THE EFFECTS OF THE PROTIST PARASITE *DERMOMYCOIDES* SP., ON THE DUSKY GOPHER FROG (*RANA SEVOSA*) AND THE SOUTHERN LEOPARD FROG (*RANA SPHENOCEPHALA*)

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LIST OF COMMONLY USED ABBREVIATIONS

Abbreviation	Explanation
svl	snout to vent length
USM	University of Southern Mississippi
GCRL	Gulf Coast Research Laboratory
PRP	Pony Ranch Pond
PCR	polymerase chain reaction
USDA	United States Department of Agriculture
USFWS	United States Fish and Wildlife Service
MS-222	Tricaine mesylate
TNC	The Nature Conservancy
R. sevosa	*Rana sevosa* (Dusky Gopher Frog)
R. sphenocephala	*Rana sphenocephala* (Southern Leopard Frog)

ABSTRACT

THE EFFECTS OF THE PROTIST PARASITE *DERMOMYCOIDES* SP., ON THE DUSKY GOPHER FROG (*RANA SEVOSA*) AND THE SOUTHERN LEOPARD FROG (*RANA SPHENOCEPHALA*)

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This research addressed whether characteristics of translocation ponds, Gosner (developmental) stage at exposure, stress caused by density, and perceived presence of predators have an effect on the susceptibly of frog tadpoles to the protozoan parasite *Dermomycoides* sp. In 2014, Dusky Gopher Frog tadpoles were headstarted in tanks and translocated to Pony Ranch Pond (N = 1168). Additional tadpoles were marked and returned to nine tanks (N = 180) or translocated to 18 screen cages (N = 62) in Pony Ranch Pond. In 2015, five Dusky Gopher Frog tadpoles were placed into each of 12 cages in each of three ponds, or into each of 10 cages in tanks used as controls. Survival to metamorphosis in 2014 was 0.09-0.26% for free-swimming translocated tadpoles, 0.12-0.18% for three natural clutches, and 15.7% for caged tadpoles. Survival to metamorphosis in 2015 was 0.03% for 18 natural-egg clutches, and for translocated, caged larvae it was 1.67% in Pony Ranch, 11.67% in TNC1 Pond, 0% in New Pond, and 22% in the control tanks. Individuals headstarted for 2 months also survived better than individuals only headstarted for 1 month. Necropsies of caged tadpoles suggest that survival of translocated and natural larvae was significantly decreased by *Dermomycoides* sp. I examined if developmental stage affected the survival of tadpoles

exposed to *Dermomycoides* sp. Southern Leopard Frog tadpoles were exposed to the parasite at different Gosner stages (replicates = 6). Survival to metamorphosis was 63% for exposure at Gosner Stage 25, 61% for Stage 30, 71% for Stage 35, 84% for Stage 40, and 80% for the control. No significant difference among treatments was found ($p = 0.19$), but mortality was higher in tadpoles exposed early in development than that in those exposed late in development. I examined whether stress from high population density or the odor of a predator (Red-spotted Newt) affected the survival or length of larval period of tadpoles exposed to *Dermomycoides* sp. Survival to metamorphosis was 62% for high density and predator odor present, 83% for low density and predator odor present, 76% for high density and predator odor absent, and 85% for low density and no predator odor. No statistical differences among the different environmental stress levels in survival or length of larval period were found ($p > 0.32$). However, mortality from infections occurred more often at higher densities than in those groups housed at low density. *Dermomycoides* sp. plays an important role in the ecology of ephemeral ponds in southern Mississippi, and it may be a hurdle to deal with as we attempt to augment the population of the Dusky Gopher Frog.

CHAPTER ONE: UNDERSTANDING DUSKY GOPHER FROG (*RANA SEVOSA*) MORTALITY RISK FROM THE PROTIST PARASITE *DERMOMYCOIDES* SP. AT TRANSLOCATION SITES

Abstract

Translocations may help recover endangered species, but data on factors affecting success are often limited. A drift fence was used to monitor survival to metamorphosis of *Rana sevosa* larvae translocated to Pony Ranch Pond (PRP). Tadpoles (N = 1168) were translocated after headstarting them for 1 or 2 months in 1000-L outdoor tanks. Additional tadpoles were marked and returned to nine tanks (N = 180) or translocated to 18 screen cages (N = 62) in the pond. In 2015 (N = 180), tadpoles were placed into 12 cages in each of three ponds: PRP, TNC1, and New Pond, and 10 cages within tanks. Survival to metamorphosis in 2014 was 0.09-0.26% for free-swimming translocated tadpoles, 0.12-0.18% for three natural clutches, and 15.7% for caged tadpoles. Survival to metamorphosis in 2015 was 0.03% for 18 natural egg clutches and 1.67% for caged larvae in Pony Ranch. Survival to metamorphosis was highest in TNC1 Pond (11.67%) and the outdoor tanks (22%). No tadpole survived to metamorphosis in New Pond. Pulses of tadpole mortality in cages corresponded with increase in water level following rain events and increases in pH in 2015. Necropsies of caged tadpoles indicate that mortality of translocated and natural larvae was caused by *Dermomycoides* sp. Thus, when additional translocation sites are being selected, it may be important to consider the stability of the hydroperiod in the pond. My results help to explain poor translocation success for *R. sevosa* at various sites, especially for those individuals translocated as larvae.

Introduction

Global amphibian decline is thought to be one of the most pressing issues in conservation biology, for it is expected that well over a third of amphibian species will go extinct in the near future (Dur-e-Ahmad et al., 2014; Pereira et al., 2013; Lannoo, 2005). While there are numerous causes for these declines in species diversity, the invasive disease Chytridiomycosis (chytrid) has caused large population crashes of amphibians globally (Blaustein et al., 2012; Kilpatrick et al., 2010; Daszak et al., 2000; Behler, 1998; Halliday, 1998).

In recent years, diseases other than the chytrid, including Ranavirus and *Dermomycoides* sp., have caused die-offs in larval amphibian populations across most of the continental United States (Gray and Chinchar, 2015; Davis et al., 2007; Green et al., 2002). Species that have been affected by habitat loss are even more susceptible to population decreases caused by these diseases (Daszak et al., 2000). Disease management has become an increasingly important factor that wildlife managers must consider when evaluating conservation projects of amphibians globally.

The Dusky Gopher Frog (*Rana sevosa*) is an endangered species once found in ephemeral ponds and longleaf pine *(Pinus palustris)* forests between Mobile Bay, Alabama and Southeast Louisiana (Young and Crother, 2001; USFWS 2001; Richter et al., 2001). The longleaf pine ecosystem has largely disappeared from the landscape and now it occupies only 3% of its former range (Aschenbach et al., 2010). Fire suppression and commercial logging in this system has degraded the remaining land,

further reducing the amount of available habitat (Aschenbach et al., 2010). Ephemeral ponds, which are breeding sites for *R. sevosa*, have also been heavily modified or destroyed over the last century further straining the species (Stein et al., 2010). The Dusky Gopher Frog population was reduced to that located in one known pond in Mississippi, and the species was listed as endangered by U. S. Fish and Wildlife Service in 2001 (USFWS 2001). It was originally listed as a distinct population of the Carolina Gopher Frog (*Rana capito*), but the Dusky Gopher Frog was later declared a separate species based on genetic differences (Young and Crother, 2001). The protist parasite *Dermomycoides* sp. has caused large die-offs in the tadpole population of the species (Cook, 2008) and negatively impacted efforts to start new populations by headstarting and translocation of tadpoles (Tupy et al., 2015; unpublished data).

Headstarting has been used in conservation programs as a method to increase populations of a threatened species (King and Stanford, 2006). The persistence of Dusky Gopher Frogs has been bolstered by a headstarting program since the initial listing as endangered in 2001. Once the headstarted frogs reach a specific age, the animals are released at the source pond or translocated to another site.

Dermomycoides sp. is found in most ponds in North America (Green et al., 2003). *Dermomycoides* sp. is a protist parasite that infects mainly the gastrointestinal (GI) tract and the liver of a developing tadpole (Cook, 2008). Normally, an infection is non-fatal. However, if the parasite reaches high densities within the body of the hosts, it can cause the animal to die (Green et al., 2003). Mortality is often a result of the animal losing liver function (Green et al., 2003; Cook, 2008).

Based on the current literature, there should be significant differences in disease prevalence and host survival to metamorphosis among ponds (Davis et al., 2007). These differences are likely tied to a variety of environmental and ecological factors. The variable pH and water level found in each pond is thought to play a major role in the ecology of *Dermomycoides* sp. Spores were most likely to hatch when water neared a pH of 6.5; whereas, the normal pH of the main Dusky Gopher Frog breeding pond averages around pH 5 (Cook, 2008; J. Tupy, personal communication, June 4, 2015). As a pond rapidly fills from rainfall and becomes more basic, *Dermomycoides* sp. spores are more likely to hatch and colonize Dusky Gopher Frog tadpoles than under typical conditions (Cook, 2008).

Community composition may also play a role in this system, in that other species of ranid frogs are susceptible to *Dermomycoides* sp., but those species succumb to the disease at different rates (Cook, 2008). Southern Leopard Frogs (*Rana sphenocephala*) are thought to be relatively tolerant of *Dermomycoides* sp. compared to other ranid frogs surviving to metamorphosis while infected 65-85% of the time (Cook 2008; present study). Thus, Leopard Frog may act as reservoir hosts, increasing the exposure of the Dusky Gopher Frog and other ranids to Dermomycoides.

Methods

Species Collection

Small portions (less than 1/3) of Dusky Gopher Frog egg masses (N = 64 in 2014, N = 18 in 2015) were collected in February from Glen's Pond, Harrison County, MS. Dusky Gopher Frogs were hatched in the USDA Forest Service Southern Research

Station Southern Institute of Forest Genetics, Harrison Experimental Forest (Saucier, MS). The time required for the eggs to hatch, young to absorb their yolk sacks, and tadpoles to grow large enough to transport was about 2 to 3 weeks.

