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PREVALENCE OF SNAKE FUNGAL DISEASE CAUSED BY OPHIDIOMYCES OPHIODIICOLA IN EAST TEXAS

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PREVALENCE OF SNAKE FUNGAL DISEASE CAUSED BY *OPHIDIOMYCES OPHIODIICOLA* IN EAST TEXAS

by

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No discovery is made alone, we all must stand on the shoulders of the brave women and men who came before us.

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In Loving Memory of John Adam "Biggs" Sanders

1982-2016

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Abstract

PREVALENCE OF SNAKE FUNGAL DISEASE CAUSED BY *OPHIDIOMYCES OPHIODIICOLA* IN EAST TEXAS

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Fungal pathogens and resultant disease are credited with the decline of many species across all branches of the tree of life. Fungal diseases such as Snake Fungal Disease (SFD), primarily caused by the fungus *Ophidiomyces ophiodiicola* (Oo), have been strongly associated with the cause of the decrease/disappearance of many snake populations in North America. To date SFD in the wild has been described as far southwest as central Louisiana. Due to similar conditions and the proximity of East Texas to central Louisiana, a survey of local snake populations provided crucial information about the spread and presence of this emerging pathogen. In total (54) snakes were sampled between July 2019 through September 2020, and four putative positives for *Ophidiomyces ophiodiicola* infections were identified.

Chapter One: Literature Review

Introduction

Snake fungal disease (SFD) is an infection of the scales and skin exclusively found in the suborder Serpentes (Burbrink et al., 2017) and has been identified as an emerging infectious disease using the Fisher model (Lorch et al., 2018). This model created by Matthew Fisher et al. in 2012 calculates the threshold of pathogen presence in a population that results in the inevitable extinction of the population. *Ophidiomyces ophiodiicola*, the pathogen primarily associated with SFD, belongs to the family Onygenaceae. This family includes the saprotroph *Uncinocarpus reesei* and human pathogens belonging to the genus Coccidioides. Herein, I will discuss the severity of this pathogen regarding natural history, the environments the fungus thrives in, along with the behavioral and physical effects of the infection.

The Natural History of *Ophidiomyces ophiodiicola*

Ophidiomyces ophiodiicola, first described in snakes in 2013 by Sigler et al. has a story that goes back further. For example, researchers have found this fungus in museum specimens dating as far back as the year 2000 (Lorch et al., 2015*c*). There has been ever changing classifications that produced an eventual change to a monophylogenetic clade. This fungus that was initially part of the *Chrysoporium* anamorph of *Nannizziopis vriessii* (CANV) complex family. The pathogen was first brought to attention to researchers in wild snakes when a population of Timber Rattlesnakes (*Crotalus horridus*) in New Hampshire in 2006 had a skin infection outbreak (Lorch et al., 2015*c*). They found that this disease resembled a superficial fungal infection that is typical of a CANV species (Allender et al., 2015*c*). In 2008, Rajeev et al. were able to isolate this fungus from Massasaugas Rattlesnakes (*Sistrurus catenatus*) specimens and sequenced its DNA. These researchers found that it was genetically different than the *Chrysoporium* that was initially determined to be the causative agent. Further sequencing of the fungus allowed for the new description and moving this organism into its own clade by Sigler et al. in 2013. After classification Allender et al. (2015*c*) ran an experiment on transmission paths. They applied fungal samples to media with different levels of carbon, nitrogen, and manganese, among other types of resources the fungus may need to thrive. Also, to replicate a more natural

growth they applied the samples to mushroom biomasses, deceased fish, locust, and demineralized shrimp. They observed that the fungus will grow on these types of detritus in vitro. The researchers also found that "isolates were positive for gelatinase activity (with subsequent medium alkalization), b-glucosidase, lipase, lipase/esterase, and keratinase" (Allender et al., 2015*b*). Lastly, they found that Uric acid and nitrate levels had a negative effect on growth, although did not completely inhibit it.

Clinical Effects of Snake Fungal Disease

Research started by Lorch et al. (2015a) set out to describe in more detail the actual progression of the disease and to correlate O. *ophiodiicola* specifically, as the pathogen that causes snake fungal disease. Describing and documenting the symptoms of this disease is scientifically important because this progression will give other researchers the ability to diagnose/treat this disease as needed. Lorch et al. (2015) asked the important questions in this research, "Does *Ophidiomyces ophiodiicola* cause snake fungal disease?" and "What clinical steps does an infection create?". They hypothesized that there is a direct link between the pathogen and disease based on Koch's postulates the researchers can determine it as the cause of SFD. Koch's postulate is as series of stages that can correlate directly pathogens to their causative organism. The stages are: 1.) The agent must be present in every case of the disease, 2.) The agent must be isolated from the host with the disease and grown in pure culture, 3.) The specific disease must be reproduced when a pure culture of the agent is inoculated into a healthy susceptible host, and 4.) The agent must be recoverable from the experimentally infected host.

