Pathways of Amphibian Chytrid Fungus Dispersal: Global Biosecurity and Conservation Implications

Thesis submitted by

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Preface

This thesis is structured as a series of connected papers that have either been published or are in preparation for publication at the time of thesis submission. Each paper was prepared as an individual, stand-alone paper and for this reason there are some unavoidable repetitions, particularly within the methods and background material.

Each chapter is comprised of publications or manuscripts as listed below. If published, the citation is included preceding the manuscript, and the manuscript is displayed in its published form.

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CHAPTER 1

Introduction and basis for the study

Paper 1: Global spread of amphibian chytrid fungus is driven by an enigmatic combination of amphibian-trade and abiotic dispersal pathways. (unpublished manuscript)

CHAPTER 2

Investigations of the presence of amphibian pathogens in the international wildlife trade

Paper 1: Kolby JE, Smith KM, Berger L, Karesh WB, Preston A, Pessier AP, Skerratt LF. (2014) First Evidence of Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) and Ranavirus in Hong Kong Amphibian Trade. PLoS ONE 9(3): e90750.

Paper 2: Kolby JE. (2014) Presence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in native amphibians exported from Madagascar. PLoS ONE 9(3): e89660.

Paper 3: Kolby JE, Smith KM, Skerratt LF, Berger L. Presence of *Batrachochytrium dendrobatidis* and Ranavirus in Bullfrogs Imported to the United States. (unpublished manuscript)

CHAPTER 3

Amphibian pathogen presence in the absence of commercial amphibian importation

Paper 1: Kolby JE, Smith KM, Ramirez SD, Rabemananjara F, Pessier AP, Brunner JL, Goldberg CS, Berger L, Skerratt LF. (2015) Rapid Response to Evaluate the Presence of Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) and Ranavirus in Wild Amphibian Populations in Madagascar. PLoS ONE 10(5):e0125330.

Paper 2: Kolby JE, Skerratt LF. (2015) Amphibian Chytrid Fungus in Madagascar neither Shows Widespread Presence nor Signs of Certain Establishment. PLoS ONE 10(10): e0139172.

Paper 3: Kolby JE. (2014) Ecology: Stop Madagascar's toad invasion now. Nature 509:563.

CHAPTER 4

The presence and distribution of amphibian pathogens in a region of high amphibian trade

Paper 1: Kolby JE, Chan SK, Smith KM, Ramirez SD, Berger L, Skerratt LF. Five Years of Surveillance to Detect the Amphibian Pathogens *Batrachochytrium dendrobatidis* (*Bd*) and Ranavirus in Hong Kong. (unpublished manuscript)

CHAPTER 5

Aerial spread of amphibian chytrid fungus

Paper 1: Kolby JE, Ramirez SD, Berger L, Griffin DW, Jocque M, Skerratt LF. (2015) Presence of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) in rainwater suggests aerial dispersal is possible. Aerobiologia 31:411-419.

CHAPTER 6

Unintentional spread of chytrid through human foot-traffic (fomites)

Paper 1: Kolby JE, Ramirez SD, Berger L, Skerratt LF. Low Risk of Amphibian Chytrid Dispersal and Establishment by Human Foot-Traffic. (unpublished manuscript)

CHAPTER 7

Terrestrial spread of chytrid fungus by amphibian locomotion

Paper 1: Kolby JE, Ramirez SD, Berger L, Richards-Hrdlicka KL, Jocque M, Skerratt LF. (2015) Terrestrial dispersal and potential environmental transmission of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*). PLoS ONE 10:e0125386.

CHAPTER 8

Suggested biosecurity actions to reduce pathogen importation to the United States

Paper 1: Kolby JE, Kraus F, Murray KA, Smith KM, van Dijk PP, Berger L, Speare R, Skerratt LF. Restricted Trade in Two Invasive Frog Species is Necessary to Mitigate Spread of Exotic Strains and Lineages of Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) into the United States. Prepared for submission to Conservation Letters.

CHAPTER 9

Infectious diseases of amphibians continue to emerge from the wildlife trade

Paper 1: Martel A, Blooi M, Adriaensen C, Van Rooij P, Beukema W, Fisher MC, Farrer RA, Schmidt BR, Tobler U, Goka K, Lips KR, Muletz C, Zamudio KR, Bosch J, Lötters S, Wombwell E, Garner TWJ, Cunningham AA, Spitzen-van der Sluijs A, Salvidio S, Ducatelle R, Nishikawa K, Nguyen TTT, Kolby JE, Van Bocxlaer I, Bossuyt F, Pasmans F. (2014) Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science, 346, 630−631.

CHAPTER 10

Conclusions and management implications

Paper 1: Kolby JE, Berger L, Skerratt LF. Risk Evaluation of Five Simultaneous Pathways of Current Global Amphibian Chytrid Fungus Dispersal. (unpublished manuscript)

Acknowledgements

I owe many thanks to my supervisors Lee Skerratt, Lee Berger, and Rick Speare for their guidance, assistance, and support throughout my academic adventure. Learning from them has made me become a better person while evolving into a better scientist. Their collective leadership and immeasurable wealth of experience helped to keep me on track amidst a series of unexpected project detours and discoveries that emerged along the way. They provided valuable editorial support as I developed my manuscripts and this greatly improved my academic writing skills and ability to communicate science to a diverse audience. Throughout my candidature, they provided the freedom and flexibility I needed to deeply explore my scientific inquiries while strengthening my foundation of knowledge in pathogen surveillance and biosecurity, data management and interpretation, and risk assessment. I am forever grateful for their mentorship.

I would also like to thank all members of the One Health Research Group including Ben Scheele, Laura Brannelly, Gerardo Martin, Rebecca Webb, Carol Esson, Alex Roberts, Jenny Laycock, Diana Mendez, among many others. This group was a valuable resource for sharing ideas, discussing research questions, and developing stronger projects.

I attribute my interest in wildlife pathogens to Katy Richards-Hrdlicka, who first taught me about amphibian chytrid fungus. Five years prior to my PhD program, Katy helped catalyse my long-term investigation of chytrid presence in Honduras, taught me how to design pathogen surveillance projects, and apply for grant funding. She tirelessly provided answers to my endless chytrid questions and encouraged me to pursue my sometimes unconventional research ideas. I truly owe much of my research success to Katy's mentorship and friendship over the past decade.

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I would like to thank all of the peer-reviewers who volunteered their time on manuscripts which I have included in this thesis. Your edits and constructive criticism helped guide my development as a writer and critical thinker. In hindsight, thank you for the rejections!

I owe deep gratitude for the support and understanding I've received from family and friends during my studies, and in particular my mom who provided countless bleary-eyed 4 AM airport drop-offs. She also managed to keep most of houseplants alive and was an exceptional foster parent to my pet lizards during my extended travels. She selflessly supported my adventures to remote research sites around the globe, often cut off from all contact with the outside world where our policy would be "no news is good news." Sorry for giving you a few extra grey hairs. I couldn't have accomplished this thesis without you!

I am especially indebted to Sara Ramirez and Merlijn Jocque - the most amazing friends and field research colleagues. I will forever cherish our months of rainforest fieldwork together, including the 2 AM cornflake breaks and endless hours waiting for the rain. You kept me motivated and inspired long after I ran out of skittles and dry socks. Last but not least, I would like to thank my partner Alyza Wiener for her endless love and support and for bearing with me during periods of frog frustration. Thank you for convincing me it's ok to rest, eat food, and take a nap once in a while!

In closing, I dedicate this thesis both to my father, Roger Kolby, and to my original field mentor, Dr. James "Skip" Lazell of The Conservation Agency. I owe my love for nature and life-long passion to protect biodiversity to my dad, who instilled upon me his fascination and appreciation for the natural world at an early age. My earliest childhood memories involve returning home from the park with my dad, mud on my face, and frog in my pocket. Then, at the age of 15, I was presented with an invaluable opportunity to join Skip on a field expedition to survey reptile and amphibian biodiversity in Hong Kong and China. This singular experience laid the foundation for my future career in field research. Twenty years and 20 expeditions later, I continue to look towards Skip as my source of inspiration. I am forever grateful for your unwavering encouragement and the many times you asked, "So, when are you finally going to get your PhD?" Onwards!

Statement on the contribution of others

My stipend and fees as a PhD student at James Cook University were funded through the James Cook University International Research Scholarship. Specific research projects were funded by grants provided to me by the National Geographic Society, United States Fish and Wildlife Service, Mohammed Bin Zayed Species Conservation Fund, Rufford Small Grants for Nature Conservation, Chicago Zoological Society, Columbus Zoo and Aquarium, and from EcoHealth Alliance through the New York Community Trust. Some travel and laboratory analysis expenses were funded in part through the Graduate Research Scheme at James Cook University and field support in Honduras was provided by Operation Wallacea.

This thesis was co-supervised by Drs Lee Skerratt, Lee Berger, and Rick Speare. Each provided advice and guidance throughout the duration of my thesis on study design, research methods, data interpretation, and analysis. Dr. Kristine Smith of EcoHealth Alliance provided guidance with wildlife trade disease surveillance. Dr. Katy Richards-Hrdlicka of the Yale School of Forestry and Environmental Science provided considerable assistance with laboratory analysis and diagnostic interpretation. Most of my projects involved collaborators that have been included as co-authors on the papers. Many additional people provided support and assistance, each of whom is named and their contribution stated in the Acknowledgements section at the end of each paper. I performed the majority of this research and was first author on all papers (except Chapter 9, Paper 1), with editing help and approval by all listed collaborators. A detailed account of each co-author's contribution is listed below, following the paper citation.

CHAPTER 2

Paper 1: Kolby JE, Smith KM, Berger L, Karesh WB, Preston A, Pessier AP, Skerratt LF. (2014) First Evidence of Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) and Ranavirus in Hong Kong Amphibian Trade. PLoS ONE 9(3): e90750.

JEK conceptualized the project. JEK, KMS, LFS and LB designed the research. JEK collected the data. JEK analysed the data. JEK wrote the manuscript and JEK, KMS, LFS, LB, WBK, AP, and APP edited the manuscript. KMS and APP provided funding, reagents and materials for the research.

Paper 2: Kolby JE. (2014) Presence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in native amphibians exported from Madagascar. PLoS ONE 9(3): e89660.

JEK conceptualized the project. JEK, LFS, and LB designed the research. JEK collected the data. JEK analysed the data. JEK wrote and edited the manuscript and provided funding for the research.

Paper 3: Kolby JE, Smith KM, Skerratt LF, Berger L. Presence of *Batrachochytrium dendrobatidis* and Ranavirus in Bullfrogs Imported to the United States.

JEK conceptualized the project. JEK, SKM, LFS and LB designed the research. JEK collected the data. JEK analysed the data. JEK wrote the manuscript and JEK, LFS, and LB edited the manuscript. KMS provided funding, reagents and materials for the research.

CHAPTER 3

Paper 1: Kolby JE, Smith KM, Ramirez SD, Rabemananjara F, Pessier AP, Brunner JL, Goldberg CS, Berger L, Skerratt LF. (2015) Rapid Response to Evaluate the Presence of Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) and Ranavirus in Wild Amphibian Populations in Madagascar. PLoS ONE 10(5):e0125330.

JEK conceptualized the project. JEK, KMS, SDR, and LFS designed the research. JEK, SDR, and FR collected the data. JEK, LFS, and LB analysed the data. JEK wrote the

manuscript and JEK, KMS, SDR, LFS, LB, and FR edited the manuscript. JEK, APP, JLB, and CSG provided funding, reagents and materials for the research.

Paper 2: Kolby JE, Skerratt LF. (2015) Amphibian Chytrid Fungus in Madagascar neither Shows Widespread Presence nor Signs of Certain Establishment. PLoS ONE 10(10): e0139172.

JEK conceptualized and designed the project. JEK and LFS analysed the data. JEK wrote the manuscript and JEK and LFS edited the manuscript. No funding was needed to perform this research.

CHAPTER 4

Paper 1: Kolby JE, Chan SK, Smith KM, Ramirez SD, Berger L, Skerratt LF. Five Years of Surveillance to Detect the Amphibian Pathogens *Batrachochytrium dendrobatidis* (*Bd*) and Ranavirus in Hong Kong.

JEK conceptualized the project. JEK, SKM, LFS and SDR designed the research. JEK and SDR collected the data. JEK analysed the data. JEK wrote the manuscript and JEK, CSK, LB, LFS, and SDR edited the manuscript. JEK and SKM provided the funding and materials for the research.

CHAPTER 5

Paper 1: Kolby JE, Ramirez SD, Berger L, Griffin DW, Jocque M, Skerratt LF. (2015) Presence of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) in rainwater suggests aerial dispersal is possible. Aerobiologia 31:411-419.

JEK conceptualized and designed the project. JEK and SDR collected the data. JEK analysed the data. JEK wrote the manuscript and JEK, GDW, LB, LFS, SDR, and MJ edited the manuscript. JEK provided funding and materials for the research.

CHAPTER 6

Paper 1: Kolby JE, Ramirez SD, Berger L, Skerratt LF. Low Risk of Amphibian Chytrid Dispersal and Establishment by Human Foot-Traffic.

JEK conceptualized and designed the project. JEK and SDR collected the data. JEK analysed the data. JEK wrote the manuscript and JEK, LB, and LFS edited the manuscript. JEK provided funding and materials for the research.

CHAPTER 7

Paper 1: Kolby JE, Ramirez SD, Berger L, Richards-Hrdlicka KL, Jocque M, Skerratt LF. (2015) Terrestrial dispersal and potential environmental transmission of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*). PLoS ONE 10:e0125386.

JEK conceptualized the project and designed the research. JEK and SDR collected the data. JEK, KLR, and MJ analysed the data. JEK wrote the manuscript and JEK, LFS, LB, KLR, SDR and MJ edited the manuscript. JEK provided funding and field materials and KLR provided reagents and materials for lab analysis.

CHAPTER 8

Paper 1: Kolby JE, Kraus F, Murray KA, Smith KM, van Dijk PP, Berger L, Speare R, Skerratt LF. Restricted Trade in Two Invasive Frog Species is Necessary to Mitigate Spread of Exotic Strains and Lineages of Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) into the United States. Prepared for submission to Conservation Letters.

JEK conceptualized and designed the project. JEK analysed the data. JEK wrote the manuscript and JEK, FK, KAM, KMS, PPV, LB, and RS edited the manuscript. No external funding was needed to perform this research.

CHAPTER 9

Paper 1: Martel A, Blooi M, Adriaensen C, Van Rooij P, Beukema W, Fisher MC, Farrer RA, Schmidt BR, Tobler U, Goka K, Lips KR, Muletz C, Zamudio KR, Bosch J, Lötters S, Wombwell E, Garner TWJ, Cunningham AA, Spitzen-van der Sluijs A, Salvidio S, Ducatelle R, Nishikawa K, Nguyen TT, Kolby JE, Van Bocxlaer I, Bossuyt F, Pasmans F. (2014) Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science, 346, 630−631.

AM and FP conceptualized the project and designed the research. AM, FP, CA, PVR, WB, MCF, RAF, BRS, UT, KG, KRL, CM, KRZ, JB, SL, EW, TWJG, AAC, AS, SS, RD, KN, TTN, JEK, IV and FB collected the data. AM and FP analysed the data. AM, FP, CA, PVR, WB, MCF, RAF, BRS, UT, KG, KRL, CM, KRZ, JB, SL, EW, TWJG, AAC, AS, SS, RD, KN, TTN, JEK, IV, FB wrote and edited the manuscript. AM and FP provided funding, reagents and materials for the research.

Abstract

Spread of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) poses the greatest emerging threat to global amphibian biodiversity. *Bd*'s low host species specificity allows the disease it causes — chytridiomycosis — to affect many of the 7,000 species of amphibians and drive population declines and extinctions worldwide. Although discovered nearly 20 years ago, the origin of *Bd* and catalyst of the seemingly recent global disease event remain obscure. Today, this international epizootic continues to advance virtually uncontrolled.

Modes of global *Bd* dispersal are not well understood, hampering the development and implementation of targeted biosecurity efforts to reduce spread. *Bd* is an aquatic pathogen most often associated with amphibian species that live in or near permanent bodies of water. It can neither survive desiccation nor extended exposure to elevated temperatures, but few environmental barriers appear to impede the spread of *Bd*. It has crossed oceans, infected terrestrial direct-developing amphibians that do not live in water, and been introduced to every continent (except Antarctica). Although low densities of *Bd* have been found in the environment outside of a host, amphibians consistently carry the highest pathogen loads and appear to be the primary host organisms that vector *Bd*.

The international trade in live amphibians transports millions of animals annually. Most previous research has focused on this anthropogenic activity as the primary pathway of global *Bd* dispersal. This is a sensible assumption—the highly visible movement of *Bd* hosts together with the lack of disease control suggests that *Bd*-positive animals are commonly transported in these shipments. Unfortunately, all previous surveillance efforts that aimed to demonstrate this phenomenon were performed in animal markets in *Bd*-positive countries where contamination from domestic *Bd* could not be excluded as a potential source of infection.

Upon close examination of global *Bd* distribution patterns, I found that regions of *Bd* presence do not exclusively overlap those of notable amphibian trade, raising questions as to the sources and pathways involved in pathogen dispersal. For instance, despite the absence of commercial amphibian importation to the islands of Dominica and Montserrat, chytridiomycosis drove the near-extinction of the Mountain Chicken frog (*Leptodactylus fallax*). Likewise, chytridiomycosis emerged in remote wilderness areas in Central America and Australia, again with no clear link to amphibian trade activity. Thus, despite the similar absence of commercial amphibian importation in Madagascar, it seems unlikely that this alone prevents the introduction of *Bd*. Meanwhile despite intensive field and market surveillance in Hong Kong — a global amphibian trade hub — *Bd* has neither been detected nor have amphibian declines been observed. Therefore, I hypothesized that additional pathways of *Bd* dispersal exist in the absence of commercial amphibian trade that can also transport this pathogen global distances.

The aims of this study were to characterize the likelihood and consequence of multiple pathways of amphibian pathogen dispersal, help identify *Bd* mitigation targets likely to reduce the greatest amount of international disease spread, and suggest potential mitigation activities. I investigated the presence of five potential *Bd* dispersal pathways that might concurrently contribute towards contemporary global pathogen spread. Because *Bd* is present both in areas heavily affected by human activity and remote wilderness areas, I explored avenues of dispersal that involved anthropogenic-assisted spread, spread by wildlife, and spread by environmental phenomena. The five pathways I evaluated include: 1. Commercial amphibian trade, 2. Amphibian hitchhikers (unintentional amphibian trade), 3. Atmospheric transport events, 4. Amphibian locomotion, and 5. Fomites. Through my series of studies, I examined high-risk

environments where each pathway was most likely to be detected if an active avenue of *Bd* spread existed.

My results demonstrate that global *Bd* dispersal does involve multiple simultaneous pathways, with all five pathways having been successfully observed, with each varying in frequency and quantity of pathogen spread. At a field site in Cusuco National Park in Honduras, I discovered evidence for both atmospheric and terrestrial dispersal of *Bd*, previously undocumented for this aquatic pathogen. *Bd* was detected in rainwater in 5% of the storm events sampled and was present at a low density (1 *Bd* zoospore/L). Because *Bd* viability cannot be confirmed from the qPCR test results alone, it remains uncertain whether *Bd*-positive rainwater is infectious to amphibians. Even in the absence of infection, aerial dispersal can produce *Bd*-positive field samples and may occasionally be responsible for enigmatic isolated records of *Bd* detection.

Another way that *Bd* spreads in the absence of human assistance, and in higher densities, is through amphibian locomotion. Although infected animals spread *Bd* to one another in captive laboratory setting, the likelihood of this pathway was not previously measured in the wild in the natural environment. Because some frogs develop high infection loads as they undergo metamorphosis from tadpole to froglet, I studied whether seasonal mass emergences from aquatic to terrestrial habitats commonly transports *Bd* across habitat boundaries. In a sample of 52 recently emerged frogs, I detected the presence of *Bd* on 76.1% (35/46) of terrestrial vegetation surfaces where a *Bd*-positive frog had been resting. As previously mentioned, the viability of *Bd* cannot be discerned from qPCR results alone, but the cool air temperature, closed canopy, and high humidity of this cloud forest provides favourable conditions for protection from desiccation and extended *Bd* persistence. Furthermore, the average *Bd* zoospore equivalent

loads that I detected on affected leaf surfaces (average = 40.48 and range was 0.12–1,040.45) compared to those measured in adjacent *Bd*-positive river water samples (average was 0.23 and range was 0.03–0.57) show that exposure to affected foliage may pose a greater threat of *Bd* transmission to terrestrial amphibians than would a comparable period of exposure to nearby river water.

I performed several studies to measure and evaluate the presence of *Bd* in amphibians commercially imported into the United States. Overall, I detected a moderately high prevalence of *Bd* in amphibians sampled immediately upon importation in the USA, validating prior studies on trade being a major route of spread. *Bd* was detected in 11.7% of 265 exotic pet trade amphibians from Hong Kong, 58.8% of 102 food trade bullfrogs from the Dominican Republic, 0/35 food trade bullfrogs sampled from Taiwan, and 0.5% of 565 exotic pet trade amphibians from Madagascar. In addition, this trade activity also caused the spread of *Bd*-contaminated shipping materials; e.g. 59.0% (62/105) of cardboard boxes carrying bullfrogs from the Dominican Republic and 5/8 bags of water carrying amphibians from Hong Kong tested positive for *Bd*.

Despite previous lack of detection, my surveillance data confirmed the presence of *Bd* in material exported from Madagascar and Hong Kong, and for the first time suggested the pathogen might already have been introduced to those countries' wild amphibian populations. In response, I designed and performed targeted rapid response investigations to determine whether *Bd* was present and identify potential introduction pathways. In 2013, I detected *Bd* in Hong Kong in Asian bullfrogs (*Hoplobatrachus chinensis*) sampled upon importation (8/26), in African clawed frogs (*Xenopus laevis*) at domestic pet stores (3/60) and in native free-ranging amphibians (2/268). In Hong Kong, I observed trade behaviors that are accompanied by a high

risk of pathogen spillover - most notably the release of non-native animals into local amphibian habitats. In contrast, I did not detect the presence of *Bd* in 508 amphibians and 68 water filter samples tested in Madagascar, although this does not preclude its presence in very limited distributions and/or very low prevalence. I inspected the wildlife trade facility from where my previous *Bd*-positive frogs originated and did not observe any obvious opportunities for non-Malagasy *Bd* contamination –animals from other countries do not enter the facility. Despite the absence of commercial amphibian importation, I identified an incursion of Asian toads likely introduced as hitchhikers inside ocean shipping containers. It remains unknown whether these toads have recently introduced foreign pathogens to Madagascar, but this invasion clearly demonstrates how the absence of intentional amphibian trade does not entirely reduce risk of exposure to *Bd*. Data produced by both rapid response field studies suggests that a virulent strain of *Bd* (e.g. *Bd*-GPL) is not yet established in either Hong Kong or Madagascar, despite evidence of *Bd* introduction pathways, presence of suitable climatic refugia, and an abundance of susceptible host species. Therefore, I believe it remains plausible to prevent *Bd*-associated declines within these countries if appropriately targeted biosecurity measures are implemented.

Applying all information developed and collated to characterize the five potential *Bd* dispersal pathways, I then performed a risk matrix analysis to compare the amount of risk associated with each in order to better assist management decision-making. Each pathway was assigned a numerical score reflecting the combined likelihood of *Bd* spread and the severity of the outcome. These values represent the estimated relative contributions of each pathway to the global emergence of *Bd*. The international trade in live amphibians creates the most consistent and predictable opportunities for long-distance spread of viable and potentially infectious *Bd* and was ranked the highest risk pathway. My data shows that in the absence of this activity, *Bd*

appears unlikely to frequently cross geographic and biotic boundaries to its survival, and that the emergence of this pandemic is likely due to human-assisted trade.

I conclude that biosecurity regulations to reduce the amount of *Bd* dispersed by the international wildlife trade is the most imperative action to slow the current global spread of *Bd* and to reduce the likelihood of additional disease emergences. Although methods to control the trade-driven spread of *Bd* were recommended by the World Organisation of Animal Health nearly five years ago, few countries have formally adopted these practices or require any actions specifically to reduce *Bd* introduction. My study confirms that the trade in live amphibians is an engine of global pathogen pollution unrivalled by other identified pathways of *Bd* dispersal and reinforces the need for disease management actions at least to the level commonly offered to livestock. Moving forward, proactive disease surveillance and biosecurity measures must target the international wildlife trade if we are to protect global biodiversity from novel emerging infectious diseases of wildlife.

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CHAPTER 1

Introduction and basis for the study

Introduction

Amphibian chytrid fungus (*Batrachochytrium dendrobatidis*, hereafter "*Bd*") is a parasitic organism responsible for chytridiomycosis, an emerging infectious disease of amphibians responsible for global amphibian declines and extinctions (Berger et al. 1998; Stuart et al. 2004; Skerratt et al. 2007). Although habitat destruction and pollution have long been known to contribute towards amphibian decline, the disappearance of amphibians from remote protected areas suggested the involvement of additional undescribed causes. Berger et al. (1998) presented evidence of a link between the presence of chytrid fungus and amphibian mortality and suggested that the disease, chytridiomycosis, was involved in certain enigmatic amphibian declines. Around the same time, Laurance et al. (1996) and Lips (1999) also reported enigmatic declining amphibian populations in remote protected areas of Australia and Panama, respectively, similarly calling attention towards disease as a potential causal agent rather than habitat degradation. Soon after, Longcore et al. (1999) described the new species of fungus observed by Berger et al. (1998) as *Batrachochytrium dendrobatidis*, the first species of chytrid known to be pathogenic to amphibians. Skerratt et al. (2007) showed that the recent spread of virulent chytridiomycosis into naïve populations has been the cause of these dramatic declines. Global amphibian surveys have since confirmed the presence of *Bd* on every continent inhabited by amphibians, but the pathways facilitating rapid global *Bd* spread have not yet been well demonstrated. It is believed that nearly one-third of all amphibian species are experiencing significant population declines with one of the major causes being chytridiomycosis (Skerratt et al. 2007; Whittaker et al 2013) and if not abated, this severe loss of biodiversity will mark the beginning of Earth's sixth mass extinction (Wake and Vredenberg 2008).

The key to understanding this global disease event and determining an appropriate conservation response to mitigate biodiversity decline first requires a stronger understanding of

Bd epidemiology. Why has *Bd* only recently emerged as a globally significant amphibian pathogen and what factors are allowing it to rapidly cross great distances? Whether *Bd* is a novel pathogen that recently spread globally or is instead a globally endemic pathogen that "suddenly" became pathogenic due to environmental changes has been debated. McCallum (2005), Pounds et al. (2006), and Wake (2007) asserted that insufficient evidence supported the characterization of *Bd* as a spreading infectious amphibian disease. They instead proposed that the influences of climate change and environmental degradation catalyzed the expression of virulence in an endemic parasite. In contrast, Skerratt et al. (2007) described *Bd* as an emerging infectious pathogen still actively spreading and causing wave-like amphibian declines as it invades new regions, as demonstrated by Lips et al. (2008). Although many questions still remain unanswered, the evidence presented by Skerratt et al. (2007) is currently widely accepted and calls attention for the need to develop measures to mitigate further *Bd* spread.

Transmission of *Bd* is mediated by exposure to aquatic uniflagellated zoospores that swim short distances (~2cm), but generally require either flowing water or the movement of amphibian hosts to move greater distances (Piotrowski et al. 2004). Development from zoospore to mature zoosporangium takes place in the amphibians' skin and the life cycle is completed in approximately 4-5 days (Berger et al. 2005). The response to infection seems to vary by species and even between individuals within the species, ranging from resistance to infection to a clinical infection, severe disease and death. In some instances, an association has been observed between host phylogenetic relationships and response to infection, whereby closely related species and genera respond to *Bd* more similarly compared with distant relatives (Knapp and Morgan 2006). In animals that develop severe chytridiomycosis, *Bd* interferes with skin structure and osmotic function, eventually leading to loss of electrolytes and death (Voyles et al.

2009). Although signs of chytridiomycosis may include lethargy, skin lesions, and loss of righting reflex, they do not always occur and laboratory tests are needed to diagnose infection and disease (Pessier et al. 1999). The application of PCR to detect the molecular presence of *Bd* on amphibian skin swabs is currently the most widely employed method for field surveillance (Annis et al. 2004; Boyle et al. 2004; Hyatt et al. 2007).

Global Dispersal of *Bd* by the Live Amphibian Trade

Current evidence suggests that the origin of *Bd* is likely South Africa or Asia, as endemic *Bd* lineages occur in these regions without causing mortality or declines (Berger et al. 2016). The lineage that has spread and caused declines has been termed the Global Pandemic Lineage (*Bd*-GPL). Although there is some debate as to the absolute global origin of this lineage, there is strong evidence that the trade in live amphibians is involved in its spread (Mazzoni et al. 2003; Fisher and Garner 2007; Schloegel et al. 2009; Catenazzi et al. 2010; Spitzen-van der Sluijs et al. 2011). Global dispersal from Africa may have been sparked by the export of African clawed frogs (*Xenopus laevis*) during the mid-1900s for their application in early human pregnancy tests (Weldon et al. 2004). Mazzoni et al. (2003) raised concern for the high likelihood of *Bd* presence in American bullfrogs (*Lithobates catesbeianus*) farmed in Uruguay for the international market and in the USA. Schloegel et al. (2009) later sampled American bullfrogs at domestic markets and found 62% of 493 amphibians infected with *Bd*. According to the sellers, these bullfrogs were imported from Southeast Asia and South America. Unfortunately, because *Bd* is already widespread in the USA and these animals were sampled from domestic markets, cross-contamination with *Bd* of domestic origin could not be ruled out. Catenazzi et al. (2010) detected a high prevalence of infected *Telmatobius* spp. frogs in domestic markets in Peru. In contrast, surveys in Europe (France, Belgium, the Netherlands, and Germany) of amphibians in

private and commercial collections demonstrated a low prevalence of *Bd* (3%) (Spitzen-van der Sluijs et al. 2011).

After *Bd* was detected in animals traded both globally and domestically, the focus of dispersal and transmission became largely fixated on the movement and presence of amphibians. Trade of live amphibians is now often assumed to be the primary mode of international and transoceanic *Bd* dispersal, although amphibians have not yet been sampled immediately upon importation from a foreign source to prove infections were present upon arrival versus contracted domestically. A robust large-scale survey to measure the infection prevalence in amphibians at the point of importation is still needed to quantify *Bd*-positive amphibians traded internationally.

Despite this focus on amphibian trade, some patterns of global *Bd* distribution cannot easily be explained by this activity alone. A growing number of remote localities continue to become exposed to *Bd*, despite being far removed from the influence of commercial amphibian importation and domestic trade. Some of the earliest recognized *Bd*-related amphibian declines were reported from wilderness regions in Panama and Australia, far from cosmopolitan areas where amphibian trade would occur. Even remote island nations seemingly protected by the added benefit of geographic isolation have become recently infected, despite the absence of commercial amphibian importation, including the islands of Montserrat (Garcia et al. 2009) and Tobago, West Indies (Alemu et al. 2008).

In addition to the enigmatic distribution of *Bd*, variable estimated rates of *Bd* spread have also raised questions as to the forces facilitating its dispersal. *Bd* was estimated to have spread across Queensland, Australia at approximately 100 km/yr since its emergence in the 1970s, a phenomenon described by McCallum (2005) as, "…implausibly high for a waterborne pathogen". In Central America, the rate of spread was estimated to have ranged from 25-282

km/yr, varying by region (Lips et al. 2008). Despite these estimations based on *Bd* presence/absence data, a complete causal pathway characterizing how *Bd* is spreading from one region to another has not yet been fully described.

Frequent *Bd* Spread Across Habitat & Species Boundaries

Although trade surveys demonstrate that *Bd*-positive amphibians are transported by human activities, none consider the subsequent likelihood of *Bd* spillover from captivity into the wild, and then from the environment to native susceptible amphibians. Species-specific behavioral patterns and ecological barriers generally define an amphibian species' distribution. Viewing *Bd* as an aquatic pathogen that primarily resides in water bodies, it would seem that the spread of *Bd* in nature should be limited by the behavior of aquatic amphibians and habitat boundaries. For instance, in a study examining 32 amphibian species, Semlitsch and Bodie (2003) determined the core terrestrial habitat utilized by these species ranged from 159 to 290 m from the edge of an aquatic site. Although exceptional long-distance movements by amphibians may occur, they are generally infrequent except for species that exhibit a high degree of independence from water bodies, such as the gopher frog (*Rana capito*), which has been found up to 691 m from the nearest breeding pond via radio telemetry (Roznik et al. 2009). Species that spend most of their time away from water bodies should serve as poor vectors of *Bd* dispersal between disparate watersheds since they are less likely to become exposed to aquatic *Bd* zoospores and more likely to cross drier warmer areas that challenge the survival of *Bd* (Lips et al. 1999; Longcore et al. 2007). In this context, the seemingly common successful dispersal of *Bd* across vast terrestrial spaces in the absence of anthropogenic influence is peculiar, considering aquatic amphibians often remain near water and are unlikely to stray great distances.

Direct physical contact between amphibian species is uncommon (Rowley and Alford 2007). Even if terrestrial amphibians were to vector *Bd* between separate aquatic habitats, the low incidence of cross-species physical contact suggests these animals would still be poor conduits of direct *Bd* transmission between species. Yet, in most locations where *Bd* is detected, multiple species with varying life history traits and behaviors are often concurrently infected. Collectively, these data suggest the presence of indirect *Bd* transmission pathways, which judging by the rate of *Bd* spread in nature, are likely to be both common and able to result in disease in new animals.

Enigmatic *Bd* Dispersal

When enigmatic patterns of disease spread and distribution emerge, it is important to consider the presence of a spectrum of potential pathogen transmission pathways, rather than focus only on the most obvious. Similar to *Bd*, other disease agents previously demonstrated patterns of spread and distribution considered "enigmatic" before their respective transmission pathways were characterized and identified. Such was the case with bovine foot and mouth disease (FMD) caused by the virus *Aphtae epizooticae*, where new outbreaks of infection were diagnosed in livestock populations that had no previous exposure to other potentially infected animals. At that time, animal-animal contact was considered to be the mode of FMD transmission. A growing collection of unusual observations challenged this understanding and thorough investigation led to the discovery of airborne FMD transmission by virus particles exhaled from infected animals and carried downwind to other animals (Garner and Cannon 1995; Schley et al. 2009).

A similar scenario describes the spread of citrus canker, a significant disease affecting citrus trees caused by the bacterium *Xanthomonas axonopodis*. This pathogen was introduced to the USA via importation of infected trees from Asia. Although contact with infected leaves was

known to spread infectious material between trees, disease was observed to spread across Florida in an erratic pattern, with new points of infection emerging in areas non-contiguous with areas that possessed infected trees. Eventually, it was confirmed that rain splash could liberate bacteria from affected leaf surfaces and create wind-swept water droplets containing infectious material. During periods of severe weather, it was found that these droplets could be transported as far as 60 km before settling and infecting trees in locations inconsistent with the anthropogenic movement of host plants, and hence, explaining the formerly enigmatic pattern of disease emergence (Irey et al 2006; Gottwald and Irey 2007).

Potential Dispersal of *Bd* via Aerial Spread

Atmospheric spread of *Bd* could help explain some of the erratic and rapid pathogen dispersal rates observed globally. Wind-based spread of other species of chytrid fungi has been recently suggested by the predominance of chytrids detected in the barren high-elevation soils of the Rocky Mountains in Colorado, USA and also the Himalayan Mountains in Nepal (Freeman et al. 2009). It is believed that the diverse chytrid communities found in these regions are likely the result of wind-based dispersal, together with the grains of pollen found in abundance in these non-vegetated landscapes, a nutrient source for many chytrid fungi. Although *Bd* was not among those chytrid species identified, the environmental forces that transported fungal spores into these isolated high-altitude locales may plausibly also influence the spread of *Bd*, though the absence of an encapsulated resting stage in *Bd* would limit its survival in unfavourable conditions (Morehouse et al. 2003). Wind-based pathogen dispersal is not uncommon in nature, and in addition to FMD and citrus canker, has been described for agents of human disease including coliforms, and *Pseudomonas spp*. (Evans et al. 2006) and plant diseases including

Sudden oak death (*Phytophthora ramorum*), Soybean rust (*Phakopsora pachyrhizi)*, and Karnal bunt of wheat (*Tilletia indica*) (http://www.aphis.usda.gov).

In many regions of the world where *Bd* is present, hurricanes and cyclones are frequent seasonal phenomena generating strong winds likely to carry microbes. This, together with the near constant generation of *Bd*-laden droplets ejected by river turbulence and waterfalls may provide frequent opportunities for *Bd* to become airborne and carried by the wind before falling from suspension elsewhere. As demonstrated by Barker and Jones (2005), water turbulence from the flushing of a household toilet generated enough energy to eject virus and bacteria-laden droplets into the air and cause at least 30 min of airborne suspension. Forces in nature will greatly surpass that of a toilet, especially during periods of severe weather, and expose ejected microbe-laden river water droplets to strong horizontal winds. In this context, wind-driven *Bd* dispersal across terrestrial zones and between separate aquatic habitats might frequently occur and warrants investigation. In extreme circumstances, it seems plausible, though unlikely, that *Bd* may even cross ecological barriers such as seas in prevailing winds; Saharan dust is carried in this fashion, landing in the Americas in as little as 1 week after leaving the African continent (Kellogg & Griffin 2007).

Potential Dispersal of *Bd* by Contaminated Water or Fomites

Infected amphibians shed *Bd* zoospores and infected skin into their surroundings, and thus the movement of contaminated environmental substrates (fomites) may transport *Bd* in the absence of amphibians. For example, after amphibians are traded, the containers and substrates used to transport *Bd*-positive animals can carry *Bd* and spread infectious material into the environment if discarded untreated. Wild fish destined for the international trade, either for ornamental or aquacultural purposes, are often collected from water bodies containing potentially

Bd-positive amphibians. Water transported with fish may provide a more obscure pathway of *Bd* dispersal in the absence of amphibian trade. Following transport, water discarded untreated into storm drains may flow directly into nearby wetlands and expose native amphibians. The spread of *Bd*-positive material is not contingent on the amphibian trade although little consideration has been given to the spread of *Bd* by the transport of fish and other non-amphibian commercial shipments.

Biological Limitations to *Bd* Survival and Dispersal

The existence of a particular *Bd* dispersal pathway does not alone assure that *Bd* zoospores will be viable when deposited at a subsequent location, and potential for fungal survival needs consideration. Many other species of chytrid fungi benefit from the ability to develop thick-walled resistant resting spores that enable survival during unfavourable environmental conditions, but no such protective encapsulation has yet been identified in *Bd* either on the amphibian host or in culture, (Longcore et al. 1999; Morehouse et al. 2003). According to Morehouse et al. (2003), a resting stage in *Bd* "could confer the ability to persist in the absence of amphibians and to be transported by wind, perhaps explaining the widespread distribution of the disease in relatively pristine areas (Berger et al. 1998; Daszak et al. 1999)." Although *Bd* is primarily clonal and the production of thick-walled resting spores is often associated with chytrid fungi that perform sexual reproduction (Sparrow 1960), the first evidence of sexual reproduction in *Bd* was recently described in an isolate from Brazil (Schloegel et al. 2012). While this suggests the possibility that resistant *Bd* spores might exist, no such confirmation has been made and *Bd* appears to remain vulnerable both to elevated temperatures and desiccation.

Laboratory experiments have shown that *Bd* can survive and reproduce in culture between 4 and 28C (Bradley et al. 2002; Berger et al. 2004) and is most active between 17 and 25C

(Longcore et al. 1999; Berger et al. 2004; Piotrowski et al. 2004). *Bd* experiences mortality within 5 min at 60C, 30 min at 47C, 4 h at 37C, and 92 h at 32C (Berger et al. 2001; Johnson et al. 2003). Although brief exposure at 29C might not cause *Bd* mortality, it does appear to halt reproduction (Longcore et al 1999). Prevalence of *Bd* measured in lowland wet forests has demonstrated dramatic depressions during warmer summer months followed by an increase in the winter; in certain instances the summer prevalence was measured at or near zero and then rose considerably to 35% in Costa Rica (Whitfield et al. 2012) and 65% in Queensland, Australia (Phillot et al. 2013). It is uncertain where in the environment this pathogen resides during periods when a prevalence of zero is measured at a site seasonally characterized as *Bd*-positive and it remains possible that infected amphibians seek out cooler microhabitats and/or harbour low *Bd* intensities that produce false-negative swab results.

Although *Bd* cannot survive complete desiccation (Berger 2001; Johnson and Speare 2003), Johnson and Speare (2005) found that sporangia survived on feathers left to dry for 1-3h. In other experiments, *Bd* in culture survived exposure to air for up to 3h when exposed to 25C at 70% relative humidity (Johnson and Speare 2003). As per these data, the aforementioned potential aerial *Bd* dispersal pathway via aerosolized river water droplets might maintain viable *Bd* zoospores for short periods, especially during times of elevated humidity as expected during tropical storms.

Biological Relevance of Exposure to Small Doses of *Bd*

The low infectious dose of *Bd* suggests that its presence in any substrate and at any concentration, whether in water, soil, or on the surfaces of shipping cartons, should be considered infectious unless proven otherwise. The ability of *Bd* to catalyze infection via minute exposure concentrations in the absence of amphibian-amphibian contact was demonstrated by

Carey et al. 2006 where Boreal toads (*Bufo boreas*) were exposed to water treatments of an estimated 1 *Bd* zoospore. Of those exposed for 24h, 38% developed infection and an extended exposure treatment of 3 days caused 100% of toads to become infected. Among infected animals, 87% developed lethal chytridiomycosis and did not survive past 42 days. Since response to *Bd* exposure varies between species, these data are not applicable to all amphibians and circumstances, but demonstrates the high infection potential of *Bd* to susceptible animals.

The relevance of a single zoospore in nature was discussed by Kriger et al. (2007) and warrants mention to prevent misunderstandings. Kriger et al. (2007) explain how the detection of DNA representing a single zoospore via qPCR analysis does not prove an amphibian is infected and Skerratt et al. (2011) further showed that PCR specificity can be less than 100%. Smith (2007) further suggested that qPCR may actually be "too sensitive" to reliably characterize chytridiomycosis and cautions against this misinterpretation of results. All of these points should be considered when interpreting field survey results, but it is important to know that small quantities of live *Bd* zoospores can indeed cause infection and chytridiomycosis. Thus, pathways of *Bd* dispersal that move small quantities of zoospores, but occur frequently, might result in establishment of *Bd* in new regions and warrants evaluation to determine the risk of spread relative to that of the commercial trade in live amphibians.

Conclusions

Although non-amphibian pathways of amphibian pathogen dispersal have been considered (Mao et al. 1999; Kiesecker et al. 2001; Johnson and Speare 2003; 2005), current biosecurity considerations to control the spread of *Bd* predominantly focus on the international trade in live amphibians. Some patterns and rates of *Bd* spread appear inconsistent with solely

anthropogenic-assisted or natural movements of amphibians and suggest the active presence of multiple simultaneous pathways of *Bd* dispersal. If some unidentified pathways exist that commonly transport viable *Bd*, but do not rely on the anthropogenic movement of amphibians, then current and future biosecurity efforts to mitigate the spread of *Bd* will be less effective than predicted. To address this question, I aimed to improve understanding of the pathways of regional and global *Bd* dispersal and provide suggestions to prevent further spread based on these new data.

The United States Fish and Wildlife Service (USFWS) is currently considering a petition to list all amphibians as injurious species under the Lacey Act unless proven free of *Bd*, a regulatory action that would potentially halt the importation of millions of animals per year. This petition submitted by Defenders of Wildlife assumes that halting the international amphibian trade will halt the introduction of *Bd* (DOW 2009). Although it is highly likely that *Bd*-positive amphibians are imported from international sources and hence this intervention is a sensible precaution, supporting evidence to strengthen this proposal involves testing amphibian shipments immediately upon importation to confirm the absence of post-arrival *Bd* contamination.

The presence of additional pathways of *Bd* dispersal that occur with or without the presence of amphibians is important to investigate in order to identify mechanisms other than amphibian trade. For instance, if water accompanying shipments of live freshwater fish contains *Bd* zoospores, this non-amphibian activity may provide additional opportunities for pathogen introduction. Although difficult to quantify the consequences of different modes of *Bd* dispersal, the spread of *Bd* through more obscure pathways might be as significant, if not more, than that by live amphibians. Therefore, it is necessary to investigate a thorough network of possible *Bd*

dispersal pathways before concluding that the most obvious pathway—international amphibian trade—is also the most important one to control.

