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# Prevalence of a Chytrid Pathogen (Batrachochytrium dendrobatidis) in Eastern Hellbender Salamanders in New York and Pennsylvania

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# Prevalence of a Chytrid pathogen (*Batrachochytrium dendrobatidis*) in Eastern Hellbender Salamanders in New York and Pennsylvania

By

Linxuan Wu

An Abstract of a Thesis
in Biology

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Arts
August 2015

Buffalo State
The State University of New York
Department of Biology

# ABSTRACT OF THESIS

Amphibian populations are currently declining globally. There are many possible causes for these declines, among which an emerging infectious disease, chytridiomycosis, has been implicated. Chytridiomycosis in the U.S.A. is mainly caused by the *Batrachochytrium dendrobatidis*. In this study, I used qPCR assays to detect the existence of this pathogen in the Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) populations in the Allegheny and Susquehanna River drainages of New York and Pennsylvania. Chytrid is most often tested by using skin swabs, but in this study, tail clips, dorsal skin, blood and eggs were tested as well. *Batrachochytrium dendrobatidis* was detected in tail clips in this study, although the tail clip samples seemed to have lower *Bd* detection sensitivities and concentrations compared with swab samples. Only three out of 41 samples that had tested positive for swabs also tested positive for tail clips, and very small tail clip samples did not result in chytrid positives, despite a relatively high known rate of infection in Pennsylvania. *Batrachochytrium dendrobatidis* was detected in 8.5% of NY and 2.4% of PA tail clip samples (out of 124 total) and from tail clips taken as early as 2004 in the NY Allegheny River drainage. This shows that archival samples, often available for genetic testing, may also be used for *Bd* detection. The *Bd* positive rate from swab samples (25 in total) was 56.0% in NY.

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# INTRODUCTION

## Chytridiomycosis

Since the late 20th century, one of the most urgent environmental problems is the global decline of amphibian populations. The 2004 Global Amphibian Assessment (IUCN Red List) found that 32% of amphibian species are threatened, and that amphibians should be considered the most threatened class of vertebrates on the IUCN Red List. The amphibian declines should get our attention because amphibians are thought to be an indicator organism of global environmental health. The possible causes for this sharp decline can be sorted into two types (Collins and Storfer 2003). Type I hypotheses represent the causes that have been in existence for over a century, such as alien species, over-exploitation and land use changes. Type II hypotheses include the more recent causes, which began during the middle of 20th century, including global change (global climate change and UV radiation), contaminants and emerging infectious diseases (Collins and Storfer 2003). Understanding the causes for amphibian declines is very important in order to seek solutions for the amphibian conservation.

Among a variety of hypotheses on the causes of global amphibian declines, the impact of emerging infectious diseases has drawn attention. Two infectious diseases, chytridiomycosis (phylum, Chytridiomycota; class, Chytridiomycetes; order, Chytridiales; genus, *Batrachochytrium*) and iridoviral infections (family, Iridoviridae; genus, *Ranavirus*) were found to result in the amphibian mass deaths in Australia, the United Kingdom, and North and Central America (Daszak et al. 1999). A study of sixty-four amphibian morbidity and mortality events in the U.S., as well as some other review studies, showed these emerging infectious diseases were playing important roles in the amphibian declines (Green et al. 2002; Skerratt et al. 2007). My research focuses on the study of fungal chytridiomycosis.

Chytridiomycosis was first described as an "amphibian pathogen" in 1998 from sick and dead adult anurans collected in the rain forests of Australia and Panama during mass mortality events accompanied by remarkable population declines (Berger et al. 1998). Two amphibian chytrid fungi, *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* sp. nov. (*Bs*) were possible pathogens for this disease (Longcore et al. 1999; Martel et al. 2013). *Batrachochytrium dendrobatidis* was considered the only species of the genus *Batrachochytrium* until the second species, *B. salamandrivorans*, was isolated from the skin of fire salamanders in the Netherlands in 2013 (Berger et al. 1998; Martel et al. 2013).

Fungi in the phylum Chytridiomycota (chytrids) are aquatic, heterotrophic, and ubiquitous (Berger et al. 1998). They can be found in moist and aquatic habitats such as lakes, bogs, moist soils, and mud puddles, and usually act as primary degraders or saprobes (Powell 1993; Berger et al. 1998). Chytrids have simple thalli and tiny zoospores that are highly mobile (Powell 1993). Chytrids can develop without hyphae, and they can degrade cellulose, chitin and keratin (Berger et al. 1998; Daszak et al. 1999). Parasitic chytrids can parasitize protists, fungi, algae, higher plants, and invertebrates while the amphibian is the only vertebrate they can infect (Powell 1993; Berger et al. 1998; Daszak et al. 1999). *Batrachochytrium salamandrivorans* and *Bd* are the only two species within parasitic chytrids that can infect vertebrates (Daszak et al. 1999; Martel et al. 2013). *Batrachochytrium salamandrivorans* has been found to be highly pathogenic and restricted to Urodela (salamanders and newts), however, it has not yet been found in the United States (Martel et al. 2014; Bales et al. 2015). Recent research showed that *Bs* originated in Asia where no obvious disease was found, but the disease broke out in Europe after its introduction there in 2010 (Martel et al. 2013;

Martel et al. 2014). Compared with *Bs*, *Bd* has been found distributed more broadly in amphibians all over the world (Martel et al. 2014; Bales et al. 2015; *Bd*-Maps).

Chytridiomycosis is an epidermal disease that causes the reddening, shedding (the shedding skin was usually described as gray-white, opaque, and tan), thickening, or even hemorrhages of skin; anorexia, convulsions, and a loss of righting reflex (Daszak et al. 1999; Nichols et al. 2001; Whittaker and Vredenburg 2011). *Batrachochytrium salamandrivorans* infects the keratinized epidermal cells of amphibians and causes a hyperkeratotic and hyperplastic response (enlargement or overgrowth of an organ or part due to increase in size of its constituent cells of the stratum corneum and stratum granulosum (Boyle et al. 2004). Skin is very important for amphibians to maintain homeostasis, take in nutrients, release toxins and breathe (Whittaker and Vredenburg 2011). One hypothesis for the pathogenicity of *Bd* is that in diseased individuals, epidermal electrolyte transport can be inhibited more than 50%, which can lead to the disruption of cutaneous function and eventual death (Voyles et al. 2009). The other explanation is that a fungal toxin is absorbed systemically in the infected amphibians (Daszak et al. 1999). These two factors may also work together.

There are two stages in the lifecycle of *Bd*; the substrate-dependent and substrate-independent (Figure 1; Rosenblum et al. 2008). *Batrachochytrium dendrobatidis* zoospores are critical in initiating the infection of amphibian tissues. They are flagellated, free-living and substrate-independent in nature. However, these zoospores have a relatively short activity period and travel relatively short distances (Piotrowski et al. 2004). Zoospores have chemotactic ability so that they have a tendency to colonize on appropriate substrates (Moss et al. 2008). The substrate-dependent portion of the life cycle begins once a zoospore encysts. Germlings (young zoosporangia) develop into zoosporangia and can produce additional zoospores. Mature zoospores are released to

the surrounding aquatic environment or can reinfect the same substrate (Berger et al. 2005a). Sporangia of *Bd* could normally be found in the stratum corneum and stratum granulosum tissues and skin smears in infected amphibians, and in the keratinized mouthparts of tadpoles (tadpoles lack epidermal keratin) (Berger et al. 1998; Daszak et al. 1999). *Batrachochytrium salamandrivorans* can grow in a wide range of temperatures, from 4 ℃ to 25 ℃, and it grows optimally from 17 ℃ to 25 ℃ (Piotrowski et al. 2004). *Batrachochytrium salamandrivorans* does not grow well above 25 ℃ and under 4 ℃ (Piotrowski et al. 2004). Lenker et al. (2014) showed that the highest average infection intensities occurred in May and October, whereas the lowest average infection intensity occurred in July in New York.

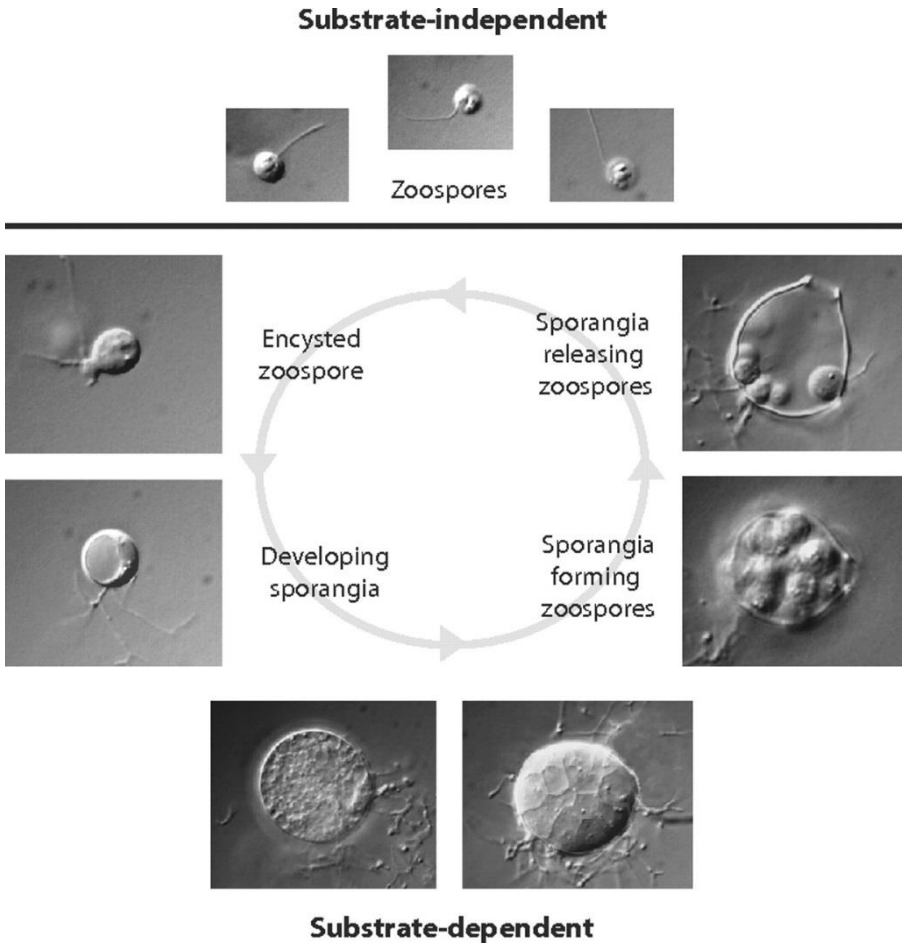


Figure 1. The lifecycle of *Batrachochytrium salamandrivorans* (Rosenblum et al. 2008). Permission granted by PNAS, copyright 2008 National Academy of Sciences, U.S.A.

The impact of *Bd* on an amphibian population could be affected by many factors. According to a model developed by Daszak et al. (1999), *Bd* is more likely to cause severe disease in the amphibian species that are stream-breeding habitat specialists, have low fecundity, and occur in montane regions (Figure 2). Some environmental cofactors such as increased UV-B, chemical pollution, climate change, or stress may predispose amphibian populations to opportunistic pathogens (Daszak et al. 1999).