They began the study by breeding corn snakes that did not have any inherent genetic issues that might confound the experiment. Once to adult age they then roughed areas on the snakes' face and body with coarse sandpaper and applied the fungal specimens from an infected snake to these areas. This was done on only half of the snakes in the experiment, the other half were inoculated without the scuffing of the scales. If snakes did not present signs of infection after a week, they reinoculated it. Then researchers observed the snakes for 47-57 days depending on the progression speed of each infection. Snake fungal disease then went through a series of worsening symptoms and one mortality during the trials. Authors of the paper were able to give evidence that *Ophidiomyces ophiodiicola* is the source of clinical SFD. They found that

the fungal growths cause didectis which keeps certain portions of the skin and scales from being removed during shedding. In 5-6 days after inoculation the formation of blistering of skin and swelling began. It is accompanied by smokey eyes and behavioral changes. Changes to infected snake's behavior include increased basking time, a loss of appetite, and exhibit overall lethargy. Knowing these symptoms and behaviors will aid field researchers in determining clinically positive snakes.

In summary, the studies presented here outline the efforts to gain better knowledge of this emergent disease. Through life history of the pathogen, exploration of clinical and genetic characteristics, and the nuances in the determination of diagnosis of the disease. With this information we gain a more in depth understanding of the fungi's growing conditions, how it manifests in snakes, and the ways we can effectively diagnose the infection. Having this preliminary understanding of the disease produced by these papers is a good starting point when undertaking novel research on this disease.

Chapter Two: Prevalence of Snake Fungal Disease Caused by *Ophidiomyces Ophiodiicola* In East Texas

Introduction

Fungal diseases are crippling or eradicating whole populations of organisms around the world. Pathogens such as *Batrachochytrium dendrobatidis* (BD or Chytrid Fungus) in amphibians and *Pseudogymnoascus destructans* (pathogen in White Nose Syndrome) in bats have been shown to devastate full populations (Blehert et al., 2009; Fisher et al., 2012). White nose syndrome is a fungal disease in which hibernating bats have their nose and eyes covered in *P. destructans.* As they fight this infection it depletes their body fat stores for overwintering. This causes them to die off from hunger or exposure (Bleher et al., 2009). In frogs and salamanders *B. dendrobatidis* causes Chytridiomycosis which infects their skin and causes complications such as: tampering their ability to osmoregulation, impair feeding behaviors, alter growth and loss of righting reflex. These symptoms get worse until death of the organism which usually happens in water and can aid in spreading the pathogen (Buck et al., 2015). This pathogen is responsible for the extinction or endangered status in 6.5% of known amphibian species since it's description in the late 1990's (Olson et al., 2013, 6th extinction). In reptiles, the *Chrysoporium* anamorph of *Nannizziopis vriessii* (CANV) species complex have been the culprit in emerging diseases in many clades of reptiles (Lorch et al., 2015; Rajeev et al., 2009). The *Nannizziopis* genus has many pathogenic species, they are the pathogen in diseases that affect different species of lizards. Yellow fungal disease in lizards for example, caused by *Nannizziopsis guarroi*, is part of this clade. Yellow fungal disease clinical symptoms are very similar to *O. ophiodiicola* and it is specific to Bearded Dragon species. It was thought to be the cause of SFD initially but was eventually determined to be genetically different than *O. ophiodiicola* (Schneider et al., 2017; Rajeev et al., 2009). It was previously called *Chrysoporium ophiodiicola* until the genetic discovery by Sigler et al. in 2013.

Ophidiomyces ophiodiicola (*O. ophiodiicola*) is a fungus, that was until 2006, part of the CANV species complex before being moved to its own phylogenetic clade (Sigler et al., 2009). *Ophidiomyces ophiodiicola* is especially deadly, with an estimated 90-100% mortality rate in wild snake populations (Lorch et al., 2016). It is currently unknown if this pathogen is invasive or

native, and its origin has puzzled scientists (Thompson et al., 2015). *Ophidiomyces ophiodiicola* is believed to be keratinophilic, it attaches to the scales of snakes and breaks down the keratin for a source of energy (Lorch et al., 2016). It has been confirmed as the primary pathogen in Snake Fungal Disease (SFD) (Allender et al., 2015*b*) and has white to yellow color and displays a unique bullseye morphology (Fig. 1). Even in individuals who do not display clinical signs of the disease it can alter the normal skin microbiota of the snakes (Allender et al., 2018). After the initial attachment, *O. ophiodiicola* can spread to subdermal tissues causing the host discomfort. Subdermal infection is characterized by lesions on the head, nose, body, and/or tail caused by dysecdysis. Additionally, there may be face swelling, clouding of the eye, and fluid-filled vesicles (Allender et al., 2015*b*). Seasonality is another dimension of this disease that, if understood, can give us valuable information about the spread of the pathogen. With climate change extending the length of warmer seasons fungal spores will increases their chances of encountering snakes. It has been found that most clinical cases are found in the late winter and early spring (McKenzie et al., 2018). This is an important factor to consider as man-made climate change extends these times of the season as we look ahead to controlling the spread of this and other devastating pathogens.

