WHAT ENVIRONMENTAL FACTORS AFFECT *BATRACHOCHYTRIUM DENDROBATIDIS* INFECTION OF GREEN FROGS (*LITHOBATES CLAMITANS*) IN MICHIGAN?

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ABSTRACT

WHAT ENVIRONMENTAL FACTORS AFFECT *BATRACHOCHYTRIUM DENDROBATIDIS* INFECTION OF GREEN FROGS (*LITHOBATES CLAMITANS*) IN MICHIGAN?

by Matthew J. Igleski

Amphibian populations worldwide have been on the decline (Blaustein and Wake 1995; Lips 1999; Wake 1991; Whitfield et al. 2007). Berger et al. (1998) linked many of the population crashes in Australia and Central America to the disease chytridiomycosis. Chytridiomycosis is caused by *Batrachochytrium dendrobatidis*, first described in 1998 (Berger et al. 1998) and recognized as a new species in 1999 (Longcore et al. 1999). The objectives of this study were to determine the presence of *B. dendrobatidis* in *Lithobates clamitans* in Michigan and examine if the prevalence of *B. dendrobatidis* infection is influenced by environmental factors. *Lithobates clamitans* were captured throughout the state, rubbed with a swab that was later used to determine infection status of individual frogs using real-time PCR. *B. dendrobatidis* was detected on 160 of the 477 frogs sampled. Only 4 of 33 sample sites did not yield a positively infected individual. The minimum level of infection was 0.08 zoospores (zsp) and the maximum level of infection was 2176 zsp. There was no significant relationship between an individual frog being infected and mass or sex. There is no significant difference between the quantity of zoospores infecting female and male *L. clamitans*. Only one independent variable, latitude, has a significant relationship with prevalence. As latitude increases, chytrid infection prevalence decreases at sites in Michigan. A possible explanation for the relationship between chytrid prevalence and latitude is that sites further north likely have

shorter seasons within the optimal growth range *B. dendrobatidis* than sites in southern Michigan.

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CHAPTER I

INTRODUCTION

Amphibian populations worldwide have been on the decline (Blaustein and Wake 1995; Lips 1999; Wake 1991; Whitfield et al. 2007). In North America, populations have been quantitatively shown as declining since at least the 1960s (Houlahan et al. 2000). Many of the contributing factors to decline are apparent, such as habitat modification/loss (Hecnar and M'Closkey 1996; Rubbo and Kiesecker 2005), pollution (Blaustein et al. 2003), introduction of nonnative species (Hecnar and M'Closkey 1996; Pilliod and Peterson 2001), and overexploitation (Lannoo et al. 1994). However, the decline of several species is considered enigmatic (Lehtinen and Skinner 2006; Stuart et al. 2004). Due to the role of amphibians as indicator species, it should be of concern to everyone that there are significant declines being tracked worldwide. Amphibians are a vital part of many ecological processes and are often tightly associated to water throughout their entire life history. Monitoring amphibian populations is important since population changes often act as a litmus for changes in the environment (Wake 1991). Therefore, significant population declines should be heavily scrutinized to assure that a much larger problem does not exist, or is incurred due to large scale decline.

Mass mortalities of individuals from several species have been observed, in some localities such as Panama, during this global amphibian decline (Lips 1999). However, it has been difficult to link these massive population declines to one specific cause or pathogen, resulting in enigmatic amphibian declines. Berger et al. (1998) linked many of the population crashes in Australia and Central America to the disease chytridiomycosis.

Chytridiomycosis is caused by a recently described species of chytridiomycete fungus *Batrachochytrium dendrobatidis*, first described in 1998 (Berger et al. 1998) and recognized as a new species in 1999 (Longcore et al. 1999). Chytrid infections have been detected in members of two amphibian orders: Anura and Caudata (Ouellet et al. 2005).

Since the discovery of this new fungal pathogen few studies have attempted to trace its origin (Weldon et al. 2004). There appear to be two major hypotheses concerning the origin of *B. dendrobatidis.* The first is that it has always been present worldwide as an endemic pathogen and an environmental cue is causing it to become lethal (Pounds et al. 2006). The second hypothesis is that it was introduced into amphibian populations as a novel pathogen through some unknown vector (Weldon et al. 2004).

An explanation offered by Weldon et al. (2004) suggested that one possible vector was through the trade of two African clawed frog species, *Xenopus laevis* and *Xenopus tropicalis*. These two species were exported to several different countries for use in pregnancy testing assays. They were also exported for the pet trade and have been shown to be resistant carriers of *B. dendrobatidis* (Weldon et al. 2004). Several 1930s museum specimens of *Xenopus* spp. have been shown to be infected with *B. dendrobatidis* (Soto-Azat et al. 2010; Weldon et al. 2004) which predates any of the known mass amphibian declines (Lips 1999; Stuart et al. 2004; Weldon et al. 2004). Another recent study has detected *B. dendrobatidis* infected museum specimens of *Andrias japonicus*, an endemic of Japan, from 1902 (Goka et al. 2009). Goka et al. (2009) noted that *Andrias japonicus* seemed to carry relatively high infection loads of *B. dendrobatidis* without exhibiting signs of chytridiomycosis; they also noted that genetic diversity of *B. dendrobatidis* in

Japan was high relative to the rest of the world. This and other evidence does appear to support the case for the fungus being a recently introduced novel pathogen to many other countries worldwide. Detection of positively infected individuals from old museum specimens in just a few countries and low genetic diversity at many sites globally certainly support the case.

