ENVIRONMENTAL ASSOCIATIONS OF *OPHIDIOMYCES OPHIODIICOLA* PRESENCE, THE CAUSITIVE AGENT OF SNAKE FUNGAL DISEASE

by

Nicholas G. Friedeman

A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Biological Sciences at Purdue Fort Wayne

Fort Wayne, Indiana

May 2022

THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Mark Jordan, Chair
Department of Biological Sciences

Dr. Evin Carter
Oak Ridge National Laboratory

Dr. Bruce Kingsbury
Department of Biological Sciences

Dr. Tanya Soule
Department of Biological Sciences

Approved by:
Dr. Jordan M. Marshall

For Alli and my family

ACKNOWLEDGMENTS

The time I have had here at Purdue Fort Wayne has been indescribable. The challenges I've overcome and all the awesome experiences I've had have been thanks to the people around me. I would not be at this point without those people.

With that I'd like to start by thanking for ___ funding this project. This project never would have been possible if it wasn't for them. It is their work that has helped fund my work and many other projects. Without their help lots of important research wouldn't be possible. My thanks extends to Matthew Kleitch, Carla Lange, Darcia Little, and Cullen Haesler with the environmental office at Camp Grayling. Their willingness to help was crucial for this project to go smoothly.

I also have to thank my parents. They have always been a call away and their continued support thought my life means the world. I cannot forget to mention my twin sis, having her close by in Detroit was something special. Even if we couldn't go home to Kansas we could still find time to visit each other. I must also thank Alli for joining me on this adventure. I know it wasn't always easy but having a partner through it all has been truly special. I wouldn't want have lived this experience with anyone else.

Of course I have to thank my field techs Owen Krauskopf, Katie Brandewie, and Zach Kellogg for their critical help during field sampling and lab work. If it wasn't for them I'd probably still be out sampling now. As well as all the unofficial field techs and fellow graduate students that came out with me. Not only was it helpful but it made for lots of fun and memories I'll cherish forever.

I need to thank my entire committee as well, Dr. Evin Carter, Dr. Bruce Kingsbury, Dr. Tanya Soule, and Dr. Mark Jordan. Their help and critiques have made this project what it is today, and without them I could never made it as far as I have. They each played major roles in the development of the project and have helped me work through hurdles I've come across.

Finally I have to thank Dr. Jordan, I am so grateful that I have gotten the opportunity to be a part of his lab. He has consistently guided me through this project at every step of the way. From him I have learned about research and professionalism, and I hope to continue to learn into the future with him as contact and friend.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Emerging pathogenic fungi have become a topic of conservation concern due to declines seen in several host taxa. One newly emerging fungal pathogen, *Ophidiomyces ophiodiicola*, has been well documented as the causative agent of Snake Fungal Disease (SFD). SFD has been found in a variety of snake species across the United States, including the Eastern Massasauga (*Sistrurus catenatus*), a federally threatened rattlesnake species. Most work to date has involved detecting SFD for diagnosis of infection through direct sampling from snakes. Attempts to detect *O. ophiodiicola* in the environment to better understand its distribution, seasonality, and habitat associations are lacking. I collected topsoil and ground water samples from four macrohabitat types in northern Michigan at a site where SFD infection has been seen in Eastern Massasauga. I used a quantitative PCR (qPCR) assay targeting the internal transcribed spacer region (ITS) developed for diagnosis of SFD after extracting DNA from samples. *Ophidiomyces* DNA was successfully detected in topsoil, with minimal to no detection in groundwater samples. The frequency in which *Ophidiomyces* was detected in a sample did not differ between habitats, but samples grouped seasonally showed higher detection occurring during mid-summer. Investigation of the correlation of environmental parameters on *Ophidiomyces* occurrence recovered no relationships. Our data suggests that season has some effect on the presence of *Ophidiomyces*. Differences between habitats may exist but are likely more dependent on the time of sampling and currently uninvestigated soil parameters. These findings build on our understanding of *Ophidiomyces* ecology and epidemiology and inform where snakes like the Eastern Massasauga may be encountering the fungal pathogen. Furthermore, they assist with developing conservation practices aimed at reducing *O. ophiodiicola* exposure in imperiled snake species.

CHAPTER 1. INTRODUCTION

1.1 The Emerging Threat of Wildlife Diseases

Global biodiversity is currently under threat from a variety of factors. Threats include climate change and anthropogenic land development, overexploitation, and the introduction of invasive species (Foden et al. 2013, Bellard et al. 2016). These activities are some of the top concerns when it comes to biodiversity preservation and lead to eventual species decline in most cases if they are allowed to persist. However, another threat that has become prominent in recent years is wildlife disease. Alone it can lead to population level declines in species, but when it is present alongside other pressures, it can become a serious threat to biodiversity.

Although wildlife disease is not something new, it has been receiving increased attention given the emergence of several novel diseases that have led to notable declines in sensitive species across the globe. Human activity has not helped this matter, as our highly motile lifestyles have led to the unintentional spread of novel diseases to certain areas through carrying it ourselves, or through the introduction of non-native species carrying novel pathogens. Climate change has also played a role in increasing the frequency and intensity of disease emergence in many cases. Most emerging diseases arise as novel pathogens, defined as a pathogen that is spread or transported via an animal or person to a new area where those organisms present have not been exposed and therefore do not have any immunity nor alternative ways to cope with its introduction (Morse 1995, Skerratt et al. 2007).

An example of this is White Nose Syndrome, a disease caused by the fungal pathogen *Pseudogymnoascus destructans*. This disease is seen in microchiropteran bats effecting at least 62 species, and has led to widespread declines in several hibernating bat species (Dzal et al. 2011, Reichard et al. 2014, Powers et al. 2016, Reynolds et al. 2016, Hoyt et al. 2021). *P. destructans* shows little differentiation in genetic markers between isolates, lending credit to a more recent emergence following the novel pathogen hypothesis. Another prominent disease is caused by the fungal pathogens *Batrachochytrium dendrobatidis* and *B. salamandrivoran*s, known to cause Chytridiomycosis in anurans and salamanders, respectively. Infection has been documented in 516 species across 52 countries, with some species exhibiting severe infection while others, such as the African Clawed Frog (*Xenopus laevis*) act as asymptomatic reservoirs (Eskew and Todd 2013).

Both fungal pathogens have widespread distributions and seem to have been spread initially via anthropogenic activity. The continued spread of *B. dendrobatidis* and *P. destructans* across the landscape is facilitated by their ability to persist in their environment within abiotic reservoirs. (Eskew and Todd 2013, Kolby et al. 2015). Alternatively, endemic pathogens can cause disease emergence on a landscape due to changes in climate, which increase virulence created by optimal conditions for pathogen growth and increased infectiousness (Skerratt et al. 2007). It is likely that climate change will lead to continued increases in the emergence of disease, allowing pathogens to colonize new geographic areas and infect novel populations.

1.2 Ophidiomyces ophiodiicola

Another recently emerged fungal pathogen, *Ophidiomyces ophiodiicola*, has been associated with Snake Fungal Disease (SFD). The disease has grown in concern as it has the potential to impact species on a population level (Clark et al. 2011, Allender et al. 2015c). *O. ophiodiicola* (formally known as *Chrysosporium ophiodiicola*) was formerly grouped within the Chrysosporium anamorph of *Nannizziopsis vriesii* (CANV) complex, a complex that contained *Nannizziopsis vriesii* and other similar isolates (Paré and Sigler 2016). Phylogenetic analysis of small sub-unit (SSU) and internal transcribed spacer (ITS) rRNA has revealed three lineages within Onygenaceae family that were once contained within CANV, those being the three now currently known genera of CANV fungi, *Nannizziopsis*, *Paranannizziopsis*, and *Ophidiomyces* (Sigler et al. 2013). These genera are known to cause dermal infection in a variety of reptilian taxa from the tuatara (*Sphenodon punctatus*) to crocodilians and a variety of squamates, however the genus *Ophidiomyces* and its sole species *O. ophiodiicola* have only been documented in snakes (Sigler et al. 2013). In the past, reports of dermal disease in reptiles have been suspected to be caused by CANV or CANV-like fungi, but the delineation of the genus *Ophidiomyces* by Sigler et al. (2013) led researchers to identify that many of these cases were caused by *O. ophiodiicola* (Allender et al. 2015c).

O. ophiodiicola infection and its link with SFD was validated by Koch's postulates through experimental infection of snakes from isolates taken from infected wild snakes (Lorch et al. 2015). Lorch et al. (2015) tracked infection and SFD progression, finding that infection is facilitated when there is an existing breach in the outer layer of the epidermis, the stratum corneum, which allows for entry of fungal spores or fungal hyphae. Up to a week after initial infection, inflammation

occurs at initial lesions along with whitening around the scale edges. Eventual hyperkeratosis results from the thickening of the keratin making up the scales and is associated with hyperpigmentation. As infection progresses necrosis begins to occur at lesions, and infection can disseminate and lead to systemic infection as fungal hyphae penetrate deeper tissue. Snakes that do manage to clear infection through ecdysis (the shedding of their skin), if exposed again, will redevelop similar lesions.

O. ophiodiicola has been shown to have robust growth on the dead tissue of several taxa, showing a wide array of metabolic activity (positive for lipase, gelatinase, keratinase activity, etc.) *in vitro*. It can grow when exposed to a wide range of pH (5-11), temperatures (7°C to 35°C), and moisture conditions. For these reasons, it has been suggested that *O. ophiodiicola* exists as a saprobe which uses open carbon sources in the environment while opportunistically infecting snakes (Allender et al. 2015c).

One of the earliest recorded observation of SFD was recovered from a museum specimen collected in 2000 from southern Illinois (Allender et al. 2016). Since then, there have been several observations of SFD in both pit vipers and colubrid species (6 families and over 30 species) across the midwestern and eastern United States, parts of Germany, the United Kingdom, and Australia (Allender et al. 2011, Clark et al. 2011, Allender et al. 2015c, Tetzlaff et al. 2015, Lorch et al. 2016, Hileman et al. 2017). *Ophidiomyces* was recently reported in California for the first time (Haynes et al. 2021) showing its presence in the western United States. Although it has been recorded causing infection in several species in the United States, it has only been associated with one population level decline, a Timber Rattlesnake (*Crotalus horridus*) population in New Hampshire, where it acted synergistically alongside high summer rainfall, and inbreeding depression (Clark et al. 2011).

The future extent to which SFD could affect snake populations is largely unknown, especially in populations that face an ever-increasing number of stressors (Hileman et al. 2017). As the climate continues to change, factors like *O. ophiodiicola* prevalence across the landscape could change as well. Therefore, it is important to uncover *O. ophiodiicola* ecology and distribution to determine which current populations are at risk of SFD exposure now and in the future. To accomplish this goal of having a more complete epidemiological understanding of *O. ophiodiicola*, further work on its presence within susceptible populations, as well as across the landscape, needs to be done. To date, most published work on *O. ophiodiicola* identifies presence

based through the direct swabbing of active infections on snakes. Information on its ecology independent of infection is largely lacking. However, a recently published paper by Campbell et al. (2021) detected *O. ophiodiicola* within the soil of snake hibernacula as well as topsoil at the level of 61% and 8% of samples, respectively. These findings from environmental studies of *O. ophiodiicola* are promising as it is important to understand where *O. ophiodiicola* exists in the environment and how it exists there. Knowledge that can help inform how snakes are developing infection and give information to managers actively surveying for it.

1.3 Eastern Massasauga

Several species of pit vipers have experienced a high frequency of infection with fungal pathogens that match the clinical signs of SFD (Cheatwood et al. 2003, Clark et al. 2011, Allender et al. 2015b, Tetzlaff et al. 2017). The Eastern Massasauga (*Sistrurus catenatus*) have been found displaying clinical symptoms of SFD since 2008 (Allender et al. 2011) and near the northern extent of their range in Grayling Michigan since 2013 (Allender et al. 2011, Tetzlaff et al. 2015). *O. ophiodiicola* presence was later confirmed in the tissue of the infected snakes (Allender et al. 2015b). This was the first observation of SFD in snakes in the state of Michigan (Tetzlaff et al. 2015), a relative stronghold for *S. catenatus*.

The Eastern Massasauga is a small species of rattlesnake with a geographic range from Illinois, east to New York and Ontario (Shoemaker and Gibbs 2010, DeGregorio et al. 2011, Ravesi 2016). It utilizes various wetland, prairie, and low-lying habitats (Syzmanski 1998, Merkling 2018) as well as drier upland habitat depending on the time of year, and physiological needs between cohorts and populations (Reinhart and Kodrich 1982, Syzmanski 1998). Eastern Massasaugas, as ectotherms occupying higher latitudes, spend half of the year overwintering. When available they use existing burrows made by crayfish to go below the frost line, and partially submerge themselves in water (Smith 2009) to provide insulation from temperature changes and prevent desiccation. Depending on geographic location, hibernacula use can be extensive, and the availability of suitable overwintering habitat can be an important limiting factor of their distribution.

Historically, the species could be found throughout its range, however due to fragmentation it has declined. Remaining populations are small and isolated due to urban and agricultural development creating unsuitable habitat (Syzmanski 1998, Johnson 2000). For this reason, the

Eastern Massasauga has been listed federally as threatened, and threatened or endangered in all states and provinces where it currently resides, apart from Michigan where it is a species of special concern (DeGregorio et al. 2011).

With reduced and isolated populations throughout its range, the additional pressure of an emerging pathogen is a concern. Fragmented and pressured populations of another pit viper, the timber rattlesnake (*Crotalus horridus*) decreased by half in the span of 15 years (1995-2010) given the presence of SFD with other stressors (Clark et al. 2011). Such an event could occur in a population of Eastern Massasauga, and the likelihood will only increase as factors like climate change and anthropogenic development continue. It is also important to consider research methods when studying the Eastern Massasauga, as researchers have the potential to increase disease prevalence by the introduction of SFD to new sites. As well as increase the ability of *O. ophiodiicola* to cause infection on an individual level, for the use of implanted radio-transmitters and other devices on fungal prevalence and infection is currently unknown (Hileman et al. 2017). For these reasons it is important to deploy management decisions that can help mitigate further population decline and susceptibility to infection by emerging diseases.

1.4 Environmental Sampling

Environmental sampling involves collecting samples from mediums such as soil or water. Recently, the environmental sampling approach has been applied for the detection of organisms in the environment via the detection of the organism itself, or DNA from environmental sources. PCR based approaches are often used to then determine presence of the species. This style of environmental sampling has been present in the field of microbial ecology since the 90's (Giovannonoi et al. 1990). Since then it has been applied to detect and quantify a variety of bacterial and fungal organisms both in the environment and for food safety (Schena et al. 2004, Nielsen et al. 2012, Lozano-Ojalvo et al. 2015, Kamoroff and Goldberg 2017, Trujillo-Gonzalez et al. 2019). In the past, traditional methods such as culture-based techniques would be used to answer questions regarding microbial ecology. However, they tend to be time consuming and cannot always generate appropriate results depending on the research goals. Additionally, in complex microbial environments like soil, such methods are not always sensitive enough to detect the species of interest (Cooke et al. 2007). Environmental sampling and DNA detection provide

an alternative route to more traditional methods for detecting microorganisms within the environment.

Environmental sampling has been successfully utilized successfully to detect fungal plant pathogens (Cullen et al. 2001, 2002, Lees et al. 2002) and insect associated pathogenic fungi (Sun et al. 2008). Aquatic, fungal pathogen detection has also applied environmental sampling with success (Kamoroff and Goldberg 2017, Trujillo-Gonzalez et al. 2019). Sampling schemes to detect pathogens environmentally can be adapted to observe temporal or spatial shifts in presence as has been applied in studying mycorrhizal and endophyte fungal communities (Davison et al. 2012, Ek-Ramos et al. 2013). This would make it possible to draw inferences on pathogen presence through time and space, to determine environmental associations that can indicate aspects about fungal activity and pathogen infection.

Quantitative PCR (qPCR) has become heavily associated with such environmental sampling for both single and multiple species. It can provide faster and more sensitive detection than conventional PCR (Lees et al. 2002, Schena et al. 2002, Shena and Ippolito 2003). With the application of qPCR for microbial species detection, findings are no longer limited to presence alone. Depending on the assay applied it is possible to quantify prevalence in the environment as well.

It is important however, to consider certain factors when using an environmental sampling scheme to detect species. False detections, or false positives, occurs when a sample that is truly negative is given as a positive results usually due to cross contamination. Such false positives can generate false data and lead to incorrect assumptions. The same principle applies for false negative detections as well, where a sample is incorrectly assigned a negative result when in truth it is positive. Therefore, it is critical to properly decontaminate sampling equipment to avoid species detection in truly negative samples, while also ensuring that the methods of sampling can detect truly positive samples (Bohmann et al. 2014). Additionally, realizing the capabilities of the assay applied is important. Not all assays can lead to appropriate quantification (Allender et al. 2015a) due to the regions they target for amplification. Therefore, it is important to choose or design an assay that can meet the desired research goals.

The inclusion of environmental sampling using highly specific and sensitive assays for detection can act as an early monitoring tool. This would allow for the detection of pathogenic

species in a new area while they are at low densities. Which can mitigate the spread of disease to new geographic areas and allowing managers to act in a timelier manner.

1.5 Objectives

O. ophiodiicola is responsible for causing SFD in snake species over a wide geographic area. Given the observation of infected *S. catenatus* displaying signs of SFD from spring egress to fall ingress (Allender et al. 2015b, Tetzlaff et al. 2015, Allender et al. 2016) in Michigan, it is possible that they are exposed to *O. ophiodiicola* while in their hibernacula. Groundwater could then be a source of infection due to *S. catenatus* tendency to partially submerge during winter (Smith 2009). Additionally, evidence persists that *O. ophiodiicola* exists as a saprophyte in soil (Allender et al. 2015c). No confirmed detection has been observed in groundwater (Baker et al. 2018) and only one attempt in soil (Campbell et al. 2021) was successful in detecting *O. ophiodiicola*.

I predict that *O. ophiodiicola* is detectable in both soil and groundwater given that it is present at our site of interest in northern Michigan, and that its presence will be linked to the surrounding habitat, its environmental parameters, and seasonality. To address the hypothesis, this study aimed to carry out several objectives. 1) Determine if *O. ophiodiicola* DNA detection is achievable in soil and groundwater samples, 2) compare detection between habitats and seasons to observe differences spatially and temporally, 3) determine environmental associations through exploratory modeling of occupancy as a function of measured parameters, and 4) determine occupancy estimates for the site, sample, and detection probability of *O. ophiodiicola*.

CHAPTER 2. METHODS

2.1 Field site

This study was conducted at the Camp Grayling Joint Maneuver Training Center, a U.S. National Guard facility west of Grayling, Michigan. The facility covers a large area over both its northern and southern locations, nearing 60,000 hectares in size (Michigan National Guard). The property consists of a mixture of training grounds that are closed to the public and public access areas for recreational activities. The area consists of a variety of habitat types, including deciduous and coniferous forests with both closed and open canopy, forested and shrub-scrub wetland, and shrub-scrub/barren areas. There are also two modified habitats, experimental clear cuts and burned areas. Clear cuts were made during 2006 in heavy canopy cover stands of red pine and quaking aspen, and monitored for Massasauga habitat suitability (DeGregorio 2008, Ravesi 2016). Burned areas occurred due to unintended spread of a burn that had been planned to take place in a small arms training area during 2010 (Ravesi 2016).

Given that Camp Grayling covers a large area and has a mixture of habitats that have remained well intact, the area hosts a wide array of wildlife, including several species protected at the state and federal level, including the Eastern Massasauga.

