Assessment of Culverts and Bridges as Roosting Habitat for Perimyotis subflavus (tri-colored bat) and Disease Transmission Corridors for Pseudogymnoascus destructans

Kelly Lutsch

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Assessment of Culverts and Bridges as Roosting Habitat for *Perimyotis subflavus* (tri-colored bat) and Disease Transmission Corridors for *Pseudogymnoascus destructans*

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*Table 1 | Species Names and Conservation Status. All mentioned species are listed with latin names, common names, abbreviations, and conservation status.*
INTRODUCTION

Fungal pathogens

In recent history, emerging fungal pathogens have threatened the survival of numerous species (Daszak et al. 2000; Fisher et al. 2012; Fisher et al. 2016). Historically, pathogenic fungi have been associated with plant health issues and crop loss, but only recently recognized as a pressing threat to animal health. Amphibian population declines in seemingly pristine areas, such as rainforests of Central America, have been attributed to the fungal pathogen *Batrachochytrium dendrobatidis*, the causative agent of chytridiomycosis (Parkes et al. 1998). North American snake populations have experienced declines due to *Ophidiomyces ophiodiicola*, the causative agent of snake fungal disease, and often characterized by facial swelling, cloudy eyes, and improper skin shedding (Last et al. 2016). Fungal pathogens have diverse and resilient dispersal mechanisms, facilitating survival in harsh environments until a suitable host is available. This has enabled the spread of several pathogens across spatial and temporal scales (Fisher et al. 2012). Among these emerging fungal pathogens is *Pseudogymnoascus destructans*, the causative agent of white-nose syndrome (WNS) in North American bats. Since its discovery in New York caves in 2006, WNS has caused precipitous declines in populations of North American bats (Blehert et al. 2009; Warneke et al. 2012).

*Pseudogymnoascus destructans*

*P. destructans* is a psychrophilic ascomycete that grows optimally between 12.5°C and 15.8°C, which makes it well-suited for inhabiting the same cave environments bats use for hibernation (Turner et al. 2011). *P. destructans* has been identified as an invasive fungal pathogen in North America and has been linked to mass mortality of bat populations in the eastern U.S. and Canada. In Eurasia, *P. destructans* has been observed infecting several bat species, although less severe disease symptoms and low mortality rates are reported (Wibblet et al. 2010; Puechmaille et al. 2011). Colonization occurs on the nose, ear, and wing tissue of bats during winter hibernation, when body temperatures are near ambient and immune responses are severely suppressed. Exposed tissue is colonized by mycelia with a fuzzy, white appearance. The fungal biomass invading tissues is characterized by prolific fungal conidia (Meteyer et al. 2009). The histology of *P. destructans* infection is characterized by cup-like erosions of
the epidermis filled with fungal hyphae (Meteyer et al. 2009). Fungal hyphae can colonize multiple
tissues such as the hair follicles, sebaceous glands, and apocrine glands (Meteyer et al. 2009). \textit{P. destructans} can be detected on an individual without associated cupping lesions, suggesting that
infection may not always lead to the development of WNS.

White-Nose Syndrome

White-nose syndrome (WNS) is the clinical manifestation of disease caused by \textit{P. destructans} in
hibernating bats. White-nose syndrome has been confirmed in 33 U.S. states and 7 Canadian provinces.\textit{P. destructans} has been detected in an additional 3 U.S. states, without documentation of WNS (Figure 1). Population models estimated that within 20 years of introduction of \textit{P. destructans}, affected North American bat species such as \textit{Myotis lucifugus} (little brown bat), \textit{Myotis septentrionalis} (northern long-eared bat), and \textit{Perimyotis subflavus} (tri-colored bat), could experience a 99% regional population
collapse resulting in the loss of over 5.5 million bats (Frick et al. 2010; USFWS 2012).

Symptoms of infection manifest as lesions, rough patches of skin, and irregular pigmentation.
Wing tissue may be more susceptible to fungal invasion and water evaporation due to its relatively
increased surface area compared to other exposed tissue. Wing tissue also plays an important role in
thermoregulation and immune function, both of which are pivotal to maintain homeostasis in a torpid
endotherm (Herreid et al. 1968; Dongaonkar et al. 2009). WNS-infected bats typically exhibit increased
torpor arousal frequency and shortened bout lengths. Premature arousal is energetically costly and
often leads to premature fat reserve depletion, dehydration, and starvation (Warnecke et al. 2012,
Warnecke et al. 2013; Lilley et al. 2016). Torpid bats lack the required inflammatory response to defend
against fungal invasion. However, post-hibernation active bats can be observed with severe emaciation
and inflammation, suggesting the potential for a rare immune response known as Immune
reconstitution inflammatory syndrome (IRIS) to present once immune function returns (Meteyer et al.
2012).

Studies have indicated that post-WNS bat foraging and hibernation behavior can be inconsistent
and often irregular compared to historical data (Reeder et al. 2012). Researchers have observed bats
foraging on the landscape during hibernation season in confirmed WNS positive areas (Grider et al.
These findings support the assertion that WNS can impact normal bat hibernation patterns, leading to abnormal arousal or inhibiting migration.

Figure 1 | Map of WNS. This map shows the spread of white-nose syndrome in North America as of June 2019. Map shows WNS positive counties and color coding indicates year of occurrence. [https://www.whitenosesyndrome.org/resource/updated-white-nose-syndrome-map](https://www.whitenosesyndrome.org/resource/updated-white-nose-syndrome-map)

Pathogen Transmission

*P. destructans* can persist at suboptimal growth temperatures and be transmitted through bat-to-bat, substrate-to-bat, or bat-to-substrate contact (Hoyt et al. 2014; Ballman et al. 2017; Lorch et al. 2011). Hibernating bat species often cluster in tight colonies during the winter months, facilitating fungal transmission throughout the hibernacula and colony. Vulnerable species such as *M. lucifugus* and *P. subflavus* are often observed sharing a hibernaculum with non-susceptible species such as *Myotis austroriparius* (southeastern myotis). *M. austroriparius* exhibit short, shallow torpor bouts allowing them to remain active during the winter months. Activities such as foraging and movement between
roosts could facilitate disease transmission between hibernating bat colonies (Lorch et al. 2011).

Findings confirm that human activities can also serve as transmission vectors as spores can remain viable on clothing and equipment (Shelley et al. 2013; Ballman et al. 2017). For example, a WNS-positive bat was recovered in King County, Washington in 2016, nearly 1,000 miles from the nearest WNS-positive county. Real-time PCR and histopathological testing confirmed the \( P. \text{destructans} \) strain isolated from the infected bat was indistinguishable from strains collected in the eastern United States (Lorch et al. 2016).

**Bats and Bridges/Culverts**

Numerous bat species are versatile in their roosting selection, choosing anthropogenic structures in addition to natural cavernous structures (Geluso et al. 2009; Allen et al. 2011; Bergeson et al. 2015). It has been hypothesized that bats select bridges for roosting due to the numerous crevices and cracks available for use (Tuttle and Keeley 1999; Allen et al. 2011). \( Tadarida \text{brasiliensis} \) (Brazilian free-tailed bats) have been documented utilizing bridges in large numbers in south-central Texas as well as \( Corynorhinus \text{rafinesquii} \) (Rafinesque’s big-eared bat) in South Carolina (Bennett et al. 2008; Allen et al. 2011). Studies have investigated anthropogenic roost-selection and bat condition compared to natural roosts, finding that some bat species in critical conditions, such as pregnant or nursing young, actually experience less stress in anthropogenic roost like a bridge. It is theorized that this is due to the escape from potential stressors related to natural hibernation structures such as resource competition or parasites (Allen et al. 2011).

More recently, researchers in Texas, Missouri, and Mississippi have observed \( P. \text{subflavus} \) roosting in roadway-associated culverts in large numbers (unpublished data Texas and Missouri DOTs). Due to the expansive nature of roadways, bridges and culverts can occur virtually anywhere on the landscape; crossing various ecoregions, topography, and natural borders.