In contrast to the USA, Australia's response to the risk of introduction of amphibian pathogens by wildlife importation was met with relatively prompt and strict action—a ban on the commercial importation of live amphibians—but potential biosecurity gaps might remain via other *Bd* dispersal pathways. For example, although amphibians can no longer be imported, the freshwater fish trade continues and may sometimes be sourced from locations where amphibians have contaminated the water with *Bd* zoospores. Australia imports approximately 8-10 million fish annually, from nearly 100 countries of origin (Brown 2006). Because infection with *Bd* is now listed as a globally notifiable disease by the World Organisation for Animal Health (OIE), the infection status of the export country should be known and the risk of *Bd* dispersal could be evaluated, if the likelihood of spread via water was better understood.

Although *Bd* has already invaded many amphibian biodiversity hotspots around the world, predictive models suggest that many more locations are suitable for *Bd* survival than are presently occupied (Ron et al. 2005; Lötters et al. 2011; Murray et al. 2011). Possibly the greatest example of this is Madagascar, where all surveys thus far have failed to detect the presence of *Bd* (Weldon et al. 2008). It is unknown whether *Bd* has not yet arrived on the island of Madagascar or if instead some biotic or abiotic condition is suppressing its establishment. Madagascar possesses habitat where *Bd* is predicted to thrive (Lötters et al. 2011), lies in proximity to several east African countries where *Bd* is present, and is visited by a large number of tourists who might introduce *Bd* on soiled footwear. The apparent absence of *Bd* in Madagascar represents an opportunity where swift proactive measures to prevent *Bd* spread and

establishment might prevent irreversible biodiversity decline in a country where several hundred species of amphibians are endemic and remain highly vulnerable.

Now is the time to strengthen our understanding of global *Bd* dispersal pathways and implement additional biosecurity efforts to reduce contemporary global spread. Although many countries are already *Bd*-positive, the spread of different *Bd* lineages and strains with potentially greater virulence continues to threaten global amphibian biodiversity. Efforts to eradicate *Bd* following establishment seem unlikely to succeed, and thus preventative measures to control further spread and establishment are essential. Additional research is urgently needed to evaluate the risks of international *Bd* dispersal via anthropogenic activity as well as by natural phenomena and to further explore patterns of spread that remain enigmatic. These data could then be applied to improve our ability to predict the pathways most likely to introduce *Bd* to particular regions and design biosecurity interventions that specifically target those pathways of greatest consequence.

Thesis Aims

Previous research on the spread of *Bd* has primarily focused on commercial amphibian importation (Mazzoni et al. 2003; Fisher and Garner 2007; Schloegel et al. 200; Catenazzi et al. 2010; Spitzen-van der Sluijs et al. 2011), but the global distribution of *Bd* also includes a diversity of regions where this activity is minimal or absent (Alemu et al. 2008; Garcia et al. 2009; Kolby et al. 2010). Despite being an aquatic pathogen, *Bd* has also been detected in a growing number of terrestrial and arboreal amphibian species without an obvious source of transmission (Caruso & Lips 2013; Cummer et al. 2005; Garcia et al. 2009; Gower et al. 2013; Kolby et al. 2010; Longo & Burrowes 2010; Weinstein 2009). Successful reduction of the

spread of *Bd* requires thorough understanding of common dispersal pathways and I believe the aforementioned observations suggest additional uncharacterized and underexplored modes of dispersal exist. This thesis aims to expand current knowledge about the opportunities for contemporary global *Bd* dispersal and provide insight to help mitigate this global biodiversity threat. It has four overarching aims:

1. To investigate pathways of *Bd* dispersal via the commercial amphibian trade.
2. To investigate pathways of *Bd* spread via natural phenomena and/or passive dispersal.
3. To compare the relative magnitudes of *Bd* spread by each of the five pathways studied in this thesis: 1. Commercial amphibian trade, 2. Amphibian hitchhikers (unintentional amphibian trade), 3. Atmospheric transport events, 4. Amphibian locomotion, and 5. Fomites.
4. To identify opportunities for risk management to reduce the spread of *Bd* by each of the aforementioned pathways evaluated.

This thesis consists of 10 chapters. Chapter One provides background information summarizing previous investigations of *Bd* dispersal and describes knowledge gaps that develop the context for this study. Chapter Two describes three studies I performed to detect and measure *Bd* presence in traded amphibians sampled immediately upon import to the United States. During these investigations, I learned that another emerging pathogen of amphibians – ranavirus – shared many nuances with the spread of *Bd* so I decided to include ranavirus surveillance into some of my studies to maximize efficiency of sampling efforts, but continued to focus primarily on *Bd* for the purpose of this thesis. Detection of *Bd*-positive animals in Chapter Two sparked rapid response field investigations where I studied the potential presence and pathways of introduction of *Bd* in Madagascar and Hong Kong, described in Chapters Three and

Five, respectively. The latter part of Chapter Three is a call to arms to halt the invasion of Asian toads in Madagascar, both acting as a devastating invasive species and a possible vector of *Bd* to the country. Chapters Five through Seven further address pathways of *Bd* dispersal not directly associated with the trade in live amphibians. Chapter Five studies the presence of atmospheric *Bd* dispersal assisted by wind and rain, which could greatly expand the diversity of species at risk of disease if the pathogen survives transport. Due to the high volume of people that pass through *Bd*-infected national parks and wilderness areas, I studied the likelihood of spreading *Bd* on footwear exposed to *Bd*-contaminated environmental substrates, such as river water, and describe this in Chapter Six. In Chapter Seven, I explore another pathway likely to occur in nature but not previously measured – the transport of *Bd* between aquatic and terrestrial locations by amphibian locomotion. Applying pathogen prevalence data described in Chapter Two, Chapter Eight proposes trade restrictions to reduce the importation of amphibian diseases into the United States by two highly invasive species, the American bullfrog and African cawed frog. During this study, a newly emerging fungal pathogen was discovered, the salamander chytrid fungus *Batrachochytrium salamandrivorans*, and this additional threat to amphibians is summarized in Chapter Nine. Chapter Ten is a risk analysis of the five different *Bd* dispersal pathways studied in this thesis and suggests management actions to reduce the global spread of *Bd* where possible.

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Chapter 2

Investigations of the presence of amphibian pathogens in the international wildlife trade

Introduction

In Chapter 2, I investigated pathways of *Bd* and ranavirus dispersal through the international commercial amphibian trade. This investigation took three different directions in order to consider the potential presence of amphibian pathogens in different species, countries of origin, and modes of transport. In all studies, I was the first person to open each shipment and handle the animals (while wearing sterile gloves) to ensure the absence of domestic cross-contamination, something that could not be said for all previous studies performed on animals post-importation at markets or pet stores.

In the first study, I examined multiple shipments of amphibians exported from Hong Kong, from different locations. I chose to investigate trade from Hong Kong for two main reasons: 1) Hong Kong is one of the main suppliers of amphibians to United States' exotic pet trade and the presence of pathogens in this high volume of trade may spread a considerable amount of infectious material, and 2) previous surveillance failed to detect *Bd* presence in the region's native and traded animals, an unexpected result for a pathogen said to be associated with amphibian trade. In addition to sampling animals from these shipments, I also filtered water collected from bags holding aquatic species to determine whether shipping substrates may also become contaminated with *Bd*. Five shipments were sampled, including four species, one of which is a known *Bd* reservoir host – the African clawed frog (*Xenopus laevis*). This study is **Paper 1** of this chapter: "First Evidence of Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) and Ranavirus in Hong Kong Amphibian Trade," which has been published in PloS ONE.

In contrast to Hong Kong, my second study investigated the presence of *Bd* and ranavirus in amphibians exported from Madagascar where, like Hong Kong, neither pathogen had previously been detected. Since multiple previous field surveys failed to detect *Bd* in Madagascar, it was thought that the absence of commercial amphibian importation might be protecting the nation from exposure. As per my hypothesis that additional uncharacterized modes of *Bd* dispersal exist independent from amphibian commerce, I was unconvinced. I assumed *Bd* was present but evaded detection, possibly due to a low prevalence in wild amphibians (<5%) and/or limited distribution. To resolve this uncertainty, I intensively sampled one entire shipment of amphibians exported from Madagascar to the United States. This study is **Paper 2** of this chapter: "Presence of the Amphibian Chytrid Fungus *Batrachochytrium dendrobatidis* in Native Amphibians Exported from Madagascar," and has been published in PLoS ONE.

In my third amphibian trade study, I investigated amphibian pathogen presence in the bullfrog trade. Approximately 50% of 5 million amphibians imported into the United States annually are American bullfrogs (*Lithobates catesbeianus*) to supply the frog leg trade (for human consumption). In addition the high trade volume, this species is recognized as both a *Bd* reservoir host and an invasive species in many parts of the world. To evaluate the role of this frequent activity in the spread of *Bd* and ranavirus, I sampled four bullfrog shipments, three originating from the Dominican Republic and one from Taiwan. This study is **Paper 3** of this chapter: "Presence of *Batrachochytrium dendrobatidis* and Ranavirus in Bullfrogs Imported to the United States," and has not yet been published.

Paper 1

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First Evidence of Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) and Ranavirus in Hong Kong Amphibian Trade

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Abstract

The emerging infectious amphibian diseases caused by amphibian chytrid fungus (*Batrachochytrium dendrobatidis*, *Bd*) and ranaviruses are responsible for global amphibian population declines and extinctions. Although likely to have been spread by a variety of activities, transcontinental dispersal appears closely associated with the international trade in live amphibians. The territory of Hong Kong reports frequent, high volume trade in amphibians, and yet the presence of *Bd* and ranavirus have not previously been detected in either traded or free-ranging amphibians. In 2012, a prospective surveillance project was conducted to investigate the presence of these pathogens in commercial shipments of live amphibians exported from Hong Kong International Airport. Analysis of skin (*Bd*) and cloacal (ranavirus) swabs by quantitative PCR detected pathogen presence in 31/265 (11.7%) and in 105/185 (56.8%) of amphibians, respectively. In addition, the water in which animals were transported tested positive for *Bd*, demonstrating the risk of pathogen pollution by the disposal of untreated wastewater. It is uncertain whether *Bd* and ranavirus remain contained within Hong Kong's trade sector, or if native amphibians have already been exposed. Rapid response efforts are now urgently needed to determine current pathogen distribution in Hong Kong, evaluate potential trade-associated exposure to free-ranging amphibians, and identify opportunities to prevent disease establishment.

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Introduction

The high volume of global trade in potentially diseased amphibians has sparked a series of investigations into its role as a primary driver of the emergence and spread of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*, *Bd*) and ranaviruses, threatening global amphibian biodiversity [1 5]. With respect to *Bd*, particular concern has been expressed regarding the transport of American bullfrogs (*Lithobates catesbeianus*), due to the species' propensity to carry infection asymptomatically and serve as a reservoir of disease [6,7]. Millions of *L. catesbeianus* are traded globally for consumption annually. High prevalence of *Bd* infection (41 62%) has been detected among this species sold in markets in the USA, imported primarily from Southeast Asia and South America [1 2]. Furthermore, *Bd*-positive water often accompanying commercial amphibian shipments likewise represent a potential source of spread [8 9].

Similarly, ranaviruses are emerging pathogens capable of causing mass mortality and localized population decline in amphibians [10], as well as reptiles and fish, and their spread shares many nuances with the global dispersal of *Bd* [11]. Transmission of viral particles occurs through direct contact with infected individuals and exposure to contaminated water or soil. Its ability to infect three classes of ectotherms and the lack of an effective therapeutic treatment warrants serious consideration. The geographic spread of ranavirus also demonstrates strong association with the trade in live amphibians, most notably the trade in tiger salamanders (*Ambystoma tigrinum*) and American bullfrogs [2,12].

Investigations for the presence of these pathogens in both traded and free-ranging amphibians in Asian countries have produced mixed results, ranging from lack of detection to widespread low prevalence [5,13 16]. Previous surveillance efforts have not detected *Bd* in Hong Kong, a global amphibian trade hub, despite substantive testing of bullfrogs imported for consumption, native free-ranging amphibians, and non-native pet species [16, Simon Chan pers. comm. 2012). Furthermore, neither amphibian mass mortality events nor enigmatic population declines have been documented in Hong Kong suggesting pathogen absence. However, pre-metamorphic and recently metamorphosed amphibians are often more susceptible to *Bd* and ranavirus [17 20], and previous surveys have only concentrated on adults, potentially reducing survey sensitivity. Surveys of imported bullfrogs (*L.*

Table 1. Summary of live amphibians imported into the USA from Hong Kong during a 5-year period (1 January 2006 -26 December 2010).

Species	Quantity
Hymenochirus curtipes	1468130
*Xenopus laevis**	673859
*Cynops orientalis**	374560
*Triturus hongkongensis**	216054
Hymenochirus boulengeri	207632
*Bombina orientalis**	190189
Hymenochirus boettgeri	102160
Cynops pyrrhogaster	83178
Xenopus sp.	82996
Triturus sp.	59065
Pachytriton brevipes	42613
Cynops sp.	27703
Non-CITES amphibian species	19027
*Paramesotriton hongkongensis**	17870
Hymenochirus sp.	8870
Physalaemus sp.	7200
Eleutherodactylus sp.	4500
Pachytriton labiatus	3584
Hyla sp.	2104
Tylototriton kweichowensis	1501
Paramesotriton chinensis	1491
Pachytriton sp.	1172
Tylototriton verrucosus	1160
Tylototriton sp.	939
Polypedates dennysii	903
Rhacophorus sp.	524
Xenopus clivii	400
Pachyhynobius shangchengensis	343
Silurana sp.	300
Bombina bombina	290
Polypedates sp.	236
Rhacophorus dennysi	226
Tylototriton shanjing	190
Hyla arborea	180
Bombina sp.	160
Bufo sp.	100
Tylototriton taliangensis	100
Paramesotriton sp.	100
Leptobrachium sp.	90
Batrachuperus sp.	76
Salamandra salamandra	20
Rana chensinensis	17
Rana sp.	10
Brachytarsophrys carinensis	4
	3601826

Amphibian trade information as recorded in the Law Enforcement Management Information System (LEMIS) maintained by the United States Fish and Wildlife Service (USFWS). Not all specimens were recorded at the species level upon importation; some only to genus and others as "Non-CITES amphibian species".

Table 1. Cont.

Data is arranged in order of decreasing trade volume by recorded classification. Asterisk denotes species sampled in the current investigation; *Triturus hongkongensis* in not a currently recognized scientific name and is herein considered synonymous with *Paramesotriton hongkongensis*.
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catesbeianus) sold for consumption in the USA have demonstrated a high prevalence of infection with ranavirus and *Bd* suggesting a foreign source [2,3]. Therefore, recognizing the current risk of pathogen pollution from regional trading partners with known presence of these pathogens, the aim of this study was to identify the potential presence of *Bd* and ranavirus in Hong Kong trade by examining amphibians commercially exported to the USA.

Methods

Ethics

This study was approved by Tufts University's Institutional Animal Care and Use Committee (Permit #G2010-85). Amphibians were sampled upon importation with permission from the United States Fish and Wildlife Service.

Amphibian trade data

A request was made to the United States Fish and Wildlife Service (USFWS) for all records describing the country's trade in amphibians from 1 January 2006 to 11 October 2011. Information characterizing the international trade in wildlife is maintained in the USFWS Law Enforcement Management Information Systems (LEMIS) and made available through the Freedom of Information Act (FOIA). Records provided by USFWS were filtered to include only specimens of live amphibians commercially exported by Hong Kong to the USA and subtotaled by species. These data were used to evaluate the potential for amphibian pathogen presence in traded species and identify which of those species were most likely to be available for sampling.

Sample Collection

Amphibians were sampled in the USA immediately upon arrival from Hong Kong International Airport between May and September 2012. Permission to sample amphibians was provided by the USFWS. Four amphibian species commonly traded in high volumes were targeted for sampling (Table 1), including those with known or expected pathogen susceptibility, to increase the likelihood of pathogen detection in each shipment. These species are typically maintained and shipped in an aqueous environment and in high densities, providing conditions likely to increase pathogen transmission for both *Bd* and ranavirus. The importation documents presented with each shipment declared that all specimens had been bred in captivity in Hong Kong. A record was made of the importation date, the total number of specimens present, substrate type, and physical condition of each sampled amphibian.

Amphibians were shipped communally in various quantities and arrived in bags of water with the exception of *Bombina orientalis*, which was shipped dry. In order to detect a minimum 10% prevalence within each shipment (with a 95% detection probability), we aimed to randomly sample 30 individuals of each species within each shipment [21]. Since this approach erroneously assumes both the sampling methodology and diagnostic test have 100% sensitivity, greater numbers were sampled when possible, although fewer were collected on occasion due to time constraints. Sampling effort was evenly distributed among all bags of

amphibians within each shipment. All amphibians were randomly selected for sampling through blinding of the sampler, often from a bag containing hundreds of individuals, and sometimes included animals that were dead on arrival. Amphibians were temporarily housed in a separate container after processing and returned upon completion to prevent re-sampling of the same individuals.

Each shipment was unsealed and sampled immediately upon arrival in the USA, eliminating the risk of domestic or iatrogenic contamination. Fresh pairs of Nitrile gloves were worn for each shipment sampled. Amphibians were sampled for *Bd* using sterile fine-tipped rayon swabs with plastic shafts. The underside of the legs, feet and ventral surface were swabbed approximately five times each [22] and the swab bud was snapped off into a dry 2 mL cryovial. Samples were maintained dry at room temperature for a maximum of seven days before being transferred to a -80C freezer pending analysis.

Water samples from bags carrying amphibians were collected from each shipment and filtered to detect the presence of *Bd* following protocols established by Kirshtein et al. (2007) [23]. Immediately after a bag was opened and before *Bd* swabbing commenced, approximately 550 mL of water were extracted and sealed in a sterile container for subsequent filtration. This water was drawn into a sterile 60 mL syringe and pumped manually through a 0.22-micron Sterivex filter capsule until the filter became nearly clogged with organic debris. Then, 50 mL of phosphate buffered saline was passed through the capsule to rinse the filter before being pumped dry. After the addition of 0.9 mL Qiagen ATL lysis buffer with a sterile 1 mL syringe, the filter capsule was sealed and stored for subsequent qPCR analysis. Most species arrived in two separate bags of water (of which only one was sampled), except for the instances where a single shipment of *Xenopus laevis* arrived in four bags of water (of which two were sampled) and a single shipment of *B. orientalis* which arrived dry. A sealed bottle of spring water was filtered onsite to serve as a negative control to assess for equipment contamination.

Ranavirus sampling was performed by cloacal swabbing as described in Gray et al. (2012) [24]. Although this technique can underestimate the incidence of ranaviral infection by as much as 22% compared to lethal methods, only non-invasive sampling was allowed. All animals were first swabbed for *Bd* immediately upon removal from the container in which they arrived. Due to time constraints, only a subset of *Bd*-tested amphibians were subsequently sampled for ranavirus while still in hand. Swab buds were snapped off into a 2 mL cryovial containing 0.5 mL Nuclisens solution and stored under the same conditions as *Bd* samples while pending analysis. Due to the overlap in sample collection between ranavirus and *Bd*, all data collection parameters previously listed for *Bd* also apply to animals tested for ranavirus.

Real-time PCR

Taqman PCR for *Bd* was generally based on the method, primers and probe of Boyle et al. (2003) [25]. For swab samples the DNA template was prepared with Prepman Ultra® (Applied Biosystems). Water filter samples were processed following the method of Kirshtein et al. (2007) [23]. Reactions used the Taqman Environmental Mastermix 2.0 (Applied Biosystems). Samples were run in triplicate on an ABI/Applied Biosystems 7900HT thermocycler using 384 well plates with an exogenous internal positive control labeled with VIC™ (Applied Biosystems) for each sample to detect PCR inhibitors. Samples that amplified at a Ct≥50 were considered negative. Samples amplifying at a Ct of <50 in 2 or more wells were considered positive. Those which produced a positive reaction in only one of three runs were considered "equivocal" and reported as negative in the data presented herein as recommended by Hyatt et al. (2007) [22] and Skerratt et al. (2011) [26] in order to maximize specificity. Quantification standards were created by growing *Bd* isolate JEL 197 on 1% tryptone agar and harvested of zoospores by rinsing plates with 1X PBS. After collection zoospores were counted three times on a hemocytometer to determine a range of zoospores ml $^{-1}$. Standard curves were generated with ten-fold serial dilutions (range 1×10^{6} to 1×10^{-2} zoospores). In addition to positive controls (quantification standards), each plate included a negative control (Taqman mastermix and no sample DNA) as well as 4 positive and negative quality assurance controls consisting of swabs either inoculated with *Bd* zoospores or sham-inoculated. The intensity of infection in positive samples was expressed as the number of zoospore equivalents per swab [27] or per liter of water.

Taqman PCR for ranavirus used primers, probes, and protocols as described by Pallister et al. (2007) [28], using the CON probe designed based on conserved segments of the ranavirus major capsid protein (MCP) gene. DNA was extracted from swabs using DNeasy Blood and Tissue Kits (QIAGEN Inc., Valencia, CA, USA) with spin columns, following the manufacturer's protocol. The assay was performed using the ABI Real-time 7900HT system as described above. Samples amplifying at Ct's of <50 in 3 or wells were considered positive. A standard curve was created by diluting a synthetic plasmid PIDTSMART-AMP (Integrated DNA Technologies, San Diego, CA) containing the ranavirus MCP gene primers and probe sequences for the conserved MCP gene region (Genbank 298256130) insert from the above. The plasmid was diluted in nuclease-free water from 10^{8} copies/5 ul in a series of eight 1:10 dilutions down to 10 copies/5 ul and run in duplicate along with a third well containing the exogenous master mix (EIC, Life Technologies).

Results

Amphibian trade activity: exports from hong kong to the USA

Approximately 720,000 live amphibians were exported from Hong Kong and imported into the USA annually from 2006–2010 (Table 1). This activity involved no less than 31 species and consisted of those utilized primarily for the pet trade. Despite this diversity, few species composed the majority of traded specimens; those four sampled in this investigation collectively represented 40.9% of the 3.6 million amphibians supplied by Hong Kong during the 5-year period examined. Although exported amphibians were primarily documented to have been bred in captivity in Hong Kong, some were first imported from other Southeast Asian countries such as China, Indonesia, Singapore, and Thailand, and then re-exported from Hong Kong to the USA.

Batrachochytrium dendrobatidis results

Five shipments of amphibians, representing four species and originating from two separate exporters in Hong Kong were examined for the presence of *Bd*. Both amphibians and water were tested from four shipments, but only water was tested in the fifth. Shipments were sampled at a single port of entry in the USA and arrived during a 19-week time period. Molecular analysis of skin swab samples by qPCR indicated the presence of *Bd* in 31/265 (11.7%) amphibians imported into the USA from Hong Kong, and in one of four shipments (Tables 2 & 3). Two of four species tested positive: *B. orientalis* and *X. laevis*. In the single shipment containing *Bd*-positive amphibians, the percentages of affected animals were 5.4% in *B. orientalis* and 70.0% in *X. laevis*. The average *Bd* zoospore equivalents per swab were consistently low, suggesting weak infection intensities.

Table 2. Cumulative *Bd* and *Ranavirus* detection in amphibians imported from Hong Kong.

Species	Common name	# *Bd*	*Bd*+	# RV	RV+	H_2O *Bd*+	Sloughing	Ulcerations	DOA
Bombina orientalis	Oriental fire-bellied toad	56	3	13	10	-	22	0	3
Cynops orientalis	Oriental fire-bellied newt	97	0	78	60	-	7	4	15
Paramesotriton hongkongensis	Hong Kong newt	72	0	54	35	+	8	0	4
Xenopus laevis	African clawed frog	40	28	40	0	+	0	0	1
		265	31	185	105		37	4	23

Number of individuals sampled (#) for either *Bd* or ranavirus (RV), number of individuals testing positive by PCR (+), and presence of pathogen in water (H_2O *Bd*+) are expressed. Animal condition recorded upon sampling is provided, including skin sloughing, ulcerations, and the number of sampled specimens that were dead on arrival (DOA).

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Filtration of water samples collected from five shipments of aquatic amphibians tested positive *Bd* via qPCR in 5/8 bags tested, demonstrating the presence of *Bd*-contaminated water in 4/5 shipments (Table 4). Each bag only contained members of a single species, and water holding two of the four species (*X. laevis* and *Paramesotriton hongkongensis*) tested positive. The same aquatic species (*X. laevis*) that tested positive by swabs also had water testing positive for *Bd*. In both shipments of *P. hongkongensis*, the water tested positive for *Bd* whereas all skin swabs tested negative. Water transporting *X. laevis* contained exceptionally high densities of *Bd*, and the average *Bd* zoospore equivalent per liter ranged from 3,390 to 16,887 in two shipments, whereas that for *P. hongkongensis* ranged from 2.9 to 5.7. Both exporters in Hong Kong supplied shipments containing *Bd*-contaminated water, whereas *Bd*-positive amphibians were only detected from one exporter. *Bd* was not detected in the water control sample processed onsite.

Table 4. Presence of *Bd* in water sampled from shipments of amphibians imported from Hong Kong.

Species	Shipment	Vol (mL)	ZSE
Cynops orientalis	1	360	ND
Paramesotriton hongkongensis	1	495	5.7
Xenopus laevis	2	515	6455
Cynops orientalis	3	480	ND
Paramesotriton hongkongensis	3	515	2.9
Xenopus laevis	4	325	3390
Xenopus laevis	4	310	16887
Cynops orientalis	5	125	ND

Volume of water processed is reflected in milliliters; *Bd* zoospore equivalents per liter (ZSE) represents the mean from three laboratory replicates; ND = *Bd* not detected.

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Ranavirus results

Molecular analysis by qPCR indicated the presence of ranavirus in 105/185 (56.8%) of amphibians and in 3/4 shipments sampled (Tables 2 & 3). Individuals from all species tested positive for infection except *X. laevis*. Cumulative percentages of affected individuals were 76.9% in *B. orientalis*, 48.6% in *P. hongkongensis*, and 76.9% in *Cynops orientalis*. Although both ranavirus and *Bd* were concurrently detected in bags of amphibians in two shipments (Shipments 1 & 4; Table 3), co-infection was not observed in any individual animal sampled. Amphibians infected with ranavirus were detected in shipments from both Hong Kong exporters.

Table 3. Presence of *Bd* and *Ranavirus* within individual amphibian shipments imported from Hong Kong.

Species	Shipment	Date of Import	Exporter	#/Shipment	# *Bd*	*Bd*+	# RV	RV+	H_2O *Bd*+
Cynops orientalis	1	05/16/2012	A	500	36	0	35	35	N
Paramesotriton hongkongensis	1	05/16/2012	A	1600	36	0	36	35	Y
Xenopus laevis	2	06/06/2012	B	500	N/A	N/A	N/A	N/A	Y
Cynops orientalis	3	06/06/2012	A	500	36	0	18	0	N
Paramesotriton hongkongensis	3	06/06/2012	A	1600	36	0	18	0	Y
Bombina orientalis	4	09/26/2012	A	1000	56	3	13	10	N/A
Xenopus laevis	4	09/26/2012	A	1200	40	28	40	0	Y
Cynops orientalis	5	09/26/2012	B	200	25	0	25	25	N
				7100	265	31	185	105	

For each importation event, the number of animals present in the shipment, number of animals sampled (#*Bd*), number of those positive by PCR for infection (*Bd*+/RV+), and presence of pathogen in water (H_2O *Bd*+) are expressed. The letter A or B reflects which exporter supplied the shipment. Note that in Shipment 2, only results for water filtration are available and not swab results for individual animals.

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Discussion

This investigation establishes the first record of *Bd* and *Ranavirus* presence in amphibian trade in Hong Kong and demonstrates an opportunity for exposure to native amphibians. In addition, this is the first report to the authors' knowledge of ranavirus detection in the three species testing positive in this study. Unlike previous amphibian trade investigations in Southeast Asia [5,29], a relatively high proportion of Hong Kong's traded animals tested positive for both of these significant pathogens. Risk of pathogen spillover and potential establishment is elevated by the regions' high volume of domestic trade in species with known pathogen susceptibility and the likelihood to persist in the wild in a wide range of habitats if released or escaped, including the Chinese bullfrog (*Hoplobatrachus rugulosus*) and African clawed frog (*X. laevis*) [4,30,31].

The previous lack of *Bd* detection in the wild and in Hong Kong trade by Rowley et al. (2007) [16] is surprising, given the findings of this study and the long-term presence of international amphibian trade in the region. Indications of amphibian escape or release from the exotic pet trade into the wild date back as far as 1977, with Japanese red-bellied newts (*Cynops pyrrhogaster*) recorded from Sha Tau Kok in the New Territories [32]. Due to the narrow diversity of native amphibian species previously evaluated for *Bd* infection and sampling bias towards post-metamorphic specimens, it remains possible that amphibian pathogens have evaded detection. Previous surveys throughout Asia have generally demonstrated widespread *Bd* distribution at low prevalence, and have provided some evidence for the presence of an endemic Asian lineage of *Bd* [5,29,31,33]. Therefore, although the possibility of an historic introduction of *Bd* and long-term presence in Hong Kong cannot be fully disregarded, the absence of both prior detection and disease-suspected population declines suggests this phenomenon would be of low conservation concern relative to the contemporary importation of exotic disease strains that typically express higher virulence than strains considered endemic to the region [20,34].

The risk of establishment of highly virulent trade-associated strains of *Bd* and ranavirus in Hong Kong depends largely on the continued importation and domestic sale of diseased amphibians. An analysis of trade activity from 2005 2006 showed the importation of nearly 4.3 million live amphibians into Hong Kong, comprised of at least 45 species originating from 11 countries [16], nine of which have reported presence of *Bd* and/or ranavirus in wild or traded herptiles [20,35,36]. The majority of this trade volume involved bullfrogs (*H. rugulosus*) intended for human consumption in Hong Kong and originated in either Thailand or China, where these pathogens have been detected in farmed and free-ranging amphibians [19,15,20,37].

The risk of pathogen spillover from trade into the wild in Hong Kong is heightened by several factors additional to those mentioned above. First, each of these pathogens may cause mortality in the host with the absence of other clinical signs or lesions prior to death, which makes visual identification of illness difficult for traders. Only 21.3% of animals testing positive for *Bd* (1/31) or ranavirus (28/105) in this study were either dead on arrival (DOA) or had some form of visible lesion upon thorough inspection, demonstrating nearly 80% of animals carrying pathogens would have passed unnoticed. Specifically with respect to those DOA, most sampled for ranavirus were infected (15/19), whereas no DOA animals were positive for Bd (0/23). Furthermore, 23.5% of all animals testing negative for *Bd* (50/234) or *Ranavirus* (5/80) did display lesions or were DOA. Therefore, identification of potential signs of illness or disease does not accurately represent risk from these pathogens and cannot be used as an effective means for traders to exclude infected animals from commerce.

Second, infectious ranavirus and *Bd* particles released by affected animals into their environment can survive for extended periods of time outside the amphibian host; ranging from at least three to seven weeks respectively, and potentially longer under optimal conditions [8,38]. This prolonged persistence extends the window of opportunity for native amphibians to become exposed to infectious particles if untreated disposal of contaminated water were to occur. For this reason, the World Organization for Animal Health (OIE) suggests the disinfection of any water, containers, or other surfaces that had contact with amphibians prior to disposal [39]. As no such measures are enforced in Hong Kong, disease outbreaks may occur even without the escape or release of infected animals. It is unknown how often facilities in Hong Kong are disposing pathogen-contaminated water in a manner exposing local wildlife, but Gilbert et al. (2013) [5] found disposal directly into the environment to be common practice among all surveyed frog-farming facilities in Vietnam. Consequently, the abundance of aquatic amphibian species traded by Hong Kong (Table 1), prolonged environmental persistence of infectious ranavirus and *Bd* particles, and employment of trade activities that neither disinfect water nor safely dispose of deceased animals creates an ideal pathway for disease transmission to native Hong Kong amphibians.

The practice of disease surveillance within the international wildlife trade is a relatively low-cost and rapid technique to detect pathogen presence in any given country of interest that engages in trade of a potential host species. Once identified, opportunities for pathogen spillover from the trade sector and subsequent exposure to native species can be investigated and managed to control spread and prevent a potential outbreak. Although the data produced remains specific to that of traded amphibians and cannot be used to draw inferences about disease status in wild amphibians, results from trade surveys can provide invaluable information about the physical presence of a pathogen in a region of uncertain status before detection in wild populations, as we have demonstrated. If soon detected in native amphibians, it will be important to discern the presence of endemic pathogen strains from those introduced by traded amphibians and remain particularly vigilant if foreign sources are suspected, as both ranavirus and *Bd* associated with commercial trade often express greater virulence [20,34].

Data produced by this investigation provides guidance for the design of surveys to determine the pathogen status of future amphibian shipments. The consistently high prevalence of ranavirus detected by cloacal swabbing suggests that relatively little sampling effort was required to identify its presence in all affected species, whereas a smaller number of skin swabs for *Bd* detection may have resulted in the false-negative classification of *B. orientalis*. Filtration of the water carrying amphibians consistently provided greater sensitivity for the detection of *Bd* than skin swabs (i.e. detection in 3/4 shipments via filtering versus 1/4 via swabs), likely due to the collective sampling of *Bd* zoospores from a larger pool of animals than those individually sampled. It is important to note the possibility that the *P. hongkongensis* tested in this study may have been shipped in *Bd*-contaminated water and not themselves been infected, but this detail is irrelevant where primary survey intent is to detect pathogen presence in a shipment rather than prevalence. Still, it is surprising that all 72 *P. hongkongensis* tested negative for *Bd*, despite their immersion in Bd-positive water and the added potential for contamination caused by the water residue on each animal sampled. In summation, these data suggest an

efficient screening method to identify pathogen presence in high volume aquatic amphibian shipments needs only to focus swabbing efforts on ranavirus detection and filter a sample of water to detect *Bd*, if knowledge of prevalence is not required.

We have demonstrated the presence of *Bd* and ranavirus in Hong Kong's trade sector and show that the risk of spillover through contaminated wastewater is particularly high. Considering these and prior findings, a limited window of opportunity exists to protect the region's 24 species of native amphibians from trade-associated pathogen exposure and potential decline. Eradication of these pathogens from wild amphibian populations is not known to be possible following establishment, calling for greater vigilance and proactive surveillance in high-risk regions where they have yet to be detected. Control over the presence of ranavirus and *Bd* in Hong Kong, a major hub of international amphibian trade, would likewise benefit global efforts to reduce the dispersal of these devastating amphibian pathogens.

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Author Contributions

Conceived and designed the experiments: JEK KMS LFS LB. Performed the experiments: JEK. Analyzed the data: JEK. Wrote the paper: JEK KMS AP APP LB LFS WBK. Processed samples: AP APP.

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Paper 2

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Presence of the Amphibian Chytrid Fungus *Batrachochytrium dendrobatidis* in Native Amphibians Exported from Madagascar

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Abstract

The emerging infectious disease chytridiomycosis is driven by the spread of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*, *Bd*), a highly virulent pathogen threatening global amphibian biodiversity. Although pandemic in distribution, previous intensive field surveys have failed to detect *Bd* in Madagascar, a biodiversity hotspot home to hundreds of endemic amphibian species. Due to the presence of *Bd* in nearby continental Africa and the ecological crisis that can be expected following establishment in Madagascar, enhanced surveillance is imperative. I sampled 565 amphibians commercially exported from Madagascar for the presence of *Bd* upon importation to the USA, both to assist early detection efforts and demonstrate the conservation potential of wildlife trade disease surveillance. *Bd* was detected in three animals via quantitative PCR: a single *Heterixalus alboguttatus*, *Heterixalus betsileo*, and *Scaphiophryne spinosa*. This is the first time *Bd* has been confirmed in amphibians from Madagascar and presents an urgent call to action. Our early identification of pathogen presence prior to widespread infection provides the necessary tools and encouragement to catalyze a swift, targeted response to isolate and eradicate *Bd* from Madagascar. If implemented before establishment occurs, an otherwise likely catastrophic decline in amphibian biodiversity may be prevented.

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Introduction

Amphibian populations are experiencing global decline in response to a storm of assaults including habitat destruction, climate change, and the emerging infectious disease chytridiomycosis caused by amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*) [1 3]. *Bd* demonstrates low host species specificity and can potentially affect the entire class Amphibia, threatening the survival of thousands of amphibian species [4]. This pathogen can be highly lethal and easily transmissible through direct physical contact with affected individuals or indirectly by exposure to water contaminated with aquatic *Bd* zoospores [5]. Despite infection, certain species can act as reservoir hosts, allowing *Bd* to persist while driving others to extinction. This, together with prolonged environmental persistence provides an optimal situation for pathogen establishment and the collapse of amphibian diversity, especially in aquatic environments [6 8].

Bd was first identified and described nearly 15 years ago [9,10], by which time it had already spread to dozens of countries, potentially through the international trade in live amphibians [11 13]. Annually, millions of live amphibians are traded globally for the exotic pet trade, biomedical research, and human consumption and this movement of potentially infected animals may be a primary driving force of global *Bd* dispersal [13 15]. The transportation of *Bd*-contaminated environmental substrates and field equipment represent additional potential dispersal pathways [6,16], suggesting that common activities such as freshwater aquaculture and mining may also contribute towards the spread of *Bd* even in the absence of amphibian movement.

Although the spread of *Bd* has continued seemingly unabated for many decades, there remain hotspots of amphibian biodiversity where this devastating pathogen is not yet established and has been presumed absent due to the lack of confirmed field detection, most notably in Madagascar. The first expansive survey for the presence of *Bd* in Madagascar failed to detect this pathogen in 527 amphibians from 79 species sampled from 2005 2006 [17]. To complement this effort, a follow-up survey of 300 animals from 53 species at 12 additional locations were sampled in 2006 and 2007 [18], and a further 56 amphibians from 12 species were sampled in the country's central highlands [19]; all results similarly demonstrated the absence of *Bd* in amphibians sampled despite covering a range of host species and environments, and employing the most sensitive diagnostic tool, the *Bd*-specific quantitative PCR (qPCR) assay. It is remarkable that *Bd* is not already widespread in Madagascar because the country possesses high diversity of amphibians likely to be susceptible to chytridiomycosis, is in close proximity to regions of *Bd* presence in continental Africa (i.e. Tanzania, Malawi, South Africa), and provides high environmental suitability for *Bd* [20,21].

Thousands of amphibians are exported annually from Madagascar and disseminated globally into the exotic pet trade. An analysis of records obtained from the United States Fish and Wildlife Service (USFWS) through a Freedom of Information Act

(FOIA) request shows 39,020 amphibians were exported from Madagascar to the United States between 2006 and 2011, from at least 31 species. Although the international movement of amphibians is believed to help spread *Bd* and thus jeopardize global animal health, we considered access to traded animals a boon to our research goals: demonstration that trade can be approached as an efficient wildlife disease surveillance tool for the rapid detection of *Bd* in Madagascar. The commercial trade in amphibians generates large and diverse sample pools from which proactive surveillance can be performed with less human and financial resources than conventional field surveys. This investigation explored the presence of *Bd* in Madagascar by examining the contents of a shipment of wild-collected endemic amphibians exported directly to the USA.

Results

In total, 565 amphibians of nine species exported from Madagascar were sampled for *Bd* detection (Table 1). *Bd* was detected in three of 565 animals and each displayed measureable amounts of *Bd* in at least two qPCR replicates, from at least two separate plates. The three species positive for *Bd* were *Scaphiophryne spinosa* (MGSS30), *Heterixalus alboguttatus* (MGHA54), and *Heterixalus betsileo* (MGHB42) (Table 1). Prior to its final qPCR with purified DNA, MGSS30 was tested in two separate qPCR plates and in each, one replicate came up positive; the zoospore loads were 0.332 and 0.040, respectively. When the purified DNA was run a final time, all three replicates of MGSS30 were negative for *Bd*. MGHA54 was tested in two plates prior to DNA purification and again, one replicate per plate came up positive for *Bd*, with zoospore loads of 0.189 and 0.089. After DNA purification, one replicate was again positive; its reported zoospore load was 0.400. MGHB42 was also tested in two plates prior to purification and unlike the other two samples, all six replicates were positive for *Bd*; the average zoospore load for the first plate was 0.395 and the average zoospore load in the second plate was 0.219. After DNA purification, all three MGHB42 replicates were again positive for *Bd*, reporting a mean value of 1.059 zoospores.

Examination of amphibians sampled for *Bd* collectively revealed ulcerations (1.8%), heavy skin sloughing (3.4%), and death on arrival (6.9%) in 68/565 animals (Table 1). Because not all deceased amphibians were sampled for *Bd*, the total number of DOA animals was greater with respect to the entire shipment (n = 99; 15.8%). No such conditions were observed in any of the three *Bd*-positive amphibians at the time of sampling.

Discussion

The presence of *Bd* has been confirmed in Malagasy amphibians for the first time. These amphibians were collected from the wild for the pet trade, exported to the USA and sampled immediately upon arrival. One sample produced a strong signal for *Bd* presence (MGHB42), and two others displayed weak indications: MGSS30 and MGHA54. Despite the low intensities, these two samples certainly displayed positive signals and, most important from separate plates, suggesting the signals were real and not due to contamination from the positive controls. It is not uncommon for the standard controls to contaminate a single replicate, but to do so across multiple plates, has never been witnessed and is unlikely. After DNA purification, all three MGSS30 replicates were negative for *Bd*. This is perplexing and could suggest that the original DNA aliquot used in the first two plates (prior to purification) was contaminated. However, it is also possible that the particular aliquot of DNA used in the final run did not actually contain *Bd* DNA, although it existed in the sample; MGSS30's measured zoospore loads were incredibly low and give some credence to this possibility. MGHA54, like MGSS30, similarly never had all replicates within a single plate turn up positive. Its zoospore load was similarly low, again suggesting that *Bd* DNA similarly might not have been present in each replicate. Because a single replicate was positive from three different plates, including the final qPCR using purified DNA, these data do suggest MGHA54 was positive for *Bd*. Although it is difficult to discern the truth about MGSS30 and MGHA54, because separate plates yielded positive replicates and contamination was unlikely, I report these samples as *Bd*-positive. Regardless, all nine replicates of MGHB42 were positive for *Bd*, undeniably confirming its presence in material from Madagascar.

The status of *Bd* in wild amphibian populations in Madagascar remains uncertain and calls for urgent targeted field surveys in regions where these *Bd*-positive animals were likely collected. The human-assisted movement of traded animals introduces an opportunity for *Bd* cross-contamination between species and collection origins prior to exportation if animals are housed in shared enclosures where direct or indirect contact is allowed. Accordingly, transmission of *Bd* between Malagasy species from

Table 1. Amphibians from Madagascar sampled for the presence of *Batrachochytrium dendrobatidis* (*Bd*).

Species	No. Sampled	Ulcerations	Sloughing	DOA	*Bd*+	Reference #	ZSE
Boophis pyrrhus	58	4	-	11	-		-
Boophis rappiodes	39	3	-	1	-		-
Boophis microtympanum	65	1	17	18	-		-
Heterixalus alboguttatus	78	-	-	1	1	MGHA54	0.089–0.400
Heterixalus betsileo	86	1	-	3	1	MGHB42	0.219–1.059
Dyscophus guineti	70	1	2	-	-		-
Scaphiophryne boribory	31	-	-	5	-		-
Scaphiophryne madagascariensis	69	-	-	-	-		-
Scaphiophryne spinosa	69	-	-	-	1	MGSS30	0.040–0.332
	565	10	19	39	3		

Conditions potentially indicative of chytridiomycosis were recorded at the time of sampling, including skin ulcerations, sloughing, and death on arrival (DOA). Number of *Bd*-positive samples (*Bd*+) is reported followed by the sample's reference number and range in average zoospore equivalents (ZSE) per run, detected by qPCR.
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different collection localities may potentially exaggerate the number of affected species in wild populations and suggested distributional range of infection.. Furthermore, identifying the source of *Bd* detected in traded animals becomes especially challenging when animals from multiple countries are also present in the trade sector. Fortunately, this is not the case in Madagascar; commercial amphibian importation does not occur in the country [17] and only those of national origin are traded. The absence of foreign-sourced amphibian species suggests my detection of *Bd* is not simply an artifact of re-exportation through the amphibian trade, but instead a reflection of *Bd* presence in the wild in Madagascar. Still, other non-amphibian wildlife trade activities may unknowingly introduce foreign infectious material to Madagascar and expose wild-collected frogs prior to exportation if housed at a shared facility (i.e. exposure to *Bd*-contaminated water accompanying freshwater fish importations). Albeit unlikely the result of such cross contamination, I employed a conservative approach by interpreting these data as confirmation of *Bd* presence in Madagascar within the amphibian trade, but not yet irrefutable evidence for *Bd* presence in wild amphibian populations, despite the strong suggestion.

