The Cutaneous Microbiome of the Eastern Red-Spotted Newt (*Notophthalmus viridescens*) and its Role as Defense Against Fungal Pathogens

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Abstract

Chytridiomycosis is a life-threatening disease which infects amphibian species worldwide. Chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* have been found to be the primary causes of chytridiomycosis. In past research, it has been found that the skin microbiome of certain amphibian species could have fungal inhibition properties, reducing the effects of the chytrid fungi. In this study, 15 eastern red-spotted newts (*Notophthalmus viridescens)* were collected, to determine if cutaneous bacteria of the newts secrete antifungal compounds. Pure cultures of each bacterium morphotype were produced from mass culture plates, with 188 morphotypes being isolated. The antifungal properties of the skin microbiome were obtained through a *Bd* assay, which was completed to find comparisons of which bacteria inhibited *Bd*.

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Introduction

Chytridiomycosis, an infectious fungal disease, has played a major role in global amphibian declines for decades. Chytridiomycosis is caused by two different fungal pathogens, *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*) (Harris et al., 2006). Multiple amphibian species extinctions have been linked to chytridiomycosis, which has been found on six of the seven continents (Weldon et al., 2004). Extinction rate of animals such as amphibians is growing at an alarming rate, amphibians generally being the most threatened group (Berger et al., 2005). These amphibian species that are in decline are integral for keeping ecosystems alive and flourishing, but *Bd* continues to negatively impact biodiversity of amphibians worldwide (Berger et al., 2005).

Many amphibian species continue to be researched to determine whether their skin microbiome has potential antifungal compounds that are able to combat the chytrid fungi. The species that is being researched in this study is the eastern red spotted newt, *Notophthalmus viridescens*, which has been identified as a species that is susceptible to chytridiomycosis through the infection of *Bd* or *Bsal* (Scheele et al., 2019; Rothermal et al., 2016). This is a common species found throughout the eastern United States in small ponds, lakes, and wetlands (Rothermal et al., 2016) including ponds in the Peaks of Otter area, Watson Pond.

Because of the potential benefits found in past bacterial studies, this research could significantly improve the health of amphibian populations by reducing the effects of

chytridiomycosis. Prior research has shown that chytridiomycosis may be inhibited through the secretions made by specific cutaneous bacteria in select species of amphibians. Since the microbiome of *N. viridescens* is still being studied, research to characterize it would provide insights into possible natural defenses against *Bd* or *Bsal*, with *Bd* being the primary focus in this study.

Batrachochytrium dendrobatidis (Bd)

Life Cycle of Bd

Batrachochytrium dendrobatidis (Bd) is a member of a group of fungi called chytrids, belonging to the Kingdom Fungi, Phylum Chytridiomycota, Class Chytridiomycetes, and Order Rhizophydiales. (Ossiboff et al., 2019). *Batrachochytrium dendrobatidis* is an aquatic organism that has two major life stages, the reproductive zoosporangium stage and the zoospore stage (Berger et al., 2005). The zoosporangium is the stationary, monocentric thallus stage which is primarily where *Bd* is able to asexually amplify and replicate (Berger et al., 2005). In this stage, the thallus creates a single zoosporangium, or sporangium, which is like container for the zoospores (Berger et al., 2005). As the thalli continues to grow and mature, mitotic division occurs and the zoosporangia becomes a complex multinucleate vessel (Berger et al., 2005). These immature sporangia are able to grow in other cells different than the keratinized cells, such as those containing pre-keratin. Keratin is the source from which the sporangium obtains nutrients, giving the sporangium the nutrients it needs to continue growing and creating more zoospores. These zoospores growing inside of the zoosporangium are then cleaved. The zoospores formed in the zoosporangium are then able to leave through the many papillae, which are developed while the sporangium grows and develops. The papillae are compartmentalized

and divided through thin septa, which are formed during the growth process of the zoosporangium. This allows for many sporangia to form, each with its own "discharge pathway" for the zoospores to be released, thus creating a type of colonial growth (Berger et al., 2005). When only a single thallus is formed, the sporangium has no dividing septa and is then characterized as monocentric (Longcore et al., 1999). From past research, this has been seen to be solely asexual reproduction, with no sexual reproduction observed (Berger et al., 2005). The zoosporangium stage occurs for 4-5 days, growing and assimilating, where it then finally releases the zoospores that were being assembled inside.

The zoospore stage occurs when the zoospores are released into the environment (Berger et al., 2005). The zoospores are motile, waterborne, and short-lived, always needing to have something to survive off in order to survive. The zoospores hold onto the keratinized layers, the stratus corneum and stratum granulosum by means of rhizoids (Berger et al., 2005). Rhizoids are filamentous extensions, allowing them to cling and assimilate (Berger et al., 2005). These zoospores are normally 3-5 µm in size, with a posterior flagellum that can be up to 19-20 µm long (Longcore et al., 1999). In the interior, a core portion of ribosomes is surrounded by a single cisterna of endoplasmic reticulum, 2-3 mitochondria, and a lipid globule complex (Ossiboff et al., 2019).

When looking at the activity of *Bd* at the zoospore level, it was found that 95% of the zoospores stopped moving within 24 hours and only moved around 2cm before encysting (Piotrowski et al., 2004). Zoospores can move by means of chemotaxis, allowing them to move towards different substances that could be present on the skin of the individual amphibian (Berger et al., 2005).

