ABSTRACT

Title of Dissertation: THE SKIN MICROBIOME OF WOODLAND SALAMANDERS AND ITS ASSOCIATION WITH HOSTS' TAXONOMY, ENVIRONMENT AND HEALTH STATUS

Carly Muletz Wolz, Doctor of Philosophy, 2016

Dissertation directed by: Dr. Karen R. Lips, Department of Biology

Microbial communities play central roles in animal health. Host species, environmental conditions and presence of pathogens can affect the diversity and composition of animal-associated microbiomes. Amphibians form integral and functionally important symbioses with microbes. The amphibian microbiome interacts with pathogens, and can protect hosts from disease, including the disease chytridiomycosis, caused by skin infection by *Batrachochytrium dendrobatidis* (Bd). The implications of amphibian-microbiome associations are difficult to predict because little is known about the factors shaping bacterial communities or their functional traits, such as anti-Bd properties. I used culture-dependent and culture-independent methods to characterize the skin microbiome of *Plethodon* salamanders in field and laboratory studies. I hypothesized that the evolutionary history, environmental conditions, and health status of the hosts shape skin bacterial community assemblages. In a field study, I sampled sympatric, congeneric salamander species (*Plethodon cinereus*, *P. glutinosus*, *P. cylindraceus*) across three localities to quantify the distribution of both anti-Bd bacteria and the entire bacterial community. I identified 50 anti-Bd bacterial OTUs and 480 bacterial OTUs overall on the salamander skin, with high prevalence and abundance of

anti-Bd bacterial genera *Pseudomonas*, *Acinetobacter* and *Stenotrophomonas*. Within a locality, co-occurring salamanders generally had similar microbiome diversity and composition patterns, but these differed among sites. This indicates that environment is more influential in shaping skin microbiome patterns than differences in host properties in these species. I sampled *P. cinereus* along an elevational gradient, as a proxy for environmental variables that co-vary with elevation. Microbiome diversity and composition changed with elevation, in which compositional changes were related to soil pH. In a laboratory experiment, I quantified the responses of *P. cinereus* and the skin microbiome to temperature (13, 17, 21 °C) and pathogen (Bd+, Bd-) exposure to determine whether the native microbiome affected survival at natural temperatures. Temperature changed the microbiome, but this did not prevent host mortality from Bd. Instead, Bd exposure changed the microbiome, and caused 78% mortality. My results demonstrate that environmental conditions and pathogen presence are important factors determining skin microbiome structure in *Plethodon* salamanders. These findings contribute to our understanding of animal-microbial symbioses, microbial community ecology, and amphibian disease ecology.

THE SKIN MICROBIOME OF WOODLAND SALAMANDERS AND ITS ASSOCIATION WITH HOSTS' TAXONOMY, ENVIRONMENT AND HEALTH STATUS

by

Carly Muletz Wolz

Dissertation submitted to the Faculty of the Graduate School of the
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Advisory Committee:

Professor Karen R. Lips, Chair
Professor Stephanie Yarwood
Professor Maile Neel, Dean's Representative
Dr. Robert Fleischer
Dr. Evan Campbell Grant

Preface

This dissertation contains an overview chapter (Chapter I), three research chapters (Chapters II-IV), a synthesis chapter (Chapter V), and an appendix. The research chapters (II-IV) represent primary work, and the appendix provides supplementary figures and tables for Chapter II and III, and supplementary methods and results for Chapter III. All chapters are presented in manuscript form and formatted depending on the journal in which they are intended to be published. A single reference section occurs at the end for literature cited throughout the dissertation.

Dedication

This dissertation is dedicated to my family: my mother, father, sister, grandparents and husband. Their love and support has given me the strength to pursue my dreams. I hope to give the same love and support to my first child that will be here soon!

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I would like to thank my advisor, Dr. Karen Lips, for her knowledge and guidance in developing, conducting and completing my dissertation. It has been a great opportunity to work with her over the years and grow as a scientist under her mentorship.

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Chapter I: OVERVIEW

Microbes are ubiquitous; they are found on nearly every surface of the globe. One reason for their success is their ability to form symbioses: permanent or long-lasting associations with other living organisms. Symbiosis is typically divided into three types of interactions: mutualistic, in which both organisms benefit; commensal, in which one organism benefits and the other is unharmed; and parasitic, in which one organism benefits and the other is harmed. Symbioses can be obligate, in which the symbiont is unable to live without its partner, or facultative where it is can be free-living. All animals form symbiotic associations with microbes, and these symbionts play vital roles in the health of their hosts. The community of host-associated microbial symbionts can consist of fungi and bacteria that potentially interact with one another through competition, predation and other forms of interactions. All of these interactions occur in the context of the environment, in which environmental conditions can change the dynamics of the symbiosis or interactions among symbionts.

Amphibian skin supports diverse communities of symbiotic bacteria that are generally hypothesized to offer protection from microbial pathogens. One of the most deadly wildlife pathogens in recorded history is the fungal pathogen of amphibians *Batrachochytrium dendrobatidis* (Bd; Skerratt et al. 2007). There is a small, but growing knowledge about the distribution, acquisition and function of amphibians' skin bacteria and how these bacteria may be used in conservation strategies to mitigate the negative impacts of Bd on amphibians. Here, I review our current knowledge of: (i) parasitism of amphibian hosts by a Bd, (ii) commensalism/mutualism between amphibian hosts and their skin microbial communities (i.e., the microbiome), and (iii) competition between the

host's resident skin microbes and invading pathogen. I consider these interactions in an environmental context (Figure 1), and highlight gaps in knowledge.

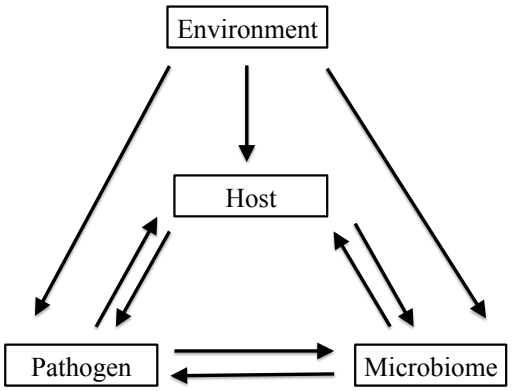


Figure 1.1 Conceptual model of interactions among host, microbiome and pathogen that are impacted by the shared environment.

The relationships among the pathogen Bd, the amphibian skin microbiome, and the amphibian host provide an excellent model to study symbiosis. These interactions occur on the amphibian skin, which provides ease of study, and the three interacting taxa are amenable to experimental manipulation. Understanding these interactions can help elucidate general principles regarding animal-microbial symbioses and disease ecology. My dissertation will provide new information concerning the specificity of amphibians' microbiome within geographic regions, between host species, and in relation to environment and disease.

I. Amphibian declines and *Batrachochytrium dendrobatidis*:

The fungal disease chytridiomycosis, a skin infection caused by Bd (Longcore et al. 1999), is of global concern as it has caused declines, extirpations and extinctions of up to 200 amphibian species worldwide (Wake & Vredenburg 2008). The pathogen

completes its life cycle inside the keratinized epithelial cells of amphibian skin (Longcore et al. 1999), producing infectious zoospores that can re-infect the host or attempt to locate a new host (Berger et al. 2005). Once a species-specific density of Bd zoospores is reached, susceptible species develop the disease chytridiomycosis (Stockwell et al. 2010). The negative effects of the disease are seen in reduced (i) developmental rates (Venesky, Parris & Storfer 2010), (ii) weight gain (Becker et al. 2010), and (iii) survival (Voyles et al. 2009). Infected individuals experience mortality due to the inhibition of electrolyte 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). Chytridiomycosis is one of the most challenging drivers of amphibian declines (Collins & Storfer 2003) as there is no proven implementable strategy in the field that can combat the disease.

Bd has the widest host range of any known pathogen in recorded history (Fisher et al. 2009) infecting over 500 amphibian species (Olson et al. 2013) with substantial variation in amphibian species- and population-level responses to infection (Smith et al. 2009, Lips et al. 2006, Crawford et al. 2010, Briggs et al. 2010). Variation in host responses to infection has been attributed to a wide variety of pathogen- (e.g., strain differences; Rosenblum et al. 2013), environment- (e.g., riparian vs. forest; Brem & Lips 2008) and host- (e.g., lifetime aquatic index; Lips et al. 2003) specific variables. One host-specific trait of particular interest is the host's skin microbiome as these communities are one of the amphibians' first lines of defense against Bd infection. The

pathogen has to pass through this microbial barrier of potential natural antagonists to infect the amphibians' skin.

II. The amphibian skin microbiome

The moist, nutrient rich mucous on amphibian skin is inhabited by diverse communities of commensal and mutualistic bacteria (Austin 2000, Lauer et al. 2007). The bacteria benefit from the available nutrients found in the mucosal layer and a suitable, protected habitat (Austin 2000). Some bacterial taxa produce inhibitory metabolites that kill fungal pathogens, providing disease mitigation benefits to the amphibian host (Harris et al. 2006, Banning et al. 2008, Becker & Harris 2010), and are therefore considered mutualistic partners with the host. For other bacterial taxa the interaction with the host is unknown, and they are defined as commensal bacteria. Other benefits such as nutritive, reproductive, developmental and other defensive benefits may exist (McFall-Ngai 2005), but have yet to be studied. Hereafter, we refer to the community of commensal and mutualistic bacteria living on amphibian skin as the microbiome.

Structure of the microbiome can depend on factors associated with the host and the environment of the host. Living in moist or aquatic environments, amphibians are exposed to environmental microbes through contact with soil, water, plants and each other. It is generally hypothesized that host factors select for microbes and the environment controls the pool of available microbes. Indeed, studies have found host species-specific microbial communities (Ley et al. 2008, McKenzie et al. 2012, Larsen et al. 2013, Kueneman et al. 2014, Amato et al. 2015, Belden et al. 2015, Sanders et al.

2015, Bik et al. 2016, Council et al. 2016, Rebollar et al. 2016), that are unique set of microbiota from their habitat (Sullam et al. 2012, Loudon et al. 2014, Walke et al. 2014, Schmidt et al. 2015, Bik et al. 2016, Rebollar et al. 2016). However, we know little about the evolutionary and ecological processes that structure these communities across species and geographic locations and how they respond to Bd or impact infection outcome, especially for closely related amphibian species and for salamanders.

III. The amphibian skin microbiome and Bd

Amphibians support diverse communities of skin bacteria that appear to generally offer protection from microbial pathogens (Harris et al. 2006). 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. We know for amphibians, skin microbes may offer protection to the host from Bd via two mechanisms: a) by physical abundance and associated competition for nutrients and adhesion sites (Becker & Harris 2010) and, b) by the production of inhibitory metabolites that can kill *Bd* zoospores (Brucker et al. 2008a, 2008b) or cause the Bd zoospores to chemotaxis away from the metabolites (Lam et al. 2011).

Manipulation of the microbiome has been championed as a conservation strategy to mitigate Bd-associated disease. Over 250 bacterial OTUs have been identified that inhibit Bd growth *in vitro* (hereafter, anti-Bd bacteria) from 37 amphibian species across 18 studies on five continents, and are compiled in an online database (Woodhams et al.

2015). These studies predominately focused on identifying the anti-Bd bacteria, and rarely, if ever, quantified how these bacteria were distributed across species and environments. A total of eight bioaugmentation trials have been conducted using some of these identified anti-Bd bacteria. Five of the studies using four different anti-Bd bacteria demonstrated successful reduction of infection, weight loss or mortality in lab (Harris et al. 2009, Harris et al. 2009, Muletz et al. 2012, Woodhams et al. 2012) or field (Vredenburg et al. 2011) experiments. Whereas, three other studies using six anti-Bd bacteria showed no effect in either lab experiments (Nebergall 2013, Becker et al. 2015) or in field mesocosms (Bletz 2013). These mixed results indicate a need to systematically quantify the geographic distribution of anti-Bd bacteria and the microbiome, as a whole, to understand the underlying processes that generate bacterial community structure and how this influences disease susceptibility.

The impact of Bd on hosts also depends on environmental context, which may be mediated by the microbiome. Associations of temperature with Bd-associated mortality are not consistent across studies (Carey et al. 2005, Knapp et al. 2011, Savage et al. 2015, Bustamente et al. 2011, Berger et al. 2005), or with the temperature optima of Bd in pure culture (17-25 °C; Piowtroski et al. 2005). This suggests that fungal properties, such as host proprieties may mediate effects of fungal growth optima, among others (life history trade-offs; Woodhams et al. 2008). Temperature-dependent proprieties of the host can include host skin shedding rate (Meyers et al. 2012, Cramp et al. 2014), host immune response (Ribas et al. 2009), and host microbiome composition (Lokmer & Wegner 2015). Yet, no study to date has examined how the amphibian microbiome changes across temperature and its subsequent impact on host disease outcome.

IV. The Study Species: *Plethodon* salamanders

In this dissertation, I used three species of Appalachian *Plethodon* salamanders as my study species. Several species of *Plethodon* species are reported to be declining across their range since the 1980s (Caruso & Lips 2013, Highton 2005). In an independent study, we tested for Bd presence on *Plethodon* salamander sampled from 1957-2011, hypothesizing that Bd was the driver of the observed declines. We found less than 1% prevalence on more than 1,400 individuals tested across the Central Appalachian range of these salamanders (Muletz et al. 2014). Therefore, it is likely that these Appalachian salamanders have some host-specific trait that limits Bd infection because (i) Bd is commonly found in the Eastern US at 10-40% prevalence and infecting all families of amphibians tested (Grant et al. 2008, Chatfield et al. 2012, Richards-Hrdlicka et al. 2013, Longcore et al. 2007, Lannoo et al. 2011, Rothermal et al. 2008), (ii) in laboratory infection trials *Plethodon* salamanders can be susceptible to Bd (Chinnadurai et al. 2009, Vazquez et al. 2009) and die from Bd infection (Becker & Harris 2010), and (iii) the environment in which *Plethodon* salamanders live is suitable for Bd (Caruso & Lips 2013). Previous research on *Plethodon* salamanders has shown that in lab experiments the native microbiome of these salamanders can reduce Bd-associated disease symptoms, such as weight loss (Becker et al. 2010) and disease prevalence (Muletz et al. 2011). I hypothesized that extremely low Bd prevalence is observed on *Plethodon* salamanders as a result of their resident skin microbiome limiting Bd infection, and that the evolutionary history, environmental conditions, and health status of the salamander hosts shape the structure of the bacterial community.

V. What are we still missing? And what does this dissertation contribute?

Despite advances in our understanding of host-associated microbial assembly and the role of microbial communities in disease within the last couple decades, many unresolved questions remain. A major roadblock to improving our understanding of these processes is the lack the data on amphibian populations and communities sampled across multiple environments, which can be used to determine host and environmental controls of the microbiome. Moreover, our knowledge of the functions of the microbial community in relation to disease is limited.

The study of animal-associated microbiomes is in its infancy with most studies focusing on the human microbiome, while only a few studies to date have examined the microbiomes of wildlife and what principles govern the observed bacterial community patterns. We know virtually nothing about how animal-associated microbes vary across environmental gradients or what scale is actually relevant for animal associated microorganisms (Nemergut et al. 2013). We do know that across broad vertebrate taxonomic groups there is some degree of host species-specific microbial community structure (mammals: Ley et al. 2008, fish: Wang et al. 2010, Sullam et al. 2012, amphibians: McKenzie et al. 2012, bats: Phillips et al. 2012) indicating that host taxonomy, representing evolutionary history, are important in shaping microbial community structure. However, environmental factors have been shown to also influence the observed microbial community (e.g., salinity: Sullam et al. 2012, e.g., population origin: Blaser et al. 2013) suggesting a somewhat dynamic nature of the host microbial community. In Chapter II and III, I sampled co-occurring species of *Plethodon* salamanders at multiple sites and sampled *P. cinereus* along an elevational gradients in

the Appalachian Mountains as proxies for environmental changes, along with host taxonomy as a proxy for evolutionary history, to determine what processes are influential in shaping observed bacterial community structure.

While it is generally regarded that the microbes benefit from symbiotic associations with animal hosts (review by McFall-Ngai 2005, also Chaston & Goodrich-Blair 2010), additional work is necessary to determine the positive functional contribution (nutritive, reproductive, developmental and/or defensive) the microbe provides to the host. In Chapter II, I identified culturable anti-Bd bacteria from *Plethodon* salamanders and determined the geographic and taxonomic distribution of these bacteria. This work focused on chemical competition between skin bacteria and Bd, and for the bacteria that inhibited Bd growth, it is suggestive of positive associations (i.e., mutualisms) between salamander host and bacteria. Given the limitations of culturing to detect all taxa that are present and to quantify abundances, I examined the distribution of putatively anti-Bd bacteria in Chapter III. I used my Chapter II database of anti-Bd bacteria along with the larger Woodhams et al. (2015) database of 255 anti-Bd bacterial OTUs to determine the prevalence and abundance of these anti-Bd bacteria in the entire skin microbial community. It would be expected that if the anti-Bd bacteria provide a service to the host, protection from Bd infection in the field, that they are then under selection to be prevalent at the population-level (Anderson & May 1999) and abundant at the individual-level to produce Bd-inhibitory metabolites (Scheuring & Yu 2012).

Growing evidence shows that animal disease dynamics may be influenced by the characteristics of the host's microbiome (Belden & Harris 2007, Roder et al. 2014, Becker et al. 2015, Lokmer & Wegner 2015, Gilbert et al. 2016). However, there has

been little experimental work conducted with wildlife species to understand how microbial communities respond to pathogen invasion and environmental factors or how microbial community structure influences disease outcome. In Chapter III, I conducted a laboratory experiment using temperature (13, 17 and 21 ˚C) and disease treatments (Bd+, Bd-) to understand whether these factors affect bacterial taxonomic and functional diversity and subsequently influence salamander susceptibility to infection and disease. This experimental approach built upon findings from Chapter II and III to better resolve how environmental factors (i.e., temperature) influence the observed bacterial community and how the resident bacteria community influences infection outcome of the host. It is important to note that in the experiment, I exposed salamanders to Bd at loads typical of an epidemic (Vredenburg et al. 2010, Catenazzi et al. 2011) that they currently do not encounter in the field (Muletz et al. 2014, Richards-Hrdlicka et al. 2013). I chose high loads because previous research demonstrated that at lower loads the microbiome is protective (Becker et al. 2011, Muletz et al. 2011), and I hypothesized that environmental conditions could alter the protective properties of the microbiome if the host was exposed to mortality-inducing loads. This has implications for the potential of future epidemics that could occur if a novel, deadly strain of Bd entered the eastern US or if Bd's sister taxon *Batrachochytrium salamandrivorans* (Bsal) reached this salamander biodiversity hotspot.

In an effort to build on our basic understanding of amphibian-microbial symbioses and Bd disease ecology, and answering the questions "what factors influence the distribution of symbiotic bacterial communities?" to "are these communities protective against Bd?," I designed a three part study that involved field research,

microbiological and molecular work, and a laboratory experiment to determine the host and environmental effects on microbial distribution (both anti-Bd and the microbiome as a whole) and to quantify how their distribution related to protection against Bd infection.

Chapter II: ANTIFUNGAL BACTERIA ON WOODLAND SALAMANDER SKIN EXHIBIT HIGH TAXONOMIC DIVERSITY AND GEOGRAPHIC VARIABILITY

Coauthors: Graziella V. DiRenzo, Stephanie A. Yarwood, Evan H. Campbell Grant, Robert C. Fleischer, and Karen R. Lips

ABSTRACT

Diverse bacteria inhabit amphibian skin, some of which inhibit growth of the fungal pathogen, *Batrachochytrium dendrobatidis* (Bd). Yet, there has been no systematic survey of anti-Bd bacteria across localities, species and elevations. This is important given geographic and taxonomic variation in amphibian susceptibility to Bd. Our sites were within the Appalachian Mountains where widespread declines in *Plethodon* salamanders have been reported, although sampling indicated low Bd prevalence. We determined the number and identity of anti-Bd bacteria on 61 salamanders (37 *P. cinereus*, 15 *P. glutinosus*, 9 *P. cylindraceus*) using culturing methods and 16s rDNA sequencing. We sampled co-occurring species at three localities and *P. cinereus* along an elevational gradient (700 – 1000 masl). We identified 50 anti-Bd bacterial OTUs and found that the degree of Bd inhibition was not correlated with relatedness. Five anti-Bd bacteria occurred on multiple species at multiple localities, but none were shared among all species and localities. Sixty-six percent (40/61) of salamanders had at least one anti-Bd bacteria. On average, each salamander had 1.7 anti-Bd bacteria; this varied among localities, but not among co-occurring species. At Shenandoah NP, VA, 96% (25/26) of salamanders had anti-Bd bacteria, averaging 3.3 per

individual, compared to 50% (7/14; average = 0.8) at Catoctin MP, MD and 38% (8/21; average = 0.4) at Mt. Rogers NRA, VA. All salamanders tested negative for Bd. Anti-Bd bacteria are diverse in central Appalachian *Plethodon* salamanders, and their distribution varied geographically. The antifungal bacteria we identified may play a protective role for these salamanders.

INTRODUCTION

The skin microbiome of vertebrates serves as a barrier against pathogens (Gallo & Nakatsuji 2011, Becker et al. 2015a, Lowrey et al. 2015), and can mediate disease risk (Rosenthal et al. 2011, Florez et al. 2015). Lower disease risk in vertebrates has been associated with different characteristics of the microbiome, such as: high bacterial richness (Chang et al. 2008, Verhulst et al. 2011, Longo et al. 2015), specific microbial community assemblages (Verhulst et al. 2011, Becker et al. 2015a, Federici et al. 2015, Schieber et al. 2015), and the presence of microbes that produce metabolites that inhibit growth of harmful microorganisms (Woodhams et al. 2007, Gallo & Nakatsuji 2011, Hol et al. 2015). For amphibians, inter- and intra-specific variation in the skin microbiome (Lauer et al. 2008, Flechas et al. 2012, Kueneman et al. 2014) may contribute to variation in responses to infection by the deadly fungal pathogen, *Batrachochytrium dendrobatidis* (Bd; Crawford et al. 2010, Piovia-Scott et al. 2015). Yet, we know little about how microbial diversity differs among amphibian host species and environments, and how this relates to Bd-associated disease risk. Characterizing these patterns is a step towards understanding evolutionary and ecological processes structuring functionally important microbial skin assemblages, which may aid in development of conservation strategies.

Bd has caused mass die-offs and extirpations of susceptible amphibians on a global scale (Berger et al. 1998, Bosch et al. 2001, Lips et al. 2006, Vredenburg et al. 2010), yet no effective treatments exist for amphibians susceptible to Bd infection in the wild. An active line of research has been to identify bacteria that inhibit Bd growth, hereafter referred to as anti-Bd bacteria, and use their geographic distribution to predict fungal disease outcome in the field (Woodhams et al. 2007, Lam et al. 2010, Kueneman

et al. 2015) or use them in trials of efficiency of bioaugmentation to mitigate Bd-associated disease symptoms (Harris et al. 2009a, Harris et al. 2009b, Vredenburg et al. 2011, Muletz et al. 2012). To date, roughly 255 anti-Bd bacterial operational taxonomic units (OTUs) have been identified from the skin of 37 amphibian species and are deposited in a reference database (Woodhams et al. 2015). Nine different anti-bacteria have been used in bioaugmentation trials (Harris et al. 2009a, Harris et al. 2009b, Woodhams et al. 2012, Nebergall 2013, Becker et al. 2015a).

