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Seasonal prevalence of *Batrachochytrium dendrobatidis* in Central Massachusetts

Lithobates catesbeianus tadpoles

Thomas Barringer

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Abstract

Batrachochytrium dendrobatidis (*Bd*) is the etiological agent of chytridiomycosis, a fungal disease that some experts believe will lead to the extinction of nearly half of the amphibian species on this planet. Limited research has been conducted on chytridiomycosis in the Northeast United States, and most of it has primarily focused on examining *Bd* in different species of amphibians. In this study, we examine the presence of *Bd* in one species, *Lithobates catesbeianus*, the American Bullfrog, which has natural resistance to the pathogenic effect of *Bd*. Examining different regions of the country, age of *L. catesbeianus*, and season may help scientists determine if environmental factors and developmental factors contribute to the spread of chytridiomycosis. This research project studied the prevalence of *Bd* in American bullfrog tadpoles at three locations in Central Massachusetts and examined if there is seasonal and spatial variation between *Bd* infection in the tadpoles. Tadpoles were swabbed during May and September 2018 and the swabs were analyzed using quantitative PCR. Positive samples were found in all three locations. Out of 136 samples, 33 were positive for *Bd* (24.3%). Twenty-six positive samples were collected during the Spring, and 7 were collected in the Fall. The variation in the density of tadpoles could lead to the significant difference between *Bd* prevalence in the Spring versus the Fall. Seasonal temperatures and the lifecycle of *Bd* could also have played a role in the variation and could be more specifically accounted for in future studies.

Introduction

Many of the living species on earth are at risk of population decline, more now than ever before. While mammalian species are often the animals people hear and care about when talking about extinction, there are thousands of other animal and plant species that go extinct, leaving many gaps in their ecosystems (Cardinale et al. 2012). The loss of biodiversity is a major concern for the health of the earth and could even rival other concerns such as pollution and climate change in terms of severity and especially irreversibility (Pope Francis 2015, Cardinale et al. 2012). But the loss of biodiversity does not have one root cause. Global climate change and deforestation are two causes affecting much of the living world, but some species of life are being affected by things that are much more specific to their nature (Cardinale et al. 2012).

“The worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and its propensity to drive them to extinction” (IUCN 2005) is currently spreading around the world. The fungal disease chytridiomycosis was only discovered in the last half-century, but its current spread could lead to the extinction of nearly half of the amphibian species on this planet. And while frogs may not seem to play a large role on a person’s life, the impact of this disease could wreak havoc on the world’s ecosystems and many levels of human society.

General Frog Biology

Frogs are vertebrates in the animal kingdom with 4,740 known species on earth and more being discovered each year (Defenders of Wildlife 2019). They fall under the class of Amphibia which also includes toads, salamanders, and caecilians, and are often referred to by the more common name of amphibians (Missouri Department of Conservation 2018). Frogs live portions of their

life both in water and on land. While many adult frogs spend time on both water and land, the reproductive cycle of frogs is dependent on water as eggs are laid in water for the hatchlings to survive. Once hatched, frogs spend the next phase of their life in water as legless tadpoles. Eventually they metamorphose into adult frogs with four legs and no tail (Missouri Department of Conservation 2018).

Frogs living in both fresh water aquatic habitats and terrestrial habitats play a large role in the health of our planet. They are found on nearly every area of the earth's land masses outside of the polar caps and a few deserts. Frogs are an important part of the food web in an ecosystem. They eat a large variety of insects, fish, birds, and even small mammals. Frogs not only eat a variety of animals but are also eaten by many animals. Being amphibious, frogs are important for transferring energy between aquatic and terrestrial ecosystems, as they play the role of predator and prey in both types of habitats during their life (National Park Service 2018).

The health of an ecosystem can often be determined by the health of the frog population as frogs take in contaminants both through their skin and their mouth (Missouri Department of Conservation 2018), making them great bioindicators. Unlike the skin of humans, frog skin is semi-permeable, allowing water, oxygen, and other essential nutrients to cross into the body through the skin. With the steady decline of frogs come a steady decline of the health of an ecosystem. Insects are a large part of most frog species' diets. A declining frog population often results in an increasing insect population due to a lack of insect predation. Insects have the potential to carry many communicable diseases, and since insects are common prey for frogs, decreased frog populations can lead to an increased disease-carrying insect population. Certain species of frogs eat mosquitoes that carry malaria, dengue, arboviruses, and the zika virus as well as other parasitic agents (Benelli et al. 2016).

Some of the most important ecosystems on earth are also the most delicate. The Amazon rainforest, for example, is one of the most biodiverse places on the planet. There are many human products that rely on plants and animals that come from the Amazon rainforest. The steady decline in frog populations, now that chytridiomycosis has reached this region of the world, could be one cause for a severe disturbance of the Amazon rainforest ecosystem, pressing many of the plants and animals humans rely on to the brink of extinction. The secretions from some frog species' skin plays a large role in medicine and other health care products for humans (Tyler 2006). Antibiotics, anti-tumor agents, analgesics, and adhesive compounds can be at least partially developed from compounds in frog skin (Tyler 2006). The prices for these products will begin to rise as the resource of frogs continues to decline.

Amphibians are currently declining at a catastrophic rate that could lead to a mass extinction of amphibians worldwide. Since 1980, nine species of frogs have gone extinct and another 122 species of amphibians are possibly extinct (Smithsonian Conservation Biology Institute, 2018). At the pace amphibians are declining, nearly 42% of all amphibian species are in danger of becoming extinct in the next 100 years (Smithsonian Conservation Biology Institute 2018). While climate change and habitat loss are considered important factors in the decline of frog populations worldwide, chytridiomycosis is considered the primary cause of decline (Skerratt et al. 2007).

Batrachochytrium dendrobatidis Biology

Chytridiomycosis, chytrid for short, is a lethal fungal disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*). *Bd* is a member of the phylum Chytridiomycota which is made up of more than 1000 species of fungi (Weldon et al. 2004). Most species have a flagellated zoospore stage and live in wet or moist areas (James et al. 2006). The ability to live in

both large bodies of water and small drops of water allow them to survive everywhere on the earth, even Antarctica (Berger et al. 1998, Rojas-Jimenez 2017). Most species in the phylum Chytridiomycota are known for being detritivores, also known as decomposers, and feed on rotting organic matter (James et al. 2006).

Chytridiomycosis was first found in Australia, but its origins are most likely in Africa. Through DNA sequencing of frogs preserved in formalin, researchers have found South Africa to be the location where chytridiomycosis originated in the early 20th Century, as the DNA from frogs archived during the 20th Century is still able to be sequenced (Smithsonian Conservation Biology Institute 2018). However, a small study found that it is possible that chytridiomycosis could have originated in North American bullfrogs (Rosenblum 2010, Huss et al. 2013). If Africa was indeed the origin of the disease, it then spread to Australia and southeastern North America (Pessier 2015). While the number of amphibians infected with chytridiomycosis has slowed in these regions of the world due to amphibian populations adapting to the fungus, the disease has now spread to South America, Central America, part of Asia, and the rest of North America (Pessier 2015). The disease is thriving in the warmer, tropical climates and continuing to spread in these areas. While *Bd* has not spread to every part of every continent, all 4740 species of frogs are at risk of coming into contact with the fungus (Pessier 2015). In 2001, chytridiomycosis was placed on the International Office of Epizootic Wildlife Diseases list, which marked the first time an amphibian disease ever made the list (Pessier 2015).

Bd is the one of two species in the phylum Chytridiomycota to infect vertebrates (Longcore et al. 1999, Smith et al. 2018). It can infect nearly all 6000 species of amphibians, resulting in death for many species (Berger et al. 1998). *Bd* takes advantage of a frog's semi-permeable skin. The skin of a frog is semi-permeable in order to absorb molecules that are

necessary to live, however it also allows an easier absorption of toxic chemicals (Voyles et al. 2009). While out of the water, frogs have two ways of respiring: through their skin and through their lungs. The mucous on their skin allows them to stay moist out of water and absorb dissolved oxygen from the air (Brown University 2018). While submerged in water, adult frogs cannot use their lungs to breathe and do not have any form of gills to perform respiration either. Instead, all gas exchange must take place through the skin. Frogs also obtain sodium, potassium, and other salts from the water that is absorbed (Voyles et al. 2009).

