ASSESSING THE EMERGENCE OF A FUNGAL PATHOGEN (*BATRACHOCHYTRIUM SALAMANDRIVORANS*) FOR THE CONSERVATION OF COSTA RICAN SALAMANDERS

by

HENRY ADAMS

(Under the Direction of Sonia M. Hernandez)

ABSTRACT

We assessed the potential impacts of the emergent fungal pathogen, *Batrachochytrium salamandrivorans* (*Bsal*), on salamander populations in Costa Rica by conducting pathogen surveillance and generating an ecological niche model (ENM) to predict areas at high risk of *Bsal* introduction. Across one year, we surveyed for *Bsal*, *B. dendrobatidis*, and Ranavirus in highland salamanders (n=100) in Cerro de la Muerte and el Parque Nacional Volcan Poas. We did not detect any of the above pathogens in our study specimens. Though we did not detect *Bsal* in our study, our ENM predicted that a large portion of Costa Rica's landmass (22.82%) is at least moderately ecologically suitable to *Bsal*. Additionally, our ENM identified multiple locations throughout Costa Rica where moderate/high *Bsal* suitability overlaps with high salamander biodiversity and/or high human visitation. We encourage extensive monitoring and research at these locations with goals of early *Bsal* detection and introduction mitigation.

INDEX WORDS:Batrachochytrium salamandrivorans, chytrid fungus, pathogensurveillance, Ecological Niche Model, salamander, conservation

ASSESSING THE EMERGENCE OF A FUNGAL PATHOGEN (*BATRACHOCHYTRIUM* SALAMANDRIVORANS) FOR THE CONSERVATION OF COSTA RICAN SALAMANDERS

by

HENRY CRAWFORD ADAMS

B.S., University of Georgia, 2015

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment

of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

© 2020

Henry Crawford Adams

All Rights Reserved

ASSESSING THE EMERGENCE OF A FUNGAL PATHOGEN (*BATRACHOCHYTRIUM* SALAMANDRIVORANS) FOR THE CONSERVATION OF COSTA RICAN SALAMANDERS

by

HENRY CRAWFORD ADAMS

Major Professor: Committee: Sonia M. Hernandez Michael Yabsley Scott Connelly Matthew Gray

Electronic Version Approved:

Ron Walcott Interim Dean of the Graduate School The University of Georgia August 2020

DEDICATION

For my family, by blood and by choice, Whose unwavering love and support echo in thunderous concert, Ringing brightly with our shared laughter and joy, Taking the dull drones of hardships and those doubtful voices, Transforming their tones to make new harmonies, Ones that resonate with lessons learned and confidence built, A symphony that has not only made me who I am, But has allowed me to see me, And love me,

For who I am.

Thank you.

I love you.

ACKNOWLEDGEMENTS

First of all, I would like to thank the National Science Foundation and their Graduate Research Fellowship program for funding my research and supporting me as a student and scientist. I would also like to thank the Latin American and Caribbean Studies Institute at the University of Georgia for their financial support. Also, an enormous thank you to the Wildlife Disease Association and Experiment for hosting the crowdfunded Challenge Grant and the many friends and family who directly contributed to my science. All of this is made possible because of my amazing community.

Second, I would like to thank CONAGEBIO for permitting and overseeing my work in Costa Rica and specifically Jose Hernandez and Melania Muñoz who worked tirelessly with me through every permitting twist and turn. An endless fount of gratitude for my incredible mentor and friend, Sonia Hernandez, as well as my fantastic committee members, Michael Yabsley, Matthew Gray, and Scott Connelly. I would like to thank my fantastic research technician, Jeremy Klank, who helped make my final field season such a triumphant experience. A tremendous thank you to my amazing collaborator, Kate Markham, who helped bring my ideas to life. And to my lab family, Andrea, Jenny, Raquel, Julia, Sebastian, Shannon, Kat, Jorge, Kayla, Brianna, Seth, Alec, Troy, Erin, your advice, laughter, and guidance along this winding road have meant the world to me.

Next, I'd like to thank my amazing friends and colleagues in Costa Rica: Bedrich, Camila, and Maureen, Esteban, Nicole, Jorge, Rodolfo, Ricardo, you all have made me feel so welcomed and cared for from the beginning of my very first field season. Your friendship and

v

generosity will always make Costa Rica feel like a home away from home. ¡Muchisimas gracias por todo, y hasta pronto!

Lastly, I can't fully express my thanks to my incomparable mother and father, Kathleen and Jonathan, who have been with me every step of the way: from holding my tiny hands as a toddler exploring stream beds to sending me off to Costa Rica for my field trips, having taught me how to walk proudly on my own two feet. Thank you to my amazingly supportive and nurturing aunt and uncle, Beverly and David, and the numerous family friends who have played enormous parts in raising me and bringing my family joy: Tommy, Nancy, Rob, Julene, Hal, Brett, Candace, Patty, Evan, Janet, Susan, Jack, Patrice, Laura. To my best friend, Savannah, and the rest of my glorious chosen family, Catie, Ty, Chas, Margaret, Elise, Nathan, Cassidy, Seth, Clara, Jett, thank you for sharing life with me.

TABLE OF CONTENTS

Page
ACKNOWLEDGEMENTS
LIST OF TABLESix
LIST OF FIGURES
CHAPTER
1 INTRODUCTION & LITERATURE REVIEW
Amphibian Conservation & Emergent Infectious Diseases1
The Role of Cutaneous Microbiome in Amphibian Conservation8
Emergence & Description of Batrachochytrium salamandrivorans (Bsal)10
Assessing and Responding to the Threats of <i>Bsal</i>
Threat of <i>Bsal</i> Emergence in Costa Rica
Significance & Rationale15
Literature Cited
2 SURVEILLANCE FOR THE PATHOGENIC FUNGI, BATRACHOCHYTRIUM
SALAMANDRIVORANS AND B. DENDROBATIDIS, AND RANAVIRUS IN
HIGHLAND COSTA RICAN SALAMANDER POPULATIONS
Abstract
Introduction
Methods
Results

	Discussion	
	Acknowledgements	40
	Literature Cited	40
3	ASSESSING THE RISK OF THE EMERGING FUNGAL PATHOGEN,	
	BATRAOCHOCHYTRIUM SALAMANDRIVORANS, TO COSTA RICAN	
	SALAMANDERS	
	Abstract	49
	Introduction	50
	Methods	54
	Results	
	Discussion	63
	Literature Cited	67
4	CONCLUSIONS	72
	Management Implications	72
	Suggestions for Further Research	72
	Literature Cited	74

LIST OF TABLES

Table 2.1: Prevalence of Batrachochytrium salamandrivorans (Bsal), B. dendrobatidis	
(Bd), and <i>Ranavirus</i> in four Costa Rican salamander species	37

LIST OF FIGURES

Page

Figure 2.1: (A) Shows the two regions of survey (Parque Nacional Volcan Poas & Cerro de la Muerte) and number of salamanders surveyed in each region. (B) Shows the five sites surveyed in Cerro de la Muerte and the number of salamanders surveyed at each site	36
Figure 3.1: Protected areas are outlined in black. Where available, visitation numbers within protected areas for 2018 are indicated by color, with darker blue indicating more visitors.	55
Figure 3.2: Salamander (left) and frog (right) alpha diversity in Costa Rica. Darker shades indicate higher alpha diversity	57
Figure 3.3: Ecological Niche Model results for <i>Bsal</i> suitability in Costa Rica. Shows high suitability especially in the Cordillera Central, Cordillera Talamanca, and northeastern Caribbean slope	58
Figure 3.4: Simplified results of our ENM. Close-up of highly suitable area for Bsal, shown in red. Protected areas are shown overlaid with diagonal lines.	59
Figure 3.5: Shows overlap of areas moderately suitable for <i>Bsal</i> and with high/very high amphibian diversity (yellow) and overlap between areas highly suitability for <i>Bsal</i> and with high/very high amphibian diversity (red). Protected areas are outlined and the number of visitors in 2018 is indicated by pattern	61
Figure 3.6: RRI values with darker red indicating salamander species found within this pixel have a restricted range across Costa Rica. High priority areas are shown in bright red. Protected areas are outlined and the number of visitors in 2018 (where available) is indicated by pattern.	62

CHAPTER 1

INTRODUCTION & LITERATURE REVIEW

Amphibian Conservation & Emergent Infectious Diseases

Since the 1970s, amphibian populations have been declining on a global scale at alarming rates, resultant in amphibians being classified the most endangered vertebrate taxa (Stuart et al. 2004, Parrott et al. 2016). Amphibians are of high ecological importance to systems across the globe. They often account for the greatest amount of vertebrate biomass in terrestrial systems relative to other taxonomic groups, especially in tropical systems, and serve as a crucial trophic base (Whiles et al. 2006). Additionally, because of the common aquatic-terrestrial duality of their life history, many amphibians fill multiple ecological roles in both terrestrial and aquatic systems throughout their lives (i.e. prey, primary consumer, secondary consumer) contributing greatly to community structure and nitrogen cycling (Whiles et al. 2006). This same complex life cycle helps make some amphibian species excellent bioindicators of ecosystem health: exposed to stressors across a wider environmental range than other taxa, they may provide insight to the status of multiple systems at once (Welsh & Ollivier 1998). Additionally, their unique physiology, specifically their highly porous skin, and specific niche requirements can make them especially sensitive to environmental pollutants and disturbances, such as siltation (Welsh & Ollivier 1998). Amphibians also provide myriad of ecosystem services, including pest and disease control, food provisioning, and medical benefits. Some amphibian cutaneous compounds have the potential to help produce highly effective painkillers, less addictive than opioids, as well as limit the spread and severity of human immunodeficiency virus (HIV) (Hocking & Babbitt

2014). Because of these services and their ecological importance, the rapid and global extirpation of amphibians is regarded as a conservation crisis (Valencia-Aguilar *et al.* 2013, Yap *et al.* 2015).

Habitat loss and degradation have been regarded as the greatest threats to amphibian communities (Wake & Vredenburg 2008). However, in recent decades emergent infectious pathogens have had increasingly significant negative impacts on wild amphibian populations (Jones et al. 2008, Wake & Vredenburg 2008, Yap et al. 2015). An emergent infectious disease is one that appears suddenly in a novel environment or host population or experiences a rapid and dramatic increase in incidence or range expansion (Jones *et al.* 2008). There are two primary hypotheses used to explain the emergence of an infectious disease: The Novel Pathogen Hypothesis and the Endemic Pathogen Hypothesis. The Novel Pathogen Hypothesis posits that a pathogen and its associated disease emerge when the pathogen is introduced from its endemic range into a novel host population, one that does not necessarily have evolutionary immunity to the pathogen and may experience increased disease incidence and intensity (Jones et al. 2008). The Endemic Pathogen Hypothesis states that an evolutionary development or environmental shift may increase a pathogen's virulence or the ways in which the pathogen interacts with host organisms, resulting in greater disease incidence and intensity in areas of historic pathogen endemism (Rachowicz et al. 2005, Jones et al. 2008).

Batrachochytrium dendrobatidis (Bd), the causative agent of chytridiomycosis, currently has had the most impact on amphibian conservation (Scheele *et al.* 2019). *Bd* belongs to the phylum *Chytridiomycota*, an ancient group of fungi that primarily parasitize invertebrates and plants (Van Rooij *et al.* 2015). *Bd* is unique in that it is one of only three species within *Chytridiomycota* that parasitizes vertebrate hosts, the other two being *Batrachochytrium*

salamandrivorans (discussed later) and *Ichthyochytrium vulgare*, a rare fish parasite (Van Rooij *et al.* 2015). *Bd* has two life stages: the first is a motile, flagellated zoospore (Berger *et al.* 1998). While environmentally persistent for roughly two weeks in ideal conditions, the *Bd* zoospore is dependent upon cool, moist environments, growing optimally between 17° and 25° C (Johnson & Spear 2005, Van Rooij *et al.* 2015) but sensitive to desiccation and/or heat. These dynamics have contributed to the development of clinical treatments for *Bd* infection as well as the natural maintenance of *Bd* within some amphibian populations through seasonal environmental variation (Weinstein 2009, Sonn *et al.* 2017).

Upon contact with a suitable amphibian host, the fungal zoospore encysts in keratinized areas of host's skin, producing germ tubes that invade the epidermal layer. Zoosporangium, Bd's second and reproductive life stage, then develop from these germ tube. In Bd, zoosporagia are typically monocentric, with one per thallus (non-colonial). The zoosporangium then produces new, infectious zoospores (Van Rooij et al. 2015). The encysting of the zoospore and development of the zoosporangium can cause clinical disease (chytridiomycosis) in susceptible species, characterized by lethargy, erythema, discoloration and sloughing of the skin, and hyperkeratinization (Van Rooij et al. 2015). Hyperkeratinization prevents the exchange of important molecules, such as electrolytes, across the host's skin, causing mortality by cardiac arrest in susceptible species (Fisher *et al.* 2009). Because *Bd* only encysts in keratinized skin layers, it can only infect the mouth parts of larval anurans (Van Rooij et al. 2015). This causes deformities and behavioral abnormalities, limiting foraging ability, and reducing overall fitness (Van Rooij *et al.* 2015). Though susceptibility to clinical disease significantly varies across species, generally anurans are considered more susceptible to *Bd* infection and clinical disease than urodelans, with *Bd* prevalences often higher in some anuran populations than sympatric

urodelan populations (Green *et al.* 2002, Rothermel *et al.* 2008, Chinnadurai *et al.* 2009). However, there are exceptions in which salamander populations have high prevalences of *Bd*. Across multiple sites in the Southeastern United States, Chatfield *et al.* (2012) found an average *Bd* prevalence of 0.34 in aquatic salamander populations. Rothermel *et al.* (2016) found *Bd* at much higher prevalences in adult eastern newts (*Notophthalmus viridescens*) than sympatric larval anurans. Additionally, *Bd* has been linked to declines in neotropical salamander populations in Mexico and Guatemala (Cheng *et al.* 2011).

Though not formally described until 1998, retrospective studies have implicated *Bd* in the highly visible amphibian declines of the late 1980s and early 1990s. These declines began as early as the 1970s when *Bd* began emerging nearly simultaneously in Central America and Australia (O'Hanlon *et al.* 2018). In these regions, *Bd* is believed to have been responsible for the extinction of the charismatic Golden Toad (*Incilius periglenes*) of Costa Rica and the two species of Gastric-brooding Frogs (*Rheobatrachus* sp.) of Australia (Crump *et al.* 1992, Berger *et al.* 1998) as well as the (at the time) enigmatic decline of numerous other amphibian species. Since its emergence, *Bd* has spread to every continent except Antarctica and has been responsible for either declines or extirpations of over 500 amphibian species (Miller *et al.* 2018, Scheele *et al.* 2019, Martel *et al.* 2020). The lasting ecosystem impacts of these mortality events are now becoming more visible across trophic levels, with recent research showing declines in tropical snake community diversity in areas of amphibian mass mortalities independent of any other apparent changes in environmental variables (Zipkin *et al.* 2020).

The emergence of *Bd* is generally attributed to the global food, pet, and pharmaceutical trade of amphibians (O'Hanlon *et al.* 2018). For years, it was unclear from where *Bd* originated, with many hypothesizing endemism to Africa, North America, and the Atlantic coasts of Brazil

(Van Rooij *et al.* 2015). O'Hanlon *et al.* (2018) recently identified East Asia, specifically likely the Korean peninsula, as the native range for *Bd*, based on the fungus' high genetic diversity in the region. Though this largely follows the Novel Pathogen Hypothesis, it is speculated that the Endemic Pathogen Hypothesis may also have played a role in the emergence of *Bd*. While the global trade of amphibians began in earnest at the turn of the 20th century, the pathogen did not truly emerge until the 1970s, leading to the belief that environmental shifts also may have contributed to the emergence of *Bd* and associated amphibian declines (Weinstein 2009, O'Hanlon *et al.* 2018).

