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Evaluation of Skin Health in Hellbenders, Cryptobranchus alleganiensis

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To the Graduate Council:

I am submitting herewith a dissertation written by Rebecca Hardman entitled "Evaluation of Skin Health in Hellbenders, Cryptobranchus alleganiensis." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Debra Miller, Major Professor

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Evaluation of Skin Health in Hellbenders, *Cryptobranchus alleganiensis*

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ABSTRACT

The Hellbender (*Cryptobranchus alleganiensis*), is a large aquatic salamander containing two subspecies, Ozark Hellbender (*C. a. bishopi*), and Eastern Hellbender (*C. a. alleganiensis*), from the Ozark mountains and eastern U.S., respectively. Both subspecies have seen population declines over the past 25 years, especially in *C. a. bishopi* which is federally endangered. Arkansas *C. a. bishopi* populations have been reduced to a single river with minimal observed juvenile recruitment. Furthermore, over the past decade biologist have observed an increase of distal limb lesions with unknown etiology. We performed yearly surveys of *C. a. bishopi* in Arkansas and *C. a. alleganiensis* in Tennessee during summers of 2011-2017, and recorded biometrics, obtained disease samples of a skin swab and tail punch biopsy as well as recorded both qualitative and quantitative details of lesions present. In 2014 we collected an additional dorsal skin swab for skin microbiome analysis. We performed qPCR to test for presence of *Batrachochytrium dendrobatidis* (*Bd*) from skin swabs and Ranavirus from tissue. From lesion data we developed a lesion scoring system from 0-7 and applied a lesion score to each hellbender blinded to any other associated information. We performed linear mixed model regressions followed by AICc model evaluation to evaluate effects of pathogen infection status and individual biometrics on lesion score. From our microbiome swabs we performed 16S amplicon sequencing and calculated both Shannon Weiner Diversity and Bray Curtis Dissimilarity scores. We discovered 93.2% of all hellbenders had distal limb lesions characterized by digit swelling often progressing towards toe-tip ulceration. In severe cases we observed digital necrosis progressing to complete digit loss. Any recaptured individuals had the same or worse lesion score with an average score increase of 1 per year. The top predictive model for lesion severity included individual mass and *Bd* infection status with a significant, albeit weak, positive effect of *Bd* on lesion severity (β=0.87; C.I.: 0.11, 1.63). Microbiome results revealed decreased skin microbial community similarity with increasing lesion score. Results demonstrate lesions are progressive, may be associated with overall skin health, and are likely multifactorial.

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INTRODUCTION

Hellbenders in Decline

We are currently experiencing a global extinction crisis and amphibians are declining at alarming rates (Stuart et al. 2004). We are obligated to understand how our growing human population is affecting the surrounding wildlife and we need to research ways to mitigate our effects. Amphibians should be a priority group; they have semi-permeable skin making them especially vulnerable to environmental change and have been proposed as indicators of ecosystem health (Welsh & Ollivier 1998). There are many benefits to maintaining robust amphibian diversity throughout the globe. Amphibians play important roles in the food chain (Burton & Likens 1975; Beard et al. 2002), help maintain insect populations (Petranka & Doyle 2010; Brodman et al. 2003) and are important models for biomedical research (Burggren & Warburton 2007; Edholm & Robert 2013). Amphibians continue to provide promising innovations for modern medicine. For example, a skin compound discovered from a poison arrow frog (*Epipedobates anthonyi*) has potential to replace addictive opioids in the hospital as a safer analgesic agent (Fitch et al. 2010). Unfortunately, this and many other amphibian species face several threats (Whitfield et al. 2016).

The Hellbender (*Cryptobranchus alleganiensis*) is a large, fully aquatic salamander that inhabits cool, rocky, well-oxygenated rivers and streams in the eastern United States (Nickerson and Mays 1973, Nickerson et al. 2003). The eastern subspecies (*C. a. alleganiensis*) encompasses a majority of the range with Ohio, Tennessee, and Mississippi River drainages while the Ozark Hellbender (*Cryptobranchus a. bishopi*) is a disjunct subspecies restricted to Ozark highland streams of Missouri and Arkansas. Hellbenders represent one of only three species from the Giant Salamander family, Cryptobranchidae. The other two species, the Japanese Giant Salamander (*Andrias japonicus*), and Chinese Giant Salamander (*Andrias davidianus*), are from Japan and China, respectively, making the Hellbender the only Giant Salamander species of North America. Individuals can grow to over two feet long with lifespan of 50+ years in captive individuals. These salamanders are top predators in aquatic ecosystems with a majority of the diet consisting of crayfish and fish as adults (Nickerson and Mays 1973). They are nocturnal and do not frequently leave the cover of their home rock to forage. However, during the breeding season, individuals, especially males, begin frequent moves to congregate near potential nest rocks. During these months, individuals can be spotted walking on the stream bottom uncovered during the day. Large covered space under boulders and within deep crevices become very important as they are required for breeding and nesting (Nickerson and Mays 1973).

Larger males will guard these nest cavities and become aggressive to conspecific males nearby. Nest cavities generally have larger spaces buried deep behind riverbanks or large boulders with only a small entrance hole providing protection from stream flow and predators. After breeding, the female leaves and the male remains to guard the eggs as they develop into larvae (Ettling et al. 2013). Juvenile growth and survival depend

strongly on sediment-free interstitial spaces in gravel (Keitzer 2007; Nickerson et al. 2003; Da Silva Neto et al. 2019).

Therefore, several life stages are vulnerable to habitat degradation, runoff, siltation, and other factors affecting stream flow dynamics and availability of stream bottom interstitial spaces. Hellbenders may also be particularly susceptible to increased sedimentation and other changes in water quality because they are fully aquatic and perform cutaneous respiration almost exclusively (Guimond and Hutchinson 1973). These features, combined with their life history and long lifespan, make Hellbenders an important sentinel species for watershed health.

Populations of both subspecies (Eastern Hellbender, *C. a. alleganiensis*, and Ozark Hellbender, *C. a. bishopi)* have been greatly reduced throughout their range (Wheeler et al. 2003; Graham et al. 2011; Hiler et al. 2013; Freake 2017). In many streams, populations have become extirpated or only large relic individuals remain (Wheeler et al. 2003; Graham et al. 2011; Lipps 2012; Miller 2013). Drastic declines have resulted in federal listing of *C. a. bishopi* as an endangered subspecies (USFWS 2011*a*) and inclusion of both *C. a. alleganiensis* and *C. a. bishopi* in appendix III of the Convention on International Trade in Endangered Species (CITES) of Wild Fauna (USFWS 2011*b*). A recent review by the US Fish and Wildlife Service (USFWS) for population status of hellbenders estimated 39.5% of the range to be extirpated, and 38.4% declining resulting in only 22.1% of the historical hellbender range to still contain healthy reproducing populations (USFWS 2018).

Streamside disturbances that result in erosion of adjacent riparian zones with subsequent sedimentation have been suggested as primary causes of *C. alleganiensis* declines (Humphries and Pauley 2005; Hopkins and DuRant 2011); and in one study in Missouri, decreased population densities of both subspecies were linked to decreased watershed buffers and associated water quality parameters (Bodinoff-Jachowski et al. 2018). In Tennessee, the best surviving populations are all within watersheds protected by national forest in the eastern part of the state (Freake 2017). Populations within areas of heavy agricultural activity in middle Tennessee are either extirpated or declining. The mechanism by which these disturbances affect population growth are not fully understood and factors affecting Hellbender health may also be involved in declines.

Populations of *C. a. bishopi,* which have experienced particularly dramatic declines, are suffering a high prevalence of ulcerative lesions of distal extremities (Hiler et al. 2005; Nickerson et al. 2011). These include feet with swollen, fused, or missing digits, and occasionally, exposed phalanges. A few cases are documented with partial to near complete foot loss. Similar but much less severe lesions have been observed in Eastern Hellbenders in Tennessee but at lower frequency (Miller and Miller 2005). An etiology for this syndrome has not been established, which highlights the need for health monitoring in Hellbenders across their range.

Disease can appear alongside other environmental issues and cause quick extirpations in already declining species such as is feared with the critically endangered dusky gopher frog in the United States (Earl et al. 2016). Furthermore, areas considered pristine and protected from habitat degradation are still vulnerable to effects from anthropogenically introduced pathogens. When investigating a wildlife disease, one must

consider effects of host, agent, and environment on manifestation of the resulting signs and lesions. Studies of stress physiology have shown differences in blood parameters (Hopkins and Durant 2011) of Hellbenders with deformities and suggested that individual immunity, whether primary or secondary to stressors, may contribute to declining hellbender health. Stress responses can be advantageous when following natural stressors by mobilizing nutrients during times of need. However, anthropogenically-induced stress such as increased pathogen burden, altered microclimates, or captive handling can over-stimulate these pathways, leading to immunosuppression and increased susceptibility to disease (see Collins 2010; Rollins-Smith 2017). In captive Eastern Hellbenders, unnatural fluctuations in water temperature has been shown to have direct negative impacts on innate immunity (Terrel et al. 2013). Therefore, habitat alteration should be of great concern in Hellbenders not only because of reduction in optimal habitat, but also because of potential to induce disease outbreaks in tenuously surviving populations. Successful conservation initiatives will require knowledge on multiple factors promoting disease to determine the most effective plan to prevent it. This will include intensive monitoring for potential pathogens, and two important amphibian pathogens that may pose a threat to wild Hellbender populations are chytrid fungi of the genus *Batrachochytrium* and Ranavirus.

Chytrid Fungi

The chytrid fungus (*Batrachochytrium dendrobatidis* or *Bd*) is a globally important pathogen implicated in several amphibian declines over the past few decades (Daszak et al. 2003; Lips et al. 2006). The initial major outbreaks and majority of amphibian extinctions from *Bd* occurred in the cooler mountainous regions of tropical Central and South America and in Australia (Lips et al. 2005). In many anuran species, introduction has resulted in mass mortality and rapid extirpations. Chytridiomycosis was shown to be responsible for 90% of amphibian species declines with up to 95% of species populations extirpated in some regions (Lips et al. 2008; Scheele et al. 2019).

This has given much deserved attention and resources to chytrid research in highly susceptible species, most notably the Panamanian golden frog (*Atelopus zeteki*) (see La Marca et al. 2005; Poole 2008). Unfortunately, how *Bd* plays a role in mortality and declines of salamander populations remains unclear. This may be in part due to the secretive nature of salamanders and therefore less apparent declines. Still, some species have experienced noticeable declines in conjunction with arrival of *Bd* (Rovito et al. 2009; Cheng et al. 2011), and salamanders should not be ignored when considering impacts of chytrid introduction. Other species may not be as highly susceptible with less obvious population effects. Some species appear to survive and maintain subclinical infections in the field with unknown sublethal consequences (Van Rooij et al. 2015; Kiemnec-Tyburczy et al. 2012). Strains in both Ozark and Eastern Hellbender populations appear to be identical to the global panzootic lineage and are, therefore, an introduced novel pathogen (Tominaga et al. 2013), with the earliest known infection from a specimen collected in1969 (Bodinoff et al. 2011).

Minimal clinical disease has been observed with *Bd* infections in wild populations even though prevalence can be as high as 33% (see Briggler et al. 2008; Goyner et al. 2011; Souza et al. 2012; Williams and Groves 2014; Bales et al. 2015). In contrast, *Bd* is considered an important threat to captive Hellbender populations and has caused sporadic mortality (Dusick et al. 2017) as well as mass mortality (Dean et al. 2016) events. Similarly, when another subclinically infected salamander species, *Batrachoceps attenuatus* was challenged in the laboratory, researchers observed morality as high as 100% (Weinstein 2009). These results insinuate that mortality due to infection is situation dependent. Therefore, other factors beyond baseline host susceptibility must play a role in whether or not *Bd* will cause disease in these salamander hosts. Environmental factors that affect host stress levels as well as pathogen variability need to be considered. For instance, seasonal fluctuations in rainfall and temperature are known to affect both chytrid prevalence and intensity within amphibian populations (Kinney et al. 2017; Ruggeri et al. 2018), and water temperatures may be affecting chytrid loads in Hellbenders. Furthermore, Hellbenders may soon rely solely on captive programs for continued survival in the event we cannot restore habitat fast enough, making *Bd*-chytridiomycosis an even more important disease to understand for Hellbender conservation.

Other *Batrachochytrium* species may also threaten Hellbenders. An outbreak of chytridiomycosis, from the emerging *Batrachochytrium salamandrivorans* (*Bsal*), another *Batrachochytrium* introduced from Asia, is affecting some salamander populations in Europe with local extirpations greater than 90% in some areas (Martel et al. 2014). Fortunately, *Bsal* is not yet found in North America, (Bales et al. 2015; Klocke et al. 2017) but poses a risk to entire salamander communities if introduced (Yap et al. 2017). Proactive monitoring and managing for this pathogen will be important to reduce its effects on our native salamanders, including Hellbenders.

Ranavirus

Ranaviruses have been identified as significant contributors to amphibian declines (Gray et al., 2009), but interactions and consequences of these diseases have not been evaluated for many salamander species (Hoverman et al. 2010). Virulence is also highly dependent upon the amphibian host and viral strain (Echaubard et al. 2014). Ranavirus is sporadically detected in many amphibians (Black et al. 2017), and in Hellbenders varies widely in prevalence between years (Souza et al. 2012).

Disease outbreaks for pond-breeding amphibians are usually identified by large numbers of dead or dying amphibians that have congregated at or are/were in the process of metamorphosing from breeding ponds. Conversely, disease-induced mortality of stream-dwelling amphibians is difficult to identify. Ranaviruses have been shown to cause mortality events in the related Chinese Giant Salamanders (*Andrias davidianus*) with a high mortality rate and lesions including skin ulceration along with necrosis of the limbs (Geng et al. 2011; Zhou et al. 2014). In a clinical trial, juvenile Eastern Hellbenders experienced 100% mortality when exposed in the laboratory to a strain of Ranavirus

(Patrick Cusaac, unpublished data). Only one previous study has evaluated Ranavirus prevalence in wild *C. alleganiensis* populations and found only subclinically infected individuals over the course of two sampling seasons (Souza et al. 2012). How Ranavirus contributes to Hellbender mortality and what role individual immune function plays in pathogenicity of this and other pathogenic organisms is still not clear; further pathogen testing alongside health assessments need to be incorporated into long-term population surveys.

Amphibian Skin Peptides

Skin health and changes in secretions have been well documented to explain differences between amphibian species' resistance to pathogens. Antimicrobial peptides, antibodies, and changes in microbial communities are factors in amphibian skin that affect an individual's ability to fight off infection. Additionally, skin health may be a better indicator of disease resistance in amphibians than classical immune parameters tested in mammals. For instance, changes in gene expression in the skin have proven more important than those seen in internal lymphoid organs when predicting chytrid resistance in frog species (Savage and Zamudio 2011). Furthermore, chytrid fungi, which are a major amphibian health threat, are cutaneous pathogens and cause disease through disruption of skin function (Voyles et al. 2009). In order to understand how salamanders succumb to disease such as chytridiomycosis, we need a baseline information of salamander skin immunity, how it is affected by stress, and how we might use that knowledge to conserve at-risk species. This concept holds true for other pathogens such as ranavirus that cause skin pathology. Even if disease is not primarily in the skin, evaluation of altered skin health parameters will still be important when investigating disease in amphibians; they rely on their skin for osmotic regulation (Shoemaker 1977).

Antimicrobial peptides (AMPs) represent a range of peptides produced in animal tissues with varying antimicrobial activity (Brogden et al. 2003). AMPs produced in granular glands of frog skin have demonstrated growth inhibition of *Bd* among other known amphibian pathogens (Woodhams et al. 2006; Rollins-Smith et al. 2006; Rollins-Smith 2009). Differences in AMP secretion can even explain some variation in certain amphibian species' resistance to *Bd* (Woodhams et al. 2006; Rollins-Smith 2009). Wild populations of Mountain Yellow-legged Frogs, *Rana muscosa*, are known to have variable resistance to chytrid fungus associated with differences in anti-microbial compounds produced on the skin (Lam et al. 2010). Salamander AMPs are hypothesized as one factor responsible for increased resistance of salamanders to *Bd* in comparison to many anuran species (Pasmans et al. 2013; Van Rooij et al. 2011). However, despite the impressive amount of information available on AMPs of anurans, there are strikingly few studies on those of salamanders. AMPs have been harvested from only a few salamander species showing varied inhibition of several pathogens such as *Bd*, *Escherichia coli*, and Ranavirus (Fredericks and Dankert 2000; Sheafor et al. 2008; Meng et al. 2013; Pereira et al. 2018) including AMPs identified in the related *A. davidianus* (Pei et al. 2017). No

published study has evaluated anti-chytrid activity from skin secretions collected from Hellbenders.

Amphibian Skin Microbial Communities

Alongside host defensive secretions, surface microbial community interactions and their contribution to host immune health is an emerging field of research proving to be very important in many complex diseases of humans and animals (Ilseung & Blaser 2012; Rodriguez Hoffman et al. 2015). The field of microbiome research is expanding rapidly as new information points to the critical importance of these communities in maintenance of host surfaces such as skin (Rodriguez Hoffman 2014) and intestine (de Vos and de Vos 2012). Associations between environmental degradation and altered microbial community are revealed in wildlife species such as black howler monkeys (Amato et al. 2013), and even invertebrate species such as coral (Krediet et al. 2013). These microbiome shifts can further change host immunity and its ability to fight infection (Glasl et al. 2016). Microbiomes, therefore, can undergo modification within one host lifetime in response to environmental triggers.

Evaluation of skin communities and investigation for causes in their alterations is important when presented with an emerging disease in any wildlife population. Amphibian microbial skin communities are known to be important in defense against pathogens including *Bd*. Both field and laboratory analyses have shown some salamander and frog species with skin communities resistant to *Bd* growth and new work suggests microbiomes are driving factors in determining amphibian host susceptibility to *Bd* (Woodhams et al. 2014; Walke & Belden 2016). *Plethodon cinereus*, a terrestrial salamander known to be resistant *Bd*, also harbors skin bacteria known to have potent anti-*Bd* activity (Brucker et al. 2008; Harris et al. 2009; Becker & Harris 2010). Skin bacteria isolated in other amphibian species have also inhibited *Bd* in vitro (Briggs et al. 2007), as well as prevented chytridiomycosis in experimental challenges (Harris et al. 2009). This can be accomplished through several mechanism such as competitive exclusion, or production of their own antimicrobial compounds. One species, *Janthinobacterium lividum* is a vibrantly purple colored bacteria due production of violacein (Pantanella et al. 2007). Violacein with other *J. lividum* products provides anti-fungal activity and is effective in preventing *Bd* growth on amphibian skin (Brucker et al. 2008, Becker et al. 2009). In Panamanian golden frogs, a species highly sensitive to *Bd*, one application of *J. lividum* on the skin prevented chytridiomycosis for approximately 6 weeks after *Bd* inoculation (Becker et al. 2011). While the bacteria did not establish permanent residence or provide permanent resistance to chytrid, it did provide resistance while present. Furthermore, another study looking at skin microbiota of the Fire Salamander, *Salamandra Salamandra*, a species very susceptible to the salamander chytrid, *Bsal*, showed that although anti-*Bsal* microbial communities existed on the skin, they were not robust enough to prevent *Bsal* colonization, subsequent community disruption, and disease (Bletz et al. 2018). This highlights the complexity of the role of the microbiome in disease resistance and justifies continued research on the diversity of

microbial organisms in amphibian skin. These skin communities can be extremely dynamic and depend on the abiotic environment provided by both the host and external factors. AMPs produced by amphibian hosts may not only serve to prevent colonization of unwanted microbial species but may also provide the right environment for growth of preferred bacterial species that then further help protect against invading pathogens. Therefore, stressors that change the physiology of the host and ultimately ecosystem of the skin surface may alter microbial community dynamics, with downstream consequences of lowered host immune response, and increased susceptibility to disease. This phenomenon has been observed in coral (Krediet et al. 2013). It will be important to identify bacterial communities on amphibian skin not only for their therapeutic properties but also as a proxy for individual health status. There are only two previously published papers describing the microbial community of Hellbenders with inconclusive results on their role in distal limb lesions (Hernandez-Gomez et al. 2017a; 2017b). However, these studies provide a necessary first step of baseline information to further investigate community shifts within populations alongside host health parameters. Increasing sampling within populations to understand the variation in microbial communities and how they change between subspecies, rivers, and other host health parameters will be needed to understand the complex and dynamic nature of the microbiome and its role in disease of wild Hellbenders.

Research Objectives

We have implemented a large-scale, coordinated pathogen surveillance program which gives us a unique opportunity to add new relevant health evaluation protocols and have them quickly adapted across a large area. This study provides essential disease information that will be used towards the long-term conservation of *C. alleganiensis*. Specifically, we applied modern molecular and protein analyses to our targeted pathogen protocols to address the issue of unknown etiology of disease in Ozark Hellbenders and provide baseline skin health information in Eastern Hellbenders. We also targeted diagnostic for the new *Bsal* chytrid fungus. These new techniques have allowed us to better understand the dynamic environment of Hellbender skin.

Results of this study will be used in conservation initiatives for hellbenders and be used to drive experimental challenges. Hellbenders are a charismatic species that once thrived in streams throughout the eastern United States and represent a unique part of our mountain ecosystems. Identifying factors in their decline is key to developing strategies to manage populations. This study is an essential step in discovering etiologic agents of disease in a species of concern in the state of Tennessee and in a federally endangered subspecies in Arkansas.

Hypotheses

1. We hypothesize that disease is more prevalent than previously recorded in Ozark Hellbenders (*Cryptobranchus alleganiensis bishopi*).

2. We hypothesize that unidentified pathogens and changes in skin microbial communities play a role in morbidity and mortality of hellbenders, particularly in ulcerative lesions of *C. a. bishopi*

Objectives

1. Sample healthy and sick hellbenders in both Arkansas and Tennessee and describe and quantify all lesions.
2. Continue ongoing targeted sampling for known amphibian pathogens, Ranavirus and chytrid fungus with the addition of the emerging *Bsal* chytrid using empirically tested sensitive qPCR protocols.
3. Collect and evaluate skin secretions and challenge against growth of amphibian fungal pathogens *Bd* and *Bsal*.
4. Apply next generation sequencing to evaluate inter- and intra-population differences in microbial skin communities based on region and lesion severity.

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CHAPTER I
GEOGRAPHIC AND INDIVIDUAL DETERMINANTS OF IMPORTANT AMPHIBIAN PATHOGENS IN OZARK AND EASTERN HELLBENDERS

Abstract

Wildlife diseases are a major threat for species conservation and there is a growing need to implement disease surveillance programs to protect species of concern. Globally, amphibian populations have suffered considerable losses due to disease, particularly from the two chytrid fungi *Batrachochytrium dendrobatids* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*), and Ranavirus. Hellbenders (*Cryptobranchus alleganiensis*) are large aquatic salamanders that have experienced precipitous declines over at least the past five decades, and emerging pathogens are hypothesized to play a role. Therefore, we surveyed Hellbender populations in Arkansas (AR), Middle Tennessee (MTN), and East Tennessee (ETN) for chytrid fungi (*Bd* and *Bsal*) and Ranavirus from swabs and tail tissue, respectively, from 2011 to 2017. Overall, we detected *Bd* on Hellbenders from 9 out of 15 rivers sampled with total prevalence of 23.7% varying regionally (AR: 32.6%, MTN: 11.1%, and ETN: 27.5%). Ranavirus prevalence was comparatively lower than *Bd* prevalence, with a 9.0% prevalence. As with *Bd*, we detected regional variation in prevalence (AR: 5.9%, MTN: 11.1%, ETN:10.1%). We did not detect *Bsal* in any Hellbender populations. Mixed model analysis of both environmental and individual factors showed a significant negative correlation between body condition score (BCS) and probability of Ranavirus infection. Evaluation of infection load of positive individuals revealed different trends than prevalence alone for both Ranavirus and *Bd*, with MTN having a significantly lower average ranaviral load than both other regions. We also documented a variety of lesions on Hellbenders located within all geographic regions that likely have multiple etiologies and evaluated *Bd* infection dynamics in recaptured individuals from AR. Our results reveal potential sublethal effects of important amphibian pathogens in Hellbenders and emphasize the need for consistent long term datasets to properly assess how disease may be affecting wild amphibian populations

Introduction

Infectious diseases are major threats to wildlife conservation (Cunningham et al. 2017). Precipitous population declines have occurred following outbreaks of emerging pathogens, including white nose syndrome in North American bats (Frick et al. 2010), Nidovirus infection in Bellinger River Turtles (Zhang et al. 2018), and bacterial septicemia in Saiga Antelope (Kock et al. 2018).

Amphibians have experienced particularly high mortality rates due to disease. Worldwide, chytridiomycosis, caused by chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), is implicated in an estimated 90 amphibian species extinctions (Scheele et al. 2019). Ranavirus represents another amphibian pathogen of concern with potential to cause extinctions in both North America (Green at al. 2002; Earl et al. 2016) and Europe (Duffus et al. 2010). Furthermore, a novel chytrid fungus, *Batrachochytrium salamandrivornas* (*Bsal*), has been discovered in Europe, and in contrast to *Bd*, is highly lethal to species of salamanders from several families (Martel et al. 2014). Wildlife

diseases can be difficult to control, and little epidemiological data exist for a majority of cases (Ryser-Degiorgis 2013). Disease monitoring programs have historically been minimal, and mortality events have been hard to predict, which makes it difficult for conservation workers to implement preventative strategies (MacPhee and Greenwood 2013). Successful conservation management plans for at-risk amphibian species need to include disease mitigation, surveillance programs, and strategies to prevent emerging pathogens from affecting population health (Kuiken et al. 2005).