Dusky Gopher Frog tadpoles were transferred to 1000-L outdoor tanks located adjacent to their source at Glen's Pond because *in situ* headstarting minimizes concerns about disease introduction and acclimation to other environments. Tanks were filled using local well water at Glen's Pond, and had a screen mesh over the top of the tanks to prevent aerial predators from eating the tadpoles. These tanks were also filled with approximately 700 g of dried leaf litter and pine needles collected from upland sites, and were inoculated with plankton collected from Glen's Pond. Additional sustenance was provided by algal wafers (Hikari Algae Wafers, Hayward, CA) that were placed in the tanks once a week. Tadpoles were raised at a density of 20 per tank for either 1 or 2 months to mimic past headstarting procedures.

Field Sites

Three ponds were selected as release sites for this experiment two within the De Soto National Forest, New Pond and Pony Ranch Pond, and TNC1 Pond, located near Ocean Springs, MS, owned by The Nature Conservancy. All three ponds were sites of previous Dusky Gopher Frog translocations. Pony Ranch Pond, was used for translocations in 2014, and all three sites were used in 2015. Pony Ranch Pond was improved for *R. sevosa* by the USDA Forest Service from 2008 to 2011 by constructing a water retention berm, clearing the pond basin, and burning the basin and uplands. Pony Ranch was colonized naturally by *R. sevosa* in 2014 following this improvement. Pony Ranch Pond was selected for translocation based on the high mortality of

translocated tadpoles seen in 2013 and in the 2014 study described here (Tupy et al., 2015).

TNC1 Pond was selected for this experiment due to its distance from the main Dusky Gopher Frog population and its close proximity to the Gulf Coast Research Laboratory. Despite a large amount of translocations, biologists have seen very limited success in establishing adult populations at TNC1 pond (J. Lee, personal communication, February 20, 2015).

New Pond was selected for this experiment as an example of a constructed pond and because there were few data on the survival to metamorphosis of tadpoles in the pond. New Pond only had a small selection of emergent wetland plants and lacked the shrubs and trees found at TNC1 Pond, Glen's Pond or Pony Ranch Pond.

Pony Ranch Pond in 2014

In 2014, once the headstarted Dusky Gopher Frog tadpoles reached either the age of 1 or 2 months old, 1168 were released into Pony Ranch Pond, Harrison Co., MS. To separate translocated tadpoles from natural individuals, tadpoles were marked with elastomer dye. Additional tadpoles (N = 62) were translocated to 18 screen cages in Pony Ranch Pond or returned to 9 tanks (N = 180) to monitor survival and mark retention more closely. Five *R. sevosa* tadpoles were headstarted in outdoor tanks for 1 month and were released into one of nine 63 X 63 X 100-cm screen-mesh cages. Five 2-month headstarted tadpoles were placed in one of two 63 X 63 X 100-cm screen-mesh cages and one 2-month headstarted tadpole was released into one of seven 63 X 63 X 100-cm screen-mesh cage. Cages receiving 5 tadpoles had a 100-cm side on the

pond bottom whereas those receiving 1 tadpole had a 63-cm side on the bottom. The cages had a fine gauge mesh that prevented predators or other competing frog species from entering and allowed for monitoring the fate of each individual

The cages were placed in the pond, each at a relatively similar water level (~40cm) with about 2/3 of the cage underwater. Each cage was checked every other day to determine the fate and survival of the enclosed tadpoles. Any individual that perished was removed from the enclosure and preserved to determine if death was caused by *Dermomycoides* sp. Cause of death was determined by detecting the pathogen or observing other agents or conditions by histological preparations and by analyzing freshly collected samples using a polymerase chain reaction (PCR; Table 1). Survival to metamorphosis of natural egg masses was determined by counting emigrating juveniles intercepted by a drift fence around Pony Ranch Pond. The initial number of naturally occurring tadpoles was determined by multiplying the average number of individuals per egg mass and the number of egg masses present in the pond. The number egg masses were determined by counting the number of egg masses that appeared after a large rain event.

It should be noted that the author of this thesis was not present at the time of the 2014 cage experiment. The experiment was designed and monitored by the student's advisor, Joseph Pechmann, and Research Assistant John Tupy.

Release Sites in 2015

Once the headstarted individuals reached either the age of 1 or 2 months old, they were released into cages into one of the three ponds to monitor mortality caused

by *Dermomycoides* sp. or other factors. Cages were also placed into 10 randomly selected outdoor tanks, also termed ‘cattle tanks,’ near Glen’s Pond; the tanks had previously been used to headstart tadpoles. These cages within tanks were used to house control groups. I randomly selected 180 individuals, 90 individuals headstarted for 1 month and 90 individuals headstarted for 2 months. These tadpoles were then placed into screen cages within the ponds. I also randomly selected 30 1-month headstarted tadpoles and 20 2-month headstarted tadpoles for the screen cages within outdoor tanks. Due to large die-offs that occurred within some outdoor tanks, only 20 2-month headstarted individuals were used. The five headstarted tadpoles were placed in a 100 X 63 X 63-cm screen-mesh cage. Six Cages were placed in each pond for each headstarting duration (1 month + 2 month, replicates = 6).

Survival to metamorphosis of natural egg masses was determined by counting emigrating juveniles intercepted by a drift fence around Pony Ranch Pond. The initial number of naturally occurring tadpoles was determined by multiplying the average number of individuals per egg mass and the number of egg masses present in the pond. The number egg masses were determined by counting the number of egg masses that appeared after a large rain event

Due to the size of the outdoor tanks, only one cage could be placed in each tank. All cages in the ponds and tanks had 300 g of leaf litter substratum added on the same day as the tadpoles. This leaf litter mixture consisted of dried pine needles and leaves from hardwood trees.

Each enclosure was checked every other day to determine the fate and survival of tadpoles. Any individual that perished was removed from the enclosure and

preserved to determine if death was caused by *Dermomycoides* sp. If individuals were thought to have been predated or escaped from the cage, they were not included in the final analysis.

While monitoring the tadpoles in each pond, I recorded environmental factors, including depth, pH, number of *R. sphenocephala* egg masses, and seasonal species composition of amphibian species within the pond.

Histology and PCR analysis

Necropsies were used to determine if *Dermomycoides* sp. had caused mortality. The liver was removed from available specimens. The liver tissue was ground up and suspended in amphibian saline solution (0.65 g NaCl/100 mL H_20) through vortexing, then 1 mL of tissue solution was removed and placed into a hemocytometer. The number of zoospores present per mL and general condition of the liver were used to make the final determination.

In 2014, PCR was used to determine if *Dermomycoides* sp. DNA was present in the liver of the specimens. All recoverable livers were removed from the specimens that had been preserved. Available DNA was extracted from the specimens using a DNeasy Kit (Qiagen Inc., Hilden, Germany), and then stored in -80°C freezers until used. Prior to PCR, each sample underwent spectrophotometric analysis (NanoDrop ND-1000) to determine the amount of DNA present per µL

I used 1 µL of DNA, 1 µL each of primers NS1 and NS8 and 22 µL of DNA free water with Illustra Puretaq ready-to-go beads (Promega, Madison, WI). The DNA then underwent 30 cycles of PCR using the primers (Table 1). The primers amplify the 18S

nu-SSU region, and the beads contain all needed components for PCR, excluding primers and initial DNA. Following PCR, each reaction sample underwent spectrophotometric analysis (NanoDrop ND-1000) to determine the amount of DNA present per µL in the amplified reaction. The difference between the two NanoDrop analyses and the control tubes was then used to determine if DNA was produced using PCR. I used agarose gel electrophoresis on a select number of samples to confirm presence/absence of the amplified 18S region.

Data Analysis

I compared survival to metamorphosis for Dusky Gopher Frog tadpoles among ponds (2015 only) and between tadpoles headstarted 1 or 2 months using a Chi-Square test in Program R 2.15 (R Core Team, 2012). A Chi-Square test was used to determine if the ponds differed in the number of *R. sphenocephala* egg masses present within each pond. The natural survival was calculated based on the average number of individuals per egg mass found by Richter et al. (2003). Retention of elastomer dye marks through metamorphosis was variable and low (average 39%) in tanks and cages, where retention could be observed. Unmarked juveniles caught at the drift fence could therefore have come from either natural eggs or translocated tadpoles. We calculated ranges for survival to metamorphosis for these groups assuming that either all or none of the unmarked juveniles came from natural eggs Values for environmental factors were compared with the percent mortality for a particular pond to determine if the factors might play a role in mortality in Dusky Gopher Frogs exposed to *Dermomycoides* sp.

Results

Pony Ranch Pond in 2014

Mark retention in Dusky Gopher Frog tadpoles through metamorphosis averaged 29% in tanks marked at 1 month, 24% in tanks marked at 2 months, 64% in cages in Pony Ranch Pond, and 39% overall. Survival to metamorphosis in 2014 was 0.09-0.26% for free-swimming translocated larvae, 0.12-0.18% for three natural egg clutches laid in Pony Ranch Pond depending on whether unmarked metamorphs were considered to have lost their marks or come from natural eggs, and 15.7% for caged larvae (Figure 1). Survival differed depending on the amount of time headstarted with those individuals headstarted for 2 months surviving to metamorphosis (35%) more than those individuals headstarted for 1 month (3%; $p < 0.02$) Survival to metamorphosis was not limited by pond drying, because Glen's Pond did not dry until September, after emigration of juveniles had ended. My necropsies and PCR analyses found that most mortality in the cages was caused by *Dermomycoides* sp.

