Characterizing the Cutaneous Microbiome of *Eurycea lucifuga* as a Potential Defense against Chytridiomycosis

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Abstract

Chytridiomycosis is an emerging infectious disease that is significantly reducing global amphibian populations. The disease is caused by *Batrachochytrium dendrobatidis* (*Bd*), a fungus that lethally modifies amphibian skin. Recent research has suggested that the cutaneous microbiome of individual amphibians may play a role in susceptibility to the pathogen. In this study, twelve cave salamanders (*Eurycea lucifuga*) were collected. Cutaneous bacteria from each salamander were isolated and identified using Sanger Sequencing. Additionally, a *Bd*-challenge assay was performed to determine each isolate's antifungal activity. Results indicated many microbial isolates possessed inhibitory capabilities against *Bd*, which may contribute to the cave salamander's lack of population decline due to chytridiomycosis. This research may contribute to an increased understanding of potential defenses against *Bd* and ultimately to the development of a treatment to mitigate the chytridiomycosis epidemic.

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Several factors have contributed to the unprecedented global decline of amphibians since the 1960s. Between one-third and half of amphibians are currently threatened with extinction, making them the most threatened class of vertebrates (Alton & Franklin, 2017). While habitat destruction, invasive species, and human influence have played a role in the deterioration of amphibian populations, 48% of population declines have occurred in physically undisturbed habitats (Stuart, 2008). These non-anthropogenic declines are attributed to one emerging infectious disease that has caused the decline of over 500 amphibian species and the extinction of 90 more (Scheele et al., 2019).

Chytridiomycosis is an infectious disease caused by *Batrachochytrium dendrobatidis* (*Bd*) and another recently discovered fungus, *Batrachochytrium salamandrivorans* (*Bsal*) (Lindauer et al., 2019). The two fungi disrupt cutaneous homeostasis in amphibians, making it difficult for salts to pass through their skin. This ultimately leads to death in nearly all infected organisms. This global wildlife epidemic, also referred to as a panzootic, has reached virtually all corners of the globe and the pathogen has been able to adapt to various hosts and environments (Weldon et al., 2004). However, some amphibian populations have been shown to be resistant to the pathogen or have asymptomatic infections (Pasmans et al., 2013). A major source of this immunity lies in the symbiotic microorganism living on the amphibian's skin. These microbes secrete antifungal compounds that prevent *Bd* from establishing a serious infection (Brucker et al., 2008). The most notable example of this connection is with the soil-dwelling bacterium, *Janthobacterium lividum*. The species secretes violacein and indole-3-carboxaldehyde, both of which confer resistance to *Bd* (Brucker et al., 2008). At this point, there are no treatment options

available to mitigate the spread of chytridiomycosis in nature, but the answer may lie in the amphibians that carry resistant microbes. It is critical for researchers to devise a remedy for the disease that is wiping out amphibians and threatening the world's amphibian biodiversity.

Eurycea lucifuga is a species of cave salamander that resides across the Midwest and southeastern United States. The species has not been thoroughly studied in relation to *Bd*, however, previous research suggests that *E. lucifuga* has not experienced a noticeable decline in population due to the chytridiomycosis epidemic (Carter et al., 2019). This indicates that they may have some sort of cutaneous defense that either prevents *Bd* infection from developing into a disease or prevents *Bd* infection altogether, as recent studies have indicated that the amphibian skin microbiome plays a crucial role in the host's survival of chytridiomycosis (Brucker et al., 2008).

There is currently a gap in knowledge regarding the impact of chytridiomycosis on the cave salamander and the species' specific cutaneous microbiome. The purpose of this study was to characterize *E. lucifuga*'s cutaneous microbiome to determine if the species hosts a microbial community capable of inhibiting *Bd* infection, or if the microbes enhance *Bd* susceptibility. Additionally, the extent to which *E. lucifuga* is at risk of morbidity and mortality due to chytridiomycosis was also investigated.

Due to the lack of decline in cave salamander populations, it is suspected that there are multiple bacterial symbionts present on the cutaneous microbiome of *E. lucifuga* that inhibit *Bd* growth. If that is the case, the following research will prompt and contribute to the efforts to develop a treatment against *Bd* using findings from amphibians already known to have immunity to the fungus.

Enigmatic Decline of Amphibians

Extinctions and species turnover have been a common occurrence throughout history. In fact, over 99% of all species that have existed on the earth are now extinct (Jablonski, 2004). However, in recent history, rapid and extensive losses of organisms have plagued the world and contributed to a loss in biodiversity. One such group of organisms is amphibians, which have experienced catastrophic declines at local and global scales over the last 50 years. Researchers have found that the current rate of amphibian extinction is over 200 times that of its historical background, and if species threatened with extinction are included, over 25,000 times the historical extinction rate (McCallum, 2007). It is difficult to disregard these numbers as a natural phenomenon, so instead it is critical to determine the causes of these unprecedented and accelerated extinction rates.

Modern amphibian loss can be associated with six major causes: commercial use, habitat loss due to land use change, chemical contaminants, invasive species, climate change, and infectious diseases (Collins, 2010). Most of these factors affect amphibian habitats and can thus be measured by their effect on the areas in which amphibians live. However, many amphibians are dying in virtually pristine or protected habitats, representing a phenomenon referred to as ‘enigmatic decline’ (Collins, 2010). Emerging infectious diseases are the culprit behind this trend and are becoming more prevalent due to environmental and ecological factors. Additionally, they are more dangerous than other diseases due to the ability of pathogens to evolve and spread quickly among hosts (Jones et al., 2008).

Two diseases are implicated in amphibian die-off: *Ranavirus* infections and chytridiomycosis. *Ranavirus* is a genus of the Iridoviridae virus family and contains at least eight strains that can infect amphibians (Collins, 2010). Upon infection, the virus can either cause a

quick host death, chronic infection, or the potential for reinfection (Collins, 2010). However, due to specific host-pathogen dynamics that fit a density-dependent disease model, *Ranavirus* infections are not responsible for amphibian extinctions (Brunner et al., 2007; Collins, 2010). This is not the case with chytridiomycosis, which is highly unusual for pathogens and warrants further investigation into this unique spectacle. The fungal disease spreads independently of population density and has reservoirs in other species as well as abiotic environmental factors. These aspects contribute heavily to global amphibian species extinction, threatening amphibian biodiversity (Lips et al., 2006).

Batrachochytrium dendrobatidis

Discovery and Origin

Batrachochytrium dendrobatidis (*Bd*) is a chytridiomycetes fungal pathogen that exclusively infects amphibian hosts. *Bd* was discovered in 1998 by Lee Berger, after she observed a mysterious decline in frog species in Australia (Berger et al., 1998). Sporangia of an unreported fungus were found on deceased museum specimens of thirteen different species of frogs from Central America (Berger et al., 1998). The novel chytrid fungus was identified through DNA analysis and ultrastructural characteristics (Berger et al., 1998). It was named *Batrachochytrium* dendrobatidis, with the new genus name stemming from the family Batrachochytriaceae (Longcore et al., 1999). The species nomenclature was derived from the genus of the blue poison dart frog, *Dendrobates azureus*, because *Bd*'s pathogenicity was confirmed through their inoculation, subsequent death, and histological analysis (Longcore et al., 1999)

Though Bd was discovered recently, it most likely originated in the early 1900s. *Bd* is theorized to have originated in Africa or Asia and spread throughout the world via African

clawed frogs (*Xenopus laevis*) (Weldon et al., 2004). The theory of African origin postulates that *Bd* was originally localized to a single or small number of anuran hosts in Western and Southern Africa that showed little clinical effects. The disease did not cause a decline in frog populations and the prevalence of *Bd* in hosts was stable over time at around 3%, representing a stable endemic infection. Evidence supporting this conclusion includes that the earliest known case of chytridiomycosis with *Bd* present was found in an African clawed frog from 1938 using retrospective histologic examination (Weldon et al., 2004).

