

2013

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Recommended Citation

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**Investigating the role of epibiotic bacteria in defense against
chytridiomycosis
in the Green Frog, *Lithobates clamitans***

Sarah Nalven

Honors Thesis 2013

Colby College, Department of Biology

**Investigating the role of epibiotic bacteria in defense against
chytridiomycosis
in the Green Frog, *Lithobates clamitans***

Honors Thesis

Presented to

The Faculty of The Department of Biology

Colby College

In partial fulfillment of the requirements for the

Degree of Bachelor of Arts with Honors

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“In nature nothing exists alone... there are intimate and essential relations between plants and the earth, between plants and other plants, between plants and animals... [Nature is] an intricate web of life whose interwoven strands lead from microbes to man.”

– Rachel Carson, *Silent Spring*

Abstract

Amphibian populations have been declining for several decades, in part due to the emerging fungal skin disease, chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). Chytridiomycosis has caused extinctions and extirpations in many parts of the world, but its influence varies across species, populations, and individuals. The Green Frog (*Lithobates clamitans*), which inhabits the eastern half of the United States, seems capable of sustaining *Bd* infections without experiencing die-offs. It is possible that the Green Frog's epibiotic bacteria are contributing to its defense against *Bd* as the epibiotic bacteria of several amphibian species are known to play a role in the amphibian innate immune system through the production of antifungal metabolites. This study sampled three populations of *L. clamitans* in Maine, and screened individuals for the presence of both *Bd* infection and epibiotic bacteria capable of inhibiting *Bd* growth. In each population, 25-40% of individuals were infected with *Bd*, all of which sustained low infection intensities of less than 10 zoospore equivalents. All individuals possessed at least one strain of anti-*Bd* bacteria. Of the 78 strains of epibiotic bacteria assayed, the metabolites of 72 inhibited *Bd* growth to some degree, and 38 completely inhibited *Bd* growth. Three inhibitory bacterial strains were identified through sequencing of the 16S rRNA gene and all were classified as *Proteobacteria*. The large proportion of anti-*Bd* bacteria isolated from *L. clamitans* in this study suggests that epibiotic bacteria likely play a role in defending individuals of the species against chytridiomycosis. In the future, anti-*Bd* epibiotic bacteria could be used in probiotics as an amphibian conservation strategy.

Introduction

All animals host extensive communities of diverse microorganisms. These communities often carry out critical functions for their hosts, but their ecology and overall importance is only beginning to be understood. Although these symbiotic microorganisms were not deemed the “microbiome” until recently (Lederberg & Mccray, 2001), they have been influencing the development and evolution of their hosts for much longer (McFall-Ngai et al. 2005). The microbiome of amphibian skin, or the “epibiome,” exemplifies such a symbiotic relationship. In light of recent, dramatic global amphibian declines, a better understanding of the role of the epibiome in the amphibian innate immune system would greatly benefit conservation efforts in the maintenance and recovery of affected amphibian populations.

Globally, amphibian populations have declined continuously for the last few decades. About 42% of all amphibian species have experienced decline and about one-third of species are threatened by extinction (Stuart et al. 2004; Lips et al. 2006; Vredenburg et al. 2010). There is no one source of these declines, but causal factors likely include atmospheric change, environmental pollutants, habitat modification, invasive species and pathogens (Hayes et al. 2010). Not only do these factors each contribute to declines, but they can interact synergistically to increase the likelihood of decline through death or decreased recruitment (Kiesecker, 2002).

Despite the body of evidence that identifies various sources of amphibian population declines, some assert that the fungal disease chytridiomycosis is directly responsible for the decline or extinction of up to 200 species of frogs (Skerratt et al. 2007). These scientists hold that only chytridiomycosis explains the rapid and global nature of amphibian declines, calling

the impact of chytridiomycosis, “the most spectacular loss of vertebrate biodiversity due to disease in recorded history,” (Skerratt et al. 2007). Caution must be used in attributing declines solely to chytridiomycosis (Navas et al. 2012), but this disease is likely a large reason for recent, unprecedented levels of amphibian population declines and disappearances.

Chytridiomycosis is caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), and has been heavily implicated in extirpations and extinctions (Stuart 2004; Wake & Vredenburg, 2008). Over 350 frog species have been reported infected, and *Bd* has been found in populations on all six of the continents that frogs inhabit (Fisher et al. 2009a). Zoospores of this aquatic fungus infect the keratinized epidermis of post-metamorphic amphibians, growing into sporangia and ultimately releasing more zoospores, which can then reinfect the host or infect new hosts (Voyles et al. 2009). The pathogenesis of chytridiomycosis is not completely known, but in species such as *Litoria caerulea*, infection is thought to cause electrolyte depletion and osmotic imbalance, which eventually leads to cardiac arrest and death (Voyles et al. 2009).

Chytridiomycosis has not caused rapid extinction in all infected frogs, however. Instead, morbidity and mortality vary at the individual, population, and species level (Berger et al. 1999; Daszak et al. 2004; Kriger 2007b; Kilpatrick et al. 2010). For instance, while certain amphibians in Central America, Australia, and the western United States have been devastated by chytridiomycosis (Berger et al. 1998; Daszak et al. 1999; Carey et al. 2003; Lips et al. 2005), populations in the eastern United States have experienced a milder response (Peterson et al. 2007; Longcore et al. 2007; Gahl et al. 2012; Richards-Hrdlicka et al. 2013). It is possible that losses have occurred to a greater extent and remain undetected, but it seems more likely that

species have either attained a certain tolerance to infections or that environmental conditions of the eastern United States tend not to produce lethal infections.

The North American Green Frog, *Lithobates clamitans*, is one such species of the eastern United States that appears relatively tolerant of chytridiomycosis. *Bd* infection is widespread among Green Frogs, yet unlike most infected species, die-offs of Green Frogs have not been documented (Longcore et al. 2007). Furthermore, when experimentally exposed to a northeastern strain of *Bd*, not a single Green Frog died (Gahl et al. 2012). It has been suggested that populations of this species are unlikely to experience *Bd*-caused declines (Longcore et al. 2007; Gahl et al. 2012; Richards-Hrdlicka et al. 2013), but the reasons for this tolerance have not yet been elucidated.

There is evidence that differences in environment, *Bd* virulence, and host immune defenses may all contribute to variation in the outcome of chytridiomycosis among different frog hosts, and one or several of these differences might explain the enigmatic response in *L. clamitans*, or lack thereof, to chytridiomycosis. Each of these factors contributing to variation is elaborated below.

Many environmental variables have been correlated with the presence of chytridiomycosis. Lower temperatures have been associated with higher prevalence of chytridiomycosis in several species in Australia belonging to the families *Myobatrachidae*, *Hylidae*, *Ranidae*, and *Bufo* (Drew et al. 2006; Kriger & Hero, 2006a,b). Lower temperatures have also been associated with increased mortality due to chytridiomycosis in 34 different Australian species (Berger et al. 2004). One study even showed experimentally that chytridiomycosis could be cleared in the Red-eyed Tree Frog, *Litoria chloris*, by just 16 hours of

elevated body temperature (Woodhams et al. 2003). Species inhabiting permanent water, especially streams, also demonstrate higher chytridiomycosis prevalence (Kriger & Hero, 2007b; Brem & Lips, 2008), as well as species that overwinter aquatically versus terrestrially (Longcore et al. 2007). The distribution of both *Bd* and fatal chytridiomycosis has also been significantly associated with annual precipitation, species richness and biome (D. Olson, D.M. Aanensen & M.C. Fisher, unpublished data). Anthropogenic stressors can also act to suppress immune response and therefore increase susceptibility to infection or mortality (Blaustein & Kiesecker, 2002).

In addition to environmental factors that influence variation in chytridiomycosis prevalence and mortality, differences among *Bd* strains in virulence and other traits may also play a role. *Bd* strains that are genetically similar have been shown to differ significantly in virulence, though the more distantly related two strains are, the more they differ in several characteristics related to virulence (Fisher et al. 2009b). A study that compared growth trajectories of zoospores and sporangia in culture also found that the sporangia of a *Bd* strain isolated from Panama (JEL423) produced significantly more zoospores than the sporangia of a strain isolated from Maine (JEL258; C.R. Bevier, unpublished data), suggesting that the strain from Panama was more infectious. This functional diversity in different *Bd* strains may be responsible for some of the variation in effects of chytridiomycosis among host species, and may also indicate that *Bd* is evolving readily to adapt to its environment.

Extrinsic factors such as environmental variables and *Bd* virulence probably play a large role in determining which frogs sustain chytridiomycosis infections, but intrinsic factors such as host immune defenses also make crucial contributions. Because chytridiomycosis is a skin

infection, immune defenses of the skin are critical for protection. Bioactive peptides secreted from granular glands are one skin defense thought to help defend against pathogens. Also known as antimicrobial peptides, they have demonstrated activity against Gram-positive and Gram-negative bacteria, fungi, protozoa, and viruses (Nicolas and Mor 1995; Zasloff 2002; Apponyi et al. 2004; Ashcroft et al. 2007). Woodhams et al. (2007c) report that after experimental infection, frog survival rates increased with increasing effectiveness of antimicrobial peptides *in vitro*, with effectiveness based on minimal inhibitory concentration (MIC) and amount of peptide recovered per 1 cm² skin surface area. Other immune defenses of the skin include lysozyme, which is contained in the mucus that coats amphibian skin (Zhao et al. 2006), and antibodies, which are capable of binding to *Bd* in the African clawed frog, *Xenopus laevis* (Ramsey et al. 2010), a sign that in this species, the adaptive immune system can be activated in response to *Bd*. The epibiotic bacteria of amphibian skin is also considered part of the innate immune system of amphibians, helping to protect their host through production of antifungal metabolites. Like other immune defenses, it is possible that differences in epibiotic communities may lead to different host responses to chytridiomycosis. In this study, I investigated the epibiotic bacteria of the Green Frog, *Lithobates clamitans*, and the role these bacteria play in protecting hosts from chytridiomycosis, aiming to elucidate whether the Green Frog's epibiome contributes to its tolerance of *Bd* infection.

Evidence from recent reports confirms that epibiotic microorganisms participate in the amphibian innate immune defense, helping their hosts combat pathogens by producing antimicrobial metabolites. Bacteria from the skin of the frog species *Lithobates pipiens*, *Rana muscosa*, and *Hyalinobatrachium colymbiphylum*, and from the salamander species

Hemidactylium scutatum and *Plethodon cinereus* have been shown to inhibit growth of *Bd* (Harris et al. 2006; Woodhams et al. 2007a,b; Walke et al. 2011). Bacteria from the two salamander species also inhibit another pathogenic fungus, *Mariannaea* sp. (Harris et al. 2006). Several studies have demonstrated that epibiotic bacteria can inhibit *Bd in vivo* as well. Juveniles of *R. muscosa* were protected from lethal *Bd* infections, for example, when they were pretreated with *Janthinobacterium lividum*, a bacterium isolated from the skin of the red-backed salamander, *Plethodon cinereus*, known to produce antifungal compounds (Harris et al. 2009a). Woodhams et al. (2007b) provide evidence supporting the protective role of epibiotic bacteria in the wild, reporting that one population of *R. muscosa* with significantly fewer *Bd*-infected individuals than a second population included significantly more frogs with at least one anti-*Bd* epibiotic species present.

In addition to producing antimicrobial metabolites, epibiotic microorganisms may provide further protection simply by out-competing pathogens for space and other resources. Bacteria have evolved a wide variety of traits to increase their competitive edge (reviewed by Hibbing et al. 2010). Traits such as rapid growth rate, the ability to sequester carbon and phosphorus, and the ability to scavenge iron, all contribute to improving nutrition. Motility allows certain bacteria to encounter the most favorable locations, and the ability to produce adhesins or receptors that bind to specific surface features allow attachment at these locations. Bacteria endowed with efficient quorum sensing strategies and modes of producing extracellular polymeric substance (EPS) may also have an easier time living epibiotically than those without these traits. But the production of antimicrobial compounds is the most intensively studied mechanism of bacterial competition, and epibiotic bacteria in particular

have been studied for this competition strategy. In fact, several epibiotic bacteria isolated from marine environments have been found to boost antimicrobial compound production when exposed to a different strain of bacteria, suggesting they compete in this way on the surface of the host (Armstrong et al. 2001).