Release Sites in 2015

Survival to metamorphosis in 2015 was 0.03% for 18 natural egg clutches laid in Pony Ranch Pond and 1.67% for caged larvae in Pony Ranch (Figure 1). Survival to metamorphosis in TNC1 Pond was 11.67% and in the outdoor tanks 22%, but no tadpole survived to metamorphosis in New Pond (Figure 1). Survival differed significantly for both ponds ($P < 0.008$), and whether individuals where headstarted 1 or 2 months ($P = 0.02$). Of those tadpoles that did metamorphose, the individual from Pony

Ranch was smaller in regards to mass and svl (Table 2). Metamorphs did not differ in size in mass or svl when comparing TNC1 Pond and the outdoor tanks.

Most pulses of tadpole mortality in cages corresponded with increases in water level and pH in 2015 (Figure 2). This phenomenon was especially prevalent at New Pond, which experiences greater fluctuations in water level than the other ponds, Pony Ranch had more Southern Leopard Frogs (*Rana sphenocephala*) egg masses ($p < 0.02$), and amphibian species present than either TNC1 Pond and New Pond (Table 3, Table 4). The low survival to metamorphosis in ponds was not due to ponds drying early in 2015. My necropsies, conducted at the USM-Gulf Coast Research Laboratory, found that most mortalities in the cages were caused by *Dermomycoides* sp.

Discussion

In 2014, the necropsies of caged tadpoles suggested that survival of translocated and natural larvae was greatly decreased by *Dermomycoides* sp. This parasite is known to cause mass mortality events in ranid frogs, but little information had been known about its chronic effects on survival (Green et al., 2002; Davis et al., 2007; Cook 2008). The survival to metamorphosis for wild and translocated tadpoles (outside of cages) was much lower than for wild tadpoles at nearby Glen's Pond. Only those individuals that were in cages came near the wild survival at Glen's. This was the impetus for the work that was conducted in 2015.

The survival to metamorphosis in 2015 of caged tadpoles and in wild tadpoles found in Pony Ranch was even lower than the previous year. The necropsies of caged tadpoles suggested that survival of translocated and natural larvae was also greatly

decreased by *Dermomycoides* sp. in 2015. These fatalities were more likely to occur just after a large increase in pond levels and/or changes in pH. This relationship is supported by the work by Cook (2008) which found that zoospores hatched best at a pH of 6.5. Thus, when additional translocation sites are being selected, it may be important to consider the stability of the hydroperiod in the pond.

Pony Ranch Pond did not see as much variation in water level as New Pond. The much larger population of Southern Leopard Frogs at Pony Ranch Pond, a species known to be able to tolerate fairly high levels on infection by *Dermomycoides* sp., may be another factor that is increasing the mortality related to this disease in Pony Ranch. In lab conditions, Southern Leopard Frogs survived *Dermomycoides* sp. infections 60-85% of the time depending on the Gosner stage; whereas this study found that Dusky Gopher Frogs are extremely vulnerable to the disease. This information indicates that Southern Leopard Frogs could be considered reservoir hosts for *Dermomycoides* sp. and Southern Leopard Frogs numbers may need to be reduced at translocation sites for Dusky Gopher Frogs. My results help to indicate why poor translocation success for *R. sevosa*, especially for those individuals translocated as larvae, has been observed at both TNC1 and Pony Ranch Pond. My results underscore that cryptic diseases like *Dermomycoides* sp. should not be underestimated.

Understanding the disease ecology of an organism is important to ensure proper management for imperiled species. The lessons learned in this series of experiments could also help inform individuals involved in translocations in other disease-threatened species systems such as the Wyoming Toad (*Bufo baxteri*) or Mountain Yellow-legged Frog (*Rana muscosa*) (Murphy et al., 2009; Fellers et al., 2007). Both species have

extensive translocation programs that have been hampered by Bd (*Batrachochytrium dendrobatidis*), a species of single-celled fungi that is thought to have caused massive anuran die-offs (Blaustein et al., 2012; Kilpatrick et al., 2010; Daszak et al., 2000; Behler, 1998; Halliday, 1998). Similar experiments to this one could be used to determine what conditions may increase the likelihood of Bd related mortality in these species, and potentially help create models of what areas should be avoided in future translocations.

The results of this experiment show the importance of monitoring ongoing translocations (King and Stanford 2006). Monitoring of the translocation success of *R. sevosa* was crucial in detecting the high mortality caused by *Dermomycoides* sp.

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Table 1. Primers used to amplify 18S DNA from *Dermomycoides* sp. using PCR.

Name	Nucleotides
NS1	GTAGTCATATGCTTGTCTC
NS8	TCCGCAGGTTCACCTACGGA

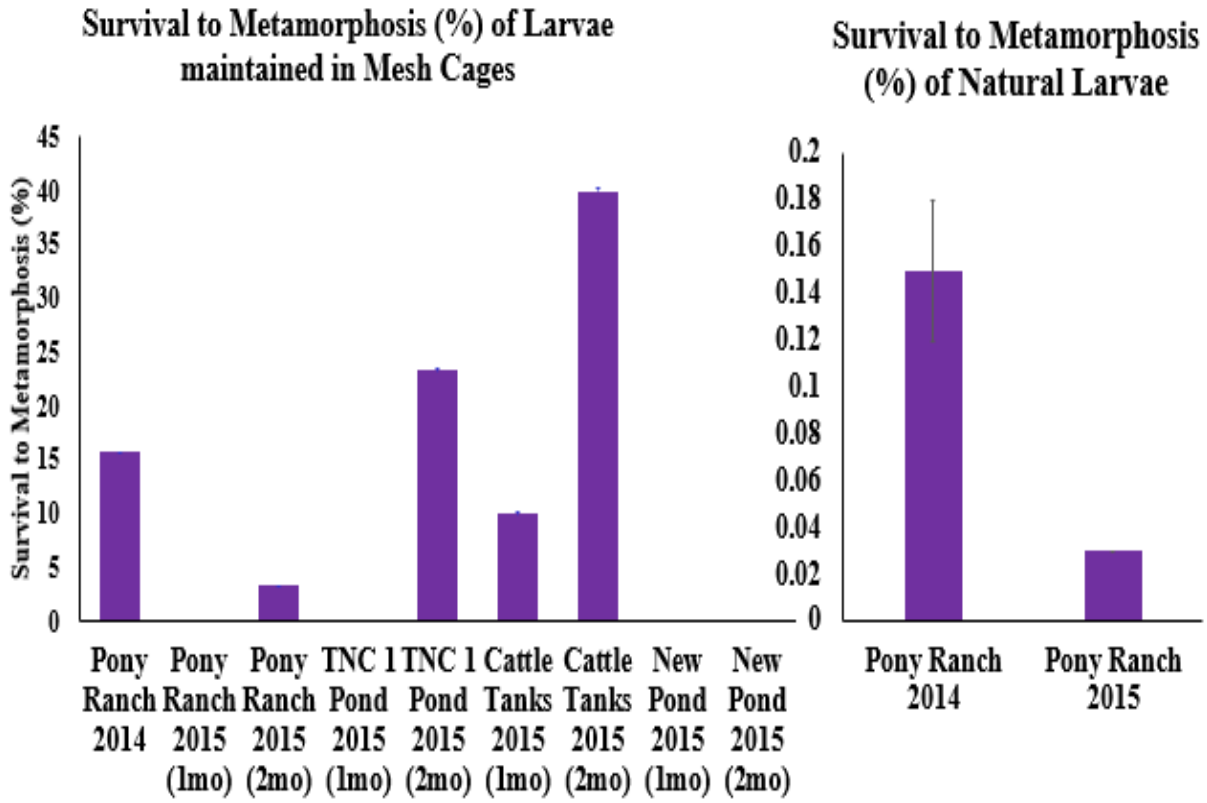

Figure 1. Percent survival of larvae translocated to cages in ponds and of free-swimming larvae of the Dusky Gopher Frog. The number of free-swimming tadpoles was calculated by counting the number of emigrating individuals using a drift fence. Percent survival in cages was monitored across three ponds and a set of cages within outdoor tanks. Percent survival in 2015 differed depending on how long the tadpoles were headstarted (1 month or 2 months), and what pond the cages were placed (replicates = 6). Individuals headstarted for 1 month are indicated as 1mo, and individuals headstarted for 2 months as indicated as 2mo.