A second, more specific tier of surveillance via targeted field sampling applying this new information, is now imperative to determine the current extent of *Bd* in Madagascar outside the trade sector. A predictive model of *Bd* distribution [20] shows that the highest climatic suitability for *Bd* overlaps particularly closely with the distributional range of *H. betsileo*, from which MGHB42 was collected. Interestingly, the distributions of *H. albuguttatus* and *S. spinosa* fall on the periphery of this climatic range and may have been collected from areas with moderate to low *Bd* suitability, potentially explaining their exceptionally low *Bd* zoospore loads compared to that detected in the specimen of *H. betsileo*. Accordingly, surveys to trace back the source of the *Bd* detected herein should commence immediately within the distributional ranges of *H. betsileo*, *H. alboguttatus*, and *S. spinosa*, target the larvae and subadults expected to exhibit increased susceptibility to infection, and include bioregions suitable for *Bd* [22] to maximize the chances of rapidly detecting *Bd* if currently present in wild populations.

The lack of *Bd* detection in previous field surveys of wild amphibians [17–19] and these newly reported *Bd*-positive animals suggest the presence of *Bd* in Madagascar is a recent phenomenon and not yet widespread. *Bd*-related die-offs have not been documented and infection prevalence is expected to still be extremely low in wild populations, if currently affected. The conditions necessary to result in *Bd* establishment in amphibian populations following exposure are poorly understood. Infection with as little as one *Bd* zoospore can result in chytridiomycosis [5], and affected amphibians have been observed to release 68 infectious *Bd* particles per minute when in an aquatic environment [23]. Therefore, the detection of *Bd* in *H. betsileo* and *H. alboguttatus* is especially concerning because these species breed in both permanent and temporary water bodies and an outbreak in wild populations may both promote extended environmental persistence and facilitate indirect transmission to nearby aquatic species, increasing the opportunity for pathogen establishment. The spread of *Bd* can occur rapidly following introduction to a naive region, estimated as much as 25–282 km/y [24], and the data presented herein provides impetus to quickly reevaluate the presence of *Bd* in Madagascar.

The confirmation of *Bd* in amphibians exported from Madagascar presents an opportunity to intervene prior to the first confirmed outbreak in wild populations – an outbreak with potentially irreparable ecological consequences. It is no longer questionable whether or not *Bd* will become introduced to Madagascar; it is now a tangible threat. Survival of the country's amphibians now requires an efficient network of proactive surveillance and rapid response to quickly identify additional introduction events and minimize exposure to wild populations [25], because pathogen eradication is considered implausible following establishment. The provenance of *Bd* detected in this investigation remains an enigma, especially considering the absence of commercial amphibian trade into Madagascar, suggesting a more insidious mechanism is responsible for the introduction. Accordingly, *Bd* may continue to arrive in Madagascar and creep closer towards establishment until the true introduction pathway is identified, targeted and controlled. Early detection now provides the opportunity to interrupt pathogen establishment, but if not acted upon with haste, disease-associated ecological decline in Madagascar may soon become inescapable.

Figure 1. One of two crates of amphibians sampled for *Batrachochytrium dendrobatidis* upon arrival from Madagascar. Amphibians were shipped sealed in wooden crates, insulated with 1/4" Styrofoam, and packed in plastic containers filled with damp sphagnum moss and leaves.
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Materials and Methods

Ethics

Amphibians were imported under United States Fish and Wildlife Service (USFWS) License No. LE65317A-0 and accompanied by a cleared USFWS Declaration for Importation of Wildlife (Form 3-177). None of the species included in this investigation are currently protected or endangered and therefore, no additional special permits were necessary. Permission to export the amphibians was granted by the Government of Madagascar with permit #'s 017/12-MEF/SG/DREF.ATS/EXPORT and 018/11-MEF/SG/DREF.ATS.

Amphibian Sampling

In February 2012, a shipment containing 565 wild-collected amphibians from Madagascar was exported and sampled for the presence of *Bd* upon importation to the United States. Of 17 endemic amphibian species commercially available from this particular supplier, nine were systematically selected for sampling to represent a potentially wide coverage of biogeographical regions and altitudinal ranges where *Bd* was expected to thrive if present. These decisions were made by comparing species distribution maps provided by the IUCN Red List of Threatened Species [26] with work that identified a predicted region of optimal *Bd* survival based on climatic suitability [20]. Sampling priority was accordingly directed towards species with distributions that overlap this

high risk zone, in addition to those vulnerable to *Bd* exposure based on life history characteristics, most importantly association with aquatic habitats [22].

Following regulatory clearance for importation into the USA, the shipment was collected from the airport and immediately transported to a small greenhouse specifically constructed to provide a controlled area for receipt and sampling of these animals. This structure had no previous exposure to amphibians and all interior surfaces were first washed with a 10% bleach solution to minimize any potential risk of domestic *Bd* contamination. The shipment remained sealed for the duration of transport from Madagascar to the USA, and was not opened until first secured inside this sampling location to further prevent opportunities for contamination. All contents of the shipment were handled exclusively with fresh pairs of Nitrile gloves.

Amphibians arrived inside two wooden crates insulated with 1/4″ Styrofoam (Fig. 1). Within these crates, the amphibians were packed in plastic containers filled with damp sphagnum moss and leaves as bedding material. Some containers housed multiple amphibians whereas others were packed individually; this varied by species and size of amphibian, but containers housing multiple individuals did not combine species. All amphibians were adults, with the exception of *S. spinosa*, for which only subadult frogs were received. Upon opening the crates, containers were arranged by species and the contents of each examined and sampled for the presence of *Bd*. A sterile fine-tipped rayon swab (Medical Wire & Equipment Co., MW113) was drawn across each amphibian's hands, feet, and pelvic patch five times each. Samples were stored in 2 mL vials filled with 1 mL 70% ethanol as preservative. To prevent cross-contamination between samples, fresh pairs of Nitrile gloves were changed each time a new amphibian was handled.

Each animal was examined immediately prior to swabbing and its condition recorded. Potential clinical symptoms of chytridiomycosis were noted, including the presence of skin ulcerations, skin sloughing, and death [27]. Most specimens of the nine target species were sampled, except for dead animals that arrived in advanced stages of decomposition (n = 60), which were excluded from this investigation. All live amphibians were swabbed individually. When multiple dead animals arrived in the same container, a single swab was used to sample all carcasses; this maneuver increases cost-efficiency of analysis, resulting in fewer swabs (n = 551) than the total number of animals actually sampled (n = 565). Following the prompt completion of sample collection for this investigation, all live amphibians were transferred back into the course of the domestic pet trade.

Molecular Analysis

Thirty swabs deemed as high priority, those most suspect of *Bd* infection based on physical examination, were first immediately shipped to the San Diego Zoo Amphibian Disease Laboratory for testing. Samples were processed via a sensitive quantitative PCR assay (qPCR) specific to *Bd* following standard methods [28,29]. Assays were run on an Applied Biosystems 7900HT thermocycler using 384 well plates with Applied Biosystems exogenous internal positive control labeled with Vic in separate wells to test for the presence of PCR inhibitors. For each sample, 5 µl of 1:10 dilution of swab DNA was added to each well for a final total qPCR volume of 20 µl. Standard curves were generated with 10-fold serial dilutions (range 10,000 to 0.001 zoospores) of laboratory cultivated *Bd* zoospores.

The remaining 521 swabs were also processed via qPCR according to established protocols [29 31] at Yale University. Samples were extracted with 150 µl Prepman Ultra (Applied Biosystems, California, USA), with a final 30 µl of supernatant removed for downstream use. An aliquot of this supernatant was diluted 1:10 in DNase-free water for qPCR. The qPCR protocol used SensiMix II Low Rox (Bioline, Massachusetts, USA) as the qPCR master mix [32]. Samples and controls were run in triplicate with three positive, standard control samples (100, 10, and 1 zoospore/well, made from JAM81 pure culture; see Boyle et al. 2004 for standard control construction) and one non-template control (DNase free, molecular-grade water). When the qPCR assay failed to detect *Bd* in all replicate wells, the sample was deemed negative for *Bd*. When one of three replicates successfully detected *Bd*, the sample was rerun (in triplicate again) in a subsequent plate. For rerun samples that had at least a total of two of six replicates positive for *Bd* (from at least two separate plates) or samples that had *Bd* in all replicates, the original DNA supernatant stock was purified and tested, at full-strength, in a final qPCR. Full-strength DNA from PrepMan Ultra inhibits qPCR, so this DNA must be cleaned-up or diluted prior to use [30]. The remaining full-strength DNA was purified using Performa DTR Gel Filtration Cartridges (Edge Biosystems, Maryland, USA). The cartridges were loaded and prepared by spinning at 750× g for two minutes. The remaining DNA was added to each column, loaded directly onto the gel matrix, and then spun for two minutes at 750× g. Five microliters of this eluted, purified, full-strength DNA was loaded into three replicate wells in a final qPCR (i.e., this DNA was not further diluted prior to qPCR). All zoospore loads described in this report have not been converted; here, reported zoospore loads come from 5 µl DNA (1:10 or full-strength), placed into 20 µl reaction volumes.

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Author Contributions

Conceived and designed the experiments: JEK. Performed the experiments: JEK. Analyzed the data: JEK. Wrote the paper: JEK.

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Paper 3

Citation: Kolby JE, Smith KM, Skerratt LF, Berger L. Presence of *Batrachochytrium dendrobatidis* and Ranavirus in Bullfrogs Imported to the United States. (Not yet published)

Abstract

Amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) and ranaviruses are globally emerging pathogens contributing towards amphibian declines and extinctions. The original catalyst of their global emergence and timeline of international dispersal remain poorly understood, but the contemporary trade in live amphibians appears to play a considerable role in recent spread. Each year, approximately 5 million live frogs are imported into the USA alone, of which nearly 50% are American bullfrogs (*Lithobates catesbeianus*). This species is native to the USA, but commonly farmed overseas to supply the USA demand for consumption of frog legs. Unfortunately, because this species can serve as a reservoir host of *Bd* and ranavirus, it can spread disease to new regions. The purpose of this investigation was to gauge the level of *Bd* and ranavirus imported by the international bullfrog trade and evaluate the risk of pathogen dispersal and spill-over by this activity. I sampled 4 shipments of bullfrogs immediately upon importation to the US and found that most animals were carrying these pathogens. Some shipments had exceptionally high infection prevalence of *Bd* (91.2%) and ranavirus (100%). In addition, shipping cartons (62/105) and water rinse samples (4/5) tested positive for *Bd* in shipments where *Bd*-positive animals were also detected, demonstrating additional opportunities for pathogen spill-over by the untreated disposal of contaminated shipping materials. In 2008, the World Organisation of Animal Health listed infection with *Bd* and ranavirus as globally notifiable diseases, but few countries responded with formal disease monitoring and control measures to reduce continued global spread. My data show that the spread of *Bd* and ranavirus through the international bullfrog trade is still a frequent phenomenon. It is likely that the risk of spread of currently unknown pathogens is also high given this lack of biosecurity. Regulatory action to mitigate pathogen dispersal is urgently needed to protect global amphibian biodiversity.

Introduction

The global wildlife trade is a common avenue of pathogen dispersal that facilitates the spread of animal diseases (McLean 2007; Schloegel et al. 2009; Kolby et al. 2014). To protect economies and reduce financial loss, most efforts to understand and mitigate this threat are directed towards controlling pathogens recognized as zoonotic and a threat to human health or those that negatively impact agriculture (Daszak et al. 2000, Grogan et al. 2014). Meanwhile, other pathogens that primarily threaten wildlife receive less attention such as the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) and ranavirus. Both of these pathogens are responsible for emerging infectious disease and contributing towards global amphibian declines (Berger et al. 1998; Lips et al. 2008; Skerratt et al. 2007). Many factors combine to catalyze global biodiversity decline, including habitat loss, pollution, and overexploitation, but the spread of disease is an increasingly common threat. *Bd* has now caused the decline or extinction of approximately 200 amphibian species, a global phenomenon said to earmark the Earth's "sixth mass extinction" (Skerratt et al. 2007; Stuart et al. 2004; Wake and Vredenburg 2008).

The American bullfrog (*Lithobates catesbeianus*) is likely the most commonly traded amphibian globally. Of nearly 5 million live amphibians imported into the USA annually, half of these are bullfrogs for the frog leg trade (Schloegel et al. 2009). This species is tolerant of infection with *Bd* and ranavirus and commonly persists with infection without disease, allowing these animals to serve as reservoir hosts (Daszak et al. 2004; Hanselmann et al. 2004; Schloegel et al. 2010a). This species is also invasive outside of its native range in the eastern USA and Canada and has established feral populations following release or escape in dozens of jurisdictions around the world (Kraus 2009). These characteristics make bullfrogs well-suited to act as vehicles of pathogen dispersal and establishment following introduction.

Bullfrogs sampled from domestic markets in the USA showed high prevalence of *Bd* (41-62%) and low prevalence of ranavirus (8%) (Schloegel et al. 2009; 2012), suggesting that the annual trade in millions of these animals may transport a considerable number of infected animals. However, these frogs were tested at markets in the USA where domestic exposure and cross contamination from adjacent containers could have occurred prior to sampling. The potential for changed infection status between importation and arrival at markets reduces certainty in the results regarding the origin of detected pathogens and the estimation of infection prevalence at the point of importation. Due to the relevance of these data in the development of trade regulations to control disease spread, irrefutable evidence of pathogen importation to the USA is needed to justify intervention. Accordingly, the purpose of this investigation was to evaluate the presence and prevalence of *Bd* and ranavirus in bullfrogs imported into the USA, prior to any opportunity for post-arrival contamination.

Methods

Ethics

This study was approved by Tufts University's Institutional Animal Care and Use Committee (Permit #G2010-85). Amphibian samples were collected upon importation with permission from the United States Fish and Wildlife Service.

Sample Collection

Bullfrogs were sampled immediately upon arrival in the USA, when unloaded from the airplane or cargo ship and remained sealed until sampling began. In total, four shipments were sampled in this study, three from the Dominican Republic transported by airplane, and one from

Taiwan transported by ocean cargo ship. Shipments arrived between 14 March 2012 and 12 December 2013. The three shipments from the Dominican Republic were exported by the same company. Live frogs transported by airplane were maintained at ambient temperature while in-transit for approximately 24 hours whereas those transported by ocean cargo ship were held inside a refrigerated container for approximately 3 weeks at 6°C. Shipments originated either from the Dominican Republic or Taiwan. Frogs arrived inside plastic netted bags, with approximately 25 frogs per bag, and either one bag per sealed cardboard box (Dominican Republic) (Figure 1) or open plastic crate (Taiwan) (Figure 2). All boxes from the Dominican Republic included a 5 cm-thick pad of wet foam to maintain humidity inside the box. At the time of sampling, records were made of the importation date, approximate total number of specimens present per shipment, type of shipping method and container type, and whether amphibians had escaped from their shipping containers during transport.

In order to detect a minimum 10% infection prevalence within each shipment (with a 95% detection probability), I approximated random sampling of 35 frogs per sampling event (Thrusfield 2005). One frog per bag was sampled to distribute the sampling effort throughout the shipment. In each bag, a frog near the center of the group was randomly selected for sampling, although upon approach frogs often moved erratically, adding to the randomization of sample collection. When sampling was complete, the frog was immediately placed back into its bag and resealed in the shipment.

Each time a shipment was examined, amphibians were first sampled for *Bd* using sterile fine-tipped rayon swabs. The underside of the hands, feet, and pelvic patch were each swabbed five times (Hyatt et al. 2007) and the swab bud was snapped off into a dry 2 mL microcentrifuge tube. Ranavirus sampling was then performed on the same animal by gently inserting a sterile

fine-tipped rayon swab into the cloaca and twirling several times (Gray et al. 2012). The swab bud was then snapped off into a 2 mL microcentrifuge tube containing 0.5 mL Nuclisens solution. Fresh pairs of Nitrile gloves were worn and changed between each animal sampled.

Swabs of the shipping boxes arriving from the Dominican Republic were collected to detect the presence of *Bd* contamination on transport materials. The interior surfaces of these boxes often accumulated a slurry of urine, feces, and skin sloughs on the foam insert (Figure 3). Immediately after a frog was swabbed, the bag of bullfrogs was momentarily lifted, and a rayon swab was drawn across the interior surface of the foam and carton, 20 times each, with approximately 10 cm-long strokes. Swab buds were snapped off into dry 2 mL microcentrifuge tubes.

Water samples were collected from two of four shipments (one each from the Dominican Republic and Taiwan) to investigate whether *Bd* was commonly shed from these frogs into their surroundings. Because frogs were shipped moist, but not in water, direct extraction of a water sample was not possible. Instead, a bag of bullfrogs was placed inside a large plastic bag and a 500 mL bottle of spring water was poured over them. This water was allowed to drip through the bullfrogs' netted bag for 60 seconds, and was collected and filtered following protocols established by Kirshtein et al. (2007). To capture any *Bd* particles present, this water was drawn into a sterile 60 mL syringe and manually pumped through a 0.22-micron Sterivex filter capsule. When the filter became nearly clogged with debris, 50 mL phosphate buffered saline was pumped through the capsule to rinse the filter, and the capsule was pumped dry. A sterile 1 mL syringe was used to add 0.9 mL Qiagen ATL lysis buffer, and then the filter capsule was sealed and stored for subsequent qPCR analysis. This process of water rinse and filtration was performed on every 7th box of frogs swabbed from that shipment, creating a series of five

samples. The water rinse was performed after the *Bd* and ranavirus swab was first collected from that box of frogs.

Although not transported internationally in water, these bullfrogs are often transferred into buckets of water when offered for sale at domestic food markets following importation (Figure 4). Because both *Bd* and ranavirus can survive without the presence of a host animal for extended periods, this water may remain infectious to amphibians for several weeks (Johnson and Speare 2003; Nazir et al. 2012). If discarded untreated into city storm drains after frogs are sold, contaminated material could be sent directly into nearby waterways where native amphibians may become exposed to these foreign pathogens (Figure 5).

Sample Analysis

Taqman PCR for *Bd* was based on the method, primers and probe of Boyle et al. (2004) following methods described in Kolby et al. (2014). DNA was extracted from swabs with Prepman Ultra® (Applied Biosystems). Water filter samples were processed for *Bd* detection following the method of Kirshtein et al. (2007), also described in Kolby et al. (2014). All *Bd* samples were run in triplicate with an exogenous internal positive control to detect PCR inhibition. Samples that amplified at a Ct≥50 were considered negative. Samples amplifying at a Ct of <50 in 2 or more wells were considered positive whereas those which produced a positive reaction in only one of three wells were reported as negative as recommended by Hyatt et al. (2007) and Skerratt et al. (2011) in order to maximize test specificity.

Taqman PCR for ranavirus used primers, probes, and protocols of Pallister et al. (2007) following the method described in Kolby et al. (2014). DNA was extracted from swabs using DNeasy Blood and Tissue Kits (QIAGEN Inc.) with spin columns. Samples amplifying at Ct's

of <50 in 2 or more wells were considered positive whereas those which produced a positive reaction in only one of three wells were reported as negative.

Results

Batrachochytrium dendrobatidis (Bd) Swab Results

Analysis of skin swabs by qPCR indicated the presence of *Bd* in 43.8% (60/137) of imported bullfrogs tested (Table 1). Frogs in all three shipments from the Dominican Republic were positive for *Bd* (58.8%, 60/102), whereas none from Taiwan were positive (0/35). The prevalence of *Bd* in affected shipments varied between sampling events and ranged from 22.9% - 91.2%. For those same shipments, *Bd* was detected on 62/105 (59.0%) of the boxes carrying bullfrogs, and from all three Dominican Republic shipments. Similarly, the proportion of boxes in each shipment that tested positive for *Bd* varied, and ranged from 25.7% - 88.6%.

Ranavirus results

Analysis by qPCR indicated ranavirus was present in all four shipments sampled and in a total of 111/140 (79.3%) of amphibians (Table 1). Prevalence of ranavirus detected by swabbing animals varied between shipments and ranged from 40.0% -100.0%. Both ranavirus and *Bd* were concurrently detected in 3/4 bullfrog shipments, and co-infection was observed in 55 animals sampled. Among those *Bd*-positive animals, 91.7% also tested positive for ranavirus (55/60), whereas only 49.5% (55/111) frogs positive for ranavirus also tested positive for *Bd*. Although the plastic crates carrying frogs from Taiwan were not tested for pathogen contamination, a slurry of urine and feces was observed dripping from all containers, and likely contained ranavirus shed from infected animals (Figure 7).

Batrachochytrium dendrobatidis (Bd) Water Filter Results

Filtered water rinse samples collected from two shipments of bullfrogs tested positive for *Bd* via qPCR in 4/10 samples (Table 2). All *Bd*-positive samples were derived from a single shipment imported from the Dominican Republic on 23 August 2012 where 4/5 boxes of bullfrogs tested positive. Of these same boxes, only 1/5 tested positive by amphibian skin swab alone, but 3/5 tested positive by box swab. All 5 water samples collected from the shipment from Taiwan tested negative for *Bd*, as did all amphibians swabbed from that shipment.

Animal Escape

In 2/4 shipments from the Dominican Republic, animals escaped through holes in the plastic netted bags during transport and leapt out when the lid was opened (Figure 6). On one occasion, I was alerted by airport personnel that several bullfrogs had already escaped from damaged boxes during the outside transfer of frogs from the plane's cargo hold into the cargo warehouse.

Discussion

This investigation provides the first direct evidence of *Bd* and *ranavirus* presence in commercial shipments of bullfrogs directly upon importation into the USA. The prevalence of *Bd* and ranavirus-positive frogs detected in these shipments was alarmingly high even though some of my sampling methods and data interpretation were conservative and likely to have underestimated the true prevalence. Swabbing for ranavirus has low sensitivity and can underestimate the incidence of ranaviral infection by as much as 20% compared to lethal methods (e.g. molecular analysis of liver samples) (Gray et al. 2012). Additionally, *Bd* and ranavirus qPCR samples which produced "equivocal" results (1/3 positive wells when run in

triplicate) were scored as negative, although some will have been legitimate weak-positive results. Therefore, the already high prevalence of *Bd* (23 - 91.2%) and ranavirus (40 - 100%) we detected via these methods clearly demonstrates the movement of significant numbers of pathogen-positive animals by the bullfrog trade.

Data produced by this investigation provides guidance for the design of surveys to determine pathogen status of future bullfrog shipments. Methods other than swabbing individual animals appear to have been similarly effective at detecting the presence of *Bd* (and possibly ranavirus). Specifically, the water rinse and filtration method identified *Bd* presence in 4/5 boxes of frogs from which the conventional skin swab method produced *Bd*-positive results in only 1/5 of those same boxes. Water rinses collectively pool the *Bd* zoospores and pieces of infected shed skin released from many animals in the box, thus forming a sample potentially representative of multiple animals and increasing the chance of pathogen detection. This method does not require direct animal handling and is also quicker to perform than swabbing individual frogs. Swabbing the interior surface of shipping cartons that carry these bullfrogs was similarly quick, minimally invasive, and likewise pooled material from many animals. This method also commonly detected *Bd* presence (62/105 cartons). *Bd* surveillance via water filtration or swabbing boxes offers considerable benefits when the goal of surveillance is to confirm pathogen presence (but not prevalence) in large commercial shipments of amphibians. Unfortunately, the common occurrence of *Bd* in shipping materials shown by these data also illustrates additional opportunities for pathogen spread if shipping materials are re-used and a high risk of pathogen spill-over when untreated shipping materials are disposed.

Amphibians housed in high densities for extended periods are likely to experience elevated rates of pathogen transmission, especially for pathogens highly transmissible through skin

contact such as *Bd* and ranavirus. In our study, pathogen prevalence was exceptionally high in most shipments, with *Bd* and ranavirus prevalence reaching 91.2% and 100%, respectively. This is not surprising, given that 78.5% of bullfrogs collectively sampled at five frog farms in Brazil tested positive for *Bd* infection (Schloegel et al. 2010a) as well as 41-62% of those tested at markets in the USA (Schloegel et al. 2009; 2012). Further, exposure to multiple pathogens simultaneously is likely to affect susceptibility, although the dynamics of *Bd*/ranavirus co-infection are still poorly understood and consistent patterns have not yet been identified (Warne et al. 2015). In the current investigation, 88.7% of bullfrogs which tested positive for *Bd* also tested positive for ranavirus (55/62), whereas only 49.5% of 111 frogs positive for ranavirus also tested positive for *Bd*. Although the relationship between *Bd* and ranaviral infection remains unclear, these data suggest that within the shipments I sampled, ranavirus was more readily transmissible than *Bd* and that infection with *Bd* might have increased the bullfrogs' susceptibility to ranaviral infection. Still, due to the possibility of DNA contamination within these crowded shipments and the absence of histological sampling, it is not possible to distinguish co-infection from co-occurrence with certainty.

Many factors are likely to influence the presence of *Bd* and ranavirus in bullfrog shipments. Potential sources of pathogen introduction to bullfrog farms include the use of infected breeding stock, contaminated water supplies, and exposure to infected native amphibians if farms are open to the outdoors. The abundance of these pathogens within bullfrog farms will be affected by husbandry conditions, as pathogen survival decreases with elevated air and water temperatures and reduced humidity (Berger et al. 2004; Granoff et al. 1966; Johnson and Speare 2005). The stocking density of frogs will impact the frequency and duration of direct skin contact between animals and thus influence rates of pathogen transmission. Factors during transport that will

further affect the number of pathogen-positive animals include the number of frogs packed together in direct contact, the air temperature, and duration of shipping. Many of these conditions are not required to be recorded by the parties trading these amphibian shipments, and consequently their disease risks cannot be predicted.

In 2008, infection with *Bd* and ranavirus were both listed as globally notifiable aquatic animal diseases by the World Organization of Animal Health (OIE) and guidelines to mitigate their spread were provided (Schloegel et al. 2010b). Specific recommendations for the trade in bullfrogs for consumption were that frogs should be immediately transported to a processing facility for slaughter and/or into quarantine upon import and that all wastewater, containers/enclosures, and other in-contact surfaces be disinfected (OIE 2016 a, b). Unfortunately, few if any countries have formally adopted the OIE recommendations to reduce amphibian pathogen dispersal and the risk of spillover remains particularly high. Our investigation has shown that the trade in live American bullfrogs remains a frequent pathway of global *Bd* and ranavirus dispersal; of the 35,075 bullfrogs collectively imported in the four shipments sampled herein, our data suggest approximately 6,713 (19.1%) may have introduced *Bd* into the USA and 24,252 (69.1%) may have introduced ranavirus. Although these pathogens already occur in the US, the introduction of different strains of *Bd* and different species of ranavirus continue to pose additional risk (Farrer et al. 2012; Schloegel et al. 2012; Brunner et al. 2015). Control over the presence of ranavirus and *Bd* in the bullfrog trade is urgently needed to mitigate the threat of disease to global amphibian biodiversity.

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Figure 1. American bullfrogs *Lithobates catesbeianus* exported from the Dominican Republic inside plastic netted bags, within cardboard cartons.

Figure 2. American bullfrogs *Lithobates catesbeianus* exported from Taiwan inside plastic netted bags, within open plastic crates.

Figure 3. Interior surfaces of American bullfrogs *Lithobates catesbeianus* boxes were commonly soiled with urine, feces, and skin sloughs.

Figure 4. Imported American bullfrogs *Lithobates catesbeianus* held in water at market.

Figure 5. Roadside storm sewer grate explaining that discarded material empties into nearby waterways where native aquatic life can be impacted.

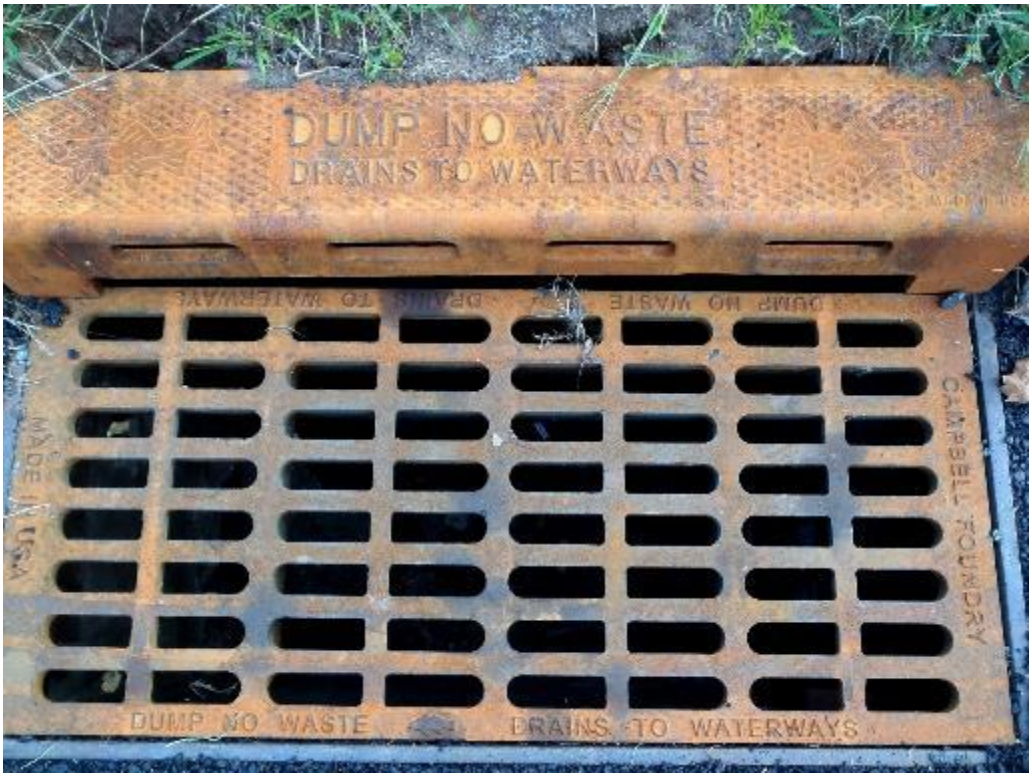

Figure 6. American bullfrogs *Lithobates catesbeianus* commonly escaped through holes in the plastic netted bags during transport from Dominican Republic. This illustrates the high risk of escape when cartons are opened upon delivery at domestic food markets. Escaped frogs are marked by red circles.

Figure 7. The plastic crates carrying American bullfrogs *Lithobates catesbeianus* from Taiwan dripped a slurry of potentially ranavirus-contaminated urine and feces onto surfaces where containers were placed.

Table 1. *Batrachochytrium dendrobatidis* (*Bd)* and *ranavirus* (RV) presence in shipments of American bullfrogs *Lithobates catesbeianus* imported into the USA. Individual bullfrog shipments sampled for *Bd* and ranavirus, including frog country of origin, total number of frogs in the shipment (Qty), number of pathogen-positive samples/ total number collected, and apparent pathogen prevalence with 95% confidence limits in parentheses, assuming Bd qPCR sensitivity and specificity as per Skerratt et al. (2011) of. 729 and .942, respectively and assuming RV qPCR sensitivity of. 80 as per Gray et al. (2012) and absolute specificity (1.0).

Shipment	Import Date	Origin	Qty	Swab *Bd*+	Swab Prev	Box *Bd*+	Box Prev	Swab RV+	Swab RV Prev
1	14 Mar 2012	Dominican Republic	3325	21/33	0.636 (0.466-0.778)	22/35	0.629 (0.463-0.768)	29/35	0.829 (0.673-0.919)
2	30 Mar 2012	Dominican Republic	4470	31/34	0.912 (0.770-0.970)	31/35	0.886 (0.740-0.955)	33/35	.943 (0.814-0.984)
3	23 Aug 2012	Dominican Republic	2280	8/35	0.229 (0.121-0.390)	9/35	0.257 (0.142-0.421)	35/35	.100 (0.901-1.00)
4	12 Dec 2013	Taiwan	25000	0/35	0 (0-0.099)	N/A	N/A	14/35	0.400 (0.256-0.564)
TOTAL			37075	60/137	0.438 (0.358-0.522)	62/105	0.590 (0.495-0.680)	111/140	0.793 (0.718-0.852)

Table 2. Presence of *Batrachochytrium dendrobatidis* (*Bd)* in water filters samples collected from two shipments of American bullfrogs *Lithobates catesbeianus* imported into the USA. Bags of bullfrogs rinsed to collect *Bd* particles, including frog country of origin, volume of water filtered, and the detection of *Bd* in either the water rinse, frog skin swab, or box swab, each collected from the same box.

Shipment	Box#	Import Date	Origin	Vol (mL)	*Bd*+ water	*Bd*+ swab	*Bd*+ box
A	1	8/23/2012	Dominican Republic	82	N	N	N
A	2	8/23/2012	Dominican Republic	49	Y	N	Y
A	3	8/23/2012	Dominican Republic	40	Y	Y	Y
A	4	8/23/2012	Dominican Republic	35	Y	N	N
A	5	8/23/2012	Dominican Republic	36	Y	N	Y
	Subtotal				(4/5)	(1/5)	(3/5)
B	1	12/18/2013	Taiwan	13	N	N	N/A
B	2	12/18/2013	Taiwan	21	N	N	N/A
B	3	12/18/2013	Taiwan	13	N	N	N/A
B	4	12/18/2013	Taiwan	14	N	N	N/A
B	5	12/18/2013	Taiwan	28	N	N	N/A
	Subtotal				0/5	0/5	N/A
	Field Blank	N/A	N/A	500	N	N/A	N/A

CHAPTER 3

Amphibian pathogen presence in the absence of commercial amphibian importation

Introduction

The results from Chapter 2 clearly show that amphibian pathogens are transported through the international wildlife trade, and at levels higher than previously confirmed. Nonetheless, regions devoid of amphibian importation still experience exposure and have likewise suffered recent outbreaks of chytridiomycosis.

In particular, the results from my Madagascar study in Chapter 2 suggested the likely presence of an unidentified *Bd* introduction pathway and these data were especially alarming with respect to amphibian conservation if *Bd* had been a recent introduction. Whether these data represented recent pathogen introduction to Madagascar or some sort of false-positive result (either trade contamination prior to export or diagnostic specificity below 100%) called for urgent ground-truth validation. I designed and performed a rapid response field surveillance project to confirm its presence and investigate the source and potential mode of *Bd* introduction to Madagascar. My study included sites, species, and life stages not previously included in Madagascar's National Monitoring Program for early *Bd* detection and also employed multiple sensitive sampling methods to increase my power of detection. Collectively, I tested 508 animals and 68 water samples for *Bd*, from 47 sites which spanned the island nearly 1000 km north to south. No *Bd* was detected, although ranavirus was found in a subsample of amphibians tested. This study is **Paper 1** of this chapter: "Rapid Response to Evaluate the Presence of Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) and Ranavirus in Wild Amphibian Populations in Madagascar," which has been published in PLoS ONE.

Although I previously detected *Bd* in the trade, I did not detect its presence in the field. Interestingly, just as **Paper 1** was about to be published, another research group reported widespread *Bd* detection in wild frogs in Madagascar (Bletz et al. 2015). This group included *Bd*-positive material found up to five years earlier, but not previously reported. The nature and context of *Bd* field data expression and interpretation can greatly affect perceived patterns of *Bd* distribution and modes of dispersal, so I combined all current *Bd* field survey results and re-examined the evidence. This study is **Paper 2** of this chapter: "Amphibian Chytrid Fungus in Madagascar neither Shows Widespread Presence nor Signs of Certain Establishment," which has been published in PLoS ONE.

Similalrly, Bletz et al. published a response to my work, titled "Consistency of published results on the pathogen Batrachochytrium dendrobatidis in Madagascar: Formal comment on Kolby et al. Rapid response to evaluate the presence of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) and ranavirus in wild amphibian populations in Madagascar". I did not formally mention this response by Bletz et al. in my thesis because this response did not specifically address my main points of concern outlined in Kolby & Skerratt 2015. The response by Bletz et al. primarily argued the consistency of results within the context of Bd presence versus absence in Madagascar on a country-wide scale. I do not disagree that we have both produced molecular evidence suggesting the presence of Bd in frogs from Madagascar. In contrast, my main concern was the problematic inconsistency in sampling results described by Bletz et al. from the same sites upon subsequent sampling using varying field and lab methods. These inconsistencies were not resolved in the response provided by Bletz et al.

As discussed in **Papers 1 & 2**, the detection of likely *Bd* presence in Madagascar brings with it more questions than answers. Regardless of the true presence, distribution, and strain of *Bd* currently in Madagascar, one thing is certain – an ongoing invasion of Asian common toads (*Duttaphrynus melanostictus*) provides a possible source of *Bd* to the country. Amidst my search for potential pathways of *Bd* introduction, I identified these toads in Toamasina, the country's ocean cargo port town. It is believed that these toads are arriving as stowaways inside ocean shipping containers from Southeast Asia. Whether or not these toads were the original source of *Bd* and ranavirus to Madagascar, they illustrate how the spread of invasive species and pathogens can occur in the absence of biosecurity. I issued an international call to arms to mount an eradication effort and increase biosecurity. This is **Paper 3** of this chapter: "Ecology: Stop Madagascar's toad invasion now," which has been published in Nature.

Paper 1

Citation: Kolby JE, Smith KM, Ramirez SD, Rabemananjara F, Pessier AP, Brunner JL, Goldberg CS, Berger L, Skerratt LF. (2015) Rapid Response to Evaluate the Presence of Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) and Ranavirus in Wild Amphibian Populations in Madagascar. PLoS ONE 10(5):e0125330.

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RESEARCH ARTICLE

Rapid Response to Evaluate the Presence of Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) and Ranavirus in Wild Amphibian Populations in Madagascar

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Abstract

We performed a rapid response investigation to evaluate the presence and distribution of amphibian pathogens in Madagascar following our identification of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*, *Bd*) and ranavirus in commercially exported amphibians. This targeted risk-based field surveillance program was conducted from February to April 2014 encompassing 12 regions and 47 survey sites. We simultaneously collected amphibian and environmental samples to increase survey sensitivity and performed sampling both in wilderness areas and commercial amphibian trade facilities. *Bd* was not detected in any of 508 amphibian skin swabs or 68 water filter samples, suggesting pathogen prevalence was below 0.8%, with 95% confidence during our visit. Ranavirus was detected in 5 of 97 amphibians, including one adult *Mantidactylus cowanii* and three unidentified larvae from Ranomafana National Park, and one adult *Mantidactylus mocquardi* from Ankaratra. Ranavirus was also detected in water samples collected from two commercial amphibian export facilities. We also provide the first report of an amphibian mass-mortality event observed in wild amphibians in Madagascar. Although neither *Bd* nor ranavirus appeared widespread in Madagascar during this investigation, additional health surveys are required to disentangle potential seasonal variations in pathogen abundance and detectability from actual changes in pathogen distribution and rates of spread. Accordingly, our results should be conservatively interpreted until a comparable survey effort during winter months has been performed. It is imperative that biosecurity practices be immediately adopted to limit the unintentional increased spread of disease through the movement of contaminated

Competing Interests: The authors have declared that no competing interests exist.

equipment or direct disposal of contaminated material from wildlife trade facilities. The presence of potentially introduced strains of ranaviruses suggests that Madagascar's reptile species might also be threatened by disease. Standardized population monitoring of key amphibian and reptile species should be established with urgency to enable early detection of potential impacts of disease emergence in this global biodiversity hotspot.

Introduction

Global amphibian biodiversity is threatened by multiple factors including the emerging infectious diseases caused by the spread of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*, *Bd*) and ranaviruses [1–4]. Madagascar possesses a wealth of endemic amphibian biodiversity, with 292 species described and over 500 believed to exist [5]. Many species are currently jeopardized by habitat destruction, exploitation for the pet trade, and climate change, and fortunately *Bd* and ranavirus have not previously been officially identified in the nation's wild amphibian populations nor have enigmatic amphibian declines been reported [6,7]. The apparent absence of *Bd* and ranavirus in Madagascar is remarkable, as these pathogens have already been detected in dozens of countries globally, including those nearby in eastern mainland Africa, and their continued spread appears certain [8–10]. Furthermore, Madagascar possesses high amphibian species richness and the climatic suitability expected to allow both pathogens to thrive [11].

Nearly a decade of field surveys for *Bd* in Madagascar have failed to produce confirmed positive results [6,12,13], suggesting either pathogen absence, severely limited *Bd* distribution, or the presence of a highly divergent *Bd* lineage that fails to react in current diagnostic protocols. Nearly a thousand amphibians comprised of dozens of species were sampled in these previous efforts, and presuming pathogen absence, a National Monitoring Plan (NMP) was recently designed and implemented to detect the arrival of *Bd* [14]. This proactive NMP called for long-term biannual surveys at eight nationally distributed sites, targeting three predicted "indicator" species at each location to serve as *Bd* sentinels. The three targeted species vary by site, but collectively includes four species of *Mantidactylus*, five species of *Heterixalus*, *Mantella cowanii* and *Ptychadena mascareniensis*. These species were selected for their widespread distribution and/or abundance and their susceptibility to *Bd* infection; at least one species per site (*M. betsilianus*, *H. betsileo*, *P. mascareniensis*) was shown to be susceptible to *Bd* in laboratory exposure trials [14]. Monitoring efforts have thus far spanned several field seasons and Weldon et al. (2013) suggested cooperation between national and international efforts would further assist early detection.

Meanwhile, the threat of ranaviruses to the biodiversity of Madagascar has attracted little attention despite their potential to drive long-term amphibian declines [3,4] and for some strains to cause disease in both amphibians and reptiles [15,16]. While some ranaviruses might be endemic to Madagascar and express relatively minimal virulence to native wildlife, targeted field surveillance efforts to evaluate ranavirus presence in the country is lacking. Due to the potentially severe disease-associated declines following introduction of non-native pathogens, we herein employ a precautionary approach and assume historical absence of both *Bd* and ranavirus from Madagascar in the absence data that may suggest otherwise.

To assist pathogen detection efforts in Madagascar, we recently sampled a commercial shipment of live amphibians exported to the USA for both *Bd* and ranavirus presence. *Bd* was detected on 3 (0.5%) of 565 wild-collected frogs upon removal from their shipping container

[17], for the first time suggesting *Bd* presence in Madagascar following an unconfirmed report in 2010 [18]. We also detected ranavirus in these exported amphibians (18 of 29 sampled), likewise demonstrating its presence in the country. Unfortunately, it was uncertain whether these pathogens originated in wild amphibian populations in Madagascar because non-Malagasy contamination within the export facility could have occurred if foreign material had previously been imported. Despite the ambiguous origin, these data provided the first confirmation of *Bd* and ranavirus presence in Madagascar and raised our concern that recent pathogen introductions may have occurred. We quickly orchestrated and performed a highly targeted surveillance project that applied multiple techniques with greater collective sensitivity than the current NMP in order to produce a snapshot of national *Bd* and ranavirus distribution.

Materials and Methods

Ethics

All amphibian handling and sample collection methods employed in this investigation were approved by the Malagasy Direction Generale Des Forets and Madagascar National Parks as part of an emergency rapid response program. This work was performed under research permit #048/14/MEF/SG/DGF/DCB.SAP/SCB provided by the Malagasy Direction de la Biodiversite et du Systeme des Aires Protegees and export permit #'s 080N-EA04/MG14 and 151c_EA04/MG14 issued by the Malagasy Ministere de l'Environment et des Forets.

Study sites and survey design

Field surveys were performed in Madagascar from 12 February to 4 April 2014. We applied a risk-based approach to the selection of survey localities and determination of which amphibian species and individuals to sample [19,20]. Regions targeted for sampling included species with predicted likelihood of pathogen exposure or susceptibility, locations in proximity to wildlife trade centers where elevated transmission rates and pathogen spillover may occur, and areas with predicted optimal environmental conditions and climatic suitability for *Bd* survival (e.g. cool wet habitats at high altitudes) [11]. Amphibians species prioritized for sampling were those described to have prolonged exposure to permanent aquatic habitats [21]. A greater diversity of water-associated species were targeted than those included by the current NMP, as susceptibility to pathogens differs between species [22,23]. Likewise, susceptibility to infection and disease varies with ontogeny, and is often higher earlier in life [24–26], so we also included pre-metamorphic animals not previously sampled by NMP activities.

Our survey efforts were performed during Madagascar's warm rainy season when most amphibian species are active, breeding, and can be readily encountered in high abundance near water bodies. Although elevated temperatures threaten the survival of both *Bd* [27] and ranavirus [28], we specifically targeted cooler habitats where conditions favored pathogen survival. Further, we believed that the frequent physical contact and water exposure during this breeding season might increase rates of transmission, the amount of time pathogens can be shed into the water, the number of amphibians that could be easily located to create a large sample pool, and hence increase detectability. Environmental conditions were measured at each survey site at the time of sampling, including water and air temperature, pH, and relative humidity.

Adult, juvenile, and larval amphibians were sampled. Tadpoles were collected by dip net and sampled by day whereas adult and juvenile frogs were primarily captured by hand, both day and night. Sampling was performed in locations where it appeared we could collect adequate numbers of amphibians (>30) in a single session to increase detection likelihood and statistical power. Throughout this investigation, we visually examined all sampled amphibians for skin lesions or other abnormalities and remained vigilant for amphibian mortality events.