It is becoming clear that the importance of the microbiome to all multicellular species cannot be overstated (McFall-Ngai et al. 2005; Peterson et al. 2009). Some have even asserted that important amphibian defenses such as antimicrobial peptide secretions may in fact be secondary to the defense provided by epibiotic bacteria (Conlon, 2011). In this study, I tested the hypothesis that the composition of the Green Frog's epibiotic community and the production of secondary metabolites by this community help infected individuals tolerate *Bd* infection, and even defend against initial infection. Through screening three populations of the Green Frog for *Bd* infection and screening their epibiotic bacteria for the ability to inhibit *Bd* growth, I predicted that if the epibiotic community indeed plays a key role in the Green Frog's immune system, there would be a relationship between presence of infection and efficacy of the epibiotic community in inhibiting *Bd* growth either among individuals or across populations.

Methods

Frog collection

Collection and handling of all frogs was approved by the Colby College Institutional Animal Care and Use Committee (#2012-07). Maine Inland Fisheries and Wildlife provided permit # 2012-261 to collect frogs from Somerset and Kennebec Counties. From September 17

to October 28, 2012, 29 adult specimens of *L. clamitans* (designated GF3 through GF33, excluding GF22 and GF31) were sampled from three populations in Somerset and Kennebec Counties, ME, USA. All available individuals were targeted; seven frogs were collected from Johnson Pond, a medium-sized pond in Waterville, five from Mercer Bog, an impounded wetland formed by a dam, and seventeen from Donihue Pond, a small pond on a farm in Waterville. Frogs were captured with gloved hands or dipnets (which were changed and rinsed, respectively, in between each capture), and placed in a sterile plastic bag until sampling.

Frog sampling

All sampling took place in the field, after which all frogs were returned to their site of capture. First, frogs were sampled for *Bd* infections by swabbing with a sterilized, wooden toothpick according to a standardized protocol (Hyatt et al. 2007). Briefly, frogs were swabbed 30 times, with special focus to the drink patch, thighs, and webbing between the toes. Toothpicks were subsequently placed into 70% ethanol and stored at room temperature. Next, frogs were rinsed with 500 mL of sterile, deionized water to remove transient bacteria. Lauer et al. (2007) demonstrated that this technique is reliable in rinsing off transient bacteria while maintaining resident epibionts. Epibiotic bacteria were sampled using a standardized swabbing technique in which sterile cotton swabs were applied to each frog 30 times, ensuring a firm, rolling motion to the same areas that were swabbed for *Bd*. Swabs were placed in tubes of sterile saline solution (0.9% NaCl) and stored on ice until cultivation, which took place upon returning to lab. A second swab for each frog was placed in an empty, sterile tube, frozen immediately in liquid nitrogen, and then stored at -80°C for future pyrosequencing analysis.

To characterize individual frogs, snout-vent length was measured to the nearest 0.1 mm using vernier calipers. Body mass was measured to the nearest 0.001 g using a portable electronic balance and sex was determined from relative size of the tympanum. Before release, frogs were marked by toe-clipping the last joint of the fourth digit on the left foot to avoid replicated samples.

Determining presence of Bd infection

Toothpicks swabbed for *Bd* were stored in ethanol at room temperature for about two months, and then shipped to Kathryn Richards-Hrdlicka (Yale University, New Haven, CT) where the presence and amount of *Bd* zoospores was determined using quantitative PCR methods outlined by Boyle et al. (2004) and Garland et al. (2010). In short, 20 ul reaction volumes included 5 ul DNA template (diluted 1:10), and were run in triplicate. If the 146 base pair fragment within *Bd*'s ribosomal RNA was successfully amplified in 2 or 3 wells, the sample was deemed positive for *Bd* (Boyle et al. 2004). If amplification only occurred in 1 well, the sample was re-run. If amplification did not occur in any of the wells, the sample was deemed negative for *Bd*.

Quantification of *Bd* load is presented in zoospore equivalents, based on the comparison of amplification in sample wells to amplification in positive control wells containing known quantities of zoospores. Values for *Bd* load of each sample represent the mean number of zoospores of the three runs per 20 ul reaction volume, and are not adjusted for dilution. These values, described in zoospore equivalents, will be used as an index of the severity of *Bd* infection.

Culturing epibiotic bacteria

Immediately upon returning from the field, tubes containing cotton swabs and saline solution (0.9% NaCl) were vortexed (1 min) to release bacteria from the swab. Each solution was diluted 1:100, of which 100 ul was cultured onto low nutrient Difco R2A media (Becton, Dickinson and Co., Sparks, MD), and incubated at room temperature. After 2 weeks, colony-forming units (CFU) were enumerated.

Each individual frog's sample of culturable bacteria was evaluated for unique phenotypes. One representative CFU for every phenotype unique to a sample was then selected for further analysis. Selected CFU were transferred to fresh Difco R2A media containing cyclohexamide in order to eliminate fungal CFU, and assigned strain numbers according to the frog they came from (e.g. The CFU 20A was the first CFU isolated from Green Frog 20). CFU were isolated from 25 out of 29 frogs, including GF3-GF10, GF12-GF28, and GF33. The sample from GF11 did not produce any CFU and cultures from GF29- GF30 appeared contaminated.

Growth inhibition assay

Seventy-eight CFU were tested for their ability to inhibit growth of *Bd*. A cultured strain of *Bd* isolated from a Wood Frog, *Lithobates sylvatica*, in Orono, ME, USA (JEL 258) was used for all assays.

Before each assay, *Bd* zoospores were harvested. This involved growing active *Bd* cultures in 1% tryptone broth, applying 1 mL of active culture to 1% tryptone agar plates, and allowing about four days of growth. Just before setting up the assay, plates were flooded with 3

mL of 1% tryptone broth, liquid was removed and centrifuged at 980 x g for 5 min, and supernatant was pooled for use as zoospore stock. To ensure an adequate harvest, another 3 mL of 1% tryptone broth was added to the plates, allowed 20 min to activate zoospores, then liquid was removed and centrifuged. Plates were then flooded a third time with 1 mL of broth, which was immediately removed and centrifuged. All supernatants from this procedure were pooled, after which zoospores were quantified using a hemocytometer and diluted to a concentration of 1.0×10^6 zoospores/mL with 1% tryptone broth. A portion of this diluted zoospore suspension was heat-killed at 60°C for 20 min for use as a negative control.

Bacterial metabolites were also harvested before the assay. CFU were first grown individually on 1% tryptone agar plates. These plates were flooded with 3 mL sterile deionized water and left for 20 min. Liquid was removed and centrifuged at 3000 x g for 5 min, after which supernatant was passed through a 0.22 μ m sterile syringe filter (Fisherbrand, 09-719A). Sterile 1% tryptone agar plates were flooded, and the supernatant was centrifuged and filtered in the same manner, for use in negative and positive controls. Metabolites were stored for up to five weeks at -20°C until the assay.

The inhibition assay was conducted in 96-well flat-bottom microtiter plates. Each well received 50 μ L of zoospores (1.0×10^6 zoospores/mL) and 50 μ L of water containing metabolites. Metabolites were tested in triplicate for inhibition of *Bd* at 100%, 50% (1:1 dilution), and 20% (1:5 dilution) their original concentrations. *Bd* cultures were assessed both immediately after setting up plates and after six days of incubation at room temperature by measuring optical density at 490 nm using the BioTek ELx808 Absorbance Microplate Reader. Change in optical density (Δ OD) over the six-day period was used as a measure of *Bd* growth.

Relating Bd infection and CFU composition

For each frog, only about three CFU were assayed on average, with a range from one to seven CFU assayed. Despite these small samples, the average number of inhibitory CFU was compared among *Bd* infected and *Bd* uninfected individuals using a Student's T-test.

Characterizing epibiotic bacteria

The 78 CFU assayed were observed under a light microscope for Gram-stain, cell morphology, arrangement, and motility. The CFU 18A, 19B, and 20D, were also selected for identification using sequencing of the 16S ribosomal rRNA gene. DNA from each isolate was amplified with the 16S rRNA primers Weisburg fD1 (AGAGTTTGATCCTGGCTCAG) and Weisburg rD1 (AAGGAGGTGATCCAGCC; Weisburg et al. 1991). Twenty-microliter PCR reactions included 2 ul 10x buffer (10 mM Tris-HCl, 50 mM KCl), 1.6 ul MgCl₂ (25 mM), 0.4 ul dNTPs (10 mM), 1 ul of each primer (10 uM), 2 U Taq polymerase, and 13.6 ul sterile water. The amplification conditions involved one step at 95°C for 5 min, then 25 cycles at 95° for 2 min, 42°C for 30 sec, and 72°C for 4 min, and then a last step of 72°C for 20 min. PCR-amplified DNA was run on a 1% agarose gel and visualized with SYBR® Safe DNA gel stain to determine whether the 16S rRNA gene was present. PCR products from all three samples were purified using the QuickClean II PCR Extraction Kit (GenScript, Piscataway, NJ), and then bidirectionally sequenced at the DNA Analysis Facility at Yale University, New Haven, CT. The Ribosomal Database Project (RDP; Cole et al. 2009) was used to taxonomically classify the isolates that the amplicons belonged to, and the BLASTn search (<http://www.ncbi.nlm.nih.gov/blast>) on the GenBank database (Benson et al. 1999) was used to identify the most similar 16S rRNA sequences.

Statistical analysis

Differences between populations were analyzed with the Kruskal-Wallis one-way analysis of variance, including differences in mass, snout-vent length, and number of CFU per frog. Regression analyses were conducted on the relationship between mass and snout-vent length, and the relationship between mass and number of CFU per frog. In analyzing data from the growth inhibition assay, as mentioned, ΔOD was used as a measure of *Bd* growth. CFU were considered inhibitory if the average ΔOD of their three replicates was less than the average ΔOD of the positive control's three replicates. To determine statistically significant inhibition, the Mann-Whitney U-Test was run on every CFU, comparing each CFU's three ΔOD values to the three ΔOD values of the positive control. A CFU was deemed completely inhibitory if ΔOD was less than 0.01, and it was said to have a dose-response curve if ΔOD at 20% concentrated metabolite was higher than ΔOD at 50%, which was higher than ΔOD at 100%. A Student's two-tailed T-test was used to compare percentage of inhibitory CFU per infected frog versus uninfected frog.

Results

Frog collection and sampling

Both juvenile and adult Green Frogs were sampled in this study (Table 1). Across all three populations, body mass of individuals ranged from 1.6 to 82.7 g, and median mass was 17.7 g. Individuals from the Donihue population had the greatest mean body mass, while individuals from the Mercer population had the lowest, but differences between populations were not significant (Kruskal-Wallis, $p = 0.4966$). Snout-vent length ranged from 3.8 to 10.1 cm

across the three populations with a median snout-vent length of 5.8 cm. Again, population differences were not significant (Kruskall-Wallis, $p = 0.752$). There was a strong, positive correlation between individual mass and snout-vent length ($r^2 = 0.9059$; $p < 0.0001$; Figure 1).

Determining presence of Bd infection

Individual frogs were infected with *Bd* in all three populations (Figure 2). In both the Johnson and Mercer populations, approximately 40% of individuals were infected, while about 25% were infected in the Donihue population. Though frogs were not scrutinized for chytridiomycosis infection based on clinical symptoms, it was apparent during sampling that GF27 had an infection, based on pink and swollen pelvic skin and toe-webbing (Figure 3). Zoospores were in fact detected from GF27 with a load of 0.106 zoospore equivalents, the second lowest *Bd* load of all infected frogs (Table 2).

