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# Investigating biometrics of Perimyotis subflavus in traditional and nontraditional hibernacula in the Southeastern United States as it relates to susceptibility to white-nose syndrome.

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# Investigating biometrics of *Perimyotis subflavus* in traditional and nontraditional hibernacula in the Southeastern United States as it relates to susceptibility to white-nose syndrome.

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by

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# ABSTRACT

*Perimyotis subflavus,* or tricolored bat, populations have declined significantly since the emergence of white-nose syndrome (WNS) and has been proposed for listing as “endangered” under the United States Endangered Species Act. Traditionally, bats use caves as hibernacula, but as anthropogenic impacts have increased so has the use of manmade structures like culverts and bridges for roosting by several bat species. The internal environment of these anthropogenic structures is influenced by external temperature and humidity differently than caves and may influence *P. subflavus* winter activity, and thus susceptibility to WNS. One of the most significant differences in *P. subflavus* roosting behavior in traditional versus nontraditional hibernacula is increased clustering during torpor while using culvert weep holes which may pose an increased risk for *Pseudogymnoascus destructans* (*Pd*) transmission, infection, and WNS development. Continued significant declines in *P. subflavus* populations due to WNS increases importance of considering potential risk factors, such as hibernacula selection or environmental factors, to guide management actions and resolve the significance of hibernacula in the life history and presence of the species. This study aimed to evaluate the differences in *P. subflavus* susceptibility to white-nose syndrome at traditional versus nontraditional hibernacula via temperature/humidity monitoring, testing for *Pd* presence, and comparisons of bat biometrics. The results suggest only fungal loads on bats in newly *Pd* positive sites experienced a significant increase between sampling sessions throughout the winter season and that surface-based anthropogenic hibernacula were more variable in temperature and humidity than were subterranean hibernacula.

# INTRODUCTION

White-nose syndrome (WNS) was first discovered on bats in a New York cave in 2006 (Blehert *et al.*, 2008), and as of 2019, has resulted in the deaths of millions of North American bats (US Fish & Wildlife Service, 2019). The causative fungus, *Pseudogymnoascus destructans* (*Pd*), invades the skin of hibernating bats through hair follicles and related glands, causing lesions and often resulting in death (Blehert *et al.*, 2008; Gargas *et al.*, 2009). Various studies suggest that WNS causes bat mortality via a pathway that results in dehydration and an increased rate of fat store usage leading to emaciation (Verant *et al.*, 2014; Mcguire, Mayberry, & Willis, 2017). Arousals during torpor account for approximately 90% of the energy used during hibernation (Thomas, Dorais, & Bergeron, 1990); an increase in these arousals, from tissue irritation or arousing to drink, was initially thought to be the cause of the increase in the rate of fat store usage (Cryan *et al.*, 2010; Willis *et al.*, 2011). It is now suspected, however, that another potential cause of increased fat store usage is an increase in metabolic rate during torpor caused by WNS (Mcguire *et al.*, 2017). Additionally, Moore *et al.* (2017) found that increased frequency of arousal from torpor is not ubiquitous with *Pd* infection, even though this had been considered a main symptom of WNS and a large cause of the accelerated use of fat stores and ultimate mortality. The psychrophilic fungus affects bats specifically during torpor due to cold temperatures in hibernacula and thus, of the bat. The temperature range at which *Pd* can grow is 0-20°C, while optimal temperatures for growth are 12.5-15.8°C (Blehert *et al.*, 2008; Gargas *et al.*, 2009; Verant *et al.*, 2012). The fungus grows optimally with a relative humidity (RH) of 81.5% or greater (Marroquin, Lavine, & Windstam, 2017). These conditions are found year-round in traditional hibernacula in some parts of North America (Blehert *et al.*, 2008). WNS has been seen in 12 of the 47 species of bats in North America, and an additional 6 species have had *Pd* detected on them without development into WNS. The disease has resulted in population declines greater than 90% in some areas (Cheng *et al.*, 2021).

One of the species that has been severely affected by WNS is *Perimyotis subflavus.* This species, also known as the tricolored bat, is a small bat that was first described by the French zoologist Cuvier in 1832 (Barbour & Davis, 1969; Fujita & Kunz, 1984). As with most bats in the family Vespertilionidae, these bats are insectivorous (Altringham, 1996). They most often roost alone but are occasionally found roosting in small clusters (Barbour & Davis, 1969). It has been estimated that 17% of colonies of *P. subflavus* have been extirpated and that remaining colonies have seen a 10 fold decrease in the number of bats (Frick *et al.*, 2015; Georgia Department of Natural Resources, 2020; Cheng *et al.*, 2021). *P. subflavus* has been observed using both traditional and nontraditional hibernacula as winter roosts for hibernation (Keeley & Tuttle, 1999; Katzenmeyer, 2016; Lutsch *et al.*, 2022) and also use trees and lichen for summer roosts (Poissant, Broders, & Quinn, 2010). Traditional hibernacula here refer to caves, mines, and other similar subterranean structures such as tunnels, while nontraditional hibernacula include culverts, bridges, and other surface-based anthropogenic structures.

In studies of *Myotis lucifugus*, also known by their common name of little brown bat, warmer hibernation temperatures of 10°C increase the likelihood of WNS mortality compared to colder temperatures of 4°C due to slower growth of *Pd* in the colder temperatures (Grieneisen *et al.*, 2015). Alongside the battle to balance temperature needs with minimizing fat store loss and *Pd* growth, humidity is another concern. Evaporative water loss, unrelated to WNS, from wing membrane and respiration through comparatively large lungs could potentially lead to dehydration in bats during hibernation (Cryan *et al.*, 2010; Marroquin *et al.*, 2017; Leivers *et al.*, 2019; Meierhofer *et al.*, 2019a). In addition to evaporative water loss (EWL) from normal physiological processes, WNS has been shown to increase evaporative water loss and may cause bats to arouse more frequently to drink (Willis *et al.*, 2011; Verant *et al.*, 2014; Mcguire *et al.*, 2017). Bats prone to greater EWL may choose microclimates with more moisture or cluster to increase microclimate RH and minimize potential for EWL (Cryan *et al.*, 2010; Marroquin *et al.*, 2017; Meierhofer *et al.*, 2019a). However, higher moisture areas may be more

prone to *Pd* growth, and clustering can increase bat to bat transmission of the pathogen (Cryan *et al.*, 2010).

Due to the needs for bats to maintain physiological temperature and humidity requirements, bats must choose a hibernaculum and microclimate that best meets their needs when they prepare for torpor. Bats have historically used caves and mines as hibernacula, but with the increase in surface-based anthropogenic structures, they have also been able to move into structures such as culverts and bridges in regions devoid of caves and mines. Bats across multiple continents have been observed roosting in culverts, though some culverts may be more suitable than others (Walker *et al.*, 1996; Keeley & Tuttle, 1999; Sandel *et al.*, 2001; Boonman, 2011; Katzenmeyer, 2016; Lutsch *et al.*, 2022). Traditional and nontraditional hibernacula have various differences that can influence the conditions within them, such as number of entrances, length, surrounding habitat, and presence of water (Katzenmeyer, 2016; Meierhofer *et al.*, 2019b).

*Perimyotis subflavus* populations have experienced precipitous declines since the discovery of WNS and still face other threats such as loss of habitat. Given this, the US Fish and Wildlife Service proposed in 2022 to list *P. subflavus* as federally protected under the Endangered Species Act (US Fish & Wildlife Service, 2022). To understand the risks to their further decline, we must consider the possible causes of increased vulnerability of these bats to WNS. This includes looking at how susceptibility to the pathogen varies in different types of hibernacula. Considering some portions of North America are still negative for *Pd*, the identification of transmission corridors that may be affected by the spread of the fungus and WNS is of critical importance. Additionally, research regarding activity and hibernation of *P. subflavus* has only gained strong interest in roughly the last decade, so most prior research has used other species such as *Myotis lucifugus* and extrapolated results onto *P. subflavus*. Cumulatively this study will increase the knowledge base on both WNS and tricolored bats as a whole, as well as provide

crucial information on the potential for *Pd* to spread through areas of North America that are critical to bat populations.

# Literature Review

## CONDITIONS EXPERIENCED DURING TORPOR

One potential difference between traditional and nontraditional hibernacula that may influence susceptibility to WNS is length or timing of hibernation season (Table 1). There are not notable differences in beginning and end timings of hibernation between traditional (caves and mines) and nontraditional hibernacula (culverts).

**Table 1: *Perimyotis subflavus* winter assemblage details**

| State | Hibernacula type | Began assembling | Peak assemblage | Began leaving | Source |
|---|---|---|---|---|---|
| Texas | Culvert | Late Sept/Oct | Nov/Dec | March | (Sandel *et al.*, 2001) |
| Arkansas | Cave | Late fall | Not discussed | Late spring | (Briggler & Prather, 2003) |
| Indiana | Mine | Early Oct | Feb | Apr/May | (Vincent & Whitaker, 2007) |
| Georgia | Culvert | Oct | Feb | March | (Lutsch *et al.*, 2022) |
| Georgia | Cave | Oct | Dec | Late Mar | (Gibizov, 2013) |
| Wisconsin | Mine | Late Oct/Nov | Not discussed | Apr | (Meyer, Senulis, & Reinartz, 2016) |

*Table 1 Details on Perimyotis subflavus winter assemblage in hibernacula gathered from literature*

The temperature of a roost is critical during torpor for maintaining low metabolic rates. For example, *Myotis lucifugus* select cooler temperatures in order to maintain deeper torpor that results in a lower metabolic rate than that of bats in warmer roosts (Boyles et al., 2007). These temperatures typically correspond with the growth range of *Pd*, leading to a cascading disease dynamic within

hibernacula (Blehert et al., 2008; Gargas et al., 2009); however, temperatures below 12.5°C decrease WNS mortalities (Langwig *et al.*, 2012; Hopkins *et al.*, 2021; Loeb & Winters, 2022; Turner *et al.*, 2022). *Perimyotis subflavus* have been found hibernating in various temperatures, but prefer to hibernate in temperatures between 12 and 15°C (Briggler & Prather, 2003; Meierhofer et al., 2019b). Vincent and Whitaker (2007) suggest that because *P. subflavus* roosts in temperatures more stable than other bats, they may be able to stay in torpor longer than other bats. The preferred temperature range places them within the optimal temperature range for the growth of *Pd* and, presumably, at a greater risk for developing WNS (Blehert et al., 2008; Gargas et al., 2009; Verant et al., 2012).

### *Differences in conditions between traditional and nontraditional hibernacula*

The availability of different microclimates within a hibernaculum can also help individuals meet their specific physiological needs during torpor. Microclimates in culverts vary more greatly with external temperature than caves, which may be a detriment to individuals roosting in these structures (Vincent & Whitaker, 2007; Leivers *et al.*, 2019; Lutsch *et al.*, 2022). Leivers (2019) found that bats select for both temperature and water vapor pressure in microclimates via distance to the nearest hibernacula entrance. *Perimyotis subflavus* were observed roosting farther from the entrance of the hibernacula in culverts than in caves, presumably to maximize stability of conditions of the roost.

Microclimates in culverts can fall within the temperature range for *Pseudogymnoascus destructans* growth, including the narrower range of optimal temperatures, as observed by Lutsch et al. (2022). Culverts often have weep holes, deep cracks, or plywood left over from construction and bats can also roost directly on the walls or ceiling. Some of these potential roosting positions are more likely to hold temperatures within the optimal range for *Pd* growth than others. Lutsch et al. (2022) showed that weep holes maintained temperatures within the optimal growth range of *Pd* for more days than did

roosts that were free hanging on walls. Weep holes were also typically warmer and had more temperature stability than the main culvert (Lutsch *et al.*, 2022).

*P. subflavus* roosts average between 10.9°C in the Northeastern US to 15.8°C in Texas, with temperatures in other locations typically falling between these (Raesly & Gates, 1987; Gibizov, 2013; Meierhofer *et al.*, 2019b). No significant difference has been found in *P. subflavus* skin temperature and roost temperature at traditional versus nontraditional hibernacula, and the temperatures have been found to be within the optimal growth range of *Pd* in both hibernacula types (Sirajuddin, 2018; Meierhofer *et al.*, 2019a).

Multiple studies have found that *P. subflavus* roosts have an average of 86.5% humidity and condensation regularly forms on fur (Raesly & Gates, 1987; Brack, 2007; Gibizov, 2013). Humidity of microclimates used in caves are similar to those in culverts, and bats modulate the humidity of their microclimate by adjusting the distance they roost from the entrance of the hibernacula (Leivers *et al.*, 2019; Meierhofer *et al.*, 2019b).

## BEHAVIOR AND ACTIVITY

Environmental conditions of hibernacula likely affect torpor patterns of *Perimyotis subflavus*. For example, Twente, Twente, and Brack (1985) found that *P. subflavus* hibernate longer in colder temperatures. While some studies noted that *P. subflavus* winter activity was rarely observed, others have seen behaviors such as flying, drinking, grooming, copulation, and moving roosting locations between bouts of torpor in multiple states throughout the species' range (Davis, 1964; Sandel *et al.*, 2001; Boyles, Dunbar, & Whitaker, 2006; Vincent & Whitaker, 2007; Gibizov, 2013).

Sirajuddin (2018) did not find any significant differences in torpor bout length or average torpid skin temperatures between WNS positive (n=1) and negative sites (n=2). Regression models suggested

that average torpor skin temperature was greater in the WNS positive site than either of the WNS negative sites. Though Sirajuddin (2018) warns that results should be interpreted with caution due to low sample days, she found that one of the WNS negative sites had more arousals than the WNS positive site according to their arousal frequency index. This directly contrasts findings by Reeder *et al.* (2012), who found that *Myotis lucifugus* in WNS positive sites had increased arousal frequency as compared to WNS negative hibernacula. Within the WNS positive site, ambient temperature and hibernacula temperature were found to correlate significantly with presence of bat activity, with the relationships being positive for ambient temperature, and negative for hibernacula temperature, respectively (Sirajuddin, 2018).

*P. subflavus* in culverts have been found clustering in weep holes with up to 10 individuals; with the combination of skin to skin contact between bats and optimal temperature for *Pd* growth for significant stretches of time, weep holes pose a risk for transmission of the fungus (Lutsch *et al.*, 2022).

## Objectives

**Goal** - Compare *Pd* burdens and WNS status of bats in traditional and nontraditional roost sites.

**Objective 1.1** - Swab bats throughout (3 seasonal samplings) winter season (November - March) in traditional and nontraditional hibernacula.

**Objective 1.2** - Use qPCR (Muller 2013 method, NWHC protocol) to quantify seasonal trends in *Pd* burden on bats.

**Objective 1.3** - Compare bat biometrics (mass, forearm length, body temperature) at traditional and nontraditional hibernacula.

**Objective 1.4** - Measure temperatures within hibernacula and on the landscape near selected roost sites. In-hibernacula data loggers will be oriented consistent with observed bat roosting behaviors (e.g. wall, ceiling, weep hole, etc.) to ascertain roost orientation microclimate differences.

**Objective 1.5** - Relate *Pd* burdens at traditional and nontraditional hibernacula to hibernacula temperature and humidity.

### Hypotheses

1. *Pd* burdens and prevalence will vary between *Pd* positive traditional hibernacula and positive nontraditional hibernacula.
2. Variability of hibernacula temperature and humidity will differ between nontraditional hibernacula than at traditional hibernacula.