**Species of Interest**

\( P. \text{subflavus} \) was once abundant across its historic range in the northeast and midwest United States but now faces significant population declines due to WNS (Fujita 1984; Briggler and Prather 2003;
This species is relatively small, with adults weighing between 4-7 g and an average forearm length of 33 mm. They are characterized by three distinct body colors; pale or yellowish fur, black wing membrane, and pink forearms. *P. subflavus* are insectivorous with a diet consisting of small insects ranging from 4-10 mm in length (Fujita 1984). In the summer months, *P. subflavus* roost in trees and often remain there throughout the May through August maternity season (Veilloux and Veilloux 2004). During the maternity season, female *P. subflavus* will carry, give birth to, and raise their young, typically giving birth to two pups (Fujita 1984; Veilloux and Veilloux 2004). During the maternity season, they have been observed roosting solitarily or in clusters of 2-3 individuals.

As ambient temperatures naturally lower in the winter season, the energy necessary to maintain a euthermic state can increase significantly. Hibernation decreases energy consumption and allows the animal to subsist on stored fat deposits until conditions become favorable for foraging. Torpor is a specialized hibernation strategy that allows a bat to reduce energy requirements to as low as ~1 % of typical metabolic activity during a season of resource scarcity (Ruf et al. 2015). To reduce energy requirements, metabolic processes decrease, waste is not excreted, immune response is suppressed, and body temperature drops to near ambient (Geiser et al. 2009; Burton 1999). During this time bats arouse periodically, depleting up to ~80% of fat reserves (Thomas et al. 1990). Some species remain torpid for weeks at a time, while others experience shallow torpor and more frequent arousals (Thomas et al. 1990). While torpor is energetically efficient it is associated with compromised immune function and dehydration related stress (Burton 1999).

*P. subflavus* have been historically documented hibernating in karst environments during the winter season. They have been consistently observed roosting individually and exhibit a preference for longer cave structures, which is thought to enhance temperature stability (Briggler and Prather 2003; Brack 2007). Records indicate large numbers of *P. subflavus* entering a hibernacula as early as August and not emerging until April. The typical hibernation season for North American hibernating bats is between December and February, suggesting *P. subflavus* are generally the first species to enter, as well as the last species to leave hibernation (Davis 1964; Fujita 1984; Briggler and Prather 2003). *P. subflavus* may be more susceptible to WNS due to their longer hibernation period, and thus greater susceptibility to infection, in addition to other factors such as small body size and presumed prolonged torpor patterns. Across their geographic range, *P. subflavus* has experienced up to 98% mortality in various states due to WNS, which has led to petitioning for government mandated protection under the Endangered Species Act of 1973. According to the Georgia Department of Natural resources, *P.*
subflavus populations have experienced a 95% population decline in Georgia since the detection of WNS in 2013.

Statement of Problem

Roadway associated bridges and culverts serve as a unique disease transmission vector with the ability to supersede unsuitable habitat. Should infected bats utilize culverts as hibernation sites, there is a potential for disease transmission to naive populations and regions. Understanding bat roosting preference is important because it has the potential to influence disease transmission.

The theory of island biogeography describes a metapopulation dispersed across habitat patches with varying degrees of connectivity (Merriam et al. 1989; Merriam et al. 1991; Opdam et al. 1991). In Georgia, the metapopulation of P. subflavus is theoretically dispersed from the traditional bat habitat in the northern portion of the state to the coast and coastal plains region. The latter region lacks the topography and associated caves suitable for bat hibernating, but shares a border with karst environments in north Florida. While patches of habitat increase movement of an organism on the landscape, they can also enhance disease transmission throughout a metapopulation (Hess 1994). Roadway culverts create corridors through patches of habitat with otherwise unsuitable features for bat roosting, foraging, or movement. Infected bats moving among these structures can facilitate transmission of P. destructans, creating a chain of potential disease reservoirs spanning across multiple states. This non-traditional interconnectivity could facilitate the spread of P. destructans to previously pathogen-free areas such as north Florida. Novel disease movement and potential development of WNS in naive populations has made such research increasingly critical (Bergeson et al. 2015).

Extensive research has been conducted to evaluate the effects of WNS on high-risk species such as M. septentrionalis and M. lucifugus (Grider et al. 2016; Pettit et al. 2017; Langwig et al. 2017). Such focused research has left a void regarding information relating to other affected hibernating bat species and habitat. Understanding how other WNS-susceptible species, such as P. subflavus, use roadway-associated structures is critical to understanding pathogen dispersal and identifying potentially vulnerable populations. The Department of Transportation is required by law to consider the potential impact of roadway structure maintenance on threatened and endangered bat species. P. subflavus is currently proposed for such listing and likely to be included as a species to be considered during
necessary roadway structure maintenance. Having thorough knowledge of seasonal *P. subflavus* use of such structures will be of benefit to any invested state and federal agencies.

Currently, there is no record of WNS in coastal Georgia. Survey efforts have been concentrated to the northern part of the state where more suitable habitat is located. Similarly, bat presence/absence survey efforts have been focused in northern Georgia. Given the numerous accounts of various bat species using bridges and culverts as roosting sites outside Georgia (including *P. subflavus*), there is a high probability of observing similar roosting patterns in coastal Georgia. The use of roadway associated bridges and culverts by *P. subflavus*, could impact the health of susceptible bats in the karst region of north Florida.

**Objectives and Hypotheses**

The objectives of this investigation were to understand the spatial spread of WNS and the roosting preference of *P. subflavus* populations potentially using roadway-associated culverts in the coastal and coastal plains regions of Georgia. We hypothesized that roadway associated culverts would have physical and ecological characteristics that would increase the likelihood of *P. subflavus* presence. We hypothesized that surveyed culverts would have characteristics favorable for the growth of *P. destructans* and the development of WNS in *P. subflavus*. Finally, we hypothesized that roadway associated culverts in the coastal and coastal plains of Georgia will have the potential to serve as a transmission corridor for white-nose syndrome from karst regions of North Georgia to *P. subflavus* occupied karst environments in North Florida. The findings from this study can help to fill the knowledge gap regarding WNS burden in coastal Georgia and presence of *P. subflavus* or other species in bridges and culverts, providing wildlife management officials with the knowledge to better manage critical bat habitat. Understanding of the spatial spread of *P. destructans* within the state and the potential for disease transmission across state lines is important for monitoring, predicting, preparing for, and potentially preventing disease movement into previously-WNS-free regions.
MATERIALS AND METHODS

FIELD METHODS

Culvert Survey and Data Collection

Roadway-associated bridges and culverts in the coastal plains and coastal region of Georgia were selected for survey based on several safety factors. Culverts with a height of less than two feet were not surveyed, as bats are not generally observed in culverts of this size (unpublished data from Texas A&M University, Missouri Department of Transportation). Additionally, culverts of this size are highly susceptible to flooding and can be a safety concern for surveyors. Bridges and culverts in areas with high vehicular traffic were assessed for suitability due to potential safety hazards.

All surveys were conducted using a standard Georgia Department of Natural Resources (GADNR) datasheet and associated guidelines (Appendix A; Appendix B). Two data sheets were completed for each survey. A GADNR sheet consisted of bridge identification, structural information, and documentation of any use of the structure by bats and birds. The second sheet documented any substrate or animal swabs acquired, including date, site number, type of swab, and associated species sampled. To collect all necessary data, surveyors thoroughly inspected all crevices, joints, and cracks of the bridge or culvert per the methods of Tuttle and Keeley 1999. Indication of bat activity included seeing bats, smelling or seeing guano, observing staining on walls, or hearing clicks or chirps. All indications of bat activity were well-documented on the bridge and culvert data sheet. If bats were observed, the roost location and orientation within the culvert were recorded. Other important culvert features, such as internal height and width, surrounding habitat, conditions under the culvert, and presence of water were also documented. Streams, forests, and vehicular traffic, which could impact bat use, were also documented per the methods of Tuttle and Keeley 1999. Photos were captured of the structure and surrounding habitat. If bats were present, photos were taken to document their location on or within the structure and for identification.