Nine frogs were infected in total (Table 2). The smallest infected frog was also the smallest sampled frog, weighing 1.6 g and measuring 3.8 cm in snout-vent length, but the largest infected frog weighed 28.1 g and measured 7.0 cm, even though frogs sampled weighed up to 82.7 g and measured up to 10.1 cm (Figures 4a-b). Of the nine individuals infected across the three populations, five had *Bd* loads below 1 zoospore equivalent, two had loads between 2 and 3, and two had loads above 6 (Figure 5).

Culturing epibiotic bacteria

CFU were isolated from 25 out of 29 frogs: GF3-GF10, GF12-GF28, and GF33. The sample from GF11 did not produce any CFU and cultures from GF29, GF30, and GF32 were unfortunately contaminated. Discounting the contaminated samples, each swab yielded an

average of 21 and a median of 13 CFU per frog (Table 3). On average the Donihue population included frogs with the most CFU, with one individual producing 146 CFU, while the Johnson population included frogs with the least. There was substantial variation among individuals, however, and differences between populations were not significant (Kruskal-Wallis, $p = 0.0623$).

There was also a very weak negative relationship between *Bd* load and number of CFU produced among frogs infected with *Bd*, though it was not significant ($r^2 = 0.1338$, $p = 0.1665$; Figure 6). There were two frogs with very high CFU counts and very low *Bd* loads, while there was one frog with a very low CFU count and a very high *Bd* load. Several frogs had low CFU counts and low *Bd* loads, but there were no frogs with high CFU counts and high *Bd* loads.

Growth inhibition assay

Out of 78 CFU whose metabolites were assayed for the inhibition of *Bd* growth, 72 inhibited *Bd* at 100% concentration, 72 inhibited *Bd* at 50% concentration, and 65 inhibited *Bd* at 20% of their original concentration (Table 4). Here, inhibition is defined as less *Bd* growth than occurred in the positive control. Of the 72 CFU that were inhibitory at 100% concentration, 60 exhibited significant inhibition (Mann-Whitney U-Test, $p < 0.05$). Of the 12 CFU whose undiluted metabolites were not inhibitory, two were found to significantly enhance growth (Mann-Whitney U test, $p < 0.05$).

In many cases, metabolites completely, or almost completely, inhibited *Bd* growth, defined as $\Delta OD < 0.01$. Thirty-eight CFU appeared completely inhibitory at 100% concentration, 18 did at 50%, and 2 did at 20% (Table 4).

There were several patterns of *Bd* growth in response to decreasing concentrations of metabolite (Figures 7a- 7d show examples of these patterns, but do not represent data for all 78 CFU). There were 49 CFU whose metabolites caused “dose-response” patterns in which less inhibition occurred when metabolites were less concentrated. Of these 49 CFU, 46 exhibited significant inhibition of *Bd* at 100% concentration (Mann-Whitney U test, $p < 0.05$). For example, 17A and 17B both caused less inhibition of *Bd* growth at lower concentrations (Figure 7a). Their curves take different shapes, however: while 17A was almost completely inhibitory at 100% and 50%, 17B was only completely inhibitory at 100% concentration. Some CFU also showed “dose-response” patterns with a wider range of *Bd* growth. The CFU 12F, for example, showed complete inhibition at 100% but no inhibition at 20%, with inhibition at 50% falling in between (Figure 7b). The CFU 12D also showed inhibition at 100%, but none at 20% (Figure 7b). Other CFU metabolites such as 8B and 9D caused similar levels of inhibition under all metabolite concentrations (Figure 7c). And yet others, such as 20A, were not the least bit inhibitory, but instead *Bd* grew more than in the positive control, and grew less with less concentrated metabolite (Figure 7d).

Relating Bd infection and CFU composition

Samples from all but one frog included at least one CFU that exhibited significant inhibition of *Bd*. The frog that did not (GF13) only had one CFU assayed, which was inhibitory but not to a significant degree. Based on the CFU that were assayed, on average each infected frog hosted about 75% inhibitory CFU, while each uninfected frog hosted about 80% inhibitory CFU. This difference, however, was not significant (Student's T-test, $p = 0.6994$).

Characterizing epibiotic bacteria

Of the 78 CFU, 64 were designated Gram-positive and 14 Gram-negative. All were spherical or rod-shaped (Figures 8a-b), but due to inadequate resolution of available equipment, it was difficult to determine arrangement and motility.

The 16S rRNA genes of three CFU, 18A, 19B, and 20D, were sequenced. Each of these CFU had demonstrated significant inhibition. Both 18A and 19B were isolated from uninfected frogs while 20D was isolated from an infected frog. Both 18A and 20D stained Gram-negative, while 19B stained Gram-positive. Analysis by the Ribosomal Database Project (RDP) revealed that all three isolates belonged to the phylum *Proteobacteria*.

According to the RDP, the CFU 18A was of the class *Betaproteobacteria*, the order *Burkholderiales*, and the family *Alcaligenaceae*. According to a BLASTn search, it was most related to the species *Advenella kashmirensis* (96% sequence similarity), *Bordetella petrii* (96%), *Bordetella bronchiseptica* (96%), and *Achromobacter xylosoxidans* (95%). It was also a little more distantly related to *Janthinobacterium sp. Marseille* (90%).

The CFU 19B was determined by the RDP to be in the class *Alphaproteobacteria*, but due to quality of sequencing, could not be confidently classified beyond its class. According to BLASTn, it was most similar to the species *Deinococcus maricopenis* (87%), *Desulfovibrio aespensis* (78%), *Desulfovibrio vulgaris* (77%), and *Deinococcus peraridilitoris* (75%).

The CFU 20D belonged to the class *Gammaproteobacteria*, the order *Pseudomonadales*, the family *Pseudomonadaceae*, and the genus *Pseudomonas*, according to the RDP. It was most similar to the species *Pseudomonas fluorescens* (96%), *Pseudomonas synxantha* (95%), and two strains of *Pseudomonas syringae* (95%) according to BLASTn.

Discussion

This study demonstrated that among three populations of *L. clamitans* in Maine, all contained individuals infected with *Bd*, a fungus known to be pathogenic to many amphibian species. These infected individuals all sustained very low intensity infections, however, and clinical signs of infection were only observed on one individual, suggesting that *L. clamitans* is probably not suffering lethal infections from these low *Bd* loads. This study also demonstrated that many bacterial species isolated from the skin of *L. clamitans* exhibit inhibitory properties against *Bd*, suggesting that these epibiotic bacteria may play a role in defending *L. clamitans* against the pathogenic effects of *Bd* infection.

Frog collection and sampling

Population sizes at the three sites were relatively small, which could account for differences between populations in male to female ratios, mass, and snout-vent length. These differences were not statistically significant, however, so samples from the three populations can be considered equivalent.

There was a strong relationship between body mass and snout-vent length. This is expected, considering that previous studies have found highly significant linear regressions of the relationship between mass and snout-vent length in the Green Frog (e.g. Deichmann et al. 2008). The ranges of both mass and snout-vent length were also not surprising. Adult Green Frogs from a population in upstate New York weigh between 30 and 70 g (Wells, 1978), and Green Frogs typically measure between 5.7- 8.9 cm in snout-vent length (Conant & Collins,

1991; Martof et al. 1980). The range of body size for frogs in this study was similar, though juveniles as well as adults were sampled, extending the lower range for body size. In addition, four frogs from the Donihue population weighed about 80 g, which extended the upper range.

Determining presence of Bd infection

Presence of *Bd* in all three populations was also not surprising. *Bd* infection has been documented in over 250 species of amphibians, including several populations of Green Frogs in Maine (Olson et al. 2013; Longcore et al. 2007). Percentages of infected specimens also seemed reasonable, considering one study, which used histology to examine Green Frogs from nine sites in Maine, found that 26% of Green Frogs sampled were infected. *Bd* prevalence in the Donihue population was almost identical, and prevalence in the other populations may have been greater due to small sample size. It is also possible that *Bd* prevalence in this study was greater because qPCR is a more sensitive method for detection than histology. Some, in fact, have criticized that qPCR is actually too sensitive for the reliable diagnosis of chytridiomycosis, and that presence of a few zoospores does not necessarily mean presence of disease (Smith, 2007).

Further, although *Bd* was detected in all three populations, detection does not indicate morbidity or mortality. Green Frogs are known to sustain infections both in the laboratory and in the wild, with little or no evidence of either mortality or population decline (Longcore et al. 2007; Gahl et al. 2012). It is also worth noting that frogs were sampled in the fall, when environmental temperatures are cooler and more conducive to *Bd* growth. As Piotrowski et al. (2004) report, *Bd* tends to grow best within 4-25° C. In some study areas, infections appear in

cooler months and clear in warmer months (Kriger & Hero, 2006a; Kriger & Hero, 2006b; Longcore et al. 2007), so it is possible that the infected frogs in this study had not been infected, or had exhibited lower *Bd* loads, just a few months earlier.

Although there were no significant differences between populations in percentage of frogs infected or average *Bd* load, there were some interesting differences between individuals. One difference was that smaller individuals were much more likely to be infected with *Bd* than larger individuals. This fits with previous observations of size-infection relationships. Kriger et al. (2007) report that frog snout-vent length was consistently the best predictor of infection levels across infected populations. Small frogs were not only more likely to be infected, but they carried more intense infections than larger frogs. Garner et al. (2009) experimentally infected toads with *Bd* just after metamorphosis, and found that body mass before exposure to *Bd* had the greatest effect on survival. Although the reason for this relationship has yet to be determined, there are several possibilities. First, smaller frogs could be more susceptible to *Bd*-related mortality, so that only uninfected frogs reach large sizes (Garner et al. 2009). Infection may also cause decreased growth-rates (Garner et al. 2009). Some have also speculated that larger frogs may be able to clear infection (Kilpatrick et al. 2010).

Another interesting difference between individuals was that many more had small *Bd* loads than large. If this pattern observed is in fact real, and not just a result of small sample size, there are a few possible explanations. First, intrinsic or extrinsic factors could be keeping *Bd* load low in Green Frog populations in Maine. Environmental factors, virulence of regional *Bd* strains, or immune defenses—either cell-mediated, antibody, or innate—could all be at work.

It is also possible that the pattern observed is due to the use of qPCR as an overly sensitive method of detection (Smith, 2007). The detection of *Bd* on the skin of a frog does not necessarily mean the frog has contracted chytridiomycosis, and smaller loads might be more prevalent in this study simply because they are figments of an overly sensitive tool, and not true *Bd* loads. Until it is known how many zoospore equivalents are necessary for producing symptoms of chytridiomycosis, results using this method must be cautiously interpreted. Furthermore, until a relationship is found between heavy *Bd* load and measured zoospore equivalents, as well as a relationship between zoospores equivalents and severity of infection, caution must be taken in adopting qPCR counts of zoospore equivalents as a measure of severity of chytridiomycosis (Smith, 2007).

It is worth noting, however, that Green Frog 27 (GF27) allows a very small glimpse into the relationship between *Bd* load measured by qPCR and actual infection. GF27 was visibly infected with chytridiomycosis in both the pelvic area and toe-webbing, but had one of the lowest zoospore counts of all infected frogs. Assuming there is a relationship between *Bd* load, measured zoospore equivalents, and severity of infection, this suggests that the frogs with lower zoospore counts are in fact sustaining chytridiomycosis infections.

Several studies have found that amphibians do not die when *Bd* infection intensities remain low (Cheng et al. 2011; Kinney et al. 2011; Retallick et al. 2004) and many believe that the key to host survival is the low infection intensities on individuals (Briggs et al. 2010; Cheng et al. 2011; Kinney et al. 2011; Vredenburg et al. 2010). In fact, a measure of >10,000 zoospore equivalents has been determined a lethal infection for species (Vredenburg et al. 2010). Low

infection intensities may be the reason Green Frogs in Maine do not experience die-offs from chytridiomycosis, but the question remains as to what factors keep their infections at bay.

