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UTILIZING MOLECULAR AND STATISTICAL MODELING METHODS TO ENHANCE WHITE NOSE SYNDROME DETECTION IN BAT HIBERNACULA

Samuel Miller
Pittsburg State University, slmiller@gus.pittstate.edu

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Samuel Miller

Pittsburg State University

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Samuel Miller

APPROVED:

Thesis Advisor _____
Dr. Peter Chung, Biology

Committee Member _____
Dr. Andrew George, Biology

Committee Member _____
Dr. Kristopher Mijares, Chemistry

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UTILIZING MOLECULAR AND STATISTICAL MODELING METHODS TO ENHANCE WHITE NOSE SYNDROME DETECTION IN BAT HIBERNACULA

An Abstract of the Thesis by
Samuel Lee Miller

White Nose Syndrome (WNS) is a fungal infection in bats caused by *Pseudogymnoascus destructans* (*Pd*). Successfully identifying hibernacula infected with WNS is essential to help control and regulate the spread of WNS. Assessing the presence of WNS in bat hibernacula is usually done by visually confirming *Pd* on hibernating bats within infected hibernacula. This can be problematic because most visual confirmation occurs during the winter when bat populations are at their peak within hibernacula. When surveys are conducted in the winter, there is an increased chance of disturbing bats. One alternative method to visually confirming WNS on bats in the winter is to test the hibernaculum environment for *Pd* DNA in the summer. This study compared a Polymerase Chain Reaction (PCR) assay for *Pd* DNA to previous visual surveys for WNS within six bat hibernacula in southwest Missouri and southeast Kansas. Six quadrant were identified and sampled within each hibernaculum. Samples were taken, DNA was extracted, and PCR was performed to DNA specific to *Pd*. Agarose gel electrophoresis was utilized to verify if there was *Pd* DNA present in the amplified PCR product. In addition, this study created a WNS predictive model to determine the probability of WNS presence Missouri counties. This study found that *Pd* DNA was present in all hibernacula previously described as WNS positive by visual confirmation, in addition, this study found that one hibernaculum was misidentified as WNS negative. This study also predicted the probability that Missouri counties had WNS. The WNS

predictive model was also tested in-field at seven different hibernacula in six different counties with 85% success.

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CHAPTER I.

INTRODUCTION

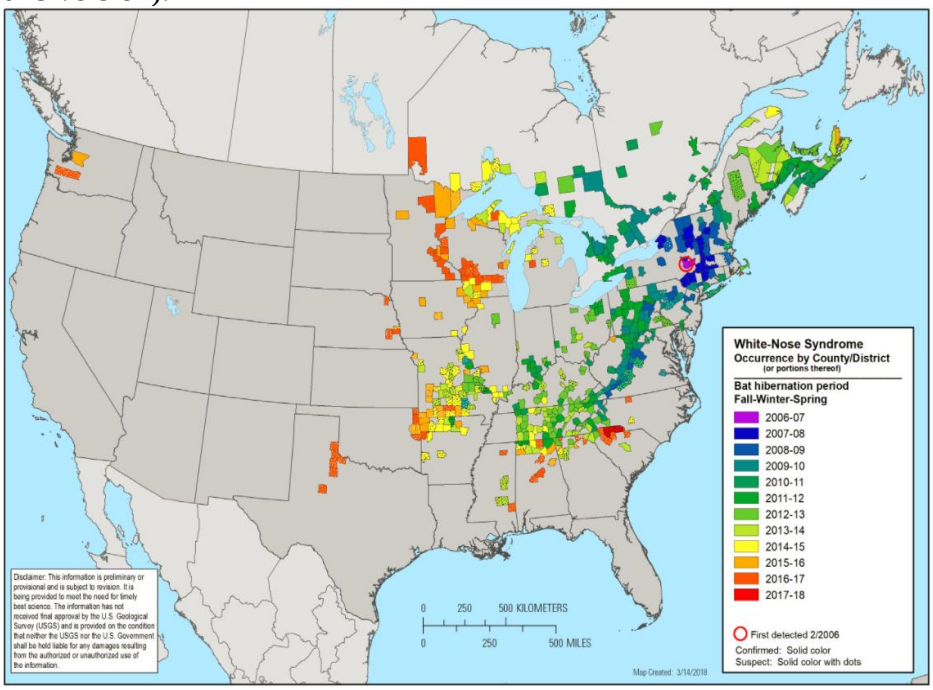
White Nose Syndrome (*Pd* Infestation): A North American Problem of European Origin

Pd is the fungal pathogen that causes White Nose Syndrome (WNS) in bats, which was first documented in the United States during the winter of 2006—2007 (Blehert *et al.* 2009; Garagas *et al.* 2009; Frick *et al.* 2010; Kunz *et al.* 2011). *Pd* was taxonomically first described as *Geomyces destructans* (Gargas *et al.* 2009; Blehert *et al.* 2009) but was taxonomically re-described later as *Pseudogymnoascus destructans* after a much closer, molecular approach was taken by scientists to examine its phylogeny (Minnis and Lindner 2013). *Pd* is now officially known as the sole causative agent of WNS in bats (Lorch *et al.* 2011; Warnecke *et al.* 2012; Raudabaugh and Miller 2013) and is responsible for an estimated six million bat deaths in the United States between 2006 and 2013 (Blehert *et al.* 2009; Gargas *et al.* 2009; Hayes 2012). It is currently devastating populations of bats (Shuey *et al.* 2013).

The current scientific literature (Micalizzi *et al.* 2017) suggests that *Pd* is not endogenous to North America but rather is an invasive species (Warnecke *et al.* 2012; Norquay *et al.* 2013). *Pd* is readily documented in bats that are endogenous to Europe (Wibbelt *et al.* 2010, Wibblet *et al.* 2011, Warnecke *et al.* 2012). However, European

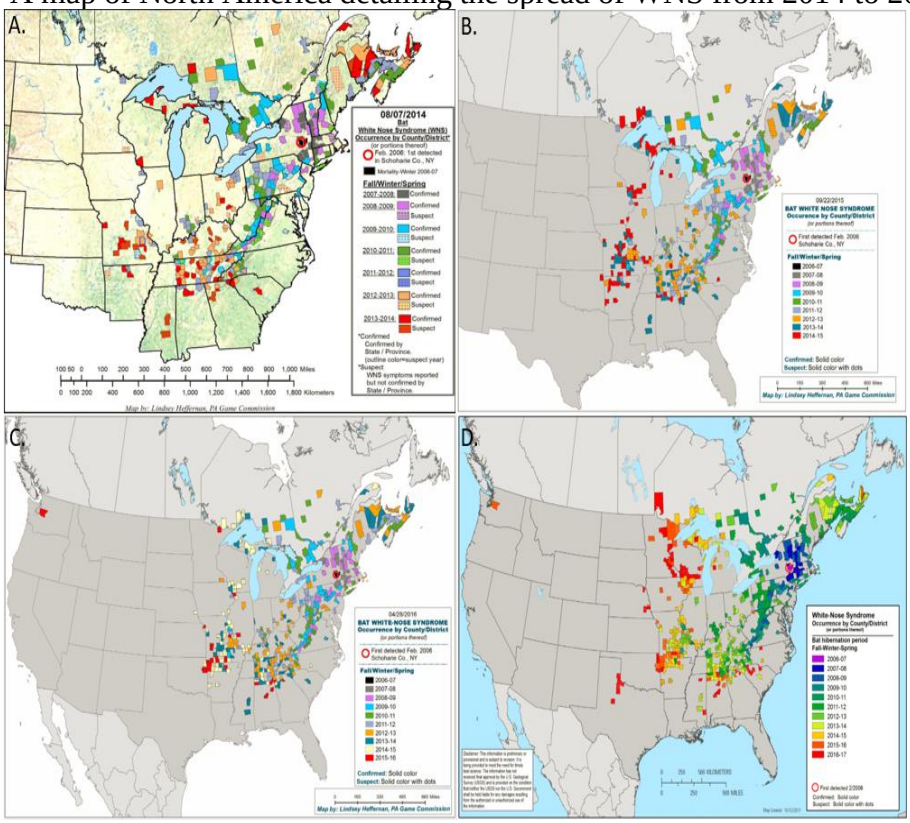
bats do not face the extreme mortality that North American bats do when infected with *Pd* (Wibbelt *et al.* 2011). It is believed that since *Pd* is endogenous to the European continent, European bats coevolved and developed immunological defenses to the *Pd* (Wibbelt *et al.* 2013). Since first documented in the State of New York in 2007, (Lorch *et al.* 2013a) near Howe Caverns, *Pd* has spread to twenty-two states in the continental United States and five Canadian Provinces by 2013 (Lorch *et al.* 2013a). As of 2018 (White-Nose Syndrome 2018), the disease has spread to thirty-one states in the continental United States and five Canadian Provinces, including Kansas (Figure 1). WNS has also been documented in several of the states that border Kansas, including Missouri. Arkansas, Nebraska, and Oklahoma. The rapid spread of WNS (Figure 2) coupled with the extreme mortality associated with bats has led to grave concerns about bat populations' and their viability.

Figure 1: A map of North America detailing the spread of WNS from 2006 to 2018 (April, 2018 version).



This figure was taken from https://www.whitenosesyndrome.org/resources/map.

Figure 2: A map of North America detailing the spread of WNS from 2014 to 2017.



A. represents the spread of WNS as of 2014. B. represents the spread of WNS as of 2015. C. represents the spread of WNS as of 2016. D. represents the spread of WNS as of 2017. This figure was taken from https://www.whitenosesyndrome.org/resources/map.

Pd: Wreaks Havoc on North American Bat Populations

According the United States Fish and Wildlife Service (USFWS) there are approximately forty-seven species of bats in North America, of which seven are federally endangered, including *Myotis grisescens* (Gray Bat), *Lasiurus semotus* (Hawaiian Hoary Bat), *Myotis sodalis* (Indiana Bat), *Leptonycteris curasoae yerbabuenae* (Lesser Long-nosed Bat), *Leptonycteris nivalis* (Mexican Long-nosed Bat), *Corynorhinus townsendii ingens* (Ozark Big-eared Bat), and *Corynorhinus townsendii virginianus* (the Virginia Big-eared Bat). According to the United States Forest Service and Muller *et al.* (2013), seven bat species in North America have had confirmed presence of *Pd* and WNS related afflictions (Table

1). However, an additional six species of bats have had confirmed presence of *Pd* without documented evidence of WNS related afflictions (Table 1).

Table 1: North American bat species: their current known WNS status and susceptibility.

Bat Species	**Common Name**	**Documented WNS Positive**	**Susceptible to WNS**
Eptesicus fuscus	Big brown bat	X	X
Myotis leibii	Eastern small-footed bat	X	X
Myotis grisescens	Gray bat	X	X
Myotis lucifugus	Little brown bat	X	X
Myotis septentrionalis	Northern long-eared bat	X	X
Myotis austroriparius	Southeastern bat	X	X
Perimyotis subflavus	Tricolored bat	X	X
Myotis yumanensis	Yuma bat	X	X
Lasiurus borealis	Eastern red bat	X	
Lasionycteris noctivagans	Silver-haired bat	X	
Corynorhinus rafinesquii	Rafinesque's big-eared bat	X	
Corynorhinus townsendii virginianus	Virginia Big-Eared Bat	X	
Myotis velifer	Cave bat	X	
Corynorhinus townsendii	Townsend's big-eared bat	X	

WNS has been documented to cause severe and sometimes fatal lesions as well as necrosis on the skin of bats during their hibernation periods. WNS is characterized by a white fungal growth on the nose and/or wings of hibernating bats (Cryan *et al.* 2010). A hallmark characteristic of WNS is that it infects multiple species of bats. All hibernating bat species in North America could potentially be infected with *Pd* and develop WNS (Turner *et al.* 2011; Langwig *et al.* 2012). However, the way in which those populations of bats would be impacted is hyper-variable and will remain so until the molecular mechanisms that enable those certain bats to remain asymptamatic carriers.

Pd: **How It Affects Bats**

The exact molecular mechanisms of how *Pd* affects bats are currently unknown. However, *Pd* can infect bats that are hibernating due to the internal body temperatures of bats being reduced to slightly above ambient cave temperatures of two degrees Celsius to ten degrees Celsius (Blehert *et al.* 2009). Infected bats will display an increased depletion of their fat reserves (O'Donoghue *et al.* 2015) leading to emaciation and death. In addition to emaciation, *Pd* can alter the ability of bats to fly by damaging their wing membranes and alter the ability of bats to respire leading to respiratory acidosis (Verant *et al.* 2014), all this in addition to promoting severe dehydration in bats (Cryan *et al.* 2010; Wibbelt *et al.* 2011).

The results of physiological studies of bats favor the conclusion that cutaneous infections on the wings of bats due to WNS are responsible for certain physical and metabolic changes (Cryan *et al.* 2010). High hematocrit levels, consistent with increased fluid loss, along with decreased levels of electrolytes have been documented in bats with

WNS (Warnecke *et al.* 2013). Bats are unable to replenish lost water and electrolytes due to a lack of sources for both within their normal hibernacula.

The body temperatures of bats while hibernating will range from two degrees Celsius to ten degrees Celsius, which is close to the optimal growth temperatures for *Pd* (Blehert *et al.* 2011). At those temperatures, the bats' immune systems are suppressed and unable to resist *Pd* infection (Hayes 2012). Bats, like most other animals, will down-regulate their immunological functions during the winter while they hibernate (Meteyer *et al.* 2009; Moore *et al.* 2011). This has been documented in conjunction with evidence that bats are unable to initiate an inflammatory response or recruit immune cells (Meteyer *et al.* 2012) if they are infected with *Pd*.

Pd affects bats by increasing the incidences of arousal during hibernation (Reeder *et al.* 2012; Warnecke *et al.* 2012). The increase in these waking events causes bats to expend unnecessary energy (Thomas *et al.*1990); bats that are infected with WNS will utilize more of their fat reserves than un-infected bats. Infected bats are unable to replenish their fat reserves due to the fact that they are hibernating. Almost all bat affected by WNS, are temperate organisms, and their diet relies heavily on the consumption of insects (Paul *et al.* 2012). This becomes a problem when bats are aroused earlier in the year and are forced leave their hibernacula more frequently than necessary when their food sources are absent in the winter months. Bats enter their hibernacula sites in the fall and are not observed outside the hibernacula until mid-spring. Bats that infrequently leave their hibernacula during the winter, and only do so to switch roosts, search for water, or search for food (Boyles *et al.* 2006). Once WNS develops, bats will exhibit abnormal winter behaviors such as daytime and cold-weather flight during their hibernating periods.

If a bat infected with *Pd* survives the hibernating months, both the bat's internal temperature and immune system will return to normal, and the *Pd* infection will subside (Meteyer *et al.* 2011). However, bats can exhibit immune reconstitution inflammatory syndrome (IRIS). IRIS develops when infected bats emerge from their hibernation period and their immune systems exhibit an overly robust reaction to the fungus present on their bodies (Chaturvedi *et al.* 2010; Puechmaille *et al.* 2011). Even if a bat can survive long enough to emerge and feed after the hibernation period, *Pd* can still contibute to the death of the bat.

Pd and *M. lucifugus* (the little brown bat)

The little brown bat (*M. lucifugus*), is one of the most wildly distributed, numerous species in North America. There has been a dramatic decline in populations of *M. lucifugus* in recent years, which has been directly attributed to *Pd* infestations in bat hibernacula (Kunz *et al.* 2011). Declines in populations of *M. lucifugus* have been as dramatic as 88% (Turner *et al.* 2011) in some areas. The population of *M. lucifugus* has suffered the greatest losses and thus the largest decline of any North American bat species (Blehert *et al.* 2009; Foley *et al.* 2011; Wilder *et al.* 2011). There have been predictions that *M. lucifugus* could go extinct within the next seven to thirty years (Frick *et al.*2010) with up to three additional bat species facing the same fate if nothing is done to slow the spread or progression of this disease. If an infected bat enters an uninfected hibernaculum, all susceptible bats within that hibernaculum will become infected within two to three years (Frick *et al.* 2017). *Pd* has been cultured in a lab setting after a five-year absence from bats, documenting that it can persist for long-term periods in the

environment (Hoyt *et al.* 2015b) and preventing bats from successfully recolonize previously infected hibernacula.

Bats and their ecological and economical impact

Bats are documented to play critical roles in many ecosystems (Moussy *et al.* 2013). Bats are ferocious predators of insect pests. A single colony of (approximately 150 bats) *E. fuscus*, has been estimated to eat approximately 1.3 million insects per year (Storm and Whitaker 2008). A single *M. lucifugus*, while weighing between five and fourteen grams can consume between four to eight grams of insects per night of active feeding (Edythe and Kunz 1990; Kurta *et al.* 1990). It has been estimated that due to a loss of bats from *Pd* an already estimated 660 to 1320 metric tons of insects per year are no longer being eaten (Boyles *et al.* 2011). Bats also eat insects that cause crop damage, in American agriculture, it is estimated that bats prevent the loss of approximately 3.7 billion US dollars annually (Boyles *et al.* 2011). Whereas, globally in Corn, bats prevent the loss of approximately 1 billion US dollars annually (Main & Boyles 2015).

Bats are also valuable for non-anthropocentric reasons. The impacts from WNS have the potential to affect numerous species of bats across North America. Bats are the second most species rich mammal order in the world (Wilson & Reeder, 2005). The USFWS categorizes cave dwelling bats as a keystone species. A keystone species is any species that is critical to the survival and persistence of other species within the same spatial ecosystem (Garibaldi & Turner 2004). They are categorized as a keystone species due to the amount of guano they produce being a driving force of energy for biologic activity inside cave ecosystems.

Managing the Spread and Regulating *Pd*

Currently, there are no chemical or biological measures developed that eliminates the growth of *Pd* in the environment or on infected bats (Boire *et al.* 2016), however, several research groups are working on methods of eliminating *Pd* growth. The USFWS recommends closing bat hibernacula to human access to prevent the spread of *Pd* into bat hibernacula that are currently not infected. However, the USFWS does consider it possible that *Pd* could colonize most applicable bat hibernacula in the United States if no measures are found to counteract its spread *Pd* can be transported into caves through numerous vectors, including: humans, weather, and small mammals but primarially bats are the main vector for the spead of WNS (Vanderwolf *et al.* 2016). Caves are known to be reservoirs for *Pd* (Raudabaugh and Miller 2013) even when bats are not hibernating (Lorch *et al.* 2013a) in the summer months. In a lab setting, *Pd* is documented as only being able to spread via direct contact and not airborne means of transmission (Lorch *et al.* 2011). . It is believed that in climates more conducive to the growth of *Pd*, in North America, that WNS will inevitably spread to all bat caves that serve as hibernacula.

Confirming the presence of *Pd* on bats in hibernacula

A confirmed case of WNS on a bat is defined by having the presence of cupping erosions on the suspected bat's skin caused by *Pd*. The determination is made via examination of tissue histopathology (Meteyer *et al.* 2009). This method is time-consuming and requires a large amount (1.5cm^2 to 3.0cm^2) of a bat's wing tissue (Lorch *et al.* 2010). Usually this test is restricted to dead bats or results in the euthanasia of bats. However, this is not always the case, as less invasive wing punches can be performed for biopsies. In addition to histopathological detection, another tool researches can is

polymerase chain reaction (PCR). Identifying *Pd* using PCR assays is widely documented in the literature (Lorch *et al.* 2010, Chaturvedi *et al.* 2010, Lorch *et al.* 2013a, Raudabaugh & Muller *et al.* 2013 Creecy et al 2015, Shuey *et al.* 2014, Young *et al.* 2017). This method can be less time-consuming and only requires $3mm^2$ to $3.3mm^2$ of bat wing tissue (Muller *et al.* 2013). Both methods have positive and negative aspects. It is most affective to combine the results of visual surveys for WNS with molecular analysis of *Pd* DNA (Muller *et al.* 2013). When utilized in conjunction with one another, these two tests work together as reliable WNS detection tools.

Ecological modeling

Modeling has become an increasingly popular tool in recent years for many scientists, especially ecologists. Ecological models are used by scientists in a variety of different ways. Some are used to predict the effects of climate change on animal populations (George *et al.* 2015). Others are used to determine management practices for curbing the spread of WNS (O' Regan *et al.* 2015). WNS models are created to gain a better understanding of where the disease might be progressing. However, most WNS prediction models are often created on a large-scale (Maher *et al.* 2012), and thus aren't always useful in determining the presence or absence of WNS on smaller scale.