2.2 Environmental Sampling

2.2.1 Soil Sample Collection

Sample collection occurred within a ~10.5 km^2 area and was developed around known massasauga habitat. A three-level sampling design involving primary units, secondary units, and replicate observations was employed following protocols designed for environmental DNA surveys aimed at detecting a single target species DNA to determine occurrence (Ficetola et al. 2015, Willoughby et al. 2016, Dorazio and Erickson 2018). For this study, the primary sampling units (locations or sites that exist within a study area) consisted of the total study area broken down into four different sampling locations (Figure 1). Sampling locations were based on the macrohabitat type present in study area, consistent with descriptions of macrohabitat characteristics. Ravesi (2016) described macrohabitats within the same study site, with forested

closed canopy areas consisting of coniferous or deciduous forest with greater than 50% canopy cover, and shrub-scrub/open as areas with low canopy cover (<30%), being comprised mostly of woody shrubbery like speckled alder and black cherry. Two other macrohabitats included as study locations were the two modified habitats that exists within the study area: the burn zone from 2010, and clear-cut areas from 2016 in both pine and aspen stands. Macrohabitat sampling locations were created as polygons through image classification and made to cover the macrohabitat type they represent. Macrohabitat coverage was checked for correct placement through inspection of aerial imagery in ArcMap (ArcGIS version 10.5, Esri), land cover data (USGS), and ground proofing on site.

At the second level of sampling (eDNA samples taken within each location) (Dorazio and Erickson 2018), an even number of random points were placed within each sampling location (Figure 2), using the create random points feature class on ArcMap (ArcGIS version 10.5, Esri). A grid feature was placed over the study locations and points were assigned to grid cells to ensure that randomly placed points had distribution across the study location. If the number of points plotted per location differed, then points were randomly removed utilizing the select random by count function in Python while in ArcMap. Ninety points were placed in each sampling location, however the number of points where samples were collected differs due to fieldwork constraints. The final level of sampling are the subsamples taken from each eDNA sample (Dorazio and Erickson 2018), which consist of PCR replicates. Each sample from a given location was run in triplicate, giving three subsamples per each location sample.

Soil samples were collected during 2020 from July to November and 2021 from April to June. Samples were grouped seasonally (spring, summer, fall) dependent on their date of collection to assess seasonality. Samples taken April-June were grouped as “spring”, July-August as “summer”, and October-November as “fall”. Each sample was taken to a depth of 10 cm and across a ~10 cm diameter of surface space at each sampling site using a Fisherbrand disposable sterile spatula. Samples were placed in sterile Fisherbrand 4 oz. specimen containers. At that depth both the O and the A horizons of soil were incorporated when present, the most organic-rich horizons of typical soil series. The logic behind focusing on these two superficial horizons is that if *Ophidiomyces ophiodiicola* is present as a free living saprophyte within soil, which has been suggested based on its ability to utilize a wide variety of carbon sources and withstand a large range of environmental conditions (Allender et al. 2015c). Then *O. ophiodiicola* is most likely

present in these substrates. Latex gloves were used at each sampling point for handling all equipment that would come into direct contact with the samples, with new gloves being used for each sample to prevent cross contamination. Soil samples were placed on ice for short-term storage (1 to 3 days) until they could be brought to the lab for DNA extraction, or frozen at -80°C for long term storage. When field housing provided proper storage, samples were kept on ice for the day of sampling and frozen until they could be brought back to the lab.

Field positive controls consisted of field collected soil that was spiked with 100,000 copies of plasmid containing the target gene to be amplified. Spiked, positive soil was placed in the Fisherbrand 4 oz. specimen containers and stored under the same storage conditions as environmental samples. For field negative samples, DNA free sand was brought into the field and collected utilizing the same approach, using the Fisherbrand spatulas and specimen containers, and stored with other samples.

2.2.2 Ground Water Sample Collection

The ground water sampling scheme was like that of the soil sampling, however the extent of the sampling area smaller. Due to the more labor-intensive process of ground water sample collection, the area sampled was limited to prior observations of massasauga overwintering sites (Figure 3). Massasauga in the area have been found with clinical signs of SFD and *O. ophiodiicola* has been isolated from such cases (Allender et al. 2011, Allender et al. 2015b, Tetzlaff et al. 2015). Therefore, it is believed that if *O. ophiodiicola* is present in the groundwater of the area, then snakes are most likely encountering it while in their hibernacula. Eastern Massasauga are known to utilize soils with high moisture, choosing sites where they can be close to the water line, staying submerged to some extent to prevent desiccation but also to take advantage of the thermal stability of water (Smith 2009, Merkling 2018).

The goal for groundwater sampling was to observe possible temporal effects on *O. ophiodiicola* presence at the site since there is only one primary sampling unit (the overwintering area). To accomplish this the Random points were plotted utilizing the same create random points feature class in Arc Map (Arc GIS) across the massasauga overwintering area. Samples were grouped seasonally like soil samples (summer, spring, fall) to assess seasonality at the site level, and potential environmental correlates were measured for each ground water sample at the sample level.

Independent of direct known hibernacula use, ground water was collected using a stainless-steel drive point well supplied by Solnist (Figure 4). The drive point well apparatus included a 30cm piezometer with holes covered in a mesh (304 S.S. 50 Mesh, 0.254 mm) to filter ground water from sand and other debris. A depth of ~1m was achieved with a 60cm extension pipe connected to the piezometer. The drive head assembly was struck with a slide hammer until the entire unit reached the proper depth. Plastic tubing provided by Solnist was attached to the piezometer and run through the drive point well where it could be attached to a tube adaptor and connected to smaller diameter, flexible tubing fitted to a peristaltic pump head. Ground water was pumped up though the well using a cordless drill that rotated the peristaltic pump head to create a vacuum. Ground water was collected by filling a sterile 500 ml plastic bottle which was sealed and labelled accordingly. Ground water samples were stored on ice for 1-2 days, or frozen at -80 °C for longer term storage.

All equipment that came into direct contact with groundwater (piezometer, extension pipes, all tubing, and adaptors) was soaked in a 3% sodium hypochlorite (50% bleach) solution for up to 20 minutes and rinsed with sterile water. All sanitized materials were brought to the field and stored in a separate container designated for clean supplies and transported to sampled sites in a separate field pack from used equipment carried between sampling points.

2.2.3 Environmental data

Alongside soil and water samples collected within the four sampling locations, environmental parameters hypothesized to be associated with *O. ophiodiicola* occurrence were taken. For each soil sample, ambient air temperature (°C) and soil temperature (°C) at 10 cm depth, was measured with a Traceable Lollipop Waterproof thermometer was measured. A ribbon test was performed to estimate the soil type at the sample point (Merkling 2018). Additionally, two soil samples were taken using a soil corer, one to determine soil pH, which involved mixing a soil core in a 1:2.5 ratio by weight with water (Kabała et al. 2016) and measured using a Quanta Hydrolab pH meter, ensuring the sensor is submerged to attain proper readings. The other soil sample was used in soil moisture and organic matter determination. Soil moisture was measured by weight change after drying, which involved taking 25 – 50 g of soil and drying it for up to 48 hours at 70°C, checking every 24 hours until mass readings remained static as suggested for organic heavy soils (Heiri et al. 2001). The initial soil weight and the final weight after drying

were used to determine the percent moisture in the soil following the equation $\%SM=((W_i-W_f)/W_i)*100$. The equation can be described as subtracting the dry weight from the wet weight to get the mass of the water in the sample, then dividing it by the weight of the wet sample and multiplying by 100. After soils were dried to determine soil moisture, organic matter was measured using the loss on ignition method. One gram aliquots from each sample were placed in 20ml porcelain crucibles and placed in a muffle furnace (Thermo Scientific Thermolyne) and exposed to 550°C for 4 hours (Figure 5, Heiri et al. 2001). Soil samples were then removed from heat and set to cool on a heat resistant plate. Samples were not cooled completely to room temperature and crucible lids were left on to prevent soils from reclaiming moisture before final measurements. After final measurements, the loss on ignition at 550 °C was calculated using the equation $LOI=((W_i-W_f)/W_f)*100$. The percentage of mass loss represents the organic carbon present in the sample.

Variables associated with seasonality were also estimated for each soil sample. Data on daily soil temperatures (10 cm depth) and daily precipitation (mm), provided by Michigan State University Weather Station Network (Kalkaska station), was used to determine cumulative growing degree days over three weeks (cGDD), and cumulative precipitation over one week prior to sample collection (weekly prcpn), respectively.

Environmental parameters collected for ground water samples include ambient air temperature (°C), ground water temperature (°C), pH, and dissolved oxygen (mg/L). Air temperature was measured using the same thermometer (Traceable Lollipop Waterproof thermometer) used when collecting soil samples, all other parameters were measured using the Quanta Hydrolab. Water parameters were measured in sterile cups separate from the sample bottles and were collected before the sample to prevent drastic changes from exposure to air.

2.3 DNA Extraction

2.3.1 Soil Samples

Upon return to the lab, soil samples were stored on ice until processing for downstream qPCR analysis. For long-term storage, samples were stored at -80 °C. DNA extraction utilized the Qiagen DNeasy Power Soil Pro DNA extraction kit, following the extraction protocol included with the kit. Bulk samples were homogenized and then 25 mg of the total sample were loaded into

power bead tubes with forceps, which were soaked in a 50% bleach solution, rinsed, and dried between samples. No further pre-processing of soil samples took place before utilization of the Power Soil Pro kit. When completed, samples were eluted into 100 µl of elution buffer and stored at -80 °C until use in qPCR.

2.3.2 Ground Water Samples

To prevent sample degradation, ground water samples were run through filtration in as timely a manner as possible once in the lab. Water samples were filtered through a Nalgene 250 ml rapid flow filter unit with a 0.45µm nitrocellulose (CN) filter membrane to capture DNA and fungal material but prevent excessive clogging that occurs with smaller filters (Eichmiller et al. 2016, Ratsch 2018). Samples were run until all water had passed through using a vacuum apparatus attached to an in-house vacuum port (Figure 6). The filtering process per sample took 5 to 12 hours depending on the sample due to the high levels of sediment present. Therefore, not all samples from a given field sampling trip were run within 24 hours, for that reason, left over samples were stored at 4°C for short term storage (up to 2 days) and longer-term storage at – 80 °C. Once filtration was complete, filters were removed from the filter unit, folded/torn with forceps, placed in a 1.7 ml micro centrifuge tube, and stored at -80°C until extraction. Used forceps were decontaminated by soaking in a 50% bleach solution and rinsing between filters.

DNA extraction of ground water samples used the same Qiagen DNeasy Power Soil Pro DNA extraction kit. The Power Soil extraction kit has shown low variation in extraction efficiency between water sources, therefore making it the most suitable for detecting across aquatic environments and quantification of DNA in the environment (Eichmiller et al. 2016). Filters were placed in power bead tubes and were run following the extraction kit protocol before elution in 100 µl of elution buffer. Ground water sample DNA extracts were stored at -80 °C.

2.4 Quantitative PCR

I used a quantitative PCR (qPCR) assay developed for more sensitive detection and diagnosis of *O. ophiodiicola* infection (Allender et al. 2015a). It has been used for detection of *Ophidiomyces* in several snake populations (Allender et al. 2016, Hileman et al. 2017) and crayfish burrow water (Baker et al. 2018). Similarly designed assays utilizing the internal transcribed spacer

(ITS) region between the rRNA genes have shown that *O. ophiodiicola* is detectable in environmental samples such as soil (Bohuski et al. 2015, Campbell et al. 2021). Therefore, the assay applied in this study offers a specific and sensitive approach for the detection of *Ophidiomyces* in environmental samples.

Reactions were run using USA Scientific TempPlate 96-Well, clear qPCR plates. Each reaction contained 0.3 µM of both the forward (OphioITS-F) and reverse (OphioITS-R) primers, and 0.2 µM of the probe (Probe-FAM) (Allender et al. 2015a). Additionally, PCR master mix (Appliedbiosystems TaqMan Environmental Master Mix 2.0) and the Internal Positive Control master mix 10X/ IPC DNA 50X (Applied biosystems TaqMan Exogenous Internal Positive Control) were added to each reaction. Finally, 2 µl of template DNA was added in to reach a total reaction volume of 20 µl. The addition of IPC functioned as a positive control to monitor for false negatives caused by PCR inhibition. Each sample was run in triplicate to increase detection. Thermo cycling parameters were carried out as in Allender et al. (2015b), with an initial step of 50 °C for 2 minutes, a denaturation step at 95 °C for 10 minutes to activate the polymerase and start the reaction, continued with 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds, and 72 °C for 10 minutes.

Each plate included a series of DNA standards run in triplicate; the template DNA of those standards was linearized plasmid with the ITS region inserted (Allender, Bunick, et al. 2015). Known concentrations of the plasmid containing the insert were made by 10-fold serial dilutions of plasmid starting at a concentration of 1.05×10^7 (10,500,000 copies of ITS insert) down to 1.05×10^1 (10.5 copies of ITS insert). A standard curve was run with all plates to determine reaction efficiency and the R^2 by plotting the known concentrations against the relative fluorescence readings they generate. Initial plates of standards were run to determine the limit of detection (LOD) for the assay, which is the lowest concentration at which 95% of the samples are positive (MIQE guidelines). LOD was determined through empirical means. Positive and negative controls were included on plates to assess contamination in negative controls, and make sure reaction is functioning as intended with the positive control. Both samples as a whole and each technical replicate were assigned a binomial indicator (positive=1, negative=0). Samples were deemed positive if at least one replicate amplified and if those that did amplify were within a Ct threshold of 40 based on suggestions made by Bohuski et al. (2015) and used in Campbell et al. (2021). This procedure is aimed at detecting very low concentrations of environmental DNA and

avoiding dismissal of potential positives based on thresholds used more for diagnostic purposes in infected snakes.

To reduce potential contamination, DNA extractions and qPCR procedures were completed using filtered pipette tips and all qPCR reagents were handled in a Class 2 Biological Safety cabinet where all surfaces were decontaminated with 50% bleach prior to use and exposed to UV radiation before and after each use. Reagents for qPCR were stored in a freezer separate from any reagents or equipment used for DNA procedures (filtration and extraction). Additionally, known *Ophidiomyces* DNA sources (the standard solutions) were stored in a separate room across the building from environmental sample handling.

Any positive replicates from qPCR analysis were Sanger sequenced (MCLAB) to verify amplification of the correct sequence from the species of interest, *O. ophiodiicola*. The primers used for the assay, both Ophio-F and Ophio-R, yielded a product of 68 base pairs in length. Sequencing attempts with these primers yielded inconclusive results due to low base quality return. Likely due to the small size of the product, as Sanger sequencing takes up to 50 bp to return quality base readings, the diagnostic primers developed for the assay were insufficient for Sanger sequencing. To remedy this, qPCR products from positive samples were purified using spin columns (Zymo Research) and run through a sequential PCR reaction using modified OphioITS-F/R primers with 5' extensions. The 5' extensions consisted of M13 standard primers M13(-21) forward and M13(-48) reverse (IDT), for the forward and reverse *Ophidiomyces* primers respectively. This lengthened the total size of the product for sequencing to 109 bp. The products were run through a 3% agarose gel, and a Qiagen gel extraction kit was used to conduct a DNA gel extraction to purify the product for sequencing.

2.5 Statistical Analysis

2.5.1 Habitat comparison analysis

All statistical analysis were conducted through RStudio version 1.4.1717 and R version 4.1.0 (R core team 2021).

All numeric covariate data (organic matter %, moisture %, soil pH, and soil temperature) was tested for normality using a Shapiro-Wilks test of normality. Variables that met the threshold of $W > 0.9$ were treated as normally distributed. Additionally, homogeneity of variance was tested

using the Levene's test. Variables that failed to meet either assumption were subject to Box cox power transformations which provided suggested transformations. Two-way analysis of variance (ANOVA) was used to observe if there were differences in the measured variables between the four habitat types as well as between the seasonal groupings. The two-way analysis also allowed for the investigation of an interaction between habitats and seasons. Tukey honest significant differences were applied as post-hoc tests to uncover the cause of any statistically significant difference in the ANOVA. All tests were performed with a significance threshold of $\alpha = 0.05$.

O. ophiodiicola DNA presence in a sample was also compared between habitats (forested, cuts, burn, and shrub-scrub open) across the entire span of sampling. Given the categorical nature of the variable for presence in a sample (1 =present, 0 = absent), a Chi-squared test of independence was used to test for whether the two variables in question (presence in a sample and habitat) are independent of each other. It also was also of interest to investigate a seasonal effect throughout the sampling effort. Samples grouped into the three seasons, "spring" (n=62), "summer" (n=104), and "fall" (n=46) were subject to the same Chi-squared test for independence.

2.5.2 Binomial Logistic Regression

Binomial logistic regression was conducted to assess the probability of *Ophidiomyces* DNA presence in a sample, including predictors (covariates) thought to inform its presence. Such logistic regression analyses have been applied to environmental DNA studies in the past on a variety of taxa such as invasive fish (Robson et al. 2016, Stoeckle et al. 2017), and the eastern hellbender (Takahashi et al. 2017) in aquatic environments. A key assumption of logistic regression is that there be little to no multicollinearity. To test for correlations among predictor variables, Kendall's Tau correlation was applied. Variables that showed high correlation ($r > 0.7$) were not run within the same model (Stulik 2015).

To determine initial relationships between the probability of *Ophidiomyces* DNA presence, the binomial variable associated with *Ophidiomyces* presence was modelled as a function of soil temperature, soil pH, soil organic matter percent, soil moisture percent, cumulative precipitation (one week before the sample was taken), and cumulative growing degree days 3 weeks prior to sampling event. A global model was created using the logistic regression function under glm (glm, "stats" R core team 2021) that included all parameters of interest. That global model was then incorporated into an automated model selection function (dredge "MuMIn", R core team 2021)

which allowed for the generation of a model selection table containing all combinations of predictors included in the original global model.

Regression models were then ranked by AIC (Akaike-Information criterion) and the model weight (w). AIC is a model selection method that ranks models based on their goodness of fit while penalizing for increased variance that comes with model complexity (Burnham and Anderson, 2002). The change in AIC (ΔAIC) and the model weight (w) are the basis for comparing models within a set. Models with a change in AIC is below two units (ΔAIC < 2) from the top candidate models share significant support for being the model of best fit, while the weight value represents the weight of evidence that the given model is the best model, with all weights summing to 1 (Burnham and Anderson 2002). Therefore, all models within the set that were within two units from the top ranked model were considered to share support for being the model of best fit. All predictors included in the top model as well as those that shared support for being the top ranked model were included as covariates of occupancy at the sample level in subsequent multiscale occupancy modeling. The sum of the weights for each variable included in modeling was generated as well to determine importance of variables (Burnham and Anderson, 2002).

A regression was done to determine initial relationships between predicting *Ophidiomyces* DNA presence in a sample and environmental parameters of interest, providing a more informed inclusion of covariates in occupancy modeling. To avoid overfitting of models a second order correction (AICc) was used as the selection criteria, due to smaller sample size given the number of explanatory variables included in modeling (Burnham and Anderson 2002). McFadden's pseudo- R^2 and a residual deviance test were used to assess the performance of the top model and how well the model fit the observed data. McFadden's pseudo-R^2 values are known as the "likelihood ratio index" and were generated by comparing the log likelihood of the model in question to the null model as a ratio (McFadden 1973, Hu et al. 2006). The residual deviance goodness of fit test is determined by using the residual deviance and corresponding degrees of freedom that come with "glm" output. One can then generate an associated p-value using the "pchisq" function in RStudio. The residual deviance itself represents the how well the response variable is predicted by the model with the included parameters. If the associated p-value is below the 0.05 threshold, then the null hypothesis that the model fits the data is rejected. Indicating that the model is not suitable for predicting the observed data.