The chytrid fungus began to spread however when a new, more accurate form of pregnancy testing was discovered in 1934. Researchers found that when female *X. laevis* frogs were injected with hormones, the extrusion of ova would occur (Elkan, 1938). Therefore, female urine concentrate with the presence of human chorionic gonadotropin hormone (hCG), a hormone exclusively produced during pregnancy, would stimulate egg deposition by the frog (Elkan, 1938). The Xenopus test was more favorable than others because the frogs required less care than other animals like mice or rabbits that were commonly used for pregnancy tests (Elkan, 1938). Additionally, the test was relatively simple to perform, did not require any animal deaths, and took less than 24 hours to receive results (Elkan, 1938). However, African clawed frogs had to be bred in Africa and shipped throughout the world in order for the tests to maintain their accuracy. At the height of Xenopus test's popularity, over 10,000 frogs were distributed in 1949 (Weldon et al., 2004). In 1970, after modern immunological pregnancy tests were invented, over 20,000 frogs were distributed as models for molecular biology and embryology (Weldon et al., 2004). The *Xenopus* frogs were ideal carriers for global chytrid dissemination because the infected frogs did not experience any clinical signs of the disease or sudden death (Weldon et al.,

2004). Thus, escaped *X. laevis* frogs in the Americas, Europe, and Asia came into contact with native frogs and *Bd* began to spread internationally.

Another more recent study conducted in 2018 concluded that East Asia was the original source of *Bd* (O'Hanlon et al., 2018). Using whole-genome sequencing, the researchers found that one Bd lineage named *Bd*ASIA-1 could be isolated to the Korean peninsula and contained genetic hallmarks of being the ancestral *Bd* population (O'Hanlon et al., 2018). They found that *Bd*ASIA-1 contained more nucleotide diversity and segregating sites than any other lineage (O'Hanlon et al., 2018). Additionally, two independent forms of testing were utilized to further investigate the assertion. Haplotype clustering found that *Bd*ASIA-1 contained the most haplotype clustering within its own lineage and between itself and other lineages (Lawson et al., 2012; O'Hanlon et al., 2018). This means that *Bd*ASIA-1 shared the most genetic diversity with other lineages. Statistical analysis demonstrated that the *Bd*ASIA-1 lineage showed the least spatial and hose radiation, indicating that it is endemic to the Korean region (O'Hanlon et al., 2018).

Continued Global Dissemination

The first documented case of chytridiomycosis outside of Africa occurred in 1961 in Saint-Pierre-de-Wakefield, Quebec, Canada (Weldon et al., 2004). The disease was found in *Rana clamitans*, a frog species native to North America (Ouellet et al., 2005). Over the next three decades, *Bd* would extend to every continent, finally reaching Europe in 1997 and Oceania in 1999 (Weldon et al., 2004).

In the United States, the first documentation of *Bd* was found in a *Rana catesbeiana* frog in 1978 (Weldon et al., 2004). This American bullfrog species has been named as an important vector in the spread of chytridiomycosis because of its usage in the international food trade

(Mazzoni, 2003). Additionally, although *R. catesbeiana* is native to the eastern United States, no large *Bd*-related die-offs have been observed in the region (Mazzoni, 2003). However, it is proposed that the introduction of the bullfrog species to the western coast of the U.S. has facilitated the current epizootic (wildlife epidemic) due to chytridiomycosis in the area (Mazzoni, 2003).

Bd entered its next continent in 1978, where it was found in Australia. The pathogen was harbored by the frog species *Litoria gracilenta*, also known as the dainty green tree frog (Weldon et al., 2004). Next, Bd made its way from the United States to Latin America in 1983, where it was found in the *Rana tarahumarae* frog species (Weldon et al., 2004). Lastly, chytridiomycosis spread to Europe and Oceania in 1997 and 1999, respectively (Weldon et al., 2004). Although it took over 60 years for *Bd* to reach every continent, by the 1990s chytridiomycosis had already become a global epidemic.

Global Geographic Distribution & Related Factors

Since the first known case of chytridiomycosis in 1938, *Bd* has spread to include over 1000 host species in 86 countries (Castro Monzon et al., 2020). Due to a variety of processes including human impact and migration, *Bd* is expanding to areas where it was previously thought to be incompatible. This is especially concerning because certain lineages that are harmless to species within their known ranges are now coming into contact with naïve species susceptible to more lethal effects of the pathogen (Becker et al., 2017). Additionally, *Bd* lineage migration to locations where another lineage is already established is now triggering the creation of hybrid lineages, which can be more virulent than either of the parent lineages (Castro Monzon et al., 2020).

Bd can be found on every continent that contains amphibians but is a temperature-dependent organism that prefers warmer climates (Xie et al., 2016). This trend can be observed in Figure 1, where *Bd* is more centralized to areas closer to the equator. Using statistical models, investigators have predicted that *Bd* will continue to disseminate into areas of higher altitudes and latitudes as climate change makes these regions more hospitable to the temperature requirements of the chytrid fungus (Xie et al., 2016). Additionally, it is projected that *Bd* occurrence will increase in 'hotspot areas' that include the coastal regions of Australia and Africa and mountainous terrains with high elevations Lastly, Bd is expected to expand in forested areas surrounding the equator, lowlands in Africa, and northeastern Brazil (Xie et al., 2016).

Distribution in the Appalachian Mountains

Significant research has been conducted regarding the environmental preferences of *Bd*. Because Bd prefers warmer, more humid climates, the Appalachian Mountains in the eastern United States represent an ideal location for chytridiomycosis to be prevalent. However, most amphibian species in this region, especially salamanders, are resistant to infection by *Bd* itself or the progression into chytridiomycosis as a disease. Some salamander populations in the region have declined in recent years, including *Plethodon glutinosus* and *Plethodon teyhaleedecline*. However, these declines are unrelated to *Bd* occurrences as only one in 665 salamander individuals tested were *Bd*-positive (Caruso & Lips, 2012).

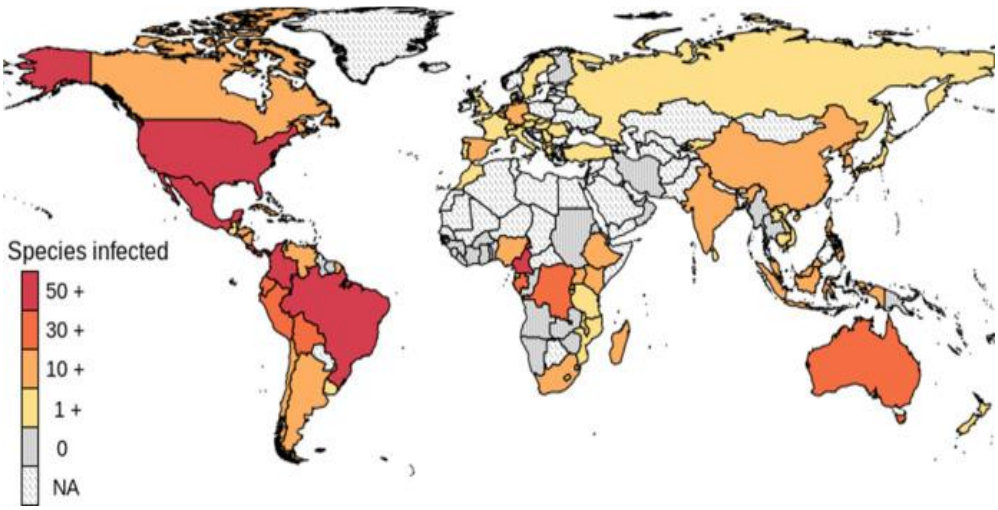

Figure 1.