While evidence for the relatively recent introduction of *B. dendrobatidis* is more compelling, there still has not been enough survey work done worldwide to establish the range of this pathogen. It is possible that the fungus has always been present and that another stressor is causing it to become a fatal disease. *B. dendrobatidis* has been detected in amphibian populations of pristine habitats, which implies it was present before the possibility of introduction (Lampo et al. 2006). Additional survey work is required to understand the historic and current range of the fungus. In addition, human impact on the distribution of *B. dendrobatidis* is unknown. With increasing human population growth there is a very good chance that more humans are inadvertently transporting *B. dendrobatidis* between water bodies, likely increasing the rate of spread within the environment.

At a coarse scale, *B. dendrobatidis* appears to be present on most continents and in most countries where survey work has been done. This distribution includes Canada and the United States, where *B. dendrobatidis* has been detected as far back as the 1960s (Ouellet et al. 2005). Finer scale studies, in terms of both geography and species specificity, have been more limited. Within North America, *B. dendrobatidis* has been detected in several species, states, and provinces. Two recent studies detected chytrid-infected frogs in Michigan, Blanchard's Cricket Frog (*Acris crepitans blanchardi*)

(Steiner and Lehtinen 2008; Zippel and Tabaka 2008) and the Wood Frog (*Lithobates sylvatica*) (Zellmer et al. 2008). *L. sylvatica* had an infection prevalence of less than 1%, present in only 2 of 239 specimens sampled (Zellmer et al. 2008). *A. crepitans blanchardi* infection prevalence ranged from 0-33% between study sites and was 16% across all individuals sampled in one study (Steiner and Lehtinen 2008).

This study focused on determining the presence of *B. dendrobatidis* in Michigan. This study examined the hypothesis that the prevalence of *B. dendrobatidis* infection would be influenced by one or several environmental factors. Determining what environmental variables could limit the growth or spread of the fungus will help establish geographic range limits. Understanding these limits and combining this information with knowledge of amphibian ecology may help to establish specific information concerning local distribution of *B. dendrobatidis*. Several factors are likely to affect growth and establishment of the fungus in different frog populations. These include: temperature, conductivity, and pH of water. Past research has indicated an optimal temperature (23°C) for *B. dendrobatidis* growth and a temperature at which growth is inhibited (29°C) (Longcore et al. 1999). The range of temperatures experienced by anurans in Michigan fluctuates above and below these cutoffs. Because of this fluctuation it is likely that the fungus may experience periods of rapid growth and dispersal, as well as periods of dormancy. Other factors that have been correlated with differences in *B. dendrobatidis* infection are latitude (Kriger et al. 2007) and elevation. Since climatic factors are variable, one point in time during sampling may not demonstrate relationships between infection differences and environmental variables when analyzing data. If a relationship

can be established between infection and one or more static factors, such as latitude and elevation, this can help link infection differences to climatic factors.

The model for this study was the Green Frog (*Lithobates clamitans*). This species was chosen because it is a habitat generalist, its range spans the entire state, and it is typically easy to locate. *Lithobates clamitans* has been documented as a carrier of the fungus in other studies and has exhibited an infection prevalence of 20% across several studies (Ouellet et al. 2005). All of these characteristics make this species an ideal model organism for determining if specific factors, not related to the host, have an effect on the infectability of *B. dendrobatidis*. By using an organism with a wide geographic range, high prevalence as a resistant carrier, and general habitat preferences a broad range of environmental variables can be compared and tested, eliminating a host affect which is created when using several different host species to test environmental variables. Since *L. clamitans* is also easy to locate in relatively higher numbers, it creates an opportunity to acquire higher sample sizes for statistical analysis and more robust conclusions can be drawn.

The objectives of this study were to determine the presence of *B. dendrobatidis* in *L. clamitans* in Michigan and examine if the prevalence of *B. dendrobatidis* infection was influenced by environmental factors. This study tested several hypotheses. One, the percentage of *L. clamitans* with positive chytrid infections at any site in Michigan has a linear relationship with: pH, temperature, and conductivity of water; latitude; and elevation. Two, there is a relationship between presence/absence of chytrid on an individual *L. clamitans* and the size and sex of the frog. Three, the chytrid infection load of male *L. clamitans* does not equal chytrid infection load of female *L. clamitans*.

CHAPTER II

METHODS

Site Selection

As shown in figure 1, 33 sites in Michigan, USA were sampled between 19 May and 20 September 2009. Without prior knowledge of *B. dendrobatidis* infection, sites were chosen based on the likely occupancy of *L. clamitans*, the spread of sites with respect to latitude, and accessibility. Most sites were permanent bodies of water ranging in size from small ponds (89 m^2) to large lakes (1214 ha).