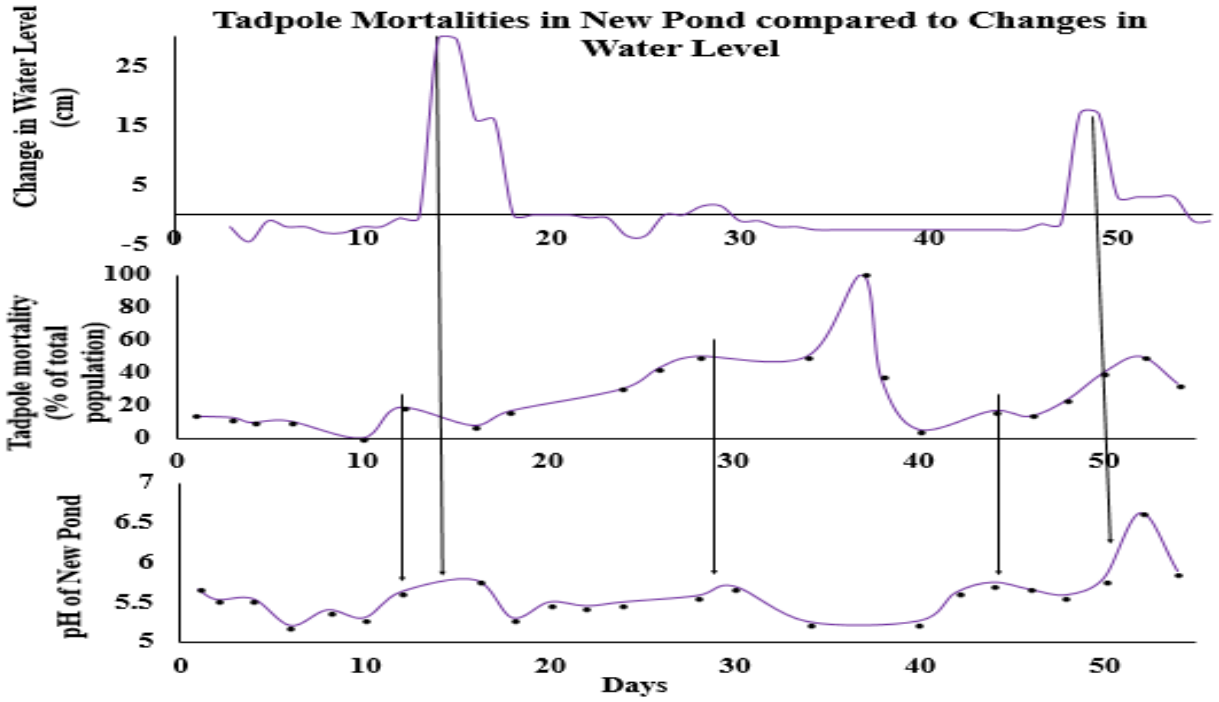

Figure 2. Changes in daily water level and pH compared with tadpole mortality (% of the remaining population) in New Pond. The tadpoles headstarted 1 month were added on day 1 and the tadpoles headstarted 2 months were added on day 37 This figure shows that as the water level and pH rise, the percent mortality increases. These moments are indicated by arrows.

Table 2. Number of tadpoles that survived to metamorphosis from cages in each pond, and the mean mass (g) and svl (mm) of those individuals that survived to metamorphosis.

Pond	Number or Metamorphs	Mean svl (mm)	Mean Mass (g)
Outdoor Tanks	12	27.88	1.87
TNC1 Pond	7	25.16	1.80
Pony Ranch	1	24.00	1.43
New Pond	0	n/a	n/a

Table 3. Presence/absence of various amphibian species associated with the Dusky Gopher Frog translocation ponds in Southern Mississippi in 2015. The presence of a species is indicated by a Y, and the absence is indicated by an N.

Species	Pony Ranch	New Pond	TNC1 Pond	Outdoor Tanks
Rana clamitans	Y	N	N	N
*Rana sphenocephala**	Y	Y	Y	N
Rana catesbiana	Y	N	Y	N
Rana sevosa	Y	Y	Y	Y
Hyla gratiosa	Y	Y	Y	Y
Hyla femoralis	Y	N	N	Y
Hyla cinerea	Y	N	N	Y
Pseudacris nigrita	Y	N	Y	N
Acris gryllus	Y	Y	Y	Y
Bufo terrestris	Y	N	Y	N
Gastrophryne carolinensis	Y	N	N	N

Table 4. The number of *Rana sphenocephala* egg masses found in three ponds and the outdoor tanks in Jackson and Harrison Co., Mississippi.

Pony Ranch Pond	New Pond	TNC1 Pond	Outdoor Tanks
>80	11	8	0

CHAPTER TWO: GOSNER STAGE MAY AFFECT SURVIVAL OF SOUTHERN LEOPARD FROGS (*RANA SPHENOCEPHALA*) EXPOSED TO THE PROTIST PARASITE *DERMOMYCOIDES* SP.

Abstract

The developmental stage of a host organism may influence how a disease affects the host. I examined the influence of Gosner stage at exposure to *Dermomycoides* sp. on mortality and length of larval period in Southern Leopard Frog tadpoles (*Rana sphenocephala*). Gravid frogs were allowed to breed within outdoor tanks to avoid unintentional exposure to *Dermomycoides* sp. The resulting eggs were reared in the laboratory until they hatched, and five tadpoles were then placed into each of 30 aquaria. These aquaria were divided into five treatment groups, with six replicate aquaria per treatment: exposures conducted at Gosner stages 25, 30, 35, and 40, and a control group that was never exposed. Survival to metamorphosis was 63% for exposure at Gosner Stage 25, 61% for Stage 30, 71% for Stage 35, 84% for Stage 40, and 80% for the control. Mortality in non-control treatments was a direct result of *Dermomycoides* sp. infection. A non-parametric Kaplan-Meir model showed no significant difference among treatments and the control in either time or survival to metamorphosis ($p = 0.19$). However, there was higher mortality of tadpoles exposed early in development than in those exposed late in development. This trend suggests that release of tadpoles headstarted for longer periods of time may help avoid mortality associated with *Dermomycoides* sp.

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Introduction

Numerous factors play a role in how a parasite may impact an individual, including host age, developmental stage, and body size (Schotthoefer et al., 2003; Webber et al., 2015; Peterson et al., 2015). Younger, less developed individuals are often more vulnerable to disease than more developed ones. This correlation exists in amphibian diseases such as Chytridiomycosis and Ranavirus (Bakar et al., 2016; Warne et al., 2011).

Frogs undergo large morphological changes in a short period of time as the larvae grow and eventually metamorphose (Altig, 1970). These changes can be classified into developmental stages known as Gosner stages (Gosner, 1960). Some Gosner stages may be more susceptible than others to pathogens such as the protozoan *Dermomycoides* sp. (Cook, 2008). Stress during critical Gosner stages can radically change the physiology and overall welfare of adult frogs (Crespi and Warne, 2013; Warne et al., 2011).

Dusky Gopher Frogs tadpoles are very susceptible to *Dermomycoides* sp., but adults appear to be not as susceptible (Cook, 2008; unpublished data). This relationship suggests that life stage, specifically Gosner stage, may affect the susceptibility of larval Dusky Gopher Frogs to the disease. This kind of relationship has been seen in other species of anurans. Gosner stage has been shown to impact the ability of a trematode parasite to affect the tadpole (Schotthoefer et al., 2003). This information can be used to create models to inform wildlife managers on how to avoid disease related mortality (Mitchell et al., 2013; Briggs et al., 2005).

I examined the susceptibility of Southern Leopard Frog tadpoles (*Rana sphenocephala*). tadpoles of different Gosner stage to *Dermomycoides* sp. infection. Based on current literature, the Gosner stages that involve the most physical changes in a short period of time, such as those that occur around the time of limb development, should be more susceptible to infections of *Dermomycoides* sp. (Warne et al., 2011; Cook, 2008; unpublished data). Any individual that survives an infection of *Dermomycoides* sp. will be more likely to have a greater time to metamorphosis than an individual that was not exposed to the disease, and would more than likely be smaller (in mass) upon the completion of metamorphosis.

Methods

Field Site and Species Collection

Southern Leopard Frog adults (four males and four females) were caught in March of 2016 by a drift fence at Glen's Pond in the De Soto National Forest near Saucier MS. These adult frogs were allowed to lay their eggs within outdoor tanks. From these adults, two egg masses were produced and collected. The egg masses were hatched in filtered well water in a laboratory. Each clutch was placed in its own plastic container. The eggs hatched and tadpoles absorbed their yolk sacks in 2 to 3 weeks. The tadpoles were then moved into two separate rooms at the Aquatic Disease Lab, a part of the University of Southern Mississippi - Gulf Coast Research Laboratory, Ocean Springs, MS.

Infection at Different Gosner Stages

Hatchling Southern Leopard Frog tadpoles were divided into 30 groups of five individuals and then assigned to treatments and spatial blocks using a stratified random design, so that each clutch was as equally represented as possible in each aquaria. Each group of five tadpoles was then placed in a 9.5-L glass aquarium filled with 3.14 L of aged tap water. All aquaria were placed in a tabletop water bath in one of the two rooms of the Aquatic Disease Lab at the GCRL in a randomized block design. These water baths were kept at approximately 25±0.5°C using bath heaters with thermostats. Tadpoles were fed algal wafers (Hikari Algae Wafers, Hayward, CA) every 2 days and a half-water change was performed after feeding.

There were four groups, each inoculated with *Dermomycoides* sp. at different times during their development, plus an unexposed control. The method for this experimental exposure was based on Cook (2008). Infected Southern Leopard Frog tadpoles were collected from Pony Ranch Pond and New Pond. Southern Leopard Frog tadpoles are found in high numbers in these ponds, and both ponds were known to have the disease (J. Tupy, personal communication, June 4, 2015). These collected tadpoles were then euthanized using MS-222, and their livers and intestines were harvested. These organs were mechanically homogenized and then suspended in 25 mL of amphibian saline solution (0.65 g NaCl/100 mL H_20). This solution was then allowed to sit in a refrigerator for a week.

Once a week had passed, the liquid part of the solution was collected and pipetted into a separate 50-mL vial. This process continued until I had obtained three, 50-mL vials. These vials were combined and concentrated to be used as the stock

solution for experimental infections. The solution of *Dermomycoides* sp. was concentrated by placing it on a 0.2-µm filter and using a vacuum pump. A small amount of amphibian saline solution was added to this filter, and the number of zoospores were counted. More zoospores or amphibian saline solution were added until the desired concentration was reached.

Once the tadpoles reached the required stage for infection, 5 mL of stock solution of *Dermomycoides* sp. was placed on 0.2-µm filter and separated using a vacuum pump, discarding the fluid. The filter containing the spores was allowed to sit for an hour in a vacuum-sealed desiccation chamber. The filter paper containing the desiccated spores was placed in a 50-mL Carolina culture bowl filled with 25 mL of distilled water adjusted to a pH level of 6.5. The spores were allowed approximately 15 minutes to rehydrate and hatch. Spores were counted to ensure they had reached a density of 100 zoospores/µL. The number of zoospores were counted by placing 1 mL of the solution into a hemocytometer. Once the solution was ready, the tadpoles to be exposed were then placed into the bowls (one aquarium of tadpoles per bowl) and allowed to sit for 1 hour. The tadpoles were rinsed in three baths of distilled water (also adjusted to a pH of 6.5) and then returned to their respective aquaria.