Global Distribution of Bd. The number of infected species varies, but there is a noticeable lack of the chytrid fungus in Africa, where the pathogen may have originated.

Life Cycle

The life cycle of *Bd* has been observed to take place over 4-5 days *in vitro*, which is assumed to be the same while the pathogen is in amphibian skin (Berger et al., 2005). The organism exists in two life stages, as a stationary reproductive zoosporangium and a motile uniflagellate zoospore about 3-5 µm in diameter that is released from the zoosporangium (Berger et al., 2005; Longcore et al., 1999). First, *Bd* enters its host amphibian from the environment by using a germ tube. Though *Bd* zoospores are motile, they can only migrate a few centimeters while active and have not been observed during the infection process. It has been proposed that the zoospores encyst on the surface of their host's skin and subsequently inject their organelles deeper into the epidermis through a germ tube for zoosporangium growth (Longcore et al., 1999). After injection, the distal end of the germ tube swells to establish a thallus (Van Rooij et

al., 2015). Additionally, the zoospores are able to use chemotaxis to travel toward specific molecules on the surface of their host, including nutritional cues like amino acids and sugars (Moss et al., 2008). This aids the pathogen in locating the mucus layer covering amphibian skin, which contains integumental free sugars like *N*-acetylneuraminic acid and β-d-*N*-acetylglucosamine (Van Rooij et al., 2015).

The zoospores contents encyst within the epidermis, specifically the stratum granulosum and stratum corneum. It has been found that zoospores that develop into younger, more immature sporangia infect the deeper, more viable epidermal cells while zoospores that quickly develop into mature zoosporangia infect the outer, dead epidermal cells that are closer to the surface of the organism (Berger et al., 2005). These differences confirm the finding that the growth of the chytrid fungus mirrors the maturation of each epidermal cell as it keratinizes and moves outward to be sloughed off. For this reason, when temperatures are above 25°C, *Bd* growth is inhibited and some amphibians can even lose the infection due to the increased rate of epidermal cell turnover that prevents the fungus from completing its entire life cycle before being shed (Piotrowski et al, 2004). Throughout the development of the fungus, *Bd* utilizes keratinolytic enzymes and esterases like trypsin and chymotrypsin to break down the keratinized epidermal cells of its host for nutrients (Symonds et al., 2008). In fact, up to three sporangia have been documented within one epidermal cell.

After settling in the epidermis, branching rhizoids grow from the length of the zoospore and it becomes an intermediate structure known as a germling (Berger et al., 2005). Through many mitotic divisions, the thallus becomes multinucleate and cleaves into many round zoospores that remain inside a newly formed sporangium (Berger et al., 2005). In some thalli, thin septa form and the thallus develops into multiple zoosporangia, which is known as colonial

growth. However, in most thalli, no divisions occur and a single zoosporangium is formed, which is referred to as monocentric growth (Longcore et al., 1999). This is an example of asexual reproduction, which is the dominant method by which *Bd* reproduces, although sexual reproduction has been observed *in vitro* (Berger et al, 2005). Within the sporangium, the dozens of zoospores each develop a 20 μm long flagellum and become motile (Longcore et al., 1999). Next, discharge tubes are formed and protrude from each zoosporangium to the surface of the host's skin, a length which ranges from 1μm to 10 μm (Berger et al., 2005). Once the sporangium determines that the environment surrounding the discharge tube is sufficiently moist, the end of the tube dissolves and the zoospores are released from the sporangium into the environment, where the infectious cycle begins anew (Berger et al, 2005).

Infection of Amphibian Larvae

The chytrid fungus has the ability to infect amphibian larvae, but a number of the processes involved are markedly different than that of juvenile or mature amphibians. The majority of research thus far has been focused on anuran larvae (tadpoles), so the following observations will be based on that order of hosts. In fact, it has been proposed that urodelan larvae cannot be infected because of their lack of keratin, which *Bd* requires for growth. Therefore, the pathogen is also limited in its infection of anuran larvae to their keratinized mouthpieces (Van Rooij et al., 2015). Because of the pathogen's inability to spread throughout the organism, most larval hosts survive the infection and only experience temporary, sub-lethal effects like mild hyperplasia, mouth depigmentation, lethargy, and poor swimming abilities (Van Rooij et al., 2015). However, larvae going through metamorphosis are especially vulnerable to the lethal effects of chytridiomycosis as they are actively keratinizing and the pathogen has the opportunity to spread throughout their changing bodies (Baitchman & Pessier, 2013).

Pathogenesis and Physiology

Bd is an especially damaging pathogen because it primarily affects the skin, which is one of the most vital organs in an amphibian and serves a variety of functions. Unlike other organisms, amphibian skin is in constant contact with a microbially rich aquatic and/or terrestrial environment. This barrier is the first defense against potentially harmful organisms, and when compromised the innate immunity of its host is weakened significantly.

Chytridiomycosis induces a variety of changes at the cellular, tissue, and behavioral levels of its host. Upon infection, *Bd* requires nutrients from the host amphibian in order to mature and reproduce. In the early stages of the disease, a rearrangement of epidermal cells as well as the dissolution of some cells is evident (Berger et al., 2005). Additionally, hyperkeratosis, or the thickening of the epidermis, occurs in response to the ongoing epidermal injury. This is caused by increased epidermal turnover, cell inflammation, and hyperplasia due to stratum basale stimulation near the site of Bd infection (Berger et al., 2005). This turnover cycle is also disrupted with multiple layers of dead keratinized cells building up before being sloughed off, furthering hyperkeratosis. At the cellular level, the pathogen induces nucleus displacement and altered fibrils in the cytoplasm of epidermal cells (Berger et al., 2005). Next, *Bd* sporangia trigger epidermal thinning as new cells fail to germinate as quickly as sloughing off occurs.

Bd also affects amphibian homeostasis through cutaneous ion transport. In healthy amphibians, the skin acts as a medium by which electrolytes pass through ion pumps and selective epithelial channels to maintain a hyperosmotic environment (Baitchman & Pessier, 2013). After *Bd* infection, epithelial ion transport is inhibited by over 50% because of hyperplasia and hyperkeratosis, among other molecular mechanisms (Baitchman & Pessier,

2013; Voyles et al., 2009). This causes a decrease in plasma electrolytes like sodium, potassium, and chloride.

The exact cause of death for Bd hosts is unclear because there is no consistent damage to internal organs due to the pathogen's epidermal-level infection (Voyles et al., 2009). However, a couple hours before death a change in the electrical activity of the heart is noted, which may be associated with the dramatic decrease of potassium in the body. This change can be compared to bradysystolic or asystolic cardiac arrest that occurs in humans. Severely diseased *Litoria caerulea* frogs that were administered oral electrolyte supplements regained some mobility and activity, supporting the theory that electrolyte imbalance is the primary source of cardiac arrest (Voyles et al., 2009). Cardiac arrest results in reduced blood flow throughout the body, myocardial contraction failure, and ultimately death.

