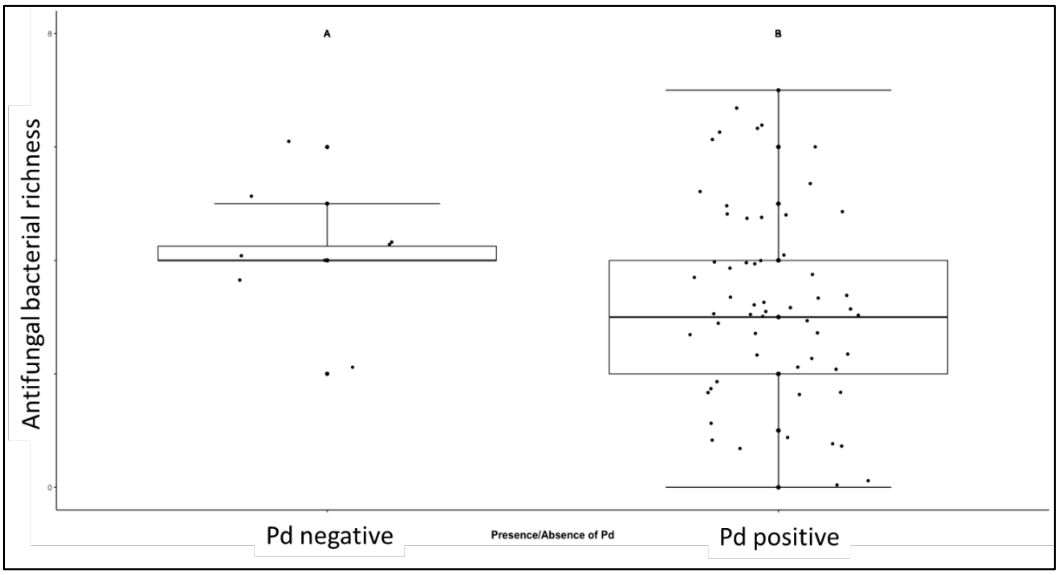


Figure 25. Comparison of antifungal richness between Pd positive and negative PESU individuals. Bats determined as Pd negative had more antifungal bacteria on their skin (LMM, □2 (1) = 4.88, $p \leq 0.05$). Figure from: Grisnik M. et al., The cutaneous microbiota of bats has in vitro antifungal activity against the white nose pathogen. FEMS Microbiology Ecology. 2020 Feb;96(2):fiz193.

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Appendix A

- A list of all WNS confirmed, suspect, or negative counties in Tennessee based on diagnostic reports.

[1]Tapelift sample taken and the bat was not euthanized; [2]Bat submitted was found dead at site; [C]WNS confirmed; [S]WNS suspect; [N]WNS Negative [SW]Only a swab sample was taken from the bat tested and was not euthanized; [N/A]Report not available.

Cave Name or Structure	County	Year	WNS Status	Species	Diagnostic Report Number
Camps Gulf Cave	Van Buren	2010	Suspect	PESU[S], MYSO[1,N]	NWHC-22984
Dunbar Cave	Montgomery	2010	Suspect	MYSE[S]	NWHC Event 15950
East Fork SLP Cave	Fentress	2010	Suspect	MYLU, MYSE[S]	NWHC Event 15979
Grindstaff Cave	Carter	2010	Confirmed	MYSE[C], PESU[C]	NWHC
Hubbards Cave	Warren	2010	Negative	MYGR[N]	NWHC
White Oak Blowhole	Blount	2010	Suspect	N/A	N/A
Worleys Cave	Sullivan	2010	Confirmed	MYSE, PESU	NWHC Event 15948
Bellamy Cave	Montgomery	2011	Negative	MYGR[N]	NWHC-23532
Camps Gulf Cave	Van Buren	2011	Suspect	PESU[S]	NWHC-23481
Cooper Creek Cave	Montgomery	2011	Confirmed	MYLU[C], MYSE[C], PESU[C]	NWHC-23444
East Fork SLP Cave	Fentress	2011	Suspect	MYLU[S]	NWHC-23482
Under a House	Polk	2011	Negative	MYGR[2]	SCWDS CC11-188
White Oak Blowhole	Blount	2011	Suspect	MYLU[N]	NWHC-23466
Austin Peay State University	Montgomery	2012	Suspect	MYLU[S]	SCWDS CC12-235
Bellamy Cave	Montgomery	2012	Confirmed	MYGR, PESU[C]	SCWDS WNS12-54, WNS12-55
Bull Cave	Blount	2012	Negative	PESU[N]	SCWDS WNS12-50
Camps Gulf Cave	Van Buren	2012	Confirmed	N/A	N/A
Cantwell Valley Cave	Hancock	2012	Confirmed	N/A	N/A