The Hellbender (*Cryptobranchus alleganiensis*) is a large, fully aquatic salamander that inhabits cool, rocky, well-oxygenated rivers and streams in the eastern United States (Nickerson and Mays 1973, Nickerson et al. 2003). Populations of both subspecies (Eastern Hellbender, *C. a. alleganiensis*, and Ozark Hellbender, *C. a. bishopi)* have been greatly reduced throughout their range (Wheeler et al. 2003; Graham et al. 2011; Applegate et al. 2018).

Streamside disturbances that result in erosion of adjacent riparian zones with subsequent sedimentation have been suggested as primary causes of *C. alleganiensis* declines (Humphries and Pauley 2005; Hopkins and DuRant 2011), but the mechanism by which these disturbances affect population growth are not fully understood. For example, Jachowski et al. (2016) found lower correlation between land use and historic disturbance with *C. alleganiensis* persistence than previously hypothesized. Other factors affecting Hellbender health and survival are, therefore, likely to be involved in declines. Additionally, populations of *C. a. bishopi*, which have experienced particularly dramatic declines, are suffering a high prevalence of ulcerative lesions of distal extremities (Hiler et al. 2005; Nickerson et al. 2011). An etiology for this syndrome has not been established, which highlights the need for disease monitoring in Hellbenders across their range.

Bd is thought to have been introduced into Hellbender populations in 1969 (Bodinof et al. 2011), and infection is reported in several *C. alleganiensis* populations with no reports of chytridiomycosis (Briggler et al. 2008; Goyner et al. 2011; Souza et al. 2012; Bales et al. 2015; Seeley et al. 2016). However, *Bd* has been reported to cause mortality in captive adults (Bruckman et al. 2014; Dean et al. 2016) and cause greater than 50% mortality in captive propagation (head-starting) programs during juvenile releases (Utrup and Mitchell 2008; Dean et al. 2016). Ranaviruses have caused catastrophic mortality events in farms of the related Chinese Giant Salamander (*Andrias davidianus*; Geng et al. 2011), presenting lesions similar to those seen in wild *C. a. bishopi.* Despite these reports, data on Ranavirus epidemiology for wild Hellbender populations are limited to one survey that reported a few infected individuals with no clinical signs of disease (Souza et al. 2012). *Bsal* has not been reported in the United States and all Hellbenders tested have been negative for this pathogen (Bales et al. 2015). Although the effect of *Bsal* on Hellbenders is not known, their susceptibility to this fungus presumably would be similar or worse than *Bd*.

Previous studies on pathogen surveillance of Hellbenders provide a necessary first step, but most are restricted by small temporospatial scales and focus primarily on *Bd* prevalence. No long-term datasets occur for all three globally important amphibian pathogens in Hellbenders and no studies have previously evaluated populations in middle

Tennessee and Arkansas. Furthermore, there are little data of disease or other health parameters associated with pathogen presence to further understand how these pathogens contribute to Hellbender health. To this end, we evaluated long-term trends in amphibian pathogen prevalence in wild Hellbender populations and provide pathogen data in previously un-surveyed portions of the range of this species. We acquired a large sampling dataset by adding pathogen and disease sampling to ongoing demographic surveys in Tennessee and Arkansas over a seven-year period (2011-2017).

Methods

Stream Collection

We sampled Hellbenders from 2011-2017 in 15 streams and rivers from the Blue Ridge ecoregion of East Tennessee (ETN), Interior Plateau of Middle Tennessee (MTN), and Ozark Highlands of Arkansas (AR). We sampled in both Tennessee ecoregions for the Eastern Hellbender (*C. a. alleganiensis*) and in Arkansas for the Ozark Hellbender (*C. a. bishopi*). We implemented disease sampling protocols in conjunction with ongoing population monitoring surveys. All sampling in Arkansas was conducted under observation of the USFWS permit TE66039A-0 issued to KJI and Arkansas Game and Fish Commission (Permits #Kelly) and with approval from Tennessee Wildlife Resources Agency (TWRA) (Permit #'s 1525,1529 (DM), 1505(MF), 1450 (BTM), and 1877 (RHH)). Most of our sampling efforts were opportunistic, based on areas of population survey history, team availability, and stream conditions from late May to early August. We performed limited sampling between late August and mid-October to prevent disturbance of breeding and nesting. In TN, and shallower water in AR, we used standard snorkeling techniques to locate individuals. In AR, we also sampled Hellbenders via artificial nest boxes (Briggler and Ackerson 2012) and sampled deep water habitats (up to 4 meters) using a hookah dive system (i.e. gasoline powered air compressor with tethered air supply lines to dive regulators). We captured any animals encountered under cover objects and nest boxes and placed them in a clean soft cotton or mesh bag. We kept bags submerged in the river before and after animals were processed. (University of Tennessee IACUC protocol # 2481-0916). We changed dive gloves between animal captures to reduce pathogen transfer and contamination of pathogen samples.

Biometrics and Pathogen Sampling

We recorded presence of lesions and external abnormalities, and observed for abnormal behaviors, such as increased lethargy or slow righting response. To sample for chytrids (*Bd* and *Bsal*), we ran a sterile cotton swab (Fisherbrand product# 23400111) five times over each body region in the following order: dorsum, ventrum, inguinal areas, and all portions of the feet including toe-tips as described by Brem et al. (2007). Although standard *Bd* swabbing protocol in frogs generally does not include the dorsum (see Brem et al. 2007), we included it because of unpublished reports of suspicious dorsal skin lesions associated with *Bd* infection in Hellbenders. We collected tissue samples for Ranavirus via a sterile punch biopsy (6mm x 3mm) or forceps and blade to remove a

small tissue section of the dorsal tailfin near the tail-tip. We immediately placed swabs and tissue in sterile 1.5 mL microcentrifuge tubes with 70% ethanol for field storage and transport.

After pathogen sampling, we recorded biometrics of total length (TL), snout-vent length (SVL), and mass. For *C. a. bishopi* in Arkansas, we marked every new capture using Passive Integrated Transponder (PIT) tags and recorded tag numbers of previously captured animals. PIT tagging was not part of all sampling in Tennessee; however, all available PIT tag data were recorded when administered. After collection of disease and biometric data, we released Hellbenders at their original point of capture. We used all new containers, bags, and gloves between individuals and cleaned all equipment (including diving gloves) with soap and soaked all equipment in 5% solution of either bleach or chlorhexidine for at least ten minutes and rinsed thoroughly before use at another site.

DNA Extraction and Quantitative PCR (qPCR)

We removed ethanol via centrifugation and dried samples under a fume hood prior to extraction. We extracted DNA from swabs and tail tissue using a DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA) and stored at -80°C. We performed separate qPCR singleplex assays for each target pathogen (Ranavirus, *Bd*, *Bsal*) with the following 15 µL protocol: 7.5 µL 2X TaqMan™ Universal PCR Master Mix (Applied Biosystems™), 1 µL Forward Primer at 10µM, 1 µL Reverse Primer at 10 µM, 0.5 µL Probe at 10 µM, 1 µL molecular grade water, and 4 µL sample DNA. Reaction temperatures were as follows for Ranavirus and *Bd*: 50°C for 2 min, 95°C for 10 min, and 45 cycles of the following: 95°C for 15s and 60°C for 60s. *Bsal* reaction temperatures were identical except with increased annealing temperatures of 62°C. We used primer and probe combinations published from Picco et al. (2007) for Ranavirus qPCR reactions and Blooi et al. (2013) for *Bd* and *Bsal* reactions. Each 15 µL reaction was performed in a 96-well plate via the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems). We performed separate singleplex assays for *Bd* and *Bsal* as opposed to the published duplex (Blooi et al. 2013) assay to avoid potential inter-assay competition and subsequent false positives (Thomas et al. 2018). We ran each sample in duplicate and reran any samples that did not have duplicates that matched within two cycle threshold (Ct) values. If a rerun continued to have a discrepancy, we removed the sample from our analysis. We determined animals to be positive for a pathogen with an average Ct value < 45.

We performed standard curves from pure cultures for all three pathogens. For chytrid fungi (*Bd* and *Bsal*), we determined zoospore genomic equivalents (GE) from DNA extracted from JEL 197 cultures of known zoospore concentrations, which were provided by the Rollins-Smith laboratory at Vanderbilt University. This gave us a Zoospore GE range of 0.5 (Ct 45.00) to 1,000,000 (Ct 16.99). For Ranavirus, recently titrated culture was not available; thus, we could not quantify plaque forming units. Instead, we created relative load scores, which represented average Ct values, where a Ct of 45 received a score of 1 with score increases of 1 for every single cycle decrease. This

resulted in a relativized load score range of 1-21, which represented Ct values from 45-25.

Infection Status Models

We performed generalized linear mixed models (binomial distribution) in RStudio (R Core Team 2013) via the lme4 package (Bates et al. 2015) to test for effects of measured parameters on animals being positive/negative for either *Bd* or Ranavirus using the fixed effects of Region (ETN, MTN, AR), Julian Day, Mass, and Body Condition Score (BCS) and included year as a random effect. We did not include TL or SVL in our models because they were highly correlated with Mass. We used Mass as our representative index of size because it is was a more reliable field measurement. We calculated BCS from residuals of the fitted polynomial plot of TL and Mass in Microsoft Excel (Microsoft Office 2016). We did not include sex as a predictor variable in GLMMs, as sexing Hellbenders is unreliable either in immature individuals or in mature individuals outside of the breeding season. We calculated z-scores for all numeric predictor variables prior to analysis with the exception of BCS.

We evaluated relative model fit from AIC_c for all possible combinations of fixed effects to determine the top performing model via the AICcmodavg package (Mazerolle 2019) and considered models competitive when ΔAIC_c was < 2.0. We model-averaged variables that appeared in more than one of the top models as recommended in Burnham and Anderson (2002) to determine relative variable contribution in those models.

Infection Intensity Models

We calculated infection intensity of pathogen- positive animals for each region across all years based on zoospore GE and load score for *Bd* and Ranavirus, respectively. We performed linear mixed model (LMM) regressions to test for effects of the same predictor variables listed for above infection status models on infection intensity of both *Bd* and Ranavirus-positive individuals. We log and square-root transformed zoospore GE and Ranavirus load scores, respectively, to obtain normal distributions prior to analysis.

Results

We surveyed 15 rivers (1 from AR, 7 from MTN, and 7 from ETN) over 7 years, with 10 of those rivers surveyed at least 3 times. We sampled 202 individual *C. alleganiensis* (86 from AR [*C. a. bishop*], 36 from MTN [*C. a. alleganiensis*] and 80 from ETN [*C. a. alleganiensis*]). We obtained a *Bd* swab from all animals and tail tissue from 200 animals (85 from AR, 36 from MTN, and 79 from ETN).

Prevalence and Temporospatial Distribution of Bd

Overall, prevalence of *Bd* across all sampled populations was 26.7% (C.I.: 20.6, 32.8; 54/202). By region, we found that AR had greatest prevalence (32.6%; C.I.: 22.7, 42.5; 28/86) followed by ETN (27.5%; C.I.: 17.7, 37.3; 22/80), and MTN (11.1%; C.I.: 0.8, 21.4; 4/36) (Fig 1). We detected *Bd* in 90% of the rivers in which we conducted

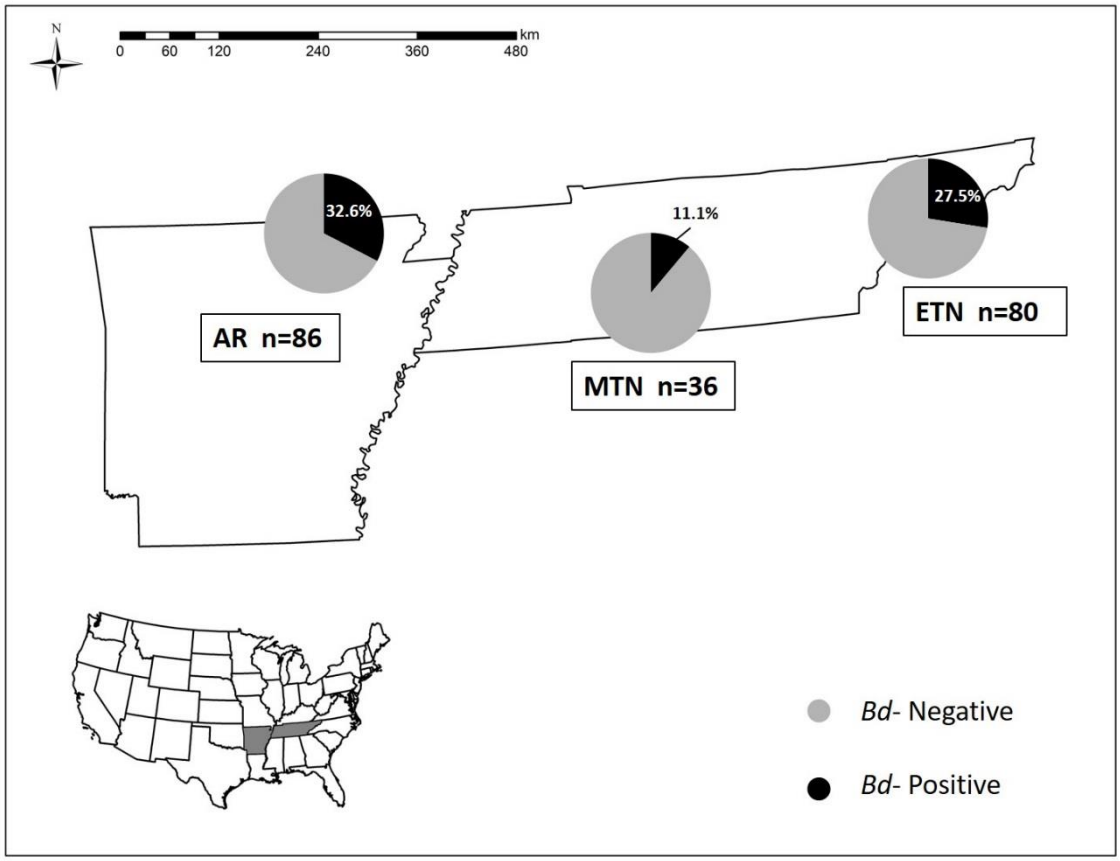

Figure 1. Prevalence of *Bd* by Region.

Map of Tennessee and Arkansas with pie charts representing overall prevalence of *Bd* from Hellbenders sampled in three ecoregions of Ozark Highlands in Arkansas (AR), Interior Plateau of middle Tennessee (MTN), and Blue Ridge ecoregion of eastern Tennessee (ETN). Black shaded portion represents percent of total animals found to be *Bd* positive from skin swabs.

repeated sampling (n=10). We did not detect *Bd* in single-visit river sites (n=5). We observed yearly fluctuations in *Bd* prevalence across all regions with greatest prevalence in 2011 when 18 of 47 (38.3%) individuals were positive for *Bd*. Other years with sampling in all three regions (2012, 2014, 2016, and 2017) had prevalence rates of 15.3%, 6.5%, 30.0%, and 32.3%, respectively (Table 1).

Prevalence and Temporospatial Distribution of Ranavirus

We detected Ranavirus sporadically throughout all three regions, with total prevalence of 9.0%. MTN and ETN had the greatest prevalence (11.1%; C.I.: 0.8, 21.4; 4/36 and 10.1% C.I.: 3.4, 16.8; 8/80, respectively), followed by 5.9% prevalence for AR (C.I.: 0.9, 10.9; 5/85) (Fig 2). We found Ranavirus-positive animals to be spatiotemporally clustered (Table 2). The greatest cluster of positive animals from a single stream (5/7; 71.4%) occurred in ETN during early July 2016. This one sampling event accounted for half of all positive individuals found in ETN across all years. Similarly, all four Ranavirus-positive animals from MTN in 2012 were from a sampling effort of 14 animals (28.6 %) in one river. Year 2012 represented the only year in which Ranavirus-positive animals were detected from all three regions (9/58 individuals; 15.5%). Six individuals (3.0 % total, 33.3% of Ranavirus-positive) were coinfected with *Bd* and Ranavirus.

Infection Status Models: Bd and Ranavirus

Of the models evaluated to explain *Bd* presence, we found greatest support for the following (Δ $AIC_c < 2$) models: 1) Region + BCS, 2) BCS Only, and 3) BCS + Region + Julian (Table 3). We did not find clear fix ed effects explaining *Bd* infection status.

Ranavirus Model evaluation for Ranavirus presence showed two top models (Δ $AIC_c < 2$), which included 1) BCS + Mass and 2) BCS + Julian (see Table 3). BCS was in both top models and had a significant negative, albeit weak, effect ($\beta = -0.13 \pm 0.06$ S.E., C.I.: -0.24, -0.02); while Mass, in the top model only, had a weak positive effect ($\beta = 0.92 \pm 0.42$ S.E., C.I.: 0.1, 1.73). Furthermore, our random effect, year, explained considerable variance, which likely reflects the site-year-specific spikes in prevalence (Var: 6.29 ± 2.51 S.D.).

Infection Intensity: Bd

Overall, we found that, of 54 individuals that tested positive for *Bd*, mean zoospore GE varied regionally (AR: 116.4 ± 34.9 S.E.; MTN: 114.1 ± 45.7 S.E.; ETN: 59.5 ± 16.3 S.E.; Fig. 3a). Five individuals had zoospore GEs at or above 1000. Of these 5 individuals, two were captured in AR and both individuals had moderately severe lesions in comparison to others seen in that river (see Ch. 2 for lesion scoring). The other three individuals were from ETN, one of which had active ulceration on the toes. Linear mixed model evaluation for *Bd* Zoospore GE of positive individuals revealed four top models 1) BCS-Only, 2) Mass-Only, and 3) Mass + Region (see Table 1). As was the case with *Bd* infection status models, we did not find clear effects explaining zoospore load.

Table 1: *Bd* Sampling by Year

List of number of Hellbender sampled by region and year and number found to be positive for *Bd* via qPCR from skin swabs

Year	AR		MTN		ETN		Total	
	sampled	*Bd*+	sampled	*Bd*+	sampled	*Bd*+	sampled	*Bd*+
2011	28	11	3	0	8	4	39	15
2012	8	1	14	1	37	7	59	9
2013	3	0	3	1	0	0	6	1
2014	33	10	8	1	5	1	46	12
2015	9	0	2	1	0	0	11	1
2016	3	1	0	0	7	2	10	3
2017	2	2	6	0	23	8	31	10
Total	86	25	36	4	80	22	202	51

Table 2: Ranavirus Sampling by Year

List of number of Hellbender sampled by region and year and number found to be positive for Ranavirus (RV) via qPCR from tissue biopsies o the dorsal tail fin.

Year	AR		MTN		ETN		Total	
	sampled	RV+	sampled	RV+	sampled	RV+	sampled	RV+
2011	28	2	3	0	8	0	39	0
2012	8	2	14	4	36	3	58	9
2013	3	0	3	0	0	0	6	0
2014	32	3	8	0	5	0	45	3
2015	9	0	2	1	0	0	11	1
2016	3	0	0	0	7	5	10	5
2017	2	0	6	0	23	0	31	0
Total	85	7	36	5	79	8	200	18

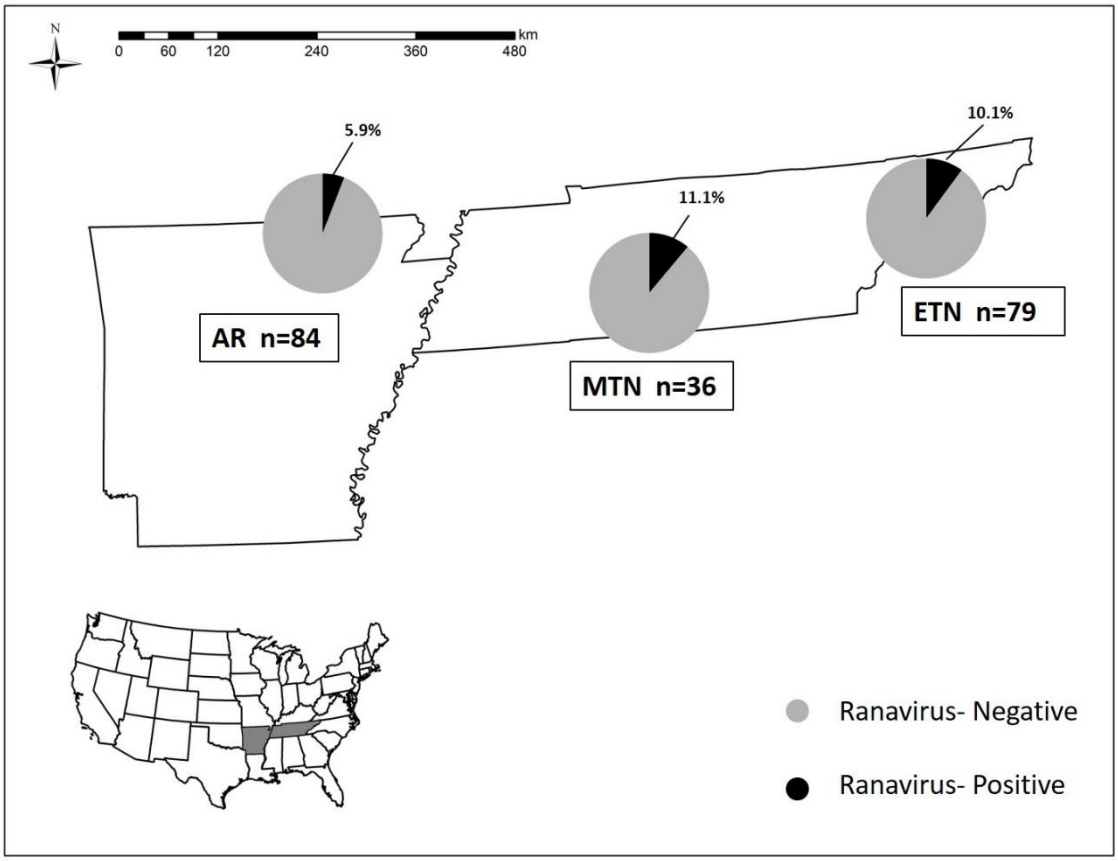

Figure 2. Prevalence of Ranavirus by Region.

Map of Tennessee and Arkansas with pie charts representing overall prevalence of Ranavirus from Hellbenders sampled in three ecoregions of Ozark Highlands in Arkansas (AR), Interior Plateau of middle Tennessee (MTN), and Blue Ridge ecoregion of eastern Tennessee (ETN). Black shaded portion represents percent of total animals found to be Ranavirus from tissue biopsies of the caudodorsal tail.

Table 3. AICc Table for *Bd* and Ranavirus Infections

Highest supported (ΔAICc < 2.0) predictive models describing infection status for *Batrachochytrium dendrobatidis* (*Bd*) and Ranavirus (0/1) and infection load for *Bd* positive individuals (zoospore GE). We did not perform AICc model evaluation for Ranavirus load scores because of small samples size of Ranavirus-positive individuals (n=18). *Bd* infection status and zoospore GE were calculated from qPCR from skin swabs and Ranavirus from qPCR of tissue biopsies.

Pathogen Infection (Status or Load)	Model	K	AICc	ΔAICc	AICcWt	Cum.Wt	LL
Bd Infection Status (GLMM)	Region + BCS + Year	5	207.4	0.00	0.33	0.33	-98.51
	BCS + Year	3	208.7	1.28	0.18	0.51	-101.3
	Region + BCS + Julian + Year	6	209.1	1.72	0.14	0.65	-98.29
Ranavirus Infection Status (GLMM)	BCS + Mass + Year	4	96.87	0.00	0.41	0.41	-44.32
	BCS + Julian + Year	4	98.75	1.88	0.16	0.57	-45.26
Bd Zoospore GE of Positive Individuals (LMM)	BCS + Year	4	99.41	0.00	0.27	0.27	-45.23
	Mass + Year	4	99.51	0.10	0.26	0.53	-45.28
	Region + Mass + Year	6	101.4	2.00	0.10	0.62	-43.66

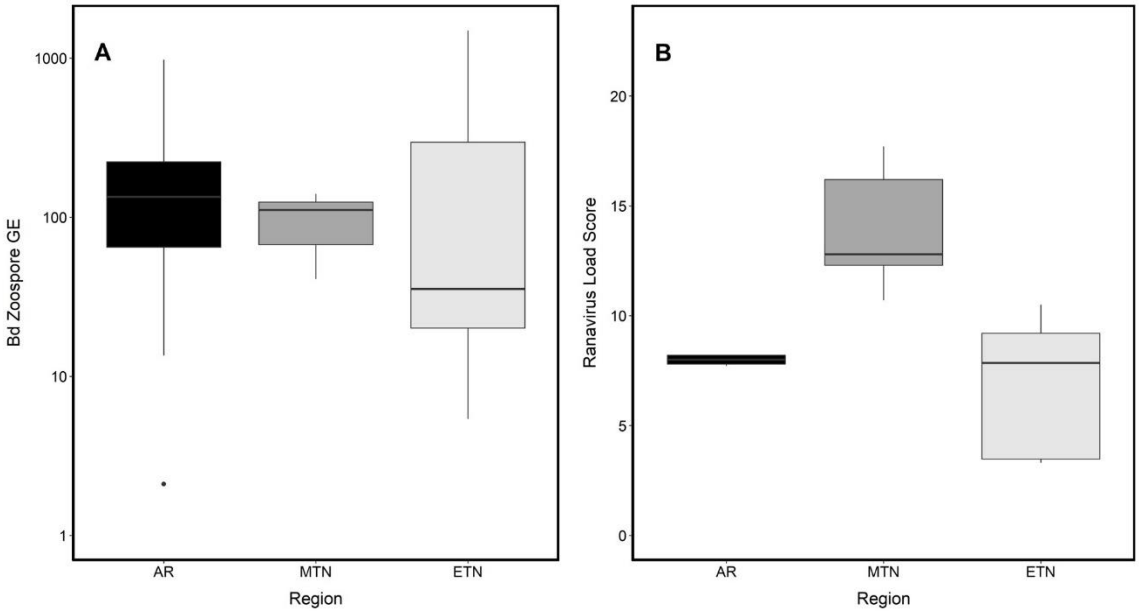

Figure 3. Infection Intensity by Region.