Another unique feature of frog skin that *Bd* takes advantage of is the various amounts of keratin it contains. Keratin is usually found on the hands and feet of frogs as well as where the legs rub against the torso. On tadpoles, keratin is usually found around the mouth. Keratin is not found abundantly in frogs because it does not allow water to be absorbed through the skin. Its primary purpose is physical protection, so it is found where a frog needs protection most. Keratin cannot be abundant on frogs or else the frog would suffer from a lack of oxygen, nutrients, and water being absorbed through the skin.

Chytridiomycosis occurs when frogs become infected with the fungus *Bd* (Collin and Crump 2009). *Bd* infects frogs by first getting into the keratin cells in the epidermis (Voyles et al. 2009). The fungus is thought to make its way into the skin of the frog through cuts and abrasions. Once inside the keratin cells, the fungus begins its reproduction cycle and spreads throughout the epidermis (Voyles et al. 2009). *Bd* causes hyperkeratosis once inside the non-keratin epidermal cells. Hyperkeratosis causes normal epidermal cells to harden and become keratinized (Voyles et al. 2009). A frog that becomes infected with *Bd* has the potential to have the fungus spread around its body causing more of the surface of their skin to become keratin than normal. Too much keratin on the body of a frog restricts the semi-permeable nature of the

frog's skin and causes the absorption of essential materials to be restricted (New South Wales: Office of Environment and Heritage 2015). Skin that becomes infected by *Bd* and undergoes hyperkeratosis begins to shed at various speeds due to the frog's body trying to maintain homeostasis (Voyles et al. 2009). However, chytridiomycosis causes multiple layers of keratin to grow in an infected frog, causing the areas of skin infected to become even more impermeable than the areas with normal keratin. Frogs that have contracted chytridiomycosis will be slow moving or even immobile due to the extra amounts of thick keratin cells spread around their body (New South Wales: Office of Environment and Heritage 2015).

While chytridiomycosis is known to be a fatal disease, the exact cause of death is not precisely known (Voyles et al. 2009). The restriction of essential materials due to much of a frog's skin being almost impermeable is one of the major reasons chytridiomycosis is deadly in some frog species (Bristow 2015). The most recent research found substantial evidence that the restriction of water and essential electrolytes due to hyperkeratosis is what leads to death in *Hyla cinerea* (Voyles et al. 2009). The study, performed by Jamie Voyles from the University of Nevada, found that electrolytes are restricted by more than 50% in green tree frogs infected with chytridiomycosis. Sodium concentrations were decreased by 20% among infected frogs, and potassium concentrations were decreased by 50%. Homeostasis could not be maintained in frogs infected due to the restriction of essential materials. Infected frogs eventually died due to systolic cardiac arrest, a type of heart attack that can occur in humans as well. This was due to low levels of sodium and potassium. Potassium is necessary for the heart to beat and sodium is necessary to retain water through creating a hyperosmotic internal environment. The results from this study are thought to be fairly applicable to other species of frogs that show susceptibility to chytridiomycosis and are symptomatic to the disease.

Bd is a parasitic fungus as it needs amphibians to continue its life cycle. The majority of the *Bd* lifecycle occurs inside of an amphibian's skin cells with the entire lifecycle lasting four to five days (Berger et al 2005). The fungus begins in a zoospore stage in open water. A zoospore is an asexual type of spore that has a flagellum for movement. It can exist in many different types of wet environments (Berger et al.2005). These environments can be as large as lakes and rivers or as small as pools and puddles of water, even wet soil. The wetter an environment, the more potential for *Bd* to spread. The *Bd* zoospore swims in water to travel between amphibian hosts.

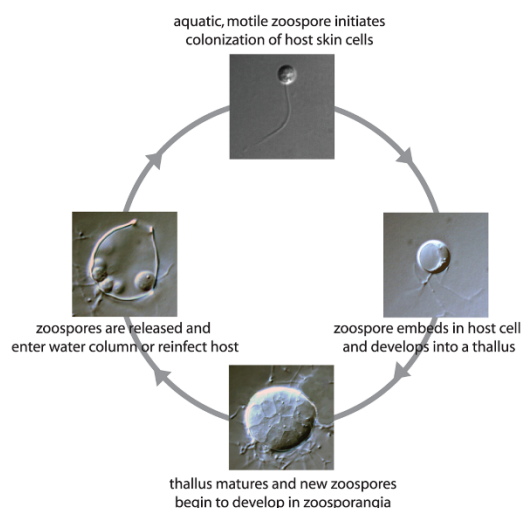


Fig 1. Life cycle of *Bd*. (Rosenblum 2010)

As previously stated, it is thought that *Bd* zoospores infect amphibians by entering the epidermis through cuts and abrasions. The other possible method of infection is by the zoospore injecting its contents into the surface of the skin through a germ tube (Berger et al. 2005). While the exact method of infection is not known, it is known that *Bd* zoospores are too large to be absorbed through the skin of an amphibian (Berger et al. 2005).

Once inside the epidermal keratin cells, the zoospore encysts after a period of motility and develops into a thallus. As a thallus, the fungus enters a dormant stage where it is less active and less mobile (Berger et al. 2005). In nature, an organism typically enters a dormant stage right before a growth stage. After the thallus stage, the fungus becomes a germling with fine branching rhizoids (Berger et al. 2005). Rhizoids are used to absorb nutrients from the fungi's surroundings and for attachment. As a germling, multiple zoospores begin to form within the mother's cell wall to form a zoosporangium, and a discharge papilla begins to form (Berger et

al.2005). Once the organelles replicate, the entire inside of the fungus begins to cleave into separate zoospores becoming a zoosporangium. A mature zoosporangium is full of pockets of zoospores, typically containing between seven and ten zoospores (Berger et al. 2005). Once a zoosporangium matures, the zoospores are released into the surrounding area through the discharge papilla (Berger et al. 2005). Zoospores that make it out of the amphibian continue into the surrounding area of water to start the life cycle over again. However not all of the zoospores always make it outside of the amphibian. The remaining zoospores can infect other non-infected cells, causing the fungus to spread to other parts of the body leading to hyperkeratosis (Berger et al. 2005). The empty discharge papilla of the zoosporangium remains open after all zoospores are released, which allows other organisms to flow into the zoosporangium. This may lead to bacterial infection in the amphibian as the zoosporangium allows a place for the bacteria to replicate without being impacted by the frog's immune system (Berger et al. 2005). *Bd* zoospores have been shown to live up to seven weeks in lake water and can live in temperatures up to 47° C (Johnson et al. 2003). They can live in pools of water and streams for extended periods of time as well. The zoospores can often find amphibian hosts as amphibians often share common sources of water. The exact way *Bd* zoospores remain alive in water for long periods of time is unknown.

While *Bd* zoospores in water are one way for a frog to contract the fungus, it is not thought to be the primary way chytridiomycosis is spread. Direct contact is the number one way the fungus is thought to be spread (Johnson et al. 2003). Direct contact typically occurs either for sexual reasons or territorial reasons. During breeding season, male frogs often fight over territory and over females (Johnson et al. 2003).

Humans have a large impact on the spread of chytridiomycosis. Frogs, including exotic, wild-caught frogs coming from tropical climates, are sent between countries and between

continents through the pet trade, the zoo trade, and food trade. *Bd* survives best in warm climates so many of these frogs are infected with *Bd* and are being traded to parts of the world where the fungus is not as prevalent (Johnson et al. 2003). The disease can make its way into an ecosystem when a pet frog infected with chytridiomycosis is released into the wild.

In captivity, chytridiomycosis has been nearly eradicated due to careful precautions taken by zoological parks. In 1997, the National Zoological Park in Washington, D.C., discovered chytridiomycosis in their population of poison dart frogs (Johnson et al. 2003). This led to zoos around the world to test for chytrid in their amphibian populations. There is a cure for chytridiomycosis, but it can only help cure amphibians in captivity. The treatment, created by the National Zoological Park, involves a series of itraconazole baths. Itraconazole is a type of antifungal, and while it has been largely successful, it still is not 100% successful (Johnson et al. 2003). These antifungals are effective but cannot help cure amphibians in nature, as introducing the large amount of antifungals into the environment that would be necessary to cure an infected amphibian would have disastrous effects on an ecosystem. Fungi play a very large role in decomposition and nitrogen fixation throughout an ecosystem, breaking down organic materials and transforming atmospheric nitrogen into an organic, useable form (Kendrick 2011). Eliminating most or all fungal species from an ecosystem would disrupt the system's function significantly.

