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A bioaugmentation approach to the prevention of chytridiomycosis

Carly Muletz
James Madison University

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Carly Muletz

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Dedication

This thesis is dedicated to my family: my dad, mom, sister, step-dad and grandparents. Their love and support has given me the strength to pursue my dreams. Sadly, my father Charles "Chill" Robert Muletz passed away while I was conducting my thesis research on October 16th, 2010. He would have been so proud of this accomplishment. He was and will continue to be a great inspiration to me.

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Abstract

Symbiosis is defined as two species living together. This association between organisms is present at all taxonomic levels making it a ubiquitious phenomenon in ecology and evolution. I studied symbiosis among three species: a mutualistic bacterium, a parasitic fungus and an amphibian host. The first goal of my research was to examine how the mutualistic bacteria of amphibians' skin are acquired. I demonstrated that a cutaneous mutualistic bacterial species, *Janthinobacterium lividum*, was transmitted environmentally, via soil, to the skin of an amphibian species, *Plethodon cinereus*. These results indicate that amphibians' mutualistic bacteria can be acquired from the environment. Based upon these results, I examined the use of soil bioaugmentation in amphibian disease prevention. I sought to determine if the environmental transmission of the mutualistic bacterial species *J. lividum* could mitigate parasite infection by the fungal pathogen *Batrachochytrium dendrobatidis* on the skin of an amphibian species, *P. cinereus*. Cutaneous infection by *B. dendrobatidis* in amphibians causes the disease chytridiomycosis, which has decimated amphibian populations and species worldwide. I found that the environmental transmission of *J. lividum* inhibited initial colonization by *B. dendrobatidis* on the skin of *P. cinereus* ($p<0.05$) five days post-infection. The use of bioaugmentation may be a feasible conservation strategy that could supplant treating amphibians individually and protect global amphibian biodiversity against declines driven by chytridiomycosis.

Introduction

Symbiosis occurs when two species live in close association with one another. Symbiosis is typically divided into three categories: mutualism, in which both organisms benefit, commensalism, in which one organism benefits and the other is unharmed, and parasitism, in which one organism benefits and the other is harmed. These three interactions play important roles in ecology and evolutionary biology. My research focused upon bacterial mutualism and fungal parasitism on amphibian hosts.

Mutualism can be observed in the diverse communities of bacteria that inhabit the moist, nutrient rich mucous on amphibians' skin (Austin 2000, Lauer et al. 2007). Studies have demonstrated that amphibians receive the benefit of disease mitigation from their cutaneous bacteria (Harris et al. 2006, Banning et al. 2008, Becker & Harris 2010). Other benefits to the amphibian may exist, but have yet to be studied. In humans, gut microbiota have been shown to direct immune system development (Mazmanian et al. 2005). Amphibian microbiota may serve the same role or potentially other beneficial roles. The other species in the mutualism, the bacteria, benefit from the available nutrients found in the mucosal layer and a suitable, protected habitat (Austin 2000).

Parasitism by the fungal pathogen *Batrachochytrium dendrobatidis* harms the infected individual amphibian and global amphibian biodiversity (Berger et al. 1998). As with the mutualistic bacteria, this pathogenic parasite is provided with a suitable, protected habitat within epidermal cells of amphibians' skin (Longcore et al. 1999). The pathogen may also use nutrients in the mucus, but this remains unclear (Piotrowski et al. 2004).

The mutualistic bacteria and parasitic fungal pathogen interact with the host and with each other. The mutualistic and pathogenic microbes use the same space and possibly the same nutrients. The principle of competitive exclusion is important in these interactions. It has been proposed that mutualistic bacteria have a direct antagonistic effect against pathogens (Chan et al. 1984, Fuller 1989, Brucker et al. 2008a, 2008b). Possible modes of action include competition for nutrients, competition for adhesion sites, production of toxic compounds (antibiotics), and stimulation of the host immune system (Fuller 1989). These mechanisms are not mutually exclusive; some microorganisms may inhibit pathogens by a single mechanism, whereas others may be inhibitory by using multiple mechanisms (Patterson & Burkholder 2003).

The relationships among cutaneous mutualistic bacteria, the parasitic pathogen *B. dendrobatidis*, and the amphibian host provide an excellent model to study symbiosis. These interactions occur on the amphibian skin, which provides ease of study. In addition, the three interacting species are all amenable to experimental manipulation. Understanding these interactions can help elucidate general principles regarding metazoan/bacterial mutualisms and disease ecology. It is increasingly recognized that disease dynamics are related to the characteristics of the ecological community, such as the community of mutualistic microbes (Belden & Harris 2007). Anthropogenic changes to microbial communities may lead to an increase in some metazoan diseases if mutualistic microbes are negatively affected. Finally, global amphibian declines present an urgency to study these basic ecological interactions with the additional hope of identifying strategies to prevent future declines.

Amphibian declines:

Scientists working around the world are documenting a loss of vertebrate

biodiversity that exceeds the normal background extinction rate (Hoffmann et al. 2010).

According to the International Union for the Conservation of Nature (IUCN) Red List,

which places species on a gradient from least concern to extinct, one-fifth of vertebrate

species worldwide are listed as threatened (Hoffmann et al. 2010). The most threatened

class of the seven classes of vertebrates is Amphibia, with 41% of extant species

classified as threatened (Hoffmann et al. 2010). This rate is an increase from 2004, when

32.5% of amphibians were classified as globally threatened (Stuart et al. 2004). The

current extinction rate for amphibians is estimated to be 211 times the background

extinction rate (McCallum 2007). The current trend in amphibian declines indicates that

there will be catastrophic future losses in amphibian biodiversity (McCallum 2007).

Furthermore, Wake & Vredenburg (2008) proposed that amphibian declines might be a

warning sign that we are in the midst of a sixth mass extinction in the history of life on

Earth, one driven by anthropogenic disturbances and infectious disease.

Ironically, both the number of described amphibian species and the number of

declining amphibian species are increasing at an unprecedented rate (Stuart et al. 2004,

Hoffmann et al. 2010). In regard to described amphibian species, this number has

increased from 5,743 species in 2004 (Stuart et al. 2004) to 6,638 species in 2010

(Hoffmann et al. 2010). In regard to amphibian declines, 662 amphibian species have

moved one Red List category closer to extinction from 1980 to 2004, while 40 of those

species have deteriorated in status by three or more categories (Hoffmann et al. 2010).

These categories listed in order of increasing concern are: least concern, near threatened,

vulnerable, endangered, critically endangered and extinct (Hoffmann et al. 2010). Since 1980 nine species have been listed as extinct, with another 122 considered 'possibly extinct', i.e., not formally 'extinct' until exhaustive studies have been performed (Stuart et al. 2004). Furthermore, the actual extinction total may be even greater since many species may reach extinction before they have been discovered. The paradox of increasing species descriptions with increasing species declines exemplifies the need for amphibian-based conservation studies.

Six major drivers of global amphibian declines have been identified: habitat loss, emerging infectious disease, introduced species, over-exploitation, contaminants and global climate change (Collins & Storfer 2003). These drivers are not mutually exclusive, but most likely interact in complex ways. For instance, increasing UV radiation and concentrations of environmental contaminants (Blaustein et al. 2003) and changing climatic conditions and agricultural practices (D'Amen et al. 2010) have been shown to act synergistically to make some amphibian populations more susceptible to decline. While all the drivers are contributing to decline, two drivers have been singled out as inflicting the most damage on amphibian biodiversity: habitat loss (Stuart et al. 2004) and the emerging infectious disease, chytridiomycosis (Skerrat et al. 2007). It has been proposed that the immunological stress inflicted on amphibian populations by anthropogenic disturbances, such as habitat loss, has given rise to the susceptibility of amphibians to disease (Blaustein et al. 2003). Chytridiomycosis has been linked to the decline or extinction of more than 200 amphibian species (Wake & Vredenburg 2008). This disease may be the most challenging driver of amphibian declines as there is no proven implementable strategy in the field that can combat this disease. However, one

potential strategy to mitigate chytridiomycosis may be the natural antagonist effects of mutualistic bacteria.

Mutualisms between metazoans and microorganisms:

As metazoans evolved so did the complexity of the microbial communities that inhabit the metazoan host (McFall-Ngai 2005). Both groups benefit from this evolutionary adaptation; the microbial species is provided nutrients and a suitable habitat and the metazoan is provided nutritive, reproductive, developmental or defensive benefits (Chaston & Goodrich-Blair 2010). An increase in fitness by the co-operating individuals sustains and modifies these interactions throughout time and space. The evolution of metazoan microbial mutualism is thought to have proceeded from binary bacterial symbioses in invertebrates to highly complex consortia of hundreds to thousands of bacterial mutualists in vertebrates (McFall-Ngai 2005). Interestingly, it has been proposed that the shift to a complex assemblage of microbes in vertebrates led to the evolution of the adaptive immune system, which is absent in invertebrates (McFall-Ngai 2005).

Metazoan microbiota can provide their host a variety of nutritive, reproductive, developmental and defensive benefits. One example of a nutritional benefit is that bacteria-free mice must eat about a third more food than mice with a natural gut microbiota to maintain the same growth rate, suggesting a role in digestion (McFall-Ngai 2005). In regards to reproductive benefits, the removal of symbiotic microbiota causes the cessation of proliferation via asexual budding in freshwater *Hydra* spp. (Fraune et al. 2009). Developmental benefits have been documented by Mazmanian et al. (2005). They found that the common human gut bacteria *Bacteroides fragilis* produces a

polysaccharide that directs the cellular and physical maturation of the mammalian immune system by aiding in T cell production, correcting T_H1/T_H2 imbalances and directing lymphoid organogenesis. Guarner & Malagelada (2003) also suggested that other ubiquitous human gut bacteria might produce immunomodulatory molecules that direct maturation of the immune system. Finally, of main interest in this study is the role of microbiota in protection from disease, as this role may be crucial in the disease dynamics of chytridiomycosis. For example, human intestinal microbiota has been shown *in vitro* to directly block attachment of pathogens to epithelial cells (Chan et al. 1984). In addition, in fish species affected by the pathogen *Vibrio anguillarum* both skin and intestinal microbes were shown to inhibit the pathogen via the production of bactericidal metabolites (Olsson et al. 1992).

Like all vertebrates, amphibians possess a diverse microbial community (Lauer et al. 2007). There is a small but growing knowledge of amphibians' mutualistic bacteria, what functions these microbes may serve and how they are attained. Most studies have only identified a partial set of microbial species on a limited number of amphibian species (Austin 2000, Culp et al. 2007, Lauer et al. 2007). However, several studies have found a role of amphibians' cutaneous bacteria in disease prevention (Lauer et al. 2007, Woodhams et al. 2007b, Harris et al. 2009a, 2009b, Becker & Harris 2010). Given this crucial role, it becomes important to determine how amphibians obtain their microbiota.

Transmission of microbiota:

Bacteria are transmitted to hosts by three methods: vertical (parent to offspring), horizontal (conspecific contact) and environmental (environment to organism). In humans, studies have found that the intestinal microbiota is initially acquired through

vertical transmission during birth (Tannock et al. 1990) and during breast-feeding (Harmsen et al. 2000). However, past infancy the intestinal microbiota changes to be composed primarily of environmentally transmitted microbiota (Xu & Gordon 2003). The squid-vibrio association provides an ideal system to study metazoan-microbial mutualisms. The light organ of sepoilid squid is colonized by the marine bacterium *Vibrio fischeri* that produces bioluminescence (McFall-Ngai & Ruby 1991). Transmission of *V. fischeri* has been shown to occur both horizontally (McFall-Ngai & Ruby 1991) and environmentally (Nishiguchi 2002), with a preference for *V. fischeri* strains from squid of the same species (Nishiguchi 2002).

It is likely that amphibians attain their mutualistic bacteria via all three mechanisms of transmission. However, the ecology of each species may dictate the predominate mode of bacterial transmission. In amphibians, vertical transmission may be common for species that attend or brood their offspring, while it is likely nonexistent for species that mature without any contact with their parent. Horizontal transmission is likely prevalent during the mating season since contact occurs during mating in amphibians. In addition, horizontal transmission may occur in social amphibian species and in individuals that share the same hibernaculum. As amphibians have their nutrient rich mucosal layer in continual contact with the environment, environmental transmission may be the most universal form of transmission.

Only a few studies have been conducted on amphibian bacterial transmission and none of these studies have used experimental manipulations. Austin (2000) found that the culturable resident and transient microbiota of *Plethodon ventralis* was 17% similar to the microbial community present in the amphibians' environment. This indicates that 17%

of bacteria isolated in this study could occupy two vastly different niches (soil/leaf litter and salamander skin), which may suggest a long history of environmental transmission. Culp et al. (2007) also found that a sub-set of bacterial species in the environment of three amphibian species, *Notophthalamus viridescens, Rana* (*Lithobates*) *catesbiana, Plethodon cinereus*, was present on the amphibians' skin. Banning et al. (2008) presented evidence that a brooding salamander species, *Hemidactylium scutatum*, transmitted cutaneous bacteria vertically in communal nests. To my knowledge, no studies have been conducted on horizontal transmission in amphibians.

Transmission of mutualistic bacteria is an important concept in disease ecology. Since mutualistic bacteria have been shown to protect their host from disease, disruption of microbiota transmission may lead to an increase in disease susceptibility. These disruptions may be caused by anthropogenic environmental disturbances such as global climate change and the widespread use of antibiotics in humans and livestock. These disturbances may cause environmental microbial community structures to change, and ecologically important microbiota to become extinct, thus making transmission impossible (Belden & Harris 2007). The emergence of chytridiomycosis may be due in part to alteration to amphibians' mutualistic bacterial community (Belden & Harris 2007). Finally, an understanding of bacterial transmission may help design conservation and restoration strategies based on the use of mutualistic bacteria to mitigate the detrimental effects of the pathogen *B. dendrobatidis*.