Boxplots showing *Bd* zoospore Genomic Equivalents (GE) for *Bd*-positive individuals (A) and Load Scores for Ranavirus-positive individuals (B), separated by region of Arkansas (AR), Middle Tennessee (MTN), and East Tennessee (ETN).

Infection Intensity: Ranavirus

Mean load score for Ranavirus-positive individuals was greatest in MTN (Score = 14.0 ± 1.4 S.E.; Ct = 32.0), followed by AR (Score = 8.0 ± 0.1 S.E.; Ct = 38.0) and ETN (Score = 6.9 ± 1.3 S.E.; Ct = 39.1) (Fig 3b). We did not perform all possible models or AICc model evaluation for RV intensity because of small sample size (n=18). Instead, we performed a linear mixed model of the global model and found MTN to have a significant positive effect on load score where load scores for MTN > ETN (β=7.96 ± 2.17 S.E., C.I.: 3.70, 12.2) and MTN > AR (β=5.87 ± 1.59 S.E., C.I.:2.75, 8.99).

Lesion Observations

In AR, we found 80 animals (93.2%) with distal limb lesions characterized by swollen, ulcerative toes, and missing toes (Fig. 4 A) (see Ch. 2). In Tennessee, 12 animals (10.3%) had abnormal toes or missing toes, but only two had toe lesions similar to ones observed in AR, one in MTN and one in ETN (Fig. 4 B, C). The remaining missing toes observed in Tennessee appeared healed with thickened, slightly fibrotic, overlying skin, one of which from MTN had two missing feet and was positive for Ranavirus (Fig. 4 D). Four of the ETN animals with toe lesions came from 2017 at the same site with greatest site prevalence of Ranavirus the year before (2016). We also observed skin lesions on the body on 14 individuals in Tennessee, 7 of which appeared to be scarring from previous trauma, 4 with open wounds or ulcerations, and 3 with extensive areas greying in the dorsal skin (Fig. 4 E).

Discussion

We found *Bd* present in all regions sampled. Our data indicate a *Bd* prevalence of 27.5% for Hellbenders inhabiting streams of ETN, which is consistent with a prevalence of 26.0% reported previously for this species in this region (Souza et al. 2012). Furthermore, these values are similar to reports of 27.9% prevalence for Hellbenders in streams in adjacent North Carolina (Williams and Groves 2014), and to that of 24.0% prevalence from a larger scale study encompassing the eastern United States (Bales et al. 2015). A slightly greater prevalence of 33% occurred in northern Georgia (Goyner et al. 2011). We also report the first *Bd* prevalence data for *C. a. bishopi* with only previous reports of positive animals (Briggler et al. 2007; 2008). Our 11.1% prevalence for Hellbenders inhabiting streams of MTN is the first report of *Bd* occurring in *C. a. alleganiensis* from that region. Both MTN and AR represent rapidly declining populations (Miller 2013; Wheeler et al. 2003) and do not have significantly different *Bd* prevalence from ETN, where populations are still considered relatively stable. However, although not significant, *Bd* zoospore loads were greater in both MTN and AR, and on average double that of ETN. Furthermore, AR populations, which harbored a high prevalence of skin lesions (93.2%), had the greatest overall *Bd* prevalence and zoospore GE of positive animals. Zoospore GEs from *C. a. alleganiensis* in MTN and ETN were much greater than loads reported previously for *C. a alleganiensis*; 12.7 in Bales et al. (2015), and 12 in Williams and Groves (2014). However, we included the dorsum in our

Figure 4. Lesions.

Distal limb lesions observed during disease surveys of Ozark (*Cryptobranchus alleganiensis* bishopi) and Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*). A majority of *C. a. bishopi* from AR had distal limb lesions that were characterized by several swollen and ulcerated toes (A). *C. a. alleganiensis* in MTN with missing toes, some swollen, and greying of skin of front left foot (B). *C. a. alleganiensis* in ETN with all toes shortened and active hemorrhage in front right foot (C). *C. a. alleganiensis* from MTN with missing front and rear left feet and positive for Ranavirus via qPCR (D). *C. a. alleganiensis* from ETN with extensive greying of dorsal skin and positive for *Bd* via qPCR (E). *C. a. alleganiensis* with intact toes but greying of palmar surface of both rear feet (F). Pictures B, E courtesy of Sherri Reinsch, and picture D courtesy of Dale McGinnity.

swabbing protocol, which makes direct comparisons with these studies difficult to interpret. Still, our zoospore GEs were generally low across all populations with more than half of all positive individuals having less than 100 zoospore GEs, and we did not see any correlation with lesions and relative *Bd* load.

Our recapture data indicates that individual Hellbenders can clear *Bd* infection at least to below detectable levels and become re-infected, which demonstrates a dynamic nature of *Bd* in AR populations. We sampled AR populations only in late summer, which represents a time of low *Bd* infection prevalence in other species of amphibians in North America, such as Crawfish Frogs (*Lithobates areolatus*, Kinney et al. 2011) and Northern Leopard Frogs (*Lithobates pipiens*, Voordouw et al. 2010). Therefore, *Bd* may be affecting a greater proportion of *C. a. bishopi* in Arkansas than our prevalence data suggest. Although we do not hypothesize *Bd* as a primary pathogen for lesions observed in Arkansas, we caution that *Bd* may play a role in lesion pathophysiology, especially in areas with high prevalence.

We also report the first record of Ranavirus in *C.a. bishopi* and in MTN for *C. a. alleganiensis*, although the relatively low occurrence (only 18 individuals tested positive) confounds attempts to interpret the effect of this pathogen on Hellbender health. Overall, we noticed sporadic temporospatial clusters of Ranavirus infection, which corroborates with Souza et al. (2012) and other studies of Ranavirus epidemiology in amphibians (Sutton et al. 2015; Brunner et al.2015). We did not notice signs of ranavirosis (disease due to ranavirus) in positive individuals; however, we recorded several swollen and one hemorrhagic lesion at an ETN site the year following a spike in prevalence at the same location. Pathogenesis of Ranavirus occurs largely through vascular and epithelial damage (Miller et al. 2011), and digital swelling and necrosis is a prominent lesion documented in Ranavirus outbreaks of the closely related Chinese giant salamander, *Andrias davidianus* (Geng et al. 2011). Therefore, the lesions we observed in Hellbenders a year later after a documented Ranavirus event possibly represent long term sequelae from vascular damage acquired during previous infection. Detection of disease consequences of a pathogen and detection of infection may be temporally separated; therefore, we suggest that repeated sampling is required to differentiate these effects.

Ranavirus may have other subtle sublethal effects on adult health emphasized by our linear mixed models that revealed a small significant negative correlation between Ranavirus infection and BCS. Ranavirus infection is likely energetically expensive and may cause changes in foraging behavior that prevent sufficient feeding. However, because our results are purely correlative, this could also suggest that animals with lower body condition are more likely to become or maintain ranaviral infections, increasing our ability to detect it. For example, Woodfrog (*Lithobates sylvaticus*) tadpole foraging and swimming phenotypes have been linked to increased Ranavirus infection rates and disease susceptibility (Araujo et al. 2016). Further disease dynamic data of Ranavirus in stream systems are needed to better understand population-level impacts.

Our findings emphasize the importance of repeated disease monitoring in Hellbenders. *Bd* was not detected in any streams with a single sampling event and Ranavirus was only detected sporadically, which illustrates both can easily be missed from single-season studies. This is important since *C. a. alleganiensis* populations are

considered to be relatively stable in certain areas such as in the Blue Ridge Ecoregion of ETN, where we discovered both *Bd* and Ranavirus to be present in a majority of streams surveyed. Both pathogens may play a subtle role in Hellbender declines and detailed disease data are required to fully understand a likely multifactorial threat to Hellbender populations. We also describe a spectrum of severity of distal limb lesions with multiple likely etiologies. Some lesions were healed, others ulcerated, some with swollen and missing toes, and others with toes intact. Future Hellbender research must describe and document all lesions in the field, and pair these observations with disease sampling to help properly diagnose morbidities that may affect population persistence. Additionally, we recommend researchers use qPCR in their study to allow evaluation of relative pathogen loads between regions and health parameters of interest. Infection burden is particularly important for *Bd* to determine subsequent individual and population health consequences (Wilber et al. 2017) and has been shown to have independent and sometimes opposite trends of prevalence in response to environmental fluctuations (Terrel et al. 2014; Ruggeri et al. 2018). We discovered different trends in pathogen load vs prevalence showing that prevalence-only data may also be misleading for how these pathogens affect Hellbenders.

Furthermore, continued disease monitoring will be important to detect any emerging or unknown disease threats, such as *Bsal*. A recent outbreak of a previously unknown virus almost resulted in extinction of the endangered Bellinger River Turtle (Zhang et al. 2018), but because of early detection and prepared wildlife disease teams, that species persists today. Our data demonstrate sporadic and easily missed occurrence of important amphibian pathogens and the presence of lesions in both subspecies of Hellbenders which should motivate increased disease monitoring in this important threatened amphibian.

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CHAPTER II
EVALUATION OF SEVERITY AND FACTORS CONTRIBUTING TO FOOT LESIONS IN ENDANGERED OZARK HELLBENDERS, *CRYPTOBRANCHUS ALLEGANIENSIS BISHOPI*

Abstract

Arkansas populations of Ozark Hellbenders, *Cryptobranchus alleganiensis bishopi* are limited to a single river and over the past decade biologists have observed an increase of distal limb lesions with unknown etiology. We performed surveys of *C. a. bishopi* in Arkansas during 2011-2014. From lesion data we developed a scoring system from 0-7 from 73 animals and applied a score to each hellbender blinded of associated information. We performed linear mixed model regressions followed by AICc model evaluation to determine associations among infection status for amphibian pathogens *Batrachochytrium dendrobatidis* (*Bd*) and Ranavirus as well as individual biometrics on lesion score. We discovered 93.2% of hellbenders had lesions characterized by digit swelling that often progressed towards toe-tip ulceration. In severe cases we observed digital necrosis progressing to digit loss. Any recaptured individuals had the same or worse lesion score from previous captures. The top predictive model for lesion severity included individual mass and *Bd* infection status with a significant, positive association of *Bd* with increased lesion severity ($\beta = 0.87 \pm 0.39$ S.E., C.I.: 0.11, 1.63). Our findings highlight a widespread and progressive disease that is an important factor to consider for the future of Ozark Hellbenders. This syndrome is likely multifactorial, and several factors of host, agent, and environment need to be studied to understand disease manifestation. We provide a summary of potential etiologies and mechanisms that may explain observed lesion distribution.

Introduction

Human alteration of habitat has been linked to increased disease prevalence in many wildlife systems (see Hing et al. 2016), and stream dwelling amphibians have been commonly used as biological indicators due to their susceptibility to a variety of anthropogenic disturbances (Southerland et al. 2004; Welsh and Ollivier 1998). Specific consequences of degraded watersheds such as chemical runoff and increased sedimentation are known to negatively impact amphibian health (see Egea-Serrano et al. 2012; Brannon and Purvis 2008).

Hellbenders (*Cryptobranchus alleganiensis*) are large, fully aquatic salamanders that inhabit cool, rocky, well-oxygenated rivers and streams in the eastern United States (Nickerson and Hays 1973). The eastern subspecies (*C. a. alleganiensis*) encompasses a majority of the range with Ohio, Tennessee, and Mississippi River drainages while the Ozark Hellbender (*Cryptobranchus a. bishopi*) is a disjunct subspecies restricted to Ozark highland streams of Missouri and Arkansas. Hellbenders may be particularly susceptible to increased sedimentation and other changes in water quality because they are fully aquatic and use cutaneous respiration almost exclusively (Guimond and Hutchinson 1973). They require large cover rocks or crevices for breeding sites (Nickerson and Mays 1973) and sediment-free interstitial spaces in gravels for larval and juvenile growth (Da Silva Neto et al. 2019; Keitzer 2007).

Over the past few decades, population numbers have decreased appreciably in

both subspecies. In many streams, populations have become extirpated or only large relic individuals remain (Wheeler et al. 2003; Graham et al. 2011). Drastic declines have resulted in federal listing of *C. a. bishopi* as an endangered subspecies (USFWS 2011*a*) and inclusion of both *C. a. alleganiensis* and *C. a. bishopi* in appendix III of the Convention on International Trade in Endangered Species (CITES) of Wild Fauna (USFWS 2011*b*). Ozark Hellbenders in Arkansas are believed to be functionally extirpated from all but one river that is located in the northern portion of the state and degraded habitat is hypothesized to be a driver behind these declines. In one study in Missouri, decreased population densities of both subspecies were linked to decreased watershed buffers and associated water quality parameters (Bodinoff-Jachowski et al. 2018). In conjunction with these declines, Ozark Hellbenders in Arkansas (and populations in Missouri) have been documented with distal limb lesions (Hiler et al. 2005). These include feet with swollen, fused, or missing digits, and occasionally, exposed phalanges. A few cases are documented with partial to near complete foot loss. Similar but much less severe lesions have been observed in Eastern Hellbenders in Tennessee but at lower frequency (Miller and Miller 2005).

Primary pathogens may also be negatively impacting *C. a. bishopi* populations and may contribute to these abnormalities, even if they are only part of a multifactorial process. Chytrid fungus (*Batrachochytrium dendrobatidis* or *Bd*) is present in *C.a. bishopi* (Briggler et al., 2008; Bodinoff et al. 2011), and *C. a. alleganiensis*, (Bales et al. 2015; Groves and Williams 2014; Goyner et al. 2011), but *Bd* prevalence is similar among both healthy and declining populations. However, in *Bd* positive individuals, infection intensity (expressed as total zoospore count), tended to be greater in declining populations, compared to relatively stable populations (Hardman, unpublished data), and in captive settings, *Bd* infection has resulted in mortality (Dean et al. 2016; Dusick et al. 2016).

Ranavirus is another emerging amphibian pathogen that is responsible for dramatic mortality events in captive colonies of the related Chinese Giant Salamander *Andrias davidianus*. (Geng et al. 2011). Lesions from Ranavirus outbreaks in *A. davidianus* are identified by distal limb swelling, necrosis, and ulceration (Zhou et al. 2014), which are similar to lesions observed in *C. a. bishopi*. Ranavirus has been found to infect wild hellbenders, but without any obvious clinical signs at the time of sampling (Souza et al. 2012). However, Ranavirus infection is negatively associated with body condition score (BCS) (Hardman, unpublished data), highlighting the possibility that this virus may have unknown sublethal effects in Hellbenders.

Bacterial pathogens, both primary and opportunistic, have the capacity to contribute to lesion manifestation. Two species, *Citrobacter freundii* and *Aeromonas hydrophila* have been cultured from some, but not all, lesions sampled in *C. a. bishopi* in Missouri (Nickerson et al. 2011). *C. freundii* is an opportunistic pathogen associated with aquatic ectotherms and is documented to produce a variety of sequelae including septicemic cutaneous ulcerative disease (SCUD) in turtles and necrotizing fasciitis in humans (Chuang et al. 2006). *A. hydrophila* is associated with mixed-pathogen infections that involves skin ulcerations and septicemia in both fish (Gudmundsdottir and Bjornsdottir 2017) and amphibians (Miller et al. 2008). When another study (Hernandez-

Gomez et al. 2017) applied culture-independent methods (16S amplicon sequencing) to identify bacteria from these lesions, they did not determine any consistent association between lesions and a particular bacterial species, further supporting this syndrome is likely of complex etiology.

Consequently, it remains unclear how host, pathogen, and environment may produce this high prevalence of distal limb lesions and no study has yet quantified lesion severity and applied an epidemiological evaluation of lesion presence. We documented lesions and collected associated individual size class data and pathogen samples in Ozark Hellbenders of Arkansas from 2011 – 2014 with the following two objectives: 1) document spatiotemporal patterns and severity of lesions present in this last remaining Arkansas Ozark Hellbender population, and 2) determine if host factors and infection status are associated with lesion severity.

Methods

Collection

Within Arkansas, Hellbenders are currently restricted to only one river, which we have chosen not to identify to prevent potential poaching. During four seasons (2011-2014), we performed one-week intensive surveys. We performed surveys within a three-week sampling window from late July to early August except for 2013 when high water levels prevented sampling, and sampling was delayed until September. Disease sampling protocols were added to ongoing surveys for population monitoring. We performed all sampling under USFWS permit TE66039A-0 issued to KJI and Arkansas Game and Fish Commission. We sampled shallow and deep-water habitats (up to 4 meters) using a hookah dive system (i.e. gasoline powered air compressor with tethered air supply lines to dive regulators). We also performed standard snorkeling to locate individuals in shallower water only. We also sampled Hellbenders via previously placed artificial nest boxes (Briggler and Ackerson 2012). We captured any animals encountered under cover objects and nest boxes and placed them in a clean soft cotton or mesh bag. We kept bags submerged in the river before and after animals were processed. (University of Tennessee IACUC protocol # 2481-0916). We changed dive gloves between animal captures to reduce pathogen transfer and contamination of pathogen samples.

Biometrics and Pathogen Sampling

Swabs for *Bd* quantitative PCR (qPCR) and tail tissue for Ranavirus qPCR were collected for another study evaluating disease prevalence (see Ch. 1) and those results were incorporated into our lesion severity analysis. In 2012 we collected additional swabs exclusively from foot lesions in a subset of animals and sent to Zoologix Inc. (Chatsworth, CA) for additional targeted qPCR for *Citrobacter freundii* and *Aeromonas hydrophila*. Standard measurements were recorded for total length (TL), snout-vent length (SVL), and mass. Captured animals were scanned for presence of a PIT tag, and captures were subcutaneously PIT tagged at the base of the tail. After processing, all individuals were released at their point of capture. New containers, bags, and gloves were

used between individuals. All equipment (including diving gloves) was cleaned with soap and soaked in 5% solution of either bleach or chlorhexidine for at least ten minutes and rinsed thoroughly before use at another site.

Lesion Description and Scoring

Based on previous surveys we knew that lesions were generally restricted to distal limbs, and this is where we focused lesion quantification. Lesions scores were based on observations of chronic, non-healing, ulcerative areas of the feet and toes. We realize that hellbenders can incur traumatic wounds to the feet from conspecific interactions during the breeding season. We minimized these types of potentially confounding wounds in adhering to our lesion score criteria (Table 4) and only evaluated non-healed lesions in the distal limbs. Using a preprinted baseline drawing, individuals, were recorded for lesion sub-type and anatomic location. Digits were noted as either "Healthy" (Fig 5a), "Bulbous" or "Swollen" (Fig 5b), "Shortened" (Fig 5c), or "Missing" (Fig 5c), with associated ulcerations on foot pads (Fig 5d), ulcerations on toe tips (Fig 5b), or completely missing limbs (picture not available).

A lesion scoring system with eight levels ranging from scores of 0 - 7 (Table 4) was created and scores were based on the number of toes affected, type of sub-lesion on each toe, and presence or absence of foot-pad ulcerations. A score of 0 denoted an individual with no digital lesions whereas a score of 7 denoted an individual with very severe lesions in all four feet. Those receiving lesion scores of 1-2 primarily had toe swelling only. Scores 3-4 had most toes shortened and some missing. Scores 5-7 had several toes or even entire feet missing with remaining toes swollen or shortened. Scores assigned to each animal were based on the standardized field drawings and were done blinded to any other information of that animal.

Lesion Severity Models

A linear mixed model was used to evaluate effects of infection status and individual factors on lesion severity. We originally chose the following fixed effect parameters: Mass, TL, and presence or absence of *Bd* and Ranavirus. We created a third individual parameter of body condition score (BCS) by calculating residuals for each individual from residuals of the fitted polynomial plot of TL and Mass. Total length was subsequently removed from our analysis because of its correlation with mass. Because of a low detection rate of Ranavirus, Ranavirus was excluded from our models. All models included a combination of our individual fixed effects listed and the following three random effects of: Individual ID, Year, and Site ID. This resulted in the following seven predictive fixed effect models: (*Bd* (0/1)+ Mass), (*Bd* (0/1)+ BCS), (*Bd* (0/1) only), (Mass only), (BCS only), (Mass + BCS), Global, Random-only. All mixed models were created from the lme4 package (Bates et al. 2015) in RStudio (R Core Team 2013).We evaluated relative model fit from AICc for all possible combinations of fixed effects to determine the top performing model via the AICcmodavg package (Mazerolle 2019) and considered models competitive when ΔAICc was < 2.0. We model-averaged variables that appeared in more than one of the top models as recommended in Burnham and Anderson (2002) to determine relative variable contribution in those models.

Table 4: Lesion Scoring System.

Twelve recaptured individuals and subsequent change in lesion score between final and initial captures. Averages for scores listed at bottom. Note all lesion score changes are positive.

Score	No. Foot Pads Ulcerated	No. Toes Missing	No. Toes Shortened	No. Toes Swollen	Description
0	0	0	0	0	No observable distal limb lesions. Healed scars may be present. No active ulcerations or swelling.
1	0	0	0	1 to 5	1-5 swollen toes in one or two feet. No ulcerations on feet or toes. No shortened, fused, or missing toes
2	0	0	0	6 to 10	6-10 swollen toes affecting two feet. Toe tip ulcerations may be present. No shortened, fused, or missing toes
3	0 to 1	0	0	11 to 18	11-18 swollen toes affecting three or four feet. Toe tip ulcerations may be present. No shortened, fused, or missing toes. May have 1 footpad ulceration
4	0 to 1	0 to 1	1 to 5	1 to 17	1-5 shortened toes with 0-1 missing toes. Remaining toes swollen. May have 1 footpad ulceration
5	0 to 2	0 to 4	1 to 16	1 to 15	1-5 shortened toes with 2-4 missing toes OR 6+ shortened toes with 0 missing toes. Remaining toes swollen. May have up to two footpad ulcerations.
6	0 to 2	5 to 18	1 to 13	1 to 13	5+ missing toes with up to 2 foot pad ulcerations
7	3 to 4	5 to 18	1 to 13	1 to 13	5+ missing toes with 3+ foot pad ulcerations.

Figure 5. Arkansas Lesions.

Examples of various levels of severity seen in foot lesions in Ozark Hellbenders *Cryptobranchus alleganiensis bishopi* in Arkansas. A. (Top Left) Foot with all toes intact slight erythema with digit III slightly swollen. B. (Top Right) Front foot with all swollen digits with a toe tip ulceration on digit IV. C. (Bottom Left) Toes have become flattened and are beginning to shorten. Digit IV is almost completely missing, and bone is beginning to protrude through the dorsal skin. Note an attached leech in the interdigital space between digits II and III. D. (Bottom Right) Digit IV is swollen and slightly erythematous with toe tip ulceration. Skin on foot pad is thin, turned white gray and slightly transparent and is close to ulcerating. Underlying tissue is swollen and erythematous with an apparent nodule forming at the medial aspect.

Results

Pathogen Prevalence

Of the 73 individuals evaluated in this analysis, 23 (31.5 %) were positive for *Bd*, four (5.5 %) positive for Ranavirus, and 0% positive for *Bsal*. Two Ranavirus positive individuals were also infected with *Bd* (50.0%). For the subset of eight lesion-only swabs tested for opportunistic bacteria, 0% were positive for both *C. freundii* and *A. hydrophila*.

Lesion Severity Distribution

A total of 73 animals were assessed during this study with an average lesion score of 4.27 and a median score of 5. Only five animals were considered lesion free (Lesion Score = 0; 6.8%), and all were negative for *Bd*. Lesion-free animals were generally smaller, falling within the ten smallest individuals captured (15^{th} percentile in mass), weighing less than 300 g. Of the 11 recaptured individuals nine had progressively greater lesion scores from the previous recapture, whereas three maintained the same score (Table 5). We recaptured one individual twice, with an initial score of 4 in 2011, followed by a lesion score of 6 in both 2012 and 2014 (Table 5).

Linear Mixed Models and AICc

Of the model sets evaluated, the Chytrid Progressive, Chytrid Individual, Chytrid, and Global model sets were competitive based on AICc evaluation (Table 6). Of the model averaged coefficients from these model sets, *Bd* infection status was the only variable to have a significant effect on lesion severity ($\beta = 0.87 \pm 0.39$ S.E., C.I.: 0.11, 1.63; Table 7). Lesions in *Bd*-positive animals had an average score of 4.78 in contrast to 4.06 in *Bd*- negative animals (Fig 6).

Only five individuals from a total of 73 (6.8%) sampled were lesion free. These apparently healthy individuals included some of the smallest individuals. Interestingly, our top predictive model included mass. Although this was not a significant effect in our model averaging analysis, mass was positively correlated with lesion severity. If this represents a true trend, it may reveal that older, larger individuals are more likely to have severe lesions. One interpretation of this phenomenon would be that lesions result from long term consequences from a single historical event such as a chemical runoff or single pathogen outbreak, and younger individuals were simply not alive during the exposure period. A second interpretation is the etiological agent(s) that is/are responsible for these lesions require(s) long-term exposure to produce disease and younger Hellbenders are not old enough to have reached a certain critical exposure time for lesion development. Both hypotheses imply a chronic and progressive disease process which is supported by our recapture data showing individuals had either the same or worse lesion scores upon successive captures.

Table 5: Lesions Scores in Recaptured Individuals

Twelve recaptured individuals and subsequent change in lesion score between final and initial captures. Averages for scores listed at bottom. Note all lesion score changes are positive.