The first group of tadpoles was inoculated at Gosner Stage 25; just after they had absorbed their yolk. The second group was inoculated around Gosner Stage 30, the beginning of limb development. The third group was inoculated during Gosner Stage 35; the completion of hind limb development. The fourth group was inoculated just prior to metamorphosis at Gosner Stage 40. The final group was the control group and was not inoculated.

The fate of each individual was monitored until metamorphosis. Any tadpoles which lost equilibrium and were swimming erratically were euthanized using MS-222 to minimize pain and stress, under the assumption that they were are about to die from an infection of *Dermomycoides* sp. These and any individuals that died were preserved to determine if death was caused by *Dermomycoides* sp. This determination was made by analyzing samples using PCR.

The amount of time it took for an individual to metamorphose, length and mass at metamorphosis, and stage-specific survival was also monitored and compared to the controls. Once the metamorphosed frogs had absorbed their tails, they were euthanized with an overdose MS-222 to determine the level of infection found in the bodies of the frogs. These frogs cannot could not be released into the wild due to their exposure to *Dermomycoides* sp.

Determining the Presence of *Dermomycoides* sp.

PCR was used to determine if *Dermomycoides* sp. DNA was present in the liver of the specimens. All recoverable livers were removed from the specimens that had been preserved. Available DNA was extracted from the specimens using a DNeasy Kit (Qiagen Inc., Hilden, Germany), and then stored in -80°C freezers until used. Prior to PCR, each sample underwent spectrophotometric analysis (NanoDrop ND-1000) to determine the amount of DNA present per µL

I used 1 µL of DNA, 1 µL each of primers NS1 and NS8 and 22 µL of DNA free water with Illustra Puretaq ready-to-go beads (Promega, Madison, WI). The DNA then underwent 30 cycles of PCR using the primers (Table 1). The primers amplify the 18S

nu-SSU region, and the beads contain all needed components for PCR, excluding primers and initial DNA. Following PCR, each reaction sample underwent spectrophotometric analysis (NanoDrop ND-1000) to determine the amount of DNA present per µl in the amplified reaction. The difference between the two NanoDrop analyses and the control tubes was then used to determine if DNA was produced using PCR. I used agarose gel electrophoresis on a select number of samples to confirm presence/absence of the amplified 18S region.

Data Analysis

To determine how survival and time to metamorphosis was affected by Gosner stage, I used a non-parametric Kaplan-Meier estimate of survival to compare exposure at a specific Gosner stage against time to metamorphosis, and I also used the Chi-Square test to compare exposure at a specific Gosner stage against survival to metamorphosis. I used an ANOVA to compare exposure at a specific Gosner stage against mass at metamorphosis, and length at metamorphosis. The Kaplan-Meier analysis, ANOVA and Chi-Square test were conducted in program R utilizing the survival package (R Core Team, 2012). A parameter was included in this test to account for the blocking design used in this experiment.

Results

All aquaria that contained inoculated tadpoles, were shown to have at least one tadpole with *Dermomycoides sp.* present. Survival to metamorphosis was 63% for exposure to *Dermomycoides* sp. at Gosner Stage 25, 61% for Stage 30, 71% for Stage 35, 84% for Stage 40, and 80% for the control (Figure 3). Using a Chi-Square test, I

found no statistical difference due to Gosner stage at infection for survival to metamorphosis ($p = 0.19$). Using a non-parametric Kaplan-Meier estimate of survival, I found no statistical difference due to Gosner stage at infection for time to metamorphosis ($p > 0.5$). Treatment groups averaged between 57-63 days to metamorphosis (Table 5). Treatments differed somewhat in the average mass (g) per recovered tadpole; the control tanks averaged 1.58 g, the tadpoles exposed at Gosner Stage 25 averaged 1.21 g, the tadpoles exposed at Gosner Stage 30 averaged 1.34 g, the tadpoles exposed at Gosner Stage 35 averaged 1.13 g, and the tadpoles exposed at Gosner Stage 40 averaged 1.40 g. However, these averages were found to not differ significantly between the different treatments.

The average snout to vent length for the tadpoles in all treatments fell between 2.4-2.7 cm (Table 5), averages which were also not significantly different. The treatments differed somewhat in the average liver mass per recovered specimen; the control tadpole's liver mass averaged 0.019 g, the tadpole's liver mass averaged 0.009 g when exposed at Gosner Stage 25, the tadpole's liver mass averaged 0.032 g when exposed at Gosner Stage 30, the tadpole's liver mass averaged 0.025 g when exposed at Gosner Stage 35, and the tadpole's liver mass averaged 0.023 g when exposed at Gosner Stage 40. However, these averages were found to not differ significantly among treatments.

Discussion

The Gosner stage of a tadpole when exposed to *Dermomycoides* sp. does seem to play a role in how the organism is able to cope with the disease. While I did not find a statistically significant difference among treatments in survival to metamorphosis, there was higher mortality from infections at younger Gosner stages than for those individuals that were exposed later in development. This information has been further confirmed in other studies of Dusky Gopher Frogs. Individuals that had been headstarted for 2 months survived to metamorphosis significantly more often than those individual tadpoles that had been headstarted only 1 month when exposed to naturally occurring *Dermomycoides* sp. (Chapter 1). This indicates that it may be more beneficial to release tadpoles that have been headstarted for a longer period of time to decrease the mortality associated with *Dermomycoides* sp. Given the survival to metamorphosis of individuals exposed at Gosner Stage 40, releasing individuals at or just prior to metamorphosis would probably be the most efficient way to bolster populations through headstarting in regards to avoiding mortality caused by *Dermomycoides* sp.

While not significant, it should be noted that the mass of recovered individuals exposed to Dermomycoides sp. was lower than that of the controls. A large difference exists between the controls and exposure at Gosner Stages 25 and 35. This relationship may be related to the stress that those particular stages place on the body of the tadpole (Holland et al, 2007). The lack of significant differences among treatments in snout to vent length (svl) was unsurprising given that svl does not change substantially in other circumstances. For example, svl does not significantly differ between individual Dusky Gopher Frogs that are raised in outdoor tanks instead of

ponds, but the mass of individuals raised in the wild is often much higher than that of tank raised tadpoles (Tupy, 2015). The lack of a significant difference among treatments regarding time to metamorphosis was somewhat surprising in that stressed or diseased individuals often are not able to allocate as much resources toward growth (Holland et al., 2007; Crespi and Warne, 2013).

This study shows the need to investigate all life stages of anurans when considering disease management, and it shows that particular emphasis should be placed on furthering the information on larval forms. A large number of changes that occur throughout the development of a tadpole have been shown to play a major role in how an individual is able to respond to infection (Crespi and Warne, 2013; Warne et al., 2011). This research, while statistically inconclusive, follows the finding of other studies that younger larval stages may be more susceptible to pathogens (Crespi and Warne, 2013; Warne et al., 2011). This correlation shows the possible importance of headstarting to counteract diseases. This notion differs from the standard reasons behind headstarting which typically focus on body size or condition to increase success in translocations (King and Stanford, 2006). In this case, this research implies the importance of raising headstarted individuals for as long as possible in order to successfully prevent mortality from disease. This supports the change in 2015 to releasing metamorphed headstarted dusky gopher frogs rather than tadpoles, to better increase the likelihood that populations avoid mortality caused by *Dermomycoides* sp. (J. Tupy, personal communication, June 4, 2015).

Understanding how a disease interacts with its host during different periods of development is crucial needed information when managing disease-imperiled systems.

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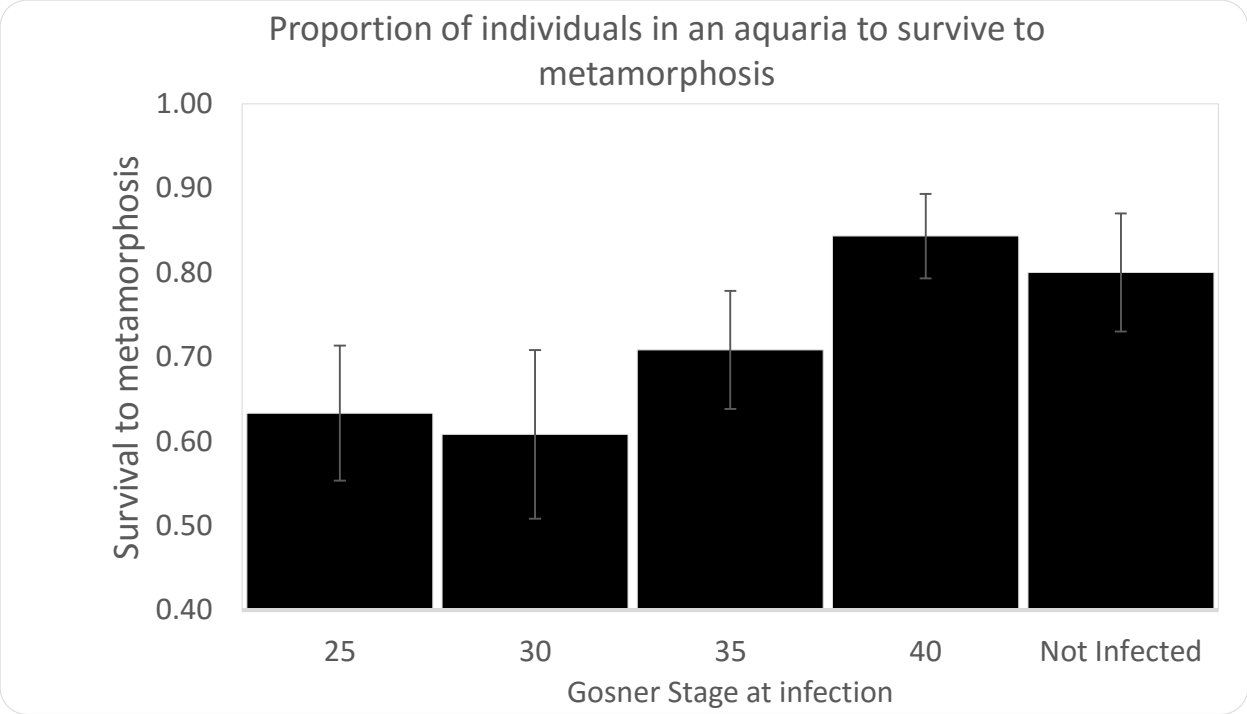

Figure 3. Proportion of individuals in a tank to survive to metamorphosis based on the Gosner stage at time of infection (replicates = 6). Error bars are 1 standard error.