2.5.3 Occupancy Analysis

Occupancy modeling has been applied widely across many taxa (Sewell et al. 2010, Campos-Cerqueira et al. 2016, Rich et al. 2016). However, to apply such an analysis to environmental DNA, alterations to classical model conditions were made to encompass three nested levels of sampling at the location, samples within the location, and subsamples or replicates of the sample (Hunter et al. 2015). These three level sampling designs have been commonly used in environmental DNA based surveys in the past (Ficetola et al. 2015, Schmelzle and Kinziger 2016, Willoughby et al. 2016, Hunter et al. 2017, Voros et al. 2017). For this reason, the occupancy analysis conducted in this study utilized the *eDNAOCCUPANCY* package in R (version 4.1.0 R Core Team, 2021) which allowed for the fitting of Bayesian, multi-scale occupancy models (Dorazio and Erickson 2018) to the *Ophidiomyces* detection data collected.

The models developed under this package estimated three probabilities, those being the occurrence probability at the site level, the occurrence probability at the sample level within a site, and the probability of detection in qPCR replicates from each sample, represented as Ψ(psi), Θ(theta), and p, respectively. Additionally, the package allows for the inclusion of covariates in model building, so interactions between environmental parameters and occurrence/detection probabilities can be observed.

All models were fit with Markov chain Monte Carlo (MCMC) algorithms and run with 11,000 iterations. Trace plots and autocorrelation plots were run on the estimates from each model output to check for convergence and autocorrelation, which was done through functions provided in the package (*eDNAOCCUPANCY*). Models consisted of a null with no covariates included, and those with covariates thought to informative of *O. ophiodiicola* presence at the site, sample, and replicate level.

To reduce the total number of models to a more manageable level, a two-step approach to modeling was done (Long et al. 2010, Yates and Muzika 2010, Stulik 2015). First, detection probability (p) models were run with occupancy at the site and sample level held constant (Ψ(),Θ()). Then the top candidate (p) model for detection was run in subsequent occupancy modeling. Covariates for detection probability included bulk DNA concentration (ng/µl), soil organic matter content, and soil pH. These variables were deemed to be informative for detection at the PCR level due to their potential effects on differential detection between PCR replicates due to inhibition. All model combinations were compared using WAIC (Watanabe-Akaike Information

criterion) and PPLC (Posterior Predictive Loss criterion) for model selection with the lowest scores representing the model with best fit.

Occupancy modeling at the sample (Θ) and site (Ψ) levels included the following covariates: soil temperature, soil pH, soil moisture, organic matter content, cGDD, and weekly precipitation (prcpn). Soil type was excluded from modeling as the variation seen in occupancy based on soil type is likely also explained by other covariates such as soil moisture and organic matter content (Burke et al. 1989, English et al. 2005, Plante et al. 2006). Additionally, the relative importance of soil type was found to be very low in logistic regression. To determine the environmental associations of *Ophidiomyces*, an exploratory analysis was run to investigate the singular effects and additive effects of all covariates in question. All combinations of covariates were run for occupancy at the sample level (Θ) with the addition of the top p model and were scored based on their WAIC and PPLC criterion scores. Estimates of both formal and derived model parameters from the optimized model were used to produce occupancy probabilities at the site (Ψ) and sample (Θ) level, and the detection probability (p).

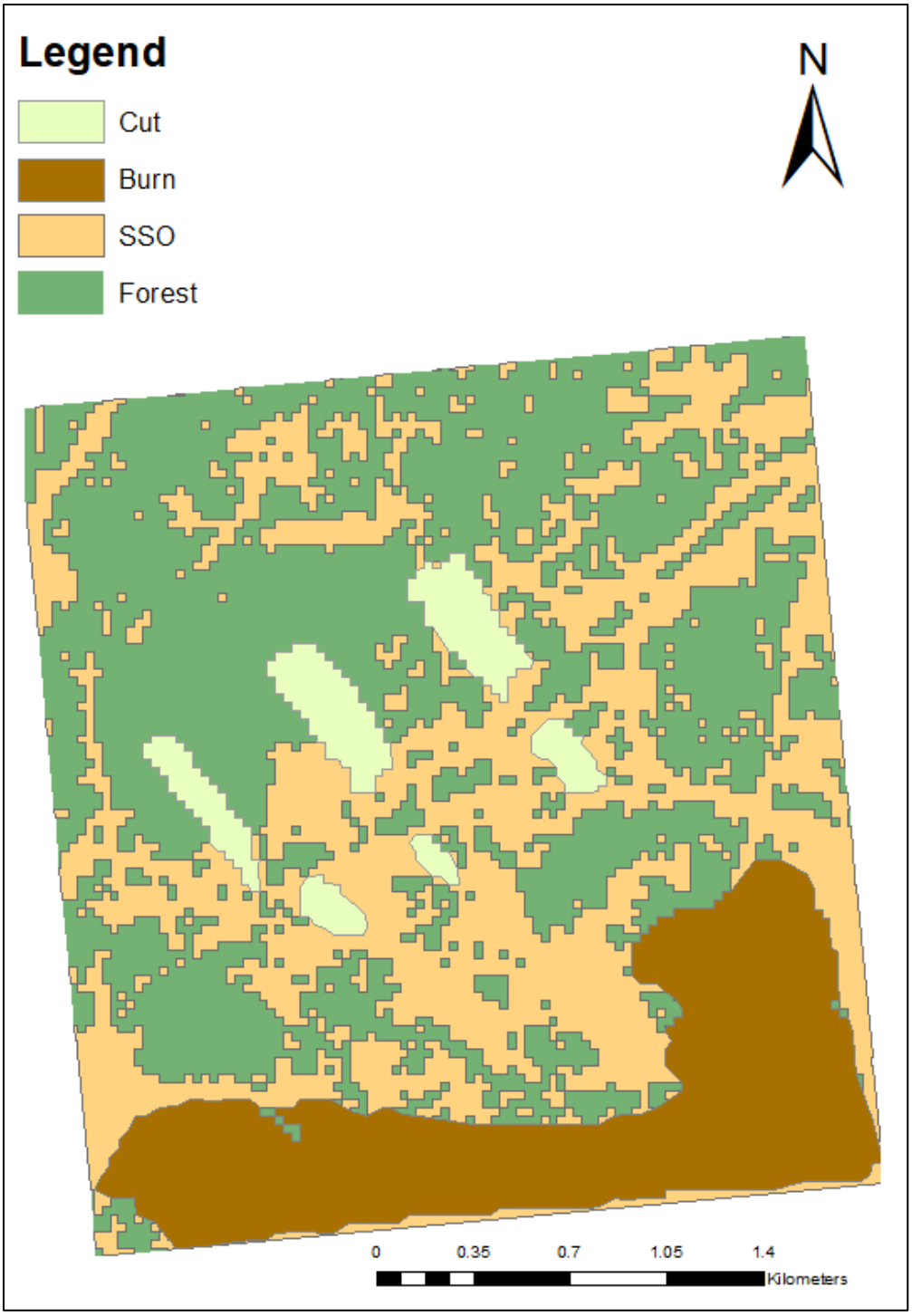

Figure 1. Map of study area (~10.5 km^2) consisting of the four macrohabitat types sampled at the primary sampling unit. Cut habitat is made up of areas subjected to clear cutting (2016), Burn represents area in the site that was burned (2010), forest is closed canopy forest with greater than 50% canopy cover, and SSO are areas with <30% canopy cover dominated by woody shrubbery. Determined through habitat classification (ArcGIS version 10.5, Esri) and cross-referenced by inspection of aerial imagery and ground proofing.

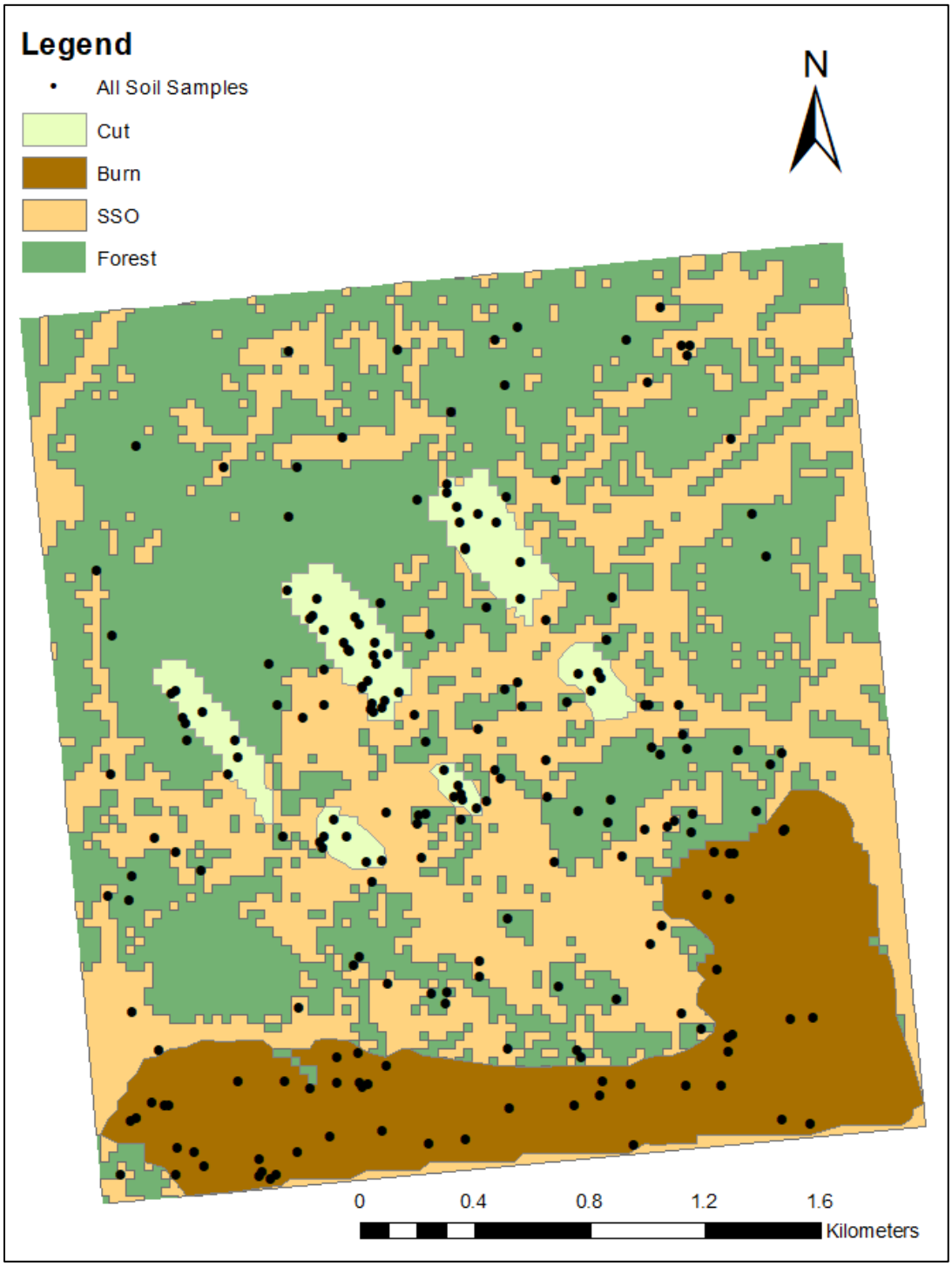

Figure 2. Map showing randomly placed sampling points, the secondary sampling unit, within each macrohabitat type (sampling location). The points represent the locations of soil samples taken during the summer sampling season.

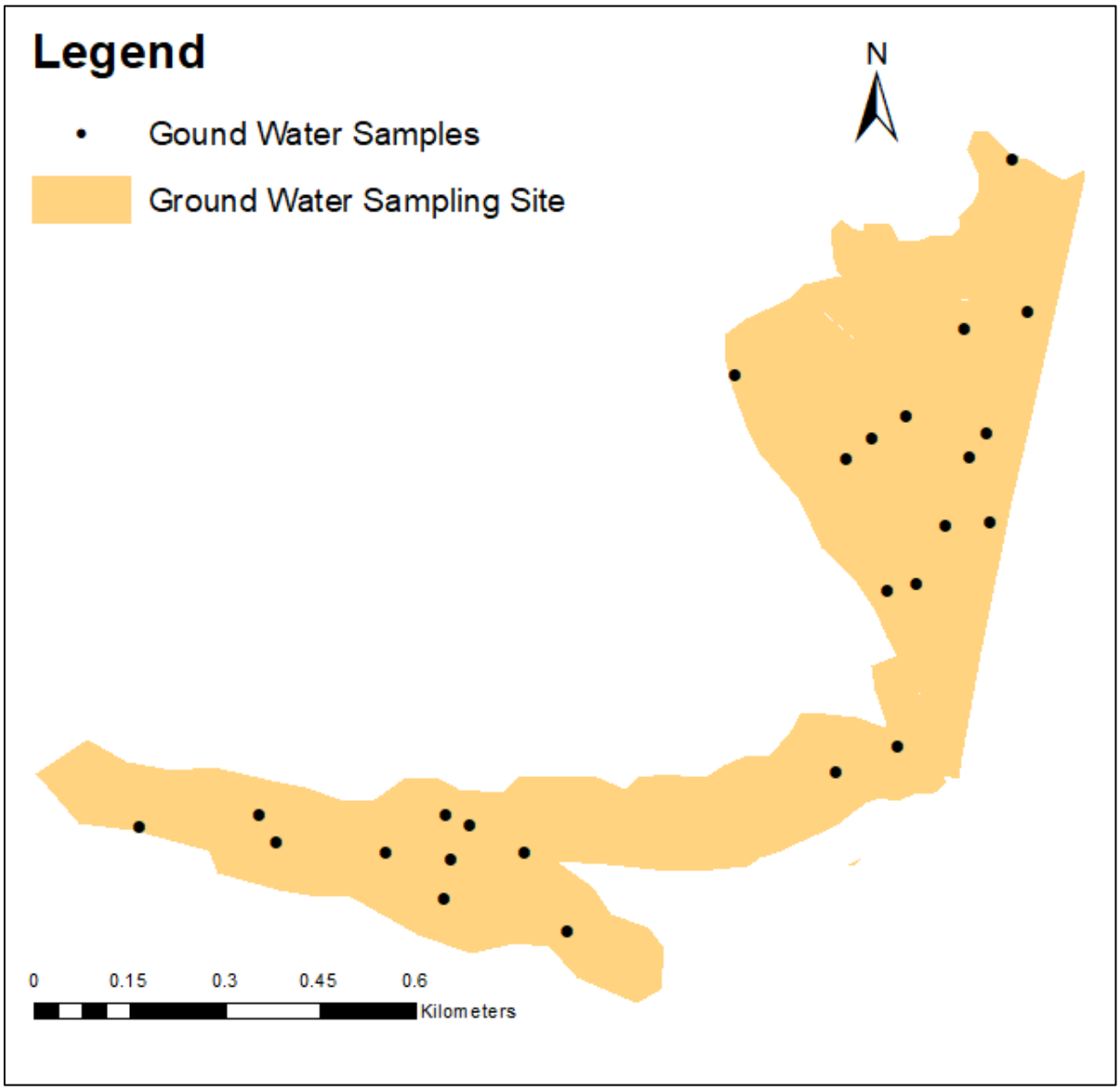

Figure 3. Map showing the sampling location for ground water samples and the corresponding random sampling points for the summer season. Sampling location was based on Massasauga hibernaculum observations and was made up of several of the macrohabitat types.

Figure 4. Drive point well apparatus used to pump ground water to the surface at each sampling point. Wells were driven to ~1 meter depth with a slide hammer. Piezometers (portion shown with openings above the tip), extension tubes, and white tubing were soaked in 50% bleach before each use.

Figure 5. Ground water filtering set up, consisting of the Nalgene filter unit connected to tubing that flows into a collection flask for flow through. The apparatus is connected to in house vacuum unit.

CHAPTER 3. RESULTS

3.1 Habitat Comparison

Of the environmental variables measured at each sampling point only soil pH and soil temperature met the normality assumption ($w > 0.90$), while all failed to meet the assumption of homogeneity of variance. Variables were subject to box-cox transformation to identify the proper transformation satisfying the assumptions so the two-way ANOVA could be applied.

Two-way analysis of variance showed that soil temperature differed between both the habitat types ($F_{3, 198} = 3.80$, $p = 0.011$) and seasons ($F_{2, 198} = 334.8$, $p < 0.0001$) (Figure 8). However, more importantly an interaction between the two independent variables (habitat and season) was observed ($F_{6, 198} = 2.485$, $p = 0.024$) due to the forest having relatively cooler temperatures in the spring and clear cuts exhibiting warmer temperatures during the summer relative to the other habitats.

When analyzing the variance in soil pH, significant differences were observed between both habitat ($F_{3, 198} = 9.09$, $p < 0.0001$) and season ($F_{2, 198 =} 62.68$, $p < 0.0001$) groupings. No significant interaction was observed between habitat and season for soil pH ($p = 0.052$) (Figure 9). Tukey post-hoc analysis showed that soil pH differed between both forest and shrub-scrub habitat ($p = 0.046$), burn and shrub-scrub ($p < 0.0001$), and cut and shrub-scrub habitat ($p=0.012$). There was also a significant difference between soil pH and all three seasons ($p < 0.0001$) with the highest pH occurring during the summer season.

Soil moisture content, like soil pH, also differed between both groups (habitat: $F_{3, 198} = 6.06$, $p = 0.001$, season: $F_{2, 198 = 28.63,} p < 0.0001$) but did not display an interaction ($p = 0.541$) (Figure 10). Tukey post-hoc results indicates moisture content differed between cut habitat and all other habitat types, forest ($p = 0.003$), burn ($p = 0.003$), shrub-scrub ($p = 0.007$). While between seasons, soil moisture content was lowest during the summer season and significantly different than both fall ($p < 0.0001$) and spring ($p < 0.0001$) seasons.

Lastly, soil organic matter content only differed between habitat types ($F_{3, 198} = 7.456$, $p < 0.0001$) with no interaction occurring between habitat and season ($p = 0.313$) (Figure 11). The differences in organic matter content between habitat types were observed between the cut habitat and the other habitat types, forest ($p < 0.0001$), burn ($p = 0.006$), shrub-scrub ($p = 0.001$). Temporal

variables (cGDD and weekly prcpn) were not subject to season grouping comparisons nor habitat level comparisons as data came from a single location outside the study location.

Confirmed positive detections differed between habitat types sampled, with forested habitat having 9 (18%), shrub-scrub open 12 (24%), clear cuts 5 (8%), and burned 6 (11%) positive samples (Figure 7). Although both shrub-scrub open and forested habitats have nearly double the number of positive samples as compared to burned and clear cuts, the presence of *O. ophiodiicola* DNA in a sample throughout the entire sampling effort was found to be independent of the habitat type from which the sample came (Chi-Squared, $X^2 = 5.406$, df = 3, $p = 0.1444$).

Findings differed when the presence of DNA was tested across the seasonal groupings regardless of habitat (Figure 12), with results suggesting that the DNA presence in a sample and the season in which the sample was taken are not independent of each other (Chi-Squared, $X^2 = 10.905$, df = 2, $p = 0.0043$). Therefore, season had some noticeable effect on whether fungal DNA is present.

3.2 Quantitative (Real-time) PCR

Both soil and groundwater extractions yielded DNA concentrations in a range of 0 to 350 ng/µl. The quantitative PCR assay developed by Allender et al. (2015) and implemented in this study utilized a standard curve based on known concentrations of linearized plasmid containing the fungal ITS insert. All plates were run with their own standard curve for which the samples on that plate were applied to. The MIQE guidelines state that limit of detection (LOD) is defined as the lowest concentration at which there is 95% amplification success (Bustin et al. 2009). The LOD for this assay was determined empirically to be 10^1 copies of the ITS gene. The dynamic range applied in this study for the standard curve on each plate was 1.05×10^7 to 1.05×10^1. This followed similar results reported previously with a dynamic range of 1.05×10^8 to 1.05×10^1 copies (Allender et al. 2015a). For simplicity in reporting, all plates were combined into a cumulative standard curve based on the mean Ct values, encompassing the interassay variation between the plates (Figure 6) as in Allender et al. (2015). The cumulative curve displayed similar Ct values at each concentration as reported previously (Allender et al. 2015a) and had an average efficiency of 119% and R-squared value of 0.96.