[1]Tapelift sample taken and the bat was not euthanized; [2]Bat submitted was found dead at site; [C]WNS confirmed; [S]WNS suspect; [N]WNS Negative [SW]Only a swab sample was taken from the bat tested and was not euthanized; [N/A]Report not available.

Cave Name or Structure	County	Year	WNS Status	Species	Diagnostic Report Number
Carlton Cave	Franklin	2012	Confirmed	PESU[C]	SCWDS WNS12-56
Fort Campbell Nerd Hole	Stewart	2012	Confirmed	PESU[C]	NWHC-23846
Grassy Cove SLP Cave	Cumberland	2012	Confirmed	MYLU[C]	SCWDS WNS12-064 A-B
Gregory Cave	Blount	2012	Negative	PESU[N]	SCWDS WNS12-50
Hubbards Cave	Warren	2012	Negative	MYGR[N]	SCWDS WNS12-067
Hurricane Creek Cave	Humphreys	2012	Negative	PESU[N], MYSO[N]	NWHC-23848
Lookout Mtn. Battlefield Pit #1	Hamilton	2012	Confirmed	PESU[C]	SCWDS WNS12-86
Lost Creek Cave	White	2012	Negative	MYGR[N,SW], MYLU[N, SW], PESU[N,SW]	SCWDS WNS12-41, WNS12-42, WNS12-43
New Mammoth Cave	Campbell	2012	Negative	MYLU[N]	SCWDS WNS12-068
Pearsons Cave	Hawkins	2012	Confirmed	MYGR[C]	SCWDS WNS12-70
Rainbow Cave	Blount	2012	Negative	PESU[N]	SCWDS WNS12-50
Upstream Cave	Hancock	2012	Confirmed	PESU[C]	SCWDS WNS12-072
White Oak Blowhole	Blount	2012	Confirmed	MYLU[C], PESU[C]	SCWDS WNS12-061, WNS12-062
Afton Cave	Greene	2013	Confirmed	PESU[C]	SCWDS WNS13-72 A-C
Big Mouth Cave	Grundy	2013	Confirmed	MYLU[C]	SCWDS WNS13-56

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Cave Name or Structure	County	Year	WNS Status	Species	Diagnostic Report Number
Blowing Cave	Hickman	2013	Confirmed	MYLU[C], MYSE[C], PESU[C]	SCWDS WNS13-38, WNS13-39, WNS13-40
Buggytop Cave	Franklin	2013	Confirmed	PESU[C]	SCWDS WNS13-103
Buis SLP Cave	Claiborne	2013	Confirmed	MYLU[C]	SCWDS WNS13-74 A-B
Cornstarch Cave	Fentress	2013	Confirmed	MYLU[C], PESU[C]	SCWDS WNS13-10, WNS13-11
Depriest Branch Cave	Lewis	2013	Confirmed	MYLU[C], MYSE[C], PESU[C]	SCWDS WNS13-46, WNS13-47, WNS48
Dunbar Cave	Montgomery	2013	Confirmed	PESU[C]	SCWDS WNS13-98, WNS13-101
East Fork SLP Cave	Fentress	2013	Confirmed	MYLU[C]	SCWDS WNS13-12
Espey Cave	Cannon	2013	Confirmed	PESU[C]	SCWDS WNS13-95
Eve's cave	Meigs	2013	Confirmed	PESU[C]	SCWDS WNS13-76
Gunter's Cave	Cannon	2013	Negative	PESU[N]	SCWDS WNS13-91
Herd O' Coons Cave	Union	2013	Confirmed	MYLU[C], PESU[C]	SCWDS WNS13-70 A-B, WNS13-71
Hubbards Cave	Warren	2013	Confirmed	PESU[C]	SCWDS WNS13-13
Hunt Cave	Dickson	2013	Confirmed	PESU[C]	SCWDS WNS13-49 A-C
Jaybird Cave	Perry	2013	Confirmed	MYLU[C]	SCWDS WNS13-44
Knob Creek Cave	Lawrence	2013	Confirmed	PESU[C]	SCWDS WNS13-54