Bioaugmentation trials using anti-Bd bacteria have had mixed success. For instance, three studies found no effect of an anti-Bd bacterial species, *Janthinobacterium lividum*, in reducing Bd-associated disease symptoms (Becker et al. 2011, Bletz 2013, Nebergall 2013), even though J. *lividum* had previously shown to be effective against Bd (Harris et al. 2009a, Vredenburg et al. 2011, Muletz et al. 2012). This suggests that a bacterial species may not be anti-Bd for all host species or in all environments.

Host and environmental factors may influence anti-Bd bacterial composition or whether specific species are inhibitory. For instance, Bresciano et al. (2015) found several anti-Bd bacteria at high-elevation sites, but did not detect these anti-Bd bacteria at low elevations, suggesting that environmental factors may impact the distribution of anti-Bd bacteria. If an amphibian population does not naturally harbor a bacterial species, that bacteria may not colonize or persist on the host if used in bioaugmentation trials (Bletz et al. 2013). Further, temperature can impact whether anti-Bd bacteria exhibit inhibitory activity, and this may depend on the species of host or bacteria (Daskin et al. 2014, Woodhams et al. 2014).

We quantified differences in the number and identity of anti-Bd bacteria on three *Plethodon* species at three localities, and along an elevational gradient in the central Appalachian Mountains. We chose *Plethodon* salamanders because previous reports described widespread declines in abundance of *Plethodon* salamanders, but sampling indicated low prevalence of Bd even though Bd often occurs on other amphibians in the region (Muletz et al. 2014). This contrasts with widespread declines caused by Bd in closely related Plethodontid salamanders in Central America (Cheng et al. 2011). The skin microbiome of *Plethodon* salamanders has been shown to limit Bd infection (Harris et al. 2009b, Becker & Harris 2010, Muletz et al. 2012, Muletz et al. 2014) and we expected anti-Bd bacteria to be prevalent. We also expected that different host species and populations would harbor different anti-Bd bacteria given known species- (Flechas et al. 2012, McKenzie et al. 2012) and population-level (Lauer et al. 2008, Kueneman et al. 2014, Bresciano et al. 2015) differences in amphibian skin bacterial communities. However, we expected a few anti-Bd bacteria, such as *J. lividum*, to be widely distributed across species and localities given previous reports of these taxa being present on *Plethodon* salamanders (Harris et al. 2006, Lauer et al. 2007). Our overall objectives were to (i) describe the geographic and taxonomic distribution of anti-Bd bacteria, (ii) determine how host and site factors influence the number of anti-Bd bacteria detected, and (iii) determine if Bd inhibition score was related to bacterial phylogenetic relatedness. The inhibitory properties of anti-Bd bacteria may inform research on other emerging fungal pathogens such as *B. salamandrivorans*, which also poses a significant threat to global amphibian biodiversity (Martel et al. 2014, Yap et al. 2015).

METHODS

Field sampling

We sampled three species of terrestrial woodland salamanders, *Plethodon cinereus* (n = 37), *P. glutinosus* (n = 15) and *P. cylindraceus* (n = 9) at sites within three localities located along a 497 km stretch in the central Appalachians in spring 2012 (Table 2.1). We chose the three localities, Catoctin Mountain Park (MP), MD, Shenandoah National Park (NP), VA, and Mt. Rogers National Recreation Area (NRA), VA, because they were within the range of localities where we had previously tested these species for Bd and found < 1% Bd prevalence (Muletz et al. 2014), and were within the distribution of *P. cinereus* and either *P. glutinosus* or its sister species *P. cylindraceus* (Petranka 1998). At Catoctin MP, we sampled *P. cinereus* (n = 7) and *P. glutinosus* (n = 7) at one site. At Shenandoah NP, we sampled 19 *P. cinereus* at six sites along an elevational gradient and sampled seven *P. cylindraceus* at four of those sites (Table 2.1). At Mt. Rogers NRA we sampled two *P. cylindraceus* and six *P. cinereus* at one site, and eight *P. glutinosus* and five *P. cinereus* at a second site (Table 2.1). We had permits from state and federal agencies for handling and swabbing live amphibians (Maryland: DNR Permit No. 50269, Virginia: VDGIF Permit No. 042151 and Shenandoah National Park: NPS Permit No. SHEN-2011-SCI-0014), and we received approval for the research from the University of Maryland Institutional Animal Care and Use Committee (R-11-11).

We collected two skin swabs from each salamander, one to culture bacteria and one to test for the presence of Bd. We used a new pair of nitrile powder-free gloves to handle each salamander, and rinsed each salamander twice for 30 seconds with sterile water to remove transient microbes (Lauer et al. 2007). We placed each individual into a

new plastic bag, and swabbed it 20 times on the left side (five strokes each: dorsal/ventral sides and front/back limbs) with a MW-113 swab (Medical Wire, UK). We stored the swab in a 1.5 ml tube on ice until returning to the laboratory where samples were stored in a -80 °C freezer until analysis for Bd quantification. Then, we swabbed the right side of each salamander 20 times, and immediately streaked the swab onto a R2-A nutrient agar plate in a zigzag fashion and wrapped the plate in parafilm in the field. We stored plates at ambient temperature (12°C to 23°C) throughout incubation.

At each site, we recorded GPS coordinates, leaf litter depth and soil pH (Kelway soil tester). For each individual, we (i) measured their substrate temperature at capture (Fluke infrared thermometer), (ii) recorded their cover object, (iii) identified their species and sex, and (iv) measured their mass and snout-to-vent length (SVL) and mass to quantify their body condition. We expected that these variables may influence the distribution of anti-Bd bacteria from previous research on their effects on microbial diversity, such as: sources of environmental microbes (e.g., leaf litter depth and cover object; (Fitzpatrick & Allison 2014, Loudon et al. 2014), pH (Shen et al. 2013), temperature (Sunagawa et al. 2015), species (McKenzie et al. 2012), sex (Krynak et al. 2016) and body condition (Ruiz-Rodriguez et al. 2009).

Microbiology procedures

We isolated morphologically distinct bacterial colonies into pure cultures based on color, form, elevation, margin, substance and opacity. We preserved isolates in 20% glycerol in a -80 °C freezer until challenge assays were conducted.

We conducted bacterial-Bd assays with Bd isolate JEL 404 (Maine, USA) using a modified protocol based on Bell et al. (Bell et al. 2013). To begin, we grew cryopreserved bacteria on 1% tryptone plates for three days, passaged the bacteria in 3 ml of 1% tryptone broth, and grew on a shaker at 100 rpm for an additional three days. Then, we made bacterial/Bd co-cultures, Bd monocultures, and negative controls in 3 ml culture tubes. For bacterial/Bd co-cultures we added 100 µl of a bacterial isolate and 100 µl of Bd to 1 ml of 1% tryptone broth. We grew bacteria and Bd together to mimic natural conditions as bacteria and Bd likely interact on amphibian skin. Nonetheless, Becker et al. (2015b) demonstrated that testing Bd/bacterial co-cultures versus bacterial monocultures against Bd had no effect on the magnitude of Bd inhibition. For Bd monocultures we added 100 µl of Bd to 1.1 ml of 1% tryptone broth. For a negative control we added 1.2 ml of 1% tryptone broth to the 3 ml tubes. We grew these cultures on a shaker at 100 rpm for three days. To obtain microbial-produced metabolites termed cell-free supernatant (CFS; Bell et al. 2013), we centrifuged the bacterial/Bd co-cultures, Bd monocultures, and negative controls at 10,000 rpm for 5 minutes. Then, we used 18 gauge hypodermic needles attached to 3 ml syringes (Bd Vacutainer No. 309657) to remove the supernatant, and filtered the supernatant through 0.22 µm filters in 13 mm syringe-filter holders (Millipore GSWP01300/SX0001300). We harvested Bd zoospores by flooding one-week old Bd plates grown at RT with 1% tryptone and filtering the broth through a sterilized coffee filter in a glass funnel.

To set up the assays, we added 50 µl of approximately $1x10^6$ zoospores/ml of JEL 404 (counted with hemocytometer; approximately 50,000 zoospores in each well) to a 96-well plate in all wells except those designated for negative controls. In bacterial-Bd

sample wells, we added 50 µl of the CFS from each bacterial-Bd sample in each of four wells. We used four controls (two negative controls and two positive controls) in each 96-well assay using four wells for each control. The positive controls were: 50 µl of Bd zoospores + 50 µl of Bd CFS (positive control: PC) and 50 µl of Bd zoospores + 50 µl of water (nutrient-depleted positive control: NDPC). The negative controls were: 50 µl of Bd zoospores heat-killed at 60°C for 60 minutes + 50 µl of Bd CFS (heat-killed Bd: HK) and 50 µl of 1% tryptone broth + 50 µl of Bd CFS. We measured absorbance (OD_{492nm}) of each well using a microplate reader on Days 0, 1, 4, 7, 8, and 10 of the experiment.

Bd inhibition score calculations

We used the day of maximum growth (Day 7 or 8) of the PC for each assay as the day to quantify Bd inhibition. To calculate Bd inhibition scores, we divided the average OD reading of the bacterial-Bd sample (n = 4) by the average OD reading of the NDPC for that assay (n = 4), after correcting for the average OD of the heat-killed Bd (HK, n = 4), and subtracted that value from one. We subtracted the OD of the HK to remove the baseline absorbance of Bd zoospores. The equation we used is as follows: $(1 - ((Sample_{OD} - HK_{OD}) / (NDPC_{OD} - HK_{OD})))$. Values greater than zero were defined as being anti-Bd bacteria, and indicated that the bacterial-Bd sample wells had less growth than the NDPC wells as measured by OD. Prior to OD readings, we conducted independent observations of each well using an inverted microscope. We coded visual observations of each set of sample wells as no, weak, moderate, strong inhibition in comparison to the NDPC wells.

To identify anti-Bd bacteria, we used a conservative approach: (i) both OD readings and visual observations had to support inhibition, and (ii) we used the NDPC rather than the PC to quantify Bd inhibition strength. We used the NDPC because the experimental wells in challenge assays contained 50 µl of CFS in which bacteria and Bd had previously been cultured, while the PC wells contained 50 µl of previously unused medium. The growth of Bd in experimental wells could have been affected by depletion of nutrients used by the bacteria and Bd. Therefore, comparing experimental wells to the NDPC accounts for the issue of potential nutrient depletion, and is a more conservative approach in identifying anti-Bd bacteria than comparing experimental wells to the PC wells.

We did not standardize bacterial population size or stage of growth among bacteria. Thus, some bacteria may have produced more secondary metabolites under the selected culture conditions and had higher Bd inhibition scores. Instead, we attempted to reduce this by allowing the bacterial culture to grow for three days so that most cultures reached the later phases of growth when inhibitory metabolites are generally produced (Berdy 2005). This is a commonly accepted practice and is the currently the best method for testing a large number of bacterial isolates (Bell et al. 2013, Daskin et al. 2014, Antwis et al. 2015, Becker et al. 2015b).

Molecular procedures

We sequenced the 16S rRNA gene of all anti-Bd bacterial isolates using Sanger sequencing. We selected a single colony of each isolate from agar plates using a sterilized toothpick, added the colony to 25 µl of sterile deionized water and boiled the mixture for

10 minutes at 95°C to extract bacterial DNA. We used PCR to amplify a 1037bp fragment of the 16s rRNA gene using the 8F/1045R primer set. Each 25-µl PCR assay consisted of 1.25U of AmpliTaq Gold DNA Polymerase (ThermoFisher) in proprietary buffer, 2.5 µM $MgCl_2$, 200 nM dNTPs, 600 nM of each primer and 2 µl DNA template. PCR conditions were 95 °C for 8 m, followed by 30 cycles of 95 °C for 45 s, 52 °C for 30 s, 72 °C for 30 s, and a final extension (72 °C for 3 m). We followed the sequencing methods outlined in Muletz et al. (Muletz et al. 2014) to sequence the cleaned PCR products. Individual sequences were assembled and edited in Sequencher 5.1 to obtain a consensus sequence per anti-Bd bacterial isolate.

We tested all sampled salamanders for Bd using qPCR. We extracted DNA from swabs using MoBio PowerSoil DNA Extraction kit following the manufacturer's protocol. We used primers developed by Boyle et al. (Boyle et al. 2004) and used iTaq supermix with Rox (Bio-Rad) following their qPCR reaction protocol. We ran all DNA samples in duplicate and used standards of 100, 10, 1, 0.1 zoospore genomic equivalents (ZGEs) developed from a Puerto Rican Bd isolate, JEL 427. If one of the duplicates returned a positive signal it was run a third time. Samples were considered positive if they amplified twice before 0.1 ZGEs.

Sequence analysis

We identified anti-Bd bacteria to the lowest taxonomic level with MacQIIME 1.9.1 (Caporaso et al. 2010) using the *pick_de_novo_otus.py* command with default parameters. This command clustered sequences into operational taxonomic units (OTUs) using UCLUST based on 97% pairwise identity and assigned taxonomy using UCLUST

(Edgar 2010) with the Greengenes reference database (DeSantis et al. 2006) 13_8 release). We aligned the bacterial isolate sequences using the Ribosomal Database Project's (http://rdp.cme.msu.edu) alignment tools following Dunitz et al. (Dunitz et al. 2015), including an archaeal outgroup in the alignment. We built a phylogenetic tree using Fasttree (Price et al. 2010) implemented in MacQIIME and used the package 'ape' (Paradis et al. 2004) in the R environment (Team 2015) to root the tree and trim the outgroup.

Comparison of identified anti-Bd bacteria to published database

We compared our anti-Bd bacterial 16s rRNA sequences to those in a published database (Woodhams et al. 2015) to identify common and novel anti-Bd bacteria. As above, we identified bacterial OTUs using the *pick_de_novo_otus.py* command with default parameters in MacQIIME 1.9.1. We then split the OTU table to only contain anti-Bd bacteria using the *split_otu_table.py* command, and filtered out zeros (not anti-Bd bacteria) using the *filter_otus_from_otu_table.py* command. We summed the number of anti-Bd bacteria OTUs in the Woodhams database, which contains a total of 255 OTUs. Next, we used a custom blast in Geneious 8.1 (Kearse et al. 2012) to query our anti-Bd bacterial isolate sequences against those 255 anti-Bd bacterial OTUs. We used a megablast program, having Geneious return results as query-centric alignment only and returning only the top hit. We considered anti-Bd bacteria in our dataset to be novel if all sequences in that OTU had < 97% pairwise identity to the OTUs in the Woodhams dataset. For common anti-Bd bacteria in our dataset, we counted the number of amphibians in the Woodhams et al. (2015) dataset that had OTUs that matched at > 97%

pairwise identity. It is important to note that while these OTUs matched the antifungal databases it does not necessarily indicate that these bacteria exhibit antifungal activity, but that they are strong candidates for exhibiting antifungal activity.

Statistical analysis

All statistical analyses were performed in R 3.1.3 (Team 2015).

To determine if the number of salamanders with at least one anti-Bd bacterial OTU differed among the three localities, we used a χ^2 contingency table test. To determine if the number of anti-Bd bacteria per salamander was related to host or environmental characteristics, we used a generalized linear mixed-effects model (GLMM) with a binomial distribution in the package 'lme4' (Bates et al. 2015). We included species and locality as the main explanatory variables, along with additional covariates of host (sex, body condition, cover object) and site (leaf litter depth, soil pH, substrate temperature). As the response variable in the model we used proportion of anti-Bd bacteria per salamander (i.e., total anti-Bd bacteria divided by total bacteria isolated) to account for variation in the number of isolates cultured per individual. Nonetheless, if we used raw counts of anti-Bd bacterial OTUs as the response variable in the GLMM with a Poisson distribution our results remained the same. We included site as a random effect in the model to account for residual variation among sites, as we sampled multiple sites at some localities. To quantify salamander body condition, we used the residuals of the linear regression of body mass on SVL for *P. cinereus* and for *P. glutinous* complex (*P. cylindraceus* and *P. glutinosus*) separately as *P. cinereus* is smaller in size than the *P. glutinosus* complex. To determine which variables were significant, we used the *Anova*

function in the 'car' package (Fox & Weisberg) with type III margin sum of squares reported. We used Tukey HSD post-hoc comparisons to perform post-hoc comparisons for significant explanatory variables using the *lsmeans* function in the package 'lsmeans' (Lenth 2015). We assessed model goodness-of-fit by visually inspecting residuals.

We used a generalized linear model to determine if the number of anti-Bd bacteria per salamander was related to elevation for *P. cinereus* sampled along an elevational gradient in Shenandoah NP. As above, we used proportion of anti-Bd bacteria per salamander as the response variable. We assessed goodness of fit for the model by visually inspecting residuals, and used a quasibinomial distribution to account for overdispersion.

We tested if Bd inhibition scores were phylogenetically conserved using a Mantel test. We examined this relationship to see if phylogenetic information could be used as a general predictive tool for Bd inhibition strength. We generated a distance matrix of Bd inhibition scores among anti-Bd bacterial isolates using Euclidean distances (Becker et al. 2015b) and compared that to the distance matrix of percent sequence identity from the anti-Bd bacterial isolate sequence alignment. We used the 'vegan' package (Oksanen et al. 2015) to conduct the Mantel test with 10,000 permutations. We visualized inhibition scores of anti-Bd bacteria on the bacterial phylogeny using iTol ((Letunic & Bork 2011); http://itol.embl.de/).

Data accessibility

We deposited the 16s rRNA sequences for the 119 anti-Bd bacterial isolates in GenBank (accession numbers: KU738912 - KU739030).

RESULTS

We isolated and tested 341 bacteria from three localities (Catoctin MP, n = 103; Shenandoah NP, n = 187; Mt. Rogers NRA, n = 51), collected from 61 *Plethodon* salamanders with an average of six bacterial morphotypes per individual (range: 0 – 17, Table 2.1). We identified 119 of those bacterial isolates as anti-Bd (Figure 2.1), which represented a total of 50 anti-Bd bacterial OTUs across all salamanders (Table 2.1). The 50 anti-Bd bacteria belonged to four bacterial phyla (Actinobacteria, n = 5; Proteobacteria, n = 33; Bacteroidetes, n = 11; Firmicutes, n = 1), with Bd inhibition ranging from 1 – 100% (Figure 2.1). We found that more closely related anti-Bd bacteria did not share similar Bd inhibition scores (Figure 2.1; Mantel: p = 0.865, Mantel statistic r: -0.023).

Most anti-Bd bacteria were rare; thirty-five anti-Bd bacteria were found on only one salamander individual. Ten anti-Bd bacteria were detected either on one salamander species or at one locality. A few anti-Bd bacteria were widespread; specifically, five anti-Bd bacteria were found in multiple localities and on multiple salamander species (Table 2.2; *Acinetobacter rhizosphaerae, Luteibacter rhizovicinus,* two *Pseudomonas* spp., and one *Stenotrophomonas* sp.). These five bacteria have also been commonly detected on other amphibian species globally (Woodhams et al. 2015; Table 2.2). *Luteibacter rhizovicinus* was the only anti-Bd bacterial OTU found at all three sampled localities, and a *Pseudomonas* sp. was the only anti-Bd bacterial OTU found on all three salamander species. No anti-Bd bacteria were found at all localities and on all salamander species.

We identified 13 novel anti-Bd bacteria that did not match any bacteria previously identified on amphibian skin (Woodhams et al. 2015). Twelve of those novel anti-Bd

bacteria occurred only on a single individual, while one was detected on two heterospecific individuals at one site.

Most salamanders had at least one anti-Bd bacteria (40/61, 66%), with a higher proportion at Shenandoah NP (25/26, 96%), than Catoctin MP (7/14, 50%) and Mt. Rogers NRA (8/21, 38%) (X^2 contingency table test: $X^2 = 12.9$, df =2, $p < 0.0001$; Table 2.1).

Locality was the only significant predictor of the proportion of anti-Bd bacteria per individual (GLMM: LR Chisq = 12.0107, df = 2, p = 0.0025). The average proportion of anti-Bd bacteria per individual was higher at Shenandoah NP, with 46% of bacteria isolated being anti-Bd bacteria (3.3/7.2), while 11% at Catoctin MP (0.8/7.3) and 17% at Mt. Rogers NRA (0.4/2.4) were anti-Bd bacteria (Table 2.1). Of the host (species, sex, body condition, cover object) and site (leaf litter depth, soil pH, substrate temperature) characteristics we examined, none were significant predictors of the proportion of anti-Bd bacteria per individual (GLMM: $p > 0.05$). On *P. cinereus* in Shenandoah NP, the proportion of anti-Bd bacteria per individual was similar among all elevations (GLM: t-value = 0.566, p = 0.58; Table 2.1).

We did not detect Bd on any of the salamanders.

DISCUSSION

Geographic and taxonomic distribution of anti-Bd bacteria

We identified a large number of anti-Bd bacterial taxa on the three *Plethodon* species, but few were widespread. Other studies have also found high diversity and little overlap in anti-Bd bacteria among individual amphibians (Woodhams et al. 2007, Lam et

al. 2010, Flechas et al. 2012). High taxonomic turnover across the landscape with functional trait redundancy have been reported in other microbial communities (Thurber et al. 2009, Sunagawa et al. 2015). Turnover in the regional pool of microorganisms is likely due to different environmental conditions driving environmental filtering of species, but not microbial traits (Fierer & Jackson 2006, Cavender-Bares et al. 2009, Sunagawa et al. 2015). This indicates that selection for certain bacteria may be driven by the function of the bacteria and not the species identity. For instance, Loudon et al. (2016) found that bacteria with anti-Bd properties are more likely to colonize a host than those that lack anti-Bd properties, suggesting that certain bacteria may be more prevalent on amphibian skin given their functional properties regardless of species identity.

Five anti-Bd bacteria were widely distributed across multiple localities and multiple salamander species, suggesting a general association between these anti-Bd bacteria and *Plethodon* salamanders. Specifically, *Pseudomonas* spp. were the most widespread in both our study and others (Woodhams et al. 2015). Several ecological and physiological reasons could explain this, including (i) their ability to utilize available glucose (Walke et al. 2011) or to produce siderophores (Jagadeesh et al. 2001), both of which would reduce resource availability for competitors, or (ii) selection by the host for pseudomonads given their capacity to produce numerous antimicrobial compounds (Leisinger & Margraff 1979, Ligon et al. 2000). If generalist anti-Bd bacteria, such as pseudomonads, are important as an amphibian defense mechanism against Bd then the abundance of these bacterial species may be a key factor in protection (Wei & Zhang 2006, Yasumiba et al. 2016). In some cases, high cell density is needed to produce inhibitory metabolites because the metabolites are only produced in high quantities when

bacteria form a biofilm and undergo quorum sensing (Barnard et al. 2007, Pantanella et al. 2007). Bd likely reduces the growth of some anti-Bd bacteria (Jani & Briggs 2014), and augmenting these bacteria on susceptible amphibians during Bd epidemics may prove helpful.