All negative controls failed to amplify, and positive controls ran consistently with their corresponding environmental samples. The internal positive control (IPC) included within each

reaction ran consistently for all samples throughout all plates, suggesting no significant PCR inhibition was present at any time.

A total of 215 soil samples were taken among the four macrohabitat types (sites) with forest, shrub-scrub open, clear cuttings, and burned areas having 50, 51, 57, and 52 samples respectively. Thirty-two (14.8%) total samples showed signs of amplification (Table 1) across all habitats (Figure 8) and were below the threshold deeming the sample positive (Bohuski et al. 2015, Campbell et al. 2021). Positive samples were confirmed through Sanger sequencing (MCLAB), with sequence data aligning properly to the ITS region of *O. ophiodiicola* (Geneious 11.1.5). Additionally, positive sample sequences all showed greater than 95% sequence identity to *O. ophiodiicola* reference sequence data across multiple strains and isolates (Genbank). DNA was only detected in a single ground water sample that was also confirmed through sequencing. This result precluded further analysis of ground water.

3.3 Binomial Logistic Regression

The top ranked model (AICc = 166.76, w = 0.0401) included the additive effects of cGDD, soil pH, soil temperature, and weekly prcpn. The estimates generated by the model suggest that only soil pH (p = 0.01842, z value = 2.357) and cGDD (p = 0.0406, z value = 2.048) had a statistically significant effect on the model with soil pH having the largest estimated effect size (Table 3). These results seem to agree with previous comparative analyses with both covariates shown to be related to *Ophidiomyces* also shown to change seasonally. Soil pH was higher during the summer than both spring and fall seasons, while cGDD is also at its highest during the summer since the days where temperatures that exists above the base (7°C) occur more frequently.

Through model selection, the top candidate model predicted that soil pH, cGDD influence the probability of presence. Weekly precipitation and soil temperature were also included within the model. Although weekly precipitation and soil temperature were shown to not influence presence, their inclusion still contributed to the model fit. There was uncertainty in model selection however (Table 2), as 18 different models were indicated as the top ranked model (ΔAICc < 2). Therefore, organic matter percentage and moisture percentage also showed evidence towards influencing presence of *Ophidiomyces* DNA in a sample. Additionally, the summed AICc weights of the variables indicated relative importance of each (Table 3). Both cGDD and soil pH had the highest summed AICc weights, 0.84 and 0.74 respectively meaning that cGDD represented 84%

of the AICc weight within the set of models and soil pH represented 74%. AICc weight was also considered for inclusion of covariates in multiscale occupancy modeling. The covariate soil type was not included in any of the models that fit within the ΔAICc threshold within 2 units, and it had the lowest of the summed weights (5.7%).

Goodness of fit of the top model was assessed through residual deviance goodness of fit test. McFadden's pseudo R^2 were also included to observe model performance. The top model of the candidate set had a residual deviance of 156.47 with 205 degrees of freedom while the null deviance was 179.26 with 209 degrees of freedom. The associated p-value from the residual deviance was shown to be significant ($p = 0.005$) indicating the model is not useful and cannot predict DNA presence well with the included predictors. Pseudo R^2 values further demonstrate poor model performance with low predictability, with models in the candidate model set having values no larger than 0.127 (Table 2).

3.4 Multiscale Occupancy Analysis

Following the two-step approach, covariate effects on detection probability models were investigated first. The complete model set for modeling detection (p) consisted of 8 total models (Table 5), which consisted of all individual and additive models between the covariates bulk DNA concentration, organic matter content, and soil pH. An additional null model p() with constant detection was included within the model set. Models were ranked off WAIC and PPLC, with the null model p() having detection held constant producing the lowest score under both selection criterion (Table 5). Therefore, any improvements in detection model fit were offset by increases in variance with the addition of covariate measures. The detection probability for *Ophidiomyces* was found to be 0.37 (0.25, 0.50) at the PCR replicate level.

Due to the exploratory nature of the occupancy analysis applied, a total of 124 models were included in the occupancy model set (Appendix B). This ensured that all potential covariate effects were investigated. Model selection uncertainty existed due to both selection criteria applied differing in their top ranking (Table 6). The top ranked model by WAIC in the candidate set included soil pH as a function of sample occupancy Ψ(),Θ(Soil pH),p() (WAIC = 69.23). Trace plots showed acceptable model convergence (Figure 13). Autocorrelation function plots also showed little autocorrelation within the model (Figure 13). The probability of occupancy at the site level (Ψ) was shown to be 0.83 (0.48, 0.99) while the conditional probability of DNA

occurrence at the sample level (Θ) ranged from between 0.04 to 0.48 as shown by the confidence interval. Estimation of the model's posterior mean and 95% credible limits (Table 7) show that the occurrence of *Ophidiomyces* DNA in a sample increases with increasing soil pH (mean = 0.434, CI = 0.196, 0.701). The relationship seen when the posterior mean, of the model's derived parameter Θ, is plotted is questionable given visualization of the probability of DNA occurrence in a sample across soil pH (Figure 14). The plot shows the no discernable trend between soil pH and the probability of occurrence within a sample.

The alternative model that was selected by PPLC (PPLC = 66.35) was the null model with all occupancy estimates for Ψ and Θ held constant Ψ(),Θ(),p(). The selection of this model via PPLC criterion indicates that the increase in fit with the addition of covariates does not outweigh the increase in variance with their addition. The occupancy probability remained the same at 0.83 (0.48, 0.99) while the conditional probability of *Ophidiomyces* DNA occurrence in a sample was 0.21 (0.14, 0.30). Trace plots and autocorrelation plots showed model convergence and little autocorrelation respectively for the null model (Figure 15).

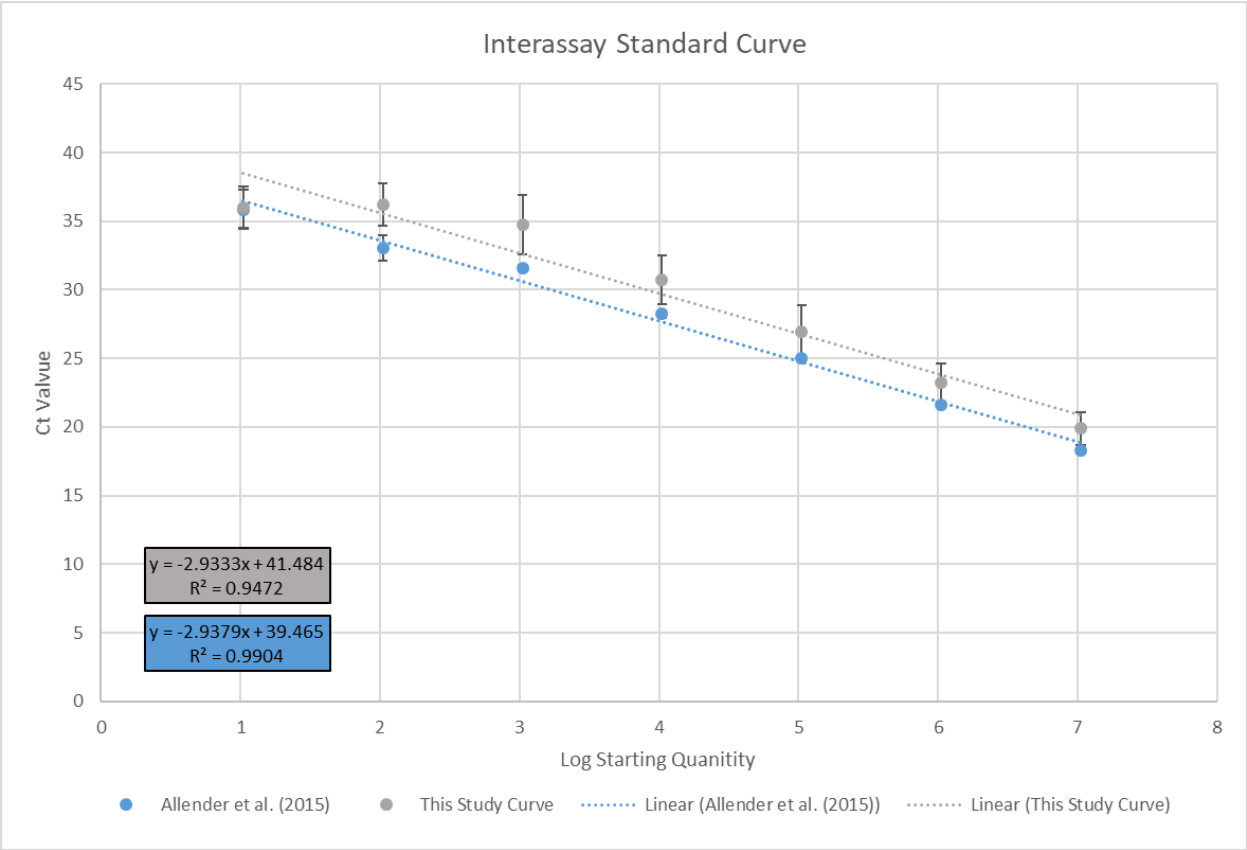

Figure 6. Cumulative Standard curve for all qPCR assays applied in this study based on interassay variation from multiple standard runs represented in gray. Interassay variation curve from data in Allender et al. (2015) is represented in blue. Standard deviations plotted for each in the form of error bars.

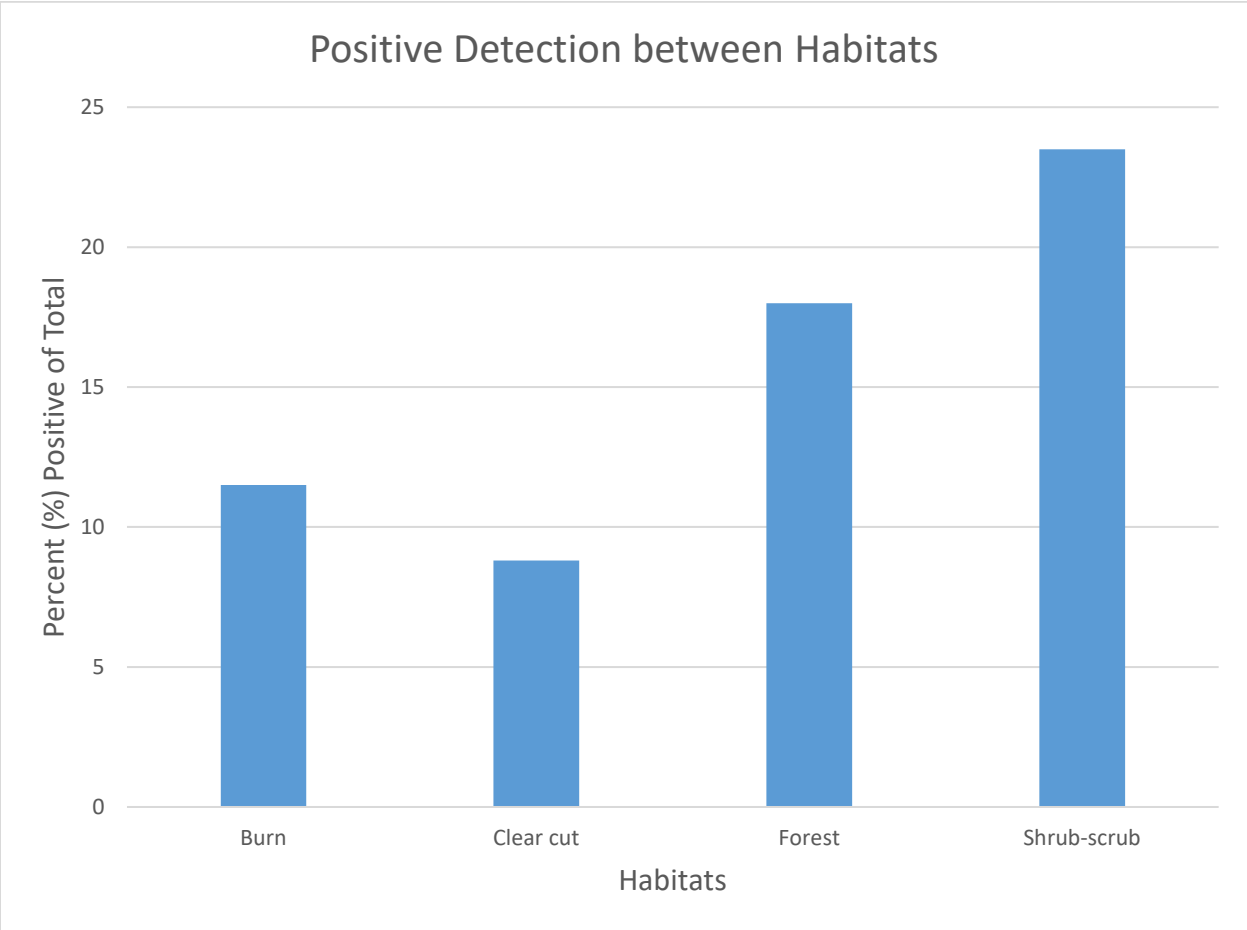

Figure 7. Percent of positive detection from the total taken in each of the habitat types sampled. Showing the difference in detection seen across Burn (n=52), Cut (n=57), Forest (n=50), and Shrub- scrub (n=51) habitats.

Table 1. List of all positive samples. Displaying sample designation, the habitat it was collected in, the number or positive replicates out of three, and the mean Ct value for that sample.

Positive Soil Samples

Sample name	Habitat	Positive Replicate Number	Ct mean
b114	Burn	1/3	36.26
b115	Burn	2/3	35.81
b201	Burn	2/3	37.23
b202	Burn	1/3	36.77
b211	Burn	1/3	35.74
b328	Burn	1/3	37.44
c105	Clear cut	1/3	36.41
c106	Clear cut	1/3	35.94
c107	Clear cut	1/3	36.24
c110	Clear cut	1/3	37.27
c201	Clear cut	1/3	36.19
f103	Forest	1/3	34.61
f108	Forest	2/3	36.89
f114	Forest	3/3	36.15
f117	Forest	2/3	36
f127	Forest	3/3	36.34
f128	Forest	1/3	35.02
f131	Forest	1/3	35.06
f132	Forest	1/3	34.54
f240	Forest	3/3	35
s105	Shrub-scrub	1/3	37.39
s108	Shrub-scrub	1/3	35.6
s111	Shrub-scrub	1/3	37.9
s112	Shrub-scrub	3/3	33.34
s114	Shrub-scrub	1/3	36.11
s121	Shrub-scrub	1/3	36.3
s123	Shrub-scrub	1/3	35.46
s126	Shrub-scrub	1/3	36.3
s133	Shrub-scrub	2/3	36.7
s201	Shrub-scrub	3/3	36.33
s202	Shrub-scrub	2/3	37.21
s328	Shrub-scrub	1/3	38.73

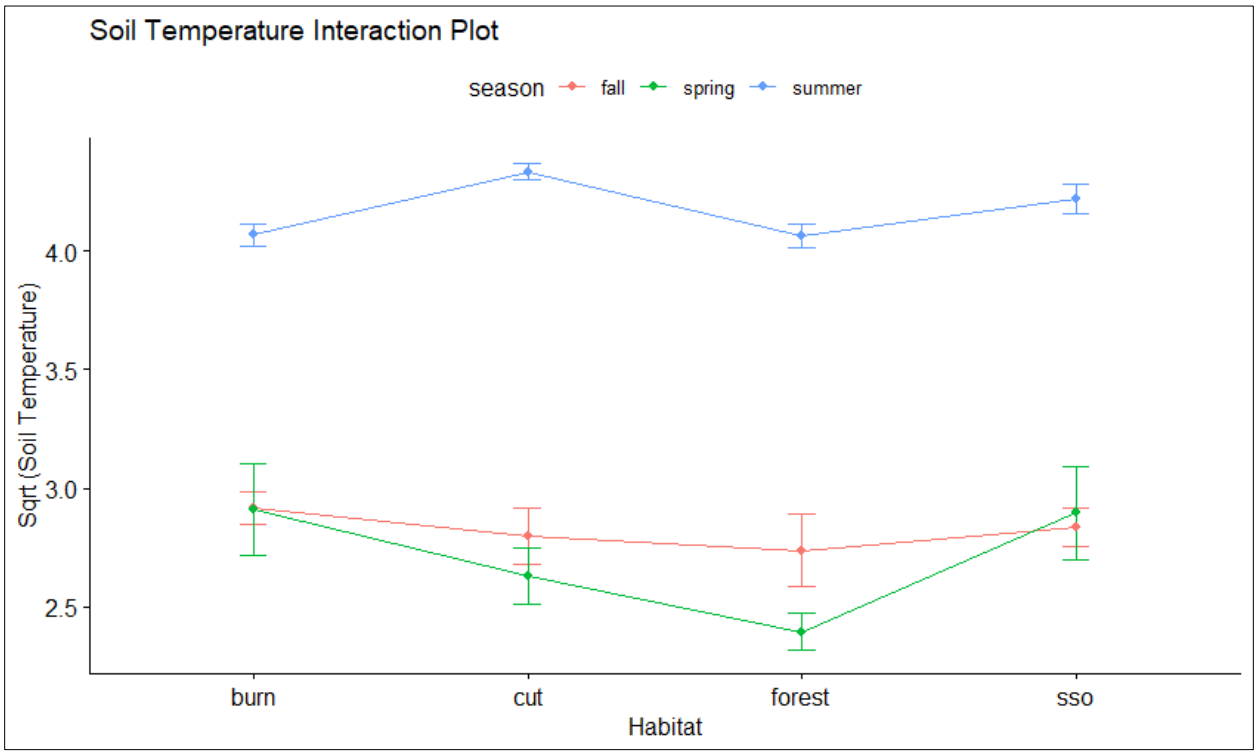

Figure 8. Interaction plot of square root transformed soil temperature across habitat and seasonal groupings. Each point represents mean values for each of the interaction groups. Error bars indicate the standard error of each mean value.

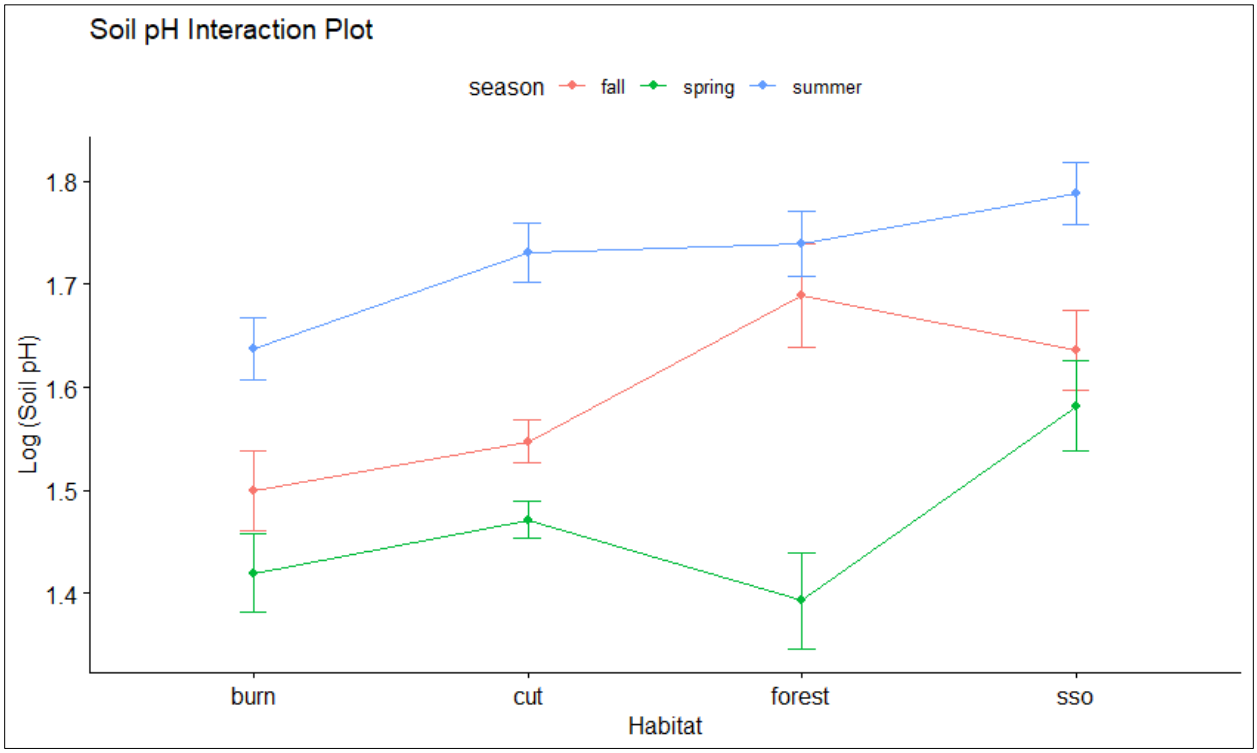

Figure 9. Interaction plot of natural log transformed soil pH across habitat and seasonal groupings. Each point represents mean values for each of the interaction groups. Error bars indicate the standard error of each mean value.