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Cave Name or Structure	County	Year	WNS Status	Species	Diagnostic Report Number
Lost Creek Cave	White	2013	Confirmed	PESU[C]	SCWDS WNS13-53 A-B
New Mammoth Cave	Campbell	2013	Confirmed	MYSE[C], MYLU[C]	SCWDS WNS13-25 A-B, WNS13-26
North Spivey Cave	Jackson	2013	Confirmed	MYLU[C]	SCWDS WNS13-94
Private Residence	Sequatchie	2013	Confirmed	PESU[C]	SCWDS WNS13-99
Pearsons Cave	Hawkins	2013	Confirmed	MYGR[2,N]	SCWDS WNS13-45
Richardson Cave	Houston	2013	Confirmed	MYLU[C]	SCWDS WNS13-02
Rose Cave	White	2013	Suspect	MYLU[S]	SCWDS WNS13-14
Sour Kraut Cave	Claiborne	2013	Confirmed	PESU[C]	SCWDS WNS13-75
Three Forks Cave	Overton	2013	Confirmed	PESU[C]	SCWDS WNS13-90
Trussell Cave	Grundy	2013	Confirmed	PESU[C]	SCWDS WNS13-55 A-C
Trussell Downstream Cave	Grundy	2013	Confirmed	PESU[C]	SCWDS WNS13-55 A-C
Virgin Falls Cave	White	2013	Confirmed	PESU[C]	SCWDS WNS13-50
Welch-Blowing Cave	Putnam	2013	Confirmed	PESU[C]	SCWDS WNS13-64
Whiteside Cave	Marion	2013	Confirmed	PESU[C]	SCWDS WNS13-63
Wolf River Cave	Fentress	2013	Confirmed	MYLU[C]	SCWDS WNS13-9
Zarathustrus Cave	Fentress	2013	Confirmed	PESU[C]	SCWDS WNS13-27
Aunt Beck Simmons Cave	Macon	2014	Confirmed	N/A	N/A
Biffle Cave	Wayne	2014	Confirmed	PESU[C]	SCWDS WNS14-10 A-C

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Cave Name or Structure	County	Year	WNS Status	Species	Diagnostic Report Number
Big Jordan Cave	Pickett	2014	Confirmed	PESU[C], MYLU[C]	SCWDS WNS14-32, WNS14-33
Bridgewater Cave	Smith	2014	Confirmed	PESU[C]	SCWDS WNS14-20 A-B
Cave Creek Cave	Roane	2014	Confirmed	PESU[C]	SCWDS WNS14-31 A-B
Corner Store Cave	Hamblen	2014	Confirmed	PESU[C], MYLU[C]	SCWDS WNS14-29, WNS 14-30
Cripps Mill Cave	Dekalb	2014	Confirmed	PESU[C]	SCWDS WNS14-9
Dunbar Cave area	Montgomery	2014	Confirmed	PESU[C]	SCWDS WNS14-13, WNS14-14, WNS14-16, WNS14-16
Gee Cave	Polk	2014	Confirmed	PESU[C]	SCWDS WNS14-53
Hubbards Cave	Warren	2014	Confirmed	MYGR[2,N]	SCWDS WNS14-7
Hurricane Creek Cave	Humphreys	2014	Confirmed	PESU[C]	SCWDS WNS14-12
Indian Cave	Grainger	2014	Confirmed	PESU[C]	SCWDS WNS14-128, WNS14-129
Leonard Cave	Clay	2014	Confirmed	PESU[C]	SCWDS WNS14-130, WNS14-131, WNS14-132
Mason Cave	Sumner	2014	Suspect	PESU[S]	SCWDS WNS14-52 A-B
Rummage Cave	Maury	2014	Confirmed	PESU[C]	SCWDS WNS14-11 A-C
Springhill SLP Cave	Anderson	2014	Confirmed	MYLU[C]	SCWDS WNS14-8 A
Ward Cave	Bedford	2014	Confirmed	PESU[C]	SCWDS WNS14-51 A-C