Table 5. The average total mass, liver mass, day of metamorphosis, and snout to vent length of recoverable specimens based on the developmental period when the individuals were exposed to *Dermomycoides* sp.

Treatment	Mean Mass (g)	Mean Liver Mass (g)	Mean svl (cm)	Mean Day of Metamorphosis
Not Infected	1.58	0.019	2.7	60
25	1.21	0.009	2.4	57
30	1.34	0.032	2.5	62
35	1.13	0.025	2.6	63
40	1.43	0.023	2.5	60

CHAPTER THREE: DOES STRESS AFFECT SURVIVAL OF SOUTHERN LEOPARD FROGS (*RANA SPHENOCEPHALA*) EXPOSED TO THE PROTIST PARASITE *DERMOMYCOIDES* SP.

Abstract

Stress is thought to impair immune response. Decreased immune response decreases the ability of an individual to fight diseases. To determine the influence of stress on mortality and length of larval period in Southern Leopard Frog tadpoles (*Rana sphenocephala*) exposed to *Dermomycoides* sp., tadpoles were exposed to the parasite under different levels of environmental stress. Gravid frogs were allowed to breed within outdoor tanks. The resulting eggs were reared in the laboratory until they hatched, and 10 or 20 tadpoles were placed into 24 aquaria. These aquaria were divided into four treatment groups: high population density and predators present (20 tadpoles per 18.9-L glass aquaria), low population density with predators present (10 tadpoles per 18.9-L glass aquaria), high population density with predators absent, and low population density with predators absent, with six replicate aquaria per treatment. Survival to metamorphosis was 62% for high population density and predators present, 83% for low population density and predators present, 76% for high population density and predators absent, and 85% for low population density and no predator. Using a non-parametric Kaplan-Meier model, I found no statistical difference among the different environmental stress levels in survival to metamorphosis ($p = 0.32$). However, mortality was higher in populations with more individuals than populations with fewer individuals. This indicates that it may more beneficial to keep stocking levels low within headstarting tanks, to reduce the mortality associated with *Dermomycoides* sp.

Introduction

Disease management has become an increasingly important factor that wildlife managers must consider when evaluating conservation projects of amphibians globally. Headstarting has been used in countless different conservation programs as a method to increase populations of threatened species of amphibians as well as other animals (King and Stanford, 2006). The persistence of Dusky Gopher Frogs, *Rana sevosa*, has been bolstered by a headstarting program since the initial status listing in 2001. Once the headstarted frogs reach a specific age, the animals are translocated to ponds in the De Soto National Forest.

Translocation of an individual from one location to another is thought to increase its amount of stress. This has been confirmed in numerous taxa including: translocated desert tortoises and Eastern Grey Squirrels (Drake et al., 2012; Bosson et al., 2013). Stress can affect the immune response in some species, such as side-blotched lizards (Lucas and French, 2012). Reeve et al., (2013) found that numerous causes of stress could lead to increased susceptibly of Wood Frogs (*Rana sylvatica*) to Ranavirus. Stress decreases the ability of an individual to properly fight diseases, potentially causing the individual to be more susceptible to diseases after translocation.

I examined the influence of environmental stress, in the form of population density and predator presence, on mortality and length of larval period in Southern Leopard Frog tadpoles (*Rana sphenocephala*) exposed to the protozoan parasite *Dermomycoides* sp.

Based on current literature, I hypothesized that individuals under stressful environmental conditions would be more likely than control individuals to perish following an exposure to *Dermomycoides* sp. Also, I predicted that any individual that survived an infection of *Dermomycoides* sp. would be more likely to need a greater time to metamorphose than an individual that was not exposed to the disease, and would more likely be smaller (in mass) upon the completion of metamorphosis.

Methods

Field Site and Species Collection

Southern Leopard Frog adults (four males and four females) were caught while monitoring a drift fence at Glen's Pond on the De Soto National Forest near Saucier, MS. These adult frogs were allowed to lay their eggs within an outdoor watering tank. From these adults, two egg masses were produced and collected. The egg masses were hatched in filtered well water in a laboratory. These eggs were allowed to hatch, and the tadpoles were allowed to absorb their yolks prior to moving these individuals. The Southern Leopard Frog tadpoles were moved into rooms at the Aquatic Disease Lab, a part of the University of Southern Mississippi Gulf Coast Research Laboratory (USM-GCRL), in Ocean Springs, MS.

The Impact of Environmentally Caused Stress

Hatchling Southern Leopard Frog tadpoles were selected using a stratified random design, so that each clutch was as equally represented as possible in each aquarium. Tadpoles were placed into 18.9-L glass aquaria filled with 6.31 L of aged tap water. The aquaria were immersed in two water baths in a randomized block design.

These water baths were kept at approximately 25±0.5°C using bath heaters with thermostats. I established four treatments: low population density (10 tadpoles per 18.9-L glass aquaria), high population density (20 tadpoles per 18.9-L glass aquaria), low population density with predator cues, and high population density with predator cues. These groups each had six replicates for a total of 24 aquaria. Ten individuals were placed into the low population density containers, and 20 individuals were placed into the high population density treatments. To create the predator cues, water from a container housing an Eastern Newt, which had been feeding on tadpoles, was collected and then added to the predator cue aquaria daily (Reeve et al., 2013; Preston et al., 2014; Carlson et al., 2014). The Eastern Newts were fed from two 18.9-l aquaria with 20 tadpoles each. The newts were fed every 3 days with one tadpole. Tadpoles were fed algal wafers (Hikari Algae Wafers, Hayward, CA) every 3 days with half of the water changed every 3 days as well.

When the tadpoles reached approximately Gosner Stage 32-35, the remaining tadpoles were infected with *Dermomycoides* sp. Infection methods were based on Cook (2008). Infected Southern Leopard Frog tadpoles were collected from Pony Ranch Pond and New Pond. Southern Leopard Frog tadpoles are found in high numbers in these ponds and both sites are known to harbor the disease (unpublished data). These tadpoles were euthanized using MS-222, and their livers/intestines were harvested. These organs were mechanically homogenized and suspended in amphibian saline solution (0.65 g NaCl/100 mL H_20). This solution was allowed to sit in a refrigerator for a week. Once a week had passed, the liquid part of the solution was pipetted into a separate 50-mL vial. This process continued until I had obtained three, 50-mL vials.

This process required extracting livers and intestines from approximately 120 tadpoles. These vials were combined, and the concentrated solution was used as the stock solution for experimental infections (5000 spores/μL amphibian saline solution). The solution of *Dermomycoides* sp. spores was concentrated by placing it on a 0.2-μm filter and using a vacuum pump. A small amount of amphibian saline solution was added to this filter, and the number of zoospores were counted. More zoospores or amphibian saline solution were added until the desired concentration was reached.

Once the tadpoles had reached the required stage of infection, 5 mL of stock solution of *Dermomycoides* sp. spores was placed on a 0.2-μm filter and separated using a vacuum pump. The filter with the spores was then allowed to sit for an hour in a vacuum-sealed desiccation chamber. The filter paper containing the desiccated spores was then placed in a 50-mL Carolina culture bowl filled with 25 mL of distilled water, adjusted to a pH level of 6.5. The spores were then given around 15 minutes to rehydrate and then counted using hemocytometer to ensure they had reached a density of 100 zoospores/μL. Once the solution was ready, the tadpoles to be exposed were then placed into the bowls (five tadpoles per 50-mL bowl), and exposed for 1 hour. Once the hour had expired, the tadpoles were rinsed in three baths of distilled water (also adjusted to a pH of 6.5) and returned to their respective aquaria.

Any tadpole which had lost equilibrium or was swimming erratically was euthanized to minimize pain and stress. This endpoint assumed that the tadpole was infected and would die from the infection of *Dermomycoides* sp. These and any individual that died were frozen to determine if death was caused by *Dermomycoides* sp. Determination of infection with the pathogen was made through histological

preparations and by analyzing samples using PCR. The amount of time it took for an individual to metamorphose, length and mass at metamorphosis, and stage-specific survival were also monitored and compared with the controls. Once the frogs had absorbed their tails, they were euthanized with an overdose of MS-222. After 125 days in the aquaria, any individual that had not undergone metamorphosis was determined to be unlikely to metamorphosis and was euthanized using methods described earlier. These frogs could not be released into the wild due to their exposure to *Dermomycoides* sp.

Quantifying the Presence of *Dermomycoides* sp.