Humans also have the ability to spread chytridiomycosis by carrying *Bd* zoospores on shoes and boots while traveling between bodies of water. Hikers traveling through wetlands and multiple bodies of water are thought to be one group of people spreading *Bd* by way of boots. Construction workers are another group of people that are possibly spreading the fungus by the use of the same pair of boots at many different construction sites (Johnson et al. 2003).

Construction around bodies of water can lead to contamination of ecosystems with *Bd* if boots and even construction equipment are not disinfected properly, preferably with bleach.

The human impact on rising global temperatures is benefiting *Bd* as the fungus is better suited to live in warmer temperatures. As global average temperatures increase, the survivability of *Bd* across the globe increases. This is causing chytridiomycosis to spread to areas of the world previously too cold for the fungus to survive (Johnson et al. 2003). The fungus has spread from its equatorial origins both North and South, surviving in areas with snowy winters. Starting approximately two decades ago, governments and environmental organizations across the globe began to attempt to limit the spread of chytridiomycosis. Limiting the global trade of amphibians along with properly quarantining traded amphibians are thought to be the best ways to prevent the spread of the disease (Department of Environmental Heritage 2006).

Chytrid Resistance and the American bullfrog

While every frog species is at risk of coming into contact with the *Bd* fungus, not every species is at risk of contracting chytridiomycosis. A number of species of frogs are resistant to the disease (Johnson et al. 2003). However, while they are asymptomatic when coming into contact with *Bd*, they still have the ability to spread the fungus in their environment. These species are known as carriers or vectors, which are organisms that transmit an infectious disease to another organism. While species resistant to chytridiomycosis help spread the disease, they also might be the key to unlocking the solution to chytridiomycosis (Johnson. et al. 2003).

There are no clear answers as to why some species are resistant to chytridiomycosis and others are not. The reason a species is resistant to chytridiomycosis is thought to be either genetic or environmental. The gene hypothesis describes the genes of a resistant species having a

resistance built to prevent the *Bd* fungus from causing symptoms in the frog's epidermis (Johnson et al. 2003). The genetic explanation of resistance could be a possible adaptation the species has made to become resistant to the disease. The environmental theory as to how some species are resistant to chytridiomycosis has multiple parts. Some species of frogs have been shown to be resistant in one ecosystem but not in another. This could point to some factor being present in one ecosystem that allows a frog to be resistant to chytridiomycosis while it is absent in another ecosystem (Johnson et al. 2003). It is also possible that there could be a difference in the virulence of the strains of *Bd* in different ecosystems (Johnson et al. 2003). Virulence is the ability of a disease to infect an organism, so it is possible that some strains of *Bd* could cause chytridiomycosis and some cannot. There are also some amphibian species, including the salamander species *Plethodon cinereus*, that carry symbiotic species of bacteria on their skin, such as *Janthinobacterium lividum*, that discourages growth of *Bd* (Harris et al. 2009). It is still possible for a frog to contract chytrid with this symbiotic species of bacteria, but the larger the volume of bacteria a frog has, the less likely it is to contract chytrid.

Another study investigated a model in which environmental conditions, species of a frog, and virulence of a strain did not have an impact on a frog population's infection rate (Briggs 2010). This model found that frogs living in dense populations along with species with extended tadpole stages were more susceptible to higher infection rates of *Bd* due to the higher infection loads seen in infected frogs. This evidence could not explain why a species would be totally resistant to chytridiomycosis, but it could help partially explain it, as some species that are resistant to *Bd* are territorial which would lead to a less dense frog population and less of a chance for one frog to transmit the disease to another frog.

The frog species *Lithobates catesbeianus*, better known as the American bullfrog, is one of ten known species of frogs and toads to live in Massachusetts (Mass Audubon 2019). Its natural range is from southeast Canada, down to Florida, and as far west as Kansas. However, it has been introduced to nearly all of the continental United States, Cuba, the Dominican Republic, Puerto Rico and Northern Colombia (Bruening 2002). Its wide modern-day range leads the individual frogs of the species to come into contact with many other species of amphibians, some naturally occurring and some not. American bullfrogs are one species of frog that is resistant to most strains of *Bd* (Gervasi et al. 2013). The American bullfrog is known to be a carrier of chytridiomycosis, spreading the disease to more



Fig 2. *Lithobates catesbeianus*. **A.** Tadpole stage **B.** Adult stage (Bruening 2002)

susceptible species of frogs. American bullfrogs can contract *Bd* but are tolerant of the fungus as they are asymptomatic when infected (Gervasi et al. 2013, Eskew 2015). Research has shown that while American bullfrogs might be carriers of chytridiomycosis, they are not long-term carriers, as the infection load of *Bd* in the skin of an American bullfrog that is infected decreases over time (Gervasi et al. 2013, Eskew 2015). While the mechanism of tolerance to *Bd* infection is not precisely known, American bullfrogs appear to be “super shedders”, shedding the epidermal layer faster than the disease can spread through the skin (Van Rooij et al. 2015).

The American bullfrog life cycle is quite long, as this species is one of the largest frogs in the world and takes a substantial amount of time to develop. Thousands of eggs are typically laid

by a female in the water and, as long as they are not eaten by predators, the eggs will hatch into tadpoles (Bruening 2002). The tadpoles hatch in the summer and spend at least an entire year developing into a froglet. Some tadpoles spend two or even three years growing to reach the froglet stage (Bruening 2002). A few months after becoming a froglet, the bullfrog becomes an adult. While frogs spend much of their time in wet environments as adults, their tadpole stage occurs entirely under water. Being submerged in water for an extended period of time leaves American bullfrog tadpoles susceptible to the *Bd* fungus, as they have keratin surrounding their mouths. Research has shown that American bullfrog tadpoles can contract the *Bd* fungus as a tadpole and still carry the fungus as an adult (Gervasi 2013).

Understanding how a carrier of the *Bd* fungus, such as the American bullfrog, can be infected with the fungus and not die could lead scientists to a cure for chytridiomycosis in the wild. But understanding how an American bullfrog can carry the fungus and be asymptomatic starts with mapping out the prevalence of the *Bd* fungus in American bullfrogs across the country. Knowing which populations of American bullfrogs in different regions of the country have a higher infection rate than other populations in other regions may point scientists to an environmental factor that allows American bullfrogs to be resistant to chytridiomycosis.

Prevalence of Chytrid

Determining the rate of infection among American bullfrog tadpoles in different ecosystems is important as well. American bullfrog tadpoles could be more susceptible to *Bd* in one ecosystem compared to another, which aligns with the environmental hypothesis. (Gahl 2012). With the American bullfrog having a tadpole stage lasting multiple seasons, there could be one season in which the tadpole is more likely to contract the fungus. The present study surveyed the prevalence of *Bd* in American bullfrog tadpoles in Central Massachusetts.

There has not been much research done on chytridiomycosis in the Northeast United States, however a few regional studies are relevant. A small study was conducted in Eastern Massachusetts and no frogs were found to have the *Bd* fungus (Global *Bd*-Mapping Project 2018). Another study performed from 2000-2002 sampled multiple species of frogs from federal lands across Maine, New York, New Hampshire, Vermont and Massachusetts. Chytridiomycosis was found to be widespread throughout the Northeast United States, with bullfrogs having an infection rate of 39.7% (Longcore et al. 2007). A third study performed in 2013 that sampled nearly 1000 frogs across Connecticut found that out 105 American bullfrog adults and tadpoles sampled, 30% were infected with *Bd* including 10.9% of tadpoles (Richards-Hrdlicka et al. 2013). Of all the frogs sampled, 28% were infected (Richards-Hrdlicka et al. 2013).

This study involved collecting samples during Spring and Autumn 2018, as prior research has shown Spring and Autumn to have the highest rates of infection among frogs (Longcore et al. 2007). This research not only studied the prevalence of *Bd* in American bullfrog tadpoles at three locations in Central Massachusetts, but also looked to determine if there is seasonal and spatial variation between *Bd* infection in the tadpoles.

Methods

Study Sites

In order to study the seasonal prevalence of *Batrachochytrium dendrobatidis* in Central Massachusetts *Lithobates catesbeianus* tadpoles, multiple locations with *L. catesbeianus* tadpoles were located. Three locations were chosen based on their shallow, reed filled tadpole-friendly waters. The three locations are shown in Figure 3.