Amphibian Swabbing

Nearly all amphibians included in this investigation were sampled for both *Bd* and ranavirus, although some were sampled only for *Bd*. Upon capture, each amphibian was first sampled for *Bd* with a sterile fine-tipped rayon swab (Medical Wire & Equipment Co. #MW113) following Hyatt et al. (2007) [29]. For adult and juvenile amphibians, the swab bud was drawn across the ventral surface of the animals' hands, feet and pelvic patch five times each. For larval amphibians, swab buds were twirled in the buccal cavity as per Retallick et al. (2006) [30]. All swab buds were snapped off into 2 mL vials containing 1 mL 70% ethanol. A fresh pair of Nitrile gloves was worn for each new amphibian handled and changed between every sample to prevent cross contamination.

Amphibians were then sampled for ranavirus following non-lethal methods [15,31]. Cloacal swabs were collected from adults by inserting sterile rayon-tipped swabs (Puritan Medical Products #P25-800R) into the cloaca and gently twirling the handle several times. For small adults, juveniles, and tadpoles, buccal swabs were collected by inserting the swab into the oral cavity and twirling. Swab buds were cut off into 2 mL vials containing 1mL 70% ethanol. Scissors were decontaminated by flaming for 5 seconds between samples. All animals were released upon completion of sampling.

It was not possible to identify tadpoles to species with certainty. Instead, oral structures and body morphology were examined and used to categorize the approximate number of species sampled per location. Variable environmental conditions (e.g. water temperature, available nutrition, disease presence), and/or injury, may be associated with deformities in a tadpole's keratinized oral structures [32,33], potentially introducing some uncertainty into our species categorizations. As prevalence of *Bd* differs between tadpole species [19,34], efforts were made to sample a diversity of species in their larval stage to improve the chance of detection.

Tadpole water

Prior to being swabbed, tadpoles at 16 sites were held aside in a 650 mL bowl of local river water for an hour to allow for the release of disease particles. This water was then collected for filtration and all tadpoles were released, except for the subsample that was then swabbed for *Bd* and ranavirus. The number of tadpoles present in each water sample and approximate number of species was recorded upon release. We performed this method to increase *Bd* and ranavirus detection probability by collecting and concentrating the pathogens off a greater number of animals than could be individually sampled.

A similar process was performed on one occasion with live crayfish (*Procambarus* spp.) in lieu of tadpoles, since *Bd* has been found within crayfish gastrointestinal tracts and these animals might serve as *Bd* reservoir hosts [35]. Approximately 500 crayfish collected by local fishermen at a site in Antananarivo were held in a large bucket, to which we added water from their habitat, and then allowed them to soak for 15 minutes. A sample of this water was then collected and filtered.

Water Filtration

Water was sampled to detect the environmental presence of *Bd* and ranavirus following methods described by Goldberg et al. (2011) [36]. This method of sampling can capture microbial material either present independently in aquatic suspension or embedded in affected amphibian tissue cells shed into the water (environmental DNA, or "eDNA"). At aquatic survey sites, three 500 mL bottles of water were each collected approximately 20 m apart and then combined into one 1500 mL bottle before processing. Samples were collected near the top of the water column, between 5 and 10 cm below the surface. At commercial trade facilities where

only one water bowl was present per enclosure housing a single species, single water collections were sampled, but where multiple species were housed communally, water was collected and combined from multiple enclosures to increase sampling efficiency. Water temperature and pH were measured with a portable handheld meter (Hannah Instruments #HI98128) at the middle of each sample locality.

Water was passed through 0.45μm Metricel (mixed cellulose esters) membrane filters held within sterile disposable filter funnels (Pall Corporation #4815) using a vacuum hand pump connected to a 1 L vacuum flask. In some instances, a battery-operated peristaltic pump was used in place of the vacuum hand pump when working with higher sample volumes, although the two methods seemed to be similarly effective and efficient. Water was processed until the filter paper became nearly clogged with debris and the flow significantly diminished, or until the total volume collected was filtered. The volume of water filtered was recorded and the filter membrane was removed with sterile forceps, folded inwards three times, and placed into a 50 mL sample tube. Each tube had a small hole in the cap covered with a pad of sterile gauze to allow air exchange, and was stored in a zip-top bag of silica gel beads to remove remaining moisture from the sample. Forceps were soaked in full-strength commercial bleach (6% sodium hypochlorite) to denature any contaminant DNA [37] and rinsed with filtered bottled spring water between samples. Water bottles used to collect the samples were also sterilized with this bleach solution and flushed with native water at each site three times immediately prior to sampling. This wastewater was discarded on land away from the water body. A fresh pair of Nitrile gloves was worn to handle each water filter sample. We sometimes processed multiple samples at a site to ensure adequate total volumes were filtered if rapid clogging of the filter membranes occurred. Water and amphibians were both concurrently sampled from the same habitat when possible, but water samples were always collected first before the team entered the water in order to prevent potential contamination and siltation of the sample.

Field biosecurity

Biosecurity measures were strictly enforced throughout this investigation to prevent accidental spread of amphibian pathogens both between amphibians and locations [38]. Fresh pairs of Nitrile gloves and plastic bags used to hold frogs when sampling were each used only once and discarded. A bleach solution (10% commercial bleach) was used to rinse all materials exposed to amphibians or environmental substrates prior to leaving each study site (e.g. dip nets, water filtration equipment, footwear). Field boots were thoroughly scrubbed to remove all sediment prior to disinfection with this bleach solution.

Sample Analysis

***Bd* swabs.** Taqman PCR for *Bd* was based on the method, primers and probe of Boyle et al. 2004 [39]. The DNA template was prepared with Prepman Ultra (Applied Biosystems) and extractions were diluted 1:10. Reactions used the Taqman Environmental Mastermix 2.0 (Applied Biosystems). Samples were run in triplicate on an Applied Biosystems 7900HT thermocycler using 384 well plates with an exogenous internal positive control labeled with VIC (Applied Biosystems) for each sample to detect PCR inhibitors. Samples that amplified at a $Ct \geq 50$ and those without amplification in any of the wells were scored as negative. Quantification standards were created by growing *Bd* isolate JEL 197 on 1% tryptone agar and harvested of zoospores by rinsing plates with 1X PBS. After collection, zoospores were counted three times on a hemocytometer to determine a range of zoospores ml^{-1}. Standard curves were generated with ten-fold serial dilutions (range 1×10^{6} to 1×10^{-2} zoospores). In addition to positive controls (quantification standards), each plate included a negative control (Taqman mastermix

and no sample DNA) as well as 4 positive and negative quality assurance controls consisting of swabs either inoculated with *Bd* zoospores or sham-inoculated.

Ranavirus swabs. Swabs were first inverted in tube so that the bud was above the ethanol and then centrifuged at 13000 rpm for 5 minutes to pellet all of the suspended material. The ethanol was carefully removed with a pipette and then 100 μL of Prepman Ultra (Applied Biosystems) was added to the swab. Samples were then centrifuged again for 2 min, the swab removed, and then incubated at 100°C for 15 min according to manufacturer's instructions. The sample was centrifuged again for 3 min and 20 μL of the supernatant containing the DNA was moved to a new sterile 1.5 mL snap cap tube and frozen until it could be screened.

The concentration of extracted DNA was measured using a NanoDrop-2000 (Thermo-Scientific) and, if necessary, diluted to approximately 20 ng DNA/μL. Extracted DNA from each sample was run full-strength and screened for ranavirus in triplicate 20 μL reactions on 96-well plates with 5 μL of DNA template (~100 ng) using a Taqman realtime polymerase chain reaction (qPCR) with primers and probe that amplify a 70-bp region within the major capsid protein of all known ranaviruses [40]. A 10-fold serial dilution of DNA extracted from a Frog Virus 3-like ranavirus grown in *Epithilium papilloma cyprinia* cells from 10^2 to 10^7 plaque-forming units (pfu's) was used as a standard against which unknown samples were quantified. Samples with amplification in two or three wells were scored as positive. Those without amplification in any of the wells were scored as negative. Ambiguous samples were re-run and if at least one well showed amplification in the second run, the sample was scored as positive. We used an Exogenous Internal Positive Control (Exo IPC, Applied Biosystems) in the third well of each sample to detect PCR inhibition of DNA, potentially caused by matter from the environment or the PrepMan Ultra (Applied Biosystems). If inhibition was detected the sample was diluted 1:10 and re-run. Viral quantities for positive samples are reported as the mean of the $\log_{10}$ (pfu) across all wells of the sample (i.e. including any zeros).

Water filters for *Bd* and ranavirus. We extracted DNA from filters using the QIAshredder/DNeasy Blood and Tissue DNA extraction kit method described in Goldberg et al. (2011) [36], in a room where no high-quality DNA extracts or PCR products had been handled and where researchers were required to shower and change clothing before entering if they had previously been in a room with PCR product. An extraction negative was created with each set of extractions and negative and positive PCR controls were included in each plate. All samples were run in triplicate. We tested for *Bd* using the assay of Boyle et al. (2004) [39] with a 6FAM-labeled probe and for ranavirus using the assay of Picco et al. (2007) [40], as above but with a NED-labeled probe, in a multiplex reaction. As part of multiplex validation, three known positive tissue samples for each pathogen were quantified in this reaction singly and in combination; Cq values were within 0.5 for each sample. Reactions were run using Quantitect Multiplex PCR Mix (Qiagen, Inc.) with recommended multiplexing concentrations (1X QuantiTect Multiplex PCR mix, 0.2 μM of each primer, and 0.2 μM of each probe) on an Applied Biosystems 7500 Fast Real-Time PCR System. Reactions were 15 μL in volume and each included 3 μL of sample. Cycling began with 15 min at 95°C followed by 50 cycles of 94°C for 60 s and 62°C for 60 s and went for 50 cycles. All reactions included an internal positive control (IC; Qiagen, Inc.). Samples showing inhibition (>3 Ct difference compared with the negative controls) were processed through a OneStep PCR Inhibitor Removal Kit (Zymo Research Corp.) and rerun in triplicate. Samples that tested positive in <3 wells in the first run were rerun in triplicate; if at least one well tested positive in each plate on the second run, that sample was considered positive.

Results

Field Surveys

We sampled a total of 508 amphibians for pathogen detection via swabbing, including 483 free-ranging amphibians and 25 wild-collected amphibians sampled at a commercial wildlife export facility in Antananarivo (Table 1). Metamorphs and adults of 37 species were sampled.

Table 1. Site summary and diversity of amphibians sampled for the presence of *Batrachochytrium dendrobatidis* (*Bd*) and ranavirus in Madagascar.

Region	Site	Desc.	Lat(S)	Long (E)	Altitude (m)	A	W	Species	T
Andasibe	1	River	18.935	48.413	952	23.6	19.2	*Anodonthyla boulengeri Boophis luteus Boophis madagascariensis Heterixalus betileo Mantidactylus betsileanus Mantidactylus femoralis Mantidactylus grandidieri Mantidactylus melanopleura Spinomantis aglavei*	0
Andasibe	2	Pond	18.933	48.413	936	N/A	N/A	*Mantidactylus betsileanus Ptychadena mascareniensis*	0
Andringitra	1	River	22.144	46.888	1729	23.1	18.5	*Mantidactylus spp.*	1
Andringitra	2	River	22.162	46.895	2050	21.4	20.5	*Mantidactylus spp. Ptychadena mascareniensis*	2
Andringitra	3	River	22.130	46.866	2111	20.5	23.9	*Boophis microtympanum Mantidactylus spp.*	1
Andringitra	4	River	22.153	46.900	1968	N/A	N/A	*Boophis goudoti Boophis microtympanum*	0
Ankarafantsika	1	Pond	N/A	N/A	N/A	31.4	28.4	*Laliostoma labrosum Ptychadena mascareniensis*	1
Ankarafantsika	2	River	16.326	46.857	125	30.7	25.9	*Mantidactylus spp. Ptychadena mascareniensis Stumpffia spp.*	0
Ankarafantsika	3	Rice Paddy	16.343	46.848	92	31.8	31.5	*Boophis spp. Ptychadena mascareniensis*	1
Ankaratra	1	River	19.333	47.263	2384	12.4	13.4	*Boophis williamsi Mantidactylus curtus Mantidactylus spp.*	1
Ankaratra	2	River	19.349	47.279	2032	13.3	13.9	*Mantidactylus curtus Mantidactylus mocquardi Mantidactylus spp.*	1
Ankaratra	3	River	19.346	47.279	2015	15.9	14.3	*Mantidactylus mocquardi Mantidactylus pauliani*	3
Antananarivo	1	Rice Paddy	18.861	47.435	1249	30.2	25.6	*Ptychadena mascareniensis*	0
Antananarivo*	2	Trade Facility	18.785	47.463	1286	N/A	N/A	*Dyscophus guineti Heterixalus madagascariensis Scaphiophryne madagascariensis*	0
Isalo	1	River	N/A	N/A	N/A	28.3	24.1	*Mantidactylus spp. Ptychadena mascareniensis*	0
Isalo	2	River	22.628	45.359	801	32.8	25.2	*Mantidactylus spp. Ptychadena mascareniensis*	0
Isalo	3	River	22.645	45.332	792	34.2	26.5	*Blommersia spp. Mantidactylus spp. Ptychadena mascareniensis*	1
Ranomafana	1	River	N/A	N/A	N/A	21.4	19.8	Unknown	2
Ranomafana	2	River	21.254	47.421	932	24.4	19.1	*Mantidactylus betsileanus Mantidactylus mocquardi*	2
Ranomafana	3	River	21.269	47.425	992	22.1	20.0	*Mantidactylus betsileanus Mantidactylus cowanii Mantidactylus majori Mantidactylus melanopleura*	3
Ranomafana	4	River	21.291	47.426	1052	23.1	19.5	*Mantidactylus cowanii Mantidactylus femoralis Mantidactylus majori*	2
Ranomafana	5	River	21.291	47.426	1053	22.3	19.6	*Mantidactylus cowanii Mantidactylus grandidieri Mantidactylus majori Mantidactylus spp.*	3
Toamasina	1	Pond	18.149	49.375	10	24.3	26.2	*Bufo melanostictus Hoplobatrachus tigerinus*	0
Zahamena	1	River	17.513	48.726	1072	20.1	19.2	*Mantidactylus sp.*	0
Zahamena	2	River	17.500	48.734	1202	18.8	17.7	*Boophis boehmi Gephyromantis moseri Mantidactylus albofrenatus Mantidactylus charlotteae Plethodontohyla notostica*	1
Zahamena	3	River	17.508	48.731	1068	21.0	18.2	*Boophis liami Boophis picturatus Mantella nigricans Mantidactylus charlotteae Mantidactylus femoralis Mantidactylus grandidieri Mantidactylus lugubris Mantidactylus mocquardi*	1

Air (A) and water (W) temperatures measured at the sample site in degrees Celsius. Approximate number of species of larval amphibians (T) included in the sample at that location.

*Amphibians sampled in Antananarivo but reported to have been collected from the wild in Fierenana.

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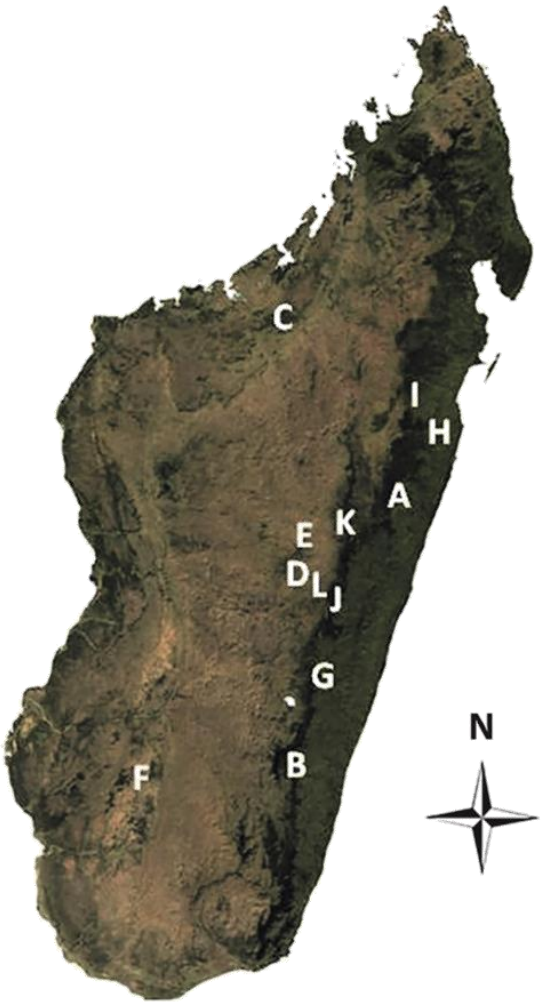

Fig 1. Locations sampled for the presence of *Bd* and ranavirus in Madagascar. Ambatolampy (A), Analamay (B), Andasibe (C), Andringitra National Park (D), Ankarafantsika (E), Ankaratra (F), Antananarivo (G), Faravohitra (H), Isalo (I), Ranomafana National Park (J), Toamasina (K), Zahamena National Park (L). The base map was obtained from www.maplibrary.org. GPS coordinates were used to identify locations on Google Earth (Google Inc., 2013) and edited onto the base map with Adobe PhotoShop CS6 (Adobe, 2012).

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Field identification of some adult animals was only possible to genus and we conservatively grouped these animals together into a single genus-level record in our results. The number of species of larvae we sampled is not known due to challenges with identification. Therefore, a greater diversity of amphibian species may have been included in this survey than expressed. All amphibian species sampled are endemic to Madagascar with the exception of the country's two exotic introduced species, *Hoplobatrachus tigerinus* and *Duttaphrynus melanostictus*.

Twelve regions were surveyed, spanning approximately 750 km from Ankarafantsika in the North to Isalo in the South, and eastward along the country's rainforest belt (Fig 1). Between one and five separate water bodies were sampled in each region of intensive sampling, with

three sampled in most instances. In total, we sampled 45 water bodies via water filtration. Volumes processed per filter ranged from 75 mL to 6000 mL, but most samples were highly turbid and clogged the filters at lower volumes (≤500 mL). In total, 66.30 L of environmental water and 4.25 L of tadpole water were filtered.

Bd Swabs

All 508 amphibians swabbed for *Bd* detection tested negative for the presence of *Bd* by qPCR and showed no obvious signs of disease (Table 2), apart from two tadpoles with severe oral deformities.

Ranavirus Swabs

Of the 508 amphibians sampled for *Bd*, 499 were also swabbed for ranavirus. Due to current funding limitations a subset of 97 ranavirus samples were prioritized for analysis. Five of these 97 samples tested positive for the presence of ranavirus (prevalence = 5.2%; 95% CI: 2.2–11.5%). Four positive amphibians were sampled from three separate water bodies in Ranomafana National Park; one adult *Mantidactylus cowanii* (cloacal swab) and three unidentified larvae (buccal swabs). The other positive sample was from an adult *Mantidactylus mocquardi* from Ankaratra (Table 3). The titers from these positive swabs were quite low (range: 2.9–12.6 pfu equivalents) and none of the animals showed clinical symptoms of infection when sampled.

Water Filters

Ranavirus was detected in the water bowls at two locations where wild-collected amphibians were temporarily held in captivity pending commercial exportation: one trade facility in Toamasina and the other in Antananarivo (Table 4). In the Toamasina facility, five amphibian enclosures were sampled; each held multiple amphibian species of the genera *Boophis*, *Heterixalus* and *Mantella* collected from unknown localities. Samples from enclosures that shared a screened wall were combined (i.e. 1 & 2, 3 & 4), producing a total of three samples. Two of three samples tested positive for ranavirus (i.e. 3 & 4 and 5). The water introduced to these enclosures was pumped on-site from an underground well. Samples of this well water tested negative for both ranavirus and *Bd*, suggesting ranavirus was introduced by infected wild-collected frogs rather than contaminated groundwater. This facility was the source of frogs previously found to be infected with *Bd* [17] and ranavirus, but very few frogs were present in these enclosures at the time of this water collection and were not included in our sampling. In Antananarivo, water from one of three enclosures, which held only tomato frogs (*Dyscophus guineti*), tested positive. Ranavirus was not detected in the other two enclosures sampled at this facility, one of which held *Heterixalus madagascariensis* and the other *Scaphiophryne madagascariensis*. The operator of this facility reported to JEK that all amphibians had been collected from Fierenana, near Moramanga. The source of water in these bowls was said to be rainwater collected onsite.

All water samples collected from natural amphibian habitats tested negative for the presence of both *Bd* and ranavirus by qPCR, although five samples from three locations demonstrated too much PCR inhibition for reliable testing, even after inhibitor removal. Environmental conditions at sampled locations fell mostly within the range suitable for *Bd* and ranavirus survival and reproduction, with air temperatures ranging from 12.4 to 34.2 C, averaging 23.3 C, and water temperatures from 13.4 to 32.0 C, averaging 21.5 C. Relative humidity was often high (average 78.3%) and water pH fluctuated little, ranging from 6.51 to 7.79, averaging 7.12. Only 3 of 45 water bodies displayed aquatic temperatures ≥ 30.0 C

Table 2. Detection of *Batrachochytrium dendrobatidis* (*Bd*) in Madagascar by quantitative PCR (qPCR) and apparent prevalence including 95% confidence limits (CL) at each location as per swab results, assuming *Bd* qPCR sensitivity and specificity as per Skerratt et al. (2011) of. 729 and. 942, respectively.

Region	Site	Prevalence (95% CI)	Water	No. Sampled	No. *Bd*+	#Spp.	Tadpole/ Adult	#Tad
Andasibe	1	0 (0–0.184)	-	17	0	9	A	0
Andasibe	2	0 (0–0.194)	-	16	0	2	A	0
Andasibe Total		**0 (0–0.104)**	-	**33**	**0**	**10**	**A**	**0**
Andringitra	1	0 (0–0.138)	-	24	0	1	T+A	15
Andringitra	2	0 (0–0.114)	-	30	0	2	T+A	15
Andringitra	3	0 (0–0.133)	-	25	0	2	T+A	17
Andringitra	4	0 (0–0.259)	-	11	0	2	A	0
Andringitra Total		**0 (0–0.041)**	-	**90**	**0**	**4**	**T+A**	**47**
Ankarafantsika	1	0 (0–0.155)	-	21	0	2	T+A	15
Ankarafantsika	2	0 (0–0.278)	-	10	0	3	A	0
Ankarafantsika	3	0 (0–0.138)	-	24	0	2	T+A	14
Ankarafantsika Total		**0 (0–0.065)**	-	**55**	**0**	**5**	**T+A**	**29**
Ankaratra	1	0 (0–0.242)	-	12	0	3	T+A	6
Ankaratra	2	0 (0–0.133)	-	25	0	3	T+A	6
Ankaratra	3	0 (0–0.114)	-	30	0	2	T+A	15
Ankaratra Total		**0 (0–0.054)**	-	**67**	**0**	**5**	**T+A**	**27**
Antananarivo*	1	0 (0–0.278)	-	25	0	1	A	0
Antananarivo	2	0 (0–0.133)	-	10	0	3	A	0
Antananarivo Total		**0 (0–0.099)**	-	**35**	**0**	**4**	**A**	**0**
Isalo	1	0 (0–0.354)	-	7	0	2	A	0
Isalo	2	0 (0 –0.299)	-	9	0	2	A	0
Isalo	3	0 (0–0.114)	-	30	0	3	T+A	8
Isalo Total		**0 (0–0.077)**	-	**46**	**0**	**3**	**T+A**	**8**
Ranomafana	1	0 (0–0.278)	-	10	0	1	T	10
Ranomafana	2	0 (0–0.143)	-	23	0	2	T+A	20
Ranomafana	3	0 (0–0.107)	-	32	0	4	T+A	20
Ranomafana	4	0 (0–0.161)	-	20	0	3	T+A	10
Ranomafana	5	0 (0–0.138)	-	24	0	4	T+A	12
Ranomafana Total		**0 (0–0.034)**	-	**109**	**0**	**8**	**T+A**	**72**
Toamasina	1	0 (0 –0.299)	-	9	0	2	A	0
Toamasina Total		**0 (0–0.299)**	-	**9**	**0**	**2**	**A**	**0**
Zahamena	1	0 (0–0.793)	-	1	0	1	A	0
Zahamena	2	0 (0–0.104)	-	33	0	5	T+A	21
Zahamena	3	0 (0–0.114)	-	30	0	8	T+A	13
Zahamena Total		**0 (0–0.057)**	-	**64**	**0**	**13**	**T+A**	**34**
National Total		**0 (0–0.008)**	-	**508**	**0**	**37**	**T+A**	**217**

Number of animals sampled, number of species represented, whether tadpoles (T) or post-metamorphic (A) animals were included, and number of tadpoles (#Tad) in the sample are reflected. Water filter results for *Bd* detection also presented. For sites where all amphibian swabs and water samples together tested negative for *Bd*, true prevalence is likely closer to the lower CL. Cumulative data for all sites sampled within the region appear in bold.
*Amphibians sampled in Antananarivo but reported to have been collected from the wild in Fierenana.

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Table 3. Detection of ranavirus (RV) in Madagascar by quantitative PCR (qPCR) and apparent prevalence including 95% confidence limits (CL) for infection as per swab results, assuming qPCR sensitivity of. 80 as per Gray et al. (2012) and absolute specificity (1.0).

Location	Site	Prev (95% CI)	Water	No. Sampled	No. Rv+	#Spp.	Tadpole/Adult	#Tad
Andringitra	2	0 (0–0.354)	-	7	0	2	A	0
Andringitra	4	0 (0–0.434)	-	5	0	2	A	0
Andringitra Total		**0 (0–0.242)**	**-**	**12**	**0**	**4**	**A**	**0**
Ankaratra	1	0 (0–0.658)	-	2	0	1	T	2
Ankaratra	4	0.042 (0.002–0.203)	-	30	1	2	T+A	15
Ankaratra Total		**0.039 (0.002–0.202)**	**-**	**32**	**1**	**3**	**T+A**	**17**
Antananarivo*	2	0 (0–0.490)	+	4	0	2	A	0
Antananarivo Total		**0 (0–0.490)**	**-**	**4**	**0**	**2**	**A**	**0**
Ranomafana	2	0 (0–0.793)	-	1	0	1	T	1
Ranomafana	3	1 (0.062–1)	-	1	1	1	T	1
Ranomafana	4	0.100 (0.028–0.301)	-	20	2	3	T+A	10
Ranomafana	5	0.060 (0.003–0.284)	-	21	1	3	T+A	12
Ranomafana Total		**0.116 (0.040–0.269)**	-	**43**	**4**	**4**	**T+A**	**24**
Toamasina	1	0 (0–0.390)	-	6	0	2	A	0
Toamasina Total		**0 (0–0.390)**	-	**6**	**0**	**2**	**A**	**0**
National Total		**0.064 (0.026–0.144)**	**+**	**97**	**5**	**15**	**T+A**	**41**

Number of animals sampled, number of species represented, whether tadpoles (T) or post-metamorphic (A) animals were included, and number of tadpoles (#Tad) in the sample are reflected. Water filter results for RV detection also presented. For sites where all amphibian swabs and water samples together tested negative for RV, true prevalence is likely closer to the lower CL. Cumulative data for all sites sampled within the region appear in bold.
*Amphibians sampled in Antananarivo but reported to have been collected from the wild in Fierenana.

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when sampled: one exposed rice paddy and two artificial bodies of water at a chelonian breeding facility, all located in Ankarafantsika.

Discussion

The presence of both *Bd* and ranavirus in Madagascar appeared to be highly localized and at low prevalence during Feb-March 2014. We detected ranavirus (5 of 97), but not *Bd* (0 of 508), in amphibians and water samples (3 of 68). The prevalence of *Bd* was below 0.8% with 95% confidence at the time of sampling (0–0.8%), using Blaker's method [41] and the *Bd* diagnostic specificity and sensitivity values reported by Skerratt et al. (2011) [42], suggesting either *Bd* absence from sample localities or a failure to detect the pathogen if prevalence and/or infection intensity in amphibians were exceptionally low. For ranavirus, the nonlethal sampling method we employed can underestimate true prevalence by 20% compared to liver samples [31], so assuming our swabs had a reduced sensitivity of 0.80, we can be 95% confident that the cumulative prevalence of infection among those processed was 6.4% (2.6–14.4%) as per Blaker's method. Also considering the very low ranavirus loads detected, it is possible that some animals which tested negative may have carried infections loads that escaped our detection, suggesting our findings might further underestimate true prevalence and distribution. Ranavirus-positive amphibians were detected at four separate rivers, three within Ranomafana National Park and one in Ankaratra.

It is unclear precisely how water filter results for *Bd* and ranavirus detection relate to concurrent prevalence of infection in amphibians, but there are two studies from North America that are relevant. Hall et al. (submitted) [43] used similar methods to ours (but with 0.22 μm filters compared to our 0.45 μm pore size) in a survey of wood frog (*Rana sylvatica*) tadpoles in

Table 4. Water filter samples processed by quantitative PCR for detection of *Batrachochytrium dendrobatidis* (*Bd*) and ranavirus (RV) in Madagascar.

Region	Site	Habitat/Source	Lat(S)	Long (E)	Air (C)	RH (%)	W(C)	pH	Swabs	W vol	T vol	T#	*Bd*	RV
Ambatolampy	1	Rice paddy	19.381	47.426	18.3	79.0	20.4	6.89	No	500 (1)	N/A	N/A	-	-
Analamay	1	Flooded dirt road	18.831	48.313	22.8	79.8	25.6	7.28	Yes	1800 (4)	N/A	N/A	-	-
Andasibe	1	River	18.935	48.413	23.6	82.8	19.2	7.46	Yes	600 (1)	N/A	N/A	-	-
Andasibe (Mitsinjo amphibian breeding facility)	3	River water provided to captive amphibians	18.933	48.413	21.4	81.8	21.4	7.79	No	1000 (1)	N/A	N/A	-	-
Andasibe (Mitsinjo amphibian breeding facility)	4	Water flushed from enclosures, (*Mantella aurantiaca*)	18.933	48.413	22.6	82.0	22.3	7.64	No	300 (1)	N/A	N/A	-	-
Andasibe (Mitsinjo amphibian breeding facility)	5	Water flushed from enclosures, *Boophis spp.* & *Mantidactylus spp.*)	18.933	48.413	22.9	82.0	21.8	7.5	No	500 (1)	N/A	N/A	-	-
Andringitra	1	River	22.144	46.888	23.1	63.1	18.5	6.51	Yes	3000 (1)	150 (1)	15	-	-
Andringitra	2	River	22.162	46.895	21.4	63.3	20.5	6.93	Yes	1500 (1)	75 (1)	160	-	-
Andringitra	3	River	22.130	46.866	20.5	52.7	23.9	7.35	Yes	450 (1)	105 (1)	58	-	-
Ankarafantsika	1	Pond	N/A	N/A	31.4	80.4	28.4	7.04	Yes	700 (2)	95 (1)	209	-	-
Ankarafantsika	2	River	16.326	46.857	30.7	70.8	25.9	6.55	Yes	375(1)	N/A	N/A	-	-
Ankarafantsika	3	Rice paddy	16.343	46.848	31.8	73.4	31.5	6.89	Yes	1400 (2)	175 (1)	22	-	-
Ankarafantsika (Durrell Chelonian Captive Breeding Centre)	4	Concrete pools, Madagascan big-headed turtles (*Erymnochelys madagascariensis*)	16.313	46.817	33.9	69.8	32.0	7.04	No	550 (1)	N/A	N/A	-	-
Ankarafantsika (Durrell Chelonian Captive Breeding Centre)	5	Water bowls, Ploughshare tortoises (*Astrochelys yniphora*)	16.313	46.817	33.3	71.5	30.0	6.85	No	1100 (2)	N/A	N/A	-	-
Ankaratra	1	River	19.333	47.263	12.4	99.9	13.4	7.45	Yes	12000 (2)	425 (2)	6	-	-
Ankaratra	2	River	19.346	47.279	13.3	99.9	13.9	7.25	Yes	5000 (2)	250 (1)	6	-	-
Ankaratra	3	River	19.336	47.281	15.9	99.9	14.3	7.16	Yes	1450 (2)*	250 (1)	20	-	-
Ankaratra	4	River	19.349	47.279	15.7	91.1	15.2	7.31	No	4800 (2) *	N/A	N/A	-	-
Antananarivo	1	Rice Paddy	18.861	47.435	30.2	65.2	25.6	6.89	Yes	600 (2)	300 (2)[c]	N/A	-	-
Antananarivo	2	Trade Facility (water bowl, *Heterixalus madagascariensis*)	18.785	47.463	24.4	87.8	23.4	6.92	Yes	150 (1)	N/A	N/A	-	-
Antananarivo	3	Trade Facility (water bowl, *Dyscophus guineti*)	18.785	47.463	24.9	92.1	22.2	7.04	Yes	450 (2)	N/A	N/A	-	+
Antananarivo	4	Trade Facility (water bowl, *Scaphiophryne madagascariensis*)	18.785	47.463	24.5	84.8	21.3	7.12	Yes	75 (1)	N/A	N/A	-	-
Antananarivo	5	Lake (crocodile farm outside trade facility)	18.785	47.463	22.8	68.8	23.0	7.54	No	700 (2)	N/A	N/A	-	-

(*Continued*)

Table 4. (*Continued*)

Region	Site	Habitat/Source	Lat(S)	Long (E)	Air (C)	RH (%)	W(C)	pH	Swabs	W vol	T vol	T#	*Bd*	RV
Antananarivo	6	Lake #1 (Tsimbazaza Zoo)	18.930	47.527	20.7	72.0	22.0	7.2	No	600 (2)	N/A	N/A	-	-
Antananarivo	7	Lake #2 (Tsimbazaza Zoo)	18.931	47.526	21.7	64.8	21.3	7.31	No	600 (2)	N/A	N/A	-	-
Faravohitra	1	Lake #1 (outdoor trout aquaculture facility)	19.359	47.316	19.1	83.1	17.5	6.89	No	1200 (1)	N/A	N/A	-	-
Faravohitra	2	Lake #2 (outdoor trout aquaculture facility)	19.359	47.316	19.3	79.1	17.9	7.16	No	1000 (1)	N/A	N/A	-	-
Faravohitra	3	River (water supplied to trout aquaculture facility	19.359	47.316	19.6	75.3	16.09	7.02	No	1500 (1)	N/A	N/A	-	-
Isalo	1	River	N/A	N/A	28.3	55.9	24.1	6.92	Yes	2800 (2)	N/A	N/A	-	-
Isalo	2	River	22.628	45.359	32.8	37.5	25.2	7.00	Yes	1100 (2)	N/A	N/A	-	-
Isalo	3	River	22.645	45.332	34.2	28.8	26.5	7.01	Yes	1700 (2)	200 (1)	8	-	-
Ranomafana	1	River	N/A	N/A	21.4	95.0	19.8	7.22	Yes	1500 (1)	500 (1)	17	-	-
Ranomafana	2	River	21.254	47.421	24.4	75.8	19.1	7.02	Yes	1500 (1)	650 (2)	45	-	-
Ranomafana	3	River	21.269	47.425	22.1	90.2	20.0	7.02	Yes	1350 (1)	400 (2)	105	-	-
Ranomafana	4	River	21.291	47.426	23.1	81.5	19.5	6.97	Yes	1500 (1)	500 (2)	53	-	-
Ranomafana	5	River	21.291	47.426	22.3	89.3	19.6	6.96	Yes	1500 (1)	350 (2)	57	-	-
Toamasina	1	Flooded grass lot	18.149	49.375	24.3	93.8	26.2	6.84	Yes	600 (2) *	N/A	N/A	-	-
Toamasina	2	Trade Facility (water bowls, enclosure #1 & 2)	18.147	49.401	n/a	n/a	n/a	n/a	No	250 (2)	N/A	N/A	-	-
Toamasina	2	Trade Facility (water bowls, enclosure #3 & 4)	18.147	49.401	n/a	n/a	n/a	n/a	No	600 (2)	N/A	N/A	-	+
Toamasina	2	Trade Facility (water bowl, enclosure #5)	18.147	49.401	n/a	n/a	n/a	n/a	No	400 (2)	N/A	N/A	-	+
Toamasina	2	Trade Facility (flooded grass lot)	18.147	49.401	n/a	n/a	n/a	n/a	No	1200 (2)	N/A	N/A	-	-
Toamasina	2	Trade Facility (well water supplied to enclosures)	18.147	49.401	n/a	n/a	n/a	n/a	No	3000 (1)	N/A	N/A	-	-
Zahamena	1	River	17.508	48.731	20.1	96.1	19.2	7.6	Yes	400 (1)	N/A	N/A	-	-
Zahamena	2	River	17.513	48.726	18.8	95.8	17.7	7.61	Yes	1500 (1)	325 (1)	21	-	-
Zahamena	3	River	17.500	48.734	21.0	88.3	18.2	7.74	Yes	1500 (1)	N/A	N/A	-	-

Environmental conditions measured at sample sites include air temperature, relative humidity, water temperature and pH. Total volume of environmental water (W vol) and tadpole water (T vol) filtered at each site is followed by the number of individual filter samples in parenthesis and number of tadpoles held in the Tvol sample (T#).

*Strong PCR inhibition was detected in these samples.

[c]Water held crayfish rather than tadpoles.

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vernal pools in Connecticut, USA and estimated the probability of detecting ranavirus DNA at 0.90 per 250 mL filtered water sample in ponds with known infection (note that all tadpoles tested from their ponds were infected). Assuming the same detection probability per 250 mL

sample a constant per mL probability, then our ability to detect ranavirus in a sample varied from a low of $1\text{-}(1\text{–}0.90)^{75mL/250mL} = 0.499$ to an average of $1\text{-}(1\text{–}0.90)^{6000mL/250mL} = 0.99$ or higher per water body. Hall et al.'s estimate comes from ponds with active die-offs, so these estimates of detection probability are likely high. For *Bd*, Schmidt et al. (2013) [44] found that each 600 mL water sample from high-elevation ponds in Arizona had a detection probability of 0.45 when present in amphibians. Although they used a different sampling design (20 mL from 30 locations in a pond) and filters (0.22 μm polyvinylidene difluoride filters), if we use their estimate as a first approximation, then our probability of detecting *Bd* in body of water would have varied with the amount of water filtered per sample from a low of $1\text{-}(1\text{–}0.45)^{75mL/600mL} = 0.072$ to a high of $1\text{-}(1\text{–}0.45)^{6000mL/600mL} = 0.997$, although on average it would have been closer to $1\text{-}(1\text{–}0.45)^{500mL/600mL} = 0.392$ per water body. It is possible that low pathogen density and/or high heterogeneity in the distribution of *Bd* may be responsible for the low detection probabilities previously reported, but the absence of *Bd* in all our 68 independent water samples does bolster the conclusion from the swab data: *Bd* was rare or absent in much of Madagascar during this survey. Furthermore, since we specifically targeted locations expected to favor pathogen presence, the lack of detection in our pathogen-negative samples provides greater certainty of pathogen absence than would similar results produced by a random sampling effort.

Our field results suggest that *Bd* and ranavirus were absent from most regions sampled in Madagascar at the time surveillance was performed, but whether these pathogens follow seasonal patterns in Madagascar warrants further investigation. Prevalence, infection intensity, and timing of disease-associated mortality events can fluctuate seasonally; some *Bd* surveys have demonstrated greater infection prevalence in cooler versus warmer months [27,45–48], whereas ranavirus appears to become more active in summer [8,49]. Seasonal patterns in adult infection with *Bd* (and perhaps ranavirus) appear to be largely temperature-driven, and the environmental conditions we recorded during this investigation fell near those optimal for growth and reproduction in culture, for both pathogens (average water and air temperatures measured 23.3 C and 21.5 C, respectively). Exposure to 32 C for four hours is lethal to *Bd* [27] and replication of the type ranavirus, FV3 ceases at 33 C [28]. Although 43/45 aquatic bodies measured less than 32 C when water was sampled during daylight hours, it is conceivable that hostile conditions may have occurred prior to our visit and influenced pathogen abundance and detectability during our survey, as suggested by Murray et al. (2013) [48]. Still, Chestnut et al. (2014) [50] investigated temporal patterns of *Bd* presence in a wetland in Oregon, USA and found that *Bd* remained detectable by water filtration year-round, including the warmer periods when fewer adult animals sometimes test positive for infection. Similarly, both Whitfield et al. (2012) [47] and Longo et al. (2010) [51] detected *Bd*-positive amphibians year-round via skin swabbing in a warm lowland site in Costa Rica and a cool upland forest in Puerto Rico, respectively, even during the warmest months when infection loads were at their lowest. Therefore, despite potentially low pathogen abundance during our survey in Madagascar, these data suggest that our *Bd*-negative water filter results together with *Bd*-negative amphibian swab results is more likely demonstrative of pathogen absence from a location rather than a seasonal false-negative characterization. This is especially relevant to our sampling efforts at cooler high elevation sites, such as Ankaratra (2,015–2,384 m elevation; Table 1), where conditions are unlikely to exceed the thermal maximum for pathogen survival, even through summer. Regardless, a series of amphibian and environmental surveys should be repeated at our sites to determine whether the strains of *Bd* and/or ranavirus present in Madagascar exhibit seasonal variation in prevalence and aquatic abundance and to what magnitude this may manifest in field results.

We previously detected *Bd* in 3 of 565 (prevalence = 0.5%; 95% CI: 0.2–1.6%) wild-collected amphibians exported from Madagascar in February 2012, although due to the possibility of exotic trade-associated contamination whilst in temporary captivity prior to export, it was difficult to confirm whether these animals were collected from the wild with *Bd* at that time [17]. Therefore, during this field study we performed an inspection at the specific wildlife trade facility in Toamasina from which the amphibians positive for *Bd* (and ranavirus) had been exported, and conducted interviews with the staff. We were unable to identify any potential sources of non-Malagasy *Bd* introduction from within this compound. Only amphibians, reptiles, and birds collected from the wild in Madagascar were temporarily housed at this facility and no exotic animals were received. Furthermore, this trade enterprise did not import or transship fresh produce or aquacultural material that might have inadvertently introduced amphibians or their pathogens. Therefore, we suspect the infected, exported amphibians reported in Kolby (2014) [17] were indeed collected from the wild in Madagascar with *Bd*.

Further, the absence of *Bd* detection in the present study does not, in fact, contradict our previous finding, assuming these exported animals had become infected in the wild. Indeed, the 95% confidence interval for *Bd* prevalence in the current study (0–0.8%) overlaps with that from Kolby (2014) [17] of 0.2–1.6%, suggesting there may have been undetected low infection prevalence in the wild during the current investigation of similar magnitude as that previously found in the exported frogs. Furthermore, combining species in high density at the trade facility prior to export was likely to have increased the opportunity for disease transmission, suggesting that true *Bd* prevalence in the source population(s) was closer to 0.2% before collection, a challenging prevalence to identify in the field especially when the location of these animals' collection remains unknown.

The detection of ranavirus in wild amphibians in Ranomafana National Park and Ankaratra, and in wildlife trade facilities in Antananarivo and Toamasina, demonstrates a potential threat to the amphibian biodiversity in these and surrounding regions. Ranavirus infection in amphibians can result in unpredictable mass mortality events, dramatic population decline, and/or local extirpation [3,4,52], and it is unknown whether the ranavirus we detected is highly virulent to native species. The surveys we performed at each site were brief and designed to identify pathogen presence, but not mortality. It is therefore possible that our single visits per location failed to capture disease-associated mortality events, especially if a highly virulent ranavirus was present that induced rapid mortality at other times. Of particular concern is the presence of ranavirus at Ankaratra, a remote area inhabited by two locally endemic critically endangered amphibian species: *Boophis williamsi* and *Mantidactylus pauliani*, the former of which is regarded to be one of the most threatened amphibians in Madagascar according to the IUCN Red List of Threatened Species (2014) [53]. Likewise true with respect to *Bd*, sufficient information to discern whether the ranavirus we detected was recently introduced or endemic to Madagascar is not available at this time. Accordingly, standardized long-term population surveys to monitor the potential impact of ranaviral infection in these two highly vulnerable species should be established with urgency.