## METHODS

### Field Methods

#### Site Selection

Both traditional and nontraditional hibernacula (n=18) in Georgia, South Carolina, Alabama, and Florida were selected for inclusion in this study based on the historical presence of *P. subflavus*, WNS status, accessibility, permitting, and proximity to participating collaborators (Fig. 1). Information on sites is presented in table 2.

| Table 2: Details on hibernacula used in study | | | | | |
|---|---|---|---|---|---|
| **Site Name** | **Abbreviation** | **County** | ***P. subflavus* Population** | **Hibernaculum Type** | **WNS status** |
| Black Diamond Tunnel | BDT | Rabun (GA) | ~200 | Traditional (Tunnel) | Confirmed positive since 2014, post collapse. |
| Stumphouse Tunnel | ST | Oconee (SC) | ~30 | Traditional (Tunnel) | Confirmed positive since 2015, post collapse |
| Weaver Cave | AW | Calhoun (AL) | ~600 | Traditional (Cave) | *Pd*+ by PCR from NWHC; visible symptoms observed |
| Bryan County Culvert | KB | Bryan (GA) | ~30 | Nontraditional (Culvert) | Confirmed negative (qPCR) in 2019 |
| Glynn County Culvert | KG | Glynn (GA) | ~35 | Nontraditional (Culvert) | Confirmed negative (qPCR) in 2019 |
| Little River Culvert | LR | Walton (GA) | ~26 | Nontraditional (Culvert) | Positive (2020/2021) No visible signs of WNS |
| Red Creek Culvert | RC | Monroe (GA) | ~50 | Nontraditional (Culvert) | Positive (2019/2020). No visible signs of WNS |
| Buck Creek Culvert SiteUWG01 | BC | Carroll (GA) | ~116 | Nontraditional (Culvert) | Positive (2019/2020). No visible signs of WNS |
| Talladega Culvert Site UWG02 | AT | Cleburne (AL) | ~80 | Nontraditional (Culvert) | Positive |
| Bullock County Culvert | BU | Bullock (AL) | ~70 | Nontraditional (Culvert) | Unknown |
| Savannah River Bridge 02-G | SW | Aiken (SC) | ~5 | Nontraditional (Bridge) | Confirmed negative (qPCR) in 2018 |
| Savannah River Bridge 03-G | SR | Aiken (SC) | ~5 | Nontraditional (Bridge) | Confirmed negative (qPCR) in 2018 |
| Clemson Gold Mine | GM | Oconee (SC) | 5-8 | Traditional (Mine) | *Pd* positive in 2016 |
| Warren's Cave | FW | Alachua (FL) | ~30 | Traditional (Cave) | Confirmed negative (qPCR) in 2019 |
| Culvert Site ALA317 | FC | Alachua (FL) | ~7 | Nontraditional (Culvert) | Presumed Negative |
| Osbourne Cave | OC | Floyd (GA) | ~50 | Traditional (Cave) | WNS Positive |

*Table 2 Details for hibernacula used in study*

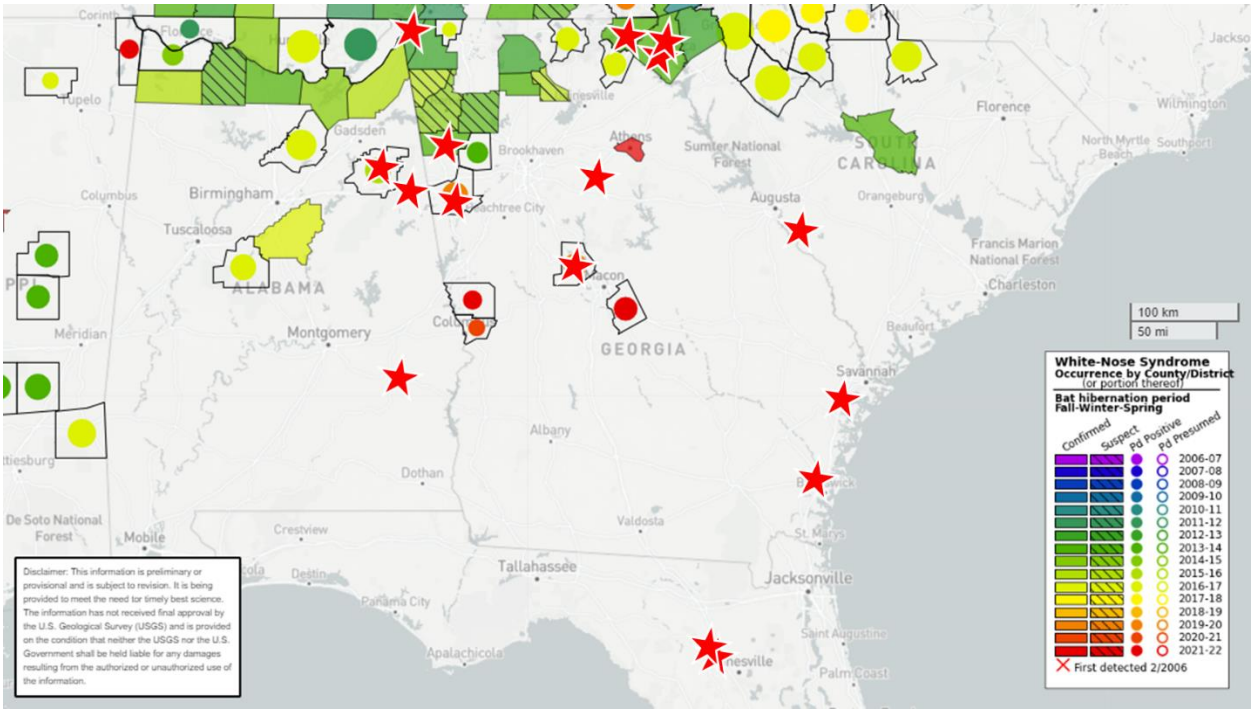



*Figure 1 Locations of study site locations overlaid on map of white-nose-syndrome spread as of November 2022. Study sites are indicated by red stars.* (White-nose Syndrome Response Team, n.d.)

## Population Surveys

All surveys were conducted using a standard Georgia Department of Natural Resources (GADNR), or comparable depending on site location, data sheet and associated guidelines. A copy of the data sheet was provided to the relevant state agency to support their WNS monitoring efforts. Multiple surveyors thoroughly inspected all crevices, joints, and cracks of bridges or culverts per the methods of Tuttle and Keeley (1999), or all accessible locations in traditional sites during each survey to count the total number of bats. Other important hibernacula features, such as internal height, length, and width, roosting substrate, surrounding habitat, and presence of water were also documented during the initial survey. Changes to sites were noted when observed across the project period. Surveys were conducted in cooperation with jurisdictional state biologists and wildlife resource managers to minimize disturbances and optimize resource allocation and effort. Surveys were performed three times during each winter season, typically in November, January, and March, but were site specific based on seasonality of *P.*

*subflavus* site utilization as well as logistical considerations. Some sites did not get surveyed mid-season due to accessibility issues or concern for increased disturbances at the discretion of the managing state biologists. Data on bat numbers and environmental data were submitted to the NABat database.

## Sample Collection

We captured accessible bats (n≤30 per site) and swabbed them and recorded biometric data at the discretion of the respective state biologist. We collected swab samples from every structure surveyed where bats were accessible. For consistency, all sampling methods followed the US Geological Survey National Wildlife Health Center protocol as well as the US Fish and Wildlife Service decontamination protocols (USGS-National Wildlife Health Center, 2019; White-nose Syndrome Disease Management Working Group, 2020).

Sterile cotton tipped swabs were kept in their original packaging until use. Before swabbing the bat, we measured the bats' skin temperature with an infrared temperature gun (Fluke Corporation, Everett, WA). To swab a bat, we held the non-dominant hand under the bat to reduce the risk of falling. We then streaked the forearm tissue five times with a sterile swab, while twisting the swab to ensure greater surface contact. The same method was used for the animal's muzzle, using the same swab. The swab was stored in a new 2 mL microcentrifuge tube. Swabs were stored in a cooler while in the field and then stored at -80 °C when available.

All bat handling was based on directions from the lead bat biologist for the appropriate state. We captured bats during the first and last surveys of each season (mid-season surveys did not include this to minimize disturbance) and placed them in a paper or cloth bag marked with an identification number. We recorded forearm length, mass, sex, reproductive status, age, wing damage index, and band number, if the individual was recaptured. We also took oral swabs and fur clippings and placed a

2.4 mm lipped metal bat band (Porzana Limited, Winchelsea, UK) on newly captured (unbanded) individuals. Each was uniquely numbered and placed on the forearm of the animal using banding pliers (Avinet Research Supplies, Portland, ME). All band numbers and associated information were submitted to the appropriate state agency. All bat handling was conducted under the State Collection Permits held by Katrina Morris (GA), Susan Loeb (SC), Andrew Edelman (AL), or Lisa Smith (FL). Methods were approved by the Institutional Animal Care and Use Committees of both Kennesaw State University and the US Forest Service.

## Temperature Monitoring

We placed two or three temperature and humidity data loggers (Hygrochron iButtons, Maxim Integrated, San Jose, CA) in each hibernaculum in an orientation mimicking the roost selection of *P. subflavus* observed in that site (e.g. weep hole, ceiling, wall). Loggers in weepholes had metal mesh blocking the opening of the weephole to exclude bats to avoid body heat changing the microclimate. In traditional sites, we placed the iButtons approximately 1/3, 2/3, and 3/3 of the distance into the hibernacula, with some variability due to differences in structures of the sites. In nontraditional sites, we placed one iButton approximately 6 m into each opening, and the third approximately in the middle in a similar roost position to the majority of the bats in the site. We affixed iButtons in the hibernacula with either an eye hook screwed into a wooden block caulked onto the substrate, or via a toggle bolt to hold it in a weephole (Fig. 2).

*Figure 2 Example of iButton placement within a culvert hibernaculum*

We programmed each logger prior to deployment to record temperature and relative humidity (RH) at 30-minute intervals. We deployed data loggers in November 2020 and October 2021 and retrieved them in March 2021 and 2022, respectively. Using a Blue Dot Receptor and USB to 1-wire adaptor (Maxim Integrated) and OneWireViewer software, we offloaded data and stored as a CSV (comma-separated value) document. For analysis, we calculated minimum, maximum, and average temperatures for each 12-hour period per day (6AM-6PM and 6PM-6AM).

## Laboratory methods

### DNA Extraction

We removed samples from storage and allowed them to thaw before adding 140 µL of TE buffer (Integrated DNA Technologies, Inc., Coralville, Iowa) to the swabs. We vortexed the tubes for ~5 seconds then centrifuged for ~5 seconds to ensure all droplets of TE buffer were drawn back into the swab. We placed a sterile 0.65 mL microcentrifuge tube with the tip cut off into a sterile, labeled 2.0 mL microcentrifuge tube. We used sterilized forceps to move the swab from the original field tube to the

0.65 mL tube inside of the 2.0 mL tube and broke off any extra stick so the swab would fit inside the tube. We centrifuged the tubes for 2 minutes at 10,000 RPM to pull any TE buffer containing sample down into the 2.0 mL tube. We pipetted any excess liquid remaining in the original 2.0 mL tube from the field into the labeled 2.0 mL tube. We removed swabs from the 0.65 mL tube and placed them back in the original field tube, which we then returned to the -80°C freezer for long term storage. We added 16 µL of Proteinase K (Qiagen, Germantown, MD) to the remaining 2.0 mL tube containing the TE buffer and any sample. We incubated tubes in a heat block at 37°C for 5 minutes, 65°C for 30 minutes, then 80°C for 20 minutes, then vortexed them on high for 10 minutes. We added an equal sample volume (156 µL) of phenol: chloroform: isoamyl alcohol (25:24:1; MilliporeSigma, Burlington, MA) to the tubes, vortexed on high for ~5 seconds until the liquid turned a cloudy white, then centrifuged them for 3 minutes. After vortexing the samples separated into 3 layers. We pipetted off as much of the top aqueous layer of liquid as possible without disturbing the lower layers, typically 45 µL, and added it to a sterile, labeled 0.65 mL microcentrifuge tube. We added an equal sample volume of 200 proof ethanol to the tubes, vortexed them on high, and incubated them at -80°C for one hour. We then removed tubes from the freezer, allowed them to thaw, and centrifuged them for 10 minutes at 10,000 RPM. We removed as much ethanol as possible, typically 50 µL, from the tubes without disturbing the DNA pellet at the bottom of the tube. We added a half sample volume, typically 22.5 µL, of 70% ethanol to the tubes, then centrifuged them for 10 minutes at 10,000 RPM. We opened the tubes to allow the sample to volatize until there was minimal liquid left, typically 24 hours, then resuspended them in 25 µL of TE buffer. Runs of DNA extractions included both a positive control, obtained by rolling a sterile cotton swab on a lawn of *Pd* and extracted as above, and a negative control, which did not contain any *Pd* DNA but allowed us to ensure reagents remained uncontaminated by *Pd*.

## Standard Curve and qPCR

We used *Pd* gBlocks DNA (Integrated DNA Technologies, Inc.; Iowa, USA) to create a standard curve for the qPCR, against which we quantified the *Pd* from field samples. A dilution series allowed for relative quantification based on the cycle threshold (Ct) value. We determined the Ct value by the cycle number necessary to achieve amplification above the threshold level. We processed this dilution series using qPCR assay parameters described by Muller et al. (2013) with volume modifications to accommodate a commercial internal control kit (QuantiFast Pathogen + PCR IC Kit, Qiagen). Reactions included 2.5 µl Internal Control Assay from the Internal Control Kit, 2.5 µl Internal Control DNA from the Internal Control Kit, 2.5 µl of a 10X solution comprised of 10 µl forward primer (5'– TGC CTC TCC GCC ATT AGT G –3'), 10 µl reverse primer (5'– ACC ACC GGCTCG CTA GGT A –3'), 10 µL TaqMan probe (5'-(FAM) CGT TAC AGC TTG CTC GGG CTG CC (BHQ-1)-3') and 70 µl sterile deionized water, 5 µl master mix from the Internal Control Kit, 7.5 µl sterile deionized water from the Internal Control Kit, and 5 µl unknown sample DNA. Forward primer, reverse primer, and probe sequences were adopted from the assay described by Muller et al. (2013). We ran all qPCR reactions using a Mic qPCR cycler (Biomolecular Systems, QLD, Australia) with the most up to date software version out at the time of run. Each qPCR run included 3 standard concentration samples, 2 no template control samples, 1 positive control sample, 1 negative control sample, and up to 41 unknown field samples. We ran a full set of standard concentration samples every 20 qPCR runs to create a standard curve and ensure it is still accurate and does not change over time. We ran samples determined to be positive for *Pd* a second time to ensure there were no false positives if the county has not previously had *Pd* detections.

## Statistical Methods

We performed all statistical analyses in Microsoft Excel (Microsoft Corporation, 2018) or R (R Core Team, 2021).

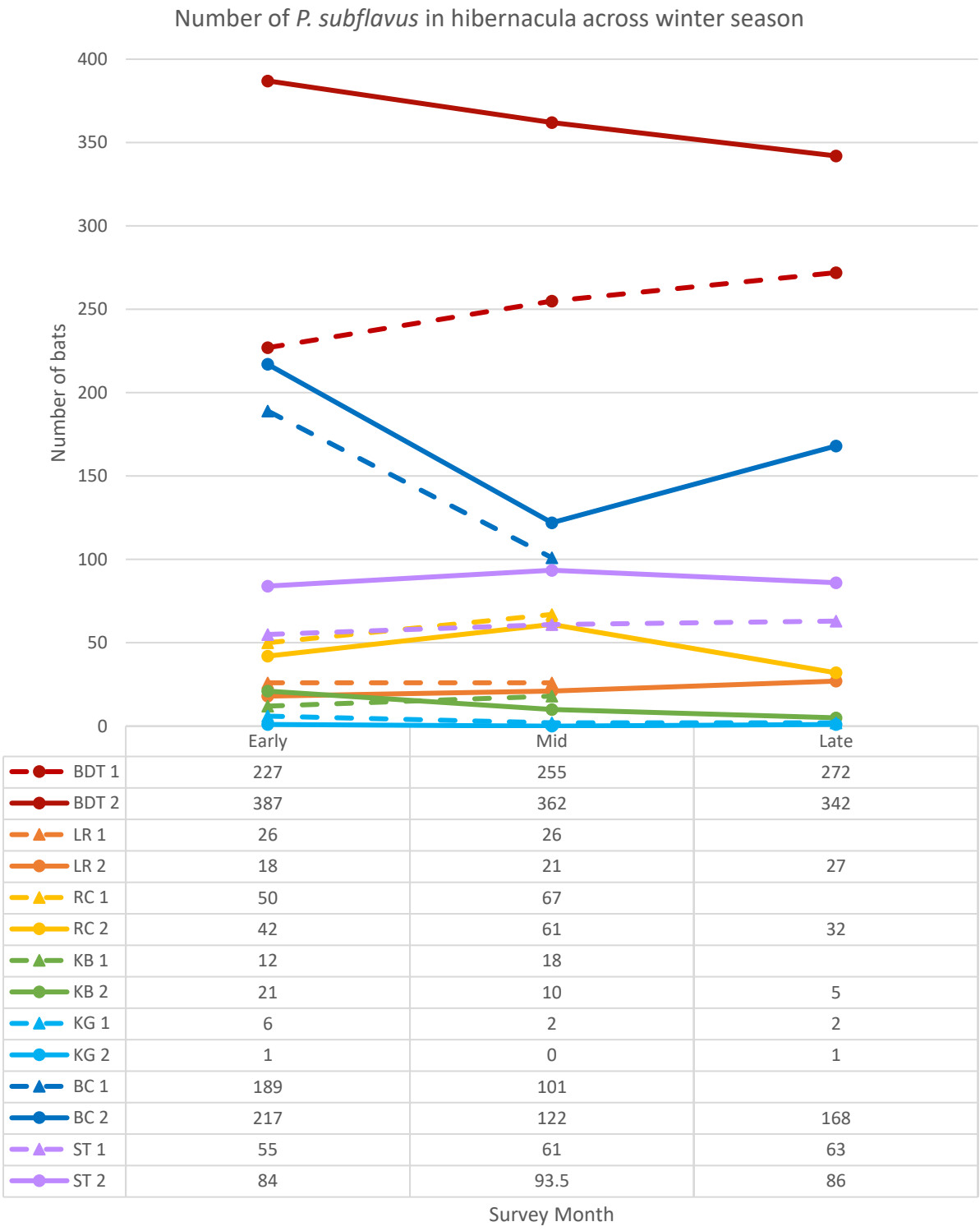
We executed one-way ANOVA tests on Ct values from qPCR to test for differences in Ct values throughout the hibernation season. We performed a separate one-way ANOVA to test for significant differences in Ct values by season, site, hibernacula type, and year.