Bd's Sister Fungus, *Batrachochytrium salamandrivorans*

Until 2013, *Batrachochytrium dendrobatidis* was the only known fungus to cause chytridiomycosis. However, researchers in the Netherlands noticed a steep decline in fire salamander (*Salamandra salamandra*) populations across Northwestern Europe in the late 2000s (Martel et al., 2013). They tested the affected salamanders, and while the lesions of the specimens were positive for chytridiomycosis, they did not contain *Bd*. The investigators isolated and characterized a new strain of chytrid fungus, named *Batrachochytrium salamandrivorans* (*Bsal*) (Martel et al., 2013).

While the newly discovered pathogen is similar to *Bd* in many regards, it is also highly divergent. *Bsal* is much more aggressive than *Bd*, and mainly causes lethal skin infections in salamanders and newts rather than anurans. Additionally, *Bsal* has a much lower temperature preference (10°C - 15°C) than that of its tropical climate-loving sister fungus (17°C - 25°C)

(Martel et al., 2013). *Bsal* also has morphological differences, including the formation of colonial thalli in contrast to the monocentric thalli that *Bd* produces (Martel et al., 2013). Currently, *Bsal* has only been found in wild and captive amphibians located in Europe, but susceptibility studies have shown that the pathogen is also lethal to some North American salamanders, including the genera *Hydromantes, Notophthalmus, and Taricha* (Martel et al., 2014). Because North American salamanders account for over 50% of global salamander diversity, it is crucial that *Bsal* not be introduced to the region so that the numerous endemic vulnerable amphibian populations are not decimated (Gray et al., 2015). A strategic plan to prevent the introduction of *Bsal* into the United States by limiting the import of commercially used amphibians is already in the works (Gray et al., 2015). Fortunately, there is a precedent to combat *Bsal* in the form of another fungal pathogen, *Pseudogymnoascus destructans*. It causes the emerging infectious disease called white-nose syndrome in bats and has killed millions in the US since it was brought from Europe in the early 2000s but has been mitigated through stringent guidelines (Brownlee-Bouboulis & Reeder, 2013).

Like *Bd*, the origin of *Bsal* is not definitively known. Using Bayesian estimation, it was proposed that *Bsal* diverged from *Bd* over 67 million years ago (Martel et al., 2014). DNA testing suggests that *Bd* was previously endemic to the East Asian countries of Japan, Thailand, and Vietnam, where there are no obvious signs of amphibian decline due to the pathogen (Martel et al., 2014). Three Asian salamander species were likely reservoirs for the fungus as tested specimens maintained the disease as a chronic infection or eliminated the infection entirely (Martel et al., 2014). Due to the geographical gap between *Bsal* identified in East Asia and Europe, its transmittance was likely mediated by humans through commercial trade routes targeting salamanders as pets.

Cave Salamander

Characteristics

This study focuses on the cave salamander, a type of salamander that primarily resides in caves across parts of the Southeast and Midwest United States. The species of cave salamander studied in this research is *Eurycea lucifuga*, also known as the spotted-tail salamander, which is a type of brook salamander. An adult *E. lucifuga* is roughly six inches long, with a dull to bright orange hue and dark spots throughout its body. Its tail makes up over 60% of its length and is prehensile, meaning that it can grasp various objects (Baken & Adams, 2019). Due to the low or nonexistent levels of light in their subterranean habitat, some cave salamanders exhibit unique adaptations that allow them to survive, including the absence of eyes or lack of pigmentation. The family Plethodontidae, which includes *E. lucifuga*, lacks lungs. Instead, they breathe by way of cutaneous respiration through their skin and the mucous membranes in their mouth. Hence, they must keep their skin moist at all times. This feature of *E. lucifuga* will become very important in the discussion related to chytridiomycosis, as the infection irreparably inhibits respiration, a vital function for the salamander's survival.

Habitat & Geographic Distribution

Contrary to their name, cave salamanders are not obligatory troglobites and can be found in areas beyond caves including crevices, exposed rock bluffs, and limestone springs (Trajano & de Carvalho, 2017). In fact, *E. lucifuga* individuals that reside in caves must venture out periodically to acquire epigean resources (Merkle & Guttman, 1977).

Cave salamander populations have been documented in states along the Appalachian Mountains and near the Mississippi River system. These include Oklahoma Missouri, Arkansas,

Illinois, Indiana, Ohio, Kentucky, Tennessee, Alabama, Georgia, West Virginia, Virginia, and North Carolina.

In Virginia, *E. lucifuga* individuals have been recorded along the western portion of the state bordering West Virginia and Kentucky. The reason for this is that limestone regions dominate the western part of Virginia, and local cave salamander populations prefer the terrain for a variety of reasons (Hutchison, 1958). While it may seem apparent that the salamander species prefers the type of rock for its chemical properties, this is not the case. Limestone possesses high solubility, making it more likely to form caves and create the twilight zone atmosphere that *E. lucifuga* salamanders favor (Hutchison, 1958).

Methods

Salamander Collection

We traveled to a cave site in Lee County, Virginia, and donned gloves prior to salamander handling. *E. lucifuga* individuals (n=12) were captured and briefly placed in individual sterile conical tubes. The specimens were rinsed with sterile RO water to remove any transient microbes so that the resident cutaneous microbiome could be accurately ascertained. The dorsal side, ventral side, and hind limbs of each salamander were thoroughly swabbed five times to collect cutaneous microbial cells for culturing (Lauer et al., 2007). Another set of skin swabs was collected to detect *Bd*. Data including snout-vent length, sex, and weight were collected for each salamander individual. A small sample of each specimen's tail was clipped for future histological studies. Salamanders were released back into the wild after testing.

Bacterial Culturing & DNA Extraction

Skin samples were streaked onto low-nutrient R2A agar plates and incubated at room temperature for several days. All incubating steps were performed at approximately 23°C

(ambient laboratory temperature) because salamanders' ectothermic nature allows their cutaneous microbial symbionts to be adapted to environmental temperatures. Fungal and bacterial colonies were separated based on morphological differences including color, margin, and elevation. Colonies were isolated via streaking onto sterile R2A agar plates (Lauer et al., 2007). Descriptions and characteristics of each pure bacterial colony were recorded.

DNA was harvested from colonies using both the "Freeze-Thaw" protocol and the QIAGEN DNeasy Blood and Tissue Kit (Lauer et al., 2007). 16S rRNA gene sequencing was performed using polymerase chain reaction (PCR) for gene amplification. Each PCR centrifuge tube contained a total of 25 µl (11.5 µl molecular grade water, 10 µl Taq 5X Master Mix, 1 µl primer 8F, 1 µl primer 1492R, 1 µl DNA extract, .5 µl BSA) according to Toffin et al. (2004) with some slight modifications. The thermocycler was programmed to the 8F 1492R protocol and the following amplification cycle was completed: one cycle at 94°C for 4 min; 34 cycles at 94°C for 1 min, 53°C for 1 min, 72°C for 90 sec; one cycle at 72°C for 10 min. Each PCR set also contained both positive and negative controls to protect against contamination and ensure proper amplification. Electrophoresis was performed in a 1% agarose to separate PCR products by molecular weight. Gel images contained a positive control and a molecular weight marker to determine which samples were correctly amplified and assayed. Samples were then sent to a commercial laboratory for Sanger Sequencing to analyze each bacterial colony's DNA sequence. 16S rRNA sequences, along with the bioinformatics software Geneious Prime and NCBI BLAST, were used to identify the genus of each bacterial isolate.

