AMPHIBIAN MICROBIAL AND MORPHOLOGICAL DEFENSES AGAINST NATURAL ENEMIES

By

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ABSTRACT OF THE DISSERTATION

Amphibian microbial and morphological defenses against natural enemies

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Amphibians face a suite of challenges to survival including predation, pollution, habitat loss, and infectious diseases. Over the last several decades, amphibian populations have been severely declining as a result of a combination of these factors. A looming threat caused by an emerging fungal pathogen has highlighted the potential importance of the amphibian cutaneous microbiome in mediating the effects of disease. In the first two chapters of my dissertation, my research focus is on understanding the diversity and function of the amphibian cutaneous microbiome in the context of resistance against this pathogen. In the last chapter, I shift my focus to exploring the role of phenotypic plasticity in protecting tadpoles of a near-threatened species from a natural predator.

In chapter 1, I used culture-based techniques to study bacteria isolated from green frog skin that inhibit the growth of the fungal pathogen, *Batrachochytrium dendrobatidis* (Bd). Despite bacteria being classified as the same operational taxonomic unit (OTU, ~bacterial species) based on 16S rRNA sequencing, I found differences in Bd inhibition capabilities among isolates. This suggests that phylogenetic relatedness alone is not a reliable predictor of whether or not a bacterial isolate can prevent Bd growth.

Furthermore, I found unique communities of anti-Bd bacteria among three populations of green frogs, suggesting functional redundancy of Bd inhibition across populations.

Because not all bacteria are readily culturable, I used culture-independent techniques in chapter 2 to explore variation in amphibian skin microbiomes among frog species, sampling sites, and individuals with and without Bd present on their skin. Through sampling skin microbiomes of six frog species, I found significant differences among frog skin microbiomes across species and sites, but not between Bd-positive and Bd-negative individuals. Additionally, putative anti-Bd OTUs made up a third of bacterial abundance among host-associated communities, and several putative anti-Bd OTUs were strongly associated with frogs based on their abundance and prevalence. The presence of anti-Bd OTUs may be offering frogs protection against Bd and may partially explain why several of the host species sampled are asymptomatic carriers of Bd. Overall, these results suggest that skin-associated microbial communities reflect host species and the environment, but not Bd status among the frogs studied here.

In my final chapter, I described phenotypic changes in tadpoles of *Hyla andersonii* exposed to the odonate predator *Anax junius.* Predator-exposed tadpoles developed darker and deeper tail fins. This response likely increases tadpole survival due to the "lure effect," where conspicuous tail morphology attracts predator attacks toward the tail, which can be regrown, and away from the vulnerable head. Given that these findings are consistent with previous documentation of conspicuous tail coloration in hylid tadpoles, I propose that this provides evidence of an adaptive syndrome among hylid tadpoles, where tadpoles develop conspicuous tails in the presence of odonate predators.

This dissertation has provided new insight on the defensive strategies employed by amphibians against their natural enemies and will help inform conservation plans in this time of significant amphibian declines.

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INTRODUCTION

Amphibians possess a suite of innate and acquired defenses that allow them to deal with threats to survival. For example, some amphibians have phenotypically plastic inducible defenses against predators (Smith & Van Buskirk 1995, McCollum & Van Buskirk 1996), while others acquire defenses through associations with microbial symbionts (Becker *et al*. 2009, Becker & Harris 2010). The aim of my dissertation research is to better understand both acquired and inducible defenses and the role they play in protecting amphibians against their natural enemies. Amphibians are ideal model systems for exploring defensive strategies because they have evolved defenses against both predators and pathogens. As such, this research has potential implications for the ecology and evolution of defenses in other systems as well.

The decline of amphibian populations worldwide is one of the largest biodiversity crises affecting our planet (Wake & Vredenburg 2008, Scheele *et al.* 2019). While amphibian population declines are likely due to a combination of factors including habitat loss and pesticide use (Kolby & Daszak 2016), the majority of declines are attributed to the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), which causes the disease chytridiomycosis (O'Hanlon *et al.* 2018, Scheele *et al.* 2019). The commercial amphibian trade is implicated as a main pathway of global transmission of the panzootic lineage of Bd after its emergence in the Korean Peninsula 50-120 years ago (O'Hanlon *et al.* 2018). Despite over two decades of research into the Bd-amphibian system, no suitable large-scale conservation strategy has been developed or implemented to curtail the spread of disease (Kolby & Daszak 2016). Many amphibians fulfill important ecological roles as both predators and prey in terrestrial and aquatic ecosystems, and their loss may have

reverberating consequences across both systems. Conservation efforts to help mitigate amphibian declines are urgently needed, and an understanding of the mechanisms of defense against natural enemies can inform those efforts.

Despite the occurrence of unprecedented amphibian declines in many parts of the world, some populations of amphibians appear to persist in the presence of Bd (Woodhams *et al.* 2007, Tobler *et al.* 2012, Rebollar *et al.* 2019). Amphibian resistance to Bd has been attributed to acquired immunity (McMahon *et al.* 2014), antimicrobial peptides secreted in granular glands (Rollins-Smith & Conlon 2005, Rollins-Smith 2009), and symbiotic microbiota that inhabit their skin (Woodhams *et al.* 2007, Becker & Harris 2010), among other factors. For the first two chapters of this dissertation, I focus on the role of symbiotic microbiota in disease resistance against Bd because the study of host microbiota has been suggested as promising avenue for wildlife conservation management (Trevelline *et al.* 2019, West *et al.* 2019).

Amphibian skin is host to resident microbes, collectively termed the microbiome, that may promote host health and prevent pathogen invasion. For this reason, there has been growing interest in describing the amphibian skin microbiome to determine how diversity or specific microbial taxa may contribute to Bd resistance. In some amphibian species, Bd inhibition may result from microbial community diversity or aggregate community functioning rather than from a single microbial taxon that strongly inhibits Bd (Bletz *et al.* 2013). The amphibian cutaneous microbiome can vary among species (McKenzie *et al.* 2012, Kueneman *et al.* 2014), habitats (Michaels *et al.* 2014, Bird *et al.* 2018), and in the presence of pathogens (Jani & Briggs 2014, Rebollar *et al.* 2016), suggesting that the role of the microbiome in Bd resistance may be context-dependent.

On a functional level, bacteria isolated from amphibian skin have been shown to reduce Bd-related mortality in susceptible amphibians (Harris *et al.* 2009). Therefore, microbiome function rather than microbial diversity per se may be essential to understanding connections between the microbiome and host health (West *et al.* 2019).

While some studies have focused on describing the overall structure of the cutaneous microbiome (Kueneman *et al.* 2014, Prado-Irwin *et al.* 2017, Bird *et al.* 2018), others have aimed to identify functionally relevant bacteria within the microbiome (Bletz *et al.* 2017, Walke *et al.* 2017, Rebollar *et al.* 2018). Because Bd resistance may be associated with the presence of bacteria that produce antifungal compounds (Becker *et al.* 2009), there has been interest in directly assessing the anti-Bd capabilities of bacteria isolated from amphibian skin (*e.g.* Harris *et al.* 2006, Bell *et al.* 2013). Such studies have led to the development of a novel antifungal isolate database that contains the DNA sequences of known anti-Bd bacteria (Woodhams *et al.* 2015), which has greatly enhanced our ability to predict microbiome function based on bacteria that are present in the microbial community. Addition of antifungal bacteria to amphibian skin has been pursued as a probiotic conservation strategy with mixed success (Becker *et al.* 2011, 2015). A more thorough understanding of the diversity and function of amphibian cutaneous microbiomes will therefore aid in the development and optimization of Bd prevention and treatment strategies.

In addition to receiving protection from microbes, some amphibians have phenotypically plastic inducible defenses that provide protection from natural enemies, a topic that is explored in the final chapter of my dissertation. Motivation for previous study on inducible defenses in amphibians has been to understand how costs and benefits

of certain phenotypes or behaviors affect fitness in the presence of a competitor or predator. This research has been very successful in describing the non-consumptive effect that predators can have on amphibian prey. For example, predators can induce defensive changes in tadpole behavior and morphology (McCollum & Van Buskirk 1996, McCollum & Leimberger 1997, Relyea 2001). Additionally, tadpoles with induced defenses can have enhanced survival with predators (McCollum & Van Buskirk 1996). Costs associated with inducible defenses include reduced growth rates and lower survival to metamorphosis (Van Buskirk 2000). These costs may explain why defensive traits are induced instead of constitutive (McCollum & Van Buskirk 1996).

Amphibians are an ideal model system for studying inducible defenses because they are easy to collect and manipulate. They also exhibit a variety of measurable phenotypic responses (Relyea 2001). Although inducible defenses have been previously studied in tadpoles, responses tend to be species-specific (Relyea 2001) and can therefore shape community dynamics in different ways. For species of conservation concern, understanding predator-induced phenotypes may lend insight to how community dynamics affect amphibian fitness. Furthermore, given recent amphibian declines due to emerging infectious diseases such as Bd, it is more crucial than ever to understand topics central to amphibian ecology that may help inform appropriate conservation strategies for threatened species.

In this dissertation, I explore two facets of amphibian defenses against enemies: (i) resident skin microorganisms and (ii) inducible defenses. In chapter one, I culture bacteria from the skin of three populations of *Lithobates clamitans* to assess the prevalence and distribution of bacteria with anti-Bd capabilities. I also test whether

bacteria classified as the same operational taxonomic unit (OTU, ~bacterial species) have similar anti-Bd capabilities, since this has implications for the use of a novel antifungal isolate database (Woodhams *et al.* 2015) for predicting the anti-Bd function based on OTUs.

In chapter two, I use culture-independent 16S rRNA sequencing to analyze the composition and diversity of microbiomes of *Lithobates clamitans* and several other species of frogs found in New Jersey. I also aim to document the prevalence of Bd in these populations and assess whether microbiome diversity, structure, and anti-Bd function is related to host species, location, and/or Bd presence.

In my final chapter, I explore a different amphibian-enemy relationship to understand how an amphibian of conservation concern responds to predator presence in artificial ponds. *Hyla andersonii* (Pine Barrens tree frog) is threatened in the state of New Jersey and much of its ecology remains unknown. I examine how tadpole survival, morphology, and behavior are affected by the presence of a probable inducible defense.

Taken together, this dissertation shows that amphibians are able to respond to threats to their survival in myriad ways, whether through associating with beneficial bacteria or through phenotypic plasticity. Understanding how amphibians deal with predators and pathogens will help prioritize management in this time of global amphibian declines.

Literature cited in Introduction

Becker, M.H., Brucker, R.M., Schwantes, C.R., Harris, R.N. & Minbiole, K.P.C. (2009). The bacterially produced metabolite violacein is associated with survival of amphibians infected with a lethal fungus. *Appl. Environ. Microbiol.*, 75, 6635–6638.

Becker, M.H. & Harris, R.N. (2010). Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. *PLoS One*, 5, e10957.

Becker, M.H., Harris, R.N., Minbiole, K.P.C., Schwantes, C.R., Rollins-Smith, L.A., Reinert, L.K., *et al.* (2011). Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. *Ecohealth*, 8, 501–6.

Becker, M.H., Walke, J.B., Cikanek, S., Savage, A.E., Mattheus, N., Santiago, C.N., *et al.* (2015). Composition of symbiotic bacteria predicts survival in Panamanian golden frogs infected with a lethal fungus. *Proc. R. Soc. B Biol. Sci.*, 282, 20142881.

Bell, S.C., Alford, R.A., Garland, S., Padilla, G. & Thomas, A.D. (2013). Screening bacterial metabolites for inhibitory effects against *Batrachochytrium dendrobatidis* using a spectrophotometric assay. *Dis. Aquat. Organ.*, 103, 77–85.

Bird, A.K., Prado-Irwin, S.R., Vredenburg, V.T. & Zink, A.G. (2018). Skin microbiomes of California terrestrial salamanders are influenced by habitat more than host phylogeny. *Front. Microbiol.*, 9, 1–14.

Bletz, M.C., Loudon, A.H., Becker, M.H., Bell, S.C., Woodhams, D.C., Minbiole, K.P.C., *et al.* (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecol. Lett.*, 16, 807–820.

Bletz, M.C., Perl, R.G.B., Bobowski, B., Japke, L., Tebbe, C.C., Dohrmann, A.B., *et al.* (2017). Amphibian skin microbiota exhibits temporal variation in community structure but stability of predicted Bd-inhibitory function. *ISME J.*, 11, 1521–1534.

Harris, R.N., Brucker, R.M., Walke, J.B., Becker, M.H., Schwantes, C.R., Flaherty, D.C., *et al.* (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.*, 3, 818–824.

Harris, R.N., James, T.Y., Lauer, A., Simon, M.A. & Patel, A. (2006). Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *Ecohealth*, 3, 53–56.

Jani, A.J. & Briggs, C.J. (2014). The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *Proc. Natl. Acad. Sci.*, 111, E5049–E5058.

Kolby, J.E. & Daszak, P. (2016). The emerging amphibian fungal disease, chytridiomycosis: A key example of the global phenomenon of wildlife emerging infectious diseases. *Microbiol. Spectr.*, 4, 1–17.

Kueneman, J.G., Parfrey, L.W., Woodhams, D.C., Archer, H.M., Knight, R. & McKenzie, V.J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Mol. Ecol.*, 23, 1238–1250.

McCollum, S.A. & Van Buskirk, J. (1996). Costs and benefits of a predator-induced polyphenism in the gray treefrog *Hyla chrysoscelis*. *Evolution*, 50, 583–593.

McCollum, S.A. & Leimberger, J.D. (1997). Predator-induced morphological changes in an amphibian: Predation by dragonflies affects tadpole shape and color. *Oecologia*, 109, 615–621.

McKenzie, V.J., Bowers, R.M., Fierer, N., Knight, R. & Lauber, C.L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *ISME J.*, 6, 588–596.

McMahon, T.A., Sears, B.F., Venesky, M.D., Bessler, S.M., Brown, J.M., Deutsch, K., *et al.* (2014). Amphibians acquire resistance to live and dead fungus overcoming fungal immunosuppression. *Nature*, 511, 224–227.

Michaels, C.J., Antwis, R.E. & Preziosi, R.F. (2014). Impact of plant cover on fitness and behavioural traits of captive red-eyed tree frogs (*Agalychnis callidryas*). *PLoS One*, 9, e95207.

O'Hanlon, S.J., Rieux, A., Farrer, R.A., Rosa, G.M., Waldman, B., Bataille, A., *et al.* (2018). Recent Asian origin of chytrid fungi causing global amphibian declines. *Science*, 360, 621–627.

Prado-Irwin, S.R., Bird, A.K., Zink, A.G. & Vredenburg, V.T. (2017). Intraspecific variation in the skin-associated microbiome of a terrestrial salamander. *Microb. Ecol.*, 74, 745–756.

Rebollar, E.A., Bridges, T., Hughey, M.C., Medina, D., Belden, L.K. & Harris, R.N. (2019). Integrating the role of antifungal bacteria into skin symbiotic communities of three Neotropical frog species. *ISME J.*, 13, 1763-1775.

Rebollar, E.A., Gutiérrez-Preciado, A., Noecker, C., Eng, A., Hughey, M.C., Medina, D., *et al.* (2018). The skin microbiome of the neotropical frog *Craugastor fitzingeri*: Inferring potential bacterial-host-pathogen interactions from metagenomic data. *Front. Microbiol.*, 9, 1–12.

Rebollar, E.A., Hughey, M.C., Medina, D., Harris, R.N., Ibáñez, R. & Belden, L.K. (2016). Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. *ISME J.*, 10, 1682–1695.

Relyea, R.A. (2001). Morphological and behavioral plasticity of larval anurans in response to different predators. *Ecology*, 82, 523–540.

Rollins-Smith, L.A. (2009). The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. *Biochim. Biophys. Acta*, 1788, 1593–1599.

Rollins-Smith, L.A. & Conlon, J.M. (2005). Antimicrobial peptide defenses against chytridiomycosis, an emerging infectious disease of amphibian populations. *Dev. Comp. Immunol.*, 29, 589–598.

Scheele, B.C., Pasmans, F., Skerratt, L.F., Berger, L., Martel, A., Beukema, W., *et al.* (2019). Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science*, 363, 1459–1463.

Smith, D.C. & Van Buskirk, J. (1995). Phenotypic design, plasticity, and ecological performance in two tadpole species. *Am. Nat.*, 145, 211–233.

Tobler, U., Borgula, A. & Schmidt, B.R. (2012). Populations of a susceptible amphibian species can grow despite the presence of a pathogenic chytrid fungus. *PLoS One*, 7, e34667.

Trevelline, B.K., Fontaine, S.S., Hartup, B.K. & Kohl, K.D. (2019). Conservation biology needs a microbial renaissance: a call for the consideration of host-associated microbiota in wildlife management practices. *Proc. R. Soc. B*, 286, 20182448.

Van Buskirk, J. (2000). The costs of an inducible defense in anuran larvae. *Ecology*, 81,

2813–2821.
Wake, D.B. & Vredenburg, V.T. (2008). Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proc. Natl. Acad. Sci. U. S. A.*, 105, 11466–11473.
Walke, J.B., Becker, M.H., Hughey, M.C., Swartwout, M.C., Jensen, R. V. & Belden, L.K. (2017). Dominance-function relationships in the amphibian skin microbiome. *Environ. Microbiol.*, 19, 3387–3397.
West, A.G., Waite, D.W., Deines, P., Bourne, D.G., Digby, A., McKenzie, V.J., *et al.* (2019). The microbiome in threatened species conservation. *Biol. Conserv.*, 229, 85–98.
Woodhams, D.C., Alford, R.A., Antwis, R.E., Archer, H., Becker, M.H., Belden, L.K., *et al.* (2015). Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecology*, 96, 595.
Woodhams, D.C., Vredenburg, V.T., Simon, M.-A., Billheimer, D., Shakhtour, B., Shyr, Y., *et al.* (2007). Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biol. Conserv.*, 138, 390–398.

CHAPTER 1

Functional redundancy of *Batrachochytrium dendrobatidis* inhibition in bacterial communities isolated from *Lithobates clamitans* skin

ABSTRACT

The cutaneous microbial community can influence the health of amphibians exposed to *Batrachochytrium dendrobatidis* (Bd), a fungal pathogen that has contributed to recent amphibian declines. Resistance to Bd in amphibian populations is correlated with the presence of anti-Bd cutaneous microbes, which confer disease resistance by inhibiting Bd growth. I aimed to determine if green frogs (*Lithobates clamitans)*, an abundant and widely distributed species in New Jersey, harbored bacteria that inhibit Bd*,* and whether the presence and identity of these microbes varied among sites. I used *in vitro* challenge assays to determine if bacteria isolated from green frog skin could inhibit or enhance the growth of Bd*.* I found that green frogs at all sites harbored anti-Bd bacteria*.* However, there were differences in Bd inhibition capabilities among bacterial isolates identified as the same operational taxonomic unit (OTU), lending support to the idea that phylogenetic relatedness does not always predict Bd inhibition status. Additionally, anti-Bd bacterial richness did not vary by site, but the composition of anti-Bd bacterial taxa was distinct at each site. This suggests that there is functional redundancy of Bd-inhibition across unique communities of anti-Bd symbionts found on frogs at different sites. These findings highlight the need to better elucidate the structure-function relationship of microbiomes and their role in disease resistance.

INTRODUCTION

Vertebrate skin is often the first line of defense against invading pathogens. In addition to being a defensive barrier, cutaneous microbial symbionts are known to promote health (*e.g.* in humans (Grice & Segre 2011; Cho & Blaser 2012; Pflughoeft & Versalovic 2012), bats (Hoyt *et al.* 2015), and amphibians (Becker & Harris 2010; Walke & Belden 2016; Bletz *et al.* 2018)). The role of the cutaneous microbiome in promoting amphibian health has gained increasing recognition with the spread of emerging infectious diseases that infect amphibian skin, such as the fungal pathogen *Batrachochytrium dendrobatidis* (Bd). Evidence suggests that symbiotic microorganisms that inhibit the growth of Bd, hereafter referred to as anti-Bd microbes, can help protect individuals from disease (Woodhams *et al.* 2007; Lam *et al.* 2010) and could be used in bioaugmentation strategies to ameliorate the effects of Bd (Harris *et al.* 2009; Bletz *et al.* 2013). As such, the study of anti-Bd microbes may help elucidate strategies that could promote Bd resistance among amphibians.

There are ongoing efforts to study the primary source of bacterial symbionts and their transmission (Belden & Harris 2007; Bright & Bulgheresi 2010; Bletz *et al.* 2013). Environmental transmission of bacteria to amphibian skin can occur (Muletz *et al.* 2012), yet amphibian cutaneous microbial communities can differ from the surrounding environment (McKenzie *et al.* 2012; Fitzpatrick & Allison 2014; Kueneman *et al.* 2014). Furthermore, different amphibian species at the same site do not have similar skin bacterial communities (McKenzie *et al.* 2012; Walke *et al.* 2014). The dissimilarity of environmental microbial communities and amphibian skin microbial communities suggests that the amphibian microbiome is not simply a reflection of microorganisms

from a regional environmental pool, and processes that influence symbiont acquisition may differ among amphibian species (McKenzie *et al.* 2012; Walke *et al.* 2014). Previous work suggests that host genetics can influence microbiome structure in amphibians (Griffiths *et al.* 2018), as seen in other animal models (Franzenburg *et al.* 2013; Goodrich *et al.* 2014). The suite of host and environmental factors that are important structuring forces driving the composition and protective function of the amphibian skin microbiome is still being explored.

Studies on the structure of host-associated microbial communities have provided important insights to how amphibians respond to Bd (Walke *et al.* 2015b; Bates *et al.* 2018). While evidence suggests that microbial community structure can be linked to disease susceptibility in amphibian-Bd systems (Walke *et al.* 2015b; Rebollar *et al.* 2016b), differences in community structure do not always translate to differences in function (Belden *et al.* 2015). Knowing the identity and function of host-associated bacteria may help determine if multiple bacterial taxa are filling the same roles, such that anti-Bd function might be maintained even if overall microbiome structure varies across space and time. Furthermore, understanding the function of specific host-associated taxa through culture-based approaches can provide insight on which members of the microbiome are particularly effective at inhibiting Bd growth and may contribute more strongly to defensive function.

Culture-based challenge assays (Harris *et al.* 2006; Bell *et al.* 2013), where bacterial symbionts are tested for the ability to inhibit Bd growth, have led to important insights in the role of antifungal bacteria protecting amphibians from Bd because inhibitory properties can be assessed *in vitro*. Furthermore, because Bd inhibition status

can differ among genetically related strains of bacteria (Antwis *et al.* 2015; Becker *et al.* 2015), assessment of the inhibition capabilities of a specific strain cultured from amphibian skin is likely more accurate than inferring inhibition status of an isolate based on phylogenetic relatedness. Widespread use of these assays has led to the development of a large database of known antifungal bacteria (Woodhams *et al.* 2015) that could serve as candidates for developing probiotic therapies to help mitigate the effects of disease (Bletz *et al.* 2013). In studies where culture-independent methods are used, this database is used to identify operational taxonomic units (OTUs) that are likely to have Bd-inhibitory function (Kueneman *et al.* 2016a, b; Bletz *et al.* 2017b).

Recent evidence suggests that the number of anti-Bd isolates present on an individual can help predict which individuals or sites may be protected from Bd (Bletz *et al.* 2017a; Bell *et al.* 2018). In addition to microbes that inhibit Bd, some microorganisms isolated from amphibian skin are able to enhance Bd growth *in vitro* (Bell *et al.* 2013; Becker *et al.* 2015; Antwis & Harrison 2018). However, whether the presence of these Bd-enhancing bacteria helps predict amphibian susceptibility to Bd is unknown. Management tools that incorporate knowledge of host-associated microbial communities (*e.g.* Woodhams *et al.* 2014) may help forecast whether a specific individual, species, or site is likely to be susceptible to Bd prior to disease invasion.

I used green frogs (*Lithobates clamitans)* as a model system to assess how the Bd-inhibitory function of bacteria on frog skin varied among sites. Green frogs are habitat generalists that are commonly found across diverse habitats in the eastern United States, and they are the most abundant species present throughout the summer in some areas of New Jersey. Green frogs can carry Bd asymptomatically and could be potential vectors of

disease spread (Gahl *et al.* 2012). Research conducted in Connecticut by Richards-Hrdlicka *et al*. (2013) found high Bd infection prevalence in green frogs, prompting these authors to propose that green frogs might serve as a focal species for other states looking to monitor for Bd. Furthermore, the first two cases of Bd in New Jersey were found in juvenile green frogs (Monsen-Collar *et al.* 2010). These lines of reasoning suggest that green frogs are a good model system for studying variation in microbial communities that may provide resistance against Bd.

I sampled the microbes present on green frog skin and in the environment at three environmentally contrasting sites and conducted culture-based challenge assays to identify anti-Bd and Bd-enhancing bacteria present on green frogs. I addressed the following questions: (i) Do green frogs harbor microbes with the ability to inhibit or enhance Bd growth *in vitro*?, (ii) Do green frogs harbor microbes that are also present in the environment, and if so, are they inhibitory to Bd?, (iii) Does the prevalence, richness, and identity of Bd-inhibitory microbes differ across sites?

MATERIALS AND METHODS

Field sampling

I sampled skin bacterial communities of adult green frogs (*L. clamitans*) in 2016 at three man-made water bodies in three different ecoregions of New Jersey (listed in parentheses): Success Pond in Colliers Mills Wildlife Management Area (WMA), Jackson, NJ (Pine Barrens), Morin Pond in Somerset, NJ (Northern Piedmont), and Imlaystown Bog in Assunpink WMA, Allentown, NJ (Inner Coastal Plain) (Fig. 1.1). Sites were selected because they had an abundance of green frogs based on surveys and

were in distinct ecoregions. By choosing environmentally contrasting sites, I aimed to encompass the variation in environmental conditions that green frogs experience across habitat types that may affect their skin microbiome. All frogs within a site were sampled in one night, but sites were sampled on different nights (Table 1.1). I collected all frogs with a dip net and handled each individual using a new pair of nitrile gloves. I transferred frogs from the dip net to a sterile Whirl-Pak®, where they were rinsed twice with sterile deionized (DI) water to remove transient matter that is not part of the skin microbiome (McKenzie *et al.* 2012; Kueneman *et al.* 2014). Individuals were then transferred to a new sterile Whirl-Pak® where they were sampled with a sterile cotton swab (Medline MDS202000).

I sampled frogs (n = 10 per site) by swabbing them five times each on their ventral surface, dorsal surface, and on both hind limbs. I also collected an environmental sample from the pond water at each site by swirling a swab at a depth of approximately 10 cm for five seconds (n = 1 per site) to determine if any anti-Bd bacteria were present in the environment. Swabs were streaked onto two R2A agar plates in the field to grow culturable microbes. Streaked plates were immediately placed into a sterilized plastic container to prevent contamination. I released frogs at their site of capture immediately following sampling. I had permission to sample amphibian skin microbes from the state of New Jersey (NJDEP Scientific Collecting Permit No. SC 2016093) and collection protocols were approved by Rutgers University's Institutional Animal Care and Use Committee (Protocol #14-080).

Growth and storage of microbial isolates

Microbes grew on R2A agar plates for three days at room temperature (RT) in the lab. I isolated morphologically distinct microbial colonies based on color, elevation, form, and margin. Subculturing was repeated until cultures consisted of a single colony type.