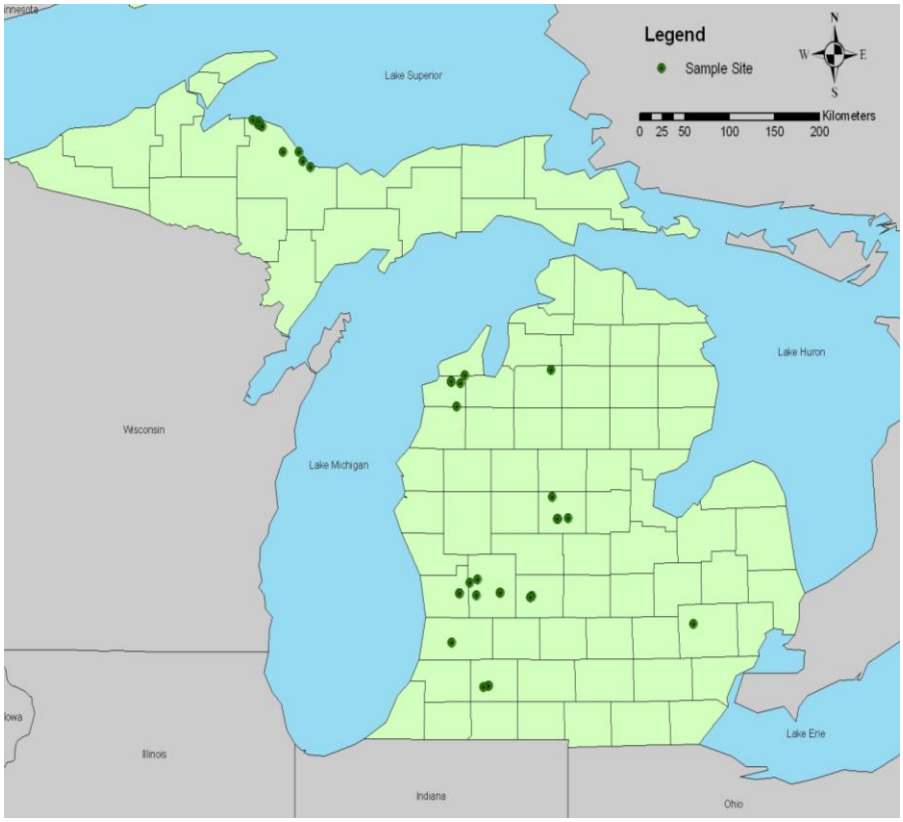

Figure 1. Location of 33 sites sampled for *Batrachochytrium dendrobatidis* on *Lithobates clamitans* in Michigan, USA, in 2009.

Several environmental variables were sampled at each site: water temperature (°C), conductivity (µMhos), pH, humidity, and air temperature (°C). Data such as GPS

coordinates and elevation (m) were also collected. Water temperature and conductivity data were collected using a YSI (Yellow Springs Instrument Co. Inc., Yellow Springs, OH) Model 33 S-C-T meter with a 3310 probe. The pH data was collected using a Double Junction pHTestr10 (Oakton instruments, Vernon Hills, IL). Air temperature and humidity were recorded from the NOAA weather website (www.noaa.gov). GPS coordinates and elevation were acquired from TOPO! version 3.4.0 (National Geographic Holdings, Evergreen, CO).

Field Methods

The shoreline of the ponds and lakes were surveyed for *L. clamitans*. Frogs were captured by gloved hands or nets. Equipment and vinyl gloves were exchanged after capture to avoid cross contamination. Each frog was kept alive and placed in a plastic bag until it could be sampled. For smaller bodies of water, as many frogs as possible were captured before swabbing, the frogs were then released back into the water. For larger bodies of water, swabbing took place after a number of frogs (8-20) were captured. In the larger bodies of water, frogs would then be released in the stretch of shoreline from which they were captured. To avoid resampling individuals, we continued surveying and capturing approximately 5 m away from the release point, in a direction away from the previous captures. This implied with reasonable certainty that we caught unique individuals. Sampling was considered complete if the entire shoreline had been covered or more than 35 frogs had been captured. Once sampling at a site was completed, all gear (boots, waders, etc.) was disinfected with a dilute (10^{-1}) bleach solution to avoid cross contamination of sites.

Each frog was handled with new gloves and swabbed with a sterile Medical Wire MW113 (Medical Wire & Equipment Co. Ltd., Corsham, Wiltshire, England) cotton-tipped, plastic shaft swab. The swab was rubbed in one direction five times on the ventral surface of the body, between the forelimb and hindlimb on each side of the body, on the ventral surface of each thigh, and once between each toe on the ventral surface of the webbing. The swab was then placed in a 1.5 mL centrifuge tube with the screw cap left open, in order to let the swab air dry. Once the swab was dry, the tube was screwed shut and stored at 4°C until it could be processed.

Frogs were identified as male, female, or immature (too young to reasonably sex). Each frog was weighed to the nearest 0.5 g using a Pesola spring scale (Kapuskasing, ON, CA). Snout to vent length (mm) was measured to the nearest 1.0 mm using SPi 2000 dial calipers (Swiss Precision Instruments, Inc., Garden Grove, CA). Any abnormalities were also noted, including abrasions, lesions, discoloration, deformities, etc. All pieces of equipment (e.g. calipers and scales) were disinfected with a dilute bleach solution in between each use.

Laboratory Methods

In order to extract DNA, 40 µL of PrepMan Ultra Sample Preparation Reagent was added to the 1.5 mL centrifuge tube containing the swab. The tube was heated to 100°C for 10 minutes and then allowed to cool for at least 2 minutes. After cooling, the tube was centrifuged for 3 minutes at 13,000 rpms. Then, a minimum of 20 µL of supernatant was transferred to a 200 µL PCR tube. Five µL of the supernatant was diluted 10^{-1} in 0.25xTE buffer (pH 7.6). The diluted samples were then stored at -20°C

until they could be analyzed using a validated quantitative real-time PCR (qPCR) assay (Boyle et al. 2004; Hyatt et al. 2007). The primers and minor groove binding (MGB) probe used in the qPCR assay were the same *B. dendrobatidis* DNA specific sequences used in Boyle et al. (2004).