Sample Collection
Initial surveys during the 2017/2018 hibernation season, swab samples were collected from every structure surveyed where bats were present. For consistency, all sampling methods were based on the United States Geological Survey and National Wildlife Health Center protocol (Appendix C). Latex gloves were worn while swabbing and changed after each swab, according to United States Fish and Wildlife Service decontamination protocols (Appendix D). Sterile swabs were kept in their original packaging until use. To swab a bat, the surveyor placed the non-dominant hand under the bat to reduce the risk of falling. The surveyor 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. For consistency, substrate swabs also required five passes using the same streak and twist method. The swab was stored in a new 2ml vial with 250 µl Ctab DNA extraction buffer (OPS Diagnostics, NJ) and labeled accordingly. Six vials were kept in the carrying tray throughout each survey season to serve as negative controls. Swabs were stored in a cooler while in the field and then stored at -80 °C when available.

Bat Handling

In the event a bat could be processed for body metric information, the USFWS recommendations for safe bat handling were followed (Range-wide Indiana Bat Survey Guidelines 2018). Standard measurements were collected, including weight, forearm length, age, sex, and reproductive status. Wings were examined for signs of damage or abnormalities, as lesions or chafing could be indicative of WNS (Meteyer et al. 2009). Active bats were only banded when appropriate and safe for the surveyor and the animal. All bat handling was based on directions from the lead bat biologist for the state of Georgia, Katrina Morris. Each band was uniquely numbered and placed on the forearm of the animal. All band numbers and associated information were submitted to Katrina Morris and the GADNR. All bat handling was conducted under the Federal Collection Permit held by Katrina Morris. Collecting body metrics can be time consuming and distressing to the animal. Stress and irregular arousal is energetically costly to a hibernating bat. For this reason, torpid bats were never disturbed for processing.

Hibernation Season Culvert Surveys
Survey efforts were focused in the coastal and coastal plains regions of Georgia, USA (Figure 2), beginning on Interstate 16 at Macon, traveling southeast to Interstate 95 and continuing south to the Florida border. Surveys in the coastal plains region began near Cordele and continued south along Interstate 75 to the Florida border. Sections of smaller roadways such as Highway 441 and 341 were also surveyed. During December, 2017 through March, 2018, Kennesaw State University (KSU), GADNR, and U.S. Fish and Wildlife Service identified and surveyed 109 roadway-associated structures in the coastal and coastal plains regions for bat presence (Figure 3). Following the USGS NWHC 2017-2018 protocol,
surveyors collected 146 swabs of bats and substrates. To further characterize *P. subflavus* seasonal use of culverts, twelve representative culverts were selected for environmental monitoring (Figure 4).
From the 109 structures surveyed for bat presence, twelve were selected for environmental monitoring based on location and bats observed during the 2017/2018 hibernation season. The twelve representative culverts are shown in blue circles. Culverts without bats observed are shown in red. Culverts with bats observed are shown in green.

Representative Culverts

Representative culverts located in the coastal and coastal plains regions were selected based on species present and roosting orientation observed during the hibernation season. The twelve culverts selected were broken further into four plots based on geographical region. Three plots represented culverts where *P. subflavus* was observed and one plot represented culverts where *M. australiriparius* was observed. Each plot contained one structure in which bats were observed roosting in weep holes (WH culverts) and one in which bats were observed free hanging on the wall or ceiling (FH culverts) and one in which no bats were previously observed (control) (Figure 5). From June, 2018 through May, 2019 each
culvert was surveyed monthly for bat presence. Due to variable weather conditions across seasons, culverts were assessed for safety and accessibility upon arrival. Surveyors walked or waded through all accessible culverts and thoroughly inspected all crevices, weep holes, and cracks of the culvert for bat presence. Swabs were collected December, 2018 through March, 2019 using the same protocol previously-described except all swabs were stored dry. During monthly surveys, all culverts including the control culverts, were swabbed in triplicate.

Figure 5 | Four Culvert Plots. The four culvert plots selected for environmental monitoring are distinguished by a unique color. Yellow shapes represent culvert plot 1, where P. subflavus were observed in the coastal plains region. Red shapes represent culvert plot 2, where P. subflavus were observed in the coastal region. Green shapes represent culvert plot 3, where P. subflavus were observed in the coastal region. Blue shapes represent culvert plot 4, where M. austroriparius were observed in the coastal plains region. Within each plot, there are three culverts. The triangles represent the WH culverts. The circles represent the FH culverts. The black squares represent the Control culverts.

Temperature Data Loggers

Three temperature data loggers (HOBO 64K Pendant, Onset Computer Corporation, MA, USA) were placed in each representative culvert, with one in the center and one on each end, to determine
potential temperature variation. Data loggers were placed in orientations mimicking the bat roosting positions previously observed. Weep hole culverts had one data logger placed in one weep hole using a bolt and toggle. Mesh was secured to the weep hole opening to prevent bats from entering and potentially affecting temperature measurements, while still allowing air to pass with minimal obstruction. To minimize disturbance to the bats, data loggers were only placed in one weep hole per culvert, leaving any others open for roosting. The other two data loggers were mounted on the culvert wall using a 6-inch wooden block, an eye screw, and a carabiner (Figure 6). The eye screw was firmly attached to the wooden block, and all-weather environmental caulk (GE Silicone) was used to adhere the block to the concrete. The data logger was then clipped to the eye using a small carabiner. Free hanging culverts and control culverts had all three data loggers placed throughout the culvert in a similar fashion. Data loggers were deployed at weep hole and free hanging culverts in June and control culverts in July, 2018 and recorded temperature until May, 2019.

Figure 6 | Data Logger Mount. All weather environmental caulk was used to adhere a 6-inch wooden block with an eyelet screw attached to the concrete culvert wall. Data loggers were attached using small carabiner.
Prior to deployment, each logger was programmed to collect temperature measurements at 10-minute intervals using the HOBO Waterproof Data Shuttle (Onset Computer Corporation) and HOBOware Pro software version 3.7.16 (Onset Computer Corporation). Upon collection during monthly surveys, all data loggers were assessed for damage and data was downloaded and stored as a CSV (comma separated values) document. Device battery capacity and free memory were monitored during this process. Loggers were reconfigured by the HOBOware Pro software prior to being returned to the same location within the culvert.

LABORATORY METHODS

DNA Extraction

Field swabs stored at -80 °C were thawed, resuspended in 140 μl TE buffer, and vortexed. A 0.6 ml vial with the bottom cut was placed inside a sterile 2 ml Eppendorf tube. The original field swab was placed into the cut 0.6 ml tube inside the 2 ml tube. If the field swab was stored in liquid, any remaining liquid was pipetted into the 2 ml tube. The 0.6 ml vial containing the field swab was sealed then centrifuged for 1 min at 10,000 rpm. This was done to ensure all liquid, potentially containing DNA, was pulled from the field swab and into the 2 ml tube. The field swab was placed back in its original vial using sterilized tweezers. All original field swabs were stored at -80 °C. Then, 16 μl of Proteinase K was added to the solution. Samples were then incubated in a heat block at 37 °C for 5 min, 65 °C for 30 min, and 80 °C for 20 min, then vortexed for 10 minutes. Equal volume to total sample volume (~156 μl) of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each sample. The samples were then vortexed and centrifuged for 2 min at 10,000 rpm. The top aqueous layer containing DNA was pipetted into a new 2ml Eppendorf tube. The sample was washed with equal volume to sample of absolute ethanol and incubated at -80 °C for one hour. Following incubation, samples were centrifuged for 10 min at 10,000 rpm. Ethanol was removed from the sample and washed with half the sample volume of 70% ethanol. Samples were centrifuged for 10 min at 10,000 rpm and left open to allow the ethanol to volatilize. Once the ethanol had volatilized, samples were resuspended in 25 μl TE buffer.
P. destructans cultures were used to create a standard curve of detection in order to quantify P. destructans from field samples. A growing culture of P. destructans was swabbed and DNA extracted using the protocol previously described. After extraction, the amount of DNA present in the sample was quantified in triplicate using a spectrophotometer (BioTek Instruments Inc). Following determination of sample concentration, dilutions were calculated for a curve ranging from 1 ng/μl to 250 ng/μl (Figure 7). A dilution series allowed for relative quantification based on the cycle threshold (Ct) value. The Ct value was determined by the cycle number necessary to achieve amplification above the threshold level. This dilution series was processed 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, 7.5 μl sterile deionized water and 5 μl unknown sample DNA. Forward primer, reverse primer, and probe sequences were adopted from the assay described by Muller et al. (2013). The standard curve was successfully reproduced several times with similar acceptable results, and yielding an R^2 of 0.98 - 0.99. The standard curve was used to quantify the 249 field swabs processed for P. destructans detection.
Figure 7 | Standard Curve. Standard curve of detection created using pure culture P. destructans. All unknown field samples were plotted against this curve to determine relative concentration. Cq value is synonymous with Ct value.