Parasitism of *Batrachochytrium dendrobatidis*:

Understanding the basic biology of *B. dendrobatidis* is crucial to developing methods to control this pathogen. To begin, *B. dendrobatidis* is a member of the phylum

Chytridiomycota, which is largely composed of primitive, saprobic fungi that live on dead and decaying matter (Longcore et al. 1999). Parasitism of protozoans and invertebrates by chytrids has previously been reported, but this species is the first chytrid known to parasitize vertebrate species (Berger et al. 1998). *B. dendrobatidis* lives in the keratin-rich internal mouthparts of tadpoles and inside the keratinized epithelial cells of adult amphibian skin (Berger et al. 1998). While *B. dendrobatidis* has only been found in keratinized cells, it still remains unclear if it is active in degradation of keratin as a nutrient source (Piotrowski et al. 2004). Piotrowski et al. (2004) proposed that *B. dendrobatidis* might live in keratinized cells since the cells are dead and easier to invade.

The fungal pathogen exhibits a dimorphic life cycle (Figure 1) with a free-living, substrate independent zoospore stage and a substrate dependent zoosporangia stage (Longcore et al. 1999). Zoospores are active for a relatively short period and travel a relatively short distance, but they are the colonists that invade amphibians' keratinized epithelial cells (Piotrowski et al. 2004). Upon entry of the zoospore into the host's epithelial cells, the development of a zoosporangium begins; zoospores are subsequently produced by mature zoosporangia (Berger et al. 2005). Zoospores are then released into the surrounding aquatic environment by mature zoosporangia and can re-infect the host or attempt to locate a new host (Berger et al. 2005). Only asexual reproduction in the life cycle has been observed (Berger et al. 2005).

aquatic, motile zoospore initiates
colonization of host skin cells

zoospore embeds in host cell
and develops into a thallus

thallus matures and new zoospores
begin to develop in zoosporangia

zoospores are released and
enter water column or reinfect host
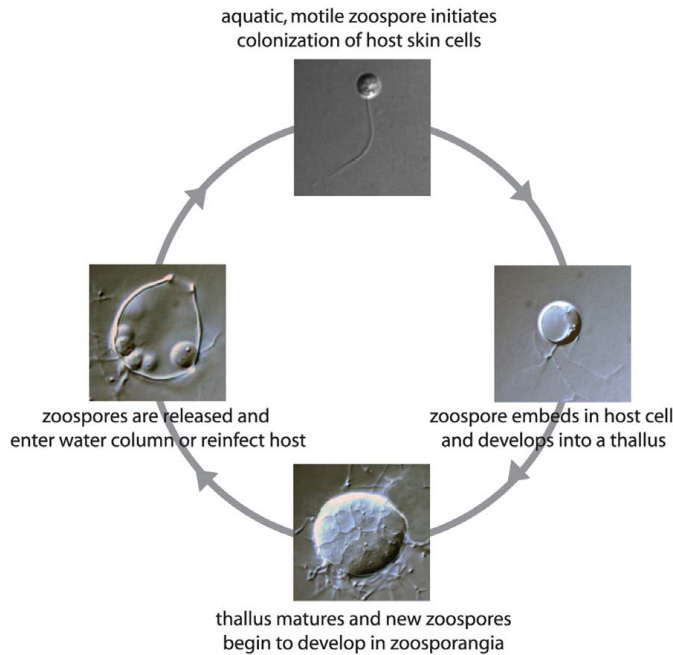
Figure 1. Life cycle of *Batrachochytrium dendrobatidis* (from Rosenblum et al. 2010).

Colonization by *B. dendrobatidis* can then result in the disease chytridiomycosis (Berger et al. 1998). There is intraspecific (Woodhams et al. 2007a, Shaw et al. 2010) and interspecific variation in susceptibility to chytridiomycosis (Blaustein et al. 2005). Once a species-specific infection density is reached, susceptible species develop the disease chytridiomycosis (Stockwell et al. 2010). Signs of the disease include lethargy, morbidity (weight loss), cutaneous erythema, irregular skin sloughing, abnormal posture, and loss of righting reflex (Voyles et al. 2009). The negative effects of the disease are seen in reduced developmental rates (Venesky, Parris & Storfer 2010) and reduced survival (Voyles et al. 2009). Infected individuals experience mortality due to the inhibition of electrolyte (notably potassium) transport across the skin, which leads to aystolic cardiac arrest (Voyles et al. 2009). At the population level, a mass mortality event is observed when a threshold density of zoospores is reached in a vulnerable population (Vredenburg et al. 2010).

Impact of chytridiomycosis on amphibian biodiversity:

Worldwide amphibian declines were first informally discussed in 1989 at the First World Congress of Herpetology (Collins & Crump 2009). In some amphibian populations, herpetologists had been observing abnormal declines since the late 1970s even in protected habitats (Collins & Crump 2009). For instance, at least 14 Australian frog species declined in numbers by more than 90% from 1979 to 1994 (Laurance et al. 1996). Laurance et al. (1996) proposed that these declines were driven by an introduced pathogen. In 1998, Berger et al. proposed that chytridiomycosis was the cause of the recently observed amphibian declines in Australia and Central America. The etiological agent for chytridiomycosis was identified as the aquatic fungus *B. dendrobatidis* (Longcore et al. 1999). The appearance of *B. dendrobatidis* in natural populations has since been directly linked to the rapid loss of amphibian biodiversity (Daszak et al. 1999, Lips et al. 2006, Woodhams et al. 2007b), probably dating back to at least the 1970s. To date, *B. dendrobatidis* has been documented to infect over 350 of amphibian species (Fisher et al. 2009). In turn, chytridiomycosis has caused the decline or extinction of more than 200 of these species (Wake & Vredenburg 2008).

There is some uncertainty in how long chytridiomycosis has been causing amphibian decline. The earliest amphibian species identified as being infected with *B. dendrobatidis* are *Xenopus fraseri* preserved in Cameroon in 1933 (Soto-Azat et al. 2010) and *Xenopus laevis* in South Africa in 1938 (Weldon et al. 2004). Both studies found a consistent low infection load in *Xenopus* spp. specimens from the 1930s to the 1990s indicating a stable, endemic disease. International trade in *Xenopus* spp. from 1934 to 1968 is likely to have been the initial cause of the global dissemination of the pathogen to

susceptible species according to the 'out of Africa' hypothesis (Weldon et al. 2004). The next documented cases of *B. dendrobatidis* infection appear in North America in 1961, Australia in 1978, Central America in 1983, South America in 1986, Europe in 1997 (Weldon et al. 2004), and Asia in 2008 (Une et al. 2008). The international trade in the resistant North American bullfrogs *Rana* (*Lithobates*) *catesbeiana* is likely to be the primary cause of the recent continual spread of the pathogen (Fisher & Garner 2007, Schloegel et al. 2010). Overall, the international amphibian trade in *Xenopus* and *Rana* has likely driven the transition of chytridiomycosis from an endemic disease in Africa to an epidemic disease worldwide (Fisher & Garner 2007).

It is crucial to study natural amphibian defense mechanisms in order to potentially prevent future losses of amphibian biodiversity due to chytridiomycosis. Some amphibian species persist despite major declines due to chytridiomycosis and other species do not decline at all (Woodhams, et al. 2007a). Amphibians' antimicrobial peptides (AMPs) produced by the innate immune system (Woodhams et al. 2007a, Ramsey et al. 2010), adaptive immune responses (Ramsey et al. 2010) and mutualistic cutaneous bacteria (Harris et al. 2009a, 2009b) are factors that can potentially explain how some amphibians eliminate or prevent infection by *B. dendrobatidis* while others do not. In terms of using these factors in hope of preventing declines, AMPs and immune responses pose challenges for successful conservation strategies. For instance, AMPs are species-specific and cannot be easily transmitted to susceptible species as a conservation strategy. The adaptive immune system of susceptible amphibians is likely compromised by a proteolytic enzyme produced by *B. dendrobatidis* that down-regulates immune response (Rosenblum et al. 2009). In addition, vaccines have proven to be ineffective for

susceptible species (Stice & Briggs 2010). However, the addition of mutualistic bacteria on amphibian skin provides a feasible control of chytridiomycosis as demonstrated in studies previously carried out in our laboratory (Harris et al. 2006, Harris et al. 2009a, 2009b, Becker & Harris 2010).

Host, mutualistic bacteria and pathogen:

Most studies that document the protective effects that indigenous microbiota provide to their host have been on the interaction of human gut microbiota with disease. For instance, the onset of atopic and asthmatic disorders is linked to aberrant immune development caused by the lack of mutualistic gut bacteria that produce immunomodulatory molecules (Mazmanian et al. 2005). Furthermore, treatment with the indigenous gut bacteria *Lactobacillus* has reduced the rate of atopic eczema in children thereby suggesting the importance of microbiota in the prevention of atopic disease (Kalliomaki et al. 2001). Finally, greater pathogen resistance in humans has been associated with certain gut microbial community composition (Dethlefsen et al. 2007). Although cause and effect remains uncertain, this finding suggests that certain species or species combinations provide more protective effects than others.

Several studies have also documented the protective effects of mutualistic microbiota in other metazoans. A vertically transmitted bacterial species *Regiella insecticola* present in a pea aphid species increases host survival rate after exposure to a fungal pathogen. The bacterial species lowers the rate of pathogen transmission by reducing the rate of successful sporulation by the fungus via an unknown mechanism (Scarborough et al. 2005) Also in a pea aphid species, it has been shown that it is not the aphid genotype, but rather the vertically and sometimes horizontally transmitted bacterial

species *Hamiltonella defensa* that confers resistance to parasitism by a parasitoid wasp (Oliver et al. 2005). According to the authors, nothing is known about the mechanism by which bacterial symbionts contribute to this resistance.

Studies on the interactions between mutualistic bacteria and pathogens have provided potential mechanisms by which the bacterial species protects the host from infection. The indigenous gut microbiota in hamsters strongly inhibits the fungal pathogen *Candida albicans* through blockage of adhesion sites (Kennedy & Volz 1985). Chan et al. (1985) proposed that the mechanism of competitive exclusion in this system is due to steric hindrance, i.e., obstruction due to the physical structure of the organism rather than specific blockage of receptor sites. In several fungus-farming ant species, a mutualistic bacterial species *Pseudonocardia* controls the fungal pathogen *Escovopis* through the production of antibiotics (Fernandez-Marin et al. 2009). In addition, bacterial species isolated from the intestinal and skin mucus of the fish species *Limanda limanda* likely inhibit a bacterial pathogen via the antibiotic properties of their metabolites (Olsson et al. 1992).

Amphibians possess indigenous bacterial species on their skin that inhibit pathogens (Harris et al. 2006, Lauer et al. 2007, Banning et al. 2008). Banning et al. 2008 proposed that embryos of *H. scutatum* in communal nests have increased survival rates due to inhibition of a fungal pathogen, *Mariannaea* spp., by mutualistic cutaneous microbiota. They found 17 resident bacterial species that directly inhibited the growth of *Mariannaea* sp. *in vitro*. On the salamander species *Plethodon cinereus*, Lauer et al. (2007) found 32 resident bacterial species that directly inhibited *Mariannaea* sp. growth *in vitro*. In addition, Harris et al. (2006) isolated seven genera of bacteria from *H.*

scutatum and three genera of bacteria from *P. cinereus* that had specific anti-*B. dendrobatidis* properties. Further studies demonstrated that the transfer of mutualistic bacteria through aquatic media in small laboratory containers to amphibian skin prevented symptoms of chytridiomycosis (Harris et al. 2009a, 2009b). The bacterial species *Pseudomonas reactans* (Harris et al. 2009a) and *Janthinobacterium lividum* (Harris et al. 2009b) produce secondary metabolites that have anti- *B. dendrobatidis* properties. *Pseudomonas spp*. produces the metabolite 2,4-diacetylphloroglucinol, among others, (Brucker et al. 2008a) and *J. lividum* produces the metabolites violacein and indole-3-carboxyaldehyde (Brucker et al. 2008b). These metabolites are one mechanism by which these bacterial species inhibit *B. dendrobatidis*.

Hypotheses:

Mutualistic bacteria in amphibians are positively associated with host fitness when pathogenic fungi are present. While mutualisms are important in ecology and evolutionary biology, their origin and maintenance are poorly understood (Hillesland and Stahl 2010). I had three questions: (1) does the amphibian host obtain mutualistic bacteria via environmental transmission, (2) does the transmission of an amphibian mutualistic bacterial species inhibit *B. dendrobatidis* infection and (3) is there evidence of interaction between *B. dendrobatidis* and the mutualistic bacteria? My a priori hypotheses are given in Table 1.

Table 1. Experimental a priori hypotheses.

Environmental Transmission
1: *J. lividum* can be introduced successfully into natural soil.
2: *J. lividum* can be transmitted environmentally to salamanders exposed to *J. lividum* in soil.
3. There is a positive correlation between *J. lividum* population densities on the salamanders and the *J. lividum* population densities in the soil.
Disease Mitigation
1: *B. dendrobatidis* prevalence will be lower on Bd-infected salamanders exposed to *J. lividum* in soil than Bd-infected salamanders not exposed to *J. lividum* in soil.
2: Bd-infected salamanders exposed to *J. lividum* in soil will experience lower morbidity and mortality than infected salamanders not exposed to *J. lividum* in soil.
Interaction between symbionts
1: Bd-infected salamanders exposed to *J. lividum* in soil will have population densities and prevalence of *J. lividum* that is different than non-infected salamanders exposed to *J. lividum* in soil.