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Cave Name	County	Year	WNS Status	Species	Diagnostic Report Number
Crumpton Creek SLP Cave	Coffee	2015	Confirmed	PESU[C]	SCWDS CC15-124
Hardin's Junkyard Cave	Davidson	2015	Suspect	MYLU[S]	Field Signs Observed, UV positive, Photos Taken
Magnussen Cave	Giles	2015	Confirmed	PESU[C]	SCWDS CC15-26
Mason Cave	Sumner	2015	Suspect	N/A	Field Signs Observed, UV positive
Petty Cave	Marshall	2015	Confirmed	PESU[C]	SCWDS CC15-123 A-C
Silvertooth Cave	Moore	2015	Suspect	PESU[N]	SCWDS CC15-125
Stark Cave	Robertson	2015	Confirmed	PESU[C]	SCWDS CC15-127
Civil War Bunker	Tipton	2016	Negative	EPFU[N], PESU[N]	SCWDS 16-92 A-B
Ball Play Cave	Monroe	2017	Suspect	PESU[SW]	CCB137
Blackmans Cave	Knox	2017	Suspect	PESU[SW]	CCB332
Ghost Cave	Loudon	2019	Suspect	PESU[SW]	CCB786, CCB787, CCB788, CCB789, CCB790, CCB791, CCB792, CCB793, CCB794
Williams Mine	Cocke	2019	Suspect	PESU[SW]	CCB1160, CCB1162

Appendix B

- 2019-2020 Winter Survey Results

County	Cave Name	Survey Date	CORA	EPFU	LANO	MYAU	MYGR	MYLE	MYLU	MYSE	MYSO	MYsp	PESU	Total Bats	Surveyors
Anderson	Springhill Slp Cave	1/13/2020		11								4	3	18	TNC, TWRA
Blount	Bull Cave	1/17/2020	1	2				1	1		33		27	65	NPS, UTK
Blount	Rich Mountain Blowhole Cave	1/16/2020												0	NPS, UTK
Blount	Snake Dance (Bull)	1/23/2020											12	12	NPS, UTK
Campbell	Linden Park Cave	1/27/2020		1									9	10	TWRA, TVA
Campbell	Norris Dam Cave	1/20/2020		4									29	33	TVA, UTK
Campbell	Norris Dam Cave NR2	1/20/2020		1									8	9	TVA, UTK
Campbell	Phillips Branch Pit	3/9/2020												0	TWRA
Carter	Grindstaff Cave	1/14/2020		19									8	27	TWRA, TNC
Carter	Laurel Creek Cave	1/14/2020		2									2	4	TNC, TWRA
Carter	Laurel Creek Karst Feature #1	1/14/2020		1										1	TNC, TWRA
Carter	Laurel Creek Karst Feature #2	1/14/2020												0	TNC, TWRA
Carter	Laurel Creek Karst Feature #3	1/14/2020												0	TNC, TWRA
Carter	Sculpture Cave	2/24/2020											19	19	TWRA, UTK
Cocke	Myers Mine (the shaft)	2/19/2020												0	TWRA
Cocke	Myers Mine w/ Cart	2/19/2020											20	20	TWRA
Cocke	Myers Mine w/gate	2/19/2020											9	9	TWRA
Cocke	Williams Mine	2/20/2020		6	1								35	42	TWRA, USGS
Cocke	Myers Mine (single portal)	2/19/2020												0	TWRA
Davidson	Hardins Junkyard Cave	2/24/2020							4				9	13	TNC, TWRA
Decatur	Swallow Bluff Cave	3/10/2020											3	3	TNC, TVA
Dekalb	Indian Grave Point Cave	2/18/2020		1									12	13	TNC, TWRA
Fentress	Buffalo Cave	1/10/2020	3	1					4				24	32	TNC, TWRA
Fentress	Coriolis Cave	2/21/2020											9	9	TWRA, TNC
Fentress	Fern Camp Cave	1/10/2020		2									21	23	TNC, TWRA
Fentress	King Cave	2/21/2020											1	1	TNC, TWRA
Fentress	Pygmalion Cave	1/7/2020	2						71		1		89	163	TWRA, TNC
Fentress	Smokin' Crack Cave	1/10/2020											1	1	TNC, TWRA