STATISTICAL METHODS

Due to the relatively small sample size, all data collected at bridges was excluded from further analyses. All analyses operated under the assumption that all tunnels at any given site had the same dimensional measurements and were treated as one culvert site. Height of the culvert, distance from the base of the structure to the ceiling, width of the culvert, distance from one wall to the opposite wall were recorded at each surveyed culvert. These measurements were used to calculate the hypotenuse or diagonal of each culvert.

The diagonal measurement of each culvert, length of the culvert, habitat surrounding the culvert, conditions inside the culvert, and presence of water were used as predictor variables in a logistic
linear regression in an effort to predict bat presence (Table 2). ‘Month’ was included as a predictor variable to account for both initial surveys and monthly surveys of representative culverts. All combinations of predictor variables were considered during model selection. The Akaike Information Criteria value (AIC) of each model was used to determine which best fit the data. Due to the relatively small sample size of this dataset (n=197 surveys), AIC values were converted into AICc values (Burnham and Anderson 2002). Model selection was then based on the delta AIC value and relative AIC weight of the individual model. Delta AIC values of 4 or less were considered to provide substantial empirical support for the individual model (Burnham and Anderson 2002). Logistic linear regression models were used to predict general bat presence, and *P. subflavus* presence. A one-tailed t-test was used to assess any differences in roosting preferences *P. subflavus* may have exhibited.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Habitat</td>
<td>Environment immediately surrounding culvert</td>
</tr>
<tr>
<td></td>
<td>Example: ‘woodland’, ‘riparian’</td>
</tr>
<tr>
<td>Conditions</td>
<td>Physical and environmental features within culvert</td>
</tr>
<tr>
<td></td>
<td>Example: ‘concrete’, ‘bare ground’</td>
</tr>
<tr>
<td>Water</td>
<td>Presence or absence of water within culvert</td>
</tr>
<tr>
<td>Month</td>
<td>Time of the year the survey was conducted</td>
</tr>
<tr>
<td>Diagonal</td>
<td>( \text{diagonal} = \sqrt{\text{height of culvert}^2 + \text{width of culvert}^2} )</td>
</tr>
<tr>
<td>Length</td>
<td>Distance between culvert entrances</td>
</tr>
</tbody>
</table>

Table 2 | Variables Included in Logistic Linear Regression. Variables were collected during each survey and included as predictor variables in a logistic linear regression.

Within each culvert, temperature data was averaged per data logger in 24-hour increments. Calculated daily averages were used for all further analyses and charts. A single factor ANOVA variance analysis was used to assess the variance observed between the three data loggers within a single culvert. For each culvert containing a data logger in a weep hole position, the daily average temperatures collected in the free hanging position were averaged and compared to the average daily temperature collected in the weep hole using a two-tailed paired t-test. A variance analysis and two-tailed paired t-test was used to compare the variances of average daily temperatures collected by data loggers deployed in weep holes and data loggers free hanging on the culvert wall. After establishing the novelty of the temperature collected inside a weep hole, a single factored ANOVA variance analysis was used to examine any variance between the average daily temperatures collected by the four data
loggers deployed in weep holes. Tukey’s test was then used to determine which of the four weep hole oriented data loggers contributed to the significant difference observed in the single-factored ANOVA. All statistical analysis was conducted in Excel or Statistical Analytical System (SAS) Studio (SAS Institute).

RESULTS

Hibernation Season Culvert Surveys

Between December of 2017 and March of 2018, 109 roadway-associated structures in the coastal plains and coastal region of Georgia were surveyed, comprising of 96 culverts and 13 bridges. While roughly half of all culverts surveyed had only one tunnel at each site (n=49), the remaining sites (n=47) contained 2 – 6 tunnels. Culverts whose entrance resembled a square or rectangle, had a prevalence of 82.29 % (n=79). Culverts whose entrance was circular, had a prevalence of 17.71 % (n=17). Culvert diagonal measurements ranged from 0.79 m – 4.15 m with a mean of 2.32 m (standard deviation of ±0.85 m). Arc GIS software (v10.6.1, Environmental Systems Research Institute, Redlands, CA) was used to measure the length of all culverts surveyed. The length of surveyed culverts ranged from 20.4 m – 92.8 m with a mean of 56.13 m (±13.91 m).

P. subflavus, M. australoriparius, and C. rafinesquii were observed roosting in surveyed culverts during the 2017/2018 winter hibernation season (Table 3). 49 P. subflavus were recorded in 12 culverts (Figure 8). 132 M. australoriparius were recorded in 31 culverts (Figure 9), and 6 C. rafinesquii were recorded in 4 culverts. 76 % of P. subflavus were observed clustered in weep holes and 81 % of M. australoriparius were observed roosting in weep holes during the 2017/2018 hibernation season.
Table 3 | Representative Culverts and Species Present. The locations of all twelve representative culverts surveyed, bat species observed, and relative number of individuals.

<table>
<thead>
<tr>
<th>Representative Culvert</th>
<th>Latitude/Longitude</th>
<th>Species Present</th>
<th>Total Number of Bat Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site 28</td>
<td>31.90822, -81.32538</td>
<td>MYAU</td>
<td>6</td>
</tr>
<tr>
<td>Site 30</td>
<td>31.869703, -81.334253</td>
<td>MYAU; PESU</td>
<td>48; 28</td>
</tr>
<tr>
<td>Site 31</td>
<td>31.766016, -81.381426</td>
<td>MYAU; PESU</td>
<td>1; 1</td>
</tr>
<tr>
<td>Site 70</td>
<td>31.78705, -83.69124</td>
<td>MYAU</td>
<td>46</td>
</tr>
<tr>
<td>Site 79</td>
<td>31.51649, -83.51678</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site 88</td>
<td>30.96825, -83.38108</td>
<td>MYAU</td>
<td>57</td>
</tr>
<tr>
<td>Site 107</td>
<td>31.228588, -81.497639</td>
<td>MYAU; PESU</td>
<td>2; 8</td>
</tr>
<tr>
<td>Site 109</td>
<td>31.35215, -81.61068</td>
<td>MYAU; PESU; CORA</td>
<td>27; 35; 15</td>
</tr>
<tr>
<td>Site 110</td>
<td>31.27178, -81.56145</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site 124</td>
<td>32.75386, -83.16170</td>
<td>MYAU; PESU; CORA</td>
<td>13; 22; 9</td>
</tr>
<tr>
<td>Site 125</td>
<td>32.68333, -83.09161</td>
<td>PESU</td>
<td>3</td>
</tr>
<tr>
<td>Site 127</td>
<td>32.66219, -83.04816</td>
<td>MYAU; PESU; CORA</td>
<td>1; 3; 2</td>
</tr>
</tbody>
</table>

Figure 8 | P. subflavus Observations 2017/2018. All 2017/2018 P. subflavus observations in the coastal plains and coastal region of Georgia. Dot size corresponds to the number of individuals observed.
While no one environmental variable had significant ability to accurately predict bat presence or \textit{P. subflavus} presence, models that best fit the data contained ‘month’ ‘habitat’ ‘water’ and ‘conditions’. Models containing only ‘habitat’ ‘conditions’ and ‘water’ provided similarly substantial support, while containing fewer variables (Table 3; Table 4).