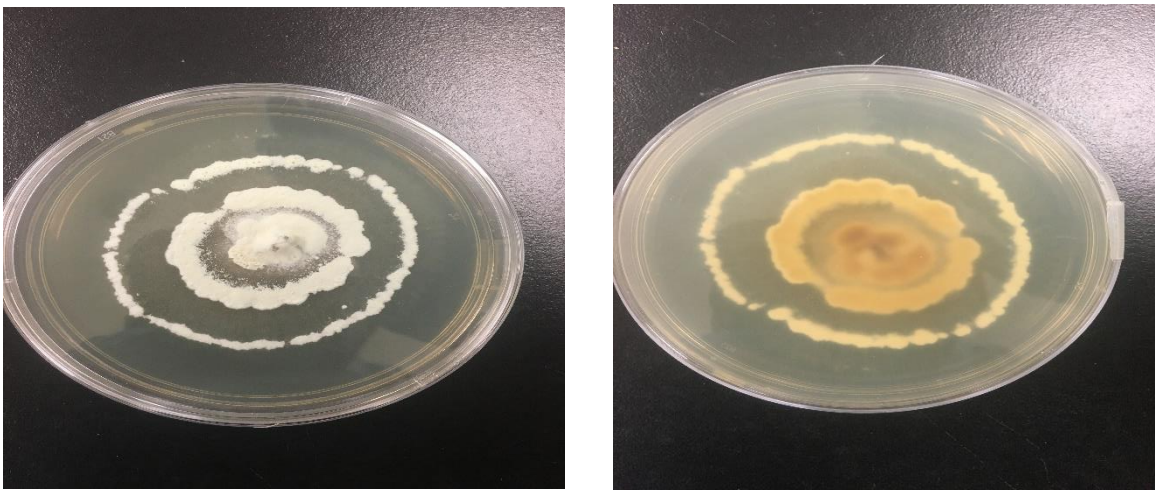

Figure 1. Top (left) and bottom (right) morphology of O. ophiodiicola grown as a control for PCR confirmations. Original specimen from a captive snake from Florida (UMAH Ontario).

Other effects noted from SFD include increased basking and decreased appetite, which are common symptoms among snakes fighting a variety of infections. While they may help them to recover, these behavior modifications make snakes easier targets for predation and they become more likely to die of malnutrition (Lorch et al., 2015). The extra shedding of the outer most layer of skin has to do with how reptiles react to skin infections. Shedding requires that they bask in the sun to raise their temperature and create the conditions to complete the shed (Stromsland and Zimmerman, 2017). This behavior puts them in the open for the ease of predators to see. Additionally, certain species hibernate or shed in communal dens which puts them at increased risk for spreading the pathogen with a 48% probability (McKenzie et al., 2020). The decrease in appetite may have been caused by the pain of opening of the mouth due to swelling and granulomas caused by infections on the head or face (Allender et al., 2015*c*). The fungus has a peak growth range of 7-35℃ in combination with being a sporophyte to make it a robust survivor (McKenzie et al., 2016). There is a conflict currently on the determination of species that are more likely to contract this pathogen. Allender et al. (2015*b*) makes the assertion that water snakes and pit vipers being more likely to get this pathogen. This conflicts with Burbink et al. (2017) using samples from across the US gave evidence that there may not be any correlation between species and pathogen.

To date there have not been any cases of SFD verifiably associated with the primary pathogen (*Ophidiomyces ophiodiicola*) in Texas. However, there have been anecdotal sightings of symptomatic individuals in East Texas and SFD found associated with *Fusarium* fungus in Southern Texas (Barber et al., 2016). The nearest researched case of SFD was in eastern Louisiana making it a priority to monitor the pathogen's spread westward (Glorioso et al., 2016). With the addition of climate change increasing the temperature of the planet, these "seasons" will become longer. Increases in temperature will extend the viability of the fungi and geographic range (McKenzie et al., 2018). The purpose of this study is to survey snakes to determine if there is a presence of the *Ophidiomyces ophiodiicola* in Eastern Texas. Then to use this data to create a monitoring program to keep a detailed record on the spread of this pathogen.