Proposed thesis project

Visual surveying is currently recommended as the procedure for identifying WNS in bat hibernacula by the USFWS WNS National Response Plan and the Canadian Wildlife Heath Cooperative WNS National Plan. This is most likely due to the low cost of visual, ecological surveys (Stallknecht *et al.* 2007 & Sleeman 2013). However, studies have increasingly been detailed surveying for the presence or absence of WNS in bat

hibernacula using visual surveying methods in conjunction with PCR methods (Linder *et al.* 2011, Lorch *et al.* 2010, Lorch *et al.* 2013a, & Muller *et al.* 2013), to provide a more enhanced method of detection. However, only visual surveying methods for the presence or absence of WNS on bats have been conducted in the past for the selected hibernacula in this study. In addition, visual surveys for the presence or absence of WNS on bats are usually conducted in the winter months when bat populations are at their peak within the hibernacula. This can be problematic, more invasive, and has the potential to negatively affect bats by accidently arousing them while inside of the hibernacula.

To confirm the results of the visual and molecular surveys in the lab, samples of suspected *Pd* are collected from bat tissues or cave surfaces and cultured. If the sample is taken from bat tissue, this usually denotes a bat mortality. DNA is then extracted, sequenced, and blasted to confirm the collected sample was indeed *Pd* DNA. This sample is then compared to a previously identified pure culture of *Pd* that has been purchased through a reputable company or university. Both the visual surveying method and the molecular method have involved actively culturing *Pd*. *Pd* is cultured at 10°C for 38 days on sabouraud dextrose agar (https://www.atcc.org/Products/All/MYA-4855.aspx#documentation) until it can be visually identified by someone trained in both mycology and microscopy (Lorch *et al.* 2010).

WNS is rapidly colonizing previously undocumented counties in the United States. This coupled with an inability to produce and implement precautions and protocols to aid in stopping and/or slowing the colonization of WNS in bat hibernacula. Prediction models have given some insight into the time that it would take WNS to colonize hibernacula within a given county. However, those models are drawn up on a

large scale, and in states like Missouri, the spread can be underpredicted (Maher *et al.* 2012). If these counties go undetected or are ignored in those models, then WNS will cause unnecessary bat deaths before it is ever detected (Ingersoll *et al.* 2016). Never have any of the SPG managed caves in this study been tested for the presence of *Pd* DNA in the cave environment via PCR. The Pittsburg Storm Sewer was tested for *Pd* DNA and results did not show that the hibernacula contained any *Pd* DNA in the summer of 2017. This allowed for a less invasive survey of WNS within the hibernacula.

This study utilized a non-culture dependent method for confirming the presence of *Pd* DNA within hibernacula. The non-culture method involved ordering a custom created GeneBlock from Integrated DNA Technologies (IDT) that was 100% identical to *Pd* DNA (Genebank ID EU884924.1). The turnaround time for WNS results was approximately three hours per cave. This study details a molecular survey for the presence or absence of *Pd* DNA in six bat hibernacula (five caves and one storm sewer) in southeast Kansas and southwest Missouri within hibernacula previously regarded as WNS positive and WNS negative.

A WNS predictor model specific for Missouri counties is needed to help slow the spread of WNS through this biologically important state. The WNS predictor model in this study utilized biologically relevant data to determine the probability that a given county would contain bat hibernacula with WNS. County cave density, median elevation, highest elevation, lowest elevation, number of adjacent WNS positive counties, average time since WNS was detected in adjacent counties, county population, the north latitude of the center of the county, the west longitude of the center of the county, and county area were use as predictor variables to generate the WNS predictor model. This model was

tested by testing for *Pd* DNA within one hibernacula within one county with a low probability of being WNS positive and testing for *Pd* DNA within one hibernacula within one county with a high probability of being WNS positive.

The goals of this study were: to confirm the presence of *Pd* DNA within bat hibernacula that had been previously described by SPG as being WNS positive by visual survey methods, to confirm the absence of *Pd* DNA within bat hibernacula that had been previously described by SPG as being WNS negative by visual survey methods, and to create and validate a WNS predictor model that would help identify counties of interest within Missouri that are currently misidentified as being WNS negative.

This study hypothesized that bat hibernacula that had been previously described by SPG as being WNS positive by visual survey methods would contain *Pd* DNA, that bat hibernacula that had been previously described by SPG as being WNS negative by visual survey methods would not contain *Pd* DNA, and that the WNS predictor model would accurately predict the presence of WNS in counties already infested with WNS and generate the probability of having WNS in any given county in Missouri.

CHAPTER II.

MATERIALS AND METHODS

Safety precautions

During this study great care was taken to ensure that no environmental contamination took place. The National White-Nose Syndrome Decontamination Protocol Version 04.12.2016 was used to ensure that no contamination occurred between hibernacula. Great care was taken to ensure the environmental safety of the bat hibernacula and the personal safety of all individuals who participated in this study. When obtaining samples from inside of bat hibernacula, personal protective equipment was always worn. This included, but was not limited to a hard hat, appropriate clothing, and appropriate footwear.

Sample site selection

This study worked cooperatively with the Springfield Plateau Grotto (SPG), according to the SPG website (http://www.spgcavers.org/):

> The SPG is made up of southwest Missourians who share an interest in the conservation, survey, and management of caves. A project-oriented grotto, our members work in tandem with public agencies and private landowners to promote a better understanding of caves and foster awareness of the importance of conserving the cave environment. SPG collaborates with the Missouri Speleogical

Society (MSS) to document, map, and study caves. SPG is a federally recognized 501©(3) non-profit organization and a member grotto of the National Speleological Society (NSS) and the MSS.

With the blessing of the SPG and under the supervision, of Jonathan Beard, former SPG President (2006-2010 and 2013), SPG secretary (2015 - present), and current SPG treasurer, this study gained access to the Missouri Cave Database (MCD), which is a state resource that contains information on over 7,000 of Missouri's caves. The information accessed from the MCD was used to select bat hibernacula. Five of the hibernacula in this study are actively managed by the SPG. Through a cooperative effort, all the necessary samples were obtained from the SPG managed hibernacula. A bat hibernaculum was defined, for the purposes of this study, as a location where bats had hibernated. Six total bat hibernacula were sampled in this study, including five caves and one storm sewer system. Five of the caves were selected for sampling using the MCD. Three of the selected caves had bats that exhibited WNS, and the two other caves did not have bats that exhibited WNS. Whether or not the bats exhibited WNS was determined by visual surveying methods. The storm sewer was treated as a hibernaculum that did not have bats that exhibited WNS.

The hibernacula selected for sampling were Breakdown Cave, Big Bear Cave, Fitzpatrick Cave, Bluff Dwellers Cave, Pittsburg storm sewer system, and Shoal Creek Cave. The hibernacula that had bats that exhibited WNS and had previously tested positive for WNS were (Table 2): Big Bear Cave, Breakdown Cave, and Fitzpatrick Cave. The hibernacula that did not have bats that exhibited WNS and had not tested positive for WNS were (Table 2): Bluff Dwellers Cave, Pittsburg storm sewer system,

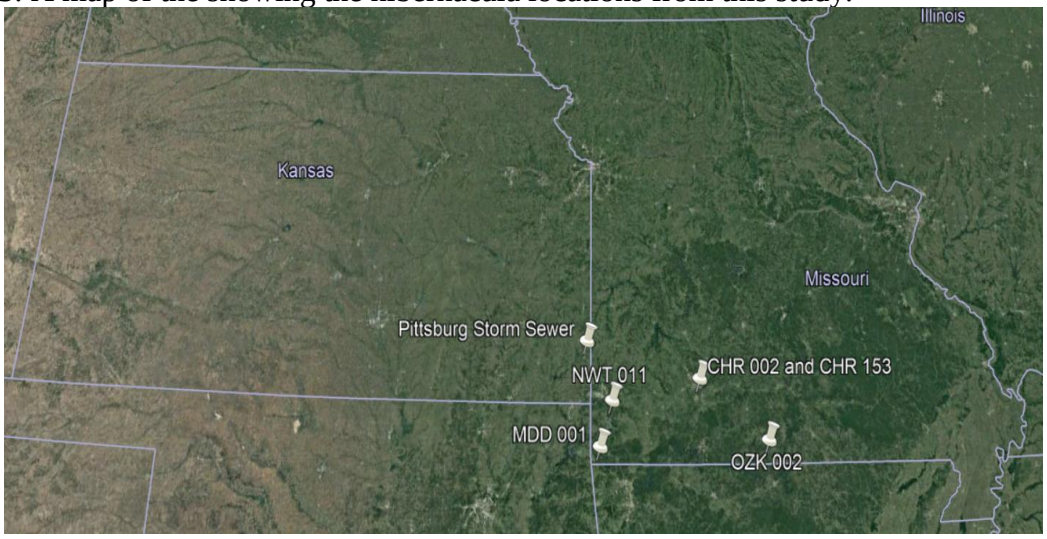
Shoal Creek Cave. The Pittsburg storm sewer system is the only hibernacula in this study from Kansas; the other five hibernacula were in Missouri (Figure 3).

Table 2: Information regarding the location of hibernacula and their current known of WNS status as of July, 2017.

Hibernacula	Cave Accession Number	State and County	Status of WNS (WNS + or WNS -)
Bluff Dwellers Cave	MDD001	Missouri, McDonald	WNS -
Pittsburg storm sewer	PSS001 *	Kansas, Crawford	WNS - **
Shoal Creek Cave	NWT011	Missouri, Newton	WNS -
Breakdown Cave	CHR153	Missouri, Christian	WNS +
Fitzpatrick Cave	CHR002	Missouri, Christian	WNS +
Big Bear Cave	OZK002	Missouri, Ozark	WNS +

Note that * and ** indicate that the criteria were unable to be met in the hibernacula. Pittsburg storm sewer does not have a cave accession number (*).Pittsburg Storm Sewer had never been tested using molecular methods for the presence of WNS until this study(**). However, due to there being no confirmed cases of WNS in the entire state of Kansas, it was considered negative for WNS.

Figure 3: A map of the showing the hibernacula locations from this study.



The counties included, in Kansas, Cherokee. The counties included, in Missouri: Christian, McDonald, , Newton, and Ozark. This figure was constructed utilizing Google EarthPro software.

Due to the sensitivity of cave ecosystems, the exact locations for the caves in this study have not been given to help protect them from unwanted, harmful exploration. However, generalized locations have been given throughout this thesis.

Caves that were selected met the following criteria (Table 3). The caves needed to be contained within the same (as similar as possible) physiogeographic region(s), be formed within the same (as similar as possible) geologic era of formation, be contained within the same (or as similar as possible) host rock, and serve as a hibernaculum for *M. grisescens, M. lucifugus,* or *P. subflavus*. The purpose of this cave selection process was to ensure that the caves sampled were as similar as possible. The three hibernacula previously considered WNS positive had some *M. grisescens, M. lucifugus,* or *P. subflavus* from 2015 to 2017 that exhibited WNS. Whereas, the three hibernacula previously considered WNS negative were did not have any *M. grisescens, M. lucifugus,* or *P. subflavus* from 2015 to 2017 that exhibited WNS.

To be considered positive for WNS, the hibernacula had to be documented with the MCD in 2015, 2016, and 2017 as having any *M. grisescens, M. lucifugus*, or *P. subflavus* surveyed exhibiting WNS. To be considered negative for WNS, the hibernacula had to be documented with the MCD in 2015, 2016, and 2017 as having all *M. grisescens, M. lucifugus,* and *P. subflavus* surveyed did not exhibit WNS whatsoever. Pittsburg Storm Sewer was considered negative due to the absence of WNS from the entire state of Kansas. It should also be noted that Pittsburg Storm Sewer has not been actively surveyed for bat abundance in the last 25 years. However, evidence from recent, preliminary studies (conducted by Dr. Andrew George, Pittsburg State University) in 2016 and 2017, have documented a presence of *M. grisescens* and *P. subflavus*.

Table 3: Criteria for cave selection.

Physiogeographic Region	Springfield Plateau or Salem Plateau
Host Rock	Limestone or Dolomite*
Geologic Era of Formation	Carboniferous period (Mississippian and Pennsylvanian subperiods)*
Serves as bat Hibernacula	Serves as Hibernacula for *M. grisescens*, or *M. lucifugus*, or *P. subflavus* for the past three years **
WNS positive	*M. grisescens*, or *M. lucifugus*, or *P. subflavus* exhibited WNS 2015-2017
WNS negative	*M. grisescens*, or *M. lucifugus*, and *P. subflavus* has not exhibited WNS 2015-2017**
Note that * and ** indicate that the criteria were unable to be met in the hibernacula. The Pittsburg storm sewer is a man-made system, and not a natural geologic process, and thus cannot have a geologic era of formation and/or host rock (*).The Pittsburg storm sewer has not been actively survived for the past 25 years, however, recent, preliminary studies (2016 and 2017) have confirmed bats are present in the Pittsburg storm sewer (**).	

Experimental design

Six total quadrant were identified within each of the hibernacula representing specific locations within each hibernaculum. The quadrant were standardized so that comparisons could be drawn between the caves sampled. All the quadrant were oriented, such that they were confined of their own respective meter squared ($1m^2$) area.

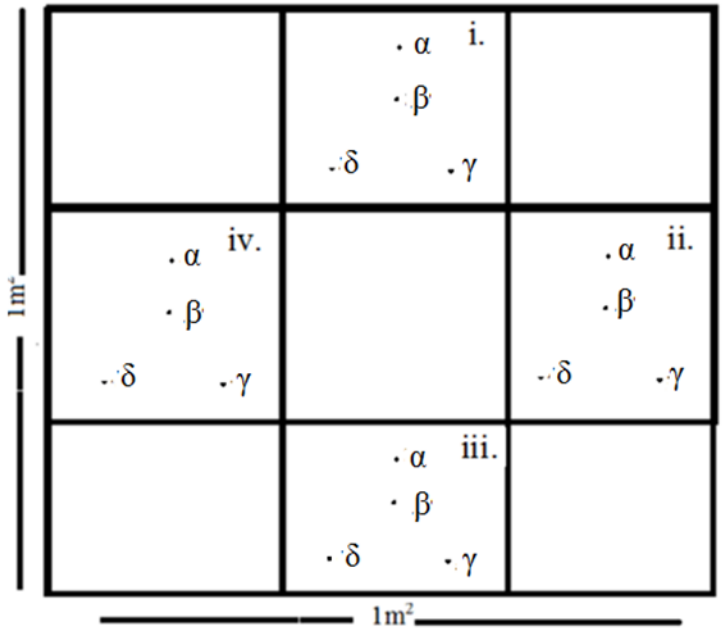
Samples from quadrant one were taken five meters inside the hibernacula's entrance. Samples from quadrant two were taken in the hibernacula's twilight zone. The twilight zone was defined as any point between quadrant one and four where there was a faint amount of light present; this quadrant also had to be at least five meters away from both quadrant one and quadrant four. Samples from quadrant three were taken where bat guano was present in the hibernacula (where applicable). Samples from quadrant four were taken where a bat roosting site was present in the hibernacula (where applicable). Samples taken from quadrant five were taken in the hibernacula's dark zone. The dark

zone was defined as any point between quadrant one and four where there was no light present; this quadrant also had to be at least 5 meters away from both quadrant one and quadrant four. Samples taken from quadrant six were taken outside of the hibernacula. Outside of the hibernacula was defined as non-subterranean in origin. This quadrant also had to be at least 5 meters away from the cave entrance. Maps of the exact quadrant locations can be found in Appendix 1.

Due to sampling during the summer months, bats were not always present in abundance within the hibernacula. Bats were most likely out actively feeding and roosting in trees and other habitat closer to their major food source, insects. This made it difficult to find evidence of bat guano and/or bat roosting sites. Where applicable, the denotation has been made to let the reader know that the sample was taken from either a bat guano deposit or a bat roosting site. If a bat guano deposit or bat roosting site was unidentifiable, then an additional dark zone sample was taken in its place.

There were four sample locations selected within each quadrant. The sample locations were selected from the top middle, bottom middle, left middle, and right middle.A total of twenty-four sample locations were selected per hibernacula. Each sample location was oriented, so that they were confined to their own respective foot squared ($1ft^2$). Four sample sites (α, β, γ, and δ) were taken from each sample location. A total of sixteen samples sites were selected per quadrant. Ninety-six samples were taken per hibernaculum (Figure 4).

Figure 4: Example of sample site orientation within a quadrant.



Note that sample locations are represented by Roman numerals (i., ii., iii., and iv.). Sample sites are represented by Greek letters (α, β, γ, and δ).

Sample collection

A field sheet (Appendix 2) was filled out for every cave entered. All samples were collected while wearing sterile gloves and flowing aseptic technique (Figure 5). Every hibernaculum swab collection started with quadrant one and sample location i., sample locations ii., iii., and iv. followed. After collecting from quadrant one; quadrant two, three, four, five, and six were sampled in order.

Figure 5: Samuel Miller, collecting swab samples from quadrant two, sample location i., sample site α inside of Fitzpatrick Cave.

Sample sites α, β, γ, and δ from a sample location within quadrant were located. A sterile 15mL falcon tube containing four sterilized cotton swabs was opened, and one swab was shaken out of the tube. The container was then closed. A sterile 50mL conical tube that contained sterile 1X phosphate buffered saline (1XPBS) was opened. 1XPBS was used as a wetting agent. The tip of the sterile cotton swab was wet in the 1XPBS, but it was never submerged in the 1XPBS. The sterile cotton swab was swirled around the inside of the conical tube to release any excess 1XPBS back into the 50 mL conical tube. The now wet swab was taken out and the conical tube closed.

The wet swab was rolled onto the surfaces of the quadrant, within the specific sample locations. The wet swabs were rolled by using the thumb and the index finger and turned in such a way that the swab rotated while contacting the desired surface. A sterile 15mL falcon tube, containing 2mL of 1XPBS, was opened. The swab that now contained sample was placed into the sterile 15mL falcon tube that contained 2mL 1XPBS solution. The swab was then shaken vigorously, by hand, for ten seconds to ensure that the sample had left the swab and dispersed into the 1XPBS solution. The swab was then swirled around the inside of the falcon tube to release any excess sample back into the falcon tube. The falcon tube then was closed. The swab was then transferred to a different sterile 15 mL falcon tube.

Sample pooling was done to ensure that there would be enough DNA present to perform molecular testing. Specifically, within the given quadrant, each sample α, β, γ, and δ was pooled from all four sample locations. The falcon tubes that contained both the samples and swabs were placed into a backpack cooler containing dry ice and were transported to Pittsburg State for processing. Until samples were processed, they were stored at -20ºC.

DNA extraction

The protocol for DNA extraction was a modified protocol from the Qiagen DNeasy Powersoil Kit (Appendix 3). The hibernacula sample containing 1.5mL of 1X PBS solution was added from the falcon tube containing sample to 2 powerbead tubes. The powerbead tubes were gently vortexed to mix by performing five inversions. Approximatly 90µL of solution C1 was added to the powerbead tubes and again gently vortexed by performing five inversions. If solution C1 was not dissolved in, it was heated

to 60ºC until it dissolved back into solution. The powerbead tubes were then placed on a table top vortex and vortexed at max speed for ten minutes.

After vortexing, the powerbead tubes were centrifuged at 10,000x gravity for 30 seconds at room temperature. The supernatant, approximately 1,800µL, was then transferred into two sterile 2mL microcentrifuge tubes. Approximatly 250µL of solution C2 was added to the two clean 2mL microcentrifuge tubes containing the supernatant. The 2mL microcentrifuge tubes were then vortexed for five seconds at max speed and then incubated at 4ºC, on ice, for five minutes. After incubation, the 2mL microcentrifuge tubes were centrifuged at 10,000x gravity for one minute at room temperature.

While avoiding the pellet, 1,800µL to 2,000µL of supernatant from the 2mL microcentrifuge tubes was transferred into four sterile 2mL microcentrifuge tubes. Approximatly 200µL of solution C3 was added to each of the four 2mL microcentrifuge tubes containing supernatant. The 2mL microcentrifuge tubes were then vortexed for five seconds at max speed and then incubated at 4ºC, on ice, for five minutes. After incubation, the 2mL microcentrifuge tubes were centrifuged at 10,000x gravity for one minute at room temperature.