We calculated the correlation coefficients between average ambient temperatures and hibernacula maximum and minimum temperatures, and for appropriate sites, weep hole maximum and minimum temperatures. These calculations were repeated for the humidity data. A one-way ANOVA was used to compare the correlation coefficients for both temperature and humidity, respectively, at traditional versus nontraditional hibernacula.

A chi-squared test for independence was performed to compare the sex ratios at traditional and nontraditional hibernacula. Two separate one-way ANOVAs were performed to compare the masses of bats at traditional versus nontraditional hibernacula with and without regards to sex, respectively.

# RESULTS

## *P. subflavus* populations




| | Early | Mid | Late |
|---|---|---|---|
| BDT 1 | 227 | 255 | 272 |
| BDT 2 | 387 | 362 | 342 |
| LR 1 | 26 | 26 | |
| LR 2 | 18 | 21 | 27 |
| RC 1 | 50 | 67 | |
| RC 2 | 42 | 61 | 32 |
| KB 1 | 12 | 18 | |
| KB 2 | 21 | 10 | 5 |
| KG 1 | 6 | 2 | 2 |
| KG 2 | 1 | 0 | 1 |
| BC 1 | 189 | 101 | |
| BC 2 | 217 | 122 | 168 |
| ST 1 | 55 | 61 | 63 |
| ST 2 | 84 | 93.5 | 86 |

*Figure 3 Trends in number of P. subflavus in hibernacula observed during three separate survey sessions over the winter season (November to March) during year 1 and 2 of the study.*

Bats in all sites, save Black Diamond Tunnel, followed a similar trend in population size across the hibernation season between year 1 and 2 of the study. Population sizes at most sites decreased slightly as the season progressed (Fig. 3). The average number of bats in Black Diamond Tunnel increased by 112.3 bats in year 2 compared to year 1, while most other sites had approximately the same number of bats between years.

| **Table 3: Percent change in hibernacula *P. subflavus* counts between winter sampling sessions** | | | |
|---|---|---|---|
| **Site by Year** | **Early to Mid** | **Mid to Late** | **Early to Late** |
| **BDT 1** | 12.3 | 6.7 | 19.8 |
| **BDT 2** | -6.5 | -5.5 | -11.6 |
| **LR 1** | 0.0 | NA | NA |
| **LR 2** | 16.7 | 28.6 | 50.0 |
| **RC 1** | 34.0 | NA | NA |
| **RC 2** | 45.2 | -47.5 | -23.8 |
| **KB 1** | 50.0 | -100.0 | -100.0 |
| **KB 2** | -52.4 | -50.0 | -76.2 |
| **KG 1** | -66.7 | 0.0 | -66.7 |
| **KG 2** | NA | NA | 0.0 |
| **BC 1** | -46.6 | NA | NA |
| **BC 2** | -43.8 | 37.7 | -22.6 |
| **ST 1** | 10.9 | 3.3 | 14.5 |
| **ST 2** | 11.3 | -8.0 | 2.4 |

*Table 3 Percent change in number of P. subflavus observed between early, mid, and late winter season hibernacula surveys. Note: year 1 and 2 are analyzed separately in this table.*

| **Table 4: Percent change in *P. subflavus* population between year 1 and 2** | | | |
|---|---|---|---|
| **Site** | **Early** | **Mid** | **Late** |
| **BDT** | 70.5 | 42 | 25.7 |
| **LR** | -30.8 | -19.2 | NA |
| **RC** | -16 | -9 | NA |
| **KB** | 75 | -44.4 | NA |
| **KG** | -83.3 | -100 | -50 |
| **BC** | 14.8 | NA | NA |
| **ST** | 52.7 | 53.3 | 36.5 |

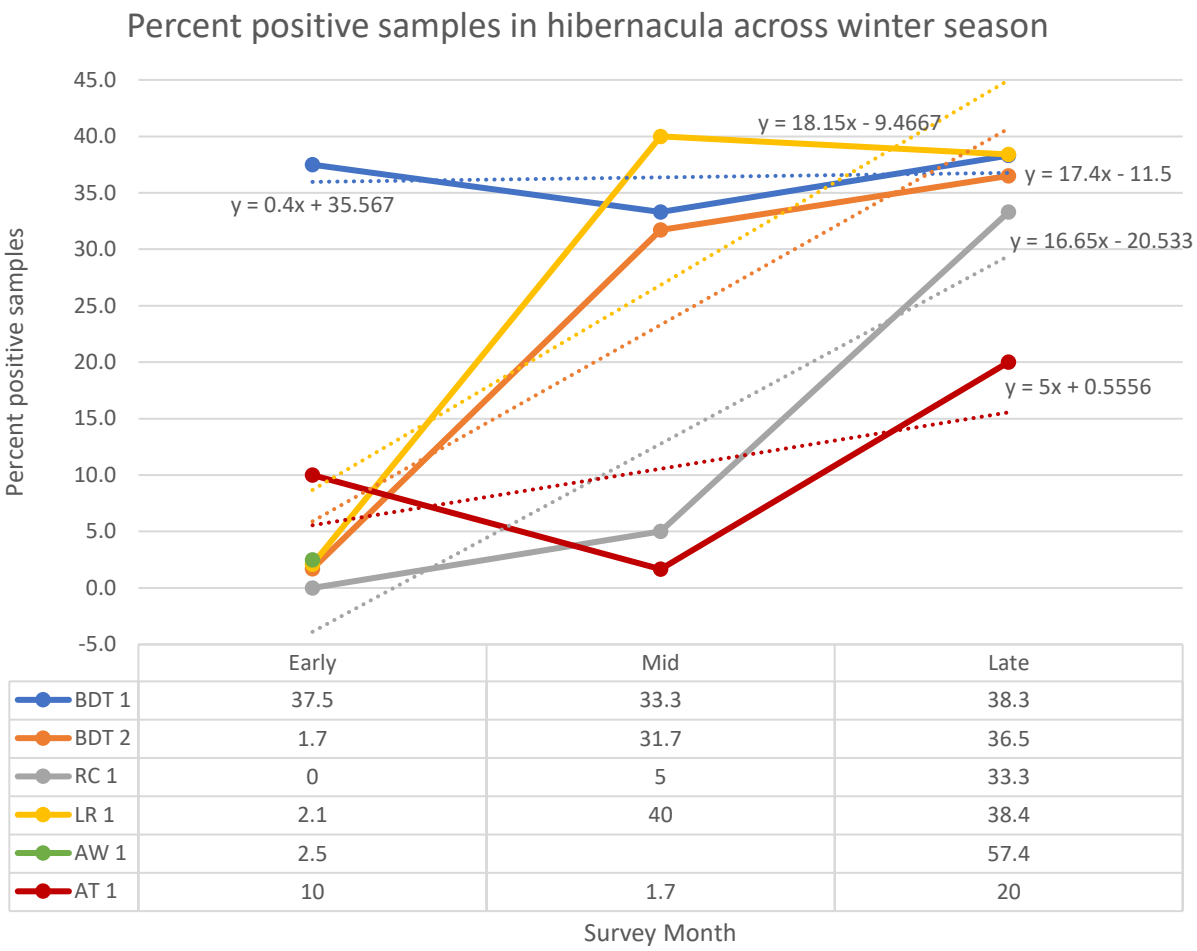
*Table 4 Percent difference in early, mid, and late season P. subflavus populations at study sites between year 1 and 2 of the study.*

There were not patterns in percent change of *P. subflavus* population sizes in hibernacula throughout the winter season (Table 3) or between year 1 and 2 (Table 4). Sites with very small populations, such as Bryan County Culvert and Glynn County Culvert, experienced large percent change in population sizes due to the larger effect of a few individuals than in sites with large populations, such as Black Diamond Tunnel and Buck Creek Culvert.

## *Pd* surveillance

| **Table 5: Sample sizes (n) of *Pd* surveillance swabs from** | | | |
|---|---|---|---|
| | **Early** | **Mid** | **Late** |
| **BDT 1** | 20 | 21 | 29 |
| **BDT 2** | 30 | 30 | 30 |
| **RC 1** | 16 | 31 | 27 |
| **LR 1** | 19 | 13 | 29 |
| **AW 1** | 20 | NA | 30 |
| **AT 1** | 20 | 30 | 30 |
| **ST 1** | 42 | 26 | 23 |
| **KB 1** | 11 | 14 | 8 |
| **KG 1** | 7 | 2 | 2 |
| **BC 1** | 19 | 30 | 23 |
| **GM 1** | 12 | NA | 3 |

*Table 5 Sample sizes of Pd surveillance swabs taken from P. subflavus at hibernacula*




| | Early | Mid | Late |
|---|---|---|---|
| BDT 1 | 37.5 | 33.3 | 38.3 |
| BDT 2 | 1.7 | 31.7 | 36.5 |
| RC 1 | 0 | 5 | 33.3 |
| LR 1 | 2.1 | 40 | 38.4 |
| AW 1 | 2.5 | | 57.4 |
| AT 1 | 10 | 1.7 | 20 |

*Figure 4 Trends in percent of positive samples taken from hibernacula at three times during the winter season (November to March) during year 1 and 2. Note: Weaver Cave (AW 1) was not sampled mid season.*

Bats at all sites experienced an increase in *Pd* prevalence throughout the winter season (Figure 4). Samples from Black Diamond Tunnel year 1 and Talladega Culvert year 1 had a lower rate of increase in *Pd* prevalence as the season progressed than did Black Diamond Tunnel year 2, Little River Culvert year 1, Red Creek year 1, or Weaver Cave year 1.

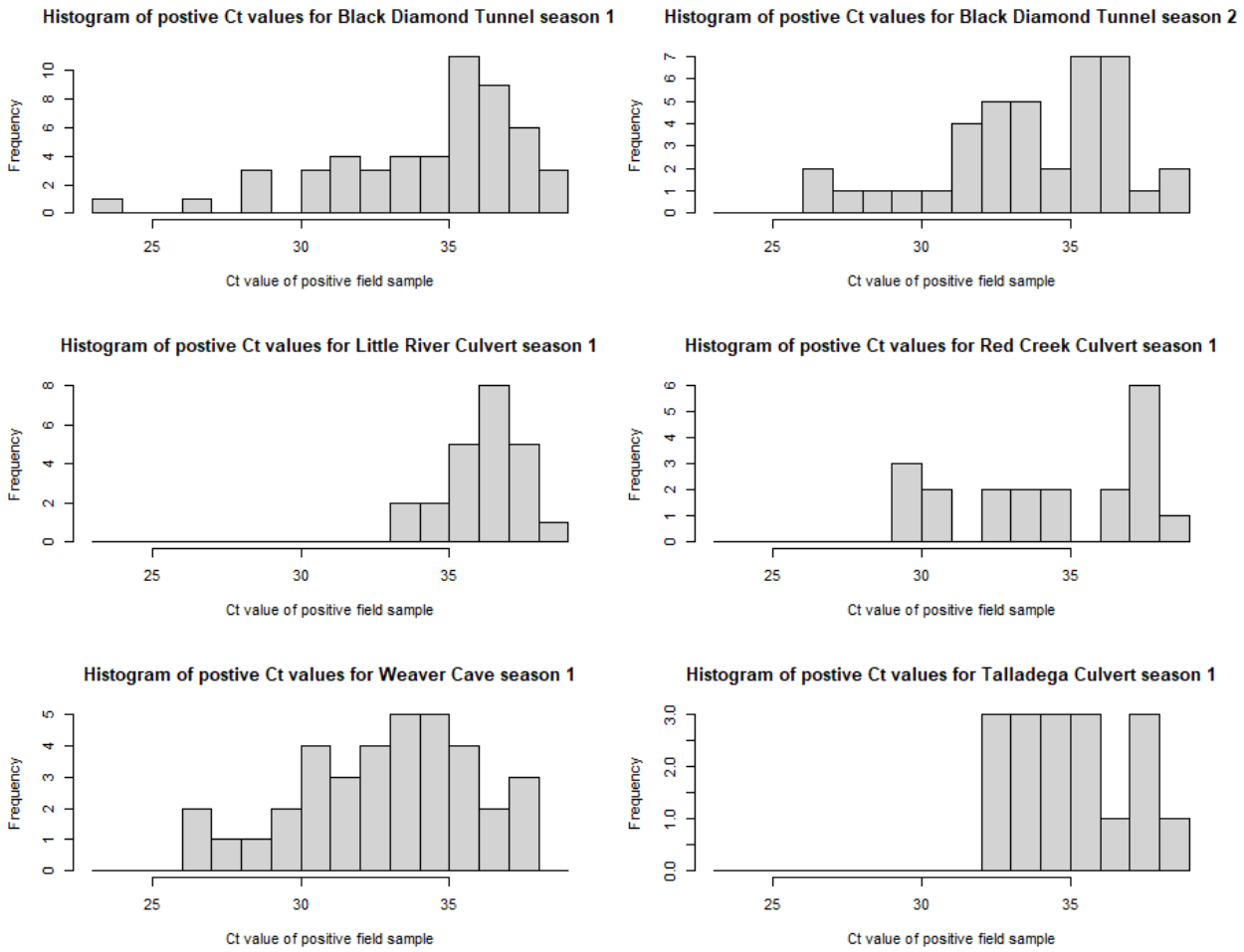



*Figure 5 Histograms of the distribution of Ct values in the hibernacula without differentiation between early, mid, or late sampling session.*

Ct values in Black Diamond Tunnel and Weaver Cave had a larger range than those in Little River Culvert, Red Creek Culvert, and Talladega Culvert (Fig. 5). The histograms of Ct values for both Little River Culvert year 1 and Talladega Culvert year 1 have samples in the higher ranges above 32. Ct values from Black Diamond Tunnel year 1 and 2 and Weaver Cave year 1 had a wider spread of values that fall into lower ranges near or below 25, but had the highest concentration of positive samples between 33 and 37.