Additional biodiversity hotspots oriented near ranavirus-positive locations, such as Andasibe-Mantadia National Park, Zahamena National Park, and Betampona Strict Nature Reserve, are threatened by pathogen exposure and warrant additional monitoring. Both Ranomafana and Andasibe are frequently visited by tourists and researchers and the movement of potentially contaminated footwear and equipment provides a likely vector for the spread of disease among and between biodiversity hotspots. Ranavirus can remain viable from days to weeks when protected from high temperatures, desiccation, and microbial action [54,55] providing considerable time for spread via fomites, and the same applies to *Bd* [56]. Even if these pathogens may be endemic to Madagascar, human-assisted introduction to naive isolated amphibian

populations or the spread of different strains to new regions may lead to more severe outcomes and increased risk of declines. Disinfection of materials exposed to amphibians or aquatic habitats is necessary to prevent increased rates of disease spread [38], and a variety of commercially available disinfectants will inactivate *Bd* and ranavirus, including bleach at 3.0% concentration or higher [57,58]. Accordingly, public education and vigilance are critically important to reduce the frequency of accidental pathogen dispersal beyond current boundaries. Furthermore, it would be prudent for Malagasy authorities to require biosecurity protocols be performed at wildlife trade facilities to reduce the risk of pathogen spillover. Since animals from disparate regions become centralized at these locations prior to exportation, the untreated disposal of pathogen-positive water, soil, or dead animals from temporary housing enclosures may expose amphibians living in proximity to centers of wildlife trade and accelerate the spread of *Bd* and ranavirus within Madagascar. Further, we cumulatively detected both ranavirus and *Bd* at the same trade facility in Toamasina, suggesting that co-infection may particularly threaten free-ranging amphibian populations near trade centers that do not employ biosecurity measures.

The current National Monitoring Plan (NMP) for the detection of *Bd* in Madagascar involves biannual swabbing surveys performed at eight fixed locations throughout the country [14], but early detection is more likely if surveillance is broadened to include additional locations, species, and sampling methods. Recognizing resource limitations and how potential seasonal influences on *Bd* might promote detectability in adult amphibians during cooler temperatures, an efficient approach would involve doubling the number of national sampling points but reducing sampling frequency to once per year, redirecting all survey efforts to the winter season. Further, the incorporation of water filtration methods would provide cost-efficient preliminary screening of amphibian habitats to detect pathogen presence before performing intensive amphibian swabbing surveys to identify infection prevalence. Regions affected by alien invasive species that might provide a vector for amphibian pathogen introduction should also be included in this sampling regime, specifically Toamasina, where an incursion of Asian common toads (*Duttaphrynus melanostictus*) was recently identified [59]. Lastly, our detection of ranavirus at both wildlife export facilities sampled demonstrates a need for the development of cooperative efforts between Malagasy authorities and these facilities. Disclosure of amphibian collection localities and permission to test the large numbers of amphibians amassed prior to export could help accelerate field detection by focusing NMP field efforts towards regions where wild populations may already be affected by *Bd* and/or ranavirus.

Ranavirus can also infect and cause disease and mortality in reptiles, especially chelonians, and transmission of novel strains from amphibians may threaten Madagascar's endangered reptile species [16, 60,61]. Nearly 40% of the country's reptile diversity faces a high risk of extinction primarily due to habitat loss and exploitation for food or pets [62]. Fortunately, we did not detect the presence of ranavirus in water sampled from enclosures of critically endangered Ploughshare tortoises (*Astrochelys yniphora*) and Madagascar big-headed turtles (*Erymnochelys madagascariensis*) at the Durrell Chelonian Captive Breeding Centre (0 of 3 samples; Table 4), but increased vigilance would benefit such reptile conservation efforts. Despite strict biosecurity measures taken by staff to prevent introduction of pathogens via their own movements, animals bred in captivity and maintained in outdoor enclosures may become exposed to ranavirus through incidental contact with local herpetofauna if able to pass through or over fences, and as demonstrated by Brenes et al. (2014) [16], transmission can occur through water shared between amphibians and reptiles. Even endemic strains of ranavirus can cause periodic mortality and threaten species that exist only in isolated limited numbers. Although ranaviral infection in reptiles is most often identified in chelonians, Malagasy lizards of conservation concern, such as *Uroplatus* spp. geckos, may also be susceptible and warrant attention [63]. Our detection of ranavirus in free-ranging amphibians sampled during this investigation

illustrates the need for preemptive surveillance among endangered and range-restricted reptile species to evaluate this potential threat.

On the morning of 26 February 2014, we recorded an enigmatic amphibian mortality event in Analamay, where 20 dead frogs (*Heterixalus* spp.) were found in a shallow pool of rainwater on a dirt road that passed through an area of forest. We were alerted to the scene and collected water filter samples for pathogen presence that same day. Unfortunately, all frog carcasses displayed advanced stages of decomposition and were unsuitable for pathological examination. However, on 3 March 2014, four additional dead frogs (*Aglyptodactylus* sp. (n = 1) and *Heterixalus* spp. (n = 3)) were found in better condition in or near the same pool, and preserved for histological and molecular analysis. All samples collected from this location tested negative for the presence of *Bd* and ranavirus both by qPCR and histology and no lesions suggestive of either chytridiomycosis or ranaviral disease were observed. The potential presence of other pathogens, environmental contaminants, or habitat degradation, might have contributed towards this mortality event, but the precise cause(s) remains unidentified. Due to the differences in sampling conditions and quality, these data are not combined with those summarized in Tables 1–3. Populations of the critically endangered Golden Mantella (*Mantella aurantiaca*) inhabit this forest area, raising concern for additional mortality events in the region. This particular event marks the first amphibian mass mortality reported to the Madagascar Chytrid Emergency Cell for rapid investigation and any future events should be similarly reported and evaluated.

Although we did not detect *Bd*-positive amphibians in Madagascar, the risk-based approach of our field surveillance activity suggests that the greatest threat posed by chytridiomycosis likely remains confined to limited regions and/or seasonal periods. Further work to identify the strain(s) of *Bd* present is needed to evaluate the risk of decline posed to native species and whether the commonly used diagnostic PCR method fails to detect a potentially highly divergent Malagasy *Bd* strain. Similarly, the distribution and dynamics of ranavirus in Madagascar, and whether it is endemic or a recent introduction, requires additional field surveillance to resolve. Therefore, it is important that standardized population monitoring of key amphibian and reptile populations be established with urgency to enable early detection of potential impacts of disease emergence in this global biodiversity hotspot before obvious declines are observed [7,64]. Risk assessments should include prediction of pathogen impacts in various habitats and prioritization of species based on their ecology and results from infection susceptibility trials [20,65,66]. Fortunately, the establishment of amphibian captive breeding initiatives by Association Mitsinjo and Madagascar Fauna and Flora Group preempted formal identification of amphibian pathogens in the country and are developing local capacity to respond with rescue activities if needed. We remain hopeful that disease-driven amphibian extinction can be prevented in Madagascar through continued monitoring of *Bd* and ranavirus distribution and spread, accurate predictions of disease impacts, and coordinated field and ex-situ management activities.

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Author Contributions

Conceived and designed the experiments: JEK KMS SDR LB LFS. Performed the experiments: JEK SDR FR. Analyzed the data: JEK LFS. Contributed reagents/materials/analysis tools: APP JLB CSG. Wrote the paper: JEK KMS SDR LB LFS JLB APP CSG FR.

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Paper 2

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FORMAL COMMENT

Amphibian Chytrid Fungus in Madagascar neither Shows Widespread Presence nor Signs of Certain Establishment

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Abstract

The global spread of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*, *Bd*) is associated with amphibian mass mortality, population decline, and extinction. Over the past decade, concern has been expressed for the potential introduction of *Bd* to Madagascar, a global hotspot of amphibian biodiversity. Following years without detection, widespread *Bd* presence in Madagascar has now been reported (Bletz et al. 2015a), raising international conservation concern. Before reacting to this finding with a significant management response, the accuracy and context of the data warrant cautious review. Re-examination of a 10-year dataset together with results from more recent surveillance (Kolby et al. 2015) does not yet demonstrate widespread *Bd* presence. Detection of *Bd* at "positive" locations in Madagascar has been inconsistent for unknown reasons. Whether *Bd* is established in Madagascar (i.e. populations are self-sustaining) or instead requires continued introduction to persist also remains uncertain. The deployment of emergency conservation rescue initiatives is expected to target areas where the distribution of *Bd* and the risk of chytridiomycosis endangering amphibians is believed to overlap. Thus, erroneous description of *Bd* presence would misdirect limited conservation resources. Standardized surveillance and confirmatory surveys are now imperative to reliably characterize the distribution, potential spread, virulence and overall risk of *Bd* to amphibians in Madagascar.

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Introduction

Two recent papers provide conflicting results on whether the globally emerging amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is now widespread and established (self-sustaining populations) in Madagascar [1,2]. Presence of *Bd* has been linked to a growing number of disease-driven amphibian mortality events and population declines since its discovery [3,4]. The global origin of *Bd*, timeline of first emergence, and reason for contemporary lethality are still unknown, although it has been present for thousands of years [5]. Despite its historic occupation in some areas and continued spread to others, certain regions appear to have

remained free of *Bd* and disease-associated amphibian decline until only very recently, most notably Madagascar—a global hotspot of amphibian biodiversity.

A series of proactive *Bd* surveillance efforts performed prior to 2010 found the pathogen to be absent in Madagascar (0% estimated prevalence with high confidence) [6–8]. Assuming *Bd* absence based on these data and considering the threat of introduction, the Madagascar Chytrid Emergency Cell was established in 2010 along with the development of the National Monitoring Plan to administer biannual surveys for *Bd* presence, performed at eight nationally distributed locations to facilitate early detection and a rapid response [9]. Soon thereafter, a first call to arms was raised when amphibians collected from the wild in Madagascar and exported to the USA pet trade in Feb 2012 tested positive for *Bd*, suggesting presence and potentially recent introduction to Madagascar [10], as no other detections had previously been confirmed.

Nearly three years later, Bletz et al. [1] now report widespread *Bd* presence in wild amphibian communities in Madagascar. Surveillance efforts described by Bletz et al. [1] span nearly a decade (2005–2014), involved the sampling of 4,155 amphibians, and were performed at 52 survey sites across the country. *Bd* was detected at 10 sites, from five locations (*i.e.* multiple sites tested positive at some locations, *e.g.* two separate *Bd*-positive stream sites within a national park). Infection prevalence reported from these sites was generally low, but reached up to 100% on occasion. Accordingly, this announcement of seemingly sudden widespread *Bd* distribution together with unpredictable high prevalence of infection ignited considerable alarm among the conservation community, as it suggests that catastrophic disease-associated decline in Malagasy amphibian biodiversity is now immediately possible.

One month following the final *Bd*-positive survey described by Bletz et al. [1] from Jan 2014, a highly intensive national surveillance program did not detect *Bd* presence in samples collected at 47 survey sites distributed throughout the country [2]. This sampling effort included 508 amphibians swabbed for *Bd* and 68 *Bd* eDNA water filter samples processed to detect the pathogen in amphibian habitats. Locations surveyed by Kolby et al. [2] included 3/5 *Bd*-positive regions described by Bletz et al. [1]. Although Bletz et al. suggested *Bd* detection is greater in the cooler dry season, this pathogen has also been detected in Madagascar during the warmer wet season and our lack of detection at "positive" locations is curious, especially since our methods incorporated multiple techniques to increase sensitivity and power of detection. Thus, field survey results presented by Kolby et al. [2] strongly conflict with the assertion that *Bd* is widely distributed in Madagascar.

Before significant conservation management decisions are developed based on the distribution of *Bd* described by Bletz et al. [1], it is important to consider and resolve the disparity in results provided by Kolby et al. [2]. Three main plausible explanations spring to mind: 1. the studies varied significantly in diagnostic sensitivity and specificity, 2. the amphibian chytrid fungus detected by PCR by Bletz et al. [1] and Kolby et al. [2] behaves differently to the *Bd* detected elsewhere in the world, and 3. non-*Bd* material is reacting with *Bd* primers and falsely suggesting its presence in Madagascar. Bletz et al. [1] partially addressed the latter hypothesis by conducting a lineage-specific PCR that suggested some of the positive samples were molecularly similar to the global pandemic *Bd* lineage, but histological confirmation of infection with *Bd* in Malagasy amphibians still does not yet exist. In considering hypothesis 1, variation in accuracy, as a possible explanation for the discordance among survey results, amphibian sampling efforts performed from 2005–2014 demonstrated an overall lack of standardization in survey design, including: the selection of locations sampled, sample collection and storage methods, and laboratory diagnostic techniques. Accuracy of the qPCR test for *Bd* detection has been shown to vary according to both field sampling and laboratory diagnostic methods [11] and Bletz et al. [12] further demonstrate that employing different DNA extraction methods

Table 1. Timeline of *Batrachochytrium dendrobatidis* (*Bd*) records of detection at affected sites in Madagascar from 2005–2014 as reported by Bletz et al. (2015a) and Kolby et al. (2015).

Location	Site	Total sample	2005–2009	2010	2011	2012	2013	2014A	2014B
Ankaratra	Tavolotara	167 (18)	–	–		X (unk)	X (18)		–
Ankaratra	Ambatolampy	64 (7)	–				X (7)		–
Ankaratra	Ambohimirandrana	42 (8)		–			X (8)		–
Ankarafantsika	Andranofasika	150 (unk)			–	X (unk)	–		–
Makay	Andranovinily	164 (3)		X (3)	–		–		
Makay	Beroroha	209 (1)			X (1)		–		
Ranomafana	Vatoharanana	117 (15)					X (15)	–	–
Ranomafana	Valohoaka	43 (1)						X (1)	–
Antoetra	Fohisokina	150 (unk)			–	X (unk)			
Antoetra	Soamazaka	9 (1)						X (1)	
Total		1115 (54)	0	3	1	unk	48	2	0

Surveys with *Bd* detection marked by "X" and those with only negative results marked by "–". The number of *Bd*-positive amphibians detected in each survey is presented in parenthesis, except where samples were pooled for analysis and the number of *Bd*-positive animals remains unknown (unk). Where multiple surveys were performed at a site within the same year, data were combined. The column "2014A" represents surveys reported by Bletz et al. (2015a) and "2014B" represents surveys reported by Kolby et al. (2015)

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will affect the detection of *Bd* from skin swabs. Given the aforementioned variation in field protocols and laboratory techniques, each study has introduced additional unique variation in diagnostic sensitivity and specificity over the 10 years of surveys. Thus, some unknown cumulative degree of uncertainty is now embedded within the description of the current presence and distribution of *Bd* in Madagascar.

Two anomalies in the data specifically deserve attention: inconsistent detection at sites reported as *Bd*-positive and dramatic fluctuation in measured *Bd* prevalence. Detection of *Bd* at specific survey sites was infrequently followed by subsequent detection (Table 1). For example, *Bd* was last detected at Makay and Ankarafantsika 3–4 yrs ago, in 2011 and 2012, respectively, and recent confirmatory survey efforts at both sites produced only negative results. If these data reflect true *Bd* absence following earlier detections, then it is plausible that *Bd* failed to become established at these locations and develop self-sustaining populations. These observations now introduce uncertainty regarding the current presence of *Bd* at most sites in Madagascar reported as "*Bd*-positive". This is because 8/10 of these sites were classified as "*Bd*-positive" based on single detection events (Table 2), where the assumption of *Bd* establishment may likewise be spurious. This sporadic pattern of *Bd* detection also raises question as to the manner in which *Bd* survey results are commonly communicated; do sites of *Bd* detection followed only by lack of detection warrant a "*Bd*-positive" label and permanent loss of freedom of disease status? The World Organisation for Animal Health (OIE) Aquatic Animal Health Code (Chapter 8.1: Infection with *Batrachochytrium dendrobatidis*) states that a country where *Bd* has been detected can be reclassified as "free from infection with *B. dendrobatidis*" if targeted surveillance has been in place for at least two consecutive years without detection [13]. If this approach was to be applied at the field site level within Madagascar, and elsewhere, it would substantially change the manner in which researchers populate *Bd* distribution maps—at present, even a single *Bd*-positive record is frequently used to symbolize *Bd* persistence at that location, with or without subsequent surveillance.

Bletz et al. [1] mention that their long-term methodological inconsistencies place a limitation on data interpretation and,". . . may confound time and season with detection method and

Table 2. Number of surveys and detections for all sites in Madagascar reported as positive for the presence of *Batrachochytrium dendrobatidis* (*Bd*) by Bletz et al. (2015a) and the most recent survey results reported by Kolby et al. (2015).

Location	Site	sample size (*Bd*+)	1 survey 1 detection	2 surveys 1 detection	≥ 3 surveys 1 detection	2 surveys 2 detections	≥ 3 surveys 2 detections	≥ 3 surveys 3 detections	Feb-March 2014
Ankaratra	Tavolotara	167 (18)					X		–
Ankaratra	Ambatolampy	64 (7)		X					–
Ankaratra	Ambohimirandrana	42 (8)					X		–
Ankarafantsika	Andranofasika	150 (unk)			X				–
Makay	Andranovinily	164 (3)			X				N/A
Makay	Beroroha	209 (1)		X					N/A
Ranomafana	Vatoharanana	117 (15)		X					–
Ranomafana	Valohoaka	43 (1)	X						–
Antoetra	Fohisokina	150 (unk)			X				N/A
Antoetra	Soamazaka	9 (1)	X						N/A
Total		1115 (54)	2	3	3	0	2	0	0

The number of surveys performed at each location is expressed together with the number of those events that resulted in *Bd* detection, as reported by Bletz et al. (2015a). Cumulative number of amphibians sampled at each site by Bletz et al. (2015a) and number of *Bd*-positive animals detected (*Bd*+) is reported, except where samples were pooled for analysis and the number of *Bd*-positive animals is unknown (unk). The final column ("Feb-March 2014") represents the most recent survey results as reported by Kolby et al. (2015), from sampling at or near sites of previous *Bd* detection. Areas not surveyed by Kolby et al. (2015) are marked with "N/A"

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could in part compromise the conclusions of the recent detection of *Bd* and seasonality trends." The exceptionally high *Bd* prevalence of 100% inferred from material collected at Ankaratra (site: Tavolotara) in Aug 2013, was both preceded and followed by complete lack of detections in Feb 2013, Dec 2013, and March 2014. Environmental conditions measured during the most recent survey at Ankaratra in March 2014 were favorable for *Bd* to thrive, with daytime air and water temperatures ranging from 12.4–15.9 C and 13.4–15.2 C, respectively. *Bd* mortality (i.e. death of the pathogen) does not normally occur until temperatures rise to 28 C and above [14], and even following death for any reason, its molecular presence will persist and extend detectability. In an aquatic habitat, *Bd* is likely to remain detectable year-round despite sometimes variable abundance, as has been observed in long-term water filtration surveys at affected wetlands [15]. Although the *Bd* in Madagascar might behave dramatically unlike that studied elsewhere, no evidence yet supports this hypothesis apart from variation in virulence observed among lineages [16]. Bletz et al. [1] hypothesize that this phenomenon at Ankaratra may be explained by a seasonal variation in *Bd* abundance caused by some unidentified factor and/or there was a changeover in the strain of *Bd* present (similar to hypothesis 2, above). Considering all available information, the law of parsimony instead suggests that samples from Aug 2013 were more likely compromised and involved some form of field or laboratory error (hypothesis 1).

In order to reduce aforementioned uncertainties, current and future *Bd* surveillance methods must be standardized to prevent continued challenges in field data interpretation and potential loss of conservation value produced by these efforts. Moving forward, variation in detection that may confound observed patterns of *Bd* presence can be minimized by establishing longer-term surveys at suspected *Bd*-positive sites and ensuring that samples are collected, stored, and analyzed following standardized established methods for *Bd*. Multiple complementary *Bd* sample collection and diagnostic techniques can also be incorporated into future surveys to increase the confidence of results, *e.g.* collection of water samples for eDNA analysis at

aquatic habitats where amphibian are simultaneously swabbed for Bd [2], and histological confirmation of *Bd* presence in an amphibian that tests positive by skin swab [17]. Water filtration can capture *Bd* particles shed from a host [15,18,19] and if detected, corroborate swab-based "*Bd*- positive" site characterizations in a highly cost-efficient manner. At the time of writing (August 2015), histological evidence of *Bd* infection in wild Malagasy amphibians still does not exist; such data would help prove that *Bd* presence is certainly causing the PCR-based detections and help rule out hypotheses 1 and 3, above. Further, fungal isolation should be performed and results verified by an OIE reference laboratory [17].

In conclusion, the uncertain distribution and potential impact of *Bd* presence in Madagascar requires additional investigation before accurate evaluations can be made. Standardized field surveillance methods and laboratory diagnostic techniques are needed to more carefully investigate the presence of *Bd* both at sites where it has and has not yet been detected. The former is necessary to build longer-term surveys that can assess whether or not Bd is established in Madagascar and the latter to monitor future *Bd* spread into potentially *Bd*-negative locations. These data can then be combined with those provided by Bletz et al. [1] and Kolby et al. [2] to more confidently assert whether *Bd* is truly present and widespread, especially in the absence of any obvious sign of disease. Still, many amphibian species in Madagascar have not been monitored in a long-term standardized fashion, and thus any *Bd*-associated decline may be occurring unnoticed. Therefore, population monitoring of key species likely to be affected warrants urgent establishment. The application of skeletochronology [20] and/or intensive mark-recapture surveys may shed light on survival patterns in amphibian communities where *Bd* has been identified or is suspected. The presence, distribution, virulence to native species and clade membership of *Bd* in Madagascar must be verified before its potential impact on Malagasy amphibians can be accurately predicted.

Author Contributions

Conceived and designed the experiments: JEK. Performed the experiments: JEK. Analyzed the data: JEK LFS. Wrote the paper: JEK LFS.

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Paper 3

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Correspondence

Stop Madagascar's toad invasion now

CHAPTER 4

The presence and distribution of amphibian pathogens in a region of high amphibian trade

Introduction

In Chapter 2, I commonly detected animals infected with *Bd* and ranavirus in the international amphibian trade. Similar to the previous case study described for Madagascar, these data became the first published records of their presence in material originating from Hong Kong. But unlike Madagascar where amphibians are not commercially imported, the high volume of amphibian trade passing through Hong Kong is a plausible avenue of pathogen introduction and spread. Interestingly, previous surveillance (Rowley et al. 2007) did not detect the presence of *Bd* in Hong Kong's wild or traded animals and I needed to explore this in greater depth.

In Chapter 4, I designed an expansive field study to better understand current patterns of *Bd* and ranavirus presence in Hong Kong and evaluate the role of trade in the introduction and spread of these pathogens. I originally designed this study to be a standalone surveillance project, but after visiting Hong Kong and collaborating with the Agriculture, Fisheries and Conservation Department (AFCD) of the Hong Kong Special Administrative Region Government, I learned that they held several additional seasons of surveillance results. We agreed to work together on a single comprehensive 5-year report of pathogen presence in Hong Kong, on which I took the lead. This study is **Paper 1** of this chapter: "Five Years of Surveillance to Detect the Amphibian Pathogens *Batrachochytrium dendrobatidis* (*Bd*) and Ranavirus in Hong Kong.," which has not yet been published.

Paper 1

Citation: Kolby JE, Chan SK, Smith KM, Ramirez SD, Berger L, Skerratt LF. Five Years of Surveillance to Detect the Amphibian Pathogens *Batrachochytrium dendrobatidis* (*Bd*) and Ranavirus in Hong Kong. (unpublished manuscript)

Abstract
In recent decades, globally emerging amphibian pathogens have been associated with dramatic amphibian population declines and extinction, most notably the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) and ranaviruses. Although both pathogens have been detected in Southeast Asia, neither was previously confirmed in free-ranging animals in Hong Kong. The international trade in live amphibians provides a common avenue of pathogen dispersal, and the high volume of amphibian trade passing through Hong Kong poses a high risk of pathogen introduction and spillover. From 2010-2014, we sampled 1,680 amphibians for *Bd* and a subset of 589 also for ranavirus, including both traded and free-ranging native amphibians. *Bd* was detected in Hong Kong in 3/5 years sampled (2012 - 2014), and in both traded and native species. Overall, *Bd* prevalence was low both in animals sampled from the wild (1.63%) and the commercial amphibian trade (6.39%). Ranavirus was detected in both years sampled (2013-14) and prevalence was notably higher in traded (36.91%) than free-ranging amphibians (10.51%). These pathogens have not yet been linked to amphibian mortality or declines in Hong Kong, which is generally consistent with previous observations in Southeast Asia where endemic strains are present. Additional research is needed to identify and characterize the strains of ranavirus and *Bd* present in Hong Kong in order to predict their impact on native species. The risk of spreading exotic pathogen strains via trade is high, requiring urgent pathogen control measures to mitigate regional and global spread of diseases through Hong Kong - a major hub of international amphibian trade.

Introduction

The emerging amphibian diseases caused by amphibian chytrid fungus (*Batrachochytrium dendrobatidis, Bd*) and ranavirus are contributing towards global amphibian population declines

and extinctions (Stuart et al. 2004; Skerratt et al. 2007; Teacher et al. 2010; Price et al. 2014). The effect of their presence in amphibian populations can vary considerably and ranges from seemingly stable endemic infection to dramatic mortality events, extirpation, and extinction (Berger et al. 1998; Lips et al. 2005; Teacher et al. 2010; Miller et al. 2011; Price et al. 2014). *Bd* and ranavirus are both pandemic in distribution and have been detected throughout Asia (Olson et al. 2013; Gilbert et al. 2013; Swei et al. 2011; Xu et al. 2010). While most of these records describe low infection prevalence in native species, the long-term impact of these pathogens on Asian biodiversity is not yet fully understood.

Hong Kong is one of a few well-studied regions where the presence of neither *Bd* (Rowley et al. 2007) nor ranavirus has been previously reported. The apparent absence of these pathogens is remarkable since Hong Kong engages in a globally significant volume of international amphibian trade, and this activity appears to provide a common pathway of amphibian pathogen spread (Mazzoni et al. 2003; Fisher and Garner 2007; Schloegel et al. 2009; Catenazzi et al. 2011; Spitzen-van der Sluijs et al. 2011; Kolby et al. 2014). Despite the absence of biosecurity measures to mitigate the risk of pathogen introduction, *Bd* was neither detected in wild nor traded amphibians sampled in Hong Kong from 2005-2006 (Rowley et al. (2007). This baseline evaluation prompted the Agriculture, Fisheries and Conservation Department (AFCD) of the Hong Kong Special Administrative Region Government to continue performing preemptive surveillance for *Bd* emergence.

From 2010 to 2014, surveys for *Bd* were conducted by the AFCD to facilitate early pathogen detection. These surveys were generally performed during the cooler, drier winter months (Sept-Jan) and involved primarily free-ranging amphibians, but also included imported amphibians upon arrival at Hong Kong International Airport and animals from domestic markets.

Meanwhile, in 2012, a trade surveillance project in the USA detected a high prevalence of *Bd* and ranavirus in amphibians arriving from Hong Kong (Kolby et al. 2014). This stimulated increased survey effort in 2013 to complement the ongoing efforts of the AFCD, and introduced ranavirus testing, environmental pathogen surveillance techniques, larval sampling, and sampling during the rainy warmer summer months (May-June).

This report details five years of pathogen surveillance to evaluate the presence and distribution of *Bd* and ranavirus in Hong Kong. We further sought to identify potential mechanisms of pathogen introduction and opportunities for exposure likely to threaten the region's biodiversity.

Materials and Methods

Ethics

All amphibian handling and sample collection activities performed in this investigation were approved and authorized by the AFCD.

Study sites and survey design

Surveys were performed in Hong Kong from 06 December 2010 to 29 December 2014. We designed a risk-based surveillance program under the assumption of low pathogen prevalence and restricted distribution, both of which would reduce our probability of detection. The majority of our surveys of wild amphibians were performed during cooler months (Sept-Jan) when *Bd* has sometimes been detected at higher prevalence (Kriger & Hero 2007; Whitfield et al. 2012; Murray et al. 2013), likely due to the reduced air and water temperatures that might increase its abundance and our chances to detect it (Skerratt et al. 2008). Traded amphibians

were also sampled to investigate their potential role in the introduction and spread of pathogens, and we assumed they would enable a higher detection rate due to transmission under crowded captive conditions. To optimise our survey efforts in detecting low prevalences and restricted distributions, we aimed to sample at least 10 amphibians per location in each survey event and amass an overall pool of at least 150+ amphibians each survey season. This approach could potentially miss a prevalence of 25-30% at each survey location in Hong Kong, but detect an overall prevalence as low as 2%, with 95% confidence (Thrusfield 2005). In instances where very few specimens were encountered and sampled despite intensive search effort, we still included these data.

Survey sites were spread throughout Hong Kong to increase the chance of detecting pathogens with limited distributions. Intensive sampling efforts encompassed each of the three main regions: Lantau Island, Hong Kong Island, and the New Territories (Figure 1). Wild amphibians were sampled from a variety of habitats ranging in quality and anthropogenic impacts from remote natural woodland habitats to artificial urban city ponds. Tai Mo Shan is the highest mountain peak in Hong Kong (957m), and was specifically targeted for surveillance since the presence of *Bd* is sometimes associated with cooler temperatures and higher elevations (Piotrowski et al. 2004; Sapsford et al. 2013).

Adopting a risk-based approach (Skerratt et al. 2008; Murray & Skerratt 2012), we directed greatest survey effort towards species with high predicted likelihood of exposure to water-associated pathogens due to their aquatic behaviour and/or those already considered a conservation concern in the region, in particular *Amolops hongkongensis, Odorrana chloronota, Quasipaa exilispinosa,* and *Quasipaa spinosa*. Additional species were sampled to increase the chance of detection in consideration of potential species-associated variation in pathogen

susceptibility (Woodhams et al. 2007). Most samples were collected from adult amphibians, but in 2013, larval amphibians were also included since they sometimes demonstrate greater prevalence of infection than adults (Skerratt et al. 2008; Langhammer et al. 2014). In this same year, surveys were also performed during the summer amphibian breeding period when increased amphibian densities in water bodies might elevate pathogen transmission and aid detectability, despite the warmer temperatures. During this survey season, environmental-DNA collection was also incorporated into surveillance to detect the presence of *Bd* shed into the water by infected animals.

Wild adult amphibians were captured by hand during both day and night, and tadpoles were collected by dip net primarily by day. Traded amphibians were sampled at Hong Kong International Airport upon importation and at domestic markets. All amphibians were tested for *Bd* presence, and a subset were also tested for ranavirus. Prior to sampling, animals were examined for gross abnormalities and signs of disease, such as skin lesions. We remained vigilant for amphibian mortality events throughout this investigation.

Amphibian Sampling

Amphibians were sampled for *Bd* primarily following the methods previously described by Rowley et al. (2007). Upon capture, each post-metamorphic amphibian was sampled for *Bd* with a sterile fine-tipped rayon swab (Medical Wire & Equipment Co. #MW113) drawn across the ventral surface of the animals' hands, feet and pelvic patch five times each (Hyatt et al. 2007). For larval amphibians, the swab bud was instead gently twirled in the buccal cavity as per Retallick et al. (2006). Swab buds were snapped off into 2 mL vials and either stored dry and frozen at -18C or at ambient temperature in vials containing 1 mL 70% ethanol (summer 2013

samples). A fresh pair of Nitrile gloves was worn for each new amphibian handled and changed between every sample to prevent cross contamination. In some instances, fresh pairs of disposable plastic bags were worn on the hands in lieu of gloves.

Amphibians were sampled for ranavirus following non-lethal methods described by Gray et al. (2009; 2012). Cloacal swabs were collected from adult amphibians by inserting a sterile rayon-tipped swab (Puritan Medical Products #P25-800R) into the cloaca and gently twirling the handle several times. For smaller amphibians and tadpoles, buccal swabs were instead collected by inserting the swab into the oral cavity and twirling several times. Swab buds were cut off into 2 mL vials containing 1mL 70% ethanol and scissors were then decontaminated by flaming with ethanol for 5 seconds before next use. All amphibians were immediately released following sampling.

Environmental DNA Sampling

During the summer 2013 survey, water samples were collected from amphibian habitats to detect *Bd* shed from amphibian skin. To capture this environmental DNA, we followed methods described by Kirshtein et al. (2007) with the exception that a peristaltic pump was used to increase the efficiency of sampling efforts by maximizing the volume of water processed. Sterile silicone rubber tubing was used in the pump, cleaned with a 10% bleach solution and then flushed with native water at each site prior to a new collection event. Water was passed through Sterivex filter capsules (0.22 micron pore size) until the flow rate greatly diminished and the total volume filtered was measured and recorded. The content of each filter capsule was then rinsed with 50 mL phosphate buffered saline solution with a sterile 60 mL syringe, pumped dry, and sealed with a bead of clay at the outlet spout of the capsule. Particles captured by the filter

were preserved by adding 0.9 mL of Qiagen ATL tissue lysis buffer with a sterile 1 mL syringe. Lastly, the inlet spout was sealed with a Luer-Lok™ screw cap and then a bead of quick-drying epoxy was applied behind the clay plug in the outlet spout to reinforce the seal during transport. Fresh pairs of Nitrile gloves were worn each time water was collected to prevent contamination. Most water samples were collected directly from amphibian habitats in the wild, but some were also collected from freshwater aquariums in commercial trade facilities that held non-native amphibian species. Whenever possible, amphibian swabs and water samples were concurrently collected from the same body of water.

Environmental and Amphibian Temperatures

Environmental conditions were measured at sample collection sites for free-ranging animals during the summer 2013 season. Records were made of the water and air temperatures and amphibian skin surface temperatures, when possible. Air temperature was measured with a Digital Thermo-Hygrometer (UEi Test Instruments, Inc. #DTH10), and accuracy was ±1˚C from -10˚- 45˚C. Amphibian skin temperature was measured using a Raytek ST81 Non-contact Infrared Thermometer (RAYST81, emissivity set to 0.95), from a distance of 0.5 m or less and water temperature was measured with the attachable RTD temperature probe. Accuracy of this thermometer was ± 1 % of reading or ± 1 °C, whichever was greater, as per manufacturer's specifications, and provided amphibian body temperature readings within 0.5 °C of cloacal temperatures (Rowley & Alford 2007).

Field biosecurity

Biosecurity measures were strictly employed throughout all field surveys to prevent accidental spread of amphibian pathogens both between animals and survey locations. Fresh pairs of Nitrile gloves and plastic bags were used to hold amphibians during sampling and discarded after single use. Depending on the survey team, either a bleach solution (10% commercial bleach) or Virkon® S was used to disinfect all field equipment exposed to amphibians or environmental substrates prior to moving to the next study site (e.g. field boots, dip nets, water filtration pump, etc.).

Sample Analysis

Bd swabs

Samples were processed via a sensitive quantitative PCR assay (qPCR) specific to *Bd* generally following the standard methods described by Boyle et al. 2004, with some exceptions. In May 2013, a subset of high priority samples were processed in Hong Kong by the veterinary team at Ocean Park via conventional PCR, using the materials and methods of Annis et al. (2004) and a SYBR safe DNA stain. Samples collected in 2014 were processed following the methods of Boyle et al. (2004), but with a "fast" protocol described by Kerby et al. (2014). All samples were run in triplicate except for those processed by conventional PCR, in singlicate. Equivocal results from samples run in triplicate were conservatively scored as *Bd*-negative to increase the specificity of results.

Ranavirus swabs

Taqman PCR for ranavirus used primers, probes, and protocols as described by Pallister et al. (2007), using the CON probe designed based on conserved segments of the ranavirus major capsid protein (MCP) gene. DNA was extracted from swabs using DNeasy Blood and Tissue Kits (QIAGEN Inc., Valencia, CA, USA) with spin columns, following the manufacturer's protocol. Samples were run in triplicate on an ABI/Applied Biosystems 7900HT thermocycler using 384 well plates with an exogenous internal positive control labeled with VICTM (Applied Biosystems) for each sample to detect PCR inhibitors. Samples amplifying at Ct's of < 50 in 2 or more wells were considered positive. A standard curve was created by diluting a synthetic plasmid PIDTSMART-AMP (Integrated DNA Technologies, San Diego, CA) containing the ranavirus MCP gene primers and probe sequences for the conserved MCP gene region (Genbank 298256130) insert from the above. The plasmid was diluted in nuclease-free water from 10^8 copies/5 ul in a series of eight 1:10 dilutions down to 10 copies/5 ul and run in duplicate along with a third well containing the exogenous master mix (EIC, Life Technologies).

Water filters for Bd

Taqman PCR for *Bd* was generally based on the method, primers and probe of Boyle et al. (2004). Water filter samples were processed following the method of Kirshtein et al. (2007). Reactions used the Taqman Environmental Mastermix 2.0 (Applied Biosystems). Samples were run in triplicate on an ABI/Applied Biosystems 7900HT thermocycler as described above. Samples amplifying at a Ct of < 50 in 2 or more wells were considered positive. Quantification standards were created by growing *Bd* isolate JEL 197 on 1% tryptone agar and harvested of zoospores by rinsing plates with 1X PBS. After collection zoospores were counted three times

on a hemocytometer to determine a range of zoospores ml^{-1}. Standard curves were generated with ten-fold serial dilutions (range $1x10^{6}$ to $1x\ 10^{-2}$ zoospores). In addition to positive controls (quantification standards), each plate included a negative control (Taqman mastermix and no sample DNA) as well as 4 positive and negative quality assurance controls consisting of swabs either inoculated with *Bd* zoospores or sham-inoculated. For *Bd*-positive samples, the number of zoospores per liter of water was estimated to reflect the approximate pathogen density in the original volume of water.

Results

Bd Swabs

The presence of *Bd* was detected in 21/1,289 amphibians sampled from the wild in Hong Kong and 25/391 animals held in captivity for commercial trade (Tables 1-2). In total, 21 species were sampled in the wild and four in captivity. Of these, *Bd*-positive amphibians included four native species (*Amolops hongkongensis, Hoplobatrachus rugulosus, Odorrana chloronota, Xenophrys brachykolos*) and one exotic species (*Xenopus laevis*). *Bd*-positive amphibians were detected at each of the three regions sampled: Hong Kong Island, the New Territories, and Lantau Island (Figure 2). The annual prevalence of *Bd* in free-ranging amphibians detected from 2010-2014 ranged from 0-5%. In traded amphibians, overall *Bd* prevalence ranged from 5.8% to 7.4% in 2012 and 2013, respectively, but was found as high as 30.8% in a shipment of *H. rugulosus* imported at Hong Kong International Airport in May 2013, where 8/26 bullfrogs tested positive.

Ranavirus Swabs

Ranavirus was detected in 10.5% (47/ 440) of free-ranging and 36.9% (55/149) of traded amphibians sampled in Hong Kong (Table 3). Ten of 16 species sampled in the wild tested positive for ranavirus, and 1/4 species sampled at commercial markets. Ranavirus-positive amphibians were found to be widespread and were present in the wild at each of the three regions surveyed, at three domestic food markets sampled, and upon importation at Hong Kong International Airport (Figure 2).

Bd Water Filters

Water was filtered to detect the presence of *Bd* at locations where amphibians were sampled. In total, 50 water filters representing 98.7 L of water were processed from amphibian habitats and 8 water filters representing 1.7 L from pet stores. *Bd* was not detected in any water samples collected from the wild, but was detected in water from 3/4 pet stores (6/8 samples). In these samples, *Bd* density ranged from 1.76 to 73.4 zoospores/liter.

Temperatures

Pathogen surveillance was performed both by day (10:00 – 19:00 hrs) and night (19:00 – 24:00 hrs). During the 2013 summer field season, night time temperatures of air, water, and amphibian dorsal surfaces ranged from 21.8–29.2°C, 21.1–28.2°C, and 21.1–30.2°C, respectively, whereas day temperatures averaged ranged from 22.2–32.4°C, 20.3–29.4°C, and 20.8–30.6°C, respectively.

Amphibian Mortality

An amphibian mass mortality event was recorded in 2013 at Shing Mun Country Park, where 17 dead and dying Chinese bullfrogs (*Hoplobatrachus rugulosus*) were encountered in a small stream feeding into the reservoir approximately 100m below. Of these, 16/17 bullfrogs tested positive for ranavirus, and all tested negative for *Bd*. Several meters away, a small bundle of recently burnt incense was found, and together with the presence of this species in high density at an atypical habitat suggested a recent Buddhist merit release event; a religious ceremony whereby animals often purchased from the food trade are saved from slaughter and released back into the wild.

Amphibian Abnormalities

In May 2013, severe skin lesions were observed on two adult Hong Kong newts (*Paramesotrition hongkongensis*) when examined prior to sampling for *Bd* and ranavirus presence (Figure 3). Both displayed necrotic facial lesions exposing bone. Neither animal tested positive for *Bd* nor ranavirus and the cause remains unknown.

Discussion

We provide confirmation of the presence of *Bd* and ranavirus in Hong Kong, a global hub of international amphibian trade. *Bd* and ranavirus were both detected in wild amphibians, traded amphibians upon importation at Hong Kong International Airport, and exotic species at domestic markets in Hong Kong. An exceptionally high prevalence of ranavirus was detected among Chinese bullfrogs sampled at Hong Kong food markets in 2013, where 75.3% (55/73) tested positive. Although *Bd* and ranavirus were found to be widespread in Hong Kong and the risk of

additional introduction is high, infections have not yet been conclusively associated with mortality and amphibian population declines have not been recorded in the region.

Genotyping is needed to identify the *Bd* lineages and ranavirus species present in Hong Kong and determine if they are endemic or recent introductions. Endemic Asian lineages of *Bd* have been identified in Japan, China, and Korea (Goka et al. 2009, Bai et al. 2012; Bataille et al. 2013), in addition to the recently spreading global pathogenic lineage. Similarly, ranaviruses occur in Asian amphibians and there have been outbreaks among both native and introduced alien species (Une et al. 2009; Xu et al. 2010). Like our results in Hong Kong, the prevalence of *Bd* previously measured in wild amphibians across Asia has been relatively low, e.g. a survey that included 3,363 animals from 14 Asian countries and Papua New Guinea demonstrated an overall prevalence of 2.35% (Swei et al. 2011). Another expansive survey that tested 2,389 animals across four Asian countries, primarily amphibians sampled from the commercial trade, detected a very low *Bd* prevalence of 0.59%, and did not detect ranavirus in the subset of 74 frogs sampled (Gilbert et al. 2013). Still, higher prevalence has been detected in introduced exotic species such as feral American bullfrogs *Lithobates catesbeianus* sampled in South Korea where 24.7% tested positive (Bataille et al. 2013). It is plausible that Asian frogs may have higher resistance to endemic Asian strains of *Bd* than frogs in countries without a similarly long history of infection (Bataille et al 2013). Furthermore, the high temperatures we measured at amphibian sample sites in Hong Kong approached the upper limits of *Bd* and ranavirus survival; exposure to 32°C for four hours is lethal to *Bd* (Berger et al. 2004) and replication of the type ranavirus, FV3 ceases at 33°C (Granoff et al. 1966). Thus, it remains plausible that periodic elevated temperatures in regions of tropical Southeast Asia might limit the reproduction and spread of these pathogens. Further efforts to obtain isolates and histological samples of *Bd* and

ranavirus present in Hong Kong are needed to enable their characterisation and predict likely impacts on amphibian populations.

Even if Hong Kong possesses endemic forms of these pathogens, it is important to reduce introduction of new strains that may demonstrate elevated virulence to native amphibians and present the risk of recombining to form unpredictable new strains. During this investigation, several high risk anthropogenic activities likely to introduce non-native pathogens were observed in Hong Kong. Firstly, the release of exotic reptiles and amphibians appears to be a common practice, both as unwanted pets and in religious merit release ceremonies. The intent to release these animals in locations where they are likely to survive not only introduce direct competition with native wildlife (e.g. red-eared sliders *Trachemys scripta scripta* are now common in protected areas throughout Hong Kong), but also provides ongoing opportunities for pathogen spread and persistence. We observed released frogs and turtles both in urban parks and in protected natural areas. Secondly, pet shops and wet markets disposed potentially pathogen-laden water directly outdoors, into roadside storm drains that often empty into the environment untreated. At some of these pet shops, aquarium tanks of water held high densities of African clawed frogs (*Xenopus laevis*) and fire-bellied newts (*Cynops orientalis* and *Pachytriton* spp.). At the wet markets, cages of imported bullfrogs were sometimes balanced over tanks of live fish providing opportunities for transmission of ranavirus from frogs to fish, which is a particular concern if these fish are purchased and become stock for outdoor ponds or aquaculture. Additionally, an invasive amphibian species (the Greenhouse frog *Eleutherodactylus planirostris*) was observed that is now widespread in Hong Kong and believed to have been introduced accidentally through the live plant trade (Lee et al. 2016). Fortunately, the *E. planirostris* we sampled in Hong Kong for *Bd* and ranavirus tested negative, but introduced

animals have tested positive for *Bd* in Florida (Rizkalla 2010) and Jamaica (Holmes et al. 2012). In the absence of more stringent import biosecurity procedures, additional animals are likely to be introduced and may carry these pathogens. Irrespective of the uncertain source and virulence of the *Bd* and ranavirus we detected in Hong Kong, biosecurity is urgently needed to protect native species from exposure to imported pathogens.