Bd is able to grow in varying circumstances, reproducing in temperatures of 4-25°C and at pH 4-8 (Piotrowski et al., 2004). *Bd* grows best in tryptone or peptonized milk, where it is able to create extracellular proteases, allowing for substances it finds using chemotaxis such as sugars, casein, and gelatin to be degraded (Piotrowski et al., 2004). The optimal temperature for protease activity and growth was at 17-25°C and at pH 6-7 (Piotrowski et al., 2004).

Physiology of Bd

While still relatively unknown, there are two hypotheses that have been developed in order to explain how *Bd* has the capability to attack and kill so many species of amphibians by just attaching to their superficial epidermis. First, *Bd* could be producing a proteolytic enzyme or other compound that is able to go through the permeable skin of the amphibian, causing long lasting damage (Berger et al., 2005). The fungus also damages the skin which results in oxygen, water, and electrolyte imbalance (Berger et al., 2005). This disrupts homeostasis of the amphibian, leaving it prone to death (Berger et al., 2005).

Bd is able to grow in the superficial keratinized levels of skin of the epidermis of the vertebrate host, settling between the stratus corneum and stratum granulosum layers of skin (Daszak et. al., 1999). The discharge papillae are seen deeper in the stratum corneum, and the papilla are able to protrude outwards. When *Bd* is present on the keratinized skin, one of the responses of the host is to increase the amount and size of the keratinized epithelial cells present. The stratum corneum is normally the width of 2 to 5 µm, but when exposed to the infectious fungus, it can increase up to around 60 µm, which is a significant difference. The cells become thick and disorganized, causing potential for homeostasis disruption. The immature sporangia grow and develop in the deeper skin cells, while the mature zoosporangia or recently released

sporangia are on the outer keratinized level (Berger et al., 2005). Due to the positioning of these sporangia, the zoospores released from the zoosporangia are able to also be released into the environment as the papillae are turned towards the outer skin surface (Berger et al., 2005).

Many recent studies have identified *Bd* or *Bsal* as the primary pathogens affecting the amphibian decline. According to past studies, when an amphibian is infected by *Bd*, its osmoregulation or respiration is disrupted across its skin, causing the amphibian to die (Carver et al., 2010). It has also been documented that *Bd* is able to release toxins into the host, causing there to be a potential metabolic rate change for the affected amphibians (Carver et al., 2010). A study was done on hylid frogs (*Litoria raniformis*), where six frogs were inoculated with *Bd* zoospores through different molecular testing in order to see the intensity of how *Bd* actually affected the frogs (Carver et al., 2010). It was found that clinical signs were noted and an increased amount of sloughing was noted, as the frogs were trying to shed their skin to normalize homeostasis levels. The frogs eventually died due to *Bd* compromising their body systems and unbalancing their water and respiration processes (Carver et al., 2010). This is a common occurrence once *Bd* is able to compromise the amphibian, creating a physiological unbalance and killing the amphibian.

Global and Non-Global Distribution of Bd

The history of how chytridiomycosis first emerged is still being debated. One proposition says that the earliest case of chytridiomycosis was discovered in 1938, where a museum specimen of African clawed frogs (*Xenopus spp.*) in South Africa was found to have succumbed to *Bd*. Around 3% of the 697 *Xenopus* species that were tested were found to have been infected by *Bd.* These were collected and identified from 1879 to 1999 by a research team investigating

these mysterious deaths (Soto-Azat et al., 2010). This species is believed to have been the original species that was infected with *Bd*, but no major increase of prevalence was noted in southern Africa after 1940 (Soto-Azat et al., 2010; Weldon et al., 2004). Others have noted that *Bd* could have originated from eastern Asia, with the prominent strain deriving from Asia and being distributed to the rest of the world (Fisher & Garner, 2020).

The *Xenopus* species was documented as being one of the first asymptomatic species to be infected by *Bd*, with widespread prevalence of *Bd* being found for this species (Soto-Azat et al., 2010). The African clawed frog was used as a major model for immunological and embryological studies, which resulted in them being shipped worldwide to different facilities. *X. laevis* also started to see an increase in exporting as the frogs were being used as one of the first types of pregnancy tests (Elkan, 1938). The female African Clawed frogs were injected with a woman's untreated urine, and the test was determined negative if the frog would produce tiny black and white spheres after around 12 hours (Elkan, 1938). This pregnancy test was called the Hogben test, which was one of the first pregnancy testing methods used around the world (Elkan, 1938). This was a great technological feat, but increased the rate of chytridiomycosis cases throughout the globe (Weldon et al., 2004). Because of the increase of *X. laevis* distribution, other amphibian species were infected as different trade routes were used and other potential amphibian vectors were infected (Weldon et al., 2004). This inevitably caused *Bd* to spread throughout the globe as the *Xenopus spp* acted as a carrier for *Bd* (Weldon et al., 2004).

The next major species that was responsible for spreading *Bd* was *Rana clamitans* in Saint-Pierre-de-Wakefield, Québec, Canada, in 1961 (Weldon et al., 2004). This species also can carry *Bd* asymptomatically, giving *Bd* another opportunity to continue spreading and infecting a

larger amphibian population (Weldon et al., 2004). Based on this information, it can be inferred that once *Bd* was first found in Quebec, it was not too long after that it made its way into the United States and started to infect other amphibians. The first chytrid infected species that was identified in the United States is thought to have been the American bullfrog (*Rana catesbeiana*) in 1978 in South Carolina (Weldon et al., 2004). Because the American bullfrog was used in food trade in America and other parts of the world, this could also be a reason as to how *Bd* was quickly transmitted throughout the United States.