Materials & Methods

Study species:

The Eastern red-backed salamander, *Plethodon cinereus*, was used as the experimental species. *P. cinereus* is a small, terrestrial salamander in the family Plethodontidae, which is composed of lungless salamanders. In order to respire through their skins these salamanders require continual inhabitance of moist environments. *P. cinereus* is commonly found in leaf litter or small burrows as well as under rocks or logs in deciduous forests. This species was chosen for study because they are common and have large local populations, previous work has been done on their skin microbes, and in their habitats, they are commonly in contact with soil that typically contains 10^6 to 10^9 bacterial cells/g. This continual interaction of moist, nutrient rich salamander skin with a bacterially rich environment likely provides transmission of microbes to salamander skin. In fact, a diverse community of bacteria has been documented to inhabit the skin of *P. cinereus* (Lauer et al. 2007, Culp et al. 2007). Some of these bacterial species are also present in the salamanders' environment (Austin 2000, Culp et al. 2007). In addition, mutualistic bacterial species isolated from the skin of *P. cinereus* have been shown to inhibit the amphibian fungal pathogens *Mariannaea* sp. (Lauer et al. 2007) and *B. dendrobatidis* (Harris et al. 2006).

The geographic range of *P. cinereus* spans most of the northeastern United States, southern Quebec, and the Maritime Provinces of Canada. While this species is abundant within its range, widespread declines have recently been reported (Highton 2003). Forest fragmentation due to logging practices is likely one cause of these declines (Kolozsvary and Swihart, 1999). While there have been no confirmed cases of declines due to

chytridiomycosis, this pathogen may still play a role in these observed declines in some parts of the Eastern red-backed salamander's range. In a recent survey in the Great Smoky Mountains National Park no declines in the salamander species were observed and all tested individuals were negative for *B. dendrobatidis* (Caruso 2011). However, in other parts of its range (Virginia and Connecticut), individuals have tested positive for infection by *B. dendrobatidis* (Harris et al. 2009b, Richards 2010). However, infection load and prevalence in these populations were low (Harris et al. 2009b, Richards 2010) and data on the status of these populations does not exist. Experimental trials have shown susceptibility of *P. cinereus* to *B. dendrobatidis* infection (Becker et al. 2009, Harris et al. 2009b, Becker & Harris 2010) and mortality due to infection has been observed (Becker et al. 2009). At low infection levels this species can clear infection by unknown mechanisms (personal observations). One potential mechanism may be by the antagonistic effect of cutaneous mutualistic bacteria against fungal pathogens (Harris et al. 2009a, 2009b).

The bacterial species *Janthinobacterium lividum* was used as the experimental mutualistic bacterium. *J. lividum* is a Gram-negative, motile, aerobic bacterium found in a variety of environmental conditions including soil (Pantanella et al. 2006) and the skin of *P. cinereus* (Lauer et al. 2007). During stationary and early death phase of growth the bacterium produces the secondary metabolite violacein that is violet in color (Pantanella et al. 2006). The production of violacein by *J. lividum* has been shown to positively influence its survival (Pantanella et al. 2006). In addition, violacein and another secondary metabolite (indole-3-carboxyaldehyde) produced by *J. lividum* can directly inhibit *B. dendrobatidis* growth (Brucker et al. 2008b).

Salamander collection and housing:

Forty-four adult red-backed salamanders were collected on Flag Pole Knob (elevation 1329m) in the George Washington National Forest in Rockingham County, Virginia in June 2010. Salamanders were housed in individual 17 x 12 x 7 cm (L x W x H) sterile plastic containers with soil collected from the site of collection until the start of the experiment. Individuals were held in incubators at a temperature of 17°C and with a 12-hour light, 12-hour dark cycle. On a weekly basis, salamanders were fed 10-15 fruit flies. Five days before the beginning of the experiment all salamanders were bathed in 25 ml of 1.5% hydrogen peroxide for 30 seconds and then rinsed in sterile artificial pond water (Wyngaard and Chinnappa 1982). The hydrogen peroxide bath was performed to reduce bacterial numbers on the salamanders' skin (Becker & Harris 2010), and to minimize variation in the microbial community on the salamanders, thereby allowing me to manipulate the presence or absence of *J. lividum* without confounding factors.

Bacterial isolation and rifampin-resistant selection:

J. lividum was isolated from the skin of the four-toed salamander *H. scutatum* in a previous study. The isolate was maintained in glycerol stock at -80°C. In order to quantify *J. lividum* present in the soil throughout the experiment, colony forming units (CFU) were counted from plated samples. Since *J. lividum* has been documented to naturally occur in soil (Pantanella et al. 2007), an antibiotic resistant strain was used to detect *J. lividum* that I added to the soil. Furthermore, many microorganisms live in soil, and by using an antibiotic resistant strain only the microorganism of interest grew on the selective media. An antibiotic resistant *J. lividum* strain was selected for by using a rifampin gradient on 1% tryptone plates. Selection for colonies that were resistant to the

highest concentration of rifampin was done for four weeks. After a resistant culture was

obtained, cultures of *J. lividum* were maintained at room temperature on 1% tryptone

containing 100 ug/l of rifampin. New cultures were made weekly.

Soil collection:

Soil was collected from three locations prior to initiation of the experiment (see

Appendix). Soil was collected from Flag Pole Knob in June 2010, from the James

Madison University (JMU) Arboretum, Harrisonburg, VA in August 2010 and from

Hone Quarry in George Washington National Forest in August and September 2010. Due

to several factors (see Discussion), *J. lividum* survival in these soils was low and did not

persist. Therefore, none of these soils were used in my experiment.

Survival and persistence of *J. lividum* was finally observed in soil collected from

the JMU Arboretum in October 2010 (see Appendix). Therefore, this soil was used to

initiate my experiment in November 2010. Soil was sieved (mesh size < 2mm, Newark

Wire Cloth Company, Clifton, NJ) to remove debris such as rocks and twigs, which led to

a uniform soil environment. Soil was stored at 17°C and soil moisture content was

maintained around 50% throughout the course of the experiment. Sterile artificial pond

water was added as necessary to maintain consistent moisture level.

Experimental design:

Each individual salamander was assigned a number and placed in treatments

using random numbers generated from www.random.org. My a priori hypotheses (Table

1) were tested using four treatments (Table 2). The salamanders in the first treatment

(n=16) were exposed to *J. lividum* in soil and also exposed to *B. dendrobatidis*

(J.liv+Bd+). The salamanders in the second treatment (n=6) were exposed to *J. lividum* in soil without being exposed to *B. dendrobatidis* (J.liv+Bd-). This treatment controlled for any possible effects that the presence of *J. lividum* in soil might have had on the salamanders. The salamanders in the third treatment (n=15) were not exposed to *J. lividum* in soil, but were exposed to *B. dendrobatidis* (J.liv-Bd+). This treatment was compared to the first treatment to determine if the transmission of *J. lividum* from soil to salamander had any effect on inhibiting the transmission and the effects of *B. dendrobatidis*. Since this comparison was of primary interest, treatments one and three had the highest sample size. The salamanders in the fourth treatment (n=6) were not exposed to *J. lividum* in soil and were not exposed to *B. dendrobatidis* (J.liv-Bd). This treatment controlled for any possible effects that the experimental procedures such as housing and handling might have had on the salamanders.

Table 2. Experimental design. J.liv+ treatment salamanders were housed throughout the experiment in soil initially inoculated with 2.9×10^7 CFUs of *J*. lividum/dry g of soil. Individuals in Bd+ treatments were exposed to five ml of 1×10^6 *B. dendrobatidis* zoospores/ml for five hours at day 8. J.liv- and Bd- treatments received sham soil inoculations and sham infections, respectively.

		Fungal Pathogen Status	
		Exposed to *Batrachochytrium dendrobatidis* (Bd+)	Not Exposed to *Batrachochytrium dendrobatidis* (Bd-)
Soil Bacterial Status	Inoculated with *Janthinobacterium lividum* (J.liv+)	J.liv+ Bd+ n=16	J.liv+ Bd- n=6
	Not inoculated with *Janthinobacterium lividum* (J.liv-)	J.liv- Bd+ n = 15	J.liv- Bd- n=6

Soil inoculation:

Bacterial suspensions of *J. lividum* used for soil inoculation were cultured in 20 ml of 1% tryptone broth at room temperature. After 24 hours of growth, the broth culture was added to 250 ml of 1% tryptone broth containing sterile three-millimeter diameter microbeads (Kimble Glass Inc., Vineland, NJ). This *J. lividum* suspension was grown at 25°C on a rotary shaker at 150rpm. After 24 hours of growth, the suspension was washed twice by centrifugation (7500rpm, 10 minutes) and re-suspended in sterile artificial pond water.

On day 0 of the experiment, 150 grams of soil were added to each terrarium and inoculated with a *J. lividum* suspension or artificial pond water. The concentration of the *J. lividum* suspension used for inoculation was determined by plating 10-fold serial dilutions and was found to be 1.4×10^9 CFUs/ml. To inoculate the soil, 1.5 ml of the *J. lividum* suspension was added to each terrarium by pipette. One gram of soil from an independent sample was dried (90°C for 24 hours) to determine the dry weight of the soil at inoculation. The concentration in the soil at inoculation was determined to be 2.9×10^7 cells/g of dry soil. Soil in the *J. lividum* negative treatments (J.liv-Bd+ and J.liv-Bd-) was inoculated with a 1.5 ml of sterile artificial pond water. Each terrarium was shaken uniformly for five seconds after introduction of the *J. lividum* suspension or the sham inoculum. The salamanders were introduced into their appropriate treatment terrarium 24 hours after introduction of the inoculums onto the soil, designated day 1.

Janthinobacterium lividum detection in soil:

Soils were sampled for *J. lividum* by plating serial dilutions of soil suspensions. The *J. lividum* negative treatment terrariums were sampled first during sampling days to prevent contamination. To detect *J. lividum* in soil, one gram of soil was removed from well-mixed soil and suspended in nine ml 0.1% sodium pyrophosphate in 15-ml Falcon tubes (Becton Dickinson, Franklin Lakes, New Jersey). The soil suspensions were shaken by hand vigorously for 15 seconds and placed on a rotary shaker at 200rpm at 25°C for 40 minutes. Serial 10-fold dilutions in 1xPBS (phosphate-buffered saline, pH 7.4) were plated onto 1% tryptone plates containing 100 ug/l cycloheximide, to inhibit fungal growth, and 100 ug/l rifampin, for selection of rifampin-resistant *J. lividum*. Plates were incubated at 26°C for 48 hours.

After incubation, colony forming units (CFU) were counted on plates. The countable range for CFUs was 25-250 colonies per plate. During some sampling events, plates containing above 250 CFU were recorded as too numerous to count (TNTC) and plates containing below 25 CFU were recorded as too few to count (TFTC). The lowest CFU count from the lowest dilution (10^{-1} plate) made the countable detection limit 2.5×10^2 *J. lividum* CFU/g of soil.

Soil *J. lividum* densities were expressed as CFUs /g of dry soil. To obtain dry soil weights, one gram of fresh soil was weighed, dried at 90°C for 24 hours and then weighed again. A proportional relationship was used to calculate the dry weight of the one gram of soil used in the soil suspension.

The *J. lividum* positive treatment terrariums were sampled on days 2, 8, 19, 30, and 41 of the experiment. During the sampling events in which TNTC and TFTC data were present, these data was omitted in order to calculate the average densities of *J. lividum* at each time point. TNTC data were present at days 2 and 8 indicating that for those dates the average densities were higher than calculated. TFTC data were present at days 30 and 41 indicating that for those dates the average densities were lower than calculated. The *J. lividum* negative terrariums were treated the same at these time points, i.e., two grams of soil were removed from well-mixed soil, but the determination of *J. lividum* presence was only conducted on days 2 and 41. An assumption was made that if these soils were negative at the beginning and at the end of the experiment then they were negative throughout the experiment.

***Batrachochytrium dendrobatidis* exposure:**

On day 8 of the experiment, J.liv+Bd+ and J.liv-Bd+ treatment salamanders were exposed to *B. dendrobatidis*. The *B. dendrobatidis* isolate, JEL 423, was obtained from Joyce E. Longcore (University of Maine). JEL 423 was isolated from El Copé, Panama on a frog in the species *Phyllomedusa lemur* on December 17, 2004. Cultures were maintained in 1% tryptone broth at 23°C and transferred weekly until the salamanders were exposed. Zoospores for exposure were prepared by growing a one week old broth culture on 1% tryptone plates. After six days of growth, plates were flooded with six ml of sterile artificial pond water. After sitting for 20 minutes, the water was removed by pipette and transferred to a 15-ml Falcon tube. The concentration of the zoospore solution was determined by duplicate counts using a hemacytometer and then calculating the average density per ml. Salamanders in treatments J.liv+Bd+ and J.liv-Bd+ were exposed to approximately 1×10^6 zoospores in five ml of solution for five hours in Falcon tubes at room temperature. J.liv+Bd- and J.liv-Bd- individuals were treated identically, but placed in five ml of a sham (sterile artificial pond water).

The initial duration of exposure to *B. dendrobatidis* was set to be eight hours; however, a temperature malfunction in the laboratory cooling system caused the room temperature to reach 30°C. One salamander experienced mortality and four salamanders displayed sub-lethal lethargy due to the temperature malfunction, and exposure was ended at five hours to avoid additional stress. At six days post-infection (day 14 of the experiment) the four salamanders that experienced sub-lethal trauma were euthanized with two g of tricaine methane sulfonate per L of sterile deionized water because they did not show signs of recovery. The data collected for these salamanders were not used.

Swabbing to sample for *Janthinobacterium lividum* and *Batrachochytrium*

***dendrobatidis*:**

Salamanders were swabbed at initial collection and thereafter on days 3, 8, 13, 20, 29 and 42. Before swabbing occurred each salamander was rinsed in sterile artificial pond water to remove transient bacteria from the skin (Lauer et al. 2007) and soil debris. During rinsing, the weight in grams of each salamander was taken. Then, on day 3, each individual was swabbed with MW100 Fine-tip swabs (Medical Wire & Equipment, Corsham, Wiltshire, England) on its ventral surface 10 times, the dorsal surface 10 times and two limbs 10 times. On day 8 immediately before infection with *B. dendrobatidis*, only two limbs were swabbed 10 times each to avoid reducing the *J. lividum* on the trunk that may occur during swabbing.