Once isolated, individual colonies picked from agar plates were grown in 500 uL of TGh broth (8 g tryptone, 2 g gelatin hydrolysate, 1 liter of DI water, autoclaved) for 48 hours, combined with 500 uL of 50% glycerol in DI water, and frozen at -80°C until challenge assays were performed.

Challenge assays

To identify anti-Bd and Bd-enhancing microbes, I used 96-well challenge assays based on the methods of Bell *et al*. (2013). Prior to challenge assays, I thawed isolates on R2A agar for three days at RT. Then, colonies were picked and grown in TGh broth in 24-well plates for 48 hours. To obtain cell-free supernatant (CFS) for use in challenge assays (Bell *et al.* 2013), I spun liquid cultures at 10,000 rpm for five minutes and collected and filtered supernatant using sterile 1mL syringes with 13mm, 0.22 μm filters (Fisher Scientific).

Bd isolate JEL423 in long-term 1% tryptone broth culture was transferred to TGh agar plates and grown for five days at RT before plates were flooded with 1% tryptone broth. To harvest Bd zoospores, this solution was collected and vacuum filtered through a sterilized 55mm, 20μm nylon filter (Spectra/Mesh®, Spectrum Labs) to remove sporangia.

Briefly, all experimental and positive control wells in a 96-well round bottom cell culture plate (Corning 3595, Fisher Scientific) received 50 uL of approximately 1.85 x 10^6 zoospores/mL of Bd (counted on a haemocytometer). All samples were run in triplicate. In experimental wells, 50uL of each bacterial isolate CFS was added to the 50 uL of Bd. Positive control wells received 50uL of TGh broth only to the 50 uL of Bd. Negative control wells contained 50 uL of Bd zoospores heat-killed at 100°C for 15 minutes and 50 uL TGh broth. Media only wells containing 100 uL of TGh broth were also included as a control. Perimeter wells of each plate were filled with 200 uL of TGh broth to prevent evaporation in inner wells. I measured absorbance using a spectrophotometer (Molecular Devices SpectraMax M3 multimode microplate reader) at an optical density (OD) of 492nm, every 24 hours for seven days. Optical density was used to measure changes in Bd growth in the assay plate. Any replicates that appeared to be contaminated upon examination with light microscopy were removed from analyses.

I calculated anti-Bd activity according to Bell *et al*. (2013) based on mean absorbance values on day 7 of the assay after correcting for background color. Briefly, mean OD in negative control wells on day 7 and mean OD in experimental wells on day 0 (to correct for differences in CFS color from assay start) were subtracted from mean OD in experimental wells on day 7 to yield the corrected sample value on the final assay day. The formula to calculate proportion inhibition or enhancement on the final assay day was: [(mean corrected sample OD ÷ mean corrected positive control OD)-1] × 100 = percent change in Bd growth relative to positive control. If Bd growth in experimental wells was less than that in negative control wells, percent inhibition relative to positive control exceeded 100%.

Isolates that showed greater than 60% inhibition of Bd relative to the positive control were considered strongly inhibitory isolates and were used in subsequent analyses of inhibitory isolates. This cut-off value is consistent with Bd inhibition values from previous studies using challenge assays to assess function of amphibian skin microbes (Bell *et al.* 2013; Daskin *et al.* 2014; Walke *et al.* 2017). Strongly enhancing isolates were classified as those displaying greater than 25% increase in Bd growth relative to the positive control, based on the frequency of isolates found to be enhancing at this level by Bell et al. (2013). Some studies have classified Bd facilitation as any increase in Bd growth relative to the positive control in challenge assays (Walke *et al.* 2017; Antwis & Harrison 2018). By setting a threshold for strong enhancement of Bd growth that does not consider slightly enhancing isolates (for example, those that increase Bd growth by 5%), I am considering isolates that have a greater impact on Bd growth. Furthermore, small changes in Bd growth in experimental wells may be the result of expected variation in Bd growth and not reflective of an actual effect of bacterial CFS on Bd.

Bacterial identification

Frozen glycerol stocks of morphologically distinct bacterial colonies that inhibited Bd were sent to Genewiz Corporation (South Plainfield, NJ) for DNA extraction and Sanger sequencing. Isolates that did not demonstrate anti-Bd activity were not sequenced. Alkaline lysis was used to extract DNA for PCR of the full 16S rRNA gene using proprietary primers. Successful amplification was confirmed using gel electrophoresis. Cleaned PCR products were sequenced using BigDye Terminator Cycle Sequencing. I used Geneious (Kearse *et al.* 2012) version 11.1.3 to assemble contigs by

combining forward and reverse reads. Within the OTU dataset I assembled, full-length 16S rRNA sequences of isolates were clustered at a sequence similarity threshold of 99%. This level was used to reduce replicate morphologically-identified isolates while attempting to retain isolates with relatively similar 16S rRNA sequences that may vary in their Bd inhibition capabilities (Becker *et al.* 2015). Sequences were then compared to the Woodhams et al. (Woodhams *et al.* 2015) antifungal isolate database using a custom BLAST in Geneious (Kearse *et al.* 2012). I classified isolates as novel if the OTUs had ≤97% pairwise identity to isolates in the antifungal isolate database. I used the RDP classifier (Wang *et al.* 2007) to assign genus-level classifications based on the closest reference sequence with sequence similarity of ≥97% (Becker *et al.* 2015). Sequences have been deposited in the antifungal isolate database (Woodhams *et al.* 2015) and GenBank (accession numbers provided in Table 1.2).

Variables of interest

Because morphological identification of microbes was performed separately for each site and does not always reflect genetic differentiation, sequencing revealed redundant anti-Bd isolates within and among sites. I used anti-Bd taxa counts based on single OTUs for all analyses. All isolate counts in analyses therefore refer to either (1) single OTUs established by sequencing anti-Bd isolates or (2) morphologically distinct colonies, but unsequenced taxa, for Bd-enhancing isolates. I calculated the prevalence of isolates that either inhibited or enhanced Bd growth among sites by dividing the number of frogs harboring each isolate by the total sample size at the site (n=10 individuals).

Statistical analyses

All analyses were conducted in R version 3.3.2 (R Core Team 2016). I used generalized linear models (GLMs) to evaluate isolate prevalence (binomial error distribution, link = logit) and isolate richness (Poisson error distribution, link = log). Model effects were summarized with Wald chi-square (type II) tests using the "Anova" function in the package *car* (Fox *et al.* 2016). In *vegan* (Oksanen *et al.* 2013), I used non-metric multidimensional scaling (NMDS) to visualize patterns in the anti-Bd OTUs across sites and analysis of similarity (ANOSIM) performed on Jaccard dissimilarity matrices (999 permutations for R statistics) of presence/absence data at each taxonomic level to determine if there were significant differences in the types of anti-Bd bacteria on frogs across sites. I compared anti-Bd isolate richness among sites using chi-squared contingency tests.

RESULTS

Challenge assays

There were 94 morphologically distinct microbial isolates from frog and environmental samples that were tested in Bd challenge assays (Table 1.1). Isolates' cell-free supernatant (CFS) tested for Bd inhibition using challenge assays had a variety of effects on Bd growth, ranging from complete inhibition to strong enhancement of Bd growth (Fig. 1.2). Of the isolates tested, 31 were identified as strongly inhibitory and 7 were strongly enhancing (Table 1.1). Mean reduction in Bd growth for strongly inhibitory isolates was 92.8% (range: 65.2% - 113%). Mean enhancement in Bd growth for strongly enhancing isolates was 42.8% (range: 28% - 77.2%).

OTU identification

I obtained high quality 16S rRNA sequences for 31 inhibitory isolates, which clustered into 17 OTUs. Only three OTUs were found at more than one site, and there were differences in mean percent Bd inhibition for two of these OTUs when isolates from each site were tested separately in challenge assays (Table 1.2). Within a site, most OTUs contained only one morphologically identified isolate, but two OTUs from Assunpink WMA and one OTU from Colliers Mills WMA contained more than one isolate. OTUs were distributed among three phyla: Proteobacteria (13), Firmicutes (2), and Bacteroidetes (2) (Table 1.2). Within the Proteobacteria, six unique OTUs belonged to the genus *Pseudomonas*. Of the 17 OTUs identified here, there were two OTUs, classified as *Brevibacillus* sp. and *Alcaligenes* sp., without consensus matches in the published anti-Bd isolate database (Woodhams *et al.* 2015). Of the remaining 15 anti-Bd OTUs that had consensus matches in the antifungal isolate database (Woodhams *et al.* 2015), 13 were also previously classified as inhibitory in the database, but two were classified as not inhibitory against Bd (Table 1.2).

Patterns in environmental isolates across sites

At all sites, there were isolates present in the environmental microbial community that were also present on green frog skin. Of the morphologically identified environmental isolates, 100% (6/6) at Morin Pond, 66.7% (8/12) at Assunpink WMA, and 66.7% (2/3) at Colliers Mills WMA were also present on frog skin. There were also

environmental isolates at each site with the ability to strongly inhibit Bd growth (Table 1.2).

Patterns in anti-Bd bacteria across sites

Anti-Bd OTUs present on green frogs differed among the three sites at all levels of taxonomic classification (ANOSIM: Phylum R = 0.2007, p = 0.002; Class, R = 0.2654, p = 0.002; Order, R = 0.5155, p = 0.001; Family, R = 0.5029, p = 0.001; Genus, R = 0.5029, p = 0.001; Fig. 1.3). When including anti-Bd OTUs present in the environment, overall anti-Bd communities were also different among sites (ANOSIM: Phylum, R = 0.2429, p = 0.001; Class, R = 0.2715, p = 0.001; Order, R = 0.4115, p = 0.001; Family, R = 0.4707, p = 0.001, Genus, R = 0.4741, p = 0.001).

Most inhibitory isolates occurred on fewer than 20% of individuals sampled at each site, and only two of the 17 inhibitory OTUs were found on more than 80% of frogs sampled at each site (Table 1.2). There were no differences in the prevalence (GLM: χ^2 = 0.038, df = 2, p = 0.98) or richness (GLM: χ^2 = 1.59, df = 2, p = 0.45) of inhibitory isolates on green frogs among sites. Similarly, there was no difference in the number of frogs possessing at least one anti-Bd isolate (chi-squared test: χ^2 = 0.29, df = 2, p = 0.87; Fig. 1.4) among sites.

Patterns in Bd-enhancing bacteria across sites

There were significant differences in the richness of Bd-enhancing isolates on frogs across sites (GLM: χ^2 = 7.58, df = 2, p = 0.023). There were a total of six enhancing isolates at Assunpink WMA compared to the other sites where there was one (Morin

Pond) or no (Colliers Mills WMA) enhancing isolates detected (Table 1.1). There were no differences in the prevalence (GLM: $\chi^2 = 0.135$, df = 1, p = 0.713) of enhancing isolates on frogs across sites.

DISCUSSION

Green frogs at all three sites harbored bacteria with the ability to inhibit Bd *in vitro*. I identified 17 unique bacterial isolates that exhibited anti-Bd activity and could potentially be used in probiotic therapy to reduce amphibian susceptibility to Bd. Two of these OTUs were previously unknown to inhibit Bd and have been added to the database of amphibian skin-associated antifungal isolates (Woodhams *et al.* 2015). The majority (76%) of inhibitory OTUs identified here were classified as Proteobacteria. Several previous studies have identified members of this phylum, specifically within the genus *Pseudomonas*, as being commonly inhibitory against Bd (Becker *et al.* 2015; Holden *et al.* 2015; Muletz-Wolz *et al.* 2017b; Walke *et al.* 2017). Some members of the second most common genus identified here, *Chromobacterium,* are known to produce violacein (Hoshino 2011), a metabolite that can prevent Bd-associated morbidity and mortality (Becker *et al.* 2009).

I predicted that there would be differences in anti-Bd OTUs across sites because a variety of local abiotic factors can influence the amphibian skin microbiome (Rebollar *et al.* 2016a). Despite sites being located in different ecoregions, anti-Bd taxa richness did not differ across sites. However, ANOSIM results indicate that there were different communities of anti-Bd bacteria at each site, and only three out of 17 inhibitory OTUs were found at more than one site. These results suggest that anti-Bd function of bacterial

communities may be conserved across sites even though there are differences in anti-Bd community composition, demonstrating functional redundancy of Bd inhibition. Despite the traditional paradigm of function being determined by community structure (Nemergut *et al.* 2013), there is evidence to suggest the link between structure and function can be weaker in microbial communities due to different taxa performing the same function (Belden *et al.* 2015; Louca *et al.* 2018). Indeed, Bletz *et al.* (2017b) found that predicted Bd-inhibitory function was maintained despite structural differences in the amphibian skin microbiome, and Belden *et al.* (2015) found that similar metabolites could be produced by different bacterial communities across amphibian hosts. It is possible that amphibian skin is a selective environment, where taxa with similar functions are more likely to thrive based on host-associated properties (Fitzpatrick & Allison 2014; Bletz *et al.* 2017b). Having functionally redundant taxa in host-associated microbial communities is important in the context of disease resistance because a beneficial bacterial species that is lost in the presence of a pathogen could be replaced by an equivalent species, thereby retaining overall defensive function for the host (Walke *et al.* 2015b).

The co-occurrence of certain microbes on both green frogs and in the environment suggests that there is some exchange of microbes between amphibians and the surrounding environment, but that the amphibian skin microbiome is not simply a reflection of the environmental microbial pool. This finding is consistent with previous work suggesting that transmission of bacteria from the environment to amphibian skin is possible (Muletz *et al.* 2012) and that amphibian skin microbiomes generally consist of bacteria that are rare in the environment (Walke *et al.* 2014). Previous evidence suggests that the environmental microbial pool influences the structure and diversity of skin-

associated microbial communities (Loudon *et al.* 2014b; Walke *et al.* 2014; Rebollar *et al.* 2016b) and that amphibian skin may select for symbionts from a local community of environmental microbes (Walke *et al.* 2014; Muletz-Wolz *et al.* 2017c). All three sites had at least one environmental microbe present that strongly inhibited Bd. Future work should investigate if the presence of anti-Bd members in the environmental microbial community influences Bd invasion success at a site.

Green frogs also harbored isolates with the ability to enhance Bd growth *in vitro*. However, enhancing isolates were only present at two sites, and only Assunpink WMA had more than one enhancing isolate. Previous studies have also noted the presence of Bd-enhancing bacteria on amphibian skin (Becker *et al.* 2015; Antwis & Harrison 2018; Varela *et al.* 2018), suggesting that researchers should continue to evaluate the presence of microorganisms that enhance Bd growth. It is important to consider Bd-enhancing bacteria in addition to Bd-inhibiting bacteria because the presence of enhancing bacteria may predispose individuals to Bd infection or cancel out the presumed beneficial effects of anti-Bd bacteria present on the skin.

Despite the presence of Bd-enhancing bacteria, mean Bd inhibition was more than twice as strong as mean Bd enhancement across sites among strongly inhibitory and enhancing isolates. This suggests that the reduction in Bd growth caused by inhibitory bacteria may outweigh the potential increase in Bd growth from enhancing bacteria. However, I did not quantify the abundance of inhibitory or enhancing bacteria on amphibian skin in this study, and microbiome function could be influenced by relative abundance of bacteria in a community. Dominant species may play an important role in determining function of the skin microbiome in amphibians (Walke *et al.* 2017), and a

single abundant isolate could have a large impact on overall microbial community function.

Furthermore, stronger anti-Bd function *in vitro* may not necessarily translate to stronger anti-Bd function *in vivo* because community function is often context-dependent (Medina *et al.* 2017a). Seasonal dynamics are known to influence the amphibian skin microbiome (Longo *et al.* 2015; Varela *et al.* 2018), so skin microbial communities may vary depending on the time of year samples are taken. Furthermore, temperature and media conditions of challenge assays can influence inhibition capabilities of bacteria (Daskin *et al.* 2014; Woodhams *et al.* 2014, 2018; Muletz-Wolz *et al.* 2017a), and they therefore may not accurately reflect inhibition status *in vivo*. Another caveat of challenge assays is that they often test inhibition status of bacteria in isolation, while bacteria on amphibian skin are part of a diverse community that likely influences microbiome function (Scheuring & Yu 2012). Using mixtures of bacteria to test inhibition status *in vitro* may more accurately reflect microbial community functioning on amphibian skin (Loudon *et al.* 2014a; Antwis & Harrison 2018).

I focused on finding culturable isolates that inhibited or enhanced Bd growth and identifying their distribution to understand how green frog susceptibility to Bd may change across sites. One limitation of focusing on culturable taxa is that most bacteria are unculturable, and culturing methods can be biased toward certain taxonomic groups (Vartoukian *et al.* 2010). Despite this restriction to culturable taxa, previous work suggests that the dominant members of the amphibian skin microbiome are culturable (Walke *et al.* 2015a). A comprehensive description of culture-independent bacterial diversity at each site will be described elsewhere (Chapter 2). Understanding the diversity

of culturable microbes that inhibit Bd is important in light of recent efforts to develop probiotics that mitigate the effects of chytridiomycosis (Küng *et al.* 2014; Kueneman *et al.* 2016a). Only culturable microbes can be assessed *in vitro* for their ability to protect amphibians from Bd and subsequently be used to develop anti-Bd probiotic therapies.

Furthermore, phylogenetically related bacteria can have divergent functional capabilities (Robinson *et al.* 2010). It is important to continue testing bacterial isolates for Bd inhibition because previous studies have found that bacterial strains in the same genus can have a range of Bd inhibition capabilities (Becker *et al.* 2015; Walke *et al.* 2017). Of the three anti-Bd OTUs identified here that were found at more than one site, two of the OTUs exhibited differences in mean percent Bd inhibition when individual isolates were tested from each site (though they were still classified as inhibitory). This highlights the substantial variation in Bd inhibition that can exist within taxa that are genetically similar, even under uniform challenge assay conditions. Similarly, two of the anti-Bd OTUs identified here had consensus matches to isolates previously classified as non-inhibitory in the antifungal isolate database (Woodhams *et al.* 2015). This indicates that 16S rRNA sequence similarity cannot always predict Bd inhibition capabilities. For this reason, and because there can be differences in Bd-inhibition capabilities of bacterial isolates depending on the Bd genotype used in challenge assays (Antwis *et al.* 2015; Muletz-Wolz *et al.* 2017a), caution should be used when inferring Bd inhibition capabilities based on 16S rRNA sequence similarity alone. More work using omics technology to identify genes that are linked to antifungal activity (Rebollar *et al.* 2016a, 2018), as well as isolate testing across various environmental conditions and community

contexts (Medina *et al.* 2017b, a) is needed to disentangle the factors that influence bacterial functioning and better understand the host-symbiont-pathogen relationship.

The culturable bacteria on green frog skin demonstrate functional redundancy of Bd inhibition despite variation in community composition of anti-Bd taxa. More studies are needed to determine how variation in microbiome structure and function relates to disease resistance. In addition to using culture-independent methods to elucidate these relationships, continuing to use culture-dependent methods to test bacteria for Bd inhibition is also important, since predicting Bd inhibition capabilities based on genetic similarity is not always reliable. Continuing efforts to understand microbiome community composition and functioning across hosts, space, and time will help improve our ability to develop effective management tools against infectious disease.

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Literature cited in Chapter 1

Antwis, R.E. & Harrison, X.A. (2018). Probiotic consortia are not uniformly effective against different amphibian chytrid pathogen isolates. *Mol. Ecol.*, 27, 577–589.

Antwis, R.E., Preziosi, R.F., Harrison, X.A. & Garner, T.W.J. (2015). Amphibian symbiotic bacteria do not show universal ability to inhibit growth of the global pandemic lineage of *Batrachochytrium dendrobatidis*. *Appl. Environ. Microbiol.*, 81, 3706-3711.

Bates, K.A., Clare, F.C., O'Hanlon, S., Bosch, J., Brookes, L., Hopkins, K., *et al.* (2018). Amphibian chytridiomycosis outbreak dynamics are linked with host skin bacterial community structure. *Nat. Commun.*, 9, 1–11.

Becker, M.H., Brucker, R.M., Schwantes, C.R., Harris, R.N. & Minbiole, K.P.C. (2009). The bacterially produced metabolite violacein is associated with survival of amphibians infected with a lethal fungus. *Appl. Environ. Microbiol.*, 75, 6635–6638.

Becker, M.H. & Harris, R.N. (2010). Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. *PLoS One*, 5, e10957.

Becker, M.H., Walke, J.B., Murrill, L., Woodhams, D.C., Reinert, L.K., Rollins-Smith, L.A., *et al.* (2015). Phylogenetic distribution of symbiotic bacteria from Panamanian amphibians that inhibit growth of the lethal fungal pathogen *Batrachochytrium dendrobatidis*. *Mol. Ecol.*, 24, 1628–1641.

Belden, L.K. & Harris, R.N. (2007). Infectious diseases in wildlife: the community ecology context. *Front. Ecol. Environ.*, 5, 533–539.

Belden, L.K., Hughey, M.C., Rebollar, E.A., Umile, T.P., Loftus, S.C., Burzynski, E.A., *et al.* (2015). Panamanian frog species host unique skin bacterial communities. *Front. Microbiol.*, 6, 1–21.

Bell, S.C., Alford, R.A., Garland, S., Padilla, G. & Thomas, A.D. (2013). Screening bacterial metabolites for inhibitory effects against *Batrachochytrium dendrobatidis* using a spectrophotometric assay. *Dis. Aquat. Organ.*, 103, 77–85.

Bell, S.C., Garland, S., Alford, R.A., Becker, M.H., Bell, S.C. & Bell, S.C. (2018). Increased numbers of culturable inhibitory bacterial taxa may mitigate the effects of *Batrachochytrium dendrobatidis* in Australian wet tropics frogs. *Front. Microbiol.*, 9, 1–14.

Bletz, M., Kelly, M., Sabino-Pinto, J., Bales, E., van Praet, S., Bert, W., *et al.* (2018). Disruption of skin microbiota contributes to salamander disease. *Proc. R. Soc. B Biol. Sci.*, 285, 20180758.

Bletz, M.C., Loudon, A.H., Becker, M.H., Bell, S.C., Woodhams, D.C., Minbiole, K.P.C., *et al.* (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecol. Lett.*, 16, 807–820.

Bletz, M.C., Myers, J., Woodhams, D.C., Rabemananjara, F.C.E., Rakotonirina, A., Weldon, C., *et al.* (2017a). Estimating herd immunity to amphibian chytridiomycosis in Madagascar based on the defensive function of amphibian skin bacteria. *Front. Microbiol.*, 8, 1751.

Bletz, M.C., Perl, R.G.B., Bobowski, B., Japke, L., Tebbe, C.C., Dohrmann, A.B., *et al.* (2017b). Amphibian skin microbiota exhibits temporal variation in community structure but stability of predicted Bd-inhibitory function. *ISME J.*, 11, 1521–1534.

Bright, M. & Bulgheresi, S. (2010). A complex journey: Transmission of microbial symbionts. *Nat. Rev. Microbiol.*, 8, 218–230.

Cho, I. & Blaser, M.J. (2012). The human microbiome: At the interface of health and disease. *Nat. Rev. Genet.*, 13, 260–270.

Daskin, J.H., Bell, S.C., Schwarzkopf, L. & Alford, R.A. (2014). Cool temperatures reduce antifungal activity of symbiotic bacteria of threatened amphibians - implications for disease management and patterns of decline. *PLoS One*, 9, e100378.

Fitzpatrick, B.M. & Allison, A.L. (2014). Similarity and differentiation between bacteria associated with skin of salamanders (*Plethodon jordani*) and free-living assemblages. *FEMS Microbiol. Ecol.*, 88, 482–494.

Fox, J., Weisberg, S., Adler, D., Bates, D., Baud-bovy, G., Ellison, S., *et al.* (2016). *Package 'car.' CRAN Repos.*

Franzenburg, S., Walter, J., Kunzel, S., Wang, J., Baines, J.F., Bosch, T.C.G., *et al.* (2013). Distinct antimicrobial peptide expression determines host species-specific bacterial associations. *Proc. Natl. Acad. Sci.*, 110, E3730–E3738.

Gahl, M.K., Longcore, J.E. & Houlahan, J.E. (2012). Varying responses of northeastern North American amphibians to the chytrid pathogen *Batrachochytrium dendrobatidis*. *Conserv. Biol.*, 26, 135–141.

Goodrich, J.K., Waters, J.L., Poole, A.C., Sutter, J.L., Koren, O., Blekhman, R., *et al.* (2014). Human genetics shape the gut microbiome. *Cell*, 159, 789–799.

Grice, E.A. & Segre, J.A. (2011). The skin microbiome. *Nat. Rev. Microbiol.*, 9, 244–253.

Griffiths, S.M., Harrison, X.A., Weldon, C., Wood, M.D., Pretorius, A., Hopkins, K., *et al.* (2018). Genetic variability and ontogeny predict microbiome structure in a disease-challenged montane amphibian. *ISME J.*, 12, 2506-2517.

Harris, R.N., Brucker, R.M., Walke, J.B., Becker, M.H., Schwantes, C.R., Flaherty, D.C., *et al.* (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.*, 3, 818–824.

Harris, R.N., James, T.Y., Lauer, A., Simon, M.A. & Patel, A. (2006). Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *Ecohealth*, 3, 53–56.

Holden, W.M., Hanlon, S.M., Woodhams, D.C., Chappell, T.M., Wells, H.L., Glisson, S.M., *et al.* (2015). Skin bacteria provide early protection for newly metamorphosed southern leopard frogs (*Rana sphenocephala*) against the frog-killing fungus, *Batrachochytrium dendrobatidis*. *Biol. Conserv.*, 187, 91–102.

Hoshino, T. (2011). Violacein and related tryptophan metabolites produced by *Chromobacterium violaceum*: Biosynthetic mechanism and pathway for construction of violacein core. *Appl. Microbiol. Biotechnol.*, 91, 1463–1475.

Hoyt, J.R., Cheng, T.L., Langwig, K.E., Hee, M.M., Frick, W.F. & Kilpatrick, A.M. (2015). Bacteria isolated from bats inhibit the growth of *Pseudogymnoascus destructans*, the causative agent of white-nose syndrome. *PLoS One*, 10, e0121329.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., *et al.* (2012). Geneious. *Bioinformatics*.

Kueneman, J.G., Parfrey, L.W., Woodhams, D.C., Archer, H.M., Knight, R. & McKenzie, V.J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Mol. Ecol.*, 23, 1238–1250.

Kueneman, J.G., Woodhams, D.C., Harris, R., Archer, H.M., Knight, R. & Mckenzie, V.J. (2016a). Probiotic treatment restores protection against lethal fungal infection lost during amphibian captivity. *Proc. R. Soc. B*, 283, 20161553.

Kueneman, J.G., Woodhams, D.C., Van Treuren, W., Archer, H.M., Knight, R. & McKenzie, V.J. (2016b). Inhibitory bacteria reduce fungi on early life stages of endangered Colorado boreal toads (*Anaxyrus boreas*). *ISME J.*, 10, 934–944.

Küng, D., Bigler, L., Davis, L.R., Gratwicke, B., Griffith, E. & Woodhams, D.C. (2014). Stability of microbiota facilitated by host immune regulation: informing probiotic strategies to manage amphibian disease. *PLoS One*, 9, e87101.

Lam, B.A., Walke, J.B., Vredenburg, V.T. & Harris, R.N. (2010). Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. *Biol. Conserv.*, 143, 529–531.

Longo, A. V, Savage, A.E., Hewson, I. & Zamudio, K.R. (2015). Seasonal and ontogenetic variation of skin microbial communities and relationships to natural disease dynamics in declining amphibians. *R. Soc. Open Sci.*, 2, 140377.