Materials and Methods

Sampling

Research was conducted in multiple areas across East Texas to include as many ecotypes from the region as possible. Traps were set out at Bloodsworth Ponds, Neches River National Wildlife Refuge (NWR), Old Sabine Bottom Wildlife Management Area (WMA), and a property belonging to the Frydenlund family. The Old Sabine Bottom WMA (Fig. 2) has a similar ecological community to the Neches River NWR with the exception that it is located along the Sabine river and has more hardwood growth. Bloodsworth Ponds is a 14.5-acre artificial wetland area created by Mike Bloodsworth and located in Chapel Hill, TX (Fig. 3). It is adjacent to Pleasant Acres Lake this site has a series of 12-14 medium sized ponds that were previously used for crayfish farming. The Neches River NWR is a U.S. Fish and Wildlife funded refuge that lies along the Neches river (Fig. 3), it is a lowland area that has many ephemeral pools. The Frydenlund land has two mostly ephemeral pools that are isolated from other properties but still was able to create habitat for tadpoles. This land is an old growth forest and looked to be a good spot to catch smaller snakes such as Western Ribbonsnakes (*Thamnophis proximus*) and Rough Earthsnakes (*Storeria dekayi*) as well as other terrestrial snakes. These were chosen for land type and additionally by the availability or accessibility of the property.

From July 2019 to September 2020 snakes were sampled using various methods including shade traps, minnow traps, road cruising, and walking encounter methods. Shade traps (coverboards) were made from plywood cut into 2’ by 3’ pieces, painted with forest green with outdoor paint to add protection, then numbered with a bright orange paint to help with visibility when sampling. These traps were placed adjacent to water where available to attract water snakes, as they are hypothesized to be disproportionately affected by the disease according to Allender et al. (2015*a*). These traps act as an artificial habitat for snakes to thermoregulate, hide, or for cover as they digest meals. Shade traps were put out during late May and early June of 2019 to allow for at least one month of habitat acclimation.

Minnow traps were placed in pools with known food sources (i.e., crayfish, small fish, tadpoles), these traps catch snakes foraging for food then because the shape of the opening coming from the inside causes the snake difficulty positioning itself to escape. They were placed with care to protect any captured animals from drowning or from heat by setting them at a depth

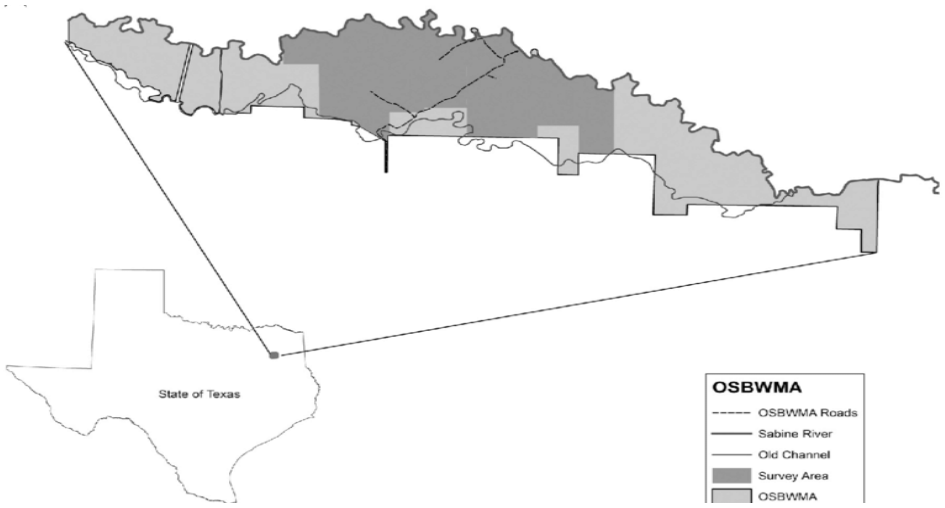

Figure 2. Map of the Old Sabine Wildlife Management Area pulled from their website where coverboards, road cruising, and minnow traps were used to create snake encounters during the project.

with plenty of room for air and in the shade. Anytime the minnow traps were set, they were checked after 2-3 days to avoid mortality. The minnow traps were used twice at the Neches River NWR and once at the Old Sabine WMA, but primarily minnow traps were left at the Bloodsworth ponds from the last week of June until the 3rd week of August 2020.

Road cruising is an additional way to encounter snakes that are trying to cross the roads or out thermoregulating. This method and the walking encounters method were used the least as they did not yield the same encounter rates as the shade and minnow traps produced. We used this method sparingly as it cost money in fuel and during this period wasn't creating many encounters.

Once a snake was encountered it was swabbed on the head and body with a sterile swab (FisherScientific, USA) that had been dipped in sterile water. We would swab over the head five times and each lateral side five times. Any lesions that were found on the snake were photographed and were individually swabbed. The swabs were most important to confirming cases, regardless of recapture. The swabs were clipped down as to fit in a 2 mL centrifuge tube and stored at -20°C until DNA extraction. To avoid inadvertent spread of pathogens Lysol wipes and 10% bleach solution were used to disinfect equipment between snakes. Additionally, clean and washed clothing was worn on each sampling trip as another safeguard to prevent the spread of the pathogen. The snakes were not clipped or implanted with marking devices. This was done

to protect the snakes from the pathogen being introduced directly to their skin. This research is to determine if the fungus has spread to east Texas and any positive samples are important, regardless of recapture.