In North America, once *Bd* entered Canada and the eastern United States, it has spread to the rest of the United States and can be found in Arizona, California, Colorado, Illinois, Indiana, Minnesota, North Carolina, South Carolina, Virginia, Wisconsin, and Wyoming (Kriger, 2007; Scheele et al., 2019). Chytridiomycosis cases continue to increase at an alarming rate, and locations with a large number of amphibian species continuing to be primarily affected (Kriger, 2007).

While the worldwide distribution of *Bd* has been recognized, some continents and countries have been impacted more than others have (Kriger, 2007). *Bd* has been found on all six inhabited continents, yet just recently in parts of Asia (Sreedharan & Vasudevan, 2021). Australia has been majorly affected, with 46 species being infected with chytrid. Located in three different locations in Australia, chytrid has devastated the eastern coast of Australia, and potential to spread still viable (Scheele et al., 2019). Along with Australia, New Zealand has recently seen an increase in cases, with Christchurch, the North and South Island, and Coromandel Range all affected (Scheele et al., 2019). In Central and South America,

chytridiomycosis has been found in countries such as Costa Rica, Mexico, Panama, Ecuador, Uruguay, and Venezuela (Scheele et al., 2019).

The different methods and studies that have been used to track the spread of *Bd*, such as through the European Union Project RACE (Risk Assessment of Chytridiomycosis to European amphibian diversity), allowed for researcher from 5 continents, 23 countries, and 62 amphibian species, to analyze and collect data on where and when *Bd* has affected different species (Fisher & Garner, 2020). Finding the evolutionary history of *Bd* and the different lineages has become doable as researchers continue to add information to this epidemiological database (Fisher & Garner, 2020).

Because of where *Bd* has spread, along with the different levels and where species have been heavily damaged, it is thought that *Bd* originally started in Asia, but was spread from South Africa to the rest of the New World (O'Hanlon et al., 2018). Believed to have originated in the Korean peninsula lineage, *Bd*ASIA-1, was discovered and thought to have originated in the early 20th century, being the starting point for the chytrid fungus (O'Hanlon et al., 2018). But, it was then determined that this was the only lineage in a mutation-drift equilibrium, which is found to be a characteristic of endemism (Fisher & Garner, 2020). A second Asian-associated lineage (*Bd*ASIA-2) was found as well in a species of North American bullfrogs that were found in Korea, causing another questioning factor as to which lineage strain was the original (O'Hanlon et al., 2018).

As of now, the locations where *Bd* currently is located in Asia are all considered cold spots, meaning that the amphibians affected are able to co-exist with *Bd* (Sreedharan & Vasudevan, 2021). New research has shown that there is a potential ancestral *Bd* lineage strain

that was identified in the Korean peninsula, but has not caused a massive impact on the multifaceted amphibian population in Asia (Sreedharan & Vasudevan, 2021). Results from another study have shown that the situation in Asia with *B. dendrobatidis* leans more towards endemism, with other lineages of *Bd* coming from this original strain (O'Hanlon et al., 2018). New data found that *Bd* is a recombining species, meaning that it can generate and change different alleles in order to manipulate its genome and have a better ability to exploit a wide variety of hosts (Fisher & Garner, 2020). There is still data pointing to Africa as the origin of *Bd*, as the different lineages found in Asia could be recombinant lineages derived from the potential original strands in Africa. However, determining the exact details as to how and why it was able to still spread throughout Asia and the rest of the world is still being investigated. With around one-third of the global amphibian species being threatened by chytridiomycosis, finding different treatment methods for *Bd* has never been more important, as amphibians are a vital part of the global biodiversity in many different ecosystems (Fisher & Garner, 2020; Bishop et al., 2012).

Distribution in the Blue Ridge Mountains

The Eastern United States has the highest diversity of salamanders in the world, so with *Bd* now found in this part of the world, it has potential for devastating destruction of the amphibian populations. The Blue Ridge Mountains specifically are known for their large salamander diversity, with over 30 different species found just in this specific Appalachian range (Green et al., 2013). Based on past research conducted on differing ecological niches in the United States, most studies displayed that *Bd* grew best in environments with higher levels of moistures in high elevations, along with cooler temperatures (Berger et al., 2005). All of these are characteristics of the Appalachian Mountains, making it prime *Bd* breeding grounds. Past

studies done by researchers Caruso and Lips (2013) showed that *Bd* has a high distribution level in parts of the Appalachian range and the Eastern United States, but *Bd* has not been the major source for amphibian decline in this part of the world. Several studies have shown that while different salamander populations have declined over the years in the Appalachian Mountain range, such as the *Plethodon* salamanders, there was no correlation found between its decline and *Bd* (Muletz et al., 2014). While there are many cases of *Bd* found in the Blue Ridge Mountains, at this time the mortality rates from *Bd* are relatively small (Muletz et al., 2014).