Louca, S., Martiny, A.C., Polz, M.F., Doolittle, W.F., Nelson, M.B., Hahn, A.S., *et al.* (2018). Function and functional redundancy in microbial communities. *Nat. Ecol. Evol.*, 2, 936–943.

Loudon, A.H., Holland, J.A., Umile, T.P., Burzynski, E.A., Minbiole, K.P.C. & Harris, R.N. (2014a). Interactions between amphibians' symbiotic bacteria cause the production of emergent anti-fungal metabolites. *Front. Microbiol.*, 5, 441.

Loudon, A.H., Woodhams, D.C., Parfrey, L.W., Archer, H., Knight, R., McKenzie, V., *et al.* (2014b). Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *ISME J.*, 8, 830–840.

McKenzie, V.J., Bowers, R.M., Fierer, N., Knight, R. & Lauber, C.L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *ISME J.*, 6, 588–596.

Medina, D., Hughey, M.C., Becker, M.H., Walke, J.B., Umile, T.P., Burzynski, E.A., *et al.* (2017a). Variation in metabolite profiles of amphibian skin bacterial communities across elevations in the neotropics. *Microb. Ecol.*, 74, 227–238.

Medina, D., Walke, J.B., Gajewski, Z., Becker, M.H., Swartwout, M.C. & Belden, L.K. (2017b). Culture media and individual hosts affect the recovery of culturable bacterial diversity from amphibian skin. *Front. Microbiol.*, 8, 1–14.

Monsen-Collar, K., Hazard, L. & Dussa, R. (2010). Comparison of PCR and RT-PCR in the first report of *Batrachochytrium dendrobatidis* in amphibians in New Jersey, USA. *Herpetol. Rev.*, 41, 460–462.

Muletz-Wolz, C.R., Almario, J.G., Barnett, S.E., DiRenzo, G. V., Martel, A., Pasmans, F., *et al.* (2017a). Inhibition of fungal pathogens across genotypes and temperatures by amphibian skin bacteria. *Front. Microbiol.*, 8, 1551.

Muletz-Wolz, C.R., DiRenzo, G. V., Yarwood, S.A., Grant, E.H.C., Fleischer, R.C. & Lips, K.R. (2017b). Antifungal bacteria on woodland salamander skin exhibit high taxonomic diversity and geographic variability. *Appl. Environ. Microbiol.*, 83, 1–13.

Muletz-Wolz, C.R., Yarwood, S.A., Campbell Grant, E.H., Fleischer, R.C. & Lips, K.R. (2017c). Effects of host species and environment on the skin microbiome of Plethodontid salamanders. *J. Anim. Ecol.*, 87, 341–353.

Muletz, C.R., Myers, J.M., Domangue, R.J., Herrick, J.B. & Harris, R.N. (2012). Soil

bioaugmentation with amphibian cutaneous bacteria protects amphibian hosts from infection by *Batrachochytrium dendrobatidis*. *Biol. Conserv.*, 152, 119–126.

Nemergut, D.R., Schmidt, S.K., Fukami, T., O'Neill, S.P., Bilinski, T.M., Stanish, L.F., *et al.* (2013). Patterns and processes of microbial community assembly. *Microbiol. Mol. Biol. Rev.*, 77, 342–356.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., *et al.* (2013). Package 'vegan.' *R Packag. ver. 2.0–8*.

Pflughoeft, K.J. & Versalovic, J. (2012). Human microbiome in health and disease. *Annu. Rev. Pathol.*, 7, 99–122.

R Core Team. (2016). *R: A language and environment for statistical computing. R Dev. Core Team*.

Rebollar, E.A., Antwis, R.E., Becker, M.H., Belden, L.K., Molly, C., Brucker, R.M., *et al.* (2016a). Using "omics" and integrated multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging infectious diseases. *Front. Microbiol.*, 7, 68.

Rebollar, E.A., Gutiérrez-Preciado, A., Noecker, C., Eng, A., Hughey, M.C., Medina, D., *et al.* (2018). The skin microbiome of the neotropical frog *Craugastor fitzingeri*: Inferring potential bacterial-host-pathogen interactions from metagenomic data. *Front. Microbiol.*, 9, 1–12.

Rebollar, E.A., Hughey, M.C., Medina, D., Harris, R.N., Ibáñez, R. & Belden, L.K. (2016b). Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. *ISME J.*, 10, 1682–1695.

Richards-Hrdlicka, K.L., Richardson, J.L. & Mohabir, L. (2013). First survey for the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in Connecticut (USA) finds widespread prevalence. *Dis. Aquat. Organ.*, 102, 169–180.

Robinson, C.J., Bohannan, B.J.M. & Young, V.B. (2010). From structure to function: The ecology of host-associated microbial communities. *Microbiol. Mol. Biol. Rev.*, 74, 453–476.

Scheuring, I. & Yu, D.W. (2012). How to assemble a beneficial microbiome in three easy steps. *Ecol. Lett.*, 15, 1300–1307.

Varela, B.J., Lesbarrères, D.A., Ibáñez, R. & Green, D.M. (2018). Environmental and host effects on skin bacterial community composition in Panamanian frogs. *Front. Microbiol.*, 9, 298.

Vartoukian, S.R., Palmer, R.M. & Wade, W.G. (2010). Strategies for culture of "unculturable" bacteria. *FEMS Microbiol. Lett.*, 309, 1–7.

Walke, J.B., Becker, M.H., Hughey, M.C., Swartwout, M.C., Jensen, R. V. & Belden, L.K. (2015a). Most of the dominant members of amphibian skin bacterial communities can be readily cultured. *Appl. Environ. Microbiol.*, 81, 6589–6600.

Walke, J.B., Becker, M.H., Hughey, M.C., Swartwout, M.C., Jensen, R. V. & Belden, L.K. (2017). Dominance-function relationships in the amphibian skin microbiome. *Environ. Microbiol.*, 19, 3387–3397.

Walke, J.B., Becker, M.H., Loftus, S.C., House, L.L., Cormier, G., Jensen, R. V., *et al.* (2014). Amphibian skin may select for rare environmental microbes. *ISME J.*, 8, 2207–2217.

Walke, J.B., Becker, M.H., Loftus, S.C., House, L.L., Teotonio, T.L., Minbiole, K.P.C.,

et al. (2015b). Community structure and function of amphibian skin microbes: An experiment with bullfrogs exposed to a chytrid fungus. *PLoS One*, 10, e0139848.

Walke, J.B. & Belden, L.K. (2016). Harnessing the microbiome to prevent fungal infections: Lessons from amphibians. *PLOS Pathog.*, 12, e1005796.

Wang, Q., Garrity, G.M., Tiedje, J.M. & Cole, J.R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.*, 73, 5261–5267.

Woodhams, D.C., Alford, R.A., Antwis, R.E., Archer, H., Becker, M.H., Belden, L.K., *et al.* (2015). Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecology*, 96, 595.

Woodhams, D.C., Brandt, H., Baumgartner, S., Kielgast, J., Küpfer, E., Tobler, U., *et al.* (2014). Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. *PLoS One*, 9, e96375.

Woodhams, D.C., LaBumbard, B.C., Barnhart, K.L., Becker, M.H., Bletz, M.C., Escobar, L.A., *et al.* (2018). Prodigiosin, violacein, and volatile organic compounds produced by widespread cutaneous bacteria of amphibians can inhibit two *Batrachochytrium* fungal pathogens. *Microb. Ecol.*, 75, 1049–1062.

Woodhams, D.C., Vredenburg, V.T., Simon, M.-A., Billheimer, D., Shakhtour, B., Shyr, Y., *et al.* (2007). Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biol. Conserv.*, 138, 390–398.

Tables

Table 1.1. Summary of microbial isolates sampled from green frog skin and the environment that were tested in Bd challenge assays.

Isolates were initially classified based on unique morphological characteristics relative to other isolates from the same site. Isolates exhibiting greater than 60% inhibition and isolates exhibiting greater than 25% enhancement of Bd growth in challenge assays were considered strongly inhibitory or enhancing, respectively (see methods for details).

Site	Sampling Date	No. of isolates tested in challenge assays	Strongly inhibitory isolates[a]	Strongly enhancing isolates
Morin Pond	5/13/16	32	6	1
Assunpink WMA	7/2/16	40	18	6
Colliers Mills WMA	7/7/16	22	7	0
	Total	94	31	7

[a]This column reflects the number of morphologically-classified isolates that were strongly inhibitory against Bd in challenge assays. Subsequent 16S rRNA sequencing of anti-Bd isolates revealed that the 31 isolates clustered into 17 OTUs.

Table 1.2. Summary of anti-Bd bacteria on green frogs (n = 10 individuals per site) identified using 16S rRNA sequencing.

Sites: AS = Assunpink WMA, CM = Colliers Mills WMA, MD = Morin Pond. OTUs found at multiple sites list the mean percent inhibition and number of frogs harboring each isolate from each site, respectively.

Anti-Bd bacteria strain	GenBank accession no.	Site	Mean % Inhibition	No. of frogs harboring OTU
Aeromonas sp.[a]	MK397522	AS	86.3%	9
Enterobacter sp.[a]	MK397523	AS	97.3%	2
Pedobacter sp.[c]	MK397524	AS	65.2%	1
Pseudomonas sp. strain 3[a]	MK397786	AS	95.4%	0 (env. only)
Stenotrophomonas sp.[a]	MK397525	AS[a], CM	105%, 108%	2, 2
Chryseobacterium sp.[a]	MK397526	AS	104%	1
Chromobacterium sp. strain 1	MK397527	CM	101%	3
Alcaligenes sp.[b]	MK397528	CM	95.0%	1
Serratia sp.[a]	MK397529	CM	72.1%	0 (env. only)
Pseudomonas sp. strain 2[a]	MK397530	CM, AS[a]	83.7%, 113%	8, 9
Chromobacterium sp. strain 2	MK397531	CM	79.3%	4
Pseudomonas sp. strain 4	MK397532	MD	91.4%	2
Pseudomonas sp. strain 1	MK397533	MD, AS	99.6%, 85.5%	6, 1
Pseudomonas sp. strain 5	MK397534	MD	102%	1
Pseudomonas sp. strain 6	MK397535	MD	93.7%	2
Bacillus sp.[ac]	MK397536	MD	81.3%	5
Brevibacillus sp.[ab]	MK397537	MD	96.8%	2

[a]These OTUs were also present in the environmental sample from the site. When the OTU was present at more than one site, the letter is listed next to the site where the OTU was present in the environmental sample. All sites had environmental bacteria that were capable of inhibiting the growth of Bd.

[b]These OTUs are novel anti-Bd isolates that were not present in the antifungal isolate database (Woodhams *et al.* 2015).
[c]These OTUs have consensus matches in the antifungal isolate database (Woodhams *et al.* 2015) that are categorized as not inhibitory to Bd, but the isolates were found to be inhibitory to Bd in the present study.

Figures

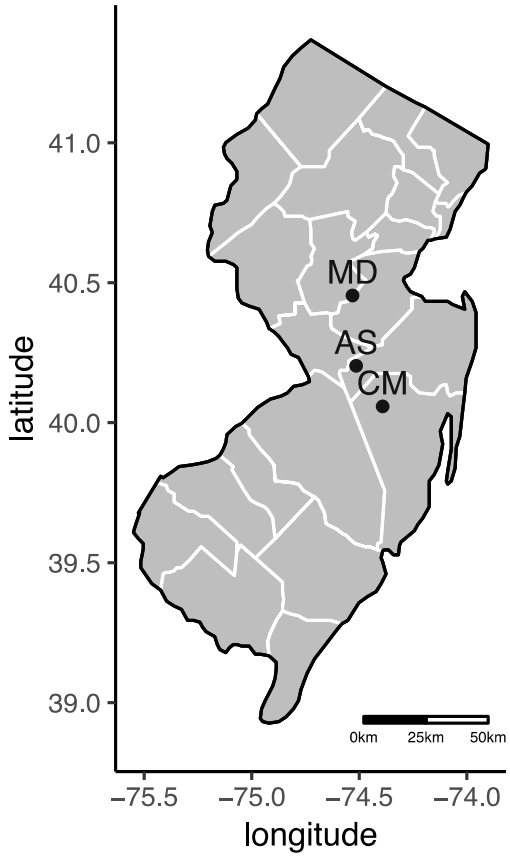

Figure 1.1. Map of New Jersey, by counties, indicating three study sites: MD = Morin Pond, AS = Assunpink Wildlife Management Area (WMA), and CM = Colliers Mills WMA.

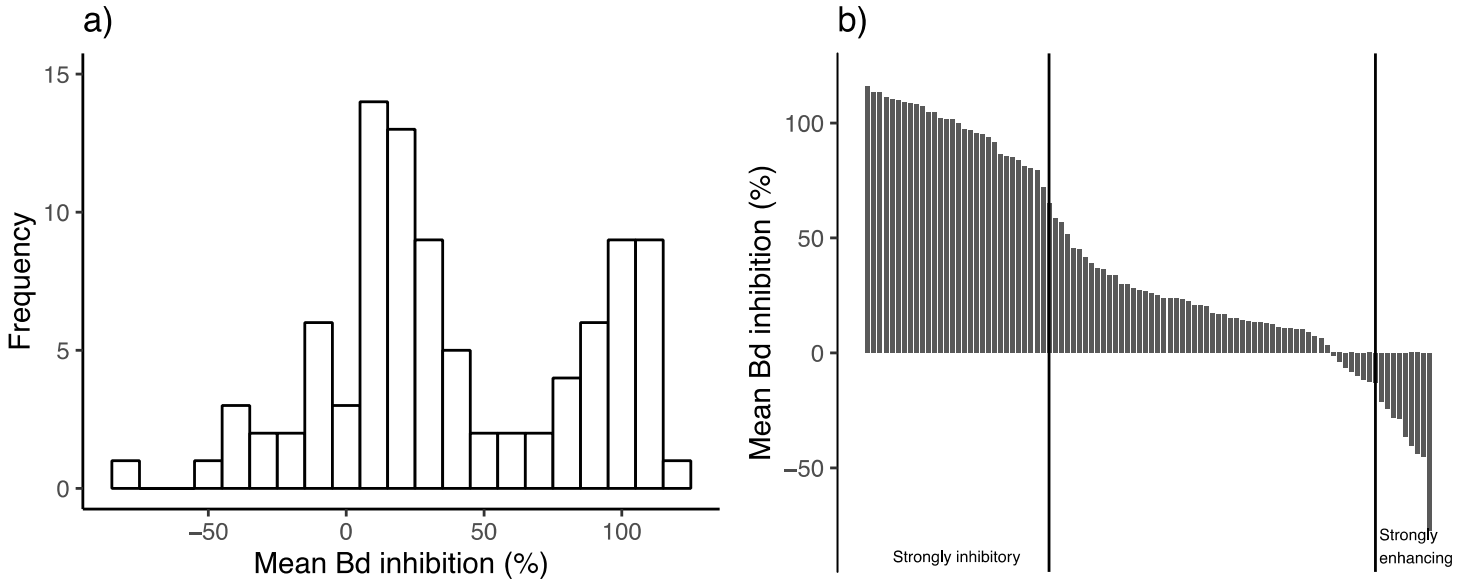

Figure 1.2. a) Frequency distribution of mean percent Bd inhibition and b) mean percent Bd inhibition of 94 morphologically identified bacterial isolates across sites.

Vertical lines in (b) correspond to cut-off values for strong Bd inhibition (≥60%) and strong enhancement (≤-25%) relative to the positive control. Mean Bd inhibition close to 0% indicates no change in Bd growth relative to the positive control when Bd was grown in the presence of isolate cell-free supernatant. Negative mean inhibition values indicate that isolates' cell-free supernatant enhanced Bd growth relative to the positive control.

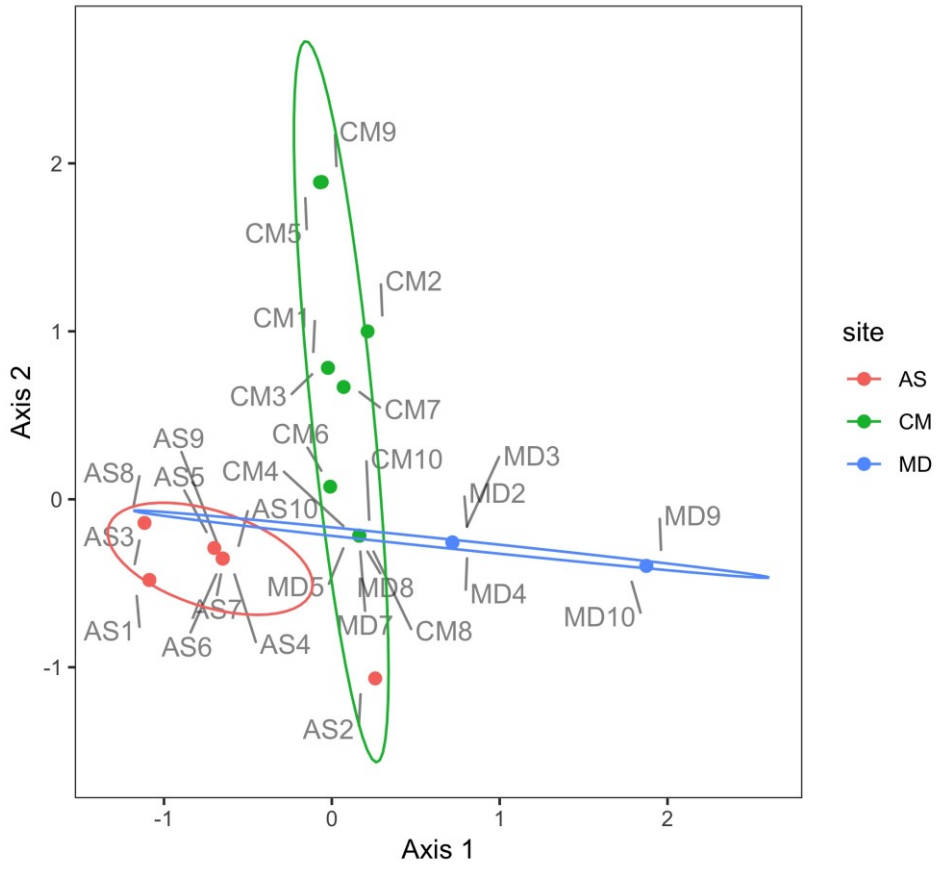

Figure 1.3. Non-metric multidimensional scaling (NMDS) plot of Jaccard dissimilarity matrix showing separation in the anti-Bd OTUs (taxonomic rank: order) on frogs across sites (stress = 0.06).

Points represent an individual's anti-Bd community and are labeled by site code and individual number (*e.g.* CM1 and CM2 = 1st and 2nd frogs sampled from Colliers Mills WMA). Points with multiple labels represent individuals with identical communities of anti-Bd bacteria. For Assunpink WMA (AS) and Colliers Mills WMA (CM), n = 10 individuals, but n = 8 for Morin Pond (MD) because two frogs sampled had no anti-Bd isolates. The composition of anti-Bd OTUs on frog skin is significantly different among sites at the order level of taxonomic classification (ANOSIM: R = 0.5155, p = 0.001).

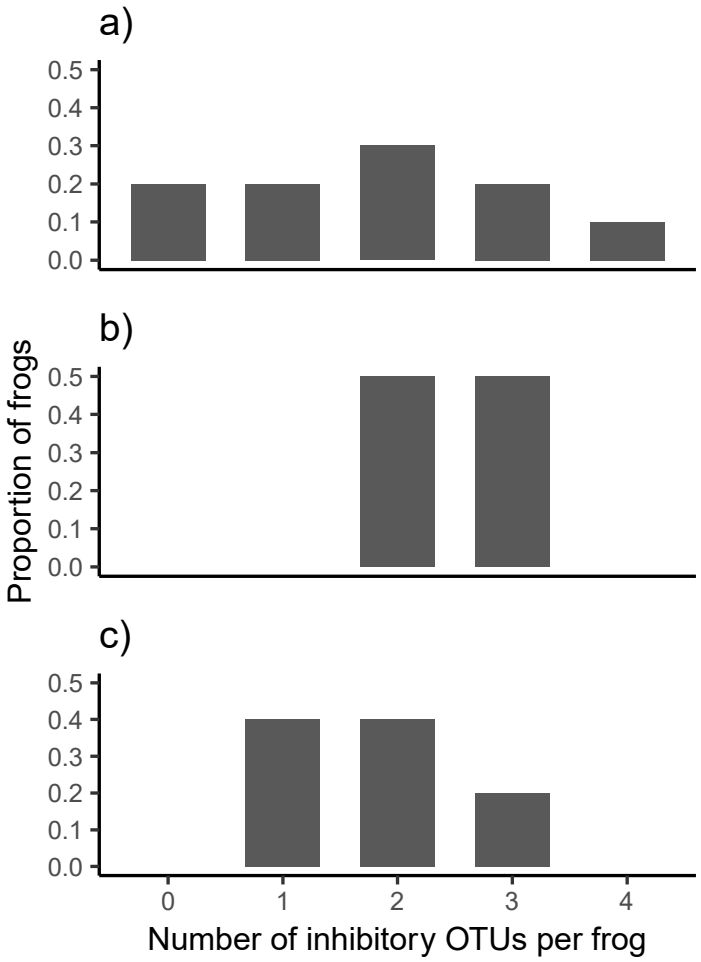

Figure 1.4. Summary of the number of inhibitory OTUs harbored by individual green frogs at a) Morin Pond, b) Assunpink WMA, and c) Colliers Mills WMA. Morin Pond is the only site where some of the individuals sampled (20%) harbored no inhibitory isolates.

CHAPTER 2

Frog skin microbiomes vary with host species and environment but not chytrid infection

ABSTRACT

Describing the structure and function of the amphibian cutaneous microbiome has gained importance with the spread of *Batrachochytrium dendrobatidis* (Bd), the fungal pathogen that can cause the skin disease chytridiomycosis. Sampling amphibian skin microbiomes is needed to characterize the current infection status of different populations and to help predict future susceptibility to Bd based on microbiome composition since some members of the microbiome have antifungal capabilities that may confer disease resistance. Here, I use 16S rRNA sequencing to describe the composition and structure of the cutaneous microbiome of six species of amphibians in New Jersey. Frog skin samples were also tested for Bd, and I found 11.8% Bd prevalence among all individuals sampled (n = 76). Frog skin microbiomes varied by host species and sampling site, but did not differ among Bd-positive and Bd-negative individuals. These results suggest that microbiome composition reflects host species and the environment, but does not reflect Bd infection among the species sampled here. Of the bacterial OTUs identified in indicator analysis as strongly associated with host taxa, significantly more than expected were putative anti-Bd bacteria, suggesting strong associations between host species and anti-Bd OTUs. This relationship may partially explain why some of these frogs are asymptomatic carriers of Bd, but more work is needed to determine the other factors that contribute to interspecific variation in Bd susceptibility. This work provides important insights on inter- and intra-specific variation in microbiome composition, putative

function, and disease dynamics in populations of amphibians that appear to be coexisting with Bd.

INTRODUCTION

The bacterial symbionts on amphibian skin can improve disease outcomes resulting from exposure to a fungal pathogen, *Batrachochytrium dendrobatidis* (Bd) (Woodhams *et al.* 2007; Harris *et al.* 2009; Becker & Harris 2010), which can cause the disease chytridiomycosis. This disease is implicated as the cause of the unprecedented loss of amphibian biodiversity that has occurred globally in recent decades (Scheele *et al.* 2019). Understanding the composition of the amphibian cutaneous microbiome is increasingly important with the spread of Bd. For example, the presence of cutaneous symbiotic microbes with anti-Bd activity has been linked to Bd-resistance in some populations (Woodhams *et al.* 2007). The recent increase in assessments of host-associated microbiota in wild populations of frogs (Belden *et al.* 2015; Rebollar *et al.* 2016b; Bletz *et al.* 2017a) and salamanders (Muletz-Wolz *et al.* 2017b; Bird *et al.* 2018) has provided valuable evidence demonstrating the role of amphibian skin microbiomes as agents of disease resistance.

Recent studies of amphibians have shown that many skin microbiomes are specific to particular host species (*e.g.*, McKenzie *et al.* 2012; Kueneman *et al.* 2014; Walke *et al.* 2014). The composition of amphibian-associated cutaneous microbes can be determined by both intrinsic factors such as host immunity and frequency of skin shedding, and extrinsic factors, such as environmental temperature and pathogen presence (Rebollar *et al.* 2016a). Interspecific variation in the skin microbiome is a

proposed mechanism for differences in Bd susceptibility (McKenzie *et al.* 2012), although a combination of factors likely influences disease susceptibility. Intraspecific variation in Bd susceptibility can also exist, with different populations of the same species showing varying responses to Bd due to population-level differences in the presence of anti-Bd skin microbes (Woodhams *et al.* 2007).

The pool of microbial taxa present in the local environment may also contribute to population-level differences in Bd susceptibility. Previous evidence suggests that the environment plays an important role in determining the structure of amphibian skin microbiomes (Muletz-Wolz *et al.* 2017b; Jani & Briggs 2018), possibly by acting as a reservoir from which potential skin microbes are recruited (Loudon *et al.* 2014). Furthermore, anti-Bd bacterial taxa can vary among localities (Muletz-Wolz *et al.* 2017a; Kruger 2019), and the prevalence of Bd-inhibitory bacteria may be related to environmental factors such as soil pH (Varela *et al.* 2018) or temperature (Muletz-Wolz *et al.* 2019). Taken together, these findings suggest that the local environment can mediate the presence and prevalence of anti-Bd microbes on amphibian skin and may therefore partially explain differences in Bd susceptibility among populations.

In addition to the influence of the local environment, infection with Bd can conceivably alter the diversity and structure of amphibian skin microbiomes (Jani & Briggs 2014; Rebollar *et al.* 2016b). Disruption of normal microbiome functioning from other stressors, such as environmental pollutants (*e.g.* Kohl *et al.* 2015), can also affect host health and disease progression (Hamdi *et al.* 2011; Thomason *et al.* 2017). Because disruption from Bd infection and disruption from external stressors that leads to Bd infection both manifest as differences in microbiome structure in assessments of wild

amphibian popualtions, it can be difficult to infer cause and effect when examing microbiome differences among Bd-infected and noninfected individuals. Additionally, these responses are not mutually exclusive (Jani & Briggs 2014; Walke *et al.* 2015). Jani *et al.* (2017) were able to tease apart the Bd-microbiome relationship by sampling pre-epizootic populations and found that microbiome differences were a result of Bd infection severity and not a cause of Bd infection. Regardless of the direction, there seems to be a clear connection between Bd infection and microbiome structure, with strong evidence that Bd infection is capable of causing changes in amphibian microbiomes. Because of interspecific differences in both host microbiomes and Bd susceptibility, more work is needed to understand how the cause and effect relationship between Bd and the skin microbiome varies among species.

Bd has been present in North America since as early as the 1960s (Ouellet *et al.* 2005) and is widespread in northeastern states (Longcore *et al.* 2007; Richards-Hrdlicka *et al.* 2013; Julian *et al.* 2019). However, there is little evidence of population declines due to Bd in this area (Longcore *et al.* 2007; Richards-Hrdlicka *et al.* 2013). Current climatic conditions may contribute to the present lack of Bd lethality, and it is possible that environmental changes associated with climate change could trigger future lethal outbreaks of disease (Rohr & Raffel 2010; Cohen *et al.* 2017). The widespread prevalence of Bd in this region is concerning given the context-dependent nature of this host-pathogen interaction. As such, documenting current infection prevalence may help determine which species are most at risk of future declines.