<table>
<thead>
<tr>
<th>Variables Included in Model</th>
<th>Raw AIC</th>
<th>AICc</th>
<th>Delta AIC</th>
<th>AICw</th>
</tr>
</thead>
<tbody>
<tr>
<td>month, water, conditions, habitat</td>
<td>241.938</td>
<td>242.146</td>
<td>0.0**</td>
<td>0.0</td>
</tr>
</tbody>
</table>
### Table 4: Best Fit Models for Predicting Bat Presence. Top logistic linear regression models.

<table>
<thead>
<tr>
<th>Variables Included in Model</th>
<th>Raw AIC</th>
<th>AICc</th>
<th>Delta AIC</th>
<th>AICw</th>
</tr>
</thead>
<tbody>
<tr>
<td>month, water, conditions, habitat</td>
<td>201.398</td>
<td>201.606</td>
<td>0.0**</td>
<td>0.0</td>
</tr>
<tr>
<td>length, habitat, month, conditions, water</td>
<td>202.534</td>
<td>202.848</td>
<td>1.136</td>
<td>0.006097</td>
</tr>
<tr>
<td>cross section, water, habitat, month, conditions</td>
<td>203.197</td>
<td>203.511</td>
<td>1.799</td>
<td>0.009655</td>
</tr>
<tr>
<td>cross section, length, habitat, month, conditions, water</td>
<td>204.29</td>
<td>204.732</td>
<td>2.892</td>
<td>0.015521</td>
</tr>
<tr>
<td>habitat, conditions, water</td>
<td>205.5</td>
<td>205.676</td>
<td>4.102*</td>
<td>0.022015</td>
</tr>
</tbody>
</table>

** indicates the model containing the lowest delta AIC value.

* indicates a simpler model containing an acceptable delta AIC value while containing less variables.

### Table 5: Best Fit Models for Predicting P. subflavus Presence. Top logistic linear regression models.

<table>
<thead>
<tr>
<th>Variables Included in Model</th>
<th>Raw AIC</th>
<th>AICc</th>
<th>Delta AIC</th>
<th>AICw</th>
</tr>
</thead>
<tbody>
<tr>
<td>month, water, conditions, habitat</td>
<td>201.398</td>
<td>201.606</td>
<td>0.0**</td>
<td>0.0</td>
</tr>
<tr>
<td>length, habitat, month, conditions, water</td>
<td>202.534</td>
<td>202.848</td>
<td>1.136</td>
<td>0.006097</td>
</tr>
<tr>
<td>cross section, water, habitat, month, conditions</td>
<td>203.197</td>
<td>203.511</td>
<td>1.799</td>
<td>0.009655</td>
</tr>
<tr>
<td>cross section, length, habitat, month, conditions, water</td>
<td>204.29</td>
<td>204.732</td>
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<td>0.015521</td>
</tr>
<tr>
<td>habitat, conditions, water</td>
<td>205.5</td>
<td>205.676</td>
<td>4.102*</td>
<td>0.022015</td>
</tr>
</tbody>
</table>

** indicates the model containing the lowest delta AIC value.

* indicates a simpler model containing a less acceptable delta AIC value, but contains less variables.

Predicting variable, ‘habitat’ was further investigated for significance within the habitat composition observed between culverts with bats present and culverts with bats absent (Figure 10). While habitat surrounding culverts surveyed varied greatly, no significant difference between surrounding habitat composition was detected.
Figure 10 | Habitat Surrounding Surveyed Culverts. Graphical representation of all habitat types documented around surveyed culverts based on observed bat presence. ‘Partially altered’ refers to habitat containing natural features and few anthropogenic features. ‘Altered’ refers to habitat containing few natural features and numerous anthropogenic features.

Representative Culverts

The diagonal measurements of representative culverts ranged from 3.72 m – 1.72 m (±0.70 m) and the mean culvert length was 53.58 m (±12.55 m). Representative culverts were surveyed for bat presence in June, September, October, November, and December of 2018, and January, February, March and May of 2019. Due to excessive flooding, culvert sites 31, 79, and 107 were inaccessible in December, culvert sites 31, 107, and 127 were inaccessible in January, and culvert site 107 was inaccessible in March.

*P. subflavus* was observed roosting in representative culverts in all months except June and September. During the February survey, there were significantly more *P. subflavus* observed roosting inside weep holes than observed free hanging on the culvert walls (p=0.03). While no other significant differences in roosting preference were observed, a consistently higher number of *P. subflavus* were
recorded clustering in weep holes then free hanging on the culvert walls for any given winter month (Figure 11).

Three individuals outfitted with unique bands were recovered over the course of the investigation. A non-reproductive male *M. austroriparius* (ID GA1969) and a non-reproductive male *P. subflavus* (ID GA0981) were banded at culvert site 109 in June of 2018 and January of 2018, respectively. A non-reproductive female *P. subflavus* (ID GA0988) was banded at culvert site 127 in March 2018. All three bats were recovered at culvert site 109 between December of 2018 and March of 2019. Bat GA1969 was recovered during both December and February surveys. Interestingly, bat GA0988 was originally banded by the U.S. Fish and Wildlife Service at culvert site 127, located near Dublin, Georgia in March of 2018 and recovered in culvert site 109, near Brunswick, Georgia, in December of 2018.

*Figure 11 | P. subflavus in Weep Holes. Percentage of all *P. subflavus* observed roosting in the weep hole orientation during December 2018, January 2019, February 2019, and March 2019. WH refers to bats observed roosting inside a weep hole. FH refers to bats observed roosting on the wall or ceiling. Consistently, a higher number of *P. subflavus* were observed roosting in the weep hole orientation than free hanging. Numbers on stacked graphs represent raw number of individuals observed.*
Temperature Data Loggers

Temperature data loggers deployed in the 12 representative culverts recorded temperature for a maximum of 321 days between June of 2018 and May of 2019 (Figures 12 – 23). Data loggers displaying less than 321 days of temperature data either malfunctioned or were added to the study at a later date.

Figure 12 | Average Daily Temperature in Culvert 28. Temperature collected by all three data loggers at culvert site 28 during deployment. Data logger location within the culvert described by “west, east, and center” labels.
**Figure 13** | Average Daily Temperature in Culvert 30. Temperature collected by all three data loggers at culvert site 30 during deployment. Data logger location within the culvert described by “west, east, and center” labels.

**Figure 14** | Average Daily Temperature in Culvert 31. Temperature collected by all three data loggers at culvert site 31 during deployment. Data logger location within the culvert described by “west, east, and WH” labels.
Figure 15 | Average Daily Temperature in Culvert 70. Temperature collected by all three data loggers at culvert site 70 during deployment. Data logger location within the culvert described by “west, east, and center” labels.

Figure 16 | Average Daily Temperature in Culvert 79. Temperature collected by all three data loggers at culvert site 79 during deployment. Data logger location within the culvert described by “west, east, and center” labels.
Figure 17 | Average Daily Temperature in Culvert 88. Temperature collected by all three data loggers at culvert site 88 during deployment. Data logger location within the culvert described by “center, east, and WH” labels.

Figure 18 | Average Daily Temperature in Culvert 107. Temperature collected by all three data loggers at culvert site 107 during deployment. Data logger location within the culvert described by “west, east, and center” labels.
Figure 19 | Average Daily Temperature in Culvert 109. Temperature collected by all three data loggers at culvert site 109 during deployment. Data logger location within the culvert described by “west, east, and WH” labels.