Data ID	Recap No.	Final Capture Year	Final Lesion Score	Years Between Recap	Total Score Δ	Score Δ per Year
151	1	2014	5	3	+0	+0.0
153	1	2015	7	4	+2	+0.5
158	1	2014	6	1	+2	+2.0
158	2	2014	6	3	+2	+0.7
161	1	2014	5	3	+0	+0.0
166	1	2012	4	3	+3	+1.0
168	1	2012	5	1	+0	+0.0
169	1	2014	7	1	+1	+1.0
177	1	2014	5	2	+4	+2.0
180	1	2015	4	1	+4	+4.0
190	1	2015	4	1	+0	+0.0
191	1	2015	7	1	+1	+1.0
Average			5.4	2	+1.6	+1.0

Table 6: AICc Table for Lesion Score Models

List of Linear Mixed Models (LMM) included in AICc analysis in order of smallest to largest ΔAICc. Response variable was lesion score (0-7). Top models of delta AICc < 2.0 are noted in bold and were used for subsequent variable model averaging.

Fixed Effects	Random Effects	K	AICc	▲AICc	AICcWt	Cum.Wt	LL
Bd (0/1) + Mass	Indv ID, Site, Year	7	290.2	0	0.26	0.26	-137.25
Bd (0/1) + BCS	Indv ID, Site, Year	7	290.9	0.66	0.18	0.44	-137.58
Bd (0/1)	Indv ID, Site, Year	6	290.9	0.7	0.18	0.62	-138.82
Bd (0/1) + BCS + Mass	Indv ID, Site, Year	8	291.6	1.34	0.13	0.75	-136.66
Mass	Indv ID, Site, Year	6	292.5	2.25	0.08	0.83	-139.6
BCS	Indv ID, Site, Year	6	292.9	2.7	0.07	0.9	-139.83
Mass + BCS	Indv ID, Site, Year	7	293.3	3.08	0.05	0.95	-138.79
----	Indv ID, Site, Year	5	293.6	3.38	0.05	1	-141.35

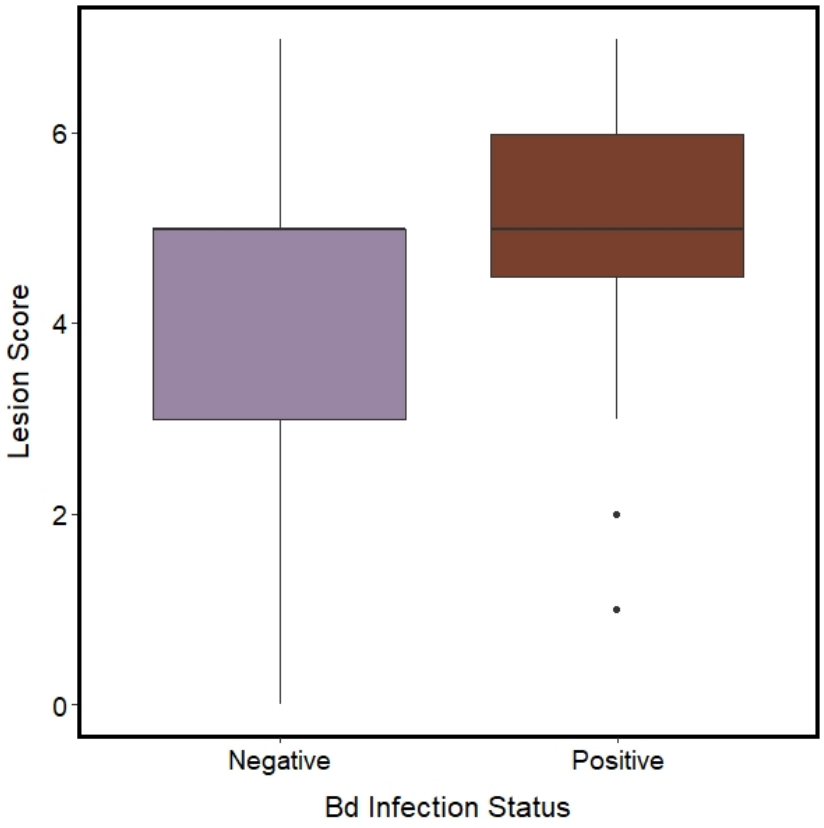

Figure 6. Lesion Score by *Bd* infection Status

Boxplots of lesions scores (ranging 0-7) from foot lesions of Ozark Hellbenders, grouped by *Batrachochytrium dendrobatidis* (*Bd*) infection status. *Bd* negative Hellbenders had an average score of 4.06 whereas *Bd* positive Hellbenders had an average score of 4.78.

Table 7: List of Fixed Effects Variables

Fixed effects variables in top models. (ΔAICc<2) listed with model-averaged results. Significant effects (based on 95% C.I.) noted in bold.

Variable	β Coeff	S.E.	95% C.I.
***Bd* (0/1)**	**0.87**	**0.39**	**0.11, 1.63**
Mass	0.35	0.21	-0.05, 0.76
BCS	0.27	0.19	-0.11, 0.65

Discussion

Our results demonstrate that distal limb lesions are prevalent (93.2 %) in *C. a. bishopi* in Arkansas. This is a larger percentage of affected animals than previously reported in Ozark Hellbenders in Missouri (USFWS 2011a). Lesions appear inflammatory in nature and do not represent typical lesions expected after traumatic events in an otherwise healthy individual. They are non-healing and progressive, and likely begin with internal inflammation within digits and develop over time to skin ulceration, loss of tissue integrity, and necrosis. In recaptured individuals, we found that lesions are maintained or worsen in severity over several years and were always restricted to the distal part of the limbs.

Our most important finding was that *Bd* infection status was in all top predictive models and represents the only statistically significant predictor to explain lesion severity. We confirmed a significant positive correlation of *Bd* infection with lesion severity. However, the causation behind this relationship remains unclear. Previous surveys of *Bd* in wild hellbenders have been of little apparent value in population assessments, as *Bd* is present in this and several other hellbender populations with no clear population health association (Bales et al. 2015; Groves and Williams 2014; Souza et al. 2012; Goyner et al. 2011). Still, *Bd* is an introduced pathogen to the United States, only to appear in Hellbenders after 1969 (Bodinoff et al. 2011) and may be causing more damage to hellbender health than we realize. In captive Hellbenders, chytridiomycosis is considered a serious health threat, and both adults and juveniles have experienced mortality due to *Bd* (Dean et al. 2016; Dusick et al. 2016). Furthermore, mortality events appeared to follow times of stress such as with juvenile captive-wild translocations (Dusick et al. 2016) where *Bd* may have been able to take advantage of weakened skin defenses. Although current literature does not support immunosuppression via chronic stress as a driver of classic *Bd* mortality events in amphibians (Grogan et al. 2017), stress may play a role in *Bd* susceptibility of more tolerant species (Rollins-Smith 2017). Laboratory trials have shown that *Bd* infection intensity and incidence of chytridiomycosis can increase after alterations in host immunity via glucocorticoid administration in plethodontid salamanders (Fonner et al. 2017). Similarly, in Northern Leopard Frogs glucocorticoids can change expression of important AMPs (Tatiersky et al. 2015). Therefore, if wild *C. a. bishopi* are experiencing immunosuppression associated with lesion severity, *Bd* may have a greater chance of maintaining infection, and should still be considered a health threat in the wild.

Our data may underestimate the impact *Bd* has on *C. a. bishopi* because of our sampling time. In Crawfish Frogs, *Lithobates areolatus*, *Bd* infection prevalence and intensity has seasonal patterns where individuals often clear infection by late summer only to become reinfected in winter and spring (Kinney et al. 2011). If *Bd* follows the same infection pattern in *C. a. bishopi*, it could be an important factor preventing wound healing and promoting progressive disease in cooler months when prevalence is much greater than when we sample in late summer. Still, we do not propose *Bd* as a primary or necessary etiologic agent for manifestation of these lesions, but stress that *Bd* may be

important in progression of lesion severity through disruption of skin function and healing, especially in more severe cases.

We hypothesize that lesion formation occurs through a multifactorial process. Synergistic effects of introduced agents and altered or degraded habitat have been shown to produce disease in an otherwise resistant population. For instance, red tide blooms can affect sea turtle immune systems and potentially drive increased prevalence of fibropapillomatosis (Perrault et al. 2017). In amphibians, increased use of the herbicide atrazine can negatively affect health directly (Hayes et al. 2011), but also indirectly though potentiating trematode infestations (Rohr et al. 2008). Ozark Hellbenders in Arkansas are experiencing heavy sediment loads from agricultural land uses and subsequent decreased watershed buffers, which could result in concomitant increases of agriculture runoff (e.g. pesticides, herbicides, contaminants of poultry litter). We cannot ignore the fact that habitat degradation is likely contributing to the lesions in Ozark Hellbenders in Arkansas and may be causing chronic stress with long term consequences of immunosuppression. Further, in laboratory experiments of another salamander species, *Desmognathus ochrophaeus*, increased plasma corticosterone was associated with increased wound healing time (Thomas and Woodley 2005), which supports a potential mechanism between chronic stress and lesions.

Ozark Hellbenders likely also suffer immunological consequences from decreased genetic diversity. Low immunogenetic diversity is linked to increased disease incidence in other wildlife species e.g., malaria in great reed warblers (Westerdahl et al. 2005), and facial tumors in Tasmanian Devils (Morris et al. 2015). *C a. bishopi* have lower MHC IIb diversity than their Eastern counter parts in Missouri (Hernandez-Gomez et al. 2018) and combined with other factors, may have reached a threshold to become less resistant to one or more pathogens.

Opportunistic bacteria are hypothesized to contribute to these digit and foot lesions once host immune systems are weakened, however, no specific opportunistic pathogen has yet been identified (Nickerson et al. 2011; Hernandez-Gomez et al. 2017). Our subset of lesion swabs was negative for both *C. freundii* and *A. hydrophila*, which lowers support for their role in lesion development. Several other pathogens including viruses and parasites still need to be considered. For instance, a case of progressive ulcerative dermatitis was reported in a captive treefrog, *Phyllomedusa bicolor*, associated both viral and microsporidial septicemia, showing potential for either to contribute to skin ulcerations (Graczyk et al. 1996).

Ranavirus should still be considered as a potential pathogen in producing these lesions despite having a low prevalence in our samples (5.5%). Lesions of ranavirosis in juveniles of related Chinese Giant Salamanders (*Andrias davidianus*) include inflammation, ulceration, and necrosis of the distal limbs (Zhou et al. 2014; Geng et al. 2010) that appear to be very similar to those observed in *C. a. bishopi*. Ranavirus infection in *C. a. bishopi* adults may result in sublethal disease with long term sequelae as opposed to dramatic mortality events observed in juvenile *A. davidianus*. If only a single Ranavirus infection event is needed to cause damage to begin lesion formation, and a typical Hellbender has a life expectancy of 20-30 years in the wild, probability of exposure and subsequent long-term sequelae may be fairly high. This concept could hold

true for many other pathogens that cause damage to skin and vasculature, making the causative agent difficult to detect without long term monitoring.

It will be critical to understand the pathophysiology of this disease to determine the most effective course of action. Examples from domestic animals shows us that complex diseases can be treated successfully if critical factors in disease manifestation are identified. For parvoviral disease in domestic dogs, the main etiologic agent is a virus (Parvoviridae), and is difficult to directly target in therapy, but mortality rate can still be significantly reduced with antimicrobial treatment to prevent effects of secondary bacterial translocation of the gastrointestinal tract (Goddard and Leisewitz 2010). Feline lower urinary tract disease (FLUTD) is a multifactorial disease in domestic cats that can result in chronic urinary tract signs. Currently, the most effective treatment is not treating the animals, but rather treating the environment by removing sources of stress at home (Westropp et al. 2019). Hellbender conservation managers should, therefore, focus attention not on a single factor but on the system. We may not be able to remove pathogens from these river systems, but we may be able to help reduce transmission between animals and reduce environmental stress. First, we can reduce artificially increased transmission and subsequent pathogen burden by adhering to aseptic sampling protocols in the field and in repatriation efforts. Second, we can focus efforts on habitat restoration. If bank restoration efforts decrease erosion and runoff, this may not only improve living and breeding space but simultaneously decrease disease incidence by reducing negative effects of environmental factors on host health.

This study documents a progressive disease affecting almost all sampled individuals in the remaining Ozark Hellbender population in Arkansas. The presentation of progressive lesions with a long differential list suggests that this disease is complex, and we highlight areas that need further research to fully understand factors in Ozark Hellbender health. Many questions still exist on the exact etiology of these lesions, and why they progress, but we do know that increased lesion severity is associated with *Bd* infection. Our lesion scoring system can be used to monitor changes in overall population health including response to restoration efforts. It can also be used to evaluate similar distal extremity lesions observed in other Ozark and Eastern Hellbender populations. Finally, Hellbenders are large, long-lived animals and may serve as sentinels for long term exposure to sublethal agents. The high prevalence of these lesions should not only motivate continued research in Hellbender health but should also raise alarms for potential health risks to other wildlife and human populations sharing these watersheds.

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CHAPTER III
HELLBENDER SKIN SECRECTIONS INHIBIT TWO SPECIES OF CHYTRID FUNGI AND ACTIVITY IS NEGATIVELY ASSOCIATED WITH PRESENCE OF LESIONS

Abstract

Hellbenders, *Cryptobranchus alleganiensis,* are large salamanders with a historical range encompassing streams and rivers throughout eastern and part of the mid-western United States. Both subspecies, the Eastern Hellbender, *C. a. alleganiensis* and Ozark Hellbender, *C. a. bishopi* have experienced considerable declines, resulting in federal listing of *C. a. bishopi*. Disease due to chytrid fungus, *Batrachochytrium dendrobatidis*, is the number one cause of amphibian declines globally and has been present in Hellbender populations for at least 50 years. Hellbenders are notorious for copious amounts of skin secretions and are colloquially called "snot otters", yet little is known about what immunity these secretions provide Hellbenders against disease and *Bd* infection. Furthermore, Ozark Hellbenders have a very high prevalence of distal limb lesions of unknown etiology. We collected skin secretions from 39 individual Hellbenders from both the Ozark subspecies in Arkansas (AR) and Eastern subspecies in middle (MTN) and eastern Tennessee (ETN) and enriched samples for small cationic peptides. We performed growth inhibition assays (GIAs) against *Bd* and *Bsal*. We performed GLMMs to compare differential inhibition of *Bd* zoospore growth at 500µg/mL p=skin peptide concentrations between regions, *Bd* infection status, Mass, and presence of toe lesions and found toe lesions to be the strongest and only significant predictor of *Bd* inhibition. We also found skin secretions to have significantly greater inhibitions of *Bsal* over *Bd*, and we also observed a potential interaction between Ranavirus and *Bd* infection status on peptide activity. MALDI-TOF mass spectrometry results reveal candidate peptides responsible for Hellbender anti-chytrid activity. These results support that Hellbender skin secretions are an important part of innate immunity and highlight a potential mechanism for sub lethal effects of chronic toe lesions observed in both subspecies.

Introduction

Amphibians are the fastest declining vertebrate group with over 41% listed as threatened or endangered by the IUCN Red List (ICUN 2019). Disease is the leading cause of amphibian extinctions across the globe and remains the major threat to extant species. Most of these disease driven declines can be attributed to the novel introduction of chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*). This global pandemic lineage (GPL) of *Bd* is endemic to Asia (O'Hanlon et al. 2018) and is estimated to be responsible for 90 extinctions in less than three decades (Scheele et al. 2019). This has given much deserved attention to highly susceptible frog species such as the Panamanian golden frog (*Atelopus zeteki*) (see La Marca et al. 2005; Poole 2008). However, other species may not be as highly susceptible with less obvious effects. Some salamander species have experienced noticeable declines in conjunction with arrival of *Bd* (Rovito et al. 2009; Cheng et al. 2011) revealing that salamanders should not be ignored when considering impacts of chytrid introduction. In fact, an emerging chytrid pathogen *B. salamandrivorans* (*Bsal*), has been introduced into Europe causing mass mortalities of

the once common Fire Salamander (*Salamandra Salamandra*) (Stegen et al. 2017), because unlike *Bd*, *Bsal* appears to affect salamanders more than frogs (Martel et al. 2014). Furthermore, Ranavirus is another important amphibian pathogen responsible for mass mortality events most notably in temperate regions of North America (Green et al. 2002) and Europe (Duffus and Cunningham 2010). While Ranaviruses have not caused extinctions like *Bd,* they have caused declines in once common species such as the Common Frog, *Rana temporaria* (Teacher et al. 2010) and have a diverse host range beyond just frogs (Chinchar and Waltzek 2014). This highlights the need for conservation efforts and baseline health information for all amphibians, including salamanders.

The Hellbender, *Cryptobranchus alleganiensis*, is a large aquatic salamander found in fast flowing streams and rivers within the eastern and midwestern United States. Two subspecies, the Eastern Hellbender, *C. a. alleganiensis* and Ozark Hellbender, *C. a. bishopi* have both experienced considerable range retraction and decreased population densities over at least the past 25 years (Wheeler et al. 2003; Graham et al. 2011; Hiler et al. 2013; Freake 2017). Ozark Hellbenders are restricted to rivers of the Ozark highlands of Missouri and Arkansas and because of rapid declines were listed as endangered by the US Fish and Wildlife Service (USFWS) in 2011 (USFWS 2011). Eastern Hellbenders, although still not listed by the USFWS as threatened or endangered, are estimated to have lost 39.5 % of their historical populations, with another 38.4% currently in decline (USFWS 2018). Hellbenders are important both genetically and ecologically. They represent one of only three species from the Giant Salamander family, Cryptobranchidae, and are the largest salamander of North America; adults can grow to over 60 cmn total length and 1 kg mass (Nickerson and Mays 1973; Miller 2013). They are important long-lived top stream predators, feeding on a variety of crayfish and fish species as adults, with a lifespan of 30+ years (Nickerson and Mays 1973).

Although habitat degradation is associated with the majority of observed declines (Humphries and Pauley 2005; Hopkins and DuRant 2011; Bodinoff-Jachowski and Hopkins 2018), an immediate mechanism into why this occurs is still unknown and there has been limited investigation into how disease may be playing a role. Unfortunately, over 90% of Ozark Hellbenders in Arkansas have a form of distal limb lesions characterized by ulceration and digital necrosis (Hiler et al. 2011; Ch 2). Although there is no definitive etiology for these chronic and progressive lesions, *Bd* infection is associated with increased severity (see Ch. 2). Manifestation of these lesions is likely a result of multiple factors, including host health; decreased immunity and decreased wound healing are hypothesized to contribute to lesion formation (Hernandez et al. 2017b; Nickerson et al 2011; Ch 3).

Bd has been detected in Hellbenders as early as 1969 (Bodinoff et al. 2011) with unknown population effects. In the past decade *Bd* has been found in both healthy and unhealthy Hellbender populations ranging from 0 to 33% prevalence (Goyner et al 2013, Souza et al 2012, Briggler et al. 2008, Bales et al 2015, Williams and Groves 2014). Although chytridiomycosis has not been confirmed in wild individuals, it has been documented in captive populations with associated mortalities (Dean et al. 2016; Dusick et al. 2017). Ranavirus has unknown effects on wild Hellbenders, however, it should still be considered a major health threat. In the closely related Chinese Giant Salamander,

Andrias davidianus, Ranavirus has devastated captive colonies causing severe hemorrhage and ulcerations and mortality (Geng et al. 2011). Ranavirus has been detected only subclinically in wild Hellbenders (Souza et al. 2012; Ch. 1), but infection is significantly associated with a slight decrease in body condition (Ch. 1) suggesting sublethal effects.

Both Ranavirus and chytrid fungi cause disease in the skin, an organ particularly important for amphibians because it is a major site for electrolyte exchange and homeostasis (Shoemaker and Nagy 1977); consequences of dysfunction can quickly be fatal. Also, because of its important physiological role and semi permeable nature, amphibian skin may be more susceptible to both environmental and systemic changes and may provide a window into individual health. This may be especially true for Hellbenders which rely almost exclusively on cutaneous respiration after larvae resorb gills around one year of age (Guimond and Hutchinson 1973). Therefore, evaluation of skin immunity in Hellbenders may be a valuable tool for assessing both individual and population health.

Granular (poison) glands are one group of specialized serous glands on amphibian skin that produce a variety of small peptides with antimicrobial activity called antimicrobial peptides (AMPs) (Rollins-Smith and Conlon 2005), and more than 2000 amphibian AMPs have been described (Xu and Lai 2015). AMPs are critical components of innate immunity in all vertebrates and represent hundreds of varied peptides produced in many animal tissues with a large spectrum of activity (Brogden et al. 2003; Brogden 2005). These cationic amphipathic 10-50 residue peptides can have direct antimicrobial activity via membrane disruption of bacteria, viral envelopes and fungi, and protozoa (Brogden 2005). Because of their non- specific targeting of cellular membranes they represent an important tool for pathogen deactivation and killing (Ageitos et al. 2017).

AMPs isolated from granular glands of frog species have demonstrated growth inhibition of *Bd* among other known amphibian pathogens (Woodhams et al. 2006; Rollins-Smith et al. 2006, 2009; Pasmans et al. 2013; Van Rooij et al. 2015). Differences in AMP secretion can even explain some variation in certain amphibian species' resistance to *Bd* (Woodhams et al. 2006; Rollins-Smith 2009). Furthermore, there is evidence of intraspecific AMP variation in frogs. *Batrachochytrium dendrobatidis* inhibition by AMPs can vary across regions (Lam et al. 2010; Tennessen 2009) and even within the same individual before and after a stress event (Tatiersky et al. 2015). Therefore, AMPs may be an important avenue through which environmental changes can impact incidence of chytridiomycosis in host amphibians.

Salamander AMPs are hypothesized as one factor responsible for increased resistance of salamanders to *Bd* in comparison to many anuran species (Pasmans et al. 2013; Van Rooij et al. 2015). However, despite the impressive amount of information available on AMPs of anurans, there are strikingly few studies on those of salamanders. Skin peptides have been harvested from only a few salamander species and show varied inhibition of several pathogens such as *Bd*, *Bsal*, *Escherichia coli*, and Ranavirus (Fredericks and Dankert 2000; Sheafor et al. 2008; Meng et al. 2013, Perieira et al. 2018; Smith et al. 2018).

Hellbenders are colloquially known as snot otters because of the copious volume of skin secretions they can produce while being handled, and these secretions are hypothesized to be an important component of Hellbender skin immunity. However, no published studies have yet evaluated what peptides may exist in Hellbender skin and whether or not they have any antimicrobial activity. Only one AMP has been characterized for the entire Cryptobranchid family (*A. davidianus*) and demonstrated anti-*Bd* and anti-bacterial activity (Pei and Jiang 2017). Given that *Bd* and *Bsal* both pose a conservation risk to Hellbenders, and various skin lesions are prevalent within Hellbender populations, we wanted to investigate if Hellbenders had skin peptides effective against both chytrid fungi and how that activity might change across regions of varying population status and across individuals of varying infection status.

Methods

Stream Collection

We sampled Hellbenders from 2015-2017 in seven streams and rivers from the Blue Ridge ecoregion of East Tennessee (ETN), Interior Plateau of Middle Tennessee (MTN), and Ozark Highlands of Arkansas (AR). We sampled in both Tennessee ecoregions for the Eastern Hellbender (*C. a. alleganiensis*) and in Arkansas for the Ozark Hellbender (*C. a. bishopi*). We implemented disease sampling protocols in coordination with ongoing population monitoring surveys. All sampling in Arkansas was conducted under observation of the USFWS permit TE66039A-0 issued to KJI and Arkansas Game and Fish Commission (Permits #Kelly) and with approval from Tennessee Wildlife Resources Agency (TWRA) (Permit #'s 1525,1529 (DM), 1505(MF), and 1877 (RHH)). Most of our sampling efforts were opportunistic, based on areas of population survey history, team availability, and stream conditions from late May to early August. We performed limited sampling between late August and mid-October to prevent disturbance of breeding and nesting. In TN, and shallower water in AR, we used standard snorkeling techniques to locate individuals. In AR, we also sampled Hellbenders via artificial nest boxes (Briggler and Ackerson 2012) and sampled deep water habitats (up to 4 meters) using a hookah dive system (i.e. gasoline powered air compressor with tethered air supply lines to dive regulators). We captured any animals encountered under cover objects and nest boxes and placed them in a clean soft cotton or mesh bag. We kept bags submerged in the river before and after animals were processed. (University of Tennessee IACUC protocol # 2481-0916). We changed dive gloves between animal captures to reduce pathogen transfer and contamination of pathogen samples.

Biometrics and Pathogen Sampling

Skin secretions were collected from a subset of Hellbenders surveyed for disease and amphibian pathogen prevalence (see Ch 1 & 2). Before collecting any samples, we placed each individual into a new, cleaned plastic tub. To sample for *Bd*, we ran a sterile cotton swab (Fisherbrand product# 23400111) five times over each body region in the following order: dorsum, ventrum, inguinal areas, and all portions of the feet including

toe-tips as described by Brem et al. (2007). Although the standard *Bd* swabbing protocol in frogs generally does not include the dorsum (see Brem et al. 2007), we included it because of unpublished reports of suspicious dorsal skin lesions associated with *Bd* infection in Hellbenders. We collected tissue samples for Ranavirus via a sterile punch biopsy (6mm x 3mm) or forceps and blade to remove a small tissue section of the dorsal tailfin near the tail-tip. We immediately placed swabs and tissue in sterile 1.5 mL microcentrifuge tubes with 70% ethanol for field storage and transport. After pathogen sampling, we recorded biometrics of total length (TL), snout-vent length (SVL), and Mass, and recorded presence of any lesions.