We expected *J. lividum* to be common as it was previously detected, using molecular approaches, on most individuals in two of three *P. cinereus* populations in Virginia (Lauer et al. 2007, Muletz et al. 2012, Loudon et al. 2014). However, using culture methods, we found anti-Bd bacterial OTUs in the *Janthinobacterium* genus on only three *P. cinereus* at two of our three sampled localities (Catoctin MP and Shenandoah NP). *Janthinobacterium* spp. may be widely distributed, but not always common across the range of *P. cinereus*. Alternatively, *Janthinobacterium* or other anti-Bd bacteria may commonly occur, but be in low abundance (Loudon et al. 2014) and less likely to be cultured.

Influence of host and site factors on anti-Bd bacterial distribution

We found no support for host species factors influencing the number of anti-Bd bacteria. Other studies have shown that skin bacterial richness is different among amphibian species (McKenzie et al. 2012, Kueneman et al. 2014, Becker et al. 2015a). However, the processes that structure the antifungal bacterial community may differ from those structuring the total bacterial community, given different selection pressures (Nemergut et al. 2013, Antwis et al. 2015). For instance, bacteria can diversify more in the presence of a parasite (Knope et al. 2012), and even a single gene locus change can modify community structure to the same extent as the loss of an apex predator (McClean

et al. 2015). Alternatively, host biology of *Plethodon* salamanders may explain the differences (Wong et al. 2013); we studied terrestrial, direct-developing amphibian species within a single genus that are ecologically similar throughout their lifetime (Petranka 1998) in contrast to previous studies that studied different families of amphibians with complex life cycles (McKenzie et al. 2012, Kueneman et al. 2014, Walke et al. 2014).

Location was a key predictor of the prevalence and number of anti-Bd bacteria indicating environmental differentiation. However, none of the site characteristics we examined (substrate temperature, soil pH and leaf litter depth) explained this pattern. Across the three localities, salamanders at Shenandoah NP had a higher number of anti-Bd bacteria than salamanders at Catoctin MP or Mt. Rogers NRA. Other environmental properties such as long-term trends in temperature may have influenced salamander bacterial diversity (Meyer et al. 2012), as both Catoctin MP and Mt. Rogers NRA have experienced faster rates of warming than Shenandoah NP (Caruso et al. 2014).

Spatial scale may explain variation in the number of anti-Bd bacteria among localities, but not along the elevational gradient. Environmental differences (e.g., temperature, moisture) from localities 150 km away (e.g. Catoctin MP to Shenandoah NP) may be more likely to drive diversity patterns than the small-scale variations in environmental conditions from 700 masl to 1000 masl within Shenandoah NP. For instance, Sunagawa et al. (2015) found temperature to be the most important factor driving ocean microbiome richness rather than other environmental factors (e.g., salinity, nutrients) or geography, but only when large temperature differences were examined. In other systems, differences in geochemistry, such as pH (Fierer et al. 2011, Sunagawa et

al. 2015), total nitrogen (Shen et al. 2013), or dissolved organic matter (Wang et al. 2011), can influence bacterial composition more than climate or elevation. We suggest future research on habitat chemistry to identify the factors driving environmental differentiation.

We did not detect Bd in this study, but Bd has been detected at all of the three localities we sampled (Hossack et al. 2010, Muletz et al. 2014, Lips, unpublished data). Bd is commonly detected in amphibian communities throughout the eastern US with prevalence estimates of 10-40% (reviewed by (Muletz et al. 2014). This suggests that these salamanders can be exposed to Bd and that anti-Bd bacteria or other factors (e.g., immunogenetic properties (Savage & Zamudio 2011, Ellison et al. 2014) may be limiting Bd infection on these salamanders. Specifically, we found evidence for herd immunity against Bd (Anderson & May 1990, Woodhams et al. 2007) at Shenandoah NP, with 96% of salamanders having at least one anti-Bd bacteria. The concept of herd immunity suggests that an infectious disease will be less likely to affect a population if the proportion of protected individuals is above a threshold value, generally between 80-95% (Anderson & May 1990). In the amphibian-Bd system, Woodhams et al. (2008) suggests that this value is approximately 80%, as a population of *R. muscosa* with 86% of individuals harboring anti-Bd bacteria persisted with Bd, whereas a population with 62% died-off following Bd invasion. We found 50% and 38% of salamanders at Catoctin MP and Mt. Rogers NRA had anti-Bd bacteria, respectively, suggesting other mechanisms of protection for those salamanders from Bd infection. Alternatively, more anti-Bd bacteria could have been present, but we were unable to detect them due to the limitations of culturing (Walke et al. 2015).

Association between Bd inhibition and anti-Bd bacterial phylogeny

Bd inhibition strength was not related to bacterial phylogeny, indicating that phylogenetic information is not informative for Bd inhibition strength. Nonetheless, certain genera contain a large proportion of anti-Bd bacteria, chiefly *Pseudomonas* and *Stenotrophomonas*, and detecting bacterial sequences in those genera likely indicate that many of those species have anti-Bd traits (Becker et al. 2015). Two possible, non-exclusive, mechanisms may explain the lack of phylogenetic conservatism of Bd inhibition: (i) genes involved in inhibition are widely distributed among bacterial phylogenies or (ii) horizontal gene transfer of antifungal genes may occur among unrelated bacteria. First, functions involving few genes are often widely distributed among bacterial phylogenies (Martiny et al. 2013), and relatively few genes can be involved in the biosynthetic pathways that produce secondary metabolites. For instance, the genes that produce the anti-Bd metabolites violacein (Brucker et al. 2008b) and 2,4-diacetylphloroglucinol (Brucker et al. 2008a) consist of five and eight genes, respectively (Sanchez et al. 2006, Moynihan et al. 2009). Second, horizontal gene transfer of genes important in Bd inhibition may be occurring among unrelated bacteria, as homologous recombination can occur among bacterial lineages as divergent as 25% in DNA (Cohan 2001). For instance, the transfer of genes encoding antifungal compounds has been observed between different bacterial species (Kinashi et al. 1987, Ravel et al. 2000). While we cannot determine which mechanism(s) operated, we found that anti-Bd bacterial traits were phylogenetically dispersed among at least four bacterial phyla, demonstrating wide taxonomic breadth of this trait.

Conservation implications

There are no proven strategies to prevent infection, mortality, or population declines of amphibians from Bd. The use of anti-Bd bacterial augmentation has been championed as a conservation strategy (Woodhams et al. 2011, Bletz et al. 2013), but results have been inconsistent, in part because environment may control bacterial communities on amphibian skin. We found that individual salamanders had a mostly unique anti-Bd bacterial profile, with a few shared bacteria, suggesting that enhancing the functional traits of the existing anti-Bd bacterial community (Mueller & Sachs 2015) or using only widely distributed anti-Bd bacteria may be beneficial bioaugmentation strategies (Bletz et al. 2013). One method to enhance the functional traits of a community is to use prebiotics, which selectively promote growth or activity in the community. For instance, polysaccharide b-glucan has been commercially used as a prebiotic to improve resistance of fish to infection by pathogens (Song et al. 2014). Additionally, using ubiquitous anti-Bd bacterial species in bioaugmentation trials, such as those identified here, may increase the likelihood that the bacteria will persist on target amphibians (Bletz et al. 2013). In two bioaugmentation studies, introduction of anti-Bd bacteria onto amphibian skin did not affect the skin microbial community (Kueng et al. 2014, Becker et al. 2015a), indicating that adding probiotics is likely not detrimental to the host in terms of their resident microbial community. Yet, augmented anti-Bd bacteria often do not persist on amphibian hosts for longer than a few months (Harris et al. 2009a, Muletz et al. 2012), and re-inoculation over time may be needed (Ackleh et al. 2016).

Inhibitory metabolites produced by anti-Bd bacteria likely have general antimicrobial properties (Leisinger & Margraff 1979) and may provide a general

defensive benefit against pathogens to the host (Lauer et al. 2007, Lauer et al. 2008, Federici et al. 2015, Kueneman et al. 2015). Therefore, our results may also be useful if the deadly, closely related fungal pathogen *Batrachochytrium salamandrivorans* (Martel et al. 2014) reaches this salamander biodiversity hotspot (Yap et al. 2015).

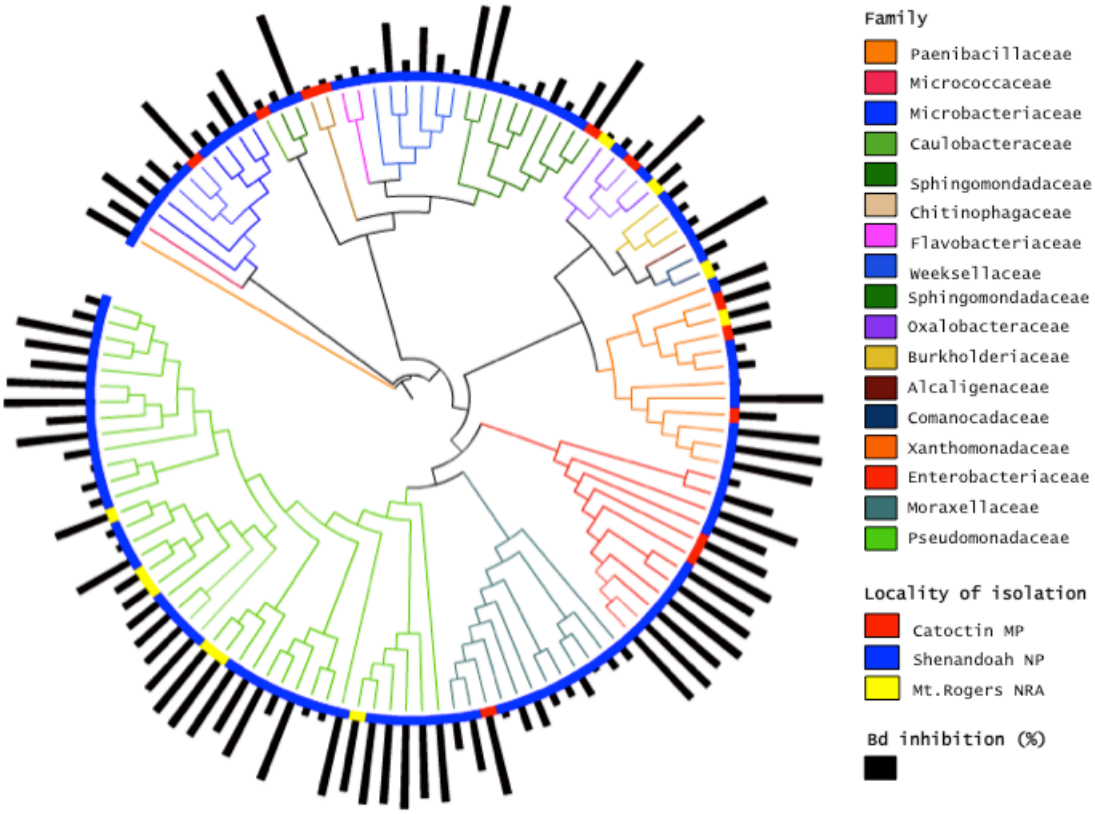
Table 2.1 Summary of salamanders sampled with the number of salamanders with at least one anti-Bd bacteria, and number of total bacterial isolates and anti-Bd bacterial isolates detected per salamander. Salamanders at Shenandoah NP had higher prevalence of anti-Bd bacteria (96%) and a higher number of anti-Bd bacteria per individual (mean = 3.3) than at Catoctin Mountain Park, MD (50%, mean = 0.8) or Mt. Rogers National Recreation Area, VA (38%, mean = 0.4), regardless of species. At Shenandoah NP, *P. cinereus* had a similar proportion of anti-Bd bacterial isolates along an elevational gradient.

Locality	Sites	Elevation (m)	Species	No. salamanders sampled	No. salamanders with anti-Bd bacteria	Avg. isolates per salamander (range)	Avg. anti-Bd isolates per salamander (range)
Catoctin	1	404	*P. cinereus*	7	4	8.9 (5 – 14)	1 (0 – 2)
Catoctin	1	404	*P. glutinosus*	7	3	5.7 (2 – 9)	0.6 (0 – 2)
			Total:	14	7	7.3	0.8
Shenandoah	1	702	*P. cinereus*	4	3	5.5 (1 – 10)	2.3 (0 – 3)
Shenandoah	2	797 ± 6	*P. cinereus*	6	6	9 (5 – 14)	3.7 (1 – 7)
Shenandoah	2	881 ± 18	*P. cinereus*	4	4	6.3 (4 – 11)	3.8 (1 – 7)
Shenandoah	1	974	*P. cinereus*	5	5	9.4 (6 – 16)	4.4 (3 – 6)
Shenandoah	4	697 – 974	*P. cylindraceus*	7	7	5.6 (2 – 8)	2.4 (1 – 4)
			Total:	25	26	7.2	3.3
Mt. Rogers	2	997, 1053	*P. cinereus*	11	5	2.7 (1 – 6)	0.6 (0 – 2)
Mt. Rogers	1	997	*P. glutinosus*	8	2	2 (0 – 3)	0.25 (0 – 1)
Mt. Rogers	1	1053	*P. cylindraceus*	2	1	2 (0 – 4)	0.5 (0 – 1)
			Total:	21	8	2.4	0.4
			TOTAL:	**61**	**40**	**5.6**	**1.7**

Table 2.2 Summary of the five common anti-Bd bacteria that we detected on at least two salamander species and at least two localities. We indicate the number of amphibian species that these same anti-Bd bacterial OTUs have been detected on in previous studies using the Woodhams et al. ((36); 37 total species) database.

Anti-Bd bacterial OTU	Mean % inhibition (range)	Locality	No. sites	Salamander species	No. salamanders	No. other amphibian species in Woodhams et al. (2015)
Acinetobacter rhizosphaerae (denovo45)	28 (1 – 99)	Catoctin, Shenandoah	6	*P. cylindraceus, P. cinereus*	9	4
Luteibacter rhizovicinus (denovo18)	35 (11 – 58)	Catoctin, Shenandoah, Mt. Rogers	4	*P. cylindraceus, P. cinereus*	4	2
Pseudomonas sp. (denovo13)	58 (4 – 100)	Shenandoah, Mt. Rogers	7	*P. cylindraceus, P. cinereus, P.glutinosus*	14	25
Pseudomonas sp. (denovo20)	63 (1 – 100)	Shenandoah, Mt. Rogers	7	*P. cylindraceus, P. cinereus*	11	26
Stenotrophomonas sp. (denovo22)	89 (45 – 100)	Catoctin, Shenandoah	4	*P. cylindraceus, P. cinereus*	4	1

Figure 2.1 Phylogenetic distribution of Bd inhibition by anti-Bd bacteria across 17 families of bacteria. Branches represent individual isolates that were cultured from *Plethodon* salamanders sampled at three localities. Each bar represents the percent Bd inhibition (1 – 100%) by each isolate. We found that Bd inhibition values were not related to the phylogenetic relationship of the anti-Bd bacteria (Mantel test: $p > 0.05$), suggesting functional trait redundancy.



Chapter III: EFFECTS OF HOST SPECIES AND ENVIRONMENT ON THE SKIN MICROBIOME OF PLETHODONTID SALAMANDERS

Coauthors: Stephanie A. Yarwood, Evan H. Campbell Grant, Robert C. Fleischer, and Karen R. Lips

ABSTRACT

The amphibian skin microbiome is recognized for its role in defense against pathogens, including the deadly fungal pathogen *Batrachochytrium dendrobatidis* (Bd). Yet, we have little understanding of evolutionary and ecological processes that structure these communities, especially for closely related species. We characterized the effects of host species and environment on skin microbiome structure (alpha-diversity, beta-diversity and bacterial OTU abundances) and quantified the prevalence and abundance of putatively anti-Bd bacteria on sympatric, congeneric salamanders. We performed 16S rRNA gene meta-barcoding sequencing on skin swabs from 100 *Plethodon* salamanders (37 *P. cinereus*, 15 *P. glutinosus*, 9 *P. cylindraceus*). Co-occurring salamanders had similar microbiome structure, but among sites beta-diversity and abundance of 28 bacterial OTUs differed. Sampling *P. cinereus* along an elevational gradient (700 – 1000 masl), we found that alpha-diversity, beta-diversity and abundance of 17 bacterial OTUs changed with elevation. Eleven putatively anti-Bd bacterial OTUs were present on 90% of salamanders and made up an average relative abundance of 83% (SD ± 8.5) per salamander. All salamanders tested negative for Bd. We conclude that environment is more influential in shaping skin microbiome structure than differences in host properties

for these congeneric species, and suggest that environmental characteristics that co-vary with elevation influence microbiome structure. High prevalence and abundance of anti-Bd bacteria may contribute to low levels of Bd infection in these populations of *Plethodon* salamanders. Describing patterns of skin microbiome structure is necessary to understand bacterial community assembly and the potential role the microbiome plays in control of diseases.

INTRODUCTION

Patterns of host and microbial associations result from evolutionary and ecological processes acting simultaneously on both host and microbe (Herre et al. 1999, McFall-Ngai 2005). Factors shaping host-associated microbiomes include (i) evolutionary history of the host (Ley et al. 2008, Larsen et al. 2013, Council et al. 2016), (ii) environmental conditions (Sullam et al. 2012, Schmidt et al. 2015, Rebollar et al. 2016) and (iii) host-microbial interactions (McFall-Ngai & Ruby 1991, Scheuring & Yu 2012). Understanding how these complex ecological and evolutionary processes contribute to host-associated community assembly remains a challenge (Nemergut et al. 2013), given the limited scope of host taxa, host body regions and environmental habitats sampled (reviewed by Colston & Jackson 2016).

Evolutionary history of the amphibian host is generally the strongest predictor of skin microbiome structure, even for co-occurring species (McKenzie et al. 2012, Kueneman et al. 2014, Walke et al. 2014, Belden et al. 2015). This suggests that host-associated bacterial community composition is not just a mere reflection of the microorganisms in a shared environment. Instead, it implies that there are deterministic mechanisms operating that structure the communities (Schmidt et al. 2015, Loudon et al. 2016), such as biological and chemical traits of the host. Host-associated traits such as chemical content of the skin mucus (Rollins-Smith et al. 2002, Conlon 2011), skin-shedding rate (Meyer et al. 2012), immune genes (Huang et al. 2016) and diet (Antwis et al. 2014) are likely drivers of selection for specific host communities in amphibians. However, it is unknown if host-associated traits that influence symbiotic communities are divergent at all evolutionary time scales. For instance, amphibian skin microbiome

studies have found that microbiome patterns differ among species from different orders (Walke et al. 2014), families (McKenzie et al. 2012, Kueneman et al. 2014, Rebollar et al. 2016) and genera (Belden et al. 2015), yet no study has examined congeneric amphibian species. If congeneric species harbored similar microbial communities, this would suggest that host traits that drive skin-associated bacterial composition might not be divergent at all evolutionary time scales.

Environment is also a strong predictor of amphibian skin microbiome structure, but to a lesser extent than differences among species (Kueneman et al. 2014, Rebollar et al. 2016). Colonization by bacterial symbionts can occur via environmental transmission (Muletz et al. 2012), in which unique sets of bacteria are selected from the regional pool of microorganisms (Fitzpatrick & Allison 2014, Walke et al. 2014, Rebollar et al. 2016). Together, these findings suggests that local environmental conditions influence the pool of potential bacterial symbionts, but that host traits influence which bacterial taxa colonize and establish on the skin. Environmental characteristics such as salinity (Sullam et al. 2012, Schmidt et al. 2015) and temperature (Lokmer & Wegner 2015) predict patterns of microbiome structure in other host-associated microbial communities. However, the environmental characteristics that influence amphibian skin microbiome structure in the wild are largely unknown (Kueneman et al. 2014, Rebollar et al. 2016), but for two aquatic frog species water surface area and conductivity explained some of the variation (Kueneman et al. 2014, Krynak et al. 2016). Surveying along environmental gradients can help determine the specific variables that shape environmental differences in host-associated microbial communities.

Host-microbial interactions that promote association between host and microbe can also influence diversity and community structure of host-associated microbiomes (McFall-Ngai & Ruby 1991, Scheuring & Yu 2012). For instance, it has been proposed that inter- and intra-specific variation in the amphibian skin microbiome relates to variation in susceptibility to a deadly, globally distributed pathogen, *Batrachochytrium dendrobatidis* (Bd; Flechas et al. 2012, McKenzie et al. 2012, Kueneman et al. 2014, Rebollar et al. 2016). Some bacteria isolated from amphibian skin can inhibit Bd growth *in vitro* (anti-Bd bacteria, hereafter) and reduce amphibian mortality or weight loss associated with Bd infection in laboratory and field experiments (Harris et al. 2009a, Harris et al. 2009b, Vredenburg et al. 2011). Loundon et al. (2016) found that environmental bacteria with anti-Bd properties are more likely to colonize an amphibian host than those that lack anti-Bd properties, suggesting a functional role that contributes to community membership. If anti-Bd bacteria were involved in resistance to Bd, we would expect these functionally important bacteria to be prevalent and abundant on amphibians rarely found infected with Bd, such as *Plethodon* salamanders (Muletz et al. 2014). At the population level, a critical fraction of the population may need a protective microbiome to prevent pathogen transmission, thus leading to herd immunity (Anderson & May 1990). For instance, Woodhams et al. (2007) found that an amphibian population with 86% of individuals harboring anti-Bd bacteria was correlated to the population persisting with Bd. At the individual level, theoretical work by (Scheuring & Yu 2012) predicted that the microbiome requires a sufficiently high population density of antibiotic producing bacteria to fend off pathogens.

We quantified the roles of evolutionary relatedness, environmental conditions, and fungal-bacterial interactions on determining skin microbiome structure (alpha-diversity, beta-diversity and bacterial OTU abundances) on three *Plethodon* salamander species (*P. cinereus*, *P. glutinosus* and *P. cylindraceus*) from the central Appalachian Mountains. We had three main objectives: (i) compare the microbiome of co-occurring species at three localities to test whether host species factors contributed more to microbiome structure than a shared environment, (ii) examine the microbiome of *P. cinereus* along an elevational gradient at one locality to determine whether elevation predicted bacterial community structure, and (iii) quantify the prevalence and abundance of putatively anti-Bd bacteria to determine if anti-Bd bacteria are dominant members of the skin bacterial community in a group of salamanders rarely infected with Bd (Muletz et al. 2014).

METHODS

Field sampling

We collected skin DNA swabs (Chapter II) from three species of terrestrial, woodland salamanders, *Plethodon cinereus* (n = 71), *P. glutinosus* (n = 17) and *P. cylindraceus* (n = 12), at sites within three localities located along a 497 km stretch in the central Appalachians in spring 2012 (Table 3.1). We chose the three localities, Catoctin Mountain Park (MP), MD, Shenandoah National Park (NP), VA, and Mt. Rogers National Recreation Area (NRA), VA, because they (i) were within the range of localities where we had previously tested these species for Bd and found < 1% Bd prevalence (Muletz et al. 2014), (ii) were within the distribution of the highly abundant species *P.*

cinereus and either *P. glutinosus* or its sister species *P. cylindraceus* (Petranka 1998), and (iii) were exact localities where we identified culturable, anti-Bd bacteria in a previous study from 61 of the same individuals (Chapter II). We recorded GPS coordinates, leaf litter depth and soil pH of the collection site. For each salamander, we recorded substrate temperature, cover object, species, sex and measured their mass and snout-to-vent length (SVL) to quantify salamander body condition (Chapter II). We had permits from state and federal agencies for handling and swabbing live amphibians (Maryland: DNR Permit No. 50269, Virginia: VDGIF Permit No. 042151 and Shenandoah National Park: NPS Permit No. SHEN-2011-SCI-0014), and we received approval for the research from the University of Maryland IACUC (R-11-11).