While avoiding the pellets, all of supernatant (approximately 2mL) from the four 2mL microcentrifuge tubes was transferred into a sterile 15mL falcon tube. Before adding solution C4, it was shaken five times via inversion technique. Appromimatly 1.4 times the final volume of supernatant in the falcon tube of solution C4 was then added to the sterile falcon tube containing the supernatant (approximately 5.6mL of solution C4). The falcon tube was then vortexed for fifteen seconds at max speed. Approximatly 675µL of the supernatant from the falcon tube at a time was passed through a sterile spin

filter at 10,000x gravity for one minute at room temperature, until there was no supernatant left in the falcon tube.

Approximatlly 500µL of solution C5 was passed through the same spin filter at 10,000x gravity for thirty seconds at room temperature. The flow through was then discarded. The spin filter was centrifuged again at 10,000x gravity for one minute at room temperature. The flow through was again discarded. The spin filter was transfered into a new, sterile 2mL microcentrifuge tube. 100µL of molecular grade sterile water was added to the center of the spin filter. The spin filter rested for one minute and then was centrifuged at 10,000x gravity for one minute at room temperature. The spin filter was then discarded and the DNA ready for use. DNA was stored at -20ºC until used for molecular analysis.

DNA quantification via nanodrop

A NanoDrop™ Lite Spectrophotometer (NanoDrop) was used to quantify the amount of DNA present in ng/µL. The following selections were made: DNA and then dsDNA (double stranded DNA) were selected. Following the selection of dsDNA, the NanoDrop was blanked. To set the control for the NanoDrop, it was wiped off with a KimTech wipe saturated with 95% ethanol, then it was dried with a KimTech wipe. Approximately 2µL of molecular grade sterile water was loaded onto the NanoDrop and blank was pressed. Once more 2µL of molecular grade sterile water was loaded onto the NanoDrop and the blank measurement was confirmed. The NanoDrop was wiped off with a KimTech wipe. Samples were then loaded onto the NanoDrop. Approximatly 2µL of each sample was added to the NanoDrop so that ng/µL of DNA could be recorded. The

average (n=3) for each of the samples was recorded. This average amount of DNA in ng/µL was used for calculating sample volume in PCR.

Validation of *Pd* primers

One set of primers were ordered from IDT; they were selected using the primer design tool from IDT, analyzed utilizing the NCBI nucleotide blast database, and amplified via PCR. Primers *Pd*Fwd and *Pd*Rev (Table 4) came lyophilized and were reconstituted in enough molecular grade sterile water to make a stock solution of 100 pmol/λ; the stock solution was stored at -20ºC. Working solutions of *Pd*Fwd and *Pd*Rev primers were made by mixing 50µL of 100 pmol/λ with 450µL of molecular grade sterile water so that the final concentration of *Pd*Fwd and *Pd*Rev primers were 10 pmol/λ.

Table 4: Primers used for molecular testing.

Primer Name	Sequence 5'-3'	Target organism	DNA Target area	Reference
*Pd*Fwd	5'- ACG TCC TAA AGC CTA CAA CAC - 3'	*Pd*	ITS1-ITS2	This Study 2017
*Pd*Rev	5' - CAT TTC GCT GCG TTC TTC ATC- 3'	*Pd*	ITS1-ITS2	This Study 2017

Fwd denotes a forward primer sequence and Rev denotes a reverse primer sequence.

A custom GeneBlock was ordered from IDT to use as the positive control for the *Pd* primers. The GeneBlock ordered (Figure 6) was identical in DNA sequence to GenBank: EU884924.1 (*Geomyces destructans* isolate 22004-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence) (Garagas *et al.* 2009). This 787bps sequence (*Pd* Pos) was chosen because it is specific for *Pd*. The GeneBlock came lyophilized and was reconstituted in enough sterile 1XTE buffer to

make a stock solution of 2.5ng/µL. The working solution was kept at -20ºC until used for making a working solution. A working solution of 1 ng/µL was made by mixing 40µL of the stock solution into 60µL of sterile 1XTE buffer.

Figure 6: GenBank: EU884924.1 sequence for *Pd*.

Geomyces destructans isolate 22004-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: EU884924.1

GenBank Graphics

>EU884924.1 Geomyces destructans isolate 22004-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

AGCACGACTGTAAATAAGTCTCCCTTCATGCAAGTCAGCACCCGCTGGCAACACGATCGAATTGACGGGG
ACGTCCTAAAGCCTACAACACCAACCCGCCCGGGAAACCGAGGCGGGGGCCCGTGCTAACTCCACGGGGT
GGTAAAAGAGTGTATGGATACTCCCTCTGGGGAACTATGGATAATCCGCAGCGAAGACCCTAAGTAGCGC
TAGCTATACGGGTAACGTTCACAGACTAAGTGGTTGTGGGTGGAGCCTAGCTCTGCTTAAGATATAGTCG
GGCCCTACGTGAAAGCGCAGGGGTGAGTCGCTACGAACTCGAAACCGTTCCGTAGGTGAACCTGCGGAAG
GATCATTACAGTAGTCGCCCGGGTTGCCGCAAGGCCTCCCGGGTAACCTACCACCCTTTGTTTATTACAC
TTTGTTGCTTTGGCAGGCCTGCCCTCGGGCTGCTGGCTCCGGCCGGCGAGCGCTTGCCAGAGGACTAAAC
TCTGTTTGTCTATACTGTCTGAGTACTATATAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGG
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
TGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTACAACCCTCAAGCT
CAGCTTGGTATTGGGCCCCGCCGACCCGGCGGGCCCTAAAGTCAGTGGCGGTGCCGTCCGGCTCCGAGCG
TAGTAATTCTTCTCGCT

For the *Pd*Fwd and *Pd*Rev primer pair a onetime denaturation was carried out at 95°C for 2 minutes, which was followed by 30 cycles of the following conditions: 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1minute. A final extension was carried out at 72°C for 5 minutes. Solutions of 25µL were prepared prior to being placed into the MyCycler Thermal Cycler. The final concentration consisted of 12.5µL of 2X Eco - Taq MasterMix, 2µL of forward primer *Pd*Fwd, 2µL of reverse primer *Pd*Rev, 7.5µL of molecular grade sterile water, and 1µL of 1ng/µL of *Pd*Pos. The PCR process was followed up by gel electrophoresis.

A 1.2% agarose gel was utilized for gel electrophoresis. Approximatly 0.6g of agarose was dissolved into 50mL of 1x TAE Buffer. The solution was boiled in a microwave until completely dissolved. Approximatly 3.0µL of ethidium bromide was

added to the agarose/1xTAE solution. It was then poured into a gel-casting tray and allowed to solidify. An 8 well comb was placed in the agarose to create 8 wells for loading samples. Approximatly 8.5µL of a 100bp DNA ladder with a concentration of 500ng/µL was pipetted into the first lane of gel. Approximatly 8.5µL of the negative control was pipetted in the third lane. Approximatly 8.5µL of the positive control was pipetted in the second lane. The gel ran at 115 volts for 50 minutes. Upon completion, the gel was placed on a transilluminator and viewed under ultra-violet light for 5 seconds prior to utilizing a FluorChem E to capture gel images

Molecular amplification of the *Pd* DNA

The samples placed in 100µL of molecular grade sterile water were utilized as the template for polymerase chain reaction (PCR) amplification applying routine thermocycler conditions. A onetime denaturation was carried out at 95°C for 10 minutes, which was followed by 40 cycles of the following conditions: 95°C for 15 seconds, 62°C for 1 minute. A final extension was carried out at 62°C for 10 minutes. Solutions of 25µL were prepared prior to being placed into the MyCycler Thermal Cycler.

One set of primers were ordered from IDT; they were selected using the primer design tool from IDT, analyzed utilizing the NCBI nucleotide blast database, and amplified using the above-mentioned PCR parameters. The primers utilized targeted the internal transcribed spacer region 1 (ITS1) in *Pd*. These primers are highly specific for *Pd*. The forward primer utilized was *Pd*Fwd (*Pd*Fwd: 5’ - ACG TCC TAA AGC CTA CAA CAC - 3’) The reverse primer utilized was *Pd*Rev (*Pd*Rev: 5’ - CAT TTC GCT GCG TTC TTC ATC - 3’) (This Study).

The final concentration for samples consisted of 12.5µL of 2X Eco - Taq MasterMix, 2µL of forward primer *Pd*Fwd, 2µL of reverse primer *Pd*Rev. The final concentration of template DNA and molecular grade sterile water varied for each reaction, due to the concentrations of DNA being different for every sample. Approximately 10ng of template DNA were in every reaction. The positive controls utilized consisted of 12.5µL of 2X Eco - Taq MasterMix, 2µL of forward primer *Pd*Fwd, 2µL of reverse primer *Pd*Rev, 6.5µL molecular grade sterile water, and 1µL of 1ng/µL *Pd*Pos**.** The negative control utilized consisted of 12.5µL of 2X Eco - Taq MasterMix, 2µL of forward primer *Pd*Pos, 2µL of reverse primer *Pd*Rev, 8.5µL molecular grade sterile water. The PCR process was followed by gel electrophoresis.

Molecular identification of the *Pd* DNA

A 1.2% agarose gel was utilized for gel electrophoresis. 0.6g of agarose was dissolved into 50mL of 1X TAE Buffer. The solution was boiled in a microwave until completely dissolved. 3.0µL of ethidium bromide was added to the agarose/TAE solution. It was then poured into a gel-casting tray and allowed to solidify. A 15 well comb was placed in the agarose to create 15 wells for loading sample. 8.5µL of a 100bp DNA ladder with a concentration of 500ng/µL was pipetted into the first lane of gel. The positive control was placed in the second lane. The negative control was placed in the third lane. 8.5µL of each sample was pipetted into sperate wells from left to right. The gel was run at 115 volts for 50 minutes. Upon completion, the gel was placed on a transilluminator and viewed under ultra-violet light for five seconds prior to utilizing a FluorChem E to capture gel images.

Creating the WNS predictor model for Missouri counties

The WNS predictor model was developed in the spring of 2018. Program R, version 3.3.2, was used to calculate all statistical values, analyze those values, and construct all the figures for this WNS predictor model using data that was z-transformed. p-values ($p < 0.05$) were considered statistically significant. All models with $\Delta AICc < 2$ were considered equally as supported, unless there were additional uninformative variables in a given model (Arnold 2010; Burnham & Anderson 2002).

Models generated were given Akaike Information Criterion that had been corrected for small sample size (AICc) values and assessed according to standard protocol in model selection. The WNS predictor model determined the probability for the presence of WNS in a given Missouri county and included the following predictor variables: highest county elevation, lowest county elevation, median county elevation, county center latitude, county center longitude, county area, county cave density, number of adjacent WNS positive counties, and the average time since WNS was detected in adjacent counties.

The county population data was taken from https://www.missouri-demographics.com/ in April of 2018. The highest county elevation, lowest county elevation, median county elevation, county center latitude, county center longitude, and county area data were taken from https://www.anyplaceamerica.com/ in April of 2018. The data on county cave density was taken from MCD with the permission of the Missouri Speleological Society (MSS), Mr. Scott House. The data on the number of adjacent WNS positive counties and average time since WNS was detected in adjacent counties were determined from https://www.whitenosesyndrome.org/ in April of 2018.

This study used generalized linear models along with an information theory approach to evaluate the relationship between biologically important predictor variables and WNS presence in Missouri counties (Burnham and Anderson 2002 & Austin 2007). This study treated the presence or absence of WNS in a given Missouri county as the response variable (WNS present = 1 & WNS absent = 0) and used a binomial distribution with a logit link function. A 'full' model was created that contained all the predictor variables. A 'null' model was created that contained only the intercept and none of the aforementioned predictor variables. A test for multicollinearity was completed prior to model fitting and only models with county center latitude, county cave density, and the average time since WNS was detected in adjacent counties were included in candidate models. Candidate models were then developed apriori and based on biological knowledge of WNS. Those models were then fited and ranked with Akaike's Information Criteria that had been corrected for small sample size (AICc) and model weights. Overdispersion was tested for by taking the ratio of the sum of squared Pearson residuals and dividing by the residual degrees of freedom. The area under the receiver operating characteristic curve (AUC) was determined to assess overall fit of the models (Fielding and Bell 1997). The best fit model was determined using a combination of these criteria. The other models were not selected since they contained insignificant predictor variables that were not contributing to the models' predictive power.

A null model containing no predictor variables, a full model containing all the predictor variables were all constructed. Overdispersion was examined using residual difference and degrees of freedom. An overdispersion ratio of less than 1.5 was considered to indicate the data was not affected by overdispersion. The significance of

the models was determined with an ANOVA using the Wald Chi^2 by comparing the significance of each predictor variable. This was utilized to determine the significance of each predictor variable. The fitness of the models was accessed utilizing the Nagelkerke (Cragg and Uhler) pseudo R^2 method.

A final model was created that only included the predictor variables selected by the stepwise model (county cave density, average time since WNS was detected in adjacent counties, and the north latitude of the center of the county). The final model also included the response variable (presence or absence of WNS). The overall model significance was evaluated with an ANOVA using the Wald Chi^2 by comparing the final model that included only the predictor variables selected by stepwise model to the null model. The overall model significance was evaluated with the likelihood ratio test.

Models were manually created to determine which predictor variable and/or combination of predictor variables best predicted the presence or absence of WNS in any given Missouri county. The models were evaluated based on their AICc values, which were examined to determine which model best predicted the presence or absence of WNS in a Missouri county. AICc and Effects tables were created to determine the best fit model. The best fit model was utilized to predict the probability that bat hibernacula within Morgan County and Taney County would be WNS positive.

Verifying the WNS predictor model for Missouri counties

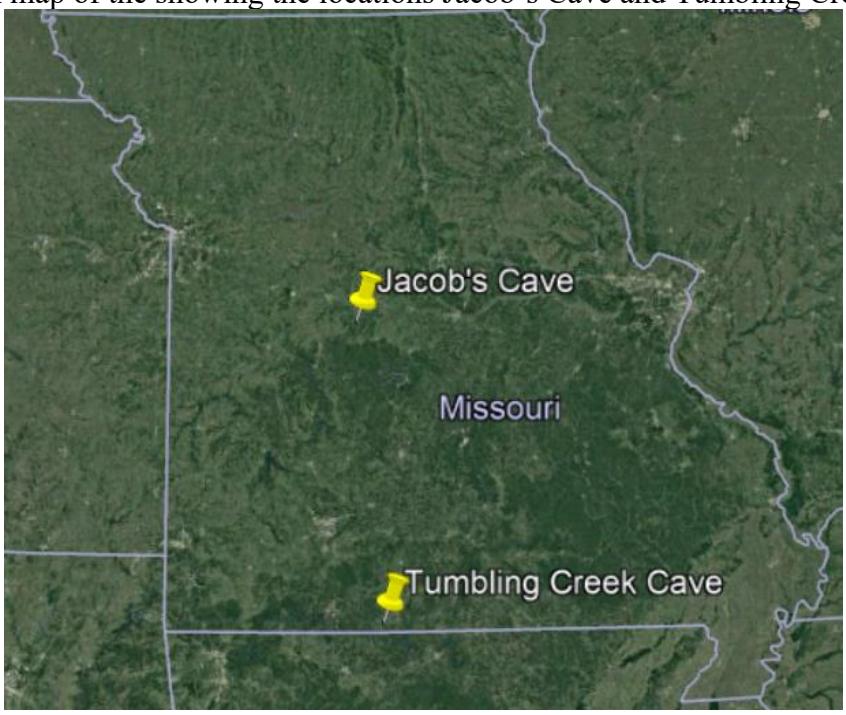
The WNS predictor model was tested in the field during the summer of 2018 by identifying two different counties of interest that had not been previously sampled in this study. Counties of interest were identified as Morgan County and Taney County. The following Missouri counties were also used to verify the WNS predictor model:

Chirstian, McDonald, Nweton, and Ozark. This was done to increase the sample size. Morgan County is in north-central Missouri (Figure 7) and was previously regarded as WNS negative (Table 5). It was regardedas Taney County is in south-central Missouri (Figure 7) and had previously been regarded as WNS Positive (Table 5). Within Morgan County, permission was obtained to study Jacob's Cave, and it was selected as the hibernacula for sampling. Jacob's Cave was considered WNS negative because there had never been any suspected incidences of WNS in the cave.Within Taney County, Tumbling Creek Cave was selected as the hibernacula for sampling. With the permission of Tom Aley, this study gained access to Tumbling Creek Cave. Tumbling Creek Cave was regardedas suspected WNS positive. It had not been confirmed in the cave, however, Tom Aley had observed bats that he thought exhibited behavior typical of bats infected with WNS. The exact same methodology and procedures used in this study were implemented with the samples that were collected from Jacob's Cave and Tumbling Creek Cave.

Table 5: Information regarding the location of hibernacula and their current known WNS status as of April, 2018.

Hibernacula	State and County	Status of WNS (WNS + or WNS -)
Jacob's Cave	Missouri, Morgan	WNS -
Tumbling Creek Cave	Missouri, Taney	Suspected WNS +

Figure 7: A map of the showing the locations Jacob's Cave and Tumbling Creek Cave.



This figure was constructed utilizing Google EarthPro software.

CHAPTER III.

RESULTS & DISCUSSION

Comparing visual surveys with PCR detection

From June 2017 to December 2017, 144 samples were taken from 6 bat hibernacula located in southeast Kansas and southwest Missouri. Of which, 5 were caves, and 1 was a storm sewer system. Of the sampled caves, 3 were previously described by SPG as being WNS negative by visual survey methods, and 3 were previously described by SPG as being WNS positive by visual survey methods.

Amplification of *Pd* DNA was not achieved in PSS001 (Figure 8), NWT011 (Figure 9). There two hibernacula that were previously described as being WNS negative by visual survey methods (Table 6). Whereas, amplification of *Pd* DNA Was achieved in MDD001 (Figure 10), CHR153 (Figure 11), CHR002 (Figure 12), and OZK002 (Figure 13). All three of these hibernacula were previously described as being WNS positive by visual survey methods except for MDD001(Table 6).

All the hibernacula previously described as WNS positive by visual survey methods were found to be WNS positive by PCR detection in this study. WNS positive was defined as *Pd* DNA was present within the hibernacula. *Pd* presence in a hibernaculum is strongly correlated with WNS presence within hibernacula (Lorch *et al.* 2013b). Only two of the

three hibernacula previously described by WNS negative by visual survey methods were found to be WNS negative by PCR detection in this study.

One hibernaculum located in McDonald, County Missouri, MD001, previously described as being WNS negative by visual survey methods was found to be WNS positive by PCR detection (Figure 10 & Table 6). This hibernaculum, MD001, had been incorrectly described in terms of its WNS status. This could have potentially been due to *Pd* recently colonizing the hibernaculum in-between the last visual survey that was conducted and the time that this study happened. With the use of PCR, this study was able to document *Pd* DNA inside of the hibernaculum in five quadrant (Figure 10 & Table 6).

MD001 is a privately-owned show cave and hosts 1,000's of visitors annually. It receives a higher number of human visitors than the other five hibernacula, as they are primarily used for research purposes by small groups of scientists. Each visitor represents a potential vector for WNS transmission. If visitors have entered other hibernacula that were WNS positive and hadn't taken the appropriate measures to decontaminate themselves and their clothing, they could unknowingly spread the disease. The hibernaculum is also home to a population of gray bats, which are believed to be one of the biggest vectors of WNS spread. Paul McKenzie of the USFWS has said, "Because gray bats hibernate together in colonies that number in the hundreds of thousands, WNS could expand exponentially across the range of the species". High visitor traffic and the population of grey bats present coupled with visual surveys only happening annually could have created a favorable environment for an outside vector to infect the hibernacula with WNS.

Figure 8: Image of agarose gel for *Pd* PCR in Pittsburg Storm Sewer.