Bd Challenge Assay

Lastly, bacterial isolates were tested to determine inhibition against *Bd*. Individual isolates were grown in broth and centrifuged to form cell-free supernatants, also known as CFS (Bletz et

al., 2013). The CFS of each isolate was combined with *Bd* in 96-well plates and incubated at room temperature for 10 days (Bletz et al., 2013). Each isolate was tested in triplicate in order to identify and minimize any error variance. Additionally, positive and negative controls were included in each plate to ensure the activity of the *Bd* solution (Bletz et al., 2013). Absorbance was measured after 0, 3, 7, and 10 days of incubation using a spectrophotometer to measure the growth of *Bd*. After 10 days, percent inhibition against *Bd* was plotted for each bacterial isolate (Bletz et al., 2013). This was performed first by calculating the slope of each isolate's absorbances from days 0-10. Then, this value was divided by the slope of the positive control to quantify *Bd* growth in the presence of each isolate. Lastly, the *Bd* growth value was subtracted from one to indicate the percent inhibition of each bacterial isolate.

Results

Bacterial Isolates

A total of 122 microbes were isolated from the skin of twelve salamander individuals, including 9 fungal colonies and 113 bacterial colonies. 92 bacterial colonies were positively identified and 40 unique bacterial genera were recorded within three different phyla, as shown in Figures 2 and 3. The vast majority of isolates were of the Actinobacteria phylum, which is typified by mostly gram-positive bacteria with diverse morphological characteristics (Barka et al., 2016). The most prevalent genera were *Streptomyces* with seventeen isolates, *Microbacterium* with fourteen isolates, *Agromyces* with nine isolates, and *Paenibacillus* with five isolates.

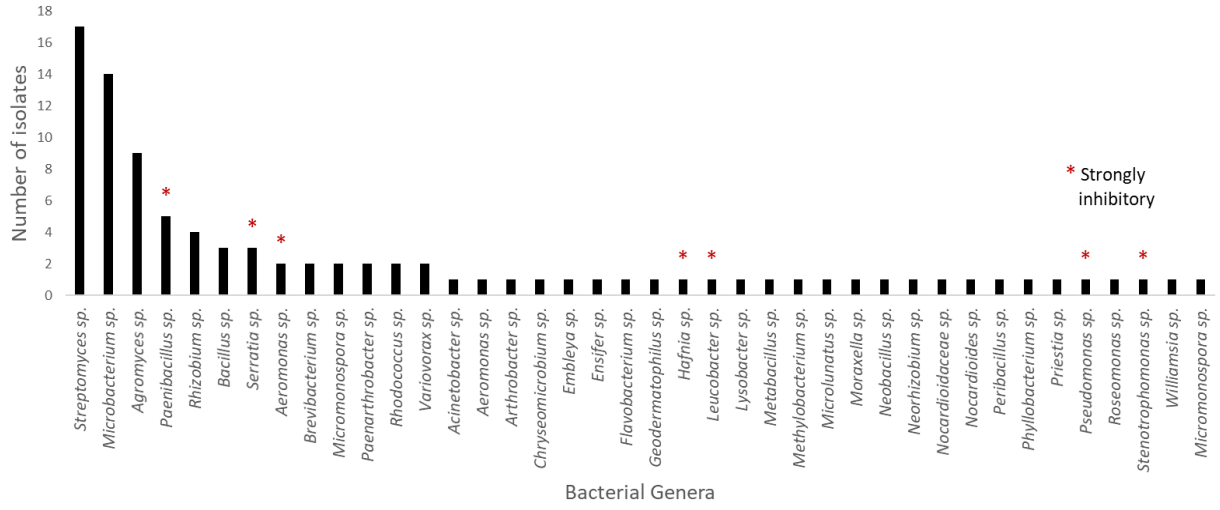

Figure 2.

Prevalence of bacterial genera. Strongly inhibitory is defined by values from .70 to 1.00.

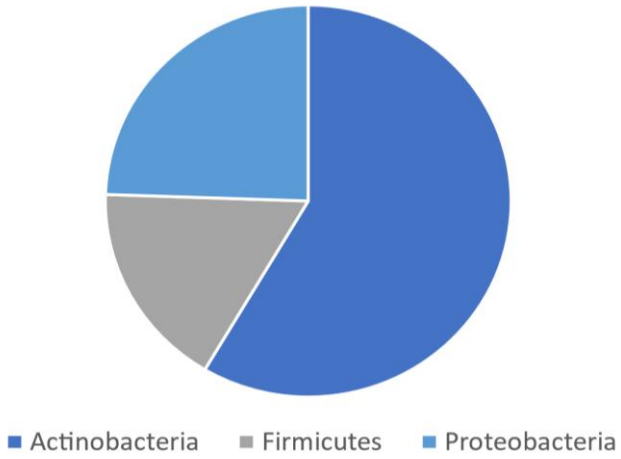

Figure 3.

Distribution of bacterial isolate phyla. The prevalence of Actinobacteria is more than double each of the other two phyla.

Bd Inhibition

Each bacterial isolate was submitted to a *Bd*-challenge assay to determine its enhancement of or inhibition against the pathogen. The results were quantified by plotting the percent inhibition of each isolate on a scale from -1.00 to 1.00. The results included values ranging from -0.65 to 1.00, as shown in Figure 4. For the purpose of this study, moderate inhibition was characterized by values ranging from 0.20 to 0.69 and strong inhibition was characterized by values ranging from 0.70 to 1.00. The median inhibition value for positive inhibitions was .32, indicating that the collective of cutaneous bacteria on this *E. lucifuga* population moderately inhibits *Bd*. Fourteen of the tested bacterial genera were moderately inhibitory against Bd, while eight were strongly inhibitory. All but one of the strongly inhibitory bacteria had inhibition values greater than 0.95. Additionally, seven of the twenty-two moderately and strongly inhibitory genera were found on multiple salamander individuals. Eight of the twelve salamander specimens carried Bd-inhibitory bacteria, as shown in Figure 5. Interestingly, one-third of the *E. lucifuga* specimens also carried *Bd-enhancing* bacteria. A comparison of the most strongly-inhibitory and strongly-enhancing bacteria is seen in Table 1. The most prevalent bacterial genus, *Streptomyces*, had a mean inhibition of 0.2, indicating low to moderate *Bd*-inhibition.

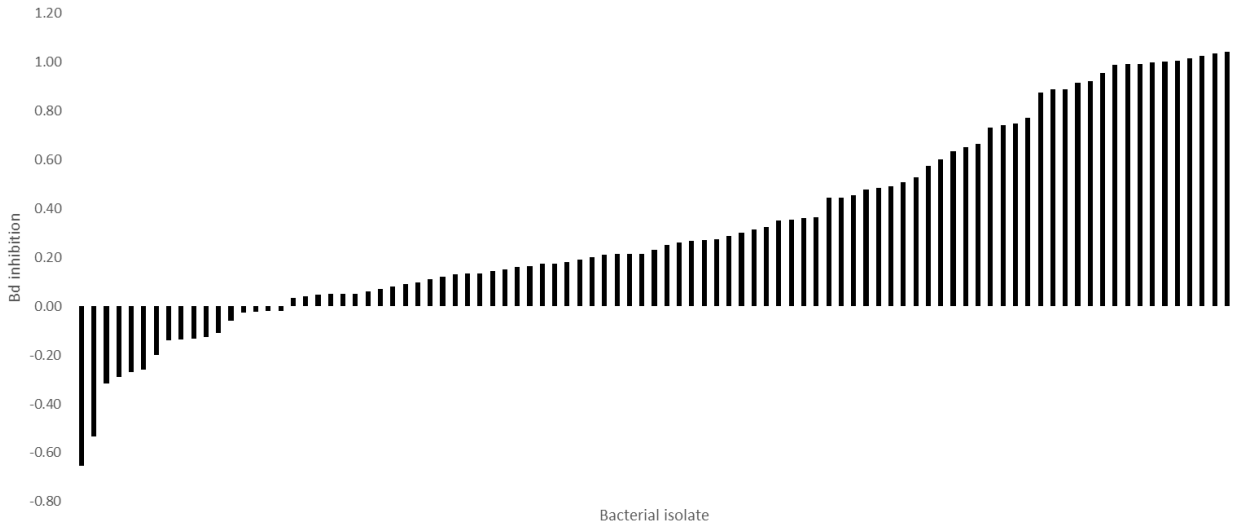

Figure 4.