Skin Secretion Collection

After targeted disease samples were collected and biometrics recorded, we returned individuals to their respective container and rinsed them with approximately 200 mL sterile distilled water. We immediately rubbed a sterile cotton swab over the dorsal skin surface for 30 seconds and placed it in a sterile 1.5 mL tube on dry ice for later microbiome analysis (see Ch 4). We then allowed individuals to sit in the sterile water bath for 10 minutes to collect any skin secretions. We gently tilted and swirled he container on each side to run water over the entire skin surface at minute 0 and 5 to collect any secretions from the dorsal surface and to also cause slight agitation to stimulate release from granular glands. Previous studies in frogs have used subcutaneous norepinephrine injections to stimulate smooth muscle contraction and subsequent granular gland release. We chose not to attempt supraphysiological stimulation of granular gland release for two reasons: 1. We did not want to completely deplete individuals of defensive peptides prior to re-release into the wild, and 2.There is limited support for the efficacy of norepinephrine for recovery of skin peptides in salamanders (Sheafor et al. 2008). We poured water/ skin secretion mixture into aliquots of sterile 50 mL tubes, added 1 mL 6M HCL, and immediately placed them on dry ice until transport to our laboratory for storage in a -80°C freezer.

To enrich secretion samples for small cationic peptides we defrosted contents of a 50 mL aliquot and pumped slowly over a C-18 Sep-Pak filter (Waters Corp, Inc) following the manufacturer's instructions and protocol provided by Goraya et al. (1998). We eluted 11 mL of final peptide mixture in a new sterile 50 mL vial and removed 1 mL for immediate peptide quantification. The remaining 10 mL were dried over a spin-vac and stored at -20°C until use for growth inhibition assays. We used a Micro BCA™ Protein Assay Kit (ThermoFisher #23235) to quantify peptides at full strength, 1:10, and 1:100 dilutions against a bradykinin standard from 0.5 – 200 µg/mL. We used Gen5 software to calculate peptide concentrations absorbance at 570 nm using a Biotek reader. Based on concentration results we resuspended dried peptides in HPLC water to at least 2,000 µg /mL to obtain a minimum volume of 500 µL. We filter sterilized all resuspended peptides immediately before use in assays.

Chytrid Culture and Zoospore Harvest

For all assays we used a *Bd* culture from the JEL 197 strain maintained at 21°C in the Rollins-Smith laboratory at Vanderbilt University that was sub-cultured weekly to

biweekly. Three to five days prior to planned zoospore harvest, we inoculated three sterile 1% tryptone agar plates infused with streptomycin and penicillin with 2-3 mL of liquid *Bd* culture each. After incubation at 21°C we visually inspected plates under a dissecting microscope for healthy *Bd* activity and for any abnormal colony formation of potential contamination; if any plates had suspected contamination, we discarded them. We then flooded each remaining plate with 3 mL sterile 1% tryptone broth, immediately recollected the liquid, and pipetted over a 20 µM sterile filter attached to a flask and vacuum. We pipetted another 3 mL over each plate and let stand for 20 minutes, covered, before recollection. We flooded plates a third and final time with immediate recollection over the vacuum filter. This process promoted release of zoospores into liquid media with filter selection for only zoospores without large debris and zoosporangia. We transferred all filtrate into a sterile 15 mL conical vial and gently vortexed for ten seconds. From the homogenized mixture we removed 10 µL into a 1.5 mL vial and created a 1:10 dilution with 90µL sterile broth. We vortexed diluted mixture and pipetted 10 µL into the well of a standard hemocytometer. We counted all zoospores observed in two opposite large grids and used the average count to calculate zoospore/mL. From this calculation we created a stock mixture of $1x10^6$ zoospore/mL with sterile 1% tryptone broth. For *Bsal,* cultures were also maintained at the Rollins-Smith laboratory. We harvested zoospores identical to *Bd* with the following exceptions: We maintained *Bsal* cultures and plates at 16°C and used TGhL (1.6% tryptone, 0.4% gelatin hydrolysate and 0.2% lactose) broth for both flask cultures and agar plates. We found *Bsal* growth to be slower than *Bd* and harvested zoospores between 5-7 days post agar plate inoculation instead of the standard 3-5 days for *Bd*.

Growth Inhibition Assays (GIAs)

To quantify peptide *in vitro* activity from each individual, we performed growth inhibition assays (GIAs) of *Batrachochytrium dendrobatidis (Bd)* zoospores. For a subset of samples with enough recovered peptides, we also performed GIAs against *Batrachochytrium salamandrivorans* (*Bsal*). GIAs were performed in sterile 96-well microtiter plates. We pipetted 50µL of zoospores (at $1x10^6$/mL; 500,000 total cells) mixed with 50µL of one of five peptide concentrations of 50, 100, 200, 500, and 1000 µg /mL in HPLC water in triplicate. To obtain more robust inhibition curves from individuals with enough recovered peptides, we tested an additional concentration of 5000 µg/mL Positive control wells contained 50µL zoospores and 50µL sterile HPLC water whereas negative control wells contained 50µL of heat-killed zoospores (90°C for 10 minutes) with 50µL sterile HPLC water. We measured change in optical density at 490 nm between day 0 and 7 for *Bd* GIAs and day 0 and 9 for *Bsal* GIAs using Biotek plate reader and Gen 5 2.01 software. We calculated % zoospore growth for each well based on change in absorbance compared to positive controls. We visually inspected all wells to confirm zoospore activity (or lack thereof) and for contamination. If a well had contamination, we removed it from our analysis. We averaged triplicate results for a single value of % zoospore growth at each peptide concentration for each sample to use for analysis.

Statistical Analysis

We performed generalized linear mixed models (GLMM) (binomial distribution) in RStudio (R Core Team 2013) via the lme4 package (Bates et al. 2015) to test for effects of the following parameters on *Bd* zoospore growth at 500 µg/ mL skin peptide concentration During data exploration, we observed a bimodal distribution so we converted % zoospore growth into a binomial response (0/1) of weak (zoospore growth > 50%) or strong (zoospore growth <50%) inhibition. We tested fixed effects of Region (ETN, MTN, AR), Mass, *Bd* infection status (positive/negative), and presence/absence of toe lesions; we included year as a random effect. We did not include TL or SVL in our models because they were highly correlated with Mass. We used Mass as our representative index of size because it was a more reliable field measurement. We did not include sex as a predictor variable in GLMMs, as sexing Hellbenders is unreliable either in immature individuals or in mature individuals outside of the breeding season. We calculated z-scores for Mass prior to analysis. We evaluated relative model fit from AIC_c for all possible combinations of fixed effects to determine the top performing model via the AICcmodavg package (Mazerolle 2019) and considered models competitive when ΔAIC_c was < 2.0 (See Table 8). We model-averaged variables that appeared in one or more top models as recommended in Burnham and Anderson (2002) to determine relative variable contribution in those models.

We did not include Ranavirus status in our models because of low number of positive individuals (n=4), but since all positives were from the same site and sampling day we reported changes in zoospore growth between Ranavirus- positive and Ranavirus-negative individuals from that sampling event (n=6). To evaluate changes between anti-*Bd* and anti-*Bsal* activity we performed a paired samples Wilcoxon test on samples we performed both *Bd* and *Bsal* GIAs.

MALDI-TOF Mass Spectrometry

We performed Matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry on skin peptide samples from 19 individuals following the protocol from Holden et al. (2015). We prepared peptide samples at 1 mg/mL at a 1:1 v/v ratio of matrix (10 mg/mL α-cyano-4 hydroxycinnamic acid (Fluka, Sigma, St. Louis, MO, USA), 60% acetonitrile, 39.6% HPLC-grade water, and 0.4% trifluoroacetic acid (v/v/v)). We used an an Ultraflex III time-offlight mass spectrometer (Bruker Daltonics, Billerica, MA, USA) for delayed extraction, positive ion, reflector mode for collection of 250 laser shots per samples and analyzed and evaluated spectra from weights of 1000 to 4000 Da using Data Explorer v4.4 software. For each individual we created a dataset of all molecular weights present with a relative intensity > 10% with corresponding isotope cluster areas. We grouped each peptide mixture into zoospore inhibition categories of 1-5 with the following cutoffs: Category 1: 91-100% zoospore growth, Category 2: 76- 90%, Category 3: 26-75%, 4: 11-25%, and Category 5: 0-10%. We summed isotope area across all samples to determine the most relatively abundant peptide weights. We performed indicator species analysis using package indicspecies ((De Caceres and Legendre 2009) in R Studio to determine peptide weights that best associated with each inhibition category.

Table 8: GLMMs for Peptide Activity

List of Generalized Linear Mixed Models (GLMM) (binomial distribution) included in AICc analysis in order of smallest to largest ΔAICc. Models were based on Hellbender skin peptide inhibition of *Bd* zoospores at 500 µg/ mL compared to positive controls categorized into weak (51-100% zoospore growth) or strong (0-50% zoospore growth) inhibition. Top models of ΔAICc < 2.0 are noted in bold and were used for subsequent variable model averaging.

Model	Fixed Effects	Random Effect	K	AICc	ΔAICc	AICcWt
Mod2	**Toe**	**Year**	**3**	**46.35**	**0**	**0.36**
Mod6	**Toe + Mass**	**Year**	**4**	**47.72**	**1.38**	**0.18**
Mod3	**Toe + *Bd***	**Year**	**4**	**48.18**	**1.84**	**0.14**
Mod4	Toe + Region	Year	4	48.83	2.49	0.1
Mod5	Toe + Mass + Region	Year	5	49.44	3.1	0.08
Mod8	Toe + Mass + *Bd*	Year	5	49.83	3.48	0.06
Mod7	Toe + *Bd* + Region	Year	5	50.85	4.5	0.04
Mod1	Mass + Region + Toe + *Bd*	Year	6	52.04	5.69	0.02
Mod9	Region	Year	3	54.83	8.49	0.01
Mod11	*Bd* + Region	Year	4	56.05	9.7	0
Mod15	Mass + Region	Year	4	56.54	10.19	0
Mod10	Mass + Region	Year	4	56.54	10.19	0
Mod17	--	Year	2	57.27	10.92	0
Mod12	Mass + *Bd* + Region	Year	5	58.21	11.87	0
Mod13	Mass	Year	3	58.24	11.89	0
Mod16	*Bd*	Year	3	58.67	12.32	0
Mod14	Mass + *Bd*	Year	4	59.37	13.03	0

Results

Bd GIAs

We performed *Bd* GIAs from skin peptides collected from 39 Hellbenders (5 from AR, 10 from MTN, and 24 from ETN). Peptides from AR had limited inhibition of *Bd* with an average % zoospore growth at 500 µg/mL of 91.7% compared with 53.4% for MTN and 43.1% for ETN (Fig 7). Arkansas also did not exhibit a typical inhibition curve with an increase in zoospore growth from 25 to 1000 µg/ mL, at which point inhibition increased (Fig 8). We performed more complete inhibition curves on 16 animals (AR=5, MTN=5, ETN=6) with an additional concentration of 2500 µg/ mL and found almost all peptides from TN animals to completely inhibit zoospores by 2500 µg/mL (22.1% for MTN,6.6% for ETN) whereas those from AR on average inhibited only just over half of zoospore growth (growth at 46.9%) (Fig 8).

AICc evaluation of GLMMs of binomial inhibition (>50% vs <50% zoospore growth at 500 µg/mL) revealed three top models with ΔAIC_c <2, Toe-Only, Toe + Mass, and Toe + *Bd* (Table 8). We model-averaged three variables present in top models (Toe, Mass, and *Bd*) and found Toe to be significantly positively correlated with *Bd* zoospore growth (β= 3.45; C.I.: 0.97, 5.92) (Table 9). Peptides of Hellbenders with no toe lesions had on average 39.9% *Bd* zoospore growth at 500 µg/mL in comparison to peptides of Hellbenders with lesions (85.0%) (Fig 9A). Peptides from Hellbenders positive for *Bd* on average had weaker inhibition of *Bd* zoospores (57.8% growth) compared to peptides from negative individuals (53.2% growth), but this was not a significant effect in our GLMMs (β= 0.07; C.I.: -1.48, 1.63)(Table 9, Figure 9B). There was no significant effect of mass on peptide activity (β= -0.54; C.I.: -1.6, 0.52) (Table 9).

For evaluation of effects of Ranavirus infection on skin peptide activity we compared peptides from six individuals collected from the same site in ETN on the same day. Two individuals were Ranavirus-positive, two co-infected with Ranavirus and *Bd*, and two negative for both pathogens. Of this group, peptides from completely negative individuals and coinfected individuals displayed full inhibition of *Bd* zoospores at 500 µg/mL with (0.0% and 0.7% zoospore growth, respectively) in comparison to Ranavirus-positive individuals with incomplete inhibition (66.7%) (Fig 10).

Bsal GIAs

We performed additional *Bsal* GIAs from 21 of the above collected peptide samples (4 from AR, 7 from MTN, and 10 from ETN) (see Fig 7, 11). Paired samples Wilcoxon Rank Sum test showed Hellbender peptides significantly inhibited *Bsal* better than *Bd* with an average drop in zoospore growth at 500 µg/mL from 50.3% to 36.0% (p= 0.034). Peptides from AR had increased but still limited inhibition of *Bsal* zoospores (71.8% growth) compared to *Bd* (91.7% growth) and remained weaker in comparison to MTN and ETN. MTN showed the greatest differential between *Bd* and *Bsal* (53.4% and 17.8%, respectively), with little change between inhibition of chytrid species for ETN (43.1% and 34.5% *Bd* and *Bsal* growth, respectively) (Fig 7, 11). Only 3/21(14.3%) individuals had peptides that displayed weaker inhibition of *Bsal* and all were from ETN.

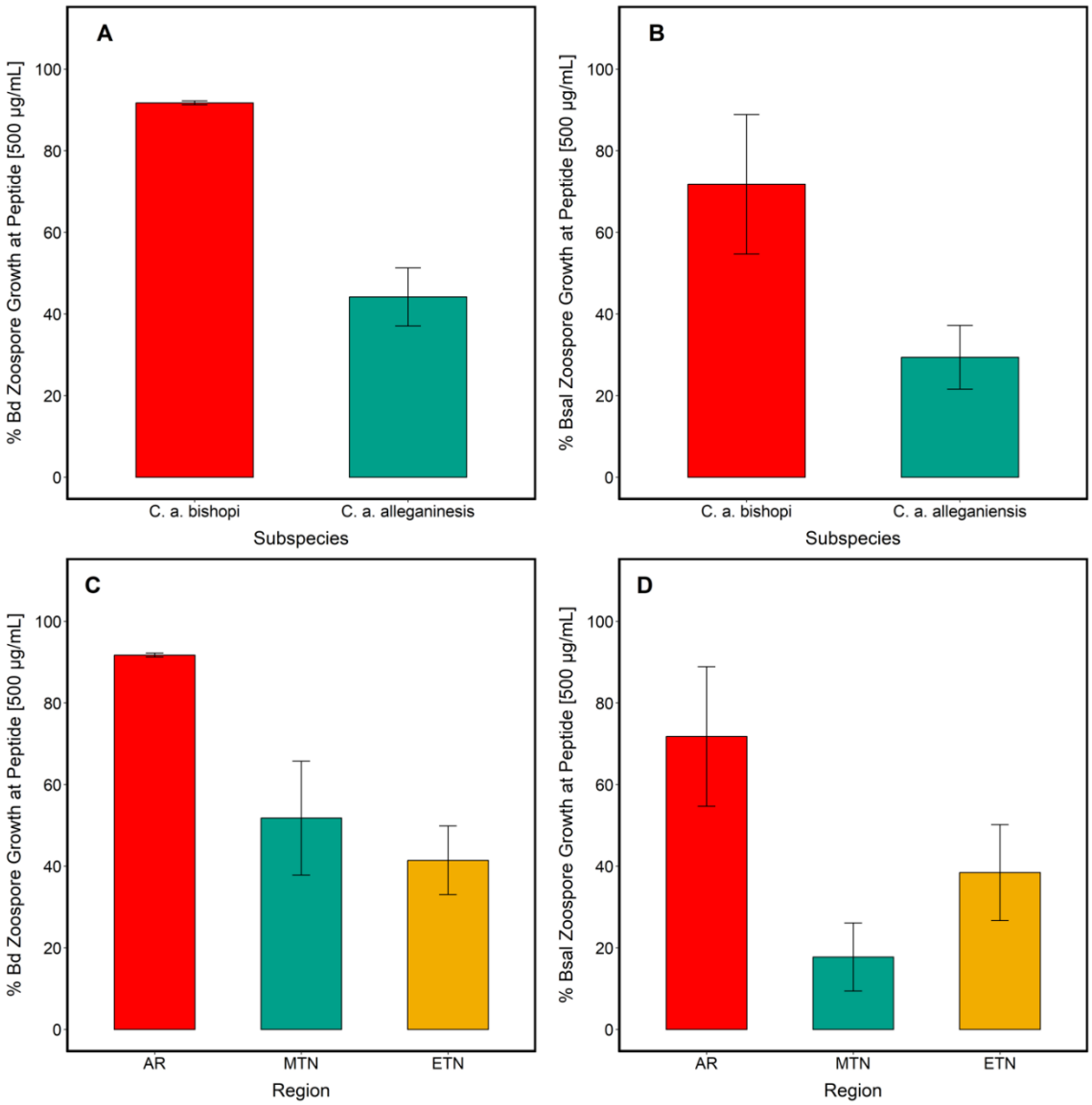

Figure 7. *Bd* and *Bsal* Zoospore Growth at 500µg/mL Peptides by Region

Bar graphs with standard error bars representing GIA results of % chytrid fungi (*Bd and Bsal*) zoospore growth at 500 µg/mL Hellbender skin peptide concentrations compared to positive controls. Note that a decrease in zoospore growth denotes an increase in zoospore inhibition. Variation in zoospore growth grouped by subspecies (A, B) and capture region (C, D). Growth of *Batrachochytrium dendrobatidis* (*Bd*) zoospores (n=39) shown in (A, C) and *B. salamandrivorans* (*Bsal*) (n=21) (B, D). Red indicates peptides collected from *Cryptobranchus alleganiensis bishopi* in Arkansas (AR), and teal / yellow represents peptides from *C. a. alleganiensis* in Tennessee regions of middle Tennessee (MTN) and eastern Tennessee (ETN).

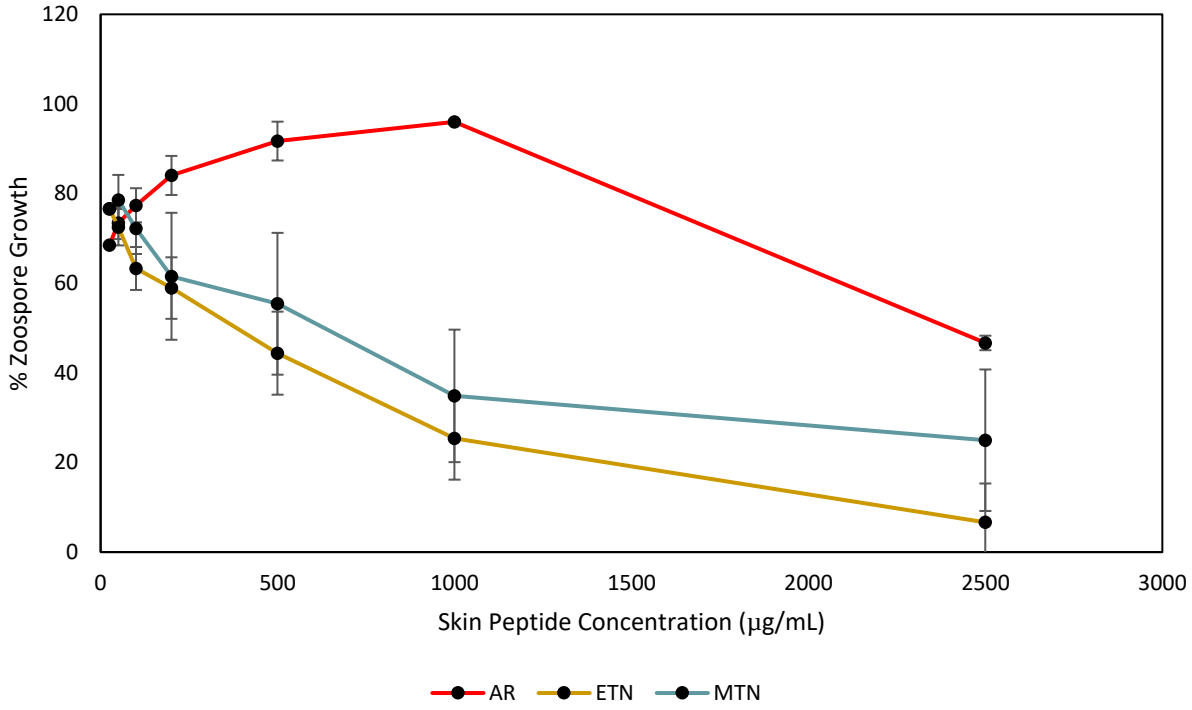

Figure 8. *Bd* Growth Inhibition Curves by Region

Line graph showing inhibition curves with standard error bars of *Bd* zoospore growth against increasing concentrations of skin peptides collected from both Hellbender subspecies (*C. a. bishopi* from Arkansas (AR; red), and *C. a. alleganiensis* from middle Tennessee (MTN; teal), and eastern TN (ETN; yellow). All individuals were tested up to 1000 µg/mL (n=39) with a subset tested out to 2500 µg/mL (n=21).

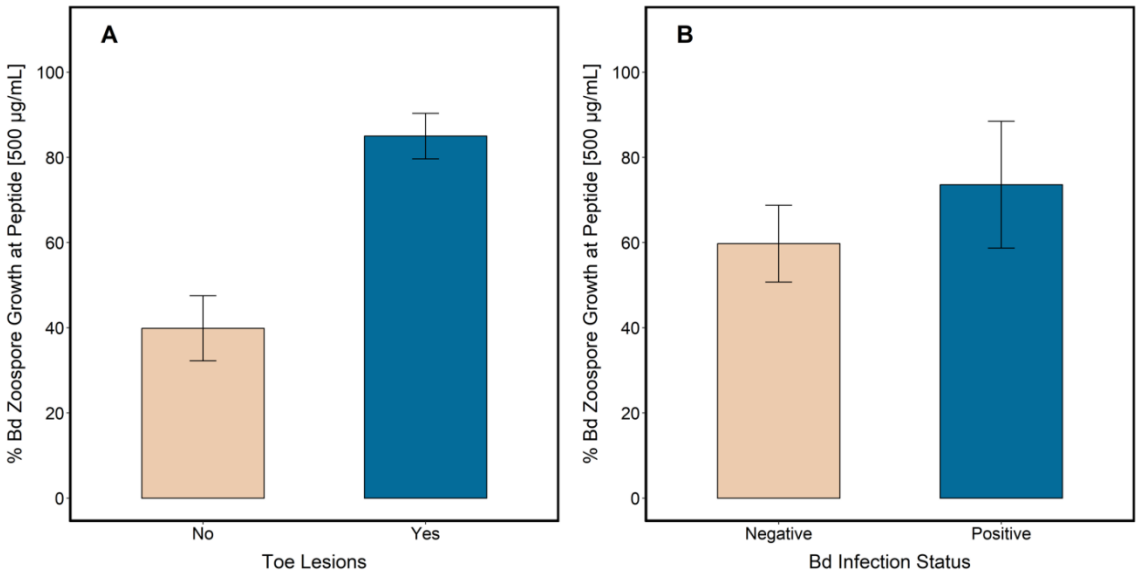

Figure 9. *Bd* Growth at 500 µg/mL Peptides by Lesion and *Bd* Infection Status

Bar graphs with standard error bars representing average % Batrachochytrium dendrobatidis (Bd), zoospore growth at 500 µg/mL Hellbender skin peptide concentrations compared to positive controls. Note that a decrease in zoospore growth denotes an increase in zoospore inhibition. Average growth compared between individuals with (85.0%; n= 10) and without (39.9%; n= 29) toe lesions present (A) and between individuals positive (57.8%; n= 14) or negative (53.2%; n= 21) for Bd via qPCR (B). GLMM AICc and model averaging results revealed a significant effect f toe lesion presence on peptide inhibition of Bd (β= 3.45; C.I.: 0.97, 5.92) but not for Bd infection status (β= 0.07; C.I.: -1.48, 1.63).

Table 9: Fixed Effects from Top GLMMs

Fixed effects variables in top models from GLMM. (ΔAICc<2) listed with model-averaged results. Significant effects (based on 95% C.I.) noted in bold.