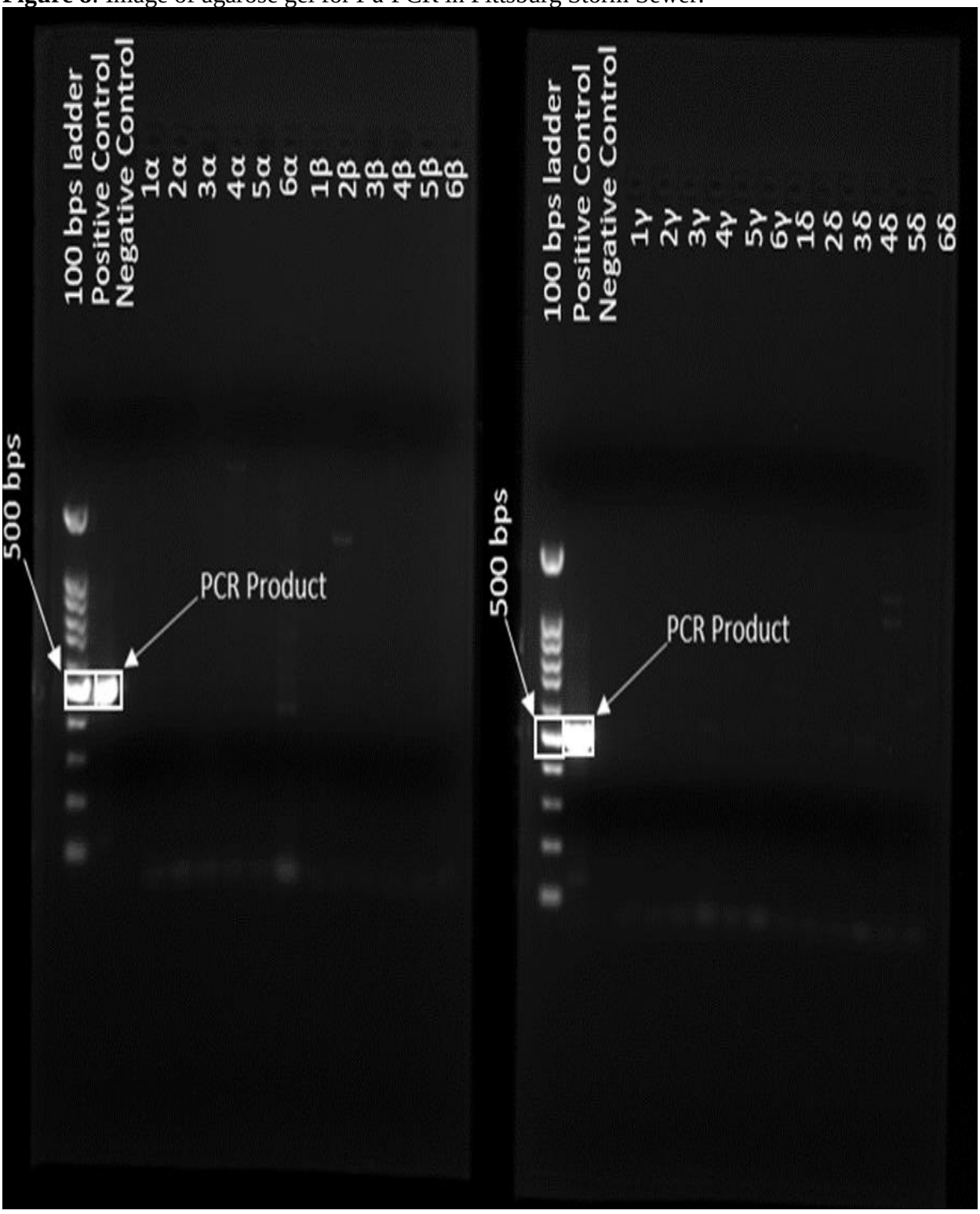


Figure 9: Image of agarose gel for *Pd* PCR in Shoal Creek Cave.

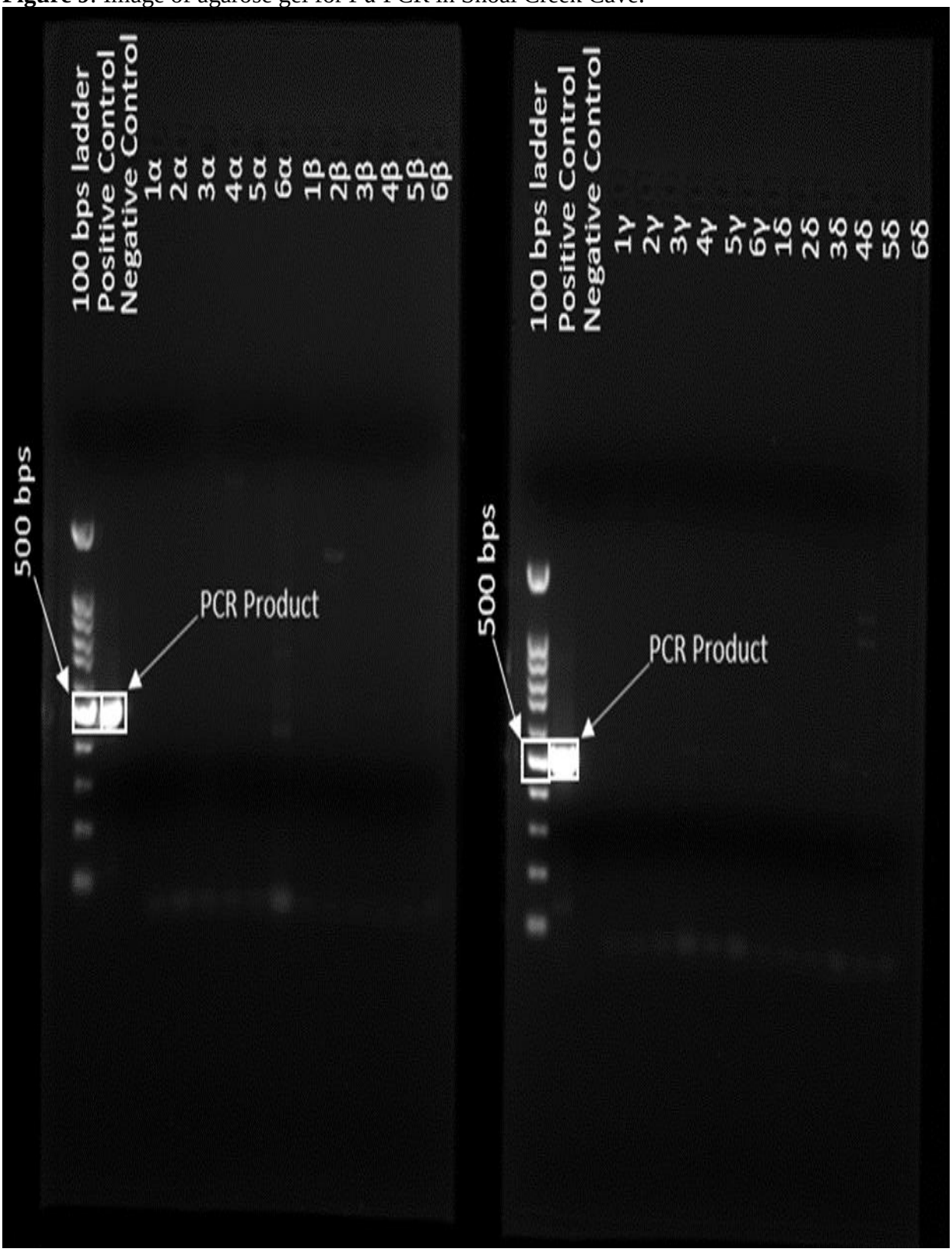


Figure 10: Image of agarose gel for *Pd* PCR in Bluff Dweller's Cave.

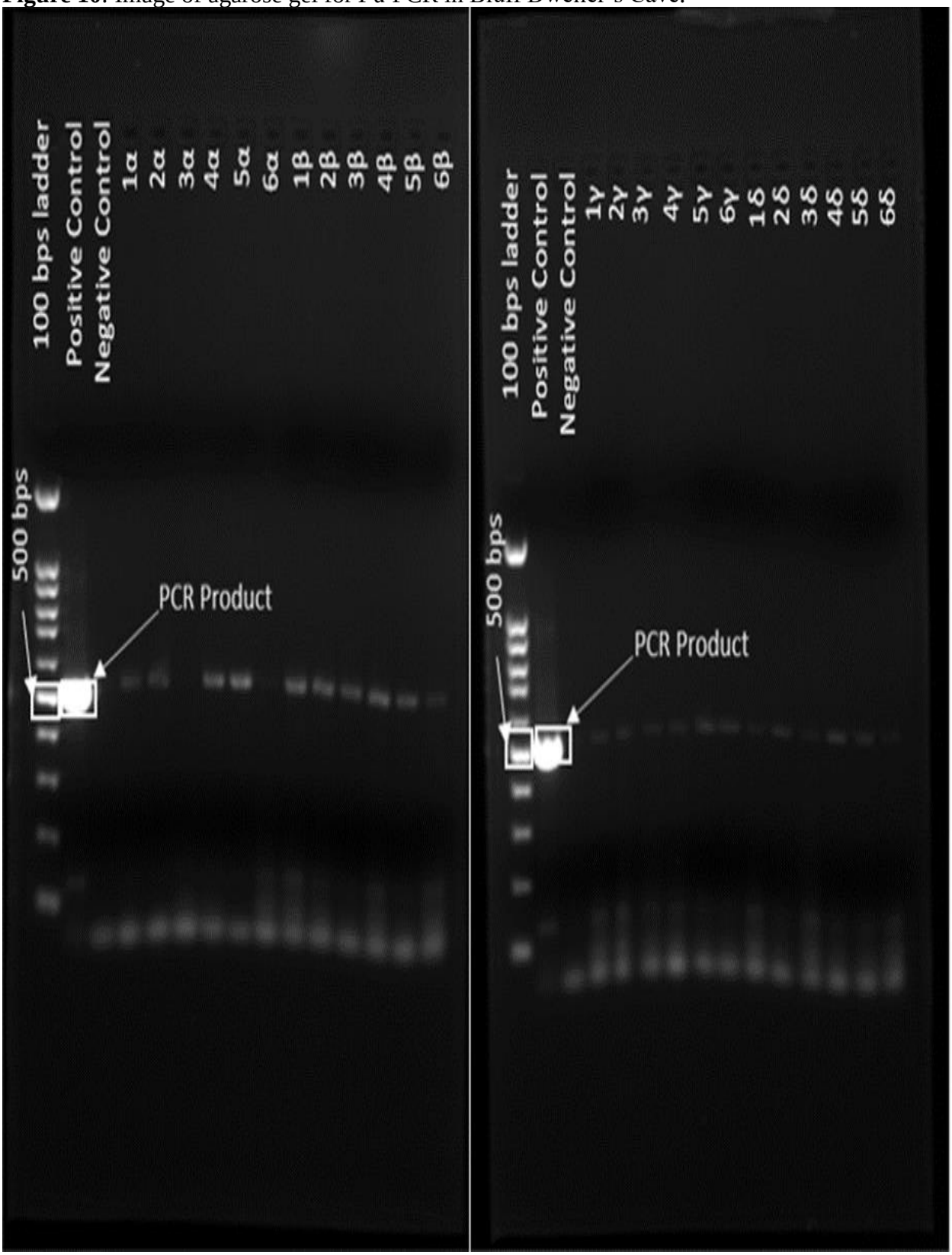


Figure 11: Image of agarose gel for *Pd* PCR in Breakdown Cave.

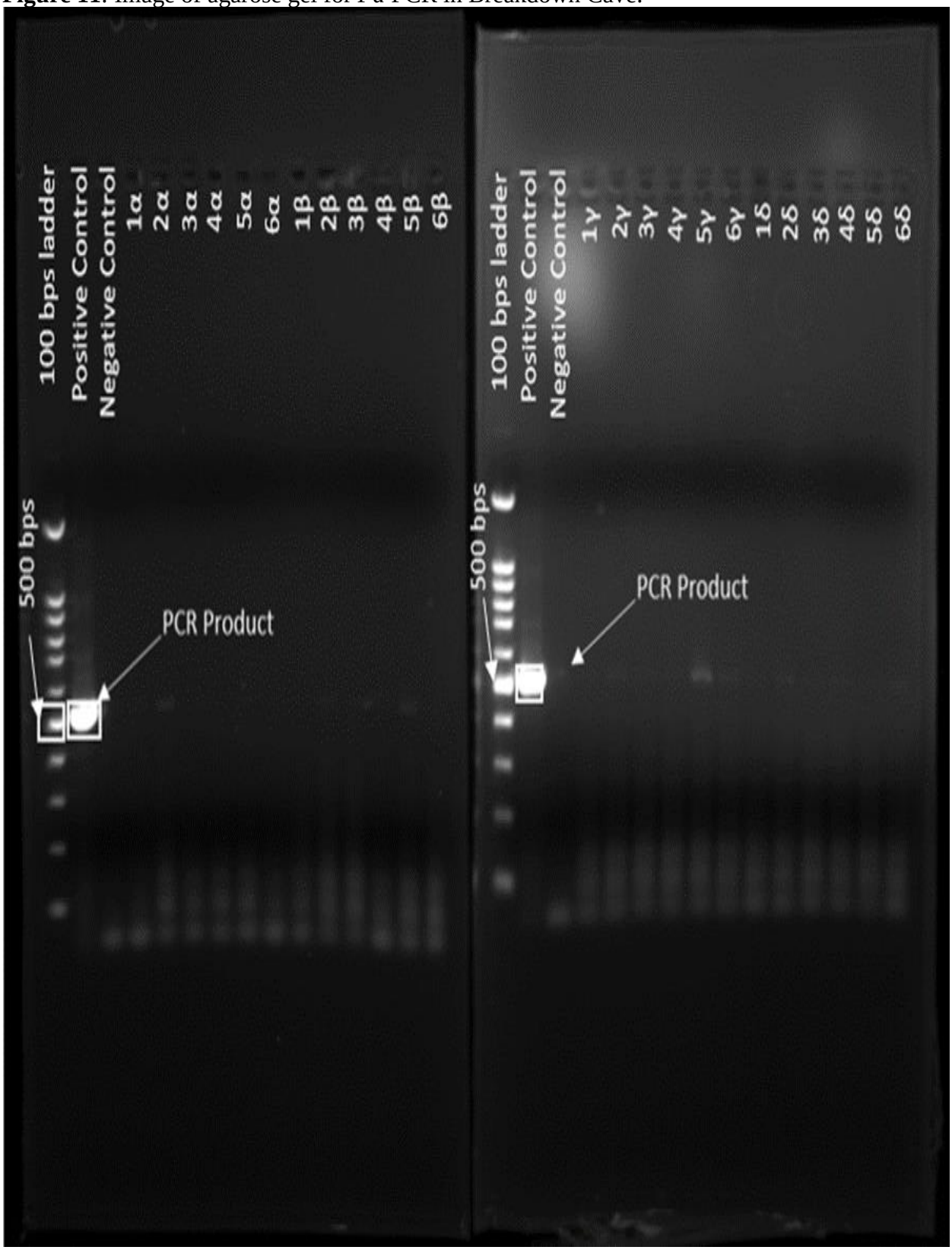


Figure 12: Image of agarose gel for *Pd* PCR in Fitzpatrick Cave.

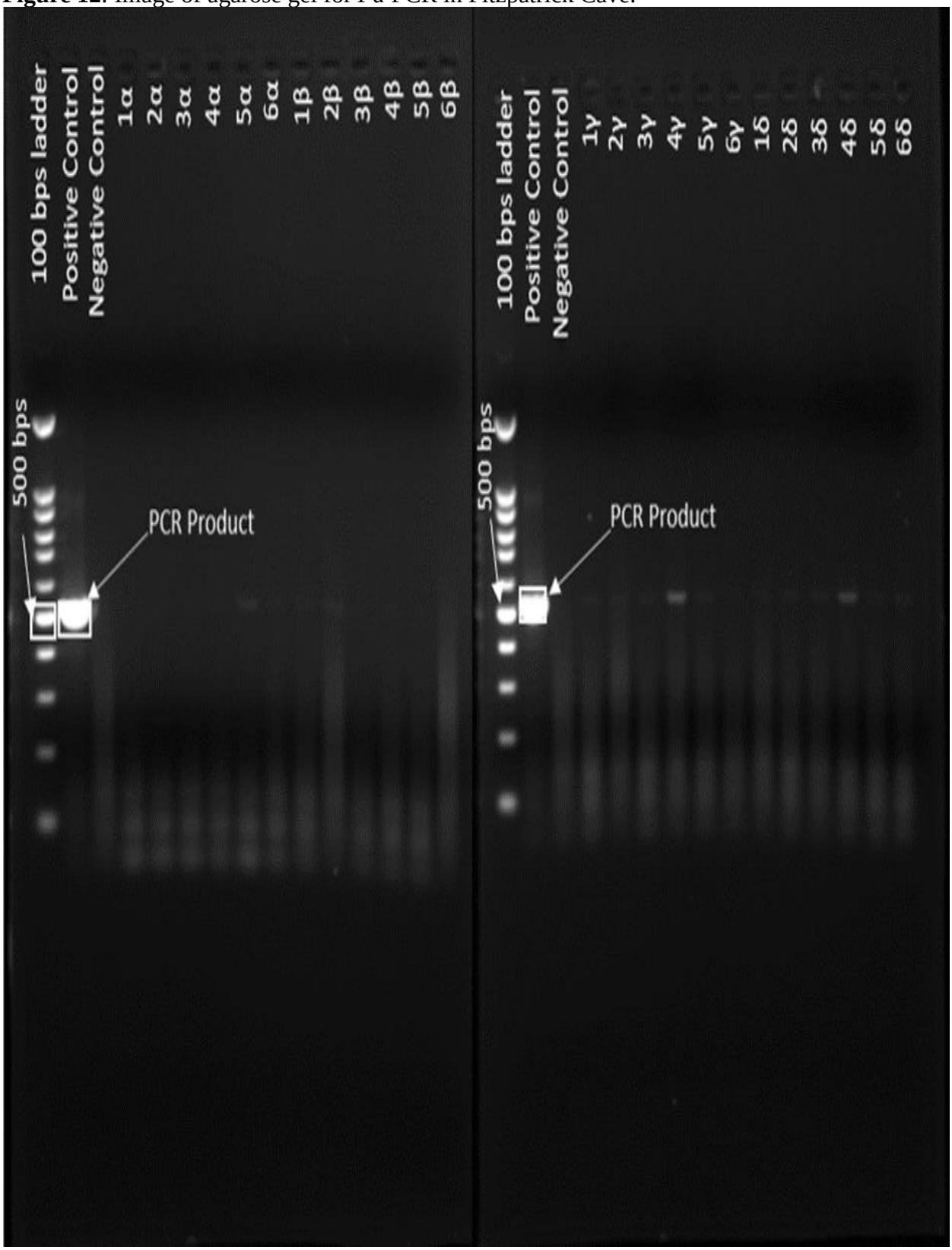


Figure 13: Image of agarose gel for *Pd* PCR in Big Bear Cave.