After exposure to *B. dendrobatidis* (days 13, 20, 29 and 42) one side of the individual was swabbed for *J. lividum* detection and the other side of the individual was swabbed for *B. dendrobatidis* detection following the procedure used on day 3. It was later determined that the DNA extraction protocol from DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD) could extract *J. lividum* and *B. dendrobatidis* DNA from the same swab, and that enough DNA was extracted to perform separate PCR reactions; only one swab was actually necessary. Swabs were frozen immediately after swabbing at -20°C until further processing.

***Janthinobacterium lividum* detection on salamanders:**

DNA was extracted from swabs using DNeasy Blood & Tissue Kit following the manufacture's protocol for Gram-positive DNA extraction. *J. lividum* is a Gram-negative bacterium, but the Gram-positive bacteria extraction protocol also extracts Gram-negative

bacteria DNA. By using this extraction protocol, the extracted DNA can then also be analyzed in the future for microbial community composition.

Salamander swabs taken before the application of *J. lividum* were analyzed to determine presence of naturally occurring *J. lividum* by performing diagnostic PCR on the DNA extracted from the swabs. The *J. lividum* specific primers and the PCR protocol as described in Harris et al. (2009a) were used. All salamanders were determined to lack *J. lividum* at the beginning of the experiment. The soil pH at the site of collection was 5. Growth of *J. lividum* does not occur below pH 5 and this may be an explanation as to why *J. lividum* was not present on these salamanders.

The number of environmentally transmitted *J. lividum* cells present on the salamanders' skin during the experiment was estimated by performing quantitative real-time PCR (qRT-PCR). Swabs from day 3 could not be analyzed by qRT-PCR due to an error in extracting DNA. For J.liv+Bd+ and J.liv+Bd- treatments, DNA was extracted from swabs on days 8, 13, 20, 29 and 42 using DNeasy Blood & Tissue Kit following the manufacture's protocol for Gram-positive bacteria DNA extraction. For J.liv-Bd+ and J.liv-Bd- treatments, DNA was extracted from swabs on days 8, 20 and 29. Population densities were calculated by dividing the number of *J. lividum* cells by the area swabbed (see below). DNA was also extracted from a positive control swab and a negative control swab at the same time as DNA extraction from the sample swabs. The positive control consisted of a swab dipped in a liquid culture of *J. lividum*. The negative control consisted of a swab dipped in sterile artificial pond water.

Amplification of each sample for *J. lividum* quantification was completed using a Roche LightCycler (Applied BioSystems, Foster City, CA). The *J. lividum* specific

primers as described in Harris et al. 2009a were used. qPCR reactions (10µl) contained 2.5µl of DNA template, 0.5µM of each primer, 2µl of LightCycler Fast Start DNA Master^{Plus} SybrGreen Master Mix (Applied Biosystems, Foster City, CA) and PCR grade H$_2$O. The LightCycler protocol included pre-incubation for 10 minutes at 95°C, followed by 50 cycles of 10 seconds at 95°C for denaturation, 5 seconds at 60°C for annealing, and 16 seconds at 72°C for extension and then a final cycle for the melting curve of 1 second at 95°C for denaturation, 30 seconds at 60°C for annealing and 0.1°C/second increase to 95°C for melting.

Quantification of samples was performed using a standard curve and the determined concentration was expressed as the number of *J. lividum* cell equivalents. A standard curve was constructed using known concentrations of 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 *J. lividum* cells. Samples were amplified in duplicate. When the difference between the cycle threshold values for the duplicate samples were greater than one, the duplicates were considered to be inconsistent, and a third sample was run. The cycle threshold value is the cycle at which a detectable increase in fluorescence (indicating amplification of DNA) is observed. The third sample allowed a better estimation of the numbers of *J. lividum* present by indicating which of the first two samples was an outlier. Positive samples were confirmed by melting curve analysis. A positive control and a negative control were run with each qRT-PCR reaction. The positive control consisted of a *J. lividum* standard. The negative control was PCR grade water.

All salamanders were measured at the end of the experiment to determine the area that was swabbed and their total length. For the area swabbed, trunk area and limb area were calculated. To calculate trunk area, the length from axilla to groin and the width at

the widest spot on the trunk were measured. To calculate limb area, the length from the elbow joint to the tip of the foot and the width at the ankle were measured for one posterior limb and one anterior limb. The area swabbed for each set of measurements was determined by multiplying length times width. The total area swabbed for day 8 was determined by adding the two limb area measurements together. The total area swabbed for days 13, 20, 29 and 42 was determined by dividing the total trunk area by two (only one side of the individual was swabbed) and adding that value to the total for the two limb area measurements. The quantitative measure of *J. lividum* abundance as determined by qRT-PCR was divided by the area swabbed to calculate the number of *J. lividum* cell equivalents per mm^2 for each salamander at each swabbing event. As the true area swabbed was probably less than the estimated area, the number of *J. lividum* cell equivalents per mm^2 was a conservative estimate. The total length was measured from the tip of the snout to the tip of the tail. This was performed to generate an index of body condition for each salamander, which is defined as mass for a given length.

***Batrachochytrium dendrobatidis* detection:**

In order to determine the presence of *B. dendrobatidis* on the salamanders' skin during the experiment, diagnostic PCR was performed. The *B. dendrobatidis* specific primers and the protocol described in Annis et al. (2004) were used to perform PCR. The DNA extracted from the swabs used for *J. lividum* quantification was also used to determine *B. dendrobatidis* prevalence on day 13 and 20 of the experiment, which were 5 days and 12 days post-infection, respectively. The extraction protocol for Gram-positive bacteria used for *J. lividum* DNA extraction was tested using known concentrations (10^3, 10^2, 10, 1) of *B. dendrobatidis*. I determined that the detection level for *B. dendrobatidis*

using this DNA extraction protocol was 10 zoospores. This detection level is sensitive and is similar to that determined for two other protocols listed in the DNeasy Blood & Tissue Handbook. During each PCR run, a positive control and a negative control were run. The positive control consisted of DNA extracted from a swab dipped in a liquid culture of *B. dendrobatidis*. The negative control consisted of PCR grade water. By 12 days post-infection, only one salamander tested positive for *B. dendrobatidis*; therefore, no further testing for *B. dendrobatidis* prevalence was performed.

Statistical analysis:

All statistical analysis was performed using SAS statistical software (SAS Institute Inc., Cary, North Carolina). To determine if there was a correlation between the density of *J. lividum* on the salamanders and the density of *J. lividum* in the soil, I analyzed data for three time points. On day 8, soils and salamanders were sampled on the same day. Soils sampled on day 19 were compared with salamanders sampled the next day. Salamanders sampled on day 29 were compared with soils sampled the next day. After day 8, it was determined that sampling soil and salamanders on one day was not practical. Day 8 soil data contained a number of soil samples with TNTC data. Day 19 soil data contained all numerical values. Day 30 soil data contained a number of soil samples with TFTC data.

Before performing the correlation analysis, I first determined whether I could pool the J.liv+Bd+ and J.liv+Bd- treatments to increase sample size at each time point. To determine if there was a difference between these treatments, I used a nonparametric Wilcoxon two-sample test for soil and salamander data separately at each time point. Soil data on days 8 and 30 that were TNTC and TFTC, respectively, were omitted to test for a

difference between treatments. The results indicated that there was no evidence of a difference in *J. lividum* density and the data were pooled from the two treatments for soil and salamander data separately to create a J.liv+ treatment at each time point.

The pooled data were analyzed with a nonparametric Spearman rank correlation test to determine if a correlation existed. Due to TNTC or TFTC soil data on days 8 and 30, respectively, the data on these days were categorized. On day 8, three ordinal categories were formed, each representing roughly one-third of the data: the one-third highest values (TNTC), the one-third middle values, and the one-third lowest values. On day 30, two ordinal categories were formed: TFTC and all counted points. Salamander data for days 8 and 29 were also categorized into three categories and two categories, respectively, so that both soil data and salamander data could be analyzed together by categorized ranks. For the salamander categorization, an even distribution of densities in each category was achieved by identifying clear separations in the data. The exact Spearman rank correlation test is appropriate for the analysis of ordinal categorical data (R. Domangue, personal communication). Soil data on day 19 contained all quantitative data. Thus, soil and salamander data for day 19/20 did not have to be categorized.

To determine if *B. dendrobatidis* prevalence on infected salamanders was related to presence or absence of *J. lividum* in soil (J.liv+Bd+ and J.liv-Bd+), I performed a Fisher's exact test. If a salamander exposed to *B. dendrobatidis* (J.liv+Bd+ and J.liv-Bd+) did not get infected, as determined by PCR analysis, then it was considered negative for *B. dendrobatidis*. Only data for day 13 were analyzed because by day 20 only one salamander of the remaining 27 that were initially infected tested positive for *B.*

dendrobatidis. No mortality (other than temperature malfunction mortality) in any treatment was observed during the experiment and was not statistically analyzed.

An analysis of variance was performed to compare the proportion of body mass lost from day 1 to day 42 of the experiment. Some salamanders lost their tail before or during the experiment due to handling; these individuals were removed from the analysis (n=5). Means presented in the results are shown as ± one standard deviation. Proportion of body mass lost was compared between J.liv+Bd- and J.liv-Bd- to determine if adding *J. lividum* to soil affected body mass of uninfected individuals. Proportion of body mass lost was compared between J.liv-Bd+ and J.liv-Bd- to determine if exposure to *B. dendrobatidis* affected the body mass of individuals. Proportion of body mass lost was compared between J.liv+Bd+ and J.liv-Bd+ to determine if the presence or absence of *J. lividum* in soil affected the body mass of individuals infected with *B. dendrobatidis*.

Body condition is an indicator of the general health of an individual (Schulte-Hostedde et al. 2005) and can be thought of as the fatness (good condition) or thinness (poor condition) of the individual relative to total length. The body condition of each salamander was examined by regressing its body mass at days 1 and 42 on its total length. Individuals that lost their tail before or during the experiment were removed from the analysis (n=5). The residuals from the regression were used as an index of body condition for the initial body condition and the day 42 body condition. In addition, an analysis of covariance (ANCOVA) was performed with body condition at day 42 as the response and the initial body condition as the covariate. Treatments were compared in the generated ANCOVA model using the same comparisons as the general linear model above.

To determine if there was an effect of *B. dendrobatidis* treatment on the density of *J. lividum* on the salamanders, I compared the J.liv+Bd+ and J.liv+Bd- treatments. In the first analysis, the *J. lividum* cell densities in these treatments at each sampling time point were compared using a Wilcoxon two-sample test. This test was used because the assumption of normality for a parametric test was not met. Secondly, *J. lividum* prevalence (presence or absence) at each sampling time point was compared between the treatments using a Fisher's exact test.

Interestingly, not all individuals in the J.liv+Bd+ tested positive for infection of *B. dendrobatidis* five days post infection, i.e., day 13 of the experiment. This led to the statistical question of whether J.liv+Bd+ individuals that tested negative should be put in the J.liv+Bd- category since it is likely that they were not infected. Consequently, salamanders in this treatment were categorized as being infected on day 13 (J.liv+Bd+(+)) or not being infected on day 13 (J.liv+Bd+(-)). A set of analyzes was performed to determine if there was an effect on *J. lividum* density on the salamanders based on these new treatments. First, the *J. lividum* cell densities in these two categories within the J.liv+Bd+ treatment were compared using a Wilcoxon two-sample test. This test was used because the assumption of normality for a parametric test was not met. Second, *J. lividum* prevalence was compared between the categories within the J.liv+Bd+ treatment using a Fisher's exact test.