Variable	β Coeff	S.E.	95% C.I.
Toe	**3.45**	**1.26**	**0.97, 5.92**
Mass	-0.54	0.54	-1.6, 0.52
Bd	0.07	0.79	-1.48, 1.63

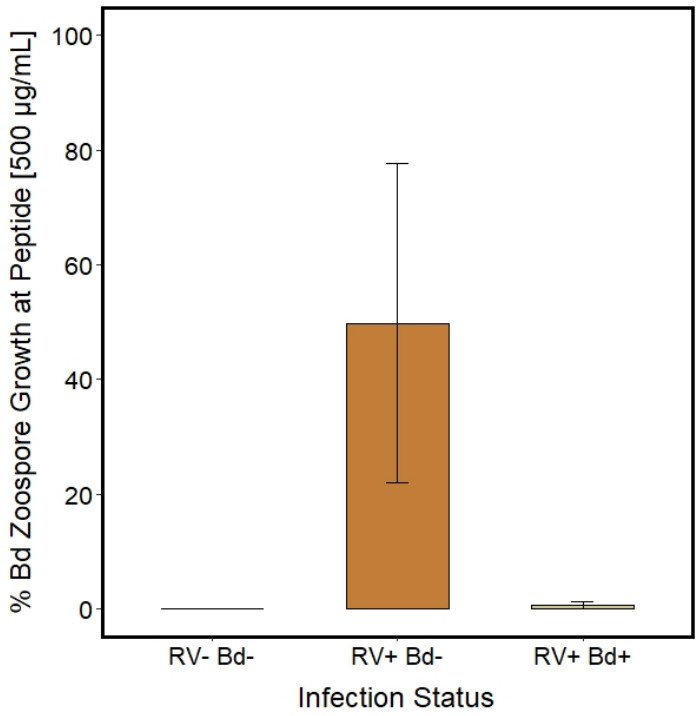

Figure 10. *Bd* Growth at 500µg/mL Peptides by Coinfection Status from a Single Site

Bar graph with standard error bars representing average % *Batrachochytrium dendrobatidis* (*Bd*) zoospore growth at 500 µg/mL Hellbender skin peptide concentrations compared to positive controls. Note that a decrease in zoospore growth denotes an increase in zoospore inhibition. Average growth compared between individuals collected from the same day at a single ETN site with varying infection status of both *Bd* and Ranavirus via qPCR of skin swabs and skin biopsy, respectively (n=6). Individuals infected with Ranavirus-only (RV+ *Bd*-) had marked decrease in zoospore inhibition (avg % growth = 49.7%; n=2) compared to those coinfected (RV+ *Bd*+) (avg % growth = 0.7%; n=2) or negative for both pathogens (RV- *Bd*-) (avg %growth = 0.0%; n=2).

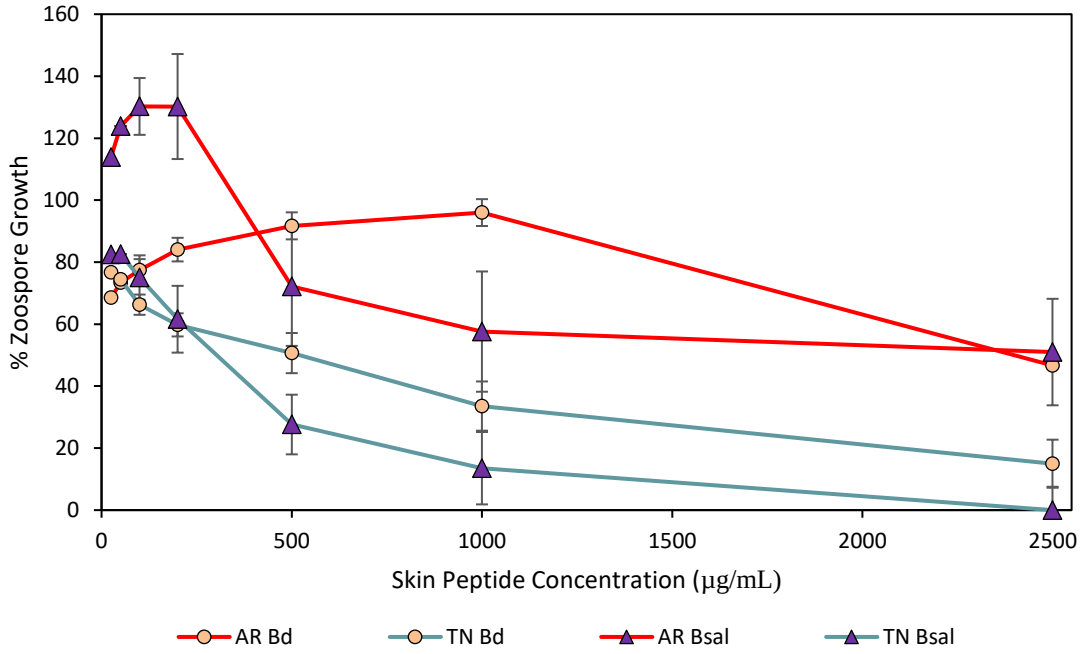

Figure 11. *Bd* and *Bsal* Growth Inhibition Curves by State (Subspecies)

Line graph showing inhibition curves with standard error bars of *Bd* (green circles) tested against Hellbender skin peptides from 25 to 1000 µg/ mL (n=39) with a subset tested out to 2500 µg/mL (n=21). Also shown are inhibition curves of *Bsal* (purple triangle; n=21) from 25 to 2500 µg/mL. Inhibition curves separated by subspecies (*C. a. bishopi* from Arkansas (AR; red) and *C. a. alleganiensis* from Tennessee (TN; teal)).

MALDI-TOF

We performed MALDI-TOF on 19 indivudals (see Fig 12 for sample profiles). We created a list of most frequent peptides with largest isotope cluster areas and found weights of 1530-1560 to be very abundant across all samples with 1547 to have the strongest relative intensity and isotope cluster area. (Table 10). Indicator species analysis revealed peaks of 8 molecular weights that best distinguished strongest inhibition of *Bd* zoospores (Category 5) from weakest inhibition (Category 1) (Table 11). Four of these candidate peptides were found exclusively and in all Category 5 samples. These samples were from individuals of four different rivers in both MTN and ETN.

Discussion

Hellbender skin peptides inhibited both *Bd* and *Bsal* zoospore growth. Wild Hellbender populations with *Bd* infections are recorded as early as 1969; however, no cases of chytridiomycosis were noted (Briggler et al. 2008; Bodinoff et al. 2011; Souza et al. 2012; Bales et al. 2015). This may be in part due to the anti-chytrid activity provided by the peptides we identified in skin secretions. In contrast, *Bd* is documented to cause chytridiomycosis and even mortality in captive hellbenders (Dean et al. 2016; Dusick et al. 2017), suggesting that skin peptides do not always provide sufficient protection against *Bd* zoospore proliferation, and environment conditions can affect production of skin secretions.

We found considerable variation in skin peptide activity between individuals. Ozark Hellbenders had skin peptides with weakest inhibition for both *Bd* and *Bsal* compared to Eastern Hellbenders of MTN and ETN. The Ozark Hellbender is a separate subspecies geographically isolated from Eastern Hellbenders, and this trend in anti-chytrid activity could be attributed to genetic or environmental difference. Furthermore, Ozark Hellbenders may be experiencing genetic bottlenecking from small remaining populations, as evidenced by their decreased immunogenetic diversity of MHC IIb (Hernandez-Gomez et al. 2018). If diversity of genes involved in adaptive immunity are reduced, those encoding products of the innate immunity, such as AMPs may also be affected. However, our GLMM results do not support either hypothesis. Region did not significantly explain inhibition and was not in the top models from AIC_c evaluation. Instead, presence of toe lesions was the only significant predictor of decreased inhibition. This would explain the apparent regional trend since all five Ozark Hellbenders sampled had toe lesions. Therefore, changes in peptide activity, even *in vitro* activity, could represent changes in host skin immunity.

AMPs can be differentially expressed in human skin affected by many factors including host health status and environmental stimuli. For example, one AMP, LL-37, can be upregulated in response to injury and accelerate wound healing (Niyonsaba et al. 2017). AMP expression changes after administration of glucocorticoids in Northern Leopard Frogs, *Lithobates pipiens,* (Tatiersky et al. 2017) and African Clawed Frogs, *Xenopus laevis* (Rollins-Smith et al. 2011), showing that a dynamic response of AMP activity also exists in amphibian skin.

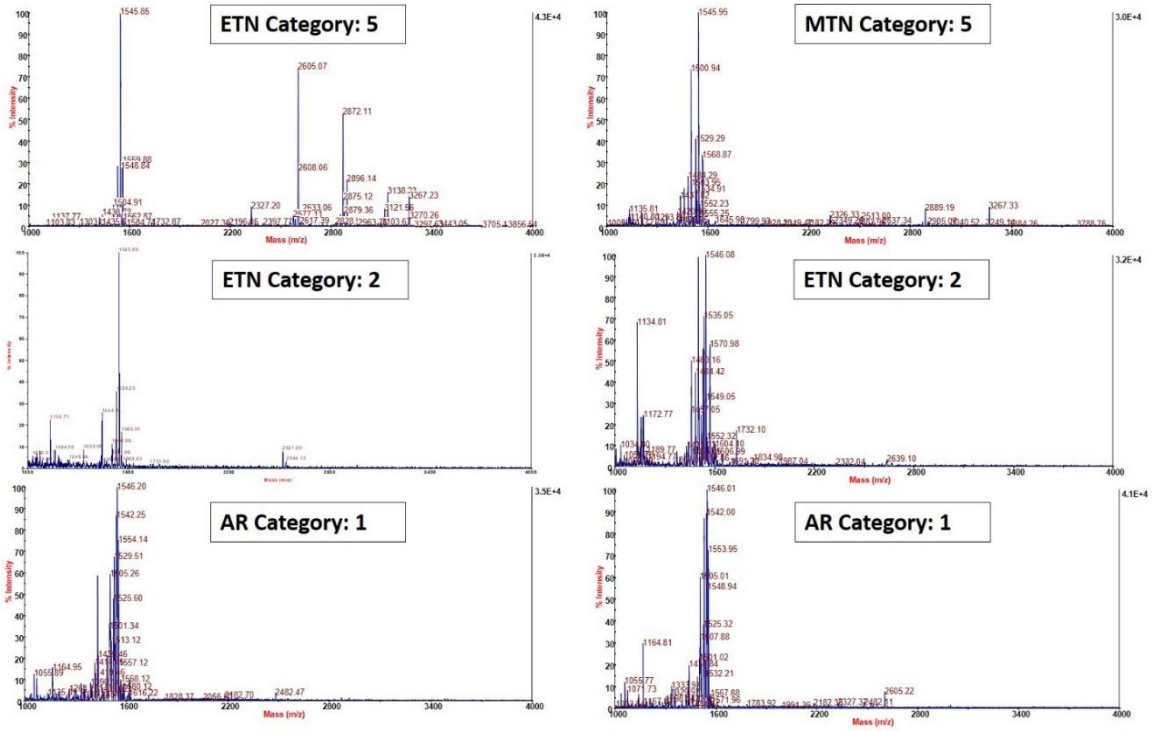

Figure 12. MALDI-TOF Profiles of Selected Individuals

MALDI-TOF profiles from 1000-4000 Da of six skin peptide samples collected passively from Hellbenders of eastern Tennessee (ETN), middle Tennessee (MTN), and Arkansas (AR). Categories are based on inhibition of *Batrachochytrium dendrobatids* at 500 µg/mL peptide concentration. A category of 1 denotes very little inhibition of *Bd* zoospore growth progressing to a category of 5 which denotes complete inhibition.

Table 10: Most Abundant Peptides by MALDI-TOF

List of peptides (identified by m/z) with largest average cluster area across all skin peptides collected from 19 Hellbenders individually analyzed via MALDI-TOF. Peptides are ranked by total average cluster area. Average cluster areas are also given for each inhibition category based on GIA results from 1-5. A category of 1 denotes very little inhibition of *Bd* zoospore growth progressing to a category of 5 which denotes complete inhibition.

Peptide	Average Cluster Area by Inhibition Category (1-5)					
Mass (m/z)	Total	**5** (n=6)	**4** (n=2)	**3** (n=4)	**2** (n=6)	**1** (n=4)
1547	448.4	533.9	722.0	53.7	504.5	493.8
1530	337.7	493.8	378.9	168.5	239.5	399.2
1529	327.2	491.8	311.0	163.7	197.4	446.4
1546	263.6	531.6	0.0	371.7	0.0	280.9
1506	196.3	230.2	265.7	65.6	159.4	297.0
1560	181.3	289.5	531.1	134.5	44.6	96.2
1543	161.3	98.2	335.3	19.2	57.8	466.2
1165	152.7	251.9	244.1	50.2	26.9	249.3
2605	139.7	323.4	393.8	31.7	0.0	54.8
1502	136.5	96.0	123.1	24.2	170.8	265.0
1439	135.0	171.4	286.2	103.4	0.0	238.8
1436	131.6	185.0	112.4	56.3	42.9	269.4
1504	124.9	183.6	133.5	0.0	57.3	258.8
1136	110.6	129.9	199.2	97.9	54.0	135.3
1122	106.8	322.0	34.0	75.7	7.9	0.0

Table 11: Indicator Peptides

List of candidate peptides identified by m/z from MALDI-TOF MS analysis of peptides collected from passive skin secretions of 19 Hellbenders and significantly associated with samples of complete inhibition (category 5). Inhibition categories were created by how well skin peptides at 500 µg/mL from each individual Hellbender inhibited *Bd* zoospore growth from greatest to least inhibition on a scale from 5-1 where Category 1 had only 0-10% growth, 2: 11-25%; 3: 26-75%; 4:76-90%; and 5: 91-100% growth.

Peptide Mass (m/z)	stat	p-value
2879	0.866	0.006
2896	0.866	0.013
1122	0.802	0.030
1105	0.791	0.037
2872	0.791	0.028

Therefore, changes in skin peptides in Hellbenders could be an indication of systemic disease manifesting as dysregulation of skin immunity. Recent studies in humans have proposed AMP dysregulation as a mechanism for manifestation of skin lesions in psoriasis and atopic dermatitis (Niyonsaba et al. 2017). Other AMPs are documented to have direct beneficial effects to wounds, including those isolated from more than one frog species (Demori et al. 2019). This provides a potential link between toe lesions and decreased anti-chytrid activity and supports peptide dysregulation as one mechanism for observed disease in Ozark Hellbenders.

Recovered peptides had greater inhibition of *Bsal* compared to *Bd*. This was an unexpected finding because Hellbender populations have had exposure to *Bd* for several decades whereas they are naïve to *Bsal* (Bales et al 2015) and *Bsal* is notorious for being particularly virulent to salamanders (Martel et al. 2014). In Fire Salamanders, *Salamandra salamandra*, a European newt species highly susceptible to *Bsal*, skin secretions were effective at killing both *Bd* and *Bsal* equally (Smith et al. 2018), whereas mucosome washes (skin products including peptides and larger proteins) had limited killing ability and were more determinant of susceptibility. Our results show that Hellbender skin secretions may provide innate immunity against *Bsal*, however, as with Fire Salamanders, other products in the skin may play a larger role in chytrid resistance, and mucosome assays from adults will be needed to better understand *Bsal* resistance in this species.

Surprisingly, *Bd* infection status did not significantly affect peptide anti-*Bd* activity. This was also an unexpected finding because studies in other free ranging species have shown variation in anti-chytrid activity associated with differences in *Bd* prevalence (Woodhams et al. 2006). Interestingly, in our evaluation of a small subset of Ranavirus-positive individuals from a single site, we found a potential interaction between how Ranavirus and *Bd* infection may affect a Hellbender's skin peptide production. Peptides from Ranavirus-positive individuals had marked decrease killing ability compared to those from Ranavirus-negative individuals, but anti-chytrid activity appeared to be restored in secretions from those also infected with *Bd*. Although these are not significant changes, they highlight potentially important host-pathogen interactions. Ranavirus is known to cause systemic change affecting fitness in developing tadpoles (Echaubard et al. 2010) and infection in adult Hellbenders is associated with decreased body condition (Ch 1). Weaker peptide activity observed from Ranavirus-only infected Hellbenders could represent a shift in AMP production as a result of systemic infection and provides further evidence that Ranavirus can have important sublethal effects in wild Hellbenders. Some AMPs from frogs can inactivate ranaviruses and reveal potential for these skin peptides in protection of ranaviral disease (Chinchar et al. 2001). Therefore, this shift in AMP activity may be a result from production of peptides more effective against viral agents as proposed to fungi. GIAs specific for ranavirus could help determine which mechanism is more likely. However, it remains unclear as to why coinfected individuals have restored anti-chytrid activity. One explanation may be that healthy Hellbenders normally produce anti-chytrid AMPs at sufficient concentrations for which *Bd* infection does not stimulate change; but when AMP production is disrupted during a Ranavirus infection, *Bd* presence may stimulate production of important

peptides back to concentrations appropriate to inhibit zoospore growth. More research will be needed to determine if these observations are repeatable.

Concentrations of 500 and 1000 µg/mL appeared to be thresholds for notable anti-chytrid activity. Most identified AMPs cause cellular damage to microbial organisms by reaching a threshold within the inner membrane leading to pore formation and membrane disruption (Brogden 2005). The 500-1000 µg/mL threshold we observed in Hellbenders likely represents the point at which one or more critical AMPs have reached that threshold on zoospore membranes. However, at lower concentrations, these same AMPs may interact with microbial organisms in different ways and may even stimulate growth. *Bsal* zoospores growing in peptides from Ozark Hellbender at low concentrations had marked increase in growth compared to positive controls. This may indicate that *Bsal* zoospores may be able to recognize salamander- specific skin peptides and respond by increasing growth rates as a potential adaptive mechanism for colonizing salamander skin. However, many other factors may also produce increased zoospore growth; when skin peptides do not achieve concentrations to cause damage, they may simply serve as an extra nutrient source.

Although we did not isolate and identify a true AMP, we have determined based on weight and isotope cluster some candidates for further investigation. All samples for which we performed MALDI had strong relative intensities for peaks of 1545-1547 m/z, and although this potential peptide likely plays an important role in Hellbender skin health, it does not explain differential inhibition of zoospores. However, peptide weights identified from indicator species analysis identified isotope clusters prominent in peptide mixtures with strong inhibition of *Bd* zoospores (categories 4 and 5) and absent in weaker inhibition categories. This association was observed regardless of capture region and even seen between individuals of differential inhibition from the same site and capture event.

Many unknowns remain about these potential AMPs. An AMP from the Chinese Giant Salamander, *Andrias davidianus* was isolated and shown to have anti-bacterial activity (Pei and Jiang), revealing that cryptobranchids do produce at least one AMP and there are likely more to be discovered. Are they produced and secreted from granular glands only? Because we collected peptides passively, a large proportion may not be from granular gland release and may be from baseline production within cells such as keratinocytes. Furthermore, Hellbenders host a rich bacterial community within the skin mucous environment (Hernandez-Gomez et al. 2017a).The skin microbiome is another part of amphibian skin immunity that is shown to be responsible for chytrid resistance (Woodhams et al. 2014), and part of this is attributed to products produced by the microbial symbionts. For instance, *Janthinobacterium lividum* is a symbiotic bacterium found in several frog species tolerant to *Bd* infection, which produces an antimicrobial compound, violacein (Pantanella 2007), and this compound is associated with increased host survival against *Bd* (Becker et al. 2009). Supernatants collected from other symbiotic bacteria from amphibian skin are shown to inhibit *Bd* (Daskin et al. 2014; Bell et al. 2018). AMPs have already been discovered to be produced by many bacterial species (Zhang et al. 2016), and it is therefore likely certain peptides on Hellbender skin may also be of bacterial origin.

Healthy amphibian skin is important to prevent dermal invasion of pathogens and our study shows that *in vitro* activity of skin peptides can vary within a species with varying skin health. Changes in pathogen dynamics and environmental stressors have potential to change amphibian skin health and more research is needed to understand how immune parameters in the skin can change with both individual and environmental factors. Further research should investigate activity of these proposed peptides found within Hellbender skin secretions to better understand which peptides drive anti-chytrid activity and if any can be associated with lesion presence. It will also be important to evaluate activity against other invading pathogens including Ranavirus to understand how this and other pathogens affect Hellbender health. This study highlights evaluation of Hellbender health and the dynamic threats from both pathogens and environmental change that will be important towards conservation of this unique stream predator with an uncertain future.

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CHAPTER IV
SKIN MICROBIAL COMMUNITY SHIFTS WITH SEVERITY OF SKIN LESIONS IN HELLBENDERS, *CRYPTOBRANCHUS ALLEGANIENSIS*

Abstract

Hellbenders (*Cryptobranchus alleganiensis*) are large aquatic salamanders from the eastern United States and have experienced considerable population declines in both Ozark and Eastern subspecies, *C. a. bishopi* and *C. a. alleganiensis*, respectively. Furthermore, ulcerative non-healing lesions have been observed with frequency in *C. a. bishopi*, and Arkansas populations are now reduced to a single river. Skin microbiomes are important components of skin health and have shown to contribute to immunity in amphibians, especially against the notorious chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*). There are only a few studies evaluating skin microbiomes of Hellbenders, none of which have included samples from Arkansas. We performed surveys of *C. a. bishopi* in Arkansas (AR) as well as a reference lesion-free and recruiting population of *C. a. alleganiensis* in eastern Tennessee (ETN) during summers of 2014-2015. We recorded biometrics, obtained a skin swab for *Bd*, and recorded lesion observations. After a sterile water rinse, we collected an additional dorsal skin-only swab for microbiome analysis. We performed qPCR for presence of *Bd* and used lesion severity scores to classify AR Hellbenders as having toe lesions to be mild, moderate or severe. We performed 16S amplicon sequencing from microbiome swabs and compared communities between Hellbenders populations (AR vs ETN) and within AR by lesion severity. We calculated the following metrics from averaged values of bootstrapped (x1000) subsamples of 1000 sequences: Hill numbers (q= 0-2) for alpha diversity and Bray-Curtis dissimilarity scores for beta diversity. We further calculated beta dispersion within each group. For taxonomic analysis we evaluated a single rarefied subsample for 1000 sequences to calculate % phylum abundance and perform indicator species analysis.

We found ETN Hellbenders to have greater bacterial diversity compared to AR, with this disparity decreasing as q order increased. Further, ETN bacterial skin communities were significantly different than those from AR, with AR communities displaying increased dispersion. Within AR, a trend of increased dispersion was observed with increased lesion severity. Taxonomic analysis revealed ETN to have greater OTU abundance of phylum Cyanobacteria_Chloroplast (24.4%) compared to AR (5.0%), and AR with increased abundance of Proteobacteria (55.4%), Firmicutes (8.1%), and Synergistetes (1.1%), in comparison to ETN (32.2%, 2.8%, 0.1%, respectively)

Results demonstrate that ETN Hellbenders have richer and less dispersed microbiomes compared to Ozark Hellbenders of AR. Although lesions are still of unknown etiology, this study is an initial step towards understand skin health in Hellbenders and its association with lesion severity and suggests that increased severity may be associated with host systemic change and skin dysbiosis.

Introduction

Hellbenders, *Cryptobranchus alleganiensis*, are fully aquatic riverine salamanders of the eastern United States and one of three species of giant salamanders (family: Cryptobranchidae). Both subspecies, the Ozark Hellbender, *C. a. bishopi* and Eastern

Hellbender, *C. a. alleganiensis*, have experienced considerable declines since the early 1990's resulting in listing of the Ozark Hellbender as endangered in 2011 (USFWS 2011). The eastern subspecies is estimated to have 77.9% of historical populations extirpated or in decline, but because the remaining 22.1% are considered relatively stable, it is not currently considered as a candidate for listing (USFWS 2018). Declines in both subspecies are linked to habitat degradation and sedimentation from eroding stream banks (Humphries and Pauley 2005; Hopkins and DuRant 2011; USFWS 2011), but disease is also hypothesized to play a role (Ch 2, 3; Nickerson et al. 2011). In Ozark Hellbenders of Arkansas, over 93% of individuals had progressive distal limb lesions that ranged from toe swelling to ulceration and digital necrosis (Ch 2; Nickerson et al. 2011). These lesions were likely linked to environmental factors, but a true etiology is unknown (Ch 1; Nickerson et al 2011).

Host factors are also hypothesized to contribute to these lesions. Hellbenders with toe lesions have changes in skin peptide *in-vitro* activity (Ch 3), and Ozark Hellbenders have decreased allelic diversity of genes important for adaptive immunity (Hernandez-Gomez et al. 2018). It is still speculative how changes in skin immunity may change with environmental factors. Several other parameters of skin function need to be evaluated to understand a likely multifactorial contribution to lesion manifestation.

The skin is the first line of defense against invading pathogens and despite a highly developed immune system, thousands of bacterial species are resident on all vertebrate skin surfaces (Grice and Serge 2011). Increasing evidence reveals the critical role these micro-organisms play in skin maintenance (Rodriquez Hoffman et al. 2015) and host immunity (de Vos and de Vos 2012; Arnolds and Lozupone 2016). Alterations in microbiomes are further associated with important diseases in humans (Ilseung & Blaser 2012) and companion animals (Rodriguez Hoffman et al. 2014). As we continue to learn more about this complex ecosystem on host surfaces, we are discovering its importance as both a medical treatment tool and indicator of host health (Shreiner et al. 2015; Kang et al. 2017).

Evaluation of altered skin microbiomes may be especially important in amphibians. They rely on their skin for osmotic regulation, which is subsequently more permeable than other vertebrate hosts (Shoemaker and Nagy 1977). Microbiome shifts may be major factors in amphibian host susceptibility to pathogens such as amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), which is a fungal pathogen responsible for an estimated 90 extinctions worldwide (Scheele et al. 2019). For instance, wild populations of the Mountain yellow-legged frog, *Rana muscosa*, have variable resistance to chytridiomycosis associated with differences in anti-*Bd* bacteria on the skin (Lam et al. 2010). *Plethodon cinereus*, a terrestrial salamander that is resistant to *Bd*, also harbors skin bacteria known to have potent anti-*Bd* activity (Brucker et al. 2008; Becker and Harris 2010). Skin bacteria isolated from other amphibian species also inhibited *Bd* in vitro (Becker and Harris 2010; Woodhams et al 2014), as well as prevent chytridiomycosis in experimental challenges (Becker et al. 2011) (reviewed in Rebollar et al. 2016).

Microbial communities, just like macrofaunal communities, are dynamic and respond to changes in their environment. On a global scale, amphibian skin microbial

community metrics are linked to climate and biosphere (Kueneman et al. 2019; Jani 2019). In terrestrial woodland salamanders, environmental bacterial assemblages were more influential on skin communities than species type (Fitzpatrick and Allison 2014). Bacterial communities on frogs in Madagascar experienced similar trends of strong associations with microhabitat type (Bletz et al. 2017). Furthermore, associations between environmental degradation and altered microbial communities were revealed in wildlife species such as black howler monkeys (Amato et al. 2013), and invertebrates such as coral (Krediet et al. 2013), which further supports important links between external environment and skin microbiome.