Figure 20 | Average Daily Temperature in Culvert 110. Temperature collected by all three data loggers at culvert site 110 during deployment. Data logger location within the culvert described by “west, east, and center” labels.
Figure 21 | Average Daily Temperature in Culvert 124. Temperature collected by all three data loggers at culvert site 124 during deployment. Data logger location within the culvert described by “west, east, and WH” labels.

Figure 22 | Average Daily Temperature in Culvert 125. Temperature collected by all three data loggers at culvert site 125 during deployment. Data logger location within the culvert described by “west, east, and center” labels.
During the winter months, temperatures in all 12 representative culverts fell within the *P. destructans* growth range of 0 – 20 °C (Blehert et al. 2009; Verant et al. 2012). Such temperatures were recorded for 163 - 200 days with a mean of 172.67 days (±11.7 days). Additionally, average daily temperatures within all 12 culverts fell between 12.5 °C – 15.8 °C, the optimal growth range of *P. destructans*. Temperatures were recorded in this range for 50 – 80 days with a mean of 57.42 days (±9.29 days). Temperatures within the *P. destructans* optimal growth range accounted for approximately 28-43 % of days spent below 20 °C, with a mean of 33.22 %. The mean days between 0 – 20 °C were similar in the coastal plains and coastal region with 176.66 and 168.66 days, respectively. Almost identical mean number of days spent in the optimal growth range were observed in culverts in the coastal plains and coastal, which were 57.16 and 57.66 days, respectively.

Temperatures inside weep holes fell within the *P. destructans* growth range of 0 – 20 °C. Growth range temperatures were recorded for 126 – 194 days with a mean of 164.75 days (±28.79 days). Additionally, temperatures collected in weep holes fell between the optimal growth range of *P. destructans*.
destrucans, 12.5 – 15.8 °C. Temperatures were recorded in this range between 36 – 112 days with a mean of 63.50 days (±34.11 days). Approximately 26 – 57% of all days spent under 20 °C, were within optimal growth range temperatures. Data loggers deployed inside weep holes in the coastal plains region collected an average of 160 days within growth range temperatures as compared to 169.5 days in the coastal region. Conversely, data loggers deployed inside weep holes in the coastal plains recorded a mean of 74 days within the optimal growth temperature range, as compared to 53 days recorded in the coastal region.

Only representative culvert 124 was found to have a significant variance of daily average temperature collected by the three data loggers (p=0.004). The four data loggers deployed inside weep holes collected significantly different temperatures from their respective free hanging counterparts (p<0.05) (Table 5). Additionally, the variance observed within average daily temperatures collected by data loggers deployed inside weep holes was consistently lower than their respective free hanging counterparts.

<table>
<thead>
<tr>
<th>Weep Hole Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
</tr>
<tr>
<td>Culvert Site 31</td>
</tr>
<tr>
<td>Culvert Site 88</td>
</tr>
<tr>
<td>Culvert Site 109</td>
</tr>
<tr>
<td>Culvert Site 124</td>
</tr>
</tbody>
</table>

*Table 6 | Weep Hole Temperature. Results of a t-test examining the average daily temperature collected by data loggers deployed within weep holes compared to the average daily temperature collected on the culvert wall.*

The weep hole data logger deployed in representative culvert site 109 collected significantly higher temperature data then the weep hole data loggers in representative culvert sites 124 and 88, but not representative culvert site 31. No significant difference was observed within temperatures collected by weep hole data loggers deployed in culverts 31 and 88 or between 88 and 124. Temperature collected by data loggers deployed in culverts 31 and 124 were less similar (Figure 24).
Figure 24 | Tukey’s Test Results. Graph depicts how similar the estimated mean temperatures collected by each weep hole are to each other. Means covered by the same bar are not significantly different. Weep hole refers to the culvert site containing a data logger deployed inside a weep hole. Estimate refers to the mean temperature estimate, based on the given data. Mean temperatures collected from June of 2018 – May of 2019 were considered for this analysis.

**qPCR**

During the course of this investigation (December of 2017 – March of 2019) Kennesaw State University (KSU), USFWS and DNR collected 249 swabs from culverts surveyed in the coastal plains and coastal region of Georgia. All 249 swabs were processed alongside positive and negative controls. No *P. destructans* positive detections were observed.

**DISCUSSION**

*P. subflavus* Ecology

Understanding how bats interact with fragmented habitat and overcome associated challenges can be useful for predicting movement and hibernation patterns. *P. subflavus* have traditionally been thought to employ a minimal geographic range, often hibernating and foraging in the same areas (Fujita and Kunz 1984). This implies that ideal *P. subflavus* habitat would contain both ample foraging area such
as bottomland forest and cavernous hibernation structures. However, more recent studies have
determined that *P. subflavus* actually migrate a much greater distance to hibernation sites then
originally assumed (Fraser et al. 2012). This strategy is believed to relieve the animal of the physiological
pressures associated with maintaining torpor during a long harsh winter and increase their chances of
survival (Fraser et al. 2012). If this latitudinal migration occurs in states like Georgia, overcoming the
challenges associated with the lack of cavernous habitat will be critical to survival. Within a patch-
corridor matrix of habitat, a non-traditional roost may be the safest option, offering a stable
environment for hibernation. Our observations could provide evidence supporting that *P. subflavus*
engage in regional latitudinal migration. In March of 2018, a non-reproductive female *P. subflavus* was
collected and given a unique band at culvert site 127, near Dublin Georgia. During the following
hibernation season, she was observed roosting at culvert site 109, near Brunswick Georgia. While her
summer foraging location is unknown, it could be assumed that she remained near culvert site 127 until
the fall months before migrating the 130 miles to the coastal region for hibernation.

Logistic linear regression models containing predicting variables ‘month’, ‘water’, ‘conditions’,
and ‘habitat’ were selected as models with the strongest ability to predict bat presence and *P. subflavus*
presence. The model containing ‘habitat’ ‘conditions’, and ‘water’ was able to provide comparable
support while including less variables. The simplest explanation is probably the most likely, thus the
model containing the least number of variables is generally selected as the model to best fit the data
(Burnham and Anderson 2002). A relatively small sample size prevented further analysis to determine if
a specific habitat or condition type influenced bat presence. Broadly, no significant difference was
observed between habitat characteristics surrounding culverts with bats present and culverts with bats
absent (Figure 10). Previous research reported an inverse relationship between the amount of non-
forested land and *P. subflavus* activity on a large geographic scale, suggesting that bat activity levels
decreased in areas that had been cleared for agriculture or residential use (Farrow et al. 2011).
Conversely, other investigators modeled bat presence in relation to landscape context, finding that *P.
subflavus* was more likely to be present in areas of sparse vegetation (Loeb and O’Keefe 2003). Based on
the literature, the landscape scale being considered is a critical component when assessing *P. subflavus*
response to habitat alteration and distribution.

Cluster Behavior
Hibernation strategies vary across species and individuals, each catering to a specific physiological need (Brack 2007). Some myotis species require a warm, thermally stable environment to minimize the amount of energy required to rewarm during arousal. Other myotis species such as *Myotis sodalis* (*Indiana bat*), are often observed roosting in small clusters in slightly cooler areas of the hibernacula (Brack 2007). *P. subflavus* have been consistently documented roosting solitarily in natural hibernacula (Fujita and Kunz 1984; Sandel et al. 2001; Brack 2007; Vincent and Whitaker 2007). During this investigation, *P. subflavus* was consistently documented roosting in clusters of 2 – 10 individuals inside weep holes. This unprecedented roosting behavior in a traditionally solitary species raises serious concerns regarding the potential transmission of *P. destructans* and development of white-nose syndrome. Pathogen transmission rates among species that roost in clusters are higher due to the constant skin-to-skin contact during the hibernation season (Hoyt et al. 2018). Species that commonly exhibit clustering behavior such as *M. lucifugus* and *M. septentrionalis* have experienced rapid disease transmission and associate population declines. In contrast, *P. subflavus* has experienced slower disease transmission, and associated population declines were not observed for several years after pathogen detection (Hoyt et al. 2018).