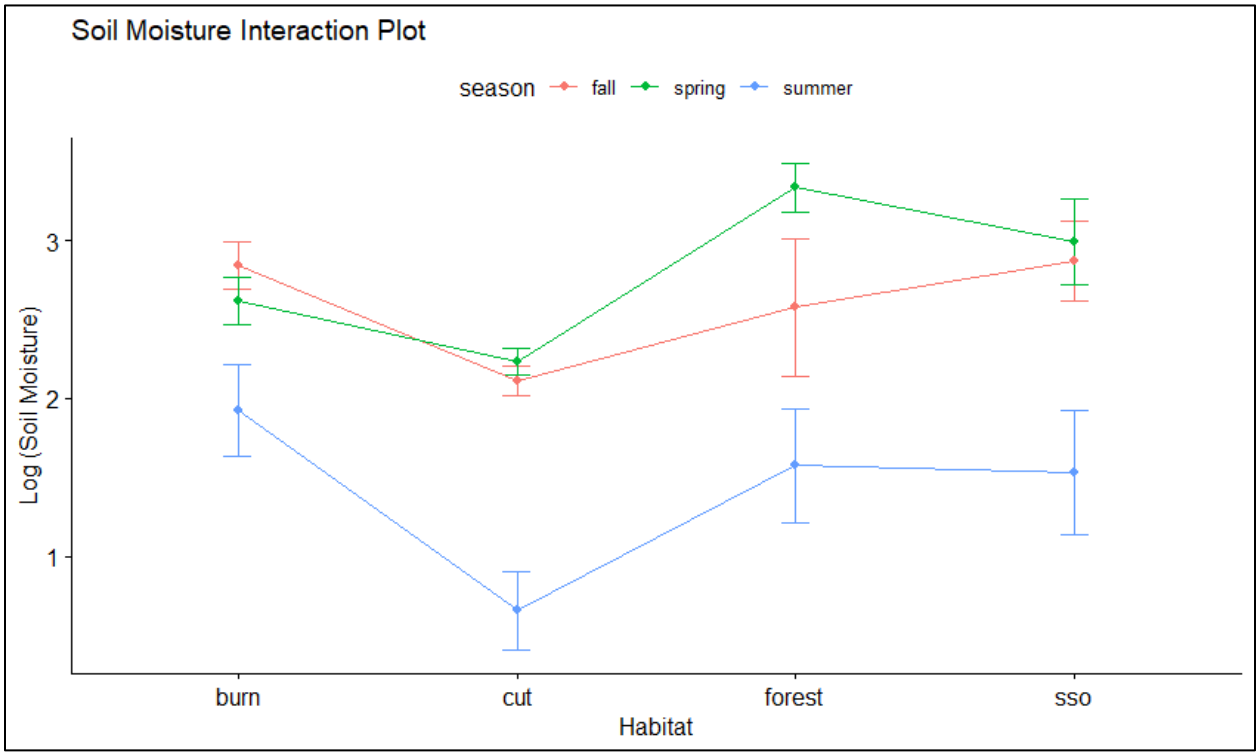

Figure 10. Interaction plot of natural log transformed soil moisture across habitat and seasonal groupings. Each point represents mean values for each of the interaction groups. Error bars indicate the standard error of each mean value.

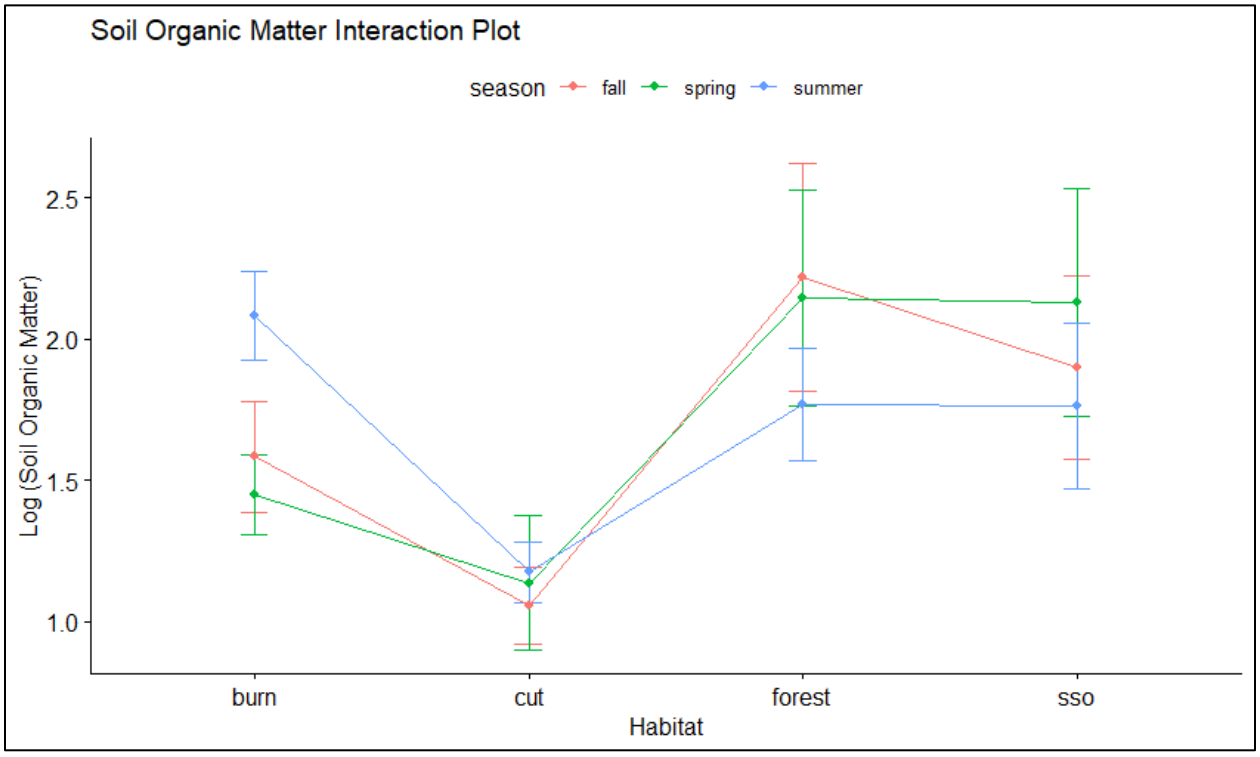

Figure 11. Interaction plot of natural log transformed soil organic matter across habitat and seasonal groupings. Each point represents mean values for each of the interaction groups. Error bars indicate the standard error of each mean value.

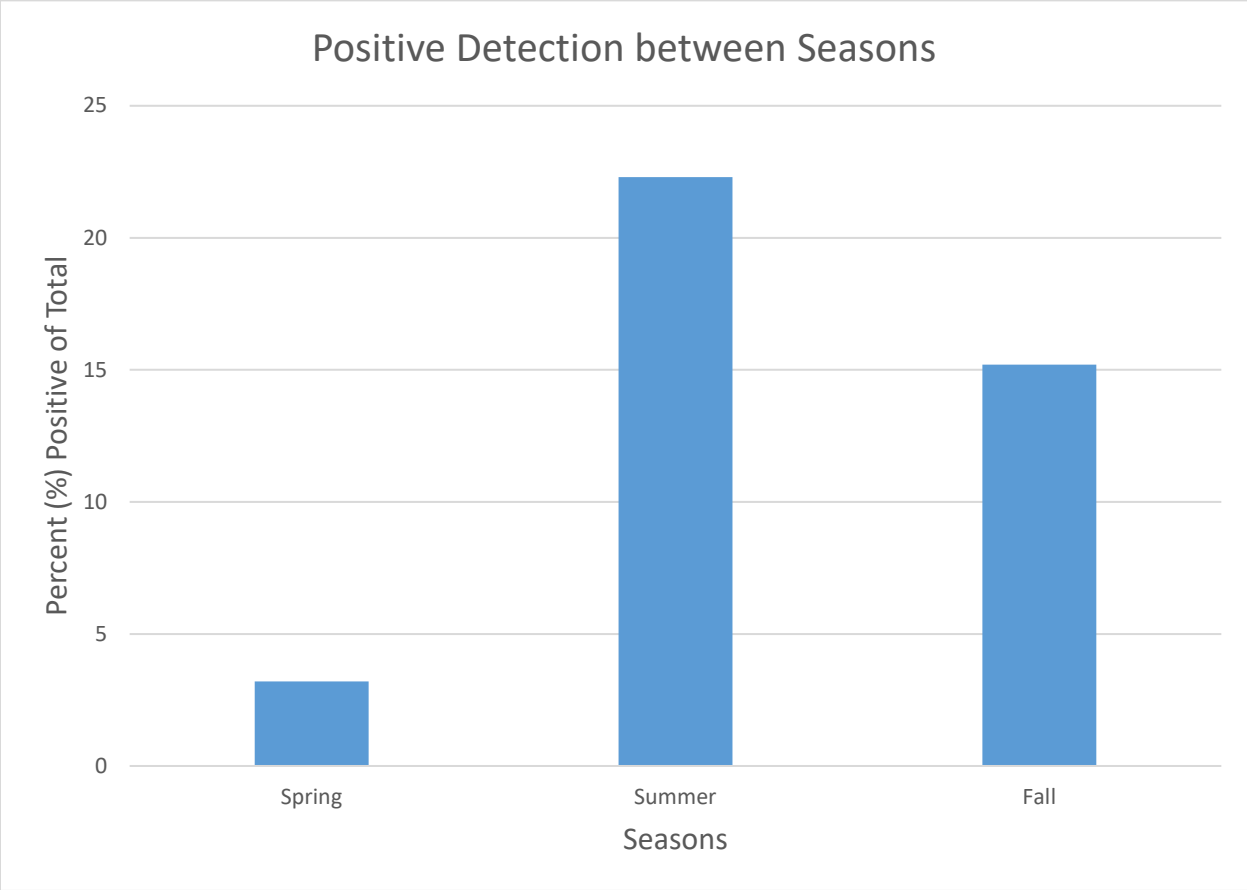

Figure 12. Percent value of positive detection between seasons fall (n=46), spring (n=62), and summer (n=103). Highest detection shown to occur during the summer season

Table 2. Binomial logistic regression output from 12 of the 124 total models ordered based on AICc. ΔAICc was used to assess model fit relative to the top model. Pseudo R^2 also included as measure of model performance. Model weight indicated by (w) shows weight of evidence that the specified model is the best model.

Binomial Logistic Regression Output												
Model	**Intercept**	**cGDD7**	**moisture**	**organic**	**Soil.pH**	**Soil.t**	**Soil.type**	**weekly.prcpn**	**Psuedo R^2**	**AICc**	**ΔAICc**	**w**
1	**-4.901810713**	**0.008**	-	-	**0.542**	**-0.151**	-	**0.016**	**0.127**	**166.76**	**0**	0.065
2	-5.060585113	0.003	-	-	0.457	-	-	-	0.104	166.8	0.036	0.064
3	-5.546896301	0.003	-	-	0.513	-	-	0.014	0.115	166.84	0.081	0.062
4	-4.97748984	0.004	-	0.016	0.386	-	-	-	0.114	167.09	0.33	0.055
5	-4.462919341	0.007	-	-	0.48	-0.127	-	-	0.113	167.2	0.435	0.052
6	-5.419018856	0.003	-	0.015	0.445	-	-	0.013	0.123	167.53	0.771	0.044
7	-3.271591584	0.005	-	0.021	-	-	-	-	0.098	167.8	1.04	0.039
8	-4.512864485	0.007	-	0.013	0.413	-0.097	-	-	0.119	168.22	1.457	0.031
9	-4.903689302	0.007	-	0.01	0.485	-0.123	-	0.015	0.131	168.25	1.492	0.031
10	-5.065461412	0.004	0.008	-	0.411	-	-	-	0.107	168.27	1.506	0.031
11	-5.522558808	0.003	0.006	-	0.475	-	-	0.014	0.117	168.57	1.807	0.026
12	-2.966654962	0.005	-	-	-	-	-	-	0.081	168.87	2.107	0.023

Table 3. The Σw represents the summed AICc weights of each variable indicating the relative importance of considered variables in predicting *Ophidiomyces* presence.

Relative Variable Importance	
	Σw
cGDD	0.842473427
Soil pH	0.74256353
Weekly prcpn	0.476704161
Organic	0.424434256
Soil temperature	0.419947769
Moisture	0.289566082
Soil type	0.056619555

Table 4. Output from the top ranked model (AICc = 166.76) of logistic regression. Estimates represent the effect size of the variable. Null and residual deviance were used to generate chi-square statistic.

Top Ranked Model (1) from Regression Output

Coefficients:	Estimate	(95% CI)	Standard error	z value	p-value
Intercept	-4.902	-7.46, -2.34	1.307	-3.751	0.00018
cGDD	0.008	0.0003, 0.015	0.004	2.048	0.0406
Soil pH	0.542	0.091, 0.993	0.23	2.357	0.01842
Soil temp	-0.151	-0.366, 0.064	0.11	-1.376	0.1689
Weekly prcpn	0.016	-0.0036, 0.036	0.01	1.609	0.1076

Table 5. Candidate models for probability of detection (p) of *Ophidiomyces* DNA in PCR replicates. All models considered with their corresponding model selection scores. Model in bold represents top ranked model used to derive probability of detection.

Model	WAIC	PPLC
Ψ(),Θ(),p()	**69.62**	**66.35**
Ψ(),Θ(),p(organic)	72.53	68.39
Ψ(),Θ(),p(DNA.con)	75.32	71.21
Ψ(),Θ(),p(Soil pH)	77.75	74.95
Ψ(),Θ(),p(DNA.con+organic)	78.31	73.44
Ψ(),Θ(),p(Soil pH+DNA.con)	78.95	74.43
Ψ(),Θ(),p(Soil pH+organic)	79.97	75.7
Ψ(),Θ(),p(DNA.con+organic+Soil pH)	81.78	75.95

Table 6. Fourteen of the 63 total models considered in occupancy analysis. Models scored based on selection criteria WAIC and PPLC with the lowest values. Bold indicates the top ranked model based on each criteria.

Model	WAIC	PPLC
Ψ(),Θ(Soil pH),p()	**69.23**	66.67
Ψ(),Θ(),p()	69.62	**66.35**
Ψ(),Θ(Soil pH+weekly prcpn),p()	69.91	67.46
Ψ(),Θ(weekly prcpn),p()	69.97	67.04
Ψ(),Θ(Soil t),p()	70.05	67.13
Ψ(),Θ(Soil pH+Soil t),p()	70.06	67.5
Ψ(),Θ(Soil t+cGDD),p()	70.27	67.58
Ψ(),Θ(Soil pH+cGDD),p()	70.4	68
Ψ(),Θ(Soil pH+Soil t+cGDD),p()	70.4	67.93
Ψ(),Θ(moisture),p()	70.48	67.05
Ψ(),Θ(Soil pH+moisture),p()	70.48	67.96
Ψ(),Θ(Soil pH+ Soil t+weekly prcpn),p()	70.8	68.29
Ψ(),Θ(weekly prcpn+moisture),p()	70.81	67.45
Ψ(),Θ(cGDD),p()	70.82	67.99

Table 7. Summaries of the parameters MCMC models. Beta, alpha, and delta represent the estimates for the site, sample, and replicate sampling levels respectively. Model Ψ(),Θ(),p() shows estimates for the null model with no covariates. B) Model Ψ(),Θ(Soil pH),p() estimates for the model with soil pH as a covariate of sample occupancy.

Bayesian Estimates of Model Parameters			
Model		Mean	95% CL
Ψ(),Θ(),p()	β (Intercept)	1.16	-0.061, 2.539
	α (Intercept)	-0.815	-1.072, -0.517
	δ (Intercept)	-0.318	-0.668, 0.017
Ψ(),Θ(Soil pH),p()	β (Intercept)	1.164	-0.072, 2.601
	α (Intercept)	-0.888	-1.167, -0.575
	α (Soil pH)	0.434	0.196, 0.701
	δ (Intercept)	-0.319	-0.666, 0.009

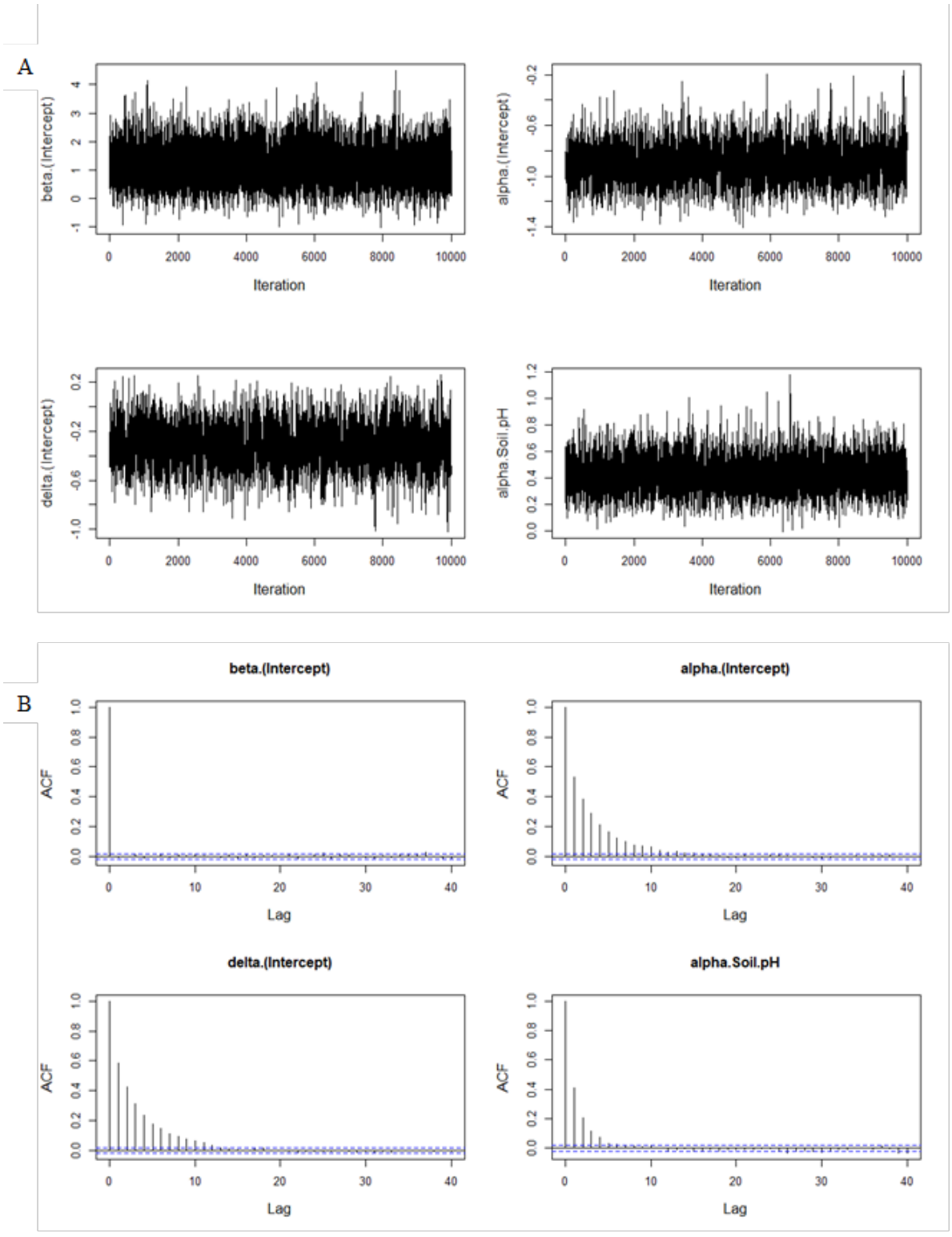

Figure 13. A) Trace plots showing model convergence along all iterations run in the model. Intercept values for each sampling level, beta (site), alpha (sample), delta (PCR replicate). Alpha.Soil.pH is the covariate soil pH at the sample level (Θ). B) Autocorrelation function plots showing the degree of autocorrelation during the Markov Chain. Each bar represents the correlation between the observation and the prior observation. The dotted blue line represents the 95% confidence limit.