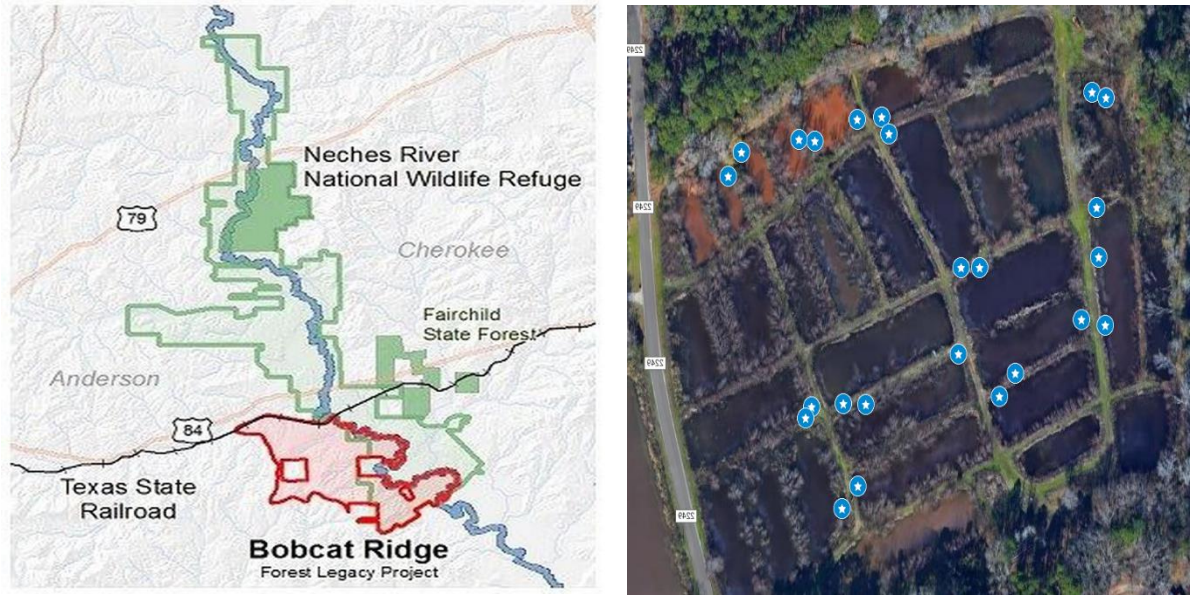

Figure 3. Map of Neches River National Wildlife Refuge (left) and Satellite map of Bloodsworth Ponds (right) with locations of minnow trap placement marked in white stars in blue circles

Sample Preparation, PCR, and Gel Electrophoresis

DNA extraction of the swabs were initiated by soaking them in lyticase (300U/mL) in microcentrifuge tubes for 1 hour to help break down the chitin walls of the fungi (Lorch et al., 2015). After the one hour they were centrifuged at 20,000 rpm for an hour to ensure all materials possible were removed from the swabs into the lyticase solution. The swabs were then removed from the remaining solution carefully and placed in another clean microcentrifuge tube and stored in a -20℃ freezer for storage. The solution in the microcentrifuge tube was then used to further extract the DNA with a Qiagen DNeasy Blood/Tissue Extraction kit using the instructions provided in the kit. The purity and quantity of DNA extracts were determined by nanodrop spectrometry (ThermoScientific, USA) and aliquots of DNA samples were stored at -80 for

further analysis. We used a SimpliAmp thermocycler (Applied Biosystems, USA) to complete polymerase chain reactions (PCR) to amplify the fungal DNA. The primers used targeted a 16S ribosomal internal transcribed spacer (ITS-2) and 23S intergenic spacer (IGS) ribosomal polymorphisms (Table 1) (Lorch et al., 2015).

Table 1. Primers and their corresponding sequences used for 18S (ITS-2) and 23S (IGS) ribosomal DNA.

Primer Name	Sequence
Oo-rt-ITS-F	5′-GAGTGTATGGGAATCTGTTTC-3′
Oo-rt-ITS-R	5′-GGTCAAACCGGAAAGAATG-3′
Oo-rt-IGS-F	5′-CGGGTGAATTACCCAGTT-3′
Oo-rt-IGS-R	5′-AGCCATCCTTCCCTACAT-3′

For PCR runs, Amplitaq Gold 360 master mix (#4398881, Thermo Fisher) was used with a SimpliAmp thermal cycler (Applied Biosystems, USA), with the following thermal profile; the starting temperature was 95℃ for 3 minutes, with denaturing temperature of 95℃ for 30 seconds, annealing temperature of 58℃ for 30 seconds for 40 cycles, an elongation cycle of 72℃ for 60 seconds, and a final cycle of 72℃ for 10 minutes. Positive control specimens purchased from The UAMH Centre For Global Microfungal Biodiversity (#10769, UAMH) and non-template controls were used as quality control throughout the runs. When PCR runs were completed, gel electrophoresis with 2-3% agarose gel (2% for IGS amplicons, 3% for ITS amplicons) was used to confirm the presence or absence of the amplicon. The voltage was set at 125V according to the distance from the positive electrode to the gel lanes then was run for 40 minutes depending on the size of the chamber used. All gels were visualized via Axygen Gel Documentation System-BL (VWR,USA) to determine confirmation.