Molecular methods

We conducted pyrosequencing of 16s rRNA gene amplicons on four Roche 454 GS Junior runs to characterize the skin bacterial community of the 100 *Plethodon* skin swabs at the Center for Conservation Genomics (National Zoological Park, DC). We extracted DNA from the skin swabs using MoBio PowerSoil DNA Extraction kit following the manufacturer's protocol, and included a negative extraction control with each set of sample extractions. We amplified the V3-V5 region of the 16s rRNA gene using the universal gene primer set 515F (5' GTGCCAGCMGCCGCGGTAA 3') and 939R (5' CTTGTGCGGGCCCCCGTCAATTC 3'). We designed fusion primers such that (i) the forward primer contained the 5' Roche Amplicon Adapter A followed by an 8-bp barcode (Hamady et al. 2008), a CA linker, and the 515F primer sequence, and (ii) the reverse primer contained the 5' Roche FLX Amplicon Adapter B followed by the

939R primer sequence. We performed duplicate PCR reactions for each sample, including the negative extraction controls, and pooled the duplicate reactions in equal volumes following PCR. Each 25-ul PCR assay consisted of 1.25U of AmpliTaq Gold DNA Polymerase (ThermoFisher) in proprietary buffer, 2.5 uM $MgCl_2$, 200 nM dNTPs, 200 nM reverse primer, 400 nM forward primer, and 3 ul DNA template. PCR conditions were 95 °C for 7 m, followed by 30 cycles of 95 °C for 45 s, 55 °C for 30 s, 72 °C for 45 s, and a final extension (72 °C for 7 m). We used SPRI-beads to clean the post-PCR products and ran a subset of samples on a Bioanalyzer chip (Agilent Techologies) to confirm removal of small DNA fragments, primers and excess nucleotides. We quantified molecules per sample using quantitative PCR with a KAPA Library Quantification Kit (Kapa Biosystems), and pooled samples in equimolar amounts. Pyrosequencing runs *WILL BE* deposited in the National Center for Biotechnology Information Sequence Read Archive (www.ncbi.nlm.nih.gov/sra).

We tested all samples for the presence of Bd using standard Bd qPCR methods (Chapter II).

Sequence analysis

We used MacQIIME 1.9.1 (Caporaso et al. 2010b) and UPARSE (Edgar 2013) to process the 454 reads. In QIIME, we assigned reads to samples based on their 8-bp barcode, discarded low quality reads using default parameters, and removed primers. We used UPARSE implemented in USEARCH v7.0 as the OTU clustering method opposed to the default QIIME method (UCLUST); UPARSE produces OTU representative sequences that are more accurate predictions of biological sequences (Edgar 2013). In

UPARSE, we truncated sequences at positions where quality score were < 15 based on Edgar (2013), and then clustered high quality DNA sequences at 97% similarity, chose a representative sequences for each bacterial OTU, and performed chimera checking. Then in QIIME, we used RDP classifier (Wang et al. 2007) to assign taxonomy with at least 80% confidence for each representative OTU using the green genes database (May 2013 version). We aligned sequences using PyNAST (Caporaso et al. 2010a) and built a phylogenetic tree from the representative sequences using FastTree (Price et al. 2010). We included an archaeal 16s rRNA sequence obtained from green genes (*Pyrodictium occultum*; GenBank accession #M21087) to root the phylogenetic tree, and then trimmed the outgroup using the package 'ape' in R (Paradis et al. 2004). OTUs that failed alignment were removed from downstream analyses. We removed one OTU assigned to the family Enterobacteriaceae that was present in the negative extraction control samples. We used QIIME, to identify the core microbiome, defined as OTUs that were present on 90% or more of individuals in total (Loudon et al. 2014).

Measures of microbiome structure

We provide a general descriptive analysis of the bacterial OTUs identified from the three host species, including description of their core microbiome. We examined microbiome structure using three indices: alpha-diversity, beta-diversity and OTU bacterial abundance. We examined two alpha-diversity metrics: total number of observed OTUs (OTU richness) and Faith's Phylogenetic Diversity (Faith's PD; Faith 1992), and two beta-diversity metrics: Jaccard and Unifrac (Lozupone et al. 2011). For bacterial abundance analyses, we performed variance-stabilizing normalization on the raw data

(Paulson et al. 2013, McMurdie & Holmes 2014), filtered the data to contain OTUs that occurred in 5% of samples to reduce spurious significance from low abundance OTUs, and reported false discovery rate (FDR) corrected p-values. All statistical analyses were performed in R version 3.2.3 (Team 2015).

We identified OTUs in our dataset that were taxonomically similar (≥ 97% sequence similarity) to known anti-Bd bacteria (Woodhams et al. 2015), using a custom blast in Geneious 8.1 (Kearse et al. 2012). We queried a database consisting of sequences of anti-Bd bacteria that we identified from 61/100 same individuals from the same *Plethodon* populations (Chapter II; GenBank accession no. KU738912 - KU739030) and the Woodhams et al. (2015) dataset, which consists of anti-Bd bacteria identified from 36 amphibian species sampled globally. We used a megablast program, having Geneious return results as query-centric alignment only and returning only the top hit.

Statistical analyses

We quantified bacterial community structure at sites in which we sampled at least two salamander species (Table 3.1) to determine the relative contribution of host species characteristics versus shared environment in shaping the skin microbiome of sympatric species. For alpha-diversity, we used generalized linear models (GLM) to examine variation in total number of observed OTUs and Faith's PD using quasipoisson and Gaussian distributions, respectively. We included species and site as the main explanatory variables, in addition to covariates of host (sex, body condition, cover object, substrate temperature) and site (leaf litter depth, soil pH). We performed deviance goodness of fit tests for each model to assess model fit, and we determined significance

of variables using the *anova* function with X^2 as the test statistic. For beta-diversity, we used PERMANOVAs (Anderson 2001) in the package 'vegan' (Oksanen et al. 2015) and included the same categorical explanatory variables as in the alpha-diversity analysis. We performed post-hoc analyses using PERMANOVAs, and corrected p-values for multiple comparisons using FDR corrections. For quantitative measurements (body condition, soil pH, substrate temperature and leaf litter depth), we examined the effects of these environmental factors on beta-diversity using distance-based linear modeling (function *capscale* in the package 'vegan') with stepwise AIC (Kueneman et al. 2014). We used principle coordinate analysis (PCoA) to visualize beta-diversity patterns using the 'phyloseq' package (McMurdie & Holmes 2013). For bacterial abundance, we determined if OTUs differed in abundance among species and sites using a zero-inflated log-normal (ZILN) mixture model in the package 'metagenomeSeq' (*fitFeatureModel* function; Paulson et al. 2015). If sites within a locality had no OTUs that were differentially abundance then we pooled the sites together at the locality level to increase statistical power.

We compared bacterial community structure among allopatric populations of *P. cinereus* sampled along the elevational gradient in Shenandoah NP (Table 3.1). For alpha-diversity, we used GLMs to examine variation in total number of observed OTUs and Faith's PD using quasipoisson and Gaussian distributions, respectively. We included elevation as the explanatory variable in addition to covariates of host (sex, body condition, cover object, substrate temperature) and site (leaf litter depth, soil pH) characteristics and assessed model fit and significance of variables as above. For beta-diversity, we computed partial Mantel correlations between compositional dissimilarity

matrices (Jaccard and Unifrac) and an elevational distance matrix after accounting for spatial distance between sites using 10,000 permutations in the package 'vegan' (Oksanen et al. 2015). To examine the effects of environment on beta-diversity, we used distance-based linear modeling with stepwise AIC as above on quantitative measurements (body condition, soil pH, substrate temperature and leaf litter depth). For bacterial abundance, we determined if OTUs were differentially abundant across elevations by comparing log transformed CSS-normalized counts (Paulson et al. 2013) using linear models (Jani & Briggs 2014). To visualize changes in significant OTUs over elevations we used 'phyloseq' and 'ggplot2' packages (Wickham 2009).

We examined microbiome structure for anti-Bd bacteria as we did above for the entire bacterial community among species, sites and elevations. For alpha- and beta-diversity, we followed the same statistical framework. For abundances of anti-Bd bacterial OTUs we denote those OTUs with an asterisk in tables that report the statistical results from the analyses of the entire bacterial community.

RESULTS

Overview of OTU diversity and core microbiome

We generated 224,503 high quality bacterial sequences (342 bp average length) from 100 *Plethodon* salamander skin samples representing 480 OTUs from 20 described bacterial phyla. The taxonomic composition of OTUs consisted predominately of bacteria in six phyla (Proteobacteria: n = 198, Bacteroidetes: n = 83, Actinobacteria: n = 76, Firmicutes: n = 30, Acidobacteria: n = 41, Planctomycetes: n = 13).

Most bacterial OTUs (377/480) were rare; they were found on fewer than five individuals, and together made up 2% of total bacterial sequences. Some OTUs were common, namely 12 OTUs, and were found on 90% of individuals (i.e., core microbiome; Table 3.2), and together made up 88% of total bacterial sequences. Some taxonomic groups were abundant, such as Proteobacteria that had an average relative abundance of 87% (SD ± 7) and Actinobacteria representing an average relative abundance of 10% (SD ± 5). Most notable were *Acinetobacter* and *Pseudomonas* within the phylum Proteobacteria, as they were widely distributed across species, sites and localities (Table 3.2) and dominant in abundance (*Acinetobacter*, average = 44%, SD ± 18, total OTUs = 5; *Pseudomonas*, average = 32%, SD ± 21, total OTUs = 7).

Co-occurring salamanders harbored similar microbiome structure, but composition differed among sites

Bacterial alpha-diversity was similar for all host species at all sites, whereas beta-diversity and bacterial OTU abundances differed among sites, but not between co-occurring species. On average, bacterial alpha-diversity was 59 OTUs per salamander (SD ±18) and a Faith's PD of 4.4 (SD ± 1.3), regardless of species or site (Figure 3.1). None of the host and site covariates tested were related to alpha-diversity (GLM: $p > 0.05$), indicating high homogeneity in alpha-diversity among salamanders from multiple species and sites. In contrast, we found a strong site effect on beta-diversity: skin microbiomes from individuals at Shenandoah NP were consistently different in bacterial composition compared to those at Catoctin MP and Mt. Rogers NRA (Jaccard and Unifrac; PERMANOVA: $p < 0.05$), regardless of host species (Figure 3.2;

PERMANOVA: $p > 0.05$). Soil pH (range 4 – 6.5) was the only significant factor correlated with changes in beta-diversity, and it explained 13% of overall variation. Bacterial abundance varied among localities for 28 OTUs and among species for 5 OTUs (Table 3.2). All 28 OTUs were differentially abundant between sites at Catoctin MP and Mt. Rogers NRA compared to sites at Shenandoah NP (Table A3.1, ZILN mixture model: $p < 0.05$). We did not find any differences in OTU abundances between Catoctin MP and Mt. Rogers NRA (Table A3.1, ZILN mixture model: $p > 0.05$). Five of the 28 OTUs were differentially abundant among salamander species (Table A3.1). However, this was primarily driven by the differences among localities, not within localities, suggesting that co-occurring species generally had similar bacterial abundances (Table 3.3; Table A3.1).

Elevation influenced P. cinereus skin microbiome composition

Alpha-diversity, beta-diversity and the abundances of 17 bacterial OTUs were correlated with elevation. Alpha-diversity increased with elevation (GLM: OTU richness, $p = 0.014$, Faith's PD, $p = 0.004$). For beta-diversity, we found a relationship with elevation, after accounting for distances among sites (Figure 3.3, partial Mantel: Jaccard, $p = 0.035$, $R^2 = 9\%$; Unifrac $p = 0.012$, $R^2 = 11\%$). Soil pH (range 4.8 – 6.3) was the only significant factor correlated with changes in beta-diversity across the gradient, though it explained 5% of overall variation. The abundances of 17 bacterial OTUs were correlated with elevation (linear models: $p < 0.05$, $R^2 = 13 – 43\%$; Table A3.2), with all but one OTU (*Pseudomonas* sp., OTU_5), increasing in abundance with elevation (Figure 3.4).

Anti-Bd OTUs dominated Plethodon skin microbiomes and had similar distributional patterns as entire bacterial community

We found 64 putatively anti-Bd bacterial OTUs in our dataset, making up an average relative abundance of 87% (SD ± 8.5) per individual. Across individuals, there was an average of 27 anti-Bd bacterial OTUs per salamander (SD ± 4). The same four anti-Bd bacterial OTUs were found on every individual. The core microbiome (90% of individuals; Table 3.3) consisted of 11 anti-Bd bacterial OTUs that were also dominant in abundance (avg. relative abundance = 83%, SD ± 8.5).

Anti-Bd bacterial community structure followed the same patterns in alpha- and beta-diversity as the entire bacterial community across species, site and elevations, except that beta-diversity on *P. cinereus* generally did not show a trend with elevation. For the co-occurring species sampled across multiple sites, alpha-diversity was similar among species and sites with an average of 27 anti-Bd bacterial OTUs per salamander (SD ± 4), and anti-Bd bacterial beta-diversity showed differences of individuals at Shenandoah NP being different in bacterial composition compared to those at Catoctin MP and Mt. Rogers NRA (Jaccard and Unifrac; PERMANOVA: $p < 0.05$). For *P. cinereus* sampled along the elevational gradient, anti-Bd bacterial alpha-diversity increased with elevation (GLM: OTU richness, $p = 0.013$, Faith's PD, $p = 0.004$), whereas anti-Bd bacterial beta-diversity changed with elevation for the Unifrac metric (partial Mantel:, $p = 0.006$, $R^2 =$ 10%), but not for the Jaccard metric (partial Mantel:, $p = 0.01$). Anti-Bd bacterial OTUs that changed in abundance among species, sites and elevations are noted in Tables 3.3 and A3.2.

DISCUSSION

We found that co-occurring *Plethodon* species harbored similar bacterial communities. Either microbiome composition is only a reflection of the bacteria in the environment or host properties that influence microbiome structure are similar among these closely related host species. The former scenario is unlikely as empirical evidence from multiple vertebrate host taxa has demonstrated that host-associated bacterial communities are a unique subset from the environmental bacterial communities (Sullam et al. 2012, Loudon et al. 2014, Walke et al. 2014, Schmidt et al. 2015, Bik et al. 2016, Rebollar et al. 2016) and that host species is generally a strong predictor of bacterial community structure (Ley et al. 2008, McKenzie et al. 2012, Larsen et al. 2013, Kueneman et al. 2014, Amato et al. 2015, Belden et al. 2015, Sanders et al. 2015, Bik et al. 2016, Council et al. 2016, Rebollar et al. 2016). This indicates that host traits select for unique sets of bacteria from the regional pool of environmental microorganisms, and that in most species sampled to date these traits are different among species. We hypothesize the main difference between our study and others is that *Plethodon* host factors related to bacterial community structure have not diverged among these closely related species. The *Plethodon* species we sampled have a smaller divergence time than previously examined of approximately 25 MYA or less (Wiens et al. 2006) compared to at least 65 MY divergence time between species for all previous amphibian skin microbiome studies (Pyron & Wiens 2013). Council et al. (2016) found similar patterns in which non-human apes shared similar skin microbiome structure (approximately 7 MY divergence time), but were different from Old World monkeys that had diverged from apes approximately 30 MYA (Perelman et al. 2011). Factors that likely influence microbiome composition

include chemical content of the skin mucus, including antimicrobial peptides (Rollins-Smith et al. 2002, Conlon 2011), skin-shedding rate (Meyer et al. 2012), immune genes (Huang et al. 2016) and diet (Antwis et al. 2014), and are important factors to examine for future work.

We provide evidence that environment is more influential in structuring the skin microbiome than differences in host properties. Co-occurring species at Shenandoah NP harbored dissimilar bacterial communities from than those at Catoctin MP and Mt. Rogers NRA. In geographic distance, Catoctin MP and Mt. Rogers NRA are most distant from one another, and it is plausible that environmental traits may drive microbiome similarity more so than geographic distance. For instance, Sunagawa et al. (2015) found that temperature was the major environmental factor shaping ocean microbial communities, with geographic distance playing a subordinate role. For terrestrial vertebrates, such as *Plethodon* salamanders, the environmental attributes that structure microbial communities are largely unknown given limited sampling of wild, terrestrial vertebrates. Therefore, to isolate the effect of environment, we sampled *P. cinereus* along an elevational gradient.

Elevation is a complex, indirect gradient along which many environmental variables change, and we detected changes in bacterial community structure with elevation, similar to other studies (Wang et al. 2011, Lear et al. 2013). Environment can influence microbiome structure by impacting bacterial properties directly, or indirectly by impacting host properties or the interactions among bacteria. Environment can impact bacterial communities directly by influencing affinity of microbes for substrates, which affects microbial growth (e.g., glutamic acid, phenol, nitrate; Nedwell 1999, Frey et al.

2013). Environmental characteristics can also impact the chemical content of amphibian skin, such as antimicrobial peptides (AMPs; Krynak et al. 2016), which could indirectly cause site-specific bacterial communities as AMPs can regulate the growth of certain microbes (Kueng et al. 2014). Finally, environmental impacts on bacterial species interactions can indirectly impact microbiome structure. For instance, Tucker & Fukami (2014) showed that priority effects and temperature alter the abundance of members of a microbial community, such that if certain microbes colonize first they can limit the abundance of other microbes, but this is dependent on temperature. While our study was not designed to disentangle these multiple processes, we do provide evidence that soil pH was negatively correlated with elevation, and explained small amounts of variation in bacterial composition. Soil pH can drive the spatial distribution of soil bacterial communities (Shen et al. 2013), which may explain the differences in microbiome structure as amphibians can acquire their bacteria environmentally (Muletz et al. 2012).

We demonstrated that the distributional patterns of putatively anti-Bd bacteria across species, sites and elevations were similar to that of the entire bacterial community. We do not know the function of all the bacteria present on amphibian skin, since the majority is not culturable (Walke et al. 2015). This limits our ability to evaluate distributional patterns, as we do not know whether unculturable bacteria provide a similar function as anti-Bd bacteria. However, we can conclude that the community assembly processes of selection, drift, dispersal and speciation events (Vellend 2010) are acting in a similar way for bacteria of known function and for those in which the function is not known.

Putatively anti-Bd bacteria were prevalent and abundant in the populations sampled, indicating that these symbionts likely serve a functional role for the host and are strong competitors with other microbial taxa. Together, both high prevalence and high abundance of anti-Bd bacteria are potential defensive mechanisms at the population- and individual- level against Bd infection, and may be mechanisms that select for certain microbiome structure profiles (Scheuring & Yu 2012). *Plethodon* salamanders are rarely infected with Bd in the wild (Muletz et al. 2014), even though Bd often occurs in regions in which they live (reviewed in Muletz et al. 2014). This suggests that their skin bacterial community may limit Bd infection (Becker & Harris 2010), yet other factors such as immunogenetics may also be involved (Ellison et al. 2015). Loudon et al. (2016) found, in an independent study, that bacteria from the environment are more likely to be overrepresented on *P. cinereus* skin if they have anti-Bd properties; they suggest that selection for anti-Bd bacteria is plausible given the strong selective pressure of Bd and other fungal pathogens, and the century-long existence of Bd in North America (Talley et al. 2015). While we cannot conclude that anti-Bd bacterial dominance is the mechanism of defense against Bd for these salamanders, we provide evidence that anti-Bd bacteria are dominant members of *Plethodon* salamander skin microbial communities. Further, these bacteria may have general antifungal proprieties that target not only Bd, but other fungal pathogens that these salamanders may encounter in the wild (Lauer et al. 2008).

CONCLUSION

We investigated patterns in the distribution of bacterial communities on *Plethodon* salamander skin across host species and environments. Co-occurring species

harbored similar microbial communities, indicating that species-specific microbial communities (Ley et al. 2008, McKenzie et al. 2012, Larsen et al. 2013, Kueneman et al. 2014, Amato et al. 2015, Belden et al. 2015, Sanders et al. 2015, Bik et al. 2016, Council et al. 2016, Rebollar et al. 2016) are not always observed and that, in some cases, host factors that select for microbial communities are similar among species. Environment was a strong predictor of skin microbiome structure across three localities and along an elevational gradient demonstrating the role of environmental filtering, driven in part by soil pH, in host-associated community assembly. Finally, the dominance of anti-Bd bacteria on *Plethodon* salamanders indicates that these antifungal bacteria may provide a functional role in protection of these salamanders from fungal pathogens. Results from this work contribute to our understanding of how skin-associated bacteria are distributed across the landscape, among host species, and their putative relationship with health and disease.

Table 3.1 Summary of locality, site and species information. We examined how bacterial community structure varied (i) among species and sites for individuals sampled at sites where two *Plethodon* species co-occurred (co-occurring dataset), and (ii) along an altitudinal gradient for *P. cinereus* at Shenandoah NP (altitudinal dataset; denoted by ^). We quantified the bacterial community and the anti-Bd bacterial community for all 100 individuals.

Locality	No. of sites	Elevation (m)	Species	No. salamanders sampled	No. salamanders co-occurring dataset
Catoctin	1	404	*P. cinereus*	7	7
Catoctin	1	404	*P. glutinosus*	7	7
Shenandoah	2	700 ± 3	*P. cinereus*	16 ^	9
Shenandoah	3	797 ± 6	*P. cinereus*	12 ^	-
Shenandoah	3	881 ± 18	*P. cinereus*	11 ^	5
Shenandoah	2	979 ± 5	*P. cinereus*	11 ^	-
Shenandoah	4	697 – 974	*P. cylindraceus*	7	5
Mt. Rogers	2	997, 1053	*P. cinereus*	14	14
Mt. Rogers	1	997	*P. glutinosus*	10	10
Mt. Rogers	1	1053	*P. cylindraceus*	5	5
			TOTAL:	**100**	**62**

Table 3.2 Core microbiome present on 90% of the salamanders sampled. Asterisks denote OTUs that were taxonomically similar to known anti-Bd bacteria.