While a copia of research has been performed on anurans and how *Bd* has affected their population, there has been a lesser focus on other amphibians such as salamanders or newts. As stated before, while amphibians have not seen an increase in mortality rates in this region of the United States yet, research is necessary in order to protect the environment against the ongoing *Bd* outbreak that is occurring. Research on the infective nature of *Bd* on two amphibian species in the Blue Ridge Mountains was accomplished by a research team in North Carolina, where the slimy salamander (*Plethodon glutinosus*) and the Blue Ridge Mountain dusky salamander (*Desmognathus orestes*), both native species to the Blue Ridge area, were infected with *Bd* zoospores (Chinnadurai et al., 2009). Over 1,000,000 zoospores were infected per amphibian, with the goal of seeing how susceptible each species was to *Bd* and how badly each of them reacted. It was found that the slimy salamanders were more prone to being infected with chytridiomycosis over the dusky salamander, with many of those salamanders not contracting *Bd* (Chinnadurai et al., 2009). With this distinctive difference, it could be inferred that either part of the microbiome of this salamander or chemical substance that the dusky salamander produces could potentially have inhibition abilities or adaptive responses (Chinnadurai et al., 2009; Muletz

et al., 2014). In the lab setting of these Blue Ridge native species, all the slimy salamanders contracted chytridiomycosis, with one dying, and the dusky salamanders only had a few contracting chytridiomycosis (Chinnadurai et al., 2009). This is encouraging to conservationists, as this gives herpetologists more time to continue doing research and finding ways to build up better protective services for the endemic caudal species found in the Blue Ridge Mountains.

Amphibian Effects

Differences in Susceptibility Among Different Species

The level of health and growth amphibians experience in a specific area is seen as a good indicator of how the overall environmental health of that location is doing (Kriger, 2007). *Bd* has been found to have infected almost 500 species of amphibians (6.5%), with over 90 species presumed to be extinct. (Sreedharan & Vasudevan, 2021). Seen as one of the greatest documented losses of biodiversity that is related to a pathogen, herpetologist specialists Scheele et al. (2019) are concerned as to how rapid infectious rates could continue if left untouched. Frogs or toads of the order *Anura* were deemed to have been the most affected, with 93% of anurans having severe decline (Scheele et al., 2019). It was found that the Neotropical genera *Atelopus, Craugastor, and Telmatobius* were most heavily affected, with around 45% of the loss connected with these genera (Scheele et al., 2019).

Finding differences in susceptibility was noted by a few different research studies throughout the past two decades. A study done in the United States took 6 different anuran species (*Pseudacris feriarum, Pseudacris triseriatea, Rana pipiens, Lithobates sylvaticus, Hyla versicolor,* and *Anaxyrus terrestris*) from different parts of the US and put them in a lab (Searle et al., 2011). They exposed each species to *Bd* for 30 days and monitored the mortality, feeding

rates, and infection levels of each amphibian. It was found that in each species, the *Bd*-exposed amphibians seemed to have had a higher mortality rate versus the control animals. *Anaxyrus terrestris* and *Lithobates sylvaticus* had the highest mortality rates, with the *Pseudacris feriarum* and *Hyla versicolor* having the lowest mortality rates (Searle et al., 2011). They stated that this could be due to the fact that the genotypes of each of the amphibian species, such as the *P. feriarum* and *H. versicolor* were found to have a closer related genome in comparison to the other two species. Another variable worth noting is the fact that the *P. feriarum* and *H. versicolor* are normally found in drier areas, whereas the other four are normally found in damper areas, which is where *Bd* tends to be found.

Besides the overall taxonomic order and family differences, other characteristics such as body size, reliance on water, and egg laying behavior of the amphibian point to differing levels of infection rate for species found in the United States (Bancroft et al., 2011). These species-level characteristics are able to greatly change the different levels of *Bd* found in each, with general substantial variation with many types of species (Bancroft et al., 2011). By looking at the composition of each microbiome and how it was affected, it can be stated that the diversity of each microbiome decreased significantly when infected with *Bd*. Other studies have shown that depending on the species, if one aggregates more, then it might actually have a stronger immunity to *Bd* in comparison to species that are more spread out and apart (Altizer et al., 2003). By finding these differences and analyzing the mortality rates and infection rates of different amphibian species for chytridiomycosis, future studies should be able to use that data to continue finding new statistics and evidence for combatting *Bd*.

Ecology of the Microbiome

The skin microbiome of amphibians has been heavily researched in herpetology. The skin of amphibians is a thin and moist layer of keratinized skin that is water permeable, allowing for gas exchange and osmoregulation to occur (Ross et al., 2019). Their skin has a sugar-rich mucosal level that is rich in nutrients for bacteria and pathogenic fungi (Ross et al., 2019). The variation of the skin microbiome of amphibians has been found to be a key determinant of how an amphibian grows and develops. The heterogeneity of the bacterial species living on amphibians has also played a key role in its proactive and protective responses against the environment (Harrison et al., 2019). The skin microbiome of amphibians has quickly become a major point of study due to the noticeable differences between the different microbiomes of those amphibians infected with *Bd* and those which are not (Ross et al., 2019).

The skin microbiome of the eastern red-spotted newt is quite complex, made up of many different species of bacteria capable of providing different functions and immunity. Researching how the microbiome reacts to different biotic and abiotic stressors has become a more prominent undertaking. Specifically with the eastern newt, it has been found that the dominant species of bacteria, or the ones that make up the majority of the relative abundance, can be key players in the defensive role against fungal pathogens (Walke et al., 2017). In a study done by Walke et al. in 2017, bacterial isolates of the eastern newts, bullfrogs, spring peepers, and American toads were collected and analyzed to determine the differences of inhibition against *Bd* in order to see if there was a possibility of the theory of dominance-function relationship (Walke et al., 2017). It was found that dominant bacteria had a higher *Bd* inhibition in comparison to the rare bacteria found on the bullfrogs and newt species (Walke et al., 2017). Over 50% of the newt and bullfrog microbiomes were relatively abundant, which differed from the microbiome of the spring peeper

and American toad. The potential for the dominant bacteria found in the microbiome to produce anti-*Bd* compounds in order to act as a competitive dominance sequence was also noted (Walke et al., 2017).