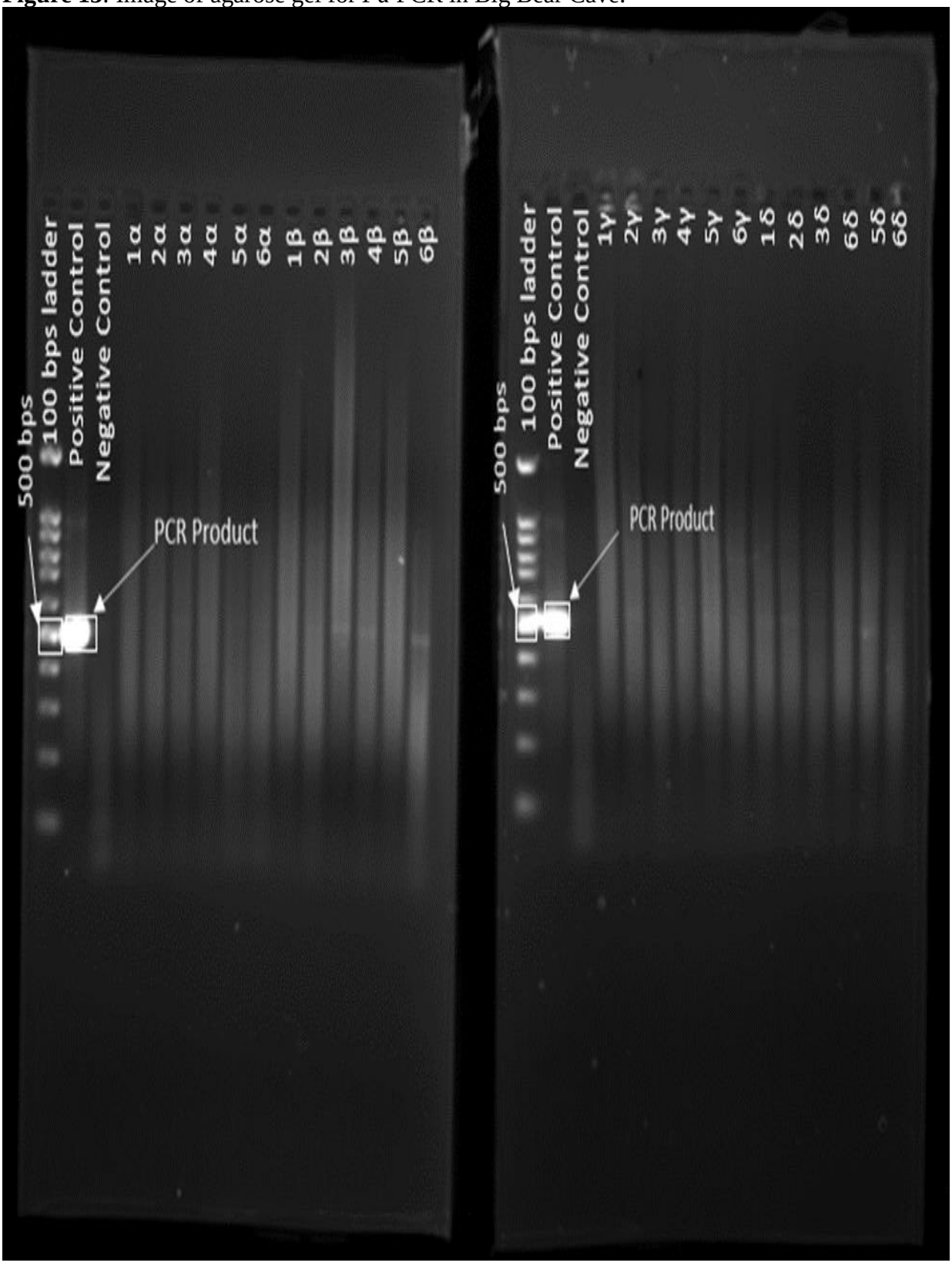


Table 6: Comparing previous visual surveys for WNS with PCR amplification of *Pd* DNA.

Pooled Samples	MDD001	NWT011	PSS001	CHR153	CHR002	OZK002
1α	Positive*	Negative	Negative	Positive	Negative∴	Positive
1β	Positive*	Negative	Negative	Positive	Positive	Negative∴
1γ	Positive*	Negative	Negative	Positive	Positive	Negative∴
1δ	Positive*	Negative	Negative	Negative∴	Positive	Negative∴
2α	Positive*	Negative	Negative	Positive	Negative∴	Positive
2β	Positive*	Negative	Negative	Positive	Positive	Negative∴
2γ	Positive*	Negative	Negative	Positive	Positive	Negative∴
2δ	Positive*	Negative	Negative	Positive	Positive	Negative∴
3α	Negative	Negative	Negative	Negative∴	Negative∴	Positive
3β	Positive*	Negative	Negative	Positive	Negative∴	Negative∴
3γ	Positive*	Negative	Negative	Positive	Positive	Negative∴
3δ	Positive*	Negative	Negative	Positive	Positive	Negative∴
4α	Positive*	Negative	Negative	Negative∴	Negative∴	Positive
4β	Positive*	Negative	Negative	Negative∴	Negative∴	Negative∴
4γ	Positive*	Negative	Negative	Positive	Positive	Negative∴
4δ	Positive*	Negative	Negative	Positive	Positive	Negative∴
5α	Positive*	Negative	Negative	Negative∴	Positive	Positive
5β	Positive*	Negative	Negative	Positive	Negative∴	Positive
5γ	Positive*	Negative	Negative	Positive	Positive	Negative∴
5δ	Positive*	Negative	Negative	Positive	Positive	Positive
6α	Negative	Negative	Negative	Negative∴	Positive	Positive
6β	Negative	Negative	Negative	Negative∴	Negative∴	Positive
6γ	Negative	Negative	Negative	Positive	Positive	Negative∴
6δ	Negative	Negative	Negative	Positive	Positive	Negative∴

Note that * indicates that the hibernaculum was previously described as WNS negative but pooled sample sites contained *Pd* DNA. Not that ∴ indicates that the hibernaculum was previously described as WNS positive but pooled sample sites did not contain *Pd* DNA.

A total of 24 samples were pooled and collected for each hibernaculum. The total number of samples that tested positive for *Pd* DNA within each hibernaculum were: CHR002 – 16, CHR 153 – 17, MDD001 – 19, OZK02 – 7, in NWT011 – 0, and in PSS01 – 0 (Table 6).

Of the six quadrant sampled within every hibernacula, there was not much variation in the presence of WNS (Table 6). The total number of samples that tested

positive for *Pd* DNA within each quadrant were: Q1 – 7, Q2 – 12, Q3 – 9, Q4 – 9, Q5 – 13, and Q6 – 9. The quadrant with the highest total number of samples that tested positive for *Pd* DNA were Q2 and Q5, which were the twilight zone, and the dark zone respectively. It is possible that Q2 and Q5 both had the highest frequencies of WNS positive samples for very different reasons. Samples from Q2 were taken from the twilight zone of the cave, which can be contained within a smaller area in some cave, thus forcing the bats that use the cave to be more likely to come into close contact with the cave surfaces. This increase in traffic and can possibly explain why 67% of samples from Q2 in WNS positive hibernacula were found to contain *Pd* DNA. Samples from Q5 were taken from a dark zone in the cave, and they were taken from the deepest areas of the cave. Bats need a suitable microclimate to hibernate, and deep areas within caves can provide just that. Taking samples from an area more conducive to bat roosting habitat and can possibly explain why 58% of samples from Q5 in WNS positive hibernacula were found to contain *Pd* DNA.

WNS predictor model

The best supported model for WNS probability prediction included the predictor variables county cave density and average time since WNS was detected in adjacent counties and had an AICc model weight of 0.5 (Tables 7, 8). The AUC for the best supported model was 0.92, which is interpreted as close to perfect (Swets 1988). The next 8 models added the county center latitude and employed various interactive effects between the predictor variables, however, these models were not well supported.

Table 7: Summary of model selection from the best ranked a priori candidate models using the effects of county cave density, average time since WNS was detected in adjacent counties, and county center latitude on the probability of WNS presence in Missouri during 2018.

Model	**K**	**AICc**	**ΔAICc**	**Weight**	**Nagelkerke R^2**
Cave_Density + Ave_Years_Adj_Counties	2	86.50	0.00	0.50	0.66
Cave_Density * Ave_Years_Adj_Counties	3	87.94	1.44	0.23	0.66
Cave_Density * GPS_N	3	88.50	2.00	0.17	0.66
Cave_Density	1	90.97	4.47	0.06	0.62
Cave_Density + GPS_N	2	91.89	5.39	0.04	0.63
GPS_N + Ave_Years_Adj_Counties	2	131.00	44.50	<0.01	0.34
GPS_N * Ave_Years_Adj_Counties	3	131.4	44.90	<0.01	0.36
Ave_Years_Adj_Counties	1	137.30	50.80	<0.01	0.26
GPS_N	1	146.80	60.30	<0.01	0.17
Null	0	160.40	73.90	0.00	0.00

Table 8: Estimated coefficients for the best supported model of the effects of county cave density and average time since WNS was detected in adjacent counties on the probability of WNS presence in Missouri during 2018.

Parameter	Coefficient	SE	Upper 95% CI	Lower 95% CI
Cave_Density	5.4253	1.3160	8.0047	2.8459
Ave_Years_Adj_Counties	0.9196	0.4140	1.7310	0.1082

The presence of WNS showed a linear response to county cave density, increasing with county cave density (Figure 14), and showed a linear response to average time since WNS was detected in adjacent counties, increasing with average time since WNS was detected in adjacent counties (Figure 15). WNS was more likely to be found in counties with higher county cave densities and in counties with a higher average time since WNS was detected in their adjacent counties.

Figure 14: Predictions of the best supported model showing the effects of county cave density on the probability of the presence of WNS in Missouri. Shaded blue area is 95 % Confidence intervals (CIs).

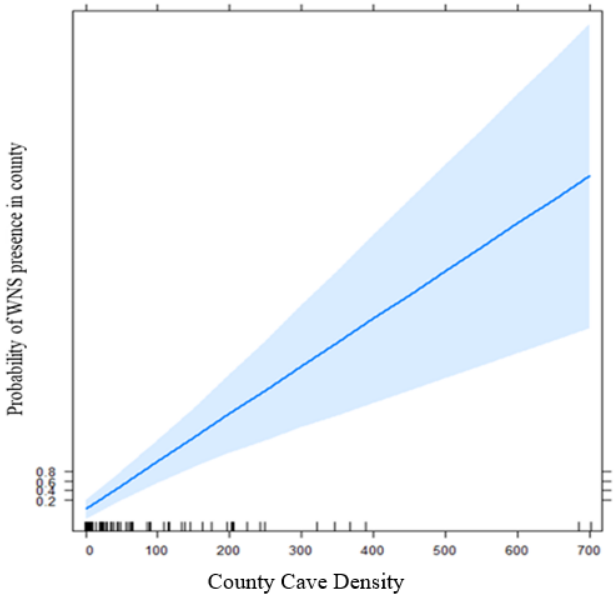
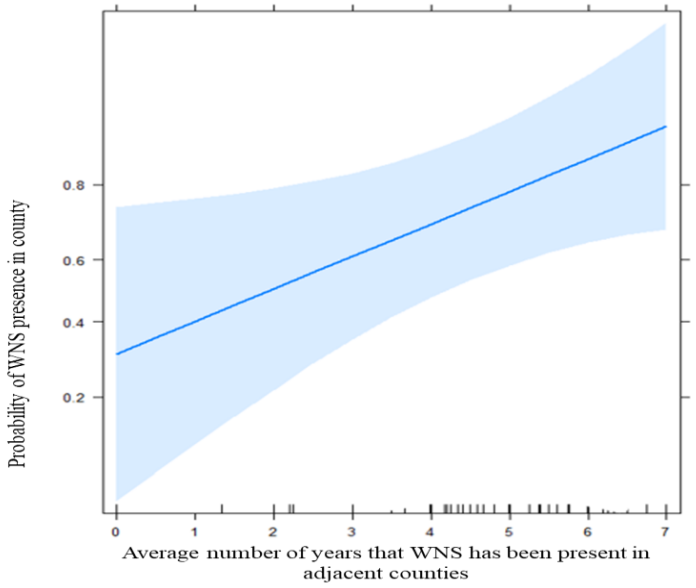


Figure 15: Predictions of the best supported model showing the effects of average time since WNS was detected in adjacent counties on the probability of the presence of WNS in Missouri. Shaded blue area is 95 % Confidence intervals (CIs)



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The best fit model produced the probability that each county in Missouri would have WNS (Table 9). A map was generated to illustrate the spatial organization of the predictions made by the model (Figure 16).

Figure 16: Visual representation of the probability that Missouri counties will be WNS Positive.

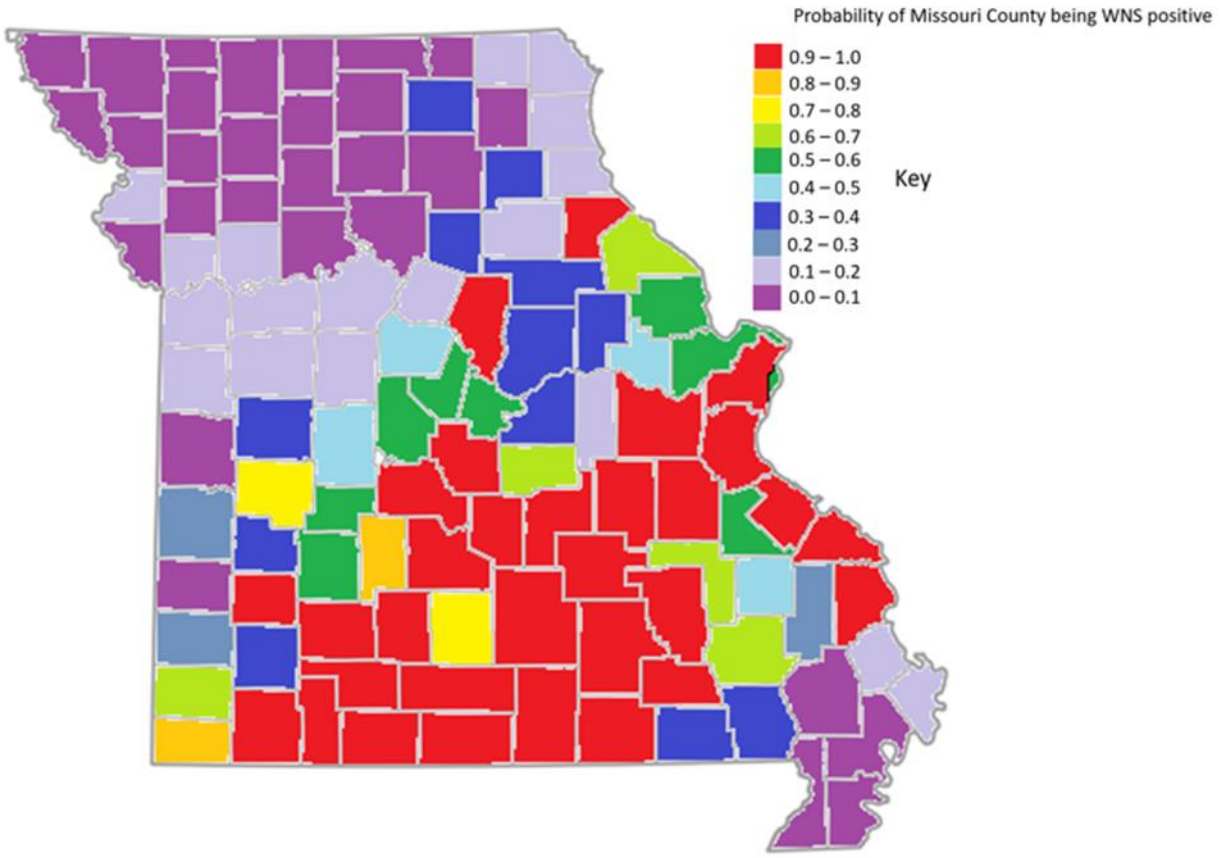


Table 9: Model generated probabilities that each county within Missouri will have WNS.

County	Model Prediction	Lower 95% CI	Upper 95% CI
Adair County	0.207822	0.089668	0.411321
Andrew County	0.050561	0.015079	0.156287
Atchison County	0.050561	0.015079	0.156287
Audrain County	0.207822	0.089668	0.411321
Barry County	0.999811	0.975735	0.999999
Barton County	0.074544	0.024681	0.204069
Bates County	0.062808	0.019900	0.181130
Benton County	0.450860	0.304277	0.606500
Bollinger County	0.338801	0.128074	0.641256
Boone County	0.916204	0.701252	0.980743
Buchanan County	0.110835	0.049976	0.228017
Butler County	0.296508	0.142494	0.516686
Caldwell County	0.022245	0.003501	0.128417
Callaway County	0.240256	0.134036	0.392500
Camden County	0.998685	0.961125	0.999957
Cape Girardeau County	1.000000	0.999987	1.000000
Carroll County	0.022245	0.003501	0.128417
Carter County	0.974029	0.857532	0.995739
Cass County	0.172921	0.082704	0.326519
Cedar County	0.270866	0.145908	0.44685

Chariton County	0.022245	0.003501	0.128417
Christian County	0.999993	0.996717	1.000000
Clark County	0.110835	0.049976	0.228017
Clay County	0.172921	0.082704	0.326519
Clinton County	0.075336	0.028983	0.181932
Cole County	0.580031	0.402919	0.73868
Cooper County	0.423989	0.295682	0.563432
Crawford County	0.999999	0.999482	1.000000
Dade County	0.999999	0.999482	1.000000
Dallas County	0.824058	0.625034	0.929380
Daviess County	0.022245	0.003501	0.128417
DeKalb County	0.050561	0.015079	0.156287
Dent County	0.982061	0.881163	0.997532
Douglas County	0.998916	0.950355	0.999977
Dunklin County	0.022245	0.003501	0.128417
Franklin County	0.982541	0.874739	0.997800
Gasconade County	0.189634	0.104481	0.319434
Gentry County	0.022245	0.003501	0.128417
Greene County	1.000000	0.999837	1.000000
Grundy County	0.022245	0.003501	0.128417
Harrison County	0.022245	0.003501	0.128417
Henry County	0.202703	0.079894	0.426737
Hickory County	0.533966	0.379773	0.681931

Holt County	0.022245	0.003501	0.128417
Howard County	0.150370	0.073757	0.282307
Howell County	0.996046	0.932185	0.999784
Iron County	0.678770	0.493502	0.820869
Jackson County	0.141076	0.070667	0.261867
Jasper County	0.362692	0.118303	0.707072
Jefferson County	0.999846	0.990325	0.999998
Johnson County	0.166444	0.078143	0.319898
Knox County	0.024335	0.003893	0.137338
Laclede County	0.994100	0.928753	0.999541
Lafayette County	0.166444	0.078143	0.319898
Lawrence County	0.295342	0.178606	0.446869
Lewis County	0.144590	0.069638	0.276258
Lincoln County	0.597405	0.419791	0.752681
Linn County	0.022245	0.003501	0.128417
Livingston County	0.022245	0.003501	0.128417
McDonald County	0.888139	0.524520	0.982801
Macon County	0.022245	0.003501	0.128417
Madison County	0.449415	0.292113	0.617529
Maries County	0.675108	0.485156	0.820855
Marion County	0.158630	0.083424	0.280859
Mercer County	0.022245	0.003501	0.128417
Miller County	0.953263	0.800452	0.990450

Mississippi County	0.110835	0.049976	0.228017
Moniteau County	0.526817	0.372837	0.675859
Monroe County	0.144590	0.069638	0.276258
Montgomery County	0.265756	0.129778	0.467643
Morgan County	0.534693	0.371525	0.690759
New Madrid County	0.022245	0.003501	0.128417
Newton County	0.671246	0.326532	0.895815
Nodaway County	0.022245	0.003501	0.128417
Oregon County	0.992819	0.924570	0.999359
Osage County	0.284187	0.178461	0.420491
Ozark County	0.999803	0.984207	0.999998
Pemiscot County	0.022245	0.003501	0.128417
Perry County	1.000000	1.000000	1.000000
Pettis County	0.135596	0.066787	0.255861
Phelps County	0.999264	0.976161	0.999978
Pike County	0.612519	0.441504	0.759673
Platte County	0.095446	0.041058	0.206373
Polk County	0.571723	0.393888	0.732778
Pulaski County	1.000000	0.999837	1.000000
Putnam County	0.022245	0.003501	0.128417
Ralls County	0.908716	0.726942	0.973838
Randolph County	0.277693	0.129044	0.499393
Ray County	0.133584	0.061986	0.264560

Reynolds County	0.923396	0.745391	0.980250
Ripley County	0.245535	0.123379	0.429394
St. Charles County	0.527678	0.371191	0.678908
St. Clair County	0.742967	0.476035	0.901928
Ste. Genevieve County	0.998124	0.963422	0.999907
St. Francois County	0.516741	0.256579	0.768134
St. Louis County	0.999128	0.974817	0.999971
St. Louis City County	0.505581	0.352738	0.657388
Saline County	0.130297	0.063085	0.250008
Schuyler County	0.022245	0.003501	0.128417
Scotland County	0.110835	0.049976	0.228017
Scott County	0.125175	0.059558	0.244302
Shannon County	1.000000	1.000000	1.000000
Shelby County	0.250891	0.110647	0.474128
Stoddard County	0.025451	0.004103	0.142033
Stone County	0.999992	0.996265	1.000000
Sullivan County	0.022245	0.003501	0.128417
Taney County	0.999703	0.978132	0.999996
Texas County	0.999945	0.993539	1.000000
Vernon County	0.392095	0.216930	0.600278
Warren County	0.486586	0.264856	0.713724
Washington County	0.997628	0.945545	0.999902
Wayne County	0.604289	0.402872	0.775608

Webster County	0.990176	0.892029	0.999188
Worth County	0.022245	0.003501	0.128417
Wright County	0.756969	0.519357	0.899781

All the previously mentioned caves in this study from Missouri were combined with Jacob's Cave and Tumbling Creek Cave to verifythe WNS predictor model with already completed field work. The WNS predictor model was able to accurately predict the presence of WNS approximately 85% of the time (Table 10). More hibernacula, in more counties will need to be sampled. The statistical model does, however, offer value to researchers, in that it demonstrates patterns in the spread of WNS within Missouri.