Fig 3. Tadpole sampling locations. **A.** Girls Inc. Worcester **B.** Ecotarium **C.** Shirley, MA. Image was provided by Google maps (Imagery ©2018 Google, Map Data ©2018 Google)

Permission was obtained from the property owner at all three locations before sampling took place.

Girls Inc. Worcester

The Girls Inc. Worcester owns a summer camp northwest of downtown Worcester.

The property contains a pond which can be seen in Figure 4 (Hereafter “INC”; 42.297243, -71.860641). INC has multiple streams that flow into it and has a small stream flowing out of it

towards the Worcester Water

Filtration plant reservoirs. The pond

is on private land and is fairly isolated from human activity outside of the summer months when camp is in session. All samples taken at this site were done outside of months when camp was running. INC is approximately 2.97 acres (1.20 hectares).

Ecotarium

The Ecotarium is a nature museum just to the east of downtown Worcester. On its property are two ponds, a large pond on the lower portion of the property, and a smaller pond on the upper part of the property. The lower pond was intended to be the sampling site at the Ecotarium, but no tadpoles were found.

Fortunately, the upper pond had a plethora of *L.*

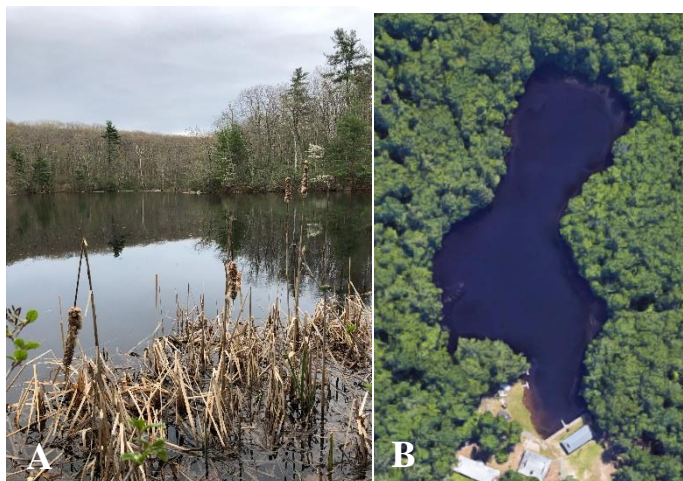


Fig 4. INC Photos **A.** Eye level view of INC in early May 2018 **B.** Overhead view of INC. Image was provided by Google maps (Imagery ©2018 Google, Map Data ©2018 Google)



Fig 5. ECO Photos **A.** Eye level view of ECO in the Fall (Ricklin 2011) **B.** Overhead view of ECO. Image was provided by Google maps (Imagery ©2018 Google, Map Data ©2018 Google)

catesbeianus tadpoles. The upper pond has one small stream flowing into it and has no outlet. The pond is shallow on the periphery and has many aquatic grasses near the water's edge. The lower pond on the Ecotarium property can be seen in Figure 5. (Hereafter "ECO"; 42.264229, -71.767954). ECO is surrounded by a parking lot, a drive way, a walkway, and a North American river otter enclosure. While the area is somewhat busy around it, human do not directly interact with ECO very often and it is on an enclosed private property, reducing the effects human activity has on the pond ecosystem. ECO is approximately 0.38 acres (0.15 hectares).

Shirley, Massachusetts

The town of Shirley, Massachusetts is north of Worcester and on public land there is a small pond that was man-made in the 1950's. This pond can be seen in Figure 6 (Hereafter "SHI"; 42.559228, -71.648749).

SHI is located next to the town's recreation center so there is moderate human activity near it,

but it is also at the border of a forest, so wildlife, including *L. catesbeianus*, can access the pond.

It has one very small inflow and no outflow. SHI is approximately 0.31 acres (0.13 hectares).

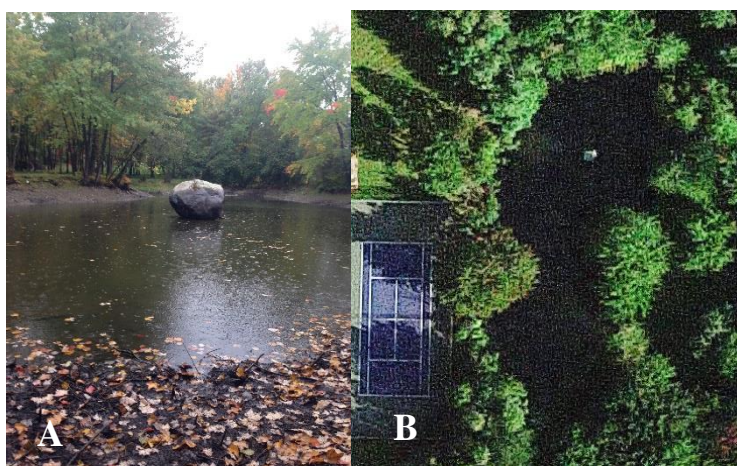


Fig 6. SHI Photos. **A.** Eye level view of SHI at low water levels (Gionet 2016) **B.** Overhead view of SHI. Image was provided by Google maps (Imagery ©2018 Google, Map Data ©2018 Google)

Sampling Times

To compare the prevalence of Bd seasonally, *L. catesbeianus* had to be sampled during two different seasons. Spring and Fall were chosen as seasons to sample tadpoles. Each study site was sampled during each season. Table 1 contains the specific days each site was sampled.

Table 1. Sampling dates for each individual study site

Study Site	First sampling date	Second sampling date	Third sampling date
INC	5/4/2018	9/15/2018	10/3/2018
ECO	5/7/2018	10/8/2018	N/A
SHI	5/8/2018	9/22/2018	N/A

Sampling all occurred during daylight hours between 10 AM and 4 PM. The number of samples collected from each site are listed below in Table 2. No samples were collected from SHI in the Fall as no tadpoles could be found.

Table 2. Number of samples collected at each sampling site

Study Site	Spring Samples Collected	Fall Samples Collected
INC	28	22
ECO	38	29
SHI	25	N/A

Swab Technique

Tadpoles primarily eat algae, so they tend to stick to shallow areas on the outskirts of a pond where rocks have algae growing on them (Miller 2010). Two primary methods were used for collecting tadpoles. The first method involved sweeping a large hand net through the water on the margins of the pond. This sweeping the net through the water was more effective in the spring when there was a plethora of tadpoles in the



Fig 7. Tadpoles feeding on algae growing on a rock in shallow waters (Tadpole Info 2017)

ponds. In the Fall, when tadpoles were not as abundant, tadpoles were often sited from above and specifically captured by using the hand net to cut the tadpoles' path off. The second method used involved using a 5-gallon bucket to scoop out the top layer of sediment from the edge of the pond and sifting through the contents for tadpoles.

Once tadpoles were collected, they were photographed next to a ruler for size measurement and swabbed around their mouth parts and on their sides following the protocol from Boyle et al. (2004), Retallick et al. (2006), Hyatt et al. (2007), and Vredenburg and Briggs (2017). Cotton-tipped swabs were used for swabbing, as they effectively collect the potentially *Bd* infected keratinized tissue on the tadpole (Retallick et a. 2006), and nitrile gloves were used to handle all tadpoles. Swab tips were broken off and stored in 1.5 mL microcentrifuge tubes. Gloves were exchanged between every tadpole that was handled in order to prevent the potential spread of *Bd* among tadpoles. All tadpoles were released back into the areas they were captured from. Once all sampling was completed at a certain site for the day, all equipment was cleaned with diluted bleach and swab samples were stored in a freezer.

Laboratory Techniques

In order to determine whether *Bd* was present in each swab sample, the procedures outlined in Boyle et al. (2004) and modified in Hyatt et al (2007) techniques were performed using Real-time quantitative polymerase chain reactions. Prepman Ultra Sample Preparation Reagent was used to extract DNA from each swab. One modification made to the DNA extraction protocol was the addition of a 1-2 cm portion of a 1-200 μ L pipet tip in the bottom of each swab vial to prevent the swab from being able to reach the bottom of the microcentrifuge tube. This allowed the extracted DNA to remain in the bottom of the vial and not be reabsorbed by the swab after centrifugation. Samples had their DNA diluted 1:10 to reduce inhibition (Hyatt et al. 2007). Taqman polymerase was used to amplify the DNA and an internal positive control (Exo IPC) was added to the samples to gauge reaction inhibition. The samples were performed singly using a CFX96 Touch™ Real-Time PCR Detection System machine.