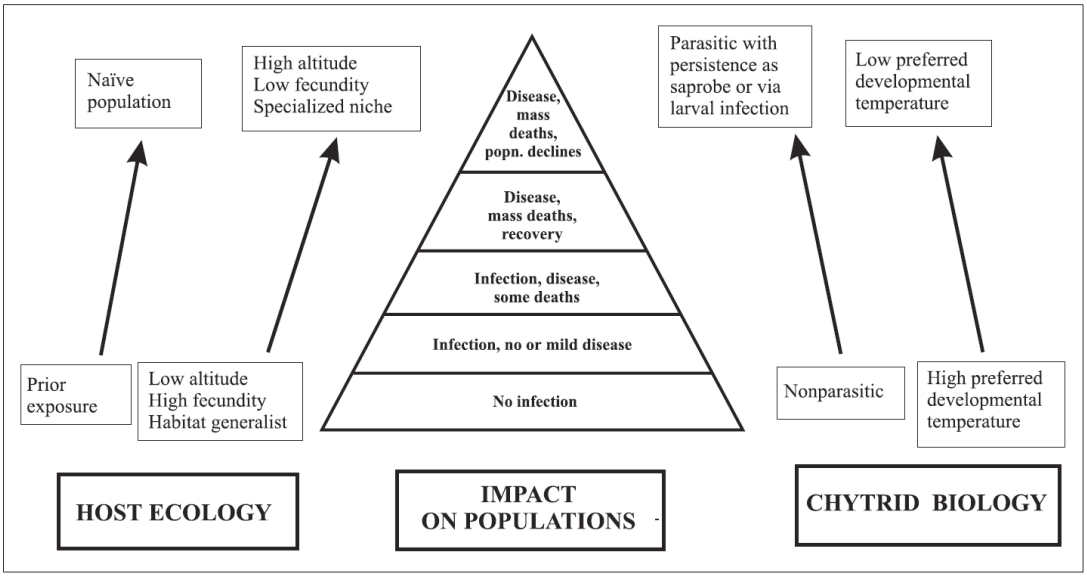


Figure 2. A model of the range of disease outcomes in populations of amphibians affected by *Batrachochytrium dendrobatidis* (Daszak et al. 1999) In this model, host ecologic traits and parasite biologic traits combine to produce declines in a specific group of amphibian species that have low fecundity, are stream-breeding habitat specialists, and occur in montane regions. These characteristics predispose them to population declines after introduction of a waterborne pathogen with a low preferred developmental temperature and ability to persist at low host population densities (Figure permission of EID).

The Global *Bd*-Mapping Project is a database that is constantly up-dated providing the locations of worldwide studies of *Bd*. A *Bd* global map shows the global prevalence of this disease (Figure 3; *Bd*-Maps). Currently, *Bd* has been found in all continents except Antarctica, including 56 out of 82 (68.3%) countries and across the three orders

of amphibians. It has been found in 520 out of 1252 (41.5%) tested species, including families of frogs, toads, and salamanders (*Bd*-Maps; Fisher et al. 2013). In the U.S., *Bd* has been detected in many states, however, there are very few studies of *Bd* prevalence in New York, and no positive results have been recorded in this map to date (Figure 3).

Similar to the debate about the reasons for global amphibian declines, two hypotheses are suggested for the origin and global spread of *Bd*; the "novel pathogen hypothesis (NPH)" (Skerratt et al. 2007) and the "endemic pathogen hypothesis (EPH)" (Rachowicz et al. 2005). The novel pathogen hypothesis states that *Bd* was recently introduced to the areas where it is causing population declines, whereas the endemic pathogen hypothesis claims that *Bd* has been a long-term endemic pathogen and population declines are more likely due to recent environmental changes, pathogen virulence, or changes in host susceptibility (Kilpatrick et al. 2010). The earliest case for chytridiomycosis was found in *Xenopus laevis* frog specimens in 1938 from Africa, where *Bd* showed stable endemic infections (Weldon et al. 2004). It is believed by some researchers that this disease originated from Africa, and spread to other places during the international trade in *X. laevis*, which began in the mid-1930s (Weldon et al. 2004). NPH is supported by the patchy distribution of *Bd* (Figure 3) and the idea that amphibian world trade is driving the spread of chytridiomycosis (Fisher and Garner 2007). EPH holds the idea that since *Bd* had already been found in amphibian populations decades before the huge declines, the sudden occurrence of chytridiomycosis is related to the environmental variables that have occurred recently (e.g. global climate change).

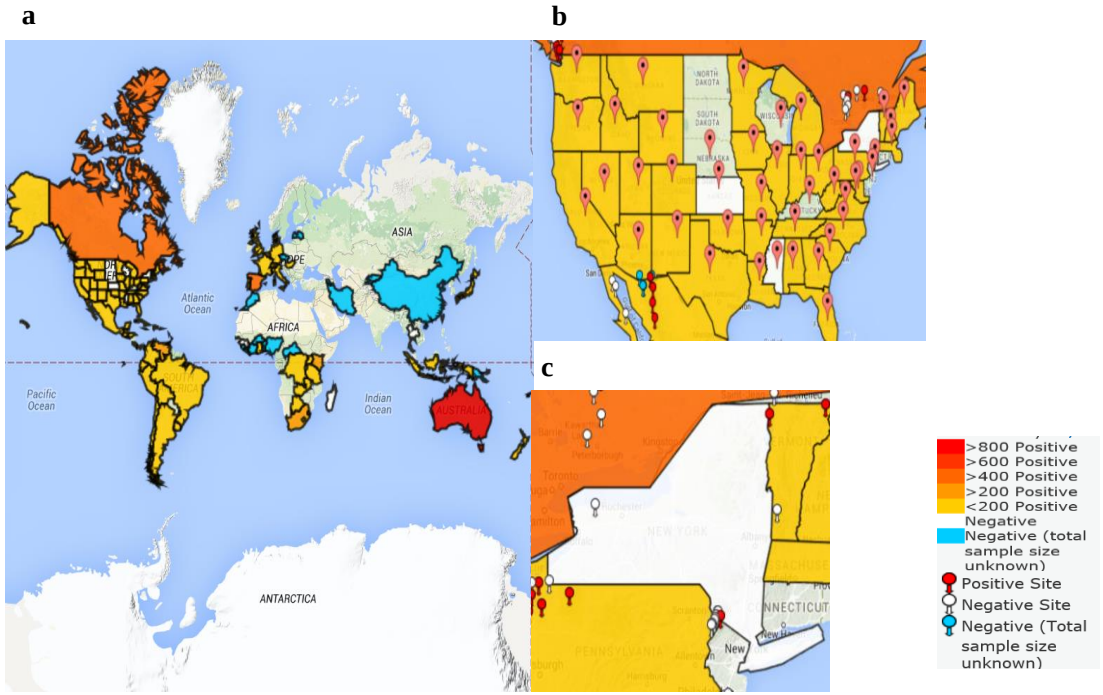


Figure 3. *Batrachochytrium dendrobatidis* (*Bd*) distribution. a. Global distribution of *Bd* by the Global Mapping Project (*Bd*-Maps). Different colors represent different positive quantities. b. The distribution of *Bd* in the U.S. c. The distribution of *Bd* in the New York State. The red dots represent the infected localities while the white and blue dots represent the negative sites.

Generally, there are two methods to detect this disease: one is the histological examination developed by Berger et al. (2002) and the other is the real-time Taqman PCR (qPCR) assay developed by Boyle et al. (2004). Histological examinations usually include the necropsies of animals, identification of *Bd* lesions, immunoperoxidase (IPX) staining, and the morphology of fungal zoospores (Berger et al. 2002). Histological examinations usually take a relatively long time, and require the whole body or a large part of the animal, which will affect the survival of diagnostic subjects and hence is disadvantageous for the conservation of amphibians. In comparison, the qPCR method is less invasive, for it only needs very small parts (tail clips, toe clips; Boyle et al. 2004) or swabs (Kriger et al. 2006a) of the individuals. It can also be used to test hundreds of samples in a single run of only two hours and it is easier to set up a unified quantitative standard (copy numbers of the target DNA per reaction) than the histology method. Studies have also shown that qPCR has a higher sensitivity, specificity, repeatability and reproducibility than histological methods in *Bd* detection (Boyle et al. 2004; Kriger et al. 2006a; Hyatt et al. 2007). Overall, qPCR is undoubtedly more convenient, with a higher accuracy and provides a more rapid way to detect *Bd*.

Various sampling types have been used in the qPCR detection assay including bathing (bathing the animals for 30 minutes) (Hyatt et al. 2007), swabbing (Hyatt et al. 2007), and toe clipping (Boyle et al. 2004). Of these, swabbing is the most common sampling type for *Bd* detection. *Batrachochytrium dendrobatidis* zoospores colonize on the stratum corneum, and are usually abundant in the ventral abdomen, hind limbs and feet of frogs (Longcore et al. 1999; Berger et al. 2005b). Swabbing from these places increases the likelihood of detection of *Bd* zoospores. Swabbing is preferred by researchers because it is more convenient, sensitive and non-invasive (Kriger et al. 2006b; Hyatt et al. 2007). However, a recent study showed individual bias exists on the

swabbing experiences done by different swabbers (Simpkins et al. 2014). Toe clips and bathing are also effective methods for the qPCR detection, however toe clips are not ideal sampling types for some ethical issues (will cause the animals unnecessary levels of pain, stress and disease), and bathing is inconvenient for the field work (Hyatt et al. 2007). To my knowledge, tail clips from salamanders have not been used for *Bd* detection. Tail clips require fewer sampling skills and do not have major negative side effects to the animals (Foster 2006; Jensen 2013). This study examined if it is possible to detect *Bd* in some other sample types, such as tail clips, dorsum tissue, blood, and eggs.

The Hellbender (*Cryptobranchus alleganiensis*)

The hellbender (*Cryptobranchus alleganiensis*) is a large and fully aquatic salamander that is endemic to the eastern United States (Petranka 1998). The hellbender is characterized by the dorsoventrally flattened body and head, keeled tail, and folded skin along each side. Its body is usually brown or gray with varying amounts of dark spots. It has small eyes without eyelids and a single open gill slit on each side of its body (Figure 4). Adult hellbenders range in size from 30 to 74 cm total length (Petranka 1998). Hellbenders usually have long lifespans; it takes 5-8 years to reach sexual maturity, and they can live more than 25-30 years in the wild (Petranka 1998). Their breeding season begins in mid-August and lasts until mid-September, during which they may move short distances to reach the breeding sites (Mayasich et al. 2003). A single female hellbender can deposit between 200 and 400 eggs, and several females may deposit eggs in the same nest (Mayasich et al. 2003). Hellbenders generally live in cool, fast-flowing, well oxygenated and rocky streams (Petranka 1998; Mayasich et al. 2003; NYSDEC). They use large flat rocks, logs or boards for cover and nests

(NYSDEC). Hellbenders mainly use their folded skin for cutaneous gas exchange instead of pulmonary respiration (Mayasich et al. 2003).

Figure 4. Eastern Hellbender physical characteristics (photo used with permission by John White).

There are two subspecies of the hellbender, the Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis)* and the Ozark hellbender (*Cryptobranchus alleganiensis bishopi*). The Ozark hellbender only exists in southeastern Missouri and adjacent Arkansas while the Eastern Hellbender exists more widely; from southern New York to northern Georgia with a west edge of Missouri (Figure 5; NYSDEC). The Ozark hellbender is an endangered species (Environmental Conservation Online System). The Eastern Hellbender is listed as endangered in Maryland, Ohio, Illinois and Indiana; is threatened in Alabama, and is listed as a special concern species of New York (NYSDEC).

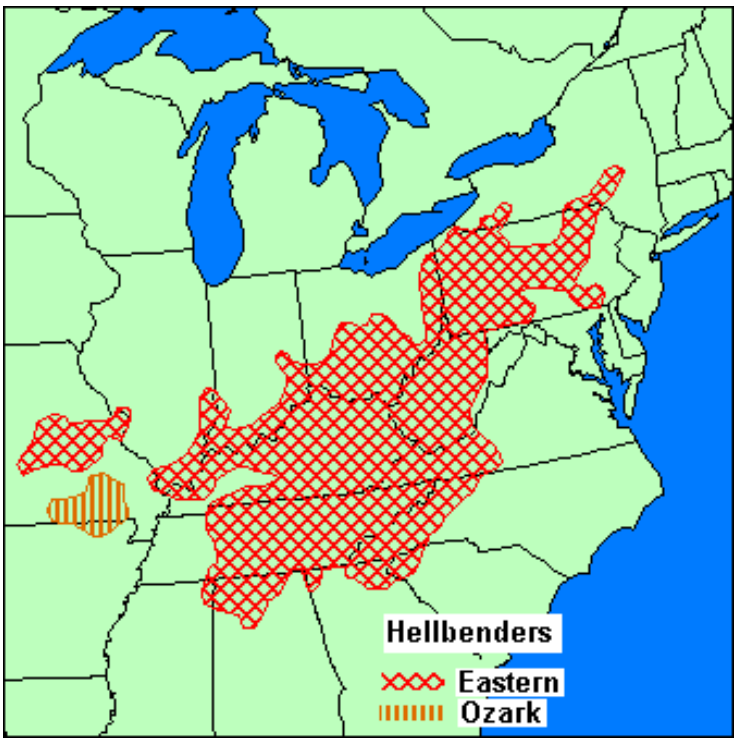


Figure 5. Range of the Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) and the Ozark Hellbender (*Cryptobranchus alleganiensis bishopi*) (NYSDEC; map used with permission of NYSDEC). Cross lines indicate the range of the Eastern Hellbender and parallel lines are the range of the Ozark hellbenders.