An ABI 7500 Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA) was used for the qPCR assay. Conditions for qPCR analysis were as follows: 2 min at 50°C, 10 min at 95°C, and 50 cycles of 15 s at 95°C, 1 min at 60°C. Each 96-well plate contained 4 standards at known zoospore concentrations of *B. dendrobatidis*, 1 negative control without DNA template, and 27 unknown samples in triplicate (triplicate = 3 aliquots from each swab sample extraction). Known DNA concentrations, provided by the Australian Animal Health Laboratory, were used to create 100, 10, 1, and 0.1 zoospore equivalent (zsp) standards. The known concentrations were then used to create a standard curve for each qPCR assay performed. Reaction volumes were 25 μL and contained 1X Taqman Environmental Master Mix 2.0 (Applied Biosystems, Inc., Foster City, CA), 900 nM concentration of each primer, 250 nM concentration of the MGB probe, and 5 μL of DNA template.

Quantification of Zoospore Infection Load

An individual frog sample was considered positive if at least one of the triplicate swab samples amplified during the qPCR assay. It was not uncommon for only 1 or 2 replicates to amplify, especially if the quantity was low. The actual quantity of zoospore equivalents for each sample was acquired by comparing critical threshold (Ct) values (the cycle in which a sample has amplified past a set threshold) of unknown samples to the Ct

values of the standard curve. The quantity for each sample represented a mean of the PCR analysis of the swab sample, if more than one replicate was positive. The prevalence of positive individuals at each site was calculated by taking the number of positive frogs and dividing that number by the number of frogs sampled. The zsp mean of each site was calculated by taking an average of only the infected individuals from that site.

Data Analysis

A correlation between average infection load (Log transformed) and site prevalence (Log transformed) was performed in Minitab 14 (Minitab Inc., State College, PA, USA). Prevalence data was Log transformed and tested for normality before and after transformation. To check for collinearity between the independent variables, a correlation matrix analysis was performed prior to the stepwise regression in Minitab 14 (Minitab Inc., State College, PA, USA) and one of two variables with a strong correlation (Pearson correlation value above 0.7) was selected. A stepwise regression was performed in Minitab 14 (Minitab Inc., State College, PA, USA) using the prevalence of positive individuals at a site as the dependent variable and several possible explanatory factors: water temperature (°C), conductivity (μMhos), pH, latitude, and elevation. A backward elimination was used to remove independent variables using an α of 0.1 as a threshold. Logistic regression was performed in Minitab 14 (Minitab Inc., State College, PA, USA) using positive or negative infection of an individual as the dependent variable and mass (g) and sex of the frog as the independent variables. Sex of the frog was actually three categories: male, female, and immature (too young to reasonably determine sex). A t-test

was performed in SYSTAT (Systat Software, Inc. Chicago, IL, USA) to determine if the infection level of males was different from females. The quantity of zoospore equivalents data was Log transformed and tested for normality before and after transformation. Anderson-Darling test for normality was used for all normality tests; these were performed using Minitab 14 (Minitab Inc., State College, PA, USA).

CHAPTER III

RESULTS

The results of this study show that *B. dendrobatidis* is widespread in Michigan. *B. dendrobatidis* was detected on 160 individual *L. clamitans* of the 477 total individuals sampled (33.5%; Table 1). Few sites were *B. dendrobatidis* negative; 4 of the 33 sites visited did not yield a positively infected frog. Positively infected individuals varied in their infection load, the minimum level of infection was 0.08 zsp and the maximum level of infection was 2176 zsp. The site average infection load (Log transformed) and site prevalence (Log transformed) were positively correlated ($r_{27} = 0.783$, $p < 0.001$; Figure 2)

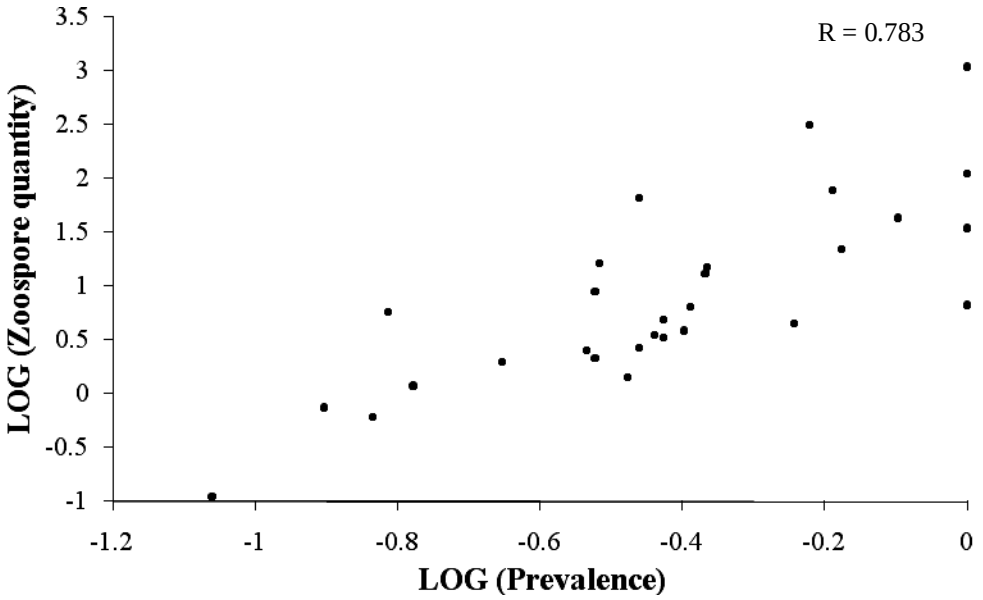

Figure 2. Correlation between prevalence (Log transformed) and average zoospore quantity (Log transformed) of *Batrachochytrium dendrobatidis* infections in *Lithobates clamitans* at 29 infected sites in Michigan, USA, in 2009 ($r_{27} = 0.783$, $p < 0.001$).