Bd Infection Quantification

A sample was considered positive if it amplified before cycle 40, or before the cycle the 0.1 standard amplified if the 0.1 standard amplified after cycle 40 (Longo et al. 2013). To determine the *Bd* infection load in each positive sample, positive standards of 0.1, 1, 10, 100, and 1000 zoospore genomic equivalents were made following the serial dilution protocol described in Boyle et al. (2004) and Longo et al. (2013). The Cq numbers of each standard were plotted and given a line of best fit. Each positive sample's Cq number was then plugged into the line of best fit equation to determine the number of zoospores in the qPCR sample. To determine the zoospore genomic equivalents (i.e., the estimated number of *Bd* zoospores) on each positive sample's swab, the number of zoospores was divided by the volume of DNA placed in the qPCR sample divided by the total extraction volume (Longo et al. 2013). This number was 5

microliters divided by 40 microliters, so each positive sample's number of zoospores was divided by 0.125 to determine the zoospore genomic equivalent in the swab.

Statistical Analysis

A two-way Analysis of Variance was performed with SPSS on the data set to compare both the infection rates and prevalence of *Bd* between the three locations and two seasons. A Least Significant Difference confidence interval adjustment was used as a part of the two-way ANOVA. A p-value less than 0.01 was used to determine significance because of unequal sample size.

Results

Bd Prevalence Results

Each swab sample from all three locations and both seasons had their Cq numbers from the qPCR interpreted to determine whether each sample was positive or negative for *Bd*. The positive samples were grouped by location and season, and they can be seen in Figure 8.

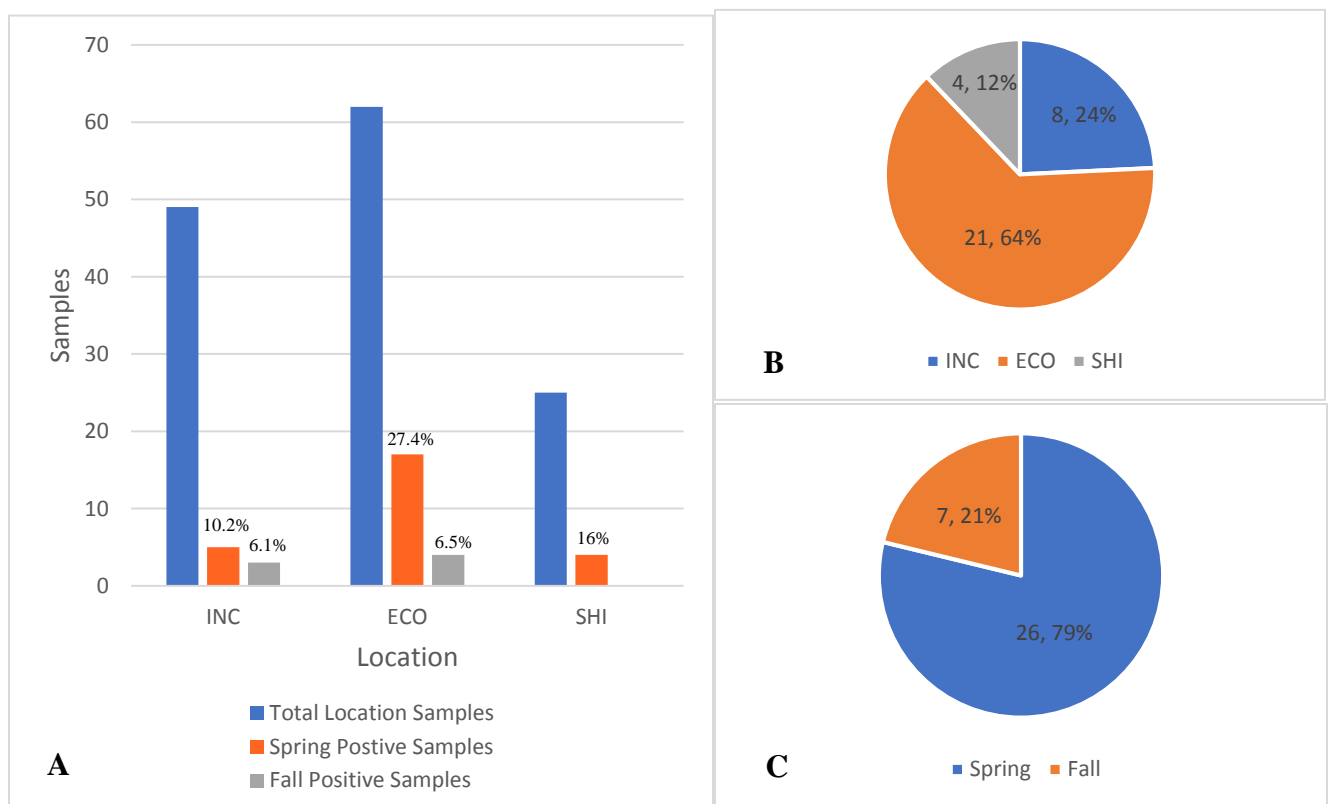


Figure 8. Prevalence of *Bd* at three locations in Central Massachusetts. A. Total number of samples at each location compared to the number of positive samples per location and season. **B.** Percentage of total positive samples per location. **C.** Percentage of total positive samples per season

A total of 136 *L. catesbeianus* tadpoles were sampled among all three locations. Thirty-three samples were positive (24.3%), and of those positive samples, 26 were collected in the Spring (78.8%) and 7 were collected in the Fall (21.2%). Eighty-five samples were collected in the Spring with 26 of those samples testing positive for *Bd* (30.6%), while 51 samples were

collected in the Fall with 7 of those samples testing positive (13.7%). Eight samples were positive (5 Spring, 3 Fall) from INC, for 16.3% of the 49 samples collected. ECO had 62 total samples collected with a positive sample rate of 33.9%, as 21 of its samples were positive (17 Spring, 4 Fall). Of the 25 samples collected from SHI, 4 samples were positive (4 Fall) for 16% of the sample. *Bd* was more prevalent in the Spring than the Fall, and ECO had the most infected tadpoles. SHI had the highest average zoospore load but there was a lot of variation in the zoospore load data leading to no significance found in the two-way ANOVA.

A Two-Way ANOVA was run on the prevalence data, however any analysis involving Fall data only included INC and ECO data as there was no SHI Fall data. A p-value less than 0.01 used to determine whether a variable was statistically significant, as unequal sample sizes were used (Kao 2008). Statistically significant data is shown below in Table 2.

Table 2. Corresponding p-values for statistical analysis of prevalence data

Relevant prevalence data	Corresponding p-value
Season	0.009
Location	0.015
ECO Spring vs INC Spring	0.002
ECO Spring vs SHI Spring	0.001

Season overall was shown to be statistically significant ($p=0.009$) while location overall was not shown to be statistically significant ($p=0.015$). In a pairwise comparison between each location per season, there was a statistically significant difference in the Spring between ECO and both INC ($p=0.002$) and SHI ($p=0.001$).

Bd Infection Load Results

The zoospore genomic equivalence on each swab sample was determined through mathematical calculations involving the Cq number. The average zoospore genomic equivalents on each swab sample for each location is shown by season in Figure 9.