Additionally important to the amphibian conservation crisis are the pathogens belonging to the genus *Ranavirus* and Class Perkinsea (Yap *et al.* 2015, Gray *et al.* 2009, Isidoro-Ayza *et al.* 2017). *Ranavirus* is a genus of DNA viruses belonging to the Iridoviridae family. Though there are multiple ranaviruses known to infect amphibians, there are only three officially recognized as species: *Frog Virus 3* (FV3), *Ambystoma tigrinum virus* (ATV), and *Bohel iridovirus* (BIV). FV3 was the first to be described after being isolated from a northern leopard frog (*Lithobates pipiens*) in 1965 (Gray & Chinchar 2015). The second ranavirus species discovered, BIV, was isolated from Australian amphibians in 1992 and ATV was discovered in larval Sonora tiger salamanders (*Ambystoma tigrinum stebbinsi*) in 1965 (Gray & Chinchar 2015). Other ranavirus variants include Common Midwife Toad Virus (CMTV), first isolated in 2007, *Rana grylio* Virus (RGV), isolated in the mid-1990s, Tiger Frog Virus (TFV), isolated in 2000, and *Rana nigromaculata* ranavirus (RNRV), described in 2016 (Gray & Chinchar 2015, Mu *et al.* 2018). Though genetically distinct, these ranaviruses are currently considered variants of named species, due to the high level of DNA sequence conservation (Gray & Chinchar 2015).

Ranaviruses most frequently cause die-offs in larval amphibians during late summer months, corresponding with the natural immune suppression associated with late stage metamorphosis (Gray & Chinchar 2015). Diseased individuals may present with erratic swimming, bloating and erythema near the gills. Ranaviruses can also cause clinical disease in adults, which can develop bloating and skin ulcerations (Gray *et al.* 2009). Ranavirus die-offs can be sparked by viral transmission from reservoir species, be them resistant amphibians or non-amphibian reservoirs, such as fish, or by environmental transmission from preserved, infected, dead host tissues or contaminated soils (Gray & Chinchar 2015, p. 71). Ranaviruses have been shown to have poor persistence in natural water bodies, i.e. those populated with other microbes. Though it can aid in transmission through one of the pathways mentioned above, it is unlikely that a ranavirus outbreak could be caused by contaminated water alone (Gray & Chinchar 2015, p. 71).

Ranavirus die-offs have been documented on every continent except for Antarctica (Duffus *et al.* 2015, Gray & Chinchar 2015). Because of the ongoing discovery of new species and the group's continued range and host expansion, ranaviruses are regarded as emergent pathogens and are reportable to the World Organization for Animal Health (OIE) (Gray *et al.* 2009). While ranaviruses are not as readily associated with large scale extirpations as *Bd*, their environmental persistence, density-independent transmission, and ability to cause sudden die-offs still make them a notable threat to amphibian populations. Multiple reports have been made of local population-level declines following ranavirus-associated epizootic events, and ranavirus-associated population declines upwards of 81% have been well documented in the common frog (*Rana temporaria*), native to the United Kingdom (UK) (Teacher *et al.* 2010). Because ranaviruses in the UK primarily affect adult anurans rather than larvae, there is an exaggerated

risk of them causing large scale species extirpation. But, even localized, larval die-offs can be extremely detrimental to population integrity, especially in already endangered populations and when outbreaks are repeated over multiple seasons (Gray *et al.* 2009, Gray & Chinchar 2015) For instance, in 2011 a unique and highly pathogenic ranavirus, similar to FV3, was isolated from captive, endangered Chinese giant salamanders (*Andrias davidianus*), creating a major conservation concern (Gray & Chinchar 2015).

Protozoan parasites belonging to the phylum Perkinsozoa (also known as Perkinsea), specifically Novel Alveolate Group 01 (NAG01), present a possible emergent threat to amphibians. This pathogenic group of Perkinsozoa was described after being identified as the causative agent in a mortality event in southern leopard frog larvae in Northeast Georgia in 2007 (Chambouvet *et al.* 2015). Diseased individuals were described as bloated and lethargic, with discoloration of the skin and livers invaded with high densities of protozoan cells (Chambouvet et al. 2015). While Perkinsoza-caused die-offs have been documented in the United States across 43 states, NAG01 organisms infectious to amphibians are more globally distributed. In a 2015 study conducted by Chambouvet et al. (2015), surveillance for Perkinsozoa across five different countries and multiple frog families detected a mean prevalence of 21%, with each country having at least one positive case. These positive cases also represented three different subclades within the NAG01. All positive cases were subclinical, leaving the NAG01 subclade found in the United States the only known to cause clinical disease in amphibians. However, there could be environmental factors or coinfection dynamics that could influence the pathogenicity of other Perkinsozoa subclades, leading to clinical infection (Chambouvet et al. 2020). More information regarding these dynamics, as well as a better understanding of susceptibility across anuran

species, urodelans and caecilians is necessary to best assess the threat these parasites pose to amphibians conservation (Chambouvet *et al.* 2020).

The Role of Cutaneous Microbiome in Amphibian Conservation

The amphibian skin plays numerous roles critical to the animal's life history beyond a physical barrier against pathogen invasion. It is responsible for gas exchange and osmoregulation, the production of anti-predation compounds, and protection against environmental toxicants and pathogens. It also supports a community of microorganisms that promote the health of the amphibian host. Increasingly, the importance of symbiotic microbiota to the health and physiology of their hosts is being recognized across multiple systems. Gut microbiomes of particular assemblages can promote different body conditions in humans, symbiotic bacteria encourage healthy coral communities, and cutaneous microbiota can increase immunity in mice against the parasite *Leishmania major* (Turnbaugh *et al.* 2009, Naik *et al.* 2012, Redford *et al.* 2012, Krediet *et al.* 2013, Becker *et al.* 2015).

The skin microbial community of an amphibian can impact its susceptibility to pathogens, such as *Bd*. For example, studies have demonstrated the ability of microbial communities to reduce host susceptibility to *Bd* infection. Specific bacteria, such as *Janthinobacterium lividum, Lysobacter gummosus, Chryseobacterium, Serratia marcescens*, and *Pseudomonas*, are associated with *Bd* inhibition and elevated host immunity (Becker & Harris 2010, Becker *et al.* 2009, Becker *et al.* 2015, Jimenez & Sommer 2017, Madison *et al.* 2017, Catenazzi *et al.* 2018). It has been shown that *J. lividum* and *S. marcescens* produce similar antifungal metabolites, violacein and prodigiosin respectively, as well extra extracellular chitinase, which presumably assists in breaking down fungal pathogens (Madison *et al.* 2017). Fungal

inhibiting bacteria have been isolated from the skin of multiple amphibian species world-wide, and the removal of these bacteria has been shown to increase susceptibility to *Bd* infection and morbidity (Becker *et al.* 2009). Conversely, the use of fungal inhibiting bacteria, specifically *J. lividum*, in probiotic therapies has been shown to successfully increase amphibian innate immunity to *Bd* through the production of the antifungal compound violacien (Becker & Harris 2010, Harris *et al.* 2009). However, the use of *J. lividum* in probiotic treatment has not been universally successful; when used in probiotic treatments of Panamanian golden frogs (*Atelopus zeteki*), the bacteria failed to colonize the skin, presumably due to a general lack of microbe-host compatibility (Becker *et al.* 2011, Becker *et al.* 2015). However, *S. marcescens* has been isolated from Costa Rican anurans, perhaps offering a more compatible option for probiotic treatments in other Central American species (Madison *et al.* 2017).

There are multiple factors that contribute to the assemblage of an amphibian's cutaneous microbial community, with various possible transmission pathways (vertical, horizontal, environmental) leading to microbial colonization (Jimenez & Sommer 2017, Albecker *et al.* 2019). Within a microbial community, there exists the "core" community. This collection of microbial Operational Taxonomic Units (OTU) is shared across >90% of individuals within a population and are specific to a certain species, though the core communities of different species may share multiple OTUs (McKenzie *et al.* 2011, Christian *et al.* 2018). These core communities are believed to help maintain essential host functionality and assist in the persistence of the entire symbiotic microbial assemblage (Loudon *et al.* 2014). While the core community is established and maintained through endogenous factors between the host and symbiotes, in amphibians, environmental variables, such as habitat, microhabitat, salinity, temperature and elevation, can

also significantly influence the cutaneous microbiome (Albecker *et al.* 2019, Apprill *et al.* 2014, Jimenez & Sommer 2017, Muletz-Wolz *et al.* 2017).

Studies have shown that certain predictions regarding the structure of microbial assemblages can be made based on spatial and environmental variables; for instance, within particular locations, the amphibian microbiome community tends to be similar to that of the host environment and of sympatric amphibians, even across species (Jimenez & Sommer 2017, Muletz-Wolz *et al.* 2017). Additionally, higher microbial alpha diversity, which is associated with a greater likelihood for the presence of fungal-inhibiting bacteria, has been associated with higher elevation (Muletz-Wolz *et al.* 2017). Catenazzi *et al.* (2018) found that across an elevational gradient in Andean anuran populations, the abundance of fungal-inhibiting bacteria followed a parabolic trend, with greater abundance at lower and higher elevations, with certain bacterial groups being present at low and high elevations, specifically *Pseudomonas* sp. Because *Bd*-caused amphibian declines in this region were greatest at middle elevations where the abundance of fungal-inhibiting microbes was lowest, it is believed that the cutaneous microbial communities played a significant role in mitigating the impact of *Bd* in certain amphibian species (Catenazzi *et al.* 2018).

Emergence & Description of *Batrachochytrium salamandrivorans*

As early as 2008, Dutch fire salamanders (*Salamandra salamandra*) began experiencing enigmatic, mass die-offs, with 96% of the population extirpated by 2013 (Martel *et al.* 2013). In 2013, Martel *et al.* (2013) isolated a new species of chytrid fungus, *Batrachochytrium salamandrivorans* (*Bsal*), from affected individuals and determined the fungus to be the causative agent in the observed population declines. This marked the discovery of the third

chytrid fungus known to parasitize vertebrate hosts and the second to affect amphibians (Van Rooij *et al.* 2015). *Bsal* and *Bd* diverged roughly 165 million years ago, though maintain great similarity (Van Rooij *et al.* 2015). *Bsal* is a saprophytic, moisture-loving fungus that, like *Bd*, has motile zoospore and reproductive zoosporangium life stages. Morphologically unique to *Bsal* is that its encysted zoospores produce tubular germ structures that more frequently produce colonial thalli, possessing more than one zoosporagium (Van Rooij *et al.* 2015). Additionally, *Bsal* is able to produce non-motile zoospores with higher environmental persistence than motile zoospores (Stegen *et al.* 2017). These adapted zoospores can endure in ideal conditions for up to 30 days, but decline quickly in the presence of micropredators (Stegen *et al.* 2017). Like *Bd*, *Bsal* causes chytridiomycosis in susceptible hosts, though it is characterized by the invasion of colonial thalli which cause erosive lesions and necrosis, not hyperkeratinization (Martel *et al.* 2013, Van Rooij *et al.* 2015). *Bsal* can persist and cause clinical disease across a fairly wide thermal niche (~10°- 24°C) (Martel *et al.* 2013, Laking *et al.* 2017, Stegen *et al.* 2017).

Phylogenetic studies point towards *Bsal* having co-evolved with *Salamandridae* host species in East Asia, specifically Vietnam where it persists in *Salamandridae* populations at low prevalence, causing limited clinical disease (Laking *et al.* 2017). The introduction of *Bsal* into Europe is attributed to the movement of amphibians through the pet trade, particularly of fire belly newts (*Cynops* spp.), a species popular in the pet trade, and natural reservoirs of *Bsal* (Martel *et al.* 2014, Laking *et al.* 2017). Roughly 2.3 million *Cynops* individuals were imported into the USA between 2001 and 2009 alone (Herrel & and van der Meijden 2014). Recent evidence has shown that anurans can support subclinical infections and transmit *Bsal* to healthy salamander hosts (Ngueyn *et al.* 2017, Stegen *et al.* 2017). While it was initially believed that anurans did not played a role in *Bsal's* emergence, it is likely that the anthropogenic movement

of east Asian anurans, such as *Bombina* sp., also contributed to the introduction of *Bsal* in Europe (Ngueyn *et al.* 2017). Since emerging in the Netherlands, *Bsal* has moved into free-living Belgian, German, and Spanish salamander populations, and additional cases of *Bsal* infections have been in captive salamanders in the UK, Germany, and Spain (Martel *et al.* 2014, Cunningham *et al.* 2015, Spitzen-van der Sluijs *et al.* 2016, Martel *et al.* 2020). It should be noted that Germany's reports of captive *Bsal* infections preceded the detection of the pathogen in the country's wild populations. Additionally, the recent emergence of *Bsal* in Montnegre i el Corredor Natural Park in Spain, nearly 1000km from the closest reported area of occurrence, has been associated with the recent introduction of an invasive newt species (*Triturus anatolicus* (Anatolian crested newt) and *Ichthyosaura alpestris* (alpine newt)) (Martel *et al.* 2020). These cases further illustrate the significance of anthropogenic movement of amphibians to the past and future emergence of this infectious pathogen.

Assessing and Responding to the Threats of Bsal

Experimental trials indicate *Bsal's* pathogenicity is fairly clade specific (Martel *et al.* 2014). However, genetic relatedness cannot always be used a predictive measure when assessing susceptibility (Carter *et al.* 2019). The fungus causes varying clinical disease in *Plethodontidae* species and is most pathogenic to *Salamandridae* (Martel *et al.* 2014, Carter *et al.* 2019). The threat of *Bsal* introduction is of particular concern for the Americas, which host the world's most diverse salamander communities, with 48% of species occurring in North America alone (Yap *et al.* 2015). Multiple factors concerning the epidemiology of *Bsal* exacerbate this risk. It has been shown experimentally that *Bsal* is a highly transmissible pathogen, with a >90% likelihood of *Bsal* transmission from direct contact between two susceptible hosts (Malagon *et al.* 2020).

Based on models developed using the epizootics in the Netherlands, it is believed that *Bsal* can persist in host populations of very low densities, though transmission has been shown to be positively associated with host density (Schmidt *et al.* 2017, Malagon *et al.* 2020). Additionally, widely distributed, densely populated, susceptible species, like *Notophthalmus viridescens* (eastern newt), may serve as super spreaders (Malagon *et al.* 2020).

In response to this threat, a consortium of organizations formed the North American *Bsal* Task Force, which has enacted ongoing *Bsal* surveillance in wild populations and susceptibility trials of North American species as research priorities (Martel *et al.* 2014, Gray *et al.* 2015, Parrot *et al.* 2016, Govindarajulu *et al.* 2017, Klocke *et al.* 2017). Based on recommendations made by this task force, the United States Fish and Wildlife Service (USFWS) imposed a ban on the import and inter-state movement of ~200 salamander species in 2016 (Grant *et al.* 2016). This ban has reportedly reduced salamander importations by 98.4% between 2015 and 2016 and the pathogen has not yet been detected in the Americas, in either captive or wild amphibians (Gray *et al.* 2015, Parrott *et al.* 2016, Klocke *et al.* 2017). However, the pathogen persists in international amphibian collections and trade, causing subclinical infections that may make infected hosts go undetected (Sabino-Pinto *et al.* 2018). This perpetuates the emergence of *Bsal* throughout Europe and may precipitate the introduction of *Bsal* into the Americas, though the level of threat may vary by country (Klocke *et al.* 2017).