Bd inhibition of bacterial isolates. The number of *Bd*-inhibitory isolates is more than quadruple that of *Bd*-enhancing isolates.

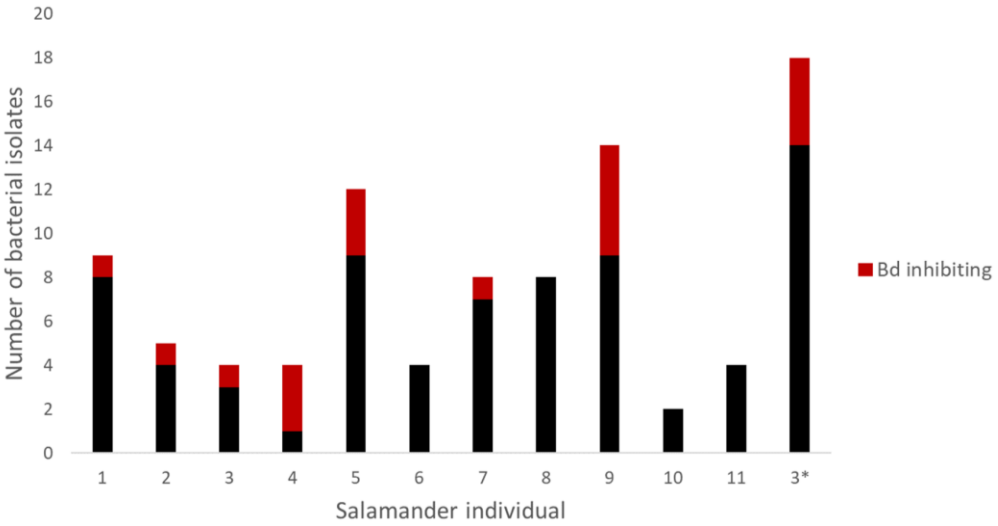

Figure 5.

Number of OTUs per salamander. The number of isolates per salamander varied from two to eighteen, with the average being 7.67 isolates per salamander. *Bd* inhibiting refers to isolates with positive *Bd*-inhibition values.

Table 1.

Strongly Bd-inhibitory & all Bd-enhancing bacterial genera.

Bacterial Strain	Mean Inhibition	No. Salamanders harboring OTU
Hafnia sp.	1.00	1
Stenotrophomonas sp.	1.00	1
Serratia sp.	0.99	2
Aeromonas sp.	0.99	1
Pseudomonas sp.	0.99	1
Leucobacter sp.	0.96	1
Paenibacillus sp.	0.71	3
Chryseomicrobium sp.	-0.14	1
Microlunatus sp.	-0.26	1
Roseomonas sp.	-0.65	1

Discussion

As mentioned previously, *E. lucifuga* salamanders have not been impacted by the current chytridiomycosis epidemic sweeping the globe. The cutaneous bacteria that the species possesses are likely the reason behind this finding. It is proposed that a synergistic effect occurs when the cutaneous bacteria are in contact with one another and their host, thereby limiting Bd to a greater extent than when tested alone (Park et al., 2014)

As a result of this research, it was determined that *E. lucifuga* salamanders carry cutaneous bacterial colonies that inhibit *Bd* growth. This is supported by evidence from other investigations that found significant fungal-inhibitory properties among diverse bacterial classes like Gammaproteobacteria and Betaproteobacteria, which are common along *E. lucifuga*'s cutaneous microbiome (Park et al., 2014). An instance of this is with the bacterial genus *Serratia*, which falls under the Gammaproteobacteria class and was the seventh-most prevalent

genus in this study. We found that the genus carried strongly inhibitory bacteria at .99 inhibition, which coincides with previous research (Park et al., 2014).

Additionally, the data is in accordance with other studies on the confirmed cutaneous microbiota of salamanders residing in the Blue Ridge Mountains, specifically the red-backed salamander, *Plethodon cinereus*. Previous research has shown that *P. cinereus* is home to *Lysobacter gummosus,* a type of bacteria that produces 2,4-diacetylphloroglucinol (Becker & Harris, 2010). 2,4-DAPG is a metabolite proven to be inhibitory to *Bd*, giving *P. cinereus* immunity to chytridiomycosis. Additionally, the infamous anti-*Bd* bacterium *Janthinobacterium lividum* was also isolated from the red-backed salamander (Becker & Harris, 2010). *J. lividium* produces violacein and indole-3-carboxaldehyde, two antifungal compounds known to repress *Bd*.

Other salamander species local to the Appalachian mountains have been observed with similar microbial populations as *E. lucifuga.* Like the cave salamander, most of the bacterial isolates found on individual *Plethodon* salamanders, including species like *P. cinereus*, *P. cylindraceus,* and *P. glutinosus,* were quite rare and not normally found among multiple individuals (Muletz Wolz et al., 2017). *Streptomyces* and *Microbacterium* were both the most prevalent genera across *E. lucifuga* individuals and had the largest number of isolates. In contrast, *Acinetobacter* and *Pseudomonas* were the most prevalent genera across *Plethodon* individuals (Muletz Wolz et al., 2017). Additionally, while most of the isolates on the skin of *E. lucifuga* salamanders were of the Actinobacteria phylum, most of the isolates on the skin of *Plethodon* salamanders were of the Proteobacteria phylum (Muletz Wolz et al., 2017).

Conclusion

Future Work

It would be beneficial to evaluate the *Bd*-inhibitory properties of two or more bacterial genera when combined, in order to determine if salamanders or other amphibians that host a variety of inhibitory bacterial species experience synergistic effects against the fungal pathogen (Park et al., 2014).

Since there are no widespread options available to help mitigate the lethal spread of chytridiomycosis, this research may contribute to the development of treatments against *Bd* using findings from amphibians already known to carry immunity against the fungus. Past research has shown that when *J. lividium* has been artificially added to the skin of amphibians susceptible to *Bd*, they have become resistant to the effects of the pathogen by resisting colonization and thus preventing the fungus from growing to lethal levels (Baitchman & Pessier, 2013). If this process could be developed into a large-scale treatment, the current chytridiomycosis epidemic in the amphibian community could come to an end.

Translational Impact

Amphibian biodiversity is not only important to the earth's ecosystem but to human health as well. During the 1980s and 1990s, a wave of chytridiomycosis spread throughout Costa Rica (Springborn et al., 2020). The epizootic then traveled to Panama in the early 2000s. The widespread amphibian declines that occurred as a result were correlated with a significant rise in malaria cases throughout the region for eight years following the outbreak (Springborn et al. 2020). The decrease in local amphibian populations likely disrupted the natural food web and caused insect populations to soar, unchecked by their natural amphibian predators. Among these insects was the mosquito, which is a vector for malaria and other human diseases. Despite

extensive research regarding how chytridiomycosis is impacting global amphibian populations, the social cost and ecosystem erosion caused by the disease is underappreciated.