Culturing epibiotic bacteria

Recent research has shown that many amphibian species harbor epibiotic bacteria on their skin, but this is the first study to focus on the epibiotic bacteria of *L. clamitans*. It is not surprising, however, that cultures produced up to 146 CFU, considering the prevalence of epibiotic bacteria in other amphibian species and the recent implications these bacteria have received in protecting the health of their hosts (Harris et al. 2006; Woodhams et al. 2007b; Harris et al. 2009a,b; Walke et al. 2011).

It is logical that the number of CFU per frog was not significantly different across populations since populations otherwise seem comparable. Individual variation in numbers of culturable bacteria, however, may be suggestive of variation *in vivo*. Though the negative relationship between number of CFU and zoospore load was not statistically significant, it is possible that the concentration of bacteria on frog skin is related to an individual's susceptibility to pathogens. Presumably, the more bacteria occupying skin surface area, the more a pathogen experiences competition for space and nutrients.

It is also worth noting that the epibiotic microorganisms isolated likely represent only a small fraction of the epibiotic community of *L. clamitans*. It has long been recognized that only a small fraction of microorganisms can grow in culture conditions (Pace, 1997). A number of factors could account for a certain microorganism's inability to be cultured (Wade, 2002). For example, a required nutrient may not be present in the culture medium, the culture medium

itself may be toxic, or other bacteria in the sample may produce substances that are inhibitory to other organisms. Additionally, one microorganism may depend on another for growth, either because it grows directly on another microorganism or it depends on another microorganism for growth initiation via production of certain compounds. For all these reasons, bacterial cultures fail to reveal the true diversity of the epibiotic community of *L. clamitans*. Even so, culturable bacteria still provide a good deal of information about these communities, especially about the dynamics between the epibiome and pathogenic organisms. As this study continues, molecular methods, such as pyrosequencing, will be employed to analyze the metagenomics of these epibiotic communities, complementing data from culture-based methods and conveying a fuller picture of the epibiome of *L. clamitans*.

Growth inhibition assay

The majority of CFU produced metabolites that were inhibitory to *Bd* growth to some degree, as represented by low ΔOD values for the *Bd* cultures over six days. There is no reason to believe that these bacteria don't also inhibit *Bd* growth *in vivo*, and that antifungal epibiotic bacteria don't play a substantial role in the host's innate immune defense.

It must be acknowledged that although the Mann-Whitney U-Test provides some context for the level of inhibition, the test proceeds using ranked data and does not account for actual values, so it is not completely reliable for samples that were run only in triplicate. Therefore, statistical significance of results should be taken into account with some skepticism since the p-values generated may be imperfect approximations. The numbers speak for themselves, however. It is almost surely no coincidence that in 72 out of 78 trials with CFU

metabolites, *Bd* grew less than the positive control. Furthermore, when metabolites were screened at 100% concentration, in almost half of all trials (38 out of 78), *Bd* growth was completely inhibited, indicating that at least these CFU produce antifungal metabolites. In future studies, growth inhibition assays should be run with more replicates so that more rigorous statistical analyses can be employed.

Dose-response patterns further indicate that these CFU are inhibitory. Presumably, as the antifungal compounds they produce become more dilute, so do their inhibitory effects. CFU 17A and 17B both exhibited dose-response curves, but their curves took on different shapes perhaps because their metabolites had different minimum inhibitory concentrations (MIC). It seems that 12F actually covered its entire range of inhibition abilities over the course of the dilutions, completely inhibiting *Bd* at 100% and surpassing its MIC at 20%. 12D also surpassed its MIC, but even at 100% concentration was not able to completely inhibit *Bd*. However, it must be noted that because of the design of this assay, it is not fair to compare CFU to each other, but instead, only fair to compare each to the positive control. Although metabolites were harvested according to a standardized protocol, depending on the CFU, metabolite concentration could vary. Because there is no practical method for adjusting metabolite concentrations so they are equal, they were simply used in the concentrations in which they were harvested, and only compared to the positive control.

Of the CFU that did not exhibit dose-response curves, there were some whose metabolites exerted the same level of inhibition at all dilutions, such as 8B and 9D. This is probably because metabolites had to be much more dilute to reach their MIC. If this assay was

performed again and metabolites were diluted further, CFU that exhibited this pattern would most likely begin to exhibit dose-response curves.

The CFU 20A was unique in that it exhibited a “reverse” dose-response curve, where *Bd* grew the most with the most concentrated metabolites, and the least with the least concentrated metabolites. This suggests that 20A produces a compound that is conducive to *Bd* growth, rather than inhibitory. Interestingly, GF20 was infected with *Bd*, and it is possible that the CFU 20A played a role in favoring conditions for chytrid growth on the skin of GF20.

This is the first evidence that *L. clamitans* hosts epibiotic bacteria with antifungal properties. Of course this is not the first time amphibian epibiotic bacteria have been found to inhibit *Bd*. Previous studies have measured *in vitro* growth of *Bd* in the presence of epibiotic bacteria or their metabolites and found inhibition (Harris 2006; Lauer et al. 2007; Lauer et al. 2008; Myers et al. 2012). Other studies have even demonstrated inhibitory effects *in vivo*. Some have experimentally infected amphibians with *Bd* after inoculation with antifungal bacteria (Harris 2009a,b), and others have found metabolites on amphibian skin in concentrations high enough to inhibit or kill *Bd* (Brucker et al. 2008b).

Although it may not be entirely surprising that *L. clamitans* harbors anti-*Bd* epibiotic bacteria, the proportion of bacteria that were found to be antifungal in this study is astonishing. In non-amphibian epibiomes, studies have found that about 17% of bacteria isolated from species of marine algae and 25% of bacteria isolated from the surface of seaweed were antibiotic-producing strains (Lemos et al. 1985; Mearns-Spragg et al. 1997). While these proportions are much higher than those found in free-living or soil-associated bacteria (Lemos et al. 1985), they do not come close to the proportions of inhibitory bacteria found on *L.*

clamitans in this study. At their original concentrations, an astonishing 72 out of 78 CFU inhibited *Bd* to some degree (92%), 60 out of 78 were significantly inhibitory according to the Mann Whitney U Test (77%), and 38 out of 78 inhibited *Bd* completely (49%).

What is more extraordinary is that the metabolites that were screened were not produced in the presence of *Bd*. Bacteria constantly alter gene expression in response to their surroundings, so it is logical that bacteria might upregulate expression of antifungal compounds in the presence of a competing fungus. In fact, there is evidence that the presence of chitin and carboxymethylcellulose, two components of fungal cell walls, induce or enhance production of antifungal compounds in cyanobacteria (Patterson & Bolis, 1997). Algal surface-associated bacteria, too, will increase production of antimicrobial compounds in response to the presence of competing organisms (Mearns-Spragg et al. 1998). It is likely that chemical defenses of amphibian epibiotic bacteria are also inducible, as anything otherwise would be wasteful of energy and therefore evolutionarily disadvantageous. If this is the case, metabolites screened in this study would only represent the baseline of potential antifungal production for these isolates, and antifungal properties might be even more potent if these isolates had been exposed to *Bd*. Some species may have even required the presence of an inducer compound of another species in order to secrete antifungal compounds all together, and were therefore evaluated as non-inhibitory when in fact they had the ability to inhibit *Bd* upon induction. It is also possible that these antifungal compounds are produced simply as a byproduct of metabolism, but that a highly competitive microbial environment selected for species that produced antifungal compounds via ordinary metabolic processes. This would explain their production in the absence of competing organisms.

Although this study has shown that compounds contained in the metabolite mixtures of each CFU are likely inhibitory, the identity of these antifungal metabolites remains unknown. The first identified antifungal compound produced by an epibiotic species of an amphibian was 2,4-diacetylphloroglucinol, produced by *Lysobacter gumosus* (Brucker et al. 2008a). Probably the best studied of amphibian antifungal epibiotic bacteria is *J. lividum*, known to produce the antifungal compounds violacein and indole-3-carboxaldehyde which inhibit *Bd* growth at relatively low concentrations (Brucker et al. 2008b). One might speculate that metabolites produced by the epibiome of *L. clamitans* would have similar structural properties to these known antifungal compounds. All are most likely low molecular weight compounds, and, like antimicrobial compounds produced by epiphytic bacteria, could be thermolabile, anionic, and unaffected by proteolytic enzymes (Lemos et al. 1985). It is also possible that these metabolites are simply acting by affecting the pH of *Bd* cultures. Since *Bd* grows optimally at a pH of 6-7 (Piotrowski et al. 2004), an increase in acidity, for example, could inhibit *Bd* growth. Whatever the identity of these compounds, it is clear that many are inhibitory of *Bd in vitro*. It is highly likely that these strains have similar effects in nature, protecting hosts from lethal *Bd* infections.

Relating Bd infection and CFU composition

Although frogs that were not infected with *Bd* possessed slightly more antifungal bacteria per individual, this difference was not significant. It may be that infected frogs have fewer or less effective inhibitory bacteria than uninfected frogs, but the current study is too limited to fully assess this relationship. For example, too few CFU were assayed per frog. If another study were to attempt to compare population trends with the presence of antifungal

bacteria, a much larger sample size of bacteria would be necessary. If these data were also complimented by metagenomic data, it might be possible to evaluate how large a role each antifungal species plays in each frog epibiome, but without molecular data we are left to speculation.

Characterizing epibiotic bacteria

A large majority of CFU were found to be Gram-positive, which may be due to bias of Gram-staining methods in which the decolorizing agent used was old and therefore potentially defective. In one of the only studies to investigate amphibian epibiotic diversity, however, McKenzie et al. (2012) found six dominant phyla present on the skin of three amphibian species, and two of these six phyla were Gram-positive, so many of these Gram-positive assignments could be correct. In addition, Lauer et al. (2007) found that rods and cocci of different sizes made up almost all of the bacteria on the skin of the Four-toed Salamander, *Hemidactylium scutatum*, which is on par with this study's observations.

Sequences of three antifungal CFU isolated from *L. clamitans* provide minimal information on the composition of the culturable members of the frog's epibiome, and a much more comprehensive study is required to gain a full appreciation for the community diversity. For instance, even though all three CFU identified belong to the phylum *Proteobacteria*, according to the RDP, the antifungal epibiome is likely more diverse than this. Lauer et al. (2008) found that the culturable bacteria with antifungal activity isolated from the skin of female *H. scutatum*, could be classified into four phyla based on 16S rRNA sequencing. In addition, McKenzie et al. (2012) found, using barcoded pyrosequencing, that among three

amphibian species, the number of unique phyla per species ranged from 10 to 18 phyla (McKenzie et al. 2012). It is therefore unlikely that the epibiome of *L. clamitans* is composed solely of *Proteobacteria*. The same study did find, however, that one of the dominant phyla across all individuals was *Proteobacteria*, and the most dominant phylotype across all three amphibian species was classified as a *Curvibacter*, from the class *Betaproteobacteria* (McKenzie et al. 2012). It is therefore possible that *Proteobacteria* are an abundant component of the epibiome of *L. clamitans*, and perhaps play an important role in its antifungal nature. Until all 78 CFU are identified, however, three sequences from three different individual *L. clamitans* cannot provide significant insight into the diversity of these communities.

Nonetheless, individual CFU can provide anecdotal insights into epibiotic diversity and function. For example, it is intriguing that 18A exhibits 90% sequence similarity to a species of *Janthinobacterium*, when another species of this genus, *J. lividum*, is one of the most effective antifungal bacteria isolated from the epibiome of an amphibian (Harris et al. 2009). It is entirely possible that 18A produces some of the same antifungal compounds produced by *J. lividum*, such as violacein and indole-3-carboxaldehyde (Brucker et al. 2008b), or produces similar compounds that possess the same antifungal mechanisms. It is also worth noting that all *Janthobacterium* species are Gram-negative, and this CFU was stained Gram-negative, supporting that Gram-stain results are accurate.