Table 10: Model generate predictions and corresponding molecular survey results.

County	Model Prediction	Molecular Survey Result Agreed with Model Prediction
Christian	Positive	Yes x2
McDonald	Positive	Yes
Morgan	Negative	Yes
Newton	Negative	Yes
Ozark	Positive	Yes
Taney	Positive	No

Two biologically important predictor variables were identified to determine the probability that a given Missouri county would be WNS positive, the average county cave density and the average time since WNS was detected in adjacent counties. The more caves that a given county has, the more hibernacula it has. Some bats do not utilize a single cave year-round, rather they migrate from cave to cave. Sometimes the caves may be in different states, and/or counties. This allows the migrating bats to pick up *Pd* in one county and carry it to another (adjacent) county with ease.

The WNS predictor model shows a pattern in the spread of WNS. Counties in Missouri with the highest probability of being WNS positive were, geographically located, in the south and east parts of the state (Figure 19). However, the model has identified several counties that do not appear to be WNS positive or suspected WNS positive according to whitenosesyndrome.org. Those counties are as follows: Cape Girardeau, Maries, Morgan, Polk, Reynolds, St. Charles, St. Louis City, Wayne, and Webster. The WNS predictor model generated probabilities for the being WNS positive that were greater than 0.5 for all these counties (Figure 17). However, Cape Girardeau, Reynolds, and Webster counties have a probability greater than 0.9 for the being WNS positive. These counties should be more heavily tested for the presence of WNS within bat hibernacula.

Figure 17: Comparing the aready known WNS positive counties (A.) in Missouri with the probabilites that counties in Missouri are WNS positive (B.).

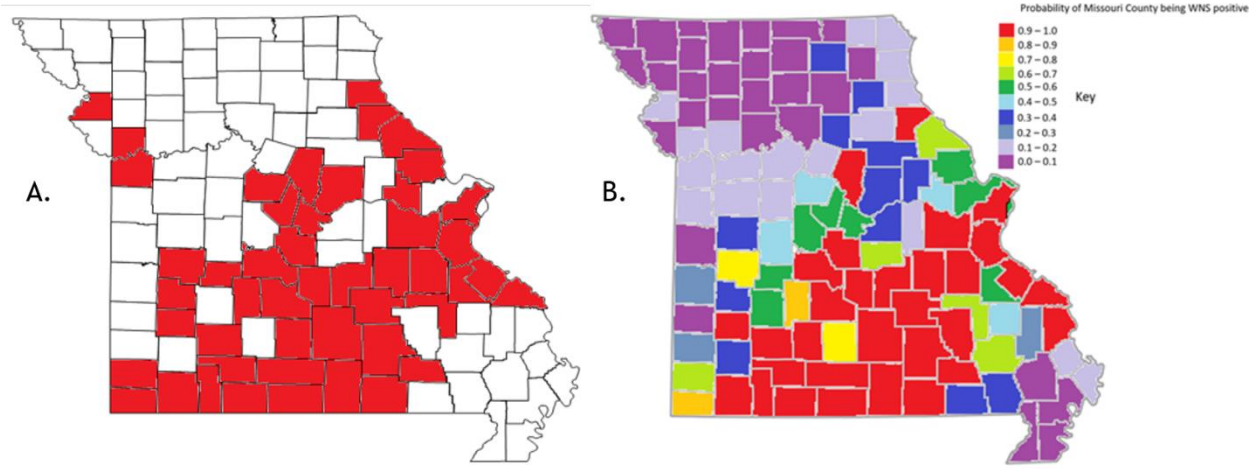


Figure 18: Image of agarose gel for *Pd* PCR in Jacob's Cave.

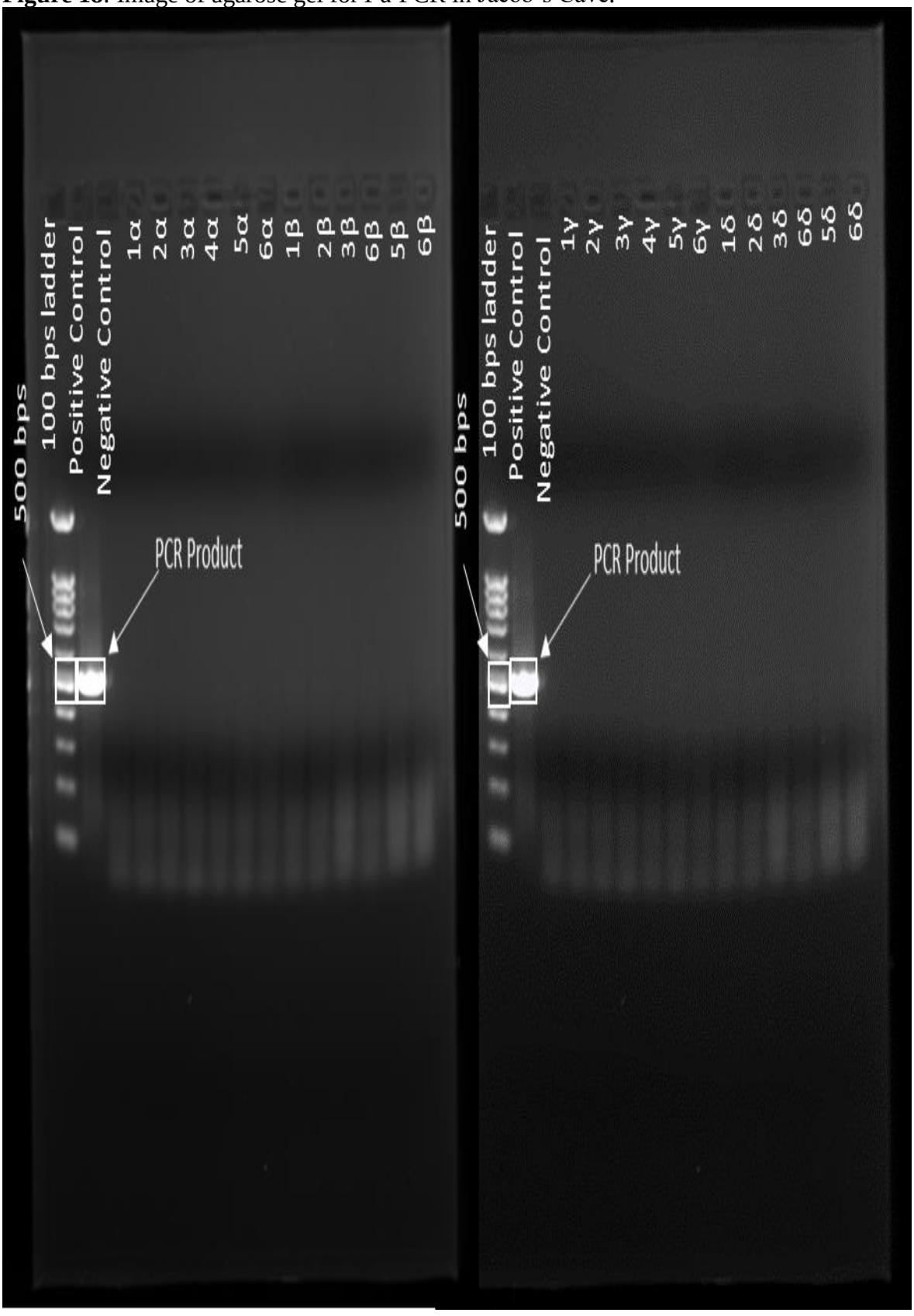
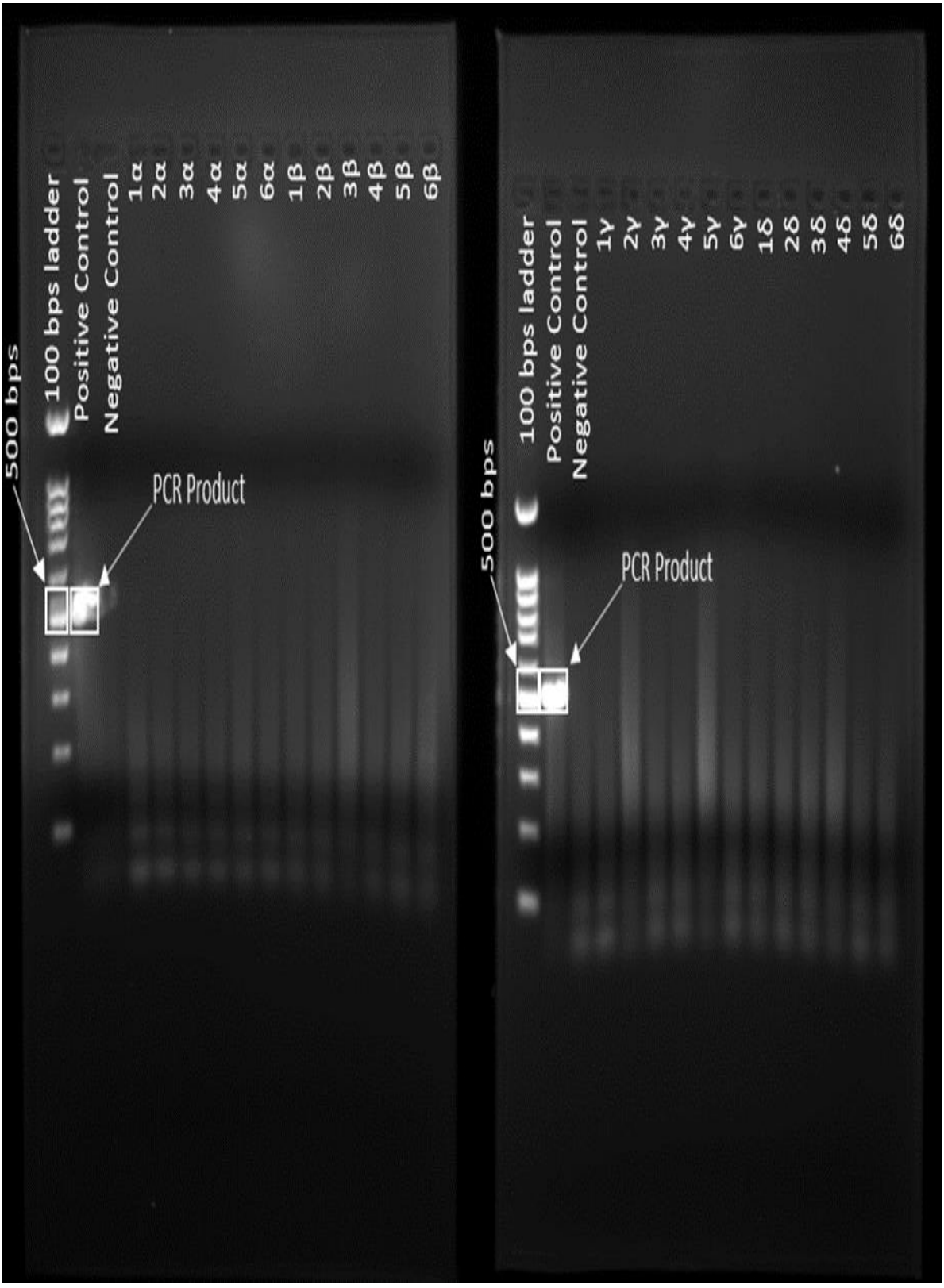


Figure 19: Image of agarose gel for *Pd* PCR in Tumbling Creek Cave.



CHAPTER IV.

CONCLUSION

This study investigated how testing for the presence of *Pd* DNA via PCR compared to visual surveys for the detection of WNS within bat hibernacula. Utilizing PCR as an assay to detect WNS within bat hibernacula is an important component of WNS research. It may prove more sensitive than visual surveys, but it should certainly be implemented in coordination with current visual surveying. *Pd* DNA was identified in all hibernacula that were previously regarded as WNS positive by visual survey methods. *Pd* DNA was successfully identified in all by one hibernaculum regarded as WNS negative by visual survey methods. Hibernaculum MD001 in McDonald County was found to be WNS positive as it contained *Pd* DNA, even though WNS had yet to be visually identified within the hibernaculum.

This study also created a WNS predictor model for counties within Missouri. While there was much similarity to the probabilities generated by the model and the WNS map (Figure 17) at whitenosesyndrome.org, there were nine counties identified that had a probability of being WNS positive (ie. greater than 0.5), and three counties had an extremely probability of being WNS positive (ie. greater than 0.9).

The spread of WNS across North America is a serious phenomenon that requires continued time and attention. WNS is limited in its range of host organisms, which are

also limited to specific habitat niches. If current WNS research can provide cave owners with accurate, rapid information regarding the presence of WNS in their caves, better management strategies could be implemented to help slow or mitigate the spread of the disease.

This was a rewarding study, however, there were some flaws with its design. If a study was to be done like this in the future, it would important to make some of these changes. Instead of investigating the isolated DNA only for the presence of *Pd* DNA, it would be beneficial to investigate the isolated DNA for bacterial and fungal communities. The cave microbiome is an area of microbiology that requires further exploration. Bacterial and fungal community analysis may lead to microbes that exhibit antagonistic relationships with *Pd*. Those communities may prove to a role beneficial to bats in terms of protection from WNS infection.

Due to this study taking place during the summer months, it made the task of identifying bat roosting sites and bat guano sites difficult. It would greatly benefit future studies if time was taken to enter bat hibernacula during the winter to identify and mark those locations, so that the hibernacula could be swabbed more accurately in the summer months when bats are not present in such high concentrations. This would also allow for continued sampling in the same locations.

This study was not a temporal study; however, it would be beneficial to sample at multiple times throughout the year in the same locations. If this was done, comparisons could be drawn, and possible patters made evident. Depending on the goals of future studies, the amount of hibernacula selected or the amount of locations within a particular hibernaculum should be done differently. If the goals of a future study are more general

and deal with WNS progression and spread, then it would be beneficial to sample in as many hibernacula as possible. If the goals of a future study are more specific to individual hibernacula and deal with wanting to understand how the microbiota within a specific hibernaculum function with microclimate and other specific, hibernaculum variables, then it would be beneficial to sample in one hibernaculum as many times as possible.

In future studies that involve modeling, it would be beneficial to analyze different predictor variables, while at the same time keeping in mind county cave density and the average amount of time that a county's adjacent counties have been WNS positive. Instead of looking at WNS distribution from a county perspective, where man has tried to draw geographic boundaries, it would be much more informative to look at WNS distribution from a natural perspective and account for things like rivers, mountain ranges, and physiogeographic boundaries.

Additionally, in future studies, it would be beneficial to model the WNS distribution with respect to each individual hibernacula. This will be difficult as that is sensitive information, and it could tell researchers much about the movement of WNS within a state, county, or area. Additionally, it would be beneficial to create individual state database like the MCD for each state. Similarly, it would be beneficial to create a national database that housed all of the aforementioned state ran databases, and then allow researches to use that resource. Data on bat hibernacula is very well guarded in both state and private entities, which makes it difficult to do research and answer questions about that environment.

Cited References

Banks, H. T., Banks, J. E., Rosenheim, J., & Tillman, K. (2017). Modelling populations of Lygus hesperus on cotton fields in the San Joaquin Valley of California: The importance of statistical and mathematical model choice. *Journal of Biological Dynamics, 11*(Sup1), 25.

Becker, M. H., Walke, J. B., Murrill, L., Woodhams, D. C., Reinert, L. K., Rollins-Smith, L. A., . . . Belden, L. K. (2015). Phylogenetic distribution of symbiotic bacteria from Panamanian amphibians that inhibit growth of the lethal fungal pathogen Batrachochytrium dendrobatidis. *Molecular Ecology, 24*(7), 1628.

Blehert, D. S., Hicks, A. C., Behr, M., Meteyer, C. U., Berlowski-Zier, B. M., Buckles, E. L., . . . Stone, W. B. (2009). Bat White-Nose Syndrome: An Emerging Fungal Pathogen? *Science, 323*(5911), 227-227.

Boire, N., Zhang, S., Khuvis, J., Lee, R., Rivers, J., Crandall, P., . . . Parrish, N. (2016). Potent Inhibition of Pseudogymnoascus destructans, the Causative Agent of White-Nose Syndrome in Bats, by Cold-Pressed, Terpeneless, Valencia Orange Oil. *PloS One, 11*(2), E0148473.

Boyles, J. G., Cryan, P. M., Mccracken, G. F., & Kunz, T. H. (2011). Economic Importance of Bats in Agriculture. *Science, 332*(6025), 41-42.

Canadian Wildlife Health Cooperative. A national plan to manage White Nose Syndrome in bats in Canada. 2015. Available: http://www.cwhc-rcsf.ca/docs/BatWhiteNoseSyndrome-NationalPlan.*Pd*f.

Cardoza, Y. J., Klepzig, K. D., & Raffa, K. F. (2006). Bacteria in oral secretions of an endophytic insect inhibit antagonistic fungi. *Ecological Entomology, 31*(6), 636-645.

Chaturvedi, V., Springer, D. J., Behr, M. J., Ramani, R., Li, X., Peck, M. K., . . . Chaturvedi, S. (2010). Morphological and molecular characterizations of psychrophilic fungus Geomyces destructans from New York bats with White Nose Syndrome (WNS). *PloS One, 5*(5), E10783.

Cornelison, C. T., Keel, M. K., Gabriel, K. T., Barlament, C. K., Tucker, T. A., Pierce, G. E., & Crow, S. A. (2014). A preliminary report on the contact-independent antagonism of Pseudogymnoascus destructans by Rhodococcus rhodochrous strain DAP96253. *BMC Microbiology, 14*(1), 246-246.

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.*BMC Biology, 8*(1), 135-135.

Dowd, S. E., Sun, Y., Secor, P. R., Rhoads, D. D., Wolcott, B. M., James, G. A., & Wolcott, R. D. (2008). Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun sequencing. *BMC Microbiology,8*(1), 43-43.

Anthony, E. L., & Kunz, T. H. (1977). Feeding Strategies of the Little Brown Bat, Myotis Lucifugus, in Southern New Hampshire. *Ecology, 58*(4), 775-786.

Fleming, T. H., Geiselman, C., & Kress, W. J. (2009). The evolution of bat pollination: A phylogenetic perspective. *Annals of Botany, 104*(6), 1017-1043.

Foley, J., Clifford, D., Castle, K., Cryan, P., & Ostfeld, R. S. (2011). Investigating and Managing the Rapid Emergence of White-Nose Syndrome, a Novel, Fatal, Infectious Disease of Hibernating Bats. *Conservation Biology, 25*(2), 223-231.

Frick, W. F., Pollock, J. F., Hicks, A. C., Langwig, K. E., Reynolds, D. S., Turner, G. G., . . . Kunz, T. H. (2010). An Emerging Disease Causes Regional Population Collapse of a Common North American Bat Species. *Science, 329*(5992), 679-682.

Garibaldi, A., & Turner, N. (2004). Cultural Keystone Species: Implications for Ecological Conservation and Restoration. *Ecology and Society*, *9*(3), 1.

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*(1), 147-154.

George, A. D., Thompson III, F. R., & Faaborg, J. (2015). Isolating weather effects from seasonal activity patterns of a temperate North American Colubrid. *Oecologia,178*(4), 1251-1259.

Hayes, M. A. (2012). The Geomyces Fungi: Ecology and Distribution. *BioScience*, *62*(9), 819-823.