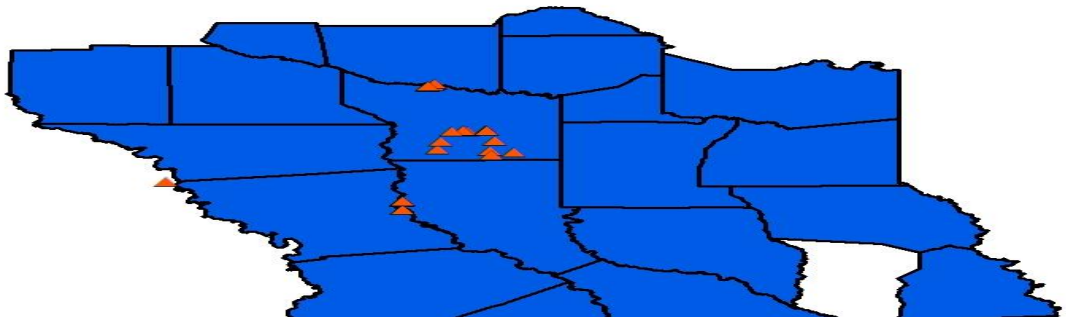

Figure 4. Map of snake encounters (orange triangles) in the East Texas region (in blue) during the research experiment.

Results

A total of 54 snakes were captured during the project (Fig. 4). The most encountered were Texas Rat snakes (*Pantherophis obsoletus,22%*) followed closely by two water snake species (*Nerodia rhombifer* 19% and *Nerodia erythrogaster*, 15%) (Table 2). Snakes from many orders were encountered during the project totaling 11 different species (Table 3). Shade traps accounted for 32% and minnow traps accounted for 20% of encounters (Table 4).

Four of the snakes sampled were found to have the pathogen in their swab, and each of these snakes were a different species Eastern Coachwhip (*Masticophis flagellum*) (Fig. 5), Eastern Copperhead (*Agkistrodon contortrix*) (Fig.6), *Pantherophis obsoletus* (Fig. 7), and Western Ribbonsnake (*Thamnophis proximus*) (Fig. 8). The swabs from the head of snakes were the only positive PCR results in the study. Additionally, only confirmation was made using the IGS primers. There were no ITS-2 amplicon positive samples that were obtained from the sampled snakes. All positive samples were replicated via PCR for confirmation of presence of the fungus (Fig. 9). None of the snakes that tested positive for the fungus showed any clinical signs or symptoms of the disease. Although there were snakes with lesions found at the same time and location as one of the positive snakes, these specimens were not PCR positive for the pathogen.

Table 2. List of species diversity throughout this project. Western Ratsnakes were the most encountered snake (12), followed closely by Diamondback Watersnakes (10).

Snake Species	Number Encountered
Texas Rat Snake	12
Diamondback Water Snake	10
Plain-bellied Water Snake	8
Dekay's Brown Snake	6
Western Ribbon Snake	5
Eastern Copperhead	4
Rough Earth Snake	2
Broad-banded Water Snake	3
Cottonmouth	1
Eastern Coachwhip	2
Rough Green Snake	1
Total	54

Figure 6. The M. flagellum that tested positive for the presence of Oo (23H) tail was mangled but tail is occluded (photo credit Neil Ford)

Figure 5. The A. contortrix (41H) which had been found to have tested positive for the fungus but no presented no clinical signs of the disease at the time of capture.

Figure 7. The P. obsoletus (45H) that was found to host the fungus but did no present any clinical signs of the disease.