The CFU 19B experienced poor sequencing quality, which probably leads to some of the conflicting information in its results. BLASTn determined that 19B was the most similar to a species called *Deinococcus maricopenis*, which is known, along with the whole *Deinococcus* genus, for its ability to tolerate UV and gamma-ionizing irradiation (Pukall et al. 2011). This

tolerance of UV radiation may be advantageous to a species living on amphibian skin, since basking in the sun is a common amphibian behavior. *Deinococcus* does not fall in the phylum *Proteobacteria*, however, even though the RDP classified 19B under *Proteobacteria*. Gram-staining also revealed that 19B was Gram-positive, and *D. maricopenis* is a Gram-positive species, whereas *Proteobacteria* are Gram-negative. It is likely that the 16S rRNA sequence of 19B closely resembles that of both *Deinococcus* and *Proteobacteria* species owing to poor sequencing quality, but the assignment of *Deinococcus* is in accordance with Gram-stain results, making 19B likely not a *Proteobacteria*. Current results are not reliable, however, and methods must be replicated to determine the true identity of this CFU.

Meanwhile, 20D was classified into the genus *Pseudomonas*, sharing 96% sequence similarity with the species *Pseudomonas fluorescens*. Species of *Pseudomonas* are notorious for producing biofilms, or matrices of extrapolymeric substance (EPS), which help cells stick to each other and to a surface (De Kievit & Iglewski, 1999). Biofilms offer numerous advantages to cells, providing environmental stability and protection from aversive compounds, and facilitating communication, nutrient uptake, and the formation of microniches (Hall-Stoodley et al. 2004). The ability to produce biofilms would be extremely advantageous to a species living on amphibian skin, and it is likely that 20D has the capacity to produce biofilms *in vivo*. It also makes sense that 20D is closely related to *P. fluorescens* as this species is normally found on the skin of the mountain yellow-legged frog (*Rana muscosa*; Myers et al. 2012). *Pseudomonas fluorescens* also produces the antifungal compound 2,4-diacetylphloroglucinol, which is not only known to inhibit *Bd*, but known to act synergistically with the antimicrobial peptides of *R. muscosa* in the inhibition of *Bd* (Myers et al. 2012). It is very possible that a similar scenario

occurs on the skin of the Green Frogs in this study. Also like 18A, all *Pseudomonas* species are Gram-negative, and 20D was stained Gram-negative, once again supporting accuracy of Gram-staining results.

Although the reasons remain unclear, the amphibian adaptive immune system is largely unresponsive to *Bd* infections in those species tested so far (Rosenblum et al. 2012; Cashins et al. 2013). One reason for this may lie in the ability of *Bd* to release immunosuppressive factors that have been shown to inhibit lymphocyte proliferation and induce of apoptosis *in vitro* (Ramsey, 2011). This lack of a productive adaptive response across amphibian species suggests that in tolerant species, the innate immune system might compensate to some degree. Epibiotic bacteria have been shown to protect hosts from *Bd* infections, so these mutualistic communities can be regarded as extensions of the host innate immune system. The species-specific nature of these bacterial communities (McKenzie et al. 2012) further suggests that they are not simply living on amphibian skin due to chance colonization, but that they have evolved, in a highly competitive environment, a specific, mutualistic relationship with their host.

Future Directions

Although it seems that the individuals of *L. clamitans* included in this study harbor a large proportion of antifungal bacteria, there are currently very few studies with which to compare these data. Future studies must evaluate the epibiotic communities of other amphibian species and then compare the epibiotic composition of vulnerable species to that of tolerant species. If epibiotic bacteria play an integral role in amphibian immunity against *Bd*, tolerant species should host higher densities or higher diversity of antifungal bacteria.

Another important avenue to continue is to further investigate the epibiotic diversity of *L. clamitans*. As mentioned, all CFU isolated in this study should be identified, and metabolites should be isolated and identified as well. It would be interesting to know if antifungal bacteria are closely related phylogenetically, physiologically, or neither. It would also be interesting to know if antifungal compounds produced by different CFU share similar structure, and inhibit fungal growth by similar mechanisms, or if the epibiome produces a wide array of antifungal compounds that are distinct both in structure and function.

The relationship between host, pathogen, and epibiome is also intriguing from an evolutionary standpoint. It would be interesting to screen epibiotic bacteria for the ability to inhibit fungi other than *Bd* in order to gain a better understanding of whether selection pressures have largely selected for the evolution of an anti-*Bd* epibiome, or whether these epibiotic bacteria have a broader spectrum of inhibition.

A method of quantifying bacteria on the skin of amphibians could also illuminate the workings of the epibiome. As *Bd* load seems to predict intensity of infection, it would be interesting to determine whether density of bacteria affects density of *Bd* on the skin.

Finally, molecular work on the epibiome of frogs is critical in gaining a better picture of epibiotic diversity and function. The culture-based methods used in this study allowed identification of the CFU's antifungal properties and other attributes, but culturable bacteria make up only a small fraction of total epibiotic diversity (Pace, 1997). A combination of culture-based techniques and molecular techniques is necessary in order to understand the overall role that culturable antifungal bacteria play in the epibiome. During fieldwork in fall 2012, samples were collected and stored for metagenomic analysis via pyrosequencing. Time constraints

prevented completion of this aspect of the study. But once this analysis is accomplished, data can be combined with the results of this study to gain a broader picture of the epibiome and how it affects amphibian host immunity.

As we gain a better understanding of this community, epibiotic bacteria may become useful in conservation strategies as a kind of cutaneous probiotic. Probiotics are a relatively cost-effective, low-risk method for protecting against infectious agents (Wargo & Hogan, 2006). If it turns out that *L. clamitans* tolerates *Bd* infection because of a protective epibiome, it is also possible that their bacteria could be harvested so that it can be used to inoculate and protect more susceptible populations. The self-propagating nature of probiotics also make them an especially promising conservation strategy, as these bacteria would not only continue to replicate to protect their individual host, but would also be transferred to each other during activities such as mating, aggressive interactions, and congregating in hibernacula.

Conclusion

As amphibian population declines and extinctions continue, many scientists have focused their studies on declining populations, especially if the source of decline appears to be driven by chytridiomycosis. Few studies, however, examine the populations that are not undergoing declines, even though studying these stable populations may provide clues to what sets them apart. Populations of *L. clamitans* in the eastern United States seem tolerant to *Bd* infections, and provide a great resource for better understanding sources of immunity. A large proportion of culturable epibiotic bacteria isolated from individual *L. clamitans* in this study produce anti-*Bd* metabolites, suggesting that the epibiome of this frog may be part of the

answer. Some of these bacteria are also related to bacteria known to protect amphibians from *Bd*, further suggesting that the epibiome of *L. clamitans* plays a role in its tolerance of *Bd*. If these bacteria in fact aid *L. clamitans* in tolerance of *Bd* infection, it is possible that amphibian species vulnerable to chytridiomycosis host a smaller proportion of antifungal bacteria than tolerant species, or that their antifungal bacteria engages in less potent antifungal activity. As *Bd* load seems to predict intensity of infection and likelihood of mortality, the epibiome might be just the factor that keeps *Bd* load low in certain tolerant species. Learning more about the synergies of this symbiotic relationship may be very useful in aiding future amphibian conservation efforts.

Acknowledgements

Many thanks to Cathy Bevier, my advisor; her guidance over the last few years has greatly influenced my growth as a scientist, and her passion for her work has inspired me. My readers, Frank Fekete and Lynn Hannum, have also been of great help, not only in this project, but as two talented professors who have made me excited about everything they've taught me. Professors Ron Peck and Jim Scott also provided critical assistance with molecular techniques and statistical analyses, respectively, and correspondences with Doug Woodhams and Katy Richards-Hrdlicka, two scientists who do similar research, were instrumental in moving my project along. I could always count on Lindsey Colby to answer a question about my cultures or raid the microbiology supplies for anything I needed, and Vanessa Silvestri was of great help when it came to the molecular part of my project. As almost everyone who knew me this past fall knows, collecting frogs was no easy feat, and it could not have been accomplished without

the help of Laramie Maxwell, Lydia Ball, Katerina Faust, Sarah Kletzer, Sarah Lake, Josie Bazemore, Jayson Ito-Adler, Adam Spierer and Shelby O'Neill, who all accompanied me at one point or another in my frog-catching endeavors. Sarah Lake, Lydia Ball, and Victoria Abel were also incredibly helpful lab assistants throughout the year. Finally, I would like to thank the Colby College Biology Department and the Colby College Dean's Special Project Fund for the necessary funding for this project. In addition, this project was supported by grants from the National Center for Research Resources' INBRE (5P20RR016463-12) and the National Institute of General Medical Sciences (8 P20 GM103423-12) from the National Institutes of Health.

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Figures

Figure 1. Relationship between mass and snout-vent length of individuals.
Figure 2. Percentage of frogs infected with *Bd* in each population.
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Table 1. Characterization of each population.
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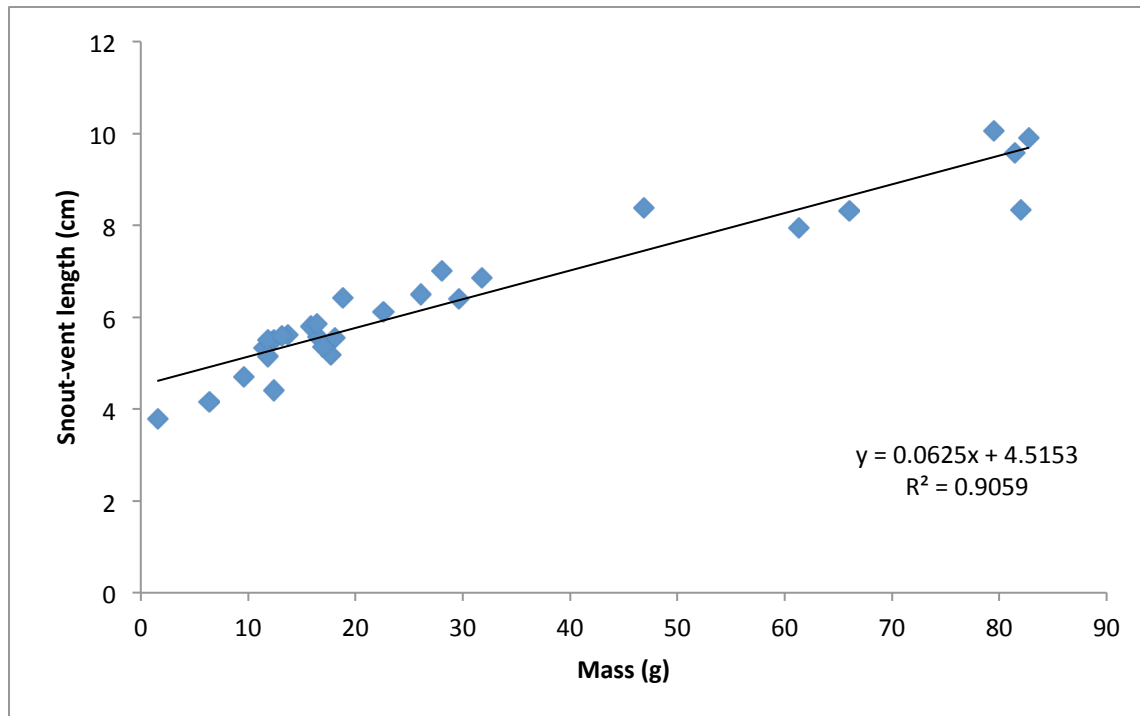


Figure 1. There was a strong, positive correlation between individual mass (g) and snout-vent length (cm; $p < 0.0001$) in sampled *L. clamitans*.