Amphibians co-infected with *Bd* and ranavirus have been found in the United States, Costa Rica, and Peru (Souza et al. 2012; Whitfield et al. 2013; Warne et al. 2015), and we report the first records among wild and captive animals in Asia. In 2013, 9/26 bullfrogs (*Hoplobatrachus rugulosus*) sampled upon importation at Hong Kong International Airport tested positive for ranavirus, and of those, 8 additionally tested positive for *Bd*. In 2014, nine frogs sampled in the wild also exhibited co-infection, including three species (*Amolops hongkongensis, H. rugulosus, Odorrana chloronota*). It is possible that infection by one pathogen may increase susceptibility to another, but patterns of *Bd*/ranavirus co-infection have not yet been linked to the infection intensity of either (Warne et al. 2015) and further investigation is needed before the additive consequences of this condition can be ascertained. Hoverman et al. (2012) found that co-occurrence of *Bd* and ranavirus was common among many amphibian communities in California, and we also observed this in Hong Kong. Co-occurrence, but not co-infection, was detected at a site of particular conservation concern - Tai Mo Shan Country Park. In a single night at this location, two adult *Amolops hongkongensis* tested positive for *Bd* and one adult *Quasipaa spinosa* tested positive for ranavirus. The concurrent detection of both pathogens in this amphibian habitat is reason for greater vigilance because *A. hongkongensis* is listed as endangered on the IUCN Red List of Threatened Species and *Q. spinosa* is in decline across its range and is only found at this single location in Hong Kong. This mountain area includes Hong

Kong's highest peak and the cooler climate might provide the most optimal year-round conditions for *Bd* and ranavirus to persist within the region. Long-term surveillance should continue to be invested in monitoring these pathogens and the status of amphibian populations at Tai Mo Shan.

Ranavirus was found to be widespread in Hong Kong and exotic strains may threaten the territory's native turtles. Hong Kong possesses five native species of chelonians (excluding sea turtles), many of which now exist in greatly reduced numbers due to previous overexploitation for the food trade. Some ranaviruses can spread from amphibians to reptiles (Brenes et al. 2014), and therefore, our detection of ranavirus-positive bullfrogs imported at Hong Kong International Airport demonstrates a risk of disease not only to native amphibians, but also to native reptiles. Proactive surveillance for ranavirus presence in the region's turtles is warranted, and ranaviral disease should be considered if chelonian mortalities are encountered.

Despite our confirmed detections of *Bd* and ranavirus in Hong Kong, disease-driven population declines do not yet appear to have impacted species in the region. This is remarkable given the frequent importation of amphibians into Hong Kong and the absence of control measures to prevent pathogen introduction and spillover. Preemptive measures are urgently needed to protect the regions' biodiversity from exotic pathogens before irreparable damage occurs. Since Hong Kong is a major hub of international amphibian trade, control measures to mitigate the spread of amphibian pathogens would provide both regional and global conservation benefits.

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Table 1. Free-ranging amphibians sampled for *Bd* presence in Hong Kong. Most amphibians were sampled from September-January except those listed under 2013A, which were sampled May-June. *Bd*-positive amphibians are listed in parenthesis.

Species	2010	2011	2012	2013A	2013B	2014
Amolops hongkongensis	31	48	56	16 (2)	42 (1)	49 (2)
Duttaphrynus melanostictus	0	30	1	47	1	0
Eleutherodactylus planirostris	0	13	65	0	39	0
Fejervarya limnocharis	0	5	2	17	1	0
Hoplobatrachus rugulosus	0	1	14	18	11	8 (1)
Hylarana guentheri	0	5	2	24	3	1
Hylarana latouchii	0	2	3	0	4	0
Kalophrynus interlineatus	0	2	2	1	1	0
Kaloula pulchra pulchra	0	14	3	6	0	0
Leptolalax liui	0	0	0	2	3	0
Limnonectes fujianensis	0	0	0	0	4	3
Liuixalus romeri	0	3	0	0	0	0
Microhyla fissipes	0	1	3	2	1	0
Microhyla pulchra	0	0	0	6	0	0
Odorrana chloronota	32	19	35	9	31	31 (6)
Paramesotriton hongkongensis	0	1	5	14	1	40
Polypedates megacephalus	0	6	2	74	2	0
Quasipaa exilispinosa	28	59	66	18	60	42
Quasipaa spinosa	11	3	17	8	10	6
Xenophrys brachykolos	3	4	12 (1)	3	3	0
Xenopus laevis	0	0	15 (8)	0	0	0
Unidentified species	0	0	0	3	0	0
Total	105	216	303 (9)	268 (2)	217 (1)	180 (9)

Table 2. Traded amphibians sampled for *Bd* presence in Hong Kong. *Bd*-positive amphibians are listed in parenthesis.

Species	2012	2013
Hoplobatrachus rugulosus	205	73 (8)
Xenopus laevis	37 (14)	60 (3)
Cynops orientalis	0	10
Pachytriton labitaus	0	6
Total	242 (14)	149 (11)

Table 3. Amphibians sampled for ranavirus presence in Hong Kong. Ranavirus-positive amphibians are listed in parenthesis.

Species	2013A	2014
Free-Ranging Amphibians		
Amolops hongkongensis	15	49 (5)
Duttaphrynus melanostictus	46 (1)	0
Fejervarya limnocharis	14 (1)	0
Hoplobatrachus rugulosus	18 (17)	8 (5)
Hylarana guentheri	24 (1)	1
Kalophrynus interlineatus	1	0
Kaloula pulchra pulchra	3	0
Leptolalax liui	2	0
Limnonectes fujianensis	0	3
Microhyla fissipes	2 (1)	0
Microhyla pulchra	6	0
Odorrana chloronota	9	31 (8)
Paramesotriton hongkongensis	14	40
Polypedates megacephalus	74 (3)	0
Quasipaa exilispinosa	18 (2)	42 (2)
Quasipaa spinosa	8 (1)	6
Xenophrys brachykolos	3	0
Unidentified species	3	0
Total	260 (27)	180 (20)
Traded Amphibians		
Hoplobatrachus rugulosus	73 (55)	0
Xenopus laevis	60	0
Cynops orientalis	10	0
Pachytriton labitaus	6	0
Total	149 (55)	0

Figure 1. Three regions intensively surveyed for *Bd* and ranavirus presence in Hong Kong: New Territories, Lantau Island, and Hong Kong Island.

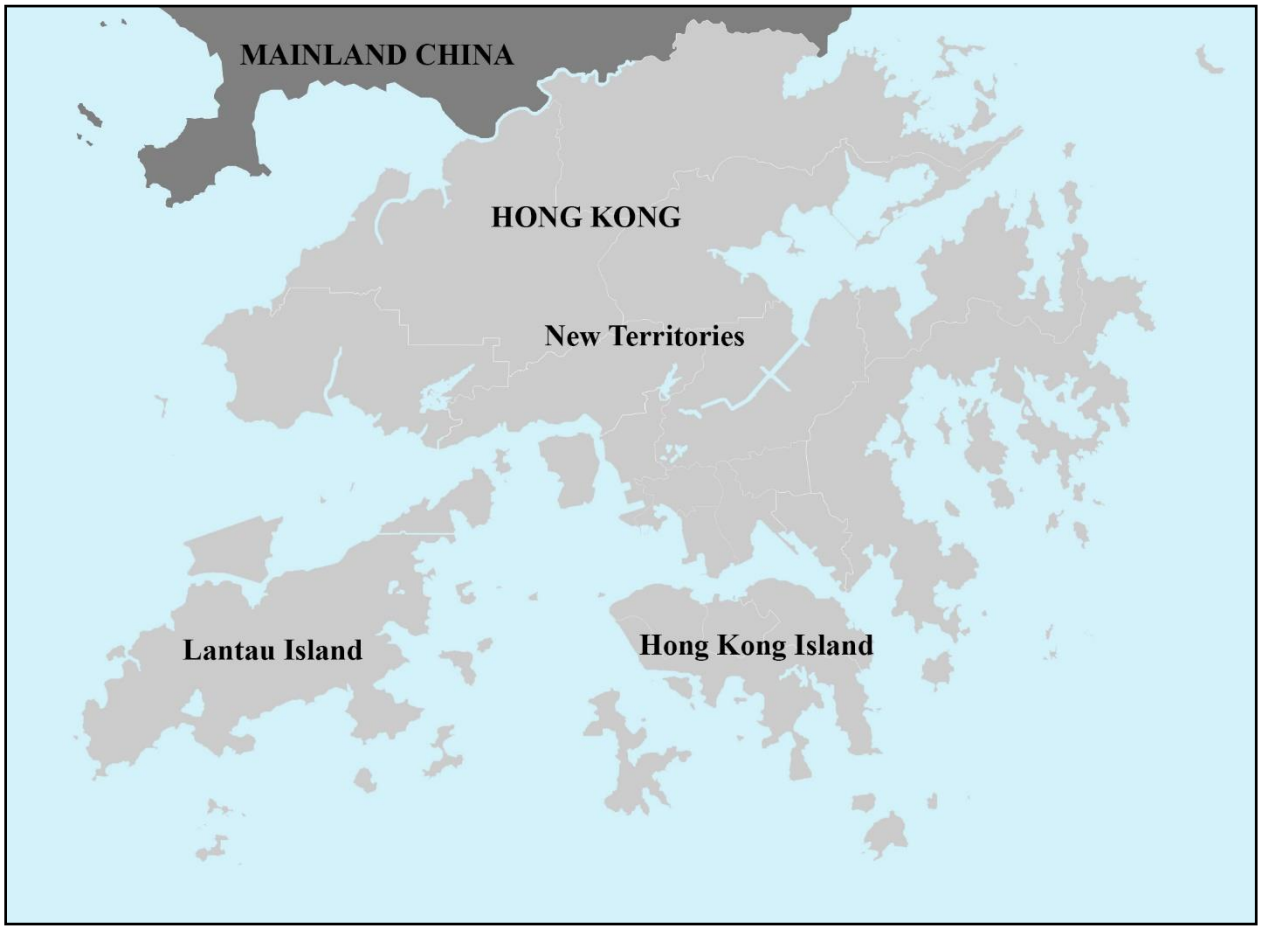

Figure 2. Distribution of *Bd* and ranavirus detected in Hong Kong from 2010-2014. Squares represent *Bd* and triangles represent ranavirus.

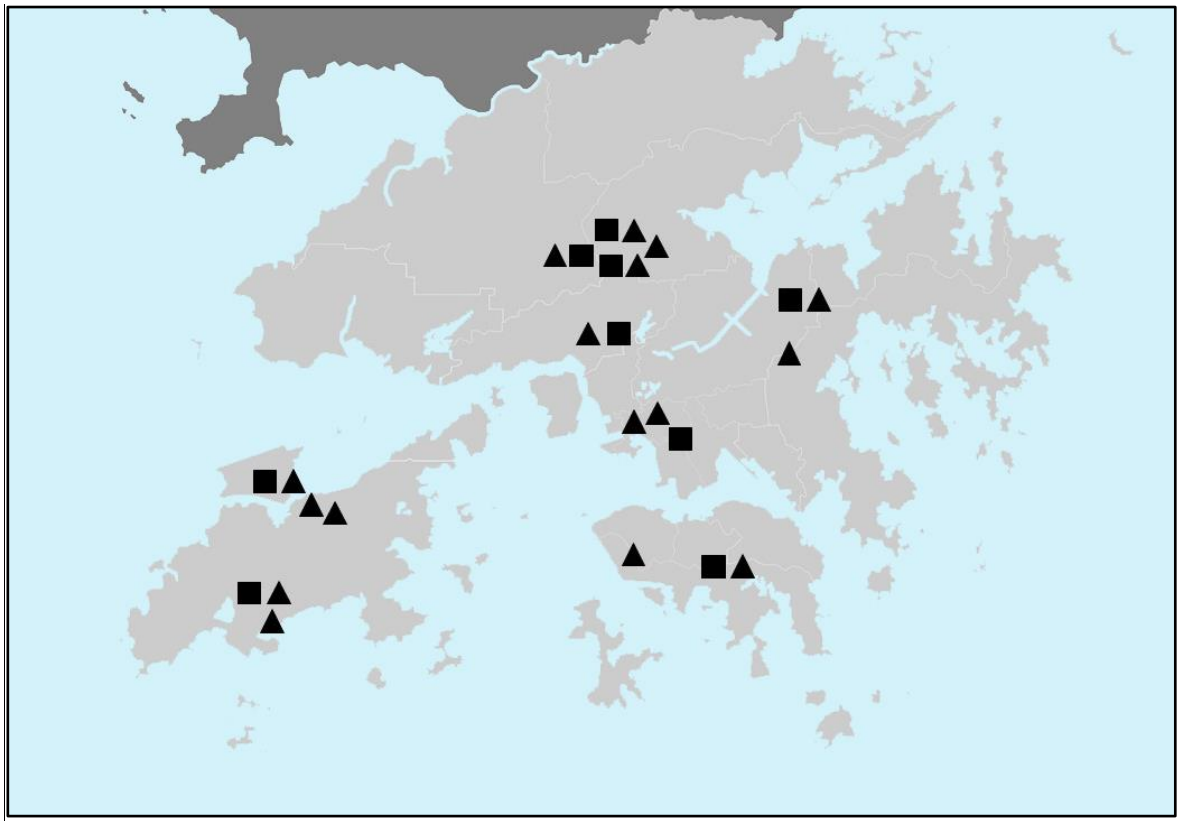

Figure 3. Severe facial lesion observed on a Hong Kong newt (*Paramesotrition hongkongensis*) in 2013. This animal tested negative for both *Bd* and ranavirus.

CHAPTER 5

Aerial spread of amphibian chytrid fungus

Introduction

Chapters 2 – 4 primarily focus on the role of human-assisted movement of amphibians in the spread of *Bd*. In contrast, Chapters 5 – 7 explore the spread of *Bd* in the absence of commercial activity. In my review of *Bd* distribution in Chapter 1, I noticed that the presence of *Bd* has also been recorded in remote wilderness areas far removed from regions of trade and other intensive human activities.

I hypothesized that *Bd* may spread into such regions via atmospheric dispersal made possible by the aerosolization of contaminated environmental substrates. I explored this idea in Cusuco National Park, a cloud forest in Honduras where waterfalls are present, a high prevalence of *Bd* in aquatic amphibians has been detected, and river water is ejected into the atmosphere. The viability of *Bd* detected by water filtration is unknown, but my primary aim was to show if the possibility of aerial spread exists. This study involved the sampling of rainwater to capture particles in the atmosphere and is **Paper 1** of this chapter: "Presence of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) in rainwater suggests aerial dispersal is possible", which has been published in Aerobiologia.

Paper 1

Citation: Kolby JE, Ramirez SD, Berger L, Griffin DW, Jocque M, Skerratt LF. (2015) Presence of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) in rainwater suggests aerial dispersal is possible. Aerobiologia 31:411-419.

*Presence of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) in rainwater suggests aerial dispersal is possible*

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ORIGINAL PAPER

Presence of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) in rainwater suggests aerial dispersal is possible

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CHAPTER 6

Unintentional spread of chytrid through human foot-traffic (fomites)

Introduction

In Chapter 5, I detected the presence of *Bd* in rainwater and demonstrated the existence of an aerial pathway of *Bd* dispersal. Unfortunately, the viability of *Bd* detected by PCR through water filtration could not be determined and it is unknown whether this mode of transport commonly results in new infections. The source of this *Bd* is also unknown but I believe the *Bd*-contaminated river water I detected could have been ejected into the atmosphere by the abundance of waterfalls in this forest.

Bd-contaminated river water might also be dispersed by terrestrial activities. For example, people frequently pass through amphibian habitats during recreational or scientific activities and *Bd*-contaminated water or sediment might adhere to objects, such as footwear. For this reason, hygiene protocols to sterilize field equipment are often observed by amphibian researchers, but the spread of *Bd* through this potential pathway has not yet been tested in the field. Further, while amphibian researchers are likely to attempt to minimize the accidental spread of *Bd*, the growing popularity of ecotourism represents a considerable number of people which regularly pass through amphibian habitats where footwear may become contaminated. To explore whether non-decontaminated footwear commonly provides a vehicle for the spread of *Bd*, I performed a study in Cusuco National Park in Honduras, where my previous research showed the presence of *Bd*-contaminated river water. This study is **Paper 1** of this chapter: "Low Risk of Amphibian Chytrid Dispersal and Establishment by Human Foot-Traffic", which has not yet been published.

Paper 1

Citation: Kolby JE, Ramirez SD, Berger L, Skerratt LF. Low Risk of Amphibian Chytrid Dispersal and Establishment by Human Foot-Traffic. (unpublished manuscript)

Abstract
Although the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*, *Bd*) has been shown to cause worldwide amphibian population declines, the global spread of this pathogen continues largely unabated. Various pathways of local and global dispersal have been theorized, focusing on human-assisted *Bd* spread such as trade in live amphibians. On a local level, attention has been directed towards the potential movement of zoospores on contaminated footwear. Disinfection of footwear is widely recommended when moving between aquatic field sites, but little empirical data exists to evaluate the risk of spread under natural conditions. From 2010 to 2011, I performed field trials in Cusuco National Park, Honduras to detect the presence of *Bd* on rubber field boots following exposure to *Bd*-positive water bodies and then walking distances of up to 60m away in the contaminated footwear. Water was concurrently sampled to establish the environmental presence of *Bd* and boots were sampled before, during, and after submersion in a river. PCR analysis confirmed the presence of *Bd* DNA on the underside of a rubber boot in 1/88 swab samples, whereas *Bd* was detected in all water filter samples. These data suggest that *Bd* is uncommonly transported between separate aquatic habitats via foot-traffic, and although plausible, does not easily explain the enigmatic establishment of *Bd* in remote montane regions. Regardless, I advocate decontamination protocols be observed to reduce this easily mitigated risk of spreading *Bd*, as well as other aquatic pathogens that could also be present.

Introduction

Spread of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) is responsible for the emerging infectious disease chytridiomycosis, now a major contributing factor in the global decline and extinction of amphibians (Skerratt et al. 2007; Stuart et al. 2004). Susceptible amphibians may experience epidermal hyperkeratosis, hyperplasia, and electrolyte imbalance

culminating in cardiac arrest (Voyles et al. 2009). Previously investigated mechanisms of *Bd* dispersal include the movement of infected amphibians, carriage of *Bd* on waterfowl feet or feathers, and the transport of contaminated water and sediment (Rowley and Alford 2009; Garmyn et al. 2012; Johnson and Speare 2005, Pauza et al. 2010). Although *Bd* can be disseminated by water currents and may be carried between separate water bodies by *Bd*-positive wildlife, most long-distance dispersal is thought to be the consequence of anthropogenic activity, particularly the international trade in live amphibians (Weldon et al. 2004; Fisher and Garner 2007, Schloegel et al. 2009).

Remote locations seemingly isolated from amphibian trade have also become exposed to *Bd*, and it remains unknown how the pathogen arrived. *Bd*-related amphibian declines were first described from wilderness regions in Panama and Australia, far from cosmopolitan areas where amphibian trade is likely to occur (Berger et al. 1998). Despite the added protection of geographic isolation, even island nations such as Montserrat (Garcia et al. 2009) and Tobago, West Indies (Alemu et al. 2008) became exposed to *Bd* in the absence of commercial amphibian importation. Not only did *Bd* arrive in these regions, but it also became established and sparked waves of disease, suggesting the unidentified *Bd* dispersal pathway is highly efficient and/or frequent. The extreme contemporary mobility of humans might assist the spread of *Bd* if recreational and scientific activities translocate *Bd*-contaminated substrates. One such potential pathway is commonly discussed —the spread of *Bd* by footwear exposed to a contaminated environment (Phillott et al. 2010) — but whether this occurs under natural field conditions has not yet been tested. To better understand the risk of *Bd* spread, I investigated the presence of *Bd* on contaminated footwear following exposure to *Bd*-positive environments and walking distances of up to 60m away in the contaminated footwear.

Methods

This study was conducted in Cusuco National Park (CNP), a fragment of montane cloud rainforest in the Merendon Mountains of northwestern Honduras. Surveys for *Bd* in CNP were first conducted in 2007, when this pathogen was found to be widely distributed throughout all watersheds sampled in the park. Certain stream-associated frog species were highly infected, with prevalence approaching 80% (Kolby et al. 2010). *Bd* surveys continued annually thereafter (2008-2011) consistently demonstrated the presence of moderate to high *Bd* prevalence (J. Kolby unpubl. data). The abundance of infected tadpoles releasing zoospores into in CNP's rivers made this location an optimal *Bd*-positive environment in which to conduct this investigation.

Fieldwork was performed from 5 June 2010 to 2 August 2010 and again on 26 June 2011 at aquatic habitats in CNP where *Bd*-positive tadpoles were detected in previous surveys. At each aquatic habitat, an aliquot of water was first collected and filtered to identify the presence of *Bd* to develop the context for the following exposure trials. Water was filtered following the method described by Kirshtein et al. (2007), with the exception that a bead of plumber's epoxy was used to reinforce the clay sealant plug in the filter capsule to reduce the risk of sample leakage during transport. Water temperature and pH were measured at each sample collection site with a portable handheld meter (Hannah Instruments #HI98128).

Boot swab samples were collected to identify the potential presence of *Bd* on the sole of a rubber Wellington field boot worn by a field assistant. Each sample was produced by dragging a rayon swab against the entire length the underside of the left boot, 20 times, moving from left to right to increase the surface area swabbed. The swab bud was then snapped off and stored in a 2 mL vial containing 1 mL 70% ethanol as preservative. This same swabbing process was

followed to produce each sample, after varying degrees of boot exposure to the environment, as described below.

Samples were collected at 6 separate rivers in CNP (Table 1, Sites A-F) in 2010 and at one site in 2011 (Site F). A series of 6 swab samples were collected per visit at each site, except for one site (Site F), where the entire 6-swab collection process was consecutively repeated 5 times to test repeatability of results. In each trial, the first swab was collected prior to river water exposure, but after the boot had been worn while walking through the forest to reach the river. This was done to make the distinction between potential *Bd*-contamination from terrestrial substrate sources (assumed to be lower risk) versus river water (assumed to be higher risk). After this first swab, the research assistant then entered the center of the river and shuffled boots through the water for a duration of 10 seconds. Standing in place, the left boot was raised and a swab sample #2 was collected. The next four swab samples (#'s 3−6) were collected after again shuffling through the water for 10 seconds, and then walking away from the rivers' edge into the forest, stopping to sample at increasing distances of 15, 30, 45, and 60m. A boot swab was collected at each stop point, and then the assistant returned to the river to begin the process again and walk the next distance. A fresh pair of Nitrile gloves was worn while collecting each sample and changed in between to prevent cross-contamination. The initial boot swab prior to entering the water was accidentally excluded from collection at Sites A & B, hence a total of 5 versus 6 samples collected at both of these locations.

Water filter samples were submitted to the United States Geological Survey (USGS) Reston Molecular and Environmental Microbiology Laboratory for analysis. Sample processing to detect and quantify the presence of *Bd* in water samples followed the methods of Kirshtein et al.

2007. Meanwhile, all boot swab samples were submitted to the Amphibian Disease Diagnostic Laboratory at Washington State University for qPCR analysis following Qiagen DNA extraction.

Results

All seven river water samples collected from CNP tested positive for the presence of *Bd* by qPCR (Table 1). The density of *Bd* in samples collected in 2010 was consistently low (< 2 zoospores/L), but higher in 2011 (23.49 zoospores/L at Site F). In total, 88 boot swab samples were collected to detect the presence of *Bd* and one sample tested positive. This sole *Bd*-positive result was collected at Site D in 2010 and was sample #2 in the series − the swab collected from the boot underside immediately following the 10-second exposure, while still standing in the river. The amount of *Bd* material present on this swab was exceptionally low (0.01 zoospore equivalent).

Discussion

Bd was rarely detected on footwear despite numerous intentional exposure events in an ecologically relevant setting of *Bd* presence. This suggests that human foot-traffic does not commonly contribute towards the spread of *Bd*. In 75 exposures to *Bd*-positive river water, *Bd* was only detected once. The *Bd* sampling method employed in the field trials was imperfect, since additional droplets of water adhered to other surfaces of the boot that were not captured by the swab. Still, our negative results of boot swabs are to be expected considering the very few *Bd* zoospores/L in the aquatic environment and the small volume of water that would be residue on the boot.

Other water bodies may have higher zoospore densities and hence pose a greater risk for contamination of fomites. For instance the highest density of *Bd* detected at our site was 23.49

zoospores/L whereas Cossel & Lindquist (2009) detected a concentration of 6,780 zoospores/L in the water that collected inside the tank of an arboreal bromeliad. Similarly, in larger bodies of stagnant water such as ponds and marshes, with high densities of infected amphibians, *Bd* zoospores may accumulate in the water (Walker et al. 2007). In addition, *Bd* prevalence in amphibians often fluctuates seasonally, usually peaking during cooler months (Berger et al. 2004; Kriger and Hero 2006; Hyman and Collins 2011), and environmental levels would fluctuate similarly. For these reasons, the risk of *Bd* dispersal by foot-traffic is expected to be variable and context-dependent.

Dispersal of *Bd* could similarly occur by the movement of wildlife through *Bd*-positive water bodies. For instance, large mammals such as tapir and peccary are present in CNP and cross these same *Bd*-positive rivers. A greater volume of water may temporarily adhere to the fur compared to that carried on the surface of human footwear and could contribute towards short-distance *Bd* dispersal, although exposure to mammalian body temperatures can kill *Bd* within hours.

Bd continues to spread globally at a rapid pace and the identification of common dispersal pathways can help illuminate potential opportunities to control further spread. Our data suggests that while possible, dispersal by contaminated footwear is an unlikely source of *Bd* emergence in remote wilderness areas like CNP. Still, before similar assumptions can made for different locations, baseline sampling to assess the abundance of *Bd* in that particular environment is highly advised. Even if environmental substrates carry low pathogen densities, field decontamination procedures should always be observed to prevent accidental *Bd* spread that can be easily avoided (Phillott et al. 2010). This is especially important for travel between sites of high biological value and high risk (Phillott et al. 2010). Further investigation is needed to better

understand patterns of *Bd* dispersal neither clearly associated with the commercial amphibian trade nor human foot-traffic.

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Table 1. Detection of *Bd* in water samples collected from sites where foot-dispersal trials were performed in Cusuco National Park, Honduras in 2010-2011. PNQ= positive but not quantitated.

Site#	Site Name	Date	Mean Zoospore equiv/L	Vol filt (mL)	T (C)	pH	Main terrestrial substrate
A	Rio Danto	5 June 2010	1.07	4620	18.8	6.7	Moist leaf litter
B	Cantiles T5 Stream	13 July 2010	PNQ	3720	15.9	6.3	Moist leaf litter
C	Rio Cantiles	13 July 2010	1.05	3000	18.0	7.0	Dry pine needles/ leaf litter
D	Rio Buenos Aires T1	23 July 2010	PNQ	3300	21.2	7.1	Moist soil
E	Rio New Eden	29 July 2010	1.08	2880	18.9	6.4	Dry sandy soil
F_1	Rio Cusuco BC	2 August 2010	1.53	4440	17.3	6.8	Moist sand/clay
F_2	Rio Cusuco BC	27 June 2011	23.49	4500	17.1	5.8	Moist sand/clay

CHAPTER 7

Terrestrial spread of chytrid fungus by amphibian locomotion

Introduction

In Chapters 5 and 6, I found evidence for *Bd* dispersal in the absence of amphibian trade, but my samples generally showed low densities of *Bd*, likely due to environmental dilution of *Bd* after it is shed from an amphibian host. While the spread of any amount of *Bd* qualifies my aim to identify the presence of a dispersal pathway, the transport of material carrying high amounts of *Bd* is more likely to transmit new infections.

Although a water-associated pathogen, *Bd* has been detected in many terrestrial amphibians and the source of infection remains unclear. Amphibians strongly associated with aquatic habitats are most likely to become exposed to *Bd*, but are generally unlikely to travel long distances away from water. Still, as tadpoles metamorphose into frogs and emerge into nearby terrestrial vegetation, their presence might sometimes overlap with amphibian species that don't normally enter the water. In this chapter, I performed a study to investigate the spread of *Bd* between aquatic and terrestrial habitats through the locomotion of infected metamorphosing frogs. This study is **Paper 1** of this chapter: "Terrestrial Dispersal and Potential Environmental Transmission of the Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*)", which has been published in PLoS ONE.

Paper 1

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RESEARCH ARTICLE

Terrestrial Dispersal and Potential Environmental Transmission of the Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*)

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Abstract

Dispersal and exposure to amphibian chytrid fungus (*Batrachochytrium dendrobatidis*, *Bd*) is not confined to the aquatic habitat, but little is known about pathways that facilitate exposure to wild terrestrial amphibians that do not typically enter bodies of water. We explored the possible spread of *Bd* from an aquatic reservoir to terrestrial substrates by the emergence of recently metamorphosed infected amphibians and potential deposition of *Bd*-positive residue on riparian vegetation in Cusuco National Park, Honduras (CNP). Amphibians and their respective leaf perches were both sampled for *Bd* presence and the pathogen was detected on 76.1% (35/46) of leaves where a *Bd*-positive frog had rested. Although the viability of *Bd* detected on these leaves cannot be discerned from our quantitative PCR results, the cool air temperature, closed canopy, and high humidity of this cloud forest environment in CNP is expected to encourage pathogen persistence. High prevalence of infection (88.5%) detected in the recently metamorphosed amphibians and frequent shedding of *Bd*-positive residue on foliage demonstrates a pathway of *Bd* dispersal between aquatic and terrestrial habitats. This pathway provides the opportunity for environmental transmission of *Bd* among and between amphibian species without direct physical contact or exposure to an aquatic habitat.

Introduction

Infection by the pathogenic amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) poses a major threat to global amphibian biodiversity [1,2]. Response to infection varies considerably between species; a minority of those tested generally carry *Bd* in the absence of

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morbidity and may serve as aclinical reservoir hosts, such as the American bullfrog, *Lithobates catesbeianus* [3], and the African clawed frog, *Xenopus laevis* [4], whereas others are highly susceptible to chytridiomycosis and have suffered dramatic decline following introduction in wild populations [5,6]. Variation in virulence has been observed, and exposure to certain isolates of the highly pathogenic *Bd*GPL clade causes mortality in amphibians more quickly than others [7,8]. *Bd* demonstrates low host species specificity and as of 2012, infection had already been reported in 516 species from 52 countries [9], and evidence suggests this pathogen is native in some parts of its range but emerging and spreading in others [10,11]. Identified 15 years ago [12], the geographic origin and subsequent pathways of global and local *Bd* dispersal remain largely speculative, although recent studies show *Bd* is now commonly spread via the international and domestic trade in live amphibians [13–16]. However, mechanisms of dispersal outside the amphibian host and in the absence of anthropogenic assistance are more obscure.

Direct and indirect modes of *Bd* dispersal and transmission within wild amphibian populations have been postulated, but few have been demonstrated. Direct contact between animals engaged in amplexus or territorial confrontation is thought to be a common mode of transmission [17]. Contact with contaminated water is another avenue, and *Bd*'s motile uniflagellated zoospores can disperse through a water body either by swimming short distances or by being carried in water currents [18]. Waterfowl might carry *Bd* between separate water bodies, either on their feathers or feet [19–21]. However, the high prevalence of *Bd* detected in terrestrial and arboreal amphibian species that infrequently contact each other and typically do not directly engage with other species or enter permanent water bodies [22–25], suggests the presence of additional avenues of *Bd* dispersal and environmental transmission. For example Burrowes et al. [26] detected a high prevalence of infection (44.1%) in *Eleutherodactylus coqui*, a direct-developing terrestrial anuran inhabiting leaf litter in the cloud forest in Puerto Rico and McCracken et al. [27] found 33% of canopy-dwelling amphibians infected in a lowland Ecuadorian rainforest. *Bd* has also been detected on 62% of terrestrial soil-dwelling caecilians sampled in Cameroon [28,29]. Collectively, the detection of *Bd* on amphibians that inhabit the forest canopy, terrestrial leaf littler, and soil suggests a common terrestrial existence where its dispersal and transmission are not constrained by the absence of permanent water.

The spread of *Bd* through Central and South America is associated with dramatic amphibian declines and extirpations [5,6,30,31] and interestingly, affected sites include remote wilderness areas and national parks where anthropogenic-assisted *Bd* spread is expected to be minimal [31–33]. Although a wave of *Bd* appears to have swept southeast through Central America during the 1980's [10,32], relatively little is known of its present distribution and ecological impact in Honduras. Infected amphibians have been reported from two locations, Pico Bonito National Park [34] and Cusuco National Park (CNP) [24], but the country boasts a mosaic of additional montane cloud forests likely to be similarly affected, but not yet surveyed. It has been estimated that nearly 50% of 111 amphibian species in Honduras have suffered declines in recent years from a combination of factors, including chytridiomycosis, and seven endemic anuran species were believed extinct [35], although one (*Craugastor milesi*) was recently rediscovered [36]. *Bd* has been detected in Honduran terrestrial anurans that undergo direct metamorphosis in leaf litter, including *Craugastor aurilegulus* and *C. rostralis* [24,34], and the source of pathogen exposure to these species remains enigmatic. Similarly, *Bd*-positive terrestrial frogs have been detected in Costa Rica (*Oophaga pumilio* and *Craugastor fitzingeri*), prompting the authors to suggest that *Bd* can survive on the moist forest floor where transmission might occur [32].

Since *Bd* occurs in the superficial skin of infected metamorphosed amphibians, there appears to be potential for infectious zoospores and sporangia within shedding skin to contaminate environmental substrates. Newly post-metamorphic anurans, in particular, often exhibit both elevated *Bd* prevalence and zoospore loads [24,37–39], so their emergence from water

might represent a considerable pathway of *Bd* dispersal into the terrestrial zone. To explore this potential avenue of terrestrial *Bd* spread we investigated whether terrestrial vegetation becomes contaminated with *Bd* following contact with recently metamorphosed amphibians under natural field conditions.

Materials and Methods

Ethics

Amphibian sampling in CNP adhered to established protocols [40] and were authorized by the Instituto Nacional de Conservacion y Desarollo Forestal Areas Protegidas y Vida Silvestre (ICF) of Honduras as part of the long-term Biodiversity Monitoring Programme performed by Operation Wallacea. Permission to export samples was granted by Honduran permit #'s 44735 and 19987.

Study Site

This investigation was performed from 9 July to 6 August 2013 in Cusuco National Park (CNP), a montane rainforest located in the Sierra de Omoa of northwestern Honduras. The altitude of CNP ranges from 550 m to 2200 m and fieldwork was performed between 1300 m and 1600 m at three different river sites (Rio Cusuco, N 15.495, W 88.213, elev. 1600 m; Rio Cortecito, N 15.523, W 88.288, elev. 1350 m; and Rio Danto, N 15.530, W 88.277, elev. 1545 m). Previous work identified widespread distribution and high prevalence of *Bd* in CNP at these locations [24] and its presence in the region for approximately two decades or greater [41]. Recently metamorphosed individuals of four tree frog species susceptible to *Bd* were targeted for sampling (*Duellmanohyla soralia*, *Plectrohyla dasypus*, *Plectrohyla exquisita*, and *Ptychohyla hypomykter*). Of these species, *P. dasypus*, previously demonstrated the highest prevalence of infection both at the species level (78%) and among recently metamorphosed individuals (94%) [24]. Most sampling was performed at night when animals were more active and likely to be encountered on riparian vegetation, although some opportunistic sampling occurred in the day. Most frogs were encountered within 5 m of the water's edge, but some were found up to 50 m from the river. Sampling was restricted to frogs resting on leaves, and not those perched on stalks or branches.

Leaf and Amphibian Sampling

Recently metamorphosed amphibians were removed from leaves by inverting them above a new plastic bag, into which the amphibian either jumped or was guided by a gentle tap on the underside of the leaf. Care was taken not to exert pressure between the frog and leaf, to prevent increased potential *Bd* shedding. Vegetation was sampled first, and then the corresponding amphibian was sampled. Nitrile gloves were worn and changed between every swab collected to reduce the risk of sample cross contamination. Leaves and frogs were each sampled with sterile fine-tipped rayon swabs (Medical Wire & Equipment Co., #MW113). For leaves, each swab was drawn across the leaf surface 20 times, where the amphibian was perched and in most instances, had left a small film of moisture visible on the leaf's surface, approximately 0.5 cm in diameter, marking the amphibians' location (Fig 1). For amphibians, the hands, feet, and pelvic patch were swabbed five times each following protocols established by Hyatt et al. [40]. Swab buds were snapped off into 2 mL microcentrifuge tubes filled with 1 mL 70% ethanol as a preservative. After sampling was completed, each amphibian was replaced to its original position in the vegetation.

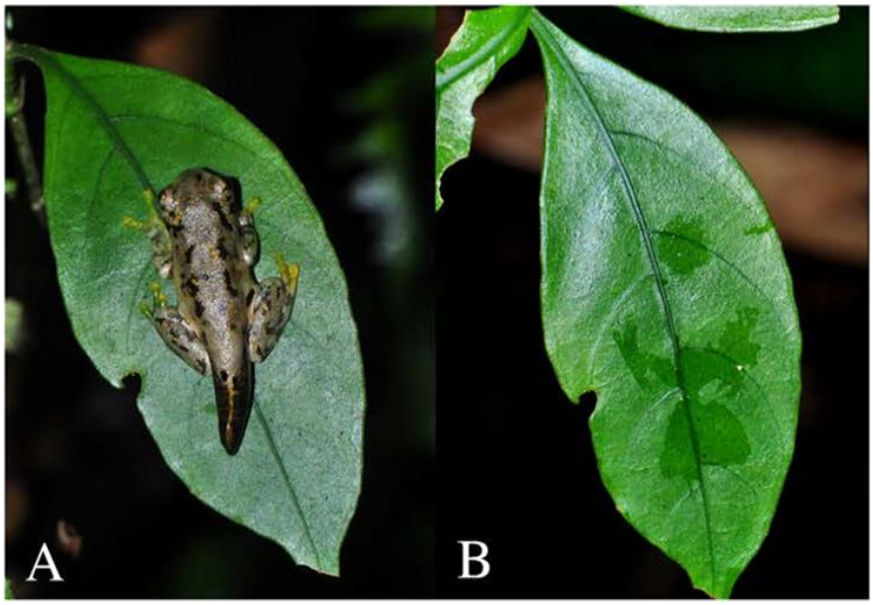

Fig 1. Recently metamorphosed *Plectrohyla dasypus* on terrestrial vegetation in Cusuco National Park, Honduras. (A) Amphibian as encountered on vegetation. (B) *Bd*-positive residue remaining on the leaf after amphibian removal.

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Temperature

Immediately upon encountering an amphibian perched on vegetation, the amphibian's dorsal body surface, the vegetation surface, and the air temperature were measured to characterize the environmental conditions *Bd* would be exposed to, if present. Temperatures were measured using a Raytek ST81 Non-contact Infrared Thermometer (RAYST81, emissivity set to 0.95), from a distance of 0.5 m or less. Accuracy of the thermometer is ± 1% of reading or ± 1°C, whichever is greater. This technique obtains amphibian body temperature readings within 0.5°C of cloacal temperatures [42]. Air temperature was measured with the attachable RTD temperature probe.

Water Sampling

Water samples from rivers at the three sites were collected and filtered for *Bd* detection. These samples were processed for *Bd* testing following established protocols [43], with the exception that a peristaltic pump was used to increase the efficiency of sampling efforts by maximizing the volume of water filtered. We used sterile silicone rubber peristaltic pump tubing and replaced a new length for the collection of each sample. Water was pumped through Sterivex filter capsules (0.22 micron pore size) until the flow rate greatly diminished. Then the volume filtered was measured and recorded. The content of each filter capsule was rinsed with 50 mL phosphate buffered saline solution and then pumped dry. A bead of clay sealant was used to plug the outlet spout of the capsule before being preserved by adding 0.9 mL of Qiagen ATL tissue lysis buffer with a sterile 1 mL syringe. Luer-Lok screw caps sealed the inlet spout of the capsules and a bead of quick-drying epoxy was applied behind each clay plug in the outlet spout to provide the seal with reinforcement during transit. Fresh pairs of Nitrile gloves were worn each time a water sample was collected. All water sampling was performed during

daytime hours. Water temperature was measured at the time of sampling using the attachable RTD temperature probe of the Raytek ST81 Non-contact Infrared Thermometer.

Real-Time PCR Analysis

Samples were processed via a sensitive quantitative PCR assay (qPCR) specific to *Bd* following the established protocol [44] and with the addition of BSA to the qPCR master mix as per Garland et al. (2010) [45]. Samples were extracted with 100 μl Prepman Ultra (Applied Biosystems, California, USA), with a final 20 μl of supernatant removed for downstream use. An aliquot of this supernatant was diluted 1:10 in DNase-free water for qPCR. The qPCR protocol used Sensi-Mix II Low Rox (Bioline, Massachusetts, USA) as the qPCR master mix [46]. For each sample, 5 μl of 1:10 dilution of swab DNA was added to each well for a final total qPCR volume of 20 μl. Samples and controls were run in triplicate with three positive, standard control samples (100, 10, and 1 zoospore/well, made from JAM81 pure culture; see Boyle et al. 2004 for standard control construction) and one non-template control (DNase free, molecular-grade water). When the qPCR assay failed to detect *Bd* in all three wells, the sample was deemed negative for *Bd*. Samples that produced a positive signal for *Bd* in either two or three wells on the first run were considered positive for *Bd*. When only one of three replicates detected *Bd*, the sample was rerun (in triplicate again) in a subsequent plate. For rerun samples that had at least a cumulative total of two of six replicates positive for *Bd* (from at least two separate plates), the sample was deemed positive for *Bd*. All zoospore loads described in this report have not been converted and reflect the actual zoospore loads present in 5 μl DNA (1:10 dilution), placed into 20 μl reaction volumes.

Data Analysis

We applied Chi-square test on a 2x2 contingency table to determine whether row and column marginal frequencies were equal. The values in the matrix included: number of *Bd*-negative frogs associated with *Bd*-negative leaves (5), number of *Bd*-negative frogs associated with *Bd*-positive leaves (1), number of *Bd*-positive frogs associated with *Bd*-negative leaves (11), number of *Bd*-positive frogs associated with *Bd*-positive leaves (35). Analysis was performed in R (R Development Core Team 2013 version 3.0.11 using package STATS (Chisq.test; version 3.0.3).

Results

Amphibian Swab *Bd* Results

Bd was detected on 46 of 52 (88.5%) amphibians and from all four species (Table 1). The average *Bd* zoospore equivalent load detected on *Bd*-positive amphibians was 103.94 and ranged from 0.06–1,574.62.

Leaf Swab *Bd* Results

Bd was detected on 36 of 52 (69.2%) leaves, 97.2% of which had a *Bd*-positive recently metamorphosed amphibian on them (35/36) (statistical significance of the association, df = 1, chi-squared = 6.23, p-value = 0.013) (Table 1). Only one *Bd*-positive leaf had an amphibian that tested *Bd*-negative. The average *Bd* zoospore equivalent load detected on *Bd*-positive leaves was 40.48 and ranged from 0.12–1,040.45.

River Water Filter *Bd* Results

The presence of *Bd* was detected in all three river water samples (Table 2). The average *Bd* zoospore equivalent load per liter of river water was 0.23 and ranged from 0.03–0.57. Daytime water temperature averaged 17.0°C and ranged from 16.3–17.5°C.

Table 1. Presence of *Batrachochytrium dendrobatidis* (*Bd*) detected on amphibians and vegetation sampled in Cusuco National Park, Honduras.