Hoyt, J. R., Cheng, T. L., Langwig, K. E., Hee, M. M., Frick, W. F., & Kilpatrick, A. M. (2015). Bacteria isolated from bats inhibit the growth of Pseudogymnoascus destructans, the causative agent of white-nose syndrome. *PloS One, 10*(4), E0121329.

Hurvich, C. M., & Tsai, C. (1989). Regression and Time Series Model Selection in Small Samples. *Biometrika, 76*(2), 297.

Janicki, A. F., Frick, W. F., Kilpatrick, A. M., Parise, K. L., Foster, J. T., & Mccracken, G. F. (2015). Efficacy of Visual Surveys for White-Nose Syndrome at Bat Hibernacula.*PloS One, 10*(7), E0133390.

Krechel, A., Faupel, A., Hallmann, J., Ulrich, A., & Berg, G. (2002). Potato-associated bacteria and their antagonistic potential towards plant-pathogenic fungi and the plant-parasitic nematode *Meloidogyne incognita* (kofoid & white) chitwood. *Canadian Journal of Microbiology, 48*(9), 772-786.

Kunz, T. H., Braun de Torrez, E., Bauer, D., Lobova, T., & Fleming, T. H. (2011). Ecosystem services provided by bats. *Annals of the New York Academy of Sciences, 1223*, 1.

Kurta, A., Kunz, T. H., & Nagy, K. A. (1990). Energetics and Water Flux of Free-Ranging Big Brown Bats (Eptesicus fuscus) during Pregnancy and Lactation. *Journal of Mammalogy, 71*(1), 59-65.

Lorch, J. M., Gargas, A., Meteyer, C. U., Berlowski-Zier, B. M., Green, D. E., Shearn-Bochsler, V., . . . Blehert, D. S. (2010). Rapid Polymerase Chain Reaction Diagnosis of White-Nose Syndrome in Bats. *Journal of Veterinary Diagnostic Investigation, 22*(2), 224-230.

Lorch, J. M., Lindner, D. L., Gargas, A., Muller, L. K., Minnis, A. M., & Blehert, D. S. (2013a). A culture-based survey of fungi in soil from bat hibernacula in the eastern United States and its implications for detection of Geomyces destructans, the causal agent of bat white-nose syndrome. *Mycologia, 105*(2), 237-252.

Lorch, J. M., Meteyer, C. U., Behr, M. J., Boyles, J. G., Cryan, P. M., Hicks, A. C., . . . Blehert, D. S. (2011). Experimental infection of bats with Geomyces destructans causes white-nose syndrome. *Nature, 480*(7377), 376-378.

Lorch, J. M., Muller, L. K., Russell, R. E., O'Connor, M., Lindner, D. L., & Blehert, D. S. (2013b). Distribution and Environmental Persistence of the Causative Agent of White-Nose Syndrome, Geomyces destructans, in Bat Hibernacula of the Eastern United States. *Applied and Environmental Microbiology, 79*(4), 1293-1301.

Maher, S. P., Kramer, A. M., Pulliam, J. T., Zokan, M. A., Bowden, S. E., Barton, H. D., Drake, J. M. (2012). Spread of white-nose syndrome on a network regulated by geography and climate. *Nature Communications, 3*(1), 1306.

Meteyer, C. U., Valent, M., Kashmer, J., Buckles, E. L., Lorch, J. M., Blehert, D. S., . . . Ballmann, A. E. (2011). Recovery of little brown bats (Myotis lucifugus) from natural infection with Geomyces destructans, white-nose syndrome. *Journal of Wildlife Diseases, 47*(3), 618.

Moussy, C., Hosken, D. J., Mathews, F., Smith, G. C., Aegerter, J. N., & Bearhop, S. (2013). Migration and dispersal patterns of bats and their influence on genetic structure. *Mammal Review, 43*(3), 183-195.

Muller, L. K., Lorch, J. M., Lindner, D. L., O'Connor, M., Gargas, A., & Blehert, D. S. (2013). Bat white-nose syndrome: A real-time TaqMan polymerase chain reaction test targeting the intergenic spacer region of Geomyces destructans. *Mycologia,105*(2), 253-259.

O. Norquay, K. J., Martinez-Nuñez, F., Dubois, J. E., Monson, K. M., & R. Willis, C. K. (2013). Long-distance movements of little brown bats (Myotis lucifugus). *Journal of Mammalogy, 94*(2), 506-515.

O'Regan, S. M., Magori, K., Pulliam, J. T., Zokan, M. A., Kaul, R. B., Barton, H. D., & Drake, J. M. (2015). Multi-scale model of epidemic fade-out: Will local extirpation events inhibit the spread of white-nose syndrome? *Ecological Applications, 25*(3), 621-633.

Piterina, A. V., & Pembroke, J. T. (2013). Use of PCR-DGGE Based Molecular Methods to Analyse Microbial Community Diversity and Stability during the Thermophilic Stages of an ATAD Wastewater Sludge Treatment Process as an Aid to Performance Monitoring. *ISRN Biotechnology, 2013,* 162645.

Piterina, A. V., Bartlett, J., & Pembroke, J. T. (2010). Molecular Analysis of Bacterial Community DNA in Sludge Undergoing Autothermal Thermophilic Aerobic Digestion (ATAD): Pitfalls and Improved Methodology to Enhance Diversity Recovery. *Diversity, 2*(4), 505-526.

Racey, P.A. & Entwistle, A.C. (2003). Conservation ecology of bats. In: *Bat Ecology* (Ed. By T.H. Kunz & M.B. Fenton), pp. 680-743. The University of Chicago, Chicago.

Raudabaugh, D. B., & Miller, A. N. (2013). Nutritional capability of and substrate suitability for Pseudogymnoascus destructans, the causal agent of bat white-nose syndrome. *PloS One, 8*(10), E78300.

Shuey, M. M., Drees, K. P., Lindner, D. L., Keim, P., & Foster, J. T. (2013). Highly Sensitive Quantitative PCR for the Detection and Differentiation of

Pseudogymnoascus destructans and Other Pseudogymnoascus Species. *Applied and Environmental Microbiology, 80*(5), 1726-1731.

Stallknecht, D. E. (2007). Impediments to wildlife disease surveillance, research, and diagnostics. *Current Topics in Microbiology and Immunology, 315*, 445.

Storm, J. J., & Whitaker Jr, J. O. (2008). Prey Selection of Big Brown Bats (Eptesicus Fuscus) During an Emergence of 17-Year Cicadas (Magicicada Spp.). *The American Midland Naturalist, 160*(2), 350-357.

Swets, J. A. (1988). Measuring the Accuracy of Diagnostic Systems. *Science,240*(4857), 1285-1293.

Symonds, M., & Moussalli, A. (2011). A brief guide to model selection, multimodel inference and model averaging in behavioural ecology using Akaike's information criterion. *Behavioral Ecology and Sociobiology, 65*(1), 13-21.

Tenorio-Salgado, S., Tinoco, R., Vazquez-Duhalt, R., Caballero-Mellado, J., & Perez-Rueda, E. (2013). Identification of volatile compounds produced by the bacterium Burkholderia tropica that inhibit the growth of fungal pathogens. *Bioengineered,4*(4), 236.

United States National White-nose Syndrome Decontamination Protocol [*PD*F]. (2016, April).

U.S. Fish and Wildlife Service. A national plan for assisting states, federal agencies, and tribes in managing White-Nose Syndrome in bats. 2011. Available: https://www.whitenosesyndrome.org/sites/ default/files/white-nose_syndrome_national_plan_may_2011.*Pd*f 22.

Vanderwolf, K. J., Malloch, D., & Mcalpine, D. F. (2016). Fungi on white-nose infected bats (Myotis spp.) in Eastern Canada show no decline in diversity associated with Pseudogymnoascus destructans (Ascomycota: Pseudeurotiaceae). International Journal of Speleology, 45(1), 43-50.

Verant, M. L., Boyles, J. G., Waldrep, J. W., Wibbelt, G., & Blehert, D. S. (2012). Temperature-dependent growth of Geomyces destructans, the fungus that causes bat white-nose syndrome. *PloS One, 7*(9), E46280.

Warnecke, L., Turner, J. M., Bollinger, T. K., Lorch, J. M., Misra, V., Cryan, P. M., Willis, C. K. (2012). Inoculation of bats with European Geomyces destructans supports the novel pathogen hypothesis for the origin of white-nose syndrome. *Proceedings of the National Academy of Sciences of the United States of America,109*(18), 6999-7003.

White-Nose Syndrome. (2018). https://www.whitenosesyndrome.org/mmedia-education/july-2-2018.

Wibbelt, G., Kurth, A., Hellmann, D., Weishaar, M., Barlow, A., Veith, M., Blehert, D. S. (2010). White-nose syndrome fungus (Geomyces destructans) in bats, Europe.*Emerging Infectious Diseases, 16*(8), 1237-1243.

Wibbelt, G., Barlow, A., Veith, M., Prüger, J., Görföl, T., Grosche, L., Blehert, D. S. (2011). Whitenose syndrome fungus (Geomyces destructans) in bats, Europe. In *Fungal Diseases: An Emerging Threat to Human, Animal, and Plant Health: Workshop Summary* (p. 368). National Academies Press.

Wibbelt, G., Puechmaille, S. J., Ohlendorf, B., Mühldorfer, K., Bosch, T., Görföl, T., . . . Forget, F. (2013). Skin lesions in European hibernating bats associated with

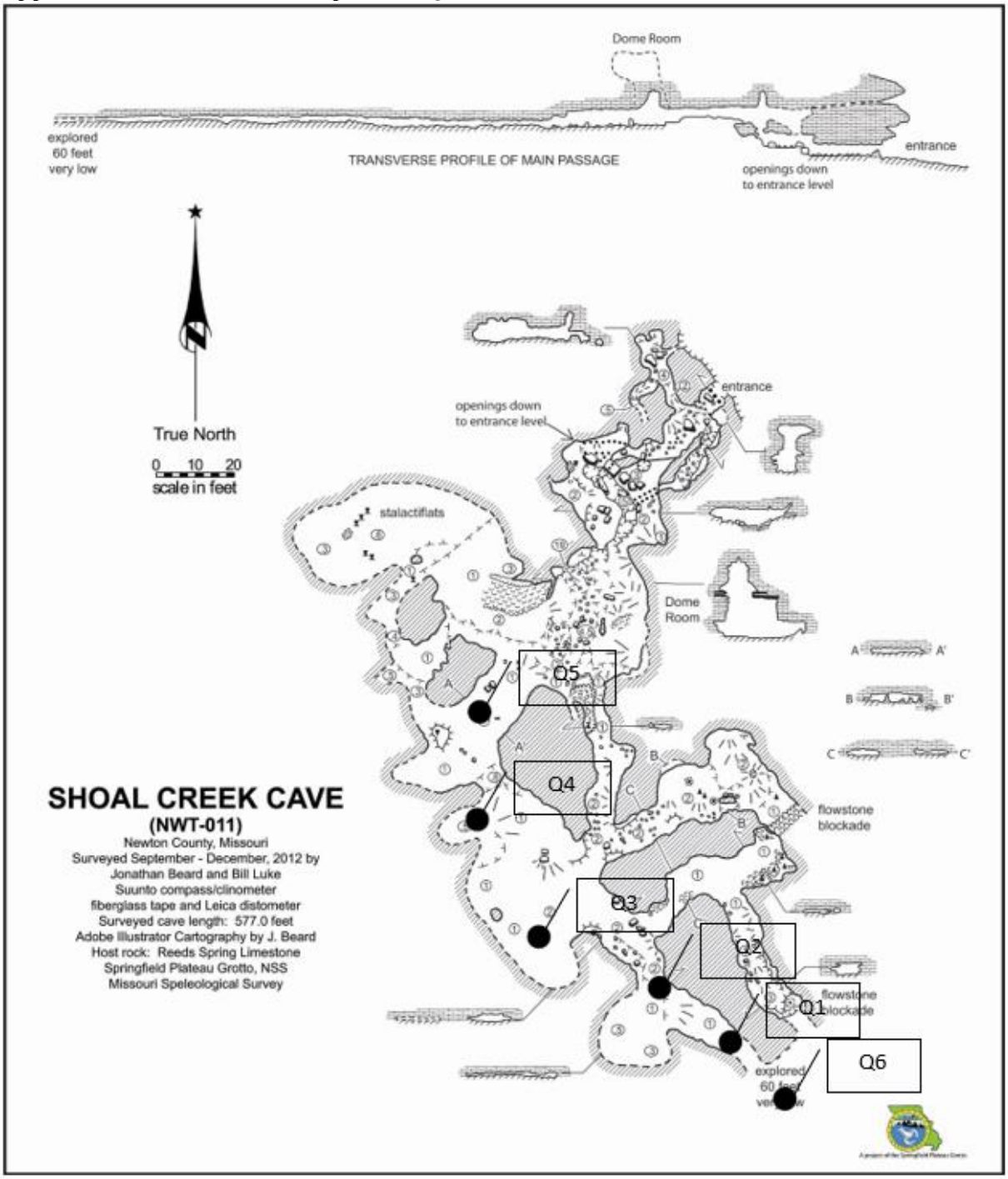
Geomyces destructans, the etiologic agent of white-nose syndrome. *PloS One,8*(9), E74105.

Wilder, A. P., Frick, W. F., Langwig, K. E., & Kunz, T. H. (2011). Risk factors associated with mortality from white-nose syndrome among hibernating bat colonies. *Biology Letters, 7*(6), 950-953.

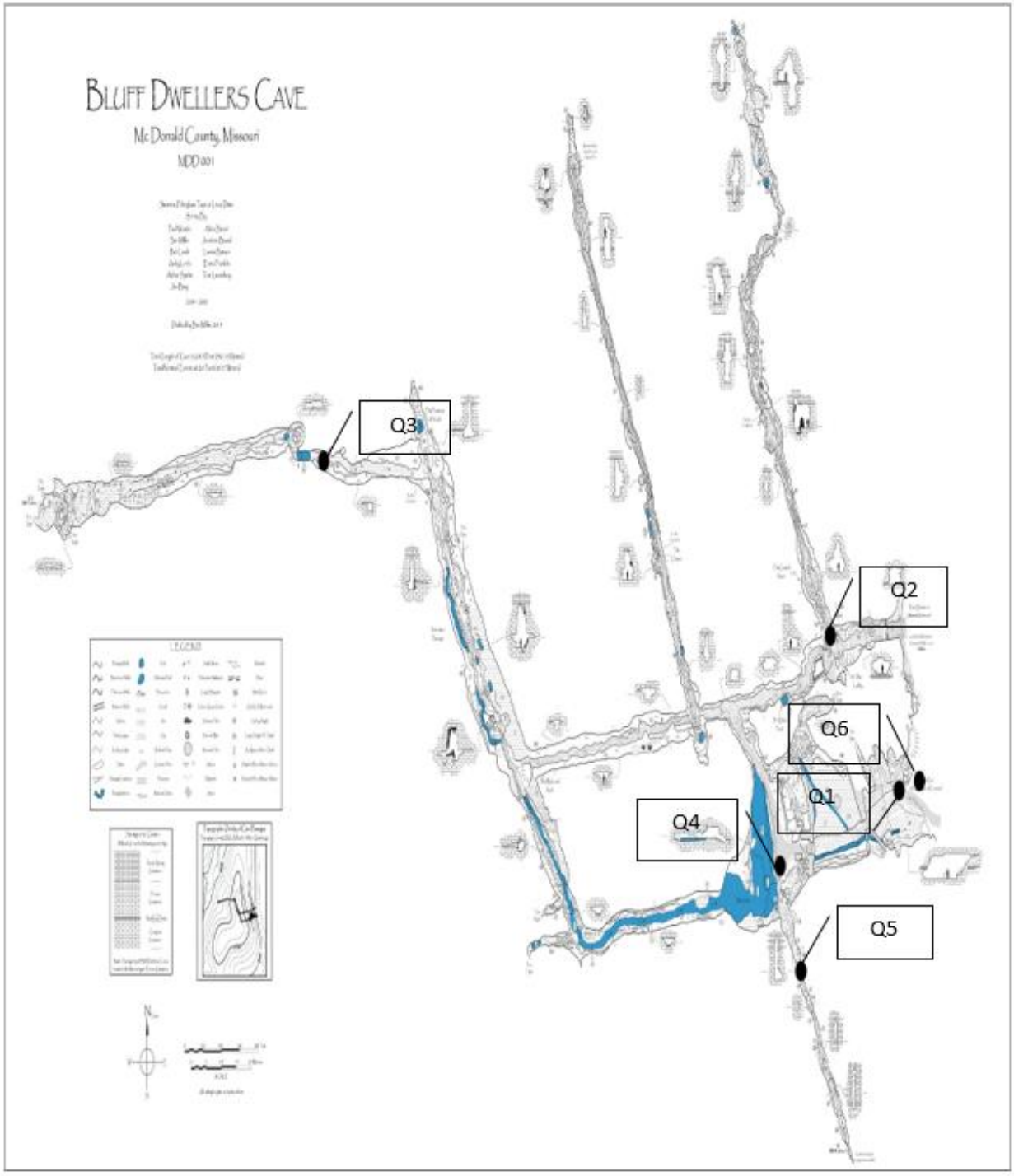
Young, J. M., Northup, D. E., Read, K. J. H., Buecher, D. C., Kimble, J. C., Valdez, E. W., . . . Winter, A. S. (2017). Skin and fur bacterial diversity and community structure on american southwestern bats: Effects of habitat, geography and bat traits. *Peerj, 5*, E3944.