Threat of Bsal Emergence in Costa Rica

Costa Rica is one country for which the introduction of *Bsal* is of particular concern, but preventative measures have yet to be implemented. Costa Rica is also country whose amphibian communities have already been marred by emergent infectious diseases. In the 1990s and 2000s,

Costa Rica experienced what were then classified as enigmatic amphibian declines, now attributed to the emergence of *Bd*. The declines reduced anuran diversity by up to 40% in areas such as Monteverde and Las Tablas (Puschendorf *et al.* 2009). One species extirpated by the *Bd* was the charismatic golden toad (*Incilius periglenes*), once a common fixture of the Monteverde amphibian community (Crump *et al.* 1992). It is hypothesized that the introduction of *Bd* and subsequent declines were due to the unintentional translocation of environmentally resistant fungal zoospores on fomites, largely through ecotourism (Cheng *et al.* 2011). Because of the similar environmental persistence of *Bsal* zoospores, it is possible the pathogen could be transported to novel environments through contaminated research or tourism equipment (Stegen *et al.* 2017).

Bsal now presents a risk similar to *Bd*, endangering Costa Rica's salamander community, the fifth most diverse in the world (Kubicki & Arias 2016). With all Costa Rican salamander species belonging to *Plethodontidae*, known to have varying susceptibility to *Bsal*, the impacts of this pathogen in Costa Rica could be disastrous (Martel *et al.* 2014, Kubicki & Arias 2016). Based on *Bsal's* thermal niche and Costa Rican salamander distributional ranges, at least 30 Costa Rican salamander species could be at risk of *Bsal* introduction, depending on their susceptibility (Savage *et al.* 2002, Cheng *et al.* 2011, Martel *et al.* 2014, Kubicki & Arias 2016, Laking *et al.* 2017). Costa Rica also has a vibrant international pet trade that lacks regulations for amphibian pathogens (pers comm Rodolfo Leiton, 2017). For example, the importation and sale of some exotic species like *Xenopus* and fire belly newts was recently banned, yet individuals are often imported in aquarium vegetation and have been recently observed for sale at a large pet store in San Jose by colleagues in Costa Rica (pers comm Rodolfo Leiton, 2017). Lastly, Costa Rica's multi-billion dollar tourism industry leads visitors, especially international visitors from

Europe and the United States (14% and 40% of annual visitors, respectively), to naïve natural areas (Instituto Costarricense de Turismo 2016). These anthropogenic and environmental factors combined provide a potential pathway for the introduction of *Bsal* into Costa Rica and subsequent spread throughout Central America and into North America.

Significance and Rationale

As a product of habitat degradation, environmental shifts, the introduction of invasive species, and emergent infectious diseases, such as those caused by Bd, Bsal, ranaviruses, and Perkinsozoa, amphibians are widely imperiled, with 18% listed as endangered or critically endangered (Chambouvet et al. 2015, Yap et al. 2015, Parrott et al. 2016). Due to their crucial ecological roles in systems across the world, the endangerment of amphibians threatens ecosystem health and integrity on a global scale (Welsh & Ollivier 1998, Whiles et al. 2006, Valencia-Aguilar et al. 2013, Hocking & Babbitt 2014, Yap et al. 2015). We have already begun to observe the collapse of certain predator communities in response to ongoing amphibian population declines (Zipkin *et al.* 2020). In order to combat these declines, especially those caused by emergent infectious pathogens and their associated diseases, proactive management measures must be designed and implemented (Grant et al. 2017, OIE 2018, Martel et al. 2020). Responses to wildlife disease crises are often highly reactive and, in some cases, inefficient. For instance, in the case of Bd, it took nearly three decades after the pathogen's initial emergence for *Bd* to be described and organized conservation action plans to be developed (Grant *et al.* 2017). In contrast, the emergence of *Bsal* has been met with fiercely proactive mitigative measures, focusing on pathogen surveillance and epidemiological research, primarily focused in North America, Europe, Asia, and Mexico (Martel et al. 2013, Martel et al. 2014, Cunningham et al.

2015, Parrot *et al.* 2016, Govindarajulu *et al.* 2017, Klocke *et al.* 2017, Laking *et al.* 2017, Nguyen *et al.* 2017, O'Hanlon *et al.* 2018, Malagon *et al.* 2020, Martel *et al.* 2020). Because *Bsal* has yet to emerge in the Americas, biologists are provided a rare opportunity to expand upon ongoing mitigative efforts and to implement such work in as many countries at risk of *Bsal* introduction as possible.

Costa Rica is one such country, whose amphibian community has already been devastated by *Bd*-related epizootics (Puschendorf et al. 2009). Additionally, the country's wealth of potentially susceptible salamander hosts and habitats suitable for the persistence of *Bsal*, combined with its flourishing ecotourism industry, place Costa Rica at risk of *Bsal* introduction (Savage et al. 2002, Cheng et al. 2011, Martel et al. 2014, Laking et al. 2017). In order to better understand how Costa Rican salamander communities may be affected by the emergence of *Bsal*, it is first important to establish the status of *Bsal* in the country through targeted surveillance or retrospective testing of archived samples, similar to actions being taken in other areas of the world (Martel et al. 2014, Gray et al. 2015, Parrot et al. 2016, Govindarajulu et al. 2017, Klocke et al. 2017). Additionally, it is crucial to determine or predict the susceptibility of various Costa Rican salamander species to *Bsal*. This could be done either through susceptibility trials of live amphibians or through the characterization of the cutaneous microbiomes of salamanders and their potential fungal inhibiting properties, to predict innate immunity to *Bsal* (Becker & Harris 2010, Becker et al. 2009, Becker et al. 2015, Jimenez & Sommer 2017, Madison et al. 2017, Catenazzi et al. 2018). Finally, it is important to identify areas that may be at high risk of introduction. Identifying these areas of high Bsal risk could inform concerted and efficient surveillance efforts, which may enable researchers to find *Bsal* in early stages of emergence and mitigate its spread throughout Costa Rica and other countries (Grant et al. 2017).

This thesis is constructed of two studies which evaluate the current status of *Bsal* and other relevant amphibian pathogens in Costa Rican salamander communities and identify areas in Costa Rica that are at high risk of *Bsal* introduction to inform future conservation action. The objective of the first study was to survey for Bsal, Bd, and ranavirus in highland salamander populations, believed to be at significant risk of Bsal introduciton. I predicted that Bsal and ranavirus would be absent from these communities but expected to find Bd at low prevalences. The objective of the second study was to create an Ecological Niche Model (ENM) designed to identify areas in Costa Rica highly environmentally suitable for the persistence of *Bsal*. Using available salamander species distribution data and national protected areas visitation data, areas of high environmental suitability, high species biodiversity, and high human visitation could be identified as areas of high *Bsal* introduction risk. I predicted that roughly 30 of Costa Rica's 49 salamander species would be in areas environmentally suitable for *Bsal*. I also predicted that environmental suitability would be highest in Costa Rica's two largest mountain ranges, the Cordillera Central and Cordillera Talamanca. Lastly, I predicted that national protected areas that fall in these mountain ranges, such as the Poas Volcano National Park, would be areas of intersection for high suitability and high human visitation.

Literature Cited

- Albecker, M.A., Belden, L.K. and McCoy, M.W., 2019. Comparative analysis of anuran amphibian skin microbiomes across inland and coastal wetlands. Microbial ecology 78(2): pp.348-360.
- Apprill, A., Robbins, J., Eren, A.M., Pack, A.A., Reveillaud, J., Mattila, D., Moore, M., Niemeyer, M., Moore, K.M. and Mincer, T.J., 2014. Humpback whale populations share a

core skin bacterial community: towards a health index for marine mammals?. PLoS One 9(3):90785.

- Becker, M.H., Brucker, R.M., Schwantes, C.R., Harris, R.N. and Minbiole, K.P., 2009. The bacterially produced metabolite violacein is associated with survival of amphibians infected with a lethal fungus. Appl. Environ. Microbiol. 75(21:6635-6638.
- Becker, M.H. and Harris, R.N., 2010. Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. PloS one 5(6):10957.
- Becker, M.H., Harris, R.N., Minbiole, K.P., Schwantes, C.R., Rollins-Smith, L.A., Reinert, L.K., Brucker, R.M., Domangue, R.J. and Gratwicke, B., 2011. Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. Ecohealth 8(4):501-506.
- Becker, M.H., Walke, J.B., Cikanek, S., Savage, A.E., Mattheus, N., Santiago, C.N., Minbiole,
 K.P., Harris, R.N., Belden, L.K. and Gratwicke, B., 2015. Composition of symbiotic bacteria predicts survival in Panamanian golden frogs infected with a lethal fungus. Proceedings of the Royal Society B: Biological Sciences 282(1805):20142881.
- Berger, Lee, Rick Speare, Peter Daszak, D. Earl Green, Andrew A. Cunningham, C. Louise Goggin, Ron Slocombe *et al.* 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proceedings of the National Academy of Sciences 15: 9031-9036.
- Carter, E.D., Miller, D.L., Peterson, A.C., Sutton, W.B., Cusaac, J.P.W., Spatz, J.A., Rollins-Smith, L., Reinert, L., Bohanon, M., Williams, L.A. and Upchurch, A., 2019. Conservation risk of *Batrachochytrium salamandrivorans* to endemic lungless salamanders. Conservation Letters.

- Catenazzi, A., Flechas, S.V., Burkart, D., Hooven, N.D., Townsend, J. and Vredenburg, V.T.,
 2018. Widespread elevational occurrence of antifungal bacteria in Andean amphibians
 decimated by disease: a complex role for skin symbionts in defense against
 chytridiomycosis. Frontiers in microbiology 9:465.
- Chatfield, M.W., Moler, P. and Richards-Zawacki, C.L., 2012. The amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, in fully aquatic salamanders from southeastern North America. Plos One 7(9):44821.
- Cheng, T.L., Rovito, S.M., Wake, D.B. and Vredenburg, V.T. 2011. Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. Proceedings of the National Academy of Sciences 108: 9502-9507
- Chinnadurai, S.K., Cooper, D., Dombrowski, D.S., Poore, M.F. and Levy, M.G., 2009.
 Experimental infection of native North Carolina salamanders with *Batrachochytrium dendrobatidis*. Journal of Wildlife Diseases 45(3):631-636.
- Christian, K., Weitzman, C., Rose, A., Kaestli, M. and Gibb, K., 2018. Ecological patterns in the skin microbiota of frogs from tropical Australia. Ecology and evolution 8(21):10510-10519.
- Crump, Martha L., Frank R. Hensley, and Kenneth L. Clark. 1992. Apparent decline of the golden toad: underground or extinct? Copeia: 413-420.
- Cunningham, A.A., Beckmann, K., Perkins, M., Fitzpatrick, L., Cromie, R., Redbond, J., O'Brien, M.F., Ghosh, P., Shelton, J. and Fisher, M.C. 2015. Emerging disease in UK amphibians. Veterinary Record. 176.

- Fisher, Matthew C., Trenton WJ Garner, and Susan F. Walker. 2009. Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. Annual review of microbiology 63:291-310.
- Govindarajulu, P., Matthews, E. and Ovaska, K. 2017. Swabbing for *Batrachochytrium* salamandrivorans on wild rough-skinned newts (*Taricha granulosa*) and pet-traded amphibians on southern Vancouver Island, British Columbia, Canada. Herp. Rev 48:564-568.
- Grant, E.H.C., Muths, E.L., Katz, R.A., Canessa, S., Adams, M.J., Ballard, J.R., Berger, L.,
 Briggs, C.J., Coleman, J., Gray, M.J. and Harris, M.C. 2016. Salamander chytrid fungus (*Batrachochytrium salamandrivorans*) in the United States—Developing research, monitoring, and management strategies. No. 2015-1233. US Geological Survey.
- Grant, E.H.C., Muths, E., Katz, R.A., Canessa, S., Adams, M.J., Ballard, J.R., Berger, L., Briggs,
 C.J., Coleman, J.T., Gray, M.J. and Harris, M.C., 2017. Using decision analysis to support
 proactive management of emerging infectious wildlife diseases. Frontiers in Ecology and the
 Environment 15(4):214-221.
- Gray, M.J., Miller, D.L. and Hoverman, J.T., 2009. Ecology and pathology of amphibian ranaviruses. Diseases of aquatic organisms 87(3):243-266.
- Gray, M.J. and Chinchar, V.G., 2015. Ranaviruses: lethal pathogens of ectothermic vertebrates. Springer Science+ Business Media.
- Gray, M.J., Lewis, J.P., Nanjappa, P., Klocke, B., Pasmans, F., Martel, A., Stephen, C., Olea, G.P., Smith, S.A., Sacerdote-Velat, A. and Christman, M.R. 2015. *Batrachochytrium salamandrivorans*: The North American response and a call for action. PLoS Pathog. 11

- Green, D.E., CoNVerSe, K.A. and SCHrADer, A.K., 2002. Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996-2001. Annals of the New York Academy of Sciences 969(1):323-339.
- Harris, R.N., Brucker, R.M., Walke, J.B., Becker, M.H., Schwantes, C.R., Flaherty, D.C., Lam,
 B.A., Woodhams, D.C., Briggs, C.J., Vredenburg, V.T. and Minbiole, K.P., 2009. Skin
 microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. The ISME journal 3(7):818.
- Herrel, A. and van der Meijden, A., 2014. An analysis of the live reptile and amphibian trade in the USA compared to the global trade in endangered species. The Herpetological Journal 24(2):103-110.
- Hocking, D.J. and Babbitt, K.J., 2014. Amphibian contributions to ecosystem services. Herpetological Conservation and Biology.

Instituto Costarricense de Turismo. 2016. Anuario Estadístico de Turismo 2016.

- Isidoro-Ayza, Marcos, Jeffrey M. Lorch, Daniel A. Grear, Megan Winzeler, Daniel L. Calhoun, and William J. Barichivich. 2017. Pathogenic lineage of Perkinsea associated with mass mortality of frogs across the United States. Scientific Reports 7(1):10288.
- Jiménez, R.R. and Sommer, S., 2017. The amphibian microbiome: natural range of variation, pathogenic dysbiosis, and role in conservation. Biodiversity and Conservation 26(4):763-786.
- Johnson, M.L. and Speare, R., 2005. Possible modes of dissemination of the amphibian chytrid *Batrachochytrium dendrobatidis* in the environment. Diseases of aquatic organisms 65(3): pp.181-186.
- Jones, K. E., Patel, N. G., Levy, M. A., Storeygard, A., Balk, D., Gittleman, J. L., & Daszak, P. 2008. Global trends in emerging infectious diseases. Nature 451(7181):990-993.