Results

Janthinobacterium lividum survival in soil:

In my experiment, all soils inoculated with *J. lividum* (J.liv+Bd+ and J.liv+Bd-) were positive for *J. lividum* at all sampling time points. The hypothesis that *J. lividum* can be successfully introduced into natural soil is supported. Following the establishment of *J. lividum* in the soil, the densities of *J. lividum* declined over time (Figure 2). For this analysis, J.liv+Bd+ and J.liv+Bd- treatments were pooled together to increase sample size. Pooling was justified because there was no difference in the number of *J. lividum* cells/dry g of soil between J.liv+Bd+ and J.liv+Bd- treatments (Wilcoxon two sample test, day 2: $W=41$ $n=5,12$ $p=0.72$; day 8: $W=88.5$ $n=3,12$ $p=0.30$; day 19: $W=54$ $n=6,15$ $p=0.35$; day 30: $W=18.5$ $n=3,5$ $p=0.23$; day 41: $W=10$ $n=3,7$ $p=0.18$). All soils inoculated with a sham (J.liv-Bd+ and J.liv-Bd-) were negative for *J. lividum* throughout the experiment. Two pilot studies conducted in April and in June of 2010 using soil collected in the James Madison University (JMU) Arboretum showed similar results in which *J. lividum* could colonize soil for at least 30 days and that the average cell density decreased over time (data not shown).
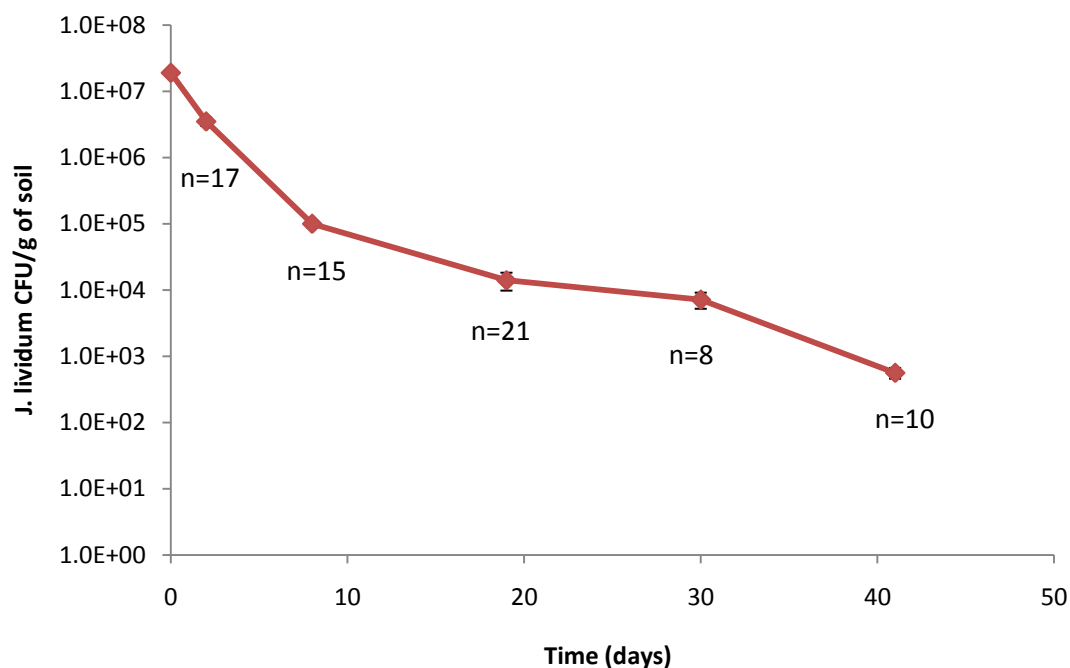
Figure 2. Average *J. lividum* soil survival over time for the pooled J. liv+Bd+ and J.liv+Bd- treatments. Soil was inoculated with 2.9×10^7 cells/dry g of soil at day 0. TNTC data points were omitted at days 2 and 8 and TFTC data were omitted at days 30 and 41. Error bars represent one standard error.

Prior to the initiation of the experiment, soil was collected from three locations in Virginia from June to September 2010 and experimentally inoculated (see Appendix). *J. lividum* survival in these soils was low. I attempted to determine what factors were affecting *J. lividum* soil survival in these soils (see Appendix). *J. lividum* soil survival remained negligible throughout these trials. The hypothesis that *J. lividum* can be successfully introduced into soil was not supported for soil collected in the summer of 2010 from these locations. In October 2010, I collected soil from the JMU Arboretum which was the soil I used for my main experiment. The fall season had begun and the local weather was cooler and moister than when the other soils had been collected. From

these results, I hypothesize that season may be an important factor in survival of bacteria introduced into soil.

Environmental transmission:

All salamanders exposed to *J. lividum* in soil were negative for *J. lividum* at the beginning of my experiment, but all salamanders became positive during the experiment. This result supports the hypothesis that environmental transmission of *J. lividum* from soil to salamander can occur. Similar results were seen in a pilot study I conducted in June 2010 in which all salamanders exposed to *J. lividum* in soil (n=7) became positive for *J. lividum* (data not shown). All salamanders not exposed to *J. lividum* in soil (J.liv-Bd+ and J.liv-Bd-) were negative for *J. lividum* throughout the experiment (day 8, 20 and 29).

J. lividum was detected on salamander skin up to the 29th day of the experiment (Figure 3 & 4). By day 42 of the experiment, *J. lividum* was no longer detected. The average number of *J. lividum* cell equivalents transmitted per mm^2 of salamander skin on days 8, 13, 20, 29 and 42 in treatments J.liv+Bd+, J.liv+Bd- is displayed in Figure 3. The average *J. lividum* density in soil had dropped from 6.3×10^3 CFU/g of soil on day 29 to below 5.6×10^2 CFU/g of soil at day 42 (Figure 2). Densities below a certain threshold may potentially impede environmental transmission.

The median number of salamander *J. lividum* cell equivalents/mm^2 in both J.liv+ treatments were fairly constant on days 8, 13 and 20, increased at day 29, then declined to zero by day 42 (Figure 3). Interestingly, on day 29 all salamanders tested positive for *J. lividum* (Figure 4) with the average density being higher than at any other sampling time point (Figure 3). On day 29, the soil *J. lividum* density was continuing to decline (Figure

2), which suggests that the densities of *J. lividum* on the salamander are independent of densities of *J. lividum* in the soil. Although salamanders in the J.liv+Bd+ treatment tended to have higher densities of *J. lividum* than those in the J.liv+Bd- treatment, especially on day 13, these differences were not statistically significant (Figure 3) at any sampling time point (Wilcoxon two-sample test; day 8: W=50 n=16,5 p=0.24; day 13: W=45 n=16,5 p=0.11; day 20: W=64 n= 15,6 p=0.90; day 29: W=64 n=15,6 p=0.90).
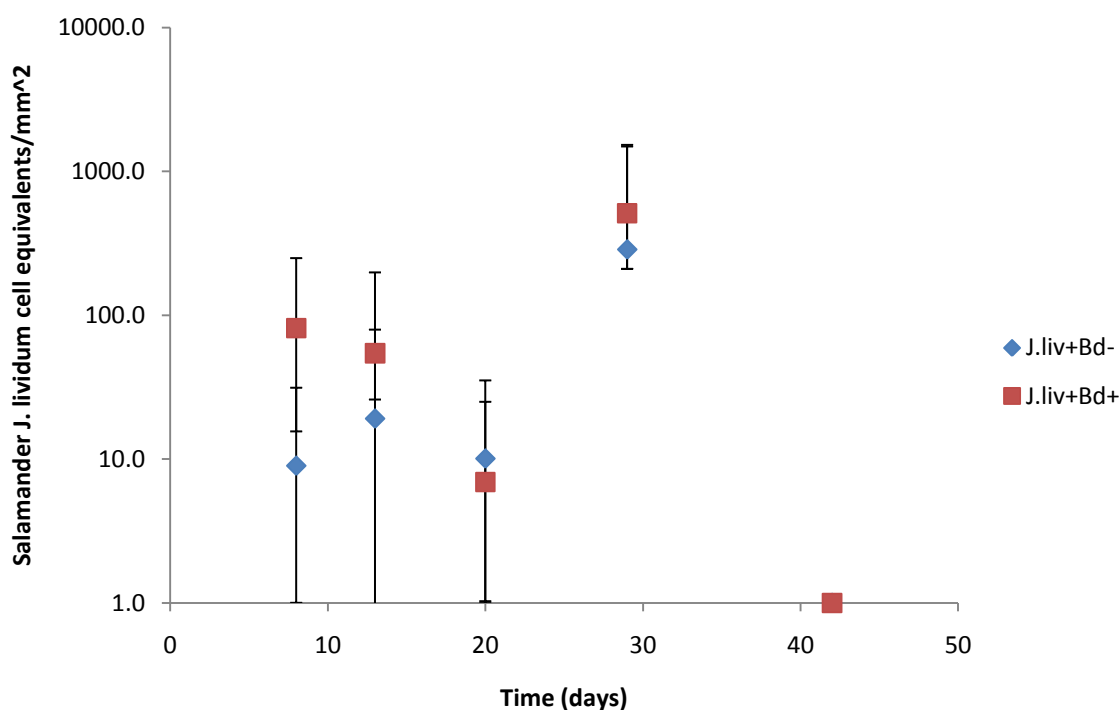
Figure 3. Transmission of *J. lividum* from soil to salamander over time for J. liv+Bd+ and J.liv+Bd-. Variation between treatments in salamander *J. lividum* cell equivalents/mm^2 is observed from day 8 to day 20. An increase amongst all salamanders was observed on day 29. By day 42 *J. lividum* was no longer detected on any salamander. No difference between treatments was observed (Wilcoxon two-sample test, p>0.05). Medians are shown with error bars representing the 25th and 75th percentiles.

Every salamander in the J.liv+ treatments tested positive at some point during the experiment, but not all salamanders tested positive for *J. lividum* at all sampling time points. The percentage of salamanders positive for *J. lividum* varied from 0-100% during

the experiment (Figure 4). There was no difference in percentage of salamanders positive

for environmental transmission between the J.liv+Bd+ and J.liv+Bd- treatments at any

sampling time point (Fisher's exact test, day 8 p=.35; day 13 p=0.07; day 20 p=1) (Figure

4). However, a strong trend on day 13 was observed in which a higher prevalence of *J.*

lividum was present on J.liv+Bd+ salamanders in comparison to J.liv+Bd- salamanders.

Both treatments followed the same cyclical trend of an increase, decrease and then

increase and decrease again in *J. lividum* prevalence. At the same time, all soils in these

treatments tested positive for *J. lividum* presence and a steady decline was observed in

the *J. lividum* soil densities (Figure 2). Therefore, the independence of *J. lividum* in soil

and *J. lividum* on salamanders is suggested.
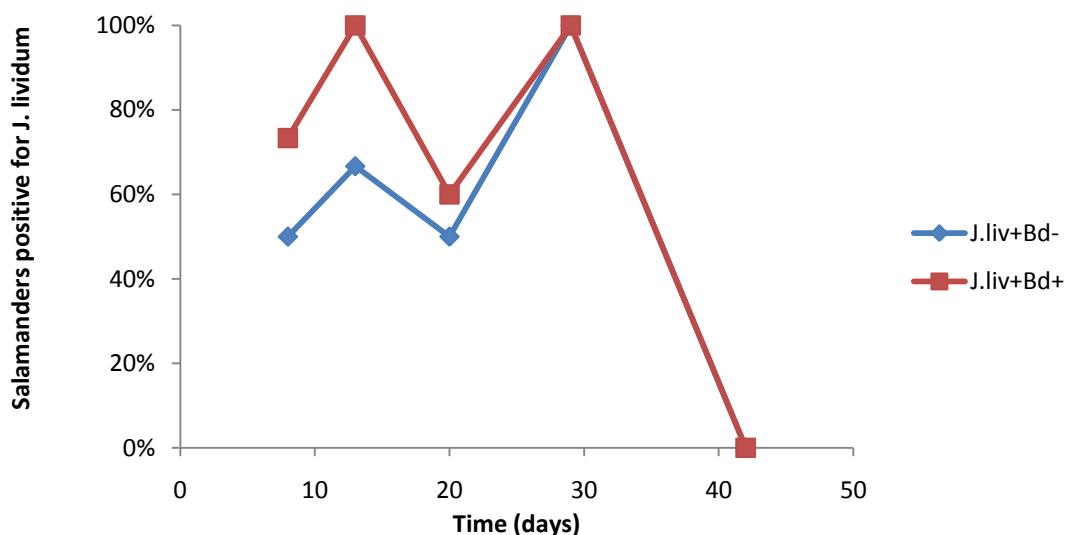
Figure 4. Percentage of salamanders positive for *J. lividum* over time for J. liv+Bd+ and J.liv+Bd-. No difference between treatments was observed (Fisher's exact test, p>0.05).

On days 8, 13 and 20, there was individual variation in the density of *J. lividum*

on salamanders exposed to *J. lividum* in soil. Interestingly, this variation on individual

salamanders could be categorized in two ways (Figures 5 & 6). The data presented in

Figures 5 and 6 is based on the trend the *J. lividum* density followed on each individual

salamander in the J.liv+ treatments. The first trend (Figure 5) was seen as negligible or

relatively low densities of *J. lividum* on the salamander (in comparison to day 13) on day

8 to higher densities on day 13 to lower densities on day 20. The second general trend

(Figure 6) was seen as relatively high densities of *J. lividum* on the salamander (in

comparison to day 13) on day 8 to lower densities on day 13 to relatively equal or still

lower densities on day 20. Two outliers in the J.liv+Bd+ treatment are not shown due to

extremely high densities of *J. lividum* cell equivalents/mm^2 ($9.2\text{x}10^2$ and $2.1\text{x}10^3$) on day

8; both followed the trend seen in Figure 6. Two outliers in the J.liv+Bd- treatment are

not shown and followed neither trend. One outlier had zero values present on days 8 and

13 and the other had zero values present at the three sampling time points.

Figure 5. One of two trends observed in the distribution of *J. lividum* cell equivalents/mm^2 over three sampling time points. The trend was seen as negligible or relatively low densities (in comparison to day 13) on day 8 to higher densities on day 13 to lower densities on day 20. Each line represents an individual salamander.