I collected 20 Southern Leopard Frog specimens from this experiment. These specimens were fixed in formalin for two weeks. The specimen fixing was done by using a scalpel to slice vertically down the abdomen to expose the organs to the formalin. Once the specimen was fixed, it was washed in tap water and with 70% ethanol. The liver and intestines were removed from the body and cut into pieces about 1-2mm thick (about 0.75 square mm or smaller if multiple pieces) with a razor blade, then put in enough clean 70% alcohol to have them remain wet. The specimens were mounted onto microscope slides by Crowder Histology, Baton Rouge, LA. Once prepared, I classified individual specimens into a category based on the amount of spores present within the tissue of the organ. Individuals that had less than 20% of their organs containing zoospores were classified as category one, between 20% and 40% were classified as category two, between 40% and 60% were classified as category three (Figure 5), between 60% and 80% were classified as category four, and if greater than 80% of the tissue was occupied by spores, this was considered category five.

PCR was used to determine if *Dermomycoides* sp. DNA was present in the liver of the specimens. All recoverable livers were removed from the specimens that had been preserved. Available DNA was extracted from the specimens using a DNeasy Kit (Qiagen Inc., Hilden, Germany), and then stored in -80°C freezers until used. Prior to PCR, each sample underwent spectrophotometric analysis (NanoDrop ND-1000) to determine the amount of DNA present per µL

I used 1 µL of DNA, 1 µL each of primers NS1 and NS8 and 22 µL of DNA free water with Illustra Puretaq ready-to-go beads (Promega, Madison, WI). The DNA then underwent 30 cycles of PCR using the primers (Table 1). The primers amplify the 18S nu-SSU region, and the beads contained all needed components for PCR, excluding primers and initial DNA. Following PCR, each reaction sample underwent spectrophotometric analysis (NanoDrop ND-1000) to determine the amount of DNA present per µL in the amplified reaction. The difference between the two NanoDrop analyses and the control tubes was then used to determine if DNA was produced using PCR. I used agarose gel electrophoresis on a select number of samples to confirm presence/absence of the amplified 18S region.

Data Analysis

To determine how survival and time to metamorphosis was affected by stress, I used a non-parametric Kaplan-Meier estimate of survival to compare exposure at a specific Gosner stage against time to metamorphosis, and I also used the Chi-Square test to compare exposure at a specific stress against survival to metamorphosis. I used an ANOVA to compare exposure at a specific environmental stress level against mass at metamorphosis, and length at metamorphosis. The Kaplan-Meier analysis, ANOVA

and Chi-Square test were conducted in program R utilizing the survival package (R Core Team, 2012). A parameter was included in this test to account for the blocking design used in this experiment.

Results

Only aquaria that had confirmed infections caused by *Dermomycoides* sp., determined using PCR or histology, were included in this analysis. Survival to metamorphosis was 62% for the group designated high population density and predators present, 83% for the group designated low population density and predators present, 76% for the group designated high population density and predators absent, and 85% for the group designated low population density and no predator (Figure 4). Using a Chi-Square test, I found no statistical difference among the different infected environmental stress levels for survival to metamorphosis ($p = 0.32$). The tadpoles differed somewhat in the average time to metamorphosis post-exposure to *Dermomycoides* sp. per treatment: 26 days for the group designated high population density and predators present, 9 days for the group designated low population density and predators present, 25 days for the group designated high population density and predators absent, and 10 days for the group designated low population density and no predator. These averages were found to not differ significantly between the different predator treatments. However, they did differ significantly in regards to population density ($p = 0.0001$, Figure 5). Recovered specimens differed somewhat in the average mass (grams) per treatment: 0.99 g for the group designated high population density and predators present, 1.40 g for the group designated low population density and predators present, 1.15 g for the group designated high population density and

predators absent, and 1.23 g for the group designated low population density and no predator. However, these averages were found to not significantly differ between the different treatments.

The average snout to vent length for all treatments fell between 2.3-2.6 cm (Table 6), which was also not significantly different between groups. The recovered specimens differed somewhat in the average liver mass per treatment: 0.004 g for tadpoles in high population density and predators present, 0.006 g for tadpoles in low population density and predators present aquaria, 0.015 g for tadpoles in high population density and predators absent aquaria, and 0.016 g for tadpoles in low population density and no predators aquaria. However, these averages were found to not be significantly different between the different treatments. The average category rating for the amount of spores present also did not differ significantly. The average ranking for the amount of *Dermomycoides* sp. spores in the livers for all treatments fell between categories three and four, and the average rankings for the amount of *Dermomycoides* sp. spores in the intestines fell between categories two and three (Table 7, Figure 6).

These results were obtained from only three of the replicates for the experiment. Near the end of the experimental exposure, a fluctuation in power resulted in the total loss of three replicates (12 aquaria), thus reducing my overall number of replicates by half.

Discussion

The environmental stress level that a tadpole is exposed to prior to infection of *Dermomycoides* sp. does seem to play a role in how the organism is able to cope with the disease. While I did not find a statistical significance in regards to survival to metamorphosis, there was higher mortality from individuals found in higher densities than those individuals that were exposed in lower densities. This result indicates that it may be beneficial to keep stocking levels low within 1000-L outdoor tanks and to avoid overstocking ponds with tadpoles, to assist in decreasing the mortality associated with *Dermomycoides* sp. This is corroborated with the results from the translocation experiment conducted in 2015 where ponds that had high overall population of Southern Leopard Frogs, had low survival to metamorphosis percentages (present study). However, the additional stress resulting from a higher population density of tadpoles may not be the only cause of the mortality seen in the experimental exposure.

The lack of significant difference between treatments and the svl was unsurprising given that svl does not change substantially in other circumstances. For example, svl does not differ significantly between individual Dusky Gopher Frogs that are raised in outdoor tanks instead of in ponds. I did see a difference in the mass of individuals that are raised in the wild compared to those raised in tanks (J. Tupy, personal communication, June 4, 2015). The significant difference between treatments regarding time to metamorphosis was not surprising in that stressed or diseased individuals often are not able to allocate as much resource toward growth (Holland et al., 2007; Crespi and Warne, 2013). However, this may be more related to the higher population density of tadpoles causing a poor allocation of resources within the aquaria.

While I did not see a significant difference in the mass at metamorphosis, there does seem to be a trend in that those individuals that were in higher densities had much lower masses. There also seemed to be a slight difference in the mass of those tadpoles that were exposed to predators. However, these trends are likely related to the stressful environmental conditions present while the tadpoles were maturing, and not related to the actual impact of the disease. This has been further corroborated by experiments conducted with raising Dusky Gopher Frog and Southern Leopard Frog tadpoles within the same 1000-L outdoor tanks (Thurgate, 2006).

Most of the results of this experiment seem to stem from the environmental stress itself, not necessarily the results of how the tadpoles coped with their exposure to *Dermomycoides* sp. Additional studies are needed to further identify the linkages between environmental stress and immunological response to *Dermomycoides* sp. However, this experiment does suggest that stress on headstarted individuals should be minimized to have better success during translocation. This notion in particular, could be extended beyond our particular system to other headstarting programs of anurans.

Understanding the impact of stress on the susceptibility to disease is incredibly important to wildlife managers. While in our experiment, the stress was environmentally caused, this is not always the case. Pesticides, and other contaminants, are thought to dramatically increase the amount of stress placed on an individual (Bryden et al., 2013; Modesto and Martinez, 2010), causing ecotoxicology and immunology to become linked. Thus, experiments like this show a need for further research into how Dusky Gopher Frogs or Southern Leopard Frogs may be able to respond to *Dermomycoides* sp. in circumstances where a pond may be impacted by contaminants. For example, we

often see that risk of infection for Ranavirus increases when amphibians are exposed to higher levels of pesticides (Forson and Storfer, 2006; Duffus, 2006). This relationship could potentially exist for the *Dermomycoides* sp.-Dusky Gopher Frog system, and could be another potential challenge for the recovery of the species or other species that face similar disease risk.

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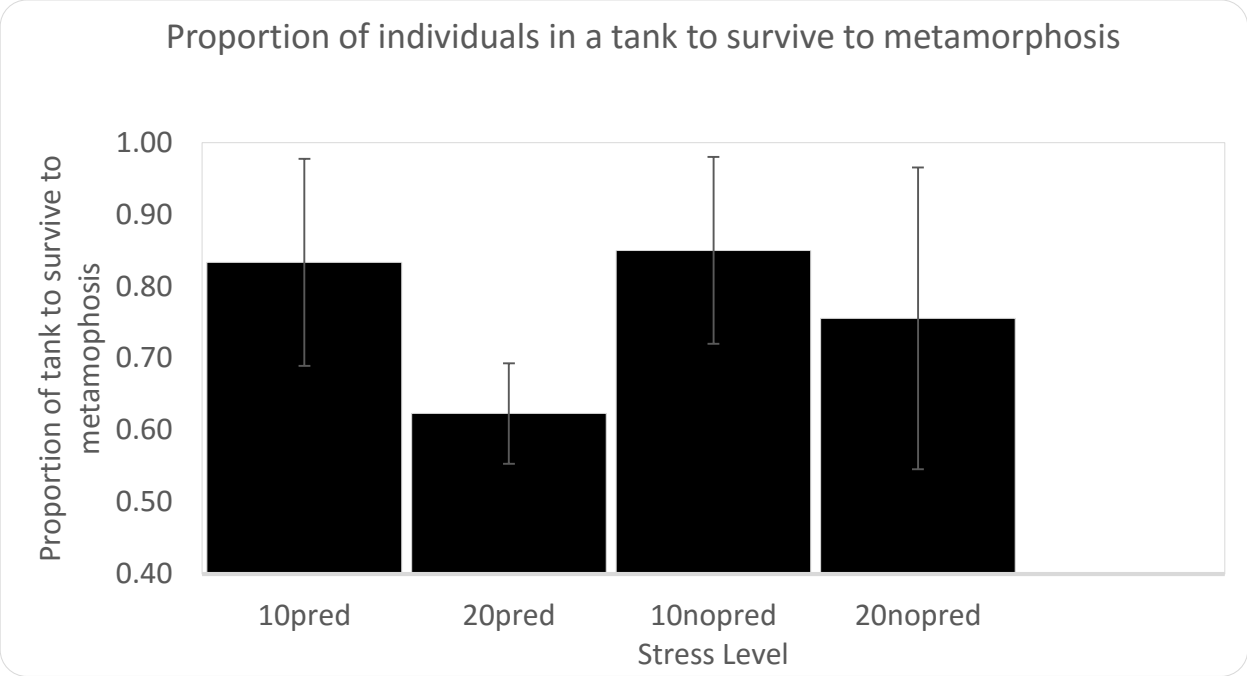

Figure 4. Proportion of individuals in a tank that survived to metamorphosis based on the level of environmental stress at the time of infection (replicates = 3). 10pred describes those aquaria with a low population density, but predators are present. 10nopred describes those aquaria with a low population density, but predators are not present. 20pred describes those aquaria with a high population density, but predators are present. 20nopred describes those aquaria with a high population density, but predators are not present. Standard error was used to create the error bars for this figure.