- Klocke, B., Becker, M., Lewis, J., Fleischer, R.C., Muletz-Wolz, C.R., Rockwood, L., Aguirre,
 A.A. and Gratwicke, B., 2017. *Batrachochytrium salamandrivorans* not detected in US survey of pet salamanders. Scientific reports 7(1):13132.
- Krediet, C.J., Ritchie, K.B., Paul, V.J. and Teplitski, M., 2013. Coral-associated microorganisms and their roles in promoting coral health and thwarting diseases. Proceedings of the Royal Society B: Biological Sciences 280(1755):20122328.
- Laking, A. E., Ngo, H. N., Pasmans, F., Martel, A., & Nguyen, T. T. 2017. Batrachochytrium salamandrivorans is the predominant chytrid fungus in Vietnamese salamanders. Scientific Reports, 7.
- Leiton, Rodolfo. 2017. Personal communication
- Loudon, A.H., Woodhams, D.C., Parfrey, L.W., Archer, H., Knight, R., McKenzie, V. and Harris, R.N., 2014. Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). The ISME journal 8(4):830.
- Madison, J.D., Berg, E.A., Abarca, J.G., Whitfield, S.M., Gorbatenko, O., Pinto, A. and Kerby,
 J.L., 2017. Characterization of *Batrachochytrium dendrobatidis* inhibiting bacteria from
 amphibian populations in Costa Rica. Frontiers in microbiology 8:290.
- Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M.C., Woeltjes, A., Bosman, W., Chiers, K., Bossuyt, F. and Pasmans, F. 2013. *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. Proceedings of the National Academy of Sciences 110:15325-15329.
- Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M.C., Farrer, R.A., Schmidt, B.R., Tobler, U., Goka, K. & Lips, K.R. 2014. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science 346:630-631.

- Martel, A., Vila-Escale, M., Fernández-Giberteau, D., Martinez-Silvestre, A., Canessa, S., Van Praet, S., Pannon, P., Chiers, K., Ferran, A., Kelly, M. and Picart, M. 2020. Integral chain management of wildlife diseases. Conservation Letters 12707.
- Malagon, D.A., Melara, L.A., Prosper, O.F., Lenhart, S., Carter, E.D., Fordyce, J.A., Peterson,
 A.C., Miller, D.L. and Gray, M.J., 2020. Host density and habitat structure influence host contact rates and *Batrachochytrium salamandrivorans* transmission. Scientific reports 10(1): 1-11.
- McKenzie, V.J., Bowers, R.M., Fierer, N., Knight, R. and Lauber, C.L., 2012. Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. The ISME journal 6(3):588.
- Miller, Courtney A., Geraud Canis Tasse Taboue, Mary MP Ekane, Matthew Robak, Paul R.
 Sesink Clee, Corinne Richards-Zawacki, Eric B. Fokam, Nkwatoh Athanasius Fuashi, and
 Nicola M. Anthony. 2018. Distribution modeling and lineage diversity of the chytrid fungus *Batrachochytrium dendrobatidis (Bd)* in a central African amphibian hotspot. PloS
 one 13(6):0199288.
- Mu, W.H., Geng, Y., Yu, Z.H., Wang, K.Y., Huang, X.L., Ou, Y.P., Chen, D.F., He, C.L.,
 Zhong, Z.J., Yang, Z.X. and Lai, W.M., 2018. FV3-like ranavirus infection outbreak in
 black-spotted pond frogs (*Rana nigromaculata*) in China. Microbial pathogenesis 123:111114.
- Muletz-Wolz, C.R., Yarwood, S.A., Campbell Grant, E.H., Fleischer, R.C. and Lips, K.R., 2018. Effects of host species and environment on the skin microbiome of Plethodontid salamanders. Journal of Animal Ecology 87(2):341-353.

- Nguyen, Tao Thien, Thinh Van Nguyen, Thomas Ziegler, Frank Pasmans, and An Martel. 2017. Trade in wild anurans vectors the urodelan pathogen *Batrachochytrium salamandrivorans* into Europe. Brill.
- Naik, S., Bouladoux, N., Wilhelm, C., Molloy, M.J., Salcedo, R., Kastenmuller, W., Deming, C., Quinones, M., Koo, L., Conlan, S. and Spencer, S., 2012. Compartmentalized control of skin immunity by resident commensals. Science 337(6098):1115-1119.
- O'Hanlon, Simon J., Adrien Rieux, Rhys A. Farrer, Gonçalo M. Rosa, Bruce Waldman, Arnaud Bataille, Tiffany A. Kosch *et al.* 2018. Recent Asian origin of chytrid fungi causing global amphibian declines. Science 360(6389):621-627.
- OIE World Organization for Animal Health. 2018. Terrestrial Animal Health Code. Retrieved from https://www.oie.int/fileadmin/Home/eng/Health_standards/tahc/2018/en_sommaire.htm
- Parrott, Joshua Curtis, Alexander Shepack, David Burkart, Brandon LaBumbard, Patrick Scimè, Ethan Baruch, and Alessandro Catenazzi. 2016. Survey of pathogenic chytrid rungi (*Batrachochytrium dendrobatidis* and *B. salamandrivorans*) in salamanders from three mountain ranges in Europe and the Americas. EcoHealth 1-7.
- Puschendorf, R., Carnaval, A.C., VanDerWal, J., Zumbado-Ulate, H., Chaves, G., Bolaños, F. and Alford, R.A., 2009. Distribution models for the amphibian chytrid *Batrachochytrium dendrobatidis* in Costa Rica: proposing climatic refuges as a conservation tool. Diversity and Distributions 15(3):401-408.
- Rachowicz, Lara J., JEAN HERO, Ross A. Alford, John W. Taylor, Jess AT Morgan, Vance T. Vredenburg, James P. Collins, and Cheryl J. Briggs. 2005. The novel and endemic pathogen

hypotheses: competing explanations for the origin of emerging infectious diseases of wildlife. Conservation Biology 19(5):1441-1448.

- Redford, K.H., Segre, J.A., Salafsky, N., del Rio, C.M. and McAloose, D., 2012. Conservation and the microbiome. Conservation Biology 26(2):195-197.
- Rothermel, B.B., Walls, S.C., Mitchell, J.C., Dodd Jr, C.K., Irwin, L.K., Green, D.E., Vazquez, V.M., Petranka, J.W. and Stevenson, D.J., 2008. Widespread occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in the southeastern USA. Diseases of Aquatic Organisms 82(1):3-18.
- Rothermel, B.B., Miller, D.L., Travis, E.R., McGuire, J.L.G., Jensen, J.B. and Yabsley, M.J., 2016. Disease dynamics of red-spotted newts and their anuran prey in a montane pond community. Diseases of Aquatic Organisms 118(2):113-127.
- Schmidt, B.R., Bozzuto, C., Lötters, S. and Steinfartz, S., 2017. Dynamics of host populations affected by the emerging fungal pathogen *Batrachochytrium salamandrivorans*. Royal Society open science 4(3):160801.
- Sonn, J.M., Berman, S. and Richards-Zawacki, C.L., 2017. The influence of temperature on chytridiomycosis in vivo. EcoHealth, 14(4):762-770.
- Spitzen-van der Sluijs, A., Martel, A., Asselberghs, J., Bales, E.K., Beukema, W., Bletz, M.C., Dalbeck, L., Goverse, E., Kerres, A., Kinet, T. and Kirst, K. 2016. Expanding distribution of lethal amphibian fungus *Batrachochytrium salamandrivorans* in Europe. Emerging infectious diseases 22.
- Sabino-Pinto, J., Veith, M., Vences, M. and Steinfartz, S., 2018. Asymptomatic infection of the fungal pathogen *Batrachochytrium salamandrivorans* in captivity. Scientific reports 8(1):1-8.
- Savage, J. M. 2002. The amphibians and reptiles of Costa Rica: a herpetofauna between two continents, between two seas. University of Chicago press.
- Scheele, B.C., Pasmans, F., Skerratt, L.F., Berger, L., Martel, A.N., Beukema, W., Acevedo,
 A.A., Burrowes, P.A., Carvalho, T., Catenazzi, A. and De la Riva, I., 2019. Amphibian
 fungal panzootic causes catastrophic and ongoing loss of biodiversity. Science 363(6434):
 1459-1463.
- Stegen, G., Pasmans, F., Schmidt, B.R., Rouffaer, L.O., Van Praet, S., Schaub, M., Canessa, S.,
 Laudelout, A., Kinet, T., Adriaensen, C., Haesebrouck, F., Bert, W., Bossuyt, F., Martel, A.
 2017. Drivers of *Batrachochytrium salamandrivorans* mediated salamander extirpation.
 Nature 544:353-354.
- Stuart, S.N., Chanson, J.S., Cox, N.A., Young, B.E., Rodrigues, A.S., Fischman, D.L. and Waller, R.W., 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306(5702):1783-1786.
- Teacher, A.G.F., Cunningham, A.A. and Garner, T.W.J., 2010. Assessing the long-term impact of ranavirus infection in wild common frog populations. Animal Conservation 13(5): 514-522.
- Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P. and Egholm, M., 2009. A core gut microbiome in obese and lean twins. Nature 457(7228):480.
- Valencia-Aguilar, A., Cortés-Gómez, A.M. and Ruiz-Agudelo, C.A., 2013. Ecosystem services provided by amphibians and reptiles in Neotropical ecosystems. International Journal of Biodiversity Science, Ecosystem Services & Management 9(3):257-272.

- Van Rooij, P., Martel, A., Haesebrouck, F. and Pasmans, F., 2015. Amphibian chytridiomycosis: a review with focus on fungus-host interactions. Veterinary Research 46(1):137.
- Wake, D.B. and Vredenburg, V.T., 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proceedings of the National Academy of Sciences 105:11466-11473.
- Weinstein, S.B., 2009. An aquatic disease on a terrestrial salamander: individual and population level effects of the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, on *Batrachoseps attenuatus (Plethodontidae*). Copeia 2009(4):653-660.
- Welsh Jr, H.H. and Ollivier, L.M., 1998. Stream amphibians as indicators of ecosystem stress: a case study from California's redwoods. Ecological Applications 8(4):1118-1132.
- Whiles, M.R., Lips, K.R., Pringle, C.M., Kilham, S.S., Bixby, R.J., Brenes, R., Connelly, S., Colon-Gaud, J.C., Hunte-Brown, M., Huryn, A.D. and Montgomery, C., 2006. The effects of amphibian population declines on the structure and function of Neotropical stream ecosystems. Frontiers in Ecology and the Environment 4(1):27-34.
- Yap, T. A., Koo, M. S., Ambrose, R. F., Wake, D. B., & Vredenburg, V. T. 2015. Averting a North American biodiversity crisis. Science 349:481-482.
- Zipkin, E.F., DiRenzo, G.V., Ray, J.M., Rossman, S. and Lips, K.R. 2020. Tropical snake diversity collapses after widespread amphibian loss. Science 367:814-816.

CHAPTER 2

SURVEILLANCE FOR THE PATHOGENIC FUNGI, *BATRACHOCHYTRIUM* SALAMANDRIVORANS AND B. DENDROBATIDIS, AND RANAVIRUS IN HIGHLAND COSTA RICAN SALAMANDER POPULATIONS¹

¹Adams, H.C., Hernandez, S.M., Gray, M.J., Connelly, S. Yabsley, M.J. 2020 to be submitted to Journal of Wildlife Diseases.

ABSTRACT

For roughly five decades, emergent infectious diseases have directly contributed to catastrophic declines in global amphibian populations. Since 2008, the emergent fungal pathogen, Batrachochytrium salamandrivorans (Bsal), has caused significant population declines in European salamander species, sparking international concern. The emergence of this pathogen has been met with significant proactive political, surveillance and research efforts in Europe, the United States, Canada and Mexico. No such work has been conducted in Costa Rica, a country: 1) rich in salamander species diversity, 2) mountainous habitat suitable for the persistence and increased pathogenicity of *Bsal*, 3) high likelihood for pathogen pollution from a multibilliondollar tourism industry. We believe Costa Rica's salamander community to be at significant risk of *Bsal* introduction, meriting proactive surveillance and research efforts. Between November 2018 and December 2019, we surveyed for *Bsal*, *B. dendrobatidis* (*Bd*), and ranavirus within highland salamander populations in the Central and Talamanca Mountains of Costa Rica, areas that would be very ecologically suitable for Bsal persistence. While we did not anticipate detecting *Bsal* or ranavirus, we did hypothesize we would detect *Bd* at low prevalences. We sampled 100 salamanders representing 4 species (Bolitoglossa subpalamata, B. pesrubra, and Nototriton abscondens) across multiple sites, including one of Costa Rica's most visited national parks. None of our target pathogens were detected. Samples were analyzed using PCR at the Southeast Cooperative Wildlife Disease Study at the University of Georgia. This was the first study to survey for *Bsal* in Costa Rica and, while we did not detect the pathogen, we encourage further surveillance efforts.

Key words: salamander, Costa Rica, pathogen surveillance, *Batrachochytrium salamandrivorans*, emergent infectious disease

Introduction

Batrachochytrium salamandrivorans (Bsal) is an emergent fungal pathogen that has caused significant population declines in European salamander populations (Martel et al. 2013, Martel et al. 2014). Like its close relative, B. dendrobatidis (Bd), Bsal can cause aggressive chytridiomycosis, characterized by invasion and erosion of the host's skin, leading to morbidity and mortality, depending on host susceptibility. In the early 2000s, *Bsal* was inadvertently introduced to the Netherlands from East Asia, presumably through the pet trade (Martel et al. 2013, Martel et al. 2014, Laking et al. 2017, Nguyen et al. 2017). Since its introduction, it has been detected in wild salamander populations in Belgium, Germany and Spain, and in captive salamanders in the UK, Germany and Spain (Martel et al. 2014, Cunningham et al. 2015, Spitzen-van der Sluijs et al. 2016, Sabino-Pinto et al. 2018, Martel et al. 2020). Although Bsal has not been detected in the Americas, its introduction is regarded as unavoidable (Grant et al. 2017). This will endanger the world's most diverse salamander communities, specifically those species belonging to Salamandridae and Plethodontidae, which have high and variable susceptibility to Bsal-caused chytridiomycosis, respectively (Martel et al. 2014, Cunningham et al. 2015, Yap et al. 2015, Parrot et al. 2016, Spitzen-van der Sluijs et al. 2016, Carter et al. 2019). As such, proactive pathogen surveillance has been prioritized with the goal of early detection (Gray et al. 2017). Early pathogen detection would best inform the development of management strategies to mitigate the impacts that Bsal may have on global salamander communities. In fact, proactive pathogen surveillance, in association with host species management, recently helped to mitigate a Bsal outbreak in a small reservoir in Spain's Montnegre i el Corredor Natural Park (Martel et al. 2020). Early detection allowed for the efficient removal of host species, drainage of the reservoir, and prevention of transmission to the surrounding area. It is important to note that, in that case, the outbreak was detected over 1000 km from the closest report of *Bsal*, likely through the introduction of an invasive salamander species, illustrating the ability of this pathogen to be moved move across large geographic areas through human activities (Martel *et al.* 2020). Given the large biodiversity and ecological significance of salamanders, enacting similar conservation measures in North and Central America is of great importance (Hocking & Babbitt 2014, Semlitsch *et al.* 2014, Yap *et al.* 2015).

Extensive surveillance has been conducted in some parts of the Americas, including Canada, the United States, and sporadic studies in Mexico, and the Peruvian Andes (Martel *et al.* 2014, Gray *et al.* 2015, Parrot *et al.* 2016, Govindarajulu *et al.* 2017, Klocke *et al.* 2017). However, such efforts have yet to be implemented in many South and Central American countries, such as Costa Rica, a country significantly impacted by emergent amphibian pathogens (Parrot *et al.* 2016). In the late 1980s and 1990s, Costa Rica experienced substantial *Bd*-associated amphibian mortality events, most notably in the Monteverde and Las Tablas regions, which included the extinction of the charismatic golden toad (*Incilius periglenes*) (Crump *et al.* 1992, Puschendorf *et al.* 2009). Additionally, *Ranavirus*, another OIE-notifiable pathogen responsible for global die-offs of amphibians (Duffus *et al.* 2015, Gray & Chinchar 2015), has been found widely distributed in lowland Costa Rican anuran populations (Whitfield *et al.* 2013). Though little is known about the epidemiology of *Ranavirus* in Costa Rica, this pathogen may negatively impact amphibian population dynamics, especially during co-infections with *Bd* (Whitfield *et al.* 2013, Warne *et al.* 2016).