County	Cave Name	Survey Date	CORA	EPFU	LANO	MYAU	MYGR	MYLE	MYLU	MYSE	MYSO	MYsp	PESU	Total Bats	Surveyors
Franklin	Keith Cave	1/6/2020											13	13	TWRA, UTK
Franklin	Lost Cove Cave	1/7/2020		6									68	74	TWRA, TDEC, UoS, UTK
Franklin	Solomen's Tunnel	2/20/2020		1					1			5	13	20	AAFB, UoS
Franklin	Solomon's Temple	2/20/2020										2	7	9	AAFB, UoS
Franklin	Walker Springs Cave	2/4/2020							5			3	18	26	AAFB, UoS
Franklin	Wet Cave	2/4/2020							1				4	5	AAFB, UoS
Franklin	Wild Woman Cave	1/6/2020											2	2	TWRA, UTK
Grainger	Neoton #1	2/21/2020		4									2	6	TVA, TWRA
Grainger	Neoton #2	2/21/2020		1										1	TVA, TWRA
Grainger	Neoton #3	2/21/2020												0	TVA, TWRA
Grainger	Neoton #4	2/21/2020												0	TVA, TWRA
Greene	Keyhole Cave	1/15/2020												0	TNC
Greene	Poplar Cave	1/15/2020		10			2						18	30	TNC, TWRA
Greene	Stillhouse Cave	1/15/2020		2									21	23	TNC, TWRA
Greene	Double Mouth Cave	1/15/2020		3									1	4	TNC, TWRA
Hamblen	Corner Store Cave	2/24/2020											5	5	TWRA, UTK
Hancock	Dingling Hole	3/11/2020	3											3	TWRA
Hickman	Blowing Cave	3/2/2020		4			2			1			24	31	TNC, TWRA
Humphreys	Hurrican Creek Cave	2/19/2020		1			16						3	20	TWRA
Jackson	North Spivey Cave	1/29/2020		7									16	23	TNC, TWRA
Knox	Blackmans Cave	3/10/2020											12	12	TWRA
Lawrance	Bailey Hollow Cave	2/6/2020											25	25	TNC, TWRA
Lawrence	Knob Creek Cave	2/6/2020		5									30	35	TNC, TWRA
Lewis	NPS Phosphate Mine (Mystery)	2/5/2020											10	10	TNC, TWRA
Lewis	Phosphate Mine	2/5/2020											10	10	TNC, TWRA
Macon	Aunt Beck Simmons Cave	1/23/2020		11									10	21	TNC, TWRA
Marshall	Petty Cave	2/5/2020			1								34	35	TWRA, TNC
Maury	Cheeks Bend Cave #1	2/7/2020											3	3	TWRA, TNC