Several independent variables did not have a significant relationship with prevalence (Table 2). None of the independent variables were strongly correlated with

each other (Table 3), however latitude and water temperature did have a significant weak relationship ($r_{27} = 0.527$; $p = 0.002$). One independent variable, latitude, did have a significant relationship with prevalence ($T_{23} = -4.15$, $p < 0.001$; Figure 3) and explained a

Table 1. Summary of *B. dendrobatidis* infection in *Lithobates clamitans* from 33 sites in Michigan, USA, in 2009. Zoospore quantities reported represent the mean, minimum (Min.), and maximum (Max.) of infected individuals only, from that site.

				Zoospore Quantity		
Site Name	**Infected**	**N**	**Prevalence**	**Mean**	**Min.**	**Max.**
Mill Pond Park	4	7	57.1	8	1.84	21.12
Deerfield Park (Pond 1)	4	4	100	35	17.52	74.72
Deerfield Park (Pond 2)	3	5	60	524	6.72	1525.6
Littlefield Pond	1	1	100	7	6.72	6.72
John Ball Park Pond	4	10	40	10	4.64	24.72
Grand Valley State Property (Pond 1)	0	2	–	–	–	–
Vickie's Marsh/Pond	2	2	100	1096	16	2176
Whalfield Mill Creek	0	1	–	–	–	–
Seidman Park (Pond 1)	3	8	37.5	9	6.48	10.24
Seidman Park (Pond 2)	0	4	–	–	–	–
Grand Valley State Property (Pond 2)	1	3	33.3	43	4.32	4.32
Ionia State Game (Pond 1)	3	10	30	7	4.72	9.2
Ionia State Game (Pond 2)	0	7	–	–	–	–
Allegan Dam Pond	3	3	100	111	38	169.76
Celery Flats (Pond 1)	11	17	64.7	122	2.56	964.8
Celery Flats (Pond 2)	2	3	66.7	33	8.88	57.04
Hampton Lake Marsh	2	9	22.2	9	7.92	9.84
Shisler Lake	9	26	34.6	213	4.64	1546.4
Shavenaugh Lake Swamp	12	28	42.9	30	2.72	127.36
Pere Marquette (Pond 1)	2	12	16.7	7	4.72	9.6
Pere Marquette (Pond 2)	3	8	37.5	13	3.28	30.08
Lindy Road Pond	9	30	30	30	0.88	146.8
Rock Cut Pond	12	33	36.4	10	0.32	75.12
Harlow Lake	9	22	40.9	16	0.08	59.28
County Road 510 Marsh	2	13	15.4	37	33.12	41.36
Westwood Mall Drainage	9	26	34.6	8	2	22
Florence Pond	2	16	12.5	6	1.6	10.4
Ives Lake	6	41	14.6	4	2.72	5.76
Trout Lake	2	23	8.7	1	0.16	2.4
Rush Creek Dam	7	23	30.4	54	7.52	207.36
Howe Lake	7	24	29.2	9	3.44	26.48
Louie's Pond	22	51	43.1	35	0.64	385.12
Big Lake Road Pond	4	5	80	54	1.68	196.24
Total/Mean	**160**	**477**	**33.5%**	**87.6**	**6.75**	**273.99**

significant proportion of the variance in prevalence between sites ($F_{5,28}$ = -0.102, r^2 = 38.94, $p < 0.001$). This relationship is significant when analyzing only the positively

Table 2. Results of a stepwise regression using Log prevalence of *Batrachochytrium dendrobatidis* infections in *Lithobates clamitans* at 29 infected sites in Michigan, USA, in 2009 as the dependent variable and 5 independent predictor variables. A backward elimination with an α of 0.1 was used to remove predictor variables.

Variable	Step	1	2	3	4	5
	Constant	3.053	3.046	3.299	3.469	4.107
Latitude	$F_{5,28}$ =	-0.085	-0.085	-0.094	-0.099	-0.102
	T_{23} =	-2.68	-2.76	-3.64	-4.03	-4.15
	p =	0.013	0.011	0.001	0.000	0.000
pH	$F_{5,28}$ =	0.054	0.051	0.054	0.06	
	T_{23} =	0.94	0.92	0.99	1.15	
	p =	0.356	0.367	0.330	0.262	
Conductivity	$F_{5,28}$ =	0.00009	0.0001	0.00008		
	T_{23} =	0.63	0.7	0.61		
	p =	0.533	0.49	0.55		
Water Temperature	$F_{5,28}$ =	-0.006	-0.006			
	T_{23} =	-0.54	-0.56			
	p =	0.593	0.579			
Elevation	$F_{5,28}$ =	-0.00026				
	T_{23} =	-0.31				
	p =	0.760				
S		0.232	0.228	0.225	0.222	0.223
R-Sq		43.7	43.46	42.72	41.87	38.94
R-Sq(adj)		31.46	34.04	35.84	37.4	36.68
Mallows C-p		6	4.1	2.4	0.7	-0.1

infected sites. Given that prevalence was 0.335 between all sites, 8 individuals would need to be sampled in order to be 96% confident that a site was absent of *B. dendrobatidis* and 12 individuals would need to be sampled to be over 99% confident that a site was absent of *B. dendrobatidis*. Since not enough individuals were collected at sites with 0% prevalence to confidently assume absence of the fungus, they were excluded from this analysis. According to the Anderson-Darling test value (AD) for the

site prevalence data, the data was not normal before Log transformation (mean = 0.4548, SD = 0.2774, AD = 1.327, p = <0.005). After Log transformation, the data was normal (mean = -0.4233, SD = 0.2805, AD = 0.453, p = 0.253).