While Costa Rica's pathogen-associated amphibian declines been have predominantly documented in anuran populations, *Bsal* now poses a threat to Costa Rica's salamander

community, the world's fifth most diverse (Kubicki & Arias 2016). All Costa Rican salamander species undergo direct development and are generally scansorial during nocturnal foraging and otherwise diurnally subterranean (Savage 2002). Because all Costa Rican salamanders belong to the family *Plethodonidae*, which is known to have variable susceptibility to *Bsal*, and with some experiencing severe mortality, the impact of *Bsal* in this naïve community could be catastrophic (Savage et al. 2002, Martel et al. 2014, Carter et al. 2019). Recent evidence has also shown that anurans can support subclinical infections and transmit *Bsal* to healthy salamanders (Ngueyn et al. 2017, Stegen et al. 2017). Thus, Costa Rica's diverse anuran community to further impact the epidemiology of the pathogen in Costa Rica. Additionally, Costa Rica is a country with habitats that are likely ecologically suitable for the persistence of *Bsal*, based on the pathogen's known thermal and moisture requirements (Cheng et al. 2011, Martel et al. 2014, Kubicki & Arias 2016, Laking et al. 2017). Lastly, Costa Rica's multi-billion-dollar tourism industry brings hundreds of thousands of visitors, in particular from Europe and the United States (14% and 40% of annual visitors, respectively), into contact with natural areas. A small portion (~0.5%) of annual visitors come from areas in which *Bsal* is endemic. This vitally important industry creates a potential pathway for *Bsal* introduction from Europe into Costa Rica and then possibly from Costa Rica into North America (Instituto Costarricense de Turismo 2016).

We believe it is crucial to initiate proactive conservation measures in Costa Rica to mitigate the impacts of *Bsal* and to avoid a repetition of the amphibian declines observed in association with *Bd*. In this study, we conducted surveillance to collect baseline data for *Bsal* prevalence in Costa Rica and additionally surveyed for *Bd* and *Ranavirus* in high montane salamander communities across multiple locations from December 2018 to December 2019. The collection of this baseline data for these pathogens in highland Costa Rican salamanders is

crucial for the identification and tracking of future outbreaks. We did not expect to find *Bsal* or *Ranavirus* in these species in this region. We did not anticipate finding *Ranavirus* in this study because of a general lack of suitable environmental conditions at our study sites (Savage 2002, Gray *et al.* 2009, Hoverman *et al.* 2011). We did anticipate finding *Bd* at low prevalences due to its proposed endemism in Costa Rica (Puschendorf *et al.* 2009).

<u>Methods</u>

Field Sampling

Between November 2018 and December 2019, we sampled salamanders at 5 locations in the Cerro del Muerte region of the Cordillera Talamanca, and within a 650-m² area along the boulevard of Poas Volcano National Park. We sampled in Poas Volcano National Park during three discrete sampling events on 4 December and 13 December 2018 and 14 June 2019. Sampling locations were selected based on previous observations of target species and historic salamander location data. At Poas Volcano National Park, we sampled in the median of the main boulevard. The median was characterized by moist soil with multiple rocks, shrubs and small trees and was roughly one meter wide and 400 m long. The median was isolated from the surrounding tropical montane forest matrix by the paved walkway on both sides. Because all surveys at this site were during the day, due to temporal restrictions to park visitation, we carefully flipped rock cover items to find salamanders.

We sampled areas in tropical moist broadleaf forests and paramo systems in Cerro de la Muerte. These tropical moist broadleaf forests were characterized by dense trees and understory with moderate to no obvious human modification. Trail cuts were the primary anthropogenic landscape modification, which exposed plant roots and allowed for the growth of large mats of

moss in many areas. In forested areas, we surveyed at night along area-constrained transects for active salamanders on moss mats, exposed root systems, and on slightly elevated vegetation, as our target species are scansorial (Level 1) (Crump & Scott 1994, p. 84-92). We predominantly found salamanders in microhabitats characterized by high amounts of lichen and moss and areas of slight disturbance, such as a trail cut. Paramo sites were characterized by typically dry soil, many rock cover items, often covered or surrounded by lichens, mosses, and other herbaceous vegetation, and shrubby plants. Surveys in paramos were conducted at day and night, during which we surveyed for salamanders on mossy embankments and underneath rocks. However, in paramos, we only successfully found salamanders during the day underneath rocks. We searched for salamanders along established area-constrained transects, surveying salamanders found on the surface and under cover items (Level 2 intensity) (Crump & Scott 1994, p. 84-92). We sampled salamander opportunistically along these transects, finding one salamander approximately every 150 cover items.

We handled all animals with powder-free nitrile gloves and placed them in individual plastic bags prior to processing (Gray *et al.* 2018). We collected standard amphibian morphometric measurements (standard length, tail length, head width and snout length) and mass. We aged (adult or juvenile) and sexed animals based on size and physical characteristics (Savage 2002). We examined each salamander for any signs of disease (righting reflex and lesions such as skin lesions, discoloration, or dysecdysis) (Gray *et al.* 2009, Martel *et al.* 2013). We swabbed each individual 30 times on the ventral side and front and back digits following methods from Parrott *et al.* (2016) using a PurFlock[®] Ultra sterile synthetic cotton applicators (Puritan Diagnostics LLC Guilford, Maine, USA). For *Ranavirus* testing, we collected a 2-5mm tail clipping using sterile razor blades from individuals with a tail length ≤20mm (Sutton *et al.*

2014). Incision sites were treated with manual pressure with a cotton gauze soaked in diluted betadine to promote clotting and reduce risk of infection. No tail clipping exceeded 15% of the total tail length (Marvin 2013, Segev *et al.* 2013). We released each animal at capture site. To avoid pathogen transmission, we disinfected all sampling equipment, changed gloves, and used new blades for sampling between individuals (Gray *et al.* 2018). All capture and sampling methods were reviewed and approved by University of Georgia's IACUC committee (AUP #: A2018 02-003-R2) and by Costa Rica's Comisión Nacional para la Gestión de la Biodiversidad (File 377, Resolution R-060-2019-OT-CONAGBIOE).

Determining Presence of Bd, Bsal, and Ranavirus

We extracted DNA from all samples using a DNeasy Blood & Tissue Kit (QIAGEN Inc., Valencia, California). Skin swabs were analyzed for presence of *Bd* and *Bsal* using conventional PCR and primers as described by Boyle *et al.* (2004) and Blooi *et al.* (2013) for *Bd* and *Bsal* respectively. Our positive control was a gBlock containing *Bd* and *Bsal* target sequences of DNA (IDT, Coralville, Iowa). Tail clippings were analyzed for *Ranavirus* using conventional PCR as described by Kattenbelt *et al.* (2000). We used DNA from a sequence confirmed *Ranavirus*-positive clinical case, provided by the Southeastern Cooperative Wildlife Disease Study (SCWDS), as our *Ranavirus* positive control.

Results

Between December 2018 and December 2019, we sampled 57 salamanders (47 *Bolitoglossa pesrubra* and 10 *B. cerroensis*) at five sites in Cerro de la Muerte and 43 salamanders (39 *B. subpalmata* and 4 *Nototriton abscondens*) in Parque Nacional Volcán Poas (Fig. 2.1, Table 2.1). All salamanders were negative for all three pathogens.



Figure 2.1 (A) Shows the two regions of survey (Parque Nacional Volcan Poas & Cerro de la Muerte) and number of salamanders surveyed in each region. (B) Shows the five sites surveyed in Cerro de la Muerte and the number of salamanders surveyed at each site.

Table 2.1 Sample size and prevalence of *Batrachochytrium salamandrivorans (Bsal)*, *B*.

	Locality	Elevation (m a.s.l.)	No. positive/No. sampled for <i>Bd/Bsal</i>	No. positive/No. Sampled for <i>Ranavirus</i>
Cerro de la Muerte			0/57	0/49
Bolitoglossa pesrubra	Iyok Ami & Route 2	2600-2830	0/14	0/12
	Paramo 84	3209-3275	0/10	0/8
	Paramo 86	3324-3403	0/7	0/5
	Paramo 88	3178-3348	0/16	0/15
B. cerroensis	Iyok Ami	2630-2830	0/8	0/7
	Paramo 84	3250-3280	0/2	0/2
Parque Nacional Volcan Poas			0/43	0/36
B. subpalmata	Volcan Poas	2500	0/39	0/35
Nototriton abscondens	Volcan Poas	2500	0/4	0/1

dendrobatidis (Bd), and Ranavirus in four Costa Rican salamander species

Discussion

None of our target pathogens, *Bsal, Bd*, and *Ranavirus*, were detected by PCR in the salamanders sampled in this study. This is encouraging, especially at Poas Volcano National Park. Historically, this is the most visited national park in Costa Rica, with ~ 400,000 tourists in 2016, ~ 50% of whom are not Costa Ricans (Instituto Costarricense de Turismo 2018). The emergence of novel pathogens has often been associated with human movement and visitation to natural areas, as was the case with White Nose Syndrome in bats and its associated pathogen, *Pseudogymnoascus destructans* (Coleman 2014). Because of this association, we believe Poas

Volcano to be an area of significant introduction risk for Costa Rica. Although visitations dropped following the Poas Volcano's 2017 eruption, the park still receives thousands of visitors annually, increasing the likelihood that *Bsal* or other pathogens could be introduced. All areas of the park, aside from the main boulevard, were closed during the course of our study, due to volcanic activity. Further surveillance should be conducted in these restricted areas upon reopening, which will concur with increased tourism and risk of introductions. While the region of Cerro de la Muerte receives fewer visitors (~28,000 visitors in 2018) than the Poas Volcano, ongoing surveillance should be conducted, given the area's general ecological suitability to *Bsal* and the presence on the Pan-America Highway, a major thoroughfare for commercial and private travel (Instituto Costarricense de Turismo 2018, Adams *et al.* submitted). Additionally, because of the significant role the international pet trade has played in the globalization of amphibian pathogens, surveillance should also be conducted in any accessible captive amphibians in Costa Rica (Martel *et al.* 2014, Nguyen *et al.* 2017, O'Hanlon *et al.* 2018, Martel *et al.* 2020).

There was little to no standing water in the areas of surveillance, reducing the ecological suitability for *Ranavirus*, which could explain our *Ranavirus* results (Gray *et al.* 2009, Hoverman *et al.* 2011). Additionally, most competent *Ranavirus* hosts are moderately to highly aquatic species and Costa Rican salamander species undergo direct development with no aquatic larval life stage (Savage 2002, Gray *et al.* 2009, Hoverman *et al.* 2011). However, it is possible that sympatric anuran species may maintain *Ranavirus* in the environment which may occasionally spill over into salamander populations (Gray *et al.* 2009).

Considering the level of endemism of *Bd* in Costa Rica since the 1980s, the lack of detection of *Bd* in this study is surprising (Lips *et al.* 2003, Stuart *et al.* 2004, Puschendorf *et al.* 2006, Whitfield *et al.* 2013). However, little is known regarding the prevalence of *Bd* in Costa

Rican salamanders, as published surveillance in Costa Rican urodelans has been nonexistent. It is important to note that *Bd* has been detected in bolitoglossine salamanders in Guatemala and has been linked to salamander declines in Mexico and Guatemala (Cheng *et al.* 2011). This implies the suitability of many Costa Rican salamander species as *Bd* hosts. It is possible that *Bd* is only able to persist in Costa Rican salamander populations at a very low prevalence, similar to other areas of the world, and thus our small sample size precluded detectability (Rothermel *et al.* 2008). Recent population data is unavailable for salamander species in Costa Rica. This makes it difficult to assess the likelihood of pathogen detected within these populations, regardless of prevalence. However, using mark-recapture data we were able to estimate the size of the salamander population in the median at Volcán Poas. We estimated 175 salamanders live in the median. Based on this population estimate and our sample size, we had a 95% chance of pathogen detection at prevalence of 10%. If pathogen prevalence was less than 10%, our chance of detection would have decreased.

To our knowledge we are the first to survey for *Bsal* in Costa Rica and fill in gaps concerning the epidemiology of three pathogens of extreme importance for amphibian conservation in Costa Rica, where amphibians have suffered population declines over the past decades. We recognize that factors such as small sample size and seasonal and site effects could have diminished the detectability of our target pathogens. However, these results still provide important baseline data for future pathogen research. The high likelihood of *Bsal* transmission from direct contact between two hosts, the ability of the pathogen to move across landscapes even with low host densities, and the potential for certain densely populated species, such as *Notophthalmus viridescens* (eastern newt), to serve as super-spreaders put New World salamanders at significant risk from *Bsal* (Hocking & Babbitt 2014, Schmidt *et al.* 2017,

Malagon *et al.* 2020). This has important implications for amphibian biodiversity and ecosystem functionality and services in the Americas (Semlitsch *et al.* 2014). Further surveillance in our study populations, sympatric anuran populations, and captive amphibians in Costa Rica could illuminate the ecology of *Bd* in Costa Rican salamanders. Additionally, retroactive studies using museum specimens (e.g., Cheng *et al.* 2011) might provide a better understanding of amphibian pathogens in Costa Rican salamander populations.

Acknowledgements

This study was made possible by funds provided by the National Science Foundation, Wildlife Disease Association, and Latin American and Caribbean Studies Institute at the University of Georgia. Permitting and overseeing of this work was provided by CONAGEBIO, specifically Jose Hernandez and Melania Muñoz. Research technician and student at the University of Costa Rica, Jeremy Klank, assisted in finding salamanders and data collection.

Literature Cited

Adams, H.C., K. Markham, M. Madden, S.M. Hernandez. Submitted. Assessing the risk of the emergent fungal pathogen, *Batraochochytrium salamandrivorans*, to Costa Rican salamanders. To be submitted

Blooi, M., Pasmans, F., Longcore, J.E., Spitzen-Van Der Sluijs, A., Vercammen, F. and Martel,
A., 2013. Duplex real-time PCR for rapid simultaneous detection of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in amphibian samples. Journal of Clinical Microbiology 51(12):4173-4177.

- Boyle, D.G., Boyle, D.B., Olsen, V., Morgan, J.A.T. and Hyatt, A.D., 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Diseases of aquatic organisms 60(2):141-148.
- Carter, E.D., Miller, D.L., Peterson, A.C., Sutton, W.B., Cusaac, J.P.W., Spatz, J.A., Rollins-Smith, L., Reinert, L., Bohanon, M., Williams, L.A. and Upchurch, A., 2019. Conservation risk of *Batrachochytrium salamandrivorans* to endemic lungless salamanders. Conservation Letters.
- Coleman, J., 2014. White-Nose Syndrome The devastating disease of hibernating bats in North America.
- Cheng, T.L., Rovito, S.M., Wake, D.B. and Vredenburg, V.T. 2011. Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. Proceedings of the National Academy of Sciences 108: 9502-9507
- Crump, Martha L., Frank R. Hensley, and Kenneth L. Clark. 1992. Apparent decline of the golden toad: underground or extinct? Copeia 413-420.
- Crump, M.L., Scott, N.J. Jr (1994) Visual encounter surveys. In: Heyer WR, Donnelly MA,
 McDiarmid RW, Hayek L-AC, Foster MS (eds) Measuring and monitoring biological
 diversity: standard methods for amphibians. Smithsonian Institute, Washington, DC,
 pp 84–92.
- Cunningham, A.A., Beckmann, K., Perkins, M., Fitzpatrick, L., Cromie, R., Redbond, J., O'Brien, M.F., Ghosh, P., Shelton, J. and Fisher, M.C. 2015. Emerging disease in UK amphibians. Veterinary Record 176.