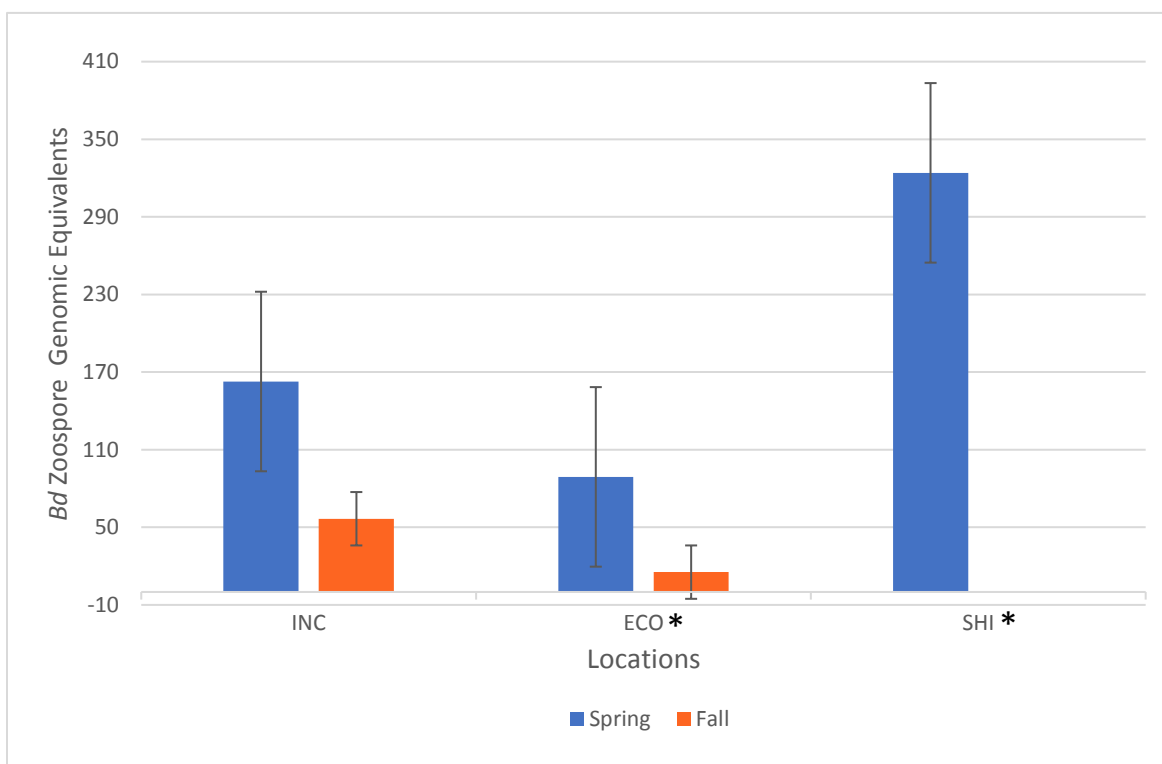


Figure 9. Average zoospore genomic equivalents per swab sample. The mean zoospore genomic equivalents per swab sample at each location is shown per season with standard error. (“*” denotes relevant but not statistically significant relationship)

INC had a mean of 162.72 (± 354.96 stdev) zoospore genomic equivalents in the Spring and 56.16 (± 94.75 stdev) zoospore genomic equivalents in the Fall. The mean zoospore genomic equivalents at ECO were less than INC at 88.96 (± 71.40 stdev) genomic equivalents in the Spring and 15.33 (± 15.93 stdev) genomic equivalents in the Fall. SHI had an average of 324.01 (± 457.18 stdev) zoospore genomic equivalents.

The Two-Way ANOVA run on the infection rate data presented no variable, season, or location as statistically significant ($p < 0.01$). The locational difference in zoospore genomic equivalents between ECO and SHI was the closest comparison to being statistically significant ($p=0.031$).

Discussion

A portion of the American bullfrog tadpoles at all three central Massachusetts locations were infected with *Bd*. Multiple positive samples were found in every season and at all three locations as well. While Location overall was not statistically significant (Table 2, $p=0.015$), ECO Spring was statistically significant compared to INC Spring (Table 2, $p=0.002$) and SHI Spring (Table 2, $p=0.001$). The significance was due to ECO having a significantly higher prevalence compared to the other locations in the Spring. The reason behind the high percentage of *Bd* prevalence at ECO is not easily explainable. The only distinguishing characteristic between ECO and the other two locations in the Spring was the density of American bullfrog tadpoles around the edges of the water at the location. While bullfrog tadpoles were not hard to find at INC or SHI in the Spring, at ECO hundreds could be seen at a time from a considerable distance from the water's edge. The surface of the pond would appear to be bubbling when in fact the disturbance of the water was from the mass amounts of bullfrog tadpoles swimming away from what they determined was a predator. This high density of tadpoles could have encouraged a more rapid spread of *Bd* within the fairly small and contained pond leading to a high prevalence of *Bd* overall in the Spring at ECO (Briggs et al. 2010).

This apparent density of bullfrog tadpoles varied by season at all locations. The ability to find and capture bullfrog tadpoles to swab was much easier in the Spring at all three locations. INC took two days in the Fall to find enough tadpoles compared to the one day it took in the Spring, and SHI had no tadpoles at the location at all. SHI has been known to have very low water levels over the Summer months, leaving tadpoles more vulnerable to other natural threats. ECO still had the highest density of bullfrog tadpoles in the Fall compared to the other two

locations, but it appeared to have fewer tadpoles compared to the Spring, as areas that seemed to have hundreds of tadpoles in the Spring had only one to five tadpoles in the Fall.

The American bullfrog life cycle likely plays into the apparent seasonal variation in bullfrog tadpole density. Bullfrogs spend one to three years as tadpoles before going through metamorphosis (Govindarajulu et al. 2005). The tadpoles cannot go through metamorphosis over the Winter in central Massachusetts, as they overwinter under the ice. The tadpoles will either begin to metamorphose once Spring comes around, or they will remain tadpoles for a longer period of time. Bullfrog tadpoles hatch from eggs in the late-Spring to Summer season as well. This leaves late-April through May with a high potential to have bullfrog tadpoles from multiple hatching years. The numbers of bullfrog tadpoles likely decline at all locations in central Massachusetts by the Fall as many of the older tadpoles have become adults during the Spring and Summer, something the tadpoles are not able to do over the Winter (Govindarajulu et al. 2005). Predation is also a potential factor that varies based on season. Predators are often coming out of a period of little activity in the Spring while they are much more active during the Fall.

While ECO had the highest prevalence of *Bd* of the three locations, it surprisingly had the lowest mean GE per swab (see Figure 9). SHI had the highest mean GE per swab in the Spring, although the *Bd* infection load varied greatly in all seasons at all locations. The standard error among infection load in the Spring was greater than the standard error in the Fall. The Fall also had lower means of GE per swab which correlated with the lower *Bd* infection rates observed in the Fall. The bullfrog tadpoles sampled generally seemed to be larger in the Spring, and body size could play a role in the infection load of an amphibian (Richards-Hrdlicka et al. 2013). A larger amphibian, in this case a larger tadpole, would have more surface area to harbor *Bd*, but the ratio of zoospores to surface area could actually be lower than a smaller tadpole with less

zoospores on its surface. In other words, infection load does not necessarily correlate with infection intensity.

The prevalence of *Bd* found among *L. catesbeianus* tadpoles in this study, along with the infection loads found, are relevant but not unusual among this species of frog. American bullfrogs are known to be carriers of *Bd*, so high prevalence of *Bd* among samples of bullfrog tadpoles is not surprising. International research studies have found *Bd* prevalence among American bullfrogs to be anywhere from 0% to 80% in certain locations (Garner et al. 2006, Martel et al. 2013). The overall prevalence of 24.3% in this study fits within the broad international range of prevalence but is also quite comparable to the other studies performed in the region. Prevalence has typically been between 25% and 40% in New England among bullfrogs (Richards-Hrdlicka et al. 2013, Longcore et al. 2007) with all other species of frogs also fitting into that range. The infection rate of this study may have been on par with the infection rates of other regional studies for the species, but among American bullfrog tadpoles, the infection rate was higher than the other regional study that also looked at *Bd* prevalence among American bullfrog tadpoles. Richards-Hrdlicka *et al.* found tadpoles of this species to have an infection rate of 10.9% (2013), less than half of the prevalence found in this study. The overall study seasons were similar, however Richards-Hrdlicka *et al.* did not report their data seasonally, so there is no comparison between Spring and Fall of the two studies (2013).

From a national and international perspective, the prevalence of *Bd* infection among bullfrog tadpoles in this study was about average or even less than the prevalence of the fungal infection in other studies. A study investigating *Bd* prevalence among the tadpoles of four different species of frogs, including American bullfrogs, across California found the *Bd* prevalence rate to be 14.7% (Padgett-Flohr and Goble 2007). That is lower than the prevalence

rate of this study but compared to the prevalence rate among the tadpoles of the other three species of frogs (1.3%), 14.7% is significantly higher. What is intriguing among the Padgett-Flohr *et al.* study is not so much the overall prevalence of *Bd* among bullfrog tadpoles, but the seasonal prevalence.