Another study done by Dr. Walke et al. (2014) also suggests how important the distinct differences in the microbiome of each amphibian are as they conducted a study on different amphibian skins and the different microbes found on each in different environments. By sampling two different species of amphibians from three specific locations, it was found that the different microbiomes of each amphibian were distinct based off of which environment they came from (Walke et al., 2014). A small percentage of overlap of the amphibians' core microbes was discovered, which led to the idea that "the relative abundances OTUs (operational taxonomic units) that were shared by amphibians and the environment were inversely related" (Walke et al., 2014, p. 2207). These findings continue to help pave the way for the understanding of the relationship between the microbiome of amphibians and how they connect and correlate with the inhibition functions against *Bd*.

Probiotics/Bioaugmentation Strategies

Bioaugmentation is the procedure of adding cultured microorganisms (such as different species of bacteria) to speed up the rate of degradation of a contaminant (Bletz et al., 2013). Amphibians are known to have symbiotic relationships with the microbes found on their skin, with each bacteria adding a different function to its symbiotic partner. Most recently, growing evidence has been found that microbes aid in the defense against pathogens and other complications that may try and affect the host amphibian (Bletz et al., 2013; Fisher & Garner, 2020). Areas that have still not been majorly exposed to *Bd* are able to be used as a location for

conservationists to implement probiotic strategies in order to continue decreasing the amounts of amphibian mortality.

A study was completed where juvenile *Rana mucosa* frogs that were not infected with *Bd* were given a probiotic, *janthinobacterium lividium* (Bletz et al., 2013). In this study, there were three treatments, with six frogs in each treatment type. In the first treatment, the frogs were given the bacteria. In the second treatment, the frogs were given both the probiotic bacteria and *Bd*. Finally, the last six frogs were given *Bd*. It was found that the frogs which had both the *Bd* and bacteria actually had a decreased mortality rate, and none of the frogs died from *Bd*, whereas at week 10 of the study, there was a decrease to four frogs surviving (Bletz et al., 2013). These numbers continued to decrease as the frogs continued to die from *Bd*, eventually reaching to only one frog alive after week 20 (Bletz et al., 2013). This is a great discovery, as it shows that probiotics do work and can be a potential treatment plan for amphibian species.

But, on the other hand, this does not mean that the same bacterial probiotic will work for every amphibian species. Another study with *janthinobacterium lividium* on the *Atelopus zeteki*, or Panamanian golden frog, was done (Harris et al., 2011). The results were not as positive as they were with the other species of frogs, as the trends for the frogs with and without the probiotic were approximately the same (Harris et al., 2011). In summary, probiotics can be very effective and help lower the mortality rate of certain amphibians if and only if it inhibits *Bd*, doesn't harm the host, and has no non-target effects (Bletz et al., 2013; Harris et al., 2011). In accordance with past studies, another study done by Bletz, in areas where *Bd* is endemic, bioaugmentation is able to extradite and move the susceptible amphibians that are maintained in assurance colonies (Bletz et al., 2013).

Purpose

The purpose of this research is to characterize the microbiome of *N. viridescens* and to determine and identify the presence of beneficial cutaneous bacteria which have antifungal properties. Past studies have shown that each individual amphibian has a diverse microbiome, with different bacteria potentially providing inhibiting properties against *Bd.* In this study, the question being posed is, what cutaneous microbiota does *N. viridescens* sustain as a potential natural defense against *Bd*? The major objective in this proposed research is to find out if there is a type of bacteria/culture of the skin microbiome of the newt that could be used to help reduce or eradicate the effects of chytridiomycosis on the eastern red-spotted newt. With the objectives of characterizing the microbiome and identifying antifungal bacteria, the goal is to find a cure for other amphibian species from chytridiomycosis.

Methods

Newt

In order to do this research study, we collected the eastern red-spotted newts, *Notophlamus viridescens,* (n=15) from Watson's Pond in Bedford, VA. We caught the newts by means of an aquatic net called a seine. We then placed the newts in sterile tubes to be rinsed with sterile RO water in order to remove any transient microbes that were still on the newt prior to microbial sampling. We swabbed the skin of each newt with a standardized technique to collect cutaneous microbial cells. The newts were swabbed five times each on the back, abdomen, and each appendage. We transferred the cells to an R2A plate, labeled 1-15 based on the newt that was streaked. The newts were streaked for isolation for each distinctive colony found on each

mass culture plate to obtain pure bacterial cultures. The plates were then incubated at 23°C for approximately 3 weeks.

After the 3-week period was completed, we analyzed and looked at each plate under a microscope. We counted the number of colonies on each plate, documenting the number found on each one. Thus far, we have isolated 188 morphotypes with an average of 12.5 isolates per newt. We documented all of this data electronically and in a lab notebook with each newt having its own section for documentation. Each isolate is currently being identified by extracting, amplifying, and sequencing the 16s rRNA gene.