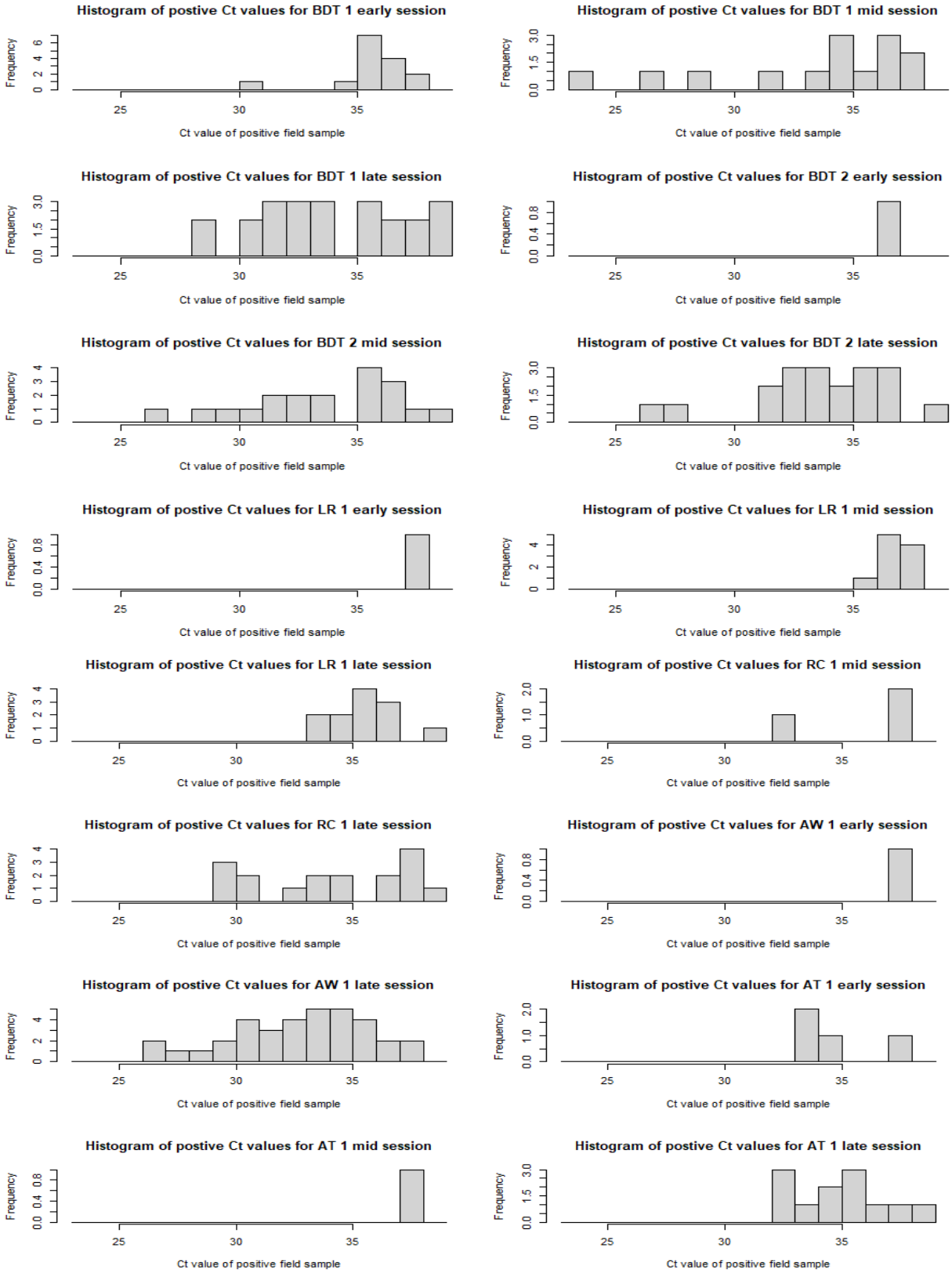



*Figure 6 Histograms of the distribution of Ct values in the hibernacula with differentiation between early, mid, or late sampling session. Note: RC 1 early session was omitted due to there being no positive samples from that survey session.*

All sets of early-mid-late session sample histograms (Fig. 6), save samples in Talladega Culvert, show both an increase in prevalence, as well as a decrease in Ct values as the season progressed. Samples from Talladega Culvert showed a similar pattern between early and late sessions, but the mid session had fewer positive samples than did the early session. Histograms for Ct values in Red Creek Culvert early sampling session and Weaver Cave mid sampling session are absent due to no positive samples and the lack of a mid season survey being performed, respectively.

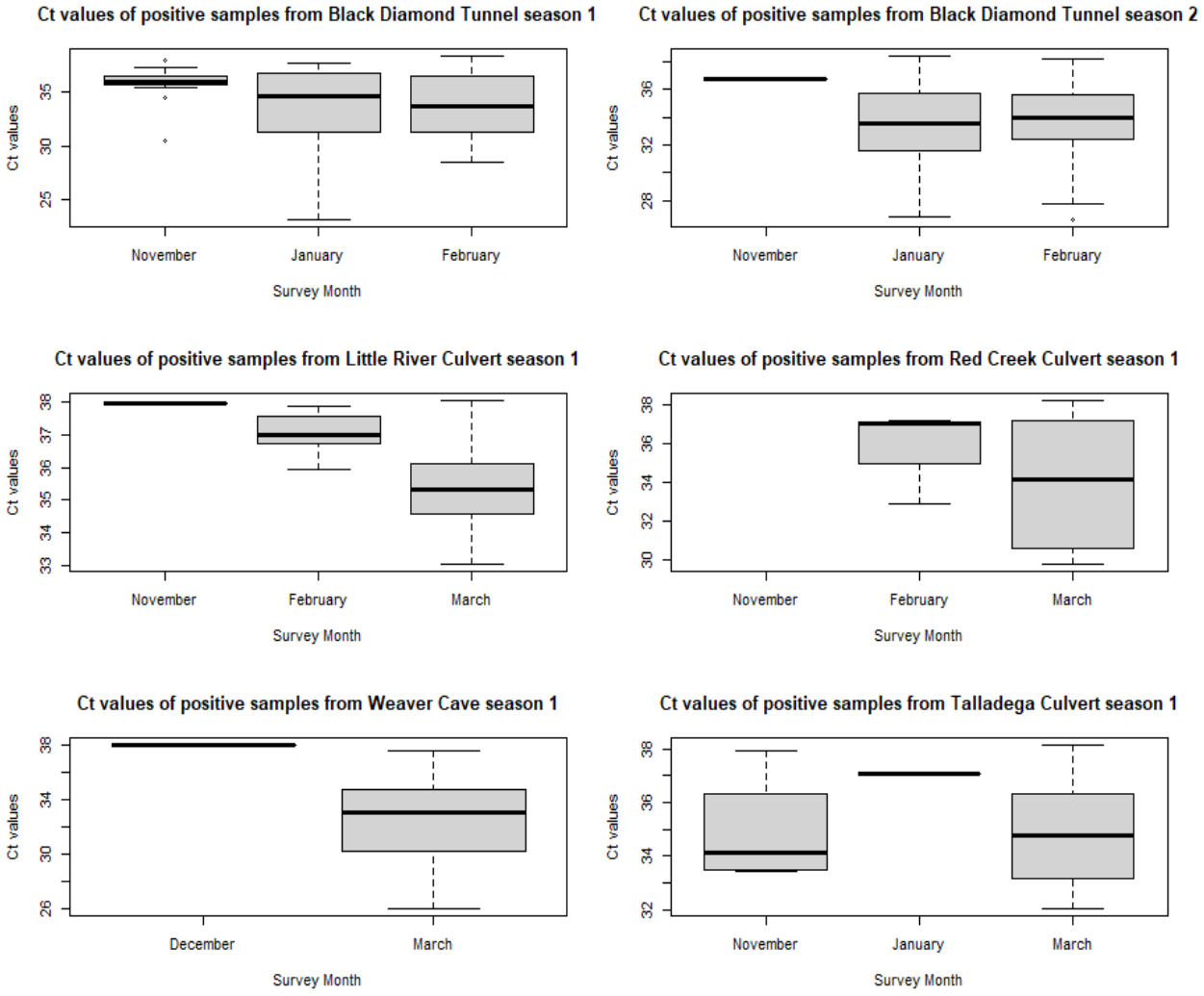



*Figure 7 Boxplots of Ct values from hibernacula at three times during winter season (November to March).*

| Table 6: Results from One-Way ANOVA test of the Ct Values by survey month for hibernacula | | | | | |
|---|---|---|---|---|---|
| | **Df** | **Sum Sq** | **Mean Sq** | **F-value** | **Pr (>F)** |
| **BDT1 by Survey Month** | 2 | 49.6 | 24.79 | 2.422 | 0.0993 |
| **BDT1 Residuals** | 49 | 501.6 | 10.24 | | |
| **BDT2 by Survey Month** | 2 | 10.8 | 5.376 | 0.57 | 0.57 |
| **BDT2 Residuals** | 36 | 339.5 | 9.429 | | |
| **LR1 by Survey Month** | 2 | 20.21 | 10.104 | 8.365 | 0.00229 |
| **LR1 Residuals** | 20 | 24.16 | 1.208 | | |
| **RC1 by Survey Month** | 1 | 6.74 | 6.745 | 0.703 | 0.413 |
| **RC1 Residuals** | 18 | 172.81 | 9.600 | | |
| **AW1 by Survey Month** | 1 | 27.67 | 27.669 | 3.065 | 0.089 |
| **AW1 Residuals** | 34 | 306.88 | 9.026 | | |
| **AT1 by Survey Month** | 2 | 4.48 | 2.239 | 0.577 | 0.574 |
| **AT1 Residuals** | 14 | 54.34 | 3.882 | | |

*Table 6 Results from the One-Way ANOVA tests of Ct values by survey month for Pd surveillance swabs taken from tricolored bats in hibernacula. Only Little River Culvert had a p-value below 0.05.*

Boxplots from most of the sites show a decrease in Ct values by site as the hibernation season progressed (Fig. 7) but are not statistically significant save Little River Culvert (Table 6). Ct values from Black Diamond Tunnel year 1 and 2, Red Creek Culvert year 1, and Talladega Culvert year 1 all have significant overlap between each sampling session. Ct values from Little River Culvert year 1 and Weaver Cave year 1 have minimal overlap in values between their respective sampling sessions.

There was a statistically significant decrease in Ct values throughout the hibernation season in Little River year 1 (Table 6, Fig. 7). Ct values in Black Diamond Tunnel year 1 and 2, Red Creek Culvert year 1, Weaver Cave year 1, and Talladega Culvert year 1 did not differ significantly among survey sessions (Table 6).

| Table 7: Results from One-Way ANOVA test of the Ct values by multiple variables for hibernacula | | | | | |
|---|---|---|---|---|---|
| | | | | | |
| | **Df** | **Sum Sq** | **Mean Sq** | **F-value** | **Pr (>F)** |
| **Season** | 2 | 60.7 | 30.336 | 3.186 | **0.0444** |
| **Season Residuals** | 137 | 1304.3 | 9.521 | | |
| **Site** | 4 | 224.8 | 56.20 | 6.654 | **6.43e-5** |
| **Site Residuals** | 135 | 1140.2 | 8.45 | | |
| **Hibernacula type** | 1 | 144.5 | 144.51 | 16.34 | **8.76e-05** |
| **Hibernacula type Residuals** | 138 | 1220.5 | 8.84 | | |
| **Year** | 1 | 6.4 | 6.362 | 0.646 | 0.423 |
| **Year Residuals** | 138 | 1358.6 | 9.845 | | |

*Table 7 One-way ANOVA results for Ct values from study hibernacula by season, site, hibernacula type, and year. Results from the comparison by year should be interpreted carefully as the only site with data from season 2 was Black Diamond Tunnel.*

There was a statistically significant difference in Ct values among survey sessions (early, mid, or late; Table 7), with Ct values decreasing as the hibernation season progressed (Figs. 6 and 7). There was also a significant difference in Ct values between sites (Table 7). A significant difference in Ct values by hibernacula type was found (Table 7), with bats from traditional hibernacula have significantly lower Ct values, and therefore greater fungal loads, than bats from nontraditional hibernacula (Figs. 6 and 7).

## Hibernacula Temperature and Humidity

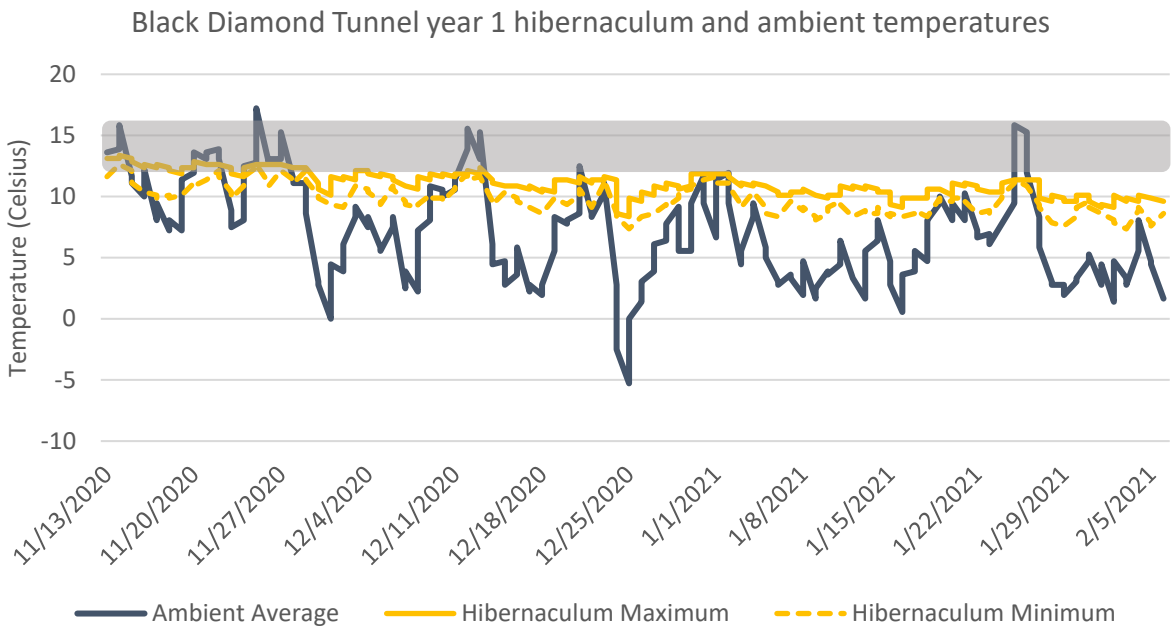



*Figure 8 Minimum and maximum temperatures inside of and ambient temperatures outside of Black Diamond Tunnel. The grey bar indicates optimal temperatures for P. destructans growth.*

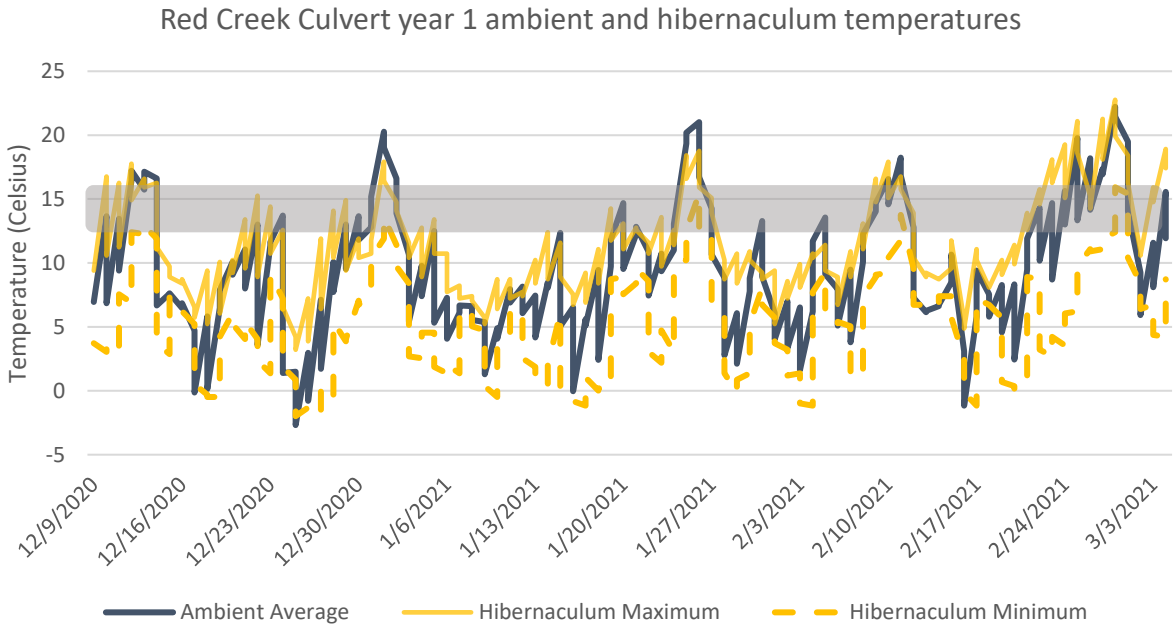



*Figure 39 Minimum and maximum temperatures inside of and ambient temperatures outside of Red Creek Culvert. The grey bar indicates optimal temperatures for P. destructans growth.*

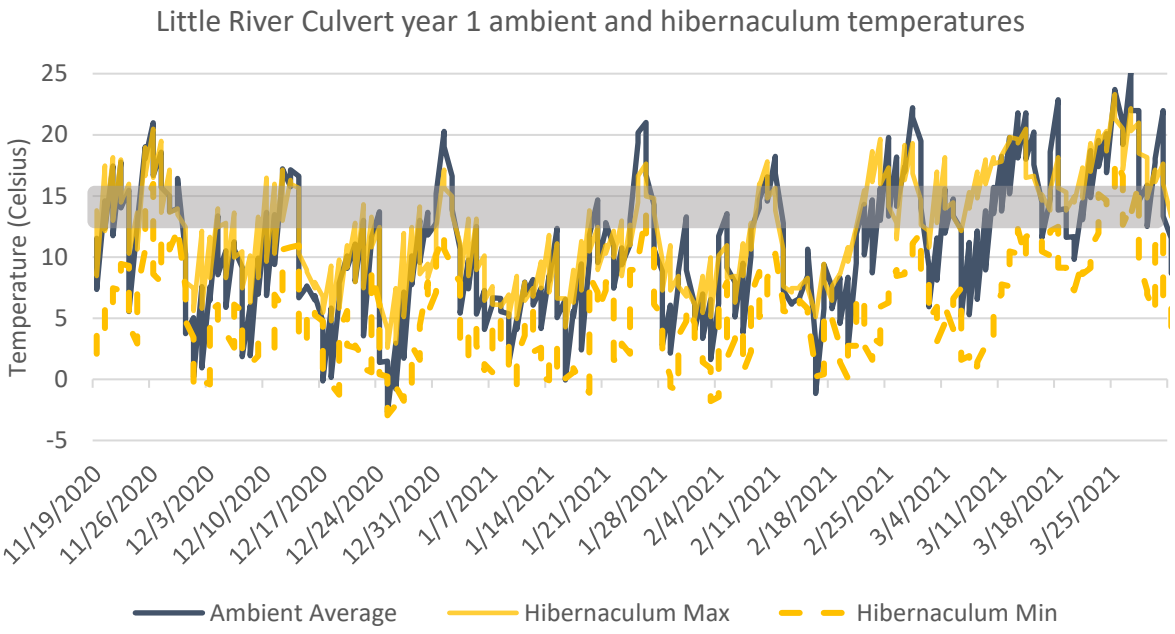



*Figure 40 Minimum and maximum temperatures inside of and ambient temperatures outside of Little River Culvert. The grey bar indicates optimum temperatures for P. destructans growth.*