Table 3. Correlation matrix of site variables used in stepwise regression to determine predictor variables for *Batrachochytrium dendrobatidis* infections in *Lithobates clamitans* at 29 infected sites in Michigan, USA, in 2009.

		Water Temperature	pH	Conductivity	Latitude
pH	r =	-0.067			
	p =	0.712			
Conductivity	r =	-0.152	0.152		
	p =	0.398	0.398		
Latitude	r =	0.527	-0.07	-0.325	
	p =	0.002	0.699	0.065	
Elevation	r =	0.134	0.16	-0.229	0.197
	p =	0.457	0.374	0.201	0.271

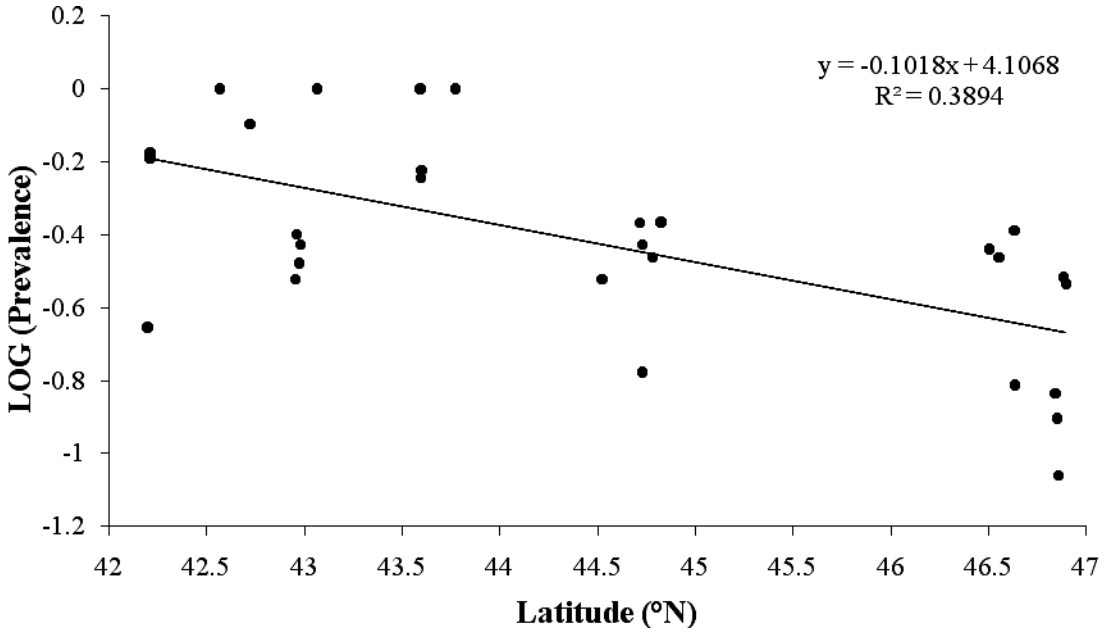

Figure 3. Relationship between latitude and prevalence (Log transformed) of *Batrachochytrium dendrobatidis* infections in *Lithobates clamitans* at 29 infected sites in Michigan, USA, in 2009 (T_{23} = -4.15, $p < 0.001$; $F_{5,28}$ = -0.102, r^2=0.3894, $p < 0.001$).

There was no significant relationship between mass (g) ($z_1 = 0.43$, $p = 0.665$) or sex ($\chi^2_2 = 0.679$, $p = 0.712$), or both ($G_3 = 0.686$, $p = 0.877$), and an individual frog being infected or not (N = 449). There is no significant difference between the quantity of zoospores infecting female and male *L. clamitans* ($T_{111} = -0.649056$, $p = 0.518$; Figure 4). According to the Anderson-Darling test value for the zoospore quantity data for females and males, the data was not normal before Log transformation (female: mean = 17.65, SD = 26.31, AD = 6.27, $p = <0.005$; male: mean = 65.13, SD = 283.7, AD = 20.953, $p = <0.005$). After Log transformation female data was normal but the male data was not (female: mean = 0.923, SD = 0.522, AD = 0.386, $p = 0.377$; male: mean = 1.000, SD = 0.670, AD = 2.115, $p = <0.005$).