DNA Extraction Protocol

As of now, each isolate has gone through DNA extraction protocol in order to get correct DNA results (Lauer et al., 2007). The boil-freeze was completed per protocol from past studies (Lauer et al., 2007). The DNA extraction process was done by using the DNeasy Blood and Tissue Kit. We preheated two incubators to 37°C and 56°C, respectively. We calculated the different amounts of lysis buffer and lysozyme mix needed to extract the DNA. We added 180 µL of lysis buffer to each swab sample and vortexed for 3-5 seconds. We incubated the samples for 1 hour at 37°C. We added 25 µL of proteinase K and 200 µL buffer AL to the sample. We vortexed the sample and incubated for 30 minutes in the 56°C incubator. We added 200µL of ethanol once the samples were removed from the incubator. We vortexed the samples. We pipetted the sample mixture into a DNeasy Mini spin column and placed in a labeled 2 mL collection tube. The total of the mixture should be close to 850 µL. Mixture was centrifuged at 8,500 rpm for one minute. We emptied the flow-through substance from the collection tube. We replaced the collection tube and 500 µL buffer AW1 was added. We centrifuged the tube again at

8,500 rpm for 1 minute. We emptied the flow-through substance from the collection tube again. We replaced the collection tube and 500 µL buffer AW2 was added. We centrifuged the tube at 14,000 rpm for 3 minutes. We discarded the flow-through and the collection tube was replaced once more. We repeated this step once more. The Mini Spin column was then placed in a sterile 1.5 mL centrifuge tube. 100µL of buffer AE was pipetted onto the membrane and incubated at room temperature for 3 minutes. The tube was centrifuged at 8,500 rpm for one minute and then stored in the freeze at -20°C, ready for PCR.

Polymerase Chain Reaction (PCR) and Sequencing

We then did PCR amplification to replicate millions of copies of the specific DNA that was extracted in the previous protocol. Based on the freeze-thaw protocol, a master PCR mix was made using a baseline of 11.5 µL molecular grade H_2O, 10 µL 5 Taq MM, 0.5 µL BSA, 1 µL 8F Primer, and 1 µL 1492R Primer. This was multiplied by the amount of isolate going through PCR, so if there are 16 isolates, the above amounts were multiplied by 18 in order to get the correct amount necessary. Once combined in a 1.5 ml centrifuge tube, 24 µL of the PCR mix was put into PCR tubes, enough for each isolate and a negative and positive control. 1 µL of sample DNA of isolate was added to each PCR tube. Each tube was centrifuged in order to confirm all reagents were combined. Samples were then placed in the thermocycler, with the amplification protocol set to the following: 1 cycle at 94°C for 4 minutes, 34 cycles at 94°C for one minute, 50°C for 1 minute, then 72°C for 90 seconds, and finally 1 more cycle at 72°C for 10 minutes. The amplified DNA was then put in a gel and analyzed. 5 µL of the dyed product (loading dye + PCR product) was placed in each well of the gel. The gel was run at 120 V for 30

minutes. The gel was then checked for amplification, with bands noted, via the imager in the cell imaging lab. A photo was taken of each gel and documented electronically.

Each bacteria sample was then shipped off to Eurofins, a company that specializes in providing testing and support services, in order to be sequenced. This allowed for the identification of the 16S DNA sequences to be found as the results from the Sanger sequencing were retrieved. Results have not yet been returned, but once the identification process is completed, we will have results to support which bacteria were able to inhibit *Bd* or cause a potential function change of *Bd.* A histogram will then be completed once the data from the identification process is obtained. By reviewing past literature and other studies, we will determine which genera or species are most commonly occurring and search for ones which have anti-*Bd* properties in other studies.

Cell Free Supernatant (CFS) Protocol and Bd Assay

Prior to *Bd* assay, the majority of the isolates collected were then processed through the cell free supernatant (CFS) protocol in order to be filtered and processed correctly. This allowed the pure isolate to be the determinant in the *Bd* assay. The goal of CFS protocol is to isolate bacterial metabolites from cultures, in order to be later used in an assay against *Bd* for identification of *Bd*-inhibitory bacteria species. CFS protocol went as follows: 50 mL tubes were filled with tryptone broth. Culture tubes were filled with 2 mL Tryptone broth using micropipettes. The number of culture tubes used were based on the number of bacterial metabolites that were being isolated. Each tube was labeled with the newt species, date, and initials. Each tube was then put in a shaker incubator at 23°C, 140 rpm for 72 hours. After the 72 hours, the samples were retrieved from the incubator. Two sets of 2 mL centrifuge tubes were

obtained, with each sample from the culture tubes transferred into the 2 mL microcentrifuge tubes. These tubes were then centrifuged for 5 minutes at 10,000 rpm. A pellet was formed at the bottom of each sample. If there was no pellet, centrifugation was necessary. A filter syringe was then used with the sample contents from the tube poured into the syringe. The sample was then slowly filtered into a new tube, seeing a steady drip of solution coming through the filter. An amount of 1 mL of CFS was the goal. The samples were then stored in -80°C for future use in the *Bd* assay.

In order for the *Bd* assay to occur, we grew *Bd* in an isolated and controlled environment. The *Bd* was given around two weeks to grow and develop. The *Bd* zoospores were then filtered by flooding the *Bd* plates with 5 mL of tryptone. The liquid was removed via a pipette and the zoospores were filtered into a new falcon tube with a coffee filter in a previously autoclaved glass funnel. The zoospores were diluted to $2x10^6$ zoospores/mL using the hemocytometer procedures as follows. 50 µL of zoospore was placed with 50 µL of Trypan Blue. The zoospores were then counted using the hemocytometer, with the equation C1V1= C2V2 being used. The desired concentration was 2,000,000 zoospores/mL and the desired volume was 15 mL, allowing for three 96-well plates to be set up properly. The heat-killed *Bd* was then prepared by placing 500 µL of quantified *Bd* into a 1.5 microcentrifuge tube. This tube was then incubated at 90°C for 30 minutes. The frozen CFS tubes were then taken from the freezer and thawed. A 96 well plate was set up with each isolate and control being replicated in triplicate. The amount of each solution for each well was as follows: the test samples had 50 µL *Bd* zoospores and 50 µL filtered bacterial CFS extract; the nutrient depleted had 50 µL zoospores and 50 µL sterile water; the positive control had 50 µL zoospores and 50 µL 1% Tryptone broth; and the negative had

100 µL 1% tryptone. This was done via a multichannel pipette, with the tips being replaced after each use. The plates were then read four separate times in the microplate reader at 492 nm. The plate was read on Day 0, Day 3, Day 7, and Day 10, with the plate being parafilmed and incubated at 21°C in between each reading.