References

Alton, L. A., & Franklin, C. E. (2017). Drivers of amphibian declines: Effects of ultraviolet radiation and interactions with other environmental factors. *Climate Change Responses*, *4*(1). https://doi.org/10.1186/s40665-017-0034-7

Baitchman, E. J., & Pessier, A. P. (2013). Pathogenesis, diagnosis, and treatment of amphibian chytridiomycosis. *Veterinary Clinics of North America: Exotic Animal Practice*, *16*(3), 669–685. https://doi.org/10.1016/j.cvex.2013.05.009

Baken, E. K., & Adams, D. C. (2019). Macroevolution of arboreality in salamanders. *Ecology and Evolution*, *9*(12), 7005–7016. https://doi.org/10.1002/ece3.5267

Barka, E. A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H.-P., Clément, C., Ouhdouch, Y., & van Wezel, G. P. (2016). Taxonomy, physiology, and natural products of actinobacteria. *Microbiology and Molecular Biology Reviews*, *80*(1), 1–43. https://doi.org/10.1128/mmbr.00019-15

Becker, M. H., & Harris, R. N. (2010). Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. *PLoS ONE*, *5*(6). https://doi.org/10.1371/journal.pone.0010957

Becker, C. G., Greenspan, S. E., Tracy, K. E., Dash, J. A., Lambertini, C., Jenkinson, T. S., Leite, D. S., Toledo, L. F., Longcore, J. E., James, T. Y., & Zamudio, K. R. (2017). Variation in phenotype and virulence among enzootic and panzootic amphibian chytrid lineages. *Fungal Ecology*, *26*, 45–50. https://doi.org/10.1016/j.funeco.2016.11.007

Berger, L., Hyatt, A. D., Speare, R., & Longcore, J. E. (2005). Life cycle stages of the amphibian chytrid batrachochytrium dendrobatidis. *Diseases of Aquatic Organisms, 68,* 51–63. https://doi.org/10.3354/dao068051

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., Slocombe, R., Ragan, M. A., Hyatt, A. D., McDonald, K. R., Hines, H. B., Lips, K. R., Marantelli, G., & Parkes, H. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences*, *95*(15), 9031–9036. https://doi.org/10.1073/pnas.95.15.9031

Bletz, M. C., Loudon, A. H., Becker, M. H., Bell, S. C., Woodhams, D. C., Minbiole, K. P., & Harris, R. N. (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: Characteristics of effective probiotics and strategies for their selection and use. *Ecology Letters*, *16*(6), 807–820. https://doi.org/10.1111/ele.12099

Brownlee-Bouboulis, S. A., & Reeder, D. M. (2013). White-nose syndrome-affected little brown myotis (Myotis lucifugus) increase grooming and other active behaviors during arousals from hibernation. *Journal of Wildlife Diseases, 49*(4), 850–859. https://doi.org/10.7589/2012-10-242

Brucker, R. M., Harris, R. N., Schwantes, C. R., Gallaher, T. N., Flaherty, D. C., Lam, B. A., & Minbiole, K. P. (2008). Amphibian chemical defense: Antifungal metabolites of the microsymbiont janthinobacterium lividum on the salamander Plethodon cinereus. *Journal of Chemical Ecology*, *34*(11), 1422–1429. https://doi.org/10.1007/s10886-008-9555-7

Brunner, J. L., Schock, D. M., & Collins, J. P. (2007). Transmission dynamics of the amphibian ranavirus ambystoma tigrinum virus. *Diseases of Aquatic Organisms, 77*, 87–95. https://doi.org/10.3354/dao01845

Carter, E. D., Miller, D. L., Peterson, A. C., Sutton, W. B., Cusaac, J. P., Spatz, J. A., Rollins-Smith, L., Reinert, L., Bohanon, M., Williams, L. A., Upchurch, A., & Gray, M. J. (2019). Conservation risk of batrachochytrium salamandrivorans to endemic lungless salamanders. *Conservation Letters*, *13*(1). https://doi.org/10.1111/conl.12675

Caruso, N. M., & Lips, K. R. (2012). Truly enigmatic declines in terrestrial salamander populations in Great Smoky Mountains National Park. *Diversity and Distributions*, *19*(1), 38–48. https://doi.org/10.1111/j.1472-4642.2012.00938.x

Castro Monzon, F., Rödel, M.-O., & Jeschke, J. M. (2020). Tracking batrachochytrium dendrobatidis infection across the Globe. *EcoHealth*, *17*(3), 270–279. https://doi.org/10.1007/s10393-020-01504-w

Collins, J. P. (2010). Amphibian decline and extinction: What we know and what we need to learn. *Diseases of Aquatic Organisms*, *92*(3), 93–99. https://doi.org/10.3354/dao02307

Elkan, E. R. (1938). The xenopus pregnancy test. *British Medical Journal*, *2*(4067), 1253–1274. https://doi.org/10.1136/bmj.2.4067.1253

Gray, M. J., Lewis, J. P., Nanjappa, P., Klocke, B., Pasmans, F., Martel, A., Stephen, C., Parra Olea, G., Smith, S. A., Sacerdote-Velat, A., Christman, M. R., Williams, J. M., & Olson,

D. H. (2015). Batrachochytrium salamandrivorans: The North American response and a call for action. *PLOS Pathogens*, *11*(12). https://doi.org/10.1371/journal.ppat.1005251

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., Lam, B. A., Woodhams, D. C., Briggs, C. J., Vredenburg, V. T., & Minbiole, K. P. (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *The ISME Journal*, *3*(7), 818–824. https://doi.org/10.1038/ismej.2009.27

Jablonski, D. (2004). Extinction: Past and present. *Nature*, *427*(6975), 589–589. https://doi.org/10.1038/427589a

Jones, K. E., Patel, N. G., Levy, M. A., Storeygard, A., Balk, D., Gittleman, J. L., & Daszak, P. (2008). Global trends in emerging infectious diseases. *Nature*, *451*(7181), 990–993. https://doi.org/10.1038/nature06536

Lauer, A., Simon, M. A., Banning, J. L., André, E., Duncan, K., & Harris, R. N. (2007). Common cutaneous bacteria from the eastern red-backed salamander can inhibit pathogenic fungi. *Copeia*, *2007*(3), 630–640. https://doi.org/10.1643/0045-8511(2007)2007[630:ccbfte]2.0.co;2

Lawson, D. J., Hellenthal, G., Myers, S., & Falush, D. (2012). Inference of population structure using dense haplotype data. *PLoS Genetics*, *8*(1). https://doi.org/10.1371/journal.pgen.1002453

Lindauer, A., May, T., Rios-Sotelo, G., Sheets, C., & Voyles, J. (2019). Quantifying batrachochytrium dendrobatidis and Batrachochytrium salamandrivorans viability. *EcoHealth*, *16*(2), 346–350. https://doi.org/10.1007/s10393-019-01414-6

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., Carey, C., Livo, L., Pessier, A. P., & Collins, J. P. (2006). Emerging infectious disease and the loss of biodiversity in a neotropical amphibian community. *Proceedings of the National Academy of Sciences*, *103*(9), 3165–3170. https://doi.org/10.1073/pnas.0506889103