OTU ID	Phylum	Family	Genus	Avg. relative abundance (%)	Standard error (%)	Range (%)
OTU_1 *	Proteobacteria	Moraxellaceae	*Acinetobacter*	32.83	1.77	4 – 77
OTU_5 *	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	15.55	2.08	0 – 68
OTU_268 *	Proteobacteria	Moraxellaceae	*Acinetobacter*	10.07	1.17	0.5 – 48
OTU_2 *	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	9.51	0.82	0.2 – 28
OTU_3	Actinobacteria	Sanguibacteraceae	*Sanguibacter*	5.4	0.44	0.07 – 22
OTU_25 *	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	4.92	0.68	0 – 38
OTU_4 *	Proteobacteria	Xanthomonadaceae	*Stenotrophomonas*	4.83	0.32	0.4 – 13
OTU_96 *	Proteobacteria	Pseudomonadaceae	--	1.77	0.21	0 – 11.5
OTU_7 *	Actinobacteria	Microbacteriaceae	*Microbacterium*	1.01	0.05	0 – 2.3
OTU_37 *	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	0.94	0.16	0 – 15.1
OTU_38 *	Proteobacteria	Xanthomonadaceae	--	0.79	0.09	0 – 4
OTU_14 *	Proteobacteria	Enterobacteriaceae	--	0.32	0.03	0 – 1.8
			TOTAL:	88		

Table 3.3 Heatmap of the average relative abundance of bacterial OTUs that were differentially abundant among localities and host species. All 28 OTUs were differentially abundant between individuals at Shenandoah NP compared to individuals at Mt. Rogers NRA and/or Catoctin MP (ZILN mixture model: $p < 0.05$). Five OTUs were differentially abundant among salamander species (highlighted in bold: ZILN mixture model: $p < 0.05$). Asterisks denote OTUs that were taxonomically similar to known anti-Bd bacteria.

OTU ID	Taxa	Catoctin *P. cinereus* n =7	Catoctin *P. glutinosus* n = 7	Shenandoah *P. cinereus* n = 16	Shenandoah *P. cylindraceus* n = 5	Mt. Rogers *P. cinereus* n = 14	Mt. Rogers *P. cylindraceus* n = 5	Mt. Rogers *P. glutinosus* n = 10
OTU_2 *	*Pseudomonas* sp.	17.71	18.31	1.95	1.81	20.05	14.78	17.85
OTU_3	*Sanguibacter* sp.	8.68	7.03	1.22	0.95	9.71	8.30	7.45
OTU_4 *	*Stenotrophomonas* sp.	7.90	8.96	1.81	1.44	8.23	5.78	7.41
OTU_8	f__Cellulomonadaceae	1.14	0.97	0.17	0.14	1.36	1.78	0.90
OTU_25 *	*Pseudomonas* sp.	0.72	0.82	8.64	6.81	0.43	0.35	0.40
OTU_5 *	*Pseudomonas* sp.	0.64	0.76	50.41	48.71	1.04	0.89	1.10
OTU_165	f__Microbacteriaceae	0.33	0.21	0.04	0.01	0.35	0.23	0.29
OTU_13	*Alicyclobacillus* sp.	0.25	0.14	0.10	0.09	0.16	0.25	0.24
OTU_19 *	*Arthrobacter* sp.	0.23	0.10	0.02	0.02	0.16	0.20	0.31
OTU_12	o__Actinomycetales	0.21	0.65	0.01	0.00	0.00	0.01	0.10
OTU_16	*Ochrobactrum* sp.	0.21	0.19	0.05	0.06	0.20	0.18	0.18
OTU_17	*Sphingobium* sp.	0.21	0.10	0.09	0.08	0.15	0.18	0.17
OTU_59 *	f__Comamonadaceae	0.18	0.10	0.07	0.02	0.07	0.08	0.07
OTU_11 *	*Delftia* sp.	0.11	0.06	0.74	0.42	0.06	0.12	0.03
OTU_34 *	*Agrobacterium* sp.	0.06	0.07	0.02	0.01	0.09	0.07	0.07
OTU_38 *	f__Xanthomonadaceae	0.05	0.07	1.57	1.08	0.27	0.15	0.33
OTU_50	*Streptococcus* sp.	0.05	0.08	0.00	0.01	0.05	0.03	0.10
OTU_249 *	*Pseudomonas* sp.	0.04	0.02	0.58	0.71	0.09	0.03	0.08
OTU_485 *	*Stenotrophomonas* sp.	0.04	0.05	0.47	0.10	0.07	0.07	0.08
OTU_158 *	*Pseudomonas* sp.	0.03	0.03	0.18	0.14	0.01	0.01	0.02
OTU_79 *	*Pseudomonas* sp.	0.03	0.02	0.62	0.77	0.10	0.08	0.05
OTU_275	*Cellulomonas* sp.	0.01	0.03	0.58	0.44	0.05	0.02	0.02
OTU_332	f__Acidobacteriaceae	0.01	0.00	0.01	0.20	0.00	0.01	0.00
OTU_449 *	*Pseudomonas* sp.	0.01	0.01	1.35	1.04	0.02	0.02	0.00
OTU_54	f__Methylocystaceae	0.01	0.02	0.07	0.09	0.06	0.05	0.19
OTU_112 *	f__Microbacteriaceae	0.00	0.00	0.17	0.08	0.00	0.01	0.00
OTU_148	o__Chlorophyta	0.00	0.04	0.03	0.11	0.01	0.01	0.00
OTU_425 *	*Curtobacterium* sp.	0.00	0.00	0.62	0.44	0.06	0.06	0.04

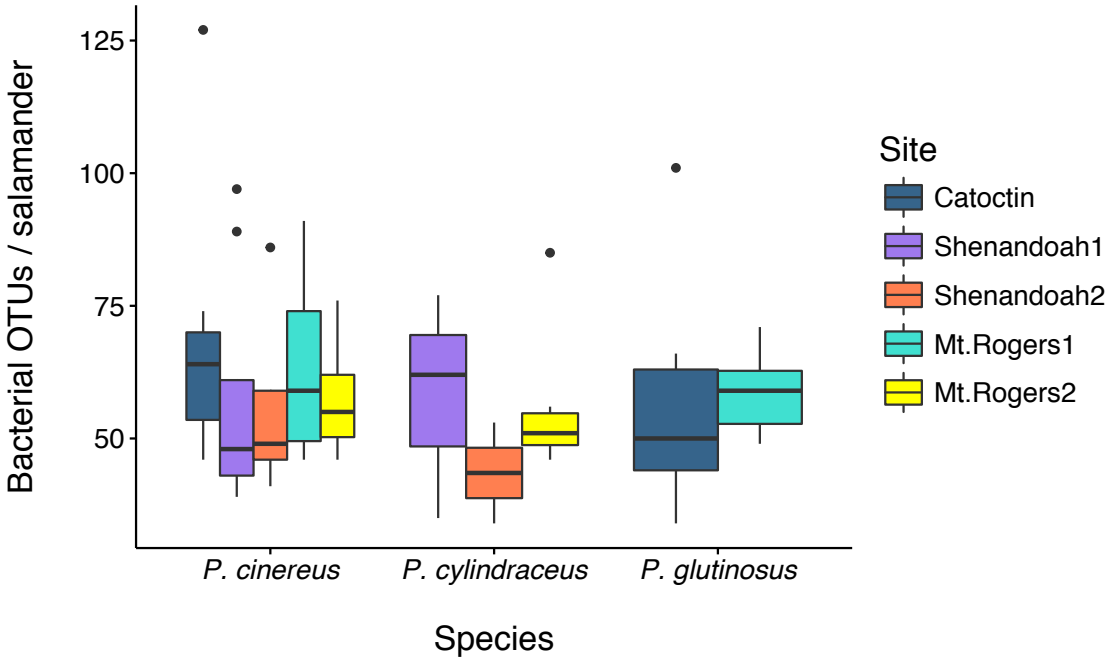


Figure 3.1 Boxplot of total number of OTUs per individual among three *Plethodon* species sampled at three localities. There was no difference in α-diversity metrics among salamander species or sites (OTU richness and Faith's PD; LMM: $p > 0.05$).

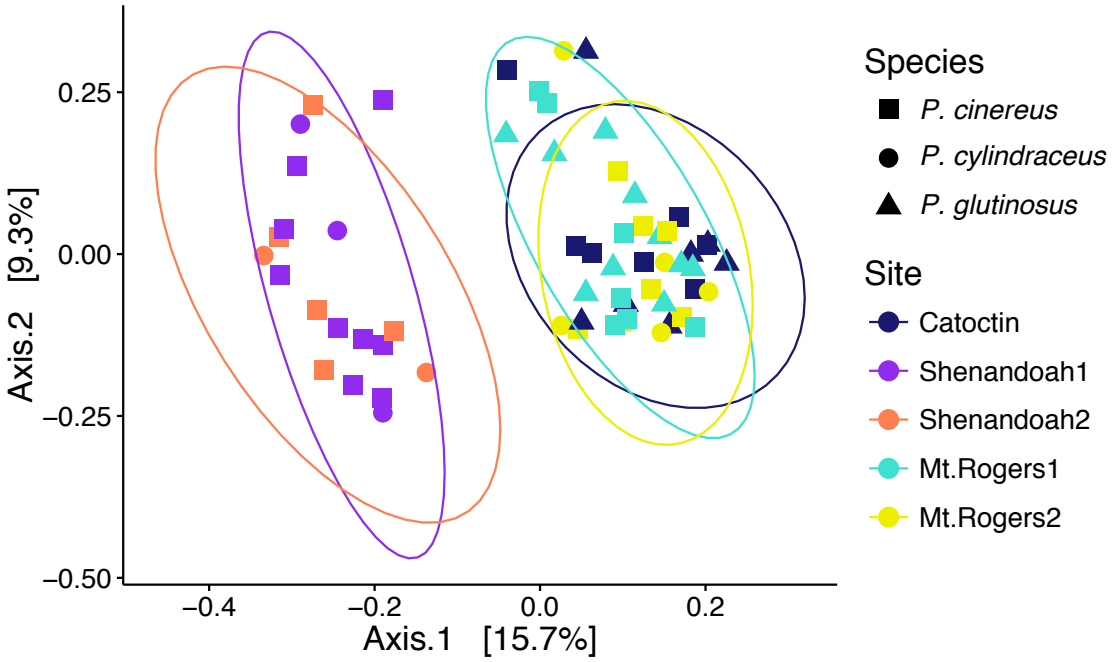
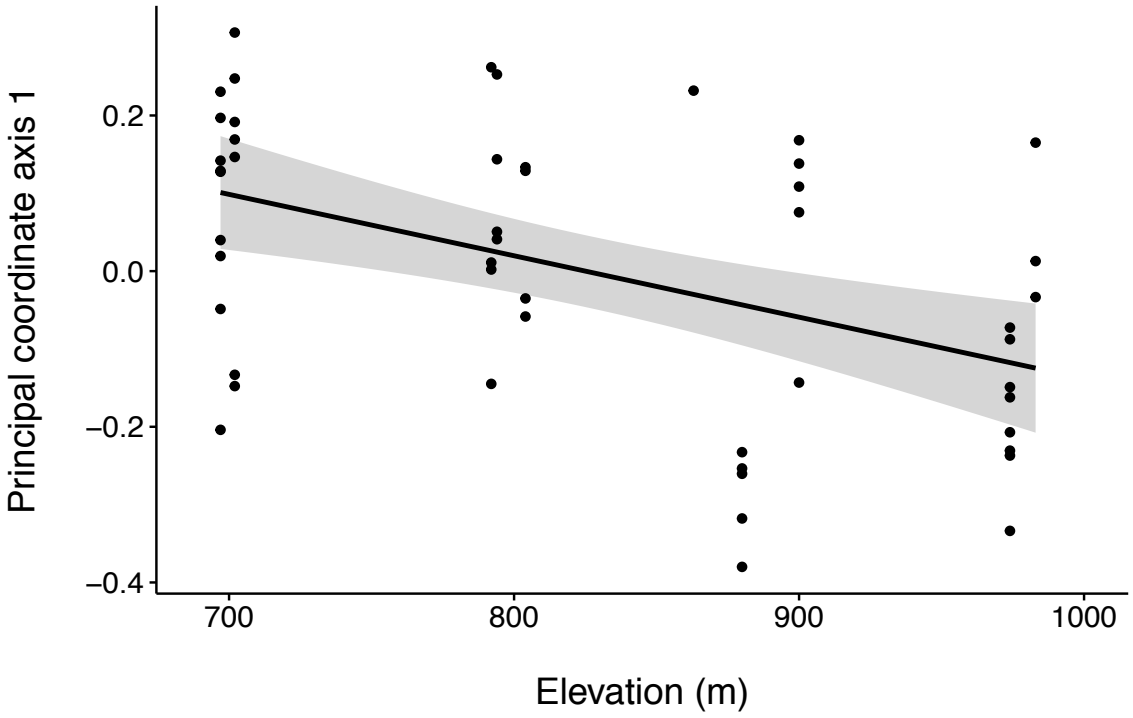


Figure 3.2 PCoA of skin bacterial beta-diversity on three *Plethodon* species sampled at three localities. Sites at Shenandoah NP had significantly different beta-diversity patterns (Jaccard and Unifrac; PERMANOVA: $p < 0.05$) than the sites at Catoctin MP and Mt. Rogers NRA, regardless of salamander species.

Figure 3.3 Relationship between elevation and principal coordinate axis 1 from Jaccard distances. Bacterial community composition (beta-diversity) changed along the elevational gradient (Partial Mantel tests, p = 0.035).



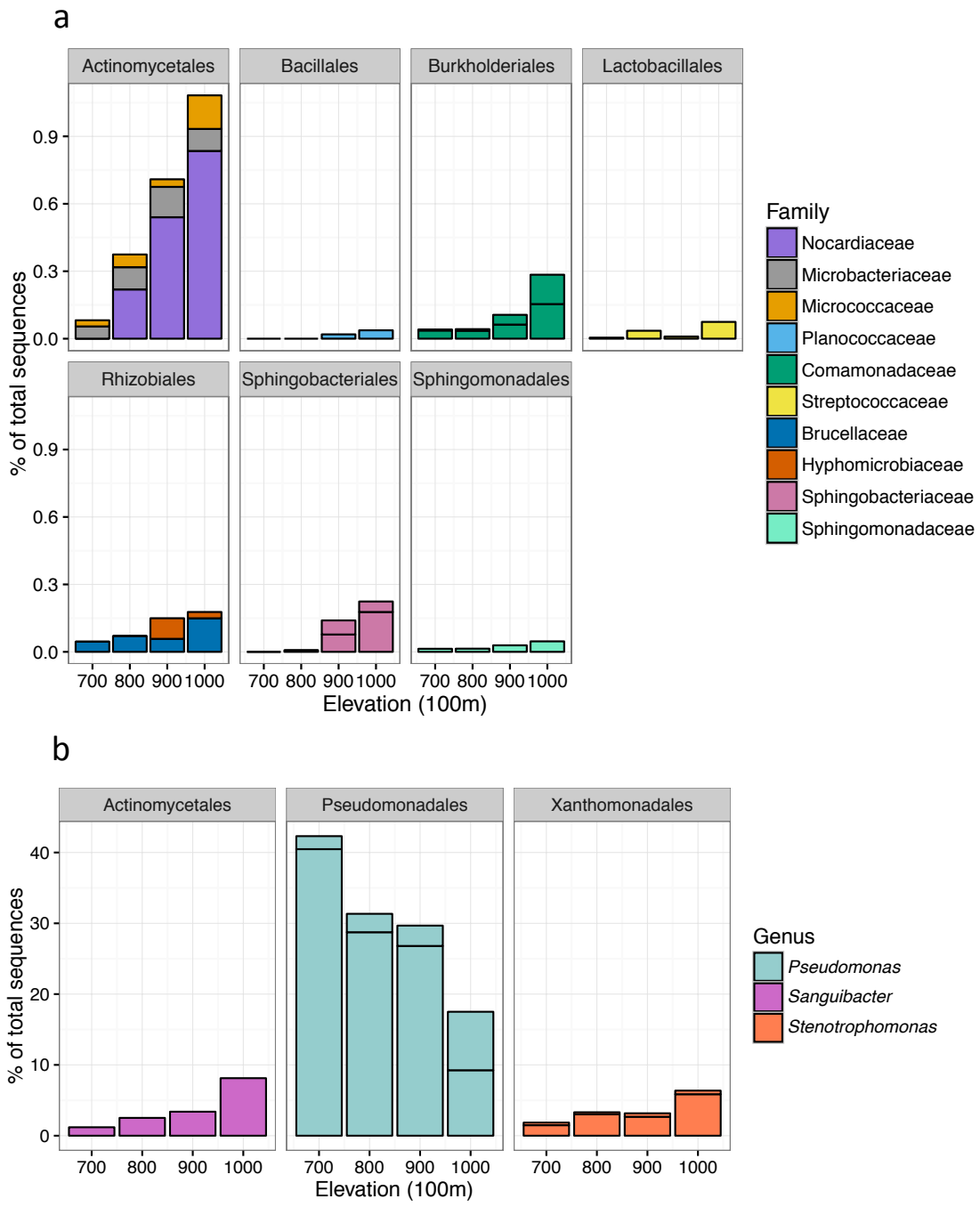


Figure 3.4 Bar plots of bacterial OTUs that showed a correlation between abundance and elevation (linear model: $p < 0.05$, $R^2 = 13 – 43\%$). Abundances are shown as relative abundances for interpretation, but were analyzed based on normalized sequence counts. Certain bar plots have multiple OTUs that belong to that order and are shown as separate stacks. Twelve OTUs were < 1% of the total sequences are shown in panel A at family level taxonomic assignment as not all OTUs could be assigned to genus level. Five OTUs were > 1% of total sequences and their lowest taxonomic assignment was genus; they are shown in panel B.

Chapter IV: PATHOGEN PRESSURE AND TEMPERATURE ALTERS SALAMANDER SKIN MICROBIOME STRUCTURE

Coauthors: Robert C. Fleischer and Karen R. Lips

ABSTRACT

Infectious diseases can cause rapid populations declines or species extinctions, such as those caused by the amphibian fungal pathogen, *Batrachochytrium dendrobatidis* (Bd). In the amphibian-Bd system, the skin microbiome has been hypothesized to protect hosts from disease. We compared host (*Plethodon cinereus*) and skin microbial responses to exposure to three temperature (13, 17, 21 °C) and two Bd levels (Bd+, Bd-) to determine whether the native microbiome affected survival at natural temperatures. We compared the microbiome before and after pathogen exposure with high-throughput sequencing of the 16s rRNA gene and quantified pathogen load with qPCR. Temperature and Bd exposure caused microbiome structure (alpha-diversity, beta-diversity, and OTU abundances) to change. However, Bd caused high host mortality, regardless of temperature or microbiome structure. Increasing pathogen load was correlated with increased changes in microbiome structure, reduction in host body condition, and increased host morality across temperatures. We show that under natural conditions the microbiome did not represent a protective effect with respect to host mortality. Instead, we found that pathogen invasion drove changes in the microbiome, and ultimately lead to host mortality. Understanding microbial response to pathogen invasion is essential for advancing disease ecology and determining how to mitigate disease.

INTRODUCTION

Global biodiversity is increasingly threatened by fungal pathogens (Fisher et al. 2012). A pathogen's impact on host populations often depends on environmental context, such as thermal conditions (Harvell et al. 2002, Lafferty 2009). For instance, amphibian biodiversity is threatened by two deadly, fungal pathogens: *Batrachochytrium dendrobatidis* (Bd; Lips et al. 2006) and its sister taxon *B. salamandrivorans* (Bsal; Martel et al. 2014), with differences in host responses that can depend on environmental temperatures (Berger et al. 2004, Bustamante et al. 2010, Murphy et al. 2011, Blooi et al. 2015). However, associations of temperature with Bd-associated mortality are not always consistent with the temperature optima of Bd growth in pure culture (17-25 °C; Piotrowski et al. 2004). This suggests that host proprieties may mediate the effects of fungal growth optima, among others (e.g., life history trade-offs: Woodhams et al. 2008). Host proprieties that may influence the course of disease in a temperature-dependent manner include: host skin shedding rate (Meyer et al. 2012, Cramp et al. 2014), host immune response (Ribas et al. 2009), and host microbiome composition (Lokmer & Wegner 2015). Yet, there is a paucity of information on how the amphibian microbiome changes across temperature and its subsequent impact on host disease outcome. Examining how temperature, pathogen and microbiome interact to impact host disease dynamics advances our understanding of disease ecology and how to mitigate disease.

Properties of the amphibian skin microbiome can mediate Bd-associated disease and may do so in a temperature-dependent manner. Reduction of the skin microbiome can increase disease symptoms such as weight loss and mortality rates (Becker & Harris 2010, Holden et al. 2015). Whereas, augmentation of amphibian skin bacteria that kill Bd

in vitro, hereafter referred to as anti-Bd bacteria, can decrease disease symptoms (Harris et al. 2009a, Harris et al. 2009b, Vredenburg et al. 2011, Muletz et al. 2012). However, manipulation of amphibian microbiomes has not always reduced the negative impacts of Bd infection (Woodhams et al. 2012, Becker et al. 2015a), suggesting context-dependency on conditions such as temperature, among others (e.g., host species). For instance, amphibian bacterial symbionts often do not exhibit Bd-inhibitory properties across all temperatures (Daskin et al. 2014, Woodhams et al. 2014). This likely relates to temperature influencing growth rate and population size of the symbionts, in which high cell density is often needed to produce inhibitory secondary metabolites (Yasumiba et al. 2016). Additionally, temperature may influence other diversity and composition proprieties of the amphibian skin microbiome, such as presence of certain bacteria (Bresciano et al. 2015).

In a variety of animal hosts, components of microbiome diversity and composition (alpha-diversity, beta-diversity and bacterial OTU abundance) can be important in predicting and/or diagnosing disease (e.g., amphibians: Becker et al. 2015a, humans: Gilbert et al. 2016, corals: Roder et al. 2014, oysters: Lokmer & Wegner 2015. For instance, Becker et al. (2015a) found abundance of certain bacterial OTUs pre-pathogen exposure could predict survival of the Panamanian golden frog, (*Atelopus zeteki*), suggesting that interactions of the microbiome with Bd can weaken the impact of Bd on the host. Alternatively, the microbiome can be diagnostic of disease post-pathogen exposure in which the pathogen alters microbial diversity and composition. For instance, Jani & Briggs (2014) found that Bd infection drove changes in microbial composition (beta-diversity) and abundance of certain OTUs, and ultimately high host mortality in

Sierra Nevada yellow-legged frogs (*Rana sierrae*). This suggests that Bd can alter the microbiome, potentially weakening its protective properties and thus increasing the negative impacts of Bd on the host. Additionally, in the coqui frog (*Eleutherodactylus coqui*), hosts with greater infection intensities had lower bacterial richness (alpha-diversity) than less infected individuals (Longo et al. 2015), suggesting that communities with higher numbers of taxa and hence more potential interactions may be more resistant to Bd invasion than communities with fewer taxa. Various indices of diversity and composition (alpha-diversity, beta-diversity and bacterial OTU abundance) can be important in predicting or diagnosing disease, and we hereafter refer to them jointly as microbiome structure.