Padgett-Flohr and Goble (2007) found only 1 out of 366 bullfrog tadpoles to be infected with *Bd* in the Fall (0.3%), but of the 68 bullfrog tadpoles sampled in the Spring, 63 were infected with *Bd* (92.6%) (Padgett-Flohr and Goble 2007). This trend of a significantly higher *Bd* prevalence in the Spring compared to the Fall was only seen in American bullfrog tadpoles. Had an equal amount of bullfrog tadpoles been sampled in both the Fall and the Spring, and the infection rate remained the same for both seasons, the overall combined infection rate for both seasons would have been closer to 45% compared to the 14.7% rate reported (2007). While the disparity between the Spring and Fall *Bd* infection rate was not quite as large in this study (30.6% in the Spring and 13.7% in the Fall), season was still a significant factor in *Bd* prevalence (Table 2, $p=0.009$), with the Spring showing a significantly higher infection rate.

A regional study determining *Bd* prevalence in New England frogs and toads had similar findings. The researchers found that the *Bd* infection rate of the frog and toad species sampled, which included bullfrogs, significantly increased during the months of April, May, and June. The percent of infected individuals decreased in June and July before increasing once again in September. While this was evident in all of the amphibians sampled, this was especially evident in primarily aquatic amphibians, which included the American bullfrog (Longcore *et al.* 2007).

Another study researching *Bd* prevalence in bullfrog tadpoles of Kanagawa, Japan sampled frogs from May through July, similar to the Spring period of this study and Padgett-Flohr and Goble. They sampled 59 bullfrog tadpoles and found an infection rate of 62.7%

(Kadearu et al. 2015). While they do not have a Fall period to compare to, they at least show another high prevalence rate of *Bd* among bullfrog tadpoles in the Spring. There are multiple cases of high *Bd* prevalence rates among bullfrogs in the Spring. Hanselmann *et al.* sampled bullfrogs in the Venezuelan Andes during March of 2002 and found an extremely high *Bd* infection rate of 96% (2004). Another study sampled bullfrogs from the Platte River of central Nebraska during late April, June, July, and September of 2010 (Harner et al. 2011). They found an overall infection rate of 41%, however the infection rate separated by sampling month showed a statistically significant seasonal trend. Bullfrogs that were sampled in April had a prevalence rate of 67% while the only other sampling month that had a positive sample was July with a 37% *Bd* prevalence rate. No positive samples were found in both June and September (Harner et al. 2011). The previously mentioned studies all seem to indicate that *Bd* prevalence rates are higher in the Spring than in the Fall, especially in American bullfrogs.

This phenomenon of bullfrogs, especially tadpoles, having a significantly higher *Bd* prevalence rate in the Spring compared to the Fall is not seen as much in frogs of other species who share the same environment with bullfrogs. Both Padgett-Flohr and Goble (2007) and Harner *et al.* (2011) observed no significance between Spring and Fall *Bd* infection rates in other species of frogs and toads. In fact, the infection rates of the other species were all less than 10%, even when bullfrog infection rate was high in the same sample area and during the same season.

There are two main hypotheses that likely both play a role into why bullfrog *Bd* prevalence varies by season. The first is that the ecological properties of the fungus are better adapted to living and reproducing during the Spring. *Bd* grows best between 17°-25° C. The fungus grows slowly below 17° C, and it grows very slowly or even stops growing at 28° C (Kadearu et al. 2015). This makes the Spring to early Summer the optimal time for *Bd* growth

in many parts of the world, including central Massachusetts. For this study, *Bd* growth is considered the ability of the fungus to go through its life cycle and the spread of the fungus in an environment. The optimal *Bd* growing temperatures fall between mid-April and mid-June in central Massachusetts (U.S. Climate Data 2019). The average September temperatures in central Massachusetts also fall within the optimal *Bd* growing range but the results of this study showed a significantly lower *Bd* infection rate for bullfrog tadpoles during September when the Fall samples were taken.

There are a few reasons why this could have occurred. The first reason is that the time period where the temperatures are optimal for *Bd* growth lasts only about a month in the Fall before it begins to become too cold. This period of time might not be long enough for the fungus to rapidly grow and reproduce before it begins to enter a period of slow growth due to the cold temperatures. Zoospore production and zoospore activity both decrease when temperatures induce a decreased growth of the fungus (Stevenson et al. 2013). The spread of the fungus by both direct contact and zoospores in open water is not very effective when growth is slowed. The approximately three-month range of optimal temperatures for *Bd* growth in the Spring allows much more time for growth and infection of bullfrogs and their tadpoles in central Massachusetts. Studies have shown *Bd* to be much more prevalent in the winter compared to the summer (Sapsford et al. 2013). While the growth and reproduction of *Bd* may slow when the temperatures dip below the optimal growth temperatures, *Bd* appears to still grow significantly better during the Winter than during the Summer. Temperatures even just slightly above the optimal *Bd* growth range lead to a halt in growth of the fungus and even die off (Berger et al. 2004). This would allow the fungus to be better suited for rapid growth and infection coming out of the Winter than it would be coming out of the Summer. This might also allow bullfrog

tadpoles to remain infected easier over the winter than over the summer, leaving more tadpoles infected with *Bd* in the Spring than in the Fall (Kadekar et al. 2015).

This point plays into the second hypothesis as to why *Bd* infection rate varies by season. The second hypothesis is that the bullfrog life cycle encourages a higher infection rate in the Spring compared to other amphibian species (Kadekar et al. 2015). The overwintering by a bullfrog tadpole is unique, as many frog species hatch in the Spring and metamorphose into an adult before the winter comes. The overwintering of bullfrog tadpoles allows them to possibly remain infected over a Winter into a Spring or even become infected over the Winter. This allows for a much higher likelihood that a bullfrog tadpole will be infected with *Bd* in the Spring compared to the tadpole of another species of frog that recently hatched. Late-Spring to early-Summer is thought to be the time of the year when bullfrog tadpoles are most likely to be infected due to the older tadpoles getting ready to go through metamorphosis and become adults. Tadpoles at the end of the tadpole phase in the late-Spring and early-Summer have spent at least a year if not more in the water which is a long period of time to potentially encounter *Bd* (Kadekar et al. 2015).

The resistance of a bullfrog to chytridiomycosis is another potential reason as to why *Bd* infection rates seem to vary by season compared to other amphibian species. The ability of a bullfrog to become infected with *Bd* and survive allows for a researcher to sample a bullfrog at any point while it is infected. This means that a positive sample could come from a bullfrog who has been infected for a day or even for multiple weeks. In other species who are not resistant to chytridiomycosis, the fungal disease often leads to death within weeks (Briggs et al. 2010). This could lead to fewer positive samples being collected by researchers as the time period to collect a living positive sample in the field is limited to potentially two to three weeks compared to the

potential one to two-month span of a bullfrog. This could especially be apparent among tadpoles as individuals infected with *Bd* are often described as lethargic and slow-moving (New South Wales: Office of Environment and Heritage 2015), and infected tadpoles may choose to spend more time deeper in ponds and lakes rather than near the edges of the body of water where samples are collected.

With the exception of Hanselmann *et al* (2004), all of the studies, including this one, occurred in temperate forest climates, where seasonal changes in temperature and rainfall occur. Hanselmann *et al.* is different in that it occurred in the highland climate of the Venezuelan Andes, but it is also similar to all of the other studies in that the area being researched goes through periods of hot temperatures and cold temperatures throughout the year. The observation of seasonal variation in *Bd* prevalence in bullfrogs may only occur in ecosystems where the climate includes significant changes in temperature between seasons and throughout the year. This is why research being done in areas where *Bd* infection rates and lethality are so high, the tropics and subtropics of Central America, South America, Africa, and Australia, are not as likely to observe the same seasonal variation of infection rates in bullfrogs or in any other species (Longcore et al. 2007). In the tropical regions where *Bd* is wreaking havoc, there are fairly optimal temperatures for *Bd* growth year-round where the *Bd* life cycle never ends or even slows down much. The temperate forest climates of the world, including central Massachusetts, benefit from the Winter and Summer periods of significant heat and cold to slow and limit *Bd* growth.