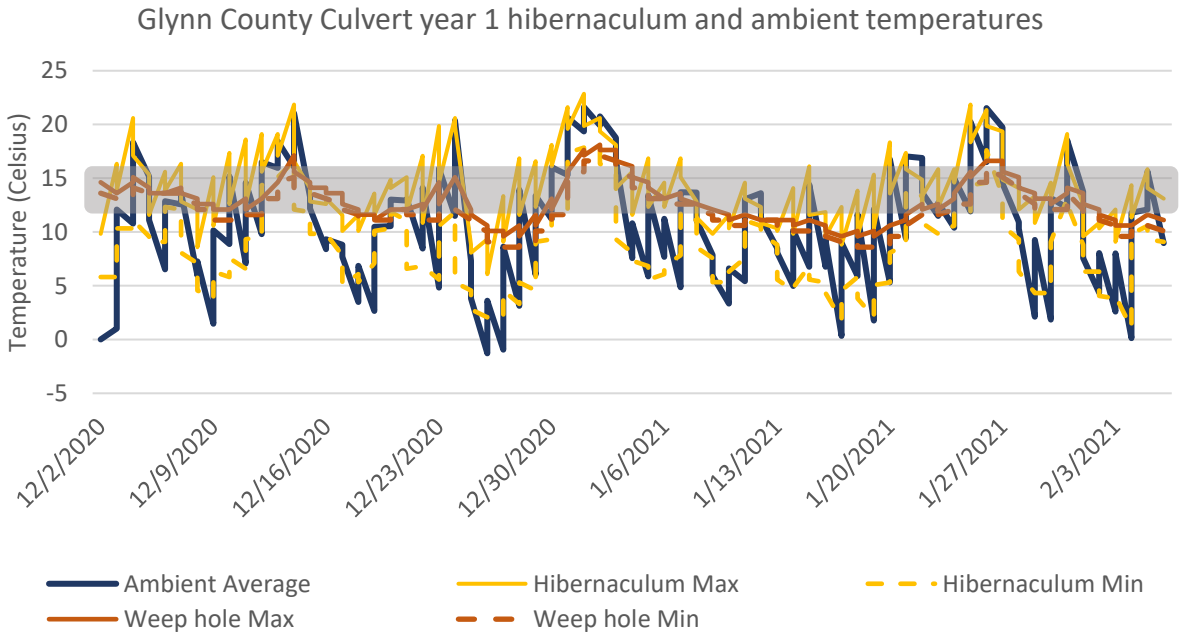



*Figure 51 Minimum and maximum temperatures inside of and ambient temperatures outside of Glynn County Culvert. Minimum and maximum temperatures for a weep hole in the middle of the structure are also included. The grey bar indicates optimal temperatures for P. destructans growth.*

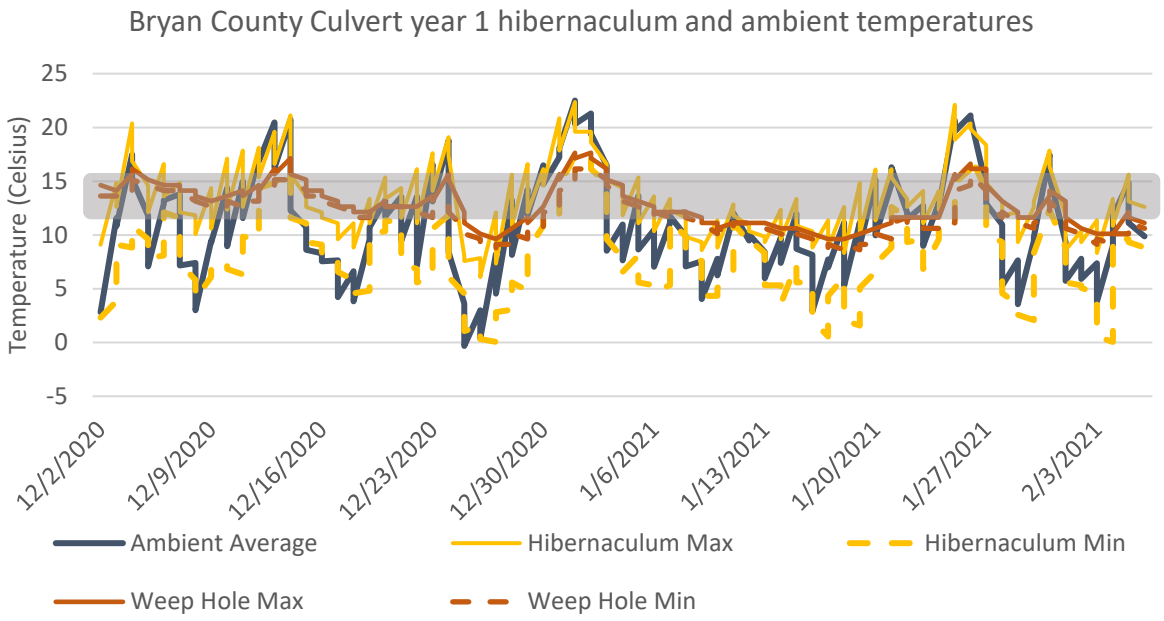



*Figure 12 Minimum and maximum temperatures inside of and ambient temperatures outside of Bryan County Culvert. Minimum and maximum temperatures for a weep hole in the middle of the structure are also included. The grey bar indicates optimal temperatures for P. destructans growth.*

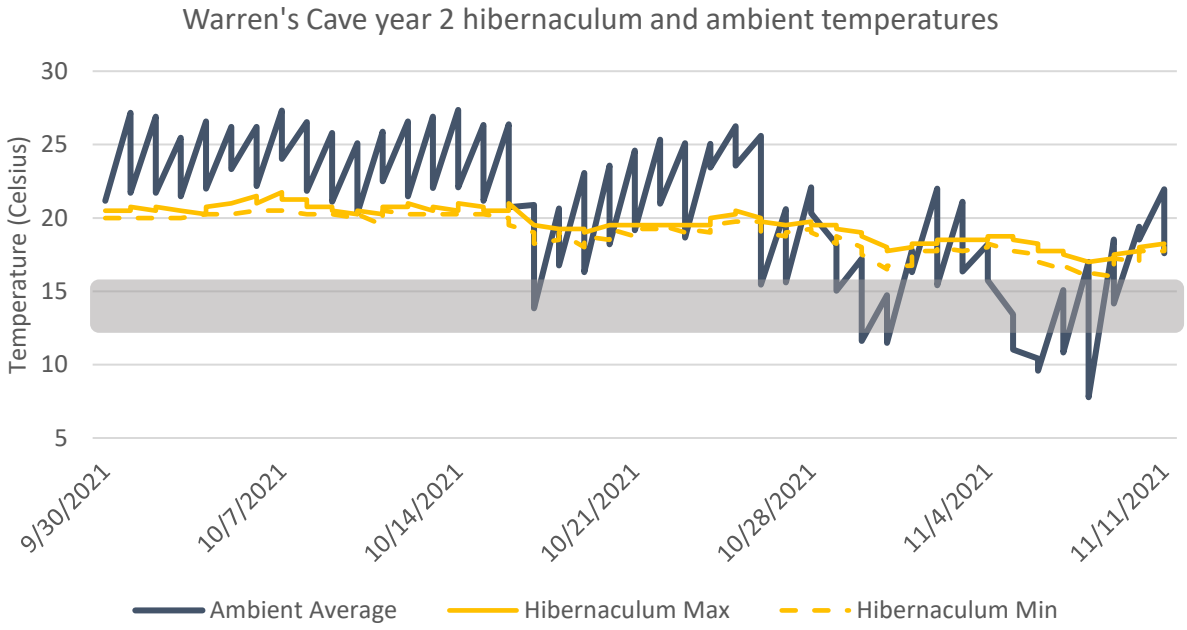



*Figure 13 Minimum and maximum temperatures inside of and ambient temperatures outside of Warren's Cave. Minimum and maximum temperatures for a weep hole in the middle of the structure are also included. The grey bar indicates optimal temperatures for P. destructans growth.*

| Table 8: Correlation coefficients between hibernaculum and ambient temperature | | | | |
|---|---|---|---|---|
| **Site** | **Ambient Max * Hibernaculum Max** | **Ambient Min * Hibernaculum Min** | **Ambient Max * Weephole Max** | **Ambient Min * Weephole Min** |
| **BDT 1** | 0.796901 | 0.89461 | NA | NA |
| **RC 1** | 0.942755585 | 0.95856 | NA | NA |
| **LR 1** | 0.918309 | 0.933374 | NA | NA |
| **KG 1** | 0.955585 | 0.919807 | 0.509418 | 0.511162 |
| **KB 1** | 0.938859811 | 0.95032088 | 0.559899659 | 0.567166229 |
| **FW 2** | 0.705644 | 0.902563 | NA | NA |

*Table 3 Correlation coefficients between ambient and hibernacula maximum and minimum temperatures. Weepholes were only measured in select hibernacula.*

| Table 9: One-way ANOVA of correlation coefficients between hibernaculum and ambient temperature | | | | | | |
|---|---|---|---|---|---|---|
| **SUMMARY** | | | | | | |
| *Groups* | *Count* | *Sum* | *Average* | *Variance* | | |
| Traditional | 4 | 3.299718 | 0.82493 | 0.008632 | | |
| Nontraditional | 8 | 7.517571 | 0.939696 | 0.000232 | | |
| | | | | | | |
| **ANOVA** | | | | | | |
| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Between Groups | 0.035124 | 1 | 0.035124 | 12.76249 | **0.005075** | 4.964603 |
| Within Groups | 0.027521 | 10 | 0.002752 | | | |
| | | | | | | |
| Total | 0.062645 | 11 | | | | |

*Table 49 Results for one-way ANOVA of Correlation coefficients between ambient and hibernacula maximum and minimum temperatures. Weep holes were not included in analysis.*

Temperatures inside Black Diamond Tunnel varied from 7°C to 13°C (Fig. 8). Temperatures in Red Creek culvert varied between -2°C and 25°C throughout the winter season (Fig. 9). Temperatures in Little River Culvert and Glynn County Culvert varied between 3°C and 24°C (Figs. 10 and 11). Bryan County Culvert temperatures varied between 0°C and 23°C (Fig. 12). Temperatures from Warren's Cave varied between 16°C and 22°C (Fig. 13). Temperatures in Glynn County Culvert and Bryan County Culvert were in the *Pd* optimal growth range of temperatures more than the other sites, with weep holes in particular experiencing significant periods in this temperature range (Figs. 11 and 12). Interestingly, in all sites but Bryan County culvert, the ambient and hibernacula minimum temperatures correlated slightly more

strongly than did the maximum temperatures (Table 8). The one-way ANOVA of correlation coefficients between hibernacula and ambient temperatures by hibernacula type (traditional versus nontraditional) was significant (P= 0.005), indicating nontraditional hibernacula were more closely correlated to ambient temperatures than were traditional hibernacula (Table 9).

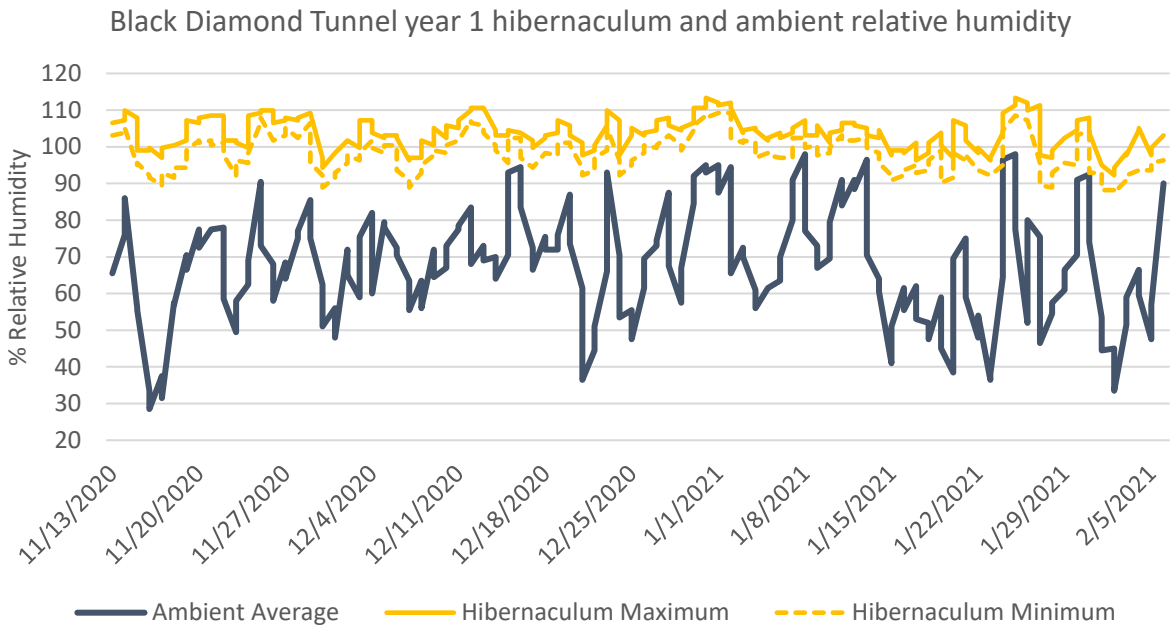



*Figure 14 Minimum and maximum percent relative humidity inside of and ambient relative humidity outside of Black Diamond Tunnel. Values above 100% were considered to be at 100% for the purpose of interpretation.*

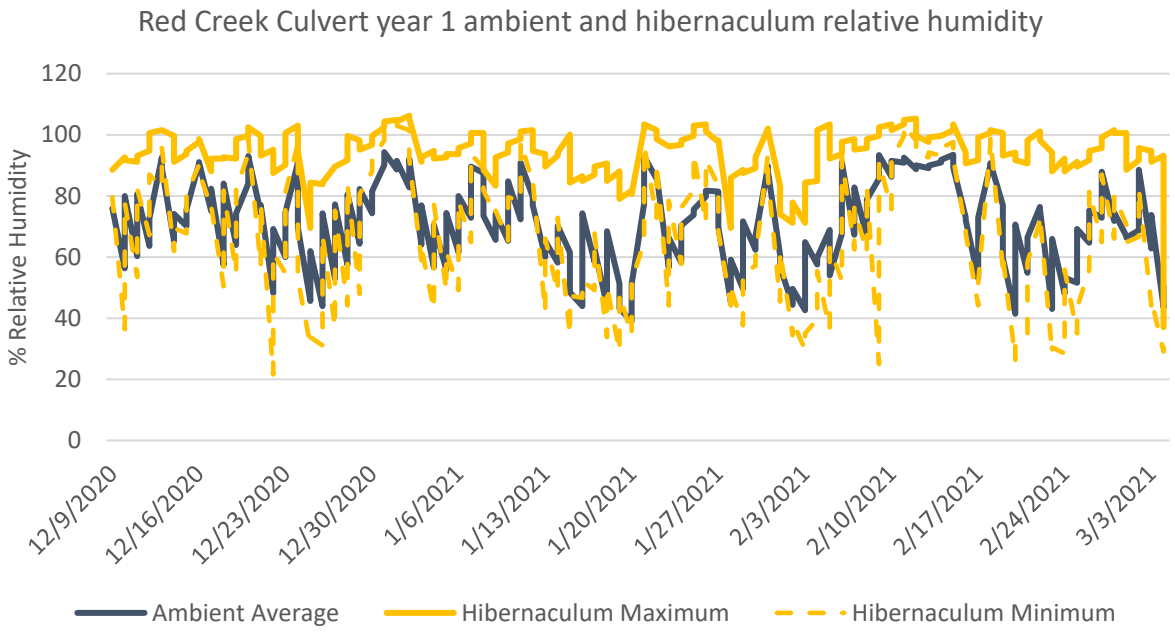



*Figure 65 Minimum and maximum percent relative humidity inside of and ambient relative humidity outside of Red Creek Culvert. Values above 100% were considered to be at 100% for the purpose of interpretation.*