We tested the effects of Bd and temperature on microbiome structure and host responses. We used a full-crossed, two-factorial laboratory experiment with three temperatures levels (13, 17, 21 °C) and two pathogen levels (Bd+, Bd-), exposing red-backed salamanders (*Plethodon cinereus*) to epidemic-like loads of Bd (Vredenburg et al. 2010, Catenazzi et al. 2011). We chose *P. cinereus* because their microbiome has been shown to protect them from Bd-associated disease symptoms (Becker & Harris 2010), likely by having high abundance of anti-Bd bacteria (Harris et al. 2009b, Muletz et al. 2012). However, Bd loads in the range of *P. cinereus* are often low (Richards-Hrdlicka et al. 2013, Muletz et al. 2014). It is unclear how epidemic-like loads of Bd, or potentially the introduction of Bsal into their native range (Yap et al. 2015), will impact these salamanders and if their microbiome can offer them protection in those instances across a range of temperatures. Our objectives were to quantify (i) the direct effects of temperature and Bd exposure on microbiome structure and host responses (survival and

body condition) and (ii) the indirect effects of pathogen and microbiome interactions on host survival across a range of temperatures. By examining Bd-microbiome interactions, we assessed if microbiome structure pre-Bd exposure could predict host survival (predictive of disease) or if Bd load was correlated to changes in microbiome structure and host mortality (diagnostic of disease), and if these relative roles were temperature-dependent. While these responses are unlikely to operate entirely independently of one another they provide a useful basis from which to test the indirect effects of skin-associated microbiome structure on disease dynamics.

METHODS

Amphibian collection and housing

We collected 87 adult *Plethodon cinereus* on May 3rd, 2014 at Cunningham Falls State Park, MD (MD DNR Permit No. 55850 and MD State Park Permit No. 2014DNR099). We turned cover objects to locate salamanders within an area approximately 300 m x 200 m. We used a new pair of sterile nitrile gloves to handle each salamander at collection and throughout the laboratory experiment. For each salamander at collection, (i) we assigned them a unique number, (ii) rinsed them twice with 25 ml sterile water in 50 ml Falcon® tubes to remove transient microbes (Lauer et al. 2007), (iii) swabbed them (five strokes each: dorsal/ventral sides and front/back limbs) with a MW-113 swab (Medical Wire, UK), (iv) measured their snout-to-vent length (SVL) and mass, (v) determined their sex following Gillette & Peterson (2001), and (vi) placed them in an individual container. From within the area of salamander collection, we collected soil and leaf litter to be used to house the salamanders with during the experiment.

In the laboratory, we housed salamanders in individual 20 x 13 x 18 cm (L x W x H) sterile plastic terrariums containing 175 g of well-mixed soil and 3 g of mixed beech, oak and maple leaves from the site of collection. Prior to adding the soil to the terraria, we sieved the soil to remove forest debris (#4 sieve, 4.75 mm opening size, Newark Wire Cloth Company, Clifton, NJ) and mixed the soil by hand wearing sterile nitrile gloves. We housed salamanders in their individual terraria in a total of six environmental chambers (Percival model DR-36VL) on a 12 h light: 12 h dark cycle and 80 ± 10% humidity throughout the experiment. On a weekly basis, we fed salamanders approximately 15 fruit flies or 10 crickets, dusted with vitamins (Rep-cal Herptivite), and misted terraria. Following feeding, we returned salamanders to their assigned environmental chamber, but placed their terrarium on a new, randomly assigned shelf. We handled all salamanders during the experiment, monitored their disease symptoms, and euthanized them according to an approved IACUC protocol (UMD Reference # R-14-04).

Experimental design

We used a 2 x 3 full-factorial experimental design with a two-level Bd factor (Bd+, Bd-) and three-level temperature factor (13, 17, 21 °C), resulting in six treatments. We randomly assigned salamanders to treatments and to one of two environmental chambers maintained at one of the three temperatures, and housed both Bd- and Bd+ treatment animals within the same chamber (Figure A4.1). Temperatures were chosen based on average spring body temperature (13 °C), average summer body temperatures (17 °C) and a higher temperature within the range of normal body temperatures (21 °C)

of *Plethodon* salamanders in the field (Caruso et al. 2014). Each treatment had a sample size of either 15 salamanders (Bd+ 13 °C, Bd+ 17 °C, Bd+ 21 °C) or 14 salamanders (Bd- 13 °C, Bd- 17 °C, Bd- 21 °C).

We acclimated salamanders to their assigned temperature for 47 days, applied the Bd factor at Day 0, and conducted the full-factorial experiment until Day 42 when most Bd+ individuals had either died or cleared infection. We used the Bd isolate JEL 423 (Lips et al. 2006), and grew 1% tryptone plates inoculated with JEL 423 for seven days at each of the three experimental temperatures. We harvested zoospores by flooding the plates with sterile water, filtered out the zoosporangia using a sterile filter, and homogenized the Bd zoospore mixture. We exposed each Bd+ level salamander to 5.3 x 10^6 zoospores/ml in 5 ml (2.6 x 10^7 zoospores; counted with a hemocytometer) in individual 15 ml Falcon® tubes. For Bd- level salamanders, we exposed them to a sham inoculation of 5 ml of sterile water, collected from flooding sterile 1% tryptone plates, in individual 15 ml Falcon® tubes. All salamanders were kept at 17 °C during exposure for 24 hours, after which they were returned to their original terrarium by emptying the 15 ml Falcon® tube into the terrarium.

We swabbed salamanders to quantify microbiome structure and Bd loads, and measured their SVL and mass at five time-points (pre-Bd exposure, and Days 5, 11, 25, and 42). We conducted pre-Bd exposure swabbing two days prior to Bd exposure. At each swabbing event, we rinsed each salamander twice with approximately 7 ml of sterile water in sterile petri dishes (Lauer et al. 2007). We stored all swabs in 1.5 micro-centrifuge tubes on ice during sampling, and thereafter stored them in a -80 °C freezer until DNA extraction was performed. We monitored salamanders daily for symptoms of

disease: abnormal posture, excess skin sloughing, loss of appetite, lethargy, loss of righting reflex and mortality. We euthanized any individual that lost their righting ability, or displayed all four of the other clinical symptoms, by applying 20% benzocaine to their dorsal side. We euthanized all surviving salamanders on Day 42 using submersion in MS-222.

Molecular methods

We extracted DNA from the skin swabs following the Qiagen BioSprint manual for Tissue Extraction with one modification. Our modification consisted of using a high-throughput cell disrupter (Mini BeadBeater 1001, Biospec) and zirconia/silica beads to increase DNA yield of Gram-positive bacteria. We added 0.2 – 0.3 g of 0.1 mm diameter sterile zirconia/silica beads to each well in a sterile 96-well plate, and exposed the plate to ultraviolet radiation for 10 min. We added swabs into each well using sterilized tweezers, added 20 ul Proteinase K and 200 ul of ATL buffer, and sealed the plate with a sterile silicone plate seal. We performed bead beating at 36 oscillations/sec (~2160 rpm) for 30 sec, followed by a centrifuge step for 5 min at 4000 rpm and incubation for 2.5 h at 56 °C. Following incubation, we centrifuged the plate for 5 min at 4000 rpm and then transferred the supernatant into a new sterile 96-well plate. Thereafter, we followed the Qiagen BioSprint manual for Tissue Extraction.

We used 454 pyrosequencing of 16s rRNA gene amplicons to quantify bacterial community structure for all individuals at collection, pre-Bd exposure and Days 5 of 11 of the experiment. We amplified the V3-V5 region of the 16s rRNA gene using the universal gene primers 515F and 939R, using custom fusion primers and the protocol as

outlined previously (Chapter III). We conducted three runs on a Roche 454 FLX+ at the Smithsonian Conservation Biology Institute-Center for Conservation Genomics.

We used qPCR to quantify Bd load, using primers and TaqMan probe developed by Boyle et al. (2005) and KlearKall Master mix (LCG) following their qPCR reaction protocol. We tested all Bd+ individuals for the presence of Bd at: pre-Bd exposure and Days 5, 11, 25 and 42. We tested all Bd- individuals for Bd at: pre-Bd exposure and Days 11 and 42 to show Bd- individuals were negative prior to, during and at the end of the experiment. We ran all DNA samples in duplicate and used standards of 100, 10, 1, 0.1 ZGEs (zoospore genomic equivalents) developed from JEL 423. If one of the duplicates returned a positive signal it was re-run a third time. Samples were considered positive if they amplified twice before 0.1 ZGEs.

Measures of microbiome structure

In all microbiome analyses, we examined microbiome structure using three indices: alpha-diversity, beta-diversity and OTU bacterial abundance. We examined two alpha-diversity metrics: total number of observed OTUs (OTU richness) and Faith's Phylogenetic Diversity (Faith's PD; Faith 1992), and two beta-diversity metrics: Jaccard and Unifrac (Lozupone et al. 2011). For OTU abundance, we performed variance-stabilizing normalization on the sequence counts (Paulson et al. 2013, McMurdie & Holmes 2014). Sequence counts are approximately quantitative for microbial OTU abundance (Amend et al. 2010); hereafter, we refer to the sequence counts as OTU bacterial abundance. For OTU bacterial abundance, we examined the overall abundance of OTUs at two levels: overall abundance of anti-Bd bacterial OTUs or overall

abundance of non-Bd inhibitory bacterial OTUs. To distinguish anti-Bd bacteria from non-Bd inhibitory bacteria, we followed methods as outlined in Chapter III to identify bacterial OTUs that were taxonomically similar (≥ 97% sequence similarity) to culturable anti-Bd bacteria (Chapter II, Woodhams et al. 2015). While these OTUs matched the antifungal databases it does not necessarily indicate that these bacteria exhibit antifungal activity, but that they are strong candidates for exhibiting antifungal activity (Becker et al. 2015b). Additionally, we examined anti-Bd bacterial abundance at the individual OTU level and report this information in the appendix.

Statistical analyses

We predominately focused host and microbiome response analyses on four sampling time points: collection, pre-Bd exposure, Days 5 and 11. We were unable to evaluate long-term effects of Bd on the microbiome because of high mortality in the Bd+ level by Day 25. All analyses were performed in R version 3.2.3 (Team 2015). For the analyses below using general and generalized linear models (ANOVAs, LM, LMM, GLMs), we determined the appropriate distributions for the data by examining distributions of the response variable and model residuals, and transformed variables when necessary to improve model fit. For those models with multiple explanatory variables we determined the significance of the fixed effects by using the car package (Fox & Weisberg) using the *Anova* function with type II SS. For significant interactions we used the *lsmeans* function in the lsmeans package (Lenth 2015) to perform Tukey HSD post-hoc analyses. When multiple comparisons were done (i.e., multiple days, and

anti-Bd bacterial OTU abundance) we corrected p-values using false discovery rate (FDR) corrections.

We quantified the effects of Bd, Temperature and their interaction on microbiome structure at collection and pre-Bd exposure, separately, and Days 5 and 11 together, with Day as a random effect. We examined collection microbiome structure to demonstrate no treatment effect prior to treatment assignment. We examined pre-Bd exposure microbiome structure to quantify temperature effects (after 45 day acclimation period). For alpha-diversity and OTU bacterial abundance, we used ANOVAs for collection and pre-Bd exposure, and LMM for Days 5 and 11. For beta-diversity, we used PERMANOVAs using the *functions procD.lm* in the package geomorph (Adams & Otarola-Castillo 2013) and the function *advanced.procD.lm* for post-hoc analyses.

We assessed host response to treatments by examining host survival and body condition. To determine if survival curves of salamanders differed among treatments, we used log rank tests (package survival, function *survdiff* (Therneau 2015)). To quantify salamander body condition, we used the residuals of a linear regression of body mass on SVL at collection, and Days 5 and 11 separately to avoid pseudo-replication. We used an ANOVA for salamander body condition at collection, and used a linear mixed-effect model (LMM) for Days 5 and 11 in the lme4 package (Bates et al. 2015) with Temperature, Bd and their interactions as explanatory variables and Day as a random effect.

We determined if there was a relationship for Bd+ level salamanders between pre-Bd exposure microbiome structure and post-Bd exposure host survival, and included Temperature as a covariate in the analyses. For alpha-diversity and OTU bacterial

abundance, we used generalized linear models (GLM) with a binomial distribution and a log link with survival outcome as the response. For beta-diversity, we used a PERMANOVA with the *adonis* function in the package vegan (Oksanen et al. 2015) and compared bacterial composition between hosts that survived from those that died.

We assessed the relationship between changes in microbiome structure with Bd load on Days 5 and 11 for Bd+ level salamanders. For alpha-diversity and OTU bacterial abundance, we used LMMs with Temperature as a covariate and Day as a random effect. For beta-diversity, we computed partial Mantel correlations between compositional dissimilarity matrices (Jaccard and Unifrac) and a Bd load (log-transformed) distance matrix after accounting for temperature distances using 10,000 permutations in the package vegan (Oksanen et al. 2015), for Days 5 and 11, separately and corrected for multiple comparisons. We calculated Euclidean distances for Bd load and temperature.

To determine if there was a relationship between (i) host survival and Bd load and, (ii) body condition and Bd load on Days 5 and 11 for Bd+ level salamanders, we used a GLMM with a binomial distribution, and a LMM, respectively. In both models, we indicated Bd load log-transformed as the explanatory variables with Temperature as a covariate and Day as random effect.

RESULTS

We generated a total of 927,114 high quality sequences (382 bp average length) from 258 samples representing 2929 OTUs from 33 described bacteria phyla. The majority of OTUs belonged to Proteobacteria, making up 75% of sequences. For anti-Bd

bacteria 69% of sequences matched known anti-Bd bacterial taxa. On average, all anti-Bd bacteria were found on 48% (SD ± 29, OTUs = 67) of individuals at all time points.

Pre-Bd exposure, temperature changed microbiome structure. Alpha-diversity differed among temperatures (OTU richness ANOVA: $F_{2,77} = 19.83$, $p < 0.001$; Faith's PD ANOVA: $F_{2,77} = 15.74$, $p < 0.001$), with 13 °C and 17 °C having higher alpha-diversity than 21 °C (Figure 4.1a, TukeyHSD: $p < 0.0008$). Beta-diversity also differed among temperatures (Jaccard PERMANOVA: Pseudo-$F_{2,77} = 3.55$, $p = 0.001$; Unifrac PERMANOVA: Pseudo-$F_{2,77} = 4.61$, $p = 0.001$) with all temperatures having dissimilar composition (Figure 4.1b, pairwise $p < 0.0008$). Overall anti-Bd bacterial abundance increased with temperature (ANOVA: $F_{2,77} = 29.5$, $p < 0.001$; TukeyHSD: $p < 0.03$), while overall non-Bd inhibitory abundance did not differ among temperatures (Figure 4.1c, ANOVA, $p > 0.05$). Initial microbiome structure at collection did not differ among treatments (alpha-diversity, ANOVA: $p > 0.05$; beta-diversity, PERMANOVA: $p > 0.05$, bacterial OTU abundances, ANOVAs: $p > 0.05$).

Post-Bd exposure, Bd, temperature and their interaction affected microbiome structure, but their effects depended on the microbiome structure index examined. For alpha-diversity, Bd- individuals had higher alpha-diversity than Bd+ individuals (OTU richness LMM: X^2 (1, n = 127) = 7.06, $p = 0.0078$; Faith's PD LMM: X^2 (1, n = 127) = 7.91, $p = 0.0049$), but among temperatures alpha-diversity was similar (Figure 4.2a; LMM: $p > 0.05$). For beta-diversity, microbial composition differed both between Bd+ and Bd- individuals (Jaccard PERMANOVA: Pseudo-$F_{1,115} = 3.18$, $p = 0.001$; Unifrac PERMANOVA: Pseudo-$F_{1,115} = 3.9$, $p = 0.001$) and among temperatures (Figure 4.2b; Jaccard PERMANOVA: Pseudo-$F_{2,115} = 1.74$, $p = 0.003$; Unifrac PERMANOVA:

Pseudo-F $_{2,115}$ =1.85, p = 0.008), in which 13 °C was dissimilar from both 17 and 21 °C (pairwise p < 0.013). Overall anti-Bd bacterial abundance was higher in Bd+ level salamanders (LMM: X^2 (1, n = 127) = 11.5, p < 0.001), but was not different among temperatures (Figure 4.2c; LMM: p > 0.05). Overall non-Bd inhibitory bacterial abundance did not differ among Bd levels (Figure 4.2c), but was different between 13 and 21 °C (LMM: X^2 (1, n = 127) = 6.8, p = 0.03, TukeyHSD: p = 0.02).

Bd exposure caused high salamander mortality and reduction in body condition across all temperatures. Survival rate varied among treatments (X^2 (5, n = 87) = 48.7: p < 0.0001), with higher mortality rates in Bd+ individuals compared to Bd- individuals (X^2 (1, n = 87) = 46.2: p < 0.001), at all temperatures (Figure 4.3; X^2: p > 0.05). For Bd+ level salamanders, average time to death was 17 days with 78% of salamanders (35/45) experiencing mortality. Bd exposure reduced salamander body condition (LMM: X^2 (1, n = 127) = 5.94: p = 0.015), regardless of temperature level. All Bd- individuals were negative for Bd throughout the experiment, prior to exposure all Bd+ individuals were negative for Bd, and body condition at collection was similar among treatments (ANOVA: p > 0.05).

Microbiome structure pre-Bd exposure did not predict host survival post-exposure (alpha-diversity GLM: p > 0.05; beta-diversity PERMANOVA: p > 0.05; anti-Bd bacterial OTU abundance GLM: p > 0.05).

Bd load was correlated with changes in microbiome structure across all temperatures. For alpha-diversity, higher Bd load was correlated with lower alpha-diversity (Figure 4.4a; LMM: X^2 (1, n = 58) = 5.16, p = 0.023). For beta-diversity, Bd loads were correlated with changes in bacterial community composition at Day 5 (Partial

Mantel: Jaccard, p = 0.05, Spearman's rank correlation coefficients = 0.17; Unifrac, p = 0.05, Spearman's rank correlation coefficients = 0.16). This result demonstrates that as Bd load increased the change in bacterial composition increased, which we represented by fitting an orthogonal linear regression to principle coordinate analysis axis 1 plotted against Bd load (Figure 4.4b). On Day 11, we observed a trend in community composition changing with Bd load (Partial Mantel: Jaccard, p = 0.072, Spearman's rank correlation coefficients = 0.14; Unifrac, p = 0.081, Spearman's rank correlation coefficients = 0.13). The overall abundance of anti-Bd bacteria increased as Bd load increased (LMM: X^2 (1, n = 58) = 11.9, p < 0.001), but the abundance of non-Bd inhibitory bacteria was not influenced by Bd load (Figure 4.4c, LMM: p > 0.05).

Individuals with higher Bd loads were more likely to experience mortality (Figure 4.5; GLMM: X^2 (1, n = 58) = 8.01, p = 0.005) and have reduced body condition (Figure A4.2; LMM: X^2 (1, n = 58) = 6.76, p = 0.009) at all temperatures.

DISCUSSION

Our results demonstrate that both abiotic filtering (temperature) and biotic interactions (pathogen pressure) are important factors determining skin microbiome structure in *P. cinereus*. In regards to disease dynamics, the temperature-induced changes in the microbiome were not predictive of host survival. Instead, increasing pathogen load was related to increased changes in microbiome structure, reduction in body condition and host mortality, regardless of temperature. This suggests that the observed link between microbiome structure changes and Bd loads across temperatures did not represent a protective effect of bacteria, at least with respect to reduction in body

condition and host mortality. Our results provide evidence for pathogen pressure driving microbiome alteration during a mimicked epidemic event.

Temperature changed microbiome structure through direct or indirect effects on bacterial abundance, bacterial presence, species interactions or host properties. Temperature can cause species tolerant of certain temperatures to increase in abundance, be lost or invade (Lokmer & Wegner 2015, Sunagawa et al. 2015, Zhou et al. 2016). For instance, we found an increase in overall abundance of anti-Bd bacterial OTUs with temperature. Anti-Bd bacteria may gain a competitive advantage at higher temperature, as they are more likely to produce inhibitory metabolites at higher temperatures (Daskin et al. 2014). This temperature-mediated change in anti-Bd bacterial abundance may have indirectly caused the reduction in alpha-diversity that we observed as temperature increased, as certain bacteria can outcompete others depending on environmental context (Tucker & Fukami 2014, Kadowaki et al. 2016). Temperature may have indirectly affected microbiome structure by causing changes in host physiology such as immune function (Maniero & Carey 1997), which can influence microbial community structure (Hooper et al. 2012). Although our data do not allow us to distinguish between these possibilities, we do provide evidence that temperature caused these changes in microbiome structure to occur. To disentangle whether bacterial species abundance/presence, host-mediated, or species-interaction effects underlie our finding, *in vitro* mock bacterial communities would need to be examined to determine how microbiome structure and function changes when exposed to different temperatures.

Bd exposure had a strong effect on microbiome structure. For instance, we found that alpha-diversity was lower on Bd+ individuals, with alpha-diversity decreasing with

increasing Bd load. When Bd colonizes amphibian skin, it disturbs skin stability, impedes electrolyte transport (Voyles et al. 2009), and changes the environment for microbial communities, which can lead to the reduction in alpha-diversity. The pathogen also interacts directly with the microbiome and may produce metabolites, prevent attachment to epithelial cells or compete for resources with bacteria. In a variety of other animal hosts, a decrease in alpha-diversity is generally observed with increasing disease severity including pulmonary (Garcia-Nunez et al. 2014) and liver diseases in humans (Ling et al. 2015), and *Vibrio*-associated disease in Pacific oysters (Lokmer & Wegner 2015). A decrease in alpha-diversity likely represents a good indicator of declining health for future disease studies. Further, we found that as Bd load increased changes in community composition increased. In mountain-yellow legged frogs, Jani & Briggs (2014) found that microbiome composition (beta-diversity) changed with Bd load, demonstrating consistencies across studies supporting a Bd-induced disturbance of the microbiome.

Overall anti-Bd bacterial abundance increased with Bd load, but this did not reduce salamander mortality. Anti-Bd bacteria may gain a competitive advantage by acting as opportunistic colonists or responding to changes in host physiology caused by Bd infection. For instance, the amphibian host may respond to Bd by producing antimicrobial peptides (AMPs), which can change microbial community structure (Kueng et al. 2014). Invading pathogens and AMP production are forms of interference competition, and theoretical work demonstrates that interference competition favors the recruitment of antibiotic-producing (and –resistant) bacteria (Scheuring & Yu 2012), such as anti-Bd bacterial taxa. Increased colonization of the skin by anti-Bd bacteria may relate to skin secretions from the amphibian during Bd-induced skin disruption (Voyles et

al. 2009), such as CO_2, that may act as a signal molecule to guide colonization of anti-Bd bacteria (Valdes et al. 2015).

While most salamanders were highly susceptible to Bd infection, some individuals cleared infection; the probability of survival increased when Bd loads were lower. This suggests that there were other factors involved in influencing host survival such as variation in host immune response, activity of the pathogen, genetic identity or unmeasured properties of the microbiome. However, since mortality was similar among temperatures, and temperature influences the host immune response (Ribas et al. 2009) and the production of infectious zoospores (Barnett 2014), we conclude that differences in host immune responses or Bd growth rate were unlikely to be directly involved in influencing host survival. However, we cannot rule out the possibility that interactions between host and pathogen may have driven a lack of differences in susceptibility among temperatures. Surviving individuals may have had unique genetic profiles (Goodrich et al. 2016) that contributed to reduction and clearance of Bd or other unmeasured properties of the microbiome may have contributed. For instance, Becker et al. (2009) found that higher concentrations of the metabolite violacein were related to some *P. cinereus* surviving Bd infection.