Additionally, *M. austroriparius* were often recorded roosting in the same culvert as *P. subflavus*. This species is commonly observed roosting in large clusters and can potentially act as an asymptomatic vector species for *P. destructans*. Only one case of a *M. austroriparius* developing white-nose syndrome has been recorded (U.S. Geological Survey 2017). These bats remain active all year rarely using deep torpor, allowing the spread of *P. destructans* spores during the winter months.

Temperature

Temperatures within roadway-associated culverts fell between the growth range of *P. destructans* for the equivalent of five months (172 days), indicating the potential for fungal growth and host infection during almost half of a calendar year. Within this time, the equivalent of almost two months (57 days) fell within the optimal growth range for *P. destructans*. During roughly one-third of the year, culverts in the coastal plains and coastal region of Georgia experience temperatures highly conducive to *P. destructans* growth. As expected, the average daily temperatures within coastal plains culverts were typically lower than culverts in the coastal region (Grider et al. 2016). Interestingly, temperatures collected in culverts in the southernmost plot and the northernmost plot were distinctly
different from the other plot in the respective region. Further investigation is required to characterize the geographic component of the temperature patterns within a culvert.

Additionally, culverts offer various microclimates. Data from this investigation suggests that bats roosting inside weep holes during the critical winter months, are exposed to significantly higher average daily temperatures than those roosting on the culvert wall or ceiling. Weep holes also offer more thermal stability, an environmental characteristic often appealing to a torpid mammal relying on fat reserves for survival (Hayman et al. 2016). While weep holes offered a warmer environment relative to the rest of the culvert, the average daily temperatures actually remained within the optimal growth range with less fluctuation (Figures 11-22), making weep holes the most susceptible microhabitat inside the culvert. Maintaining temperatures within 12.5 – 15.8 ºC for a multi-week duration provides the ideal environment for efficient *P. destructans* growth within the weep hole microhabitat (Verant et al. 2012).

**Disease Triangle**

The disease triangle is a concept that suggests an inherent relationship between the host organism, pathogen, and environment in which they interact (McNew 1960). Initially used as a general framework, it is now a fundamental tool for understanding and predicting epidemics in plants, animals, and humans. Epidemics such as *Batrachochytrium dendrobatidis* infection in amphibians, Malaria in humans, and the Panama disease in *Musa* spp., have been evaluated within the disease triangle framework (Scholthof 2007; James et al. 2015). In each context, the disease triangle model was used to explain how host susceptibility, environmental conditions and variation, and pathogen virulence influenced varying disease outcomes. The disease triangle model has only been applied to the white-nose syndrome epidemic in a laboratory context, investigating mortality in captive bats, or a theoretical context, building predictive models (Johnson et al. 2014; Hayman et al. 2016). Broadening the application of this concept to management activities could aid in identifying susceptible populations, predicting transmission corridors and preemptively implementing mitigation efforts.

Roadway-associated culverts can be evaluated for the potential to serve as disease transmission corridors within the context of the disease triangle. Our data confirms such structures provide an acceptable roosting environment for a highly susceptible host species, *P. subflavus*, during the hibernation season. Roadway-associated culverts in the southern region of Georgia can also provide an
acceptable environment for the survival of *P. destructans* and the development of white-nose syndrome. Data collected during this investigation suggests that the average daily temperature within culverts are highly conducive to *P. destructans* growth. Further, the data suggests that bats roosting inside weep holes located in the culvert ceiling could be at the highest risk for developing clinical WNS as the temperatures remain within the optimal growth range for weeks at a time. Also, disease severity could be exacerbated due to the novel clustering behavior observed in *P. subflavus* roosting under these conditions. Because this observation is unique to culverts, the risk of disease transmission may be higher than within natural hibernation structures. While *P. destructans* was not detected during the course of this investigation, it is evident that roadway-associated culverts provide ideal host and pathogen conditions for pathogen establishment and disease development.

Conclusions

In the southern region of Georgia, cavernous hibernations structures are sparse. In the absence of such structures, we hypothesized that non-traditional habitat, roadway-associated culverts, could serve as *P. subflavus* hibernacula. During this investigation, *P. subflavus* were observed roosting in roadway-associated culverts during the critical winter months, fall swarm months and one individual during the maternity season. Documenting *P. subflavus* over two consecutive years directly addressed this objective. We hypothesized that surveyed culverts would have characteristics favorable for the growth of *P. destructans* and the development of WNS in *P. subflavus*. Characterizing the temperature within culverts, documenting temperatures within the optimal growth range for *P. destructans*, directly addressed this objective. Finally, we hypothesized that roadway associated culverts in the coastal and coastal plains of Georgia will have the potential to serve as a transmission corridor for white-nose syndrome from karst regions of North Georgia to *P. subflavus* occupied karst environments in North Florida. Observing *P. subflavus* in culverts located along roadways in the coastal plains and coastal region during the winter months, roosting in clusters, and temperature characterization of microhabitats within culverts allowed us to directly address this objective. These novel observations across multiple months imply that roadway-associated culverts play a vital and persistent role in *P. subflavus* ecology and disease transmission in Georgia.
This investigation integrated methods and principles from multiple fields. The primary objectives were to understand the spatial spread of WNS and the roosting preference of *P. subflavus* populations potentially using roadway-associated culverts in the coastal and coastal plains regions of Georgia. The spatial distribution of a population is a primary characteristic of a species ecology. Knowledge of species-specific ecological niche, allows research to pose specialized questions regarding movement patterns or disease susceptibility. Understanding disease susceptibility requires monitoring of the host population and the pathogen. In this investigation the pathogen was monitored using methods commonly associated with microbiology and biochemistry related fields. Useful application of methods such as DNA extraction and qPCR require an integrated knowledge of the internal reaction, and the implications for various results. This investigation required methods often associated with community ecology, microbiology, and biochemistry. Information gleaned using principles from one field of study, can inform another field of study to ultimately address a complex question.


REFERENCES Linked references 71:475–479.


Appendix A. Bridge Survey Data Sheet

GA DNR, Nongame Conservation Section, 2065 US HWY 278 SE, Social Circle, GA 30025 Ph: 770-918-6411

GEORGIA BATS IN BRIDGES DATASHEET

Investigator Name(s): ____________________________________________
Phone: ___________________________ Email: __________________________
Date: ___________________________ County: __________________________
Lat: ___________________________ Long: __________________________

Bridge Location:

GDOT Structure ID # ________ GDOT PL No ________

Bridge Type: (check one)  
- ☐ Parallel Box Beam  ☐ Steel I-beam  ☐ Concrete
- ☐ Pre-stressed Girder  ☐ Flat Slab / Box  ☐ Corrugated Steel
- ☐ Cast in Place  ☐ Trapezoidal Box  ☐ Other: _____________
- ☐ Culvert – Box  ☐ Culvert – Round

Road Type: (check one)  
- ☐ Interstate  ☐ U.S. Highway  ☐ State Road  ☐ County Road

Surrounding Habitat: (check all that apply)
- ☐ Residential  ☐ Agriculture  ☐ Commercial  ☐ Woodland  ☐ Grassland  ☐ Ranching  ☐ Riparian  ☐ Mixed Use

Conditions Under Bridge: (check all that apply)
- ☐ Bare Ground  ☐ Concrete  ☐ Rip rap  ☐ Flowing Water  ☐ Standing Water
- ☐ Open Vegetation (not obstructing flight path)  ☐ Closed Vegetation (may obstruct flight path)
- ☐ Two Lane Road  ☐ Four (or more) Lane Highway  ☐ Dirt Road  ☐ Railroad

Bat indicators: (check all that apply)  
- ☐ Visual  ☐ Smell  ☐ Sound  ☐ Staining  ☐ Guano