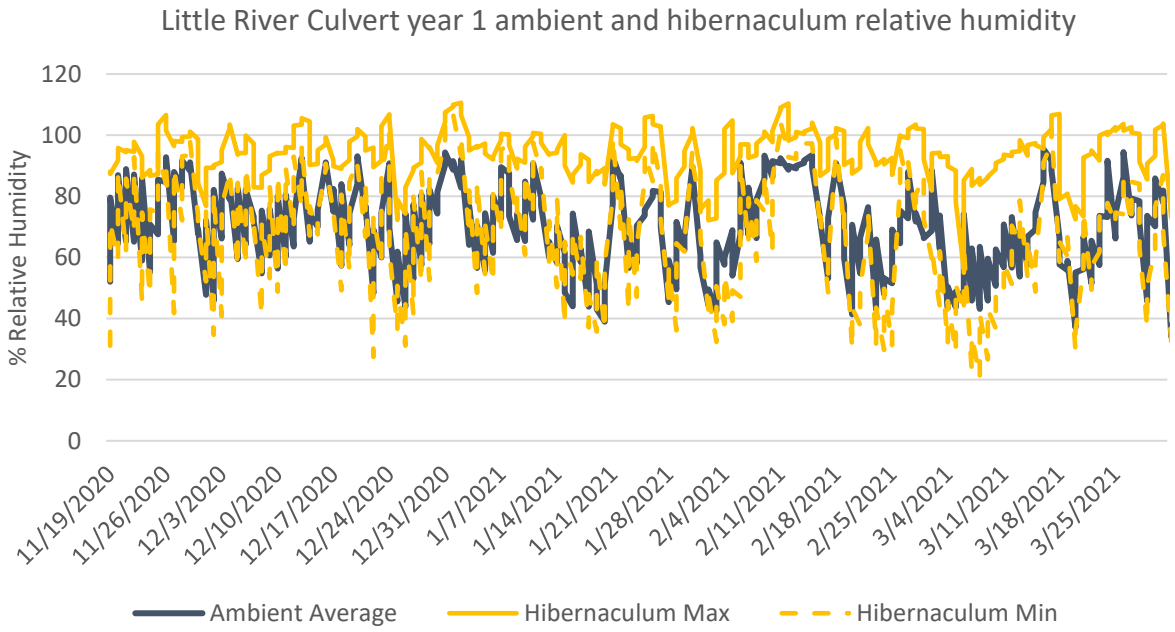



*Figure 16 Minimum and maximum percent relative humidity inside of and ambient relative humidity outside of Little River Culvert. Values above 100% were considered to be at 100% for the purpose of interpretation.*

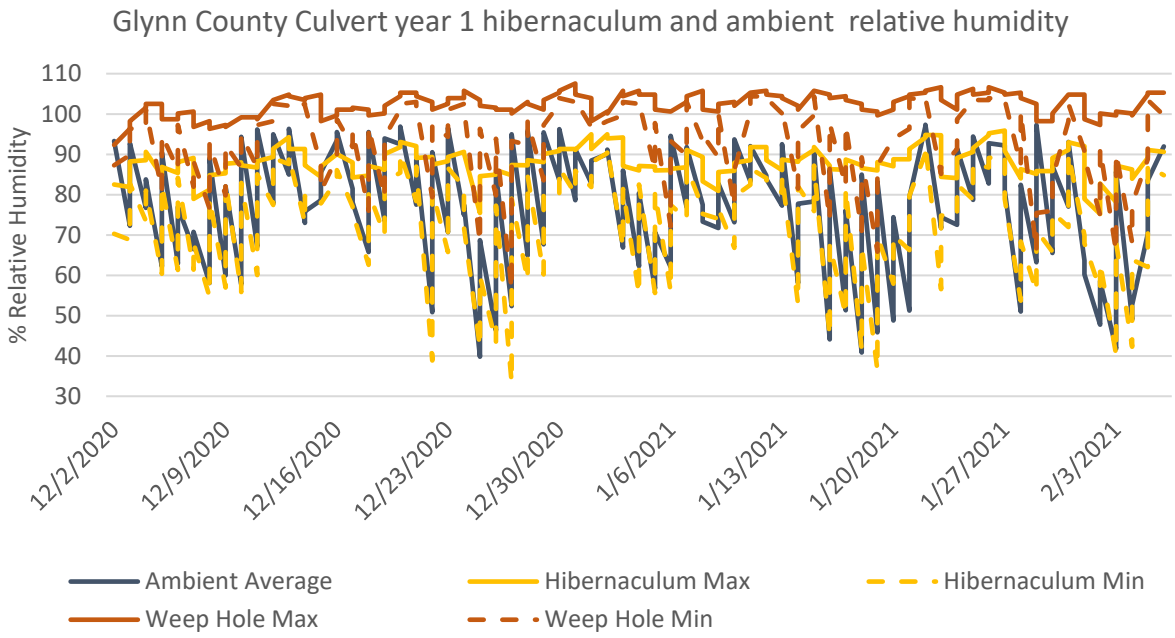



*Figure 77 Minimum and maximum percent relative humidity inside of and ambient relative humidity outside of Glynn County Culvert. Minimum and maximum relative humiditiess for a weep hole in the middle of the structure are also included. Values above 100% were considered to be at 100% for the purpose of interpretation.*

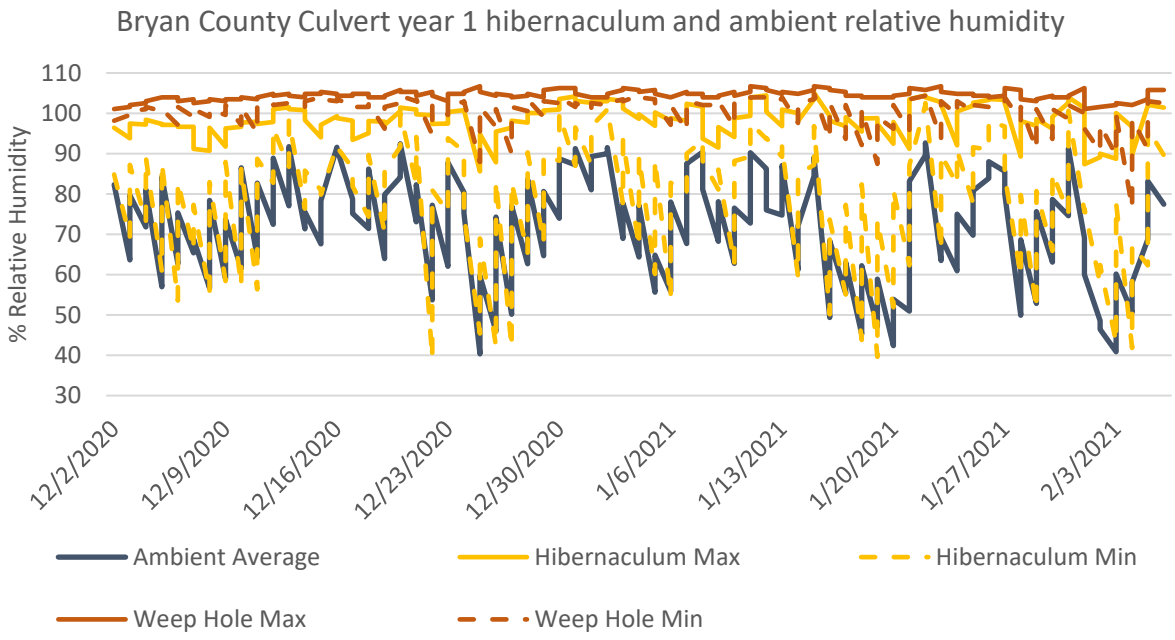



*Figure 88 Minimum and maximum percent relative humidity inside of and ambient relative humidity outside of Bryan County Culvert. Minimum and maximum relative humidities for a weep hole in the middle of the structure are also included. Values above 100% were considered to be at 100% for the purpose of interpretation.*

| Table 10: Correlation coefficients between ambient and hibernacula percent relative humidity | | | | |
|---|---|---|---|---|
| **Site** | **Ambient Max * Hibernaculum Max** | **Ambient Min * Hibernaculum Min** | **Ambient Max * Weep Hole Max** | **Ambient Min * Weep Hole Min** |
| **BDT 1** | 0.681175 | 0.663368 | NA | NA |
| **RC 1** | 0.670254208 | 0.88656 | NA | NA |
| **LR 1** | 0.65981 | 0.864162 | NA | NA |
| **KG 1** | 0.623576 | 0.901081 | 0.186946 | 0.642262 |
| **KB 1** | 0.728993423 | 0.887180655 | 0.263516996 | 0.670599675 |

*Table 50 Correlation coefficients between ambient and hibernacula maximum and minimum percent relative humidity. Weep holes were only measured in select hibernacula.*

RH nearly always stayed at or above ambient RH in all hibernacula, with minimum RH in all hibernacula save Black Diamond Tunnel and weep holes regularly falling to ambient levels. Black Diamond Tunnel maximum RH consistently stayed near 100%, while the minimum rarely dropped below 90% (Fig. 14). RH of the weep hole in Bryan County Culvert consistently stayed at or just below 100% (Fig. 18), while the weep hole in Glynn County Culvert experienced maximum RH consistently around 100%, but the minimum regularly fell to near the ambient RH (Fig. 17). In weep holes, minimum RH varied more closely with ambient RH than did maximum RH, which consistently stayed at or near 100% (Table 10, Figs. 17 and 18). Interestingly, in all sites but Black Diamond Tunnel, the ambient and hibernacula minimum temperatures correlated slightly more strongly than did the maximum temperatures (Table 12).

## Bat Biometrics

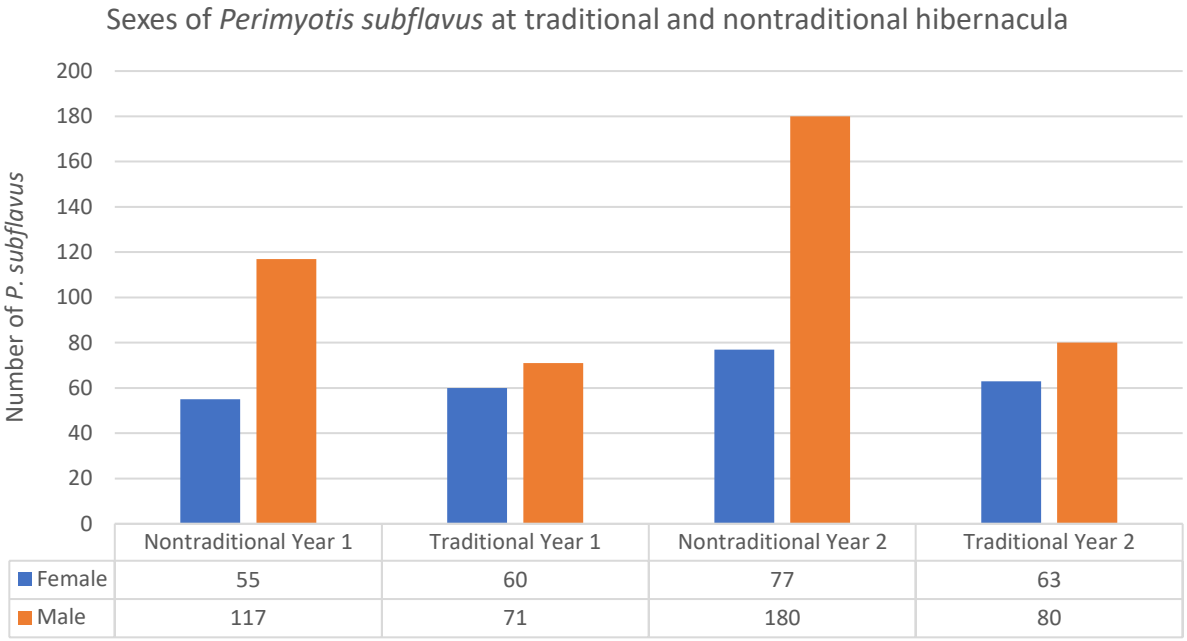



*Figure 919 Bar graph of the total number of female and male P. subflavus captured at traditional and nontraditional hibernacula during year 1 and year 2 of the study.*

| **Table 11: Chi-square test for independence for bat sex by hibernacula type** | | | | |
|---|---|---|---|---|
| **Actual Values** | | | | |
| | **Nontraditional Year 1+2** | **Traditional Year 1+2** | **Total** | |
| **Female** | 132 | 123 | 255 | |
| **Male** | 297 | 151 | 448 | |
| **Total** | 429 | 274 | 703 | |
| | | | | |
| **Expected Values** | | | | |
| | **Nontraditional Year 1+2** | **Traditional Year 1+2** | **Total** | **Proportion** |
| **Female** | 156 | 99 | 255 | 0.36 |
| **Male** | 273 | 175 | 448 | 0.64 |
| **Total** | 429 | 274 | 703 | |
| **Proportion** | 0.61 | 0.39 | | 1 |
| **P-value =0.000145912** | | | | |

*Table 611 Female to male ratio of P. subflavus captured at traditional vs nontraditional hibernacula*

Nontraditional sites had nearly twice as many male *P. subflavus* compared to females, while traditional sites had nearly equal numbers of female and male bats (Figure 19). The difference in sex ratios between hibernaculum types was statistically significant (P = 0.0001).

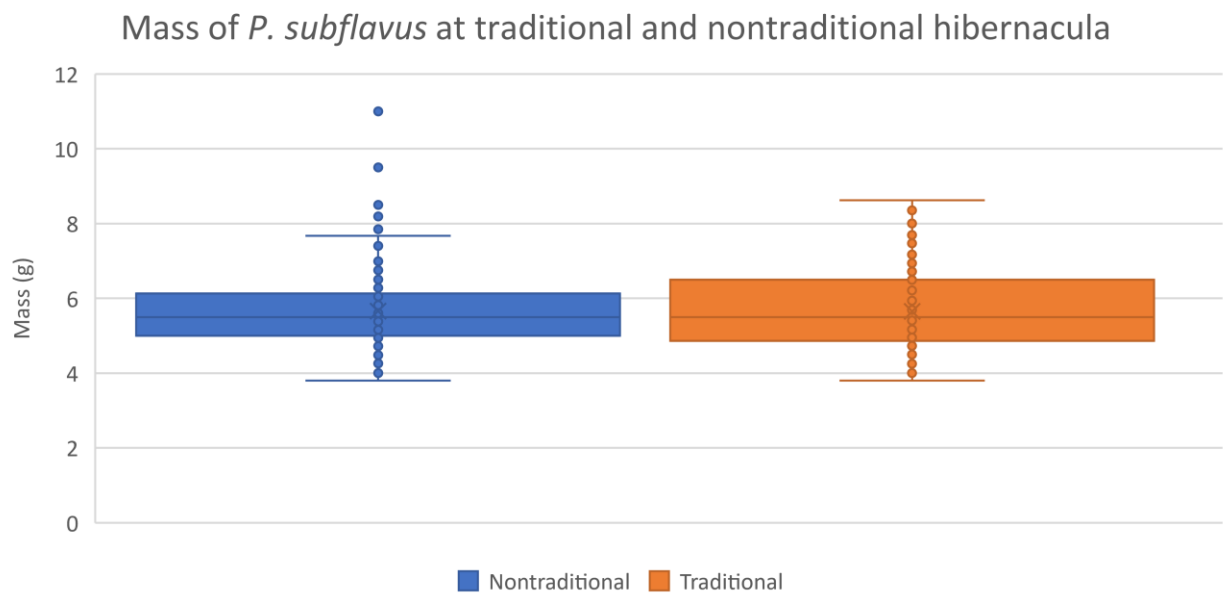



*Figure 100 Boxplots of the mass (g) of P. subflavus captured at traditional and nontraditional hibernacula*

**Table 12: One-way ANOVA of body mass of *P. subflavus* at traditional and nontraditional hibernacula**

| | | | | | | |
|---|---|---|---|---|---|---|
| **SUMMARY** | | | | | | |
| *Groups* | *Count* | *Sum* | *Average* | *Variance* | | |
| Y1+2 NT F+M | 384 | 2173.45 | 5.660026 | 0.799635 | | |
| Y1+2 T F+M | 262 | 1480.09 | 5.649198 | 1.072255 | | |
| | | | | | | |
| | | | | | | |
| **ANOVA** | | | | | | |
| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Between Groups | 0.018258 | 1 | 0.018258 | 0.020061 | **0.88741** | 3.855939 |
| Within Groups | 586.1186 | 644 | 0.910122 | | | |
| | | | | | | |
| **Total** | 586.1369 | 645 | | | | |

*Table 712 Results of single factor ANOVA on body mass (g) of P. subflavus captured at traditional vs nontraditional hibernacula*