Results

Due to the ongoing nature of this research, final results have yet to be tabulated. What we have obtained so far are the amounts of bacteria that were studied and the *Bd* assay information. From the 15 newts that were caught, 188 morphotypes were found in total. On average, around 12.5 bacteria were found per newt. Each newt's gender was documented, with 12 males and 3 females caught. Each newt morphotype was described and documented in the lab notebook physically and electronically. The results of the number of bacteria on each individual newt is shown in Figure 1.

The resulting individualized bacterium from each eastern newt were swabbed and streaked for isolation. Each individual plate had to be clear of any potential contaminations prior to being used for the next step in the research. The findings of the individual streaking were captured and shown in Figure 2. Later on, during PCR and sequencing, it was found that many of the different isolates that were obtained happened to be fungi. These individual isolates were then documented and thrown out of the results, as these are unable to be used in the treatment with the probiotic solution.

Figure 1

Quantity of Bacterial Morphotypes per Newt

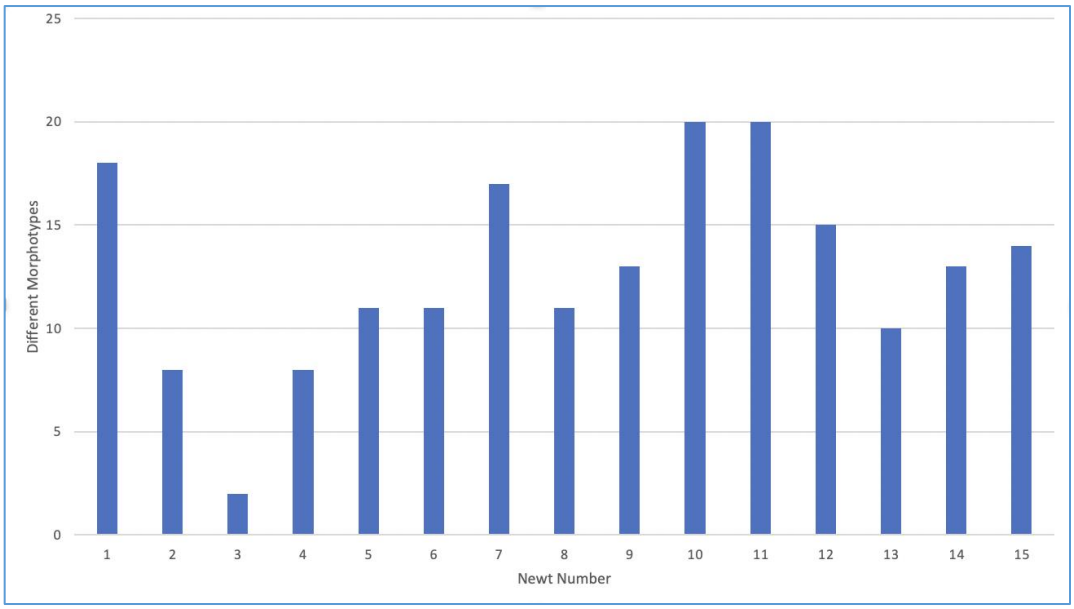
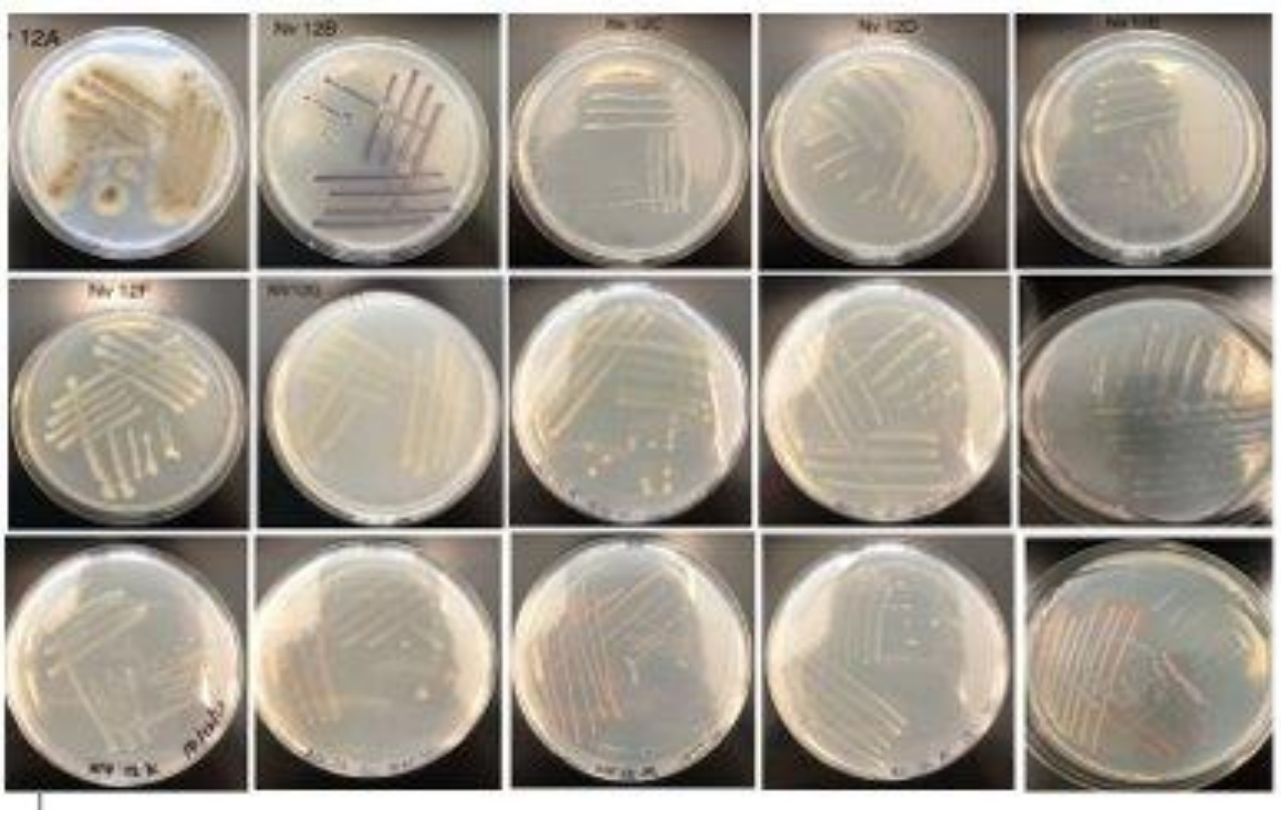