Just like many amphibians, hellbender populations have been declining widely and sharply for decades, including in the Missouri River Drainage (Wheeler et al. 2003), Allegheny River Drainage (Foster et al. 2009), and southern Indiana (Burgmeier et al. 2011). In New York State, the hellbender exists only in the Allegheny and Susquehanna drainage basins (Figure 6; NYSDEC). Research conducted by Foster (2006) has shown apparent declines of the Eastern Hellbender in the Allegheny River drainage of New York in the past twenty years (Foster 2006; Foster et al. 2009). More recent work indicates that the New York Susquehanna populations may be functionally extirpated (Foster pers. comm.). Specific reasons for this decline are still not well-defined. Potential threats to hellbenders are habitat loss (siltation, changes in water quality, and water impoundment), overutilization by people (for education, entertainment or

commercial purposes), predation, lack of reproduction and recruitment, and some environmental issues (Mayasich et al. 2003).

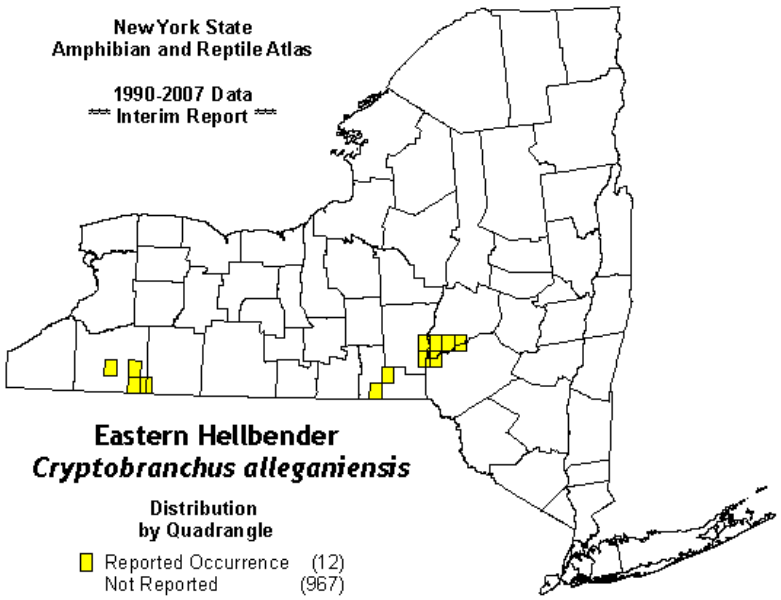


Figure 6. Eastern Hellbender distribution map in New York State as reported by the Amphibian and Reptile Atlas Interim Report (NYSDEC; map used with permission of NYSDEC). Data collection was from 1990-2007.

The occurrence of *Bd* infection in the Ozark hellbender in 1969 in Missouri is the earliest reported occurrence in hellbenders (Bodinof et al. 2011). *Batrachochytrium dendrobatidis* has also been found in hellbender populations of Georgia (Gonynor et al. 2011), Arkansas (Briggler et al. 2008), Eastern Tennessee (Souza et al. 2012), Ohio (Bales et al. 2015), Virginia (Bales et al. 2015), Kentucky (Lipps 2009; *Bd*-Maps), Pennsylvania (Regester et al. 2012; Bales et al. 2015) and New York (Bales et al. 2015) (Figure 7).

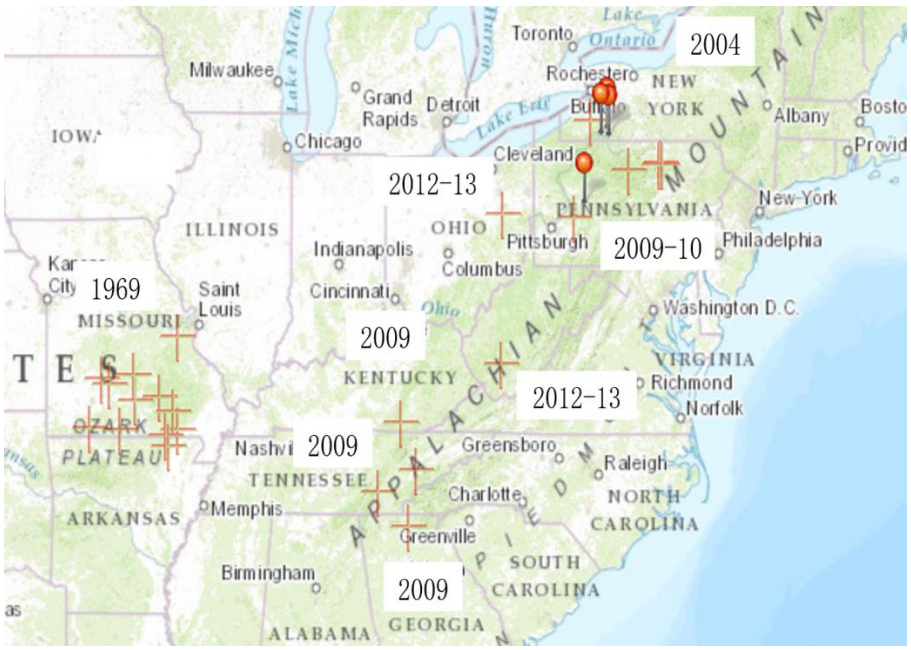


Figure 7. Map of former studies about *Batrachochytrium dendrobatidis* prevalence on hellbenders. Red crosses indicate *Bd* (+) locations, with the earliest year of the positive samples found in relevant state. Red pins show the positive sites in this study, with an earliest year of 2004. Map was developed by ArcGIS online (ArcGIS Online) using details of *Bd* sites from Briggler et al. 2008; Lipps 2009; Gonynor et al. 2011; Souza et al. 2012; Regester et al. 2012; Bales et al. 2015.

There have been very few studies about *Bd* prevalence in NY. In this study, qPCR assays were applied to detect the existence of *Bd* in the Eastern Hellbender samples collected between 2003 and 2014 in the Allegheny River and Susquehanna River watersheds of New York and Pennsylvania, to partially explore the prevalence of *Bd* in NY and PA and the efficacy of using archived tail clip samples for historical detection. This study could also contribute to the conservation of hellbenders (*C. alleganiensis*). This salamander exists only in a few states in the U.S., so that not many groups are doing the relative studies. This study would undoubtedly provide some information for the epidemicity of chytridiomycosis in this animal, and its relationship with population declines.

## OBJECTIVES

The objectives of this study are to:

1) First utilize archived samples collected for genetic analysis and determine if they can be used for *Batrachochytrium dendrobatidis* detection in hellbenders.

2) If these samples are viable for *Bd* detection, provide baseline data of *Bd* prevalence in the Allegheny and Susquehanna River watersheds in New York and Pennsylvania.

3) Compare *Bd* detection sensitivities among different sample types, including blood samples, swabs, tissue samples (tail clips and dorsum skin samples), and eggs.

# MATERIALS AND METHODS

## Samples and Positive controls

A total of 157 hellbender samples including skin tissues (both tail and dorsum), swabs, blood, and eggs collected between 2004 and 2014 were provided by different groups (Table 1). Most of the samples were collected between June and September. Negative samples were swab samples from *Xenopus laevis* frogs housed in the SUNY Buffalo State Biology Department and $ddH_2O$.

Table 1. Sample sources and sample types

| Sources | Sample types |
|---|---|
| Robin Foster and Meghan Jensen, SUNY Buffalo State | 59 tail clips, 22 swabs, 5 blood samples, 1 egg sample |
| Elizabeth Marion Bunting, Cornell University | 2 tail tissues, 2 dorsum skin |
| Peter Petokas, Lycoming College | 18 tail clips |
| Kenneth Roblee, NYSDEC | 21 tail clips, 3 swabs |
| Tom Hayes, Pittsburgh Zoo | 2 tail clips |
| Eric Chapman, Western PA Conservancy | 22 tail clips |

Four areas within the Allegheny River and Susquehanna River drainages in New York (NY), and Pennsylvania (PA) were studied (Figure 8). Blood samples were collected if a hellbender bled during the PIT (Passive Integrated Transponder) tag insertion by blotting on a filter paper and allowing to air dry. These samples were stored at room temperature until DNA extraction. Tail tissue samples were clipped from the end of the captured hellbenders' tails with sterile scissors and placed in alcohol (Foster 2006). Swab samples were collected following a surface swabbing protocol (Appendix A). Swab and tail clip samples were immediately placed on ice after collection in the field and kept cold until they were further processed in the lab.

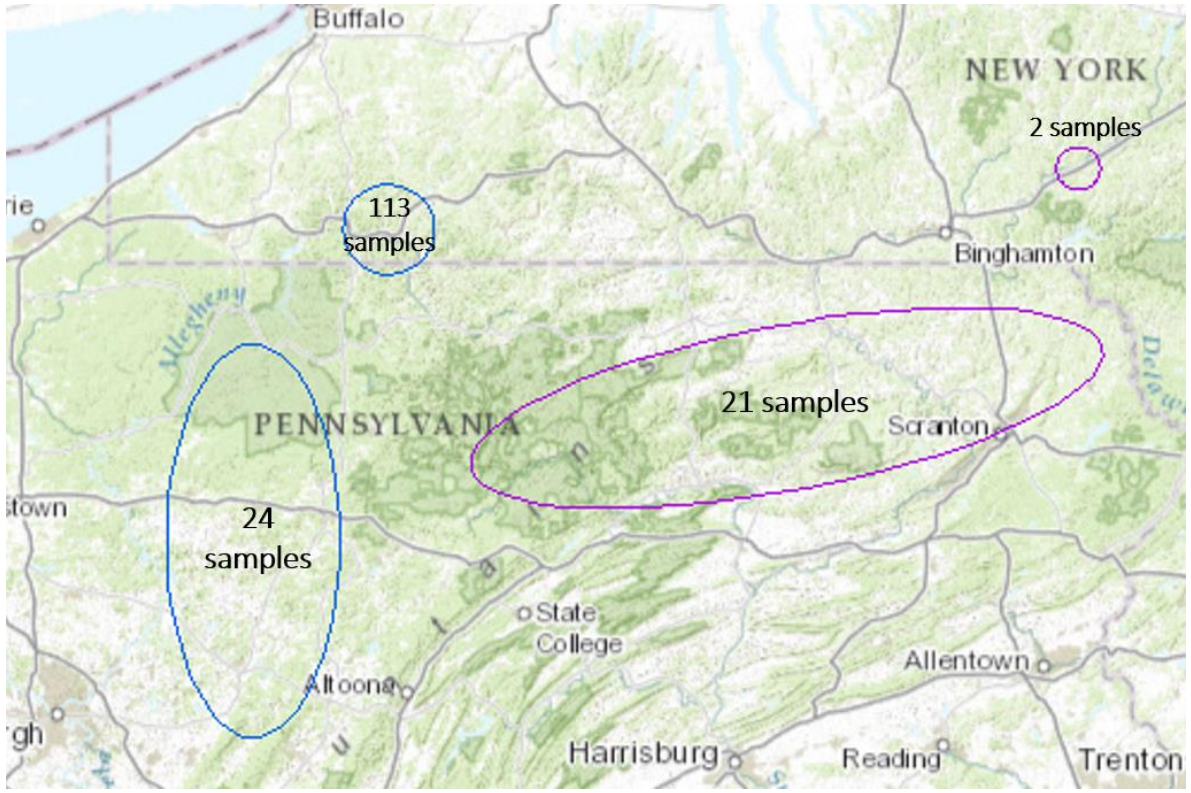


Figure 8. Map of study areas in New York and Pennsylvania. Blue circles indicate the Allegheny River drainage, purple circles indicate the Susquehanna River drainage. Numbers are the number of samples collected in each area. Map was developed by ArcGIS online (ArcGIS Online).