Longcore, J. E., Pessier, A. P., & Nichols, D. K. (1999). Batrachochytrium dendrobatidis gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia*, *91*(2), 219–227. https://doi.org/10.2307/3761366

Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M. C., Farrer, R. A., Schmidt, B. R., Tobler, U., Goka, K., Lips, K. R., Muletz, C., Zamudio, K. R., Bosch, J., Lötters, S., Wombwell, E., Garner, T. W., Cunningham, A. A., Spitzen-van der Sluijs, A., Salvidio, S., … Pasmans, F. (2014). Wildlife disease. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science (New York, N.Y.)*, *346*(6209), 630–631. https://doi.org/10.1126/science.1258268

Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M. C., Woeltjes, A., Bosman, W., Chiers, K., Bossuyt, F., & Pasmans, F. (2013). Batrachochytrium salamandrivorans sp. nov. causes lethal chytridiomycosis in amphibians. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(38), 15325–15329. https://doi.org/10.1073/pnas.1307356110

McCallum, M. L. (2007). Amphibian decline or extinction? current declines dwarf background extinction rate. *Journal of Herpetology*, *41*(3), 483–491. https://doi.org/10.1670/0022-1511(2007)41[483:adoecd]2.0.co;2

Mazzoni R. (2003). Emerging pathogen of wild amphibians in frogs (Rana catesbeiana) farmed for international trade. *Emerging Infectious Diseases*, *9*(8), 995–998. https://doi.org/10.3201/eid0908.030030

Merkle, D. A., & Guttman, S. I. (1977). Geographic variation in the cave salamander Eurycea lucifuga. *Herpetologica*, *33*(3), 313–321. http://www.jstor.org/stable/3891947

Muletz Wolz, C. R., Yarwood, S. A., Campbell Grant, E. H., Fleischer, R. C., & Lips, K. R. (2017). Effects of host species and environment on the skin microbiome of plethodontid salamanders. *Journal of Animal Ecology*, *87*(2), 341–353. https://doi.org/10.1111/1365-2656.12726

Moss, A. S., Reddy, N. S., Dortaj, I. M., & San Francisco, M. J. (2008). Chemotaxis of the amphibian pathogen batrachochytrium dendrobatidis and its response to a variety of attractants. *Mycologia*, *100*(1), 1–5. https://doi.org/10.3852/mycologia.100.1.1

O'Hanlon, S. J., Rieux, A., Farrer, R. A., Rosa, G. M., Waldman, B., Bataille, A., Kosch, T. A., Murray, K. A., Brankovics, B., Fumagalli, M., Martin, M. D., Wales, N., Alvarado-Rybak, M., Bates, K. A., Berger, L., Böll, S., Brookes, L., Clare, F., Courtois, E. A., … Fisher, M. C. (2018). Recent Asian origin of chytrid fungi causing global amphibian declines. *Science*, *360*(6389), 621–627. https://doi.org/10.1126/science.aar1965

Ouellet, M., Mikaelian, I., Pauli, B. D., Rodrigue, J., & Green, D. M. (2005). Historical evidence of widespread chytrid infection in north american amphibian populations. *Conservation Biology*, *19*(5), 1431–1440. http://www.jstor.org/stable/3591111

Park, S. T., Collingwood, A. M., St-Hilaire, S., & Sheridan, P. P. (2014). Inhibition of Batrachochytrium dendrobatidis caused by bacteria isolated from the skin of boreal toads, anaxyrus (bufo) boreas boreas, from Grand Teton National Park, Wyoming, USA. *Microbiology insights*, *7*, 1–8. https://doi.org/10.4137/MBI.S13639

Pasmans, F., Van Rooij, P., Blooi, M., Tessa, G., Bogaerts, S., Sotgiu, G., Garner, T. W., Fisher, M. C., Schmidt, B. R., Woeltjes, T., Beukema, W., Bovero, S., Adriaensen, C., Oneto, F., Ottonello, D., Martel, A., & Salvidio, S. (2013). Resistance to chytridiomycosis in European plethodontid salamanders of the genus Speleomantes. *PLoS ONE*, *8*(5). https://doi.org/10.1371/journal.pone.0063639

Piotrowski, J. S., Annis, S. L., & Longcore, J. E. (2004). Physiology of Batrachochytrium dendrobatidis, a chytrid pathogen of amphibians. *Mycologia*, *96*(1), 9–15. https://doi.org/10.2307/3761981

Scheele, B. C., Pasmans, F., Skerratt, L. F., Berger, L., Martel, A., Beukema, W., Acevedo, A. A., Burrowes, P. A., Carvalho, T., Catenazzi, A., De la Riva, I., Fisher, M. C., Flechas, S. V., Foster, C. N., Frías-Álvarez, P., Garner, T. W., Gratwicke, B., Guayasamin, J. M., Hirschfeld, M., … Canessa, S. (2019). Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science*, *363*(6434), 1459–1463. https://doi.org/10.1126/science.aav0379

Springborn, M. R., Weill, J. A., Lips, K. R., Ibáñez, R., & Ghosh, A. (2020). Amphibian collapses exacerbated malaria outbreaks in Central America. *MedRXiv*. https://doi.org/10.1101/2020.12.07.20245613

Stuart, S. N. (Ed.). (2008). *Threatened amphibians of the world*. Lynx Edicions.

Symonds, E. P., Trott, D. J., Bird, P. S., & Mills, P. (2008). Growth characteristics and enzyme activity in Batrachochytrium dendrobatidis isolates. *Mycopathologia, 166*(3), 143–147. https://doi.org/10.1007/s11046-008-9135-y

Toffin, L., Webster, G., Weightman, A. J., Fry, J. C., & Prieur, D. (2004). Molecular monitoring of culturable bacteria from deep-sea sediment of the Nankai Trough, Leg 190 Ocean Drilling Program. *FEMS Microbiology Ecology*, *48*(3), 357–367. https://doi.org/10.1016/j.femsec.2004.02.009

Trajano, E., & de Carvalho, M. R. (2017). Towards a biologically meaningful classification of subterranean organisms: A critical analysis of the Schiner-RACOVITZA system from a historical perspective, difficulties of its application and implications for conservation. *Subterranean Biology*, *22*, 1–26. https://doi.org/10.3897/subtbiol.22.9759

Van Rooij, P., Martel, A., Haesebrouck, F., & Pasmans, F. (2015). Amphibian chytridiomycosis: A review with focus on fungus-host interactions. *Veterinary Research*, *46*, 137. https://doi.org/10.1186/s13567-015-0266-0

Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W. F., Dinudom, A., Cook, D., Webb,

R., Alford, R. A., Skerratt, L. F., & Speare, R. (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science*, *326*(5952), 582–585. https://doi.org/10.1126/science.1176765

Weldon, C., du Preez, L. H., Hyatt, A. D., Muller, R., & Speare, R. (2004). Origin of the amphibian Chytrid fungus. *Emerging Infectious Diseases*, *10*(12), 2100–2105. https://doi.org/10.3201/eid1012.030804

Wu, N. C., Cramp, R. L., Ohmer, M. E., & Franklin, C. E. (2018). Epidermal epidemic: Unravelling the pathogenesis of chytridiomycosis. *Journal of Experimental Biology*, *222*(2). https://doi.org/10.1242/jeb.191817

Xie, G. Y., Olson, D. H., & Blaustein, A. R. (2016). Projecting the global distribution of the emerging amphibian fungal pathogen, Batrachochytrium dendrobatidis, based on IPCC Climate Futures. *PLOS ONE*, *11*(8). https://doi.org/10.1371/journal.pone.0160746