We exposed salamanders to high pathogen loads, in which salamanders harbored infections of Bd at levels typical of epidemics (Vredenburg et al. 2010, Catenazzi et al. 2011). In other experiments, *P. cinereus* was exposed to Bd at lower loads to the same Bd isolate, and in those experiments Bd did not cause mortality and individuals cleared infection within a few weeks (Becker & Harris 2010, Muletz et al. 2012). This suggests context-dependency on pathogen load on whether individuals survive or die. When the

pathogen is present at lower levels the microbiome may offer greater protection in those instances (Becker & Harris 2010, Muletz et al. 2012). For example, when pathogen exposure was 50% less than our exposure amount the skin microbiome on *P. cinereus* mediated weight loss by Bd (Becker & Harris 2010). It is increasingly recognized that microbiome structure can affect animal host disease dynamics, yet our findings indicate that in terms of fatal disease the native microbiome did not provide protection for the salamanders. Examining microbiome response across a range of pathogen loads would be a useful next step to determine if differences in pathogen load can cause differences in the microbiome response to pathogen invasion.

Our findings are directly relevant to amphibian conservation efforts: Bd is a leading cause of amphibian declines, and methods to manage disease in wild amphibians are needed. Given the opportunity to manipulate the amphibian microbiome by applying probiotics for restoration and protection of many amphibian species globally, it is important to develop effective management protocols that consider *in vivo* Bd-microbiome dynamics. Probiotic application on amphibian skin generally does not persist for longer than a couple months (Harris et al. 2009a, Muletz et al. 2012). Considering the effects of temperature and Bd exposure on the bacterial community are important for advancing these conservation tools. For example, the effects of temperature on the abundance of targeted probiotics and the Bd-induced disturbance of the microbiome should be considered. Re-application of probiotics during disease epidemics will likely be needed given the chance of Bd-induced microbiome disturbance, which is corroborated by theoretical work (Ackleh et al. 2016). Additional studies in other amphibian hosts in

laboratory and field experiments will be useful to achieve an integrated understanding of microbiome variability and stability and its role in disease protection.

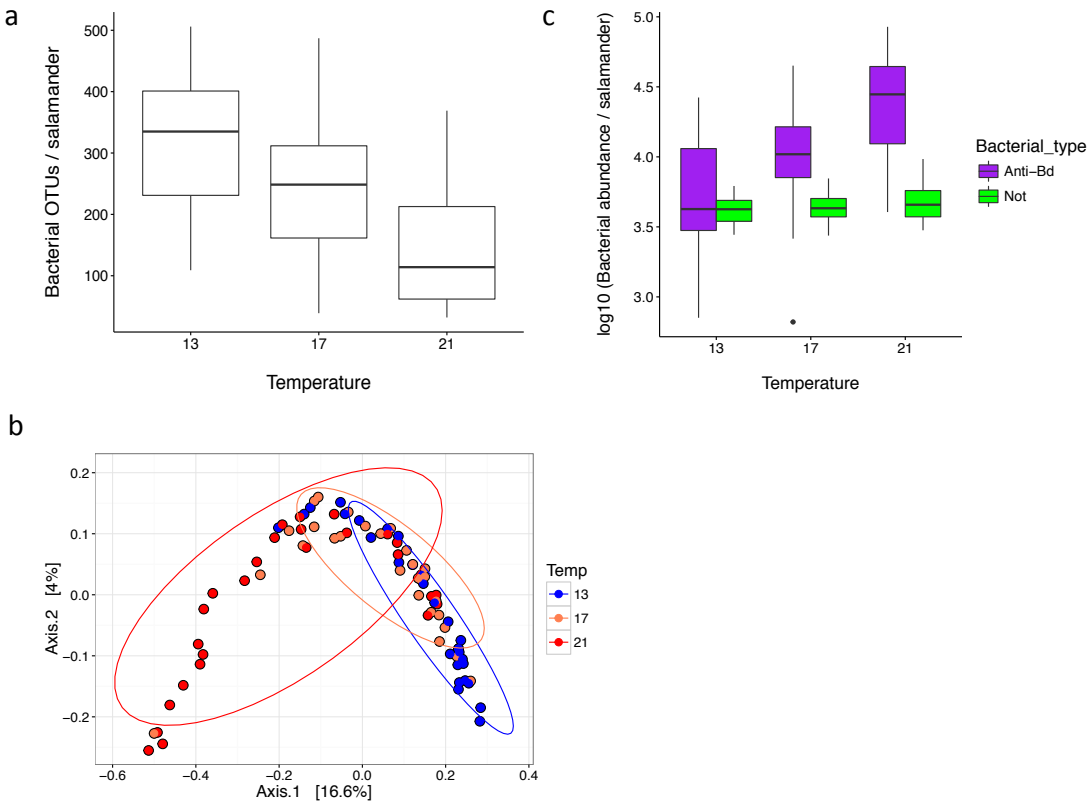


Figure 4.1 Skin microbiome structure pre-Bd exposure. Increased temperature caused reduction in alpha-diversity (a), changes in beta-diversity (b; PCoA of Jaccard distances shown with 80% confidence ellipses), and increase in abundance of anti-Bd bacteria, but no effect on non-Bd inhibitory bacteria (c).

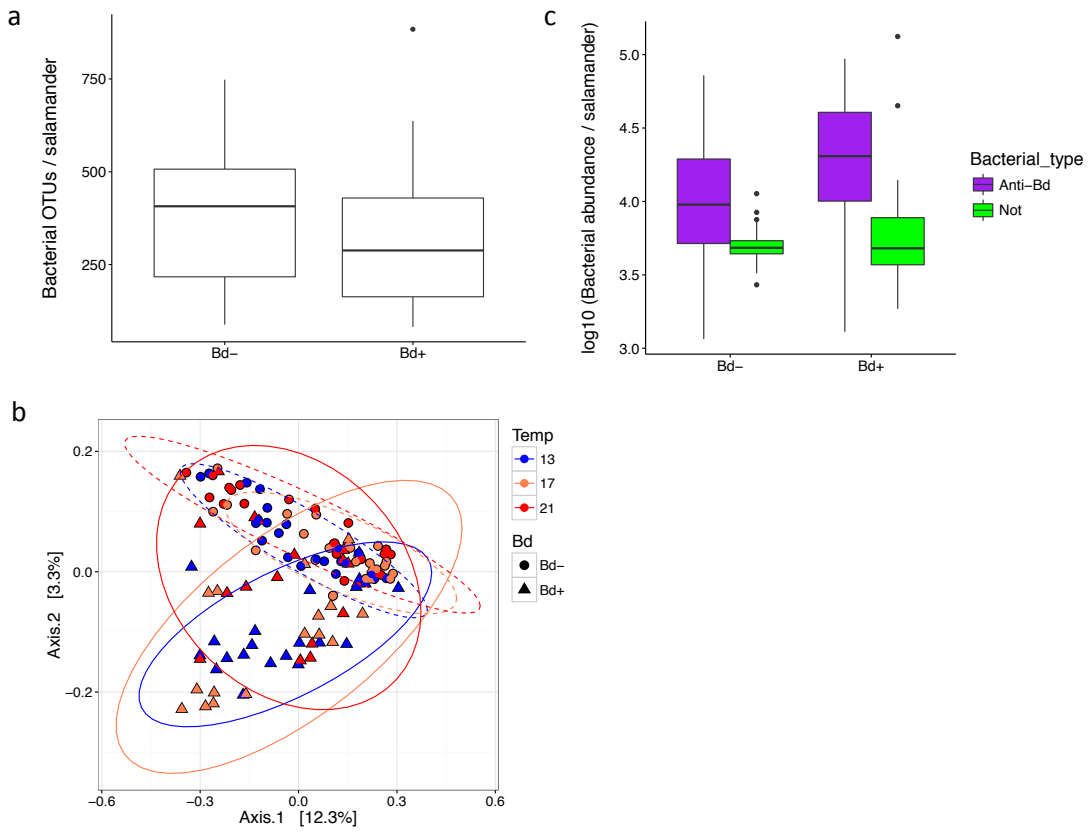


Figure 4.2 Skin microbiome structure post-Bd exposure. Bd caused a reduction in alpha-diversity (a), changes in beta-diversity (b; PCoA of Jaccard distances shown with 80% confidence ellipses), and an increase in abundance of anti-Bd bacteria, but no effect on non-Bd inhibitory bacteria (c). Only for beta-diversity (b) was there an effect of temperature on community composition.

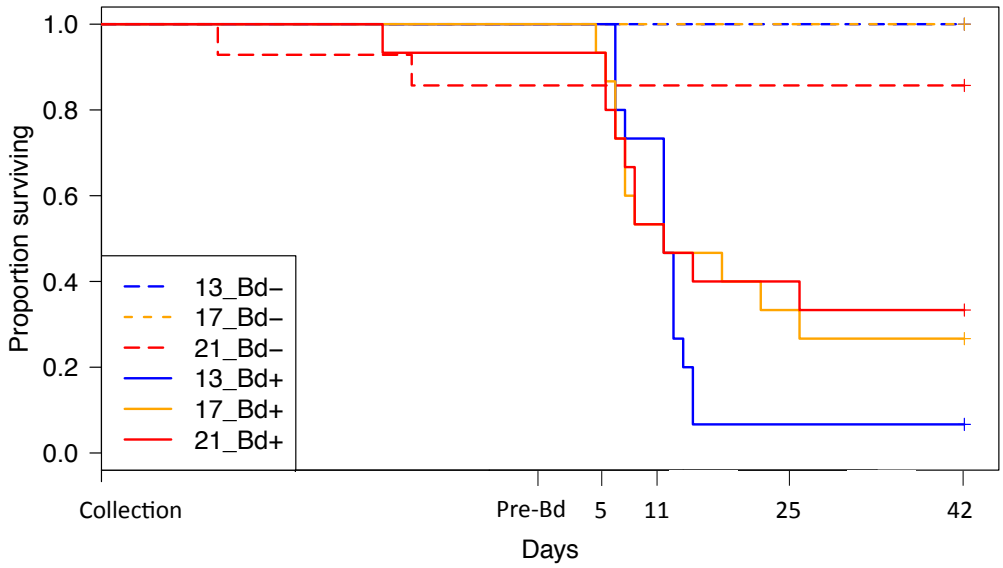


Figure 4.3 Survival curves by treatment. Salamanders in the Bd+ level experienced higher mortality rate than those in the Bd- level, regardless of temperature. Each sampling event is indicated on the x-axis.

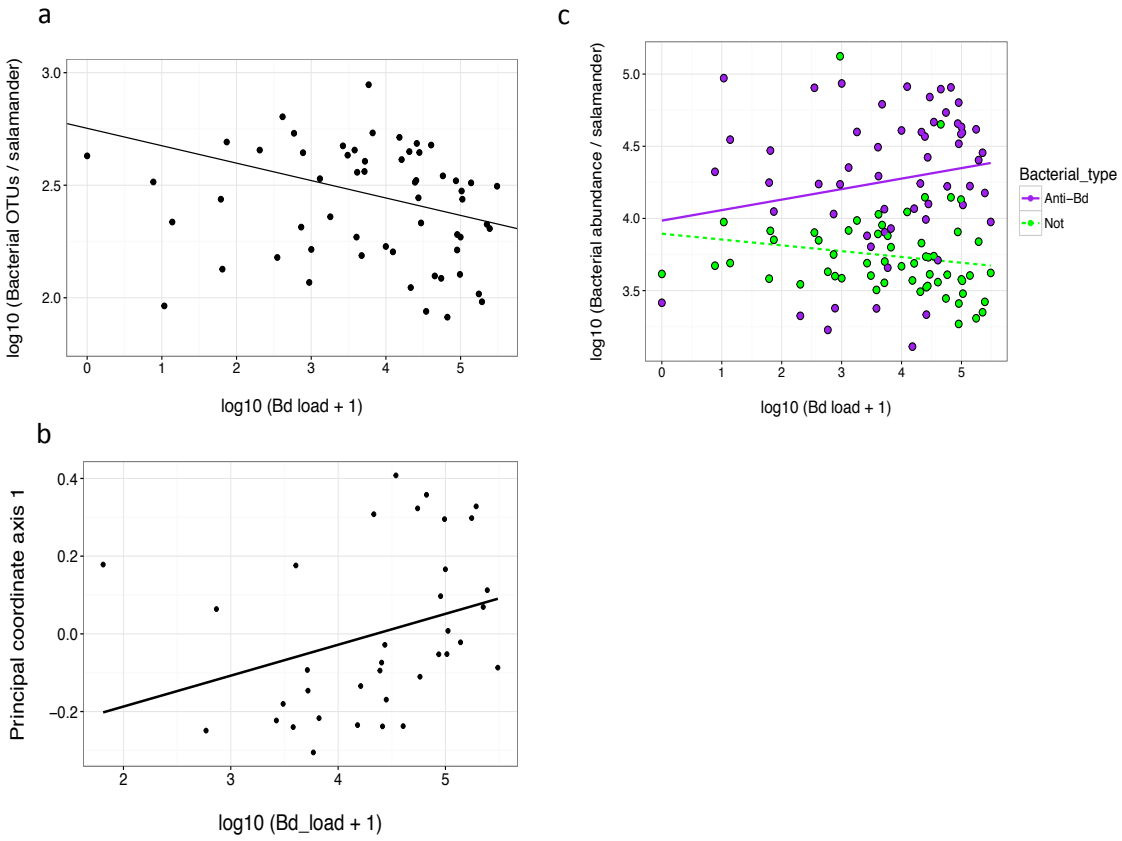


Figure 4.4 Correlations between Bd load and microbiome structure. For Bd+ level salamanders, Bd load was correlated to reduction in alpha-diversity (a), changes in beta-diversity (b), and increase in abundance of anti-Bd bacteria, but no effect on non-Bd inhibitory bacteria (c) across temperatures.

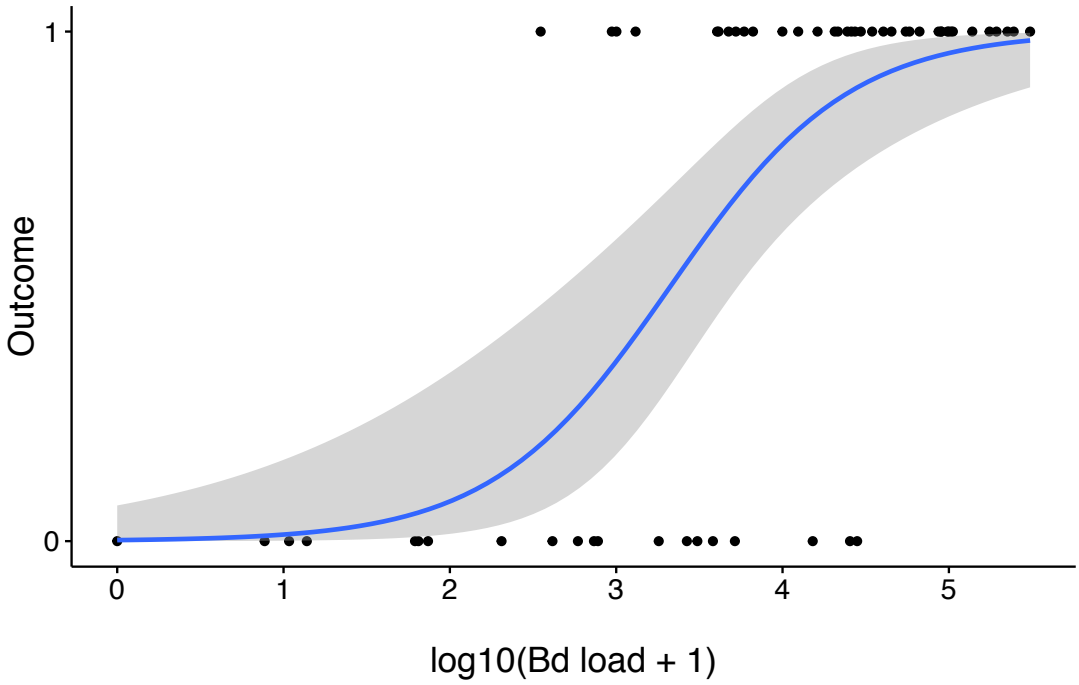


Figure 4.5 The relationship between Bd load and survival outcome (0 = survival, 1 = mortality). Individuals with higher Bd loads were more likely to experience mortality for Bd+ level salamanders across temperatures.

Chapter V: SYNTHESIS

In this dissertation, I addressed several questions about the ecology and evolution of animal-microbial symbioses in the salamander-Bd-microbiome system (Chapter I). I employed both microbiological and molecular techniques in field and laboratory studies using *Plethodon* salamanders as the focal host species. I synthesize results, explaining how they fit together and in a larger scientific context.

I. Taxonomic distribution of anti-Bd bacteria on *Plethodon* salamanders

The use of anti-Bd bacteria as conservation treatment of Bd-associated disease has been championed as a conservation strategy. Yet, we know little about the taxonomic distribution of these bacteria. I identified anti-Bd bacteria across multiple species of salamanders across multiple locations in the eastern US. I described a diverse group of bacteria from 4 bacterial phyla, and identified 13 new anti-Bd bacteria that did not match any bacteria previously identified on amphibian skin.

Several anti-Bd bacterial taxa were widespread, being present at multiple sites and on multiple species. In both Chapter II and III, I found high abundance and prevalence of OTUs belonging to the Proteobacteria phyla; the majority of which I found to either have anti-Bd properties or have high sequence similarity to anti-Bd bacterial OTUs. Within this phylum, three bacterial genera were common in our study, *Pseudomonas*, *Acinetobacter* and *Stenotrophomonas*. Both *Pseudomonas* and *Stenotrophomonas* are genera that contain high percentages of anti-Bd bacteria, while *Acinetobacter* contains a moderate percentage of anti-Bd bacteria (Becker et al. 2015, Woodhams et al. 2015). All three genera are well known for producing antifungal secondary metabolites that inhibit

plant pathogens (Walsh et al. 2001, Gulati et al. 2009, Hayward et al. 2010), and are common on many amphibians globally (Woodhams et al. 2015). They may be good colonizers of amphibian skin, or amphibians may have a long history of hosting such bacteria. The dominance these microbial partners suggests that these symbionts serve a functional role for the host and are strong competitors with other microbial taxa (Scheuring & Yu 2012).

II. Host and environmental impacts on microbiome structure

Understanding how animal-bacterial associations are formed, modified and sustained is important in understanding the ecology and evolution of animal-bacterial symbioses. More than 90% of microbiome studies have characterized mammalian microbiomes, particularly humans (reviewed by Colston & Jackson 2016), and therefore we have little understanding of the ecological and evolutionary factors that shape the microbiome of wild animals. I studied the amphibian skin microbiome of sympatric, congeneric species across multiple sites (Chapter II and III); this contributes to determining general principles of host-associated community assembly and the role host-associated microbes play in health and disease of the host.

Major factors that are generally hypothesized to shape microbial community structure are host species and environment. I attempted to isolate the effect of host species from confounding site environmental factors by sampling co-occurring species at multiple sites. I discovered that all co-occurring species had similar microbial communities in terms of anti-Bd bacteria (Chapter II and III) and their entire bacterial community (Chapter III). This indicated that innate biological and chemical properties of

host species that structure microbial communities are similar among co-occurring *Plethodon* salamanders. This finding is important because it demonstrates that species-specific microbial communities that have been reported in many animal systems (Amato et al. 2015, Belden et al. 2015, Bik et al. 2016, Council et al. 2016, Kueneman et al. 2014, Larsen et al. 2013, Ley et al. 2008, McKenzie et al. 2012, Rebollar et al. 2016, Sanders et al. 2015) are not always observed and that, in some cases, host factors that select for microbial communities are similar among species. It might be argued that host factors are inconsequential and microbiome composition is only a reflection of the bacteria in the environment, but many studies have shown that host communities are a unique set of bacteria from environmental communities (Bik et al. 2016, Loudon et al. 2014, Rebollar et al. 2016a, Schmidt et al. 2015, Sullam et al. 2012, Walke et al. 2014). To better understand the host factors that are driving the similarities among co-occurring species, the next steps would be to examine the chemical content of the skin mucus, such as antimicrobial peptides (Rollins-Smith et al. 2002, Conlon 2011), skin-shedding rate (Meyer et al. 2012), immune genes (Huang et al. 2016) and diet (Antwis et al. 2014), which may influence microbiome structure.

Both the structure of the entire bacterial community and anti-Bd bacterial community differed among sites (Chapter II and III). This suggested that environment plays some role in determining microbiome diversity and composition on salamander skin. To isolate the effect of environment from host species, I specifically examined microbiome structure along an elevational gradient for one species at Shenandoah NP. For the entire bacterial community, I found that (Chapter III) varied in a predictable way along the gradient. I hypothesized that temperature was an important factor as it has been

shown to influence microbiome structure (Sunagawa et al. 2015, Lokmer & Wegner 2015). Therefore, in Chapter IV I used temperature in a laboratory experiment to examine its impact on microbiome structure. I demonstrated that temperature impacted microbiome structure. This was the first study to examine the interaction of temperature, Bd and microbiome and provides insight into the role of environment in impacting Bd disease dynamics.

III. Is the microbiome protective?

Here I pose the question: "is the microbiome or anti-Bd bacteria specifically protective against Bd?" My field data suggest that there are at least 50 species of anti-Bd bacteria present on *Plethodon* salamanders in the wild (Chapter II) and these anti-Bd bacteria are prevalent and abundant (Chapter III). If anti-Bd bacteria are important, then these salamanders may be protected from Bd. Limited data and published studies prevent the comparison of prevalence and abundance of anti-Bd bacteria between *Plethodon* salamanders and other amphibian species. First, more sampling of microbiomes along a gradient of amphibian species that are susceptible to resistant to Bd is needed to identify correlations between high prevalence and abundance of anti-Bd bacteria and protection from Bd. Second, experimental tests are needed to show causation. Therefore, I conducted a laboratory experiment for Chapter IV to determine if certain properties of the host microbiome were protective of the salamander against Bd.

I showed that the microbiome was not protective against Bd for *P. cinereus*, although anti-Bd bacterial abundance increased with increasing Bd loads. These salamanders were collected from the same region in which we conducted field sampling

for Chapter II and III, and had high prevalence and abundance of anti-Bd bacteria. I exposed the salamanders to Bd at loads typical of epidemics (Vredenburg et al. 2010, Catenazzi et al. 2011). If an epidemic were to occur, the native microbiome alone is unlikely to provide these salamanders protection. However, anti-Bd bacteria may provide these salamander defenses against Bd (Becker et al. 2011, Muletz et al. 2012) under current conditions in the wild, where Bd levels are much lower (Richards-Hrdlicka et al. 2013, Muletz et al. 2014). We provide evidence that these bacteria are abundant and prevalent members of *Plethodon* salamander skin microbial communities. Other properties of the host may explain the low prevalence of Bd detected on these salamanders (Muletz et al. 2014), including host immunogenetics (Savage & Zamudio 2011, Ellison et al. 2015). The next steps to determine if *Plethodon* salamanders are combating Bd infection via immune genes would be to quantify the relationship between immunogenetic response and Bd clearance using transcriptomics. This type of information would help predict the potential for evolutionary resistance or tolerance in *Plethodon* salamanders or other wild populations.