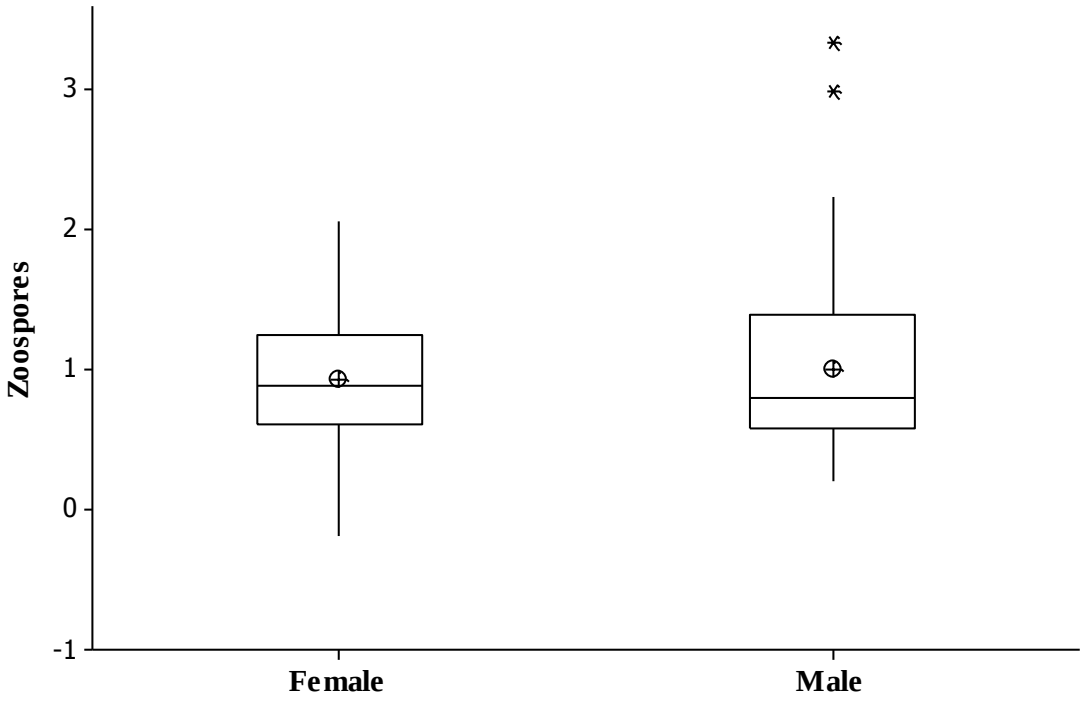

Figure 4. Comparison of *Batrachochytrium dendrobatidis* zoospore infection load (Log transformed) between male (N = 69) and female (N = 44) *Lithobates clamitans* sampled in Michigan, USA, in 2009 ($T_{111} = -0.649056$, $p = 0.518$). Data is shown as a box plot, the crosshairs represent the mean, and the asterisks represent outliers.

CHAPTER IV

DISCUSSION

It is evident from this study that *B. dendrobatidis* can be found in *L. clamitans* at most sites in Michigan. Few sites did not yield at least one infected individual. Among those sites that did not, it was impossible to determine absence due to low sample sizes. There were two reasons for low sample sizes. One reason was that there did not appear to be very many frogs to catch at some sites. Generally, *L. clamitans* is a conspicuous species preferring the shallow edge of water bodies. Thus, after hours of searching at some sites without finding any more new frogs, sampling was ended and the assumption was that few frogs were using that site. The other reason was that the habitat characteristics, such as dense vegetation at the margin of some water bodies, made it difficult to catch more than a few frogs.

Although there was not an obvious reason for some sites to have only a few *L. clamitans* inhabiting them, it is possible that sites with few individuals could have been subject to a recent chytrid introduction. If this were the case then the population could be in the process of recovery, possibly repopulating the site with chytrid resistant individuals. Elsewhere, species thought to be extirpated or even extinct, possibly due to chytrid, have now been rediscovered in their historic ranges (Rodriguez-Contreras et al. 2008). Without ongoing monitoring over a long period of time it is difficult to detect a recovery event. So it would be difficult to determine if recovery is the case at sites with low sample sizes in this study.

Several environmental factors were explored in this study to determine if they correlated with infection loads or infection prevalence. One factor, latitude, was determined to have a significant relationship with infection prevalence. As latitude increases, chytrid infection prevalence decreases at sites in Michigan. This is not entirely surprising since latitudinal variation has been linked to chytrid infection prevalence and intensity in other studies (Kriger et al. 2007; Roedder et al. 2008). Kriger et al. (2007) found that a significant relationship between chytrid infection intensity and latitude was likely due to changes in precipitation, along a large scale latitudinal gradient (15-40°S). The latitudinal gradient explored in this study was at a much smaller scale, between 42-47°N. A significant relationship at such a small scale is interesting and deserves further exploration to determine the exact cause of this relationship. Since latitude itself does not have a direct effect on infection, there is likely one or more environmental factors not explored in this study influencing infection at Michigan sites. Future studies should still consider latitude, even at small scales, as a variable when trying to determine differences in prevalence between sites. Since latitude has been successfully used to link infection differences to environmental variables in past studies, latitude can be a useful first step when exploring infection gradients.

In other studies, it has been determined that temperature does have an effect on chytrid infection (Andre et al. 2008). Conditions of optimal growth and temperatures causing arrested development have been identified (Longcore et al. 1999). In this study, the temperature of the water, at the time a sample was taken, did not correlate with any infection results. Since water temperature was not a significant factor it is possible that another temperature variable could be influencing chytrid infection. One temperature

variable that changes with latitude is average yearly temperature at a site. Sites further north have a lower average yearly temperature than sites in southern Michigan. This provides a possible explanation for the decrease in chytrid prevalence correlated to an increase in latitude.