In this study, I describe the composition, predicted anti-Bd function, and Bd status of frogs' skin microbiomes across several localities in New Jersey. There has been some

evidence of Bd in New Jersey (Monsen-Collar *et al.* 2010; Chu *et al.* 2014), but more work is needed to comprehensively determine Bd prevalence in the state. It is especially important to monitor abundant and potential reservoir species because these individuals may amplify pathogen transmission and therefore have a disproportionate impact on communities if infected with Bd.

Using culture-independent methods, the objectives of this study were to (i) characterize the composition and diversity of the skin microbiomes of frogs at several localities in New Jersey, (ii) compare amphibian skin microbiomes to environmental bacterial communities, (iii) determine whether Bd infection is associated with differences in skin microbiomes, and (iv) examine the prevalence of potentially Bd-inhibiting bacteria among species and sites. I gathered information on potential Bd-inhibitory bacteria from an antifungal database containing 16S rRNA sequences of bacteria that have demonstrated anti-Bd activity using *in vitro* assays (Woodhams *et al.* 2015). I predicted that communities of putative anti-Bd bacteria would be unique across sites based on previous results obtained using culture-dependent methods (Kruger 2019).

MATERIALS AND METHODS

Field sampling

I sampled the cutaneous microbiomes of spring peepers (*Pseudacris crucifer)*, wood frogs (*Lithobates sylvaticus)*, green frogs (*Lithobates clamitans),* bullfrogs (*Lithobates catesbeianus),* and Pine Barrens tree frogs (*Hyla andersonii)* across five sites: Success Pond in Colliers Mills Wildlife Management Area (WMA), Jackson, NJ, Albertson Bog in Wharton State Forest, Hammonton, NJ, Morin Pond and Kai Pond in

Somerset, NJ, and Imlaystown Bog in Assunpink WMA, Allentown, NJ (Fig. S2.1). Frog species were selected based on preliminary visual surveys, such that there was a sufficiently large population at each site to make sampling feasible. Sites were further selected based on permit regulations and pond accessibility. Two species – green frogs and bullfrogs – were sampled at more than one site. Frogs were sampled in the spring and summer of 2016 and 2017, and I aimed to sample 10 individuals of target species at each site (Table 2.1). Sampling methods were as previously described (Kruger 2019). Briefly, each frog was handled with new nitrile gloves and rinsed twice with sterile deionized water to exclude transient matter that is not part of the skin-associated microbiome (McKenzie *et al.* 2012; Kueneman *et al.* 2014). I swabbed each frog with a sterile cotton swab (Medline MDS202000) 20 times (five streaks each on the dorsal side, ventral side, and each hind limb). All amphibians were released immediately after sampling. Environmental bacteria were sampled by swirling a swab in pond water at 10cm depth for five seconds (n = 9). Swabs were placed in sterile centrifuge tubes on ice and subsequently preserved at -80°C until DNA extraction. The New Jersey DEP approved this protocol for sampling amphibian skin bacteria (NJDEP Scientific Collecting Permit No. SC 2016093 & 2017053), and amphibian sampling methods were approved by Rutgers University's Institutional Animal Care and Use Committee (Protocol #14-080).

16S rRNA amplicon sequencing

I extracted DNA from swabs using the Qiagen DNeasy PowerSoil kit following kit instructions with modifications according to the Earth Microbiome Project for low DNA amounts (Berg-Lyons *et al.* 2018). After extraction, an initial round of PCR of the

full 16S gene with 27F and 1492R primers was performed to deal with low DNA concentrations. Each 20 μL reaction contained 0.02 U/uL Phusion® DNA polymerase, 200 μM dNTPs, 0.5 μM forward primer, 0.5 μM reverse primer, and 1μL DNA template. The mixtures were amplified at 98°C for 30 seconds, followed by 35 cycles of 98°C for 10 seconds, 62°C for 30 seconds, 72°C for 45 seconds and a final elongation for 7 minutes. I checked products for successful amplification using gel electrophoresis. PCR products were cleaned using the AxyPrep Mag PCR cleanup kit per kit instructions and sent to the Integrated Microbiome Resource (IMR) lab at Dalhousie University (Halifax, Canada) for library preparation and sequencing. A second round of PCR of the V4-V5 region using the 515F and 926R primers was performed, and pooled PCR amplicons were sequenced by an Illumina MiSeq using 2x300 bp paired-end v3 chemistry. Sequencing procedural details were as described elsewhere (Comeau *et al.* 2017).

Sequence processing

Sequence assembly was performed by the IMR lab at Dalhousie University according to the Microbiome Helper standard operating procedures workflow (Comeau *et al.* 2017) using QIIME (Caporaso *et al.* 2010). Briefly, sequences were clustered into operational taxonomic units (OTUs) based on 97% sequence similarity and taxonomy was assigned using the Greengenes database (DeSantis *et al.* 2006). Sequences without taxonomic matches were clustered *de novo* at the 97% sequence similarity level. Sequences were quality filtered such that low-confidence OTUs making up <0.1% of reads and chimeric reads were removed. Samples were rarefied at 2500 reads to standardize sampling effort while maximizing sample inclusion, and samples that did not

reach this threshold were removed from subsequent analyses. After filtering, 76 frog skin samples and 8 environmental samples remained (Table 2.1). Sequences will be deposited in the SRA database upon publication of this manuscript.

Bd testing

I sent extracted DNA to Pennsylvania State University-Altoona College for Bd-testing using qPCR, where primers developed by Boyle *et al.* (2004) were used. All samples were run in triplicate with positive and negative controls as described by Julian *et al.* (2016). Individuals were considered Bd-positive if at least two out of three qPCR wells were positive. If one of the three wells was positive, the reaction was run in triplicate again. If at least one of the wells was positive on the second run of PCR, this was considered a positive detection of Bd.

Microbiome structure metrics

In the context of microbiome studies, alpha diversity refers to microbiome diversity of an individual host. Beta diversity refers to the differences in microbiome composition among hosts and is calculated using a distance matrix to compare bacterial taxa dissimilarity between all pairs of individuals (Kumar *et al.* 2014). Shannon index, Chao1, Faith's phylogenetic distance (Faith 1992), and observed OTU richness alpha diversity metrics were calculated with QIIME (Caporaso *et al.* 2010). Beta diversity was calculated using weighted and unweighted Unifrac distances (Lozupone *et al.* 2011), Bray-Curtis dissimilarity (Bray & Curtis 1957), and Jaccard indices in the phyloseq package (McMurdie & Holmes 2013) in R. UniFrac metrics encompass information on

phylogenetic relatedness (Lozupone *et al.* 2011), while Jaccard and Bray-Curtis do not. Jaccard and unweighted UniFrac use only presence/absence data, while Bray-Curtis and weighted UniFrac use data on relative abundances of OTUs sampled on each individual. I visualized results using non-metric multi-dimensional scaling (NMDS).

I compared the bacterial OTUs identified in this study to OTUs in a published database containing bacterial isolates known to inhibit or enhance Bd activity (Woodhams *et al.* 2015). I used a custom blast search in Geneious ver. 11.1.3 (Kearse *et al.* 2012) to determine which potentially Bd-inhibitory and enhancing OTUs were present in my dataset based on >97% sequence identity. I used the megablast tool and had Geneious return only the top hit from the antifungal isolate database (Muletz-Wolz *et al.* 2017b).

Comparison of culture-independent and dependent techniques

In the previous chapter, I identified anti-Bd OTUs on green frog skin using culture-based techniques (*i.e*., agar plating). Here, I describe the skin microbiome of the same green frog individuals using culture-independent techniques (*i.e.,* 16S rRNA amplicon sequencing). By analyzing microbiomes of the same individuals using two different techniques, I am able to assess whether the results of these two methods of describing microbiome diversity are comparable. I used Geneious (Kearse *et al.* 2012) to compare the OTUs identified using the culture-independent methods of the present study to OTUs identified previously using culture-based techniques (Kruger 2019).

Indicator analysis

I conducted indicator species analyses using the "multipatt" function in the *indicspecies* package (De Cáceres & Legendre 2009) in R. This analysis identifies species that characterize a group of sites, or in this case, bacterial OTUs that characterize different frog species, based on their relative abundance and frequency of occurrence (Dufrene & Legendre 1997). OTU tables were filtered before analyses to exclude environmental bacterial samples, thereby only including amphibian-associated bacteria. I identified OTUs that had high association values (IndVal > 0.7, Becker *et al.* 2017) and were significantly ($p < 0.05$) associated with host species. I also performed an indicator analysis between Bd positive and negative individuals to determine which OTUs were strongly associated with individuals based on Bd status. I compared the indicator OTUs to the antifungal isolate database (Woodhams *et al.* 2015) to determine how many indicator OTUs were putative anti-Bd bacteria.

Statistical analysis

All statistical analyses were performed using R ver. 3.5.1 (R Core Team 2016). I determined if alpha diversity metrics were normally distributed with Shapiro-Wilk tests and analyzed the effect of site, host species, and Bd infection status on alpha diversity metrics using GLMs. Observed OTU data were over-dispersed, so a quasi-Poisson distribution was used. Faith's phylogenetic distance and Chao1 indices were square root-transformed to meet assumptions of normality, and GLMs with Gaussian normal errors were used. I used Kruskal-Wallis tests followed by FDR-corrected Wilcoxon tests to determine if bacterial community alpha diversity varied among Bd status, sample type (frog vs. environment), and populations of green frogs across sites.

I determined the relative importance of site, host (frog species), and Bd status in influencing cutaneous microbiome beta-diversity using permutational multivariate analysis of variance (PERMANOVA) with the "adonis" function (999 permutations) in *vegan* (Oksanen *et al.* 2013). When testing the importance of frog species in influencing community beta-diversity, the "strata" argument accounted for sampling site. I also used a PERMANOVA to compare beta diversity between environment and frog-associated bacterial communities. Green frogs were the only species sampled at more than two sites, so I also analyzed patterns in green frog microbiomes across sites to determine if there were population-level differences in green frog microbiome structure. Pairwise PERMANOVAs compared significant effects. I used the false discovery rate (FDR) procedure to correct for multiple comparisons (Benjamini & Hochberg 1995). The "betadisper" function in *vegan* (Oksanen *et al.* 2013) was used to test for homogeneity of dispersions based on species and site and Tukey's HSD test was used for post-hoc analyses. Only significant betadisper results are reported, since heterogeneous dispersion among groups can influence PERMANOVA results.

I analyzed the diversity of potentially anti-Bd bacteria using similar methods described above for entire bacterial communities. I used Kruskal-Wallis tests to determine if the number and relative abundance of OTUs varied by host species, site, and Bd status. I compared the proportion of putative anti-Bd OTUs, enhancing OTUs, and OTUs with no Bd function among all frog-associated OTUs to the proportion of these OTUs among indicator taxa using a Chi-square goodness of fit test to determine if more indicator OTUs were inhibitory or enhancing than expected based on their prevalence among all frog-associated OTUs.

RESULTS

There were a total of 2371 bacterial OTUs representing 24 phyla identified in frog and environmental bacterial samples. The five most abundant phyla present in frog skin samples by percent composition were Proteobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, and Acidobacteria (Table 2.2), and the dominant phylum differed among frog species and the environment (Fig. 2.1).

Alpha diversity

All alpha diversity (within sampling unit = individual frogs) metrics yielded similar results, so Chao1, commonly used for measuring microbial diversity, and OTU richness are presented for simplicity. OTU richness was variable across individuals, ranging from 4 to 312 OTUs per frog. Both host species and sampling site significantly affected OTU richness (Fig. 2.2; GLM – Species: $\chi^2 = 15.28$, df = 4, $p = 0.004$; Site: $\chi^2 = 19.95$, df = 3, $p < 0.001$) and Chao1 index (GLM – Species: $\chi^2 = 11.06$, df = 4, $p = 0.026$; Site: $\chi^2 = 23.24$, df = 3, $p < 0.001$), indicating that both host species and sampling location influenced individual-level microbiome diversity.

Beta diversity – dissimilarity among samples

Bray-Curtis, Jaccard, unweighted UniFrac, and weighted UniFrac yielded consistent results, so only Bray-Curtis results are presented for simplicity. Host species (Fig. 2.3a; Pseudo-F = 5.34, df = 5, $R^2 = 0.26$, $p = 0.001$) and site (Fig. 2.3b; Pseudo-F = 5.36, df = 4, $R^2 = 0.21$, $p = 0.001$) were significantly associated with microbiome beta-

diversity differences among individuals. Microbiome compositional differences among sites were significant between Colliers Mills and Assunpink, Morin Pond and Assunpink, and Morin Pond and Colliers Mills (pairwise PERMANOVA: $p < 0.01$). Compositional differences among frog host species' microbiomes were significant between bullfrogs and green frogs, bullfrogs and spring peepers, and wood frogs and green frogs (pairwise PERMANOVA: $p < 0.05$).

I found significant differences in beta dispersion (distance to centroid) among both sites ($F_{4,71} = 10.94$, $p = 0.001$) and species ($F_{5,70} = 14.03$, $p = 0.001$). These differences were driven by the relatively small distance to centroid observed among Pine Barrens Tree Frogs at Albertson Pond (Fig. 2.4), indicating that Pine Barrens tree frogs' skin microbiomes were more compositionally similar to one another than individuals of other species. This pattern is also evident in the NMDS plots of Bray-Curtis distances (Fig. 2.3), where Pine Barrens tree frog individuals cluster closely in ordination space and have a relatively narrow confidence ellipse.

Bd prevalence

No clinical signs of chytridiomycosis were observed during frog swabbing. Nine individuals tested positive for Bd (Table 2.1), making Bd prevalence 11.8% across all individuals sampled ($n = 76$). One individual, a green frog at Morin Pond, yielded an inconclusive result with only one of three PCR wells testing positive for Bd. Because there was not enough DNA to re-test this individual, it was conservatively considered negative for Bd.

Bacterial alpha diversity did not differ between Bd-positive and Bd-negative individuals (KW – OTU richness: $\chi^2 = 1.89$, $p = 0.17$; Chao1: $\chi^2 = 2.02$, $p = 0.15$). Among spring peepers, the only species with roughly equal numbers of Bd positive (n = 4) and negative (n = 3) individuals, there were also no differences in alpha diversity based on Bd status (KW – OTU richness: $\chi^2 = 0.8$, $p = 0.37$; Chao1: $\chi^2 = 1.13$, $p = 0.29$), suggesting that this trend is not simply an artifact of unbalanced sample sizes.

Bd status explained very little variation in community composition and was not significant in PERMANOVA results (Pseudo-F = 1.17, df = 1, R^2 =0.02, $p = 0.75$). Furthermore, Bd-positive individuals were nested within overall community structure in NMDS plots (Fig. 2.3c). Taken together, these results indicate that differences among skin microbiomes were not related to Bd status.

Comparison to antifungal isolates database

Of the 2194 frog-associated OTUs, 11.9% (260) matched inhibitory isolates in the antifungal isolate database. These putative anti-Bd OTUs made up 32.6% of bacterial abundance among host-associated bacterial communities. The mean number of anti-Bd OTUs across individuals was 21 (SD +/- 14), and the majority (80%) of anti-Bd OTUs were classified as Proteobacteria, although members of Bacteroidetes, Actinobacteria and Firmicutes were also present.

There were significant differences in the number of anti-Bd OTUs across sites (Fig. 2.5a) and Bd status (Fig. 2.5b), but not host species (GLM –Site: $\chi^2 = 21.1$, df = 3, $p < 0.001$; Bd status: $\chi^2 = 4.07$, df = 1, $p = 0.04$; Species: $\chi^2 = 5.97$, df = 4, $p = 0.2$). However, these differences were no longer significant when considering the relative

abundance of anti-Bd OTUs among sites (Fig. 2.5c), Bd status (Fig. 2.5d), and host species (GLM –Site: χ^2 = 6.81, df = 3, p = 0.08; Bd status: χ^2 = 0.63, df = 1, p = 0.43; Species: χ^2 = 4.31, df = 4, p = 0.37). This indicates that OTU richness is not always directly related to bacterial abundance in a community.

PERMANOVA results indicated that site (Pseudo-F = 4.98, R^2 = 0.2, df = 4, p = 0.001) and host species (Pseudo-F = 4.25, R^2 = 0.21, df = 5, p = 0.001) significantly influenced anti-Bd community beta-diversity (Fig. S2.2). However, Bd status had no significant effect on anti-Bd community beta-diversity (Pseudo-F = 1.12, df = 1, R^2 = 0.015, p = 0.8). Similar to the overall beta diversity results, there were significant differences in beta dispersion for anti-Bd OTU communities among both sites ($F_{4,71}$ = 7.59, p = 0.001) and species ($F_{5,70}$ = 10.4, p = 0.001). Once again, these differences were driven by the relatively small dispersion among Pine Barrens Tree Frogs at Albertson Pond. These trends suggest that patterns in anti-Bd community diversity reflect patterns in overall community diversity.

In addition to identifying the presence of anti-Bd OTUs, there were eight OTUs that had consensus matches in the antifungal isolate database to isolates with Bd-enhancing activity. While these putative Bd-enhancing OTUs were found at three of the five sites (Colliers Mills, Kai Pond, and Morin Pond), they comprised, on average, less than 1% of total relative abundance of frog skin bacterial communities.

Indicator species

There were 71 OTUs strongly associated (IndVal > 0.7) with at least one frog species (Fig. 2.6; Table S2.1). Of these indicator taxa, 30 (42.3%) were putative anti-Bd

OTUs, which is significantly more than expected based on the proportion of putative anti-Bd OTUs among the 2194 OTUs in the larger dataset ($\chi^2 = 62.94$, df = 2, $p < 0.001$). The majority (84.5%) of indicator OTUs belonged to the phylum Proteobacteria. Only 13 of all indicators were associated with more than one frog species.

In the indicator analysis based on host Bd status, six OTUs were highly associated with Bd-positive individuals. All six OTUs belonged to the family Comamonadaceae (Phylum: Proteobacteria, Class: Betaproteobacteria, Order: Burkholderiales). Three out of six of these OTUs were putative anti-Bd isolates based on consensus matches in the antifungal isolate database, indicating that several potential anti-Bd OTUs were strongly associated with Bd-positive individuals.

Comparison to environment

Within the environmental samples, there were no significant differences in OTU richness (KW $\chi^2 = 1.83$, $p = 0.77$) or Chao1 index (KW $\chi^2 = 2.75$, $p = 0.6$) among sites. Mean OTU richness was significantly higher in environmental samples compared to frog samples (KW $\chi^2 = 4.68$, $p = 0.03$), but there were no differences in Chao1 index (KW $\chi^2 = 3.8$, $p = 0.051$). Beta diversity was significantly different between environmental and frog samples (PERMANOVA – Pseudo-F = 1.63, df = 1, $R^2 = 0.02$, $p = 0.02$) when samples were pooled across species and sites. Taken together, these results suggest differences in community structure between frog and environmental bacterial communities.

Green frog results

Here, I compare results from the previous chapter that analyzed patterns in culturable anti-Bd bacteria to results obtained from 16S amplicon sequencing of microbiomes of the same individuals. Using 16S amplicon sequencing, there were significant differences in alpha diversity (KW – OTU richness: $\chi^2 = 10.02$, $p = 0.007$; Chao1 $\chi^2 = 10.29$, $p = 0.006$) and beta diversity (PERMANOVA – Pseudo-F = 4.28, $R^2 = 0.25$, df = 2, $p = 0.001$) of host-associated bacterial communities among the three green frog populations.

I previously published a list of 17 anti-Bd OTUs that were cultured from green frog skin and subsequently identified using Sanger sequencing (Kruger 2019). When compared to the present database obtained via culture-independent techniques, 15/17 OTUs had matches at >97% sequence similarity. While culture-based approaches yield a fraction of the OTUs present in the community, they can accurately represent a subset of bacterial community diversity. Within the culture-independent dataset, 13.3% (193/1447) of all green frog OTUs had consensus matches to putative anti-Bd bacteria. The majority (86.5%) of these OTUs were Proteobacteria, as were the majority (13/17) of OTUs identified using culture-based approaches.

There were significant differences in the number (KW $\chi^2 = 12.81$, $p = 0.002$), relative abundance (KW $\chi^2 = 10.67$, $p = 0.005$), and beta diversity (PERMANOVA – Pseudo-F = 4.79, $R^2 = 0.27$, df = 2, $p = 0.001$; Fig. 2.7) of putative anti-Bd OTUs among the three populations of green frogs. Additionally, seven green frog OTUs matched Bd-enhancing isolates listed in the antifungal isolate database. However, these putative Bd-enhancing isolates were only found on two individuals at Colliers Mills and three individuals at Morin Pond. Mean relative abundance of Bd enhancing OTUs across sites

was extremely low (<1%), and mean relative abundance of anti-Bd OTUs was significantly higher than mean relative abundance of Bd-enhancing OTUs (KW χ^2 = 46.02, $p < 0.001$). Bd enhancing isolates were present when using culture-based approaches in the previous chapter, but were also relatively rare.

DISCUSSION

In this survey of skin microbiomes of six species of amphibians distributed across multiple sites, I found that host species and sampling site, but not Bd status, influenced microbiome alpha and beta diversity. Additionally, the beta diversity, but not alpha diversity, of putative anti-Bd bacteria varied among host species and sites when considering differences in OTU relative abundance. There were also distinct bacterial communities found on frogs and in the environment, with environmental bacterial communities having greater mean OTU richness compared to frog bacterial communities.

Host species and sampling site contributed to differences in skin-associated bacterial communities among individuals. Furthermore, indicator analysis suggested that amphibian host species harbored OTU indicators that were mostly unique to a single host species. These results provide additional evidence that amphibian skin microbiomes tend to be host species-specific (McKenzie *et al.* 2012; Kueneman *et al.* 2014; Walke *et al.* 2014) and can vary among sites (Kueneman *et al.* 2014; Hughey *et al.* 2017; Muletz-Wolz *et al.* 2017a, b). Despite differences among host species, Proteobacteria were common among skin microbiomes of all frog species, making up 53.6% of OTUs and having a mean relative abundance of 63.5% across frog samples. Previous studies have found Proteobacteria to dominate skin microbiomes of amphibians (Walke *et al.* 2014;

Kueneman *et al.* 2016; Rebollar *et al.* 2018), and the majority of known Bd-inhibitory isolates are classified as Proteobacteria (Woodhams *et al.* 2015), suggesting that this diverse group may play a key role in amphibian skin microbiome function.

In addition to differences in bacterial alpha and beta diversity among host-associated bacterial communities, I found that there were significant differences in bacterial community dispersion, the average distance of group members to the group centroid based on a Bray-Curtis distance matrix, among both species and sites. This pattern was driven by the relatively low distance to centroid among Pine Barrens tree frogs at Albertson Bog, suggesting that all the Pine Barrens tree frogs sampled had very similar skin microbiomes. Because Pine Barrens tree frogs were only sampled at one site, more information is needed to determine if host factors, environmental factors, or a combination of both factors drive this response. Because all other host species and site pairs had similar dispersion values, the differences among species and sites observed in beta diversity analyses are likely due to true differences in microbiome beta diversity and are not simply an artifact of heterogeneous dispersion among groups.

Comparison of frog-associated bacterial communities and environmental bacterial communities showed there were significant differences in the composition of the two assemblages, which supports previous findings that amphibian microbial communities can contain a subset of bacteria found in the environment but are compositionally distinct from the environment (Loudon *et al.* 2014; Walke *et al.* 2014; Rebollar *et al.* 2016b). While the small number of environmental samples analyzed here (n = 8) makes it difficult to draw conclusions about the role of the environment in serving as a reservoir for potential skin microbes, the fact that there were common OTUs found in both

communities suggests some environmental transmission of bacteria to frog skin is occurring. However, the environmental samples gathered here were only from pond water, and amphibians interact with many other habitats throughout their lifetimes. For example, Rebollar *et al.* (2016b) found the majority of frog skin-associated OTUs were also present on environmental perches, and Kueneman *et al.* (2014) found that amphibians shared many OTUs with soil samples. These results indicate the need for boarder environmental sampling across the microhabitats used by hosts to more accurately assess the role of the environment in structuring amphibian skin microbiomes.

The presence of cutaneous anti-Bd bacteria can be used to predict potential Bd risk across different hosts (Bletz *et al.* 2017b). As such, elucidating patterns in anti-Bd bacteria among host species may help inform use of conservation resources to prevent Bd spread or mitigate the effects of disease. Using a reference antifungal isolate database (Woodhams *et al.* 2015), 11.8% of OTUs were classified as potential anti-Bd bacteria, and these OTUs comprised nearly a third of total bacterial abundance across samples. Patterns in anti-Bd OTU richness did not necessarily reflect patterns in anti-Bd OTU relative abundance. For example, mean anti-Bd OTU richness among Pine Barrens tree frog individuals was less than 10 OTUs, but those OTUs comprised greater than 75% of bacterial community relative abundance on average, suggesting that these may be key OTus in terms of defense. Furthermore, significantly more indicator OTUs were putative anti-Bd taxa than would be expected based on the proportion of anti-Bd OTUs among all frog OTUs, suggesting that anti-Bd OTUs are integral members of bacterial communities based on their prevalence and abundance in these populations. Based on this strong

association with host amphibians, it may be possible that selection for these OTUs is occurring based on their abilities to inhibit Bd growth.

There were no differences in the relative abundance of anti-Bd OTUs across sites, host species, or Bd status. However, beta-diversity of the anti-Bd community varied among host species and sites. This result demonstrates that taxonomically unique antifungal bacterial communities occur across host species and sites. Similar findings have been reported previously (Lam *et al.* 2010; Flechas *et al.* 2012) and provide another line of reasoning, in addition to anti-Bd OTU prevalence among indicator OTUs, that amphibians may opportunistically acquire potentially beneficial bacteria, rather than rely upon a distinct subset of bacterial taxa that are common across sites.

I found Bd-positive individuals harbored significantly more anti-Bd OTUs than Bd-negative individuals. This result is opposite from what might be expected if individuals with more anti-Bd OTUs are less likely to be susceptible to Bd due to bacterial inhibition of Bd growth. However, Muletz-Wolz *et al.* (2019) also found an increase in the abundance of anti-Bd bacteria on amphibians after Bd infection, and Walke *et al.* (2017) found more anti-Bd OTUs among populations with higher Bd prevalence. This pattern may be due to selection for anti-Bd bacteria after Bd colonizes the skin as a mechanism to fight infections (Walke *et al.* 2017) or cutaneous disruption from Bd infection (Voyles *et al.* 2009) leading to a more favorable environment for some anti-Bd bacteria (Muletz-Wolz *et al.* 2019). Alternatively, Bd presence at sites may have already selected for frogs that harbor more anti-Bd bacteria (Belden *et al.* 2015), as Bd-positive individuals without these bacteria may have experienced Bd-related mortality.

Differences between Bd positive and negative individuals were no longer present when anti-Bd OTU relative abundances were considered, and could imply that the increase in anti-Bd OTU richness among Bd-positive individuals does not translate to an increase in anti-Bd function if function is better predicted by dominance rather than OTU richness. Because Bd prevalence was low among most of the species sampled here, there may not be strong selection for anti-Bd bacteria to dominate these communities (Walke *et al.* 2017). Rather, the presence of some anti-Bd OTUs may allow individuals to persist asymptomatically with low infection burden instead of eliminating infection entirely (Bresciano *et al.* 2015). This could be problematic if individuals remain reservoirs of infection and continue to transmit Bd to co-occurring amphibians that do not harbor anti-Bd OTUs or are more susceptible to mortality from Bd.