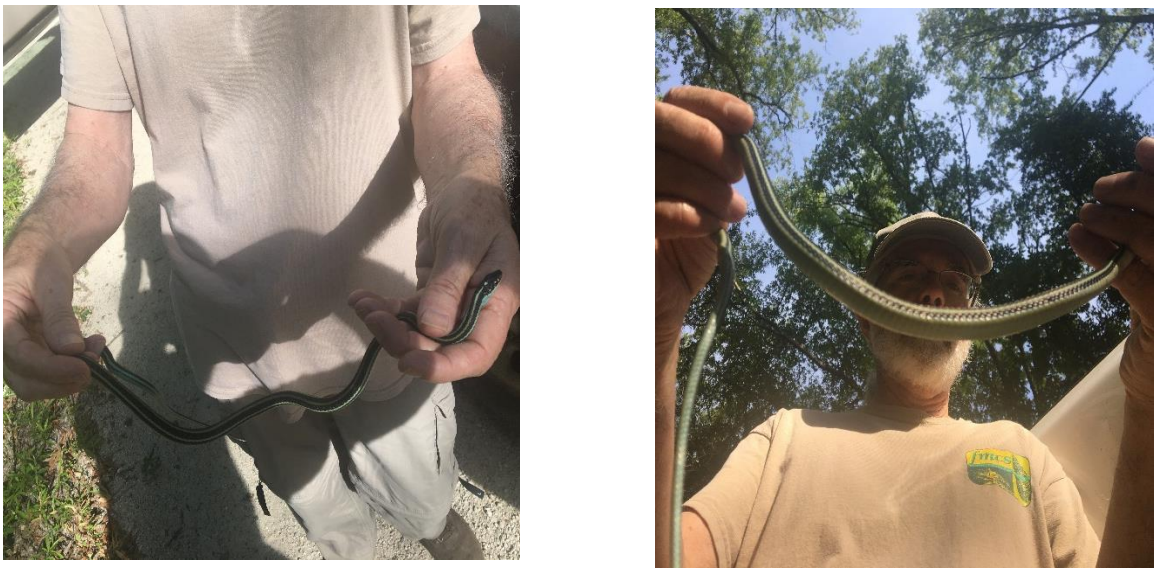

Figure 8. The T. proximus (39H) found positive for the fungus but showed no clinical signs of the disease. Pictured is both dorsal (left) and ventral (right) sides of the positive snake with no signs of disease.

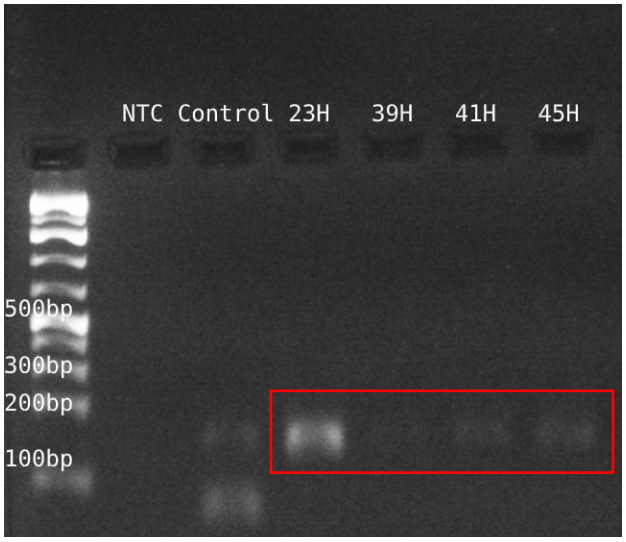

Figure 9. Gel electrophoresis confirmation of the 133bp IGS amplicons from the study. NTC.) Non-template control, Control.) Used from acquired sample from UAMH, 23H*.) Texas Ratsnake, 39H.) Copperhead, 41H.) Eastern Coachwhip, and 45H.) Western Ribbonsnake (located in boxed area). Doubling of the control's banding is hypothesized to be the result of primer dimerization or contamination. *The letter H denotes sample obtained from head of snake.

Discussion

This study found that there is evidence of *Ophidiomyces ophiodiicola* is present in East Texas. It was found in two wildlife management areas (Old Sabine Bottom WMA and the Neches River Federal Wildlife Refuge). The quantity of samples collected is comparable to studies that have established presence of the pathogen in other regions, so it is a reasonable conclusion that the pathogen is present in the East Texas region as well (Glorioso et al., 2016; Chandler et al., 2019). However, obtaining more data will allow for better forecast modelling of this pathogen. Because we have four positive samples it did not provide enough data to generate accurate models. These models will be important to study as changes in climate come changes the suitable habitat of many different organisms (McKenzie et al., 2018). Changes that extend seasons and ranges for pathogens to include fungal pathogens. Research has shown that there is a correlation between average yearly temperature, rainfall, and the growth of ranges of other fungal diseases such as but not limited to: Coccidioidomycosis, Cryptococcosis, and Histoplasmosis (Rickerts, 2019). By increasing our data, we can create models that forecast the increase in seasonality as the warming of the planet increases and likelihood which can help fight the spread by knowing where these areas of increased seasonality will be at to survey them properly. It is also important to acknowledge that this study was conducted in mostly wetland/river areas. Which makes monitoring so important as it has been found in other fungal pathogens that they can be passed through water via spores (Olsen et al., 2020). As the climate becomes warmer and wetter will only aid in this method of spread.