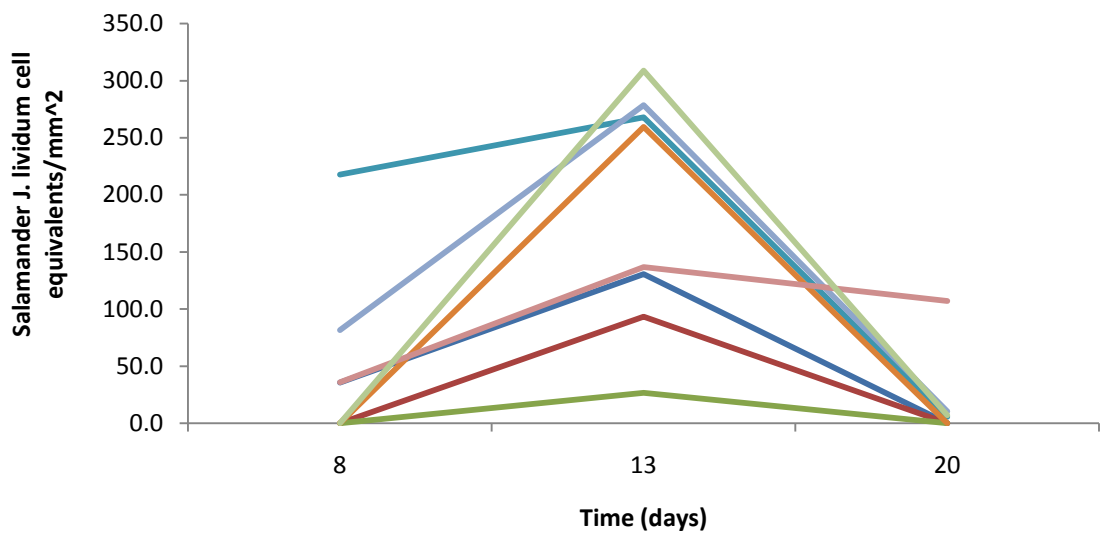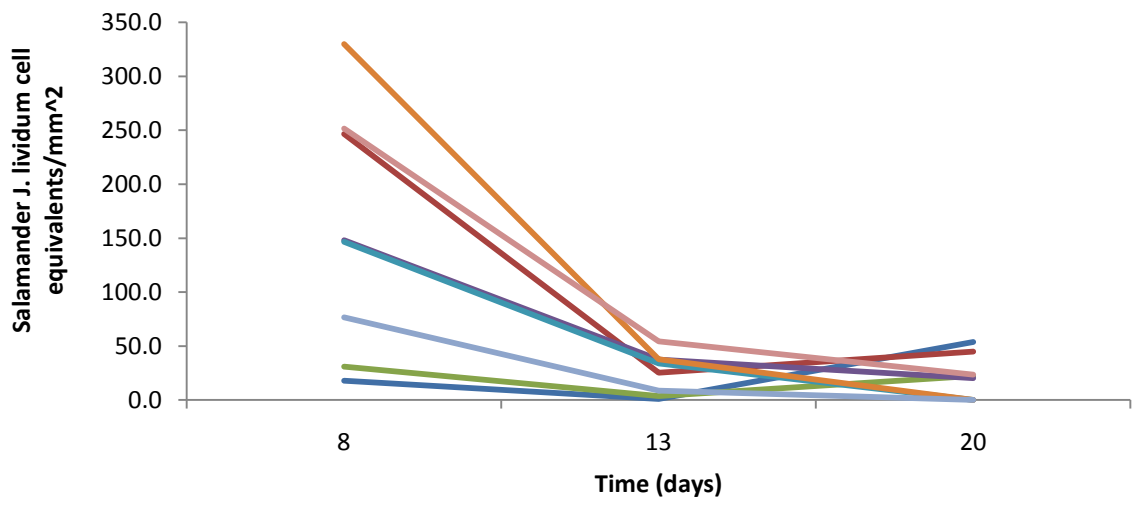Figure 6. The second of two trends observed in the distribution of *J. lividum* cell equivalents/mm^2 over three sampling time points. The trend was seen as relatively high densities (in comparison to day 13) on day 8 to lower densities on day 13 to relatively equal or still lower densities on day 20. Each line represents an individual salamander.

Correlation between *Janthinobacterium lividum* found in soil and on salamanders:

The densities of *J. lividum* in the soil and on the salamander were not correlated on three sampling dates (Spearman rank correlation, day 8: r=0.21 n=21 p=0.42; day 19/20: r=-0.06 n=21 p=0.78; day 29/20: r=0.07 n=21 p=1). Based on these results, the hypothesis that there is a positive relationship between *J. lividum* densities in soils to *J. lividum* densities on the skin of the salamanders is not supported. This conclusion is also evident by comparing Figures 2 with Figures 3 and 4. This result suggests that populations of *J. lividum* on the salamander have population dynamics that are independent of population patterns on *J. lividum* in the soil.

***Batrachochytrium dendrobatidis* prevalence:**

An a priori one-tailed hypothesis was generated before data collection that *B. dendrobatidis* prevalence in the J.liv+Bd+ treatment will be lower than *B. dendrobatidis* prevalence in the J.liv-Bd+ treatment. This is based on previous findings that mutualistic bacteria (Harris et al. 2006, Woodhams et al. 2007b, Harris et al. 2009b, Lam et al. 2010) and specifically *J. lividum* (Brucker et al. 2008b, Becker et al. 2009, Harris et al. 2009b, Becker & Harris 2010) inhibit *B. dendrobatidis*.

The inoculation of soil with *J. lividum* halved the prevalence of *B. dendrobatidis* on salamanders (Fisher's exact test, two-tailed p=0.047, one-tailed p=0.028) five days after the salamanders were exposed to *B. dendrobatidis* (day 13 of the experiment) (Table 3). In the J.liv+Bd+ treatment 40% of salamanders (n=15) were infected with *B. dendrobatidis*, whereas, 83% of salamanders (n=12) were infected with *B. dendrobatidis* in the J.liv-Bd+ treatment. This result supports the hypothesis that the exposure of salamanders to *J. lividum* in soil will decrease the prevalence of *B. dendrobatidis*. By 12

days post-infection (day 20), only one salamander in the J.liv+Bd+ treatment tested

positive for *B. dendrobatidis*. Based on this result no further testing for *B. dendrobatidis*

was performed.

Table 3. Disease outcome at five days post exposure to *B. dendobatidis*. Significantly fewer salamanders were infected with *B. dendrobatidis* in the treatment exposed to *J. lividum* in the soil (Fisher's exact test, two-tailed p=0.047, one-tailed p=0.028).

		Disease Outcome	
		Batrachochytrium dendrobatidis positive	*Batrachochytrium dendrobatidis* negative
Salamander Bacterial Status	Exposed to *Janthinobacterium lividum* in soil	6	9
	Not exposed to *Janthinobacterium lividum* in soil	10	2

Morbidity and mortality effects caused by *Batrachochytrium dendrobatidis* infection:

The hypothesis that infected salamanders would experience lower morbidity and

mortality in *J. lividum* augmented soil was rejected. Over the course of the experiment all

salamanders on average lost the same relatively small amount of weight from day 1 to

day 42 in all treatments (ANOVA, p>0.05; J.liv-Bd- mean=-0.09±0.06, n=5; J.liv-Bd+

mean=-0.05±0.04, n=10; J.liv+Bd- mean=-0.06±0.07, n=5; J.liv+Bd+ mean=-0.06±0.09,

n=13). The slight reduction in weight over time was likely due to a diet of only fruit flies. In addition, the body condition of all salamanders did not differ when the body condition at day 42 was adjusted for the body condition at day 1 (ANCOVA, p>0.05). Of the 27 salamanders infected with *B. dendrobatidis* all of them with the exception of one salamander had cleared the infection by day 20. All salamanders survived during the experiment.

Interaction between infection status and *Janthinobacterium lividum* on salamanders:

Densities of *J. lividum* tended to be higher on salamanders that were *B. dendrobatidis* negative. As indicated above, there was no difference in salamander *J. lividum* cell equivalents/mm^2 (Figure 3) or in salamander *J. lividum* prevalence (Figure 4) when the J.liv+Bd+ and J.liv+Bd- treatments were compared. However, not all individual salamanders in the J.liv+Bd+ treatment became infected with *B. dendrobatidis* as determined by PCR five days post infection (day 13). Therefore, I categorized the J.liv+Bd+ treatment as being infected with *B. dendrobatidis* (J.liv+Bd+(+)) or not being infected with *B. dendrobatidis* (J.liv+Bd+(-)) on day 13. No difference in salamander *J. lividum* cell equivalents/mm^2 was observed before infection on day 8 (Wilcoxon two-sample test, W=59 n=6,9 p=0.93) (Figure 7). A trend on day 13 was observed in which a higher density of *J. lividum* cell equivalents/mm^2 were present on J.liv+Bd+(+) salamanders (Wilcoxon two-sample test, W=78 n=6,9 p=0.11) (Figure 7). A significantly higher density of *J. lividum* cell equivalents/mm^2 on J.liv+Bd+(+) salamanders was observed on day 20 (Wilcoxon two-sample test, W=69 n=6,9 p=0.026) and on day 29 (Wilcoxon two-sample test, W=67 n=6,9 p=0.047) (Figure 7).
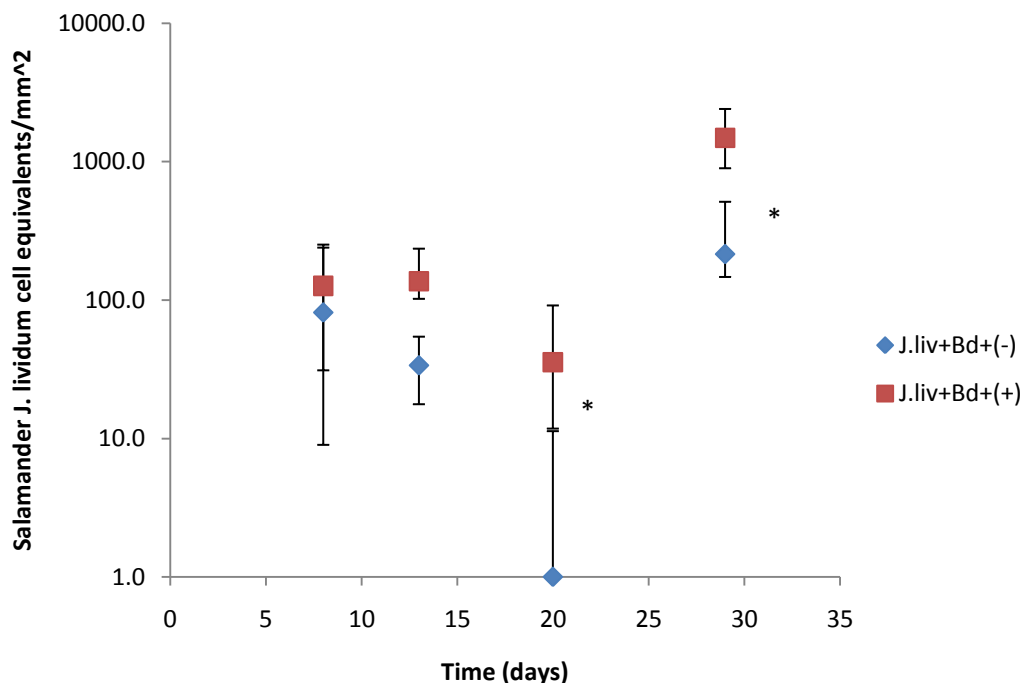
Figure 7. Transmission of *J. lividum* from soil to salamander over time based on the categories of being infected (J.liv+Bd+(+)) or not being infected with *B. dendrobatidis* (J.liv+Bd+(-)) on day 13 within the J. liv+Bd+ treatment. A difference between treatments as indicated by an asterisk(*) was observed on days 20 and 29 (Wilcoxon two-sample test, p<0.05). Medians are shown with error bars representing the 25th and 75th percentiles.

B. dendrobatidis presence was associated with a higher proportion of salamanders having *J. lividum* on day 20 (Figure 8). The categories (J.liv+Bd+(+)) and J.liv+Bd+(-)) within the J.liv+Bd+ treatment were analyzed at each time point by salamander *J. lividum* prevalence. No difference in salamander *J. lividum* prevalence (Figure 8) was observed on days 8, 13 and 29 (Fisher's exact test, days 8, 13 and 29 p=1). A significantly higher number of salamanders tested positive for *J. lividum* in the J.liv+Bd+(+) category on day 20 (Fisher's exact test, p=0.03) (Figure 8). In sum, the *B. dendrobatidis* infection status of the salamander positively affects the salamander *J. lividum* density on days 20 and 29 (Figure 7) and the salamander *J. lividum* prevalence on day 20 (Figure 8). These results

support the hypothesis that infection status causes a difference in *J. lividum* density and

prevalence. In addition, while it is controversial to pool J.liv+Bd+(-) with J.liv+Bd-, if

pooling is done, I obtain the same results when comparing this pooled treatment to

J.liv+Bd+(+).

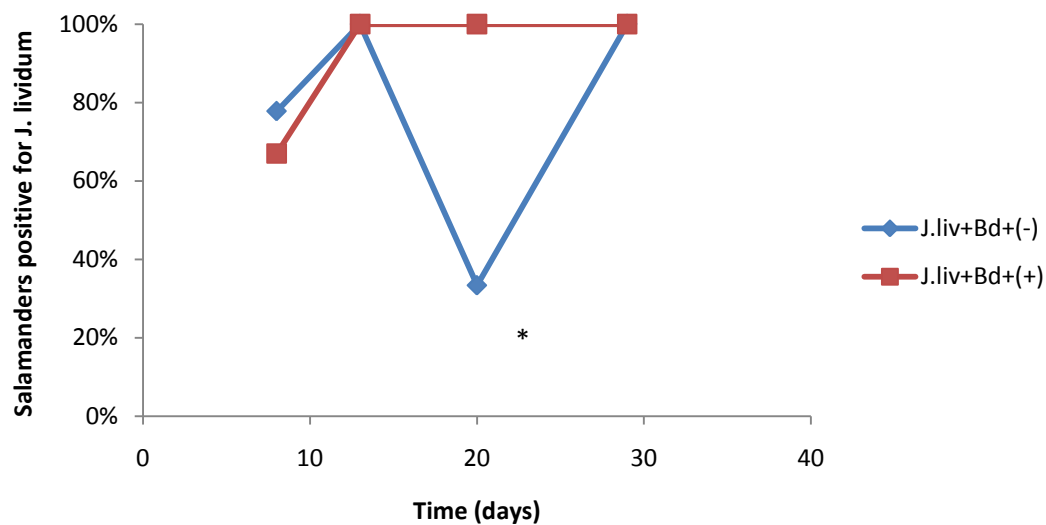Figure 8. Percentage of salamanders positive for *J. lividum* over time based on the categories of being infected (J.liv+Bd+(+)) or not being infected with *B. dendrobatidis* (J.liv+Bd+(-)) on day 13 within the J. liv+Bd+ treatment. A difference between treatments as indicated by an asterisk(*) was observed on day 20 (Fisher's exact test, p=0.03).