Bats Present  ☐ YES  ☐ NO

Species Present
- Myotis septentrionalis (Northern long-eared)
- Myotis sodalis (Indiana)
- Myotis leibii (Eastern small-footed)
- Myotis lucifugus (Little brown)
- Myotis grisescens (Gray)
- Myotis auroriparius (Southeastern)
- Lasiusurus borealis (Eastern red)
- Lasiusurus seminolus (Seminole)
- Lasiusurus intermedius (Northern yellow)
- Lasiusurus cinereus (Hoary)
- Lasiusurus noctivagans (Silver-haired)
- Perimyotis subflavus (Tri-colored)
- Eptesicus fuscus (Big brown)
- Nycticeius humeralis (Evening)
- Tadarida brasiliensis (Braz. free-tailed)
- Corynorhinus flavescens (Rafinesque's)
- UNKNOWN

Roost description (if known, check all that apply):  
- ☐ Day Roost  ☐ Nursery Roost  ☐ Night Roost

*Please submit this data online at: [https://n3mqg.enierto.kobotoolbox.org/webform](https://n3mqg.enierto.kobotoolbox.org/webform) v20160421
Number of roosts ________________________________

Roost design: (check all that apply)

☐ Crack/crevice/expansion joint: underside of bridge  ☐ Crack/crevice/expansion joint: top side of bridge
☐ Plugged drain  ☐ Under/along the main bridge structure  ☐ Other: ________________________________

Human disturbance or traffic under bridge or at structure? ☐ High  ☐ Low  ☐ None

Evidence of bats using bird nests? ☐ Yes ☐ No (if yes, please describe and photograph nest location)

Areas Inspected: (check all that apply)

☐ Vertical surfaces on I-beams  ☐ Vertical surfaces between concrete end walls and bridge deck
☐ Expansion joints  ☐ Rough surfaces  ☐ Guardrails  ☐ Crevices  ☐ Other: ________________________________

Areas NOT Inspected because of safety or inaccessibility:

____________________________________________________________________________________

Additional Comments / Sketch:

____________________________________________________________________________________

Is there evidence of migratory birds using the structure? ☐ Yes ☐ No

If yes, what species (excluding pigeons) are present, what evidence is there, and locations (check all that apply)?

☐ Barn Swallow
  ☐ Old Nest  ☐ Adults  ☐ Building  ☐ Complete Nest  ☐ Eggs  ☐ Young  ☐ Unknown Stage
  ☐ concrete beam  ☐ steel beam  ☐ cap  ☐ pile/bent  ☐ rails  ☐ under deck, exterior sides  ☐ under deck, interior

☐ Cliff Swallow
  ☐ Old Nest  ☐ Adults  ☐ Building  ☐ Complete Nest  ☐ Eggs  ☐ Young  ☐ Unknown Stage
  ☐ concrete beam  ☐ steel beam  ☐ cap  ☐ pile/bent  ☐ rails  ☐ under deck, exterior sides  ☐ under deck, interior

☐ Eastern Phoebe
  ☐ Old Nest  ☐ Adults  ☐ Building  ☐ Complete Nest  ☐ Eggs  ☐ Young  ☐ Unknown Stage
  ☐ concrete beam  ☐ steel beam  ☐ cap  ☐ pile/bent  ☐ rails  ☐ under deck, exterior sides  ☐ under deck, interior

☐ Other: ________________________________

☐ Old Nest  ☐ Adults  ☐ Building  ☐ Complete Nest  ☐ Eggs  ☐ Young  ☐ Unknown Stage
  ☐ concrete beam  ☐ steel beam  ☐ cap  ☐ pile/bent  ☐ rails  ☐ under deck, exterior sides  ☐ under deck, interior
### Appendix B. Swab Collection Data Sheet

<table>
<thead>
<tr>
<th>Date</th>
<th>Culvert Site #</th>
<th>Swab ID #</th>
<th>Bat</th>
<th>Substrate</th>
<th>Species CODE</th>
<th>Band #</th>
<th>Notes</th>
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APPENDIX E - Protocol for Non-lethal Swab Sampling of Bat Skin for Detection of Pseudogymnoascus destructans (Pd)


Purpose: The following procedure is designed to detect the presence of Pd while minimizing harm to the sampled bat. This technique will NOT confirm White-nose Syndrome (WNS) on bats and should not be used as the sole sampling methodology in areas where WNS has not been previously confirmed in the bat population.

Materials
Provided by NWRC:
- Sterile and individually wrapped Puritan Pur-Wrap polyester-tipped swabs with plastic shafts (27)
- Sterile, nuclease-free, pre-labeled 2 ml microcentrifuge tubes, each containing 150 μl of sterile nuclease free water (25)
- 2 Ziplock bags (1 quart-size) for vial storage & trash
- Datasheet
- 1 Ziplock bag (1 gallon-size) for clean outer storage & packaging of sample vials and datasheet (do not carry bag inside hibernaculum)
- Insulated shipper box with 2 ice packs (for return shipment only; do not carry inside hibernaculum)

Needed:
- Disposable exam gloves
- Pencil or indelible ink pen
- Clipboard
- Decontamination supplies
- Cooler with ice for sample transport

Bat Swab Collection Protocol:
1. Persons collecting swab sample from bats or handling sample tubes should wear disposable exam gloves. It is not necessary to change gloves between each bat/sample tube provided the persons performing these tasks do not directly contact individual bats or inside rim of sample vial lid.
2. Identify a bat to be sampled.
3. Remove a pre-labeled sample tube from the Ziplock bag and record the requested individual bat information on the provided data sheet. Remember to include the unique Swab Vial # from the selected sample tube.
4. Tap sample tube to ensure all liquid is pooled at the bottom of the tube.
5. Remove a swab from its packaging without touching the polyester tip.
6. Dip the tip of the swab into the sample tube containing sterile water to moisten the tip (most water will be absorbed by the swab).
7. Bats may be sampled without removing them from their roosting location. If direct handling of the bat is required for other work, hold bat face down with one wing pulled slightly away from the body at the elbow.
8. 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.
9. Roll the same swab across the muzzle of the same bat 3 times.
10. Place the swab tip into the same sample tube used to moisten the swab, and break off the protruding plastic shaft of the swab without touching the rim of the tube or inside of lid with your fingers. Close the lid of tube tightly.
11. Place sample tube containing swab into the second Ziplock bag (1 quart-size) maintained at ambient cave temperature.
12. Dispose of swab handles, paper wrappers and contaminated exam gloves as necessary into third Ziplock bag (1 quart-size).
13. Repeat the above process for each bat sampled.
14. Upon exiting the hibernaculum but prior to leaving the area, place the datasheet inside of the emptied Ziplock bag (1 quart-size). Decontaminate the outer surfaces of all Ziplock bags taken inside the hibernaculum following current USFWS Decontamination Guidelines. Place the Ziplock bags containing all sample tubes and datasheet inside the clean Ziplock bag (1 gallon-size) for storage and shipment. Ensure all excess air is removed from the bags.
15. Following removal of collected samples from the hibernaculum, store them on ice for transport to an office refrigerator or freezer.

Sample Storage:
Hold swab samples chilled (4°C) if they are to be shipped within 2 days following collection; otherwise freeze samples at -20°C until they are shipped. If you are sampling multiple sites, samples can be stored frozen to facilitate batch shipping at your convenience. Avoid multiple freeze-thaw cycles.

Sample Shipment:
Package bagged samples between frozen ice-packs for shipment by overnight courier to the USGS – National Wildlife Health Center. Ensure that ice-packs are frozen solid prior to sealing the package for shipment. Ship early in the week to avoid weekend deliveries (DO NOT ship on Fridays or the day before a holiday). Notify Anne Ballmann (608-270-2445; aballmann@usgs.gov) with the courier service and package tracking number of the return shipment.

Ship samples to:
USGS – National Wildlife Health Center
Necropsy Loading Dock
Diagnostic Microbiology
6006 Schroeder Road
Madison, WI 53711
608-270-2400 (emergency contact number)
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!

A. 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. As stated earlier, all clothing layers should still be decontaminated or otherwise cleaned and dedicated after every use.

Disposable items, regardless of condition, should not be reused. 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.

B. 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 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.