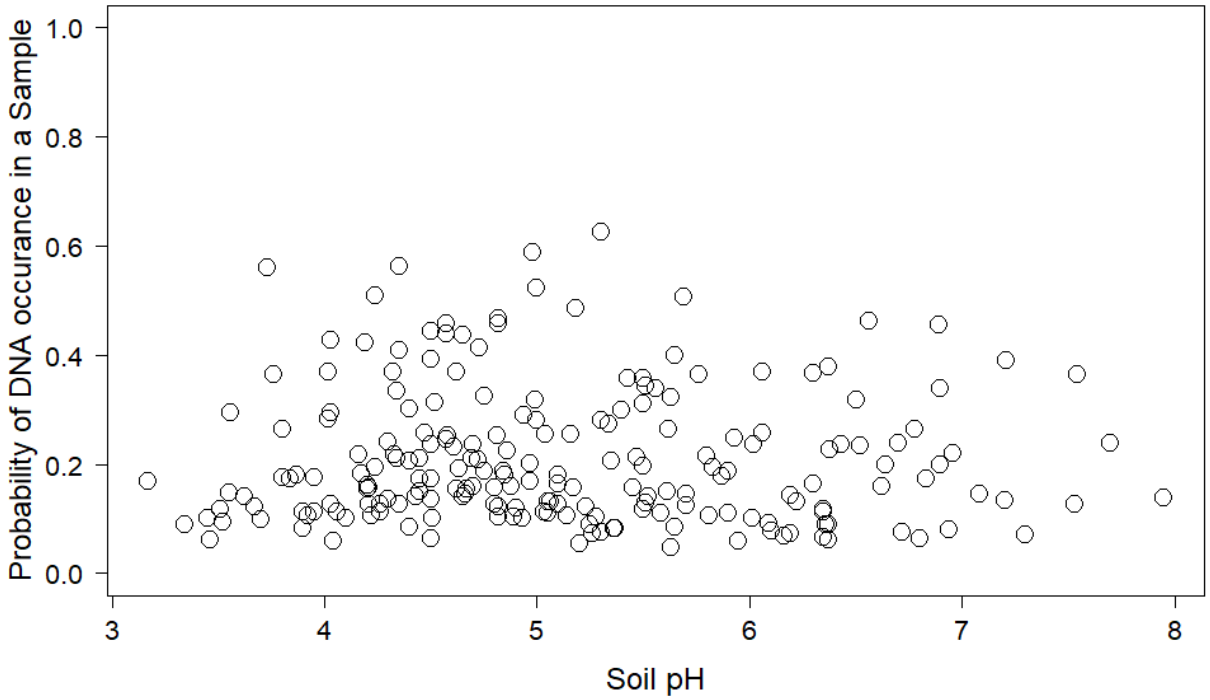

Figure 14. Relationship between the probability of *Ophidiomyces* DNA occurrence in a sample and the soil pH. Each point represents the posterior mean estimate from a sample (occupancy probability Θ) at a given pH. No discernable relationship is seen between occupancy probability and soil pH. 95% limits showed high variability in some estimates, not included for ease of viewing

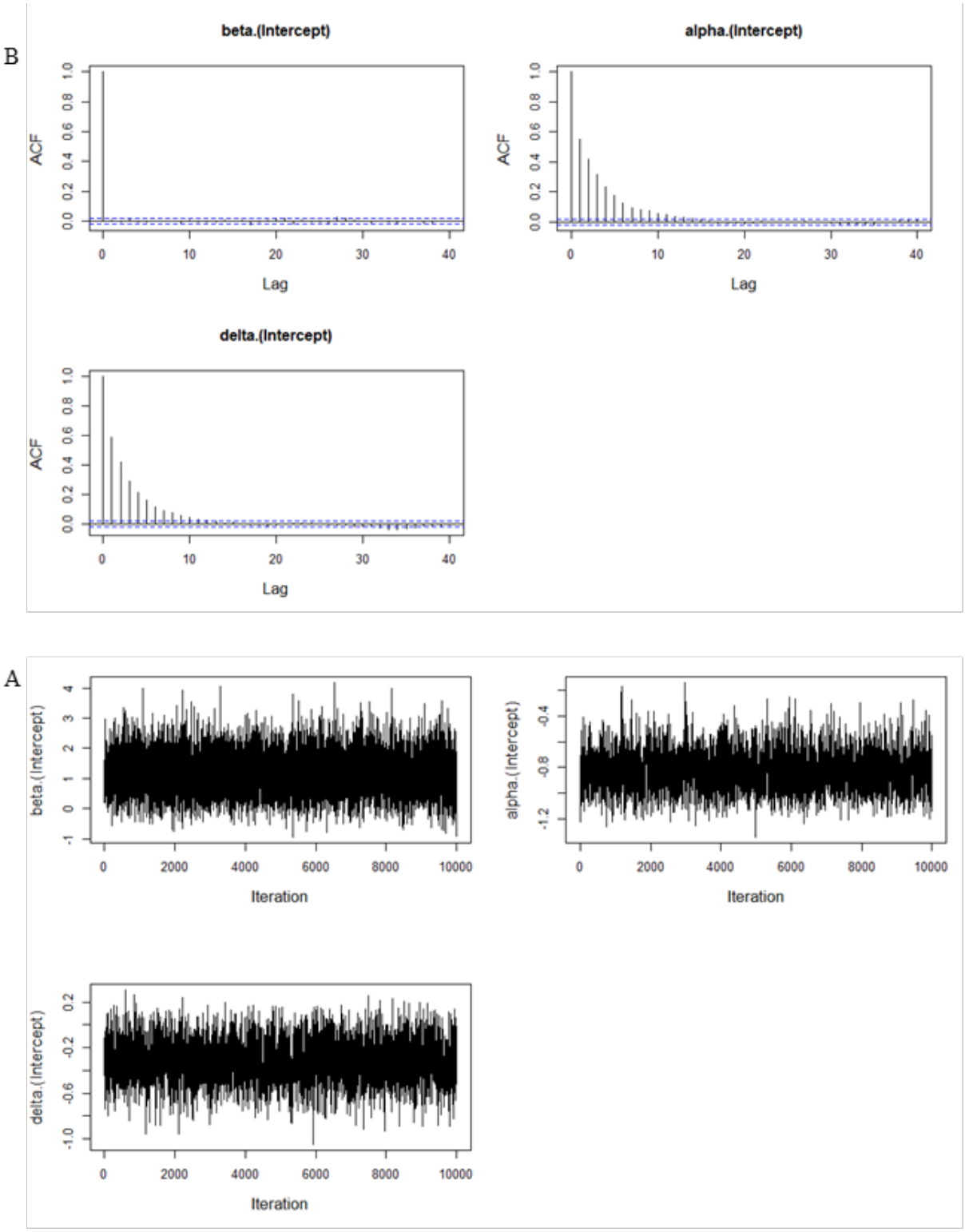

Figure 15. A) Trace plots showing model convergence along all iterations run in the model. Intercept values for each sampling level, beta (site), alpha (sample), delta (PCR replicate). B) Autocorrelation function plots showing the degree of autocorrelation during the Markov Chain. Each bar represents the correlation between the observation and the prior observation. The dotted blue line represents the 95% confidence limit.

CHAPTER 4. DISCUSSION

Since the identification of Snake Fungal Disease and its causative agent *Ophidiomyces ophiodiicola,* it has become a relevant topic of conservation concern. Many studies have been conducted which survey for the presence of *Ophidiomyces* within populations of susceptible species across the United States (Allender et al. 2015b, Chandler et al. 2019, Licitra et al. 2019, Haynes et al. 2020, Haynes et al. 2022). Surveys of snakes and active sampling of individuals for disease and infection, have spurred the design of highly specific assays for diagnosis of infection (Allender et al. 2015a, Bohuski et al. 2015), improving our ability to monitor susceptible species for signs of infection. This work provides critical insight into not only disease progression in individuals, but also key epidemiological information on species at risk of disease and how infection can cause population level effects (Clark et al. 2011).

Although many pieces of the puzzle regarding *Ophidiomyces* have been discovered, there remains a gap in information with respect to literature on the general ecology of *O. ophiodiicola*. Proper management of susceptible species requires a more complete understanding of *Ophidiomyces* and its habitat associations, seasonality, and distribution in the environment independent of hosts. This study represents one of the few attempts to detect *Ophidiomyces* through an environmental sampling approach and aims to determine where it exists in an area it is known to infect the federally threatened Eastern Massasauga (*Sistrurus catenatus*).

The approaches applied here were successful in detecting *Ophidiomyces*, but there were limitations. There was only questionable evidence towards environmental predictors and *Ophidiomyces,* making it difficult to draw inferences on its environmental associations. The variables thought to be informative may have been too broad, where true differences in occupancy or detection could exist within more specific aspects of variables. Additional, differing variables that were not included could have also have an effect. The development of methods and the collection of data in this study had occurred when there was still no successful detection of *Ophidiomyces* in the environment from which more specific hypotheses about informative variables could be drawn. Additionally, field sample storage constraints and soil/ ground water volumes during extraction could have also impeded detection.

Even with such limitations I am still confident in this data generated in this study. I was able to apply a specifically targeted qPCR assay (Allender et al. 2015a) for the detection of *O.*

ophiodiicola in environmental samples. I found that detection was reliable in topsoil samples and observed a seasonal effect, with most detections occurring during the summer. Minimal to no detection occurred in groundwater samples, and I was not able to determine any meaningful correlations between *Ophidiomyces* presence and considered environmental variables.

4.1 *Ophidiomyces* DNA Detection

The application of the ITS targeted real time PCR (qPCR) assay (Allender et al. 2015) provided a specific approach for detection of *Ophidiomyces* in environmental samples. Detection was primarily achieved in topsoil samples taken from the study site. A total of 14.8% of soil samples showed signs of *Ophidiomyces* DNA amplification across all four habitat types sampled and each was confirmed by DNA sequencing of the assay target. Although there were differences in the number of detections among the habitat types sampled, no statistical difference was observed.

In contrast, detection did differ when samples were grouped by season (spring, summer, and fall) with the highest detection occurring during the summer season. This could be caused by increased soil temperatures allowing for optimized growing conditions for *Ophidiomyces*. *In vitro* culturing of *Ophidiomyces* found that the optimal temperature for growth was 25°C, while growth significantly slowed and completely stopped at 14°C and 7°C, respectively (Allender et al. 2015c). At the study site, soil temperatures climbed closer to optimal growing temperatures during the summer season while most samples taken during the spring and fall groupings were considerably lower and decreased below the base temperature (7°C) for growth. Similarly, Allender et al. (2015) found that the pH range for growth was 5-11 with optimal growth at a pH of 9. Soil pH at the study site showed seasonal trends with significantly higher soil pH occurring during the summer (mean = 5.67), while pH dropped during both fall (mean = 4.96) and spring (mean = 4.37). Seasonal trends in soil pH are the result of many factors including geography and climate, and macro- and microorganisms that interact with the soil (Yan et al. 1996, Yamashita et al. 2011). The higher detection seen during the summer season could be affected by these seasonal differences, as the time of year provides *Ophidiomyces* with conditions more like optimal conditions determined *in vitro*. Soil moisture content was lowest during the summer as seen in both our data and the MSU weather station network. The fact that detection was at its highest when soil moisture was at its lowest is counterintuitive. Generally, soil moisture (matric potential) is a limiting factor for soil fungi, and most soil fungi can only tolerate matric water stress down to

a certain level (Magan and Lynch 1986, Deacon 2006). However, *Ophidiomyces* has been shown to have a higher tolerance of matric water stress (-5 MPa) (Allender et al. 2015c). Therefore, although there is seasonality in latent soil moisture (capillary and hydroscopic moisture, not due to precipitation), it is likely not contributing to the seasonal detection differences seen here.

Only one groundwater sample yielded a positive result, or 0.2% of the total sample collected. Such low levels of detection in water samples from an area where it is known to occur on snakes could indicate a failure to collect enough DNA through sampling or that *Ophidiomyces* has little to no presence in ground water at the site. Fungal DNA detection from water samples has been successful in the past when surveying for *Batrachochytrium dendrobatidis* (Kolby et al. 2015), which displays an aquatic stage in its lifecycle. *Ophidiomyces* is known to display filamentous growth (Sigler et al. 2013, Allender et al. 2015c) and has no known aquatic life stage, which may explain the lack of detection within water. Other published work attempting to detect *Ophidiomyces* DNA in water samples exhibited no detection, specifically from water in crayfish burrows (Baker et al. 2018). The results of this study might have been an artifact of the small volume of water sampled, but the volume in our study was 10 times (500ml) higher and again detection was minimal. Although, the sample protocol cannot be ruled out as a cause of low detection, our results suggest that *Ophidiomyces* may be at low levels or largely absent from ground water microhabitats.

The detection seen in topsoil marks one of the first successful attempts to detect *Ophidiomyces* environmental through eDNA based approaches. Campbell et al. (2021) were successful in detecting the fungus in both topsoil samples (8% of samples) and soil collected from snake hibernacula. Our results found nearly double the level detection seen within topsoil (14.8%). However, the detection probability in soil samples observed in this study was still low (0.37). This could likely be improved if larger quantities of soil are extracted. Increasing the amount of *Ophidiomyces* DNA present in each qPCR reaction. Which is discussed as a limitation. Taking multiple extractions of a sample per point could also lead to increased detection probability.

In any case, the rates in topsoil seen in both studies were considerably lower than those seen from soil within snake hibernacula (61%) in Campbell et al. (2021). It is difficult to determine if the higher detection within hibernacula is a result of the increased contact that soil has with snakes or if conditions for growth are better. Both this study and that by Campbell et al. (2021) represent successful detection within soil, providing further evidence for the claim that *Ophidiomyces*

function as a saprotrophic fungi in soil (Allender et al. 2015c) that opportunistically infects susceptible snakes.

4.2 Environmental Associations

Simply knowing where *Ophidiomyces* exists helps inform focused approaches for future surveying, however if a more complete picture is to be obtained it is important to understand parameters within its environment that could be influencing its presence and prevalence on the landscape. I evaluated several environmental (both spatially and temporally dependent) variables hypothesized to be associated with its presence. Factors that vary spatially between points like soil pH, organic matter and moisture all have well known effects on *Ophidiomyces* and general fungal/microbial ecology within soils (Magan and Lynch 1986, Yan et al. 1996, Blazewicz et al. 2014, Allender et al. 2015c). Temporal effects on soil microbes have been investigated as well with cGDD, or thermal time, offering a unique temporal measurement that can be more useful for plants and fungi than regular time measurements (Lovell et al. 2004a, Lovell et al. 2004b, Lione et al. 2021). Precipitation also has been shown to cause significant fungal growth and activity following soil wet up events (Blazewicz et al. 2014).

Since *Ophidiomyces* ecology has largely been studied *in vitro*, little is known of its environmental associations. Our modeling initially showed that some of variables investigated contribute to explaining presence in both logistic regression and multiscale occupancy modeling. The top ranked regression model found that soil pH and cGDD had effects on DNA presence. This aligns with what was seen when comparing detections seasonally, as both parameters that had an effect were at their highest (closest to *Ophidiomyces* optimal growth conditions) during the summer where the most detections were observed. However, goodness of fit tests found that the model had poor fit and did not match the observed data well. Therefore, proper inferences about what influences *Ophidiomyces* presence within a given sample are difficult to draw.

Results were similar when covariate effects were investigated in multiscale occupancy modeling at the sample and detection level through eDNAOCCUPANCY. Occupancy at the site level, which was assumed to be constant, was relatively high (0.83). Although the confidence interval for this estimate had a lower bound of 0.48, the estimate still shows that occupancy across the site is roughly greater than 50%. Soil pH as a function of occupancy within a sample was ranked highly and was shown to affect the probability of occurrence in a sample. However, the

relationship was questionable at best when observed visually. Therefore, no strong conclusions could be drawn about pH effects on occupancy in a sample. The null model was used for determining occupancy estimates due to the lack of relationship seen with covariates. The constant conditional probability of occupancy at the sample level was only 0.21 indicating that there is only a 21 percent chance a given sample will be occupied if the *Ophidiomyces* is present at the site. No covariates for detection probability were found to have an effect as the top ranked model was the null model.

The variables included as predictors in logistic regression and multiscale occupancy analysis appear to have little relationship to *Ophidiomyces* growth within samples. Similarly, no relationships of abiotic parameters to fungus presence and growth were found in the only other study to investigate soil substrates (Campbell et al. 2021). *Ophidiomyces* does exhibit broad capacity for growth in many abiotic parameters (Allender et al. 2015b), which might cause the lack of strong correlations between its presence and environmental variables. The soil variables included in this study, while numerous, were more general variables that are made up of many factors. The inclusion of differing variables, like biotic variables, or those with asymptotic qualities might lead to a correlation, in contrast to those in this study.

Initial growth of *Ophidiomyces* has been shown to be completely inhibited in soils that have diversified microbial communities relative to sterilized soils (Campbell et al. 2021). Soil microbial communities are quite complex and there is competition among species, which often results in the suppression of growth in certain species and a general lack of available nutrients. Complex soil communities can prevent colonization and growth of pathogenic species (Weller et al. 2002, Agtmaal et al. 2017, Schlatter et al. 2017). *Ophidiomyces* may be a poor competitor, limiting its presence in soil with diverse communities of soil flora. Biotic, rather than abiotic, variables might have more of an effect on the presence and prevalence of *Ophidiomyces* in soils. Campbell et al. (2021) provided useful initial results towards biotic effects on *Ophidiomyces* presence and growth. If studies on addressing biotic variables continue, focus should remain on soil microbiomes. The methods applied in Campbell et al. (2021) could be expanded to new areas within the known range of *Ophidiomyces,* to see if there are different trends in the observed correlations in different locations. Additionally, there should be continued investigation into microbial suppression and *Ophidiomyces* presence in soil. Determination of specific microbial groups or community compositions associated with suppression could be investigated through DNA metabarcoding.

4.3 Eastern Massasauga and Disease Development

The Eastern Massasauga has displayed clinical signs of SFD, and molecular investigation into such cases has confirmed the presence of *Ophidiomyces* in those cases (Allender et al. 2015b, Tetzlaff et al. 2015). *Ophidiomyces* has been known to exist at the study site, but it has not been clear on how snakes develop infection, whether snakes are the main reservoir, or if the fungus resides in the environment. This study has shown that it is detectable in topsoil samples throughout the site independent of snakes, but at much lower levels or absent in groundwater (Baker et al. 2018). In addition to it found within snake hibernacula at high levels (Campbell et al. 2021), snake exposure in soils appears to be the most likely source of environmental infection. Groundwater survey methods may not be adequate, or levels of *Ophidiomyces* in groundwater may be incidental and insufficient for snakes to develop infection.