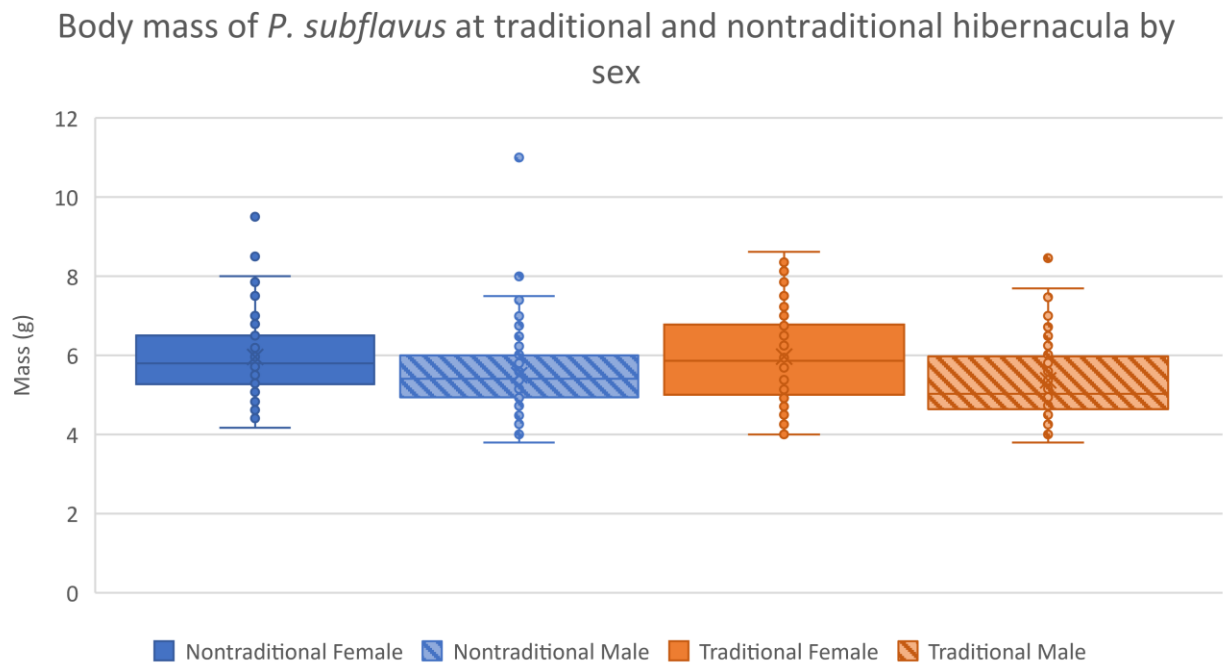



*Figure 21 Boxplots of the mass (g) of male and female P. subflavus captured at traditional and nontraditional hibernacula.*

The box plots of body mass of *P. subflavus* at traditional and nontraditional hibernacula showed complete overlap between the two groups with bats in traditional hibernacula having a greater range in body mass (Fig. 20).There was no significant difference in the body mass between traditional and nontraditional hibernacula (Table 12). Males in both hibernaculum types had slightly lower body mass than females (Fig. 21).

# DISCUSSION

## Population Counts

The similarity in the hibernation season population counts between season 1 and 2 suggests that the populations were relatively stable (Fig. 3). There were no discernible patterns in the population counts throughout the season between sites (Table 3). However, Black Diamond Tunnel averaged 112.3 more

bats in season 2 compared to season 1. The increase in population size at Black Diamond Tunnel between season 1 and 2 suggests a population rebound after colony collapse due to WNS (Table 4).

## *P. destructans* Surveillance

The increase in positive samples as the season progressed indicates an increase in the percentage of bats in each hibernaculum that were infected with *Pd* (Fig. 4). The increase in low Ct value samples as the season progresses indicates an increase in fungal load on bats throughout the hibernation season (Figs. 6 and 7). Both of these patterns are to be expected as *Pd* growth on bats is restricted to the temperatures that typically only occur during the winter season, and bats not previously infected with *Pd* must come in contact with the fungus, potentially via an environmental reservoir or other individuals, before the fungal load can increase on the body (Frick *et al.*, 2017). The nonconformity of Ct values from Talladega Culvert bats to the same patterns as bats from Black Diamond Tunnel, Weaver Cave, Little River Culvert, and Red Creek Culvert may be due to sampling error such as bias in what bats are swabbed or less bats present in the hibernaculum for swabbing during the mid season session.

The early sessions had fewer, higher Ct samples compared to later sessions, indicating an increase in number of colonized individuals as well as overall increased fungal loads as the season progresses (Figs. 6 and 7). The positive slopes of the trendlines for percentage of positive samples throughout the winter season indicates an increase in percentage of *Pd* positive bat swabs over the winter season. Interestingly, Black Diamond Tunnel year 1 and 2 had distinctly different rates of change of the percentage of positive samples over the winter season (Fig. 4), which could potentially be affected by ongoing WNS mitigation efforts using volatile organic compounds (Gabriel *et al.*, 2022). Frick *et al.* 2017 showed that prevalence of *Pd* on bats in hibernacula new contaminated with *Pd* more slowly reaches 100% than in hibernacula more than 2-3 years post-*Pd* introduction, but that nearly all

hibernacula reach 100% prevalence by the end of winter. The results of this study support the difference in rate of prevalence increase, however none of the hibernacula we studied reached 100% prevalence. Frick et. al 2022 showed that in captive settings where *P. subflavus* collected from a Mississippi culvert were held in a variety of temperature and humidity conditions, only 33% of individuals tested positive for *Pd* by qPCR after approximately 85 days. Prevalence of *Pd* on bats in many of the hibernacula used in this study ranged between 30 and 40%, which is much more similar to the captive study in 2022 than to the *in-situ* study by Frick et. al in 2017.

Bats swabbed at Little River Culvert year 1 and Talladega Culvert year 1 both had positive samples only in the greater Ct value range, indicating lesser fungal load on the bats (Fig. 5). This is to be expected at Little River Culvert as year 1 was the first year that *Pd* was detected, thus has not had time to grow in the hibernacula and on the bats. The status of *Pd* at Talladega Culvert was previously unknown, and the fungus may only have been introduced in recent years. Bats from hibernacula that had been positive for the fungus for more hibernation seasons such as Black Diamond Tunnel, Weaver Cave, and Red Creek Culvert, all had lesser Ct values than bats from Little River Culvert and Talladega Culvert, indicating greater fungal loads. This is likely due to having more time for the fungus to grow in the hibernacula and on the bats. Additionally, bats from Black Diamond Tunnel and Weaver Cave, both of which are subterranean hibernacula, had the least Ct values, thus the greatest fungal loads.

Ct values by survey session from Little River Culvert year 1, Red Creek Culvert year 1, Talladega Culvert year 1, Weaver Cave year 1, and Black Diamond Tunnel year 1 and 2 show a decrease in Ct value as the hibernation season progressed with the greatest proportion of positive samples remaining between 34 and 37 Ct (Figs. 6 and 7). This lowering of Ct values indicates an increase in fungal load on bats over the hibernation season. Ct values at Little River Culvert had the narrowest range and Little

River Culvert had the most recent detection of *Pd*. Bats at sites that have been positive for the fungus for more years have greater fungal loads.

The minimal overlap in Ct values between the boxplots for Little River Culvert year 1 and Weaver Cave year 1 suggest there may be a statistically significant difference in the Ct values throughout the winter season (Fig. 7). The only site with a statistically significant difference in Ct values by survey month was Little River Culvert as was determined by a one-way ANOVA (Table 6). This is a distinct opposition to the results of Frick *et al.*, 2017, which found a greater rate of increase in fungal loads on *P. subflavus* throughout the hibernation season as more years passed since initial detection of *Pd.*

## Hibernaculum Temperature and Humidity

Black Diamond Tunnel year 1 and Warren's Cave year 2 had little change in hibernaculum temperature and were minimally affected by variation in ambient temperatures (Figs. 8 and 13). Black Diamond Tunnel year 1 temperatures remained just below the optimal range for *Pd* growth, while Warren's Cave year 2 temperatures were well above the optimal range. In contrast, the hibernaculum temperatures in Red Creek Culvert year 1, Little River Culvert year 1, Glynn County Culvert year 1, and Bryan County Culvert year 1 varied greatly throughout the hibernation season, with multiple periods in which hibernaculum temperatures were in the optimal range for *Pd* growth (Figs. 9-12). Lutsch et. al 2022 observed similar trends in temperature variation and significant portions of time in which culverts were within the optimal growth range for *Pd.* Weep hole temperatures in both Glynn County Culvert and Bryan County Culvert had little variation throughout the hibernation season and spent the majority of the season in the optimal temperature range for *Pd* growth (Figs. 11 and 12). This is corroborated by Lutsch's findings of weep hole temperatures that varied significantly less than culvert temperatures. This

indicates that weep holes may react to ambient temperatures more similarly to traditional hibernacula and therefore be more likely to become “hotspots” for *Pd* growth within these hibernacula. Additionally, the weep hole in Bryan County Culvert, which has a greater box length and a deeper weep hole than the Glynn County Culvert, had more stable temperatures.

Comparatively, all of the nontraditional hibernacula had more time during the hibernation season in which temperatures were within the optimal range for *Pd* growth, but also had long spans of time where hibernaculum temperatures were well outside of the optimal range (Figs. 9-12). This is reflected in the correlation coefficients between ambient and hibernacula temperatures which were significantly higher in nontraditional hibernacula than traditional hibernacula, indicating that nontraditional hibernacula more closely followed ambient temperature than do traditional hibernacula as we hypothesized in this study (Table 8). This was confirmed by a one-way ANOVA that resulted in a p-value of 0.005 (Table 9).

RH was nearly always greater than ambient and regularly neared or exceeded saturation inside all hibernacula save Glynn County Culvert, which varied above and below 90% RH (Fig. 17). Black Diamond Tunnel unlike the nontraditional hibernacula, maintained a minimum RH that stayed well above ambient and rarely dropped below 90% (Fig. 14). In the field this was often seen via condensation forming on the fur of the bats. Weep holes in Glynn County Culvert and Bryan County Culvert reliably maintained a RH greater than the overall hibernacula and did not vary as closely with ambient RH as the rest of the hibernacula. As with temperature, the RH in the Bryan County Culvert weep hole, which is a deeper weep hole in a longer culvert, was more stable and did not vary as closely with ambient RH as did the Glynn County Culvert weep hole (Figs. 18 and 17). RH in all hibernacula is conducive to the growth of *Pd,* particularly so in Black Diamond Tunnel.

The results for temperature and RH should be interpreted with caution, as the iButtons measuring these factors did not run the for entire length of the hibernation season at all sites due to equipment malfunction. Additionally, these loggers cannot accurately measure RH near saturation.

## Bat Biometrics

Nontraditional hibernacula were shown to have a significantly greater proportion of male bats to female bats than did traditional hibernacula (Table 11; $P<0.05$). While traditional hibernacula had approximately equal proportions of sexes, nontraditional hibernacula had nearly twice as many males compared to females (Fig. 19). This is supported by the thrifty female hypothesis formed by Jonasson and Willis (2011), as males can better withstand the fluctuations in temperature and humidity within nontraditional hibernacula, where they also may be able to respond to favorable weather conditions and arouse from torpor to forage. In contrast, females must retain as much energy, in the form of fat stores, as possible to best prepare them for gestation and raising young, thus they stay in deeper torpor, which is easier in climatically stable traditional hibernacula.

There were not statistically significant differences in body mass in *P. subflavus* at traditional versus nontraditional sites. The box plots of body mass of *P. subflavus* at traditional and nontraditional hibernacula showed complete overlap between the two groups with traditional hibernacula having a greater range in body mass (Fig. 20). The one-way ANOVA for this comparison indicated no significant difference in mass between traditional and nontraditional hibernacula ($P = 0.33$; Table 12). Additionally, while there was no difference between masses by hibernaculum type, both types showed slightly lesser body mass for males compared to females (Fig. 21).

Sampling could not be guaranteed to be random due to the complex nature of hibernacula. This may introduce added error due to the bias of accessible bats during sampling. Error may also have been introduced via differences in measuring tools and between observers.

## STATEMENT OF INTEGRATION

This topic integrates microbiology, physiology, ecology, and conservation biology. The ecology includes both behavioral ecology and disease ecology. Mycology and conservation biology are essential to the understanding of the topic because the pathogen is fungal and has caused significant population reductions in multiple bat species, including some that were of concern before the emergence of the epizootic in North America. Climate science, geology, geography, and environmental science are critical to understand the abiotic factors of this problem. The integration of subfields within biology and fields outside of it are crucial to analyzing this problem and its real-world applications.

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# REFERENCES

ALTRINGHAM, J. (1996) *Bats: Biology and Behavior*. Oxford University Press, New York.

BARBOUR, R.W. & DAVIS, W.H. (1969) *Bats of America.* In *University of Kentucky Press, Lexington KY* p.

BLEHERT, D.S., HICKS, A.C., BEHR, M., METEYER, C.U., BERLOWSKI-ZIER, B.M., BUCKLES, E.L., COLEMAN, J.T.H., DARLING, S.R., GARGAS, A., NIVER, R., OKONIEWSKI, J.C., RUDD, R.J. & STONE, W.B. (2008) Bat White-Nose Syndrome : An Emerging Fungal Pathogen ?, 5–6.

BOONMAN, M. (2011) Factors determining the use of culverts underneath highways for bats. *Lutra* **54**, 3–16.

BOYLES, J.G., DUNBAR, M.B., STORM, J.J. & BRACK, V. (2007) Energy availability influences microclimate selection of hibernating bats, 4345–4350.

BOYLES, J.G., DUNBAR, M.B. & WHITAKER, J.J. (2006) Activity following arousal in winter in North American vespertilionid bats. *Mammal Review* **36**, 267–280.

BRACK, V.J. (2007) Temperatures and Locations Used by Hibernating Bats , Including Myotis sodalis ( Indiana Bat ), in a Limestone Mine : Implications for Conservation and Management. *Environmental Management*.

BRIGGLER, J.T. & PRATHER, J.W. (2003) Seasonal Use and Selection of Caves by the Eastern Pipistrelle Bat ( Pipistrellus subflavus ) **149**, 406–412.

CHENG, T.L., WELLER, T.J., BENNETT, A.B., FELLER, D.J., HICKS, A.C., KING, R.A., REICHARD, J.D., THOGMARTIN, W.E., COLEMAN, J.T.H., REICHERT, B.E., CAMPBELL, J., ETCHISON, K., BRODERS, H.G., GEBOY, R., HEMBERGER, T., ET AL. (2021) The scope and severity of white-nose syndrome on hibernating bats in North America, 1586–1597.

CRYAN, P.M., METEYER, C.U., BOYLES, J.G. & BLEHERT, D.S. (2010) Wing pathology of white-nose syndrome in bats suggests life-threatening disruption of physiology.

DAVIS, W.H. (1964) Winter Awakening Patterns in the Bats Myotis lucifugus and Pipistrellus subflavus Author ( s ): Wayne H . Davis Published by : American Society of Mammalogists Stable URL : https://www.jstor.org/stable/1377349 REFERENCES Linked references are available on. *Journal of Mammology* **45**, 645–647.

FRICK, W.F., CHENG, T.L., LANGWIG, K.E., HOYT, J.R., JANICKI, A.F., PARISE, K.L., FOSTER, J.T. & KILPATRICK, A.M. (2017) Pathogen dynamics during invasion and establishment of white-nose syndrome explain mechanisms of host persistence. *Ecology* **98**, 624–631.

FRICK, W.F., PUECHMAILLE, S.J., HOYT, J.R., NICKEL, B.A., LANGWIG, K.E., FOSTER, J.T., BARLOW, K.E., BARTONIČKA, T., FELLER, D., HAARSMA, A.J., HERZOG, C., HORÁČEK, I., VAN DER KOOIJ, J., MULKENS, B., PETROV, B., ET AL. (2015) Disease alters macroecological patterns of North American bats. *Global Ecology and Biogeography* **24**, 741–749.

FUJITA, M.S. & KUNZ, T.H. (1984) Pipistrellus subflavus. *Mammalian Species*, 1–6.

GABRIEL, K.T., MCDONALD, A.G., LUTSCH, K.E., PATTAVINA, P.E., MORRIS, K.M., FERRALL, E.A., CROW, S.A. & CORNELISON, C.T. (2022) Development of a multi-year white-nose syndrome mitigation strategy using antifungal volatile organic compounds. *PLoS ONE*, 1–15.

GARGAS, A., TREST, M.T., CHRISTENSEN, M., VOLK, T.J. & BLEHERT, D.S. (2009) Geomyces destructans sp. nov. associated with Bat white-nose syndrome. *Mycotaxon* **108**, 147–154.