Figure 2.

Streaking for Bacterial Isolation Morphology



Note. Each individual bacteria were swabbed from each mass culture plate to its own separate plate in order to isolate each individual bacterium. In the figure above, it can be noted that each individual bacterium is different, with different colors, shapes, texture, and arrangements. While it can be seen that each is different, sequencing is still necessary to determine the specific species of bacterium present.

Once each individual has been identified through the sequencing process, then results will be able to be compiled and selected from the *Bd* assay inhibition data. The results have been delayed due to the unavailability of retrieving the sequence data at this current time. The *Bd*

assays have been completed at this time for newts 1-11, with the data currently being analyzed and compiled. Once the sequencing data is completed, then that information will be used to investigate the inhibition levels of each bacterium that has been identified.

Discussion

The main objective of this study was to determine whether there was a relationship between the microbiome of the skin of the eastern red-spotted newt and the ability of the bacteria to potentially inhibit the growth of *Bd*. We found that there were specific bacteria that were able to inhibit the growth of *Bd* on the amphibian skin, leading to the conclusion that some of the bacteria from the skin of the eastern red-spotted newt has potential for becoming probiotics for either the newt or other species. While some produced inhibition and anti-*Bd* properties, others did not. Reasoning behind this could be because of the symbiotic effects that the bacterial microbiome already creates due to the mutualistic relationship between the amphibian host and bacteria (Becker & Harris, 2010).

With the continual study on the microbiome of the newts in place, the focus of finding out more about the probiotic properties that can help inhibit *Bd* will continue to be the main focus. Due to our results showing potential for *Bd* inhibition, it is possible that the same bacteria could be used as potential probiotics and used *in vivo* in other organisms. With this data, testing the efficacy and the ability of using these bacterial samples is very important. Considering how other studies have been used to support and gain traction for greater opportunities in herpetology research, the ideal is to have the microbiome and the inhibition properties of the eastern red-spotted newt be documented as well. By focusing on the microbiome and more so on the bacterial aspects of the newts, other characteristics of the newts were not documented, such as

size or age. The gender was documented for each newt, with the idea of future research that could be done looking at the differences in microbiomes of male and female amphibian species. As *Bd* has not yet overtaken the *N. viridescens* population and has not infested the Blue Ridge Mountain range, there is still hope for this ecosystem and community to continue to thrive and institute protective measures against *Bd*.

Conclusion

Future Work

In the future, each bacteria isolate that was discovered on each newt will be run through a sequencing program in order to determine what the bacteria is. By discovering the species of bacteria and comparing each of them to the *Bd* assay, we are able to determine the inhibitory properties of each isolate. By doing so, we can identify what bacteria can and cannot inhibit the growth of the fungi. Some of the next steps that can help catapult and increase the awareness of this situation include other research success stories and finding more information and data on the microbiome of other species and the bacteria's potential inhibition properties against *Bd*. With our future findings, we hope to discover an effective treatment of amphibians affected by chytridiomycosis and contribute to the conservation of amphibians.

Impact

The native amphibian species found in the Blue Ridge Mountains have just recently been examined for the potentiality of *Bd*, even though it is a prime location for *Bd* to spread quickly due to its prime breeding grounds. In the past, the majority of the *Bd* research that occurred was found in places that had large ecological organizations in place, such as universities and governmental agencies. With the large resources from these organizations, they are able to

consistently maintain a high presence in the environment and have a large impact on amphibians. It is essential for herpetologists and surveyors to continue to be on the lookout for new locations that *Bd* might take over, as they are able to hopefully act prior to this ever occurring. Because *Bd* is such a prevalent and dangerous pathogen to the amphibian world, continual advocacy and research is so important in order to ensure amphibian conservation continues and that these fragile amphibian species are protected and safe.

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