DNA was extracted using a modified extraction protocol of the DNEasy Blood and Tissue kit by Qiagen (Valencia, CA, Appendix B, C). Quantitation of DNA was then performed on the BioRad VersaFluor Fluorometer or the Implen P-Class Nano Photometer (Foster 2006; Jensen 2013). DNA was placed in 1X TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0) and stored at 4 °C.

Real time PCR (qPCR) Method

The real time PCR (qPCR) method developed by Boyle et al. (2004) was performed for the samples. The ChytrMGB2-probe, primers ITS1-3 and Chytr5.8S were used to amplify IST-1/5.8S junction of rDNA to detect *Batrachochytrium dendrobatidis* (Table 2; Boyle et al. 2004). Amplification conditions for this reaction were 50 °C for 2 min, 95 °C for 10 min followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min.

A Taqman probe was used in this study. The Taqman probe has a gene-specific sequence and can bind the target sequence between the two PCR primers (Life Technologies 2012). The 5' end of the Taqman probe is a "reporter", which is a fluorescent dye that can report the amplification of the target. On the 3' end of the probe is a quencher that quenches the fluorescence of the reporter when the probe is intact. When the probe is damaged during the PCR, the reporter will be released and fluoresce. There are two kinds of Taqman probes, minor groove binder (MGB) and non-MGB. The MGB probe was used in this study, which has a higher specificity compared with the traditional TAMRA probe (Life Technologies 2012).

Table 2. Primers and probe sequences (Boyle et al. 2004).

| Primers | Sequence (5'-3') | Probe Sequence | Target Sequence Length |
|---|---|---|---|
| ITS1-3 Chytr | CCTTGATATAATACAGTGTGCCATATGTC | ChytrMGB2-probe 6FAM-CGAGTCGAACAAAAT-MGBNFQ | 146bp |
| 5.8S Chytr | AGCCAAGAGATCCGTTGTCAAA | | |

A standard curve was developed using linearized synthetic plasmids constructed by Pisces Molecular (Boulder, CO). The plasmid contains the rDNA internal transcribed spacer (ITS) region of *Bd* strain JEL 270. The *Bd* ITS region contains the target sequences for both the forward and reverse primers of the Boyle qPCR *Bd* assays. The plasmid was linearized and serially diluted in 0.1×TE by Pisces Molecular. Dilutions were from $2.1\times10^{6}$ to $2.1\times10^{-2}$ molecules/µL. DNA of two swabs from two *Xenopus laevis* (that were not infected with *Bd*, based on comparing with standards) and dd$H_2O$ were used as negative controls in each run.

Because I used 5 µL of DNA per reaction (Appendix D), the ITS-1 copies for the standard dilutions were from $1.05\times10^{7}$ to $1.05\times10^{-1}$ molecules/reaction (concentrations per microliter times five). After preliminary tests using all of the standard dilutions and some of the samples, a tenfold serial dilution series ranging from 1,050 to 1.05 copies of the target sequence was used as a standard curve. A series of standard dilutions was run on each plate. Each unknown sample was tested in triplicate in three separate runs, and samples were considered positive only if the results were positive at least twice. Samples were run in a 25 µL reaction (Appendix D). Negative controls were 5 µL dd$H_2O$ with the TaqMan Universal Master Mix II (Life Technologies, Grand Island, NY, Appendix D). Negative DNA controls were 5 µL frog swabbed DNA or 5 µL

dd$H_2O$ with the Master Mix. Each reaction plate contained 10% negative controls (half frog-swab negative control and half water negative control).

Assays were performed on a CFX96 Real-Time System, with a C1000 Touch Thermal Cycler (BioRad Laboratories, Hercules, CA). Real-time PCR efficiency, slope, and $R^2$ values were calculated with Bio-Rad CFX Manager V3.1 (BioRad Laboratories), with the baseline-subtracted curve-fit setting. Slope and $R^2$ were calculated with the standard curves determined with the quantitation standards described earlier. Efficiency is calculated as $10^{(-1/slope)}$ -1 (Life Technologies 2012) and indicates how efficient the PCR reaction proceeded based on the concentrations of the standard dilutions. The baseline of the real-time PCR reaction is the signal level during the initial cycles of PCR (usually 3 to 15 cycles), and can be set automatically. The threshold of the reaction is the signal level that reflects a statistically significant increase over the calculated baseline signal. It is usually automatically set as 10 times the standard deviation of the fluorescence value of the baseline (Life Technologies 2012). Thresholds can also be manually defined. Since the target DNA concentration was relatively low for most of the samples, the auto-set threshold was so low that it may lead to some false positives. In this study, the threshold was set manually to be conservative about what was called a positive result. It was set in the middle or slightly above the middle of the geometric phase where all of the amplification curves were straight and parallel to one another (the highest precision) for each run while viewing the data in a logarithm scale (Ask TaqMan). The second threshold setting criteria was that it should be higher than the plateau of any negative water samples (these were all lower than the half-way point of the geometric phase). Pathogen prevalence was calculated using the number of infected animals divided by the total sample size and included 95% binomial confidence intervals.

# RESULTS

A standard curve was generated for each of the 96-well plate reactions (Figure 9). The efficiencies of the reactions were all within 80-110%, with $R^2$ greater than 0.970.

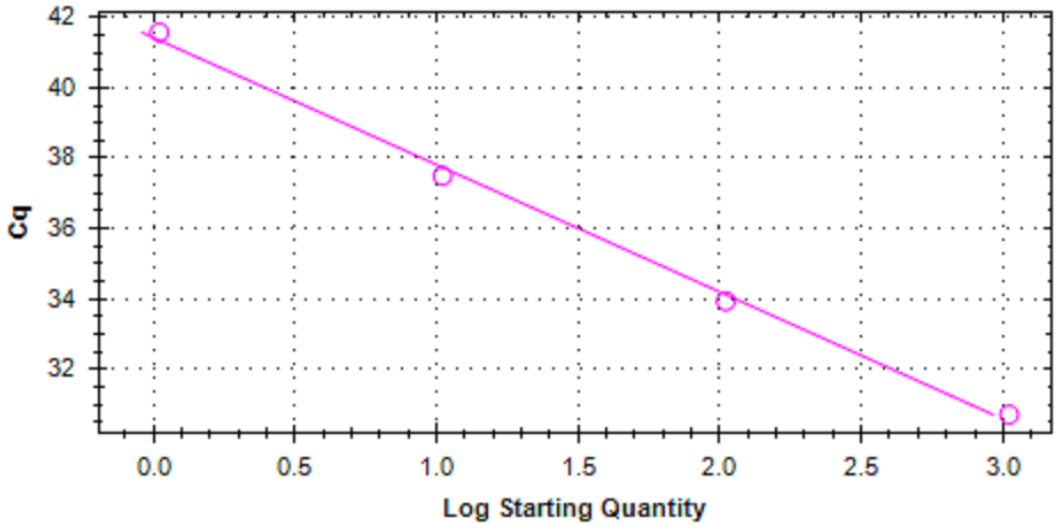


Figure 9. An example standard curve for qPCR reactions. Cq-quantification cycle, cycles needed to reach the threshold. E=89.1%, $R^2$=0.997, Slope=-3.615, y-int=41.432 (for definitions of these numbers, see the Methods section). Each run contained a standard curve.

The amplification curve was used to calculate the DNA content during the PCR reaction (Figure 10). The curves that rose above the manually set threshold were considered positives while the negatives never reached the threshold. The initial DNA concentration was then calculated by its linear relation with Cq. All positive samples were above the threshold for each of three runs (with a relatively low standard deviation; Figure 11).

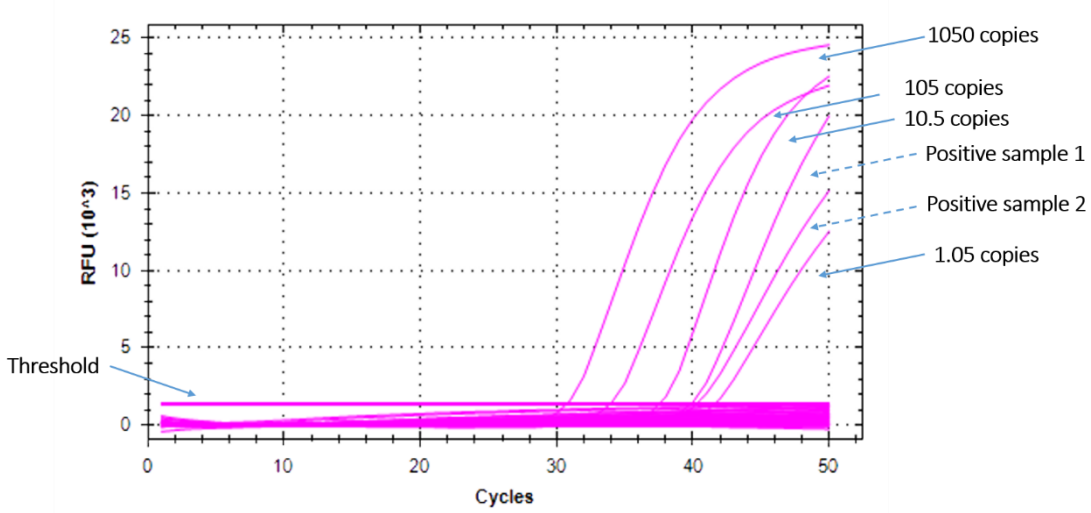


Figure 10. The amplification curve of some of the samples. RFU- Relative Fluorescence Units. This shows two positive samples with four known positive dilutions (initial ITS-1 copy numbers from 1.05 to 1050 per reaction). Solid arrows point the amplification curves of standard dilutions, and dotted arrows point the tested samples.

Twenty-four of 157 (prevalence = 15.3±5.7%, 95% CI) samples were found to be *Batrachochytrium dendrobatidis* positive in this study. The concentration of ITS-1 DNA (copies/μL) in the samples ranged from less than one copy to tens of thousands. Average concentrations of positive samples were converted to napierian logarithm forms as shown in Figure 11. The lowest concentration that could be detected was 0.016 copies/μL (data not shown). Samples with negative natural logarithmic results (No. 18 to 24, Figure 11) were considered as positive suspicions that have a high probability of being positive for the following reasons. First, their concentrations were very low (less than one ITS-1 copy/μL), which might lead to some technical errors. The lowest standard dilutions, which were equivalently low concentrations (0.21 molecules/μL), even turned out to be negative in a few cases. Second, the lab frog swabbed samples turned out to be slightly positive (less than one copy/μL) in a few cases. However, these slightly positive results were not consistent in three runs, which means if the frog swab

turns out to be positive one time this does not mean it will be positive in the next run. And the concentrations of positive samples were all higher than the frog swab concentrations in the same runs, and were at least one copy/µL. Finally, the shape of the amplification curves for these suspects were smooth and performed just like other positives. Although the exact copy number results were not reliable due to the precision of qPCR, they should be considered as positives qualitatively.

The quantification cycles (Cq, cycles needed to reach the threshold) for the tested samples ranged from 24 to 45. ITS-1 gene copy numbers per microliter (for the positive samples) are shown in Appendix E, with 95% confidence intervals. The coefficient of variation (CV) of results varied from 6% to 116%. The coefficient of variation was very high (90%-116%) for samples 22, 23, 24 (the lowest concentration samples) but the CV of most of the others ranged from 6% to 50%.