Indicator analysis showed that there was a strong association between Bd-positive individuals and putative anti-Bd bacteria. Three out of six indicator OTUs for Bd-positive individuals were present in the antifungal database. All six indicator OTUs belong to the family Comamonadaceae, which has previously been noted for having members with anti-Bd properties (Becker *et al.* 2015b; Woodhams *et al.* 2015). There is also evidence to suggest that frogs harboring bacteria from this group may be better equipped to clear Bd infection (Becker *et al.* 2015a). Walke *et al.* (2015) found that the relative abundance of a Comamonadaceae OTU was significantly higher in Bd-exposed frogs compared to non-exposed frogs and proposed that this shift in abundance was due to selection for this OTU after Bd exposure. My results provide supporting evidence that Comamonadaceae could play an important role in regulating Bd infection in amphibians. Given recent interest in probiotic therapies against Bd (Bletz *et al.* 2013), results from assessments of

wild amphibian populations should be used to help inform probiotic selection because they identify groups of bacteria that are integral members of host-associated microbial communities.

It is difficult to disentangle the cause and effect relationship between the microbiome influencing Bd susceptibility and Bd infection impacting microbiome structure, but experimental evidence suggests that both outcomes are possible and not mutually exclusive (Jani & Briggs 2014; Walke *et al.* 2015). I hypothesized that Bd-positive and Bd-negative individuals would harbor distinct bacterial communities because of the previously documented connection between Bd and host microbiota, but I did not find evidence to support this. However, I found relatively few Bd-positive individuals (9/76), and these individuals were spread across multiple host species and sites. Because of this, it is difficult to accurately conclude whether or not there is a relationship between Bd status and host microbiota in these populations. However, there were roughly equal numbers of Bd-positive and Bd-negative spring peepers, and there were no observable differences in microbiome composition among these individuals. These results could suggest that relatively stable microbiomes that include anti-Bd bacteria could allow species to tolerate Bd infection. Belden *et al.* (2015) also found no link between microbiome structure and Bd infection status, and Longo *et al.* (2015) found evidence that environmental variables were more important in structuring the skin microbiome than Bd status among species with enzootic Bd infections.

Additionally, microbiome-Bd interactions may vary depending on host species susceptibility to Bd. Several species sampled in this study, such as spring peepers, green frogs, and bullfrogs, can live with Bd asymptomatically (Gahl *et al.* 2012). Bullfrogs are

believed to serve as reservoirs of Bd infection (Daszak *et al.* 2004) and can transmit Bd to wood frogs, a species that is susceptible to mortality from Bd (Greenspan *et al.* 2013). To my knowledge, the susceptibility of carpenter frogs and Pine Barrens tree frogs to Bd is unknown. Asymptomatic carriers of Bd may be able to persist with Bd infection due to microbiomes that contain anti-Bd bacteria, which could prevent amphibian hosts from developing disease. Despite a lack of clinical signs of chytridiomycosis in this study, Bd presence is still worrisome because of the complex nature of host, disease, and environmental interactions that influence disease dynamics. For example, changes in climate are predicted to increase amphibian susceptibility to disease (Rohr & Raffel 2010). Furthermore, other anthropogenic stressors, such as habitat loss, may interact with disease and contribute to amphibian population declines (Blaustein *et al.* 2011). Finally, theory predicts that pathogen virulence can increase over time as a result of host population structure (Boots *et al.* 2004) or novel pathogen mutations (Bull 1994), which could result in future amphibian declines due to Bd. Describing current patterns in both amphibian microbiomes and Bd prevalence is necessary to discern disruptions or changes that may lead to future disease outbreaks.

Of the individuals that tested positive for Bd, none were from the two southernmost sites (Colliers Mills and Albertson Bog) located in the New Jersey Pine Barrens. These results may reflect the fact that some samples were collected in warmer months (Table 2.1). Bd detection rates may change seasonally (Korfel & Hetherington 2014; Julian *et al.* 2016) and can be lower in summer months (Julian *et al.* 2016) due to temperatures exceeding the optimal growth range of Bd (Piotrowski *et al.* 2004). A previous study conducted in the Pine Barrens in 2008 also found no evidence of Bd (Di

Leo 2010), however the sampling was performed during the summer. A lack of Bd in this area could alternatively be due to the low pH of ponds in this habitat. Johnson & Speare (2005) found minimal Bd growth from pH range 4-5, which is characteristic of Pine Barrens ponds. It is possible that the low pH environment in this region prevents Bd persistence. Future studies should focus on Bd sampling in early summer before temperatures increase beyond the range suitable for Bd growth (Julian *et al.* 2016) or across seasons to maximize detection of Bd across landscapes. Additionally, identifying environmental variables associated with Bd presence or absence could help determine sites that should be prioritized for Bd monitoring.

In the previous chapter, I described the differences in communities of culturable anti-Bd and Bd-enhancing bacteria among three populations of green frogs (Kruger 2019). The same individuals were analyzed in the present study using culture-independent techniques to determine if results from studies using culture-dependent and culture-independent methodologies are comparable. I previously found no difference in the number of culturable anti-Bd isolates, but differences in the taxonomic composition (beta diversity) of these bacteria among green frog populations. Using culture-independent techniques in the present study, I found significant differences in both alpha and beta diversity of anti-Bd bacterial communities among green frog populations. These results suggest that while culture-based methods may not be reliable for estimating bacterial richness because most bacteria cannot be readily cultured (Vartoukian *et al.* 2010), culture-based approaches may be sufficient to determine if antifungal community structure differs among populations. Proteobacteria were the most common anti-Bd OTUs using both methods, suggesting that culture-based approaches can be reliable in

describing bacterial diversity, albeit at a smaller scale than culture-independent methods. Bd-enhancing OTUs were also identified using both methods, but were found at extremely low abundance using culture-independent methods. However, predicting Bd-enhancing function in this manner is likely unreliable since the antifungal isolate database (Woodhams *et al.* 2015) is dominated by Bd-inhibitory isolates and contains relatively few Bd-enhancing isolates. Continuing to identify and document patterns in Bd-enhancing isolates will allow us to predict overall microbiome function in a more holistic manner.

As the prevalence of emerging infectious diseases continues to increase, it is important to identify the key drivers of microbiome structure and function in systems where host-associated microbes may play a role in disease resistance. While this study provides valuable support for host species and site-level variation in amphibian skin microbiomes, it also identifies a gap in our understanding of why microbiome composition is related to Bd infection in some cases but not others. Exploring other aspects of host immunity, such as antimicrobial peptides (Rollins-Smith *et al.* 2002), may provide insight on the mechanisms that allow some species to persist with Bd without developing chytridiomycosis. As such, studies focusing on identifying patterns in a single aspect of host immunity (*e.g.* microbiome composition *or* antimicrobial peptides) may not be sufficient to understand patterns in Bd infection. A more comprehensive approach to studying amphibian immune defenses may be crucial to discovering ways to ameliorate the spread of infectious disease and prevent mortality of at-risk amphibians.

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Literature cited in Chapter 2

Becker, C.G., Longo, A. V., Haddad, C.F.B. & Zamudio, K.R. (2017). Land cover and forest connectivity alter the interactions among host, pathogen and skin microbiome. *Proc. R. Soc. London B Biol. Sci.*, 284, 20170582.

Becker, M.H. & Harris, R.N. (2010). Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. *PLoS One*, 5, e10957.

Becker, M.H., Walke, J.B., Cikanek, S., Savage, A.E., Mattheus, N., Santiago, C.N., *et al.* (2015a). Composition of symbiotic bacteria predicts survival in Panamanian golden frogs infected with a lethal fungus. *Proc. R. Soc. B Biol. Sci.*, 282, 20142881.

Becker, M.H., Walke, J.B., Murrill, L., Woodhams, D.C., Reinert, L.K., Rollins-Smith, L.A., *et al.* (2015b). Phylogenetic distribution of symbiotic bacteria from Panamanian amphibians that inhibit growth of the lethal fungal pathogen *Batrachochytrium dendrobatidis*. *Mol. Ecol.*, 24, 1628–1641.

Belden, L.K., Hughey, M.C., Rebollar, E.A., Umile, T.P., Loftus, S.C., Burzynski, E.A., *et al.* (2015). Panamanian frog species host unique skin bacterial communities. *Front. Microbiol.*, 6, 1–21.

Benjamini, Y. & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B*, 57, 289–300.

Berg-Lyons, D., Lauber, C.L., Humphrey, G., Thompson, L., Gilberg, J.A., Jansson, J.K., *et al.* (2018). EMP DNA Extraction Protocol. *protocols.io*.

Bird, A.K., Prado-Irwin, S.R., Vredenburg, V.T. & Zink, A.G. (2018). Skin microbiomes of California terrestrial salamanders are influenced by habitat more than host phylogeny. *Front. Microbiol.*, 9, 1–14.

Blaustein, A.R., Han, B.A., Relyea, R.A., Johnson, P.T.J., Buck, J.C., Gervasi, S.S., *et al.* (2011). The complexity of amphibian population declines: understanding the role of cofactors in driving amphibian losses. *Ann. N. Y. Acad. Sci.*, 1223, 108–119.

Bletz, M.C., Archer, H., Harris, R.N., McKenzie, V.J., Rabemananjara, F.C.E., Rakotoarison, A., *et al.* (2017a). Host ecology rather than host phylogeny drives amphibian skin microbial community structure in the biodiversity hotspot of Madagascar. *Front. Microbiol.*, 8, 1–14.

Bletz, M.C., Loudon, A.H., Becker, M.H., Bell, S.C., Woodhams, D.C., Minbiole, K.P.C., *et al.* (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecol. Lett.*, 16, 807–820.

Bletz, M.C., Myers, J., Woodhams, D.C., Rabemananjara, F.C.E., Rakotonirina, A., Weldon, C., *et al.* (2017b). Estimating herd immunity to amphibian chytridiomycosis in Madagascar based on the defensive function of amphibian skin bacteria. *Front. Microbiol.*, 8, 1751.

Boots, M., Hudson, P.J. & Sasaki, A. (2004). Large shifts in pathogen virulence relate to host population structure. *Science*, 303, 842–844.

Boyle, D.G., Boyle, D.B., Olsen, V., Morgan, J.A.T. & Hyatt, A.D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Dis. Aquat. Organ.*, 60, 141–148.

Bray, J.R. & Curtis, J.T. (1957). An ordination of the upland forest communities of

southern Wisconsin. *Ecol. Monogr.*, 27, 325–349.
Bresciano, J.C., Salvador, C.A., Paz-y-Miño, C., Parody-Merino, A.M., Bosch, J. & Woodhams, D.C. (2015). Variation in the presence of anti-*Batrachochytrium dendrobatidis* bacteria of amphibians across life stages and elevations in Ecuador. *Ecohealth*, 12, 310–319.
Bull, J.J. (1994). The evolution of virulence. *Trends Microbiol.*, 2, 73–75.
De Cáceres, M. & Legendre, P. (2009). Associations between species and groups of sites: indices and statistical inference. *Ecology*, 90, 3566–3574.
Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., *et al.* (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods*, 7, 335–336.
Chu, T.-C., Wu, M., Pohren, L., Haghjoo, B., Soman, C. & Lee, L.H. (2014). Molecular identification of a fungal pathogen *Batrachochytrium dendrobatidis* and its impact on urbanized New Jersey. *Adv. Microbiol.*, 4, 1164–1173.
Cohen, J.M., Venesky, M.D., Sauer, E.L., Civitello, D.J., McMahon, T.A., Roznik, E.A., *et al.* (2017). The thermal mismatch hypothesis explains host susceptibility to an emerging infectious disease. *Ecol. Lett.*, 20, 184–193.
Comeau, A.M., Douglas, G.M. & Langille, M.G.I. (2017). Microbiome Helper: A custom and streamlined workflow for microbiome research. *mSystems*, 2, e00127-16.
Daszak, P., Strieby, A., Cunningham, A.A., Longcore, J.E., Brown, C.C. & Porter, D. (2004). Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. *Herpetol. J.*, 14, 201–207.
DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., *et al.* (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.*, 72, 5069–5072.
Dufrene, M. & Legendre, P. (1997). Species assemblages and indicator species: The need for a flexible asymmetrical approach. *Ecol. Monogr.*, 67, 345–366.
Faith, D.P. (1992). Conservation evaluation and phylogenetic diversity. *Biol. Conserv.*, 61, 1–10.
Flechas, S. V, Sarmiento, C., Cárdenas, M.E., Medina, E.M., Restrepo, S. & Amézquita, A. (2012). Surviving chytridiomycosis: differential anti-*Batrachochytrium dendrobatidis* activity in bacterial isolates from three lowland species of *Atelopus*. *PLoS One*, 7, e44832.
Gahl, M.K., Longcore, J.E. & Houlahan, J.E. (2012). Varying responses of northeastern North American amphibians to the chytrid pathogen *Batrachochytrium dendrobatidis*. *Conserv. Biol.*, 26, 135–141.
Greenspan, S.E., Calhoun, A.J.K., Longcore, J.E. & Levy, M.G. (2013). Transmission of *Batrachochytrium dendrobatidis* to wood frogs (*Lithobates sylvaticus*) via a bullfrog (*L. catesbeianus*) vector. *J. Wildl. Dis.*, 48, 575–582.
Hamdi, C., Balloi, A., Essanaa, J., Crotti, E., Gonella, E., Raddadi, N., *et al.* (2011). Gut microbiome dysbiosis and honeybee health. *J. Appl. Entomol.*, 135, 524–533.
Harris, R.N., Brucker, R.M., Walke, J.B., Becker, M.H., Schwantes, C.R., Flaherty, D.C., *et al.* (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.*, 3, 818–824.
Hughey, M.C., Pena, J.A., Reyes, R., Medina, D., Belden, L.K. & Burrowes, P.A. (2017).

Skin bacterial microbiome of a generalist Puerto Rican frog varies along elevation and land use gradients. *PeerJ*, 5, e3688.

Jani, A.J. & Briggs, C.J. (2014). The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *Proc. Natl. Acad. Sci.*, 111, E5049–E5058.

Jani, A.J. & Briggs, C.J. (2018). Host and aquatic environment shape the amphibian skin microbiome but effects on downstream resistance to the pathogen *Batrachochytrium dendrobatidis* are variable. *Front. Microbiol.*, 9, 1–17.

Jani, A.J., Knapp, R.A. & Briggs, C.J. (2017). Epidemic and endemic pathogen dynamics correspond to distinct host population microbiomes at a landscape scale. *Proc. R. Soc. B Biol. Sci.*, 284, 20170944.

Johnson, M.L. & Speare, R. (2005). Possible modes of dissemination of the amphibian chytrid *Batrachochytrium dendrobatidis* in the environment. *Dis. Aquat. Organ.*, 65, 181–186.

Julian, J.T., Brooks, R.P., Glenney, G.W. & Coll, J.A. (2019). State-wide survey of amphibian pathogens in green frog (*Lithobates clamitans melanota*) reveals high chytrid infection intensities in constructed wetlands. *Herpetol. Conserv. Biol.*, 14, 199–211.

Julian, J.T., Gould, V.A., Glenney, G.W. & Brooks, R.P. (2016). Seasonal infection rates of *Batrachochytrium dendrobatidis* in populations of northern green frog *Lithobates clamitans melanota* tadpoles. *Dis. Aquat. Organ.*, 121, 97–104.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., *et al.* (2012). Geneious. *Bioinformatics*.

Kohl, K.D., Cary, T.L., Karasov, W.H. & Dearing, M.D. (2015). Larval exposure to polychlorinated biphenyl 126 (PCB-126) causes persistent alteration of the amphibian gut microbiota. *Environ. Toxicol. Chem.*, 34, 1113–1118.

Korfel, C.A. & Hetherington, T.E. (2014). Temperature alone does not explain patterns of *Batrachochytrium dendrobatidis* infections in the green frog *Lithobates clamitans*. *Dis. Aquat. Organ.*, 109, 177–185.

Kruger, A. (2019). Functional redundancy of *Batrachochytrium dendrobatidis* inhibition in bacterial communities isolated from *Lithobates clamitans* skin. *Microb. Ecol.* doi.org/10.1007/s00248-019-01387-7.

Kueneman, J.G., Parfrey, L.W., Woodhams, D.C., Archer, H.M., Knight, R. & McKenzie, V.J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Mol. Ecol.*, 23, 1238–1250.

Kueneman, J.G., Woodhams, D.C., Van Treuren, W., Archer, H.M., Knight, R. & McKenzie, V.J. (2016). Inhibitory bacteria reduce fungi on early life stages of endangered Colorado boreal toads (*Anaxyrus boreas*). *ISME J.*, 10, 934–944.

Kumar, R., Eipers, P., Little, R.B., Crowley, M., Crossman, D.K., Lefkowitz, E.J., *et al.* (2014). Getting started with microbiome analysis: Sample acquisition to bioinformatics. *Curr. Protoc. Hum. Genet.*, 82, 18.8.1-18.8.29.

Lam, B.A., Walke, J.B., Vredenburg, V.T. & Harris, R.N. (2010). Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. *Biol. Conserv.*, 143, 529–531.

Di Leo, K. (2010). An assessment of the correlation between amphibian populations, chytridiomycete communities, and the ecological integrity of the habitat. Master's

Thesis. Rutgers University.
Longcore, J.R., Longcore, J.E., Pessier, A.P. & Halteman, W. A. (2007). Chytridiomycosis widespread in anurans of northeastern United States. *J. Wildl. Manage.*, 71, 435–444.
Longo, A. V, Savage, A.E., Hewson, I. & Zamudio, K.R. (2015). Seasonal and ontogenetic variation of skin microbial communities and relationships to natural disease dynamics in declining amphibians. *R. Soc. Open Sci.*, 2, 140377.
Loudon, A.H., Woodhams, D.C., Parfrey, L.W., Archer, H., Knight, R., McKenzie, V., *et al.* (2014). Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *ISME J.*, 8, 830–840.
Lozupone, C., Lladser, M.E., Knights, D., Stombaugh, J. & Knight, R. (2011). UniFrac: An effective distance metric for microbial community comparison. *ISME J.*, 5, 169–172.
McKenzie, V.J., Bowers, R.M., Fierer, N., Knight, R. & Lauber, C.L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *ISME J.*, 6, 588–596.
McMurdie, P.J. & Holmes, S. (2013). Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*. 8, e61217.
Monsen-Collar, K., Hazard, L. & Dussa, R. (2010). Comparison of PCR and RT-PCR in the first report of *Batrachochytrium dendrobatidis* in amphibians in New Jersey, USA. *Herpetol. Rev.*, 41, 460–462.
Muletz-Wolz, C.R., DiRenzo, G. V., Yarwood, S.A., Grant, E.H.C., Fleischer, R.C. & Lips, K.R. (2017a). Antifungal bacteria on woodland salamander skin exhibit high taxonomic diversity and geographic variability. *Appl. Environ. Microbiol.*, 83, 1–13.
Muletz-Wolz, C.R., Yarwood, S.A., Campbell Grant, E.H., Fleischer, R.C. & Lips, K.R. (2017b). Effects of host species and environment on the skin microbiome of Plethodontid salamanders. *J. Anim. Ecol.*, 87, 341–353.
Muletz-Wolz, C.R., Fleischer, R.C. & Lips, K.R. (2019). Fungal disease and temperature alter skin microbiome structure in an experimental salamander system. *Mol. Ecol.*, 2917–2931.
Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., *et al.* (2013). Package 'vegan.' *R Packag. ver. 2.0–8*.
Ouellet, M., Mikaelian, I., Pauli, B.D., Rodrigue, J. & Green, D.M. (2005). Historical evidence of widespread chytrid infection in North American amphibian populations. *Conserv. Biol.*, 19, 1431–1440.
Piotrowski, J.S., Annis, S.L. & Longcore, J.E. (2004). Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia*, 96, 9–15.
R Core Team. (2016). *R: A language and environment for statistical computing. R Dev. Core Team*.
Rebollar, E.A., Antwis, R.E., Becker, M.H., Belden, L.K., Molly, C., Brucker, R.M., *et al.* (2016a). Using "omics" and integrated multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging infectious diseases. *Front. Microbiol.*, 7, 68.
Rebollar, E.A., Gutiérrez-Preciado, A., Noecker, C., Eng, A., Hughey, M.C., Medina, D., *et al.* (2018). The skin microbiome of the neotropical frog *Craugastor fitzingeri*: Inferring potential bacterial-host-pathogen interactions from metagenomic data.

Front. Microbiol., 9, 1–12.

Rebollar, E.A., Hughey, M.C., Medina, D., Harris, R.N., Ibáñez, R. & Belden, L.K. (2016b). Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. *ISME J.*, 10, 1682–1695.

Richards-Hrdlicka, K.L., Richardson, J.L. & Mohabir, L. (2013). First survey for the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in Connecticut (USA) finds widespread prevalence. *Dis. Aquat. Organ.*, 102, 169–180.

Rohr, J.R. & Raffel, T.R. (2010). Linking global climate and temperature variability to widespread amphibian declines putatively caused by disease. *Proc. Natl. Acad. Sci. U. S. A.*, 107, 8269–8274.

Rollins-Smith, L.A, Doersam, J.K., Longcore, J.E., Taylor, S.K., Shamblin, J.C., Carey, C., *et al.* (2002). Antimicrobial peptide defenses against pathogens associated with global amphibian declines. *Dev. Comp. Immunol.*, 26, 63–72.

Scheele, B.C., Pasmans, F., Skerratt, L.F., Berger, L., Martel, A., Beukema, W., *et al.* (2019). Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science,* 363, 1459–1463.

Thomason, C.A., Mullen, N., Belden, L.K., May, M. & Hawley, D.M. (2017). Resident microbiome disruption with antibiotics enhances virulence of a colonizing pathogen. *Sci. Rep.*, 7, 1–8.

Varela, B.J., Lesbarrères, D.A., Ibáñez, R. & Green, D.M. (2018). Environmental and host effects on skin bacterial community composition in Panamanian frogs. *Front. Microbiol.*, 9, 298.

Vartoukian, S.R., Palmer, R.M. & Wade, W.G. (2010). Strategies for culture of "unculturable" bacteria. *FEMS Microbiol. Lett.*, 309, 1–7.

Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W.F., Dinudom, A., *et al.* (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science*, 326, 582–585.

Walke, J.B., Becker, M.H., Hughey, M.C., Swartwout, M.C., Jensen, R. V. & Belden, L.K. (2017). Dominance-function relationships in the amphibian skin microbiome. *Environ. Microbiol.*, 19, 3387–3397.

Walke, J.B., Becker, M.H., Loftus, S.C., House, L.L., Cormier, G., Jensen, R. V., *et al.* (2014). Amphibian skin may select for rare environmental microbes. *ISME J.*, 8, 2207–2217.

Walke, J.B., Becker, M.H., Loftus, S.C., House, L.L., Teotonio, T.L., Minbiole, K.P.C., *et al.* (2015). Community structure and function of amphibian skin microbes: An experiment with bullfrogs exposed to a chytrid fungus. *PLoS One*, 10, e0139848.

Woodhams, D.C., Alford, R.A., Antwis, R.E., Archer, H., Becker, M.H., Belden, L.K., *et al.* (2015). Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecology*, 96, 595.

Woodhams, D.C., Vredenburg, V.T., Simon, M.-A., Billheimer, D., Shakhtour, B., Shyr, Y., *et al.* (2007). Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biol. Conserv.*, 138, 390–398.

Tables

Table 2.1. **Summary of frog and environmental sampling by site.**

"N" indicates the number of individuals swabbed from each population, while the "N" in parentheses includes the number of individuals retained in analyses after quality control filtering during bioinformatics. "Bd-positive" indicates the number of samples that tested positive for Bd in qPCR assays. Environmental samples were not tested for Bd.

Site	Sample origin	Date sampled	N (N after filtering)	Bd-positive
Assunpink WMA				
	Bullfrog	5/22/17	10 (10)	2
	Green frog	7/2/16	10 (10)	1
	Environment	5/22/17, 7/22/17	2 (2)	--
Kai Pond				
	Bullfrog	8/24/17	3 (3)	0
	Spring peeper	4/25/17	7 (7)	4
	Environment	4/25/17, 8/24/17	2 (2)	--
Morin Pond				
	Green frog	5/13/16	10 (9)	0
	Wood frog	3/9/16	10 (10)	2
	Environment	5/13/16, 3/9/16	2 (2)	--
Colliers Mills WMA				
	Carpenter frog	7/27/17	10 (8)	0
	Green frog	7/7/16	10 (10)	0
	Environment	7/7/17, (7/27/17)	2 (1)	--
Albertson Bog				
	Pine Barrens tree frog	4/30/17	10 (9)	0
	Environment	4/30/17	1(1)	--

Table 2.2. **Phylum-level summary of OTU distribution in frog microbiome samples.** Proportion of OTUs was calculated out of the total number of OTUs found on frog skin across samples (n = 2194). Mean relative abundance is across all individuals sampled, regardless of species or site (n = 76). Proteobacteria made up the majority of OTUs in the dataset and are further broken down by class. Phyla with less than 0.1% relative abundance are excluded.

Phylum	**Proportion OTUs**	**Mean relative abundance**
p__Proteobacteria	0.536	0.635
c__Alphaproteobacteria	*0.145*	*0.063*
c__Betaproteobacteria	*0.306*	*0.481*
c__Gammaproteobacteria	*0.062*	*0.087*
c__Deltaproteobacteria	*0.021*	*0.005*
c__Other	*0.003*	*0*
p__Bacteroidetes	0.177	0.157
p__Cyanobacteria	0.064	0.110
p__Firmicutes	0.057	0.025
p__Acidobacteria	0.042	0.018
p__Actinobacteria	0.024	0.004
p__Planctomycetes	0.021	0.008
p__Verrucomicrobia	0.015	0.022
p__Chloroflexi	0.006	0.001
p__Armatimonadetes	0.005	0.002
p__Gemmatimonadetes	0.005	0.001
p__[Thermi]	0.004	0.002
p__Chlorobi	0.003	0.001
p__OP3	0.001	0.001
p__GN02	0.001	0.001
p__Deferribacteres	0	0.001
Unclassified	0.030	0.011

Figures

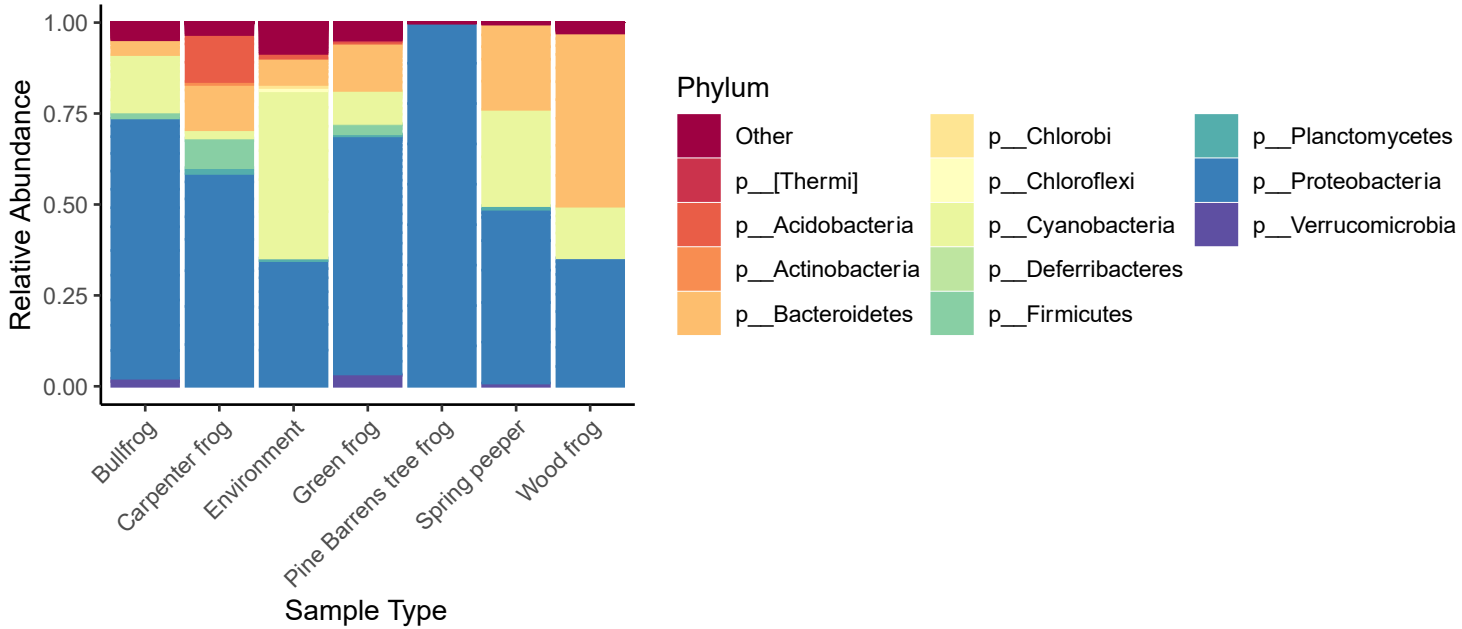

Figure 2.1. Taxonomic bar plots of mean relative abundance of bacteria (phylum level) across species and in the environment. Phyla comprising less than 5% relative abundance are grouped together in the "other" category. Sample sizes for each category are reported in Table 2.1.