County	Cave Name	Survey Date	CORA	EPFU	LANO	MYAU	MYGR	MYLE	MYLU	MYSE	MYSO	MYsp	PESU	Total Bats	Surveyors
Maury	Cheeks Bend Cave #2	2/7/2020												0	TWRA, TNC
Maury	Cheeks Bend Cave #3	2/7/2020												0	TWRA, TNC
Maury	Rummage Cave	2/3/2020											8	8	TWRA, TNC
Meigs	Eaves Cave	2/18/2020											15	15	TVA
Montgomery	Dunbar Cave	3/3/2020											9	9	TNC, FORT
Moore	Silvertooth Cave	2/7/2020		5									16	21	TWRA, TNC
Overton	Bailey's Webb Cave	3/11/2020												0	TNC, TWRA
Overton	Webb Cave	3/11/2020	1	1									10	12	TNC, TWRA
Perry	Blowing Caves	3/2/2020							1				13	14	TNC, TWRA
Pickett	Bunkum Cave	1/6/2020		2				2					62	66	TNC, TWRA
Roane	Marble Bluff Cave	1/17/2020		1									16	17	TVA
Robertson	Whiskey River Cave	1/22/2020		5									17	22	TNC, TWRA
Rutherford	Herron/Herring Cave	3/9/2020					1						24	25	TNC
Sevier	Duncan Cave	3/13/2020		1									1	2	TVA
Sevier	Hammer Cave	3/13/2020											1	1	TVA
Sevier	Turtle Tomb Pit Cave	3/13/2020											6	6	TVA
Smith	Bridgewater Cave	1/23/2020		2									5	7	TNC, TWRA
Sullivan	Worley's / Morrell Cave	1/14/2020		5			2						5	12	TWRA, TNC
Sumner	Mason Cave	1/22/2020		3									4	7	TNC, TWRA
Tipton	Civil War Magazine	1/28/2020											18	18	TWRA
Unicoi	Bumpus Cove Mine #2	2/20/2020											2	2	TWRA, USFS, UTK
Unicoi	Bumpus Cove Mine #3	2/20/2020											2	2	TWRA, USFA, UTK
Unicoi	Bumpus Cove Mine #1	2/20/2020											12	12	TWRA, USFS, UTK
Union	Asmus Well	2/25/2020										7	4	11	TVA, TWRA
Union	Boxed Wine Cave	1/23/2020		2										2	TWRA, UTK
Union	Oaks Cave (Jenny Oaks Cave)	1/21/2020					5						18	23	TWRA, UTK
Union	Oaks Cave (Jenny Oaks Cave)	3/9/2020		3			50						5	58	TWRA
Union	Wright Cave	1/13/2020											14	14	TNC, TWRA

County	Cave Name	Survey Date	CORA	EPFU	LANO	MYAU	MYGR	MYLE	MYLU	MYSE	MYSO	MYsp	PESU	Total Bats	Surveyors
Union	Herd O Coons	1/21/2020		4					1				10	15	TWRA, UTK
Van Buren	Measles Gulf Cave	1/28/2020	78	1										79	TWRA, TNC
Van Buren	White's Tater Cave	1/27/2020											7	7	TNC, TWRA
Warren	Big Bone Cave	1/27/2020	12	27				1					24	64	TNC, TWRA, TDEC
Warren	Hazel Ward Cave	1/8/2020											15	15	TNC, TWRA
Warren	Jaco Spring Cave	1/8/2020					5						20	25	TNC, TWRA
Warren	Little Bat Cave	1/28/2020	101	1									5	107	TNC, TWRA
White	Big Boy Canyons Cave	2/20/2020												0	TNC, TWRA
White	Crafty Commie Cave	2/20/2020											2	2	TNC, TWRA
White	Indian Cave	1/27/2020											23	23	TNC, TWRA
White	Lockwood Cave	2/19/2020											26	26	TWRA, TNC
White	Mill Hole Cave	3/11/2020								1			43	44	TNC, TWRA