While temperature certainly plays a role in the external growth and reproductive cycles of *Bd*, temperature could also play a key role in the amphibian internal growth of the fungus. As previously stated, *Bd* growth slows significantly or even stops once temperatures exceed 25° C

(Kadokaru et al. 2015). Thermoregulatory behavior, such as basking, seen in amphibians that allow them to raise their body temperature may allow an infected amphibian to raise its body temperature high enough to slow or halt the growth of the fungus on its own body (Berger et al. 2004, Longcore et al. 2007). While slowing the growth of the fungus may not on its own cure an amphibian from chytridiomycosis, it could help other potential healing factors such as the super-shedding that is theorized to occur in bullfrogs (Van Rooij et al. 2015). The slowing of the growth of *Bd* by an increase in body temperature might even be necessary for a bullfrog to completely shed away the fungus. Tadpoles have a harder time thermoregulating as they cannot leave the water to bask in the sun on land. The lack of efficient thermoregulation seen in their adult counterpart could be one reason why bullfrog tadpoles developed specific cardiac adaptations, such as an increase in ventricular mass to support a higher volume of pumped blood with less energy usage, to promote chytridiomycosis resistance (Salla et al. 2015).

However, amphibians are ectotherms, or cold-blooded in more common terms. This inability to self-regulate their body temperature forces them to find features in their environment to control their temperature, and this includes if they want to increase their body temperature while infected with *Bd* (Longcore et al. 2007). It is possible that activities such as long periods of basking in the sun could potentially be a sign of an amphibian that is infected with *Bd*. Once the weather of the Northeastern United States warms up in the late-Spring and Summer, the temperatures in the sun could be hot enough to cause thermal setbacks to the fungus on the skin of some species of amphibians that typically suffer lethal effects from *Bd* (Longcore et al 2007). This could cause some species of amphibians to be resistant to the disease in temperate climates during the Summer while the same species remains susceptible to the disease in climates where seasonal temperatures never become as hot (Longcore et al 2007). Amphibian-diverse regions

such as Madagascar are at risk to catastrophic chytrid infection due to an environment with an ideal climate for *Bd* growth (Kolby and Skerratt 2015).

As far as infection loads go among American bullfrogs and their tadpoles, there is a wide range of GEs. In this study, the infection loads ranged 1.32 GEs to 992.02 GEs, with most of the infection loads falling between 1 GE and 200 GEs. Similar regional, national, and international studies also observed a wide range of infection loads. Richards-Hrdlicka *et al.* had a range of 0.068 GEs to 535.00 GEs among bullfrogs in Connecticut (2013). While their maximum infection load was less than the largest infection load among this study for bullfrogs, other species of their study had upwards of 29,000 GEs. Compared to infection loads of other species, 992.02 GEs seems relatively low.

Martel *et al.* (2013) also observed infection loads among bullfrogs. The bullfrogs they sampled in Belgium and the Netherlands had infection loads ranging from 1.6 GEs to 368 GEs. Garner *et al.* (2006) studied the prevalence of *Bd* in bullfrogs primarily in Canada, but also sampled bullfrogs from the United States, Europe, South America, and Japan. This global study of *Bd* prevalence in bullfrogs also included a wide array of infection loads. The GEs of this study ranged from 0.1 to 211222.4 GEs. The extremely high infection loads of this study occurred primarily in South America with a few high GEs in Canada, while Europe had quite low GEs among the bullfrogs sampled.

While *Bd* infection loads among bullfrogs are shown to be quite broad in both this study and many other studies, the implications of infection load are not quite clear. Species, habitat, climate, and elevation have all shown no correlation or relationship with *Bd* infection load (Grundler *et al.* 2012). The highest GEs of *Bd* that a bullfrog carries is likely during the middle of the period it is infected. The *Bd* infection load among bullfrogs gradually decreases overtime

which is part of the mechanism allowing them to be resistant to chytridiomycosis (Gervasi et al. 2013). This could mean that the bullfrogs with low infection loads in this study and in other studies have either recently been infected with *Bd* at the time they were swabbed, or they have been infected with *Bd* for a longer period of time and the infection load has already decreased.

Bd infection load on its own might not be able to determine how long a bullfrog has been infected but combined with an observation of mouthpart deformities, infection time might be able to be determined. Hyperkeratosis of the mouth occurs with *Bd* infection among bullfrog tadpoles. There have been debates over whether macroscopic observation of a bullfrog's mouthpart deformities on its own can diagnose *Bd* infection (Kadekaru et al. 2015, Padgett-Flohr and Goble 2007, Fellers et al. 2001), but a bullfrog tadpole recently infected with *Bd* is less likely to have mouthpart deformities compared to a tadpole that has been infected for weeks. A bullfrog tadpole with a low *Bd* infection load and little to no mouthpart deformities might indicate a recently infected tadpole, while a tadpole with a low infection load and obvious mouthpart deformities might have already past the peak of its *Bd* infection and is slowly seeing its infection load decrease. Many healthy mouthparts were observed amongst tadpoles in this study, as well as a few certainly deformed mouth parts in some tadpoles. While accurate records of mouthparts were not kept throughout this study, more healthy mouthparts were observed than deformed mouth parts.

High prevalence and high infection loads of *Bd* promote efficient long-term carriers of *Bd*. While American bullfrogs certainly can have both, the lack of research showing any correlation between the two factors leaves the role of bullfrogs as long-term carriers somewhat blurred (Martel et al. 2013). High levels of prevalence with lower levels of infection load compared to other species appears to be more of the norm rather than the exception among

bullfrogs worldwide, especially in more temperate climates. While low levels of *Bd* infection load may not pose as much of a threat to other amphibian species sharing a habitat with a bullfrog compared to sharing a habitat with an amphibian with a high infection load, a large population or density of bullfrogs in an ecosystem might leave other species more susceptible with the high *Bd* prevalence levels among bullfrogs and their tadpoles. Low infection loads seem likely to be sufficient for maintaining *Bd* in an ecosystem (Martel et al. 2013), and if that is the case, then the current bullfrog habitat range that nearly blankets the globe could leave many other amphibian species threatened.

Studies have questioned how much of a role bullfrogs play as a reservoir species of *Bd*, as many other amphibian species and even non-amphibian sources, such as crayfish, waterfowl, and terrestrial reptiles, have been identified as reservoirs for the disease (Martel et al. 2013, McMahon et al. 2013). The generally low *Bd* infection loads causes researchers to question the role of bullfrogs as a primary reservoir species, but even a small *Bd* infection load can have disastrous consequences for susceptible amphibian species in novel ecosystems. This is why the American bullfrog plays such a large role in the global spread of chytridiomycosis. American bullfrogs and their tadpoles spread to new ecosystems by natural and human ways, and the ability of the species to carry *Bd* for weeks at a time without dying allows them to introduce the fungus to novel environments, even if it never ends up playing the role of the primary reservoir. Bullfrogs have lead areas previously untouched by chytridiomycosis, such as China, Japan and Korea, to have *Bd* found in their native amphibians and ecosystems (Yang et al. 2009, Bai et al. 2010, Kaddekaru et al. 2015). The high prevalence of *Bd* among the species, and especially its tadpoles, allows any bullfrog entering a novel environment a much greater chance of introducing the fungus to the ecosystem. Tadpoles who are infected late in the tadpole stage even have the

ability to carry the fungus with them for a period of time as an adult (Gervasi 2013), potentially bringing *Bd* to multiple bodies of water within an ecosystem.

While bullfrogs may be spreading this deadly disease across the globe, hopefully they can also be a part of the cure. Understanding how a bullfrog resists chytridiomycosis and appears to shed *Bd* from its epidermis could hold the key to the solution for this amphibian pandemic (Johnson and Speare 2003). The newest finding regarding the cure for chytridiomycosis may even lie in electrolytes. It has been shown that a restriction of electrolytes from hyperkeratosis of the epidermis leads to cardiac arrest and death in amphibians susceptible to chytridiomycosis (Voyles et al. 2009). While salt baths have been known to help cure frogs with chytrid in captivity for years, a new study has shown that even a slight increase in the salinity of an aquatic ecosystem through added electrolytes leads to a significantly lessened mortality rate among *Bd* infected amphibians and a reduced transmission rate of the fungus (Clulow et al. 2018). If this study shows substantial benefit for environments with prevalent *Bd* infection, electrolytes could be added to central Massachusetts aquatic ecosystems in the future. Changing the salinity of an environment affects more than just the amphibians, but if adding natural sea salts to aquatic ecosystems proves to lower the transmission rate of *Bd* and does not drastically affect other organisms, an increase in salinity could be the solution to chytridiomycosis worldwide.

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