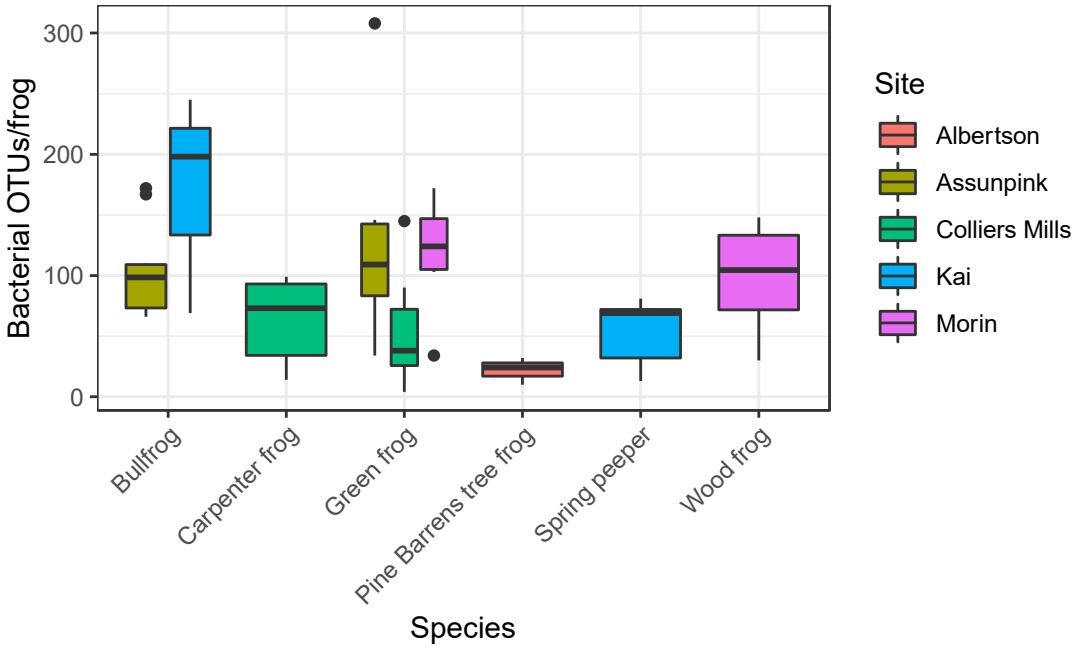

Figure 2.2. Alpha diversity (OTU richness) varied among host species and sampling site (GLM $p < 0.05$).

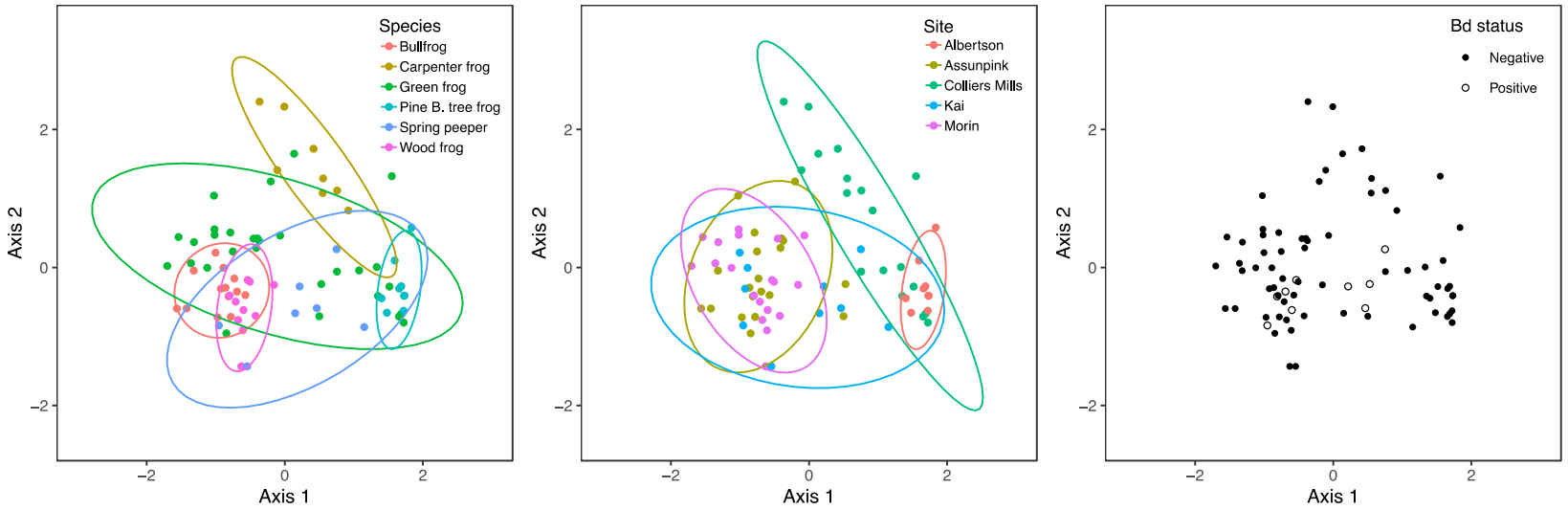

Figure 2.3. NMDS plots of bacterial communities based on Bray-Curtis distances (k = 3, stress = 0.15) and grouped by a) frog species, b) sampling site, and c) Bd status.

Each point represents an individual amphibian's skin bacterial community. PERMANOVA results indicated that site and species ($p \leq 0.05$) but not Bd status ($p > 0.05$) influenced bacterial community beta-diversity.

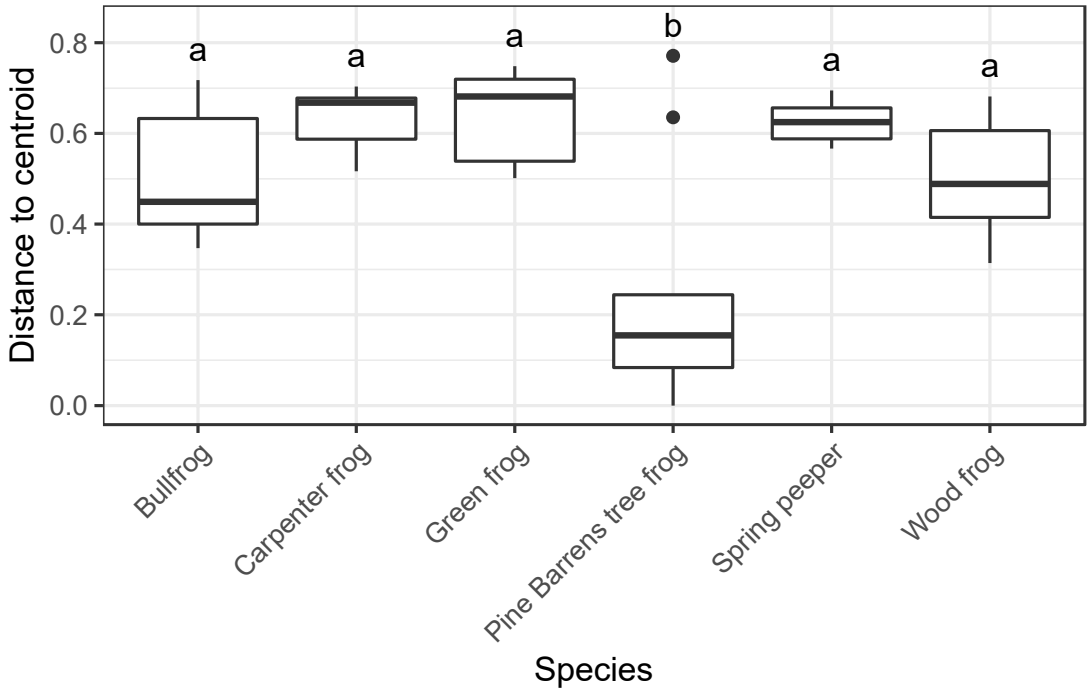

Figure 2.4. **Dispersion among skin bacterial communities of each of the six species of frogs sampled based on Bray-Curtis dissimilarity matrix.**

Different letters represent species with significant differences in group dispersion based on a permutational test of dispersion (FDR-corrected $p < 0.05$).

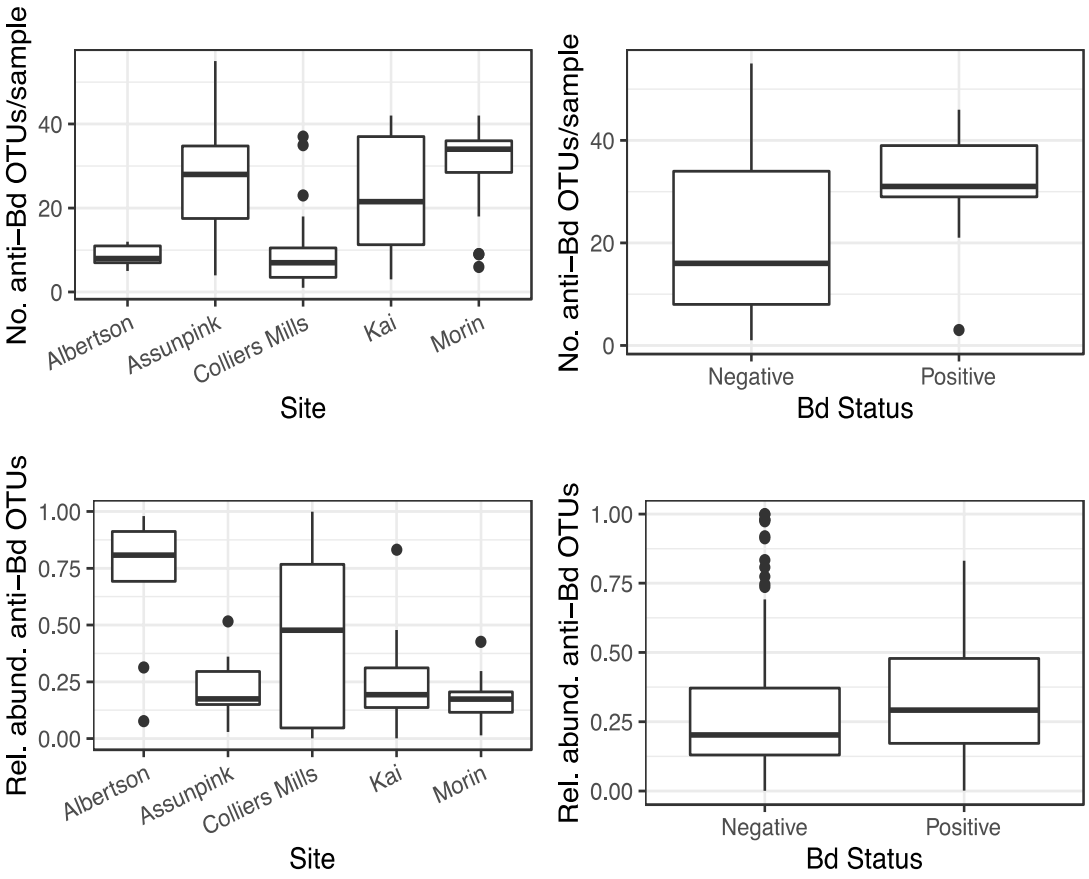

Figure 2.5. The number of putative anti-Bd OTUs varied among a) sites and b) Bd status. (GLM $p < 0.05$).

There were no differences in putative anti-Bd OTUs across c) sites or d) Bd status once relative abundances of bacteria were considered (GLM $p > 0.05$).

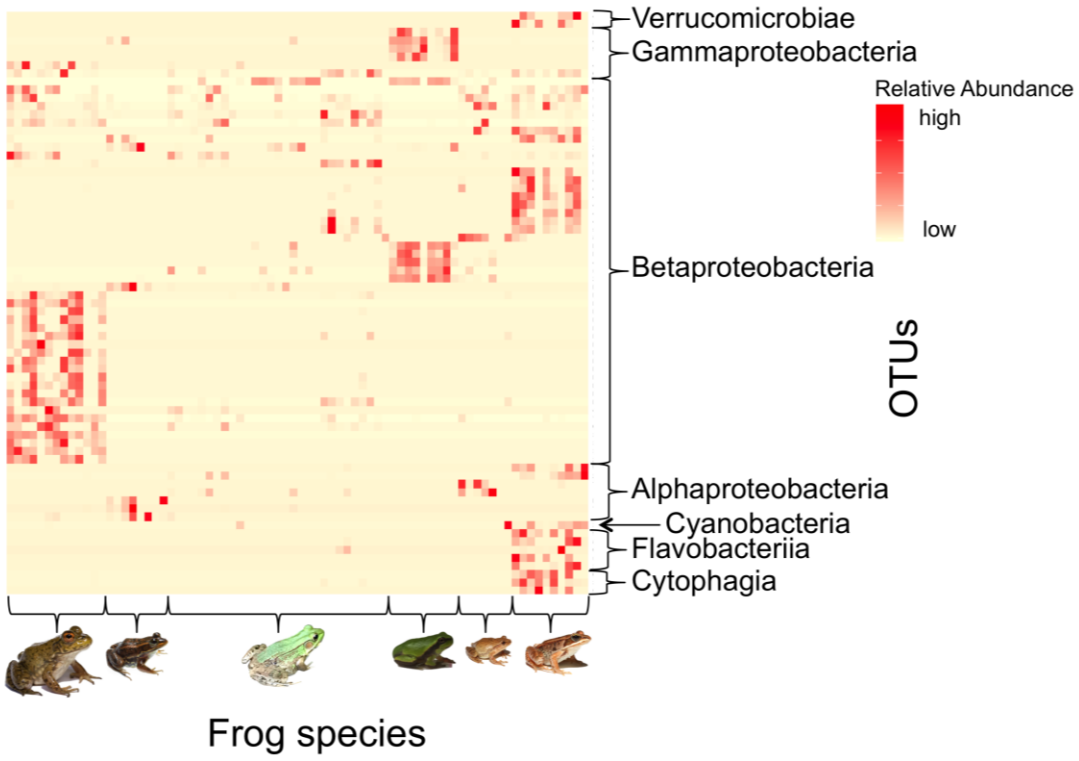

Figure 2.6. Heatmap depicting the relative abundances of indicator bacterial taxa (IndVal > 0.7) across frog host species.

Each row depicts a unique indicator OTU (Class taxonomic level displayed) and each column indicates an individual frog (Frog species from left to right: American bullfrog, carpenter frog, green frog, Pine Barrens tree frog, spring peeper, and wood frog). There were 71 OTUs that were strong indicators for at least one frog species (see Table S2.1), and most indicators were unique among frog species. [Photo credits: Bullfrog, spring peeper, and wood frog – Brian Gratwicke; Carpenter frog – Brady Beck; Green frog – Peter J. Morin; Pine Barrens tree frog – A.K.]

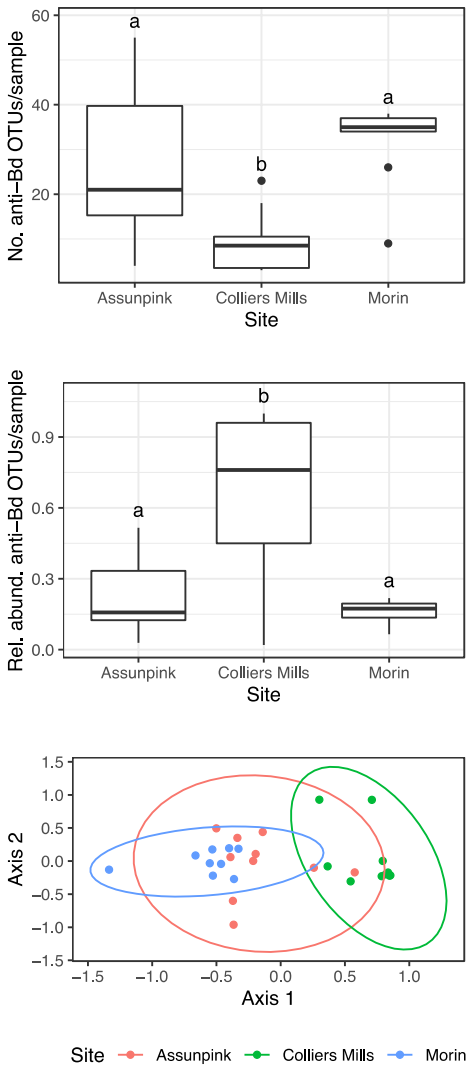

Figure 2.7. Summary of putative anti-Bd OTU community metrics among three green frog populations.

The a) number and b) relative abundance, and c) beta diversity (NMDS: k = 2, stress = 0.151) of putative anti-Bd OTUs varied significantly among green frog populations. Letters in (a) and (b) denote significant differences between sites (FDR-corrected $p < 0.05$). Pairwise PERMANOVAs indicated that there were significant differences in anti-Bd beta-diversity between all site pairs ($p < 0.05$).

Supplementary Information Chapter 2

Table S2.1. List of indicator OTUs found on frog skin, including the OTU ID, IndVal statistic, p-value indicating significance, and frog host species for which the OTUs were considered significant (IndVal > 0.7, p < 0.05) indicators.

OTUs marked with an asterisk (*) had consensus matches in the antifungal isolate database (Woodhams *et al.* 2015) at a 97% sequence similarity threshold.

OTU	Indval-stat	p-value	Host species	Taxonomy
974797	0.739	0.009	bullfrog+green	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__ s__
798634*	0.8	0.003	bullfrog+green+peeper	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__ s__
672144*	0.759	0.005	bullfrog+green+peeper+wood	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__ s__
818450*	0.705	0.024	bullfrog+green+wood	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__ s__
576785*	0.795	0.002	bullfrog+peeper+wood	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__ s__
576928*	0.874	0.001	bullfrog+peeper+wood	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__ s__
578572*	0.784	0.001	bullfrog+peeper+wood	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__ s__
614969*	0.721	0.005	bullfrog+wood	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Rhodocyclales f__Rhodocyclaceae g__ s__
222554	0.92	0.001	bullfrog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Methylophilales f__Methylophilaceae g__ s__
306546	0.877	0.001	bullfrog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Methylophilales f__Methylophilaceae g__ s__
312987	0.832	0.001	bullfrog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Methylophilales f__Methylophilaceae g__Methylotenera s__mobilis
322972	0.999	0.001	bullfrog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Methylophilales f__Methylophilaceae g__ s__
661442*	0.7	0.002	bullfrog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__ s__
777498	0.95	0.001	bullfrog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Methylophilales f__Methylophilaceae g__Methylotenera s__mobilis
4315164	0.704	0.005	bullfrog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae NA NA
4397450	0.723	0.001	bullfrog	k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Methylococcales f__Crenotrichaceae g__Crenothrix s__
New.CleanUp.ReferenceOTU0*	0.806	0.001	bullfrog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__ s__
New.ReferenceOTU1509	0.734	0.001	bullfrog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Methylophilales f__Methylophilaceae g__Methylotenera s__mobilis
New.ReferenceOTU1536*	0.83	0.001	bullfrog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__Paucibacter s__

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New.ReferenceOTU3981	0.734	0.001	bullfrog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__ s__
New.ReferenceOTU4424*	0.797	0.001	bullfrog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__ s__
New.ReferenceOTU4804	0.734	0.001	bullfrog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Methylophilales f__Methylophilaceae g__ s__
New.ReferenceOTU5397	0.734	0.001	bullfrog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Methylophilales f__Methylophilaceae g__ s__
New.ReferenceOTU6735	0.783	0.002	bullfrog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__ s__
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749696	0.7	0.003	carpenter_frog	k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Sphingomonadales f__Sphingomonadaceae g__ s__
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New.ReferenceOTU0*	0.868	0.001	green+pb_treefrog+peeper	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Alcaligenaceae g__Achromobacter s__
38733	0.796	0.001	peeper+wood	k__Bacteria p__Cyanobacteria c__Chloroplast o__Stramenopiles f__ g__ s__
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558264*	0.817	0.001	Pine_Barrens_tree_frog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Alcaligenaceae g__Achromobacter s__
922761	1	0.001	Pine_Barrens_tree_frog	k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Enterobacteriales f__Enterobacteriaceae NA NA
4403542*	0.924	0.001	Pine_Barrens_tree_frog	k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Enterobacteriales f__Enterobacteriaceae g__ s__

4451045*	0.97	0.001	Pine_Barrens_tree_frog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Alcaligenaceae g__Achromobacter s__
New.ReferenceOTU1763	0.87	0.001	Pine_Barrens_tree_frog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Alcaligenaceae g__ s__
New.ReferenceOTU6067	0.816	0.001	Pine_Barrens_tree_frog	k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Enterobacteriales f__Enterobacteriaceae g__Erwinia s__
New.ReferenceOTU72	0.926	0.001	Pine_Barrens_tree_frog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Alcaligenaceae g__ s__
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972719	0.707	0.001	Wood_frog	k__Bacteria p__Bacteroidetes c__Cytophagia o__Cytophagales f__Cytophagaceae g__Flectobacillus s__
998905	0.892	0.001	Wood_frog	k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Caulobacterales f__Caulobacteraceae g__Mycoplana s__
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4329804*	0.76	0.001	Wood_frog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Rhodocyclales f__Rhodocyclaceae g__ s__
4340146	0.888	0.001	Wood_frog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__ s__

4351336*	0.834	0.001	Wood_frog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__Hydrogenophaga s__
4389007	0.707	0.001	Wood_frog	k__Bacteria p__Verrucomicrobia c__Verrucomicrobiae o__Verrucomicrobiales f__Verrucomicrobiaceae g__Luteolibacter s__
4429445	0.774	0.001	Wood_frog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__Hydrogenophaga s__
4430221	0.768	0.001	Wood_frog	k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae g__Hydrogenophaga s
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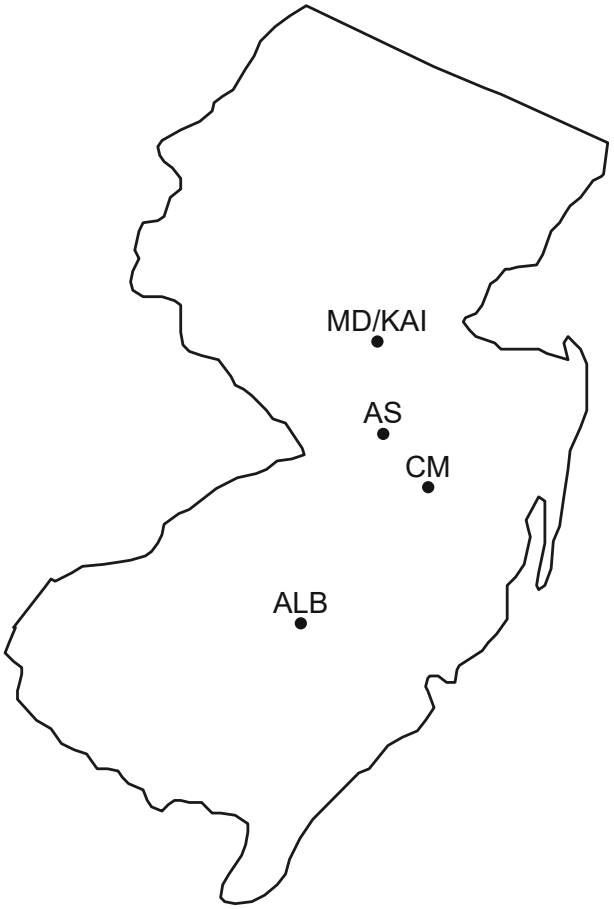

Figure S2.1. **Map of sampling sites in New Jersey.**

ALB = Albertson Bog, CM = Colliers Mills WMA, AS = Assunpink WMA, MD = Morin Pond, and KAI = Kai Pond. Morin Pond and Kai Pond are located approximately 0.5 miles apart from each other, so are listed together on the map.

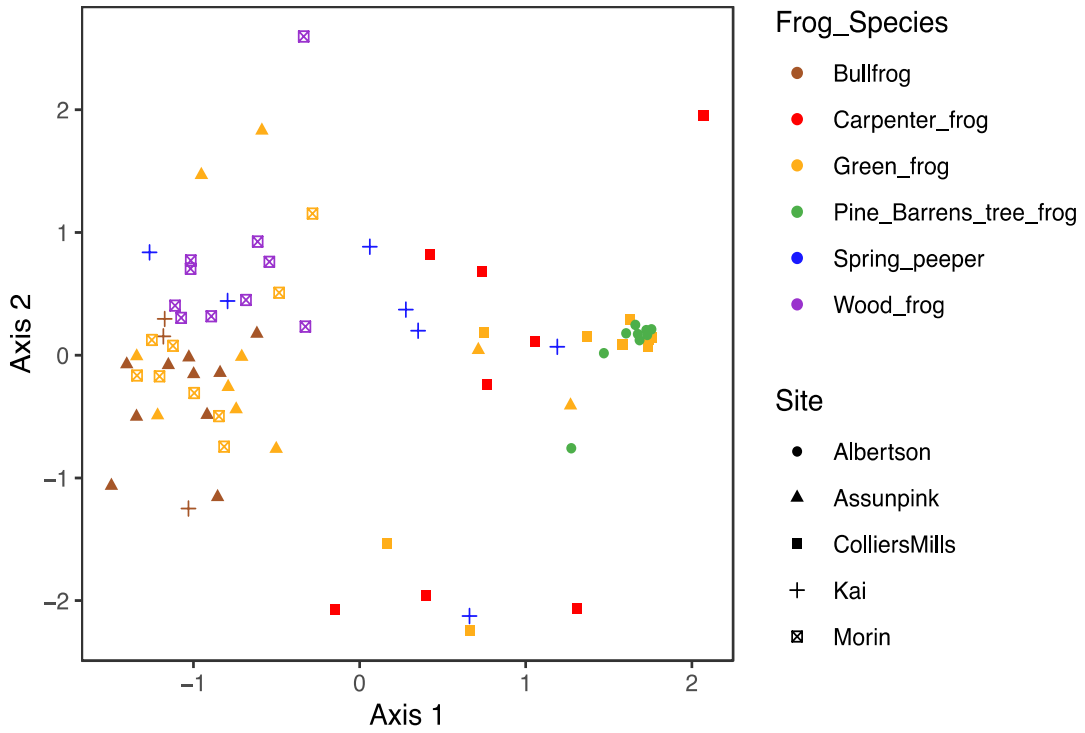

Figure S2.2. **NMDS ordination of putative anti-Bd bacterial communities across sites and species based on Bray-Curtis dissimilarity matrix (k = 3, stress = 0.16).** PERMANOVA results indicated that both site and species significantly influenced anti-Bd bacterial community beta-diversity ($p < 0.05$).