The study showed that the four species while not necessarily closely related do not seem to correlate to the found *O. ophiodiicola* cases (Table 3). This was to be expected as Burbink et al. found in 2017 using 100's of samples from all over the Eastern United States to show that there is no innate species that this disease disproportionally effects more. Additionally, this study warns that due to its evolutionary history that the pathogen may have other fungi in its clade which could possibly affect other reptiles. The four PCR positive samples do suggest the presence of this fungus in East Texas. The last published account of SFD caused by O. ophiodiicola in this region was in Eastern Louisiana in 2016 (Chandler et al., 2019), and data collected in this study have allowed the range to be expanded in this region to include Smith and Cherokee counties. Monitoring and tracking pathogens is necessary to establish and enact a control and mitigation

strategy. This study focused solely on wild snake populations, but one of the primary means of invasive species introduction is the pet trade (Thompson et al., 2018); poor conditions and cramped enclosures are an incubator for pathogens, especially when the animals are a newer monoculture. By monitoring positive cases both in captivity and in the wild we can gain a better understanding of both how anthropomorphic and natural based infections spread. Doing so will allow researchers time to come up with solutions to this deadly fungus and future pathogens like it.

Table 3. List of species found with positive test for *O. ophiodiicola* and the percent of snakes in that species in relation to the 54 snakes encountered.

Species Name	Positive	Negative	Total	Percent Positives
Masticophis flagellum	1	1	2	50
Agkistrodon contortrix	1	3	4	25
Thamnophis proximus	1	4	5	20
Pantherophis obsoletus	1	11	12	8.33
Nerodia rhombifer	0	10	10	0
Nerodia erythrogaster	0	8	8	0
Storeria dekayi	0	6	6	0
Haldea striatula	0	2	2	0
Nerodia fasciata	0	3	3	0
Akistriodon piscivorus	0	1	1	0
Opheodrys aestivus	0	1	1	0

The trapping methodology used in this study is relatively cost effective and does not have to be personnel intensive. Using artificial shade (coverboards) and looking under other materials near habitat that contains food were the most effective in generating encounters with snakes with approx. 41% of encounters (Table 4). The next effective was minnow traps and Low Grass/Open both with 24.07% (Table 4). The lack of encounters during cruising or pit traps may be attributed to low target mobility on hot days or bias in selection of areas as the summer and fall had decreased rainfall during this period. Lack of encounters under logs can be hypothesized to be due the lack of accessible downed trees or ones they may have been under being too large to turn over.

Table 4. Effectiveness of all methods of encounters during the research. Under cover was the highest encounter rate with an almost 41% of encounters.

Cover Type	Found	Percent
Under Cover (coverboard, tin, etc.)	22	40.74
Minnow Trap	13	24.07
Low Grass/Open	13	24.07
Road	3	5.56
High Grass	1	1.85
Log	1	1.85
Pit Trap	1	1.85

Using the primers designed by Lorch et al. (2015) for their TaqMan protocol and running them on 2-3% agarose, depending on primer amplicon provides another cost-effective sampling strategy. The lack of amplification of the ITS-2 protocol in this experiment is still informative. Having no ITS-2 amplification may be due to the IGS protocol being more effective in identifying strains associated with wild caught snakes. The ITS-2 primers are selective for the strains common to captive snakes (Lorch et al., 2015), thus possibly not at all amplifying for the wild caught control or samples. IGS confirmation is still a significant indicator for detection as it captures strains that the ITS-2 primers may not amplify.

Future Studies

Presence of the pathogen has been positivity confirmed in four specimens in two locations in East Texas. The next step in documenting this species in east Texas would be to start a monitoring program at these, and surrounding locations, to get more specific estimates about the spread of *Ophidiomyces ophiodiicola.* Yearly sampling in these areas will significantly expand understanding of the epidemic, and we can begin to assess any damage to snake populations along with severity of individual infections. Furthermore, gaining more data can aid in creating models to predict suitable habitat and potential spread based on the increase in season lengths caused by the current climate change. It will be vital to keep tabs as data has shown that eastern Texas has been becoming increasingly warmer and more wet (WorldClim, 2020). This is

explicitly important in these locations, as they are state and federally funded areas whose purpose is to monitor and create safe areas possible for the wildlife inside of their borders. A spread of the fungus has been shown to decimate populations in a few years, and to this point removing snakes with clinical signs out from the populations has been the best defense that can be posed until viable treatments are available. Like White Nose Syndrome in bats this fungus is making its way westward in the wild. For this reason, the establishment of a larger scale survey is crucial to accurately assessing the extent of infected individuals in the East Texas region; with this added data, can make informed decisions in management of the wildlife. Increased funding would allow for more widespread use of advanced molecular diagnostic methods to assess the presence more accurately and concretely in the east Texas snake populations.

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Appendix: 1

Photographs of All Snakes That Presented Lesions (none tested positive)

Appendix: 1 (continued)

Appendix: 1 (continued)