Even if a reason for the infection differences between sites cannot be determined, the fact still remains that most sites in this study had at least a few positively infected individuals. This leads me to believe that there are several likely scenarios for chytrid in Michigan. One is that the fungus has been around for a while (possibly introduced by humans decades ago) and that there are now very few sites unexposed. Ouellet et al. (2005) identified two museum specimens of *L. clamitans* as being the oldest known cases of chytrid infection in North America, dating back to 1961. Ouellet et al. (2005) also identified infected museum specimens of different species scattered throughout North America from several decades. Since chytrid was detected in several states in past decades it may be possible that chytrid was also in Michigan several decades ago. Without testing museum specimens it is impossible to determine when chytrid made it to Michigan. However, it is now known that chytrid appears to be widespread in *L. clamitans* in Michigan.

Another likely scenario is that regardless of how long *B. dendrobatidis* has been in Michigan, it currently has been introduced to most sites that are easily accessed by humans. Since this study did not visit many sites without direct access to humans, it is reasonable to think that chytrid has had the opportunity to infect frogs at most sites with direct access. This scenario would not be easy to distinguish from the first scenario, it

only serves as an explanation for chytrid introduction and explains the high prevalence of infected sites in this study.

From a conservation standpoint, identifying the origin of the pathogen and modes of transport are interesting to try and understand what can be done to prevent further spread, if there are still uninfected sites or populations. Also, without further chytrid testing it is difficult to determine if there are indeed still naïve populations of not only *L. clamitans* but any amphibian species in Michigan. Since these types of unknowns remain for not only *B. dendrobatidis* but several waterborne pathogens, it would be better to play it safe and convince the general public to be mindful of cross contaminating bodies of water due to recreational activities.

This study expands the knowledge of *Batrachochytrium dendrobatidis*. Several features of the pathogen, including life history, seem to be relatively well-known at this point. However, finer scale details such as range and virility have been difficult to wholly establish. This study fills in gaps of broad and local geographic range as well as, establishs variables for detection probabilities. This study implies that, even at a relatively small scale, prevalence of infected individuals at a site may change relative to latitude. One explanation offered in this study was that average yearly temperature may be an influence of prevalence differences at different latitudes. However, like many environmental variables influencing biology there may be some other undetectable synergistic effect between several variables causing the infection differences between sites.

APPENDIX A

COORDINATES (DECIMAL DEGREES) AND COUNTY LOCATIONS FOR 33 SITES IN MICHIGAN, USA WHERE *LITHOBATES CLAMITANS* WAS SAMPLED FOR *B. DENDROBATIDIS* INFECTION.

Site Name	**Sampling Date**	**County**	**N**	**W**
Mill Pond Park	19 May 2009	Isabella	43.593466°	-84.893624°
Deerfield Park (Pond 1)	19 May 2009	Isabella	43.590699°	-84.895050°
Deerfield Park (Pond 2)	20 May 2009	Isabella	43.598672°	-84.788758°
Littlefield Pond	20 May 2009	Isabella	43.770861°	-84.948215°
John Ball Park Pond	26 May 2009	Kent	42.959130°	-85.705680°
Grand Valley State Property (Pond 1)	28 May 2009	Ottawa	42.972240°	-85.877908°
Vickie's Marsh/Pond	29 May 2009	Kent	43.063600°	-85.773432°
Whalfield Mill Creek	29 May 2009	Kent	43.089351°	-85.695526°
Seidman Park (Pond 1)	30 May 2009	Kent	42.981054°	-85.468707°
Seidman Park (Pond 2)	30 May 2009	Kent	42.981527°	-85.470234°
Grand Valley State Property (Pond 2)	30 May 2009	Ottawa	42.972472°	-85.878730°
Ionia State Game (Pond 1)	2 June 2009	Ionia	42.954040°	-85.151256°
Ionia State Game (Pond 2)	3 June 2009	Ionia	42.943729°	-85.167543°
Allegan Dam Pond	4 June 2009	Allegan	42.564650°	-85.952683°
Celery Flats (Pond 1)	5 June 2009	Kalamazoo	42.210483°	-85.584717°
Celery Flats (Pond 2)	5 June 2009	Kalamazoo	42.210000°	-85.585350°
Hampton Lake Marsh	5 June 2009	Kalamazoo	42.196267°	-85.634533°
Shisler Lake	8 June 2009	Leelanau	44.780748°	-85.821673°
Shavenaugh Lake Swamp	9 June 2009	Benzie	44.713317°	-85.865181°
Pere Marquette (Pond 1)	10 June 2009	Benzie	44.728417°	-85.958478°
Pere Marquette (Pond 2)	10 June 2009	Benzie	44.729219°	-85.958696°
Lindy Road Pond	11 June 2009	Benzie	44.520913°	-85.905132°
Rock Cut Pond	15 June 2009	Marquette	46.504683°	-87.370450°
Harlow Lake	17 June 2009	Marquette	46.629550°	-87.488733°
County Road 510 Marsh	18 June 2009	Marquette	46.632300°	-87.644383°
Westwood Mall Drainage	19 June 2009	Marquette	46.552750°	-87.449367°
Florence Pond	22 June 2009	Marquette	46.850617°	-87.870700°
Ives Lake	23 June 2009	Marquette	46.839450°	-87.854050°
Trout Lake	24 June 2009	Marquette	46.857433°	-87.892433°
Rush Creek Dam	25 June 2009	Marquette	46.883683°	-87.886350°
Howe Lake	26 June 2009	Marquette	46.894529°	-87.951718°
Louie's Pond	4 August 2009	Antrim	44.823567°	-84.956617°
Big Lake Road Pond	2 September 2009	Oakland	42.721200°	-83.528812°

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