CHAPTER 3

Predators induce morphological changes in tadpoles of *Hyla andersonii*

ABSTRACT

Predators can affect the development, fitness, and behavior of prey species in myriad ways. In response to the threat of predation, tadpoles can alter growth rate, phenotype, and foraging behavior. Changes to tadpole development have the potential to alter life history characteristics and are therefore of interest in species of conservation concern. Using experimental mesocosms, we explored how non-lethal predators affected the larval development of the Pine Barrens tree frog, *Hyla andersonii*, a near-threatened species in the United States. Predator-induced changes in morphology occur in some hylid tree frogs, but had not been explored in *H. andersonii*. We found that caged dragonflies (*Anax junius)* induced darker tail coloration and deeper tail fins in *H. andersonii* tadpoles, but did not affect tadpole activity level, survival, or size at metamorphosis. Nonlethal predator presence also induced greater within population variation in the tail color trait compared to populations without predators. This result suggests that there may be underlying genetic variation in the ability to express phenotypically plastic traits, a concept that should be explored further because it has implications for the evolution of inducible defenses. These findings support the existence of an adaptive syndrome among hylid tadpoles, where tadpoles express tail flagging in response to larval dragonfly predators.

INTRODUCTION

Phenotypic plasticity, the ability of an organism to develop different phenotypes based on external stimuli, is a strategy that can benefit organisms living in heterogeneous environments because different phenotypes may be favored under different environmental conditions (Relyea 2004). For prey species, the flexibility to express a defensive phenotype may be advantageous when predatory risk is variable. Inducible defenses are traits that are expressed in predator presence that can reduce prey susceptibility to predation (Harvell 1990). These traits may be plastic rather than fixed because of fitness trade-offs associated with behavioral, morphological, or physiological changes that can occur in the presence of predators (Van Buskirk & Schmidt 2000; Wilson *et al.* 2005).

Tadpoles have been used as a model system for studying inducible defenses, predator avoidance, and fitness trade-offs for decades. Past research suggests that predator presence can greatly influence tadpole behavior, growth, and morphology (McCollum & Van Buskirk 1996; Van Buskirk 2000; Relyea 2001). In addition to predator presence causing decreased activity levels in tadpoles, which can lead to decreased growth rates (Werner & Anholt 1993; Relyea 2002), there can be phenotypic differences in tadpoles raised in predator-free ponds compared to those raised in ponds with predators (McCollum & Van Buskirk 1996; Relyea 2001). The defensive phenotype expressed by prey can differ based on the species of predator present (Relyea 2001; Hoverman *et al.* 2005). For example, *Lithobates pipiens* tadpoles had deeper tail fins in the presence of fish predators but not in the presence of larval odonate predators, suggesting that tadpoles can distinguish among predator species (Relyea 2001). The

ability to accurately respond to different predators is likely advantageous when a phenotype is protective in the presence of one species of predator but not another. Carfagno *et al.* (2011) demonstrated that *Acris blanchardi* tadpoles lose the conspicuous tail spot that is displayed in the presence of larval odonate predators when they are reared in the presence of fish predators. While the tail spot may offer an advantage in the presence of larval odonates, it may make tadpoles more susceptible to predation from fish, which attack their prey differently from dragonfly larvae (Carfagno *et al.* 2011).

Although an inducible defense may provide an immediate benefit to the prey species (*i.e.*, predator avoidance), potential trade-offs may ultimately lead to fitness costs for the prey (DeWitt *et al.* 1998; Van Buskirk 2000). For example, some tadpoles produce conspicuously pigmented tails in response to predators, including larval dragonflies (Smith & Van Buskirk 1995; McCollum & Van Buskirk 1996). Plastic phenotypes that involve significant tail coloration may result in trade-offs because of high energetic costs associated with producing pigmentation (Grether *et al.* 2001). Because costs related to reduced fecundity cannot be measured in tadpoles, it is difficult to quantify fitness costs as a result of an inducible defense (LaFiandra & Babbitt 2004). Prey growth rate and survival to metamorphosis are often used to determine fitness costs in anurans (McCollum & Van Buskirk 1996; Relyea 2002) because costs of inducible defenses are likely incurred in terms of resource allocation (Van Buskirk 2000).

Despite theory predicting that inducible defenses must be associated with fitness costs or else traits would be fixed rather than plastic, some studies have found no evidence of reduced fitness among tadpoles as a result of predator-induced changes during development (Van Buskirk & Saxer 2001; Relyea & Hoverman 2003).

Furthermore, *A. blanchardi* tadpoles still express their tail spot in the absence of predatory aeshnid larvae (Carfagno *et al.* 2011), suggesting the cost of this phenotype is negligible under some conditions.

Predator-induced phenotypic plasticity has been demonstrated in tadpoles of many hylid species. *Hyla versicolor* (Van Buskirk & McCollum 2000) and *Hyla chrysoscelis* (McCollum & Van Buskirk 1996) tadpoles develop morphological changes in tail color and shape that reduce attacks by predators. Furthermore, *A. blanchardi* tadpoles with dark tail tips co-occur more frequently in ponds with high densities of aeshnid larvae (Caldwell 1982), suggesting that although this trait seems to be polymorphic, its induction may depend on environmental variation in predator presence. It is clear that tail flagging, a phenotype that consists of conspicuous tail markings or coloration, is a common response of hylid tadpoles to larval odonate predators (*e.g.,* McCollum & Van Buskirk 1996, Van Buskirk & McCollum 2000, LaFiandra & Babbitt 2004). However, closely related hylid species may differ in whether or not they manifest inducible defenses (Smith & Van Buskirk 1995) such as tail flagging. Conspicuous tadpole tails may direct predatory dragonfly attacks toward the tail, which can be a non-lethal injury, as opposed to potentially lethal attack on the vulnerable tadpole body (McCollum & Van Buskirk 1996; Van Buskirk *et al.* 2003). Interactions with predators and competitors during larval development can greatly impact fitness at other stages in the anuran complex life cycle (Benard & Fordyce 2003; Relyea & Hoverman 2003). Therefore, understanding how tadpoles deal with stress from predation threats may be just as critical as understanding how stressors such as habitat loss affect anuran species.

The Pine Barrens tree frog (*Hyla andersonii)* is a habitat specialist with an unusual relictual geographic distribution, with populations in southern New Jersey, the Carolinas, and Alabama and the Florida panhandle. Despite this disjunct range, recent genetic evidence suggests that the taxon should still be considered a single species (Warwick *et al.* 2015). Due to its limited range and relative rarity, knowledge of the ecology of *H. andersonii* remains rather incomplete. Adults breed primarily in acidic, fish-free temporary water in shrub bog habitats in eastern USA. They are an IUCN Red List near-threatened species and are considered to be at risk of decline because of habitat loss (IUCN 2016). *Hyla andersonii* is also listed as a threatened species in New Jersey (NJDEP 2019), a species in need of management in South Carolina (Bennett & Buhlmann 2015), and is considered imperiled in Florida (Florida Fish and Wildlife Conservation Comission 2018). Adult *H. andersonii* breed asynchronously from mid-April to mid-July in New Jersey (Morin *et al.* 1990), and populations in New Jersey may be larger than in the rest of the species' range (Warwick *et al.* 2015). *Hyla andersonii* tadpoles are known to potentially compete with other aquatic organisms including herbivorous insects and other anuran larvae (Morin *et al.* 1990; Pehek 1995). *Hyla andersonii* tadpoles are also readily eliminated from pond communities by predatory fish and salamanders (Kurzava & Morin 1998). Larval odonates co-occur in ponds across the range of *H. andersonii,* and are known to prey upon tadpoles. In a previous study of *H. andersonii* (Morin *et al.* 1990), we noticed that tadpoles sometimes manifested conspicuous darkly pigmented tails when they co-occurred with aeshnid dragonfly larvae in natural and artificial ponds. Here we test the hypothesis that this change in morphology was the result of an inducible defense expressed in response to predatory odonate larvae.

We experimentally tested whether *H. andersonii* tadpole behavior, morphology, and development changed in response to the nonlethal presence of an important predator, larvae of the odonate *Anax junius.* Tadpoles were reared in mesocosms with or without caged *A. junius* larvae to determine whether *H. andersonii* displayed phenotypically plastic traits. We hypothesized that tadpoles raised in ponds with odonate predators would display changes consistent with an inducible defense including the development of a conspicuous tail, reduced activity levels, and slower growth compared to conspecifics reared in predator-free ponds.

MATERIALS AND METHODS

Artificial ponds

We constructed 12 artificial ponds/mesocosms using 359-liter cylindrical polyethylene stock tanks that were housed at the Hutcheson Memorial Forest of Rutgers University (Somerset County, New Jersey). The tanks were filled with well water on 27 April 2018. Each tank received 200 g of dry grassy plant litter raked from the surrounding area to add habitat complexity, plus 15 g of Purina® Rabbit Chow as an added source of nutrients. We added 700 ml of a mixture of phytoplankton and zooplankton collected from a single pond in the NJ Pine Barrens to each tank on 2 May 2018 to provide an inoculum for food web development and subsequently added another 400 ml of plankton from the source pond where breeding frogs were collected to each tank on 3 May 2018. Each artificial pond was covered with a square lid constructed from wood and fiberglass screening to prevent colonization of ovipositing insects. In each

tank, we provided three “ladders,” pieces of fiberglass screening draped over the side of the tank, so froglets had a path for emergence from the water once metamorphosis began.

Experimental design

Our experimental design included a predator treatment (n = 6 artificial ponds) and a predator-free control (n = 6 artificial ponds). Treatments were spatially randomized among artificial ponds using a block design. Our predator treatment included three individually caged larval odonates (late instar *Anax junius*), and the predator-free mesocosms contained three empty cages to control for possible effects of the cages alone. Odonate larvae were individually caged to prevent cannibalism. Our field sampling indicated that *A. junius* larvae can be abundant in pond-edge vegetation, and although not quantified here, the density of *A. junius* used in mesocosms likely reflects natural conditions. Cages were made of fiberglass screening with plastic tubing at both ends to create a cylinder that was approximately 50 centimeters in length and 15 cm in diameter. These cages allowed larval dragonflies to move vertically in the water column and were permeable to zooplankton, which served as a food source. Cages were suspended at mid depth at equidistance points around the circumference of the mesocosms by a strand of monofilament fishing line. This approach, which has been used in previous studies of other anuran species (Van Buskirk 2001; Relyea & Hoverman 2003), allowed us to analyze the effect of nonlethal predator presence on tadpole development without confounding effects of reduced survival or density that might result from direct dragonfly predation. Larval odonates were collected from ponds in Bass River State Forest (Ocean

County, NJ) and Somerset County, NJ. *Anax junius* were added to mesocosms on 9 May 2018.

We collected six breeding pairs of *Hyla andersonii* on 3 May 2018 from a single pond in the NJ Pine Barrens as a source of hatchling tadpoles. The frogs were placed in covered plastic containers where they deposited eggs overnight. After oviposition, frogs were returned to the site of capture (<48 hours later). The eggs subsequently hatched on 10 May 2018 (Day 0), and hatchlings were counted for addition to the tanks on 11 May 2018. Each mesocosm received a total of 270 hatchling tadpoles pooled from the six clutches of eggs. Mesocosms received supplemental additions of 10 g of rabbit chow on 29 May, 25 June, 15 July, and 7 August 2018 to provide continued support for the pond food webs. We returned all froglets to the field site where their parents were collected after they completed metamorphosis.

Morphological Responses

We measured tadpole morphology at three time points during development 32, 39, and 46 days after tadpoles hatched. These days were picked to 1) allow ample time for phenotypic changes to occur in the presence of the predator, 2) allow tadpoles to be large enough that they could be safely sampled with replacement, and 3) to determine if morphology differed across time until tadpoles metamorphosed. On each sampling day, we haphazardly collected (using hand nets) 25 tadpoles from each pond to photograph their phenotype for subsequent analysis. The only exception to this sampling effort occurred on Day 46 for ponds two (n = 2 tadpoles, -predator treatment) and three (n = 15 tadpoles, -predator treatment), when the onset of tadpole metamorphosis made it

impossible to collect 25 tadpoles from those ponds. Tadpoles were sampled with replacement on each sampling day. Tadpoles were individually placed into a narrow aquarium with a standard grid in the background and were photographed using a Sony alpha a6000 digital camera. All photos were taken with F/7.1 aperture, a $1/320^{th}$ second exposure time, and ISO 3200. Artificial light conditions were held constant across individuals and the three sampling dates.

We analyzed tadpole tail color and morphology using ImageJ 1.51p (Rasband 2012). Tail color was analyzed by outlining the tadpole tail using the polygon tool and measuring the mean gray value of the photos' pixels. This unitless value is an indicator of how light or dark an image is, with lower values indicating there are more dark pixels present in the photo, and higher values indicating there are more light pixels in the photo (range: 0-255). Tadpole tail length (TL), body length (BL), tail muscle depth at the base of the tail (TMD), maximum tail fin depth (TFD), and total tadpole length (TTL) were measured after setting the scale within each photo based on the background grid. Measurements of tadpole tail length, body length, tail muscle depth, and tail fin depth were divided by total tadpole length to determine what proportion of total tadpole length each response variable accounted for. These standardized measures relative to total tadpole length were used as response variables in all analyses.

Behavioral responses

Tadpole activity level was measured at four time points on days 28, 32, 40, and 42 of larval development. Before counting, all mesocosm lids were removed and tadpoles were allowed to acclimate after this disturbance for approximately five minutes. Artificial

ponds were approached slowly and from a direction that ensured no shadows were cast across the water surface. Tadpole activity was measured by counting the number of tadpoles visible (*i.e.*, those not hiding in leaf litter). We repeated this count for a total of two counts per mesocosm for each sampling day. The mean number of tadpoles visible on each day was standardized by dividing by the number of individuals that survived to metamorphosis in each mesocosm to correct for possible differences in tadpole counts due to survival differences among ponds.

Life history responses

Artificial ponds were checked daily for metamorphosing froglets starting on day 41. Froglets that had emerged from the water were collected and housed in plastic containers where they completed metamorphosis, as determined by complete tail resorption. We measured wet weight and snout-urostyle length of each froglet after tails had been fully resorbed. We calculated larval period as days from tadpole hatching (day 0) to when tail resorption was complete. Overall survival was calculated as the percent of individuals surviving to metamorphosis. Growth index was calculated as wet mass at metamorphosis divided by larval period.

Statistics

All statistical analyses were conducted in R version 3.3.2 (R Core Team 2016). We used population (*i.e.,* mesocosm) means for analyzing response variables to retain independence when testing for the effect of predator presence. We used linear mixed effects models (LMMs) with normal error distribution using the lme4 package (Bates *et*

al. 2014) to test for differences in tadpole morphology. When testing for changes across time, repeated measurements of mesocosms nested within the spatial blocks were used as random effects to account for non-independence (Pinheiro & Bates 2000). Predator presence and sampling date were used as fixed effects. Block was included as a random factor in the model, and this term was excluded if it did not improve model fit as assessed by likelihood ratio tests. LMMs were also used to test for differences in within pond tail color variance by using mean within pond variance as a response variable. We used the "Anova" function in the car package (Fox *et al.* 2016) to run Wald chi-square tests to determine confidence in model estimates. To test for differences in tadpole activity between treatment groups and over time, we used a generalized linear mixed effects model (GLMM). Repeated measurements of mesocosms were treated as random effects.

To determine overall predator effects, we used MANOVAs on tadpole morphological measurements and metamorphosis measurements. Block interaction effects were not significant in either model, so these degrees of freedom were pooled with the error term. We then conducted univariate ANOVAs to determine which response variables contributed to the multivariate responses. We used a principal components analysis (PCA) for different tail morphological measurements (tail color, tadpole length, and standardized measures of body length, tail length, muscle depth, and fin depth) and a separate PCA for metamorphosis measurements (mass and length at metamorphosis, survival to metamorphosis, and length of larval period) to determine which components explained the most variation between differences in predator and predator-free treatments. We used Pearson correlation matrices to compare principal component scores to original variables to interpret the principal components. We also used a Pearson

correlation matrix to determine how tadpole development traits were related to one another. We applied a Bonferroni correction to adjust for multiple comparisons when comparing trait correlations.

RESULTS

Tail morphology

Tadpoles in ponds with predators had significantly darker tails (Fig. 3.1) on all three sampling days (LMM: $\chi^2 = 16.2$, df = 1, $p < 0.001$; Fig. 3.2). There was no effect of sampling date ($\chi^2 = 2.34$, df = 2, p = 0.31) or treatment by date interaction ($\chi^2 = 0.25$, df = 2, p = 0.88) on tail color. Within population variance in tail color was significantly greater in predator treatments (LMM: $\chi^2 = 5.7$, df = 1, p = 0.017; Fig. 3.3), and there was no effect of sampling date ($\chi^2 = 4.01$, df = 2, p = 0.13) or treatment by date interaction ($\chi^2 = 4.15$, df = 2, p = 0.13).

Tadpoles in mesocosms containing predators developed significantly greater standardized tail fin depths (LMM: $\chi^2 = 10.8$, df = 1, $p < 0.001$; Fig. 3.4). Standardized tail fin depth also significantly decreased over time as tadpoles grew ($\chi^2 = 38.4$, df = 2, $p < 0.001$), but there was no treatment by date interaction ($\chi^2 = 2.04$, df = 2, p = 0.36). Predators did not affect tadpole body length, tail length, tail muscle depth, or total tadpole length, but these variables did change significantly over time as tadpoles grew and developed (Table S3.1; Fig. 3.4).

Patterns in multivariate analysis of tadpole morphological data were consistent across sampling days, so Day 32 results are presented for simplicity. The first two principal components accounted for 80.5% of the variation in tadpole morphology based

on Day 32 measurements (Table 3.1), and the principal components plot shows that populations clustered in ordination space based on the presence or absence of predators (Fig. 3.5). A MANOVA on tadpole morphological traits from Day 32 confirms a significant effect of predators on *H. andersonii* morphology that was driven by effects on tail coloration and standardized tail fin depth (Table 3.2).

Tadpole behavior

Predators had no detectable effect on tadpole activity level (GLMM: $\chi^2 = 0.82$, df = 1, p = 0.36), but tadpole activity significantly decreased over time ($\chi^2 = 526.4$, df = 3, p < 0.001).

Life history measurements

Tadpoles metamorphosed between days 41 and 166, with 73% of tadpoles across ponds metamorphosing within 70 days and 90% of tadpoles metamorphosing within 100 days. A total of 1923 froglets emerged across all 12 mesocosms, making mean survival to metamorphosis among artificial ponds 59.4% (range: 17.8-77.8%; Table 3.3). There was a significant overall multivariate effect of predators and block on metamorphosis measurements, but there were no significant predator effects in any of the univariate tests for mass and length at metamorphosis, larval period, and survival to metamorphosis (Table 3.4). The first two principal components accounted for 97.6% of the variation in the metamorphosis measurements PCA (Table S3.2). Population density (*i.e.,* the number of survivors per mesocosm) was significantly negatively correlated with size at metamorphosis (Table 3.5).

DISCUSSION

Our findings document an important feature of the poorly known ecology of *Hyla andersonii* and contribute additional support for the existence of an adaptive syndrome in hylid tadpoles that represents convergent phenotypically plastic responses (tail flags) to a specific class of aquatic predators. In response to larval aeshnid dragonflies, *Hyla chrysoscelis* (McCollum & Van Buskirk 1996; McCollum & Leimberger 1997; Richardson 2006), *Hyla versicolor* (Van Buskirk & McCollum 2000), and *Hyla femoralis* (LaFiandra & Babbitt 2004) also develop tail flagging. *Acris blanchardi* tadpoles lose their tail flags that are expressed both in the presence of *Anax* sp. and in no-predator controls when exposed to fish (Carfagno *et al.* 2011), suggesting that the flagging phenotype is not beneficial against all aquatic predators. A strategy such as unpalatability might be expected in tadpoles that live in permanent ponds and encounter different classes of predators, which seems to be the case in bullfrog tadpoles and fish predators (Kats *et al.* 1988; Werner & McPeek 1994).

In this study, tadpoles exposed to predators also had deeper tail fins, a response to larval dragonflies observed in other larval amphibians (Van Buskirk & Relyea 1998; Van Buskirk & Saxer 2001). For example, *Pseudacris triseriata* have larger tail fins and greater tail muscle depth when exposed to odonates (Smith & Van Buskirk 1995). Though not directly tested here, deeper tail fins can produce greater burst swimming speeds in some species (Dayton *et al.* 2005), which may facilitate escape from predators. Furthermore, deeper and more conspicuously pigmented tails may increase survival by encouraging non-lethal predator attacks of the tail (Van Buskirk *et al.* 2003). It has also

been suggested that increased burst swimming speed may help facilitate tail ripping to escape predators after attack (Dayton *et al.* 2005). Taken together, the deeper- and darker-tailed tadpoles observed in predator treatments are consistent with the "lure effect," where changes in tadpole morphology in the presence of dragonfly larvae lure predator attacks toward the tadpole tail and away from the vulnerable tadpole head (Van Buskirk *et al.* 2003). These changes may facilitate the ability of tadpoles to tear away from a predator's grasp (Hoff & Wassersug 2000) and survive with ripped tails that can be regrown.

Inducible defenses can be generated from visual, mechanical, and/or chemical cues produced in the presence of the predator. Chemical cues seem to be particularly important for predator detection in aquatic animals (Stauffer & Semlitsch 1993; Eklöv 2000). Chemical cues can arise from predator odor (Eklöv 2000), the cues produced from conspecific digestion (Stabell *et al.* 2003; LaFiandra & Babbitt 2004), or alarm pheromones generated by attacked conspecifics (Hews 1988; Schoeppner & Relyea 2005). For example, *H. chrysoscelis* develop tail flagging in response to caged dragonfly larvae that are fed conspecifics, but do not develop flagging when caged predators are starved (McCollum & Leimberger 1997). We found that *H. andersonii* tadpoles exposed to caged dragonflies on a planktivorous diet developed tail flagging, suggesting that predator odor is sufficient to elicit this response. If zooplankton were producing alarm cues during predation, it is probable that *H. andersonii* would be unable to recognize these cues given previous research suggesting that prey only respond to alarm cues produced by conspecifics or closely related heterospecifics (Schoeppner & Relyea 2009). However, it is possible that general predator digestion cues, which are absent when

predators are starved, are adequate to trigger a phenotypic response. Taken together, these results suggest that non-contact cues are sufficient to induce tail flagging in hylids. More research is needed to identify the specific metabolites that trigger tadpoles to display an inducible defense and determine if the metabolites that elicit the defense vary across species.

Despite changes in morphology during *H. andersonii* development, we did not observe any costs in terms of reduced survival or size at metamorphosis in tadpoles reared in the presence of predators. Theory predicts that there should be some cost associated with expressing an inducible defense, otherwise the trait would be expected to be expressed constitutively (McCollum & Leimberger 1997; Relyea 2002). However, costs associated with inducible defenses have previously been difficult to document in tadpoles (Van Buskirk & Saxer 2001; Benard 2004). Our results are consistent with previous studies showing that caged predators rarely elicit smaller size or shorter time to metamorphosis in their tadpole prey (Relyea 2007; Relyea & Rosenberger 2018), suggesting that such costs of inducible defenses are uncommon (Benard 2004). Because costs of defenses are likely greatest when food is scarce and limited resources are allocated to the defensive trait instead of growth (Van Buskirk 2000), costs may be difficult to document in artificial settings where resources are often abundant. It is also possible that costs associated with the expression of a plastic trait during larval development may not materialize until later life stages, such as in adult survival or performance (Van Buskirk & Saxer 2001; Benard & Fordyce 2003; Relyea & Hoverman 2003). Despite the lack of predator effects on tadpole growth and survival, tadpole density (*i.e.,* number of survivors) in each pond was significantly negatively correlated

with froglet size at metamorphosis. This is consistent with previous work suggesting that frog body size at metamorphosis can be influenced by tadpole density (Wilbur & Collins 1973).

Spatial orientation of the artificial ponds in the field (*i.e.,* blocks) influenced larval development. Specifically, mesocosms closer to the forest edge of the field experienced more shade, which could have contributed to differences in productivity across mesocosms (Morin *et al.* 1990). While we did not observe differences in tadpole behavior between predator and predator-free treatments, overall tadpole activity decreased over time in all ponds. We suggest that this decrease in tadpole activity could have been due to a shift in resources in the pond. At the start of the experiment, tadpoles spent time grazing on the side of the tank closer to the surface. Eventually, the periphyton resources on the side of the tank could have become depleted, and consequently tadpoles may have shifted to acquiring resources at the bottom of tank, making them less visible during the activity census.

Another pattern that emerged was that the overall within population variation in the expression of tail pigmentation was greater when predators were present. Tadpoles in each mesocosm were a mixture of six different sibships, so it is possible that the observed phenotypic variation within populations represents genetic variation in the inducible defense. If darker tails have a selective advantage in the presence of predators, the variation in phenotypic expression of darker tails observed here may eventually lead to selection for this trait. However, decreased variation in the trait or lack of predator pressure makes it likely that selection will not act on the trait in populations where the predators are absent. Moderate levels of plasticity, which may manifest as variability in

plastic expression of a trait, may have the ability to promote genetic evolution (Price *et al.* 2003), given that individuals expressing a trait are more likely to survive and reproduce and plasticity has some genetic basis. Variance in the ability to express a trait may be highest in communities that have temporal or spatial variability in predator presence, and these communities may be most likely to experience plasticity-first evolution. The concept of plasticity-first evolution posits that initially plastic traits displayed due to environmental variation can lead to evolutionary adaptation because selection acts on phenotypes that have some genetic basis (West-Eberhard 2005). Theoretical work has shown that phenotypic plasticity can affect heritable trait variation and subsequent evolution (Draghi & Whitlock 2012), and recent empirical evidence supporting plasticity-first evolution (Levis & Pfennig 2016; Levis *et al.* 2018) suggests this topic deserves further evaluation in natural systems.

Our study documents the presence of a probable inducible defense in *Hyla andersonii*, a species of conservation concern throughout its fragmented range. Phenotypically plastic tail flagging appears to be an adaptive syndrome that is a common feature in hylid tadpoles exposed to odonates. More research is needed to determine if increased trait variation seen in the presence of predators represents genetic variation in the expression of the inducible trait. A study evaluating the responses of tadpoles of different sibships to predator presence would provide valuable insight to the role of genetic variation in inducible defenses in this system. Future work should also continue to explore if the tail flag adaptive syndrome is a general feature of hylid tadpoles that regularly encounter odonate predators in fish-free ponds.