Date	Sample#	Species	Site	Frog qPCR	Leaf qPCR	Frog ZSE	Leaf ZSE
Jul 9 2013	HN13BD107	*Plectrohyla dasypus*	CO	+	-	0.45	n/a
Jul 9 2013	HN13BD109	*Ptychohyla hypomykter*	CO	+	-	0.54	n/a
Jul 9 2013	HN13BD110	*Plectrohyla dasypus*	CO	+	+	24.39	17.81
Jul 9 2013	HN13BD111	*Plectrohyla dasypus*	CO	+	-	2.38	n/a
Jul 9 2013	HN13BD112	*Plectrohyla dasypus*	CO	+	+	29.19	12.25
Jul 9 2013	HN13BD114	*Plectrohyla dasypus*	CO	+	+	2.38	16.11
Jul 9 2013	HN13BD115	*Plectrohyla dasypus*	CO	+	+	3.04	14.57
Jul 9 2013	HN13BD116	*Plectrohyla dasypus*	CO	+	+	2.10	0.81
Jul 9 2013	HN13BD117	*Plectrohyla dasypus*	CO	+	+	93.05	4.70
Jul 9 2013	HN13BD118	*Plectrohyla exquisita*	CO	+	+	53.94	1040.45
Jul 9 2013	HN13BD120	*Plectrohyla dasypus*	CO	+	+	0.39	1.52
Jul 10 2013	HN13BD121	*Duellmanohyla soralia*	CO	+	-	16.75	n/a
Jul 10 2013	HN13BD122	*Plectrohyla dasypus*	CO	+	-	0.35	n/a
Jul 10 2013	HN13BD123	*Duellmanohyla soralia*	CO	-	-	n/a	n/a
Jul 10 2013	HN13BD124	*Plectrohyla dasypus*	CO	-	-	n/a	n/a
Jul 10 2013	HN13BD125	*Duellmanohyla soralia*	CO	+	+	0.64	0.34
Jul 10 2013	HN13BD128	*Plectrohyla dasypus*	CO	+	-	3.00	n/a
Jul 10 2013	HN13BD129	*Duellmanohyla soralia*	CO	-	-	n/a	n/a
Jul 10 2013	HN13BD130	*Plectrohyla dasypus*	CO	-	+	n/a	0.12
Jul 10 2013	HN13BD131	*Plectrohyla dasypus*	CO	+	+	2.00	0.64
Jul 10 2013	HN13BD132	*Ptychohyla hypomykter*	CO	+	+	28.77	8.39
Jul 10 2013	HN13BD133	*Plectrohyla dasypus*	CO	+	+	13.79	1.97
Jul 10 2013	HN13BD134	*Plectrohyla dasypus*	CO	+	+	23.76	0.93
Jul 10 2013	HN13BD135	*Plectrohyla dasypus*	CO	+	+	33.18	1.68
Jul 10 2013	HN13BD136	*Plectrohyla dasypus*	CO	+	-	2.11	n/a
Jul 10 2013	HN13BD137	*Duellmanohyla soralia*	CO	+	+	22.42	23.06
Jul 11 2013	HN13BD144	*Plectrohyla dasypus*	CO	+	+	11.82	1.07
Jul 11 2013	HN13BD145	*Plectrohyla dasypus*	CO	+	+	1085.68	43.30
Jul 14 2013	HN13BD161	*Duellmanohyla soralia*	CO	+	-	0.06*	n/a
Jul 14 2013	HN13BD164	*Ptychohyla hypomykter*	CO	+	+	660.54	49.09
Jul 15 2013	HN13BD166	*Plectrohyla dasypus*	CO	-	-	n/a	n/a
Jul 15 2013	HN13BD170	*Plectrohyla dasypus*	CO	+	+	236.29	139.30
Jul 15 2013	HN13BD171	*Duellmanohyla soralia*	CO	+	+	1.20	0.47
Jul 15 2013	HN13BD172	*Plectrohyla dasypus*	CO	+	+	58.50	0.79
Jul 15 2013	HN13BD173	*Plectrohyla dasypus*	CO	+	+	57.69	4.38
Jul 15 2013	HN13BD174	*Plectrohyla dasypus*	CO	+	+	17.44	40.30
Jul 15 2013	HN13BD175	*Plectrohyla exquisita*	CO	+	+	38.66	1.00
Jul 15 2013	HN13BD177	*Plectrohyla dasypus*	CO	+	+	18.10	0.32
Jul 15 2013	HN13BD178	*Plectrohyla exquisita*	CO	+	+	281.79	10.93
Jul 15 2013	HN13BD179	*Ptychohyla hypomykter*	CO	+	+	0.64	0.25
Jul 15 2013	HN13BD180	*Ptychohyla hypomykter*	CO	+	-	1.37	n/a
Jul 15 2013	HN13BD181	*Duellmanohyla soralia*	CO	+	+	4.06	3.36
Jul 16 2013	HN13BD183	*Plectrohyla dasypus*	CO	+	+	1574.62	3.90
Jul 16 2013	HN13BD249	*Ptychohyla hypomykter*	CO	+	+	39.07	0.23
Jul 18 2013	HN13BD261	*Plectrohyla dasypus*	DA	+	+	147.26	7.84
Jul 14 2013	HN13BD323	*Plectrohyla dasypus*	DA	-	-	n/a	n/a
Aug 5 2013	HN13BD389	*Plectrohyla exquisita*	CU	+	-	27.10	n/a

(*Continued*)

Table 1. (*Continued*)

Date	Sample#	Species	Site	Frog qPCR	Leaf qPCR	Frog ZSE	Leaf ZSE
Aug 5 2013	HN13BD390	*Plectrohyla exquisita*	CU	+	+	34.02	0.44
Aug 5 2013	HN13BD391	*Plectrohyla exquisita*	CU	+	+	48.13	1.97
Aug 6 2013	HN13BD407	*Plectrohyla dasypus*	CU	+	+	25.23	0.29
Aug 6 2013	HN13BD408	*Plectrohyla exquisita*	CU	+	+	1.03	2.63
Aug 6 2013	HN13BD409	*Plectrohyla exquisita*	CU	+	-	52.55	n/a

Survey sites include Rio Cortecito (CO), Rio Danto (DA), and Rio Cusuco (CU). Average zoospore equivalent (ZSE) per qPCR reaction is reflected for all *Bd*-positive samples. Asterisk denotes the single sample that produced a positive reaction in 2/6 wells; all other samples produced *Bd*-positive reactions in 2/3, 3/3, or 3/6 wells.

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Amphibian and Vegetation Temperatures

Most animals were sampled during nocturnal surveys, from 20:00–2:00 hrs (n = 45), although some were occasionally encountered and sampled during the day, from 10:45–15:00 hrs (n = 7). Night temperatures of the frogs' dorsal surfaces, leaf surfaces, and air averaged 17.0°C, 17.1°C, and 16.9°C and ranged from 15.2–18.9°C, 15.8–19.1°C, and 15.3–17.8°C, respectively, whereas day temperatures averaged 20.8°C, 21.0°C, and 20.2°C and ranged from 18.4–26.6°C, 18.8–7.0°C, and 19.4–21.9°C, respectively.

Discussion

We frequently detected *Bd* on leaf surfaces after removal of recently metamorphosed *Bd*-positive frogs, indicating their emergence does contribute towards the spread of *Bd* from aquatic into terrestrial locations. Average zoospore loads detected on leaf surfaces were comparable to those from corresponding amphibian skin swabs, and sometimes greater. The presence of *Bd* on riparian vegetation allows exposure to occur in the absence of direct physical contact with *Bd*-positive animals or contaminated water. Accordingly, this pathway of *Bd* dispersal and terrestrial exposure provides one possible explanation for the source of infection previously detected in amphibians that do not demonstrate a strong association with water.

This pathway of *Bd* spread may occasionally facilitate transmission between aquatic and terrestrial species and from juvenile to adult frogs, if foliage maintains infectious *Bd* loads. On 11 July 2013, both a recently metamorphosed and adult *Plectrohyla dasypus* were observed perched together on the same plant at the same time, approximately 5 cm apart (Fig 2). The skin swab sample collected from this juvenile frog (HN13BD145) exhibited a considerable zoospore load (1,085.68), as did the leaf swab (43.30), demonstrating a high risk of exposure to the

Table 2. Presence of *Batrachochytrium dendrobatidis* (*Bd*) detected in water filter samples collected from amphibian survey sites in Cusuco National Park, Honduras.

Sample#	Site	Vol (ml)	T (°C)	ZSE/L
HN13W01	CO	11000	17.5	0.08
HN13W02	DA	2700	17.1	0.03
HN13W03	CU	4600	16.3	0.57

Survey sites include Rio Cortecito (CO), Rio Danto (DA), and Rio Cusuco (CU). Volume of water filtered, water temperature, and average *Bd* zoospore equivalent (ZSE) per liter of river water is reflected for all samples.

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Fig 2. Adult and recently metamorphosed *Plectrohyla dasypus* resting in close proximity in Cusuco National Park, Honduras. The skin swab collected from this juvenile (HN13BD145) tested positive for *Bd* infection and exhibited a considerable zoospore load (1,085.68 ZSE), as did the leaf swab (43.30 ZSE), demonstrating the risk of exposure to the nearby *Bd*-negative adult through contact with contaminated vegetation.

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nearby adult which tested *Bd*-negative at the time of sampling. Following metamorphosis, this species leaves the aquatic habitat and moves into arboreal vegetation, reducing the likelihood of subsequent *Bd* exposure from contaminated river water. The high prevalence of infection in *P. dasypus* juveniles detected in this and previous surveys [24] suggests that their seasonal emergence *en masse* may release a substantial quantity of *Bd* into the riparian zone shared with amphibians that approach the water's edge, but do not typically enter it.

Although we identified a potential mechanism of pathogen exposure to terrestrial amphibians, the role of contaminated vegetation in *Bd* transmission remains in question. Detection of *Bd* via qPCR indicates DNA presence, but does not reveal condition at the time of sampling. A lack of experimental work to evaluate the persistence and detectability of *Bd* DNA following cell death makes it difficult to discern whether we likely detected viable *Bd* or instead DNA fragments from expired cells that continued to react with *Bd* qPCR primers. This interpretation limitation is not exclusive to environmental *Bd* swab samples, but likewise applies to amphibian skin swabs; a positive qPCR result does not independently demonstrate the viability of *Bd* on that animal. Still, environmental conditions observed at all sampling localities in CNP were similar to those in the laboratory where *Bd* survived outside a host [19] and may aid persistence of *Bd* on leaf surfaces. Temperatures recorded in the field were all within or near the range for optimal in vitro growth of *Bd* (17–25°C) and well below its thermal maximum of 28°C [47], although optimal temperature regimes may vary between *Bd* isolates [48] and none from Honduras have yet been characterized. Desiccation poses the other well-defined abitoic limitation to *Bd* survival [19,49] but the presence of both high relative humidity and a dense forest canopy preventing direct sun exposure is correlated with higher *Bd* prevalence and infection loads [50]. These conditions are typical of CNP, a montane cloud forest, and expected to prolong

drying. Lastly, laboratory experiments have shown that when maintained under suitable temperature and moisture levels (and without bacteria), *Bd* can survive in the absence of a host for at least two months in water or moist sand [19]. Thus, additional laboratory work is needed to test the survival times of cultured *Bd* on leaf surfaces to identify the potential duration of this form of environmental persistence and evaluate the average *Bd* loads needed to cause successful transmission under naturalistic conditions.

Previous efforts to illustrate environmental *Bd* transmission have mainly focused on exposure to permanent water bodies inhabited by *Bd*-infected amphibians [18,19]. Laboratory trials demonstrated transmission of *Bd* between experimentally-infected and uninfected tadpoles of *Rana muscosa* and also from tadpoles to post-metamorphic animals, when occupying a shared water source [18]. Successful transmission required a 2–3 week duration of exposure, likely impeded by dilution of the pathogen in a naturalistic environment, similar to the low densities of *Bd* detected in the water samples collected at our survey sites in CNP (Table 2). To encourage transmission after short-term exposure, laboratory experiments have often employed highly concentrated inoculates of approximately 100 million *Bd* zoospores delivered in less than 100 mL of water [51–53] whereas the highest concentration detected in a natural body of water has been 3 million zoospores L^{-1} and less than 100 zoospores L^{-1} is common [54]. In this context, the concentrated *Bd* loads we detected on leaf surfaces in CNP relative to the adjacent *Bd*-positive river water suggests that contact with affected foliage might pose a greater threat of exposure and transmission to terrestrial amphibians than would a splash of water from these rivers.

We detected the presence of *Bd* on vegetation in the understory, but periods of heavy rain are expected to also flush *Bd* into the soil and leaf litter below. Surveys in CNP have identified the presence of live aquatic crustaceans (copepods and ostracods) inhabiting terrestrial water films on forest floor leaf litter [55,56], suggesting moisture persistence in this limnoterrestrial habitat. The persistence of these water films in humid rainforest environments would help protect *Bd* from desiccation in a seemingly terrestrial habitat, and also allow exposure to amphibians that occupy leaf litter and burrow into the ground. Accordingly, this mode of *Bd* dispersal and indirect exposure may explain the origins of infection documented in species of soil-dwelling salamanders [22,23,25] and caecilians [28,29].

Numerous biologic and abiotic factors are expected to influence the frequency of *Bd* dispersal from aquatic into terrestrial habitats and potential consequences. The prevalence and intensity of *Bd* detected in amphibian populations often demonstrates fluctuations due to seasonal changes in environmental conditions and these factors will affect the amount of zoospores available to be shed into the terrestrial environment [49,54,57]. Rowley et al. [58] investigated the presence of *Bd* in terrestrial retreat sites of two aquatic stream frog species (*Litoria lesueuri* and *L. nannotis*), and did not detect *Bd* in 122 environmental swab samples. As suggested by the authors, the observed *Bd* absence may have been influenced by the low prevalence and infection loads concurrently detected in the adult amphibians sampled at these locations. Our results show that in a locality where both *Bd* prevalence and infection loads are high, it is common for *Bd* to be shed into terrestrial locations, including amphibian retreat sites.

The presence of *Bd* in terrestrial habitats should be considered when identifying potential threats to amphibian species of concern. Although it has been suggested that *Bd* poses the greatest risk of infection to amphibians breeding in permanent streams [59], we caution against this generalization and encourage additional surveillance in terrestrial and arboreal amphibian habitats where animals continue to test positive for *Bd*, despite pathways of exposure being more obscure. The frequency of *Bd* exposure from terrestrial substrates is unknown but may be considerable where optimal environmental conditions are present, especially if it can survive as a saprobe as previously suggested [12]. An improved understanding of *Bd* dispersal and

persistence in the natural environment is essential to better explain and predict the continued spread of this pathogen in regions where the anthropogenic-assisted exposure to *Bd*-positive amphibians or substrates is unlikely.

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Author Contributions

Conceived and designed the experiments: JEK. Performed the experiments: JEK SDR. Analyzed the data: JEK MJ. Contributed reagents/materials/analysis tools: KLR. Wrote the paper: JEK SDR LB LS MJ KLR.

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CHAPTER 8

Suggested biosecurity actions to reduce pathogen importation to the United States

Introduction

In Chapter 8, I begin to apply the data produced in this thesis and suggest policy interventions to reduce the spread of *Bd* into the Unites States – the most common global destination of the international amphibian trade. In Chapter 2, I created new data about the presence of *Bd* in the amphibian trade and it supported the need for increased biosecurity, as previously suggested in a petition submitted to the United States Fish and Wildlife Service by Defenders of Wildlife. At this time of writing, no decision to take action in response to this petition has been announced.

In this chapter, I apply these data and reccommend a streamlined multiphase risk-reduction approach to more quickly reduce the risk of importing new pathogen strains into the USA through the amphibian trade. This study is **Paper 1** of this chapter: "Restricted Trade in Two Invasive Frog Species is Necessary to Mitigate Spread of Exotic Strains and Lineages of Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) into the United States", which has been prepared for submission to Conservation Letters.

Paper 1

Citation: Kolby JE, Kraus F, Murray KA, Smith KM, van Dijk PP, Berger L, Speare R, Skerratt LF. Restricted Trade in Two Invasive Frog Species is Necessary to Mitigate Spread of Exotic Strains and Lineages of Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) into the United States. Prepared for submission to Conservation Letters.

Abstract: The spread of amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), through international trade is contributing towards global amphibian declines and extinctions. In 2009, Defenders of Wildlife submitted a petition to the United States Fish and Wildlife Service (USFWS) to list all live amphibians as injurious species under Lacey Act authority to prohibit their trade in the USA. After five years of deliberation, no action has yet been taken by USFWS. We propose an alternate, multi-phase regulatory approach to more rapidly reduce the continued introduction and spread of exotic strains and lineages of *Bd*. Two highly traded frog species pose an exceptional threat of *Bd* introduction because they are widely, but aclinically, infected with *Bd*: the American bullfrog, *Lithobates catesbeianus*, and the African clawed frog, *Xenopus laevis*. Collation of US import records from 2006 to 2010 showed about 12.64 million *L. catesbeianus* and about 0.80 million *X. laevis* were imported, representing 50.2% and 3.2% of all live amphibian imports, respectively. Rather than waiting for approval to list all amphibians, we call for immediate injurious-species listing of these two commonly infected *Bd* reservoir hosts. Following regulation of these high-risk infection sources, additional amphibian species should be evaluated for risk and potential regulation.

Introduction

The pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), causes the lethal disease chytridiomycosis, which is causing massive population declines and extinctions worldwide (Berger et al. 1998; Stuart et al. 2004; Skerratt et al. 2007; Vredenburg et al. 2010; Cheng et al. 2011). *Bd* is transmitted through direct contact with infected individuals or by exposure to contaminated water or substrates (Rachowicz & Vredenburg 2004; Carey et al. 2006). Its low host specificity allows *Bd* to potentially infect thousands of amphibian species, making it a uniquely devastating pathogen. Chytridiomycosis has already decimated several

native species in the United States, including California's yellow-legged frogs (*Rana muscosa* and *Rana sierrae*) (Vredenburg et al. 2010) and the Wyoming toad (*Bufo baxteri*) (Odum & Corn 2005). As of 2013, 42% of 1,240 amphibian species tested globally were found to be susceptible to infection (Olson et al. 2013).

Although chytridiomycosis is often lethal to amphibians, a minority of species consistently survive with aclinical infection and are regarded as *Bd* reservoir hosts (Daszak et al. 2004; Woodhams et al. 2007; Reeder et al. 2012). These animals tolerate *Bd* infection without morbidity and can become efficient dispersers of the pathogen across traditional ecological barriers upon release into the wild (Daszak et al. 2004; Weldon et al. 2004; Garner et al. 2006). The most prominent reservoir hosts are the American bullfrog, *Lithobates catesbeianus* (Daszak et al. 2004; Hanselmann et al. 2004; Gervasi et al. 2013), and the African clawed frog, *Xenopus laevis* (Rollins-Smith et al. 2009; Ramsey et al. 2010), and both are highly invasive alien species in the USA and elsewhere (Kraus 2009; Measey et al. 2012). In amphibian communities containing these reservoir hosts, the extended environmental persistence of *Bd* and its low host specificity promote the extinction of highly susceptible native amphibians and the persistence of *Bd* (Daszak et al. 1999; Johnson & Speare 2003; Schloegel et al. 2006; Mitchell et al. 2007; Reeder et al 2012).

The global spread of *Bd* is closely associated with the international trade in live amphibians, which moves millions of animals annually (Weldon et al. 2004; Fisher & Garner 2007; Schloegel et al. 2009, 2012; Kolby et al. 2014). Shipping boxes and water used to transport infected amphibians can provide additional sources of infection long after amphibians have been sold (Johnson & Speare 2005; Pauza et al. 2010). Although *Bd* is already present in the USA, a diversity of lineages exist globally (Farrer et al. 2011; Schloegel et al. 2012; Rosenblum et al.

2013) and differ dramatically in virulence (Berger et al. 2005; Fisher et al. 2009; Gahl et al. 2012, Gervasi et al. 2013). The introduction of alien *Bd* lineages to American amphibians is nearly certain to occur if unregulated importation of infected amphibians continues. The precise consequences of such introductions are currently unknown and extremely difficult to predict. However, history suggests that the potential of increased threat to amphibian biodiversity is very high given that "the impact of chytridiomycosis on frogs is the most spectacular loss of vertebrate biodiversity due to disease in recorded history" (Skerratt et al 2007).

In September 2009, in order to reduce the risk of importing *Bd* into the USA, Defenders of Wildlife (DOW) submitted a petition to USFWS requesting the agency to list all live amphibians as injurious under the Lacey Act, except for animals certified to be free of *Bd* (DOW 2009). One year later, USFWS published a Notice of Inquiry in the Federal Register seeking additional information (USFWS 2010), and the Acting Director expressed concern: "The worldwide decline of amphibians is of great concern to us. Chytrid is attributed as a major cause of this amphibian mortality. We understand that halting the spread of the fungus or eradicating it will take more than just regulating importation and transportation of infected amphibians, but it is a major step in the right direction." (Fellows 2010).

Five years have now passed (October 2015), and USFWS has still not acted on the DOW petition. I inquired as to the source of this delay, and the USFWS Branch of Aquatic Invasive Species stated: "The scope of a class-level injurious listing action encompassing over 7,000 amphibian species has complicated the USFWS's ability to identify an appropriate risk management response under the injurious wildlife provisions of the Lacey Act. The USFWS may list as injurious the host of a pathogen but does not have the authority to list the pathogen

(such as *Bd*) under the injurious provisions of the Lacey Act. This can complicate any proposed risk management action, especially when applied to an entire class of organisms."

We agree that regulation of the amphibian trade is required to avert potentially greater impacts from *Bd* in the USA. The DOW petition could be improved by targeting the most significant *Bd* introduction pathways and, hence, excluding unnecessary listing of species of low risk; this would result in less economic harm to private industry, less onerous policy, and more realistic enforcement requirements for governments. Therefore, we propose an alternative, multi-staged risk-management regulatory approach that allows USFWS to first list high-risk species posing the greatest threat of spreading alien lineages of *Bd* and follows that with additional listings as new data become available. Two species in particular – the American bullfrog, *L. catesbeianus,* and the African clawed frog, *X. laevis* – are traded in disproportionately high numbers, are known to serve as aclinical *Bd* reservoir hosts, and are invasive pests outside their native ranges. *Lithobates catesbeianus* are imported primarily for the frog leg trade and *X. laevis* for the exotic pet trade. Both pose a very high risk for establishment of additional strains of *Bd* relative to other traded species, and both warrant immediate listing as injurious under the Lacey Act. For all other amphibians, which are less frequently traded or whose role in the spread of *Bd* is less certain, regulatory evaluation is only warranted following action on the above two high-risk species. Below we provide data justifying this proposal.

USA amphibian import analysis

We used import records for USA trade in all live amphibians maintained in the USFWS Law Enforcement Management Information System (LEMIS) to investigate the diversity and quantity of traded amphibians over a five-year period (1 January 2006 to 31 December 2010) (Table 1). Amphibian trade was recorded by USFWS in various units of measure (kilograms, pounds, or

numbers of individuals). We standardized these data by converting all records into the single unit of number of animals for estimating the total number of traded amphibians. For each shipment recorded by weight, we estimated the number of animals present by dividing that value by the approximate mass of one adult individual of that species (Table 2). We used adult masses because it was not possible to verify age or snout-vent-length of traded animals. This conservative approach may underestimate number of animals traded because inclusion of smaller animals would require higher numbers to achieve similar total masses. One shipment, containing 32 kilograms of amphibians, was excluded from analyses because no species information was recorded. Additionally, another said to contain "0.35 *Hymenochirus curtipes*" was rounded up to a quantity of one animal for inclusion in our trade-volume analyses.

LEMIS data involve varying levels of taxonomic specificity, ranging from species to the uninformative class-level description of "Amphibian." We tallied all unique classifications present in these data and estimated the minimum number of traded species. To estimate the quantities of *Bd*-positive *L. catesbeianus* and *X. laevis* imported, we multiplied the total number of animals imported by the highest prevalence of infection measured in these species among traded animals (62% prevalence in *L. catesbeianus* [Schloegel et al. 2009]; 70% prevalence in *X. laevis* [Kolby et al. 2014]) (Table 1).

Amphibian trade summary

From 2006-2010, 25,165,417 live amphibians were recorded entering the USA, or approximately 5 million annually (Table 1). Documented trade involved ~300 of the 7400+ described amphibian species. This trade was taxonomically disproportionate, with ten species accounting for 91.5% of all amphibians imported. Two *Bd* reservoir host species—*L. catesbeianus* and *X. laevis*—were among those ten and represented 50.2% and 3.2% of all

amphibians imported during the study period, respectively (Table 1). We estimate as many as 7,833,792 infected *L. catesbeianus* and 556,707 infected *X. laevis* were imported into the USA from 2005-2010.

Discussion

Proposed trade restriction

We propose that *L. catesbeianus* and *X. laevis* be immediately listed as injurious wildlife under Lacey Act authority and that all live animals of each species be prohibited from importation unless a permit to import from USFWS is obtained. Permits could be provided for good reason, such as for *bona fide* scientific, medical, educational, or zoological purposes, or if the risk of introduction and spread of alien lineages of *Bd* has been mitigated (see Potential Trade Exceptions below).

Impact of listing

Our proposed regulation would considerably reduce the two greatest sources of *Bd* introduction to the USA. We expect this action to prevent the importation of approximately 1 million live *Bd*-infected frogs annually and reduce the risk of *Bd* transfer to native amphibians either in captivity or in the wild following successful establishment of feral populations.

Additionally, the manner in which these two species are shipped provides an abundance of *Bd*-contaminated cardboard cartons and bags of water that allow *Bd* to spread even when imported animals remain captive. In one study, 59% of cartons used to transport *L. catesbeianus* into the USA were contaminated with *Bd* (Chapter 2C), and water carrying *X. laevis* accumulated exceptionally high pathogen densities (3,390-16,887 *Bd* zoospores/L) (Kolby et al. 2014).

Annually, approximately 100,000 cartons carrying *L. catesbeianus* and hundreds of gallons of water containing *X. laevis* are imported into the USA. Because *Bd* can remain infectious in the absence of a host (Johnson & Speare 2003), these commonly contaminated materials pose a high risk of spreading *Bd* to other animals within importers' facilities, to additional traded amphibians should materials be reused for future shipments, and to the environment when discarded untreated.

"Bd-free" health certification

Improved biosecurity to reduce risk of spreading pathogens through commercial activity are needed, are practicable, and should be encouraged. Eventually, full import prohibitions of *L. catesbeianus* and *X. laevis* might be unnecessary to prevent *Bd* spread if certain amphibian-breeding/housing/distribution facilities could be certified as "free of disease." The World Organisation for Animal Health (OIE) has provided guidelines to ensure amphibians can be traded *Bd* free (OIE 2014). Operations might qualify were they to eradicate the pathogen from founding animals, maintain a closed system to prevent *Bd* introduction, and follow best practices to maintain sanitation. Similar risk-mitigation programs are commonly implemented in the livestock industry to control disease spread: the United States Department of Agriculture only allows importation of goats after five years of isolation from animals potentially affected by scrapie and cattle from premises free of bovine tuberculosis for the previous two years. Many additional regulatory requirements are listed under 7 U.S.C. 8303; 9 CFR 93.

Potential trade exceptions

Our proposed import restriction is not meant to preclude continued trade of *L. catesbeianus* and *X. laevis* that are domestically produced or collected. Novel foreign *Bd* strains are potentially more virulent to North American amphibians and represent a greater threat to native biodiversity than the spread of *Bd* strains already widespread (and potentially native) in the USA (Longcore et al. 2007; Farrer et al. 2012; Schloegel et al. 2012). Therefore, disease risk posed by trade in foreign-sourced amphibians warrants distinction from that of domestic origin. Shifting commercial focus towards domestic production instead of foreign-sourced animals could ameliorate any potential detriment to industry from an import prohibition in live animals. Further, the majority of imported wild-collected *X. laevis* originate from invasive populations in Chile, and similar harvest from the feral populations in California and Arizona may be explored as a surrogate source of safer animals.

Importing skinned *L. catesbeianus* legs rather than live animals could reduce risk of *Bd* importation without obstructing the frog-meat industry. Bullfrogs traded for human consumption are commonly transported either as live animals or as freshly prepared frog legs, and the latter are either chilled or frozen (Gratwicke et al. 2009). Because *Bd* inhabits amphibian skin, live or freshly butchered whole animals may still carry active infections upon importation, whereas skinned frog legs have been literally stripped of this threat. Therefore, risk of *Bd* infection on skinned frog legs would likely be negligible and could be allowable; this is similar to the trade exception allowing import of listed salmonid fish that have been eviscerated or filleted (18 U.S.C. 42; 50 CFR 16.13(c)) to avoid vectoring disease to domestic fisheries. The financial and human resources required to enforce this type of trade exception by visual inspection are trivial compared with those needed to perform diagnostic sampling of live bullfrogs, which varies from $13 to $40 per swab sample (Kriger et al. 2006). Therefore, reasonable biological and economic

arguments exist to provide a trade exception for importing skinned frog legs without requiring diagnostic testing for *Bd*.

Research needed for additional injurious-species evaluations

Our recommendation to immediately prohibit importation of live *L. catesbeianus* and *X. laevis* via injurious-species listing is neither meant to exclude additional species from future listing nor to suggest that other species do not also pose risk. Rather, we believe that existing biological and trade-surveillance information shows that unrestricted trade in these two species presents the highest risk of *Bd* introduction and is unwarranted. Additional research is needed to evaluate the risk posed by other highly traded species (Table 1) that are susceptible to *Bd*, such as *Bombina orientalis* (Bataille et al. 2013) and *Hymenochirus boettgeri* (Raverty & Reynolds 2001). Less is known about their ability to serve as *Bd* reservoir hosts, and relatively few individuals have tested positive for *Bd* upon importation (3/56 *B. orientalis*; 0/48 *H. boettgeri*) (Kolby et al. 2014; Kolby unpubl. data), although screening has been limited. Both *Hymenochirus curtipes* (Groff et al. 1991; Carey et al. 2003) and *H. boettgeri* (Raverty & Reynolds 2001) appear vulnerable to lethal chytridiomycosis and might serve as poor long-term *Bd* carriers, but they may still pose a considerable risk of *Bd* importation.

Additional emerging amphibian pathogens

Our analysis of the risk of *Bd* introduction to the USA and suggested regulatory response is based on 15 years of data collection on *Bd*, but ideally a precautionary regulatory approach to prevent disease establishment would incorporate the risk of new diseases emerging. Biosecurity that limits animal movement is the only approach to mitigate this threat, as surveillance and

quarantine work best for known diseases. For wildlife, knowledge of diseases is inadequate, and many remain undiscovered. A prime example is the recently described salamander chytrid, (*Batrachochytrium salamandrivorans,* "*Bsal*") (Martel et al. 2013), a pathogen neither native to the USA nor yet detected in the country. Establishment of *Bsal* would cause the near-certain decline and potential extinction of salamanders in the USA— the world's global hotspot for salamander diversity (Petranka 1998). In May 2015, a petition to ban importation of all salamander species was submitted to the US Department of the Interior by the Center for Biological Diversity and SAVE THE FROGS! (2015). Although this ban is supported by overwhelming evidence (Martel et al. 2014; Yap et al. 2015), and is urgent while there is still a chance to prevent entry to the USA, action has not yet eventuated despite it being an exotic disease. Hence, to hasten regulatory action, it may be worth banning the highest-risk species first, as we have proposed for *Bd*. Because most species known to be susceptible to *Bsal* are within the family Salamandridae (Martel et al. 2014), banning trade at this taxonomic level may be a more effective initial approach, with greater likelihood of approval, than an emergency salamander-wide import moratorium. As salamanders are primarily traded only as pets, and many domestic species are already available, the cost and inconvenience of a ban appears negligible compared with the environmental catastrophe of a *Bsal* outbreak.

Conclusion

The greatest risk of continued novel *Bd* introduction events in the USA lies with importation of live *L. catesbeianus* and *X. laevis*. Immediate injurious-species listing of these two species under the Lacey Act is necessary to reduce this severe ongoing threat to native amphibian diversity. Targeted trade regulation in these two heavily traded frogs will significantly lower the threat of disease. This simple action could be approved more rapidly by the US government.

Additional amphibian species can also vector *Bd* and other emerging pathogens that threaten native amphibians, and future regulatory consideration should aim to improve wildlife biosecurity at least to the level offered to livestock.

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Table 1. Total live amphibian imports into the USA from 2006-2010 as per USFWS LEMIS declaration records. Estimated total number of amphibians is the combined sum of records originally reported in quantity (Raw #) and mass (Raw Kg) in kilograms (Table2). For the 10 most traded species, we list the primary purpose of trade (Purpose), whether that species has been found susceptible to *Bd* infection under natural conditions (*Bd*+), is an invasive species in the USA (Inv.) and records of *Bd* prevalence for amphibians sampled from the wild (*Bd* % W) and from a captive environment (*Bd* % C). N/A = not available.

Species	**Total [% total]**	**Total /yr**	**Raw #**	**Raw Kg**	**Purpose**	***Bd*+**	**Inv.**	***Bd* % W (95% CI)**	***Bd* % C (95% CI)**
Lithobates catesbeianus	12,635,149 [50.2%]	2,527,030	8,555,415	2,039,867	Food	Yes	Yes	24.7 (18.7–33.5) (feral population, Bataille et al. 2013) 100.0 (81.6–100.0) (invasive population, Goka et al. 2009)	100 (95.7–100.0) (farmed, Schloegel et al. 2010)
Hymenochirus curtipes	4,168,874 [16.6%]	833,775	4,168,874		Exotic pet			N/A	N/A
Lithobates forreri	1,745,481 [6.9%]	349,096	1,019,600	30,487	Research	Yes		22.2 (2.3-60.0) (native population, Zumbado-Ulate et al. 2014)	N/A
Bombina orientalis	1,616,825 [6.4%]	323365	1,616,825		Exotic pet	Yes		16.7 (13.9–20.3) (native population, Bataille et al. 2013)	5.4 (1.8–14.6) (captive bred, Kolby et al. 2014)
Xenopus laevis	795,295 [3.2%]	159,059	795,295		Research	Yes	Yes	13.0 (4.5–32.1) (feral population, Vredenburg et al. 2013)	70.0 (54.6–81.9) (captive bred, Kolby et al. 2014) 20.3 (14.3–28.1)

							2.6 (1.5–4.2) (native population, Weldon et al. 2004)	(wild collected, Goka et al. 2009)
Lithobates pipiens	636,881 [2.5%]	127,736	47,140	26,749	Research		N/A	N/A
Cynops orientalis	620,167 [2.5%]	124,033	620,167		Exotic pet		N/A	N/A
Hymenochirus boettgeri	343,170 [1.4%]	68,634	343,170		Exotic pet	Yes	N/A	N/A
Hymenochirus boulengeri	260,464 [1.0%]	52,093	260,464		Exotic pet		N/A	N/A
Litoria caerulea	216,064 [0.9%]	43,213	216,064		Exotic pet	Yes	N/A	N/A
Subtotal	**23,038,370 [91.5%]**	**4,608,034**	**17,643,014**	**2,097,013**				
All other spp.	2,127,047 [8.5%]	425,049	2,111,163	577.7				
Total	**25,165,417**	**5,033,083**	**19,754,177**	**2,097,680.7**				

Table 2. All amphibians imported into the USA from 2006-2010 reported in units of mass (kilograms) in USFWS LEMIS declaration records and converted into approximate numbers of animals.

Species	Mass Imported (Kg)	Kg/animal	Qty. imported (#)	Source of mass estimate
Lithobates catesbeianus	2,039,867	0.500	4,079,734	Woodward & Quinn 2011
Lithobates forreri	30,487	0.042	725,881	Chatfield et al. 2013 (estimated from *L. pipiens*)
Lithobates pipiens	26,749	0.042	636,881	Chatfield et al. 2013
Bufo marinus	411	0.300	1,370	Zug & Zug 1979
Osteopilus spp.	159	0.011	14,455	McGarrity & Johnson 2010 (estimated from *O. septentrionalis*)
Rana chensinensis	7.7	0.130	59	Lu et al. 2008 (estimated from *R. kukunoris*)
Total	**2,097,680.7**		**5,458,380**	

CHAPTER 9

Infectious diseases of amphibians continue to emerge from the wildlife trade

Introduction

During my preparation of this thesis, a novel emerging infectious amphibian pathogen was identified – *Batrachochytrium salamandrivorans* (*Bs*). While *Bd* typically affects frogs, this novel pathogen appears to primarily affect salamanders. This threat to amphibian biodiversity provides a good example of how pathogens will continue to emerge in the absence of biosecurity measures to control their spread, and like *Bd*, this agent appears to be spreading via the international trade in amphibians. Chapter 9 summarizes the threat posed by the spread of Bs and provides evidence suggesting its spread is associated with the exotic pet trade. This study is **Paper 1** of this chapter: "Recent introduction of a chytrid fungus endangers Western Palearctic salamanders" which has been published in Science.

Paper 1

Citation: Martel A, Blooi M, Adriaensen C, Van Rooij P, Beukema W, Fisher MC, Farrer RA, Schmidt BR, Tobler U, Goka K, Lips KR, Muletz C, Zamudio KR, Bosch J, Lötters S, Wombwell E, Garner TWJ, Cunningham AA, Spitzen-van der Sluijs A, Salvidio S, Ducatelle R, Nishikawa K, Nguyen TTT, Kolby JE, Van Bocxlaer I, Bossuyt F, Pasmans F. (2014) Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science, 346, 630−631.

WILDLIFE DISEASE

Recent introduction of a chytrid fungus endangers Western Palearctic salamanders

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CHAPTER 10

Conclusions and management implications

Introduction

In the previous Chapters, I studied five different modes of potential *Bd* dispersal and evaluated each in their own right. In Chapter 10, I now compare all data produced by my studies and develop a risk matrix to characterize the relative threat of *Bd* introduction posed by each pathway. I then analyse the conditions likely to promote successful pathogen transport and identify actions that could reduce the spread of *Bd*. This study is **Paper 1** of this chapter: "Risk Evaluation of Five Simultaneous Pathways of Current Global Amphibian Chytrid Fungus Dispersal", which has not yet been published.

Paper 1

Citation: Kolby JE, Berger L, Skerratt LF. Risk Evaluation of Five Simultaneous Pathways of Current Global Amphibian Chytrid Fungus Dispersal. (unpublished manuscript)

Abstract
Discovered nearly 20 years ago, the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), continues to spread largely unabated, and pathways of spread remain poorly understood. The recent global emergence of chytridiomycosis, is causing amphibian population declines contributing towards a global amphibian extinction crisis. Eradication of *Bd* following establishment in a new area is not generally feasible. The development of targeted control efforts to mitigate *Bd* spread are urgently needed, and this requires an evaluation of the most relevant dispersal pathways. Hence I reviewed the best available information and estimated the relative likelihood and consequences of five modes of *Bd* dispersal, including both anthropogenic and natural mechanisms. I assessed the likelihood of viable *Bd* passage between non-contiguous aquatic amphibian habitats and highlighted opportunities to reduce *Bd* transport to new zones. Although I determined that all five pathways can transport *Bd* to some extent, it was clear that one pathway – the international trade in live amphibians – poses a greater risk of spreading disease than the other four combined. My analysis reinforces the urgent need for improved biosecurity to reduce the presence of *Bd* in commercial amphibian shipments. As global connectivity continues to increase, additional wildlife diseases are expected to emerge posing risks to biodiversity, livestock and human health, and hence stricter trade regulations will have broad international benefits.

Introduction

Amphibians have survived on this planet for millions of years, but are now are experiencing rapid global population decline and extinction (Stuart et al. 2004). Many contributing factors have been identified as consequences of human activity, most notably the widespread use of pesticides toxic to amphibians (e.g. Atrazine) (Hayes et al. 2010) and the conversion of rainforest

to agricultural land (Stuart et al. 2004). The spread of infectious disease is a much more insidious catalyst of decline that has become increasingly prominent. Since multiple threats to amphibian survival are likely to act in synchrony in nature, it is important to tease apart proximate and ultimate causes of decline, and identify conservation intervention opportunities most likely to reduce additional biodiversity loss.

The greatest modern threat to global amphibian biodiversity is the disease chytridiomycosis, caused by infection with amphibian chytrid fungus (*Batrachochytrium dendrobatidis*, *Bd*) (Skerratt et al. 2007). *Bd* has been detected in over 500 amphibian species and is potentially capable of infecting thousands of the 7,000+ known species. It has also been detected at 48% of localities surveyed around the world and on all continents, except Antarctica (Olson et al. 2013). Presence of *Bd* is often associated with cool freshwater aquatic habitats, as this pathogen can neither survive complete desiccation nor extended exposure to elevated temperatures (Berger et al. 2004; Johnson & Speare 2005). Despite these environmental limitations to survival, *Bd* has successfully traversed seemingly hostile regions and spanned global distances. Methods to safely eradicate *Bd* from affected environments and to control infection in wild amphibians are being tested but have not yet been proven (Woodhams et al. 2011).

The geographic origin of *Bd*, mechanisms of pre-modern transoceanic dispersal, and reason for recent disease emergence despite a long history of *Bd* presence in some countries, remain enigmatic (Rosenblum et al. 2013). The contemporary global spread of *Bd* is commonly attributed to the international trade in live amphibians—an activity that transports millions of amphibians around the world annually and includes animals infected with *Bd*, primarily for use as exotic pets (Spitzen-van der Sluijs et al. 2011; Kolby et al. 2014), food (i.e. frogs legs)

(Garner et al. 2006; Fisher & Garner 2007; Schloegel et al. 2009; 2012), and biomedical research (Weldon et al. 2004).

Although amphibian trade represents an important opportunity for global *Bd* dispersal, patterns of *Bd* emergence and distribution do not exclusively overlap regions and periods of notable commercial amphibian trade. *Bd* has been detected in numerous countries where deliberate amphibian importation is minimal or absent, including Madagascar (Kolby 2014a; Bletz et al. 2015), Honduras (Puschendorf et al. 2006; Kolby et al. 2010), and Montserrat (Garcia et al. 2009). Additionally, recent surveillance for *Bd* in archived museum specimens shows an historic spread of *Bd* to four continents without the aid of modern amphibian trade. These data illustrate the presence of *Bd* in the USA by 1888 (Talley et al. 2015), Brazil in 1894 (Rodriguez et al. 2014), Japan in 1902 (Goka et al. 2009), and Cameroon in 1933 (Soto-Azat et al. 2010). Although the current trade in amphibians undoubtedly plays an important contemporary role in the global spread of *Bd*, these observations suggest the presence of multiple *Bd* dispersal pathways of variable frequency and consequence.

In order to better understand the forces currently driving this panzootic event and explore opportunities to reduce continued *Bd* spread, I investigated five potential *Bd* dispersal pathways. These include pathogen transport facilitated by: 1. Commercial amphibian trade, 2. Unintentional amphibian trade, 3. Atmospheric spread, 4. Amphibian locomotion, and 5. Fomites. Integrating literature reviews and results from my original field investigations, I assess the likelihood and consequence of each pathway. Based on these data, I then suggest management activities expected to reduce the risk of *Bd* spread and disease emergence in non-contiguous locations.

I acknowledge that the establishment of *Bd* and emergence of chytridiomycosis in populations of wild amphibians following exposure is difficult to predict and likely to be affected by

numerous variables of unknown relative importance. For instance: the degree of susceptibility to infection of those animals exposed, number of amphibians exposed within a certain time period, the dose of *Bd* zoospores presented to those amphibians, the duration of *Bd* exposure, water and air temperatures during exposure, virulence of the *Bd* strain involved, and so on. Thus, to streamline my analysis, I evaluate the relative impact of each *Bd* dispersal pathway within the assumed context that frequent amphibian exposure to a high density of *Bd* will result in *Bd* establishment and disease emergence in a population of susceptible amphibians in the natural environment. Throughout this paper, when calculating risk, I assume the consequence of *Bd* spread to be high.

Bd Dispersal Pathways

Commercial Trade in Live Amphibians

Each year, millions of live amphibians are traded internationally and the majority are traded either as exotic pets or for human consumption as frogs' legs. Much smaller quantities are traded for biomedical research or for zoological and educational purposes. The United States imports approximately 5 million live amphibians annually without pathogen screening requirements (Schloegel et al. 2009) and is likely the greatest consumer nation of these animals. Unlike internationally traded amphibians, centralized data recording the nature of domestic amphibian trade is rarely available. All intentional human-assisted movements of amphibians may help spread *Bd*, although most of our focus will be directed at international trade. Hereafter, we collectively refer to all commercial trade in live amphibians as the "amphibian trade."

I examined amphibian import records maintained by the United States Fish and Wildlife (USFWS) Law Enforcement Management Information System (LEMIS) to identify the most

highly traded species over a five-year period (1 January 2006 to 31 December 2010) (as discussed in Chapter 8). Although a high diversity (300+ species) of amphibians was imported, nearly 90% of animals were comprised of just 10 species among which considerable variation in *Bd* prevalence was detected (Table 1; adapted from Chapter 8). Of these, the most frequently imported was the American bullfrog (*Lithobates catesbeianus*), a species both well-known to be a *Bd* reservoir host (Daszak et al. 2004; Hanselmann et al. 2004; Gervasi et al. 2013) and highly invasive outside its native range (Kraus 2009). I sampled three separate shipments of bullfrogs immediately upon arrival in the USA from the Dominican Republic and detected *Bd* prevalence ranging from 23–91% (Chapter 2). These three shipments also tested positive for the presence of ranavirus, another emerging pathogen of international concern, which ranged in prevalence from 83–100%. At live animal food markets in the USA, detected *Bd* prevalence in bullfrogs was as high as 62% and 8% for ranavirus (Schloegel et al. 2009). Despite the extended post-import survival likely for *Bd* reservoir host species such as bullfrogs, any species of traded amphibian can potentially carry this pathogen and serve as a vehicle for dispersal.

Bullfrogs are commonly transported within plastic netted bags packed inside cardboard boxes. These frogs often escaped from their bags during transport and became loose inside the cardboard boxes, as we discovered when we opened boxes to collect *Bd* samples (Figure 1). Upon opening boxes, these frogs often sprung out, and large holes were observed in the plastic bags. These frogs are likely to escape when these boxes are opened in commercial markets. In one of three shipments, a cardboard box became damaged in-transit and frogs had already escaped through a hole in this box prior to its arrival for our examination. Because the American bullfrog is a highly mobile and adaptable invasive species, animals that escape following

importation pose high risks of both *Bd* spread and establishment, especially if the airport is situated near local wetlands, as is often the case (Figure 2).