Discussion

The results of this study showed that the mutualistic bacterial species *J. lividum* could be introduced into soil, that environmental transmission of *J. lividum* from soil to the skin of *P. cinereus* occurred, and that this transmission inhibited infection by the pathogen *B. dendrobatidis*. After introduction into natural soil, *J. lividum* survival was observed for 41 days. Persistence of environmentally transmitted *J. lividum* on salamander skins was observed to the 29th day of the experiment. No relationship or correlation between soil *J. lividum* densities and salamander *J. lividum* densities was observed, suggesting the population dynamics of *J. lividum* in soil and on the salamanders were independent. A steady decline in soil *J. lividum* density was observed during the experiment. Once the soil *J. lividum* density dropped to an average of 5.6×10^2 CFU/g of soil at day 41, *J. lividum* was not detected on salamanders, suggesting that environmental transmission no longer occurred. Environmental transmission of *J. lividum* to salamander skin decreased the prevalence of *B. dendrobatidis* five-days post infection. The presence of *B. dendrobatidis* on salamander skin was associated with an increased density of *J. lividum* on salamanders at 12 and 21 days post infection and increased the prevalence of *J. lividum* on salamander skin at 12 days post infection.

Survival of introduced bacteria into soil:

In the experiment, all soils inoculated with *J. lividum* showed colonization and survival by the bacteria for 41 days. In preliminary trials (see Appendix), *J. lividum* did not survive long enough to colonize the soil. For several decades, bacteria have been introduced into soil to improve the growth and health of crops (Gentry et al. 2004). Recently, the introduction of bacteria into soil has also been used to degrade

environmental contaminants (bioremediation) (Gentry et al. 2004). The survival of

bacterial inoculants in soil in these applications typically varies over time and space (Van

Elsas & Heijnen 1990, Van Veen et al. 1997, Gentry et al. 2004). Similarly, my

preliminary trials and experiment showed that survival of *J. lividum* after introduction

into soil varied.

Survival of *J. lividum* in soil depends on multiple factors, as revealed by studies

of agricultural and bioremediation applications. Abiotic factors that have been suggested

to govern survival of introduced bacteria into soil include: soil moisture (Postma et al.

1989), soil temperature (Vandenhove et al. 1991, Zogg et al. 2007), soil type (Latour et

al. 1999), soil pH and salinity (Kästner et al. 1998). Biotic factors that have been

suggested to govern survival include: predation by protozoa (Clarholm 1981), microbial

antagonism and competition (Postma et al. 1990) and the physiological status of the

introduced bacteria (Vandenhove et al. 1991, Van Veen et al. 1997). I examined soil

moisture, temperature, pH, and the physiological status of the bacteria in my preliminary

study (see Appendix). I found that in altering these factors in the laboratory, *J. lividum*

survival remained negligible in soil collected in the hot, dry summer of 2010. However,

J. lividum did survive in soil collected in the fall of 2010. This soil was moister, cooler

and had a higher pH than similar soil collected from the same location in the summer of

2010 (see Appendix). Two pilot studies also demonstrated that *J. lividum* could survive

after introduction into soil collected during moister, cooler conditions in the spring of

2010 (data not shown). Changes in climate due to season or anthropogenic disturbances

affect many abiotic and biotic factors in the soil (Waldrop & Firestone 2006, Castro et al.

2010), which include most of the factors mentioned above. Thus, seasonality or climate may be strong determinants of introduced bacterial survival.

I observed an exponential decrease in *J. lividum* soil density during the 41 days of detectable persistence (Figure 2). The decline may have been due to limited nutrients, high levels of predation or other factors that were not examined. Similar declines in bacterial numbers after introduction into soil have been observed in other studies (Postma et al. 1990, Van Veen et al. 1997). However, in one study, the bacterial density in different soil types reached an equilibrium density 50-100 days after introduction in which silt loam soil reached a higher equilibrium density earlier than loamy sand (Postma et al. 1990). In my experiment, *J. lividum* was below the detectable limit by day 41 so determination of equilibrium status of the soil bacteria was not feasible. Future work should be pursued to determine if *J. lividum* reaches an equilibrium state when inoculated into different soil types or at higher densities than I used.

Environmental transmission:

My study demonstrated that environmental transmission of *J. lividum* from soil to salamander skin could occur. The presence of environmentally transmitted *J. lividum* on salamanders was detected to the 29th day of the experiment, although I could not distinguish environmental transmission from bacterial survival and reproduction after the first positive sampling date. Environmental transmission of symbiotic bacteria occurs throughout the animal kingdom with examples including marine bivalves (Gros et al. 1996), terrestrial isopods (Wang et al. 2007), dolphins (Goldman et al. 2009) and humans (Xu & Gordon 2003). For amphibians, Austin (2000) suggested that environmental transmission of soil microbiota to salamander skin likely occurs. However, to my

knowledge, this study is the first empirical demonstration of environmental transmission of a beneficial bacteria species to the skin of an amphibian species under simulated natural conditions.

The population densities of *J. lividum* on the salamander were independent of those in the soil. In the experimental soil, it appears that over time mortality was greater than reproduction, and numbers declined in a steady exponential decay (Figure 2). On the salamander, the median density of *J. lividum* remained relatively constant over the first 20 days, then spiked to its highest density on day 29 and then dropped to zero on day 42 (Figure 3). Decreases in bacterial density likely reflected high mortality on the salamanders, reduced or absent transmission and possibly emigration from the salamanders. Increases in density likely reflected reproduction on the salamanders and continued transmission. These population processes may help explain the variation seen in the densities of *J. lividum* on the salamanders (Figure 3), in the prevalence of *J. lividum* on the salamanders (Figure 4), and in the two population trends of *J. lividum* observed at the individual salamander level (Figures 5 & 6). In this study, I was unable to distinguish bacterial reproduction from transmission or mortality from emigration. A future study to distinguish continual transmission from survival and reproduction on salamander skins would be of interest.

Mortality and emigration or reproduction and transmission of *J. lividum* are likely due to the conditions of the soil and the salamander. Bacterial mortality on the salamander and emigration from the salamander occur due to lack of nutrients, microbial competition, or immune defenses by the salamander. For instance, an increase of *J. lividum* to 100% prevalence in all salamanders, and an increase to the highest density

across all other sampling time points occurred on day 29. At this time point, *J. lividum* in the soil had declined to relatively low densities. Wang et al. (2007) proposed that it is advantageous for microbes to colonize the hindgut of terrestrial isopods, as the hindgut is a more favorable environment than soil and leaf litter (Wang et al. 2007). It is possible that the soil environment was becoming inhospitable for *J. lividum* (increased microbial competition, decreased nutrients) so that the bacteria were colonizing the skin of the salamanders (less competition, more nutrients) to escape mortality in the soil, which led to continual environmental transmission. It is also possible that conditions for survival and reproduction of *J. lividum* were optimal at that time point. For example, immune defenses by the salamanders may have been down regulated. Ramsey et al. (2010) found variability in the number and quantity of antimicrobial peptides (AMPs) produced by individual frogs in the species *Xenopus laevis* suggesting that amphibians may produce varied levels of skin defenses. Therefore, salamanders may vary in their innate immune responses to the presence of *J. lividum* leading to variation in colonization, reproduction, emigration and mortality rates of *J. lividum*.

J. lividum on salamanders was no longer detected by day 42. The average density of soil *J. lividum* density had dropped from 6.3×10^3 CFU/g of soil at day 29 to 5.6×10^2 CFU/g of soil at the point in which transmission was not detected. These results imply that if *J. lividum* is present in densities higher than 6.3×10^3 cells/g of soil then it is likely that transmission from soil to salamander will occur, but below this point transmission will likely be absent. A study conducted by Doring et al. (1993) in children's hospitals found that concentrations greater than 10^5 *Pseudomonas aeruginosa* CFU/ml in sink

drains were more likely to result in hand contamination (environmental transmission) during hand washing than lower concentrations (Doring et al. 1993).

Disease mitigation:

The presence of *J. lividum* in the soil decreased the prevalence of *B. dendrobatidis* infection at five days post infection. The proportion of *B. dendrobatidis* infected salamanders in soil augmented with *J. lividum* was half that of salamanders in non-augmented soil. This suggests that the presence of *J. lividum* in soil helped the salamanders clear the infection earlier than the salamanders in the non-augmented soil. Brucker et al. (2008b) demonstrated that two metabolites produced by *J. lividum*, violacein and indole-3-carboxaldehyde, directly inhibited *B. dendrobatidis* growth at relatively low concentrations. They also found that *J. lividum* naturally present on *P. cinereus* skins produces these metabolites at high enough concentrations to be inhibitory. Thus, the production of antifungal bacterial metabolites may be one mechanism by which *J. lividum* protected its amphibian host from disease.

At 12-days post infection regardless of soil augmentation status all salamanders except one individual tested negative for *B. dendrobatidis*. It is likely that infection by *B. dendrobatidis* did not persist long enough to cause chytridiomycosis. Thus, no morbidity or mortality effects of chytridiomycosis were observed. In other experimental studies, morbidity (Harris et al. 2009b, Becker & Harris 2010) and mortality effects (personal observation, Becker et al. 2009) in *P. cinereus* infected individuals have been observed. In my study, I ended pathogen exposure at five hours, so pathogen load at exposure may not have been high enough to cause morbidity and mortality effects.

The rapid clearing of infection may be due to several factors other than low infection load. *P. cinereus* have been shown to possess 32 bacterial species with antifungal properties (Lauer et al. 2007) and three genera of bacteria with anti-*B. dendrobatidis* properties (Harris et al. 2006). The presence of these or other mutualistic bacterial species may explain the observed clearing of infection in all experimental salamanders. In field studies, the presence of multiple species of anti-*B. dendrobatidis* bacteria is associated with persistence of *Rana muscosa* populations (Woodhams et al. 2007b, Lam et al. 2010). Moreover, the adaptive immune system of *X. laevis* has been shown to be activated by *B. dendrobatidis* infection (Ramsey et al. 2010). Thus, like *X. laevis*, *P. cinereus* may have an adaptive immune response, although the presence of adaptive immunity against *B. dendrobatidis* remains controversial (Rosenblum et al. 2009). Finally, caudates in general have been found to be less vulnerable to the effects of chytridiomycosis than many other anurans (Lips et al. 2006).

The results of experimental infection trials in the Lature can be challenging to interpret which makes it difficult to design an infection study. In experimental trials exposure times have varied from 4 hours (Shaw et al. 2010) to 48 hours (Blaustein et al. 2005). Furthermore, studies have shown that pathogen densities at exposure (Chinnadurai et al. 2009), the amphibian species being exposed (Blaustein et al. 2005, Chinnadurai et al. 2009, Vazquez et al. 2009), and the strain of *B. dendrobatidis* being used for exposure (Retallick & Miera 2007) are factors that affect outcomes. Thus, systematic study of durations of exposure, exposure loads, amphibian species and experimental strains of *B. dendrobatidis* are important projects to continue to pursue in the future. Therefore, to

determine the generality of my conclusion, it is important to conduct my study again with a pathogen load that produces morbidity and mortality effects.

Interaction between symbionts:

For the salamanders in the J.liv+Bd+ treatment, a higher density and prevalence of *J. lividum* were observed on salamanders that tested positive for *B. dendrobatidis* than those that did not. AMPs that are part of the innate immune system of amphibians may be a factor in this observed difference. First, there may be a co-evolutionary relationship between the salamander and the bacteria in which AMPs target *B. dendrobatidis* and other non-protective microbial species and not *J. lividum*. If so, this targeted response would provide *J. lividum* with more space and available nutrients. Protective bacteria and AMPs could work in synergy to combat infection by *B. dendrobatidis*. Meyers (2011) found that a metabolite produced by an amphibian symbiotic bacterial species and AMPs work in synergy against *B. dendrobatidis*. Second, *B. dendrobatidis* may suppress AMP production that would lead to enhanced bacterial growth of most or all bacterial species. Third, infected salamanders are sick and have less energy to produce AMPs, which would lead to an increase in bacterial growth. The higher density and prevalence of *J. lividum* on infected salamanders were seen at time points after the salamanders had cleared infection. Population growth is an exponential process so that small changes in growth rate early in the experiment would have been exaggerated later in the experiment.

Microbial composition change leads to increase in disease:

Environmental factors can contribute to an increase in disease prevalence. Climate change is a one environmental factor that may influence disease outbreaks. The direct link between climate change and chytridiomycosis remains inconclusive (Lips et

al. 2008, Rohr et al. 2008). However, Longo et al. (2010) proposed an indirect effect of climate change in which drought conditions likely induced behavioral changes in amphibians, i.e., crowding in moist areas, which led to an increase of chytridiomycosis. Further research is merited to examine such complex effects of climate change on chytridiomycosis. For instance, it is possible that climate change and other anthropogenic changes may affect microbial communities, which may lead to an increase of chytridiomycosis (Belden & Harris 2007).

Climate change could alter the composition of microbial communities (Zogg et al. 1997). My study demonstrated that the presence of a mutualistic bacterial species could inhibit prevalence of a disease-causing species. What happens if the presence naturally occurring protective bacteria is altered? Zogg et al. (1997) proposed that large seasonal variation in soil temperature or small annual increases due to global climate change would alter the structure and function of soil microbial communities. If seasons are shifting due to climate change, then microbial compositions in soil may be temporally altered and environmental transmission of anti-*B. dendrobatidis* microbes may be occurring at an inappropriate time. Alternatively, microbial composition in the soil or on the skin of amphibians could be spatially altered such that anti-*B. dendrobatidis* species would be present in reduced densities or completely absent in all seasons. Studies have found that incidences of chytridiomycosis vary by season (Berger et al. 2004, Kriger et al. 2007a, 2007b, Conradie et al. 2011). Higher prevalence are typically found in cooler, moister months (Berger et al. 2004, Kriger et al. 2007a, 2007b), but a recent study found a higher prevalence during warmer, drier months (Conradie et al. 2011). Local changes in skin microbial community may be an alternative explanation for these observed

differences in prevalence. No published research has been conducted on the variation of amphibian skin microbiota throughout seasons or the relationship of microbial community structure to weather patterns. Future work on seasonal variations in microbial community structure on amphibian skin is warranted.