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Literature cited in Chapter 3

Bates, D., Maechler, M., Bolker, B., Walker, S., Christensen, R.H.B., Singmann, H., *et al.* (2014). Package "lme4." *Compr. R Arch. Netw.*

Benard, M.F. (2004). Predator-induced phenotypic plasticity in organisms with complex life histories. *Annu. Rev. Ecol. Evol. Syst.*, 35, 651–673.

Benard, M.F. & Fordyce, J.A. (2003). Are induced defenses costly? Consequences of predator-induced defenses in Western toads, *Bufo boreas*. *Ecology*, 84, 68–78.

Bennett, S. & Buhlmann, K.A. (2015). *Pine Barrens Treefrog*. *South Carolina Dep. Nat. Resour. State Wildl. Action Plan*. http://www.dnr.sc.gov/cwcs/pdf/PineBarrensTreefrog.pdf (accessed 18 April 2019).

Caldwell, J.P. (1982). Disruptive selection: a tail color polymorphism in *Acris* tadpoles in response to differential predation. *Can. J. Zool.*, 60, 2818–2827.

Carfagno, G.L.F., Carithers, J.M., Mycoff, L.J. & Lehtinen, R.M. (2011). How the cricket frog lost its spot: The inducible defense hypothesis. *Herpetologica*, 67, 386–396.

Dayton, G.H., Saenz, D., Baum, K.A., Langerhans, R.B. & DeWitt, T.J. (2005). Body shape, burst speed and escape behavior of larval anurans. *Oikos*, 111, 582–591.

DeWitt, T.J., Sih, A. & Wilson, D.S. (1998). Costs and limits of phenotypic plasticity. *Trends Ecol. Evol.*, 13, 77–81.

Draghi, J.A. & Whitlock, M.C. (2012). Phenotypic plasticity facilitates mutational variance, genetic variance, and evolvability along the major axis of environmental variation. *Evolution*, 66, 2891–2902.

Eklöv, P. (2000). Chemical cues from multiple predator-prey interactions induce changes in behavior and growth of anuran larvae. *Oecologia*, 123, 192–199.

Florida Fish and Wildlife Conservation Comission. (2018). *Pine Barrens treefrog*. Available at: https://myfwc.com/wildlifehabitats/profiles/amphibians/pine-barrens-treefrog/ (accessed 20 April 2019).

Fox, J., Weisberg, S., Adler, D., Bates, D., Baud-bovy, G., Ellison, S., *et al.* (2016). *Package 'car.' CRAN Repos.*

Grether, G.F., Hudon, J. & Endler, J.A. (2001). Carotenoid scarcity, synthetic pteridine pigments and the evolution of sexual coloration in guppies (*Poecilia reticulata*). *Proc. R. Soc. London B Biol. Sci.*, 268, 1245–1253.

Harvell, C.D. (1990). The ecology and evolution of inducible defenses. *Q. Rev. Biol.*, 65, 323–340.

Hews, D.K. (1988). Alarm response in larval western toads, *Bufo boreas*: Release of larval chemicals by a natural predator and its effect on predator capture efficiency. *Anim. Behav.*, 36, 125–133.

Hoff, K. vS. & Wassersug, R.J. (2000). Tadpole locomotion: Axial movement and tail functions in a largely vertebraeless vertebrate. *Am. Zool.*, 40, 62–76.

Hoverman, J.T., Auld, J.R. & Relyea, R.A. (2005). Putting prey back together again: Integrating predator-induced behavior, morphology, and life history. *Oecologia*, 144, 481–491.

IUCN. (2016). IUCN Red List of Threatened Species. Version 2016-2. *Fourth Quart.*

Kats, L.B., Petranka, J.W. & Sih, A. (1988). Antipredator defenses and the persistence of amphibian larvae with fishes. *Ecology*, 69, 1865–1870.

Kurzava, L.M. & Morin, P.J. (1998). Tests of functional equivalence: Complementary

roles of salamanders and fish in community organization. *Ecology*, 79, 477–489.
LaFiandra, E.M. & Babbitt, K.J. (2004). Predator induced phenotypic plasticity in the pinewoods tree frog, *Hyla femoralis*: Necessary cues and the cost of development. *Oecologia*, 138, 350–359.
Levis, N.A., Isdaner, A.J. & Pfennig, D.W. (2018). Morphological novelty emerges from pre-existing phenotypic plasticity. *Nat. Ecol. Evol.*, 2, 1289–1297.
Levis, N.A. & Pfennig, D.W. (2016). Evaluating "plasticity-first" evolution in nature: Key criteria and empirical approaches. *Trends Ecol. Evol.*, 31, 563–574.
McCollum, S.A. & Van Buskirk, J. (1996). Costs and benefits of a predator-induced polyphenism in the gray treefrog *Hyla chrysoscelis*. *Evolution*, 50, 583–593.
McCollum, S.A. & Leimberger, J.D. (1997). Predator-induced morphological changes in an amphibian: Predation by dragonflies affects tadpole shape and color. *Oecologia*, 109, 615–621.
Morin, P.J., Lawler, S.P. & Johnson, E.A. (1990). Ecology and breeding phenology of larval *Hyla andersonii*: The disadvantages of breeding late. *Ecology*, 71, 1590–1598.
New Jersey Department of Environmental Protection (NJDEP). (2019). *Online Field Guide for Reptiles and Amphibians*. Available at: https://www.state.nj.us/dep/fgw/ensp/fieldguide_herps.htm (accessed 20 April 2019).
Pehek, E.L. (1995). Competition, pH, and the ecology of larval *Hyla andersonii*. *Ecology*, 76, 1786–1793.
Pinheiro, J.C. & Bates, D.M. (2000). Fitting nonlinear mixed-effects models. In: *Mixed-effects models in S and S-PLUS*. Springer New York.
Price, T.D., Qvarnström, A. & Irwin, D.E. (2003). The role of phenotypic plasticity in driving genetic evolution. *Proc. R. Soc. B Biol. Sci.*, 270, 1433–1440.
R Core Team. (2016). *R: A language and environment for statistical computing. R Dev. Core Team*.
Rasband, W.S.W. (2012). *ImageJ. U. S. Natl. Institutes Heal. Bethesda, Maryland, USA*.
Relyea, R.A. (2001). Morphological and behavioral plasticity of larval anurans in response to different predators. *Ecology*, 82, 523–540.
Relyea, R.A. (2002). Costs of phenotypic plasticity. *Am. Nat.*, 159, 272–282.
Relyea, R.A. (2004). Fine-tuned phenotypes: Tadpole plasticity under 16 combinations of predators and competitors. *Ecology*, 85, 172–179.
Relyea, R.A. (2007). Getting out alive: How predators affect the decision to metamorphose. *Oecologia*, 152, 389–400.
Relyea, R.A. & Hoverman, J.T. (2003). The impact of larval predators and competitors on the morphology and fitness of juvenile treefrogs. *Oecologia*, 134, 596–604.
Relyea, R.A. & Rosenberger, D. (2018). Predator effects on metamorphosis: The effects of scaring versus thinning at high prey densities. *Copeia*, 106, 457–467.
Richardson, J.L. (2006). Novel features of an inducible defense system in larval tree frogs (*Hyla chrysoscelis*). *Ecology*, 87, 780–787.
Schoeppner, N.M. & Relyea, R.A. (2005). Damage, digestion, and defence: The roles of alarm cues and kairomones for inducing prey defences. *Ecol. Lett.*, 8, 505–512.
Schoeppner, N.M. & Relyea, R.A. (2009). When should prey respond to consumed heterospecifics? Testing hypotheses of perceived risk. *Copeia*, 1, 190–194.
Smith, D.C. & Van Buskirk, J. (1995). Phenotypic design, plasticity, and ecological

performance in two tadpole species. *Am. Nat.*, 145, 211–233.
Stabell, O.B., Ogbebo, F. & Primicerio, R. (2003). Inducible defences in *Daphnia* depend on latent alarm signals from conspecific prey activated in predators. *Chem. Senses*, 28, 141–153.
Stauffer, H.-P. & Semlitsch, R.D. (1993). Effects of visual, chemical and tactile cues of fish on the behavioural responses of tadpoles. *Anim. Behav.*, 46, 355–364.
Van Buskirk, J. (2000). The costs of an inducible defense in anuran larvae. *Ecology*, 81, 2813–2821.
Van Buskirk, J. (2001). Specific induced responses to different predator species in anuran larvae. *J. Evol. Biol.*, 14, 482–489.
Van Buskirk, J., Anderwald, P., Lüpold, S., Reinhardt, L. & Schuler, H. (2003). The lure effect, tadpole tail shape, and the target of dragonfly strikes. *J. Herpetol.*, 37, 420–424.
Van Buskirk, J. & McCollum, S. (2000). Functional mechanisms of an inducible defence in tadpoles: morphology and behaviour influence mortality risk from predation. *J. Evol. Biol.*, 13, 336–347.
Van Buskirk, J. & Relyea, R.A. (1998). Selection for phenotypic plasticity in *Rana sylvatica* tadpoles. *Biol. J. Linn. Soc.*, 65, 301–328.
Van Buskirk, J. & Saxer, G. (2001). Delayed costs of an induced defense in tadpoles? Morphology, hopping, and development rate at metamorphosis. *Evolution,* 55, 821–829.
Van Buskirk, J. & Schmidt, B.R. (2000). Predator-induced phenotypic plasticity in larval newts: Trade-offs, selection, and variation in nature. *Ecology*, 81, 3009–3028.
Warwick, A.R., Travis, J. & Lemmon, E.M. (2015). Geographic variation in the Pine Barrens Treefrog (*Hyla andersonii*): Concordance of genetic, morphometric and acoustic signal data. *Mol. Ecol.*, 24, 3281–3298.
Werner, E. & McPeek, M. (1994). Direct and indirect effects of predators on two anuran species along an environmental gradient. *Ecology*, 75, 1368–1382.
Werner, E.E. & Anholt, B.R. (1993). Ecological consequences of the trade-off between growth and mortality rates mediated by foraging activity. *Am. Nat.*, 142, 242–272.
West-Eberhard, M.J. (2005). Developmental plasticity and the origin of species differences. *Proc. Natl. Acad. Sci.*, 102, 6543–6549.
Wilbur, H.M. & Collins, J.P. (1973). Ecological aspects of amphibian metamorphosis. *Science,* 182, 1305–1314.
Wilson, R.S., Kraft, P.G. & Van Damme, R. (2005). Predator-specific changes in the morphology and swimming performance of larval *Rana lessonae*. *Funct. Ecol.*, 19, 238–244.

Tables

Table 3.1. Principal component analysis of Day 32 morphological traits of *H. andersonii* tadpoles reared with or without predators.

PC1 and PC2 indicate the first two principal components, and they accounted for 80.5% of the variance between treatments. The values in the table are the correlation coefficients of each morphological trait with the principal component scores.

Response variable	PC1	PC2
Tail color Day 32	-0.24	-0.78
Standardized tail fin depth	0.43	0.82
Standardized tail muscle depth	-0.65	-0.39
Standardized body length	0.76	-0.59
Standardized tail length	-0.76	0.59
Total tadpole length	-0.93	-0.12
% of variance explained	44.8%	35.7%

Table 3.2. Summary of MANOVA and univariate ANOVAs analyzing the effect of predator treatment and block on *H. andersonii* morphological measures, total tadpole length (TTL), standardized tail muscle depth (sTMD), standardized tail fin depth (sTFD), and tail color (col1) on Day 32.

Block and treatment interactions were not significant, so the degrees of freedom were pooled with the error term. Bolded values indicate statistical significance ($p < 0.05$).

	Wilk's λ	F	DF	p
MANOVA				
Predator Treatment	0.11	8.15	1, 7	**0.03**
Block	0.26	0.6	3, 7	0.8
Univariate ANOVAs				
TTL Treatment		0.64	1, 7	0.45
Block		1.95	3, 7	0.21
sTMD Treatment		0.18	1, 7	0.69
Block		0.68	3, 7	0.59
sTFD Treatment		30.0	1, 7	**<0.001**
Block		0.76	3, 7	0.55
col1 Treatment		16.35	1, 7	**0.005**
Block		0.50	3, 7	0.70

Table 3.3. Summary statistics for survival, larval period, and size at metamorphosis for *H. andersonii* reared either in the absence (-predator) or presence (+predator) of dragonfly larvae.

Growth index is calculated as wet mass at metamorphosis divided by larval period.

Predator treatment	N	Survival %	Mass (mg)	Larval period (days)	Growth index (mg/days)
			Mean +/- 1 standard deviation		
- Predator	6	52.4 +/- 17.9	206.1 +/- 55.31	67.73 +/- 20.53	3.45 +/- 1.78
+ Predator	6	66.3 +/- 9.5	189.61 +/- 15.57	61.59 +/- 6.14	3.1 +/- 0.5

Table 3.4. Results of overall MANOVA and univariate ANOVAs analyzing the effect of predators on mass and length at metamorphosis, larval period, and survival to metamorphosis.

Block and treatment interactions were not significant, so the degrees of freedom were pooled with the error term. Bolded values indicate statistical significance ($p < 0.05$).

	Wilk's λ	F	DF	p
MANOVA				
Predator Treatment	0.10	8.98	1, 7	**0.028**
Block	0.008	4.55	3, 7	**0.009**
Univariate ANOVAs				
mass Treatment		0.62	1, 7	0.46
Block		1.86	3, 7	0.22
length Treatment		1.22	1, 7	0.30
Block		3.5	3, 7	0.08
period Treatment		1.99	1, 7	0.20
Block		11.18	3, 7	**0.005**
survival Treatment		2.99	1, 7	0.13
Block		1.2	3, 7	0.38

Table 3.5. Pearson correlation matrix showing relationships between *H. andersonii* response variables.

Population density refers to the number of individuals surviving to metamorphosis in each mesocosm. Growth index is calculated as mass at metamorphosis divided by larval period. Bolded values indicate correlation coefficients that were significant after applying a Bonferroni correction adjusting for multiple comparisons.

	Tail color Day 32	Mass at metamorphosis	Length at metamorphosis	Larval period	Growth index
Mass at metamorphosis	0.14				
Length at metamorphosis	0.21	**0.97**			
Larval period	0.19	-0.63	-0.70		
Growth index	0.08	**0.97**	**0.96**	-0.78	
Population density	-0.26	**-0.89**	**-0.85**	0.37	**-0.82**

Figures

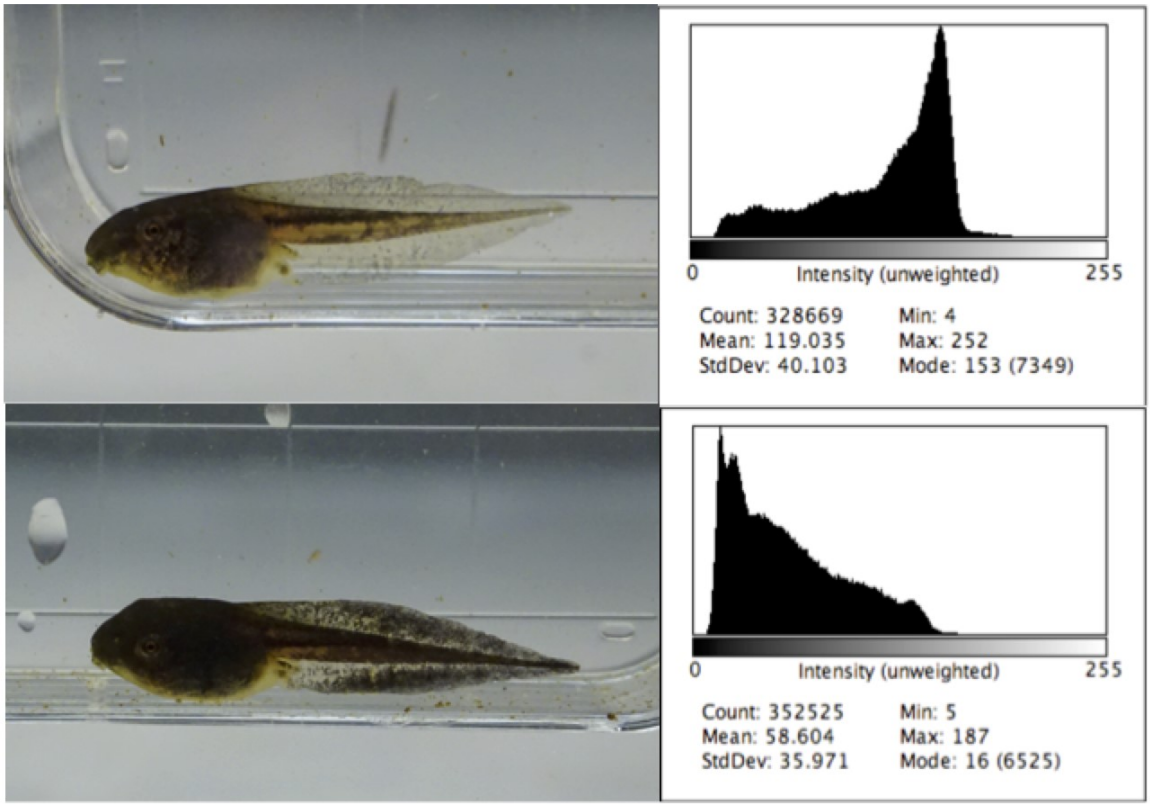

Figure 3.1. Representative photos of *H. andersonii* tadpoles reared without predators (top) and with predators (bottom) and their corresponding ImageJ pixel color histograms.

Lower values indicate darker pixels, and higher values indicate lighter pixels in the image. The y-axis of the histograms indicates the frequency of pixels for each darkness score.

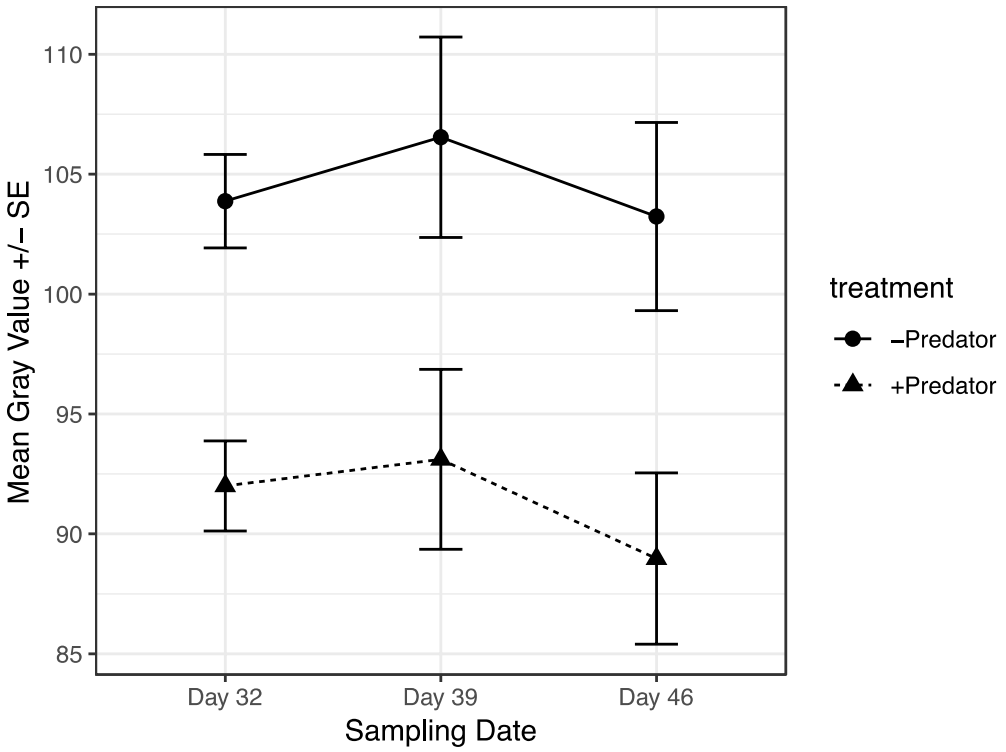

Figure 3.2. Tadpole tail color (mean gray value) was significantly lower (= darker tails) in predator treatments compared to non-predator controls (LMM: $\chi^2 = 16.2$, df = 1, $p < 0.001$).

Sampling date had no effect on tail color ($\chi^2 = 2.34$, df = 2, p = 0.31).

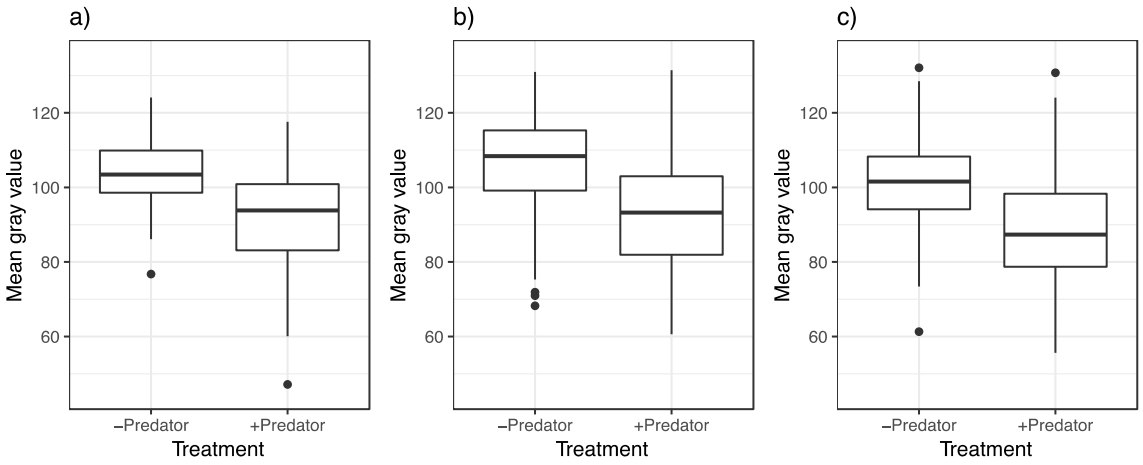

Figure 3.3. Boxplots showing variation in tadpole tail mean gray value (a measure of tail darkness) on sampling dates a) Day 32, b) Day 39, and c) Day 46 (individuals pooled across ponds within treatments on each sampling date).

Lower mean gray values indicate darker tail coloration.

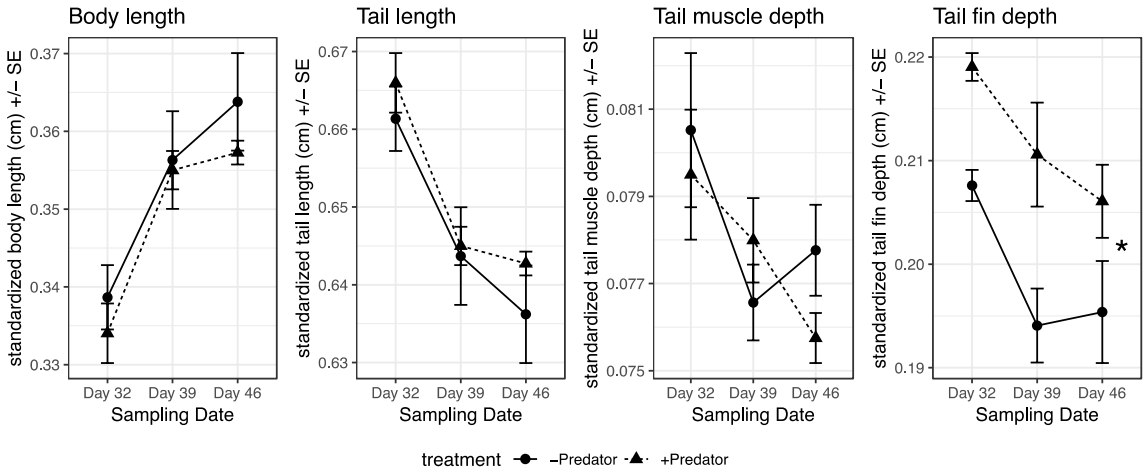

Figure 3.4. Comparison of relative tadpole size across sampling dates and predator treatments.

All measurements were standardized against total tadpole length to account for size differences among individual tadpoles. * $p < 0.05$ between treatments.

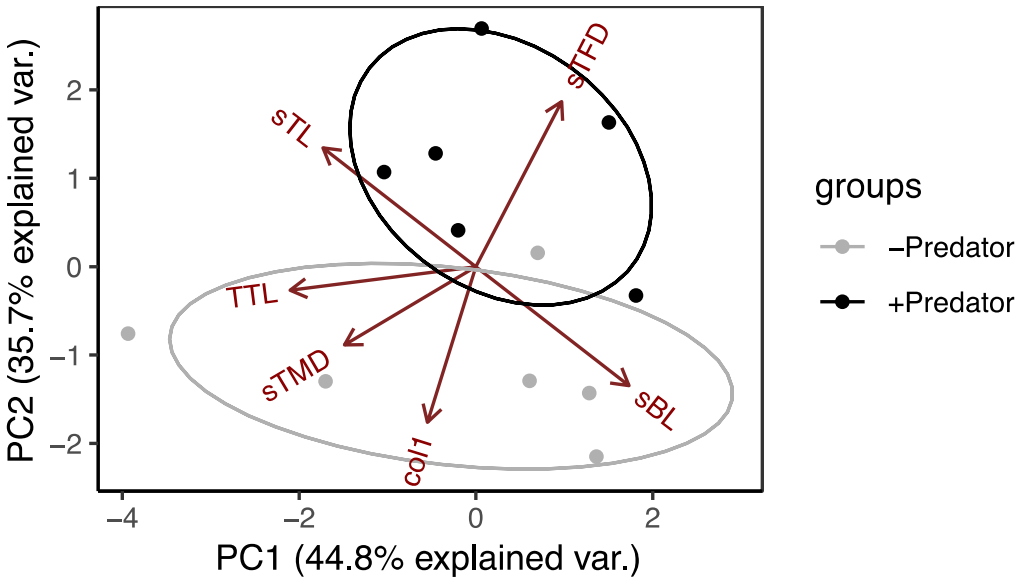

Figure 3.5. Principal components analysis of the tail morphological traits total tadpole length (TTL), tail color (col1), and standardized body length (sBL), standardized tail muscle depth (sTMD), standardized tail length (sTL), and standardized tail fin depth (sTFD) sampled on Day 32.

Supplementary Information Chapter 3

Table S3.1. Mixed effects models results for tadpole tail measurements across time.

Including block as a random effect did not improve model fit, so this term was excluded. Bolded values indicate statistical significance ($p < 0.05$).

	Body length			**Tail length**			**Tail muscle depth**			**Total tadpole length**		
	Df	χ^2	p	Df	χ^2	p	Df	χ^2	p	Df	χ^2	p
Treatment	1	0.60	0.44	1	0.60	0.44	1	0.2	0.65	1	0.48	0.49
Date	2	199	**<0.001**	2	199	**<0.001**	2	17.5	**<0.001**	2	8.9	**0.012**
Treatment x Date	2	2.13	0.35	2	2.13	0.35	2	4.5	0.1	2	7.9	**0.019**

Table S3.2. Principal component analysis of *H. andersonii* metamorphosis variables for tadpoles reared with or without predators.

PC1 and PC2 indicate the first two principal components, and they accounted for 97.6% of the variance between treatments. The values below are the correlation coefficients of each morphological trait with the principal component scores.

Response variable	PC1	PC2
Weight	-0.98	0.11
Length	-0.99	0
Larval period (days)	0.73	0.68
Survival	0.88	-0.43
% of variance explained	81.2%	16.4%