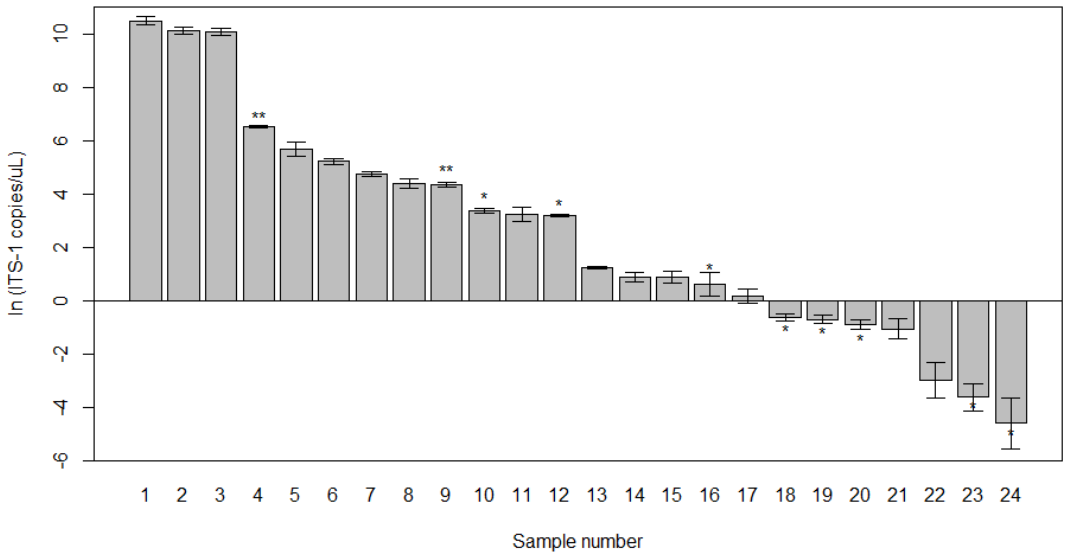


Figure 11. Napierian logarithm of the average ITS-1 concentrations (± standard errors of three runs) for *Batrachochytrium dendrobatidis* positive samples. Samples labeled with two asterisks (**) were dorsal tissues, with one asterisk (*) were tail clips, and the rest were swabs.

Two positive samples were from before 2010 and were tail clips (No. 18 and 23, Table 3); the earliest of these was collected in 2004 in an Allegheny River tributary (No. 18, Table 3). There were a total of 54 samples collected before 2010 with a *Bd* prevalence of 3.7±5.2%, 95% CI and 103 samples collected after 2010 with a *Bd* prevalence of 21.4±8%, 95% CI.

*Batrachochytrium dendrobatidis* positive rate was higher in recent years compared with before 2010, which were all tail clip samples. Where there were both swabs and tail clips in the same time period (post-2010), the positive rate for swabs was higher than that of tail clips (Table 4). No swab samples were available from before 2010.

Most of the available samples were tail clips (124), some were dorsum skin, swabs and blood samples, and only one egg sample (Table 5). Ten out of 126 (95% CI: 8.0±4.3%) tissue samples turned out to be positive for *Bd*, while 14 out of 25 (95% CI: 56.0%±20.5%) swab samples were positive. The two dorsal tissues from dead hellbenders were both *Bd* positive (No. 4 and 9 in Table 3 and Figure 11). None of the blood and egg samples had detectable amounts of *Bd* (Table 4 and Table 5). Over half (14 out of 24) of the positive samples were swabs although over two thirds of the available samples were tail clips. Most of the high *Bd* concentrations were detected in swab samples while tail clip samples have relatively low concentrations (Figure 11). These results suggest that *Bd* is more likely to be detected in swabs and at higher concentrations. However, since the date collected and individuals varies in the tests, further evidence is needed to draw this conclusion.

Table 3. Sampling type, collection date and location for *Batrachochytrium dendrobatidis* positive samples. Allegheny Trib (1-3) and Allegheny main are from the New York Allegheny watershed, Allegheny Trib PA is a tributary in the Pennsylvania Allegheny watershed, Susquehanna Trib is from a tributary in the New York Susquehanna watershed.

| Sample No. | Sample Type | Capture Time | Capture Place |
|---|---|---|---|
| 1 | swab | Sep, 2014 | Allegheny Trib 1 |
| 2 | swab | Sep, 2014 | Allegheny Trib 1 |
| 3 | swab | Sep, 2014 | Allegheny Trib 1 |
| 4 | dorsum | Aug, 2014 | Released to Allegheny Trib 2 |
| 5 | swab | 2013-2014 | Allegheny Trib 2 |
| 6 | swab | 2013-2015 | Allegheny Trib 2 |
| 7 | swab | June, 2013-2014 | Allegheny Trib 2 |
| 8 | swab | Sep, 2014 | Susquehanna Trib |
| 9 | dorsum | Aug, 2014 | Allegheny Trib 2 |
| 10 | tail | Sep, 2014 | Dead animal – exposed to Allegheny main water |
| 11 | swab | Aug, 2013 | Allegheny Trib 2 |
| 12 | tail | Sep, 2014 | Dead animal – exposed to Allegheny main water |
| 13 | swab | June, 2013-2014 | Allegheny Trib 2 |
| 14 | swab | June, 2013-2014 | Allegheny Trib 2 |
| 15 | swab | June, 2013-2014 | Allegheny Trib 2 |
| 16 | tail | Aug, 2012 | Allegheny Trib 3 |
| 17 | swab | Aug, 2013 | Allegheny Trib 2 |
| 18 | tail | Sep, 2004 | Allegheny Trib 2 |
| 19 | tail | Aug, 2012 | Allegheny main |
| 20 | tail | Sep, 2012 | Allegheny Trib PA |
| 21 | swab | Aug, 2013 | Allegheny Trib 2 |
| 22 | swab | June, 2013-2014 | Allegheny Trib 2 |
| 23 | tail | Sep, 2005 | Allegheny Trib 2 |
| 24 | tail | Aug, 2012 | Allegheny main |

Table 4. Results of *Batrachochytrium dendrobatidis* detection classified by year and sample types. P-Positives, N-Negatives. Positive rates were in the parentheses.

| | Total Number | Tail | | Swab | | Dorsal tissue | | Others (Blood and Egg) | |
|---|---|---|---|---|---|---|---|---|---|
| | | P | N | P | N | P | N | P | N |
| Pre-2010 | 54 | 2(4.2%) | 46 | 0 | 0 | 0 | 0 | 0 | 6 |
| Post-2010 | 103 | 6(7.7%) | 72 | 14(56%) | 2(100%) | 0 | 0 | 0 | 0 |

Table 5. Number of *Batrachochytrium dendrobatidis* positives and negatives for different sample types. P-Positives; N-Negatives.

| | Total | P | N |
|---|---|---|---|
| Tail Clip | 124 | 8 | 116 |
| Dorsal Tissue | 2 | 2 | 0 |
| Swab | 25 | 14 | 11 |
| Blood | 5 | 0 | 5 |
| Egg | 1 | 0 | 1 |

To further explore the difference among different sample types, for 41 individuals, both tail clip and swab samples were collected. Researchers at Cornell University used the same qPCR method to test the swabs, where the same animal tissue samples were tested in this study. The sensitivities for these two sample types were different. For 43.9% samples, swabs and tail clip turned out to have the same results, which means that results were both positive or both negative for the two sampling types; twenty-one of the swab samples were positive while the tissue samples showed negative results; two tail clips were positive (low concentrations) while no *Bd* was detected in the swab samples (Table 6; No. 19 and 24, Figure 11). Overall, the *Bd* positive rate for these samples using tissue samples was 12.2% (5 out of 41); the *Bd* positive rate for these samples using swabs were 58.5% (24 out of 41). An additional two individuals had both dorsal tissues and swab samples, and for both of these the results were *Bd* positive.

Table 6. Comparison of *Batrachochytrium dendrobatidis* results for both swabs and tail clips. P-Positives; N-Negatives.

| | P-tail | N-tail |
|---|---|---|
| P-swab | 3 | 21 |
| N-swab | 2 | 15 |

Including all of the sampling types, *Bd* exists in the Allegheny River drainage of NY (prevalence = 19.5%, 22 out of 113) and PA (prevalence = 4.8%, one out of 21),

and in the Susquehanna River drainage of NY (prevalence = 50%, one out of two), but not PA (21 samples tested). By the detection of only tail clip samples, *Bd* prevalence in NY was 8.5 ±6.1% (seven out of 82; 95% CI); in PA was 2.4 ±4.8% (one out of 42; 95% CI). By the detection of swab samples, *Bd* prevalence was 56.0 ±20.5% (14 out of 25; 95% CI) in NY. The *Bd* prevalence distribution by river drainages is shown in Figure 12 a and b. In the Allegheny River drainage, New York, seven out of 81 samples were positive (8.6%); In the Allegheny River drainage, Pennsylvania, one out of twenty-one samples (4.8%) turned out to be *Bd* positive; no positives were found in the Susquehanna river drainage in NY and PA by testing the tail samples (Figure 12a). As for the swab samples, 13 out of 24 (54.2%) samples were positive in the Allegheny River basin, one (100%) sample was positive in the Susquehanna River basin (Figure 12b). The positive rate in NY detected with swab samples was much higher than it with tail clips.

Most of the *Bd* positive animals did not show significant clinical signs such as shedding skin, discoloration, or lethargy when they were caught (Foster, DEC and Cornell University pers. comm.). Only one was found lethargic and markedly blue in color. Four of the hellbenders were known dead (sampling was done after death), and the dead all tested as *Bd* positive (No. 1, 2, 10, 12, Figure 11).

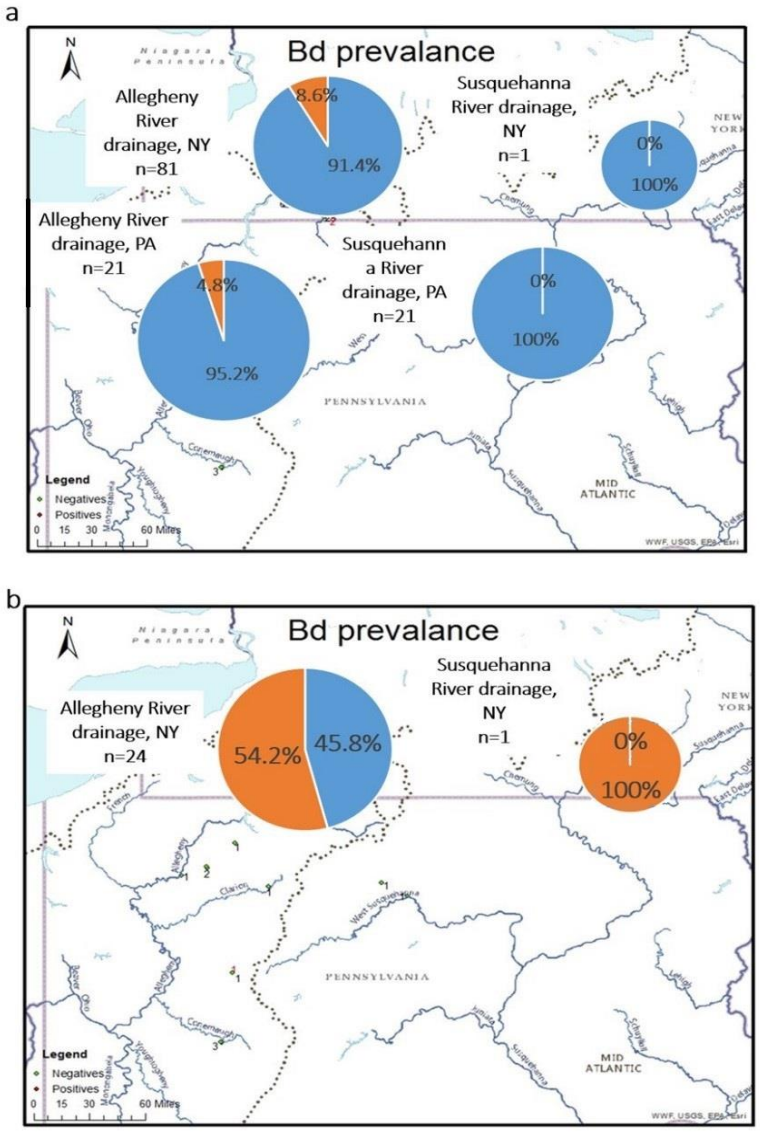


Figure 12. Map of *Batrachochytrium dendrobatidis* prevalence on Eastern Hellbenders in Pennsylvania and New York. a. *Bd* prevalence in tail clip samples; b. *Bd* prevalence in swab samples. *Batrachochytrium dendrobatidis* has been found in both the Allegheny and Susquehanna River drainage. Positive and negative proportions are indicated by pie charts. Orange indicates positive proportions while blue indicates negative proportions. Map was developed by ArcGIS Version 10.2.2 on a world hydro basemap.