GEORGIA DEPARTMENT OF NATURAL RESOURCES (2020) 2020 White-nose Syndrome Season Summary.

GIBIZOV, K.K. (2013) Breeding behavior of the tri-colored bat (Perimyotis subflavus): Relevance for development of a captive breeding program. University of Georgia.

GRIENEISEN, L.E., BROWNLEE-BOUBOULIS, S.A., JOHNSON, J.S. & REEDER, D.M. (2015) Sex and hibernaculum temperature predict survivorship in white-nose syndrome affected little brown myotis ( Myotis lucifugus ) Subject Category : Author for correspondence :

HOPKINS, S.R., HOYT, J.R., WHITE, J.P., KAARAKKA, H.M., REDELL, J.A., DEPUE, J.E., SCULLON, W.H., KILPATRICK, A.M. & LANGWIG, K.E. (2021) Continued preference for suboptimal habitat reduces bat survival with white-nose syndrome. *Nature Communications* **12**. Springer US.

KATZENMEYER, J.B. (2016) Use of highway culverts, box bridges, and caves by winter-roosting bats in Mississippi, 70.

KEELEY, B.W. & TUTTLE, M.D. (1999) Bats in American bridges.

LANGWIG, K.E., FRICK, W.F., BRIED, J.T., HICKS, A.C., KUNZ, T.H. & MARM KILPATRICK, A. (2012) Sociality, density-dependence and microclimates determine the persistence of populations suffering from a novel fungal disease, white-nose syndrome. *Ecology Letters* **15**, 1050–1057.

LEIVERS, S.J., MEIERHOFER, M.B., PIERCE, B.L., EVANS, J.W. & MORRISON, M.L. (2019) External temperature and distance from nearest entrance influence microclimates of cave and culvert-roosting tri-colored bats (Perimyotis subflavus). *Ecology and Evolution* **9**, 14042–14052.

LOEB, S.C. & WINTERS, E.A. (2022) Changes in hibernating tricolored bat ( Perimyotis subflavus ) roosting behavior in response to white-nose syndrome. *Ecology and Evolution*, 1–9.

LUTSCH, K.E., MCDONALD, A.G., GABRIEL, K.T. & CORNELISON, C.T. (2022) Roadway-Associated Culverts May Serve As a Transmission Corridor for Pseudogymnoascus Destructans and White-Nose Syndrome in the Coastal Plains and Coastal Region of Georgia, Usa. *Journal of Wildlife Diseases* **58**, 322–332.

MARROQUIN, C.M., LAVINE, J.O. & WINDSTAM, S.T. (2017) Effect of humidity on development of pseudogymnoascus destructans, the causal agent of bat white-nose syndrome. *Northeastern*

*Naturalist* **24**, 54–64.

MCGUIRE, L.P., MAYBERRY, H.W. & WILLIS, C.K.R. (2017) White-nose syndrome increases torpid metabolic rate and evaporative water loss in hibernating bats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 680–686.

MEIERHOFER, M.B., JOHNSON, J.S., LEIVERS, S.J., PIERCE, B.L., EVANS, J.E. & MORRISON, M.L. (2019a) Winter habitats of bats in Texas. *PLoS ONE* **14**, 1–16.

MEIERHOFER, M.B., LEIVERS, S.J., FERN, R.R., WOLF, L.K., JR, J.H.Y., PIERCE, B.L., EVANS, J.W. & MORRISON, M.L. (2019b) Structural and environmental predictors of presence and abundance of tri-colored bats in Texas culverts, 1–8.

MEYER, G.A., SENULIS, J.A. & REINARTZ, J.A. (2016) Effects of temperature and availability of insect prey on bat emergence from hibernation in spring. *Journal of Mammalogy* **97**, 1623–1633.

MICROSOFT CORPORATION (2018) Microsoft Excel. Https://office.microsoft.com/excel.

POISSANT, J.A., BRODERS, H.G. & QUINN, G.M. (2010) Use of lichen as a roosting substrate by Perimyotis subflavus, the tricolored bat, in Nova Scotia. *Écoscience* **17**, 372–378. Taylor & Francis.

R CORE TEAM (2021) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Https://www.r-project.org/.

RAESLY, R.L.. & GATES, J.. E. (1987) Winter Habitat Selection by North Temperate Cave Bats. *The University of Notre Dame* **118**, 15–31.

SANDEL, J.K., BENATAR, G.R., BURKE, K.M., WALKER, C.W., LACHER, J. & HONEYCUTT, R.L. (2001) Use and selection of winter hibernacula by the eastern pipistrelle (Pipistrellus subflavus) in Texas. *Journal of Mammalogy* **82**, 173–178.

SIRAJUDDIN, P. (2018) Vulnerability of tri-colored bats (Perimyotis subflavus) to white-nose syndrome in the southeastern United States. Clemson University.

THOMAS, D.W., DORAIS, M. & BERGERON, J.-M. (1990) Winter Energy Budgets and Cost of Arousals for Hibernating Little Brown Bats, Myotis lucifugus. *Journal of Mammalogy* **71**, 475–479.

TURNER, G.G., SEWALL, B.J., BITZ, D., JOHNSON, J.S., SCAFINI, M.R. & LILLEY, T.M. (2022) Cooling of bat hibernacula to mitigate white-nose syndrome, 1–11.

US FISH & WILDLIFE SERVICE (2019) White-Nose Syndrome The devastating disease of hibernating bats in North America.

US FISH & WILDLIFE SERVICE (2022) Service proposes to list the tricolored bat as endangered under the Endangered Species Act Ongoing spread of white-nose syndrome is primary threat, increasing risk of extinction. Https://www.fws.gov/press-release/2022-09/proposal-list-tricolored-bat-endangered.

USGS-NATIONAL WILDLIFE HEALTH CENTER (2019) Protocol for Non-lethal Sampling Methods for Pseudogymnoascus destructans (Pd) Surveillance.

VERANT, M.L., BOYLES, J.G., JR, W.W., WIBBELT, G. & BLEHERT, D.S. (2012) Temperature-Dependent Growth of Geomyces destructans , the Fungus That Causes Bat White-Nose Syndrome **7**.

VERANT, M.L., METEYER, C.U., SPEAKMAN, J.R., CRYAN, P.M., LORCH, J.M. & BLEHERT, D.S. (2014) White-nose syndrome initiates a cascade of physiologic disturbances in the hibernating bat host. *BMC Physiology* **14**, 1–10.

VINCENT, E.A. & WHITAKER, J.O. (2007) HIBERNATION OF THE EASTERN PIPISTRELLE , PERIMYOTIS SUBFLAVUS , IN AN ABANDONED MINE IN VERMILLION COUNTY , INDIANA , WITH SOME INFORMATION ON MYOTIS LUCIFUGUS, 58–65.

WALKER, C.W., SANDEL, J.K., HONEYCUTT, R.L. & ADAMS, C. (1996) Winter utilization of box culverts by vespertilionid bats in southeast Texas. *Texas Journal of Science*. .

WHITE-NOSE SYNDROME DISEASE MANAGEMENT WORKING GROUP (2020) National White-Nose Syndrome Decontamination Protocol [2020-Oct]. Https://www.whitenosesyndrome.org/static-page/decontamination-information.

WHITE-NOSE SYNDROME RESPONSE TEAM (undated) Where is WNS Now? Https://www.whitenosesyndrome.org/where-is-wns [accessed 12 April 2022].

WILLIS, C.K.R., MENZIES, A.K., BOYLES, J.G. & WOJCIECHOWSKI, M.S. (2011) Evaporative Water Loss Is a Plausible Explanation for Mortality of Bats from White-Nose Syndrome **51**, 364–373.

# APPENDICES

## Appendix A. Bat and Sample Data Sheet

**Culvert Monitoring Bat Data**

**Surveyer(s)**_____

| # | Site # | Box Lett | Bat # | Roost | Date | Species | Age | Sex / Repr. | Mass (g) | FA (mm) | Samples Taken (Circle All Taken) | Visible Signs of WNS | Bat Temp | WDI | Band # | Comments (Band New or Recap, *Other, etc.) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 2 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 3 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 4 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 5 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 6 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 7 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 8 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 9 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 10 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 11 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 12 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 13 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 14 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 15 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 16 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 17 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 18 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 19 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |
| 20 | | | | | | | | / | | | WP BS ES G H O | Y / N | | | | |

| Codes for Bat Roosts | | | |
|---|---|---|---|
| SW | Side Wall | CK | Crack |
| HC | Hanging Ceiling | O* | Other |
| WH | Weep Hole | | |

| Samples Taken Key to Codes | | | |
|---|---|---|---|
| WP | Wing Punch | BS | Bat Swab |
| ES | Enviro. Swab | G | Guano |
| H | Hair | O* | Other |

| Wing Damage Index (WDI) | | | |
|---|---|---|---|
| 0 | fewer than 5 small scar spots, membranes intact | 3 | holes ≥5mm, necrotic tissue, membrane loss |
| 1 | <50% pigment loss | P | physical impact damage |
| 2 | > 50% scarring/splotching, holes <5mm | | |

* Describe other in comments

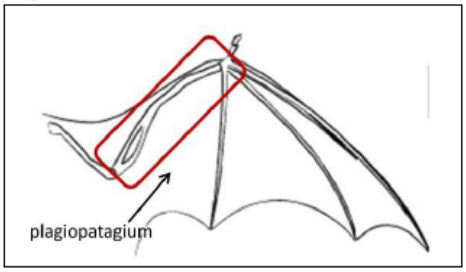
# Appendix B. USGS National Wildlife Health Center Swabbing Protocol

**D. BAT SKIN SWAB COLLECTION PROTOCOL**

1. Persons collecting swab samples from bats or handling sample vials should wear disposable exam gloves. It is not necessary to change gloves between each bat/sample vial provided the persons performing these tasks do not directly contact individual bats or the environmental substrate.
2. Identify a bat to be sampled.
3. Record the individual bat information on the Individual Specimen Datasheet. Remove a pre-labeled sample vial from the "SWAB VIALS" bag.
4. **Pull the Datasheet Label from the sample vial and place on datasheet. Remove and dispose of end tab to allow label to lie flat.**
5. Tap sample vial to ensure all liquid is pooled at the bottom.
6. Remove a swab from its packaging **without touching the polyester tip**.
7. Dip the tip of the swab into the sample vial to moisten (most water will be absorbed by swab).
8. Bats may be sampled without removing them from their roosting location. If direct handling of the bat is necessary, hold bat face down with one wing pulled slightly away from the body at the elbow.

4

9. Sample one of the bat's forearms and adjacent wing tissue between the elbow and wrist (see diagram) by gently **ROLLING** the swab across the surface of skin (three passes back & forth). Rolling the swab as it is moved along the skin prevents abrading the delicate wing skin and maximizes contact with the swab surface.
10. Roll the same swab across the muzzle of the same bat 3 times.
11. After collecting the sample, transfer swab to the same sample vial used to moisten it. Break off the shaft **near the applicator tip**. Avoid touching the vial rim or inside of lid with your fingers. Screw closed the vial lid tightly.
12. Place swab sample vials into the "SAMPLES" bag and follow instructions for sample handling and storage (see I. Sample Handling & Storage).
13. Dispose of swab handles, wrappers, end tabs of Datasheet Labels, and contaminated exam gloves as necessary into "TRASH".
14. Repeat the above process for each bat sampled.



## Appendix C. US Fish and Wildlife Service White Nose Syndrome Decontamination Protocol

**VI. EQUIPMENT AND ACTIVITY SPECIFIC RECOMMENDATIONS:**

***It is the responsibility of the users of this protocol to read and follow the product label and SDS. The product label is the law!***

1) **Clothing & Footwear:**

**IMPORTANT: All clothing (*i.e.*, inner and outer layers) and footwear should be decontaminated after every site visit using the most appropriate Application/Product in Table 1 or otherwise cleaned and dedicated for use at individual sites or areas as determined appropriate in Section IV.**

Use of a disposable suit (*e.g.*, Tyvek® or ProShield®) or site-dedicated, reusable suit (*i.e.*, coveralls) is an appropriate strategy to minimize sediment/soil accumulation on clothing during a cave/mine or bat research activity. All clothing layers should still be decontaminated or otherwise cleaned and dedicated after every use.

Contain all used equipment in plastic bags upon final exit from a site, separating disposable materials from reusable equipment. Seal and store plastic bags in plastic containers until trash can be properly discarded, and/or exposed reusable equipment can be properly decontaminated off site.

*While significantly more comprehensive than the National WNS Decontamination Protocol, Centers for Disease Control provide more information about proper use of protective equipment here:* https://www.cdc.gov/hai/prevent/ppe.html ("The resources on this [CDC] page are intended to promote patient safety and increase the safety of the healthcare work environment through improved use of personal protective equipment (PPE) by healthcare personnel.")

**Cave/Mine and other Subterranean Equipment:**

Dedicate, as necessary, or decontaminate all cave/mine equipment (*e.g.*, backpacks, helmets, harness, lights, ropes, etc.) using the most appropriate guidance in Section V. Most types of equipment, including but not limited to, technical and safety equipment, have not undergone manufacturers' consented testing for safety and integrity after decontamination. Therefore, carefully review and adhere to the manufacturer's care and use standards to maintain equipment functionality and safety protective features. If the application/product options in Table 1 are not approved by the manufacturer's care and use standards for the respective type of equipment, clean and inspect equipment according to manufacturer's specification and dedicate to similarly classified caves/mines/bat roosts and only reuse in progressively more contaminated caves/mines/bat roosts as determined appropriate in Section IV.

3) **Scientific Equipment:**

***Only properly trained, vaccinated, and, where necessary, authorized personnel should handle bats!***

Consider the use of disposable scientific equipment and materials that can be refreshed between contact with individual bats, especially in the Intermediate and At-Risk management areas. All disposable scientific equipment (*e.g.*, work surfaces, containers/envelopes, exam gloves, etc.) should only be used to process one bat, then discarded after use. Similarly, reusable equipment (e.g., cotton holding bags, gear bags, gloves, wing punches, banding pliers, rulers, and other field instruments) should only be used to process one bat prior to initiating procedures for decontamination. Any bag used to hold bats must be breathable and safe for the animals.

Use the guidance in Section V to determine the relevant procedure for decontamination of all work surface area(s) and equipment (*e.g.*, light boxes, banding pliers, holding bags, rulers, calipers, scale, scissors, wing biopsy punches, weighing containers).

Autoclaving non-submersible equipment is an acceptable sterilization measure, if feasible and permissible for the equipment, although this method has not been tested directly for *Pd*.

**NOTE: In situations where disposable items (*i.e.*, nitrile or latex gloves) are in limited supply or unavailable, disinfecting them between bats may be allowable. Appropriate products must be selected to insure they are being used in accordance with label specifications, as would be done with any reusable equipment. As with any items that will come in contact with bats, disinfected gloves must be dry and free of residue that may be harmful to the bats before another animal is handled.**

4) **Mist-Nets & Harp Traps:**

Dedicate, as necessary, or decontaminate all netting and harp trapping equipment (*e.g.*, netting, tie ropes, poles, stakes, trap bags, lines, trap frame and feet) using the most appropriate guidance in Section V for the particular equipment. This is only necessary after each night of use when the net and/or trap equipment come in contact with one or more bats OR enter a cave/mine/bat roost. Disposable harp trap bags or liners can be also be used to reduce transmission risks and should be discarded at the end of each night if any bats have come in contact with the bag.

5) **Acoustic Monitor, Camera, and Related Electronic Equipment:**

For electronic equipment and accessories used within bat roosts, dedicate, as necessary, or decontaminate all acoustic monitoring, camera, and related electronic equipment (*e.g.*, detector, camera, tablets, cell phones, laptops, carrying case, lenses, microphone(s), mounting devices, cables) using the most appropriate guidance in Section V for the particular equipment. The material composition of this equipment requires careful review and adherence to the manufacturer's care and use standards to maintain their functionality and protective features. If application/product options in Table 1 are not approved by the manufacturer's care and use standards for the respective type of equipment, clean equipment accordingly and dedicate to similarly classified caves/mines/bat roosts or only reuse in progressively more contaminated caves/mines/bat roost as determined appropriate in Section IV. Electronic devices used as lower-risk terrestrial equipment, and not used in bat handling work, pose a negligible risk of transmission (*i.e.*, driving transects or fixed point detector surveys not associated with a cave/mine/bat roost entrance).

Equipment used in a cave/mine/bat roost may be placed in a sealed plastic casing, plastic bag, or plastic wrap to reduce the potential for contact/exposure with contaminated environments. The outer surfaces of plastic protective covers should be cleaned after leaving the cave, mine, or roost and prior to removing the equipment. Plastic wraps should then be discarded (if disposable) or further treated (if reusable) using the most appropriate guidance in Section V.