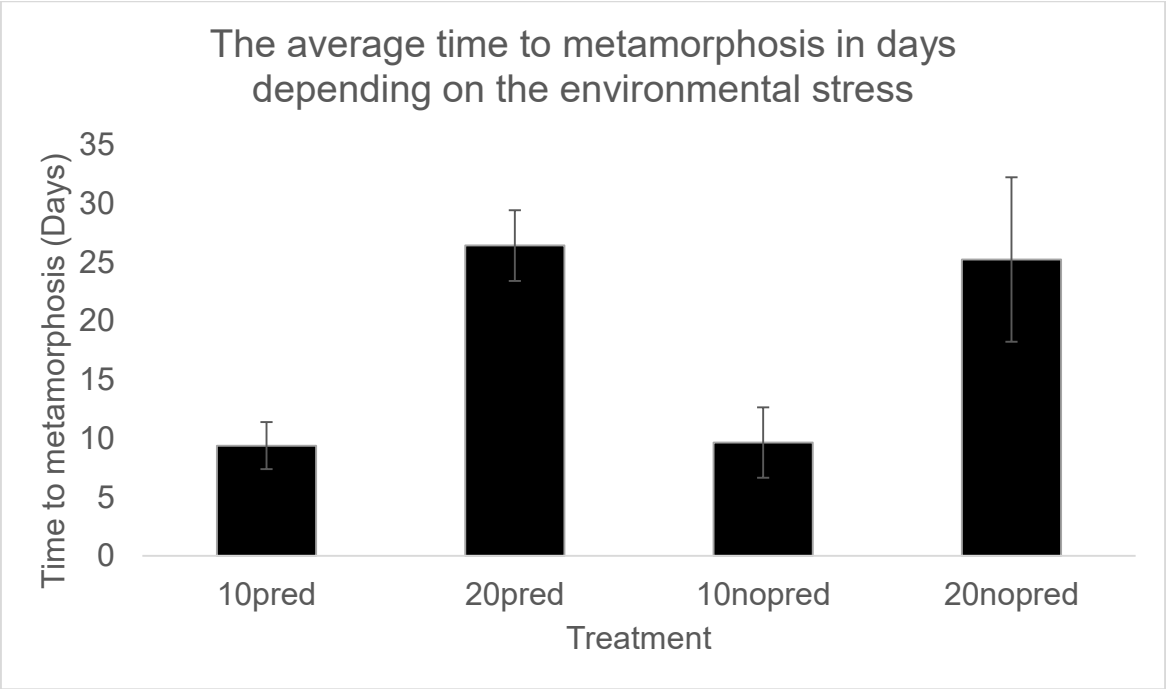

Figure 5. The average time to metamorphosis post-exposure to *Dermomycoides* sp. based on the level of environmental stress at the time of infection (replicates = 3). 10pred describes those aquaria with a low population density, but predators are present. 10nopred describes those aquaria with a low population density, but predators are not present. 20pred describes those aquaria with a high population density, but predators are present. 20nopred describes those aquaria with a high population density, but predators are not present.

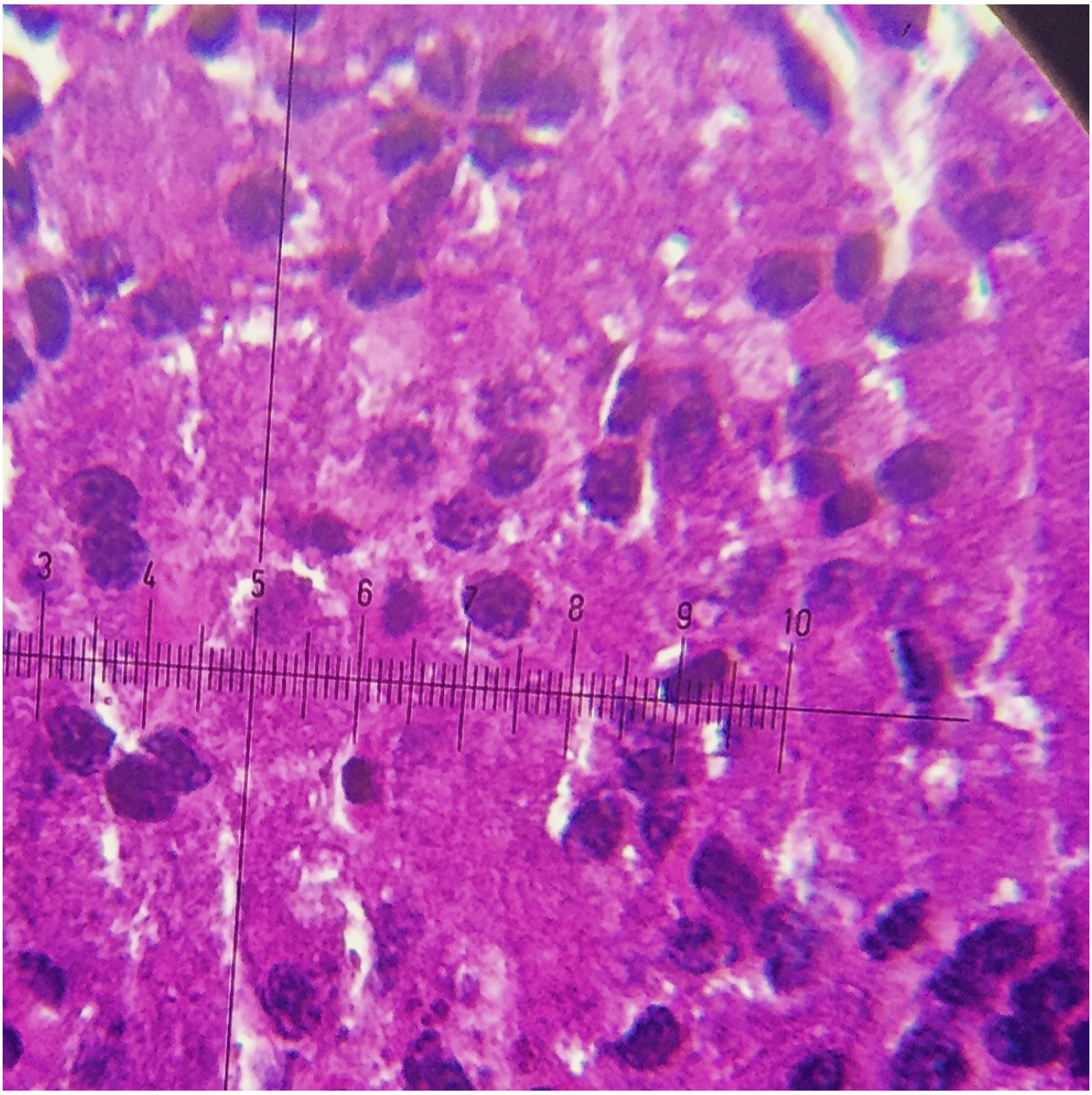

Figure 6. This shows an example of what would be classified as a category three infection (40-60%) based on the amount of zoospores present in the liver tissue. The purple seen in this picture are the zoospores found within the tissue, and the pink is the liver tissue.

TABLE 6. The average total mass, liver mass, day of metamorphosis, and snout to vent length (svl) of recoverable specimens based on the environment the individuals were subjected to prior to being exposed to *Dermomycoides* sp. (replicates = 3). 10pred describes those aquaria with a low population density, but predators are present. 10nopred describes those aquaria with a low population density, but predators are not present. 20pred describes those aquaria with a high population density, but predators are present. 20nopred describes those aquaria with a high population density, but predators are not present.

Treatment	Mean Mass (g)	Mean Liver Mass (g)	Mean SVL (Cm)	Day of Metamorphosis Post-exposure*
10non	1.23	0.016	2.4	9
20non	1.15	0.015	2.3	26
10pred	1.40	0.006	2.6	10
20pred	0.99	0.004	2.4	25

Table 7. The average ranking for amount of zoospores present in specimen tissue based on environmental stress and type of tissue (replicates = 3). Individuals that had less than 20% of their organs containing zoospores were classified as category one, between 20% and 40% were classified as category two, between 40% and 60% were classified as category three, between 60% and 80% were classified as category four and if greater than 80% of the tissue was occupied by spores, this was considered category five. 10pred describes those aquaria with a low population density, but predators are present. 10nopred describes those aquaria with a low population density, but predators are not present. 20pred describes those aquaria with a high population density, but predators are present. 20nopred describes those aquaria with a high population density, but predators are not present.

Treatment	Tissue	Category
10non	Liver	3
10pred	Liver	4
20non	Liver	4
20pred	Liver	3
10non	Intestines	3
20non	Intestines	3
20pred	Intestines	2