Intrinsic factors related to the amphibian host are also important to colonization and maintenance of communities (Jimenez and Sommer 2017). Frog assemblages in temperate ponds were found to have skin microbiomes that cluster with species, regardless of site. (McKenzie et al. 2011). Furthermore, experimental challenges of repeated bacterial washes on the skin of healthy amphibian hosts revealed maintenance of original microbial communities and resistance to colonization (Küng et al. 2014). Microbiomes, therefore, appear to associate with their host, but can be further shaped by the surrounding environment (Jani and Briggs 2018). If changes from either host or environment are maintained, communities may experience permanent shifts leading to decreased microbial stability and ecosystem function (dysbiosis), which further affects host surface immunity (Glasl et al. 2016).

This circular dynamic of host, microbiome, and environment is complex but important to consider during evaluation of skin disease in any vertebrate. In humans, microbial community alterations are also associated with chronic skin wounds (Grice and Segre 2012; Scales et al. 2012) and chronic skin diseases such as psoriasis (Thio 2018), which suggests that communities may provide more than just antimicrobial activity towards skin health. However, there is a paucity of research on how microbiomes may be related to wounds and skin immunity in amphibians.

To understand the role these microbiomes play in skin health of Hellbenders, we need to first understand how they are associated with both environmental and host parameters. Previous research has revealed that skin microbiomes of Eastern Hellbenders vary between rivers, however, small sample size limited statistical power to understand the extent of variation within each site (Obed-Hernandez et al. 2017a). In addition, microbiomes between foot lesions and dorsal skin of Ozark Hellbenders showed an increased richness in wounds but not a significantly different microbial community (Hernandez et al. 2017b). These studies are a first step in evaluating skin microbiomes of Hellbenders, but it is still unknown how Hellbender skin microbiomes vary within populations of a single river and how these communities may be associated with health. Toe lesions of Ozark Hellbenders are likely a product of a multifactorial process, and more information is needed on how skin microbiomes may be associated with not only presence but also severity of lesions. Therefore, we evaluated skin microbiomes of Hellbender populations from two rivers of different conservation status: a stable population of Eastern Hellbenders in eastern Tennessee (ETN) and a declining and diseased population of Ozark Hellbenders in Arkansas (AR). We evaluated microbial

community differences within and between these systems and if variation in AR could be explained by lesion severity.

Methods

We sampled Hellbenders from 2014-2015 in two rivers, one from the Blue Ridge ecoregion of East Tennessee (ETN), the other in the Ozark Highlands of Arkansas (AR). We sampled in ETN for the Eastern Hellbender (*C. a. alleganiensis*) and in AR for the Ozark Hellbender (*C. a. bishopi*). All sampling in AR was conducted under observation of the USFWS permit TE66039A-0 issued to KJI and Arkansas Game and Fish Commission (Permits #Kelly) and in Tennessee with approval from Tennessee Wildlife Resources Agency (TWRA) (Permit # 1877 (RHH)). Sampling occurred from mid-July to mid-August. In TN, and shallower water in AR, we used standard snorkeling techniques to locate individuals. In AR, we also sampled Hellbenders via artificial nest boxes (Briggler and Ackerson 2012) and sampled deep water habitats (up to 4 meters) using a hookah dive system (i.e. gasoline powered air compressor with tethered air supply lines to dive regulators). We captured any animals encountered under cover objects and nest boxes and placed them in a clean soft cotton or mesh bag. We kept bags submerged in the river before and after animals were processed. (University of Tennessee IACUC protocol # 2481-0916). We changed dive gloves between animal captures to reduce pathogen transfer and contamination of samples. Skin microbiome samples were collected from a subset of Hellbenders surveyed for disease and amphibian pathogen prevalence (see Ch 1 & 2). All individuals encountered from AR for microbiome analysis had toe lesions and received a lesion score based on severity. Lesion scores were created for a separate study quantifying toe lesions of AR and each Hellbender received a score from 0-7 ((See Ch 2 for scoring system methods). Because of low sample size within AR and subsequent inability to compare across all 8 lesions scores, we binned scores of 0-7 into three biologically relevant severity levels of mild (score 0-2; toe swelling only), moderate (3-4; toe swelling and some shortened) and severe (5-7; most toes shortened and/or missing).

We placed each individual into a new, cleaned plastic tub and rinsed them with approximately 200 mL sterile distilled water. We immediately rubbed a sterile cotton swab (Fisherbrand product# 23400111) over the dorsal skin surface for 30 seconds and placed it in a sterile 1.5 mL tube on dry ice for transport to a -80°C freezer.

16S Sequence Preparation

We extracted DNA from skin swabs using ZR Fungal/ Bacterial DNA Miniprep kits (Zymo Research Corporation, Irvine, CA, USA) and sent extracted DNA to Hudson Alpha (Huntsville, AL) for 16S rDNA amplicon sequencing of the V3-V4 region of 350 bp length. Bioinformatic preparation of the resulting sequence data was performed in the program MOTHUR, following the MiSeq protocol (Schloss et al. 2009). We created an additional oligo file to recognize our particular primer specific for the V3-V4 region which was different than the program default. We assigned operational taxonomic units

(OTUs) to each sequence, using a phylotype approach based on the silva v128 reference database. We removed rare sequences (< 5 total or occurring in < 3 samples). We also removed samples with less than 4000 sequences based on cutoffs determined from frequency histogram of sequence populations.

To compare alpha diversity among samples we used packages vegetarian (Charney and Record 2012) and vegan (Oksanen et al. 2015) in RStudio (R Studio Team 2016) to calculate an averaged bootstrap subsample of1000 sequences from 1000 iterations for the following Hill numbers: relative richness (q=0), exponential of Shannon's index (q=1) and the inverse Simpson's index (q=2). We used Hill numbers as opposed to more traditional alpha diversity metrics as they provide better evaluation of both richness and evenness (Chao et al. 2014). We performed the same bootstrap technique to calculate beta diversity distance matrices of Bray-Curtis dissimilarity.

Statistical Analysis

We performed all statistical analyses in package vegan (Oksanen et al. 2019) using RStudio (R Studio Team 2016). We performed either a one-way ANOVA or Kruskal-Wallis rank sums based on Shapiro-Wilks test of normality to test differences in Hill numbers order q0, q1, and q2 for the following comparisons: Subspecies (Eastern Hellbenders of ETN vs Ozark Hellbenders of AR), and lesion severity (mild, moderate, or severe) within AR only. We performed adonis tests (999 permutations) to test for effects of the same groups on beta diversity (Bray-Curtis). We also quantified beta dispersion (community consistency among individuals) by calculating the multidimensional area of minimum convex polygons fit to clusters of subspecies and lesion severity using 'betadisp' function based on distances created from PCoA analysis from previously calculated Bray-Curtis values. We performed PERMANOVAs to test for statistical differences in beta dispersion between subspecies and lesion severity groups.

Community Visualization

For visualization of alpha diversity differences between skin microbiomes from Eastern Hellbenders and Ozark Hellbenders of varied lesion severity, we created boxplots for each Hill number by either subspecies or by within-AR lesion severity of Ozark Hellbenders (mild, moderate, severe) via package ggplot2 (Wickham 2016). For visualization of beta diversity between these same groups we created NMDS plots based on Bray-Curtis distance matrices via packages vegan and ggplot2.

Taxonomic-based Analysis

We calculated and graphed OTU % abundance by phylum for a single subsample of 1000 sequences for each sample and compared within subspecies and toe lesion severity in package phyloseq (McMurdie and Holmes 2013). We identified significant indicator OTUs for each subspecies as well as each lesion severity level within AR via the indicspecies package (De Caceres and Legendre 2009).

Results

We sampled 63 individuals, 37 from AR and 26 from ETN. All animals sampled were adults of unknown sex except two of subadult size class from ETN. We recovered 2,804,796 sequences assigned to 911 OTU phylotypes after sequence preparation in MOTHUR. Sequence counts per sample ranged from 3 to 379,352. Subsampling and sequence removal resulted in subsequent removal of 25 samples with less than 4000 sequences, with 42 samples remaining for analysis (17 AR and 25 ETN). Within the 17 AR samples, two were from individuals with mild toe lesions, six with moderate lesions, five with severe lesions, and four without an assigned lesion severity score. Two individuals with severe toe lesions (Severe #2 and Severe #3) had very dispersed outlier microbiomes and were removed from all analyses and NMDS plots to compare among the remaining sample pool (n=40).

Alpha Diversity

Eastern Hellbenders of ETN had greater relativized richness (Hill number q=0) ($\bar{X}$=140.00 +/- SD 17.79) than Ozark Hellbenders of AR ($\bar{X}$=111.38 +/- SD 23.94) (p=.0003) (Fig 13, Table 12). Hill numbers order q=1 and q=2 were also greater for Eastern compared to Ozark Hellbender skin communities, but disparity between groups decreased with increasing order (p= 0.24 and 0.71, respectively) (Fig 13, Table 12). Within AR, relativized richness was greatest for individuals with mild lesion severity (mild=138.74 +/- SD 11.04) and lowest for those with moderate lesions (122.63 +/- SD 27.42) with those from sever lesion slightly greater than moderate (135.06 +/- SD 23.22) (p=0.25) (Fig 13, Table 13). Opposite for subspecies comparisons, disparity between communities of mild and moderate/severe lesion groups increased with higher q orders with the ranking remaining as mild>severe> moderate (see Fig 13, Table 13)

Beta Diversity

Beta diversity was significantly different between subspecies based on Bray-Curtis (p=0.001), but not significantly different between lesion severity groups within Ozark Hellbenders (p=0.168). Beta dispersion was also significantly greater for AR compared to ETN (p=0.002) (Fig 15). Within AR, dispersion increased with increasing severity, but was not significant (p=0.322) (Fig 15). NMDS plots of Bray-Curtis revealed grouping between subspecies and was in concordance with increased skin microbial community similarity (decreased dispersion) within ETN Hellbenders compared to those of AR (Fig 14). NMDS of lesion severity groups show increased dispersion despite small sample size (n=11).

Taxonomic

For taxonomic analysis we compared groups based on 473 OTUs remaining from a single regenerated 1000 sequence rarefied subsample for each of 42 samples. We identified phylotypes from 20 bacterial phlya (Table 14). Ozark and Eastern Hellbenders shared 325 out of 473 OTUs, with 110 unique to Eastern Hellbenders and 38 unique to

Table 12: Alpha Diversity between Subspecies

Alpha diversity results based on Hill numbers for each order (q=0,1,2) for each subspecies of Eastern Hellbenders of eastern Tennessee (Eastern) and Ozark Hellbenders of Arkansas (Ozark). P-values were calculated from either Kruskal-Wallis or ANOVA based on Shapiro-Wilk test for normality.

Hill Number q order	Test	p-value	Eastern (avg +/- SD)	Ozark (avg +/- SD)
0	Kruskal-Wallis	0.0003	140.00 +/- 17.79	111.38 +/- 23.94
1	ANOVA	0.24	45.39 +/- 13.51	40.25 +/- 12.30
2	Kruskal-Wallis	0.71	19.03 +/- 8.92	20.1 +/- 8.49

Table 13: Alpha Diversity between Lesion Severity Groups within AR

Alpha diversity results based on Hill numbers for each order (q=0,1,2) for Ozark Hellbenders of Arkansas with mild, moderate, or severe toe lesions. P-values were calculated from either Kruskal-Wallis or ANOVA based on Shapiro-Wilk test for normality.

Hill Number q order	Test	p-value	Mild (avg +/- SD)	Moderate (avg +/- SD)	Severe (avg +/- SD)
0	ANOVA	0.25	138.74 +/- 11.04	122.63 +/- 27.42	135.06 +/- 23.22
1	ANOVA	0.01	47.41 +/- 11.96	39.05 +/- 12.35	39.07 +/- 13.50
2	Kruskal-Wallis	0.10	21.38 +/- 10.75	17.00 +/- 7.26	22.66 +/- 8.93

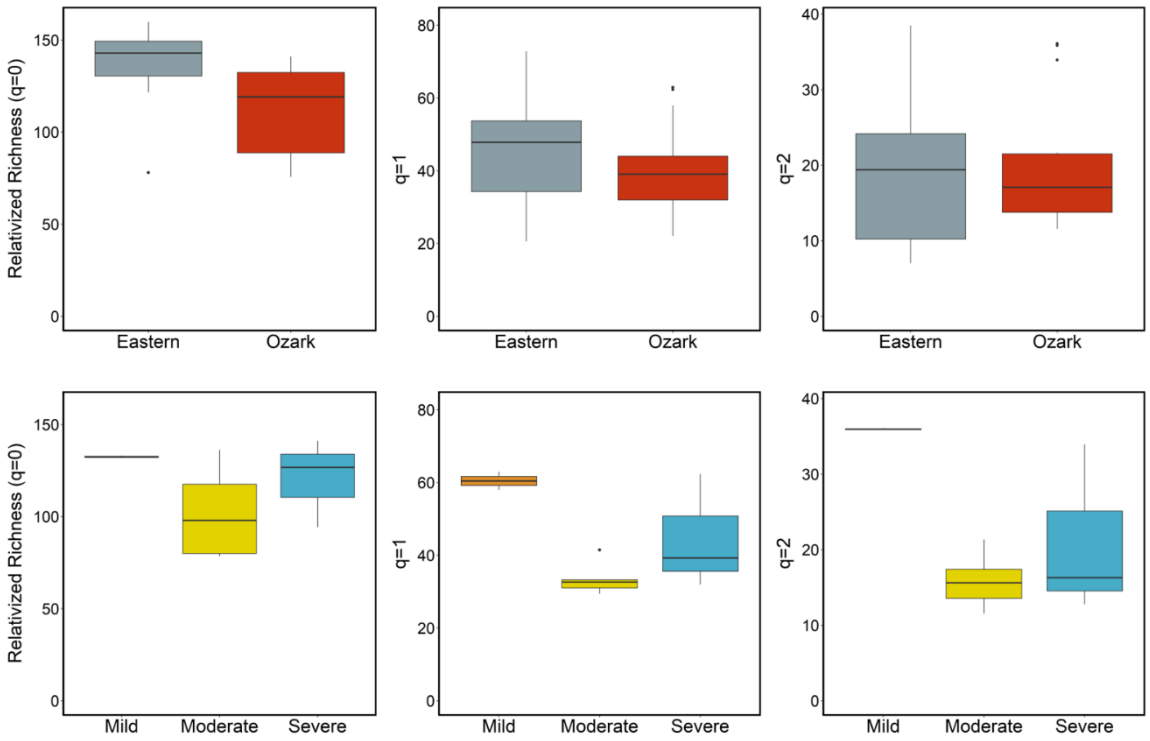

Figure 13. Alpha Diversity

Alpha diversity comparison using Hill numbers from order q=0 (relativized richness), q=1 (Exponential of Shannon Index) and q=2 inverse Simpson. Comparison between Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis)* of eastern Tennessee and Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) of Arkansas in top row. Comparison within Ozark Hellbenders with varying toe lesion severity (mild moderate and severe) in bottom row.

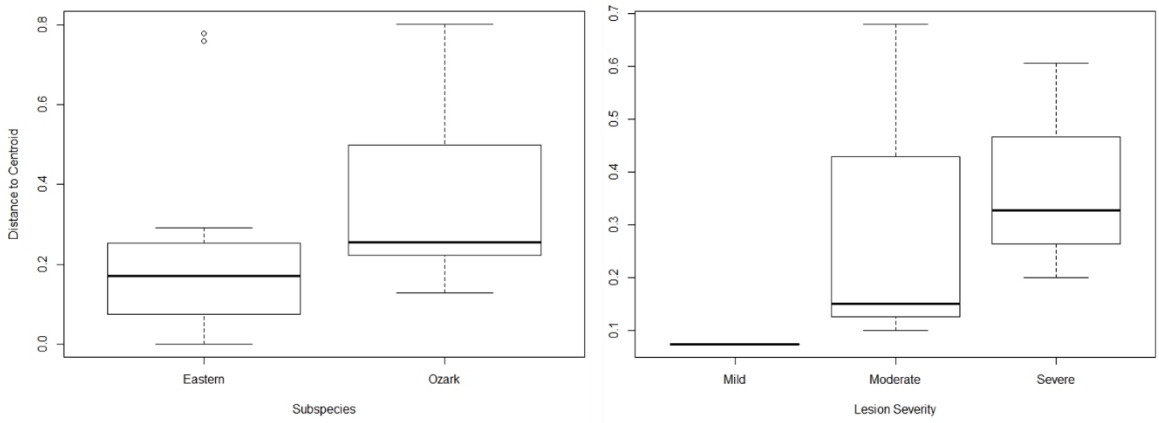

Figure 14. Beta Dispersion

Box plot illustrating beta dispersion of Hellbender skin microbiomes by subspecies of Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis)* of eastern Tennessee and Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) of Arkansas (left), and within Ozark Hellbenders with toe lesions observed to be mild, moderate, or severe (right).

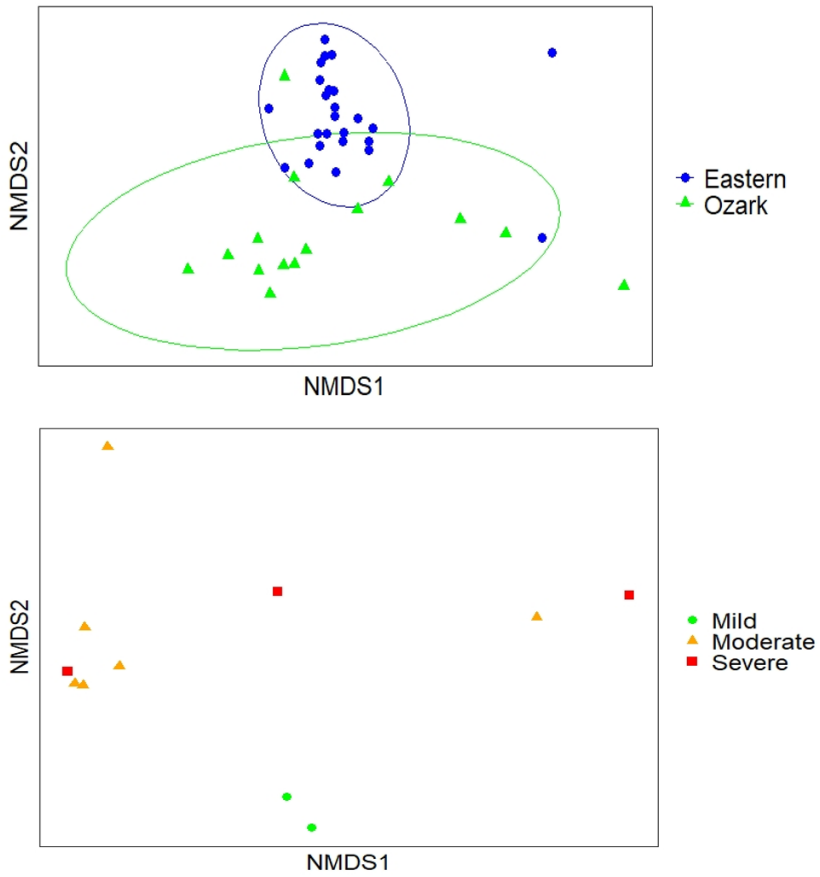

Figure 15. NMDS of Bray-Curtis Dissimilarity

NMDS ordination plots of hellbender skin microbial communities based on Bray-Curtis dissimilarity. Each point represents a single skin bacterial community sample. Top plot grouped by subspecies of Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis)* of eastern Tennessee (blue) and Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) of Arkansas (green) with associated ellipses base on standard error. Bottom is Ozark Hellbenders only grouped by toe lesion severity of Mild (green), Moderate (orange), or severe (red).

Table 14: Phylum % Abundance

Average % phylum abundance listed by group of Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) of eastern Tennessee (Eastern) and Ozark Hellbenders (*Cryptobranchus alleganiensis bishopi*) of Arkansas (Ozark). Ozark Hellbenders a further separated by severity of toe lesions of mild, moderate, and severe.

Phylum	Eastern	Ozark	Mild	Moderate	Severe
Spirochaetes	0.17	0.03	0.10	0.05	0.00
Cyanobacteria_Chloroplast	24.44	5.02	8.55	4.35	3.18
Armatimonadetes	0.98	0.22	0.20	0.28	0.18
Nitrospira	0.14	0.04	0.10	0.00	0.04
Gemmatimonadetes	0.16	0.04	0.15	0.00	0.02
WS3	0.18	0.05	0.10	0.02	0.08
OD1	0.78	0.27	0.95	0.23	0.16
Acidobacteria	2.62	1.17	2.15	1.57	0.86
Chlorobi	0.01	0.01	0.05	0.00	0.00
Verrucomicrobia	3.83	1.88	4.40	2.22	1.44
Planctomycetes	4.33	2.35	5.95	3.12	1.44
Chlamydiae	0.22	0.14	0.70	0.05	0.08
Bacteria_unclassified	7.39	4.90	6.80	6.88	2.54
Chloroflexi	0.56	0.43	0.75	0.50	0.36
Bacteroidetes	11.91	10.27	18.70	7.87	9.16
Actinobacteria	4.15	4.01	5.55	5.13	2.14
TM7	0.86	0.91	0.65	1.42	0.28
BRC1	0.02	0.02	0.15	0.02	0.00
Deinococcus-Thermus	1.79	2.63	2.35	2.50	1.44
Proteobacteria	32.24	55.38	40.10	55.83	67.72
SR1	0.02	0.04	0.05	0.05	0.04
Lentisphaerae	0.14	0.34	0.00	0.60	0.08

Table 14. Continued.

Phylum	Eastern	Ozark	Mild	Moderate	Severe
Firmicutes	2.76	8.15	1.20	5.38	7.32
Fusobacteria	0.17	0.58	0.25	0.30	0.18
Synergistetes	0.12	1.03	0.00	1.48	1.22
Deferribacteres	0.00	0.04	0.00	0.08	0.00
Elusimicrobia	0.00	0.03	0.00	0.03	0.00
Fibrobacteres	0.00	0.00	0.00	0.00	0.00

Ozark Hellbenders. Between lesion severity groups of Ozark Hellbenders of AR, 23 OTUs were unique to animals with mild lesion, 51 to moderate, and 34 to severe. Eastern Hellbenders had on average greater OTU abundance of phylum Cyanobacteria_Chloroplast (24.4%) compared to Ozark Hellbenders (5.0%) (see Fig 16,17, Table 14). Ozark Hellbenders had a greater average relative abundance of Proteobacteria (55.4%), Firmicutes (8.1%), and Synergistetes (1.1%), in comparison to Eastern Hellbenders with (32.2%, 2.8%, 0.1%, respectively) (Fig 16,17, Table 14). This increased abundance in the latter two phyla was driven by moderate and severe lesion groups (Fig 16,18,Table 15). Skin communities of Ozark Hellbenders with mild lesions had very low abundance of Firmicutes and no OTUs from Synergistetes, similar to Eastern Hellbenders (1.2% and 0.0 %, respectively) (Fig 17,Table 14). Further evaluation of these phlya reveal the greater abundance of Synergistetes in moderate and severe lesion animals to be attributed to one OTU of the genus *Cloacibacillus*, and in Firmicutes to one unclassified Clostridiales.

Indicator species analysis produced 32 OTUs from 13 phlya significantly distinguishing Eastern Hellbender skin communities and only six OTUs from three phlya for Ozark Hellbenders (Table 15). Within Ozark Hellbenders,14 OTUs were indicative of individuals with mild toe lesions, two for those with moderate lesions, and none for those with severe lesions (Table 16). Five of the 14 OTUs distinguishing mild lesions within AR where also indicators for Eastern Hellbender skin. Although no indicator taxa were significant for individuals with severe toe lesions, microbiomes from the two outlier individuals with severe toe lesions (Severe #2 and Severe #3) were dominated (>90%) by one OTU from genus *Mesorhizobium,* phylum Proteobacteria (Fig 16).