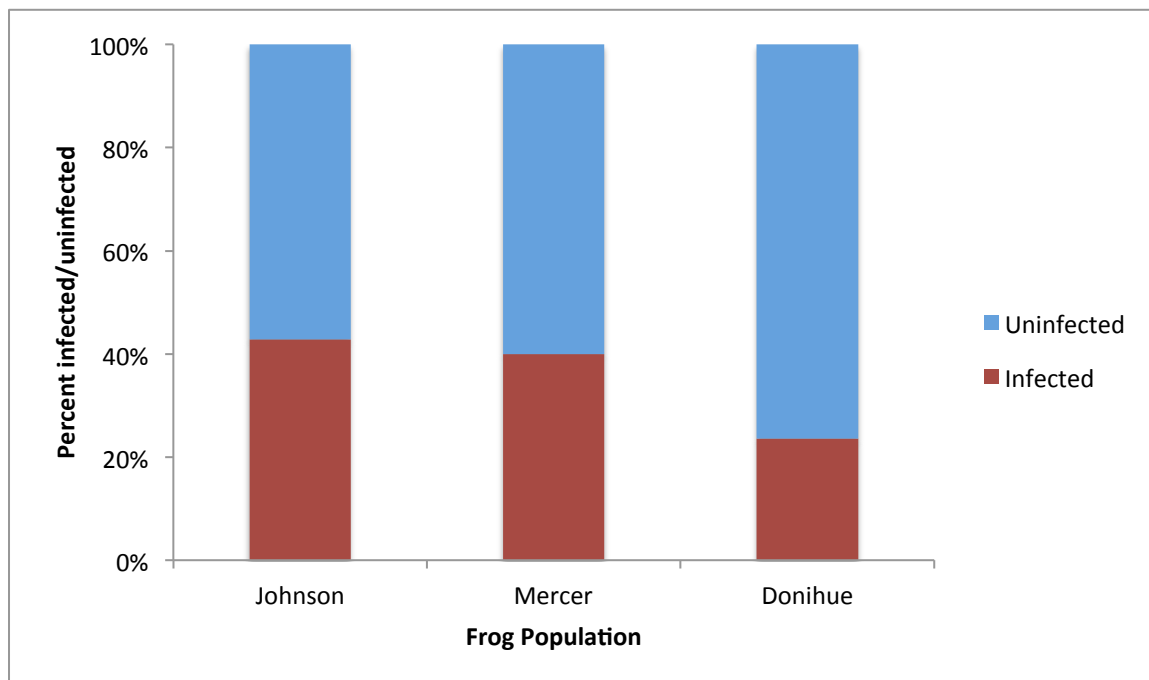


Figure 2. Percentage of frogs infected with *Bd* in the Johnson (n=7), Mercer (n=5), and Donihue (n=17) populations.



Figure 3. Green Frog 27 had a visible chytridiomycosis infection, displaying swelling and redness of pelvic skin.

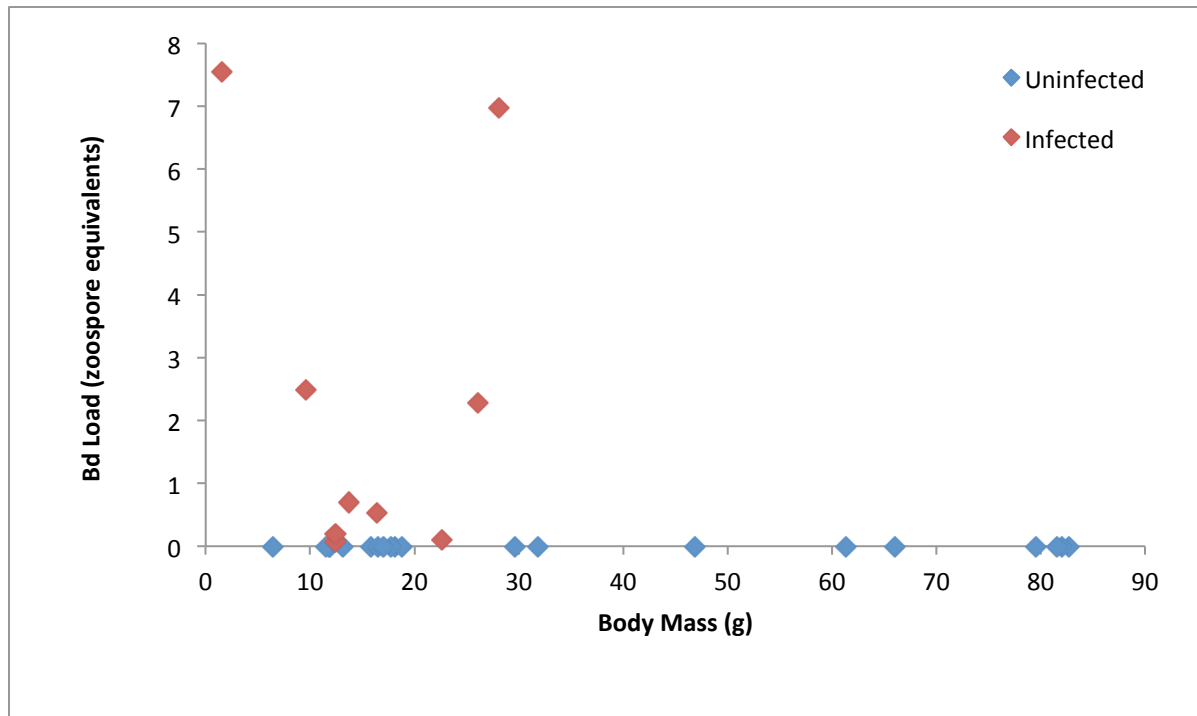


Figure 4a. Relationship between body mass (g) and *Bd* load (zoospore equivalents) for individual *L. clamitans*.

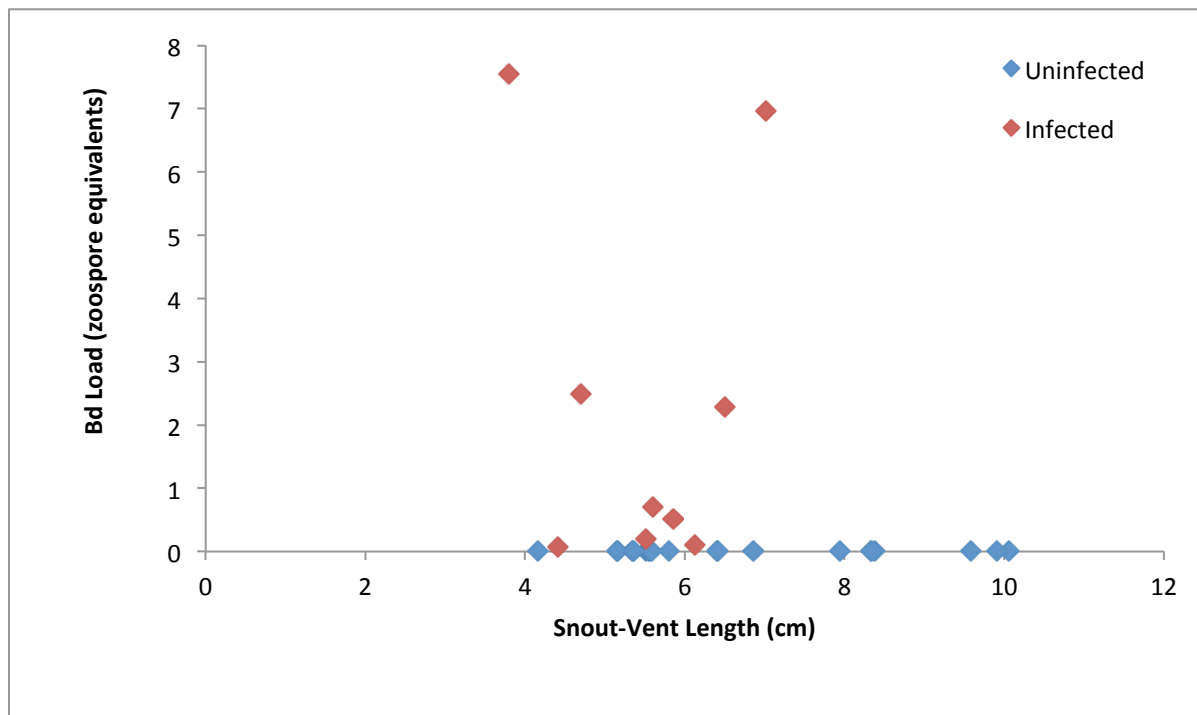


Figure 4b. Relationship between snout-vent length (cm) and *Bd* load (zoospore equivalents) for individual *L. clamitans*.

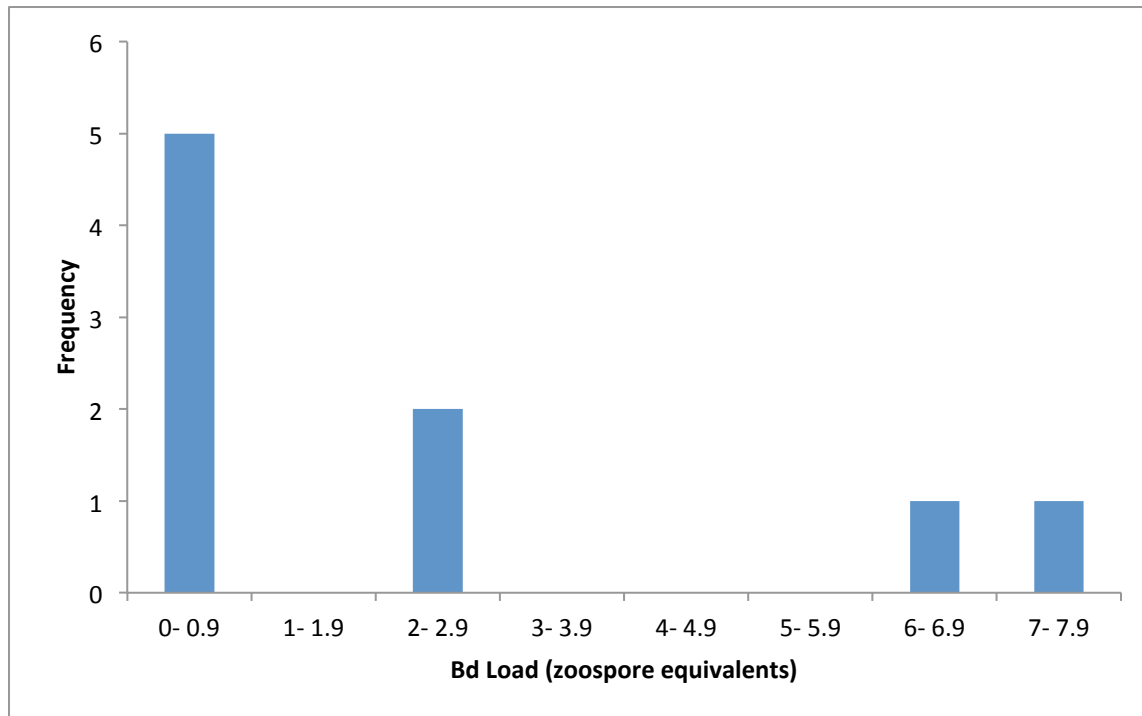


Figure 5. Frequency of *Bd* loads (zoospore equivalents) among the nine infected frogs.