- Govindarajulu, P.U., Matthews, E.L., and Ovaska, K.R. 2017. Swabbing for Batrachochytrium salamandrivorans on Wild Rough-skinned Newts (*Taricha granulosa*) and Pet-Traded Amphibians on Southern Vancouver Island. British Columbia, Canada. Herp. Rev 48:pp.564-568.
- Gray, M.J., Miller, D.L. and Hoverman, J.T., 2009. Ecology and pathology of amphibian ranaviruses. Diseases of aquatic organisms 87(3):243-266.
- Gray, M.J., Lewis, J.P., Nanjappa, P., Klocke, B., Pasmans, F., Martel, A., Stephen, C., Olea, G.P., Smith, S.A., Sacerdote-Velat, A. and Christman, M.R., 2015. *Batrachochytrium salamandrivorans*: the North American response and a call for action. PLoS pathogens 11(12).
- Gray, M.J. and Chinchar, V.G., 2015. Ranaviruses: lethal pathogens of ectothermic vertebrates. Springer Science+ Business Media.
- Gray, M.J., Duffus, A.L.J., Haman, K.H., Harris, R.N., Allender, M.C., Thompson, T.A.,
 Christman, M.R., Sacerdote-Velat, A., Sprague, L.A., Williams, J.M. and Miller, D.L., 2017.
 Pathogen surveillance in herpetofaunal populations: guidance on study design, sample
 collection, biosecurity, and intervention strategies. Herpetological Review 48(2): 334-351.
- Gray, M.J., Spatz, J.A., Carter, E.D., Yarber, C.M., Wilkes, R.P. and Miller, D.L., 2018. Poor biosecurity could lead to disease outbreaks in animal populations. PloS one 13(3): p.e0193243.
- Hoverman, J.T., Gray, M.J., Haislip, N.A. and Miller, D.L. 2011. Phylogeny, life history, and ecology contribute to differences in amphibian susceptibility to *Ranaviruses*.EcoHealth 8(3):301-319.
- Instituto Costarricense de Turismo. 2016. Anuario Estadístico de Turismo 2016.

Instituto Costarricense de Turismo. 2018. Visitas áreas protegidas 2011 - 2018

- Kattenbelt, J.A., Hyatt, A.D. and Gould, A.R., 2000. Recovery of *ranavirus* dsDNA from formalin-fixed archival material. Diseases of Aquatic Organisms 39(2):151-154.
- Klocke, B., Becker, M., Lewis, J., Fleischer, R.C., Muletz-Wolz, C.R., Rockwood, L., Aguirre,
 A.A. and Gratwicke, B., 2017. *Batrachochytrium salamandrivorans* not detected in US survey of pet salamanders. Scientific reports 7(1):1-5.
- Kubicki, B., & Arias, E. 2016. A beautiful new yellow salamander, genus *Bolitoglossa* (Caudata: *Plethodontidae*), from the northeastern slopes of the Cordillera de Talamanca, Costa
 Rica. Zootaxa 4184:329-346.
- Laking, A. E., Ngo, H. N., Pasmans, F., Martel, A., & Nguyen, T. T. 2017. Batrachochytrium salamandrivorans is the predominant chytrid fungus in Vietnamese salamanders. Scientific Reports 7.
- Lips, K.R., Green, D.E. and Papendick, R., 2003. Chytridiomycosis in wild frogs from southern Costa Rica. Journal of Herpetology 215-218.
- Malagon, D.A., Melara, L.A., Prosper, O.F., Lenhart, S., Carter, E.D., Fordyce, J.A., Peterson,
 A.C., Miller, D.L. and Gray, M.J., 2020. Host density and habitat structure influence host contact rates and *Batrachochytrium salamandrivorans* transmission. Scientific reports 10(1):1-11.
- Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M.C., Woeltjes, A., Bosman, W., Chiers, K., Bossuyt, F. and Pasmans, F. 2013. *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. Proceedings of the National Academy of Sciences 110:15325-15329.

- Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M.C., Farrer, R.A., Schmidt, B.R., Tobler, U., Goka, K. & Lips, K.R. 2014. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science 346:630-631.
- Martel, A., Vila-Escale, M., Fernández-Giberteau, D., Martinez-Silvestre, A., Canessa, S., Van Praet, S., Pannon, P., Chiers, K., Ferran, A., Kelly, M. and Picart, M. 2020. Integral chain management of wildlife diseases. Conservation Letters 12707.
- Marvin, G.A., 2013. Critical tail autotomy for reduction of maximal swimming performance in a plethodontid salamander (*Desmognathus quadramaculatus*). Journal of Herpetology 47(1):174-178.
- Nguyen, T. T., Van Nguyen, T., Ziegler, T., Pasmans, F., and Martel, A. 2017. Trade in wild anurans vectors the urodelan pathogen *Batrachochytrium salamandrivorans* into Europe. Brill.
- O'Hanlon, Simon J., Adrien Rieux, Rhys A. Farrer, Gonçalo M. Rosa, Bruce Waldman, Arnaud Bataille, Tiffany A. Kosch *et al.* 2018. Recent Asian origin of chytrid fungi causing global amphibian declines. Science 360(6389): 621-627.
- Parrott, J.C., Shepack, A., Burkart, D., LaBumbard, B., Scimè, P., Baruch, R., and Catenazzi, A..
 2016. Survey of pathogenic chytrid rungi (*Batrachochytrium dendrobatidis* and *B. salamandrivorans*) in salamanders from three mountain ranges in Europe and the Americas. *EcoHealth* : 1-7.
- Pounds, J.A., Fogden, M.P., Savage, J.M. and Gorman, G.C., 1997. Tests of null models for amphibian declines on a tropical mountain. Conservation Biology 11(6):1307-1322.

- Puschendorf, R., Bolaños, F. and Chaves, G., 2006. The amphibian chytrid fungus along an altitudinal transect before the first reported declines in Costa Rica. Biological Conservation 132(1):136-142.
- Puschendorf, R., Carnaval, A.C., VanDerWal, J., Zumbado-Ulate, H., Chaves, G., Bolaños, F. and Alford, R.A., 2009. Distribution models for the amphibian chytrid *Batrachochytrium dendrobatidis* in Costa Rica: proposing climatic refuges as a conservation tool. Diversity and Distributions 15(3):401-408.
- Rothermel, B.B., Walls, S.C., Mitchell, J.C., Dodd Jr, C.K., Irwin, L.K., Green, D.E., Vazquez, V.M., Petranka, J.W. and Stevenson, D.J., 2008. Widespread occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in the southeastern USA. Diseases of Aquatic Organisms 82(1):3-18.
- Sabino-Pinto, J., Veith, M., Vences, M. and Steinfartz, S., 2018. Asymptomatic infection of the fungal pathogen *Batrachochytrium salamandrivorans* in captivity. Scientific reports 8(1):1-8.
- Savage, J. M. 2002. The amphibians and reptiles of Costa Rica: a herpetofauna between two continents, between two seas. University of Chicago press.
- Schmidt, B.R., Bozzuto, C., Lötters, S. and Steinfartz, S., 2017. Dynamics of host populations affected by the emerging fungal pathogen *Batrachochytrium salamandrivorans*. Royal Society open science 4(3):160801.
- Segev, O., Polevikove, A., Blank, L., Goedbloed, D., Küpfer, E., Gershberg, A., Koplovich, A. and Blaustein, L., 2015. Effects of tail clipping on larval performance and tail regeneration rates in the near eastern fire salamander, *Salamandra infraimmaculata*. PloS one, 10(6).

- Semlitsch, R.D., O'Donnell, K.M. and Thompson III, F.R., 2014. Abundance, biomass production, nutrient content, and the possible role of terrestrial salamanders in Missouri Ozark forest ecosystems. Canadian Journal of Zoology 92(12):997-1004.
- Spitzen-van der Sluijs, A., Martel, A., Asselberghs, J., Bales, E.K., Beukema, W., Bletz, M.C., Dalbeck, L., Goverse, E., Kerres, A., Kinet, T. and Kirst, K. 2016. Expanding Distribution of Lethal Amphibian Fungus *Batrachochytrium salamandrivorans* in Europe. Emerging infectious diseases. 22.
- Stegen, G., Pasmans, F., Schmidt, B.R., Rouffaer, L.O., Van Praet, S., Schaub, M., Canessa, S.,
 Laudelout, A., Kinet, T., Adriaensen, C., Haesebrouck, F., Bert, W., Bossuyt, F., Martel, A.
 2017. Drivers of *Batrachochytrium salamandrivorans* mediated salamander extirpation.
 Nature 544: 353-354.
- Stuart, S.N., Chanson, J.S., Cox, N.A., Young, B.E., Rodrigues, A.S., Fischman, D.L. and Waller, R.W., 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306(5702):1783-1786.
- Sutton, W.B., Gray, M.J., Hoverman, J.T., Secrist, R.G., Super, P.E., Hardman, R.H., Tucker,J.L. and Miller, D.L., 2015. Trends in ranavirus prevalence among plethodontid salamandersin the Great Smoky Mountains National Park. EcoHealth 12(2): 320-329.
- Warne, R.W., LaBumbard, B., LaGrange, S., Vredenburg, V.T. and Catenazzi, A., 2016. Coinfection by chytrid fungus and ranaviruses in wild and harvested frogs in the tropical Andes. PLoS One 11(1): 0145864.
- Whitfield, S.M., Geerdes, E., Chacon, I., Rodriguez, E.B., Jimenez, R.R., Donnelly, M.A. and Kerby, J.L., 2013. Infection and co-infection by the amphibian chytrid fungus and ranavirus in wild Costa Rican frogs. Diseases of aquatic organisms 104(2):173-178.

Yap, T. A., Koo, M. S., Ambrose, R. F., Wake, D. B., & Vredenburg, V. T. 2015. Averting a North American biodiversity crisis. Science 349:481-482.

CHAPTER 3

ASSESSING THE RISK OF THE EMERGING FUNGAL PATHOGEN,

BATRAOCHOCHYTRIUM SALAMANDRIVORANS, TO COSTA RICAN SALAMANDERS²

²Adams, H.C., K. Markham, M.J. Gray, M. Madden, S.M. Hernandez. 2020 to be submitted to Journal of Wildlife Diseases

ABSTRACT

Implementing proactive conservation strategies in wildlife disease management is crucial. Essential to the success of these proactive strategies is pathogen surveillance and accurate epidemiological predictions. The emergence of the fungal pathogen, *Batrachochytrium* salamandrivorans (Bsal), which initially caused significant declines in Dutch fire salamander (Salamandra salamandra) populations in 2008, has been met with largely proactive mitigation efforts. The continuation of these proactive measures is crucial, especially in naïve environments in which no mitigative efforts against Bsal have been enacted, such as Costa Rica. Some surveillance for *Bsal* has been conducted in Costa Rica, but, in order to inform continued targeted surveillance, we developed an Ecological Niche Model (ENM) using MAXENT in ArcGIS to identify areas ecologically suitable for *Bsal* in Costa Rica. We sought to identify areas at high risk of *Bsal* introduction by finding where areas of ecological suitability overlap with high human visitation and high salamander biodiversity. We predicted suitability would be highest in Costa Rica's Cordillera Central and Cordillera Talamanca, that 30 salamander species would live in areas suitable for *Bsal*, and that protected areas in the aforementioned mountain ranges would be points of high suitability and visitation. We found that $\sim 23\%$ of Costa Rica's landmass is suitable for Bsal, that 27 salamander species, including 4 endangered species, live in areas suitable for *Bsal*, and identified eight areas in at which suitability intersected with high biodiversity and/or human visitation. Many of these locations were within the Cordillera Central and Cordillera Talamanca and we encourage pathogen surveillance, research, and potentially government implemented biosecurity in these areas.

Key words: Ecological niche model, risk assessment, *Batrachochytrium salamandrivorans*, Costa Rica, salamanders

Introduction

Emergent infectious pathogens and their associated diseases pose an increasing threat to global wildlife biodiversity (Daszak et al. 2000). Unfortunately, responses to wildlife epizootics are often more reactive than proactive (Voyles *et al.* 2014). In the case of the fungal pathogen Batrachochytrium dendrobatidis (Bd), one of the two causative agents in amphibian chytridiomycosis, decades passed between initial emergence, formal description, and subsequent unified action. Before being described in 1998, nearly two decades after its initial emergence, Bd caused extensive declines and even extinctions in many Central American and Australian species, including the Golden Toad (Incilius periglenes) of Costa Rica and the Gastric-brooding Frogs (Rheobatrachus) of Australia (Crump et al. 1992, Berger et al. 1998, O'Hanlon et al. 2018, Scheele et al. 2019). It was not until 2005 and 2006 that Australia and the United States, respectively, developed conservation action plans to combat the fungus (Grant et al. 2017). Now, Bd has been implicated in the decline of over 500 amphibian species, with 40% of species lost in certain regions (Puschendorf et al. 2009, Miller et all 2018, Scheele et al. 2019, Martel et al. 2020). A dramatic increase in proactivity was seen in the case of the emergence of *Pseudogymnoascus destructans (Pd)*, the causative agent of White-Nose Syndrome: action plans had been drawn within two years of its description in 2008, only two years after the pathogen's initial emergence in Howes Cave near Albany, New York (Blehert et al. 2009, Voyles et al. 2014).

Proactive management strategies, which are far more common in human and livestock disease outbreaks, are known to mitigate negative outcomes, reduce costs, and promote efficiency of response (Grant *et al.* 2017, OIE 2018, Martel *et al.* 2020). Adequate pathogen surveillance and the ability to make epidemiological predictions are required for the success of

proactive responses (Grant *et al.* 2017). Such responses are more crucial than ever, especially in light of the emergence of the fungal pathogen pathogen, *Batrachochytrium salamandrivorans* (*Bsal*). *Bsal*, the third chytrid fungus known to parasitize vertebrate hosts (in addition to *Bd* and *Ichthyochytrium vulgare*), is a saprophytic fungus with an affinity for cool, moist environments. It persists at a fairly wide thermal range (\sim 5°-25°C), being most pathogenic between 10°-15°C, and presents a major threat to amphibians globally, specifically urodelans (Martel *et al.* 2013, Martel *et al.* 2014, Stegen *et al.* 2017, Carter *et al.* 2019). *Bsal* first emerged in the Netherlands in 2008, where it caused enigmatic die-offs in local fire salamander (*Salamandra salamandra*) populations. By the time Martel *et al.* (2013) formally described the pathogen, Dutch fire salamanders had experienced upwards of 94% population declines.