APPENDIX

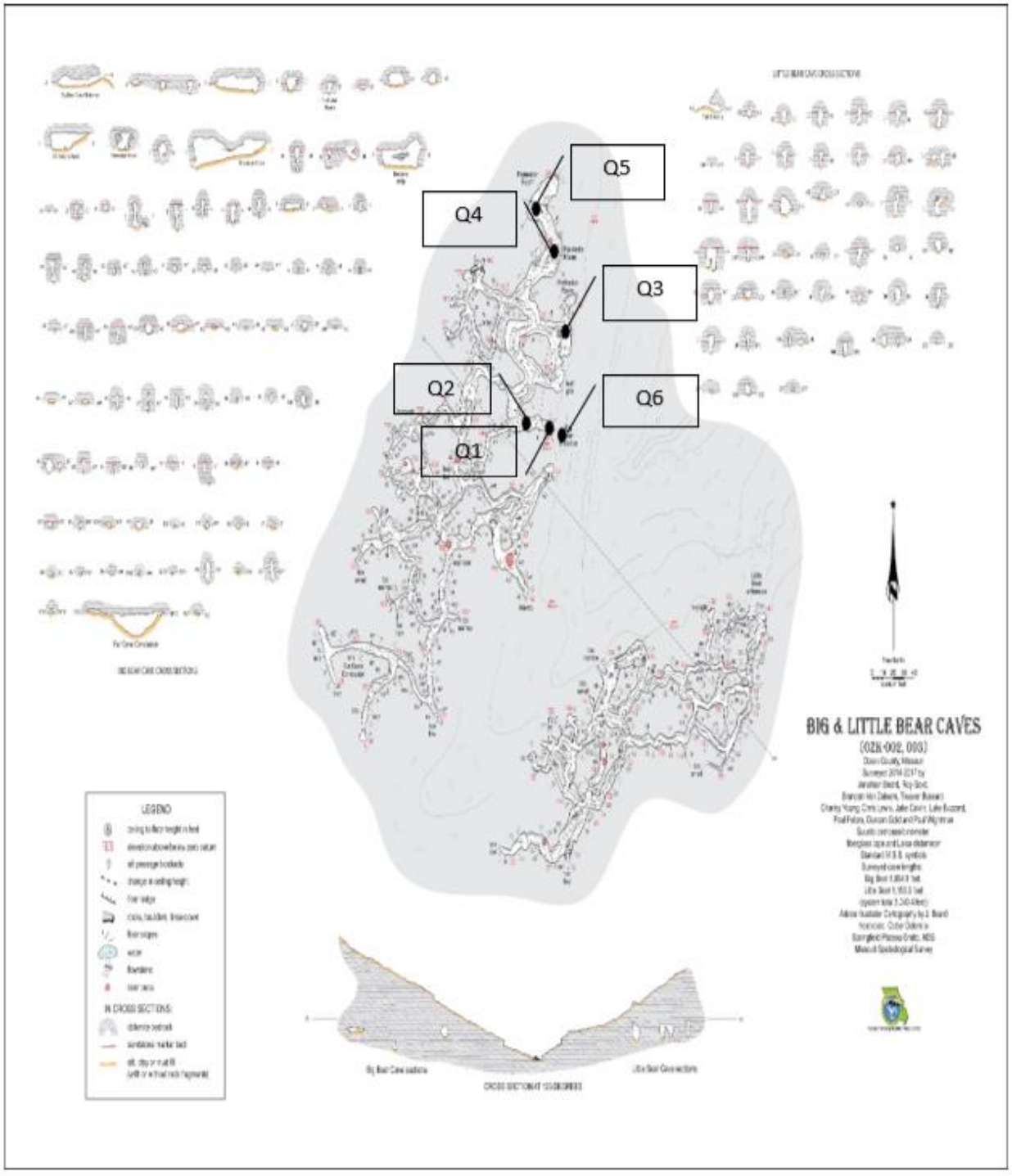
Appendix 1: Hibernacula Maps with Quadrant Locations



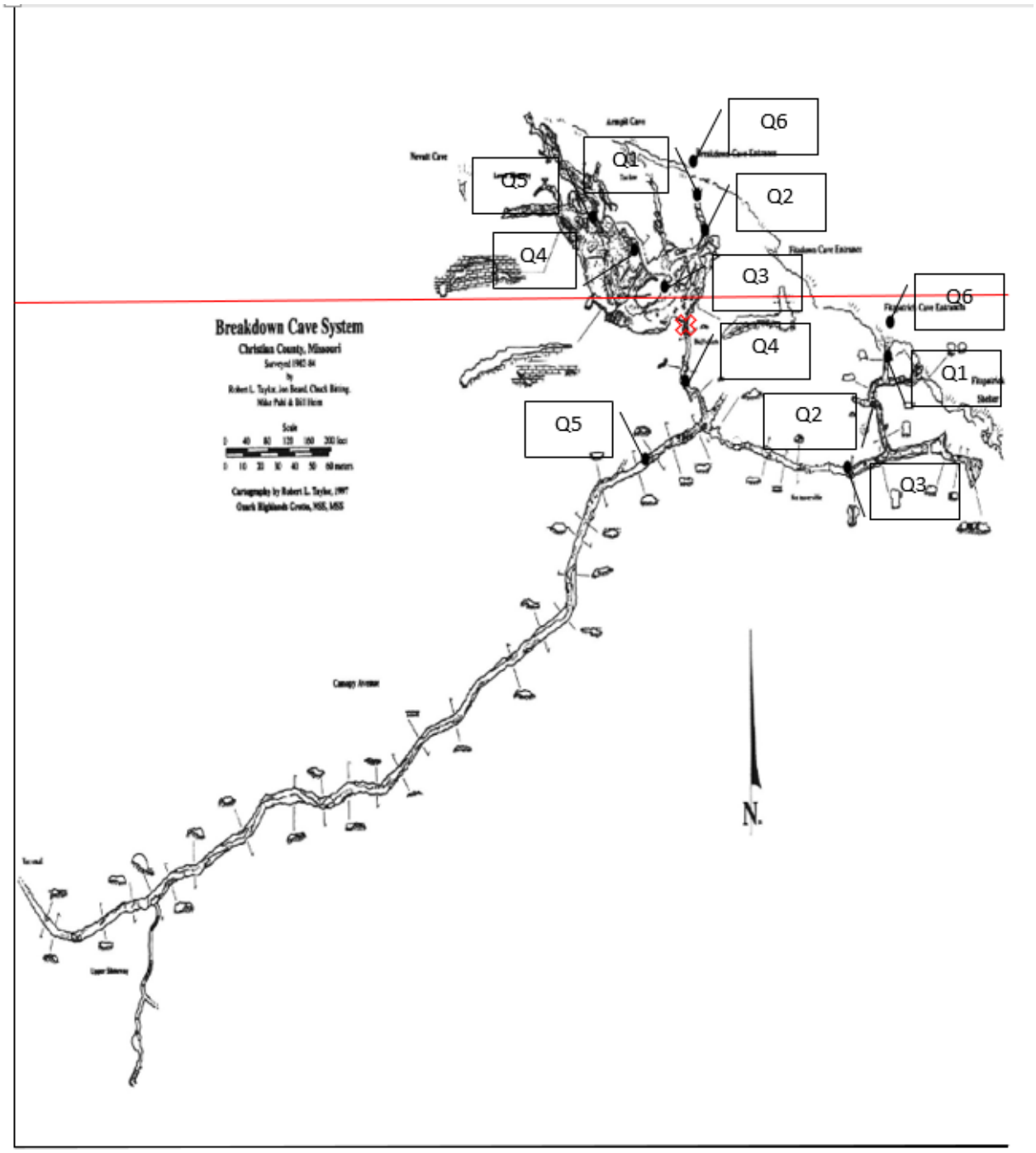
A map of Shoal Creek Cave, sample locations are represented with black dots: Quadrant 1 (Q1), Quadrant 2 (Q2), Quadrant 3 (Q3), Quadrant 4 (Q4), Quadrant 5 (Q5), Quadrant 6 (Q6). This map was created by Jonathan Beard and Bill Luke.



A map of Bluff Dweller's Cave, sample locations are represented with black dots: Quadrant 1 (Q1), Quadrant 2 (Q2), Quadrant 3 (Q3), Quadrant 4 (Q4), Quadrant 5 (Q5), Quadrant 6 (Q6). This map was created by Ben Miller.



A map of Big Bear Cave and Little Bear Cave (Only Big Bear Cave was sampled), sample locations are represented with black dots: Quadrant 1 (Q1), Quadrant 2 (Q2), Quadrant 3 (Q3), Quadrant 4 (Q4), Quadrant 5 (Q5), Quadrant 6 (Q6). This map was created by Jonathan Beard.



A map of Breakdown Cave and Fitzpatrick Cave c(Both Breakdown Cave and Fitzpatrick Cave were sampled), sample locations are represented with black dots: Quadrant 1 (Q1), Quadrant 2 (Q2), Quadrant 3 (Q3), Quadrant 4 (Q4), Quadrant 5 (Q5), Quadrant 6 (Q6). The red line represents the separation between the two caves. This map was created by Robert Tayler.

Appendix 2: Cave Field Sheets

Date: 06/01/2017

Cave: Pittsburg Storm Sewer

Location (County, State): Crawford, Kansas

Quadrant	Where
Quadrant 1	5m inside entrance
Quadrant 2	Twilight Zone
Quadrant 3	Dark Zone
Quadrant 4	(Bat Guano if applicable) Dark Zone
Quadrant 5	(Bat Roosting Site if applicable) Dark Zone
Quadrant 6	5m outside entrance

Was a bat guano site identified for sampling of quadrant 3?

YES NO

Was a bat roosting site identified for sampling of quadrant 5?

YES NO

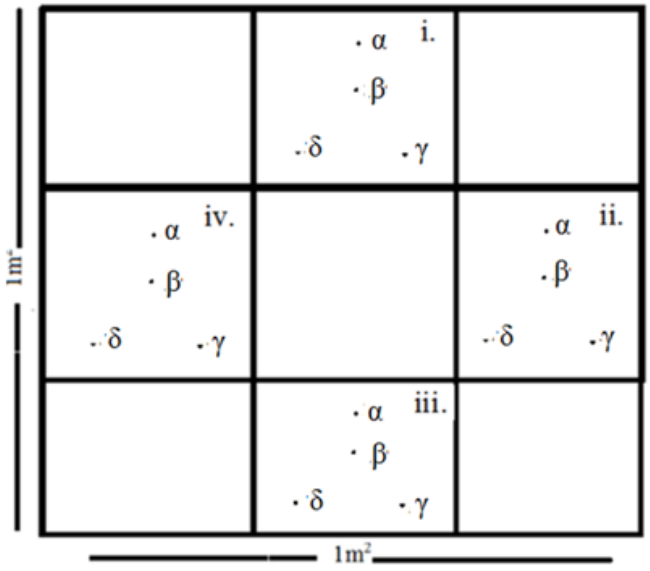
Were bats spotted in cave today?

YES NO

Other notes:

Hundreds of bats present

Example Quadrant



Date: 06/15/2017

Cave: Breadown Cave

Location (County, State): Christian, Missouri

Quadrant	Where
Quadrant 1	5m inside entrance
Quadrant 2	Twilight Zone
Quadrant 3	Dark Zone
Quadrant 4	(Bat Guano if applicable) Dark Zone
Quadrant 5	(Bat Roosting Site if applicable) Dark Zone
Quadrant 6	5m outside entrance

Was a bat guano site identified for sampling of quadrant 3?

YES NO

Was a bat roosting site identified for sampling of quadrant 5?

YES NO

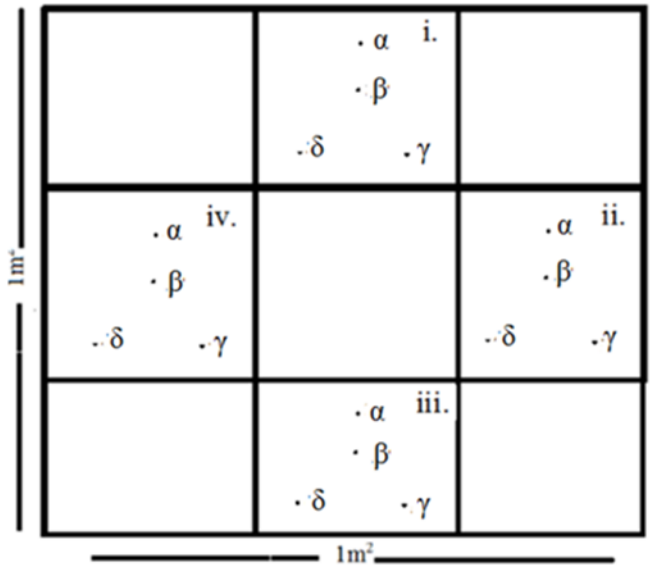
Were bats spotted in cave today?

YES NO

Other notes:

Only a few bats were spotted <5

Example Quadrant



Date: 06/15/2017

Cave: Fitzpatrick Cave

Location (County, State): Christain, Missouri

Quadrant	Where
Quadrant 1	5m inside entrance
Quadrant 2	Twilight Zone
Quadrant 3	Dark Zone
Quadrant 4	(Bat Guano if applicable) Dark Zone
Quadrant 5	(Bat Roosting Site if applicable) Dark Zone
Quadrant 6	5m outside entrance

Was a bat guano site identified for sampling of quadrant 3?

YES NO

Was a bat roosting site identified for sampling of quadrant 5?

YES NO

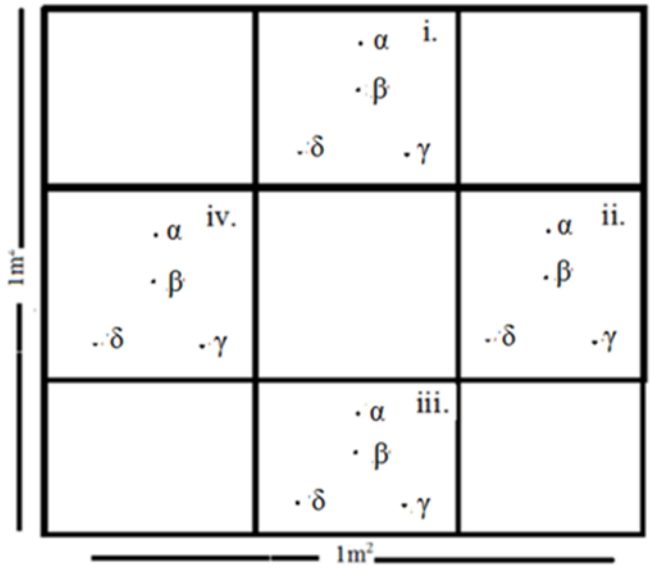
Were bats spotted in cave today?

YES NO

Other notes:

Only a few bats were spotted <5

Example Quadrant



Date: 06/22/2017

Cave: Bluff Dweller's Cave

Location (County, State): McDonald, Missouri

Quadrant	Where
Quadrant 1	5m inside entrance
Quadrant 2	Twilight Zone
Quadrant 3	Dark Zone
Quadrant 4	(Bat Guano if applicable) Dark Zone
Quadrant 5	(Bat Roosting Site if applicable) Dark Zone
Quadrant 6	5m outside entrance

Was a bat guano site identified for sampling of quadrant 3?

YES NO

Was a bat roosting site identified for sampling of quadrant 5?

YES NO

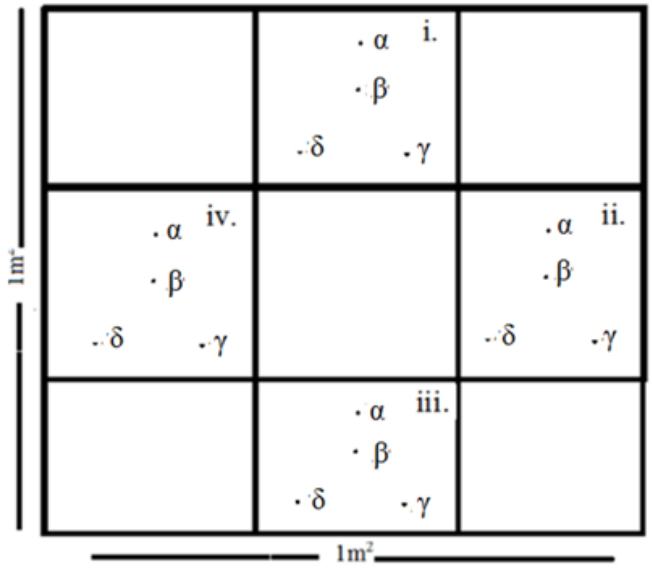
Were bats spotted in cave today?

YES NO

Other notes:

Only a few bats were spotted <5

Example Quadrant



Date: 07/04/2017

Cave: Shoal Creek Cave

Location (County, State): Newton, Missouri

Quadrant	Where
Quadrant 1	5m inside entrance
Quadrant 2	Twilight Zone
Quadrant 3	Dark Zone
Quadrant 4	(Bat Guano if applicable) Dark Zone
Quadrant 5	(Bat Roosting Site if applicable) Dark Zone
Quadrant 6	5m outside entrance

Was a bat guano site identified for sampling of quadrant 3?

YES NO

Was a bat roosting site identified for sampling of quadrant 5?

YES NO

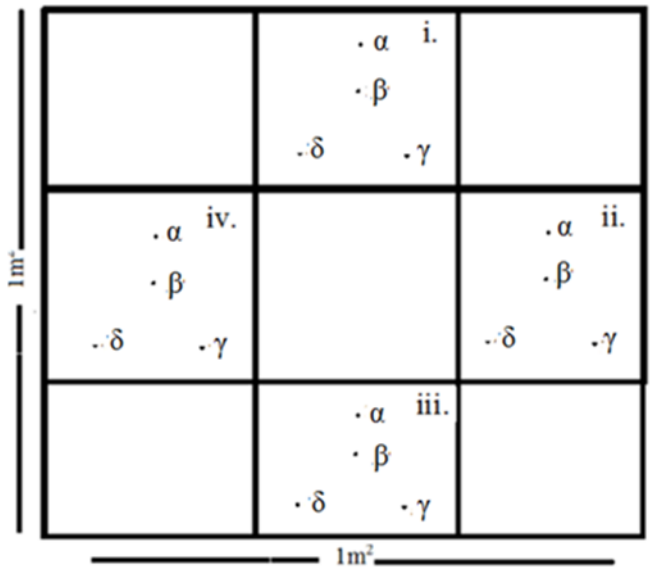
Were bats spotted in cave today?

YES NO

Other notes:

NA

Example Quadrant



Date: 12/7/2017

Cave: Big Bear Cave

Location (County, State): Ozark, Missouri

Quadrant	Where
Quadrant 1	5m inside entrance
Quadrant 2	Twilight Zone
Quadrant 3	Dark Zone
Quadrant 4	(Bat Guano if applicable) Dark Zone
Quadrant 5	(Bat Roosting Site if applicable) Dark Zone
Quadrant 6	5m outside entrance

Was a bat guano site identified for sampling of quadrant 3?

YES NO

Was a bat roosting site identified for sampling of quadrant 5?

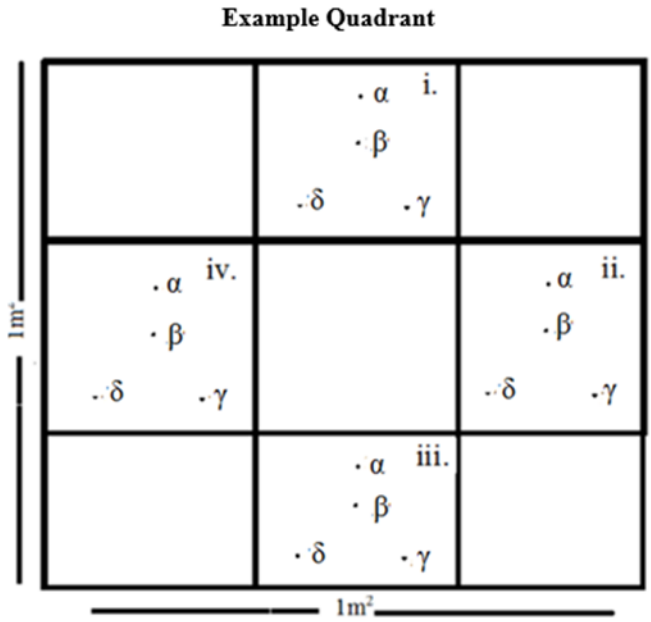
YES NO

Were bats spotted in cave today?

YES NO

Other notes:

Only a few bats were spotted <5



APPENDIX 3: Modified Qiagen DNeasy Powersoil Kit Protocol

1. Add 750µL of 1x PBS solution containing cave swab samples into 2 PowerBead Tubes (total volume used is 1.5mL)
2. Gently vortex PowerBead Tubes for 5 seconds
3. Make sure solution C1 is dissolved, if not supply heat until precipitate re-dissolves
4. Add 90µL of solution C1 to each of the two PowerBead tubes
    a. Vortex 5 seconds to mix
5. Vortex PowerBead tubes on table top vortex for 10 minutes at maximum speed
6. Centrifuge PowerBead tubes at 10,000 times gravity for 1 minute at room temp
7. While avoiding the pellet, collect all the supernatant (approximately 900µL)

a. Transfer equally into two microcentrifuge tubes

8. Add 250µL of solution C2 to each tube
    a. Vortex 5 seconds to mix
    b. Incubate the two tubes on ice (4ºC) for 5 minutes
9. Centrifuge each of the microcentrifuge tubes at 10,000 times gravity for 1 minute at room temp
10. While avoiding the pellet, collect all the supernatant (approximately 1,800µL to 2,000µL)
    a. Place 450µL to 500µL of supernatant into 4 microcentrifuge tubes
11. Add 200µL of solution C3 to each of the 4 microcentrifuge tubes
    a. Vortex 5 seconds to mix
    b. Incubate the four tubes on ice (4ºC) for 5 minutes
12. Centrifuge each of the microcentrifuge tubes at 10,000 times gravity for 1 minute at room temp
13. While avoiding the pellet, collect all the supernatant from the 4 tubes and collect them into 1 falcon tube
14. Invert solution C4 4 times before use
    a. Guestimate the approximate volume inside the falcon tube and multiply by a factor of 1.4 and add that much of solution C4 to the pooled supernatants in the falcon tube
15. Load 675µL from the falcon tube onto a spin filter column
    a. Centrifuge the spin filter at 10,000 times gravity for 1 minute at room temp

b. Discard the flow through

c. Repeat until no supernatant remains in the falcon tube

16. Add 500µL of solution C5 to the spin filter

    a. Centrifuge the spin filter at 10,000 times gravity for 30 seconds at room temp

17. Discard the flow through

18. Centrifuge the spin filter again at 10,000 times gravity for 1 minute at room temp

19. Place spin filter into clean microcentrifuge tube

20. Add 100µL molecular grade water (DNA/RNA free water) to the center of the spin filter

    a. Let it set 1 minute on the spin filter

21. Centrifuge the spin filter at 10,000 times gravity for 30 seconds at room temp

22. Label and store DNA at -20ºC until needed