# DISCUSSION

*Batrachochytrium dendrobatidis* broadly exists in hellbender populations (Figure 7). The earliest record of the occurrence of *Bd* infection in the Ozark Hellbender was in Missouri in 1969 (Bodinof et al. 2011). Recently, several studies have shown broad *Bd* prevalence in hellbenders throughout the U.S., including New York (Bales et al. 2015), Pennsylvania (Regester et al. 2012; Bales et al. 2015), Ohio (Bales et al. 2015), Virginia (Bales et al. 2015), Kentucky (Lipps 2009; *Bd*-Maps), Tennessee (Souza et al. 2012), Georgia (Gonynor et al. 2011), Missouri (Briggler et al. 2008; Bodinof et al. 2011) and Arkansas (Briggler et al. 2008). As for the river drainages involved in this study, Bales et al. (2015) found *Bd* positive hellbenders from swab samples (sampled in 2012 and 2013) in the Allegheny River drainage of NY and PA. Regester et al. (2011) found *Bd* positives in pooled swab samples of the Allegheny-Ohio and Susquehanna River drainage, PA (sampled in 2009 and 2010).

From my study of the hellbender samples from the last ten years (2004-2014), the overall *Bd* positive rate in NY and PA area was 15.3%, with 24 out of 157 samples testing positive. *Batrachochytrium dendrobatidis* has been found in the Allegheny River drainage of PA and NY, and Susquehanna River drainage of NY (Figure 12); no *Bd* positives were found in the Susquehanna River drainage of PA in this study. By comparing the tail samples, the prevalence of *Bd* increased from 4.2% before 2010 to 7.7% after 2010 (Table 4). This may due to the different sample types I used.

The two earliest positive samples I tested were from 2004 and 2005 from the Allegheny River drainage, New York (No. 18 and 23, Table 3, Figure 11). This showed that *Bd* pathogen has existed in the Eastern Hellbender populations in the Allegheny River drainage of NY for at least 10 years. To my knowledge, 2004 is the earliest record of *Bd* existence on the Eastern Hellbender in NY.

Another important finding in this research is some tissue samples can also be used for *Bd* detection. In this study, swabs, blood, eggs, tail clips and dorsum tissues were used for *Bd* analysis. Most of the researchers are now using swabs to test for *Bd* by qPCR. The reason tail clips were used in this study was that all of the hellbender samples in the lab (from before 2010) were archived as tail clips. A limited number of egg and blood samples were tested but *Bd* was not detected in these samples (Table 5). The lack of Bd in the egg and blood samples was expected because *Bd* is an epidermal disease. However, the results of this study show that *Bd* can be detected in dorsum and tail tissues. Dorsum tissues of frogs usually do not contain many *Bd* zoospores, which probably is due to the serous glands that produce antifungal peptides on frog dorsum skin (Berger et al. 2005b). However, hellbenders do not have serous glands, which may increase the existence of *Bd* zoospores in the dorsal tissues. Currently, *Bd* detection in skin tissue samples is still done by histological methods (Bodinof et al., 2011), which requires more time and is not as sensitive as qPCR methods (Hyatt et al. 2007). In contrast, many tissue specimens collected in previous studies can be scanned rapidly for *Bd* using the real-time PCR method.

However, the sensitivities of qPCR *Bd* detection are different among different sample types. Tail clip samples tend to have lower estimations of the *Bd* prevalence compared with swabs. The total positive rate (number of total samples divided by positive numbers) for dorsum tissues was 100% (just two samples), tail clips was 6.5% (8 of 124), and swab samples was 56.0% (14 of 25). To evaluate the detectability of *Bd* from tail clips and swabs, samples from 41 animals from which both swabs and tissue samples had been collected were compared. For this group of individuals, the *Bd* incidence percentage estimated by tail clips was 12.2%, significantly lower than the estimation of 58.5% by the swabs (Table 6). In addition to this, for my study of the

Allegheny River drainage, NY, the *Bd* infection rate estimated by swab was 54.2%, which was much higher than the infection rate estimated by tail clips of 8.6%, despite having many more tail clip samples to test than swab samples (Figure 12). Bales et al. (2015) found a *Bd* prevalence of 10% by using ten swab samples in the same area sampled for the current study. Furthermore, although I did not find any *Bd* positives from the tail clip samples from Susquehanna River drainage in PA, another researcher has found a high *Bd* infection rate of 40.4% in swab samples from the same area (Petokas pers. comm.). One possible factor is that these tail clips were very small tissue samples compared to those collected in the NY drainage (McMillan pers. comm.) and may not contain as much dermal tissue. These issues all indicate that tail clips have lower positive rates compared with swab samples, however, which sample type is more accurate in estimating the real *Bd* infection prevalence rate remains unclear. Even in the positive samples, swabs usually turned out to have higher *Bd* concentrations compared with tail clips (Figure 11). The difference between swabs and tail clips may due to the uneven distribution of *Bd* on the hellbender bodies. *Batrachochytrium dendrobatidis* is usually abundant in the ventral abdomen, hind limbs and feet of frogs, where the animals were swabbed (Longcore et al. 1999; Berger et al. 2005b; Appendix A). Swabbing from these places increases the likelihood of detection of *Bd* zoospores but might not indicate actual infection. Interestingly, the lowest average detected prevalence of *Bd* in NY occurred in July, and remain lowest until September (Lenker et al. 2014), while most of the current study samples were collected from June to September, which may mean the current study samples were at the lowest concentrations of *Bd,* and thus, the lowest detection potential. Lenker et al. (2014) found the highest *Bd* infection peaked in May and October, which suggests tail clips collected during this time might be more effective at *Bd* detection.

Based on my research, future studies can use the museum specimens for *Bd* detection (although the prevalence may be underestimated), which can help researchers have a better understanding of the historical origin of this disease. Specimens stored in alcohol should be used, because DNA for the TaqMan assay cannot be extracted from samples stored in formalin (Hyatt et al. 2007).

The Allegheny watershed (NY) and Susquehanna watershed (PA) both have high *Bd* prevalence based on Petokas (pers. comm.) and my study on the hellbender swab samples. The high *Bd* prevalence in these two river drainages coincide with the hellbender declines that have been found in NY and PA (Foster et al. 2009; Petokas pers. comm.). It is possible, among other potential causes, that *Bd* contributed to the hellbender declines in these areas. *Batrachochytrium dendrobatidis* is known to be related to global amphibian declines, and can cause severe declines to fully-aquatic species with low fecundity (Daszak et al. 1999; Skerratt et al. 2007). The study by Foster (2006) found very few juvenile hellbenders during their breeding season, which probably indicates a low reproductive success for hellbenders in the Allegheny River drainage of New York.

In studies by Pessier et al. (1999) and Bales et al. (2015), no obvious clinical signs and specific body conditions were found for most *Bd* positive individuals. Likewise, for most of the *Bd* positive individuals in this study, no specific *Bd* symptoms were observed at the time they were sampled. Only one hellbender was found lethargic and markedly blue in color. Four of the *Bd* positive animals were known dead, and then sampled for *Bd* detection. One reason for low observed symptoms may be that most of the animals were caught early in their infectionand released after sampling, so the ultimate fate of these animals is unknown. Another possible reason may be that *Bd*

symptoms occur in the later infectious stages, and hellbenders will die soon after severe clinical signs occur (Nichols et al. 2001; Whittaker and Vredenburg 2011).

The qPCR results showed the copy numbers of ITS-1 DNA per microliter, but not actual zoospore numbers. Some former studies transformed the qPCR results to zoospore numbers by dividing them by the ITS-1 copy numbers per zoospore. However, in a recent study, a high strain variability in ITS-1 copy number was found (Longo et al. 2013). ITS-1 copy numbers range from 10 to 144 copies per single zoospore among different *Bd* strains (Longo et al. 2013), which makes it difficult to unify the results among different labs or locations. Using single ITS1 PCR-amplicons as the absolute standard in conjunction with current quantitative assays is a better method to determine copy number variation and provide universal estimates of pathogen zoospore loads from field-caught amphibians (Longo et al. 2013).

The initial ITS-1 copies/reaction (calculated by copies/μL times five, since 5 μL of DNA sample was used per run) for some samples were less than 1 copy/reaction (No. 22-24, Figure 11; Appendix E), which does not have actual meaning. The quantities might not be very reliable for these extremely low concentrations for they have very large CV. But these low positives showed the sensitivity of the qPCR assay, for it can show positive even at very low DNA concentrations. The CV of middle ITS DNA concentration samples (tens and hundreds copies/μL) were small, suggesting that these higher concentrations had more consistent results. The threshold was manually set conservatively (in an attempt to avoid false positives), so that even results with very low ITS-1 concentrations should be considered as positives.

However, a *Bd* positive result does not directly indicate that the positive animals have chytridiomycosis. This is especially true for the positive ones with low ITS-1 DNA concentrations, the animals could just be the carriers of *Bd* zoospores, without

the development of zoosporangia. "Vredenburg's 10,000 Zoospore Rule" suggests that for most amphibian species, animals will die when they reach the *Bd* threshold of 10,000 zoospore equivalents/swab (Briggs et al. 2010; Kinney et al. 2011). Despite the zoospore loads, the susceptibility of different individuals under different conditions also varies, so that more evidence (maybe histological analysis) will be needed to tell if a hellbender was infected. The chytrid susceptibility of an animal could be influenced by species (Blaustein et al. 2005; Bancroft et al. 2011; Searle et al. 2011), environmental temperature (Maniero and Carey 1997; Woodhams et al. 2003), life stage (Lenker et al. 2014), sex, and maybe even other health conditions. Bancroft et al. (2011) found different susceptibilities between species was dependent on body size at maturity, egg laying behavior, taxonomic order and family, and reliance on water. In the same species, the *Bd* susceptibility varies between larvae and adults (Bancroft et al. 2011). As for the environmental factors, cool seasons and high-elevation are more likely to lead to the death of hosts (Bancroft et al. 2011).

An idea for future study is to attempt to detect both *Batrachochytrium salamandrivorans* and *Bd* from environmental DNA (eDNA) samples collected in the Allegheny and Susquehanna watersheds to have a better understanding of the distribution of these pathogens. Recent work on aquatic animals uses eDNA for detection of some rare species (Environmental DNA). Environmental DNA is DNA found in the environment. This is based on the fact that all aquatic animals can leave DNA in the water through feces, urine or skin cells (Environmental DNA). This DNA dissolves in the water and becomes diluted as it spreads over a larger area. *Batrachochytrium dendrobatidis* zoospores may also be detected in these water samples. The McMillan Lab at Buffalo State collected extensive eDNA samples throughout both of the Allegheny and Susquehanna watersheds in NY and PA during the summers of

2014 and 2015. Samples were collected by using the protocol developed by Santas et al. (2013). Water was collected from the stream, run through a fine filter, and total DNA was extracted from the filters for each sample (Foster pers. comm.). A duplex real-time PCR method was developed recently to detect two chytrid pathogens, *Bd* and *Bs* at the same time (Blooi et al. 2013). Future researchers could try the eDNA samples with this duplex real-time PCR to figure out the distribution of *Bd* and *Bs* rapidly.