Prior to the advent of modern commercial trade, the movement of *Bd*-positive frogs across biogeographic boundaries is likely to have been severely limited. With the speed of commercial transport, even moribund *Bd*-positive animals may be provided with opportunities to traverse longstanding physical and temporal barriers.

Amphibian trade also generates and transports fomites—objects capable of carrying infectious *Bd* particles— such as bags of water and shipping boxes. These materials accumulate *Bd* zoospores and sporangia shed from infected animals and under suitable conditions they may grow and may remain infectious for days to weeks, and possibly longer. The spread of *Bd* by fomites can occur without amphibians being present, and carries unique risks compared with the movement of amphibians. For this reason, I treat the spread of *Bd* via fomites as a distinct pathway of *Bd* dispersal and specifically address it later.

Risk Summary

A) *Frequency of Bd spread*: **Very High**

- Amphibian trade is a commonly recurring activity
- High quantities of amphibians are often traded in each instance
- *Bd* prevalence has reached 100% in traded species

B) *Density of Bd transported*: **Very High**

- Highest densities of *Bd* are found on amphibian hosts
- Amphibians are often shipped en masse, encouraging increased rates of *Bd* transmission among traded animals

Potential *Bd* Mitigation Activities

- Restrict international trade in *Bd* reservoir host species such as *Lithobates catesbeianus* and *Xenopus laevis*, as per Chapter 8
- Raise standards of biosecurity associated with trade import, for example place pet amphibians into biosecure facilities for quarantine prior to entry into commerce
- Improve security of shipping containers to reduce likelihood of amphibian escape
- Pack amphibians in lower densities to reduce rates of *Bd* transmission experienced during transport
- Discard amphibian shipping materials after single use to prevent *Bd* cross contamination between shipments
- Slaughter bullfrogs traded for frog leg consumption either prior to exportation from the source country or immediately following importation to prevent accidental escape of invasive *Bd* reservoir hosts
- Greater public education and awareness to discourage the intentional release of traded amphibians into the wild (e.g. pets no longer wanted, laboratory research animals meant to be euthanized following experimentation, animals purchased from food markets and released in compassion rituals, etc.)
- Encourage exporters and buyers to introduce testing for *Bd* prior to shipping

Unintentional amphibian trade

Even in the absence of amphibian trade, amphibians may still be transported as hitchhikers within other types of commercially traded products. In particular, this applies to goods and materials collected from or held outdoors prior to transport, such as vegetation (e.g. fresh

produce, potted plants, cut flowers), building materials and landscaping substrates (e.g. soil and rocks, lumber, cinder blocks), and heavy machinery (e.g. stacked rubber tires, heavy machinery, and automobile wheel wells). Without rigourous inspection, amphibians may become stowaways. Further, dark cavernous habitats such as shipping containers are often attractive to amphibians. If loading doors remain open at night prior to transport, local amphibians may be transported, regardless of the type of cargo loaded into the container. For the purpose of this analysis, we regard "unintentional amphibian trade" as the transportation of any non-amphibian cargo where amphibians were accidental stowaways.

Although the quantity and frequency of amphibians unintentionally transported is much less than by commercial amphibian trade, it is not without ecological consequence. For example, since 1937 thirteen species of non-native amphibians have been documented on Guam. These animals are believed to have been introduced through the aquaculture trade, horticulture trade, and as stowaways inside air and ocean vessels, and at least six of these species have already become established (Christy et al. 2007). In particular, the trade in ornamental plants appears to be a common pathway of international amphibian dispersal (Kraus et al. 1999; Heinicke et al. 2011; Somma 2015; Somma and Neilson 2015). The greenhouse frog, *Eleutherodactylus planirostris*, is native to Cuba, the Cayman Islands and northern Bahamas but persistent feral populations have been documented in the USA, Hong Kong, Mexico, Honduras, Panama, Jamaica, Grenada, and Guam (Hedges et al. 2004; Christy et al. 2007; Kraus 2009; Somma 2015; Lee et al. 2016). This species is susceptible to *Bd* and has contributed towards the introduction and establishment of *Bd* in Hawaii, despite the island possessing no native amphibians (Beard and O'Neill 2005).

Similarly, the coqui frog, *Eleutherodactylus coqui*, is native to Puerto Rico, but is now found on St. Croix, St. John and St. Thomas, U.S. Virgin Islands, Dominican Republic, Culebra, and Vieques, and in the USA (Somma and Neilson 2015). Like the greenhouse frog, the coqui is also susceptible to *Bd* but do not often develop severe chytridiomycosis, and infected animals are likely transported in commercial shipments of greenhouse nursery plants (Beard and O'Neill 2005). Patterns of population genetics identified among invasive E. coqui in Hawaii show that human-assisted "jump dispersal" is a significant pathway of frog dispersal on Hawaiian islands compared to natural diffusion dispersal from locally established populations (Everman and Klawinski 2013). This also suggests that early detection and the rapid implementation of an eradication program may both prevent establishment of invasive amphibians and the pathogens they carry.

The potential emergence of *Bd* in Madagascar was recently reported (Kolby 2014a, Kolby et al. 2015a; Kolby & Skerratt 2015; Bletz et al. 2015) and its origin and mode of introduction proved especially enigmatic due to the absence of commercial amphibian importation. Interestingly, an incursion of Asian toads (*Duttaphrynus melanostictus*) was concurrently identified in Madagascar (Kolby 2014b). These amphibians are believed to have arrived as hitchhikers inside ocean shipping containers that originated in Southeast Asia and arrived in Toamasina, Madagascar's coastal ocean port city that lies near the epicenter of this invasion. It is unknown how many animals were imported, but there are now estimated to be 4 million toads occupying an area of at least 100 km^2 (McClelland et al. 2015). It remains unknown whether these toads have introduced *Bd* to Madagascar, but this incursion currently poses a likely source and pathway for *Bd* importation. Unfortunately, neither eradication nor import biosecurity

efforts have yet been implemented nationally, and this route provides an ongoing opportunity for the introduction of additional *Bd*.

Another instance of suspected *Bd* introduction by unintentional amphibian trade is the chytridiomycosis outbreak on the island of Montserrat that nearly caused the extinction of the Mountain chicken frog, *Leptodactylus fallax*. Again, amphibians are not commercially imported here, yet a *Bd*-driven disease event swept across the island a few years after a similar disease event swept across the nearby island of Dominica and caused the rapid decline and extirpation of *L. fallax* (Fa et al. 2004). According to Adams et al. (2014), eleutherodactylid frogs are frequently transported internationally within bananas and other fresh produce, and since much of Montserrat's fresh produce is imported from Dominica, a known *Bd*-positive country, this agricultural trade represents the most likely source of *Bd* introduction to Montserrat.

An extensive collection of global herpetological interception records has been collated (Kraus 2009), and many more instances likely evade detection and /or documentation. A nationally coordinated biosecurity effort to intercept the arrival of stowaway reptiles and amphibians is employed in New Zealand (Gill et al. 2001), but this program is globally unparalleled. Most countries neither systematically monitor these incursions nor record these data if observed. In New Zealand, vegetable and banana imports were often associated with the introduction of *Litoria* spp., *Bufo* spp., and *Duttaphrynus* spp. (Maria 2014) and it is likely that this phenomenon occurs in many other countries despite the paucity of empirical data.

Risk Summary

A) *Frequency of Bd spread*: **Moderate**

- Amphibian presence in non-amphibian shipments is much less common than in amphibian trade shipments, but most common source of frogs to countries without amphibian trade
- *Bd* prevalence highly variable depending on species and source involved

B) *Density of Bd transported*: **High**

- Highest density of *Bd* is found on amphibian hosts
- Amphibians present in very low densities
- In-transit spread of infection unlikely to increase *Bd* prevalence (compared with commercial amphibian shipments with high amphibian densities)

Potential *Bd* Mitigation Activities

- Targeted inspection of cargo held outdoors and potentially exposed to amphibians
- Removal and containment of intercepted amphibians to reduce opportunities for introduction and establishment following importation
- Closure of ocean shipping container doors when not actively loading cargo to reduce likelihood of amphibian entry
- Fumigation/chemical/ heat treatment of horticultural shipments to euthanize stowaway amphibians
- Greater public education to report sightings of non-native amphibians to the local authorities responsible for invasive species control

Aerial Bd dispersal (weather and wildlife)

Bd zoospores must first cross terrestrial boundaries in order to spark new waves of disease in non-contiguous aquatic habitats. In water, flagellated *Bd* zoospores can swim short distances and/or become transported by water currents (Johnson and Speare 2005), but dynamics of terrestrial spread are more obscure. *Bd* does not possess protective encapsulation, leaving zoospores vulnerable to desiccation and prolonged exposure to temperatures above 29 C (Berger et al.2004; Longcore et al.1999). The movement of *Bd* on infected amphibians can carry the pathogen away from water and provide some avoidance of conditions lethal both to amphibians and *Bd*, although independent amphibian movements are themselves limited by species-specific habitat boundaries. However, the detection of *Bd* in terrestrial and arboreal amphibian species that typically do not enter permanent water bodies (Cummer et al. 2005; Weinstein 2009; Kolby et al. 2010), suggests the presence of non-aquatic avenues of *Bd* dispersal and transmission.

Pathogenic microorganisms are commonly aerosolized by naturally occurring environmental phenomena involving wind and rain (Kellogg and Griffin 2006; Griffin 2007). Previous investigations have primarily focused on weather-driven dispersal of agricultural pathogens, such as *Aphtae epizooticae*, the virus responsible for bovine foot and mouth disease (FMD), and *Xanthomonas axonopodis pv. citri*, the bacterial agent of citrus canker (Gloster 1982; Gottwald and Irey 2007; Irey et al.2006). Powerful tropical storms are seasonally common in many regions occupied by *Bd*-positive amphibians and it is possible that these and other aerial phenomena have assisted the spread of *Bd* beyond obstacles that would otherwise limit terrestrial dispersion. For our analysis, we define aerial *Bd* dispersal to include any movement of *Bd* that occurs while suspended above the ground, but specifically exclude aerial movement caused by anthropogenic activity (i.e. *Bd*-positive amphibians transported by airplane).

A low level of *Bd* was recently detected in rainwater in Cusuco National Park, Honduras, collected from a location naturally devoid of forest canopy cover by a landslide (Kolby et al. 2015b). It's uncertain where this *Bd* originated and how far it traveled while suspended, but the surrounding landscape was populated by waterfalls generating clouds of aerosolized river water particles likely to contain *Bd*. Previous field surveys showed these rivers were occupied by a high prevalence of *Bd*-positive tadpoles (Kolby et al. 2010), and some *Bd* zoospores shed by these animals are expected to become ejected into the air as water crashes into waterfall plunge pools. Even in fully terrestrial locations, the impact of raindrops against arboreal *Bd*-positive amphibians may dislodge zoospores that then become integrated into wind-swept precipitation. The influence of weather on the dispersal of aerosolized *Bd*-positive water droplets is variable and unpredictable; small breezes may commonly carry water droplets short distances whereas hurricanes may quickly carry this material much further, and in extreme circumstances may even be strong enough to allow wind-driven dispersal of live *Bd*-positive amphibians.

The flight of wildlife also contributes towards aerial *Bd* dispersal. The feet of 15% of geese sampled tested positive for *Bd* and when tested in the laboratory, *Bd* demonstrated chemotaxis towards the birds' keratinous toe scales (Garmyn et al. 2012). Even in the absence of chemotaxis, small volumes of *Bd*-positive water and sediment may be carried on birds' feet and feathers when leaving one aquatic habitat and be introduced to a noncontiguous water body following flight. Similiarly, it is also possible that the flight of aquatic invertebrates between watersheds might spread *Bd*. Although evidence of insect-driven *Bd* dispersal has not yet been produced, *Bd* grew and reproduced on autoclaved crayfish carapace (McMahon et al. 2013), and the chitinous exoskeletons of other arthropods might similarly prove attractive to *Bd*.

Risk Summary

A) *Frequency of Bd spread*: **High**

- Near-constant aerosolization of *Bd*-positive water by river water splash and waterfall plunge pools
- Weather events accompanied by strong wind and rain are especially prevalent in tropical regions where *Bd*-positive amphibians are present
- Frequent flight of birds between watersheds particularly during seasonal migrations

B) *Density of Bd transported*: **Very Low**

- *Bd* subjected to extreme environmental dilution factors after shed from an amphibian host
- Aerial translocation of high density *Bd* sources (e.g. *Bd*-positive amphibians carried by hurricanes or birds) possible but likely uncommon

Potential *Bd* Mitigation Activities

- None; the spread of *Bd* by weather and wildlife are natural phenomena that cannot feasibly be controlled.

Amphibian locomotion

Because amphibians are the major habitat of *Bd*, amphibian locomotion provides opportunities for the transport of *Bd*. *Bd* is highly transmissible through direct contact between amphibians and is likely to be spread during amplexus or territorial combat. Additionally, as infected amphibians move through an aquatic habitat, they shed *Bd* zoospores into the water that

can infect nearby amphibians (Rachowicz and Vredenburg 2004; Reeder et al. 2012). Further, as infected amphibians travel through terrestrial vegetation, they often leave trails of *Bd*-positive residue that may prove infectious to passer-by amphibians that take refuge in the same patches of vegetation (Kolby et al. 2015c). Thus, the autonomous patterns of movement and behavior of *Bd*-positive amphibians is expected to bear strong influence on the spread and distribution of *Bd*. In our analysis of this pathway, we define amphibian locomotion to include all self-directed amphibian movements and exclude movements caused by human activity, other wildlife, and weather events.

Varying combinations of biotic and abiotic constraints delineate natural boundaries to amphibian dispersal. These species-specific limitations characterize patterns of species distributions and determine the extent to which animals are likely to spread *Bd* following introduction. Further, transmission of *Bd* through direct physical contact would generally restrict the spread of *Bd* to a low diversity of species because amplexus and territorial disputes infrequently engage members of different species. However, direct contact does not appear to be a key driver of spread of *Bd* — waves of chytridiomycosis often indiscriminately infect multiple species in an area simultaneously (Lips et al. 2008; Crawford et al. 2011). Thus, it appears that indirect *Bd* transmission from infectious material shed into the environment as amphibians disperse is a significant source of disease spread, despite high environmental dilution of zoospores outside a host.

Patterns of autonomous amphibian dispersal vary considerably between species and even individuals. Some are sedentary with high site fidelity whereas others may travel great distances in a night foraging for food or seeking mates. For example, a mark-recapture study of White-bellied frogs (*Geocrinia alba*) in Australia found that 90% of adult male frogs dispersed less than

20 m over one year (Driscoll 1997). On the other extreme, radio-tracked cane toads (*Bufo marinus*) along an invasion front in Australia traveled an average of 264 m in a single night, and as much as 21.8 km over a 30-day period (Phillips et al. 2007). Besides species-specific tendencies, amphibian movements vary with habitat type, animal size and sex, and in particular the weather (McGarrity and Johnson 2010). Despite species dispersal limits, the common presence of multiple amphibian species in suitable habitats provides the opportunity for species ranges to overlap and for *Bd* to move in waves across species boundaries.

Although we exclude human-assisted amphibian movements from this dispersal pathway, the introduction and spread of invasive species caused by the amphibian trade warrants mention. Two amphibian species in particular – the American bullfrog, *Lithobates catesbeianus*, and the African clawed frog, *Xexopus laevis* – are globally traded in disproportionately high numbers, are aclinical *Bd* reservoir hosts, and have a tendency to disperse following escape or release. Feral populations of one or both now exist on every continent (except Antarctica) (Kraus 2009) and the spread of these highly mobile invasive species following anthropogenic introductions has undoubtedly removed certain longstanding boundaries to the dispersal of *Bd* driven by independent amphibian locomotion.

Risk Summary

A) *Frequency of Bd spread*: **Very High**

- Near-constant autonomous movements of amphibians in nature
- Invasive amphibians commonly spread by human activities into areas likely inhabited by more sedentary amphibian species

B) *Density of Bd transported*: **Very High**

- Highest density of *Bd* is found on infected amphibian hosts

Potential *Bd* Mitigation Activities

- Encourage regrowth of native vegetation to slow advancement of amphibians via anthropogenic clearings such as roads
- Restrict trade in the American bullfrog, *Lithobates catesbeianus*, and the African clawed frog, *Xexopus laevis* and/or improve containment methods to reduce the likelihood of escape from trade
- Greater public education to discourage the release of unwanted non-native amphibian pets into the wild, especially those that are invasive species
- For naïve areas containing endangered species, build exclosures to prevent direct contact with *Bd*-positive amphibian which may arrive

Fomites (anthropogenic-generated & dispersed)

Animals infected with *Bd* commonly shed zoospores and infected skin moults into the environment where they may contaminate substrates and prove infectious to other amphibians (Rachowicz and Vredenburg 2004; Reeder et al. 2012). These *Bd*-positive materials act as fomites—vehicles that carry infectious zoospores or sporangia from one location to another and serve as potential sources of transmission. Examples of *Bd* fomites include *Bd*-contaminated rocks, vegetation, water, and amphibian trade shipping cartons. Although fomites can also be spread by wildlife (e.g. *Bd*-contaminated water droplets carried in the fur of mammals or in the

feathers of waterfowl), I consider only those fomites created and transported by humans in my analysis of this *Bd* dispersal pathway.

Human activities frequently occur in *Bd*-contaminated locations and/or involve the handling of infected amphibians, creating an abundance of opportunities for *Bd* fomites to become generated and transported. Most notably, live amphibians are frequently traded en masse and transported inside cardboard boxes, plastic containers, and bags of water, all of which may accumulate *Bd* zoospores or sporangia shed from infected animals. Approximately 68 zoospores per minute were released from Pacific chorus frogs (*Pseudacris regilla*) into the water in laboratory trials (Reeder et al. 2012). International shipments of amphibians often keep animals confined inside their shipping containers for approximately 12 hours to three weeks, depending if they are transported as air or ocean cargo. This long holding period provides ample time for *Bd* to be shed onto shipping materials.

Bd fomites were commonly detected in amphibian shipments sampled upon importation to the USA. *Bd* was found in water sampled from bags carrying amphibians in 4/5 shipments exported from Hong Kong (Kolby et al. 2014). Skin swabs for *Bd* were concurrently collected from animals in these same bags of water and 70% of *Xenopus laevis* tested *Bd*-positive, whereas all *Paramesottriton hongkongensis* tested negative despite detection of *Bd* in their water. Water filtered from bags containing *X. laevis* demonstrated exceptionally high densities of *Bd*, with average *Bd* zoospore equivalents per liter ranging from 3,390 to 16,887 between two shipments. Those for *P. hongkongensis* were considerably lower, and ranged from 2.9 to 5.7 *Bd* zoospore equivalents per liter, but still represent levels potentially infectious to other animals.

The containers used to transport "dry" amphibians can also serve as *Bd* fomites. Live American bullfrogs (*Lithobates catesbeianus*) are often shipped in plastic netted bags sealed

inside cardboard boxes. I collected swab samples of these boxes from three shipments of frogs exported from the Dominican Republic to the USA (Chapter 2). Swabs were collected from the interior bottom surface of the cardboard boxes, upon which the bags of frogs rested. A high prevalence of *Bd* was detected on boxes in all three shipments and ranged from 25.7% - 88.6% of boxes tested. Cumulatively, 62/105 (59.0%) of boxes from the three shipments tested *Bd*-positive. Although these frogs were shipped in the absence of water, approximately 25-30 animals were packed tightly into each box and produced a slurry of urine and feces that created a wet environment inside the cartons, expected to extend the survival of *Bd* by preventing rapid desiccation.

In the natural environment, humans passing through amphibian habitats can likewise transport objects that become contaminated with *Bd*, such as footwear, recreational equipment, and research equipment exposed to amphibians and/or aquatic environments. In the absence of decontamination with bleach or other disinfectants (Cashins et al. 2008), *Bd* might be transported by foot, car, or plane and then transferred to subsequent aquatic habitats if field activities are soon repeated. If kept cool and moist, *Bd*-contaminated objects and substrates might remain infectious for days to weeks (Johnson and Speare 2003). Due to the exceptionally high densities of *Bd* that accumulate in amphibian trade substrates compared with extremely dilute amounts of *Bd* typically found in natural environmental substrates, I separately evaluate the risk of disease spread associated with: A) Amphibian trade and B) All other human activities.

Potential *Bd* Mitigation Activities

Amphibian trade

- Sterilization of all potentially *Bd*-contaminated shipping materials prior to disposal via exposure to disinfectants or incineration.

- Disposal of all shipping materials (cartons, bags, and water used to transport amphibians) after single use

All other human activities (scientific/recreational)

- Foot-bath cleaning stations positioned at the entrance to popular eco-tourism destinations where *Bd* is known or suspected to be present
- Disinfection of all research equipment used in aquatic habitats with bleach by all researchers (not just amphibian researchers)
- Gloves worn when handling amphibians and then disinfected and discarded after single use to prevent transmission between animals and locations
- Drain water from fishing boat live wells and ballast water compartments upon leaving an aquatic area
- Scrubbing and/or removing mud and vegetation from equipment when leaving an aquatic site
- Greater public education & awareness of the potential presence of *Bd* in environments utilized by the public

Risk Summary

Amphibian trade

A) *Frequency of Bd spread*: **Very High**

- High rate of *Bd* zoospore and sporangia shedding from infected amphibians into their surroundings
- Long holding periods of amphibians in-transit allows ample time to accumulate shed *Bd* zoospores

- Amphibians frequently transported in large numbers, involving high quantities of shipping cartons and volumes of water

B) *Density of Bd transported*: **Moderate**

- *Bd* zoospores become dilute after shed from a host, but will accumulate in confined artificial environments (e.g. inside bags of water and cardboard boxes)

All other human activities (scientific/recreational)

A) *Frequency of Bd spread*: **Moderate**

- High rate of *Bd* zoospore and sporangia shedding from infected amphibians into their surroundings

B) *Density of Bd transported*: **Low**

- *Bd* shed from amphibian hosts into the natural environment typically become subject to extreme environmental dilution (although initial density of *Bd* present may be higher if a sick animal is handled)
- Small volumes of *Bd*-contaminated environmental material commonly spread by recreational and scientific activities (although the quantity will by acitivity)

***Bd* Dispersal Risk Matrices**

The precise sequence of events requisite for *Bd* to cause disease and/or become established in new a new location following introduction is poorly understood and hinges upon any number of varibles. Notwisthstanding these uncertainties, certain pathways of *Bd* dispersal are likely associated with geater risk of disease and decline than others, based on their respective frequency

of ocuurence, density of *Bd* transported, distance *Bd* is transported, and viability of *Bd* upon introduction. I have assumed that the consequence of introduction of *Bd* to a naïve population is severe and so risk is driven by the likelihood of introduction. In order to visibly compare these risks to better assist management decision-making, I have developed risk matrices that include all five *Bd* dispersal pathways evaluated above. In the following matrices, each pathway is given a numerical score which is the product of variables which affect the ability of *Bd* to spread. The first matrix estimates the frequency of each pathways' activity and the amount of *Bd* likely to be transported in each instance (Figure 3). The second matrix estimates whther the pathway carries *Bd* far from the point of origin (i.e. is better at dispersing *Bd*) and the likelihood that *Bd* is infectious upon introduction (Figure 4). Lastly, the scores produced in Figures 3-4 are combined to illustrate the estimated relative contribution of each pathway in the recent global emergence of *Bd* (Figure 5).

Discussion

I found evidence supporting the existence of all five *Bd* dispersal pathways investigated, and my analysis suggests that international trade in live amphibians is the highest risk pathway. This activity creates the most consistent and predictable opportunities for long-distance spread of viable *Bd* likely to result in new establishments (Figures 3-5). This activity concurrently produces an abundance of fomites, facilitating the second most significant pathway of *Bd* dispersal we identified—fomites created and spread by the amphibian trade. These fomites accumulate higher densities of *Bd* zoospores than those transported by scientific or recreational activities and are more likely to deliver viable *Bd* due to the measures taken to keep traded amphibians alive. In the absence of modern amphibian trade, *Bd* is unlikely to quickly spread

across oceans in high densities, a phenomenon that may have historically suppressed the emerging panzootic event.

Certain assumptions and generalizations were made here that warrant mention. Both the distances and viability of *Bd* dispersed are expected to be highly variable between each instance of transport in each of the five pathways considered. I made my best effort to characterize the most likely "average" expectations for these parameters, but too few quantitative measurements were available with which to make my judgements quantitative. For instance, we detected the presence of *Bd* in rainwater in 1/20 sampling events (Kolby et al. 2015b), but to say that *Bd* can be expected to be present in rainwater in 5% of storm events is erroneous considering our sample size and the enormity of the natural environment. Similarly, a high degree of species-specific variation has been reported in the average distances traveled by amphibians (Driscoll 1997; Phillips et al. 2007), and the majority of species have not been closely monitored to determine typical rates of dispersal. Thus, it is not possible to calculate an average dispersal rate for the "amphibian independent locomotion" *Bd* dispersal pathway that would reliably bear relevance across nature. Of all pathways and scenarios considered, that of the commercial amphibian trade provided the most consistent long-distance spread of *Bd* material, despite shipment-to-shipment variation in prevalence.

Although the amphibian trade might appear to be the obvious catalyst for global *Bd* spread, a more complex history has recently become evident. Retrospective surveys for *Bd* in museum archives show that it was already present on four continents prior to the mid 1930's, when transoceanic amphibian trade via commercial flights first began. Although historical avenues of *Bd* dispersal remain obscured, it was present in the USA by 1888 (Talley et al. 2015), Brazil by 1894 (Rodriguez et al. 2014), Japan by 1902 (Goka et al. 2009), North Korea by 1911 (Fong et

al. 2015), and Cameroon by 1933 (Soto-Azat et al. 2010) (Figure 6). Exportation of *Xenopus laevis* from South Africa beginning around 1935, for use in human pregnancy tests, was proposed to be the catalyst for global *Bd* emergence (Weldon et al. 2004), although it is more likely that at least two global spread events have occurred – this recent one via amphibian trade and an ancient one. This is further supported by the presence of endemic *Bd* lineages on some continents where the highly virulent *Bd*GPL lineage has been more recently introduced via the bullfrog trade (Goka et al. 2009; Bataille et al. 2013; Rosenblum et al. 2013). Accordingly, I assert that the global distribution of *Bd* as observed today is the cumulative result of multiple dispersal pathways of varying consequence, one of which is the amphibian trade.

Long-distance *Bd* dispersal pathways that predate amphibian trade emergence do exist—aerial transport by weather (Kolby et al. 2015b) and wildlife capable of flight (Garmyn et al. 2012) —although current data suggests *Bd* survival following such dispersal events are infrequent. Still, future outcomes of atmospheric pathogen transport are unpredictable, especially as global climate patterns become disrupted by anthropogenic climate change and shift weather conditions that may affect airborne pathogen survival. Also prior to the advent of human flight, it is plausible that stowaway amphibians were carried by ocean vessels and may have provided opportunities for early transoceanic *Bd* spread.

Identification and characterization of pathogen dispersal pathways is imperative to design effective disease mitigation efforts. Multiple simultaneous opportunities for *Bd* spread exist, obscuring a clear distinction of the most frequent source of new introduction and establishment events. Making such distinctions is critical in order to ensure that biosecurity efforts target the weakest link in the chain of events that most often result in disease emergence. Of the five *Bd* dispersal pathways examined herein, biosecurity interventions must target the international

amphibian trade in order to prevent continued *Bd*-associated global amphibian decline and extinctions.

Bd is already global in distribution, but recent studies have shown that a diversity of lineages exist (Farrer et al. 2011; Schloegel et al. 2012; Rosenblum et al. 2013) which differ dramatically in virulence (Berger et al. 2005; Fisher et al. 2009; Gahl et al. 2012, Gervasi et al. 2013). During my thesis, I generally referred to *Bd* as a single organsm rather than as specific strains. This is because the technology to differentiate *Bd* strains from skin swab samples has only recently become available. Moving forward, identification of *Bd* strains from field swabs should be performed wherever possible in order to help reveal the strains and sources of *Bd* commonly associated with certain dispersal pathways. These data may likewise provide insight into whether *Bd* collected in the field was a regional endemic strain, or an exotic strain associated with introduction and spillover from the international wildlife trade. This same information will also aid predictions of Bd infection outcome, judging from strain's relative degree of virulence.

Methods to control the trade-driven spread of *Bd* have been recommended by the World Organisation of Animal Health (OIE) and are described in the Aquatic Animal Health Code (OIE 2015). If shipments of live amphibians are imported from countries not declared "free from infection with *Bd*", Article 8.1.8 of this Code suggests the following biosecurity actions should be implemented:

- the direct delivery to and lifelong holding of the [amphibian] consignment in biosecure facilities for continuous isolation from the local environment
- the treatment of water and equipment used in transport and of all effluent and waste materials in a manner that inactivates *B. dendrobatidis*.

Further to these actions, additional requirements and parameters pertaining to specific end-uses are also described, such as the importation of live amphibians for human consumption or to establish new amphibian breeding stocks. Unfortunately, while these recommendations were developed by the OIE nearly five years ago (Schloegel et al. 2010a), few countries have formally adopted these practices or require any actions specifically to reduce *Bd* introduction.

Other commercial animal trades have long been closely regulated to protect human and livestock health, but relatively little regulatory enforcement is directed towards the protection of wildlife. For instance, in the USA, certain species of rodents and bats are restricted from commercial importation by the Centers for Disease Control and Prevention (CDC) to prevent the introduction of zoonotic pathogens, such as monkeypox and Nipah virus, which threaten public health (McLean 2007). Similarly, the US Department of Agriculture (USDA) administers a comprehensive risk-management program to protect the economic security of the nation's livestock industry by controlling disease. For example, it is forbidden to import a goat prior to five years of isolation from any animal potentially affected by scrapie, and cattle can only be imported from premises free of bovine tuberculosis for the previous two years (USDA regulatory requirements 7 U.S.C. 8303; 9 CFR 93). In just one instance, the USFWS introduced trade restrictions to prevent disease spread in wildlife, although the main intent was to protect commercial fisheries from diseases vectored by salmonid fish (18 U.S.C. 42; 50 CFR 16.13), rather than the inherent protection of a wildlife.

Due to the severity and scope of an impending global amphibian extinction event, the scientific and NGO communities called for a full ban on the importation of live amphibians into the USA and formally requested emergency action by the USFWS (DOW 2009). After five years, no decision has yet been announced by USFWS. I also performed a detailed risk

evaluation of amphibian importation into the USA and concluded that even in the absence of a complete trade ban, a targeted multi-phase approach to end the import of known *Bd* reservoir host species, i.e. the American bullfrog (*Lithobates catesbeainaus*) and African Clawed Frog (*Xenopus laevis*), would offer rapid far-reaching disease reduction benefits (Chapter 8).

Many wildlife species are reservoirs of pathogens that threaten domestic animal and human health (Daszak et al. 2000), but it is uncommon for wildlife disease reduction efforts to be implemented unless severe financial consequences of inaction emerge. To protect global biodiversity, a paradigm shift is critically needed in the way animal health is prioritized. This shift has been embodied by the "One Health" approach that recognizes the inextricable links between human, wildlife, and environmental health (Karesh & Cook 2005). Preemptive wildlife trade disease surveillance is central to this theme and greater collaboration between researchers and governments is necessary to develop pathogen monitoring programs. Proactive efforts to protect wildlife health would reduce species extinctions while also safeguarding public health and economic security.

Rapidly increasing globalization with inadequate biosecurity ensures the continued emergence of pathogens with the potential to cause dramatic biodiversity decline and extinctions. The absence of proactive disease screening and control allows undiscovered wildlife diseases to be dispersed by the wildlife trade long before formal identification, just as with *Bd*. The recently described chytrid, (*Batrachochytrium salamandrivorans*, "Bsal") (Martel et al. 2013), a fungal pathogen that causes chytridiomycosis in salamanders, is now in the early stages of global emergence. This pathogen is believed to be of East Asian origin and recently invaded salamander populations in Belgium, Germany, and the Netherlands, likely through spillover from the exotic salamander pet trade (Martel et al. 2014; Sabino-Pinto et al. 215). In the Netherlands,

where it was first discovered, Bsal has already driven the rapid near-extinction of fire salamanders (*Salamandra salamandra*) with estimated 95% population decline (Spitzen-van der Sluijs et al. 2013). Recent surveys suggest this pathogen has not yet reached the USA —the world's global hotspot for salamander diversity (Petranka 1998) —where consequences of Bsal invasion are predicted to be severe and irreversible (Yap et al. 2015). In May 2015, a petition to ban importation of all salamanders into the USA to prevent a Bsal outbreak was submitted to the US Department of the Interior and is currently under review (C*BD* 2015). Based on my analyses of *Bd* global dispersal pathways and similarities between the spread and transmission of *Bd* and Bsal, I agree that biosecurity efforts to limit the movement of Bsal-positive animals are imperative to mitigate this threat and prevent an otherwise near-certain outbreak in the USA.

Future Directions

Although I applied the best available data to my evaluation of *Bd* dispersal pathways (as of December 2015), I recommend the framework provided herein be continually updated as new information emerges. Laboratory investigations to measure the survival of *Bd* zoospores and zoosporangia in a wide variety of environmental conditions have been limited, and additional investigations could help refine estimates of *Bd* persistence when spread independent from amphibian hosts. In particular, while it is known that exposure to 32 C for four hours is lethal to *Bd* (Berger et al. 2004), *Bd* viability in water and sediment maintained at lower temperatures is not well-defined. Further, desiccation poses a threat to zoospore survival and laboratory exposure trials to investigate the infectiousness of *Bd* post-aerial dispersal have not yet been performed.

Improvements in tracing previous and current movements of particular *Bd* strains could also help improve dispersal pathway evaluation. Refined methods are still needed that better identify

the global origin or clade membership of single, individual samples of *Bd* - be it from freshly captured and swabbed animals, museum preserved specimens, or water filter samples. For instance, it is unknown whether the *Bd*-positive frogs collected 100+ years ago in North and South America, Asia, and Africa were all infected with *Bd*GPL when sampled, currently the most globally dispersed strain, or instead with *Bd* from sister clades potentially endemic to each region and expressing low virulence to native species at that time. The ability to disentangle historic changes in global *Bd* distribution from potential changes in the distribution of certain *Bd* strains may significantly help elucidate *Bd* origin and dispersal patterns.

The spread of an emerging fungal pathogen through time and space via enigmatic pathways is not a phenomenon unique to *Bd*. For instance, bats in the northeastern United States have been severely impacted by the recent emergence (in 2006) and rapid spread of white nose syndrome, caused by the fungal pathogen *Pseudogymnoascus destructans*. Like *Bd*, *P. destructans* appears to persist in the environment, spreads freely with the movement of host animals, and sometimes makes dramatic dispersal leaps, such as the recent identification of *P. destructans* in the Western United States (Washington State) – nearly 2,100 km from the nearest previous detection (Lorch et al. 2016). According to Lorch et al. (2016), this detection "appears inconsistent with the previously documented and predicted pattern of pathogen spread" and the mode of dispersal remains unknown. No effective method to halt its continued spread has been identified and recent studies have shown that arthropods (harvestmen and wing mites) sharing hibernacula with bats can also carry and potentially spread this bat pathogen (Lucan et al. 2016; Vanderwolf et al. 2016). This phenomenon demonstrates the complexity in identifying the full spectrum of possible dispersal pathways of an emerging fungal pathogen after it has already become widespread and established in a new environment. This is much like the case with *Bd*, which can

similarly infect and be spread by the movement of non-amphibian arthropod hosts, such crayfish (*Procambarus* spp. and *Orconectes virilis*) (McMahon et al. 2013; Brannelly et al. 2015) and the nematode worm *Caenorhabditis elegans* (Shapard et al. 20102). Thus, interventions to control these pathogens' dispersal must be initiated as quickly as possible following introduction before they are able to exploit a variety of alternate hosts that provide additional obscure opportunities for dispersal. Major advances in the study of *Bd* dispersal pathways and identification of opportunities to safely control this pathogen are likely to offer insight that can help mitigate the impact of other emerging infectious fungal diseases that severely threaten biodiversity.

Bd was discovered and described nearly 20 years ago (Berger et al. 1998; Longcore et al. 1999), and disease-driven declines and extinctions continue to pose one of the greatest amphibian conservation challenges in recorded history. The modern international trade in live amphibians provides an engine of global pathogen pollution historically unrivaled and warrants disease management at least to the level commonly offered to livestock animals. Biosecurity efforts must now co-evolve alongside increasing opportunities for disease spread fueled by globalization, before additional wildlife taxa become subject to irreversible extinction crises akin to that of amphibians from *Bd*.

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Table 1. Total live amphibian imports into the USA from 2006-2010 as per USFWS LEMIS declaration records. For the 10 most traded species, I list the primary purpose of trade, whether that species has been found infected with *Bd*, and highest published measures of *Bd* prevalence for amphibians sampled from the wild and captivity. N/A = not available.

Species	**Total Qty [% total]**	**Purpose**	***Bd*+**	***Bd* % Wild (95% CI)**	***Bd* % Captive (95% CI)**
Lithobates catesbeianus	12,635,149 [50.2%]	Food	Yes	24.7 (18.7–33.5) (feral population, Bataille et al. 2013) 100.0 (81.6–100.0) (invasive population, Goka et al. 2009)	100 (95.7–100.0) (farmed, Schloegel et al. 2010b)
Hymenochirus	4,168,874	Exotic		N/A	N/A

curtipes	[16.6%]	pet			
Lithobates forreri	1,745,481 [6.9%]	Research	Yes	22.2 (2.3-60.0) (native population, Zumbado-Ulate et al. 2014)	N/A
Bombina orientalis	1,616,825 [6.4%]	Exotic pet	Yes	16.7 (13.9–20.3) (native population, Bataille et al. 2013)	5.4 (1.8–14.6) (captive bred, Kolby et al. 2014)
Xenopus laevis	795,295 [3.2%]	Research	Yes	13.0 (4.5–32.1) (feral population, Vredenburg et al. 2013) 2.6 (1.5–4.2) (native population, Weldon et al. 2004)	70.0 (54.6–81.9) (captive bred, Kolby et al. 2014) 20.3 (14.3–28.1) (wild collected, Goka et al. 2009)
Lithobates pipiens	636,881 [2.5%]	Research		N/A	N/A
Cynops orientalis	620,167 [2.5%]	Exotic pet		N/A	N/A
Hymenochirus boettgeri	343,170 [1.4%]	Exotic pet	Yes	N/A	N/A
Hymenochirus boulengeri	260,464 [1.0%]	Exotic pet		N/A	N/A
Litoria caerulea	216,064 [0.9%]	Exotic pet	Yes	N/A	N/A
Subtotal	**23,038,370 [91.5%]**				
All other spp.	2,127,047 [8.5%]				
Total	**25,165,417**				

Figure 1. American bullfrogs (*Lithobates catesbeanus*) escape from plastic netted bags during transport from Dominican Republic. Loose frogs are marked by red circles.

Figure 2. Proximity of major international airports to local wetlands where escaped *Bd*-positive American bullfrogs could invade following importation. Red circles mark airports and red "X" marks nearby wetlands. (A) Newark Liberty International Airport, (B) Hartsfield-Jackson Atlanta International Airport.

(A)

(B)

Figure 3. Matrix of risk scores for pathways. Score = estimated frequency of transport x estimated *Bd* density transported.

***Bd* Density Transported**						
	Very High 5	**5**	**10**	**15**	**20**	**25** Amphibian Trade; Amphib. Locomotion
	High 4	**4**	**8** Unintent. Amphib Trade	**12**	**16**	**20**
	Moderate 3	**3**	**6**	**9**	**12**	**15** Fomites (Amphib. Trade)
	Low 2	**2**	**4**	**6** Fomites (Other)	**8**	**10**
	Very Low 1	**1**	**2**	**3**	**4** Aerial Transport	**5**
		Very Low 1	**Low 2**	**Moderate 3**	**High 4**	**Very High 5**
		Frequency of pathway presence				

Figure 4. Matrix of risk scores for pathways. Score = estimated distance of transport x estimated viability of *Bd* transported.

***Bd* Viability post-dispersal**						
	Very High 5	**5**	**10** Amphib. Locomotion	**15**	**20**	**25** Amphibian Trade; Fomites (Amphib. Trade)
	High 4	**4**	**8**	**12**	**16**	**20** Unintent. Amphib Trade
	Moderate 3	**3**	**6**	**9**	**12** Fomites (Other)	**15**
	Low 2	**2**	**4**	**6**	**8** Aerial Transport	**10**
	Very Low 1	**1**	**8**	**12**	**16**	**20** Unintent. Amphib Trade
		Very Low 1	**Low 2**	**Moderate 3**	**High 4**	**Very High 5**
		Distance of *Bd* transport				

Figure 5. *Bd* Dispersal Cumulative Risk Evaluation

Total Risk Score = (Figure 3. Frequency x *Bd* Density) + (Figure 4. Distance x *Bd* Viability). It is assumed that consequence of introduction of *Bd* to a naïve population is severe.

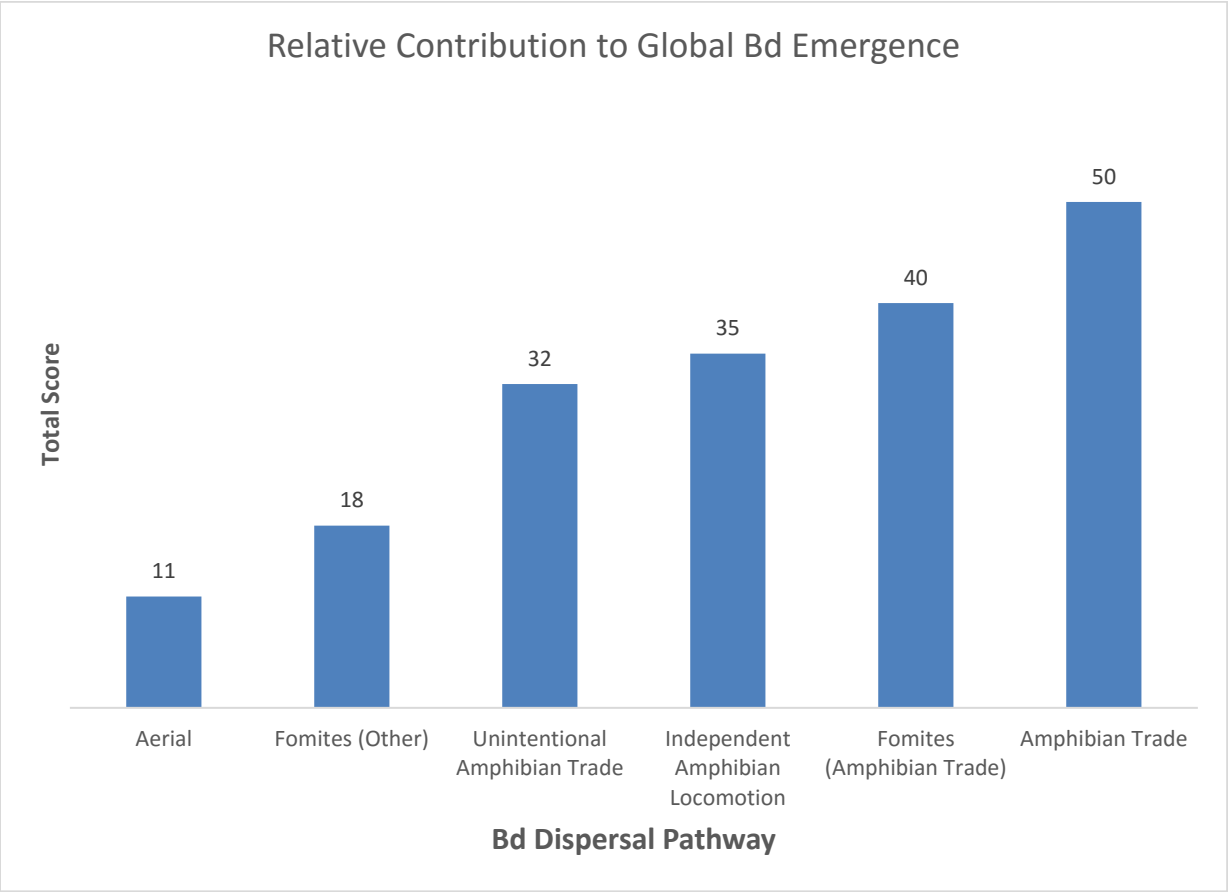

Figure 6. **Minimum global distribution of amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) pre-1934, after which commercial *Xenopus laevis* exportation from Africa began.** Black shading represents *Bd* detection in archived museum specimens. Shaded countries and year of earliest *Bd* presence include: USA (1888), Brazil (1894), Japan (1902), North Korea in (1911), and Cameroon (1933).