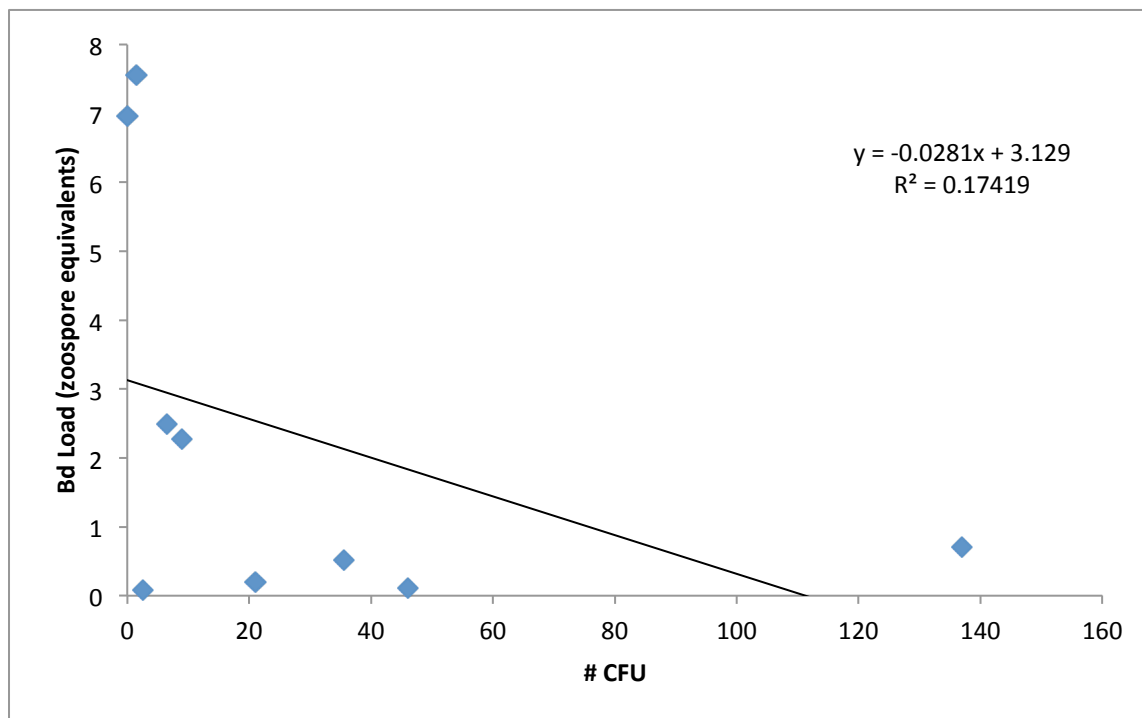


Figure 6. Relationship between number of CFU and *Bd* load (zoospore equivalents) of each individual *L. clamitans*, exhibiting a very weak negative relationship.

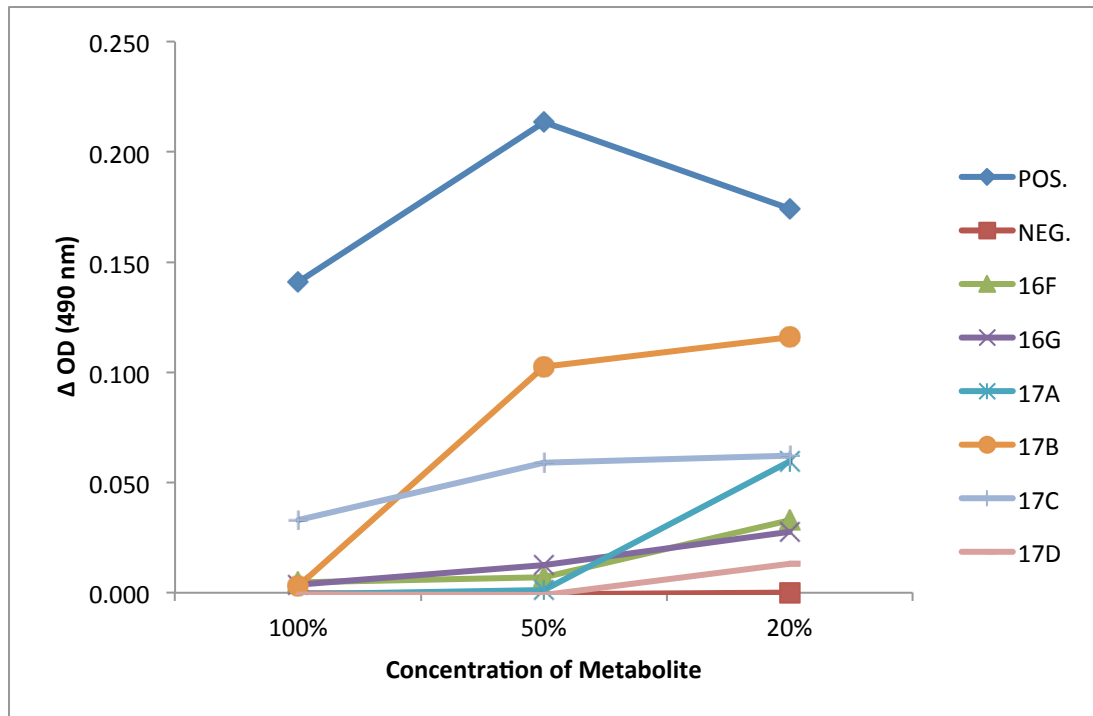


Figure 7a. Representative results from the growth inhibition assay. Notice that the metabolites of the CFU 17A and 17B both caused less inhibition of *Bd* growth at lower concentrations.

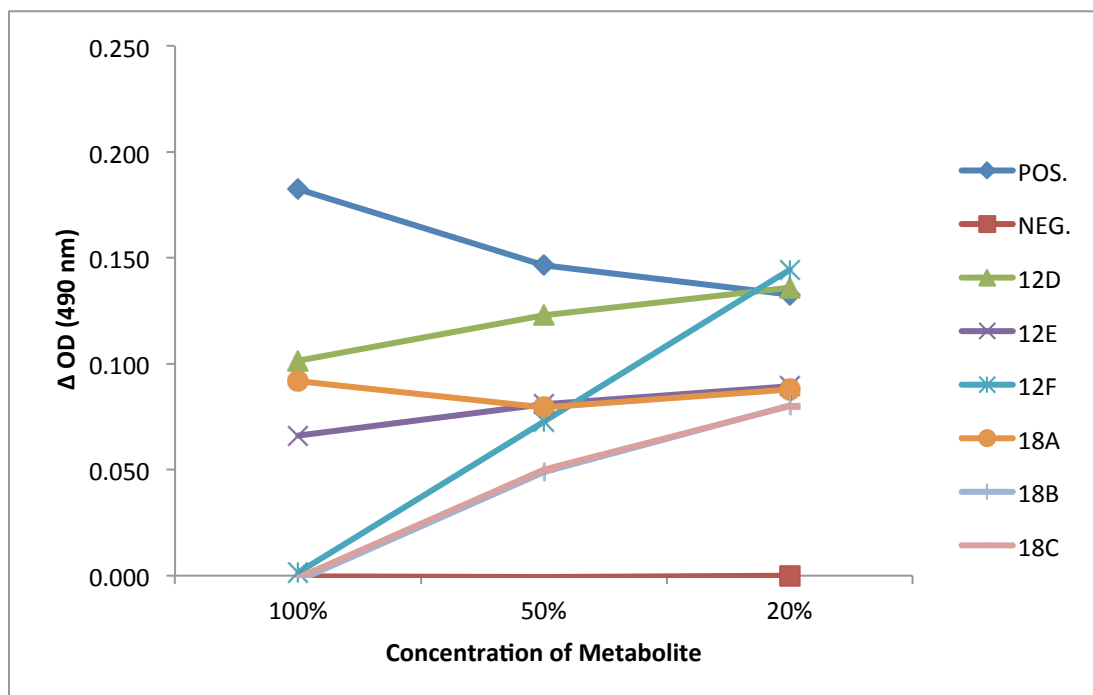


Figure 7b. Representative results from the growth inhibition assay. Notice that the dose-response curve for the CFU 12F covers a wide range of *Bd* growth.

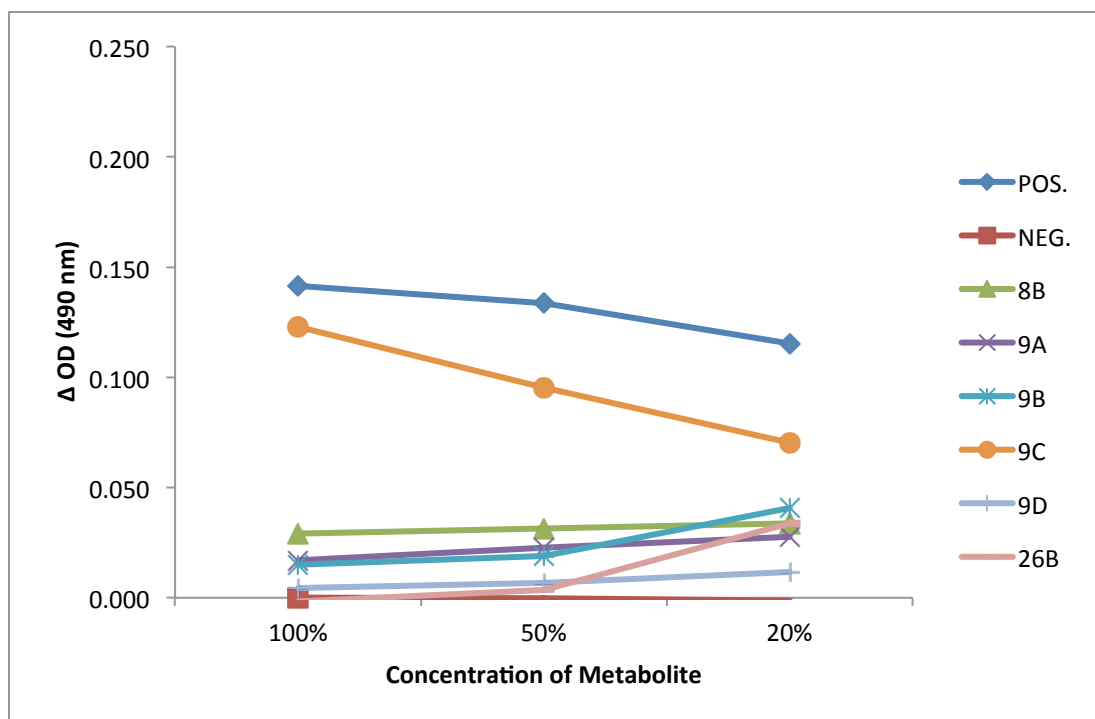


Figure 7c. Representative results from the growth inhibition assay. Notice that the metabolites of the CFU 8B and 9D caused similar levels of inhibition under all metabolite concentrations.

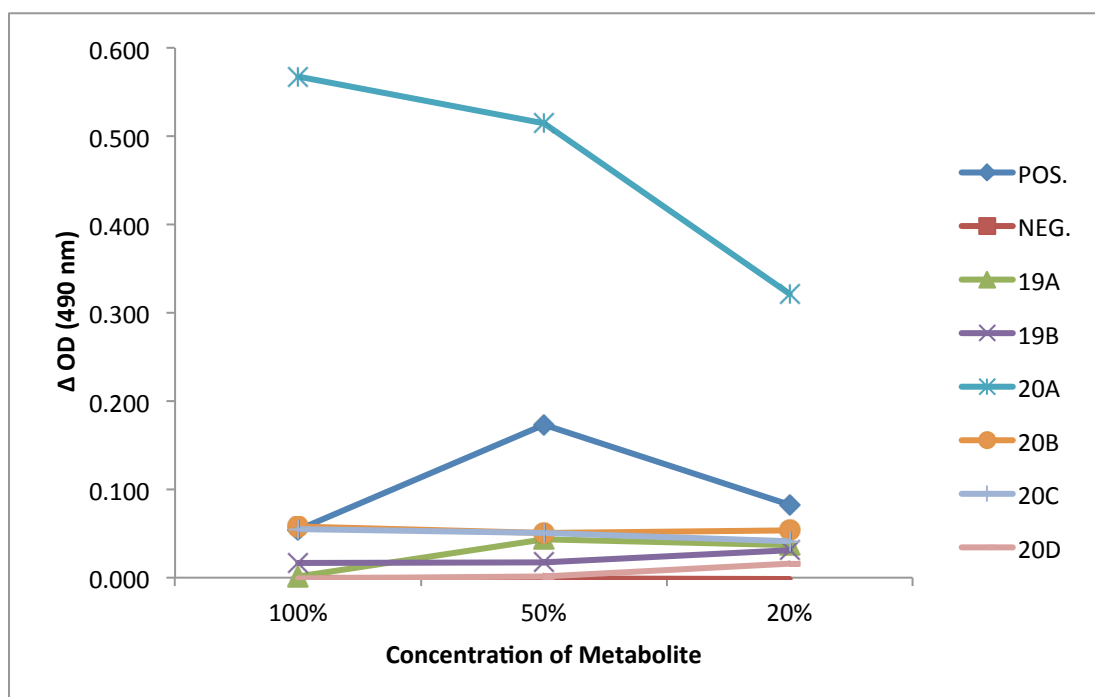


Figure 7d. Representative results from the growth inhibition assay. Notice that the metabolites of the CFU 20A caused *Bd* to grow more than the positive control.