# CONCLUSION

Some people believe that a sixth major extinction event is underway (Wake and Vredenburg 2008), with a phenomenon of amphibian population declines and extinctions at unprecedented rates. An emerging infectious fungal disease, chytridiomycosis, may be one of the main reasons for these amphibian declines. This disease is mainly caused by *Batrachochytrium dendrobatidis*. In this study, I have detected this pathogenic fungus in the Eastern Hellbender populations from the Allegheny River drainage in New York and Pennsylvania, and the Susquehanna River drainage in New York using a qPCR assay. From this study, I found that *Bd* has existed in the hellbender populations of the Allegheny River drainage in NY from as early as 2004. The existence of this disease may correlate with the apparent hellbender population declines (over the last 20 years) in the Allegheny River drainage of NY (Foster et al. 2009). In addition, I have successfully detected *Bd* in different sample types other than the commonly used swabs. I detected *Bd* in tail clip samples, dorsal tissues, and swab samples but not in eggs and blood samples. Compared with swab samples, tail tissues seemed to be less sensitive in *Bd* detection, and may underestimate the prevalence of *Bd*. In the analysis of 124 tail clips, I found *Bd* prevalence to be 8.5% in NY and 2.4% in PA. In 25 swab samples, I found the *Bd* prevalence in NY of 56.0%. This study has provided some baseline data on the *Bd* prevalence in NY and PA, and will contribute to the future study of the relationship between chytridiomycosis and amphibian population declines in these areas.

# LITERATURE CITED

ArcGIS Online [Internet]. Environmental Systems Research Institute [cited 2015 June 4]. Available from: https://www.arcgis.com/home/.

Ask TaqMan® Video Gallery [Internet]. Thermo Fisher Scientific Inc; c2015 [cited 2015 July 13]. Available from: http://www.lifetechnologies.com/us/en/home/life-science/pcr/real-time-pcr/qpcr-education/ask-taqman/ask-taqman-video-gallery.html.

Bales, E. K., O. J. Hyman, A. H. Loudon, R. N. Harris, G. Lipps, E. Chapman, K. Roblee, J. D. Kleopfer, K. A. Terrell. 2015. Pathogenic chytrid fungus *Batrachochytrium dendrobatidis*, but not *B. salamandrivorans*, detected on Eastern Hellbenders. PLoS ONE 10(2):e0116405.

Bancroft, B. A., B. A. Han, C. L. Searle, L. M. Biga, D. H. Olson, L. B. Kats, J. J. Lawler, A. R. Blaustein. 2011. Species-level correlates of susceptibility to the pathogenic amphibian fungus *Batrachochytrium dendrobatidis* in the United States. Biodivers Conserv 20:1911-1920.

*Bd*-Maps [Internet]. Imperial College London [cited 2015 May 12]. Available from: http://www.bd-maps.net/.

Berger, L., A. D. Hyatt, R. Speare, J. E. Longcore. 2005a. Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. Dis Aquat Org 68:51-63.

Berger, L., A. D. Hyatt, V. Olsen, S. G. Hengstberger, D. Boyle, G. Marantelli, K..Humphreys, J. E. Longcore. 2002. Production of polyclonal antibodies to *Batrachochytrium dendrobatidis* and their use in an immunoperoxidase test for chytridiomycosis in amphibians. Dis Aquat Org 48:213-220.

Berger, L., R. Speare, L. F. Skerratt. 2005b. Distribution of *Batrachochytrium dendrobatidis* and pathology in the skin of green tree frogs *Litoria caerulea* with severe chytridiomycosis. Dis Aquat Org 68:65-70.

Berger, L., R. Speare, P. Daszak, D. E. Green, A. A. Cunningham, C. L. Goggin, R. Slocombe, M. A. Ragan, A. D. Hyatt, K. R. Mcdonalds, H. B. Hines, K. R. Lips, G. Marantelli, H. Parkes. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. PNAS USA 95:9031-9036.

Blaustein, A. R., J. M. Romansic, E. A. Scheessele, B. A. Han, A. P. Pessier, J. E. Longcore. 2005. Interspecific variation in susceptibility of frog tadpoles to the pathogenic fungus *Batrachochytrium dendrobatidis*. Conserv Biol 19:1460-1468.

Blooi, M., F. Pasmans, J. E. Longcore, A. Spitzen-van der Sluijs, F. Vercammen, A. Martela. 2013. Duplex real-time PCR for rapid simultaneous detection of

*Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in amphibian samples. JCM 51(12):4173-4177.

Bodinof, C. M., J. T. Briggler, M. C. Duncan, J. Beringer, J. J. Millspaugh. 2011. Historic occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in hellbender *Cryptobranchus alleganiensis* populations from Missouri. Dis Aquat Org 96:1-7.

Boyle, D. G., D. B. Boyle, V. Olsen, J. A. T. Morgan, A. D. Hyatt. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time taqman PCR assay. Dis Aquat Org 60:141-148.

Briggler, J. T., K. A. Larson, K. J. Irwin. 2008. Presence of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) on hellbenders (*Cryptobranchus alleganiensis*) in the Ozark Highlands. Herpetol Rev 39:443–444.

Briggs, C. J., R. A. Knapp, V. T. Vredenburg. 2010. Enzootic and epizootic dynamics of the chytrid fungus pathogen of amphibians. PNAS 107(21):9695-9700.

Burgmeier, N. G., S. D. Unger, T. M. Sutton, R. N. Williams. 2011. Population Status of the Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) in Indiana. Journal of Herpetology 45(2): 195–201.

Collins, J. P. and A. Storfer. 2003. Global amphibian declines: sorting the hypotheses. Diversity and Distributions 9: 89–98.

Daszak, P., L. Berger, A. A. Cunningham, A. D. Hyatt, D. E. Green, R. Speare. 1999. Emerging infectious diseases and amphibian population declines. Emerging Infectious Diseases 5(6): 735-748.

Environmental Conservation Online System [Internet]. U.S. Fish and Wildlife Service [cited 2015 August 2]. Available from: http://ecos.fws.gov/speciesProfile/profile/speciesProfile?spcode=D032.

Environmental DNA [Internet]. Environmental DNA; c2015 [cited 2015 June 2]. Available from: http://www.environmental-dna.nl/en-us/environmentaldna.aspx.

Fisher, M. C., D. A. Henk, C. J. Briggs, J. S. Brownstein, L. C. Madoff, S. L. McCraw, S. J. Gurr. 2013. Emerging fungal threats to animal, plant and ecosystem health. Nature 484: 186-194.

Fisher, M. C., T. W. J. Garner. 2007. The relationship between the emergence of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species. Fungal Biology Reviews 2 I: 2-9.

Foster, R. L. 2006. A study of the Hellbender Salamander (*Cryptobranchus alleganiensis*) in the Allegheny River drainage of New York state: Examination of population trends, assessment of capture methods, and

development of genetic techniques. Master's thesis: State University of New York Buffalo State.

Foster, R. L., A. M. McMillan, K. J. Roblee, 2009. Population status of Hellbender Salamanders (*Cryptobranchus alleganiensis*) in the Allegheny river drainage of New York state. Journal of Herpetology, 43 (4):579-588.

Gonynor, J. L., M. J. Yabsley, J. B. Jensen. 2011. A preliminary survey of *Batrachochytrium dendrobatidis* exposure in hellbenders from a stream in Georgia, USA. Herpetol Rev 42 (1): 58-59.

Green, D. E., K. A. Converse, A. K. Schrader. 2002. Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996-2001. Ann NY Acad Sci 969: 323-339.

Hyatt, A. D., D. G. Boyle, V. Olsen, D. B. Boyle, L. Berger, D. Obendorf, A. Dalton, K. Kriger, M. Hero, H. Hines, R. Phillott, R. Campbell, G. Marantelli, F. Gleason, A. Colling. 2007. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendribatidis*. Dis Aquat Org 73: 175-192.

IUCN Red List [Internet]. Amphibian Specialist Group and Amphibian Survival Alliance; c2014 [cited 2015 April 6]. Available from: http://www.amphibians.org/redlist/process/.

Jensen, M. 2013. Conservation genetics of New York's giant salamander: the Eastern Hellbender (*Cryptobranchus alleganiensis*). Master's thesis: State University of New York Buffalo State.

Kilpatrick, A. M., C. J. Briggs, P. Daszak. 2010. The ecology and impact of chytridiomycosis: an emerging disease of amphibians. TREE 25(2):109-118.

Kinney, V. C., J. L. Heemeyer, A. P. Pessier, M. J. Lannoo. 2011. Seasonal pattern of *Batrachochytrium dendrobatidis* infection and mortality in *Lithobates areolatus*: affirmation of Vredenburg's "10,000 Zoospore Rule". PLoS ONE 6 (3):e16708.

Kriger, M. K., H.B. Hines, A.D. Hyatt, D. G. Boyle, J.-M. Hero. 2006a. Techniques for detecting chytridiomycosis in wild frogs: comparing histological with real-time taqman PCR. Dis Aquat Org 71:141-148.

Kriger, M. K., J.-M. Hero, K. J. Ashton. 2006b. Cost efficiency in the detection of chytridiomycosis using PCR assay. Dis Aquat Org 71:149-154.

Lenker, M. A., A. E. Savage, C. G. Becker, D. Rodriguez, K. R. Zamudio. 2014. *Batrachochytrium dendrobatidis* infection dynamics vary seasonally in upstate New York, USA. Dis Aquat Org 111: 51-60.

Life Technologies™. 2012. Real-time PCR handbook. 2nd ed.

Lipps, G. 2009 [Internet]. Unpublished. *Bd*-Maps. Imperial College London [cited 2015 May 12]. Available from: http://www.bd-maps.net/maps/.

Longcore, J. E., A. P. Pessier, D. K. Nichols. 1999. *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. Mycologia 91(2): 219-227.

Longo, A. V., D. Rodriguez, D. da Silva Leite, L. F. Toledo, C. M. Almeralla, P. A. Burrowes, K. R. Zamudio. 2013. ITS1 copy number varies among *Batrachochytrium dendrobatidis* strains: implications for qPCR estimates of infection intensity from field-collected amphibian skin swabs. PLoS ONE 8(3): e59499.

Maniero, G. D. and C. Carey. 1997. Changes in selected aspects of immune function in the leopard frog, Rana pipiens, associated with exposure to the cold. J Comp Physiol B 167: 256−263.

Martel, A., A. Spitzen-van der Sluijs, M. Blooi, W. Bert, R. Ducatelle, M. C. Fisher, A. Woeltjes, W. Bosman, K. Chiers, F. Bossuyt, F. Pasmans. 2013. *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. PNAS 110 (38): 15325-15329.

Martel, A., M. Blooi, C. Adriaensen, P. Van Rooij, W. Beukema, M. C. Fisher, R. A. Farrer, B. R. Schmidt, U. Tobler, K. Goka, K. R. Lips, C. Muletz, K. R. Zamudio, J. Bosch, S. L ötters, E.Wombwell, T.W. J. Garner, A. A. Cunningham, A. Spitzen-van der Sluijs, S. Salvidio, R. Ducatelle, K. Nishikawa, T. T. Nguyen, J. E. Kolby, I. Van Bocxlaer, F. Bossuyt, F. Pasmans. 2014. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science 346: 630-631.

Mayasich, J., D. Grandmaison, C. Phillips. 2003. Eastern Hellbender status assessment report. Duluth (MN) and Champaign (IL): Natural Resources Research Institute and Illinois Natural History Survey Center For Biodiversity. NRRI/TR-2003/09.

Moss, A. S. M., N. S. Reddy, I. M. Dortaj, M. J. San Francisco. 2008. Chemotaxis of the amphibian pathogen *Batrachochytrium dendrobatidis* and its response to a variety of attractants. Mycologia 100: 1–5.

Nichols, D. K., E. W. Lamirande, A. P. Pessier, J. E. Longcore. 2001. Experimental transmission of cutaneous chytridiomycosis in *Dendrobatid* Frogs. Journal of Wildlife Diseases 37(1): 1-11.