Discussion

Skin microbiomes of ETN were significantly different from those of AR based on beta diversity analysis which was further illustrated by NMDS ordination. This is not surprising since host populations are from different streams of different ecoregions and are separate subspecies. Furthermore, the ETN Hellbender population we sampled is considered healthy and stable and surrounded by forested land whereas the AR Ozark population is in danger of extirpation with little surrounding forest buffer. Therefore, several factors including both environment and host genetics may contribute to these differences as has been hypothesized in a previous study by Hernandez-Gomez et al. (2017). Alpha diversity at all three Hill numbers orders were greater for Eastern Hellbenders compared to Ozark Hellbenders, but was only significant for relativized richness (q=0). This indicates that difference in diversity between these subspecies is dominated by presence of relatively rarer OTUs, even after subsampling and removing rare OTUs ($n < 5$). Interestingly, these findings of greater microbial diversity on the skin of Eastern Hellbender is opposite to what is reported in the only two previous studies comparing Eastern and Ozark Hellbenders, both of which are from samples within Missouri populations (Obed-Hernandez et al. 2017a, b). Eastern Hellbenders span a large

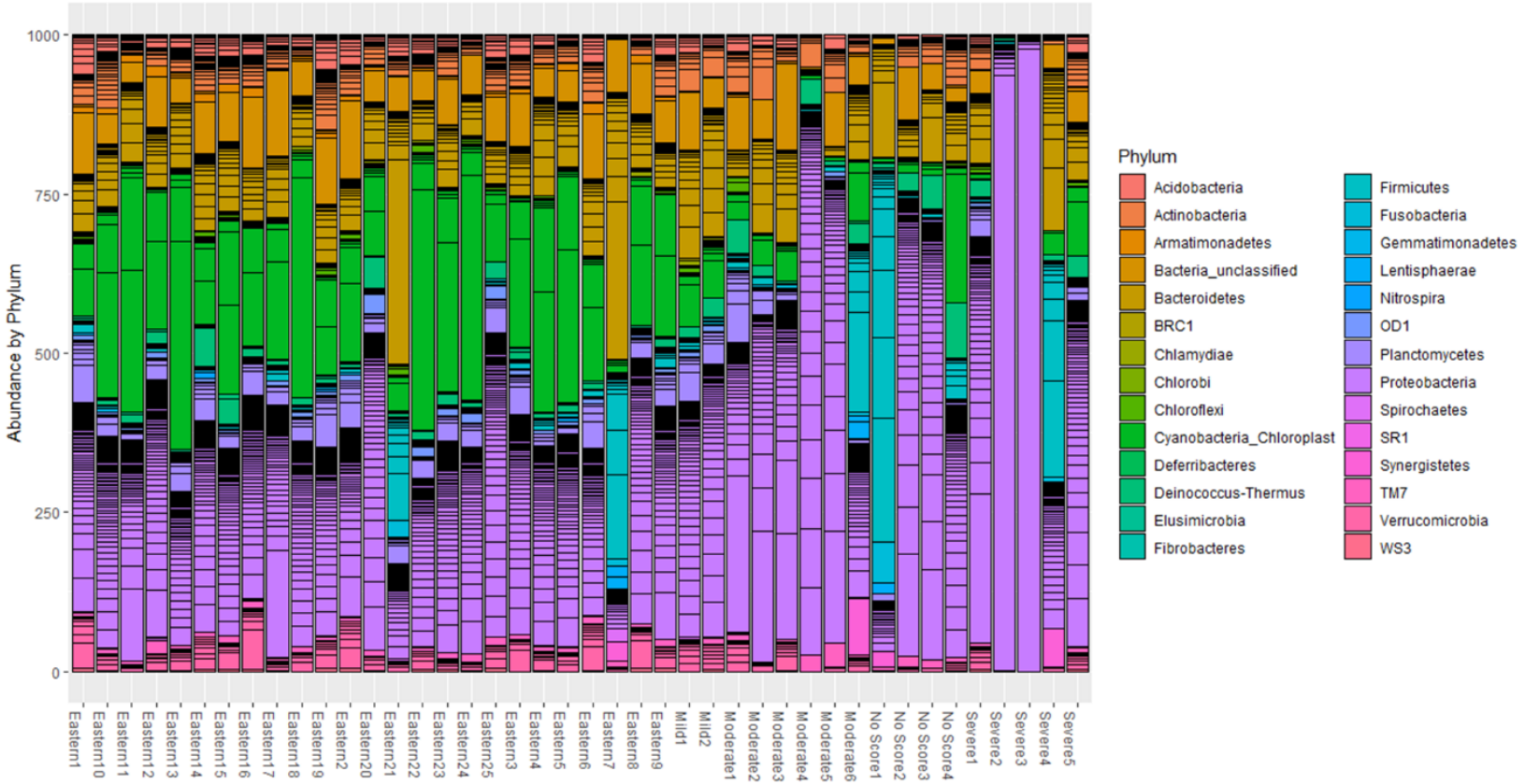

Figure 16. Sample Phylum Abundance

Phylum abundance from single subsample of 1000 sequences for each individual sampled. Labels represent samples from one of four groups of: Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis)* of eastern Tennessee (Eastern) and Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) of Arkansas with toe lesions observed to be (Mild), (Moderate), or (Severe).

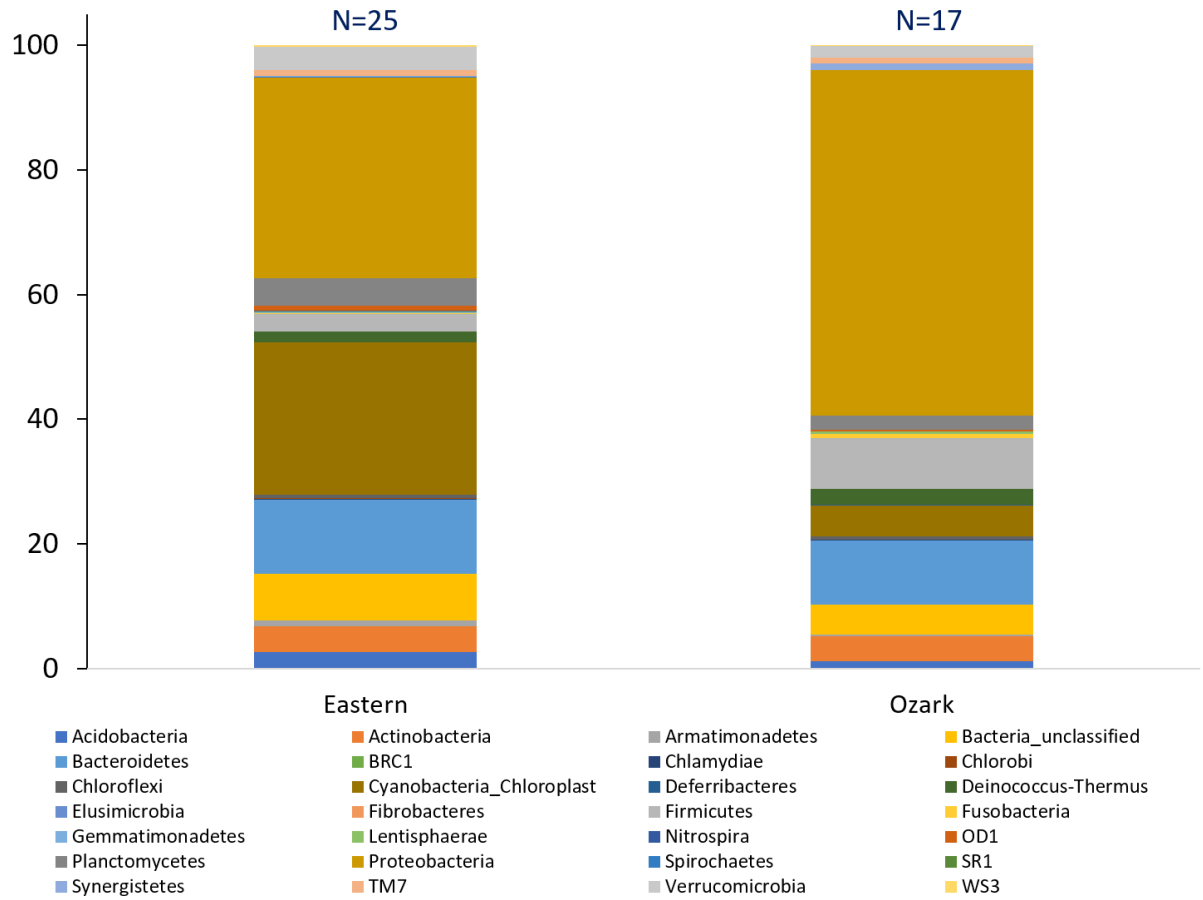

Figure 17. Average % Phylum Abundance by Subspecies

Average relative abundance of each phylum of Hellbender skin microbial communities for each of four groups: Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis)* of eastern Tennessee (Eastern) and Ozark Hellbenders (*Cryptobranchus alleganiensis bishopi*) of Arkansas (Ozark).

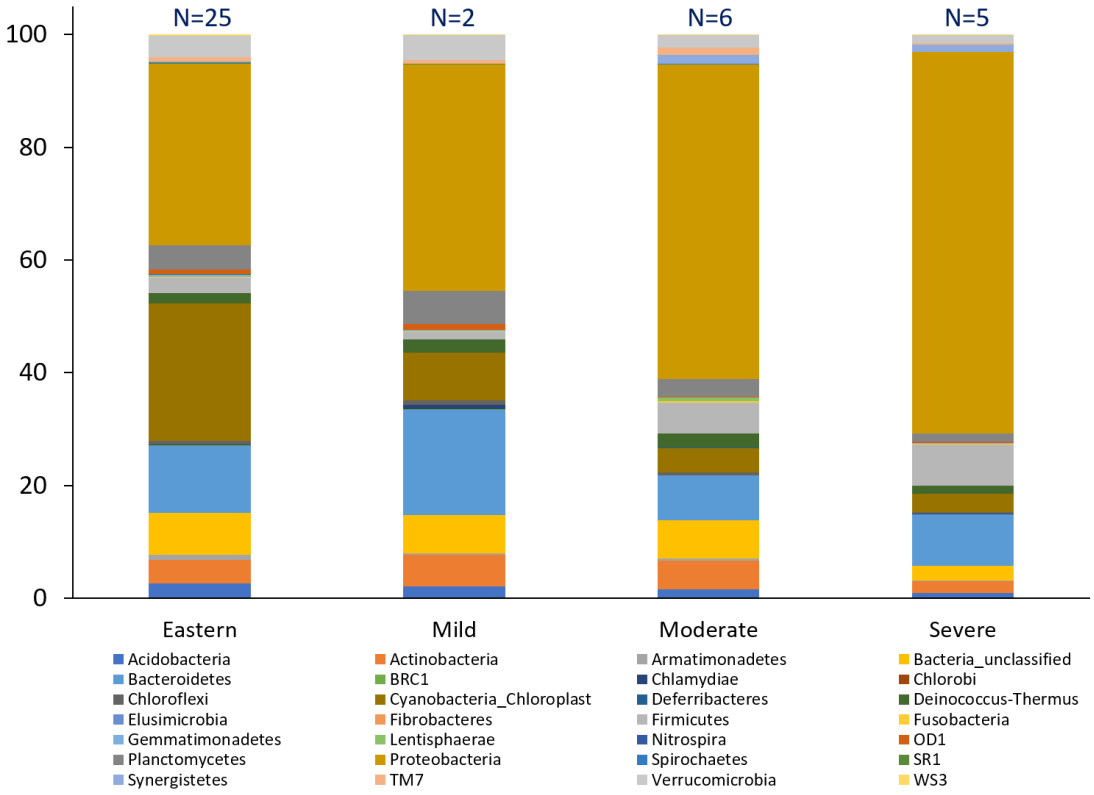

Figure 18. Average % Phylum Abundance by Lesion Severity

Average relative abundance of each phylum of Hellbender skin microbial communities for each of four groups: Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis)* of eastern Tennessee (Eastern) and Ozark Hellbenders (*Cryptobranchus alleganiensis bishopi*) of Arkansas with toe lesions observed to be (Mild), (Moderate), or (Severe).

Table 15: Indicator Taxa for Subspecies

List of top five indictor taxa significantly associated with each subspecies group of Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) of eastern Tennessee (Eastern) and Ozark Hellbenders (*Cryptobranchus alleganiensis bishopi*) of Arkansas (Ozark) based on indicator species analysis. Seventy-one OTUs were significantly associated with Eastern Hellbenders and twenty with Ozark Hellbenders.

Region	stat	p-value	Phylum	Genus
Eastern	0.958	0.001	Cyanobacteria_Chloroplast	*Cyanobacteria_order_incertae_sedis_unclassified*
Eastern	0.943	0.001	Bacteroidetes	*Ferruginibacter*
Eastern	0.934	0.001	Verrucomicrobia	*3_genus_incertae_sedis*
Eastern	0.910	0.001	Cyanobacteria_Chloroplast	*GpI*
Eastern	0.902	0.001	Actinobacteria	*Acidimicrobiales_unclassified*
Ozark	0.906	0.001	Proteobacteria	*Limnohabitans*
Ozark	0.853	0.002	Proteobacteria	*Aeromonas*
Ozark	0.835	0.037	Proteobacteria	*Acinetobacter*
Ozark	0.809	0.001	Actinobacteria	*Microbacteriaceae_unclassified*
Ozark	0.806	0.013	Bacteroidetes	*Cytophagaceae_unclassified*

Table 16: Indicator Taxa for Ozark Individuals with Varying Lesion Severity

List of top five indictor taxa for Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) of Arkansas significantly associated with individuals of varying toe lesion severity (mild, moderate, or severe) based on indicator species analysis. Twenty OTUs were significantly associated with mild lesions, one for moderate and none for severe lesions.

Severity	stat	p-value	Phylum	Genus
Mild	1.000	0.014	Bacteroidetes	*Ferruginibacter*
Mild	1.000	0.014	Bacteroidetes	*Hymenobacter*
Mild	1.000	0.014	Bacteroidetes	*Emticicia*
Mild	0.973	0.020	Cyanobacteria_Chloroplast	*Chloroplast_unclassified*
Mild	0.968	0.014	Acidobacteria	*Gp7*
Moderate	0.799	0.020	Proteobacteria	*Burkholderiales_unclassified*

geographic range where Missouri populations are disjunct from all other Eastern Hellbenders and are phylogenetically distinct from ETN (Hime 2017). Conflicting results may be due to genetic or regional differences within this subspecies. In addition, Eastern Hellbenders of Missouri are experiencing severe declines whereas those from ETN are considered healthy and relatively stable (USFWS 2018). Regions and populations are also confounded by population health. Interestingly when alpha diversity within Ozark Hellbenders was separated by lesion severity groups, a trend of decreased diversity was seen with increased lesion severity. Although this trend was not significant it implies that some variation in decreased alpha diversity could be indicative of decreased host health. Along with greater alpha diversity, skin microbiomes of ETN Hellbenders also had decreased beta dispersion suggesting greater similarity among individuals. Further, the two ETN individuals with greatest dispersion were also two of the smaller individuals sampled and considered subadults, revealing the importance of examining age class as a cofactor when comparing skin microbiomes. This is especially relevant for evaluation of Hellbender microbiomes because rivers with Hellbender declines also have reduced recruitment and decreased abundance of younger age classes, increasing the chance of unbalanced age-class sampling between healthy and unhealthy populations. Still, despite the divergence of two younger individuals, ETN skin microbiomes had significantly less dispersion between communities than those of AR for which only adults were sampled. Associated lesion severity scores helped explain interindividual community variation within AR as individuals with severe lesion scores had greater dispersion between communities, followed by those of moderate lesions. This increased dispersion persisted even with removal of two outlier communities which were both from animals with severe lesion scores.

These distal limb lesions are progressive and can take several years to increase in severity (Ch 2) and the same factors affecting lesion manifestation may also be affecting skin community composition. Increased variation between Ozark skin communities could represent a decreased ability to recruit and maintain an optimal microbial community as disease progresses in an individual. Interestingly, this association is between the dorsal skin microbiome and severity of lesions restricted to distal limbs. This supports a hypothesis that lesions are associated with overall skin health changes and that these changes are strong enough to affect the microbial community. This relationship between dorsum and toe lesions is also supported by a previous study which found minimal difference between OTUs of wounds and dorsum in Ozark Hellbenders of Missouri (Hernandez Gomez et al. 2017b). One mechanism to explain this trend is that local microbial changes due to chronic lesions can eventually generate changes over the entire skin surface as has been seen with ulcerations due to leishmaniasis in humans (Gimblet et al. 2017). However, shifts in microbial communities are also implicated in the manifestation of lesions and lesion severity may be a direct result of dysbiosis. Third, toe lesions and skin microbiomes dispersion are a result of the same disease processes and neither causes the other.

Examples from ecosystem ecology show how healthy and diverse communities can be resilient to disturbance events (Folk et al. 2004). However, if a community is

pushed beyond a threshold, it may not be able to return to its original state (Brook et al. 2013). This same phenomenon can occur in microbial communities. One example is with rumen acidosis. This often occurs after a cow is given a diet high in short chain carbohydrates, which shifts bacterial metabolism, resulting in acidosis, leading to death of important rumen microbial organisms, and causing ultimate rumen failure from dysbiosis (Chen et al. 2012). Microbial community disturbance can also be primarily host driven. Skin microbiomes are altered and less stable in patients with primary immunodeficiencies and hypothesized to occur because of increased permissiveness and less control of the host to recruit a healthy microbiota (Oh et al. 2013). If these skin communities shift far enough, they can further decrease skin immunity and wound healing (Pang et al. 2019). For instance, beneficial commensal bacteria in human skin such as *Staphylococcus epidermidis* are known to directly affect host antimicrobial peptide production which in turn regulates overgrowth of pathogens including *Staphylococcus aureus* (Lai et al. 2010); if these species are reduced, a positive feedback loop could initiate a dysbiotic event.

Although our results are purely correlative, they do support the hypothesis that Ozark Hellbenders are experiencing decreased skin immunity leading to decreased maintenance of beneficial bacteria, and overgrowth of undesirable species. Interestingly, Ozark Hellbenders and Eastern Hellbenders with toe lesions have skin peptides with marked decrease in anti-*Bd* activity in comparison to Eastern Hellbenders with healthy toes (Ch 3), revealing a potential link between altered microbiomes and innate immunity. Regardless of the true mechanism behind this association, the fact that lesion severity is strongly associated with decreased community similarity shows that individuals do not merely have toe lesions but are undergoing a systemic disease process with potentially more unnoticed sequelae.

Indicator species analysis produced OTUs associated with each group, except individuals with severe lesions (Tables 12,13). ETN had 71 indicator taxa with only twenty from AR further highlighting the fact that Eastern Hellbenders from this region have greater similarity and a larger number of shared OTUs. Interestingly, Ozark Hellbenders with mild lesions had 20 indictor taxa in comparison to one for moderate and none for severe lesion Hellbenders. Although we were only able to evaluate two individuals with mild lesions, several of the resulting indicator taxa that distinguished mild lesioned animals were the same OTUs that distinguished Eastern from Ozarks, which included the two mild individuals. This shows that differences between these two populations may be more driven by skin health and other factors of subspecies and region. Further, the two indicator taxa for moderate lesions (*Aeromonas* and unclassified Burkholderiales) are both reported as opportunistic pathogens in amphibian skin. *Aeromonas* is commonly involved in lesions of *Bd* and Ranavirus infections (Miller et al. 2008) and was cultured from several lesions of Missouri Ozark Hellbenders (Nickerson et al. 2011). Experimental infections of *Aeromonas* in the Chinese giant salamander, *Andrias davidianus*, a species closely related to Hellbenders, stimulates a systemic immune response further implicating it as pathogenic (Qi et al. 2016). The second OTU is unclassified within Burkholderiales, an order recently shown to increase in abundance

on skin of Sierra Nevada yellow-legged frogs, *Rana sierrae*, experimentally infected with *Bd* (Ellison et al. 2019). Therefore, both could be also be opportunistic pathogens and represent disrupted skin of Hellbenders. Additionally, two bacterial phyla (Firmicutes and Synergistetes) had increased abundance in Ozark Hellbenders with moderate and severe lesions and were scarce in in both Ozark Hellbenders with mild lesions and Eastern Hellbenders. Furthermore, this increased abundance was primarily represented by single genera in each phylum (an unclassified Clostridiales from Firmicutes and a *Cloacibacillus* from Synergistetes). Several bacterial species from Clostridiales (genus *Clostridium*) are notorious for colonizing wounds and producing toxins leading to further necrosis (Hatheway 1990). Little is known about bacteria from the genus *Cloacibacillus* but at least two species have been associated with septicemia in humans (Domingo et al. 2015). A third mucin-degrading species was identified from pig gastrointestinal tracts (Looft et al. 2013). Both OTUs, therefore have the ability to colonize a disrupted skin surface with an abundance of mucins such as a Hellbender skin wound.

Although microbiomes from Ozark Hellbenders with severe lesions did not have indicator taxa, one OTU from the genus *Mesorhizobium* dominated skin communities in two of six individuals. In fact, these two microbiomes were so dominated by this one OTU, that these samples were removed as outliers for diversity analyses. The majority of species in this bacterial genus are associated with mycorrhizal communities in plant root systems, but species have been identified from deep marine environments (Yuan et al. 2016; Fu et al. 2017), and more importantly, associated with amphibian hosts. One study found *Mesorhizobium* to be significantly more abundant in the gut of tadpoles in warmer versus cooler water (Kohl and Yahn 2016), while another found this genus to significantly define differences in the skin of adult coqui frogs, *Eleutherodactylus coqui*, from varied elevations (Hughey et al. 2017). However, because they were such striking outliers, we recommend further studies to confirm that this genus may be sporadically abundant on Ozark Hellbender skin before inferring its role in these skin communities.

We did not find any single bacterial OTU to be consistently associated with unhealthy Hellbender skin and similar conclusions were made from both culture-based (Nickerson et al. 2011) and culture-independent (Hernandez-Gomez et al. 2017b) evaluations of bacterial communities of toe lesions in Ozark Hellbenders of Missouri. However, we have identified a few potentially opportunistic pathogens that may be able to increase in abundance after a disruption on the skin. Each taxon deserves further attention and has potential to be an indicator of Ozark Hellbender health.

Regardless of the immediate mechanism shaping skin microbiomes, evaluation of skin microbiomes, when added to traditional amphibian disease surveillance, may provide a more comprehensive assessment of wild population health. We found a correlation between decreased community consistency and richness within Ozark Hellbenders compared to Eastern and further saw this decreased trend with increased severity of toe lesions. This supports a hypothesis that Ozark Hellbenders are experiencing chronic disease and associated dysbiosis. Hellbenders are long lived river giants and host immunity could be affected by various environmental factors, but we do know that declines are associated with habitat degradation. More intensive sampling will

be required in both healthy and unhealthy populations of both subspecies to disentangle factors affecting these important skin microbial communities.

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CONCLUSION

Wildlife health investigation has become a critical component of conservation research but requires collaboration from many research fields. This project occurred at a junction between wildlife biology and clinical medicine and is a critical step in merging medical diagnostics and conservation management. We evaluated skin health in both clinically sick and healthy Hellbenders from two subspecies by measuring several skin parameters. Evaluation of skin peptides, skin microbial communities combined with targeted molecular assay of amphibian pathogens allowed us to better understand changes in disease state and innate immune status. We found that toe lesions in Hellbenders are associated with changes in several of these parameters and these results added to the baseline knowledge of how classical amphibian pathogens affect Hellbender populations.

We documented a progressive disease affecting almost all sampled individuals in the remaining Ozark Hellbender population in Arkansas. The presentation of progressive lesions with a long differential list suggests that this disease is complex, and we highlight areas that need further research to fully understand factors in Ozark Hellbender health. Many questions still exist on the exact etiology of these lesions, and why they progress, but we do know that increased lesion severity is associated with *Bd* infection. Our lesion scoring system can be used to monitor changes in overall population health including response to restoration efforts. It can also be used to evaluate similar distal extremity lesions observed in other Ozark and Eastern Hellbender populations. We also describe a spectrum of severity of distal limb lesions with multiple likely etiologies in apparently health Eastern Hellbender populations. Some lesions were healed, others ulcerated, some with swollen and missing toes, and others with toes intact. Future Hellbender research must describe and document all lesions in the field, and pair these observations with disease and skin health sampling to help properly diagnose morbidities that may affect population persistence.

Our amphibian pathogen prevalence study found *Bd* present in all regions sampled, but not in every stream, although detection appeared to be limited to sample sizes less than 5. We also did not see any clear correlation between infection and presence of disease. However, within Ozark Hellbenders populations experiencing a high prevalence of distal limb lesions, lesion severity was associated with *Bd* presence indicating this pathogen may have negative effects on Hellbender hosts synergistically with other factors. Ranavirus detection was low and sporadic and we only discovered it in clinically normal individuals. However, we caution that it still may have negative health consequences, especially because of observations of increased distal lesions at a site a year after a spike in Ranavirus prevalence. Furthermore, a sampling from this site of skin peptides showed marked decrease in anti-chytrid activity from individuals with active Ranavirus infections.

We also discovered different trends in pathogen load vs prevalence showing that prevalence-only data may also be misleading for how these pathogens affect Hellbenders. Our prevalence findings emphasize the importance of repeated disease monitoring in Hellbenders. Both *Bd* and Ranavirus may play a subtle role in Hellbender declines and detailed disease data are required to fully understand a likely multifactorial threat to

Hellbender populations. Furthermore, continued disease monitoring will be important to detect any emerging or unknown disease threats, such as *Bsal*.

Our study shows that *in vitro* activity of skin peptides can vary within a species with varying skin health and may be an indication of susceptibility to disease. We showed that evaluation of skin microbiomes, when added to traditional amphibian disease surveillance, may provide a more comprehensive assessment of wild population health. We found a correlation between decreased community consistency and richness with increased severity of toe lesions. This supports a hypothesis that Ozark Hellbenders are experiencing chronic disease and associated dysbiosis. Hellbenders are long lived river giants and host immunity could be affected by various environmental factors, but we do know that declines are associated with habitat degradation. More intensive sampling will be required in both healthy and unhealthy populations of both subspecies to disentangle factors affecting these important skin microbial communities.

Finally, healthy amphibian skin is important for both homeostasis and prevention of pathogen invasion, and our study provides evidence of this important link. Furthermore, Hellbenders are large, long-lived animals and may serve as sentinels for long term exposure to sublethal agents. The high prevalence of these observed toe lesions should not only motivate continued research in Hellbender health but should also raise alarms for potential health risks to other wildlife and human populations sharing these watersheds. Potentially increased prevalence of similar toe lesion in Eastern Hellbenders indicate that more research is required to fully understand what threatens these populations and this research provides a platform to intensify disease investigation in Hellbenders to help prevent the Eastern Hellbender from undergoing the same fate as the endangered Ozark subspecies.

VITA

Rebecca Hardman graduated with a BA in Biology with Specialization in Ecology and Conservation Biology from Boston University in 2006. She worked as a herpetological field technician at Alabama A&M University before pursuing her Master's in Biology at Western Carolina University which she completed in 2014. She then moved on at the University of Tennessee College of Veterinary Medicine where she completed a DVM in 2016 and stayed on as a dual degree student to pursue her PhD from 2016-2020.