Evolution:

Chytridiomycosis is expected to drive evolutionary processes in amphibian and bacterial natural defenses. Evolution in susceptible amphibians could occur via the evolution of AMPs that specifically target *B. dendrobatidis* or the evolution of the adaptive immune system in which recognition of infection by *B. dendrobatidis* would occur. Alternatively, amphibians and anti-*B. dendrobatidis* bacteria could co-evolve in which amphibians' AMPs do not target the anti-*B. dendrobatidis* bacteria and the bacteria colonize the amphibian's skin. Another possibility is that amphibian symbiotic cutaneous bacteria evolve to combat the pathogen since the bacteria are under strong selection to do so. Vorburger et al. (2010) found that a common endosymbiont of aphids had *evolved the trait to* confer resistance to a parasitoid wasp. This result suggested that the ability of symbiotic bacteria to protect their host against natural enemies could evolve readily. Furthermore, James et al. (2009) proposed that due to low genetic diversity of *B. dendrobatidis*, the species might not be able to co-evolve with adapting amphibian populations. The time needed for these or other evolutionary processes to occur are unknown. However, the use of probiotics or protective bacteria (bioaugmentation) may provide susceptible amphibians the necessary time to evolve natural defenses or for evolution to drive the extinction of *B. dendrobatidis*.

Bioaugmentation & probiotics:

Bioaugmentation is the introduction of bacteria by humans to the environment or an organism to produce a beneficial effect. Probiotics are live active cultures of bacteria introduced for beneficial effects. Bioaugmentation has been used in legume production dating back to the 1800s, and probiotics have been consumed for centuries by humans. As more research documents the health benefits of indigenous bacteria, the use of bioaugmentation with probiotics in mitigating disease is increasing. In recent decades, bioaugmentation has been used for disease mitigation in fish (Olsson et al. 1992), poultry (Patterson & Burkholder 2003), peaches (Restuccia et al. 2006), and humans (Cherif et al. 2009) and has been suggested for use in coral reef disease mitigation (Teplitski & Ritchie 2009). The use of bioaugmentation may be a strategy to combat declines in amphibians driven by chytridiomycosis.

A bioaugmentation strategy for combating disease in amphibians can be implemented by adding bacteria to water, soil or other biotic materials present in amphibians' environment. To date, bacteria have been added to amphibians by placing them in small solutions of protective bacteria in water, which has mitigated symptoms of chytridiomycosis in *R. muscosa* (Harris et al. 2009a) and in *P. cinereus* (Harris et al. 2009b, Becker et al. 2009) *in vitro*. This method may be a promising conservation strategy. However, in the field, amphibians need to be located, captured and treated individually. Using soil augmentation would potentially be less time consuming and costly if the use of soil augmentation proves to be effective and safe to the environment. It is important in soil inoculation trials to consider the various methods that have been designed by other researchers to increase survival of bacterial inoculants into soil (Van

Elsas & Heijnen 1990, Van Veen et al. 1997, Gentry et al. 2004). One such method is the

use of carrier materials such as peat and encapsulation of the bacterial cells in alginate

beads (Van Elsas & Heijnen 1990, Van Veen et al. 1997).

An important question regarding the use of bioaugmentation is the safety of the

ecosystem as a whole. For example, it is important to maintain the biodiversity of the soil

and the amphibians' skin microbiota. The introduction of a microbial species could

potentially shift the microbial community composition of the soil. A study was conducted

by Edel-Hermann et al. (2009) to assess the alteration of the soil microbial community

after the introduction of the biocontrol agent *Fusarium oxysporum*. They demonstrated

that little to no community structure change was observed after introduction of a

beneficial fungal strain of *F. oxysproum*. Although encouraging, this work needs to be

repeated in amphibian systems before large-scale bioaugmentation is considered.

If bioaugmentation is to be pursued further it is important to select appropriate

bacterial strains as probiotics. The characteristics needed for a bacterial strain to serve

effectively as a probiotic include the capability to adhere to amphibian epithelial cells, to

colonize the skin for the time needed for protection, and to produce molecules that inhibit

the growth of the pathogen. Furthermore, the use of an indigenous strain that is dominant,

competitive and adapted to local conditions may improve survival rates and maintains the

biointegrity of the environment (Paau 1989). It may also be critical to find a probiotic that

works synergistically with amphibians AMPs. In addition, the skin microbial community

of amphibians may be species specific (Lauer et al. 2007) suggesting that appropriate

probiotics will likely vary amongst species. However, *Pseudomonas* spp. may be good

candidates for bioaugmentation trials. They are found naturally in the environment and

on several amphibian species in Virginia (Culp et al. 2007, Lauer et al. 2007) and in Australia (Alford 2010). Furthermore, one species of *Pseudomonas*, *P. reactans*, has been transmitted to amphibian skin via bacterial baths and has provided salamanders protection from chytridiomycosis (Harris et al. 2009b).

Field trials are important to determine if bioaugmentation can work in nature. Lips et al. (2003) found that amphibians associated with water are more likely to be susceptible to chytridiomycosis than terrestrial amphibians in Central America. Thus, an aquatic or semi-aquatic amphibian species susceptible to *B. dendrobatidis* would be a good candidate for field trials. In addition, breeding season is likely a time of high transmission of the pathogen. Thus, it would be of interest to implement bioaugmentation directly before breeding. First, an appropriate bacterial species would be identified using the criteria above. Then, the riparian zone of a breeding location would be bioaugmented. Finally, it would need to be determined if the environmentally transmitted bacteria provide the susceptible amphibian additional protection from the pathogen during and after breeding. A field trial is currently underway in California in which *R. muscosa* were captured, placed in bacterial baths of *J. lividum*, and released to determine if these baths can protect the frogs from chytridiomycosis and early results are encouraging (Rex 2010).

Additional work should be pursued in developing strategies for bioaugmentation. Comparing the efficacy of bacterial baths versus soil inoculations would provide insight into appropriate strategies to use for amphibians species. Species that vary in on life history, geographical range and distribution should be examined as well. In soil trials, the compositional change of the soil microbial community could be examined. In addition,

the microbiota of amphibian skin before and after the introduction of bacteria could be determined. Testing the soil and amphibian microbial community structures will indicate if the introduction of the bacteria is shifting the natural microbial composition. In addition, future work should be directed towards the use of a combination of microbes for bioaugmentation. The augmentation of multiple species could increase the chance of successful colonization of the amphibians' skin and inhibition of the pathogen. However, the proper choice of multiple species cocktails would have to be carefully done, e.g. so that natural competitors are not chosen. The use of bioaugmentation in amphibian disease prevention is a new concept that should be explored as it is the only strategy proposed to date that has the potential to halt amphibian extinctions within a reasonable time frame. It may prove to be an implementable conservation strategy to combat chytridiomycosis driven amphibian declines. Regardless, in the process of exploring bioaugmentation, a fundamental understanding of amphibian bacterial mutualisms will be gained.

Appendix

Introduction

Initial studies of *J. lividum* survival in soil demonstrated both survival and absence of survival. This observed difference in survival related to changes in season. Pilot studies using soil collected in spring 2010 demonstrated both survival of *J. lividum* at high density in soil and high rates of environmental transmission of *J. lividum* from soil to salamander (data not shown). Preliminary trials using soils collected in summer 2010 did not demonstrate long-term survival of *J. lividum* or environmental transmission of *J. lividum*. A preliminary trial using soil collected in fall 2010 again showed survival of *J. lividum* at high densities. The data presented here describes the trials conducted in summer and fall of 2010.

Methods & Results

Flag Pole Knob soil:

Soil was collected from Flag Pole Knob in June 2010 when salamanders were collected. The soil was inoculated with 3.3×10^6 *J. lividum* cells/dry g of soil. Six days post-inoculation only 18% of soil samples (n=22) tested positive for *J. lividum* and those positive had low cell densities of *J. lividum* ($< 5 \times 10^2$ CFU/dry g of soil).

Another inoculation trial using the Flag Pole Knob soil was performed to determine if culture duration of the isolate affected survival of *J. lividum*. The *J. lividum* isolate being used had been cultured weekly on plates for over one year in the laboratory. A new *J. lividum* culture from -80°C stock was started to use for inoculation. Colonization of *J. lividum* in the soil in this trial was low (10%, n=22). Soil pH was then determined to be the main factor that inhibited colonization by *J. lividum*. The soil pH for

the Flag Pole Knob soil was 5. Testing of pH levels in this soil and subsequent soils was performed using a Rapitest pH meter (Luster Leaf Products, Woodstock IL). According to Bergey's Manual of Systematic Bacteriology the optimal pH for *J. lividum* is 7-8, and no growth occurs below pH of 5.

James Madison University Arboretum soil:

Soil was collected with a pH above 5 from James Madison University (JMU) Arboretum (pH 6) in August 2010 to determine if inoculum density affected *J. lividum* survival. Different inoculum densities ($1x10^6$, $5x10^6$, $1x10^7$, $5x10^7$, $1x10^8$ *J. lividum* cells/g) were inoculated into the soil. Inoculum density has been shown to be a factor that affects bacterial survival in soil (Postma et al. 1990, Vandenhove et al. 1991). Two replicates per inoculum density were conducted (n=10). Regardless of inoculum density by three days post-inoculation survival was either minimal or undetectable.

Hone Quarry soil:

Survival of *J. lividum* in soil was observed in soil collected from Hone Quarry in August 2010 (pH 6.5), but varied based upon bacterial isolate and inoculation density. Three *J. lividum* isolates were used: an isolate from the skin of *H. scutatum*, and two isolates from separate soil samples and five different inoculum densities ($4x10^6$, $7x10^6$, $1x10^7$, $2x10^7$, $3x10^7$ *J. lividum* cells/g) were tested to see if isolate or density affected survival. One replicate per isolate per inoculum density was conducted (n=15). There was *J. lividum* survival at six days post-inoculation in the soil for all three isolates, but survival across inoculum densities varied. The isolate from the skin of *H. scutatum* was the only isolate to exhibit survival across all inoculum densities. The inoculum density of $2x10^7$ *J. lividum* cells using the *H. scutatum* isolate exhibited the

highest survival rate (2.5×10^5 CFUs/g) at six days post-inoculation. Only one replicate was performed so no statistical analysis could be performed. Survival in relatively high densities for the *H. scutatum* isolate was encouraging; I ended this trial to begin a new trial to test environmental transmission.

In the second trial, I used the soil collected in Hone Quarry in August and incorporated salamanders into the design of this trial. The *J. lividum* isolate from the skin of *H. scutatum* was inoculated into soil. The starting density after inoculation was 2×10^7 *J. lividum* cells/dry g of soil. Salamanders were introduced one day after inoculation. By six days post-inoculation 55% of the soils (n=22) tested positive for *J. lividum.* Those that were positive had a cell density generally less than 3×10^3 CFUs/g of soil. Environmental transmission from soil to salamander was detected in 1 of 22 salamanders that were exposed to *J. lividum* in soil at six days post inoculation. The density of *J. lividum* in the soil in which the salamander obtained the bacteria contained the highest amount of *J. lividum* of all 22 soils tested (1.8×10^4 CFU/dry g of soil). All other positive soils sampled at this time point had a density of less than 2.5×10^3 *J. lividum* CFU/dry g of soil on average. Therefore, I hypothesized that without relatively high numbers of *J. lividum* in soil ($>2.5 \times 10^3$ *J. lividum* CFU/dry g) environmental transmission will likely not occur. Due to the inability to test further hypotheses additional trials were conducted.

The physiological stage of the bacteria was tested to see if it was a factor causing the limited survival of *J. lividum* in these soils. A study by Vandenhove et al. (1991) found that the physiological stage of bacteria at inoculation affects bacterial survival in soil. Soil with a pH of 6.8 was collected from Hone Quarry in September 2010 to examine the role of this factor in survival. I tested late exponential phase and stationary

phase (three replicates per phase). There was initial survival, but it was not consistent and by nine days post-inoculation *J. lividum* was undetectable.

Survival of *Janthinobacterium lividum* in fall soil:

Survival and persistence of *J. lividum* was finally obtained in soil collected from JMU Arboretum in October 2010 (pH 6.7). During soil collection, five *P. cinereus* individuals were seen in the location indicating that the soil was collected in a natural *P. cinereus* environment. A trial was conducted where soil moisture, sieving of soil, and inoculum densities ($5x10^6$, $1x10^7$ and $2x10^7$ *J. lividum* cells/g) were varied (n=12). Relatively high densities of *J. lividum* were detected in all categories over 15 days of testing. By 15 days post-inoculation moist, sieved soil with an inoculum density of $2x10^7$ *J. lividum* cells/g of soil displayed the highest cell density count ($1.2x10^4$ CFU/g). Thus, these soil and bacterial characteristics were used as a basis for my main experimental design. One replicate was conducted for each soil moisture level, sieve status and inoculum density combination so no statistical analysis could be performed.

Discussion

Negligible survival in the preliminary trials conducted in summer 2010 may have been related to the soil being collected in the hot, dry summer climate of 2010 in Virginia. Smit et al. (2001) found that the soil bacterial community present in summer was an outlier when compared to relatively similar bacterial communities from fall, winter and spring in the Netherlands. It is feasible that the ecological niche of *J. lividum* was not present in these summer soils and thus survival was not possible. The possible seasonal variability in the presence of *J. lividum* has implications for bioaugmentation.

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