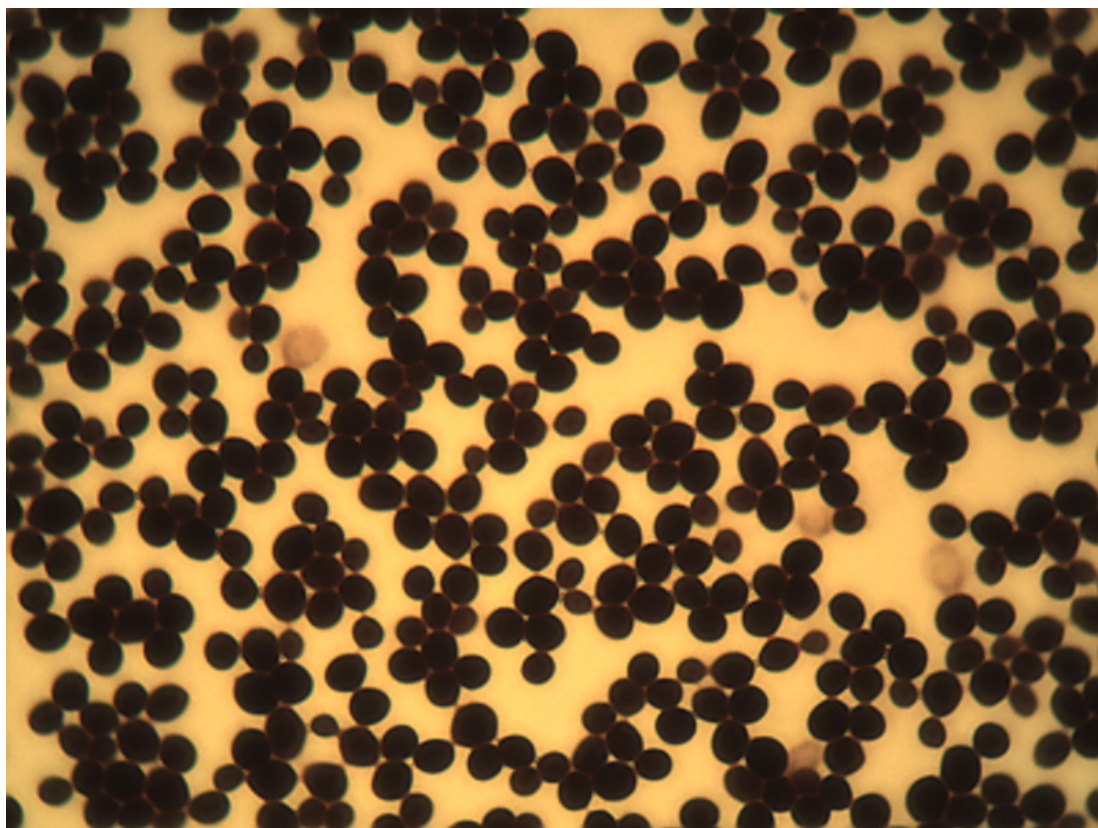


Figure 8a. An example of Gram-stain results for CFU 12A, which was Gram-positive.

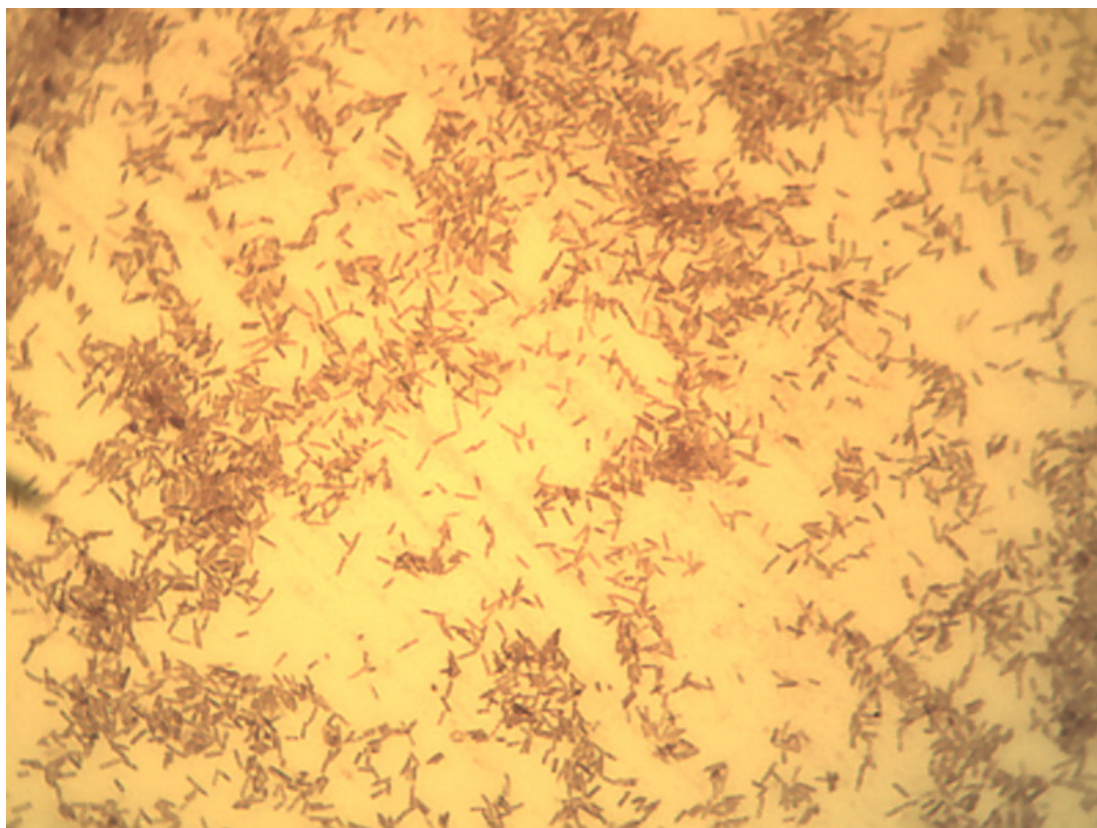


Figure 8b. An example of Gram-stain results for CFU 12B, which was Gram-negative.

Population	Johnson	Mercer	Donihue	Total
<i>Sample Size</i>				
Adult Females	5	2	6	13
Adult Males	2	0	2	4
Juveniles	0	3	9	12
Total	7	5	17	29
<i>Mass (g)</i>				
Mean	26.5	19.8	34.0	26.8
Median	26.1	12.4	17	17.7
Range	1.6- 66.0	9.6- 46.9	6.4- 82.7	1.6- 82.7
<i>Snout-vent length (cm)</i>				
Mean	6.0	6.1	6.6	6.2
Median	6.4	5.5	5.8	5.8
Range	3.8- 8.3	4.7- 8.4	4.2- 10.1	3.8- 10.1

Table 1. Characterization of each population including sex, weight and snout-vent length of each individual *L. clamitans*.

Green Frog	<i>Bd</i> Load (zoospore equivalents)
<i>Johnson</i>	
GF9	0.072
GF10	7.553
GF11	2.276
<i>Mercer</i>	
GF20	0.199
GF33	2.489
<i>Donihue</i>	
GF5	6.963
GF15	0.7
GF24	0.52
GF27	0.106

Table 2. The nine infected frogs and their *Bd* loads.

	Johnson	Mercer	Donihue	Total
<i>Number of CFU</i>				
Average	6	13	42	21
Median	3	14	35	13
Range	1- 16	0- 26	0- 146	0- 146
<i>Number of Phenotypes</i>				
Average	5	4	3	4
Median	3	3	3	3
Range	1- 12	2- 6	1- 7	1- 12

Table 3. Characteristics of cultured bacteria from each individual *L. clamitans*.

CFU	100% Inhibitory	50% Inhibitory	20% Inhibitory	Dose- Response	Mann Whitney	100% growth<0.01	50% growth<0.01	20% growth<0.01
3A	X	X	X	X	X	X		
4C	X	X			X			
5B	X	X	X	X	X	X		
5C	X	X		X	X	X		
6A	X			X	X	X		
6B	X	X	X	X	X	X		
7A	X	X		X	X			
7B	X	X	X	X	X	X		
7C	X	X	X					
7F	X	X	X		X	X		
7G	X	X	X	X	X			
7J	X	X	X	X	X			
7L	X	X	X		X	X		
8A	X	X	X	X	X			
8B	X	X	X	X	X			
9A	X	X	X	X	X			
9B	X	X	X	X	X			
9C	X	X	X					
9D	X	X	X	X	X	X	X	
10A	X	X	X					
10B	X							
10E	X	X	X		X			
10F	X	X	X					
12A								
12C		X	X					
12D	X	X		X	X			
12E	X	X	X	X	X			
12F	X	X		X	X	X		
13A	X	X	X					
14A	X							
14B	X	X	X	X	X			
15A	X	X	X	X	X			
16A	X	X	X	X	X			
16B	X		X					
16C	X	X	X					
16D	X	X	X					
16E	X	X	X		X	X		
16F	X	X	X	X	X	X	X	
16G	X	X	X	X	X	X		
17A	X	X	X	X	X	X	X	
17B	X	X	X	X	X	X		
17C	X	X	X	X	X			
17D	X	X	X	X		X	X	
18A	X	X	X		X			
18B	X	X	X	X	X	X		

18C	X	X	X	X	X	X		
19A	X	X	X		X	X		
19B	X	X	X	X	X			
20A								
20B		X	X					
20C		X	X					
20D	X	X	X	X	X	X	X	
20E	X	X	X		X			
20F	X	X	X		X			
21A	X	X	X	X	X			
21B		X	X	X	X			
21C	X	X	X	X	X			
21D	X	X		X				
21E	X	X	X		X			
23A	X	X	X		X	X	X	X
23B	X	X	X	X	X	X		
23C	X	X	X	X	X	X	X	
23D	X	X	X	X	X	X		
24A	X	X	X		X	X		
25A	X	X	X	X	X			
25B	X	X	X	X	X	X		
26A	X	X	X	X	X	X	X	
26B	X	X	X	X		X	X	
26C	X	X	X	X	X	X	X	
26D	X	X	X	X	X	X		
26E	X	X	X	X	X	X	X	
27A	X	X	X	X	X	X	X	
27B	X	X	X		X	X	X	X
28A	X	X	X		X	X	X	
28B	X	X	X	X	X	X	X	
28C	X	X	X	X	X	X	X	
33A	X	X		X	X	X	X	
33B	X	X		X	X	X	X	
Total	72	72	65	49	60	38	18	2

Table 4. Growth inhibition assay results for each CFU.