Bsal's emergence was met with fiercely proactive research and mitigative measures: Martel *et al.* (2014) initiated wide scale surveillance for *Bsal*, surveying over 5,000 individual animals, as well as experimental infection trials. This research illuminated general taxonomic trends for *Bsal* susceptibility and predicted *Bsal* to have originated in East Asia, dispersed through the international pet trade (Martel *et al.* 2014). These predictions concerning *Bsal's* native range and the role of the pet trade in its emergence were later corroborated by further research (Cunningham *et al.* 2015, Laking *et al.* 2017, Nguyen *et al.* 2017, O'Hanlon *et al.* 2018). By 2016, both the European Union and the United States had passed legislation limiting the international and internal movement of injurious salamander species (Grant *et al.* 2016, 2017). Further, susceptibility research has continued to illuminate the nuances of *Bsal* susceptibility in salamanders (e.g., Carter *et al.* 2017, Stegen *et al.* 2017). Ongoing surveillance work in the Americas and Europe has catalogued the continued expansion of *Bsal's* European range, which has now been detected in Belgium, Germany, and Spain in wild salamander populations (Martel *et al.* 2014, Cunningham *et al.* 2015, Gray *et al.* 2015, *et al.* 2016, Spitzen-van der Sluijs *et al.* 2016, Govindarajulu *et al.* 2017, Klocke *et al.* 2017, Martel *et al.* 2020). *Bsal*'s recent detection in Spain's Montnegre i el Corredor Natural Park, 1000 km away from the nearest known area of occurrence, was extremely fortuitous and due to opportunistic disease screening in conjunction with ongoing invasive species eradication efforts (Martel *et al.* 2020). The detection sparked immediate collaborative efforts between regional authorities and scientist, leading to effective outbreak containment. This narrative further illustrates the necessity for conscious *Bsal* surveillance whenever possible and the efficacy of proactive disease management measures implemented (Martel *et al.* 2020). Though *Bsal* has yet to be detected in the Americas, home to the world's most diverse salamander communities, the continued execution and expansion of such proactive conservation measures are of immense importance (Parrot *et al.* 2016, Govindarajulu *et al.* 2017).

Adams *et al.* (submitted) initiated surveillance for *Bsal* in Costa Rica, which possesses the fifth most diverse salamander community in the world as well as a wealth of habits ecologically suitable for *Bsal* persistence (Savage 2002, Martel *et al.* 2013, Laking *et al.* 2017, Stegen *et al.* 2017). Additionally, Costa Rica has a thriving ecotourism industry with ca. 14% (~280,000) of annual visitors originating from Europe and 40% (~800,000) originating from the United States. Roughly 0.5% of annual visitors (~10,000) originate from areas in which *Bsal* is endemic (Instituto Costarricense de Turismo 2016). This provides a potential pathway of introduction from Europe into Costa Rica, and then from Costa Rica to the United States, threatening both Central and North American salamander communities. Ecotourism and other anthropogenic activities are believed to have exacerbated the emergence of *Bd* in Costa Rica

during the 1980s and 1990s (Cheng *et al.* 2011, O'Hanlon *et al.* 2018). Though Adams *et al.* (submitted) did not find *Bsal* in their study, their study was somewhat limited to small geographic areas and had small sample sizes, especially in the Cordillera Talamanca. Therefore, further surveillance is necessary.

Ecological Niche Models (ENM) are extremely valuable tools for generating efficient and targeted surveillance efforts and have been employed to pinpoint areas in Costa Rica of high ecological suitability to Bd (Puschendorf et al. 2009). Basanta et al. (2019) recently generated a similar model that predicted areas of high suitability for *Bsal* in Mexico, using climatic data from locations in Bsal's native range. They identified 13 hotspots for Bsal introduction, where ecological suitability overlapped with high salamander species biodiversity. These locations primarily fell in Central and Southern Mexico (Basanta et al. 2019). Here, we created a similar ELM to be used to develop future targeted surveillance strategies in Costa Rica. In this study, we generated an ENM that 1) identifies areas in Costa Rica that are ecologically suitable for the persistence of Bsal, 2) have salamander species diversity, and receive large amounts of human visitation. Based on known salamander ranges and associated ecological characteristics in those ranges, we predicted that roughly 30 of Costa Rica's 49 salamander species would be in areas environmentally suitable for *Bsal* (Savage 2002). We also predicted that environmental suitability would be highest in Costa Rica's two largest mountain ranges, the Cordillera Central and Cordillera Talamanca, based on ecological characteristics, such as rainfall and temperature (Savage 2002). Lastly, we predicted that national protected areas that fall in these mountain ranges, such as the Volcán Poas National Park, would be areas of intersection for high suitability and high human visitation (Savage 2002, Instituto Costarricense de Turismo. 2016). We have

characterized areas in which our three criteria overlap as areas at high risk of *Bsal* introduction and encourage they be prioritized for future conservation research.

Methods

We first used calculated biodiversity measures for Costa Rican amphibians using TerrSet's Habitat and Biodiversity Modeler using polygons of species extent from the IUCN Red List. Ranges of 44 species of salamander (of Costa Rica's 49) and 174 frog species were available. Alpha diversity and the Range Restriction Index (RRI), which compares the area a species is found with the entire study region, were calculated for available salamander species. Higher RRI values suggest that the majority of species present have relatively restricted ranges or are highly endemic. Salamander diversity was reclassified into four categories: low (zero to two), medium (two to four), high (four to six), and very high (six to eight). Because of their potential to act as carrier hosts for *Bsal*, anuran alpha diversity was also calculated (Ngueyn *et al.* 2017, Stegen *et al.* 2017).

We estimated visitation using data provided by the Costa Rican Ecotourism Institute. Visitation data from 2018 were available for forty-six out of one hundred and sixty-four protected areas (Figure 3.1). Visitation data includes both national and international tourists.



Figure 3.1 Protected areas are outlined in black. Where available, visitation numbers within protected areas for 2018 are indicated by color, with darker blue indicating more visitors.

To identify potential suitable habitat in Costa Rica, ecological niche modeling for *Bsal* was conducted using presence points in areas of *Bsal* endemism. This model was then used to predict suitability in Costa Rica. The maximum entropy model (MAXENT) was used due to its ability to model distribution using presence only data and its high predictive accuracy (Elith *et al.* 2006). Thirty-four presence points of *Bsal* within its natural range were used. Following

Basanta *et al.* (2019), the following bioclimatic variables were included in the model: mean diurnal range, maximum temperature of warmest month, temperature annual range, precipitation seasonality, precipitation of warmest quarter, and precipitation of coldest quarter. Correlation between variables was checked and all variables scored below 0.7. We performed MAXENT analyses following methods outlined by Basanta *et al.* (2019). We used a parametrization of regularization multiplier of 2.5 and a linear, quadratic and product feature combination. MAXENT produced a map ranging from zero to one with one indicating the most highly suitable habitat. We reclassified the suitability map into low (less than 0.5), medium (0.5-.75), and high (over 0.75) suitability categories. To identify high priority areas where monitoring efforts should be focused, we examined areas where high/very high alpha diversity and high suitability overlapped. We also compared these high priority areas with the RRI to identify species with particularly restricted range (i.e. at higher risk of local extinctions).

<u>Results</u>

Alpha diversity for salamanders was greatest in the central part of Costa Rica as well as along the Panamanian border, and lowest in northwestern Costa Rica (Fig. 3.2). Frog alpha diversity followed similar patterns, with high diversity along the eastern portion of the country overlapping areas of high salamander diversity.



Figure 3.2 Salamander (left) and frog (right) alpha diversity in Costa Rica. Darker shades indicate higher alpha diversity.

The raw results of our ENM is shown in Figure 3.3, showing highest suitability in the Cordillera Talamanca, Cordillera Central, and Northern Caribbean Coast. Of Costa Rica's roughly 51,000 km² of land, 39,440 km² (77.33%) are classified as low suitability for *Bsal* (scores <0.50), 11,585 km² (22.72%) are classified as medium suitability (scores 0.5-0.75), and 79 km² (0.15%) are considered highly suitable (scores >0.75) (Fig. 3.4).



Figure 3.3 Ecological Niche Model results for *Bsal* suitability in Costa Rica. Shows high suitability especially in the Cordillera Central, Cordillera Talamanca, and northeastern Caribbean slope.



Figure 3.4 Simplified results of our ENM. Close-up of highly suitable area for *Bsal*, shown in red. Protected areas are shown overlaid with diagonal lines.

The majority of land predicted to be highly suitable area for *Bsal* (almost 73 square kilometers) is within protected areas (Fig. 3.4), including Cordillera Volcanica Central Biological Reserve, Braulio Carrillo National Park, La Amistad National Park, Chirripo National Park, and Tapanti-Macizo de la Muerte National Park. Brauilo Carrillo National Park had over

25,000 visitors and Tapanti-Macizo de la Muerte National Park had nearly 29,000 visitors in 2018. La Amistad National Park had 744 visitors in 2018. Of land considered highly suitable for *Bsal*, 3.16% (2.5 km²) contained between six and eight salamander species and 17.1% (13.5 km²) contained between four to six species. Fifteen species of salamander were found in areas predicted to be highly suitable. Four of those species are endangered. Nearly 44% (35 km²) of land predicted to be highly suitable areas for *Bsal* do not contain any salamander species.

Of the 11,585 square kilometers considered moderately suitable for *Bsal*, 56.13% (6,503 km²) are within protected areas (Fig. 3.4), including parks with considerable visitation in 2018: Tortuguero National Park (135,000 visitors), Brauilo Carrillo National Park, Volcan Irazu National Park, (420,000 visitors) and Volcán Poas National Park (50,000 visitors). Of land considered moderately suitable for *Bsal* 2,084.91 square kilometers contain between six and eight species of salamander 4,982 square kilometers contain between four to six species. Thirtyseven salamander species are found within moderately suitable habitat including the critically endangered *Nototriton major* and thirteen other species listed as endangered by the IUCN.

To suggest high priority area for monitoring, we identified areas of overlap between areas predicted to be moderately-highly suitable for *Bsal*, with high/very high salamander diversity (i.e. four or more species), and with notable human visitation (Fig. 3.5). Seventeen square kilometers of land met these criteria (Fig. 3.5). This high priority area falls within Braulio Carrillo National Park, Cordillera Volcanica Central Reserve, and the Talamanca Range of La Amistad International Park near Limón, with some high priority area extending just beyond the boundary of the park. Salamander species found within high priority areas include *Bolitoglossa alvaradoi, B. colonnea, B. robusta, O. alfaroi, O. cyclocauda, O. gracilis, O. poelzi, and O. uniformis*.



Figure 3.5. Shows overlap of areas moderately suitable for *Bsal* and with high/very high amphibian diversity (yellow) and overlap between areas highly suitability for *Bsal* and with high/very high amphibian diversity (red). Protected areas are outlined and the number of visitors in 2018 is indicated by pattern.

The high priority area within Brauilo Carrillo and Cordillera Volcanica Central Biological Reserve also have high RRI values, indicating that salamander species found there are
highly endemic within Costa Rica (Fig. 3.6). The high priority area within La Amistad International Park has relatively low RRI levels.



Figure 3.6. RRI values with darker red indicating salamander species found within this pixel have a restricted range across Costa Rica. High priority areas are shown in bright red. Protected areas are outlined and the number of visitors in 2018 (where available) is indicated by pattern.

Discussion

Our ecological niche model predicted that ecological suitability for *Bsal* follows the general trends that we hypothesized, with suitability being highest in Costa Rica's mountain ranges. However, our model also shows that suitability is high in the Caribbean tropical wet and moist forests of northeastern Costa Rica (Fig. 3.3). We did not anticipate this due to the region's high temperatures, but, the area's high annual rainfall could mitigate the influence of high temperatures, increasing ecological suitability for Bsal in those areas. A large portion of Costa Rica's landmass (22.67%) is moderately ecologically suitable (*Bsal* suitability score 0.5-0.75) for the persistence of *Bsal*. This area of moderate suitability is generally limited to the Caribbean slope of Costa Rica, including the premontane and montane forests of the Talamanca and Central mountain ranges as well as the Caribbean tropical wet and moist forests (Fig. 3.4). A total of 37 salamander species out of the 44 species available (>75% of Costa Rica's total salamander species) live in areas moderately suitable for Bsal (Bolitoglossa alvaradoi, B. bramei, B. cerroensis, B. colonnea, B. compacta, B. diminuta, B. epimela, B. gracilis, B. lignicolor, B. marmoreal, B. minutula, B. nigrescens, B. obscura, B. pesrubra, B. robusta, B. schizodactyla, B. sombra, B. sooyorum, B. striatula, B. subpalmata, B. tica, Nototriton abscondens, N. gamezi, N. guanacaste, N. major, N. picadoi, N. richardi, N. tapanti, Oedipina alfaroi, O. carablanca, O. collaris, O. cyclocauda, O. gracilis, O. grandis, O. poelzi, O. pseudouniformis, O. uniformis). 13 of those species are listed as endangered by the ICUN (Bolitoglossa alvaradoi, B. compacta, B. marmoreal, B. minutula, B. nigrescens, B. sooyorum, B. subpalmata, B. tica, Oedipina carablanca, O. gracilis, O. grandis, O. poelzi, O. pseudouniformis) and a single species, Nototriton, major, is listed as Critically Endangered. Within these areas of moderate ecological

suitability, there are large patches containing high salamander alpha diversity: 43% of moderately suitable areas contain 4-6 six salamander species and 20% contain 6-8 species, with 8 being the greatest alpha diversity in any given place in Costa Rica.

These areas of moderate suitability and high salamander biodiversity are clustered in the northern and very southern Cordillera Talamanca, the Cordillera Central, two points in the Cordillera Guanacaste, one point in the Cordillera Tilaran, and a large portion of the Caribbean wet and tropical wet and moist forests (Fig. 3.5). While experimental susceptibility trials have yet to specifically demonstrate the susceptibility of Costa Rican salamanders to Bsal, all Costa Rican species belong to the family *Plethodontidae*. This family has been shown to have variable susceptibility to the fungus, with some species being highly susceptible (Martel et al. 2014, Carter et al. 2019). With such a large proportion of the country's salamander species living in areas ecologically suitable for *Bsal*, we encourage a robust surveillance program is established, especially for the 14 endangered species located in high-risk areas. Additionally, based on the available natural area visitation data, we encourage surveillance specifically in the Volcán Poas National Park, Volcán Irazu National Park, Braulio Carillo National Park, Tortuguero National Park, Tapantí-Macizo de la Muerte National Park, and Chirripo National Park. These areas have considerable tourist visitations per year (Fig. 3.5), which when combined with the high salamander biodiversity and moderate *Bsal* suitability located in these Parks, puts them at high risk of Bsal introduction.

Our model identified 78.62 km² (0.15% of Costa Rica's landmass) as highly ecologically suitable for *Bsal (Bsal* suitability score >0.75) (Fig. 3.4). Most of these areas of high suitability fall within natural protected areas, specifically the Volcanica Central Biological Reserve, Braulio Carrillo National Park, La Amistad National Park, Chirripo National Park, and Tapanti-Macizo

de la Muerte National Park, with only a few areas of high suitability falling closely outside of protected areas. This could be advantageous, in that visitation to these areas could monitored, restricted even, for management purposes, should the need arise. There are 15 salamander species that fall in areas of high suitability, four of which are listed as Endangered (*Bolitoglossa alvaradoi, B. subpalmata, Oedipina. gracilis, O. poelzi*). There are only three areas of high ecological suitability in Costa Rica that also contain high salamander alpha diversity. These are located in Braulio Carillo, La Amistad, and Volcanica Central Biological Reserve. Of these three protected areas, Braulio Carillo is a park with notable human visitation, so we encourage prioritizing monitoring at the hotspot at the southwest of the park. However, other areas of high suitability, regardless of salamander diversity, should not be discounted. Primarily located in the Cordillera Talamanca (Fig. 3.4), these areas of high suitability could provide important footholds for *Bsal* in Costa Rica, potentially allowing the pathogen to spill over into more specious areas. Additionally, the species in these areas of Costa Rica have highly restricted ranges (Fig. 3.6), putting them at significant risk of extirpation in the event of *Bsal* introduction.