Although no environmental associations were observed, there was seasonality found in its detection. This could suggest higher exposure during the warmer summer when the snakes are most active. Interestingly, snakes have been reported showing signs of infection after emergence from hibernacula which occurs several weeks prior to the peak period of *Ophidiomyces* detection. Snakes may first be exposed to *Ophidiomyces* through contact with soil containing the fungus, or other infected individuals during their overwintering, when snakes would be most immunocompromised. Following emergence, it is known that Massasaugas can achieve body temperatures much higher than environmental temperatures through thermoregulation. Such thermoregulation may provide more optimal growing temperatures for *Ophidiomyces* on snakes than in the surrounding environment in the spring. This is one proposed mechanism of infection at the site in northern Michigan based off findings in this study. To test such a hypothesis would require early monitoring of snakes directly following emergence, and confirmation of *Ophidiomyces* presence/ infection. Repeated measurements of individual snakes for *Ophidiomyces* and disease development could be paired with massasauga temperature data to monitor infection status as thermoregulatory opportunities improve.

However, snakes could also develop infection independent of hibernacula. It may be less likely due to the difference in detection seen between topsoil and hibernaculum soil (Campbell et al. 2021), but still probable given the wide spatial coverage of positive detection in the study site. This degree of spread could lead to a chance of exposure throughout the active season as snakes move beyond hibernacula and encounter topsoil occupied by *Ophidiomyces*. Given *Ophidiomyces*

low ability to compete in complex soil microbial communities (Campbell et al. 2021) it may be difficult for it to spread spatially in soil itself. Therefore, snakes themselves may facilitate its spread across the landscape. Positive detection of *Ophidiomyces* in samples had a large, random spread across the study site, a site where SFD has been observed for almost decade (Allender et al. 2015b, Tetzlaff et al. 2015, Allender et al. 2016).

4.4 Suggestions for Management and Future Work

The successful application of specific qPCR assays for detecting *Ophidiomyces* in environmental samples, as shown here, is a promising step in answering ecological and epidemiological questions. If surveying for *Ophidiomyces,* environmental sampling can provide specific and sensitive detection, using qPCR assays like the ITS assay applied here and in Campbell et al (2021). Topsoil offers a reliable method if sampling is to be done through environmental mediums. Soil within snake hibernacula has yielded even higher detection than topsoil (61%) and is another route that can be taken for environmental surveys.

Groundwater, whether it is from hibernacula like crayfish burrows (Baker et al. 2018) or independent of snake activity is not a reliable medium in which to survey for the presence of *Ophidiomyces*. Although snakes are suspected to emerge with infection from hibernacula where they were in contact with ground water, attempts to detect DNA in water have failed (Baker et al. 2018). Suggesting it is not a viable means for determining presence of the fugal pathogen.

Analysis showed that there seems to be evidence of seasonal trends in the ability to detect *Ophidiomyces* in topsoil samples regardless of the macrohabitat. In northern Michigan specifically, success might be higher during the warmer points of the year. This seasonal trend may change based on the geographic area and its seasonal trends, but it offers a good starting point for managers looking to develop a monitoring scheme.

To that point, most monitoring for *Ophidiomyces* and SFD infection relies on snake surveying and swabbing. While this method is very reliable way of determining active infection within populations and can give you an indication of *Ophidiomyces* presence in the area, it is more involved and unpredictable than environmental sampling. Environmental DNA approaches are known to offer benefits over more traditional methods, they are easier to accomplish, less demanding, and less invasive for the animal (Dejean et al. 2012, Davy et al. 2015, Spear et al. 2015). Such methods like those applied here and in Campbell et al. (2021) have been shown to be

effective at detecting low amounts of *Ophidiomyces* free of snakes. If one wants a complete picture of *Ophidiomyces* presence on the landscape both surveys of infected snakes and *Ophidiomyces* are required.

Since no predictors were found to be all that useful, and there was no statistically significant difference in detection between the macrohabitat types sampled, no suggestions can be made regarding habitat level management for *Ophidiomyces* across the landscape. From what is known so far about *Ophidiomyces* it is likely important to have intact soil microbiomes to aid in suppression of its growth (Campbell et al. 2021) and continue to monitor its status across the United States as well as those threatened species susceptible to infection.

This project provides a useful start to answering questions on *Ophidiomyces* ecology. I provide a reliable means for the detection of *Ophidiomyces* environmentally and show initial evidence of trends in detection. Continued research is needed and should be geared towards further surveying for *Ophidiomyces* within soil and other environmental mediums.

REFERENCES

Agtmaal, M., A. Straathof, A. Termorshuizen, S. Teurlincx, M. Hundscheid, S. Ruyters, P. Busschaert, B. Lievens, and W. Boer. 2017. Exploring the reservoir of potential fungal plant pathogens in agricultural soil. Appl Soil Ecol **121**:152-160.

Allender, M. C., D. Bunick, E. Dzhaman, L. Burrus, and C. Maddox. 2015a. Development and use of a real-time polymerase chain reaction assay for the detection of *Ophidiomyces ophiodiicola* in snakes. J Vet Diagn Invest **27**:217-220.

Allender, M. C., M. Dreslik, S. Wylie, C. Phillips, D. B. Wylie, C. Maddox, M. A. Delaney, and M. J. Kinsel. 2011. *Chrysosporium* sp. infection in eastern massasauga rattlesnakes. Emerg Infect Dis **17**:2383-2384.

Allender, M. C., E. T. Hileman, J. Moore, and S. Tetzlaff. 2016. Detection of *Ophidiomyces*, the Causative Agent of Snake Fungal Disease, in the Eastern Massasauga (*Sistrurus catenatus*) in Michigan, USA, 2014. J Wildl Dis **52**:694-698.

Allender, M. C., E. T. Hileman, J. A. Moore, and S. J. Tetzlaff. 2015b. *Ophidiomyces* detection in the Eastern Massasauga in Michigan. Wildlife Epidemiology Laboratory.

Allender, M. C., D. B. Raudabaugh, F. H. Gleason, and A. N. Miller. 2015c. The natural history, ecology, and epidemiology of *Ophidiomyces ophiodiicola* and its potential impact on free-ranging snake populations. Fungal Ecology **17**:187-196.

Baker, S. J., M. L. Niemiller, A. J. Stites, K. T. Ash, M. A. Davis, M. J. Dreslik, and C. A. Phillips. 2018. Evaluation of environmental DNA to detect *Sistrurus catenatus* and *Ophidiomyces ophiodiicola* in crayfish burrows. Conservation Genetics Resources **12**:13-15.

Bellard, C., P. Cassey, and T. M. Blackburn. 2016. Alien species as a driver of recent extinctions. Biol Lett **12**:20150623.

Blazewicz, S. J., E. Schwartz, and M. K. Firestone. 2014. Growth and death of bacteria and fungi underlie rainfall-induced carbon dioxide pulses from seasonally dried soil. Ecology **95**:1162-1172.

Bohmann, K., A. Evans, M. T. Gilbert, G. R. Carvalho, S. Creer, M. Knapp, D. W. Yu, and M. de Bruyn. 2014. Environmental DNA for wildlife biology and biodiversity monitoring. Trends Ecol Evol **29**:358-367.

Bohuski, E., J. M. Lorch, K. M. Griffin, and D. S. Blehert. 2015. TaqMan real-time polymerase chain reaction for detection of *Ophidiomyces ophiodiicola*, the fungus associated with snake fungal disease. BMC Vet Res **11**:95.

Burke, I. C., C. M. Yonker, W. J. Parton, C. V. Cole, K. Flach, and D. S. Schimel. 1989. Texture, Climate, and Cultivation Effects on Soil Organic Matter Content in U.S. Grassland Soils. Soil Sci Soc Am J **53**:800-805.

Burnham, K. P., and D. R. Anderson. 2002. Model Selection and Mulitmodal Inference: A practical information-theoretic approach. Springer Science & Buisness Media.

Bustin, S. A., V. Benes, J. A. JGarson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele, and C. T. Wittwe. 2009. The MIQE Guidelines Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin Chem **55**:611-622.

Campbell, L. J., J. Burger, R. T. Zappalorti, J. F. Bunnell, M. E. Winzeler, D. R. Taylor, and J. M. Lorch. 2021. Soil Reservoir Dynamics of *Ophidiomyces ophidiicola*, the Causative Agent of Snake Fungal Disease. J Fungi (Basel) **7**.

Campos-Cerqueira, M., T. M. Aide, and K. Jones. 2016. Improving distribution data of threatened species by combining acoustic monitoring and occupancy modelling. Meth Ecol Evol **7**:1340-1348.

Chandler, H. C., M. C. Allender, B. S. Stegenga, E. Haynes, E. Ospina, and D. J. Stevenson. 2019. *Ophidiomycosis* prevalence in Georgia's Eastern Indigo Snake (*Drymarchon couperi*) populations. PLoS One **14**:e0218351.

Cheatwood, J. L., E. R. Jacobson, P. G. May, T. M. Farrell, B. L. Homer, D. A. Samuelson, and J. W. Kimbrough. 2003. An outbreak of fungal dermatitis and stomatitis in a free-ranging population of pigmy rattlesnakes (*Sistrurus miliarius barbouri*) in Florida. J Wildl Dis **39**:329-337.

Clark, R. W., M. N. Marchand, B. J. Clifford, R. Stechert, and S. Stephens. 2011. Decline of an isolated timber rattlesnake (*Crotalus horridus*) population: Interactions between climate change, disease, and loss of genetic diversity. Biol Con **144**:886-891.

Cooke, D. E. L., L. Schema, and S. O. Cacciola. 2007. Tools to Detect, Identify and Monitor Phytophthora Species in Natural Ecosystems. J Plant Path **89**:13-28.

Cullen, D. W., A. K. Lees, I. K. Toth, and J. M. Duncan. 2001. Conventional PCR and real-time quantitative PCR detection of *Helminthosporium solani* in soil and on potato tubers. Europ J Plant Path **107**:387-398.

Cullen, D. W., A. K. Lees, I. K. Toth, and J. M. Duncan. 2002. Detection of *Colletotrichum coccodes* from soil and potato tubers by conventional and quantitative real-time PCR. Plant Path **51**:281-292.

Davison, J., M. Opik, M. Zobel, M. Vasar, M. Metsis, and M. Moora. 2012. Communities of arbuscular mycorrhizal fungi detected in forest soil are spatially heterogeneous but do not vary throughout the growing season. PLoS One **7**:e41938.

Davy, C. M., A. G. Kidd, and C. C. Wilson. 2015. Development and Validation of Environmental DNA (eDNA) Markers for Detection of Freshwater Turtles. PLoS One **10**:e0130965.

Deacon, J. W. 2006. Fungal Biology. 4 edition. Blackwell Publishing.

DeGregorio, B. A. 2008. Response of the Eastern Massasauga Rattlesnake (*Sistrurus catenatus*) to Clear-Cutting. Purdue Univeristy.

DeGregorio, B. A., J. V. Manning, N. Bieser, and B. A. Kingsbury. 2011. The Spatial Ecology of the Eastern Massasauga (*Sistrurus c. catenatus*) in Northern Michigan. Herpetologica **67**:71-79.

Dejean, T., A. Valentini, C. Miquel, P. Taberlet, E. Bellemain, and C. Miaud. 2012. Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog *Lithobates catesbeianus*. J Appl Ecol **49**:953-959.

Dorazio, R. M., and R. A. Erickson. 2018. EDNAOCCUPANCY: An r package for multiscale occupancy modelling of environmental DNA data. Mol Ecol Resour **18**:368-380.

Dzal, Y., L. P. McGuire, N. Veselka, and M. B. Fenton. 2011. Going, going, gone: the impact of white-nose syndrome on the summer activity of the little brown bat (*Myotis lucifugus*). Biol Lett **7**:392-394.

Eichmiller, J. J., L. M. Miller, and P. W. Sorensen. 2016. Optimizing techniques to capture and extract environmental DNA for detection and quantification of fish. Mol Ecol Resour **16**:56-68.

Ek-Ramos, M. J., W. Zhou, C. U. Valencia, J. B. Antwi, L. L. Kalns, G. D. Morgan, D. L. Kerns, and G. A. Sword. 2013. Spatial and temporal variation in fungal endophyte communities isolated from cultivated cotton (*Gossypium hirsutum*). PLoS One **8**:e66049.

English, N. B., J. F. Weltzin, A. Fravolini, L. Thomas, and D. G. Williams. 2005. The influence of soil texture and vegetation on soil moisture under rainout shelters in a semi-desert grassland. J Arid Environ **63**:324-343.

Eskew, E. A., and B. D. Todd. 2013. Parallels in Amphibian and Bat Declines from Pathogenic Fungi. Emerg Infect Dis **19**.

Ficetola, G. F., J. Pansu, A. Bonin, E. Coissac, C. Giguet-Covex, M. De Barba, L. Gielly, C. M. Lopes, F. Boyer, F. Pompanon, G. Raye, and P. Taberlet. 2015. Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. Mol Ecol Resour **15**:543-556.

Foden, W. B., S. H. Butchart, S. N. Stuart, J. C. Vie, H. R. Akcakaya, A. Angulo, L. M. DeVantier, A. Gutsche, E. Turak, L. Cao, S. D. Donner, V. Katariya, R. Bernard, R. A. Holland, A. F. Hughes, S. E. O'Hanlon, S. T. Garnett, C. H. Sekercioglu, and G. M. Mace. 2013. Identifying the World's Most Climate Change Vulnerable Species: A Systematic Trait-Based Assessment of all Birds, Amphibians and Corals. PLoS One **8**:e65427.

Giovannonoi, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field. 1990. Genetic diversity in Sargasso Sea bacterioplankton. Nature **345**.

Haynes, E., H. C. Chandler, B. S. Stegenga, L. Adamovicz, E. Ospina, D. Zerpa-Catanho, D. J. Stevenson, and M. C. Allender. 2020. Ophidiomycosis surveillance of snakes in Georgia, USA reveals new host species and taxonomic associations with disease. Sci Rep **10**:10870.

Haynes, E., K. Stanford, M. Gramhofer, K. Vivirito, K. Durante, A. Wright, C. Varga, and M. C. Allender. 2022. Epidemiology of Ophidiomycosis in Lake Erie Watersnakes (*Nerodia Sipedon Insularum*). J Wildl Dis **58**:100-113.

Heiri, O., A. F. Lotter, and G. Lemcke. 2001. Loss on Ignition as a method for Estimating Organic and Carbonate Content in Sediments: Reproducibility and Comparibility of Results. J Paleolimnol **25**:101-110.

Hileman, E. T., M. C. Allender, D. R. Bradke, L. J. Faust, J. A. Moore, M. J. Ravesi, and S. J. Tetzlaff. 2017. Estimation of *Ophidiomyces* prevalence to evaluate snake fungal disease risk. J Wildl Manag **82**:173-181.

Hoyt, J. R., A. M. Kilpatrick, and K. E. Langwig. 2021. Ecology and impacts of white-nose syndrome on bats. Nat Rev Microbiol **19**:196-210.

Hu, B., J. Shoao, and M. Palta. 2006. Pseudo-R 2 in Logistic Regression Model. Stat Sinica **16**:847-860.

Hunter, M. E., R. M. Dorazio, J. S. Butterfield, G. Meigs-Friend, L. G. Nico, and J. A. Ferrante. 2017. Detection limits of quantitative and digital PCR assays and their influence in presence-absence surveys of environmental DNA. Mol Ecol Resour **17**:221-229.

Hunter, M. E., S. J. Oyler-McCance, R. M. Dorazio, J. A. Fike, B. J. Smith, C. T. Hunter, R. N. Reed, and K. M. Hart. 2015. Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive burmese pythons. PLoS One **10**:e0121655.

Johnson, G. 2000. Spatial Ecology of the Eastern Massasauga (*Sistrurus c. catenatus*) in a New York Peatland. J Herp **34**:186-192.

Kabała, C., E. Musztyfaga, B. Gałka, D. Łabuńska, and P. Mańczyńska. 2016. Conversion of Soil pH 1:2.5 KCl and 1:2.5 H2O to 1:5 H2O: Conclusions for Soil Management, Environmental Monitoring, and International Soil Databases. Polish J Environ Studies **25**:647-653.

Kamoroff, C., and C. S. Goldberg. 2017. Using environmental DNA for early detection of amphibian chytrid fungus *Batrachochytrium dendrobatidis* prior to a ranid die-off. Dis Aquat Organ **127**:75-79.

Kolby, J. E., S. D. Ramirez, L. Berger, K. L. Richards-Hrdlicka, M. Jocque, and L. F. Skerratt. 2015. Terrestrial Dispersal and Potential Environmental Transmission of the Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*). PLoS One **10**:e0125386.

Lees, A. K., D. W. Cullen, L. Sullivan, and M. J. Nicolson. 2002. Development of conventional and quantitative real-time PCR assays for the detection and identification of *Rhizoctonia solani* AG-3 in potato and soil. Plant Path **51**:293-302.

Licitra, D., D. P. Quinn, J. E. Reeder, T. Gavitt, J. Dickson, B. Hess, B. J. Mangold, A. D. Tuttle, A. Rosas-Rosas, S. Frasca, Jr., and S. M. Szczepanek. 2019. Snake Fungal Disease in Colubridae Snakes in Connecticut, USA in 2015 and 2017. J Wildl Dis **55**:658-662.

Lione, G., L. Giordano, F. Sillo, F. Brescia, and P. Gonthier. 2021. Temporal and spatial propagule deposition patterns of the emerging fungal pathogen of chestnut *Gnomoniopsis castaneae* in orchards of north-western Italy. Plant Pathology **70**:2016-2033.

Long, R. A., T. M. Donovan, P. MacKay, W. J. Zielinski, and J. S. Buzas. 2010. Predicting carnivore occurrence with noninvasive surveys and occupancy modeling. Landscape Ecology **26**:327-340.

Lorch, J. M., S. Knowles, J. S. Lankton, K. Michell, J. L. Edwards, J. M. Kapfer, R. A. Staffen, E. R. Wild, K. Z. Schmidt, A. E. Ballmann, D. Blodgett, T. M. Farrell, B. M. Glorioso, L. A. Last, S. J. Price, K. L. Schuler, C. E. Smith, J. F. Wellehan, Jr., and D. S. Blehert. 2016. Snake fungal disease: an emerging threat to wild snakes. Philos Trans R Soc Lond B Biol Sci **371**.

Lorch, J. M., J. Lankton, K. Werner, E. A. Falendysz, K. McCurley, and D. S. Blehert. 2015. Experimental Infection of Snakes with *Ophidiomyces ophiodiicola* Causes Pathological Changes That Typify Snake Fungal Disease. MBio **6**:e01534-01515.

Lovell, D. J., T. Hunter, S. J. Powers, S. R. Parker, and F. Van den Bosch. 2004a. Effect of temperature on latent period of septoria leaf blotch on winter wheat under outdoor conditions. Plant Path **53**:170-181.

Lovell, D. J., S. J. Powers, S. J. Welham, and S. R. Parker. 2004b. A perspective on the measurement of time in plant disease epidemiology. Plant Path **53**:705-712.

Lozano-Ojalvo, D., A. Rodriguez, M. Cordero, V. Bernaldez, M. Reyes-Prieto, and J. J. Cordoba. 2015. Characterisation and detection of spoilage mould responsible for black spot in dry-cured fermented sausages. Meat Sci **100**:283-290.

Magan, N., and J. M. Lynch. 1986. Water Potential, Growth and Cellulolysis of Fungi Involved in Decomposition of Cereal Residues. Microbiology **132**:1181-1187.