IV. Conservation Implications

There is a pressing need for development of conservation strategies to combat emerging infectious diseases. Not only is Bd a threat to amphibian biodiversity, but its closely related sister taxon, *Batrachochytrium salamandrivorans* (Bsal) is now also of major conservation concern. Bsal has only been detected in Europe and Asia (Martel et al. 2014), but the risk of Bsal entering the US is possible through wildlife trade. Bsal preferentially infects salamanders, and the US is a hotspot of salamander biodiversity.

One potential action strategy assessed by the USGS is to apply probiotics to salamanders (Grant et al. 2016). In an independent study, we found that five species of anti-Bd bacteria also showed anti-Bsal properties (Almario 2016), demonstrating that knowledge about anti-Bd bacteria will be useful in Bsal probiotic conservation strategies also.

A number of factors deserve consideration when developing an effective probiotic. These include, but are not limited to, interactions between the bacteria and its host, environment and other microbiota, including the pathogen. Further, the use of an indigenous strain that is dominant, competitive and adapted to local conditions may improve survival rates and maintain the biointegrity of the environment (Paau 1989). In Chapter II and III, I identified widely distributed and highly inhibitory anti-Bd bacteria that may be useful not only for *Plethodon* salamanders if needed, but for other species. *Pseudomonas* spp. may be good candidates for bioaugmentation trials. They are found naturally in the environment and on at least 25/37 amphibian species sampled globally for their anti-Bd bacteria (Chapter II, Woodhams et al. 2015). 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). In Chapter IV, I showed that Bd altered the microbial community, indicating that applying bioaugmentation continually through an epidemic is likely needed for the bacteria to have any effect. This is corroborated by probiotic research in other systems showing that the protection offered against pathogens is often only effective when the probiotic is regularly applied (Verschuere et al. 2000, Fric 2007).

I provide new information concerning the specificity of amphibians' microbiome within geographic regions, between host species, and in relation to environment and

disease. Results are interesting from both a scientific and conservation viewpoint because they contribute to understanding microbiome composition and distribution and how Bd and environment are likely to affect bacterial and amphibian biodiversity.

Appendix

Table A3.1 Changes in abundance among bacterial OTUs among localities and species.

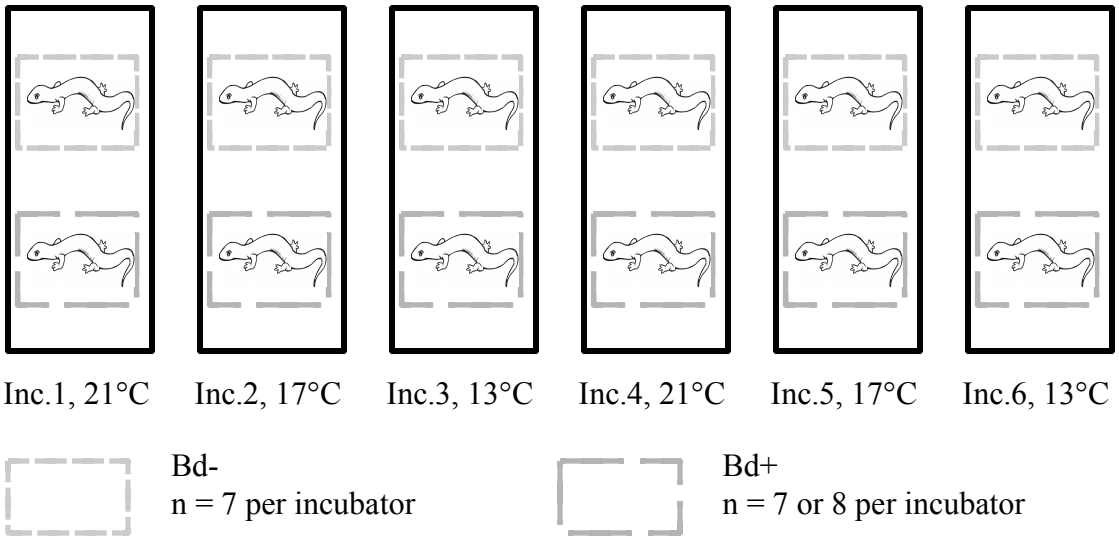
OTU ID	logFC	se	FDR p-value	Comparison
OTU_11	1.94	0.23	<0.001	Mt. Rogers to Shenandoah
OTU_449	4.00	0.38	<0.001	Mt. Rogers to Shenandoah
OTU_79	1.73	0.20	<0.001	Mt. Rogers to Shenandoah
OTU_249	2.01	0.26	<0.001	Mt. Rogers to Shenandoah
OTU_425	1.87	0.25	<0.001	Mt. Rogers to Shenandoah
OTU_275	2.16	0.33	<0.001	Mt. Rogers to Shenandoah
OTU_5	3.62	0.57	<0.001	Mt. Rogers to Shenandoah
OTU_19	-2.86	0.56	<0.001	Mt. Rogers to Shenandoah
OTU_25	2.81	0.58	<0.001	Mt. Rogers to Shenandoah
OTU_2	-2.65	0.55	<0.001	Mt. Rogers to Shenandoah
OTU_16	-1.36	0.32	<0.001	Mt. Rogers to Shenandoah
OTU_158	1.89	0.46	<0.001	Mt. Rogers to Shenandoah
OTU_3	-2.37	0.61	<0.001	Mt. Rogers to Shenandoah
OTU_112	3.13	0.82	0.001	Mt. Rogers to Shenandoah
OTU_4	-1.84	0.57	0.005	Mt. Rogers to Shenandoah
OTU_34	-1.57	0.49	0.006	Mt. Rogers to Shenandoah
OTU_17	-0.87	0.30	0.012	Mt. Rogers to Shenandoah
OTU_50	-2.41	0.83	0.013	Mt. Rogers to Shenandoah
OTU_485	0.78	0.27	0.013	Mt. Rogers to Shenandoah
OTU_332	2.29	0.88	0.030	Mt. Rogers to Shenandoah
OTU_148	1.87	0.76	0.043	Mt. Rogers to Shenandoah
OTU_449	4.41	0.58	<0.001	Catoctin to Shenandoah
OTU_249	2.37	0.34	<0.001	Catoctin to Shenandoah
OTU_165	-2.29	0.33	<0.001	Catoctin to Shenandoah
OTU_5	3.97	0.63	<0.001	Catoctin to Shenandoah
OTU_79	2.76	0.46	<0.001	Catoctin to Shenandoah
OTU_8	-1.99	0.35	<0.001	Catoctin to Shenandoah
OTU_19	-2.42	0.62	0.001	Catoctin to Shenandoah
OTU_2	-2.56	0.70	0.002	Catoctin to Shenandoah
OTU_13	-1.31	0.36	0.002	Catoctin to Shenandoah
OTU_158	1.50	0.43	0.003	Catoctin to Shenandoah
OTU_275	2.34	0.68	0.003	Catoctin to Shenandoah
OTU_38	2.42	0.74	0.005	Catoctin to Shenandoah
OTU_12	-3.30	1.01	0.005	Catoctin to Shenandoah
OTU_3	-2.30	0.71	0.005	Catoctin to Shenandoah
OTU_25	2.32	0.74	0.007	Catoctin to Shenandoah
OTU_59	-1.08	0.35	0.007	Catoctin to Shenandoah

OTU_50	-2.58	0.92	0.017	Catoctin to Shenandoah
OTU_4	-1.89	0.70	0.022	Catoctin to Shenandoah
OTU_17	-0.88	0.34	0.026	Catoctin to Shenandoah
OTU_485	1.19	0.46	0.026	Catoctin to Shenandoah
OTU_54	2.04	0.81	0.033	Catoctin to Shenandoah
OTU_79	-1.34	0.39	0.031	*P. cinereus* to *P. glutinosus*
OTU_449	-3.20	0.96	0.031	*P. cinereus* to *P. glutinosus*
OTU_425	-1.85	0.57	0.031	*P. cinereus* to *P. glutinosus*
OTU_50	1.08	0.34	0.031	*P. cinereus* to *P. glutinosus*
OTU_11	-1.25	0.41	0.035	*P. cinereus* to *P. glutinosus*
OTU_449	-3.27	0.88	0.013	*P. cylindraceus* to *P. glutinosus*
OTU_79	-1.47	0.44	0.029	*P. cylindraceus* to *P. glutinosus*

Table A3.2 Bacterial OTUs that showed correlations of abundances with elevation. Asterisks denote OTUs that were taxonomically similar to known anti-Bd bacteria.

OTU ID	Family	Genus	FDR p-value	adjusted r-sq	slope	Panel i Figure
OTU_6	Nocardiaceae	*Rhodococcus*	0.001	0.31	0.014	A
OTU_165	Microbacteriaceae	--	0.043	0.13	0.006	A
OTU_19 *	Micrococcaceae	*Arthrobacter*	0.036	0.15	0.007	A
OTU_382 *	Planococcaceae	--	0.010	0.20	0.005	A
OTU_10 *	Comamonadaceae	--	0.034	0.15	0.007	A
OTU_367	Comamonadaceae	--	0.001	0.28	0.008	A
OTU_50	Streptococcaceae	*Streptococcus*	0.003	0.24	0.007	A
OTU_16	Brucellaceae	*Ochrobactrum*	0.010	0.20	0.008	A
OTU_93	Hyphomicrobiaceae	*Rhodoplanes*	0.043	0.13	0.004	A
OTU_26 *	Sphingobacteriaceae	*Sphingobacterium*	0.001	0.31	0.009	A
OTU_40	Sphingobacteriaceae	*Sphingobacterium*	0.039	0.14	0.005	A
OTU_41 *	Sphingomonadaceae	*Sphingomonas*	0.043	0.13	0.005	A
OTU_3	Sanguibacteraceae	*Sanguibacter*	0.001	0.32	0.006	B
OTU_2 *	Pseudomonadaceae	*Pseudomonas*	0.001	0.30	0.005	B
OTU_4 *	Xanthomonadaceae	*Stenotrophomonas*	< 0.001	0.43	0.005	B
OTU_5 *	Pseudomonadaceae	*Pseudomonas*	0.034	0.15	-0.008	B
OTU_61	Xanthomonadaceae	*Stenotrophomonas*	0.044	0.13	0.006	B

Figure A4.1 Incubator set-up. We randomly assigned two chambers to one of three temperatures (13, 17, 21 °C), and maintained them at that temperature, a 12 h light: 12 h dark cycle and 80 ± 10% humidity throughout the experiment. We randomly assigned salamanders to an environmental chamber such that Bd- and Bd+ salamanders were both housed within each chamber. On a weekly basis salamander shelf number was randomly changed.



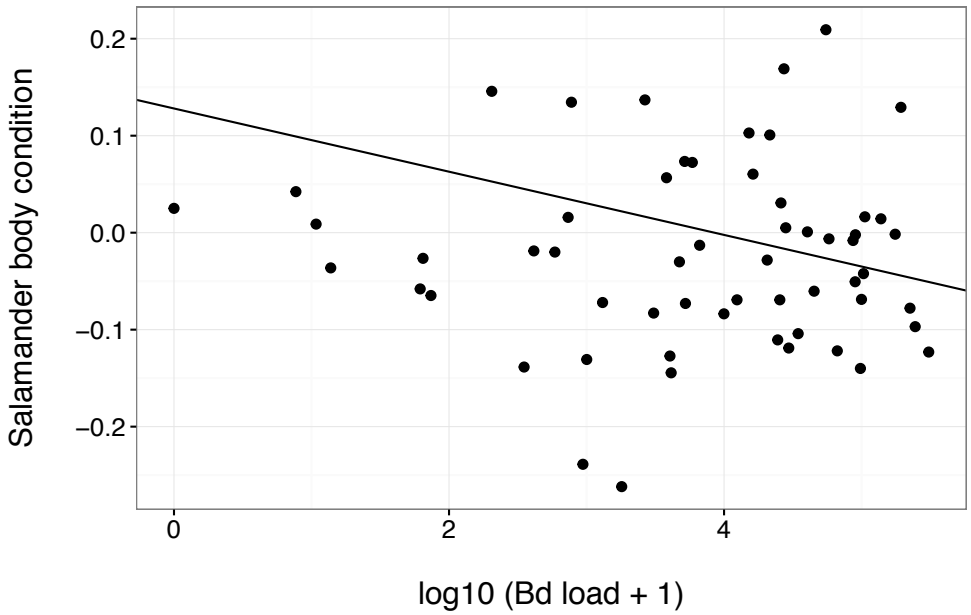


Figure A4.2 Bd load was correlated to reduction in body condition for Bd+ level salamanders across temperatures.

Supplementary methods and results for Chapter IV

We quantified the effects of Bd, Temperature and their interaction on anti-Bd bacterial abundance. We used ANOVAs for pre-Bd exposure, and LMM for Days 5 and 11 with Day as a random effect. For anti-Bd bacterial OTUs that were significantly different among temperatures we quantified the directional effect of temperature on their abundance using linear models, and report the statistics for those OTUs. Pre-Bd exposure, we found that the abundance of 21 anti-Bd bacterial OTUs differed among temperatures; 13 increased linearly with temperature, and the other 8 decreased (Table A4.1; ANOVA: $p < 0.05$). Post-Bd exposure, we found that the abundance of 15 anti-Bd bacterial OTUs differed among temperatures (9 linearly decreasing with temperature, and the other increasing; Table A4.2), 44 anti-Bd bacterial OTUs differed between Bd+ and Bd- individuals (LMM: $p < 0.05$, Table A4.3), and 7 showed a Bd x Temperature interaction (Table A4.3).

We determined if there was a correlation of anti-Bd bacterial abundance with Bd load on Days 5 and 11 for Bd+ level salamanders using LMMs with Temperature as a covariate and Day as a random effect. For anti-Bd bacterial OTUs that were significantly different among Bd levels, we examined if there was a correlation between the abundance of these bacterial OTUs with Bd load using linear models, and report the statistics for those OTUs. We found that of the 44 OTUs examined, 37 were correlated to Bd load, with 27 increasing in abundance with increasing Bd load, and the other 10 decreasing (Table A4.3).

Table A4.1 21/67 anti-Bd bacterial OTUs showed a relationship with temperature pre-Bd exposure, which is 45 days of acclimation to 13, 17, 21 C.

OTU_ID	adjusted p-values	adjusted R-squared	model slope	% change /unit Temp	Phylum	Genus	Species
OTU_10	0.001	0.14	0.15	16	Actinobacteria	*Microbacterium*	--
OTU_26	0.002	0.11	-0.13	-12	Bacteroidetes	*Flavobacterium*	*succinicans*
OTU_93	0.004	0.09	-0.09	-9	Bacteroidetes	*Flavobacterium*	--
OTU_94	0.001	0.12	-0.14	-13	Bacteroidetes	*Pedobacter*	--
OTU_105	0.004	0.09	0.13	14	Proteobacteria	--	--
OTU_222	< 0.001	0.17	-0.19	-18	Proteobacteria	*Burkholderia*	--
OTU_429	0.017	0.06	-0.08	-8	Proteobacteria	*Burkholderia*	--
OTU_7	< 0.001	0.26	0.19	21	Proteobacteria	*Delftia*	--
OTU_408	0.004	0.09	-0.13	-12	Proteobacteria	*Dyella*	--
OTU_23	< 0.001	0.25	0.22	25	Proteobacteria	*Erwinia*	--
OTU_17	< 0.001	0.20	-0.18	-16	Proteobacteria	*Janthinobacterium*	*lividum*
OTU_1	< 0.001	0.27	0.20	22	Proteobacteria	*Pseudomonas*	*viridiflava*
OTU_145	0.001	0.13	0.14	16	Proteobacteria	*Pseudomonas*	--
OTU_1761	0.002	0.11	0.16	17	Proteobacteria	*Pseudomonas*	--
OTU_2064	< 0.001	0.28	0.28	33	Proteobacteria	*Pseudomonas*	*viridiflava*
OTU_3	< 0.001	0.24	0.16	18	Proteobacteria	*Pseudomonas*	*veronii*
OTU_61	< 0.001	0.23	0.20	22	Proteobacteria	*Pseudomonas*	--
OTU_2462	0.002	0.11	-0.13	-12	Proteobacteria	*Rhodanobacter*	--
OTU_1200	0.003	0.10	0.16	18	Proteobacteria	*Stenotrophomonas*	--
OTU_2	< 0.001	0.26	0.19	20	Proteobacteria	*Stenotrophomonas*	--
OTU_6	< 0.001	0.28	0.17	19	Proteobacteria	*Variovorax*	*paradoxus*

Table A4.2 15/67 anti-Bd bacterial OTUs showed a relationship with temperature on Day 5 and 11.

OTU_ID	adjusted p-values	adjusted R-squared	model slope	% change /unit Temp	Phylum	Genus	Species
OTU_5	0.036	0.03	-0.17	-16	Bacteroidetes	*Chryseobacterium*	--
OTU_93	0.005	0.06	-0.11	-10	Bacteroidetes	*Flavobacterium*	--
OTU_1189	0.009	0.05	-0.15	-14	Bacteroidetes	*Flavobacterium*	--
OTU_26	0.005	0.06	-0.22	-20	Bacteroidetes	*Flavobacterium*	*succinicans*
OTU_29	0.003	0.07	-0.19	-17	Bacteroidetes	*Pedobacter*	--
OTU_112	0.007	0.05	-0.13	-12	Bacteroidetes	*Pedobacter*	--
OTU_73	0.000	0.15	-0.20	-18	Bacteroidetes	*Pedobacter*	*cryoconitis*
OTU_22	0.000	0.12	0.28	32	Bacteroidetes	*Sphingobacterium*	*multivorum*
OTU_715	0.001	0.10	0.18	20	Bacteroidetes	*Sphingobacterium*	*multivorum*
OTU_15	0.073	0.02	0.12	12	Bacteroidetes	*Wautersiella*	--
OTU_18	0.000	0.15	0.27	31	Proteobacteria	*Comamonas*	--
OTU_7	0.013	0.04	0.08	8	Proteobacteria	*Delftia*	--
OTU_17	0.001	0.09	-0.22	-19	Proteobacteria	*Janthinobacterium*	*lividum*
OTU_80	0.011	0.05	-0.07	-7	Proteobacteria	*Janthinobacterium*	--
OTU_2895	0.000	0.13	0.15	16	Proteobacteria	*Sphingomonas*	--

Table A4.3 44/67 anti-Bd bacterial OTUs showed a relationship with Bd (including 7 showing Temperature interaction) on Day 5 and 11. When examined as a linear function with Bd load, 37/44 showed a linear relationship.

OTU_ID	adjusted p-values Bd level	adjusted p-values Bd x Temp	adjusted p-values Bd load	model slope (% change/ unit Bd)	Phylum	Genus	Species
OTU_32	< 0.001	--	0.034	0.26	Actinobacteria	*Arthrobacter*	--
OTU_3218	< 0.001	--	< 0.001	-0.32	Actinobacteria	*Chitinophaga*	--
OTU_2571	0.003	--	0.005	-0.24	Actinobacteria	*Curtobacterium*	--
OTU_10	< 0.001	--	< 0.001	-0.44	Actinobacteria	*Microbacterium*	--
OTU_111	0.000	--	0.013	0.38	Bacteroidetes	*Chryseobacterium*	--
OTU_5	< 0.001	0.004	< 0.001	0.61	Bacteroidetes	*Chryseobacterium*	--
OTU_11	< 0.001	--	0.109	NS	Bacteroidetes	*Chryseobacterium*	--
OTU_400	< 0.001	--	0.006	0.40	Bacteroidetes	*Flavobacterium*	*succinicans*
OTU_1189	< 0.001	--	< 0.001	0.55	Bacteroidetes	*Flavobacterium*	--
OTU_26	< 0.001	0.020	< 0.001	0.75	Bacteroidetes	*Flavobacterium*	*succinicans*
OTU_112	< 0.001	--	0.003	0.37	Bacteroidetes	*Pedobacter*	--
OTU_94	< 0.001	--	< 0.001	0.46	Bacteroidetes	*Pedobacter*	--
OTU_29	< 0.001	--	< 0.001	0.57	Bacteroidetes	*Pedobacter*	--
OTU_73	< 0.001	< 0.001	0.064	NS	Bacteroidetes	*Pedobacter*	*cryoconitis*
OTU_715	< 0.001	0.003	0.002	0.35	Bacteroidetes	*Sphingobacterium*	*multivorum*
OTU_22	< 0.001	0.003	< 0.001	0.59	Bacteroidetes	*Sphingobacterium*	*multivorum*
OTU_15	< 0.001	--	< 0.001	0.64	Bacteroidetes	*Wautersiella*	--
OTU_45	< 0.001	--	0.020	0.24	Firmicutes	--	--
OTU_312	< 0.001	--	< 0.001	0.43	Firmicutes	--	--
OTU_263	< 0.001	--	0.021	-0.08	Proteobacteria	--	--
OTU_105	0.026	--	0.497	NS	Proteobacteria	--	--
OTU_2891	0.001	--	0.032	0.24	Proteobacteria	*Acinetobacter*	*guillouiae*
OTU_1345	0	--	0.023	0.28	Proteobacteria	*Acinetobacter*	--
OTU_1485	< 0.001	--	< 0.001	0.42	Proteobacteria	*Acinetobacter*	--
OTU_249	0.000	--	0.001	0.46	Proteobacteria	*Acinetobacter*	--
OTU_14	< 0.001	--	0.003	0.50	Proteobacteria	*Acinetobacter*	--
OTU_2043	< 0.001	--	0.307	NS	Proteobacteria	*Acinetobacter*	--
OTU_3054	0.006	--	0.033	0.23	Proteobacteria	*Comamonas*	--
OTU_18	< 0.001	0.001	0.008	0.38	Proteobacteria	*Comamonas*	--
OTU_7	< 0.001	0.007	0.001	-0.18	Proteobacteria	*Delftia*	--
OTU_17	< 0.001	--	< 0.001	0.71	Proteobacteria	*Janthinobacterium*	*lividum*
OTU_177	0.001	--	0.256	NS	Proteobacteria	*Janthinobacterium*	--
OTU_80	0.010	--	0.656	NS	Proteobacteria	*Janthinobacterium*	--

OTU_61	< 0.001	--	< 0.001	-0.60	Proteobacteria	*Pseudomonas*	--
OTU_2064	< 0.001	--	< 0.001	-0.43	Proteobacteria	*Pseudomonas*	*viridiflava*
OTU_8	0.008	--	0.006	-0.14	Proteobacteria	*Pseudomonas*	*viridiflava*
OTU_1	0.003	--	0.022	-0.13	Proteobacteria	*Pseudomonas*	*viridiflava*
OTU_145	< 0.001	--	0.003	0.24	Proteobacteria	*Pseudomonas*	--
OTU_142	0.001	--	< 0.001	0.36	Proteobacteria	*Pseudomonas*	--
OTU_3	< 0.001	--	< 0.001	0.36	Proteobacteria	*Pseudomonas*	*veronii*
OTU_42	< 0.001	--	0.028	0.22	Proteobacteria	*Serratia*	*marcescens*
OTU_1200	0.011	--	0.007	0.37	Proteobacteria	*Stenotrophomonas*	--
OTU_2	0.016	--	0.052	NS	Proteobacteria	*Stenotrophomonas*	--
OTU_6	0.026	--	0.001	-0.16	Proteobacteria	*Variovorax*	*paradoxus*

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