Amphibian communities in Costa Rican have already been enormously impacted by *Bd* related epizootics, with some areas experiencing upwards of 40% decline in species (Puschendorf *et al.* 2009). In eastern newts co-infected with *Bd* and *Bsal*, it has been shown that *Bd* infection can increase susceptibility to *Bsal* (Longo *et al.* 2019). Co-infection dynamics between *Bd* and *Bsal* are largely unknown, it is presumable that their combined impacts on host populations would be detrimental. Based on the predictions of our ENM and a similar ENM produced by Puschendorf *et al.* (2009), many areas ecologically suitable for *Bsal* in Costa Rica are also ecologically suitable for *Bd*. Such overlap exists in regions that have already experienced significant *Bd*-related amphibian population declines, specifically Monteverde and

Las Tablas. Monitoring for *Bsal* in these areas could be crucial to the preservation of these already degraded amphibian communities. Recent research has begun to illuminate the greater role that anurans may play in *Bsal* epidemiology (Ngueyn *et al.* 2017, Stegen *et al.* 2017). While it was initially believed that *Bsal* could not infect anurans, studies have shown that some anuran species, such as midwife toads (*Alytes* sp.) and *Bombina* sp. can maintain subclinical infections and infect naïve urodelan hosts. It would be prudent to test the susceptibility of anuran species in these areas using controlled dose-response experiments (e.g., Carter *et al.* 2019), as some may be able to function as infectious reservoir hosts, allowing for continued spillover into salamander populations. Similarly, understanding the susceptibility of salamander species across Costa Rica, specifically those species living in high-risk areas (*Bolitoglossa alvaradoi, B. colonnea, B. diminuta, B. epimela, B. gracilis, B. pesrubra, B. robusta, B. subpalmata, Nototriton abscondens, N. richardi, Oedipina alfaroi, O. cyclocauda, O. gracilis, O. poelzi, and O. uniformis) is needed.*

In this study, we used ecological niche modeling, salamander distribution data, and protected natural area visitation data to identify areas ecologically suitable for the persistence *Bsal* and where these areas overlapped with regions of high salamander diversity as well as high human visitation. In comparison with *Bsal* suitability in Mexico, there is a much larger proportion of the Costa Rican landmass predicted to be suitable for *Bsal* (Basanta *et al.* 2019). We have identified that ~23% of Costa Rica is moderately to highly suitable for *Bsal* and contains a high amount of salamander diversity, with 37 salamander species, including 14 endangered species, living in areas at least moderately suitable for *Bsal*. Range data for five of Costa Rica's salamander species were not available. As such these species could also reside in areas of high risk. While we encourage monitoring throughout areas of suitability, we have identified eight locations (Poas, Irazu, Tortuguero, Volcanica Central Biological Reserve,

Braulio Carrillo National Park, La Amistad National Park, Chirripo National Park, and Tapanti-Macizo de la Muerte National Park) as potential hotspots for *Bsal* introduction because of their high suitability to *Bsal*, high diversity of salamander species, proximity to an area of high human visitation, or a combination of all three of those factors. We encourage that pathogen surveillance as well as characterizing the susceptibility of salamander and anuran species at these locations be considered research priorities. Additionally, especially in areas with high human visitation, we encourage the development of educational tools designed to inform the public on amphibian conservation, diseases, and easy steps they can take to limiting pathogen transmission, such as regularly cleaning recreational equipment.

Literature Cited

- Adams, H.C., Hernandez, S.M., Gray, M.J., Connelly, S. Yabsley, M.J. Submitted. Surveillance for the pathogenic fungi, batrachochytrium salamandrivorans and B. Dendrobatidis, and Ranavirus in highland costa rican salamander population. To be submitted
- Basanta, M.D., Rebollar, E.A. and Parra-Olea, G., 2019. Potential risk of *Batrachochytrium salamandrivorans* in Mexico. PloS one, 14(2).
- Berger, Lee, Rick Speare, Peter Daszak, D. Earl Green, Andrew A. Cunningham, C. Louise
 Goggin, Ron Slocombe *et al.* 1998. Chytridiomycosis causes amphibian mortality
 associated with population declines in the rain forests of Australia and Central
 America. Proceedings of the National Academy of Sciences 15: 9031-9036.
- Blehert, D.S., Hicks, A.C., Behr, M., Meteyer, C.U., Berlowski-Zier, B.M., Buckles, E.L., Coleman, J.T., Darling, S.R., Gargas, A., Niver, R. and Okoniewski, J.C., 2009. Bat whitenose syndrome: an emerging fungal pathogen?. Science 323(5911):227-227.

- Cheng, T.L., Rovito, S.M., Wake, D.B. and Vredenburg, V.T. 2011. Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen
 Batrachochytrium dendrobatidis. Proceedings of the National Academy of Sciences 108: 9502-9507
- Crump, Martha L., Frank R. Hensley, and Kenneth L. Clark. 1992. Apparent decline of the golden toad: underground or extinct? Copeia: 413-420.
- Cunningham, A.A., Beckmann, K., Perkins, M., Fitzpatrick, L., Cromie, R., Redbond, J., O'Brien, M.F., Ghosh, P., Shelton, J. and Fisher, M.C. 2015. Emerging disease in UK amphibians. Veterinary Record. 176
- Daszak, P., Cunningham, A.A. and Hyatt, A.D., 2000. Emerging infectious diseases of wildlife-threats to biodiversity and human health. Science 287(5452):443-449.
- Govindarajulu, P., Matthews, E. and Ovaska, K., 2017. Swabbing for *Batrachochytrium* salamandrivorans on wild rough-skinned newts (*Taricha granulosa*) and pet-traded amphibians on southern Vancouver Island, British Columbia, Canada. Herp. Rev 48:564-568.
- Elith*, J., H. Graham*, C., P. Anderson, R., Dudík, M., Ferrier, S., Guisan, A., J. Hijmans, R., Huettmann, F., R. Leathwick, J., Lehmann, A. and Li, J., 2006. Novel methods improve prediction of species' distributions from occurrence data. Ecography 29(2):129-151.
- Grant, E.H.C., Muths, E., Katz, R.A., Canessa, S., Adams, M.J., Ballard, J.R., Berger, L., Briggs,
 C.J., Coleman, J.T., Gray, M.J. and Harris, M.C., 2017. Using decision analysis to support proactive management of emerging infectious wildlife diseases. Frontiers in Ecology and the Environment 15(4):214-221.

Gray, M.J., Lewis, J.P., Nanjappa, P., Klocke, B., Pasmans, F., Martel, A., Stephen, C., Olea, G.P., Smith, S.A., Sacerdote-Velat, A. and Christman, M.R. 2015. *Batrachochytrium salamandrivorans*: The North American response and a call for action. PLoS Pathog 11

Instituto Costarricense de Turismo. 2016. Anuario Estadístico de Turismo 2016.

- Laking, A. E., Ngo, H. N., Pasmans, F., Martel, A., & Nguyen, T. T. 2017. Batrachochytrium salamandrivorans is the predominant chytrid fungus in Vietnamese salamanders. Scientific Reports 7.
- Longo, A.V., Fleischer, R.C. and Lips, K.R., 2019. Double trouble: co-infections of chytrid fungi will severely impact widely distributed newts. Biological Invasions, 21(6): 2233-2245.
- Klocke, B., Becker, M., Lewis, J., Fleischer, R.C., Muletz-Wolz, C.R., Rockwood, L., Aguirre, A.A. and Gratwicke, B., 2017. *Batrachochytrium salamandrivorans* not detected in US survey of pet salamanders. Scientific reports 7(1):13132.
- Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M.C., Woeltjes, A., Bosman, W., Chiers, K., Bossuyt, F. and Pasmans, F. 2013. *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. Proceedings of the National Academy of Sciences 110:15325-15329.
- Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M.C., Farrer, R.A., Schmidt, B.R., Tobler, U., Goka, K. & Lips, K.R. 2014. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science 346:630-631.
- Martel, A., Vila-Escale, M., Fernández-Giberteau, D., Martinez-Silvestre, A., Canessa, S., Van Praet, S., Pannon, P., Chiers, K., Ferran, A., Kelly, M. and Picart, M. 2020. Integral chain management of wildlife diseases. Conservation Letters p.e12707.

- Nguyen, Tao Thien, Thinh Van Nguyen, Thomas Ziegler, Frank Pasmans, and An Martel. 2017. Trade in wild anurans vectors the urodelan pathogen *Batrachochytrium salamandrivorans* into Europe. Brill.
- O'Hanlon, Simon J., Adrien Rieux, Rhys A. Farrer, Gonçalo M. Rosa, Bruce Waldman, Arnaud Bataille, Tiffany A. Kosch *et al.* 2018. Recent Asian origin of chytrid fungi causing global amphibian declines. Science 360(6389): 621-627.
- OIE World Organization for Animal Health (2018). Terrestrial Animal Health Code. Retrieved from https://www.oie.int/fileadmin/Home/eng/Health_standards/tahc/2018/ en_sommaire.htm
- Parrott, Joshua Curtis, Alexander Shepack, David Burkart, Brandon LaBumbard, Patrick Scimè,
 Ethan Baruch, and Alessandro Catenazzi. 2016. Survey of pathogenic chytrid rungi
 (*Batrachochytrium dendrobatidis* and *B. salamandrivorans*) in salamanders from three
 mountain ranges in Europe and the Americas. EcoHealth: 1-7.
- Puschendorf, R., Carnaval, A.C., VanDerWal, J., Zumbado-Ulate, H., Chaves, G., Bolaños, F. and Alford, R.A., 2009. Distribution models for the amphibian chytrid *Batrachochytrium dendrobatidis* in Costa Rica: proposing climatic refuges as a conservation tool. Diversity and Distributions 15(3):401-408.
- Scheele, B.C., Pasmans, F., Skerratt, L.F., Berger, L., Martel, A.N., Beukema, W., Acevedo,
 A.A., Burrowes, P.A., Carvalho, T., Catenazzi, A. and De la Riva, I., 2019. Amphibian
 fungal panzootic causes catastrophic and ongoing loss of biodiversity. Science 363(6434):
 1459-1463.
- Spitzen-van der Sluijs, A., Martel, A., Asselberghs, J., Bales, E.K., Beukema, W., Bletz, M.C., Dalbeck, L., Goverse, E., Kerres, A., Kinet, T. and Kirst, K. 2016. Expanding distribution

of lethal amphibian fungus *Batrachochytrium salamandrivorans* in Europe. Emerging infectious diseases 22.

- Stegen, G., Pasmans, F., Schmidt, B.R., Rouffaer, L.O., Van Praet, S., Schaub, M., Canessa, S.,
 Laudelout, A., Kinet, T., Adriaensen, C., Haesebrouck, F., Bert, W., Bossuyt, F., Martel, A.
 2017. Drivers of *Batrachochytrium salamandrivorans* mediated salamander extirpation.
 Nature 544: 353-354.
- Van Rooij, P., Martel, A., Haesebrouck, F. and Pasmans, F., 2015. Amphibian chytridiomycosis: a review with focus on fungus-host interactions. Veterinary Research 46(1):137.
- Voyles, J., Kilpatrick, A.M., Collins, J.P., Fisher, M.C., Frick, W.F., McCallum, H., Willis, C.K.,
 Blehert, D.S., Murray, K.A., Puschendorf, R. and Rosenblum, E.B., 2015. Moving beyond
 too little, too late: managing emerging infectious diseases in wild populations requires
 international policy and partnerships. EcoHealth 12(3):404-407.

CHAPTER 4

CONCLUSIONS

Management Implications

It is encouraging that *Bsal* was not detected in our studies, especially at a national park as well visited and ecologically suitable for *Bsal* as Volcan Poas. However, as clearly indicated by our ENM, salamanders in Costa Rica are still at significant risk of *Bsal* introduction: roughly 23% of the country's landmass is ecologically suitable to the pathogen and 37 of Costa Rica's 49 salamander species live in areas of ecological suitability. Some of these species are already listed as endangered by the IUCN or possess extremely restricted ranges, putting them at significant risk of extinction. It is crucial that surveillance for *Bsal*, as well as *Bd* and ranavirus, be continued in Costa Rica, especially within the eight high risk areas previously identified, which include our areas of surveillance. Early detection of *Bsal* is crucial to mitigating the impacts it may have on Costa Rican salamander communities. Simultaneously surveying for *Bd* and ranavirus would not only expand the epidemiological knowledge within salamanders in Costa Rica the epidemiological knowledge within salamanders in Costa Rica to be potentially informative of co-infection dynamics with *Bsal*.

Future Research

Crucial to the management of species in the context of emergent infectious diseases is understanding susceptibility to the target pathogen. As has been previously stated, all salamander in Costa Rica belong to the family *Plethodontidae*. While it is known that this family possesses variable susceptibility to *Bsal*, with some species experiencing high mortality rates, susceptibility

of the genera specific to Costa Rica is unknown (Martel *et al.* 2014). Even species within the same genera have been known to experience completely different susceptibility to *Bsal* (Carter *et al.* 2019). So, in order to develop the most informed management protocols possible, we would recommend testing at least a representative species for each of Costa Rica's three genera (*Bolitoglossa, Oedipina*, and *Nototriton*), while aiming to expand susceptibility testing to as many species as possible, targeting those present in the aforementioned eight high risk areas. Additionally, because of increasing evidence that anurans may act as carrier hosts for *Bsal*, the susceptibility of anuran species sympatric with "at risk" salamander species should also be investigated. These recommendations are made with the understanding that experimental susceptibility trials can be very logistically challenging and require fatal collections. As such, this research may not be possible or even permitted in certain species, specifically those that are particularly cryptic and/or endangered.

An alternative to experimental infection trials could be the characterization of a salamander species' cutaneous microbiome and the associated fungal inhibiting properties. Depending on logistical feasibility, cutaneous microbiomes could be sampled such that bacteria could be cultured and challenged in a fungal killing assay or simply sequenced, describing the community. The database of amphibian cutaneous bacteria and their fungal inhibiting properties, cultivated by Woodhams *et al.* (2015), could be referenced in order to identify the fungal inhibiting properties of any bacteria groups identified.

It was initially a research goal of this thesis to test the susceptibility of at least one Costa Rican salamander species. However, the logistics of such an undertaking, in compliance with the requests of our permitting agency, prevented us from accomplishing this goal. As a proxy for susceptibility, we decided to characterize the cutaneous microbial communities of the

salamanders we sampled during pathogen surveillance. The appropriate samples to characterize cutaneous microbiome were collected during the pathogen sampling process, following protocols defined by Muletz Wolz *et al.* (2017). Unfortunately, due to COVID-19 lab closures, we have not able to complete the appropriate analyses in time for the completion of this thesis. Once it can be conducted safely, we look forward to completing this aspect of our work, as it could provide important insight regarding the susceptibility of Costa Rican salamander species to *Bsal*.

Literature Cited

- Carter, E.D., Miller, D.L., Peterson, A.C., Sutton, W.B., Cusaac, J.P.W., Spatz, J.A., Rollins-Smith, L., Reinert, L., Bohanon, M., Williams, L.A. and Upchurch, A., 2019. Conservation risk of *Batrachochytrium salamandrivorans* to endemic lungless salamanders. Conservation Letters.
- Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M.C., Farrer, R.A., Schmidt, B.R., Tobler, U., Goka, K. & Lips, K.R. 2014. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science 346:630-631.
- Muletz Wolz, C.R., Yarwood, S.A., Campbell Grant, E.H., Fleischer, R.C. and Lips, K.R., 2018. Effects of host species and environment on the skin microbiome of Plethodontid salamanders. Journal of Animal Ecology 87(2):341-353.
- Woodhams, D.C., Alford, R.A., Antwis, R.E., Archer, H., Becker, M.H., Belden, L.K., Bell, S.C., Bletz, M., Daskin, J.H., Davis, L.R. and Flechas, S.V., 2015. Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens: Ecological Archives E096-059. Ecology 96(2):595-595.