McFadden, D. 1973. Conditional logit analysis of qualitative choice behavior. Front Econometrics:105-142.

Merkling, J. L. 2018. Development of an Environmental DNA Assay for Eastern Massasauga. Purdue Fort Wayne.

Morse, S. S. 1995. Factors in the Emergence of Infectious Diseases. Emerg Infect Dis **1**.

Nielsen, L. K., J. D. Jensen, A. Rodriguez, L. N. Jorgensen, and A. F. Justesen. 2012. TRI12 based quantitative real-time PCR assays reveal the distribution of trichothecene genotypes of *F. graminearum* and *F. culmorum* isolates in Danish small grain cereals. Int J Food Microbiol **157**:384-392.

Paré, J. A., and L. Sigler. 2016. An Overview of Reptile Fungal Pathogens in the Genera Nannizziopsis, Paranannizziopsis, and Ophidiomyces. J Herp Med Surg **26**.

Plante, A. F., R. T. Conant, C. E. Stewart, K. Paustian, and J. Six. 2006. Impact of Soil Texture on the Distribution of Soil Organic Matter in Physical and Chemical Fractions. Soil Sci Soc Am J **70**:287-296.

Powers, K. E., R. J. Reynolds, W. Orndorff, B. A. Hyzy, C. S. Hobson, and W. M. Ford. 2016. Monitoring the Status of Gray Bats (*Myotis grisescens*) in Virginia, 2009–2014, and Potential Impacts of White-Nose Syndrome. Southeast Nat **15**:127-137.

Ratsch, R. A. 2018. Efficacy of using Environmental DNA (eDNA) to detect Kirtland's Snakes (*Clonophis kirtlandii*). Purdue Fort Wayne.

Ravesi, M. J. 2016. Timber Harvest and Prescribed Fire as Tools for Massasauga Conservation. Purdue Fort Wayne.

Reichard, J. D., N. W. Fuller, A. B. Bennett, S. R. Darling, M. S. Moore, K. E. Langwig, E. D. Preston, S. von Oettingen, C. S. Richardson, and D. S. Reynolds. 2014. Interannual Survival of *Myotis lucifugus* (Chiroptera: Vespertilionidae) near the Epicenter of White-Nose Syndrome. Northeast Nat (Steuben) **21**:N56-N59.

Reinhart, H. K., and W. R. Kodrich. 1982. Movements and Habitat Utilization by the Massasauga, *Sistrurus catenatus catenatus*. J Herp **16**:162-171.

Reynolds, R. J., K. E. Powers, W. Orndorff, W. M. Ford, and C. S. Hobson. 2016. Changes in Rates of Capture and Demographics of *Myotis septentrionalis* (Northern Long-eared Bat) in Western Virginia before and after Onset of White-nose Syndrome. Northeast Nat **23**:195-204.

Rich, L. N., D. A. W. Miller, H. S. Robinson, J. W. McNutt, M. J. Kelly, and M. Hayward. 2016. Using camera trapping and hierarchical occupancy modelling to evaluate the spatial ecology of an African mammal community. J Appl Ecol **53**:1225-1235.

Robson, H. L., T. H. Noble, R. J. Saunders, S. K. Robson, D. W. Burrows, and D. R. Jerry. 2016. Fine-tuning for the tropics: application of eDNA technology for invasive fish detection in tropical freshwater ecosystems. Mol Ecol Resour **16**:922-932.

Schena, L., F. Nigro, and A. Ippolito. 2002. Identification and detection of *Rosellinia necatrix* by conventianal and real-time Scorpion-PCR. European Journal of Plant Path **108**:355-366.

Schena, L., F. Nigro, A. Ippolito, and D. Gallitelli. 2004. Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. Europ J Plant Path **110**:893-908.

Schlatter, D., L. Kinkel, L. Thomashow, D. Weller, and T. Paulitz. 2017. Disease Suppressive Soils: New Insights from the Soil Microbiome. Phytopathology **107**:1284-1297.

Schmelzle, M. C., and A. P. Kinziger. 2016. Using occupancy modelling to compare environmental DNA to traditional field methods for regional-scale monitoring of an endangered aquatic species. Mol Ecol Resour **16**:895-908.

Sewell, D., T. J. C. Beebee, and R. A. Griffiths. 2010. Optimising biodiversity assessments by volunteers: The application of occupancy modelling to large-scale amphibian surveys. Biol Con **143**:2102-2110.

Shena, L., and A. Ippolito. 2003. Rapid and sensitive detection of *Rosellinia necatrlxin* roots and soils by real time scorpion-PCR. J Plant Path **85**:15-25.

Shoemaker, K. T., and J. P. Gibbs. 2010. Evaluating Basking-Habitat Deficiency in the Threatened Eastern Massasauga Rattlesnake. J Wildl Manag **74**:504-513.

Sigler, L., S. Hambleton, and J. A. Pare. 2013. Molecular Characterization of Reptile Pathogens Currently Known as Members of the *Chrysosporium* Anamorph of *Nannizziopsis vriesii* Complex and Relationship with Some Human-Associated Isolates. J Clin Microbiol **51**:3338-3357.

Skerratt, L. F., L. Berger, R. Speare, S. Cashins, K. R. McDonald, A. D. Phillott, H. B. Hines, and N. Kenyon. 2007. Spread of Chytridiomycosis Has Caused the Rapid Global Decline and Extinction of Frogs. Ecohealth **4**.

Smith, C. S. 2009. Hibernation of the Eastern Massasauga rattlesnake (*Sistrurus catenatus catenatus*) in northern Michigan. Purdue Fort Wayne.

Spear, S. F., J. D. Groves, L. A. Williams, and L. P. Waits. 2015. Using environmental DNA methods to improve detectability in a hellbender (*Cryptobranchus alleganiensis*) monitoring program. Biol Con **183**:38-45.

Stoeckle, M. Y., L. Soboleva, and Z. Charlop-Powers. 2017. Aquatic environmental DNA detects seasonal fish abundance and habitat preference in an urban estuary. PLoS One **12**:e0175186.

Stulik, E. 2015. Amphibian Occupany and Habitat Use in a System of Restored Wetlands. Purdue Fort Wayne.

Sun, B.-D., H.-y. Yu, A. J. Chen, and X.-Z. Liu. 2008. Insect-associated fungi in soils of field crops and orchards. Crop Protec **27**:1421-1426.

Syzmanski, J. 1998. Status Assessment for Eastern Massasauga (*Sistrurus c. catenatus*) 1998. U.S. Fish & Wildlife Service.

Takahashi, M. K., M. J. Meyer, C. McPhee, J. R. Gaston, M. D. Venesky, and B. F. Case. 2017. Seasonal and Diel Signature of Eastern Hellbender Environmental DNA. J Wildl Manag **82**:217-225.

Tetzlaff, S., M. C. Allender, M. J. Ravesi, J. Smith, and B. A. Kingsbury. 2015. First report of snake fungal disease from Michigan, USA involving Massasaugas, *Sistrurus catenatus* (Rafinesque 1818). Herp Notes **8**:31-33.

Tetzlaff, S. J., M. J. Ravesi, M. C. Allender, E. T. Carter, B. A. DeGregorio, J. M. Josimovich, and B. A. Kingsbury. 2017. Snake Fungal Disease Affects Behavior of Free-Ranging Massasauga Rattlesnakes (*Sistrurus catenatus*). Herp Con Biol **12**:624-634.

Trujillo-Gonzalez, A., R. C. Edmunds, J. A. Becker, and K. S. Hutson. 2019. Parasite detection in the ornamental fish trade using environmental DNA. Sci Rep **9**:5173.

Voros, J., O. Marton, B. R. Schmidt, J. T. Gal, and D. Jelic. 2017. Surveying Europe's Only Cave-Dwelling Chordate Species (*Proteus anguinus*) Using Environmental DNA. PLoS One **12**:e0170945.

Weller, D. M., J. M. Raaijmakers, B. B. Gardener, and L. S. Thomashow. 2002. Microbial Populations Responsible for Specific Soil Suppressiveness To Plant Pathogens. Annu Rev Phytopathol **40**:309-348.

Willoughby, J. R., B. K. Wijayawardena, M. Sundaram, R. K. Swihart, and J. A. DeWoody. 2016. The importance of including imperfect detection models in eDNA experimental design. Mol Ecol Resour **16**:837-844.

Yamashita, N., S. Ohta, H. Sase, B. Kievuttinon, J. Luangjame, T. Visaratana, and H. Garivait. 2011. Seasonal changes in multi-scale spatial structure of soil pH and related parameters along a tropical dry evergreen forest slope. Geoderma **165**:31-39.

Yan, F., S. Schubert, and K. Mengel. 1996. Soil pH Increase Due to Biological Decarboxylation of Organic Anions. Soil Biol. Biochem. **28**:617-624.

Yates, M. D., and R. M. Muzika. 2010. Effect of Forest Structure and Fragmentation on Site Occupancy of Bat Species in Missouri Ozark Forests. J Wildl Manag **70**:1238-1248.

APPENDIX A. POSITIVE SAMPLE DATA

Table consisting of all positive samples, the habitat they were taken from, the number of positive replicates out of three, and the variable data collected for each sample.

Sample name	Habitat	Positive Replicates	Ambient temp. (°C)	Soil temp. (°C)	Soil pH	Soil type (Texture)	Percent Moisture
b328	burn	1/3	18.1	5.7	3.56	LSn	35.74
s328	sso	1/3	22.2	8	4.3	LSn	9.02
b114	burn	1/3	29.2	16.9	4.73	SnL	8.35
b115	burn	2/3	30.9	18.3	4.5	SnL	11.05
c105	cut	1/3	28.9	17.8	7.54	LSn	0.24
c106	cut	1/3	28.9	17.8	7.08	LSn	0.97
c107	cut	1/3	29.5	18.5	6.78	LSn	0.27
c110	cut	1/3	20.5	19.5	5.7	SnL	0.6
f103	forest	1/3	22.2	14.4	4.45	SnL	12.4
f108	forest	2/3	22.8	18.5	4.75	LSn	0.67
f114	forest	3/3	23	16.3	5.83	LSn	0.91
f117	forest	2/3	24.4	14.1	4.45	SnL	2.24
f127	forest	3/3	22.7	12.6	7.2	LSn	9.19
f128	forest	1/3	23.6	14.3	6.9	L	64.97
f131	forest	1/3	25.1	13.4	4.9	L	42.76
f132	forest	1/3	28	18.4	5.56	LSn	15.26
s105	sso	1/3	21.7	18.6	6.8	Sn	8.4
s108	sso	1/3	26.4	17.7	5.93	LSn	1.19
s111	sso	1/3	25	20.5	6.62	SnL	0.67
s112	sso	3/3	30.3	27.5	5.95	SnL	0.76
s114	sso	1/3	26.1	19.1	7.21	SnL	0.56
s121	sso	1/3	19	16	6.35	CL	40.99
s123	sso	1/3	20.5	16	6.94	SnCL	21.85
s126	sso	1/3	19.7	14.8	6.9	SiCL	58.18
s133	sso	2/3	23.2	18.5	5.04	LSn	0.81
b201	burn	2/3	8	9.1	4.97	LSn	12.03
b202	burn	1/3	11.1	10.2	4.35	LSn	16.88
b211	burn	1/3	13.3	10.9	5.65	SnL	14.84
c201	cut	1/3	7.5	10.3	4.62	SnL	11.67
f240	forest	3/3	5	7.7	6.19	SnCL	76.2
s201	sso	3/3	8.2	10.5	5.35	SnCL	52.78
s202	sso	2/3	7.2	10	6.16	SnCL	44.45

Continued. Table consisting of all positive samples, the habitat they were taken from, the number of positive replicates out of three, and the variable data collected for each sample

Sample name	Percent Organic	DNA Concentration	Date	Season	Cumulative Growing Degree Days	Weekly Precipitation
b328	4.03	25.9	13-May	spring	21.85	7.37
s328	48.85	56.5	24-Apr	spring	46.05	3.55
b114	21.93	16.1	15-Jul	summer	405.95	60.96
b115	7.55	7	15-Jul	summer	405.95	60.96
c105	1.62	65.3	8-Jul	summer	385.05	0
c106	5.64	29.6	13-Jul	summer	396.75	2.54
c107	1.76	11.8	13-Jul	summer	396.75	2.54
c110	2.3	20.9	13-Jul	summer	396.75	2.54
f103	6.07	26.5	4-Aug	summer	360.8	7.36
f108	2.89	53.2	11-Jul	summer	395.7	2.54
f114	3.93	8.8	14-Jul	summer	400.6	2.54
f117	2.88	33.2	23-Jul	summer	402.15	30.74
f127	5.28	17.4	5-Aug	summer	355.65	7.36
f128	68.58	1.1	5-Aug	summer	355.65	7.36
f131	36.46	13.5	5-Aug	summer	355.65	7.36
f132	7.45	24.3	8-Jul	summer	385.05	0
s105	0	22.2	8-Jul	summer	385.05	0
s108	5.55	53.4	11-Jul	summer	395.7	2.54
s111	2.83	13.2	15-Jul	summer	405.95	60.96
s112	5.57	11.9	15-Jul	summer	405.95	60.96
s114	2.4	19.2	15-Jul	summer	405.95	60.96
s121	40.26	1	4-Aug	summer	360.8	7.36
s123	10.38	60.3	4-Aug	summer	360.8	7.36
s126	32.3	59.6	5-Aug	summer	355.65	7.36
s133	3.49	53.2	11-Jul	summer	395.7	2.54
b201	2.95	34.7	4-Oct	fall	164.45	40.14
b202	3.55	14.5	4-Oct	fall	164.45	40.14
b211	3.79	4.7	13-Oct	fall	132.1	20.32
c201	5.11	61.2	4-Oct	fall	164.45	40.14
f240	67.39	25.4	4-Oct	fall	164.45	40.14
s201	25.72	90.1	4-Oct	fall	164.45	40.14
s202	23.24	8.3	4-Oct	fall	164.45	40.14

APPENDIX B. OCCUPANCY MODEL SET

Complete occupancy model set for determining covariate influences on the conditional probability of *Ophidiomyces* occupancy in a sample.

Model	WAIC	PPLC
Ψ(),Θ(Soil pH),p()	**69.23**	66.67
Ψ(),Θ(),p()	69.62	**66.35**
Ψ(),Θ(Soil pH+weekly prcpn),p()	69.91	67.46
Ψ(),Θ(weekly prcpn),p()	69.97	67.04
Ψ(),Θ(Soil t),p()	70.05	67.13
Ψ(),Θ(Soil pH+Soil t),p()	70.06	67.5
Ψ(),Θ(Soil t+cGDD),p()	70.27	67.58
Ψ(),Θ(Soil pH+cGDD),p()	70.4	68
Ψ(),Θ(Soil pH+Soil t+cGDD),p()	70.4	67.93
Ψ(),Θ(moisture),p()	70.48	67.05
Ψ(),Θ(Soil pH+moisture),p()	70.48	67.96
Ψ(),Θ(Soil pH+ Soil t+weekly prcpn),p()	70.8	68.29
Ψ(),Θ(weekly prcpn+moisture),p()	70.81	67.45
Ψ(),Θ(cGDD),p()	70.82	67.99
Ψ(),Θ(Soil pH+weekly prcpn+moisture),p()	71.07	68.51
Ψ(),Θ(organic),p()	71.07	67.8
Ψ(),Θ(Soil pH+Soil t+cGDD+weekly prcpn),p()	71.34	69.24
Ψ(),Θ(Soil t+weekly prcpn),p()	71.55	68.57
Ψ(),Θ(cGDD+moisture),p()	71.6	69.02
Ψ(),Θ(Soil pH+Soil t+moisture),p()	71.77	69.23
Ψ(),Θ(Soil t+moisture),p()	71.77	69.06
Ψ(),Θ(weekly prcpn+organic),p()	71.88	68.8
Ψ(),Θ(Soil pH+cGDD+weekly prcpn),p()	71.89	69.64
Ψ(),Θ(Soil t+cGDD+moisture),p()	71.91	69.33

Continued. Complete occupancy model set for determining covariate influences on the conditional probability of Ophidiomyces occupancy in a sample.

Model	WAIC	PPLC
Ψ(),Θ(Soil pH+cGDD+moisture),p()	72.11	69.68
Ψ(),Θ(cGDD+weekly prcpn),p()	72.11	69.46
Ψ(),Θ(Soil pH+Soil t+cGDD+moisture),p()	72.2	69.77
Ψ(),Θ(Soil t+cGDD+weekly prcpn),p()	72.24	69.58
Ψ(),Θ(cGDD+organic),p()	72.38	70.12
Ψ(),Θ(Soil pH+weekly prcpn+organic),p()	72.44	70.21
Ψ(),Θ(Soil t+cGDD+organic),p()	72.57	70.34
Ψ(),Θ(Soil pH+Soil t+cGDD+organic),p()	72.68	70.61
Ψ(),Θ(Soil pH+Soil t+weekly prcpn+moisture),p()	72.69	70.48
Ψ(),Θ(Soil pH+Soil t+organic),p()	72.72	70.8
Ψ(),Θ(Soil pH+moisture+organic),p()	73.01	70.7
Ψ(),Θ(Soil pH+cGDD+weekly prcpn+moisture),p()	73.09	71.07
Ψ(),Θ(Soil t+organic),p()	73.25	71.08
Ψ(),Θ(Soil pH+weekly prcpn+moisture+organic),p()	73.38	71.54
Ψ(),Θ(Soil t+weekly prcpn+moisture),p()	73.38	70.77
Ψ(),Θ(Soil pH+Soil t+cGDD+weekly prcpn+moisture),p()	73.4	71.27
Ψ(),Θ(cGDD+moisture+organic),p()	73.55	71.28
Ψ(),Θ(Soil pH+cGDD+organic),p()	73.82	71.6
Ψ(),Θ(organic+moisture),p()	74.02	71.71
Ψ(),Θ(Soil pH+Soil t+weekly prcpn+organic),p()	74.07	72.37
Ψ(),Θ(Soil t+cGDD+moisture+organic),p()	74.28	72.06
Ψ(),Θ(cGDD+weekly prcpn+moisture),p()	74.4	71.92

Continued. Complete occupancy model set for determining covariate influences on the conditional probability of *Ophidiomyces* occupancy in a sample.

Model	WAIC	PPLC
Ψ(),Θ(weekly prcpn+moisture+organic),p()	74.44	71.87
Ψ(),Θ(Soil pH+cGDD+weekly prcpn+organic),p()	74.49	72.86
Ψ(),Θ(Soil t+moisture+organic),p()	74.62	72.45
Ψ(),Θ(Soil pH+cGDD+moisture+organic),p()	74.63	72.65
Ψ(),Θ(Soil pH+Soil t+cGDD+moisture+organic),p()	74.68	72.8
Ψ(),Θ(Soil pH+Soil t+cGDD+weekly prcpn+organic),p()	74.76	73.02
Ψ(),Θ(Soil pH+Soil t+moisture+organic),p()	75.13	72.96
Ψ(),Θ(cGDD+weekly prcpn+organic),p()	75.24	73.06
Ψ(),Θ(Soil t+cGDD+weekly prcpn+moisture),p()	75.72	73.35
Ψ(),Θ(.),p()	75.74	74.12
Ψ(),Θ(Soil t+weekly prcpn+organic),p()	76.27	74.19
Ψ(),Θ(cGDD+weekly prcpn+moisture+organic),p()	76.51	74.81
Ψ(),Θ(Soil t+cGDD+weekly prcpn+organic),p()	76.73	74.8
Ψ(),Θ(Soil t+weekly prcpn+moisture+organic),p()	77.48	75.51
Ψ(),Θ(Soil t+cGDD+weekly prcpn+moisture+organic),p()	77.97	76.09
Ψ(),Θ(cGDD+weekly prcpn+moisture+organic),p()	78.54	76.58