[NYSDEC] New York State Department of Environmental Conservation [Internet]. New York State Department of Environmental Conservation; c2015 [cited 2015 May 12]. Available from: http://www.dec.ny.gov/animals/7160.html, http://www.dec.ny.gov/animals/44550.html.

Pessier, A. P., D. K. Nichols, J. E. Longcore, M. S. Fuller. 1999. Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and White's tree frogs (*Litoria caerulea*). J Vet Diagn Invest 11:194–199.

Petranka, J. W. 1998. *Salamanders of the United States and Canada*. Washington and London: Smithsonian Institution Press. 139 p.

Piotrowski, J. S., Seanna L. Annis, Joyce E. Longcore. 2004. Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. Mycologia 96:9–15.

Powell, M. J. 1993. Looking at mycology with a Janus face: a glimpse at chytridiomycetes active in the environment. Mycologia 85 (1): 1-20.

Rachowicz, L. J., J.-M. Hero, R. A. Alford, J. W. Taylor, J. A.T. Morgan, V. T. Vredenburg, J. P. Collins, C. J. Briggs. 2005. The novel and endemic pathogen hypotheses: competing explanations for the origin of emerging infectious diseases of wildlife. Conservation Biol 19: 1441-1448.

Regester, K. J., H. Simpson, E. J. Chapman, P. J. Petokas. 2012. Occurrence of the fungal pathogen *Batrachochytrium dendrobatidis* among Eastern Hellbender populations (*Cryptobranchus a. alleganiensis*) within the Allegheny-Ohio and Susquehanna River drainages, Pennsylvania, USA. Herpetol Rev 43(1): 90–93.

Rosenblum, E. B., J. E. Stajich, N. Maddox, and M. B. Eisen. 2008. Global gene expression profiles for life stages of the deadly amphibian pathogen *Batrachochytrium dendrobatidis*. PNAS 105(44):17034-17039.

Santas, A. J., T. Persaud, B. A. Wolfe, J. M. Bauman. 2013. Noninvasive method for a statewide survey of Eastern Hellbenders *Cryptobranchus alleganiensis* using environmental DNA. International Journal of Zoology 2013:1-6.

Searle, C. L., S. S. Gervasi, J. Hua, J. I. Hammond, R. A. Relyea, D. H. Olson, and A. R. Blaustein. 2011. Differential host susceptibility to *Batrachochytrium dendrobatidis*, an emerging amphibian pathogen. Conservation Biology 25(5): 965-974.

Simpkins, C. A., M. V. Sluys, J.-M. Hero. 2014. Swabber effect: swabbing technique affects the detectability of *Batrachochytrium dendrobatidis*. Herpetol Rev 45(2): 443-445.

Skerratt, L. F., L. Berger, R. Speare, S. Cashins, K. R. McDonald, A. D. Phillott, H. B. Hines, N. Kenyon. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. EcoHealth 4: 125–134.

Souza, M. J., M. J. Gray, P. Colclough, D. L. Miller. 2012. Prevalence of infection by *Batrachochytrium dendrobatidis* and *Ranavirus* in Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) in Eastern Tennessee. Journal of Wildlife Diseases 48 (3): 560-566.

Voyles, J., S. Young, L. Berger, C. Campbell, W. F. Voyles, A. Dinudom, D. Cook, R. Webb, R. A. Alford, L. F. Skerratt, R. Speare. 2009. Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. Science 326: 582-585.

Wake, D. B., V. T. Vredenburg. 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proc Natl Acad Sci USA 105:11466–11473.

Weldon, C., L. H. du Preez, A. D. Hyatt, R. Muller, R. Speare. 2004. Origin of the amphibian chytrid fungus. Emerging Infectious Diseases 10 (12): 2100-2105.

Wheeler, B. A., E. Prosen, A. Mathis, R. F. Wilkinson. 2003. Population declines of a long-lived salamander: a 20+-year study of hellbenders, *Cryptobranchus alleganiensis*. Biological Conservation 109: 151-156.

Whittaker, K. and V. Vredenburg. 2011. An overview of chytridiomycosis. AmphibiaWeb: Information on amphibian biology and conservation [Internet]. University of California, Berkeley; c1995-2015 [cited 2015 May 12]. Available from: http://www.amphibiaweb.org/chytrid/chytridiomycosis.html.

Woodhams, D. C., R. A. Alford, G. Marantelli. 2003. Emerging disease of amphibians cured by elevated body temperature. Dis Aquat Org 55: 65−67.

# APPENDICES

Appendix A. Amphibians Swabbing Protocol.
(modified from http://amphibiaweb.org/chytrid/swab_protocol.html)

Procedure:

1. Preferably, capture amphibians by hand. Wear gloves when swabbing animals and change gloves between animals. If you are using a dip net, be aware that *Batrachochytrium dendrobatidis* zoospores could be caught on the net and transferred between individuals, therefore, use different nets whenever possible, or disinfect the net as often as you can (there is no perfect solution to this problem).
2. Swab the underside or ventrum of adult/metamorphs 30 times. Remember you are in effect scraping small amounts of tissue from the skin. Some pressure must be applied, but this does not mean that you must squash the animal.

   1. For Frogs: Areas to target are the inguinal areas, thighs and webbing between the toes. Zamudio lab has standardized our swabbing as follows: 5 swabs each on R/L inguinal region, 5 swabs on each of 4 feet.

   2. For Salamanders: Areas to target include the underside of the tail and the backside of each of the limbs. Swab the back of each leg 5 times (20 total), the underside of the tail 5 times and the underside of the pelvic region 5 times for a total of 30.
3. Break swab ~3cm from tip and drop into empty screw cap tube. The swab stick should not touch or bump against the top of the vial. Screw the cap on the vial and store in the shade.
4. Samples can be kept a room temperature for a week or maybe longer, but it is best to keep the samples cool and placed as soon as possible in a 4 degree C freezer (the kind you have at home is fine). Avoid extreme high temperature and direct sunlight. Samples may be stored in a freezer for many months without problems.
5. Analysis of swabs: We use quantitative PCR methodology as described by Boyle et al. (2004).

Appendix B. DNA Extraction Protocol for swab samples.

Swab and Filter Paper Extraction Procedure (Modified by McMillan, 2014):

1. Add filter/swab to 1.5mL microcentrifuge tube.
2. Add 180 µL ATL buffer.
3. Add 20 µL Proteinase K, vortex for 10 sec.
4. Put into 56 °C water bath (90 oscillations) for 1.5-3 hrs. Vortex occasionally during incubation, vortex 15s directly before proceeding to step 5.
5. Move each sample to a Qiashredder spin column by moving the filter/swab with clean forceps and pipetting the rest of the liquid.
6. Spin Qiashredder column 5min at 8000rpm.
7. Replace Qiashredder columns with caps from the Qiashredder kit, label caps.
8. Remove caps and add 200 µL AL buffer, vortex 10 sec.
9. Incubate at 56 °C for 10min.
10. Add 200 µL of 100% ethanol and vortex for 10 sec.
11. Pipette liquid into DNA easy mini spin column making sure to suck out as much liquid from the gauze as possible! (really suck it dry).
12. Centrifuge at 8000 rpm for 1 minute, then discard collection tube.
13. Add 500 µL AW1 buffer.
14. Centrifuge at 8000 rpm for 1 minute, then discard collection tube.
15. Add 500 uL AW2 buffer.
16. Centrifuge at 14,000 rpm for 3 minutes.
17. Discard bottom collection tube and place the filter part into a clean 1.5 mL microcentrifuge tube.
18. Add 100 µL AE buffer, allow to sit 5 minutes.
19. Centrifuge at 8000 rpm for 1 minute. KEEP THE LIQUID!!
20. Add 50 µL AE buffer and allow to sit 5 minutes (this could change depending on the extraction).
21. Centrifuge at 8000 rpm for 1 minute.
22. Discard filter part and store microcentrifuge tube in 4 °C cooler.

Appendix C. DNA Extraction Protocol for tissue samples.

Protocol for extracting DNA from Ethanol Preserved Tissue (Modified from Rayman, 2010 and JL, 2013):

1. Cup up to 25mg from samples. Blot ethanol on kimwipe to remove ethanol.
2. Chop tissue into several pieces with razor blade. Use new razor blade for each sample to avoid cross contamination.
3. Place pieces into labeled 1.5 mL microcentrifuge tubes.
4. Add 180 µL ATL Buffer.
5. Add 20 µL Proteinase K thoroughly by vortexing and incubate at 56 °C in shaking water bath. Samples can be lysed overnight or a minimum of 3 hours. Add an additional 10 µL Proteinase K to each tube if large tissue pieces still present. Continue incubating and check periodically for complete lysing of tissue.
6. Vortex for 15 s. Add 200 µL AL Buffer. Vortex to mix thoroughly.
7. Transfer to 2 mL DNeasy Mini Spin Column. Spin 1 minute at 8000rpms. Discard collection tube and flow through (NOT SPIN COLOMN).
8. Place spin column in new collection tube. Add 500 µL AW1 Buffer. Centrifuge 1 minute at 8,000 rpm. Discard /low-through and collection tube.
9. Place spin column in a new 2 mL collection tube. Add 500 µL AW2 Buffer. Centrifuge 3 minutes at 15,000 rpm to dry DNeasy membrane. Discard flow-through and collection tube.
10. Place spin column in a clean 1.5 mL tube and pipette 100 µL AE Buffer directly onto the DNeasy membrane. Incubate at room temperature for 5 minutes. Centrifuge for 1 minute at 8,000 rpm to elute. Keep the liquid.
11. Repeat step 10.
12. Discard DNeasy Spin Column and store microcentrifuge tubes in 40 °C cooler.

Appendix D. The qPCR Reaction System for a single plate (modified from Boyle et al. 2004).

| Composition | Volume/ μL | Final concentration/volume |
|---|---|---|
| Universal master mix (2×) | 12.5 | 1× |
| Primers/10 μM | 2.25 each primer | 10 μM |
| MGB Probe/10 μM | 0.625 | 900 nM |
| DNA Samples/ddH2O/negative controls/positive plasmids | 5 | 250 nM |
| Total volume (per reaction) | 25 | 25 μL |

Appendix E. Mean (copies/μL), 95% Confidence Intervals (CI), and Coefficient of Variation (CV) of positive samples based on triplicate runs.

| Sample no. | mean(copies/μL) | CI (copies/μL) | CV (%) |
|---|---|---|---|
| 1 | 37108.24 | [0,85513.61] | 30.31 |
| 2 | 24993.54 | [0,51661.64] | 24.80 |
| 3 | 24080.75 | [0,50643.84] | 25.64 |
| 4 | 682.38 | [436.17,928.59] | 8.39 |
| 5 | 314.21 | [0,1010.1] | 51.48 |
| 6 | 184.93 | [49.40,320.46] | 17.03 |
| 7 | 115.73 | [28.73,202.74] | 17.47 |
| 8 | 82.45 | [0,179.92] | 27.48 |
| 9 | 77.18 | [35.18,119.17] | 12.65 |
| 10 | 29.06 | [6.88,51.24] | 17.74 |
| 11 | 26.17 | [0,67.50] | 36.71 |
| 12 | 24.10 | [16.94,31.27] | 6.91 |
| 13 | 3.44 | [2.46,4.42] | 6.61 |
| 14 | 2.50 | [0,5.62] | 28.97 |
| 15 | 2.46 | [0,6.02] | 33.56 |
| 16 | 2.14 | [0,7.44] | 57.51 |
| 17 | 1.24 | [0,3.48] | 41.74 |
| 18 | 0.53 | [0,1.1] | 24.60 |
| 19 | 0.51 | [0,1.07] | 26.11 |
| 20 | 0.42 | [0,0.99] | 31.64 |
| 21 | 0.39 | [0,1.37] | 57.55 |
| 22 | 0.08 | [0,0.47] | 115.36 |
| 23 | 0.03 | [0,0.17] | 93.46 |
| 24 | 0.02 | [0,0.09] | 95.55 |